PORTUGAL

MINISTÉRIO DA AGRICULTURA DO DESENVOLVIMENTO RURAL E DAS PESCAS
DIRECÇÃO-GERAL DE PROTECÇÃO DAS CULTURAS

Report prepared in the context of the application for first inclusion of dodine in Annex I of the Council Directive 91/414/EEC

DODINE

Volume 3-2

Annex B

Section B6

Summary, evaluation and assessments of the data.

List of tests and studies relied upon

2006

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B.6 Toxicology and metabolism

B.6.1 Absorption, distribution, excretion and metabolism (Annex IIA 5.1)

B.6.1.1 Single dose, second single dose, and repeated dose, oral route, rats

The Residue Kinetics of n-Dodecylguanidine Acetate (Dodine) in the Rat

Cameron, B.D., Milner, N.P. and Dunsire, J.P. (December 1985) – IRI Report No. 4066. Performed by Inveresk Research International, Musselburgh, EH21 7UB, Scotland.

Guidelines and GLP:

The study was performed according to GLP and was conducted with a standard method before OECD Guidelines were released. The design, treatments and analysis satisfied in principal all the requirements listed in FIFRA guidelines for rat metabolism study (F-85-1) at the time the study was performed: using radiolabelled dodine for the determination of absorption, distribution, excretion (including volatiles), comparison of males and females, single and repeated doses, high and low doses, oral and intravenous treatments and the required observation period (i.e. until >90% was excreted). Deviations from the recommended test guideline – method B.36 of EU or OECD 417 were the following:

1. Three males and 3 females were used when guidelines recommends at least 4 animals per sex and dose.

2. Blood concentrations were measured only in the plasma, the RBC was discarded.

3. Identification of the metabolites was not done. The metabolites were separated enough to rule out the presence of any significant amount of parent material.

4. Quantification of the metabolites is not acceptable; each product was presented as major, minor or intermediate.

5. For repeated dosing, animals received seven daily oral doses of ^{14}C dodine instead of at least 14 days of "cold" dodine administration.

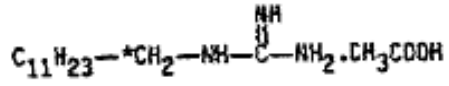
6. Volatile C^{14} was measured only in 4 animals (2 rats from each 2 groups oral and intravenous) but in view of the low quantity found and the consistency between the values found for each of the 4 treatments, this deviation is not considered significant.

7. Tissue residues were not characterized.

Materials and methods:

Groups of 3 male and 3 female rats (Sprague Dawley CD from Charles River (UK), bodyweight 160-240 g) were dosed with a single oral dose of 5 or 50 mg/kg bw, a single intravenous administration of 5 mg/kg bw, or seven daily oral doses of 5 mg/kg bw of ^{14}C-dodine.

The test material – batch No. CFQ 3930, specific activity 2 mCi/mmole, total activity 2.5 mCi - was labelled as shown in the following figure. The radiochemical purity was greater than 95%.



[1-^{14}C]-Dodecylguanidine acetate {* = Site of label}

For oral administration, ^{14}C-dodine was suspended in 0.5% carboxymethylcellulose and administered as a 1 ml target dose volume by gastric gavage. There were no adverse effects observed following administration of oral doses.

For intravenous administration, ^{14}C-dodine was dissolved in ethanol, each animal received 50 µl of dose solution. Immediately following intravenous administration, the animals showed signs of ethanol intoxication. In most cases, the animals recovered, although in one case, a rat died and was replaced by a substitute. In addition, some animals passed blood in their urine during the first hour post dose.

Dosing groups consisted of:

Group A: single oral administration at low dose (5 mg/kg bw) by gastric gavage;

Group B: single intravenous administration at low dose (5 mg/kg bw);

Group C: 7 oral daily doses at low dose (5 mg/kg bw) of ^{14}C-dodine;

Group D: single oral administration at high dose (50 mg/kg bw).

The study was performed in 5 major phases:

Phase 1 (3 rats/sex/group): Plasma levels of radioactivity were recorded from the 4 groups. Following single oral administration (Groups A and D) blood samples were removed from the tail veins at the following times post dose: 0.5, 1, 2, 4, 6, 8, 12 and 24 h. Following intravenous administration (Group B) blood samples were taken additionally at 5 and 15 min post dose. Following repeated oral administration (Group C) blood samples were taken daily at 4 h after dosing (peak plasma time) and at the same times described above following the seventh dose. Plasma was separated from the red cells by centrifugation. Plasma was sampled for total radioactivity and the cells discarded.

Phase 2 (3 rats/sex/group A and B): The excretion of radioactivity was monitored in groups A and B. Both groups of animals were housed in all glass metabolism cages after ^{14}C-dodine administration. Urine and faeces were collected at 24 h intervals for 96 h post dose. In addition, expired $C0_2$ over the first 48 h post dose was collected from 4 of the animals (2 from each group). At 96 h the total radioactive residue in the gastro-intestinal tract and remaining carcass were measured. Cage wash radioactivity was also measured.

Phase 3 (3 rats/sex/group A and 4 rats/sex/group C) Tissue distribution study following single and repeated oral dosing: One animal from each group was sacrificed at each of the following times post dose: 4 h (male and female), 8 h (male), 12 h (female), 24 h (male) and 96 h (female). In addition, one animal in Group C was sacrificed immediately prior to the penultimate dose (-24 h (male)) and another immediately prior to the final dose (0 h (female)).

Immediately after sacrifice a sample of blood was removed and the following tissues and organs dissected: bone marrow, brain, fat, heart, spleen, skeletal muscle, skin, testes/ovaries, liver, eyes, lung, thyroid, kidney, gastro-intestinal tract and residual carcass.

Radioactivity was measured separately in all the tissues and organs removed and in the residual carcass and plasma.

Phase 4 (1 rat/sex/group A) Protein binding study: Two animals (1/sex) were administered a single oral dose of 5 mg/kg bw ^{14}C-dodine. The animals were sacrificed at peak plasma level (4 h post dose) and a blood sample taken from the vena cava. Protein binding was determined by equal volume equilibrium dialysis of the plasma samples from the above animals.

Phase 5: The profiles of metabolites in selected samples of urine and faeces obtained from phase 2 and 3, and of plasma from phase 4 were described.

Results:

Plasma levels of radioactivity:

Results obtained in the 4 treatment groups are presented in Table 6.1.

Following single oral administration (group A), levels of radioactivity observed in plasma were low. At 4 h post dose, levels of radioactivity reached a maximum of ca 0.19 µg equiv./ml. During the next 8 h radioactivity fell to ca 0.05 µg equiv./ml. Thereafter, levels of radioactivity fell more slowly to 24 h post dose.

When administered intravenously (group B), 5 min post dosing, levels of ca 1.3 µg equiv./ml were recorded in plasma which corresponds to ca 1% of the administered dose indicating a rapid elimination from plasma, either by rapid excretion or rapid uptake into the tissues. After an initial minor peak of radioactivity, levels of radioactivity declined at a moderate rate until 8-12 h, and thence more slowly until 24 h at which time point levels of ca 0.06 µg equiv./ml were recorded in plasma.

When administered for 7 days (group C), at 4 h post first dose (the first period of measurement) levels of ca 0.17 µg equiv./ml were recorded in plasma. Following progressive doses peak plasma levels (4 h post dose) increased slightly to ca 0.22 µg equiv./ml at 4 h post dose 6. After the fifth dose, peak plasma levels remained approximately constant.

The overall profile of plasma radioactivity levels after the seventh dose was similar to that observed after a single oral dose.

Following oral administration of a high dose of ^{14}C-dodine (group D), levels of radioactivity were also low. During the following 4 h, the levels increased ca 4-fold to peak plasma levels, a similar rate of increase to that observed after low dose administration. Peak plasma level of radioactivity was maintained from 4 h until 12 h. Thereafter, radioactivity levels decreased slowly to 24 h.

Table 6.1 – Plasma levels of radioactivity following oral and intravenous administration of [^{14}C]-dodine to rats at low and high doses levels after a single and repeated oral doses

Time	Group A # (5 mg/kg, oral, single)			Group B # (5 mg/kg, intravenous)			Group C # (5 mg/kg, oral repeated)			Group D # (50 mg/kg, oral, single)		
	male	female	mean	male	female	mean	male	female	mean	male	female	mean
Dose 1*							0.1579	0.1868	0.1723			
Dose 2*							0.1380	0.1907	0.1643			
Dose 3*							0.1686	0.2312	0.1999			
Dose 4*							0.1764	0.2486	0.2125			
Dose 5*							0.1277	0.2810	0.2043			
Dose 6*							0.1701	0.2697	0.2199			
5 min				1.1895	1.4799	1.3347						
15 min				1.0996	1.2209	1.1602						
0.5 h	0.0371	0.0544	0.0457	1.3251	1.2554	1.2902	0.0761	0.1380	0.1070	0.3329	0.3357	0.3343
1 h	0.0619	0.1164	0.0891	0.7457	0.9463	0.8460	0.0915	0.1521	0.1218	0.4903	0.5816	0.5359
2 h	0.1038	0.1599	0.1318	0.4323	0.6485	0.5404	0.1341	0.1899	0.1620	0.7603	0.9796	0.8699
4 h	0.1320	0.2522	0.1921	0.2233	0.3668	0.2950	0.1619	0.2488	0.2053	1.0685	1.4318	1.2501
6 h	0.1292	0.2115	0.1703	0.1871	0.2333	0.2102	0.1669	0.2417	0.2043	1.0629	1.4203	1.2416
8 h	0.0932	0.1570	0.1251	0.1325	0.2143	0.1734	0.1455	0.2091	0.1773	1.0706	1.5829	1.3267
12 h	0.0519	0.0556	0.0537	0.0910	0.1161	0.1035	0.0892	0.1683	0.1287	0.8997	1.3244	1.1120
24 h	0.0262	0.0248	0.0255	0.0526	0.0632	0.0579	0.0602	0.0810	0.0706	0.3067	0.3583	0.3325

Results expressed as µg equiv. dodine/ml

* Oral repeated administration: 4 hours post dosing

Excretion of radioactivity

Following oral administration of ^{14}C-dodine, radioactivity was eliminated almost equally via the urine and faeces (approx 45% each) whereas the majority of the radioactivity was eliminated in the urine (approx 70%) in the case of intravenous administration. Expired $^{14}CO_2$ was a minor route of elimination (ca 0.5% in both type of administration). Radioactivity remaining in the residual carcass was higher by intravenous injection (8%) compared to oral administration (0.6%). In both cases, excretion was rapid. Urine and faeces accounted for more than 80% of radioactivity after 96 h.

Table 6.2 – Recovery of total radioactivity following oral and intravenous administrations of [^{14}C]-dodine to rats

Sample		Group A (5 mg/kg bw, oral) % of dose recovered			Group B (5 mg/kg bw, intravenous) % of dose recovered		
		male	female	mean	male	female	mean
Urine	0-24h	40.22	45.63	42.92	57.11	51.24	54.17
	0-96h	42.67	48.49	45.55	70.24	66.59	68.36
Faeces	0-24h	37.77	38.34	38.05	8.07	9.91	8.99
	0-96h	49.67	47.24	48.43	12.73	15.64	14.18
CO_2	0-48h	0.75	0.34	0.54	0.31	0.72	0.51
Carcass		0.69	0.50	0.59	7.77	8.70	8.23
GI tract		0.08	0.06	0.07	0.33	0.44	0.37
Cage Wash		0.88	0.98	0.93	3.80	2.28	3.04
Total		94.74	97.61	96.11	95.18	94.36	94.69

Tissue distribution of radioactivity following single and multiple oral dose administration

Following single oral administration of ^{14}C-dodine, radioactivity was distributed unevenly to the tissues (see Table 6.3 and Table 6.4). Whilst radioactivity in most tissues rapidly declined over 96 h post dose; the levels in fat tissue declined only slowly. The ovaries, thyroid and skin showed some degree of radioactive retention similar to that of the fat tissue. However, the overall radioactivity in the animal was rapidly eliminated.

As in the case of single oral dose administration, the majority of tissues showed a steady decrease in radioactivity after the final daily dose of the multiple dose administration (see Table 6.5). However, the rate at which radioactivity decreased in the tissues and in the plasma was much slower than after single dose. As demonstrated following single dose administration, fat tissue, ovaries, thyroid and skin retain considerable quantities of radioactivity.

Table 6.3 – Tissue Distribution of radioactivity following oral administrations of [^{14}C]-dodine to rats (single dose of 5 mg/kg bw) - Results expressed as µg equiv./g

Tissue	Time of sacrifice animal number (sex)					
	4 h 33 (male)	4h 38 (female)	8h 34 (male)	12h 36 (female)	24h 35 (male)	96h 37 (female)
Liver	1.9339	2.8906	1.9210	0.8889	0.2117	0.0311
Kidney	1.4156	2.2214	1.1195	0.6121	0.1317	0.0252
Lung	0.1732	0.3546	0.1725	0.1330	0.0402	0.0134
Heart	0.2196	0.4929	0.1673	0.1626	0.0393	0.0102
Spleen	0.1087	0.1975	0.1126	0.1105	0.0557	0.0147
Brain	0.0134	0.0039	0.0151	0.0124	0.0048	0.0013
Testes	0.0776	-	0.0568	-	0.0174	-
Ovaries	-	0.3211	-	0.2629	-	0.0523
Eyes	0.0439	0.0875	0.0689	0.0541	0.0160	0.0093
Thyroid	0.2815	0.5992	0.4021	1.1903	0.1237	0.0000
Fat	0.1588	0.2173	0.1489	0.0814	0.1508	0.0513
Skeletal Muscle	0.1169	0.1183	0.0855	0.0526	0.0299	0.0104
Skin	0.0877	0.1279	0.1504	0.0763	0.0736	0.0052
Bone Marrow	0.1097	0.1403	0.1396	0.0089	0.0933	0.0000
Plasma*	0.2012	0.3412	0.1391	0.0835	0.0223	0.0014

* Results expressed as µg equiv./ml

Table 6.4 – Tissue Distribution of radioactivity following oral administrations of [^{14}C]-dodine to rats (single dose of 5 mg/kg bw) - Results expressed as total % dose in whole organ

Tissue	Time of sacrifice animal number (sex)					
	4 h 33 (male)	4h 38 (female)	8h 34 (male)	12h 36 (female)	24h 35 (male)	96h 37 (female)
Liver	2.0852	2.1907	1.6506	0.6286	0.2374	0.0284
Kidney	0.2862	0.3526	0.2350	0.0997	0.0301	0.0041
Lung	0.0210	0.0366	0.0190	0.0138	0.0051	0.0015
Heart	0.0179	0.0365	0.0148	0.0130	0.0035	0.0008
Spleen	0.0061	0.0100	0.0049	0.0045	0.0025	0.0008
Brain	0.0021	0.0039	0.0027	0.0021	0.0009	0.0002
Testes	0.0188	-	0.0140	-	0.0044	-
Ovaries	-	0.0033	-	0.0027	-	0.0006
Eyes	0.0009	0.0023	0.0017	0.0013	0.0004	0.0000
Thyroid	0.0002	0.0006	0.0003	0.0011	0.0001	0.0000
Fat	-	-	-	-	-	-
Skeletal Muscle	-	-	-	-	-	-
Skin	-	-	-	-	-	-
Bone Marrow	-	-	-	-	-	-
Residual carcass	4.4926	6.0662	2.5809	1.9617	1.0074	0.9063
Total % dose * remaining in body	6.9310	8.7027	4.5239	2.7285	1.2918	0.9427
GI tract	74.11	71.58	63.37	25.56	4.11	0.87

* These values do not take into account small quantity of radioactivity contained in the plasma taken for analysis (ca 2 ml) and the small amounts of fat, skeletal muscle, skin and bone marrow also taken for analysis

Table 6.5 – Tissue Distribution of radioactivity following multiple oral administrations of [^{14}C]-dodine to rats (7 daily doses of 5 mg/kg bw) - Results expressed as µg equiv./g

Tissue	Time of sacrifice animal number (sex)							
	-24 h 19 (m)	0 h 23 (f)	4 h 20 (m)	4h 24 (f)	8h 21 (m)	12h 25 (f)	24h 22 (m)	96h 26 (f)
Liver	0.4565	0.5613	2.8275	2.7800	2.3647	1.7029	0.6341	0.1208
Kidney	0.2622	0.3541	1.6587	5.9312	1.2059	1.1962	0.3520	0.1113
Lung	0.1332	0.1368	0.3236	0.4806	0.2851	0.2694	0.1580	0.0612
Heart	0.0987	0.1215	0.3386	0.4192	0.2448	0.2982	0.1213	0.0484
Spleen	0.1360	0.1610	0.2650	0.4262	0.2336	0.2002	0.1672	0.0685
Brain	0.0243	0.0223	0.0387	0.0499	0.0362	0.0313	0.0371	0.0227
Testes	0.0564	-	0.1216	-	0.1015		0.0604	-
Ovaries	-	0.5397	-	1.4026	-	0.5837	-	0.2336
Eyes	0.0466	0.0427	0.0863	0.1060	0.0677	0.0715	0.0403	0.0142
Thyroid	0.1562	0.4367	0.6835	2.3792	0.8472	0.9371	0.6758	0.1167
Fat	0.8511	0.6658	1.4324	0.6792	1.1562	0.5046	1.2542	0.4379
Skeletal Muscle	0.0973	0.0984	0.1602	0.2247	0.1737	0.1261	0.1062	0.0574
Skin	0.3096	0.1736	0.5838	0.2713	0.4639	0.3160	0.3537	0.1435
Bone Marrow	0.1185	0.1095	0.1866	0.2869	0.2064	0.1624	0.0685	0.0253
Plasma*	0.0579	0.0712	0.2531	0.2791	0.1929	0.1314	0.0839	0.0171

* Results expressed as µg equiv./ml

Plasma protein binding

The control experiments demonstrated that a high percentage of unchanged dodine binds to plasma protein (94-99%). The dialysis of plasma obtained from rats 4 h post oral dose of 5 mg/kg bw, showed considerably less protein binding of radioactivity (34-39.5%). This indicates that radioactivity at peak plasma levels was, at least in part, in a form that binds less well to plasma protein than unchanged.

Examination of the nature of radioactivity

The profiles of metabolites in selected samples were described but the metabolites were not identified. Analysis of the metabolites indicates that ^{14}C-dodine was rapidly metabolised to a number of unidentified polar components which were chromatographically dissimilar to dodine, dodecylamine and dodecylurea.

Conclusions:

Following administration of ^{14}C-dodine by intravenous or oral route, radioactivity was eliminated via urine and faeces. Elimination via the faeces was found to be more important after oral administration than after intravenous administration. This indicates either low absorption of the compound or increased biliary elimination following oral administration. $^{14}CO_2$ levels in expired air were low, suggesting that complete degradation is limited. Overall elimination following intravenous administration was markedly slower than that observed after oral administration.

In most tissues, radioactivity levels decreased rapidly after administration. However, substantial amounts of radioactivity were retained by the fat tissue and to a lesser degree by the skin, ovaries and thyroid. As the fat tissue constitutes a high proportion of the overall body weight of the animal, the total amount retained by the fat tissue may be considerable. Indeed, high radioactivity levels remained in the carcass following intravenous administration. This could well be due to retention by fatty tissues. However, significant retention by the fat is not observed following oral administration.

The plasma protein binding study indicates that dodine is rapidly metabolised to more water soluble metabolites with less affinity to plasma proteins.

Analysis of the metabolites indicates that ^{14}C-dodine was rapidly metabolised to a number of unidentified polar components, which were chromatographically dissimilar to dodine, dodecylamine and dodecylurea.

Disposition and Metabolism of ^{14}C-Labeled Dodine in Rats (Preliminary and Definitive Study)

Reddy, V., Litle, L. and Murrill, E. (September 10, 1992) – MRI Report No. 9938-F. Performed by Midwest Research Institute, Kansas City, Missouri 64110, USA.

Guidelines and GLP:

The study was performed according to GLP and US EPA FIFRA F-85-1 guideline for rat metabolism study.

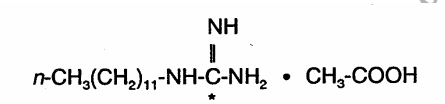
The study is acceptable.

Materials and methods:

In the preliminary study, Sprague-Dawley rats (1 male and 1 female) were given a single oral dose of 40 or 400 mg/kg bw ^{14}C-dodine (in corn oil) by gavage and were housed in glass metabolism cages for assessment of radioactivity in expired air, urine, faeces, and tissues. Intravenous dosing was considered as not possible due to dodine's insolubility in vehicles suitable for intravenous administration. Urine, faeces and expired air were collected at 4, 8, 12, 24, 48 and 72 hours post-dosing. All animals were sacrificed at 72 hours.

Definitive study: Groups of 5 male and 5/6[1] female Sprague Dawley rats [Crl:CD BR] from Charles River Breeding Laboratories Inc. (Portage, Michigan Facility), bodyweight 195-273 g (males) and 183-215 g (females), 56-72 days (males), and 62-84 days (females) old, were dosed by gavage with single oral doses of 40 or 400 mg/kg bw (at 4ml/kg bw), or 14 oral daily doses of 40 mg/kg of [^{14}C]-dodine.

The test material – Lot No. 910225, specific activity 56.5 mCi/mmole -, was labelled as shown in the following figure. The radiochemical purity was greater than 99%. Nonlabelled dodine (Lot No. FF1/88) was indicated to be 99.8% pure.



* position of the ^{14}C label

Following administration of the radiolabelled dose, urine and faeces were collected at 4, 8, 12, 24, 48, 72, 96, and 120 h. After 120 h, all animals were anesthetized and exsanguinated. The tissues examined were the following: liver, kidneys, lungs, brain, heart, spleen, ovaries, testes, uterus, adrenals, fat (retroperitoneal), skin, muscle (thigh), bone (femur), GI tract and residual carcass. Samples of whole blood, plasma and red blood cells were analyzed for radioactivity; aliquots of total weights of urine, cage rinse and expired air trapping solutions were counted for radioactivity, as well as homogenized faeces.

[1] In the multiple dose group, a total of six females were used. One female rat sustained a mechanical injury and was replaced by two female rats.

Table 6.6 – Dosage and treatment with dodine

Study	Sex	No. of animals	Nominal dose (mg/kg)	Actual dose (mg/kg)	Radioactivity administered (µCi/kg)	Specific activity (dpm/µg)
Preliminary						
40 mg/kg	M	1	40	27.5	152.1	12.296
	F	1	40	28.5	158.1	12.296
400 mg/kg	M	1	400	397.1	157.8	882
	F	1	400	415.0	165.9	882
Definitive						
Single P.O	M	5	40	36.2	176.3	10.819
	F	5	40	36.6	178.2	10.819
Multiple P.O	M	5	40	37.5	175.1	10.366
	F	6	40	37.4	174.8	10.366
Single P.O	M	5	400	377.2	211	1242
	F	5	400	375.4	210	1242

Results:

***Preliminary study**:*

The preliminary data indicated that most of the radioactivity was eliminated in the urine (approx. 40% and 44% for males and females respectively) and faeces (approx. 56 and 44% for males and females) at 72 hours post-dosing in the low-dose group. Only trace amounts of dodine were expired as volatiles or CO_2. Total radioactivity recovery for the low dose group was 100% and 92% in male and female rats, respectively.

In the high-dose group excretion of dodine through urine was 30% and 37% and through faeces 23% and 35% for the male and female rat, respectively. A significant amount of radioactivity was recovered in the GI tract (including GI contents), 37% and 12% of the administered dose for male and female rats, respectively. Very little radioactivity was detected in other tissues. Less than 1.5% of the total radioactivity remained in the carcass. Total radioactivity recovery was 96% and 89% in male and female rats, respectively.

As the preliminary data suggested that elimination of radioactivity was incomplete by 72 hours in the high dose group, the definitive study was conducted up to 120 hours. Less than 1% of the total dose was eliminated through the expired air, therefore CO_2 and expired air were not collected in the definitive study and the study was conducted using stainless steel metabolism cages.

***Definitive study**:*

Excretion of radioactivity

There were no unusual clinical signs in any of the test animals during the study.

The major portion of the low oral dose (single or multiple) was eliminated within 48 h, in urine and faeces of male and female rats. In the high dose (oral) group, the elimination of radioactive dose was slower than the low dose group rats; and was complete in 120 h.

With a single low dose (40 mg/kg) in males, urinary elimination of radioactivity averaged 40.5% of the administered dose. A similar percent (42.4%) of excretion was observed in female rats. The greatest proportion of the dose was excreted in urine within 48 h (approximately 39.2% and 39.4% for males and females, respectively). Only minimal amounts were excreted in urine between 48 and 120 h (~1.3% and 3% for males and females, respectively). Faecal elimination of ^{14}C-dodine between 0-120 h was higher than the urinary excretion in both male and female rats. Elimination of radioactivity through faeces averaged 59.7% and 55.1% for male and female rats, respectively. The largest proportion of the dose in faeces was excreted within 48 h, only minimal amounts were excreted between 48 and 120 h (3.2% and 4.4% for males and females, respectively). The

cumulative excretion in urine and faeces was approximately 100% and 97.5% of administered dose for both male and female rats, respectively (see Table 6.7).

Table 6.7 – Radioactivity excreta following single or multiple oral administration of ^{14}C-dodine to rats (mean cumulative percent of dose)

Excretum	Time after dosing (h)	40 mg/kg x 1		40 mg/kg x 15[b]		400mg/kg x 1	
		Male[a]	Female[a]	Male[a]	Female[g]	Male[a]	Female[a]
Urine	4	1.78	2.34	1.59	2.97	1.31	1.49
	8	12.07	8.89	11.16	10.88	3.58	3.04
	12	25.69	19.14	25.78	21.70	5.03	4.80
	24	36.35	35.15	39.94	38.34	11.94	10.03
	48	39.19	39.43	43.49	42.41	24.43	20.17
	72	40.06	41.30	44.62	43.91	35.72	31.11
	96	40.38	42.06	45.08	44.53	39.68	40.60
	120	40.55	42.42	45.35	45.00	41.91	43.11
Faeces	4	-[c]	-[c]	0.01[d]	0.00[e]	-[c]	-[c]
	8	-[c]	-[c]	0.01	-[c]	-[c]	-[c]
	12	4.54	7.12[h]	4.09[e]	15.11[e]	-[c]	1.40[d]
	24	47.88	34.66	42.22	29.41	7.18	4.76[f]
	48	56.46	50.74	52.99	49.93	17.94	14.52
	72	58.92	54.20	55.47	52.66	36.08	26.68
	96	59.40	54.79	55.94	53.54	45.89	42.37
	120	59.69	55.14	56.18	53.74	50.50	47.63
Total	4	1.78	2.34	1.60	2.97	1.31	1.49
	8	12.07	8.89	11.16	10.88	3.58	3.04
	12	30.23	21.99	29.05	24.22	5.03	5.64
	24	84.23	69.81	82.16	67.74	19.12	14.80
	48	95.66	90.17	96.48	92.34	42.38	34.69
	72	98.98	95.50	100.09	96.57	71.82	57.79
	96	99.78	96.85	101.02	98.07	85.60	82.97
	120	100.25	97.56	101.52	98.74	92.42	90.74

[a] : Mean of five rats per group, except where indicated.
[b] : Rats received 14 nonlabelled doses of dodine followed by 15th dose of ^{14}C-dodine.
[c] : No faeces during this time point.
[d] : Mean of three rats during this time point.
[e] : One rat from this group had faeces.
[f] : Four rats from this group had faeces.
[g] : Mean of six rats per group, except where indicated.
[h] : Mean of two rats during this time point.

Both male and female rats treated with series of single daily doses (40 mg/kg x 14) of nonlabelled dodine followed by a 15th dose of radiolabelled dodine, excreted 45% of the administered dose in the urine. Similar to single low dose group, the greatest amount of the dose found in urine was excreted within 48 h (approximately 43.4% and 42.4% for male and females, respectively). Only minimal amounts were excreted in urine between 48 and 120 h (~1.9% and 2.6% for males and females; respectively). Excretion of dodine-derived radioactivity in faeces was higher than urinary excretion in both male and female rats (56.1% and 53.7% for male and female rats, respectively). The largest proportion of the dose found in faeces was excreted within 48 h, only little amounts were excreted between 48 and 120 h (~3.2% and 3.8% for male and female rats, respectively. The total dose excreted through urine and faeces was 101 % and 98.7% for males and females, respectively.

In the high dose (400 mg/kg), both male and female rats excreted about 41.9% and 43.1% of administered dose in the urine. However, urinary excretion profile was different from single or

multiple low dose rats, only 24.4% and 20.1 % (male and female, respectively) of the dose was excreted within 48 h; i.e., approximately 50% of the total radioactivity in the urine was excreted during this time. Approximately 17.5% and 22.9% of the administered dose was excreted in urine between 48 h and 120 h. In low single and multiple dose groups more than 90% of the total urinary dose was eliminated during 48 h. Faecal elimination profiles in the high-dose group were similar to urinary excretion profiles. Total dose eliminated in faeces was higher than that in urine for both male and female rats (50.5% and 47.6% for both male and female rats, respectively). Total dose eliminated through urine and faeces was 92.4% and 90.7% for males and females, respectively (Table 6.7).

Blood and tissue radioactivity levels

The recovery of radioactivity in blood and tissues of rats treated with ^{14}C-dodine expressed as percent of the administered dose is presented in Table 6.8. Very low amounts of radioactivity were recovered from tissues and carcass. The recovery in all tissues together ranged from 0.62% to 3.34% of the administered dose. The overall distribution pattern was similar in all dose groups.

Table 6.8 – Radioactivity in blood, tissues and excreta following single or multiple oral administration of ^{14}C-dodine to rats (mean percent of dose) [a]

Tissue/Excretum	40 mg/kg x 1		40 mg/kg x 15[b]		400mg/kg x 1	
	Male	Female	Male	Female	Male	Female
Blood [c]	0.02	0.01	0.01	0.01	0.01	0.01
Plasma [c, d]	0.00	0.00	0.00	0.00	0.00	0.00
RBCs [c, d]	0.01	0.01	0.01	0.00	0.00	0.01
Liver	0.13	0.08	0.09	0.05	0.11	0.06
Kidneys	0.01	0.02	0.01	0.01	0.01	0.01
Lungs	0.03	0.03	0.00	0.00	0.00	0.00
Brain	0.01	0.01	0.00	0.01	0.00	0.01
Heart	0.00	0.00	0.00	0.00	0.00	0.00
Spleen	0.01	0.00	0.00	0.00	0.00	0.00
Testes/ovaries	0.01	0.00	0.01	0.00	0.01	0.00
Uterus	-	0.01	-	0.00	-	0.00
Adrenals	0.00	0.00	0.00	0.00	0.00	0.00
Fat [c]	0.07	0.08	0.04	0.05	0.02	0.02
Skin [c]	0.18	0.21	0.11	0.15	0.06	0.07
Muscle [c]	0.30	0.61	0.24	0.32	0.20	0.22
Bone [c]	0.04	0.04	0.01	0.02	0.04	0.04
GI tract	0.57	0.29	0.16	0.30	1.14	0.56
GI contents	-	-	-	-	1.75	1.86
Carcass [d]	0.75	0.66	0.79	0.88	0.61	0.93
Urine	40.55	42.42	45.35	45.00	41.91	43.11
Faeces	59.69	55.14	56.18	53.74	50.50	47.63
Total [d]	101.64	98.96	102.20	99.65	95.74	93.62

[a] : Mean and S.E. of five rats per group.
[b] : Rats received 14 nonlabelled doses of dodine followed by a 15th dose of ^{14}C-dodine.
[c] : Percent of dose calculations based on 7, 11, 16, 40, and 8% of body weight for blood, fat, skin, muscle, and bone, respectively. Plasma and RBC calculations based on 60 and 40% of blood volume, respectively.
[d] : Carcass values not included in recovery estimates (see text).

Metabolic profile

The sample preparation for HPLC analyses involved filtration of the urine samples or faecal homogenate extracts, followed by HPLC assay. Urine samples exhibiting low levels of radioactivity were typically concentrated prior to assay. This method was found to recover >90% of the radioactivity from the urine and the faecal homogenate extracts.

In all dose groups, most of the dodine-derived radioactivity in the urine was eliminated as metabolites. No significant amounts of the parent compound were detected. In general, the metabolic profile was similar in both sexes and in all dose groups. In the urine profiles, four major peaks were typically observed by HPLC: M2, M3, M4 and M5 (see Table 6.9.).

M2 was identified as an alcohol of dodine, dodecylanolguanidine - DOLG - (hydroxydodecylguanidine), an omega-oxidation product. This was the major peak in the urine, accounting for 11% - 23% of the cumulative percent of dose excreted in the urine sample (120 h following administration). Females contained slightly higher amounts of this metabolite in the urine than did males.

M5 (retention time ~1.5 min) was identified as urea and accounted for 3% - 5% of the cumulative percent of dose excreted in the urine sample.

The second and third major peaks in the chromatogram (**M4 and M3**, retention times of ~25 and ~34 min, respectively) were not identified. These two regions (M4 and M3), typically comprised 4%-13% of the cumulative percent of dose excreted in the urine sample. M4 was however tentatively identified as a mixture of acidic products produced by beta-oxidation of the alkane side chain of dodine.

Overall, the 4 major urine peaks outlined in Table 6.9 typically accounted for 33% to 48% of the dose.

Incubation of urine samples with hydrolytic enzymes indicated no glucuronide or sulphate conjugates.

In the faecal samples, the parent compound was identified by mass spectrometry as the major component (**M1** peak, retention time ~34 min), representing 42% - 63% of the cumulative percent of dose excreted in faeces, with slightly higher amounts excreted in faeces of animals in the single high-dose group than animals in the single or repeated low-dose group. Negligible amounts of metabolites were also detected. According to the author of the study, the presence of predominantly dodine in the faeces may be solely the result of poor intestinal absorption or in part may be due to excretion of the parent compound into intestinal lumen through bile, which was not examined for radioactivity; inhibition of GI tract motility (peristaltic movements) may have resulted in the prolonged excretion of dodine in the high dose group following first-order absorption process.

Table 6.9 – Metabolic profiles observed in urine and faeces 120 h following single or multiple oral administration of ^{14}C-dodine to rats (cumulative percent of dose administered)

Dose, sex	% of dose in the urine sample	Urine peaks (retention time)				% of dose in the faeces sample	Faeces (retention time)
		M5 (~1.5 min)	M4 (~25 min)	M3 (~34 min)	M2 (~40 min)		M1 [a] (~34 min)
40 mg/kg x 1, Male	39.50	5.14	9.90	7.46	10.98	62.90 [b]	55.07 [b]
Female	42.14	5.20	3.70	9.37	20.56	58.51 [b]	51.94 [b]
40 mg/kg x 15, Male	57.23	5.40	12.85	11.15	18.62	53.54 [b]	47.62 [b]
Female	49.43	3.78	7.62	9.49	23.52	49.78 [c]	42.23 [c]
400mg/kg x 1, Male	45.65	3.28	13.48	8.50	11.87	42.39 [c]	39.22 [c]
Female	44.25	3.95	8.71	11.79	15.61	50.93 [b]	42.58 [b]

[a] : Free base of dodine
[b] : No faeces were collected 4 and 8 h following administration
[c] : No faeces were collected 4, 8 and 12 h following administration

Metabolic pathway:

Urine analysis using HPLC indicated four major peaks. The peak designated as M5 was identified as urea in all samples. Urea may be formed as a result of the action of arginase, a cytosolic enzyme, on dodine or one or more of its metabolites, resulting in urea and an amine (not containing the ^{14}C-label, see Figure 1). The peak designated as M2 was identified as the alcohol of dodine, hydroxy dodecylguanidine. The peak designated as M4 was tentatively identified as a mixture of acidic products produced by beta oxidation of the alkane side chain of dodine. Because of the presence of the hydroxy dodecylguanidine (M2) and the other tentatively identified acids in the M4 peak, the author of the study postulates that, the metabolism of dodine follows a beta oxidation pathway similar to that of medium- or long-chain fatty acids. Upon entering the liver cell, dodine may be activated by formation of a CoA derivative. With the help of a carrier (similar to carnitine) it may be entering the mitochondrial matrix, and being oxidized by a sequence of reactions in which the alkyl chain of dodine is shortened by two carbon atoms at a time (beta oxidation). The proposed flow chart for oxidation of alkyl chain of dodine is presented in Figure 6.1. This series of reactions may also be catalyzed by a monooxygenase that requires NADPH, O_2, and cytochrome P450.

The absorbed dodine probably enters the liver through the portal circulation and is metabolized to hydroxydodecylguanidine and other intermediate products with shorter chain lengths which are then eliminated through the urine. Urea may also be formed in the liver as a result of the action of arginase on dodine and/or one or more of its metabolites and eliminated through the urine.

The possible metabolic pathway of dodine in Sprague-Dawley rats is presented in Figure 6.1.

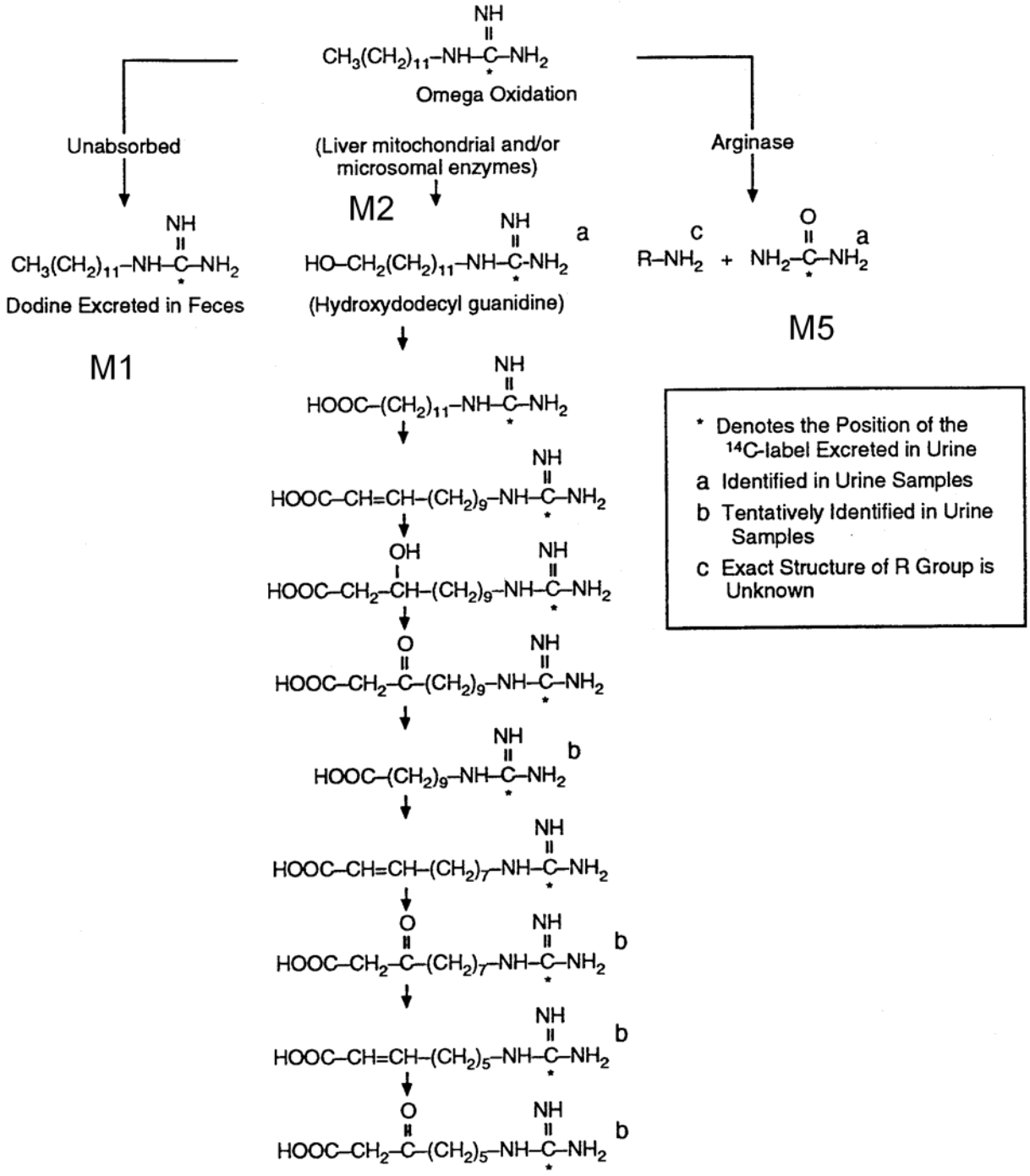


Figure 6.1 – Possible metabolic pathway of dodine in Sprague-Dawley rats.

Conclusions:

The present study indicated that dodine-derived radioactivity was eliminated within 120 h in both male and female rats. The major portion of the radioactivity in the single or multiple low oral dose (40 mg/kg) groups was eliminated in 48h, almost equally in urine and faeces. The high dose group rats eliminated radioactivity slower than the low dose group, but elimination was essentially completed within 120 h. Analysis of the ^{14}C-content of expired air during the preliminary study

indicated that less than 1% of the initial dose was recovered as either $^{14}CO_2$ or volatiles throughout the 72-h period.

Tissue distribution and excretion data indicated that gastrointestinal absorption and metabolism were similar in both sexes. At the high dose, data indicated that the excretion pattern was affected by the dose but not the metabolic profile. In all dose groups, very low concentrations of radioactivity were detected in tissues or carcass samples at 120 h after dosing. The overall distribution pattern was similar in all dose groups and there was no evidence for accumulation of dodine or its metabolites in tissues after single or multiple exposures.

Analysis of the faecal extracts using mass spectrometry indicated that dodine-derived radioactivity excreted by the faecal route was mainly the parent compound (M1) representing 42% - 63% of the dose. Most of the dodine-derived radioactivity in the urine was eliminated as metabolites. Urine analysis using HPLC, indicated four major peaks, M2, M3, M4 and M5, which together accounted for 33% to 48% of the dose.

In general, after oral administration, approximately 45% of dodine was absorbed from the GI tract; the remainder was unabsorbed and was eliminated in faeces.

B.6.2 Acute toxicity including irritancy and skin sensitization (Annex IIA 5.2)

B.6.2.1 Acute oral toxicity (Annex IIA 5.2.1)

Acute Oral Toxicity Study of Dodine Technical Material in Albino Rats

Kern, T.G. (April 1999) – Report No. WIL 21130; performed by WIL Research Laboratories, Inc. 1407 George Road, Ashland, OH 44805-9281, USA.

Guidelines and GLP

The study was performed according to GLP, and EPA OPPTS Guideline 870.1100 (1998), OECD Guideline 401 (1987) and EC's Method B1 (1992).

The study is acceptable.

Materials and methods

Test substance: Dodine Technical Material, off-white powder, lot no. 032246 (synthesis batch no. 3044 in Certificate of Analysis), purity 967 g/kg.

Animals: Albino rat, strain Crl:CD®(SD)IGS BR from Charles River Laboratories, Raleigh, NC, body weight from 196 to 228g, 8-12 weeks old at initiation of dosing.

Prior to initiation of the main study, 2 range-finding experimentations were conducted. In the first one, groups of 1 animal/sex were administered by gavage, test material at dose levels of 500, 1000, 2000, 3500 and 5000 mg/kg. Nine of 10 animals died. Therefore, a second test was conducted in which groups of 1 animal/sex were administered test material at levels of 50, 100, 200, 300 and 400 mg/kg bw. Based on the results (not specified in the study), 450 mg/kg bw was selected as the first dose level on the main study.

Main study: Test material diluted in 0.5% methylcellulose was administered once orally via gavage to groups of 5 male and 5 female fasted albino rats at dose levels of 450, 761 and 1285 mg/kg bw in a dose volume of 10.0 ml/kg.

The rats were observed at approximately 1, 3 and 4 hours post-dosing on day 0 and once daily thereafter for mortality and clinical observations. Body weights were recorded on study days -1, 0 (initiation), 7 and 14 (termination). Upon termination, major organ systems of the cranial, thoracic and abdominal cavities were examined for all animals.

Results

Mortality: Mortality (see Table 6.10) was 0/10, 4/10 and 9/10 for the 450, 761 and 1285 mg/kg bw groups respectively. 11/13 animals died within 7 days, and 1 male and 1 female in the high dose group were found dead on day 11. In addition, one 761 mg/kg bw male and two 1285 mg/kg bw males were euthanized *in extremis* on days 6, 5 and 7.

Clinical observations: Clinical findings were observed in all dosage groups. The most frequent observations were abnormal defecation (decreased defecation, diarrhoea, mucoid faeces and/or white material in faeces), various discoloured areas due to discharges/excretions (around the eyes, nose, mouth, forelimbs, hindlimbs, base of tail anogenital and/or urogenital areas), and hypoactivity. Six animals had a prolapsed penis. Five animals exhibited impaired muscle coordination. Three animals were noted with reddened base of tail and/or hypothermia. Other findings noted at a low incidence included hair loss (on the anogenital, urogenital, ventral trunk areas), pale body, rales and clear ocular discharge. With the exception of discoloured areas and hair loss, all surviving animals appeared normal by day 12.

Body weights: 4 surviving animals in the 761 and 1285 mg/kg bw groups lost 5.6-18.9% of their initial body weight during the first week of the study. These animals regained and/or surpassed their initial body weight by study termination. In general, male body weight gains in the 450 and 761 mg/kg bw during the first week of the study were reduced when compared to the second week. Weight losses were noted prior to death for all animals that were euthanized *in extremis* or found dead after day 7.

Table 6.10 – Mortality of rats treated orally with dodine

Dose level (mg/kg bw)	Sex	Number of dead / number of investigated	Time of death (range)	Observations
450	male & female	0/10	-	0% mortality
761	male	1/5	Days 4-7	40% mortality both sexes combined
761	female	3/5	Days 0-7	
1285	male	5/5	Days 0-14	90% mortality both sexes combined
1285	female	4/5	Days 0-14	
LD_{50} value (95% confidence limits)	male	830 (731-942) mg/kg bw		
	female	817 (501-1333) mg/kg bw		
	combined	851 (658-1100) mg/kg bw		

Necropsy findings: All animals euthanized *in extremis* were observed to have gastrointestinal abnormalities. These findings included distension of the stomach or gastrointestinal tract, yellow mucosa of the stomach, abdominal cavity adhesions (attached to the liver, spleen, pancreas and/or abdominal wall), an emaciated abdominal cavity and/or thick white material present in the abdominal cavity. A prolapsed penis was noted for 2 animals euthanized *in extremis*. Additional findings noted in individual animals included a white area on the kidney, an enlarged lymph node, yellow discolouration of the liver, pancreas and spleen, and soft small testis. In addition, various external brown, red and/or yellow matting were noted.

8/10 animals found dead were observed to have gastrointestinal abnormalities as described for animals euthanized *in extremis*. Additional findings included dark red stomach content, 8 animals were noted with various external brown, red and/or yellow matting, a prolapsed penis, reddened enlarged adrenal gland and clear fluid within the abdominal cavity.

At the scheduled necropsy, single animals were noted with abdominal cavity adhesions of the liver, spleen, intestine and abdominal wall and dilated renal pelvis.

Conclusions

The oral LD_{50} of dodine technical was found to be 851 mg/kg bw with 95% confidence limits of 658-1100 mg/kg bw for male and female rats combined. There were no significant differences between male and female.

The author of the study noted that in other studies conducted with this test material, a high degree of potential primary irritation has been observed. Indications of dosing with a severely irritating material can include abnormal defecation, hair losses in the lower abdominal area, delayed onset and recovery from clinical abnormalities and gastrointestinal abnormalities, especially adhesions noted in the abdominal cavity. Findings noted in this study were considered consistent with findings expected from dosing with a severely irritating test material.

In accordance with the provisions of Commission Directive 2001/59/EC the test substance requires labelling with the risk phrase R22 “Harmful if swallowed”.

The Acute Oral Toxicity of Dodine in Mice

Spanjers, M.Th, Til, H.P. (May 1985) – Report No. V 85.169/250208 (Project No. B 85-0208), performed by TNO, Division for Nutrition and Food Research TNO, P.O. Box 360, 3700 AJ Zeist, Netherlands.

Guidelines and GLP

Although no GLP certificate of compliance was obtained at TNO performing laboratory before 1989, TNO laboratory began to perform studies according to GLP in 1981 with the "Council of Decision on the Mutual Assessment of Data in the Assessment of Chemicals – OECD Principles of GLP", and had supervision by the Dutch government in November 1986 and inspection by the GLP committee in 1987. No guideline is specified (in-house method); the study was primarily meant to be a dose range-finding study for a subsequent mutagenicity study. Main deviations from B.1 method of EU were that 2 dose levels were used (instead of 3).

This study is considered as additional data.

Materials and methods

Test substance: dodine, technical quality, white powder, code no. KG 8507, purity 98%.

Animals: SPF-bred albino mice, (Cpb:SE; Swiss random) from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, NL; body weight of males: 26-31 g, and of females: 26-31 g, the animals were about 6 weeks old at study initiation.

Since no exact value of LD_{50} was needed for the objective of this study, only two dose levels of 250 and 500 mg/kg bw were chosen.

Test material was administered by gavage as a 10.0% (w/v) suspension in propylene glycol to groups of 5 male and 5 female fasted mice in one single dose volume of 2.5 or 5.0 ml/kg bw.

After treatment the mice were observed frequently for signs of intoxication during the 1st 4 hours and thereafter at least once daily throughout an observation period of 14 days. Animals found dead were removed and autopsied, if possible. Body weights were recorded on study days 0, 7 and 14. At the end of the observation period, the surviving mice were killed and examined grossly.

Results

During the first 4 hours after dosing and throughout the remaining of the observation period, no signs of intoxication were observed in either dose levels. No death occurred and all mice looked quite healthy at the end of the observation period.

The individual body weights were normal for mice of this age and strain.

Macroscopic examination did not reveal any treatment-related gross alteration.

Conclusions

From these results, the oral LD_{50} of dodine technical for male and female mice exceeds 500 mg/kg bw.

B.6.2.2 Acute percutaneous toxicity (Annex IIA 5.2.2)

Acute Dermal Toxicity Study of Dodine Technical Material in Albino Rats

Kern, T.G. (February 1999) – Report No. WIL-21131, performed by WIL Research Laboratories, Inc., 1407 George Road, Ashland, OH 44805-9281, USA.

Guidelines and GLP

The study was conducted according to GLP, and OECD Guideline OECD No. 402, OPPTS Guideline 870.1200 and EU method B.3 (1992).

The study is acceptable.

Materials and methods

Test substance: Dodine Technical Material, off-white powder, lot no. 032246 (synthesis batch no. 3044 in Certificate of Analysis), purity 967 g/kg.

Animals: Albino rat, strain Crl:CD®(SD)IGS BR from Charles River Laboratories, Raleigh, NC, body weight from 215 to 295 g, 8-12 weeks old at initiation of dosing.

Test material was moistened with approximately 1.1 ml of deionised water and administered directly to clipped unabraded skin of 5 rats/sex at a dose level of 5000 mg/kg bw. Doses covered approximately 15-19% of total body surface. Upon completion of the 24-hour, occluded exposure, the bandages were removed and the sites were wiped with disposable paper towels moistened with tepid tap water.

The rats were observed at approximately 1, 3 and 4 hours post-application on day 0 and once (for clinical observations) or twice (for mortality) daily thereafter for 14 days. The application sites were examined for erythema, oedema and other dermal findings beginning approximately 30-60 minutes after bandage removal and daily thereafter for 13 days. The areas of application were clipped free of hair to facilitate dermal observations on study days 4, 7, 10 and 14. Body weights were recorded on study days 0 (initiation), 7 and 14 (termination). Upon terminal sacrifice, the major organ systems of the cranial, thoracic and abdominal cavities were examined for all animals.

Results

Mortality: There were no deaths during the study.

Clinical observations: All rats had various discoloured areas due to discharges/excretions [described as wet and/or dried red and/or yellow material around the mouth, nose, eye(s), ventral neck, forelimb(s), hindlimb(s), anogenital and/or urogenital area]. All animals appeared normal by day 11 and throughout the remainder of the study. There were no other clinical findings.

Dermal observations: Severe erythema, very slight to slight oedema, eschar, exfoliation and desquamation were noted on all animals. Focal eschar was noted on five animals and completely subsided by day 11. Dermal findings that persisted through day 14 (study termination) consisted of very slight to severe erythema on two females, desquamation on five animals and eschar and exfoliation on a single female.

Body weights: Two males lost 6 g to 11 g and two females lost 1 g to 7 g of initial (day 0) body weight during the first week of the study. All animals regained and surpassed their initial (day 0) body weights by study termination (day 14).

Necropsy: One female had thickening and scabbing of the application site at the terminal necropsy. There were no other gross necropsy findings observed.

Conclusions

The dermal LD_{50} of dodine technical was found to be greater than 5000 mg/kg bw when administered once for 24 hours to the clipped, unabraded skin of male and female albino rats.

In accordance with the provisions of Commission Directive 2001/59/EC, classification is not required relating to the dermal acute toxicity of dodine.

B.6.2.3 Acute inhalation toxicity (Annex IIA 5.2.3)

Dodine Technical: Acute (Four-Hour) Inhalation Study in Rats

Kenny, T., Fensome, Z. (March 1999) - Report No. RNP 605/992051, performed by Huntingdon Life Sciences Ltd., P.O. Box 2, Huntingdon, Cambridgeshire, PE18 6ES, England. Report issued: March 5th 1999; amended Report issued: May 4th 1999.

Guidelines and GLP

The study was conducted according to GLP, and was designed in compliance with EEC, OECD, US-EPA and J-MAFF test guidelines for acute inhalation studies.

The study is acceptable.

Materials and methods

Test substance: dodine technical, white waxy solid (referred as white powder in Certificate of Analysis), batch no. 032246 (synthesis batch no. 3044 as referred in Certificate of Analysis), purity 967 g/kg.

Animals: albino rats (Sprague-Dawley in origin), from Charles River UK Limited, Manston Road, Margate, Kent, England, approximately 7-8 weeks old on receipt, body weight from 244 to 279 g (males) and 203 to 227 g (females) on day of dosing.

Four groups of 5 rats/sex were exposed for 4 hours, snout-only, to a particulate aerosol generated from the test substance at concentrations of 0 (clean air only), 0.25, 0.34, or 0.51 mg/l of air.

The rats were observed continuously for signs of reaction to the test substance during exposure and at least twice daily throughout the observation period. The clinical signs were recorded at the end of the 5-minute chamber equilibration period, at 0.25, 0.5 and 1.0 hours, then hourly during the remainder of exposure. Clinical signs were recorded immediately post-exposure and then at 1 and 2 hours post-exposure. During the observation period, the clinical signs were recorded once in the morning. The rats were checked for survival later in the day.

All rats were weighed at least twice during the week prior to exposure, immediately prior to exposure (Day 0) and weekly during the observation period. In addition rats exposed to 0 (controls) and 0.51 mg/l were weighed on Day 3, 4, 5, 6, 7, and 14. The daily mean food intakes and water consumption were recorded for each cage (5 rats/cage) during the observation period.

All rats that died as a result of the exposure and all those killed at the end of the 14-day observation period were subjected to a detailed macroscopic examination. The lungs (including the larynx and trachea), liver and kidneys were removed, dissected clear of surrounding tissue, and the weights recorded. The kidneys were weighed together. The tissues were then discarded.

The concentration of the test substance likely to cause death in 50% of exposed rats following a single 4-hour exposure was calculated by the log probit method of Miller and Tainter (1944).

The study was first reported on 5 March 1999. Amendment became necessary when it was found that certain revisions requested by the Sponsor had not been incorporated. The revisions included:

- Clarification of systems airflow to indicate that 15 l/min was the total flow through the chamber to which the animals were attached.
- Addition of % (w/w) of particles <3 µm.
- Addition of statement reporting times of observation of ataxia in female rats exposed to 0.51 mg/l.
- Separate LC_{50} values are reported for each sex.

Results

Chamber atmosphere conditions are summarized in Table 6.11.

Table 6.11 – Chamber atmosphere conditions

Group	Gravimetric analysed (mg/l) (sd)	Nominal concentration† (mg/l)	MMAD (µm) (σg)	% respirable (<7µm)	% respirable (<3µm)	Temperature (°C) (sd)	Relative Humidity (%) (sd)
1	-	-	-	-	-	20.7 (0.43)	55 (6.6)
2	0.25 (0.019)	0.68	3.2 (2.43)	81	46	20.4 (0.17)	46 (9.9)
3	0.34 (0.035)	0.93	3.3 (2.59)	78	45	20.4 (0.46)	33 (12.0)
4	0.51 (0.035)	1.28	3.0 (2.62)	80	49	20.6 (0.42)	29 (9.9)

MMAD Mass median aerodynamic diameter
σg standard geometric deviation
sd standard deviation

† Nominal concentration: $\frac{\text{Canister weight loss during exposure (mg)}}{\text{Total air flow through system during exposure (l)}}$

The analysed concentrations of dodine were approximately 37%, 37% and 40%, of the nominal concentration for Groups 2 to 4 respectively.

The mass median aerodynamic diameter (MMAD) of the airborne dust was 3.2, 3.3 and 3.0 µm for Groups 2 to 4 respectively. Approximately 81, 78, 80% of the particles were less than 7 µm in diameter and considered of a respirable size for Groups 2 to 4 respectively.

Mortality: 1 male of Group 3 was found dead on Day 2 of observation, 3 males and 4 females of Group 4 died post exposure, until Day 5 (see Table 6.12).

Clinical signs: During the exposure, exaggerated breathing was noted in test rats at all exposure levels. During the 14 day post exposure observation period signs noted in a proportion of test rats included whole body cold to touch, pilo-erection, slow breathing rate, gasping, noisy respiration, swollen abdomen, brown staining and/or brown crusty staining around snout and/or jaws, wet and matted fur. Exaggerated breathing, immobility, lethargy and unsteady gait were noted in a smaller proportion of the test rats.

Body weights and food consumption: The rate of bodyweight gain and food consumption by Day 14 of the observation period for the surviving test rats was lower than that of the control rats. Lower water consumption observed on Day 1 in test rats was followed by recovery, so water consumption of test animals was similar to controls after 14 days.

Macroscopic pathology: Severe congestion in all lobes of the lungs and congested areas of the intestines were noted in a proportion of the test rats exposed to 0.51 mg/l. Enlarged heart and grossly distended stomach, caecum and large and small intestines were noted in a proportion of the female test rats exposed to 0.51 mg/l only.

Severe congestion in all lobes of the lungs, grossly distended stomach, caecum and intestines and a large pale area in the left lobe of the liver were noted in one male test rat exposed to 0.34 mg/l. Small dark foci on the left lung were noted in one female test rat exposed to 0.34 mg/l.

There were no macroscopic findings noted in rats exposed to 0.25 mg/l.

Organ weights: Lung weights of decedent rats were greater than that of the control rats. The lung weight of the surviving female rat exposed to 0.51 mg/l was markedly greater than that of control

rats, otherwise the organ weight values for surviving test rats were generally similar to that of the values of the control rats.

Table 6.12 – Mortality data of dodine administered by inhalation to rats

Exposure concentration (mg/l air)	Sex	Number of dead / number of investigated	Time of death (range)	Observations
0.25	male & female	0/10	-	0% mortality
0.34	male	1/5	Day 2	10% mortality in both sexes combined
	female	0/5	-	
0.51	male	3/5	Day 0-1	70% mortality in both sexes combined
	female	4/5	Days 1-5	
LC_{50} value (95% confidence limits)	male	0.47 (0.24 – 0.69) mg/l air		
	female	0.44 (0.29 – 0.59) mg/l air		
	combined	0.45 (0.34 – 0.57) mg/l air		

Conclusions

The estimated LC_{50} (4-hour) for dodine technical was 0.45 mg/l air with 95% confidence limits of 0.34 to 0.57 mg/l air.

In accordance with the provisions of Commission Directive 2001/59/EC the test substance requires labelling with the risk phrase R23 "Toxic by inhalation".

B.6.2.4 Skin irritation (Annex IIA 5.2.4)

Acute Dermal Irritation Study of Dodine Technical Materiel in Albino Rabbits

Kern, T.G. (February 1999) - Report No. WIL-21132, performed by WIL Research Laboratories, Inc., 1407 George Road, Ashland, OH 44805-9281, USA.

Guidelines and GLP

The study was conducted according to GLP, and in compliance with, US-EPA OPPTS Guideline 870.2500, OECD Guidelines No. 404 and EEC Guideline B.4.

The study is acceptable.

Materials and methods

Test substance: dodine technical, off-white powder, lot no. 032246 (synthesis batch no. 3044 in Certificate of Analysis), purity 967 g/kg.

Animals: Young adult albino rabbits, strain New Zealand White, from Covance Research Products, Inc. Denver, PA, USA, body weight from 3451 to 3648 g.

Single 0.5-g doses of the test material, moistened with 0.4 millilitres of deionised water, were applied to an area of approximately 2.5 x 2.5 cm of clipped, unabraded skin of 3 albino rabbits (2 males and 1 female) under occlusive dressings for a 4-hour exposure period. At completion of exposure, the bandages were removed and the sites washed with disposable paper towels moistened with deionised water.

Application sites were evaluated in accordance with the method of Draize at approximately 30-60 minutes and 24, 48 and 72 hours after patch removal and daily through day 14. In order to facilitate

dermal observations, the areas of application were clipped free of hair approximately 2 hours prior to collecting the 72-hour dermal scores and on day 10.

The rabbits were observed twice daily for mortality, body weights were recorded on day 0 (initiation) and at each rabbit's termination from the study.

Results

There were no deaths during the study.

The test material induced slight to moderate erythema and desquamation on all animals. One animal was observed with very slight oedema at 24 hours. The erythema and desquamation persisted though study termination, with the exception of one animal in which both subsided by day 14. There were no other dermal findings (see Table 6.13).

There were no remarkable changes observed in body weights during the study period.

Table 6.13 – Acute dermal irritation of dodine in albino rabbits (individual and average scores)

individual/average scores	time	Erythema	Oedema
individual scores, rabbits Nos. 26320M / 26321M / 26353F Draize scores (0 to maximum 4)	*0.5-1 h*	0/1/1	0/0/0
	24 h	2/1/2	0/0/1
	48 h	2/1/2	0/0/0
	72 h	2/2/2	0/0/0
other times	*Day 5*	3/3/2	0/0/0
	Day 8	2d/2d/1d	0/0/0
	Day 14	1d/0/1d	0/0/0
average score/animal investigated	24h, 48h, 72h	2/1.3/2	0/0/0.3
reversibility: *		n c	c
average time for reversibility		n c after 14 days	24 h

M: male
F: female
d: desquamation
* c : completely reversible
n c : not completely reversible
n : not reversible

Conclusions

Although erythema was not completely reversible at the end of the 14-day observation period, it can be observed that scores tended to decrease with time and it is reasonable to expect that, at the end of a longer period of observation, irritation scores would be completely reversible.

Considering the average scores for the 24, 48 and 72 hours, obtained for each rabbit, 2/3 animals showed positive irritating effects. So according to the provisions of Commission Directive 2001/59/EC the test substance requires labelling with the risk phrase R38: "Irritating to skin".

B.6.2.5 Eye Irritation (Annex IIA 5.2.5)

Acute Eye Irritation Study of Dodine Technical Material in Albino Rabbits

Kern, T.G., (February 1999) – Report No. WIL-21133, performed by WIL Research Laboratories, Inc., 1407 George Road, Ashland, OH 44805-9281, USA.

Guidelines and GLP

The study was conducted according to GLP, and in compliance with, US-EPA OPPTS Guideline 870.2400, OECD Guidelines No. 405 and EEC Guideline B.5.

The study is acceptable.

Materials and methods

Test substance: dodine technical, off-white powder, lot no. 032246 (synthesis batch no. 3044 in Certificate of Analysis), purity 967 g/kg.

Animals: Young adult female albino rabbit, strain New Zealand White, from Covance Research Products, Inc., Denver, PA, USA, body weight 3024 g at initiation of dosing.

A single 47-mg dose (based on the weight equivalent volume of 0.1 ml) of the test material was instilled into the lower conjunctival sac of the right eye of one albino rabbit. Initially, a screen animal was dosed to evaluate the primary ocular irritation potential of the test material. This single rabbit had the test material instilled into the right eye and the eyelid was held closed for approximately one second and released. The left eye was manipulated in a similar manner as the right eye and served as a contralateral control. The control (left) and test (right) eyes were washed out with approximately 60 millilitres of sterile saline for approximately 30 seconds following the 24-hour observation. Due to the severe ocular damage observed, the study was terminated on day 7 and no further animals were used to evaluate the ocular irritation potential of the test material.

The eye was examined for ocular reactions in accordance with the method of Draize at approximately one, 24, 48 and 72 hours after dosing and on days 4 and 7. Sodium fluorescein was used to aid in revealing possible corneal damage at 72 hours and on day 7. Body weights were recorded on study day 0 (initiation) and at study termination (day 7).

Results

The screen rabbit did not vocalize upon instillation of dodine technical. The test material induced severe corneal; iridal and conjunctival irritation that persisted through study termination (day 7, see Table 6.14). Therefore, the test material is considered to be severe eye irritant. Other ocular findings were purulent discharge from the 24-hour observation onward, haemorrhage at the 4- and 7-day observations, and neovascularization at the 7-day observation. Observation with fluorescein at the 72-hour and 7-day showed a 25% of cornea retaining stain.

The left (control) eye was free of evidence of ocular irritation and other findings for the duration of the study.

A slight loss of 21 g (1.0%) of initial body weight was noted, which was not considered as uncommon by the author of the study.

Table 6.14 – Eye irritation of dodine in albino rabbit (individual ocular irritation scores)

	Cornea	Iris	Conjunctiva	
			redness	chemosis
score (1 animal investigated)	0 to 4	0 to 2	0 to 3	0 to4
60 min	0	0	1	4
24 h	4a	2a	2	4
48 h	4a	2a	3	4
72 h	4	2	3	4
Day 7 (termination)	4	2	3	4
average 24h, 48h, 72h	4	2	2.7	4
reversibility *	n	n	n	n
time for reversion	-	-	-	-

a : unable to be determined due to severe chemosis, maximum score applied

* c : completely reversible
n c : not completely reversible
n : not reversible

Conclusions

According to the provisions of Commission Directive 2001/59/EC the test substance requires labelling with the risk phrase R41: "Risk of serious damage to eyes".

B.6.2.6 Skin sensitization (Annex IIA 5.2.6)

Dodine Technical: Skin Sensitization Test in Guinea-Pigs (Maximization Method of Magnusson, B. and Kligman, A.M.)

Manciaux, X., (January 1999) - Report No. 17473 TSG, performed by CIT, Centre International de Toxicologie, Miserey - 27005 Evreux, France.

Guidelines and GLP

The study was conducted according to GLP, and in compliance with OECD Guidelines No. 406 (1992), the maximization method of Magnusson & Kligman, and EC Guideline B.6 (1992).

The study is acceptable.

Materials and methods

Test substance: dodine technical, white powder, batch no. OP9750142 (synthesis batch no. 3044 in Certificate of Analysis), purity 967 g/kg.

Vehicle: the test substance was not soluble in 0.9% NaCl. The vehicle chosen was corn oil: a homogeneous suspension was obtained at the maximum concentration of 40% (w/w). The test substance formulation which could pass through a needle and into the dermis had a maximum concentration of 0.1% (w/w).

Animals: Dunkin-Hartley guinea-pigs, from Charles River France, 76410 Saint-Aubin-les-Elbeuf, France; on day -l, the animals were approximately 3 months old and had body weights ranging from 317 to 358 g (males) and from 316 to 360 g (females).

A preliminary test was conducted to determine the concentrations to be tested in the main study which showed that a concentration of 0.1% was well-tolerated by intradermal route. This application produced signs of irritation at the 24 hours observation and a slight irritation after 48

hours. When applied with Freund's Complete Adjuvant, signs of irritation were present at both observation time. When applied by cutaneous route neither concentration of 20% nor of 40% (w/w) did produce signs of irritant effects at both observation time of 24 and 48 hours.

Main study: Thirty guinea-pigs were allocated to two groups: a control group 1 (5 animals/sex) and a treated group 2 (10 animals/sex).

On day 1, intradermal injections of Freund's complete adjuvant (FCA) mixed with the test substance (treated group) or the vehicle (control group) were performed in the interscapular region.

On day 7, the same, region received a topical application of sodium lauryl sulfate in vaseline (10% w/w) in order to induce local irritation.

On day 8, the test substance (treated group) or the vehicle (control group) was applied to the same test site which was then covered by an occlusive dressing for 48 hours.

On day 22, after a rest period of 12 days, all animals of the treated and control groups were challenged by a cutaneous application of the test substance to the right flank. The left flank served as control and received the vehicle only. Test substance and vehicle were maintained under an occlusive dressing for 24 hours.

Skin reactions were evaluated approximately 24 and 48 hours after removal of the dressing.

Test substance concentrations were as follows:

Induction (treated group)

- intradermal injections: DODINE TECHNICAL at the concentration of 0.1% (w/w) in corn oil;
- topical application: DODINE TECHNICAL at the concentration of 40% (w/w) in corn oil.

Challenge (all groups)

- topical application: DODINE TECHNICAL at the concentration of 40% (w/w) in corn oil.

At the end of the study, animals were killed without examination of internal organs. No skin samples were taken from the challenge application sites.

The sensitivity of the guinea-pigs in CIT experimental conditions was checked with a positive sensitizer, 2,4-dinitrochlorobenzene (DNCB). During the induction period, the reference substance DNCB was applied at the concentrations of 0.1% (w/w) (day 1) and 1% (w/w) (day 8) in corn oil. For the challenge application, the reference substance DNCB was applied at the concentration of 1% (w/w) in corn oil.

Results

No clinical signs and no deaths related to treatment were noted during the study. Body weight gain was normal compared to that of the control animals.

No cutaneous reactions were observed after the challenge application (all scores were zero).

The species and strain which were used showed a satisfactory sensitization response in 90% animals treated with DNCB.

Table 6.15 – Study design and results

	GPMT		Observations/Remarks
	day of treatment	application	
induction 1	1	intradermal injection of FCA + test substance (test)/vehicle (control)	Irritation results available only from preliminary test
pre-treatment to induce local irritation	7	topical application of sodium lauryl sulphate in vaseline (10% w/w)	Irritation results available only from preliminary test
induction 2	8	topical application occluded for 48h	
challenge	22	topical application occluded for 24h	
scoring 1	24		No dermal reaction
scoring 2	25		No dermal reaction

Conclusions

According this Magnusson and Kligman test substance dodine technical does not induce delayed contact hypersensitivity in guinea-pigs.

In accordance with the provisions of Commission directive 2001/59/EC classification is not required.

B.6.3 Short-term toxicity (Annex IIA 5.3)

B.6.3.1 Oral 28-day toxicity (Annex IIA 5.3.1)

B.6.3.1.1 Oral in the Rat

A 4 Week Oral (Gavage) Toxicity Study of Dodecylguanidine Acetate (Dodine) in the Albino Rat

Batham P. (January 14, 1994a) - Project No. 84568, performed by Bio-Research Laboratories Ltd., Senneville, Quebec H9X 3R3, Canada. Dates of Experimental Work: December 4, 1991 to March 20, 1992.

Guidelines and GLP

The study was conducted according to GLP, and in compliance with US EPA FIFRA 40 CFR Part 158 (Subdivision F/82-1).

Deviations were: The high level of deaths among the high dose group (15 animals on 20) required termination of treatment and euthanasia of the remaining 5 animals after 12 days of dosing instead of 28 days.

As a range finding study, the study is acceptable.

Materials and methods

Test substance: dodine technical, fine white powder, APA batch no. 303/90, purity: 94.07% (w/w) was prepared weekly as an aqueous suspension with 0.5% methylcellulose solutions.

Test animals: Sprague-Dawley rats (Crl:CD^{R}(SD)BR) received from Charles River Canada were approximately 28 ± 1 days old. Body weights ranged from 188.6 to 225.5 g (males) and 138.1 to 173.2 g (females) at start of treatment.

Dodine was administered daily (by gavage) to groups of l0 rats/sex at dose levels of 0, 75, 100 or 200 mg/kg bw/day in a dosing volume of 5 ml/kg bw/day. Control group received 0.5% methylcellulose solution alone at the same dose volume as the treated rats. For high dose animals, treatment was terminated and the remaining animals euthanized after the 12th day of dosing due to the high incidence of deaths observed among these animals.

All animals were examined twice daily for mortality and signs of ill health or behavioural changes. In addition, detailed clinical examinations were performed once weekly. Individual body weights and food consumption were measured weekly commencing 1 week prior to treatment start.

Blood was collected from all surviving high dose animals at week 2 of treatment and from the remaining animals from all dose groups at week 4 for evaluation of haematology and clinical chemistry parameters.

All surviving high dose animals were sacrificed after l2 days of treatment and all surviving animals from the remaining dose groups were sacrificed after 4 weeks of treatment. Gross pathological examination was performed for all animals, with assessment of organ weights and histopathological examinations of the liver, kidney, spleen, adrenal, heart, lungs, testes, thyroid lobes and any gross lesions observed macroscopically were performed on Group l and 4 animals regardless of mode of death. Because of findings in the high dose animals, the histopathological examination was extended to stomach, duodenum, jejunum, ileum, cecum and colon of animals of the lower dose groups.

Individual data including body weights, body weight gains, food consumption, haematology, clinical chemistry and organ weights were subjected to calculation of group mean values with standard deviations. The data were analyzed for homogeneity of variance using Bartlett's test. Homogeneous data were analyzed using analysis of variance and the significance of intergroup differences was assessed using Dunnett's "t" test. Heterogeneous data were analyzed using Kruskal-Wallis test and the significance of the intergroup differences was assessed using Dunn's test.

Results:

Stability and homogeneity of test article suspensions assessed prior to commencement of treatment and on weeks 1 and 4 of the study were considered acceptable.

Mortality: Treatment-related deaths were observed throughout the 4-week treatment period with dodine at 100 and 200 mg/kg bw/day (see Table 6.16).

Table 6.16 – Mortality data

Dose group	Dse level (mg/kg bw/day	males		females	
		Mortality	Time of death (days post start of dosing)	Mortality	Time of death (days post start of dosing)
1	0	0/10	-	0/10	-
2	75	0/10	-	1/10	5
3	100	0/10	-	4/10	23, 24, 25, 25
4	200	9/10	6, 6, 7, 7, 9, 9, 9, 12, 12	6/10	4, 4, 4, 6, 7, 12

High incidence of deaths (15/20) was observed among high dose males and females beginning on the 4th day of treatment, which required termination of treatment and euthanasia of the remaining animals (1 male and 4 females) on the 13th day of dosing.

In addition, 4 mid dose females (100 mg/kg bw/day) were found dead during the 4th week of dosing. One low dose female (75 mg/kg bw/day) was also found dead in the first week of dosing. Clinical signs consisted of salivation and respiratory problems. No abnormal findings were noted at the gross pathology examination: The cause of death for this animal remains uncertain.

Clinical signs: Salivation was observed in all treated groups throughout the treatment period. Fewer or smaller faeces were also noted in high dose animals.

Treatment-related increased incidence of deteriorating health status (including reduced activity; hunched posture, pale or partly closed eyes, blue skin tone and decreased body temperature) and of respiratory distress (wheezing; gasping and abnormal sounds) together with firm abdomens, pale and/or soft faeces on occasion mixed with mucoid material and staining of the head and urogenital regions were noted among mid (100 mg/kg/day) and high dose (200 mg/kg/day) animals.

Clinical signs observed for low dose animals (75 mg/kg/day) consisted mainly of respiratory problems (wheezing or abnormal sounds), salivation and/or staining of the fur.

Body weights (see Table 6.17):

A significant effect on growth was noted in the high dose animals (200 mg/kg/day). The overall mean body weight gained noted among these animals for the treatment period was significantly lower when compared to the control group. Significantly lower ($p<0.01$) weekly mean body weights were also noted.

The weight gain of the mid dose animals was also significantly depressed, particularly the males, where weekly body weight and overall mean body weight gain values were significantly reduced when compared to corresponding control values. In the females; although there was a trend of slightly depressed values, statistical significance was noted in week 4 only.

The growth of the low dose animals was slightly affected by the administration of dodine. However, the difference was not statistically significant except in the 4th week of treatment for the males:

Table 6.17 – Group mean body weight gains over 2 (high dose)/4 (low and mid-dose) weeks of treatment

Group	Period of treatment	Mean Body weight Gains (g) ± S.D.	
		males	females
(1) Control, vehicle **	Week 2	55.3 ± 8.08	23.0 ± 4.51
(4) Dodine 200 mg/kg bw/d**		-37.0^{B} ± 5.49	-3.3^{B} ± 20.47
(1) Control, vehicle	Week 4	134.5 ± 18.22	56.4 ± 15.11
(2) Dodine, 75 mg/kg bw/d		107.8^{A} ± 24.49	48.0 ± 15.40
(3) Dodine, 100 mg/kg bw/d		69.8^{B} ± 21.67	38.0 ± 26.35

** : Mean values for Group 1 (vehicle control) obtained on Day 14 of treatment and for Group 4, on Day 12.
Significantly different from control Group 1: A : $P<0.05$; B : $P<0.01$ (Dunnett's)

Food consumption and food efficiency:

Treatment with dodine produced decreased food intake at all dose levels. Weekly values were statistically significant throughout the respective treatment periods for mid (100 mg/kg bw/day) and high (200 mg/kg bw/day) dose animals and low dose females, except in week 3 for mid dose animals and week 2 for low dose females.

For low dose males, the weekly group mean food consumption values were slightly lower than those of controls throughout the treatment period but only attained statistical significance in the first week of dosing (see Table 6.18).

Table 6.18 – Total mean food intakes over 2 (high dose)/4 (low and mid-dose) weeks of treatment

Group	Period of treatment	Total mean food consumption (% of control Group 1 values)	
		males	females
(1) Control, vehicle **	Week 2	423 (-)	293 (-)
(4) Dodine 200 mg/kg bw/d**		153 (36%)	159 (54%)
(1) Control, vehicle	Week 4	887 (-)	613 (-)
(2) Dodine, 75 mg/kg bw/d		792 (89%)	529 (86%)
(3) Dodine, 100 mg/kg bw/d		650 (73%)	509 (83%)

** : Mean values for Group 1 (vehicle control) obtained on Day 14 of treatment and for Group 4, on Day 12.
Significantly different from control Group 1: A : P<0.05; B : P<0.01 (Dunnett's)

Feed efficiency could not be clearly determined for high dose animals throughout the treatment period and for mid dose animals during week 4 due to loss of weight. Reduction in feed efficiency as shown by higher food consumption ratios was noted at group 3 in week 1 males and females, and low dose males in week 4 and low dose females in weeks 1 and 3.

Haematology (see Table 6.19):

High dose animals sacrificed at week 2 of treatment presented haematological values that were essentially within normal range. Exceptions included slightly elevated platelet counts in males and females. Slightly higher white blood cell counts (WBC), segmented neutrophils and haematocrit values and slightly lower lymphocyte counts were also noted for some of these females.

The week 4 data showed similar changes in white blood cell parameters for mid dose males which included significant increases in WBC counts and segmented neutrophils with corresponding decreases in lymphocytes. In addition, significant increases in red blood cell (RBC) parameters were noted which consisted of elevated RBC counts, haemoglobin (Hb), haematocrit (Ht) and red cell distribution width (RDW) for mid dose males and to a lesser degree among mid dose females. Statistical significance was attained for all these parameters among mid dose males but only segmented neutrophils, lymphocytes and RDW for mid dose females and RDW for low dose males were statistically significant.

Clinical chemistry (see Table 6.19):

Administration of dodine at 200 mg/kg bw/day for 12 days produced serum chemistry changes, when compared to normal ranges, which included increases in BUN, total bilirubin, AST, ALT, phosphorus and decreases in glucose, ALP, total protein, albumin, globulin and chloride.

Similar intergroup differences were observed for mid dose animals after 4 weeks of treatment with the exception of the changes noted in phosphorus and chloride levels. Additional changes included decreased calcium level and increased sodium level among mid dose males. Statistical significance was attained for most but not all parameters at week 4. Notably; ALT (> 3x control value), glucose, total protein, albumin, globulin, A/G ratio (females only), calcium and sodium levels were statistically significantly different from control values for mid dose males and/or females. Statistical differences for ALT, total protein, globulin and albumin (females only) levels were also attained among low dose animals.

Some of these changes in the clinical chemistry, primarily the decreases in glucose, total protein level, albumin and globulin were considered to be an indirect effect of the decrease in food intake and body weight gain noted for treated animals.

Table 6.19 – Statistically significant changes in haematology and clinical chemistry parameters of rats treated with dodine for 4 weeks

Parameters	males			females		
	Group 1 control	Group 2 75 mg/kg/d	Group 3 100 mg/kg/d	Group 1 control	Group 2 75 mg/kg/d	Group 3 100 mg/kg/d
Haematology						
WBC ($x10^3$)	11.7	13.9	19.1 $\uparrow^B$	10.8	13.9	15.1
Neut Seg (%)	8.5	6.9	17.3 $\uparrow^A$	8.2	8.5	29.3 $\uparrow^D$
Lymph (%)	89.6	90.2	79.7 $\downarrow^A$	88.3	88.9	66.3 $\downarrow^C$
RBC ($x10^6$)	7.80	7.99	8.46 $\uparrow^B$	7.75	8.02	8.14
Hb (g/dl)	16.0	16.5	17.4 $\uparrow^B$	16.3	16.7	16.6
Hct (%)	45.5	46.6	49.0 $\uparrow^B$	45.1	46.2	46.7
MCHC (g/dl)	35.3	35.4	35.5	36.0	36.0	35.6 $\downarrow^A$
RDW (%)	12.5	13.2	14.0 $\uparrow^B$	12.1	12.5	12.9 $\uparrow^B$
Plt ($x10^3$)	1096.5	1183.8	1135.0	1070.2	1191.0 $\uparrow^A$	1173.2
MPV (Um^3)	8.4	8.2	8.3	8.6	8.3 $\downarrow^A$	8.5
Clinical chemistry						
BUN (mg/dl)	15.0	16.3	18.0	17.0	16.6	20.1
Glucose (mg/dl)	139.9	127.1	102.4 $\downarrow^A$	120.6	114.4	82.7 $\downarrow^B$
AST (U/l)	131.0	119.2	159.6	124.6	127.4	146.7
ALT (U/l)	36.8	54.3 $\uparrow^D$	115.0 $\uparrow^E$	34.6	74.7 $\uparrow^E$	104.7 $\uparrow^D$
Ca^{++} (mg/dl)	9.2	9.2	8.9 $\downarrow^A$	10.1	10.0	10.0
Total protein (g/dl)	6.6	6.2 $\downarrow^A$	5.9 $\downarrow^B$	6.7	6.2 $\downarrow^B$	5.7 $\downarrow^B$
Albumin (g/dl)	3.2	3.1	2.9 $\downarrow^B$	3.3	3.1 $\downarrow^A$	3.0 $\downarrow^B$
A/G ratio	0.92	0.99	1.00	0.96	1.01	1.13 $\uparrow^B$
Globulin (g/dl)	3.4	3.1 $\downarrow^A$	2.9 $\downarrow^B$	3.4	3.1 $\downarrow^B$	2.7 $\downarrow^B$
Na^+ (meq/l)	143.4	142.7	145.0 $\uparrow^A$	142.1	142.8	143.3

↑↓: Significantly different from control (Group 1) value : A : $p<0.05$; B : $p<0.01$ (Dunnett's)
C : $p<0.05$; D : $p<0.01$; E : $p<0.001$ (Dunn's)
WBC – White blood cells; Neut Seg – Neutrophil segmented; Lymph – lymphocytes; RBC – Red blood cells; Hb – Haemoglobin; Hct – Haematocrit; MCHC – Mean corpuscular haemoglobin concentration; RDW - Red cell distribution width; Plt – Platelet count; MPV - Mean platelet volume; BUN – Blood urea nitrogen; AST - Aspartate aminotransferase; ALT – Alanine aminotransferase; Ca^{++} - Calcium; A/G ratio – Albumin/Globulin ratio; Na^+ - Sodium

Organ weights:

Individual organ weight data obtained for high dose animals after 12 days of dosing were recorded, but in the absence of comparative control data, the significance of these values are uncertain and are not reported here.

Several intergroup differences in absolute and relative organ weights were observed among mid dose animals sacrificed at week 4. The significance of the differences noted in absolute organ weights remains uncertain but were thought to be related to the decrease in growth observed in mid dose animals. The apparent differences in relative organ weights were considered to be due to the disparity in body and brain weight seen between treated and control animals. The absolute organ weight data revealed significantly lower liver, lung and brain weight for mid dose males and significantly lower brain weight for low and mid dose females.

Table 6.20 – Statistically significant changes in organ weights of rats treated with dodine for 4 weeks

Organ weighs	males			females		
	Group 1 control	Group 2 75 mg/kg/d	Group 3 100 mg/kg/d	Group 1 control	Group 2 75 mg/kg/d	Group 3 100 mg/kg/d
Absolute (g)						
Terminal bw (g)	346.3	312.3 $\downarrow^{A}$	259.9 $\downarrow^{B}$	203.7	184.8	170.9 $\downarrow^{B}$
Liver	12.336	11.225	9.642 $\downarrow^{B}$	6.727	6.660	7.190
Lungs	1.553	1.463	1.381 $\downarrow^{B}$	1.096	1.067	1.009
Brain	2.070	2.008	1.960 $\downarrow^{B}$	1.878	1.797 $\downarrow^{A}$	1.755 $\downarrow^{B}$
Relative (g%)						
Liver	3.584	3.584	3.699	3.304	3.602	4.202 $\uparrow^{B}$
Lungs	0.454	0.470	0.533 $\uparrow^{B}$	0.541	0.580	0.592
Adrenals	0.018	0.019	0.024 $\uparrow^{A}$	0.030	0.037	0.041 $\uparrow^{B}$
Kidneys	0.864	0.842	0.952	0.852	0.895	0.978 $\uparrow^{B}$
Gonads	0.964	1.101	1.265 $\uparrow^{B}$	-	-	-
Brain	0.609	0.646	0.759 $\uparrow^{B}$	0.932	0.978	1.031

↑↓: Significantly different from control (Group 1) value : A : $p<0.05$; B : $p<0.01$ (Dunnett's)

Gross and histopathological findings:

Treatment with dodine at 200 mg/kg bw/day was associated with macroscopic and microscopic changes in the stomach, spleen, thymus, adrenal glands and intestines.

The histopathological changes observed for high dose animals were extensive and included hyperkeratosis, hyperplasia, erosion and ulceration of the forestomach, atrophy of the white pulp, haemorrhage and lymphoid necrosis in the thymus, haemorrhage in the adrenal gland and vacuolization of the apical villi and/or haemorrhage of the lamina propria in the small and large intestines.

Examination of the gastrointestinal tract of animals treated at 75 and 100 mg/kg bw/day showed oedema, mixed cell infiltration, hyperplasia of the squamous mucosa of the stomach at both dose levels and, hyperkeratosis, erosion and ulceration of the forestomach in animals at 100 mg/kg bw/day.

Conclusions

Treatment with dodine during daily administration (by gavage) for a maximum of 12 days for high dose animals (200 mg/kg bw/day) and for 4 weeks for the remaining dose groups (75 or 100 mg/kg bw/day) was associated with deaths, treatment-related clinical signs and produced changes in body weight, body weight gains, food consumption, feed efficiency and/or clinical pathology at all dose levels and extensive tissue changes at 200 mg/kg bw/day and histopathological changes, in the stomachs of animals dosed at 75 and 100 mg/kg bw/day. It is concluded that the maximum tolerated dose (MTD) was considered to be less than 75 mg/kg bw/day.

No NOAEL could be established.

A 4 Week Oral (Diet) Toxicity Study of Dodecylguanidine Acetate (Dodine) in the Albino Rat

Batham P. (January 14, 1994b) - Project No. 84569, performed by Bio-Research Laboratories Ltd., Senneville, Quebec H9X 3R3, Canada. Dates of Experimental Work: December 4, 1991 to February 24, 1992.

Guidelines and GLP

The study was conducted according to GLP, and in compliance with US EPA FIFRA 40 CFR Part 158 (Subdivision F/82-1), with no significant deviations.

The study is acceptable.

Materials and methods

Test substance: dodine technical, fine white powder, APA batch no. 303/90, purity: 94.07% (w/w).

Test animals: Sprague-Dawley rats (Crl:CD^{R}(SD)BR) received from Charles River Canada were approximately 28 ± 1 day old. Body weights ranged from 181.8 to 227.7 g (males) and 131.0 to 172.8 g (females) at start of treatment.

Dodine was administered in the diet to groups of l0 rats/sex at dose levels of 0 (untreated basal diet), 500, 750 or 1000 ppm. Diets were freshly prepared weekly. The stability and homogeneity of test article was evaluated prior to commencement of treatment, and concentration of the test article in the diet was checked during the feeding period during weeks 1 and 3 of treatment.

All animals were examined twice daily for mortality and clinical signs. In addition, detailed clinical examinations were performed once weekly. Individual body weights and food consumption were measured weekly commencing 1 week prior to treatment start.

During week 4, haematology and clinical chemistry were performed on all surviving animals from each dose groups.

Gross pathology was performed on each animal found dead during the conduct of the study and all rats sacrificed at completion of the treatment period. The following organs were weighed: liver, adrenals, lungs, brain, testes, kidneys, thyroid lobes (and parathyroids). Tissues and organs were fixed and preserved and histopathological examination was performed for Group 1 and 4 animals only on the liver, kidney, spleen, adrenal, heart, lungs, testes, thyroid lobes and any tissues showing gross lesions at necropsy from all animals.

Individual data including body weights, body weight gains; food consumption, haematology, clinical chemistry and organ weights were subjected to calculation of group mean values with standard deviations. The data were analyzed for homogeneity of variance using Bartlett's test. Homogeneous data were analyzed using analysis of variance and the significance of intergroup differences was assessed using Dunnett's “t” test. Heterogeneous data were analyzed using Kruskal-Wallis test.

Results:

Stability and homogeneity of test article were considered acceptable. Diet samples from weeks 1 and 3 were analysed to be within 85% and 115% of nominal claim.

Test article achieved intake: Overall mean average achieved intakes of dodine throughout the treatment period were as follows:

Table 6.21 – Test article achieved intake (mg/kg bw/day)

	Group 2 500 ppm	Group 3 750 ppm	Group 4 1000 ppm
Males	47	71	87
Females	50	72	92

Mortality and clinical signs: There were no deaths related to treatment with dodine at any dose level and no clinical signs were considered to be directly related to treatment.

Body weights: Significantly lower weekly mean body weights and overall mean body weight gains were noted among animals treated with dodine at 750 or 1000 ppm. Lower overall mean body weight gains were noted also for low dose males and females (500 ppm) compared to the controls, 8% and 12 %, respectively.

Table 6.22 – Mean body weight gains (g) over the 4-week treatment period with dodine

	Group 1 Control	Group 2 500 ppm	Group 3 750 ppm	Group 4 1000 ppm
Males	114.6	105.6	97.5 ↓[B]	88.4 ↓[B]
Females	53.3	47.1	46.5	40.7 ↓[A]

↓: Significantly different from control Group 1 – [A] : $p<0.05$; [B] : $p<0.01$

Food consumption: Significantly lower weekly mean food consumption values were seen for mid and high dose animals. Statistical significance was attained for high dose animals throughout the 4-week treatment period for all treatment weeks but week 3 for mid dose animals and on one occasion (week 1) for low dose females (500 ppm).

Table 6.23 – Mean food intake

	Group 1 Control		Group 2 500 ppm		Group 3 750 ppm		Group 4 1000 ppm	
	Total	(%)*	Total	(%)*	Total	(%)*	Total	(%)*
Males	748	-	717	96	685	92	601	80
Females	536	-	504	94	463	86	433	81

*: % of control Group 1 value

Haematology (see Table 6.24): A statistically significant increased mean haemoglobin value was observed for high dose females at week 4. However, it was considered that there were no toxicologically significant differences in the haematology parameters between treated and control animals that could be attributed to treatment with dodine.

Clinical chemistry (see Table 6.24): Treated males and high dose females showed significant decreases in alanine aminotransferase (ALT) levels at week 4. Decreases in aspartate aminotransferase (AST) levels were also noted for treated females. Statistical significance for AST levels was attained only for low (500 ppm) and high (1000 ppm) females. These decreases in AST and ALT were not considered to be of any toxicological relevance.

A statistically significant decrease in glucose levels for high dose males and increases in electrolytes (sodium and/or chloride) for mid and/or high dose animals were noted. These changes were considered to be related to the decreases in food intake noted for these animals.

Table 6.24 – Statistically significant changes in haematology and clinical chemistry parameters of rats treated with dodine for 4 weeks in diet

Parameters	males				females			
	Group 1 control	Group 2 500 ppm	Group 3 750 ppm	Group 4 1000ppm	Group 1 control	Group 2 500 ppm	Group 3 750 ppm	Group 4 1000ppm
Haematology								
Hb (g/dl)	15.9	16.0	15.7	15.8	15.7	15.7	15.7	16.2 ↑[A]
Clinical chemistry								
Glucose (mg/dl)	106.3	112.9	102.8	90.7 ↓[A]	107.3	114.4	104.3	109.1
AST (U/l)	143.2	133.8	116.8	149.2	155.6	112.8 ↓[B]	135.0	121.5 ↓[B]
ALT (U/l)	40.0	32.5	30.7 ↓[B]	32.7 ↓[B]	35.5	30.7	30.7	29.3 ↓[A]
Na^+ (meq/l)	143.3	143.2	144.5	144.9 ↑[A]	140.7	140.0	142.3 ↑[B]	141.9 ↑[A]
Cl^- (meq/l)	100.6	101.7	103.0 ↑[B]	102.3 ↑[A]	101.9	102.8	103.1	103.3

↑↓: Significantly different from control (Group 1) value : A : $p<0.05$; B : $p<0.01$ (Dunnett's)

Organ weights: Kidney weights of males and females treated at 1000 ppm (absolute and relative to brain) were significantly lower than those of the controls. High dose males also revealed significantly lower mean absolute lung weight.

Significantly higher organ weights relative to body weight were noted for mean gonadal weight among treated males, brain weight among high dose animals and lung weight among high dose females. These differences were considered to be related to the disparity in body weights between treated and control animals.

Gross and histopathological findings: The gross and histopathological examinations revealed no macroscopic or microscopic changes in the tissues examined that could be attributed to treatment with dodine up to 1000 ppm.

Conclusions:

According to the author of the study, treatment with dodine during dietary administration for 4 weeks produced limited changes at the 750 and 1000 ppm levels and was considered to be well tolerated at all dose levels, the NOAEL was considered to be 500 ppm.

However, RMS has the opinion that reduced body weight gain of 8 to12% in males and females respectively at the low dose level of 500 ppm (corresponding to 47 and 50 mg/kg bw/day for males and females, respectively), without associated significant reduced food consumption is a marked sign of toxicity, even though no other treatment related effects were noted at this dose level. No NOAEL could be established in this study.

Dodecylguanidine Acetate (Dodine) - 28-day Toxicity Study in the Rat by Dietary Administration

Dange M. (June 3, 1997) - Report No. SA 94448, performed by Rhône-Poulenc Agrochimie, Centre de Recherche, 06903 Sophia Antipolis Cedex, France. Dates of Experimental Work: January 18, 1995 to August 18, 1995.

Guidelines and GLP

The study was conducted according to GLP. As a range-finding study, no specific guidelines were followed, deviations from a standard study were that 2 dose levels were used instead of, at least, 3.

No haematological/blood chemistry were performed. However, taking in consideration this study together with the former 28-day study, also conducted via the diet, the study is considered acceptable.

Materials and methods

Test substance: dodine technical, a white powder, batch no. DA717, purity: 986 g/kg.

Test animals: Sprague-Dawley rats received from Iffa-Credo, L'Arbresle, France were 5 to 6 weeks old on arrival; body weights ranged from 186 to 220 g for males and 146 to 170 g for females at start of treatment.

Dodine was administered continuously in the diet to groups of l0 rats/sex at dose levels of 0 (untreated basal diet), 200 or 800 ppm for 28 days. The stability, homogeneity and concentration of the test article in the diet were verified at both dose levels.

All animals were checked twice daily for mortality, morbidity and clinical signs. Detailed physical examinations were performed at least weekly during the treatment period. Body weights and food consumption were determined weekly. On study Day 29, all surviving animals were sacrificed, all animals, including those found dead and those euthanatized on schedule, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Kidneys and liver were weighed. Samples for histology were taken from stomach, duodenum, jejunum, ileum, cecum, colon, rectum and anus from all animals from all groups. Histological sections prepared from these tissues were examined microscopically.

Statistical analysis: Body weight, body weight gain, food consumption, organ weight, organ to body weight ratio and organ to brain weight ratio were compared for the treated and control groups by use of:

- Bartlett's test for homogeneity of variance between groups;
- If Bartlett's test indicated homogeneous variances, any significant differences were identified by using the combination of analysis of variance (ANOVA) and Dunnett's test.
- If Bartlett's test indicated heterogeneous variances, significant differences were identified by using the combination of the Kruskal-Wallis one-way analysis of variance by ranks followed by the Mann-Whitney's test if the KRUSKAL-WALLIS test was significant.

Results:

The stability (after a three week frozen period and a week in the animal study room), homogeneity and concentrations were within the limits of 100 ± 10% of the nominal concentrations.

Achieved dosages: The mean achieved dose levels expressed in mg/kg bw/day of dodine throughout the treatment period were as follows:

Table 6.25 – Test article achieved intake (mg/kg bw/day)

	Mean achieved dietary intake of dodine (weeks 1- 4)	
	200 ppm	**800 ppm**
Males (mg/kg bw/day)	17.7	67.7
Females (mg/kg bw/day)	19.2	76.7

Mortality and clinical signs: There were no unscheduled mortalities and no treatment-related clinical signs during the study.

Body weights: There was a statistically significant decrease in body weight on Days 8 and 15, in male animals at 800 ppm. Body weight gain was significantly decreased in both sexes at 800 ppm between Days 1 and 8. There was no effect on body weight at 200 ppm.

Food consumption: Food consumption was significantly decreased between Days 1 and 15 in males at 800 ppm. Food consumption was not decreased for males and females at 200 ppm and for females at 800 ppm.

Organ weight: A statistically significant ($p<0.1$) decrease in absolute (-16%) and relative to body (-11%) liver weight was seen in the females exposed to 800 ppm of dodine.

Gross pathology: There were no treatment-related gross changes.

Microscopic pathology: There were no treatment-related histopathological changes in the gastrointestinal tract.

Conclusion:

The dose level of 800 ppm in the diet produced no mortalities and no treatment-related clinical signs, but the body weight of males was significantly decreased on Days 8 and 15, the body weight gain was significantly decreased in both sexes between Days 1 and 8 and food consumption was significantly decreased in males during the first two weeks of the study.

At necropsy, no treatment-related abnormalities were observed. A statistically significant decrease in absolute and relative to body liver weight was observed in females at 800 ppm. The microscopic examination of organs of the gastrointestinal tract revealed no treatment-related changes.

The NOAEL was 200 ppm in both sexes.

Dodecylguanidine Acetate (Dodine) - Assessment of Gut Motility Following Dietary Administration of Dodine in the Rat

Dange M. (July 4, 1996) - Report No. SA 94453, performed by Rhône-Poulenc Agrochimie, Centre de Recherche, 06903 Sophia Antipolis Cedex, France. Dates of Experimental Work: January 18, 1995 to February 24, 1995.

Guidelines and GLP

The study was conducted according to GLP. No specific guidelines are available for the objective of this study, i.e., to assess the gut motility in rats following continuous dietary administration of dodine for 7 and 28 days, so an in-house method was used. The study is considered acceptable.

Materials and methods

Test substance: dodine technical, a white powder, batch no. DA717, purity: 986 g/kg.

Test animals: Sprague-Dawley rats received from Iffa-Credo, L'Arbresle, France were 5 to 6 weeks old on arrival; body weights ranged from 186 to 220 g for males and 146 to 170 g for females at start of treatment.

Dodine was administered continuously in the diet to groups of l0 rats/sex at dose levels of 0 (untreated basal diet), 200 or 800 ppm for 7 and 28 days. This study was performed during the same period of time as the 28-day rat toxicity study by dietary administration reported above (Report SA94448); the same diet mixtures were used for both studies, therefore the analytical report is the same for both studies.

All animals were checked twice (once on week-ends or public holidays) daily for mortality, morbidity and clinical signs. Body weights and food consumption were determined weekly.

One group of 5 animals/sex/dose was sacrificed after 7 days of treatment and the remaining 5 animals/sex/dose were sacrificed after 28 days of treatment. Gut motility was assessed on all animals after an oral administration (gavage) of a charcoal suspension followed by a 2-hour observation period (1 hour for animals sacrificed after 28 days) during which the animals had access to water only. At the end of the observation period, all animals were sacrificed. The abdominal cavity was opened and the gastro-intestinal tract was removed and extended to its full length. The distance from the pyloric sphincter and the proximal and distal traces of the charcoal was measured. In addition, the length of the major portions where the charcoal was located along the gastro-intestinal tract was measured. Also, the intensity of the charcoal in the major portions was visually evaluated (from 1: low intensity to 4: strong intensity).

Results:

On all occasions, results for homogeneity, concentrations and stability were within the acceptable ranges.

Mortality and clinical signs: There were no mortalities and no treatment-related clinical signs.

Body weights: The body weight and body weight gain of male and female animals treated at 800 ppm were slightly inferior to those of controls.

Food consumption: Males treated at 800 ppm had food consumption slightly inferior to that of controls during the first three weeks of the study. Females at 800 ppm had a lower food consumption when compare to control during the first week only.

Gut motility assessment: The location of the charcoal along the gastro-intestinal tract was not modified either after 7 or 28 days of dodine administration at 200 and 800 ppm.

Conclusion:

Normal gut motility was seen following continuous dietary administration of dodine for 7 and 28 days in rats at dose levels of 200 or 800 ppm.

B.6.3.1.2 Oral in Mice

Dodine : 8 Week Dietary Dose Range Finding Study in Mice

Mulhern, M., Perry C.J., Snodgrass E. (August 5, 1988) - Report No. 5411, IRI Project No. 436893, performed by Inveresk Research International, Musselburgh, Scotland, UK.

Guidelines and GLP

The study was conducted according to GLP; as a dose range finding study, an in-house protocol was followed. Main deviations from OECD guideline no. 407 (Repeated dose oral toxicity – rodent: 28-day or 14-day study) included that no haematological or clinical biochemistry were performed

The study is considered as additional information.

Materials and methods

Test substance: dodine technical, APA Batch 92/88/2, purity: 95%.

Test animals: Four groups of 5 males and 5 females CD-1 mice were used in this study, obtained from Charles River (UK) Limited, Margate, Kent, England. On arrival, they were ca 4 weeks of age and weighed 18-21 g.

Dodine was administered via the diet at constant concentrations of 0, 100, 250 and 625 ppm. After 3 weeks dosing the concentration given to the group that had received 100 ppm was increased to 1250 ppm, due to no obvious toxic effects being observed. Viability was checked twice daily. Any

changes in clinical signs were noted. At least once each week every animal was examined and palpated and its detailed health status recorded. Body weights of each animal and food consumption were recorded before the start of treatment and weekly thereafter. Water consumption was monitored by visual inspection throughout the treatment period. All mice were killed after 8 weeks of dosing. The following organs were weighed: heart, lungs, kidneys, spleen and liver; tissues from the same organs and any abnormal tissue were fixed. All tissues fixed were examined from all animals in the Control and 100/1250 ppm dodine dose groups. The livers were re-evaluated in random order (blind) to check for the presence of "eosinophilia" (a change noted in the cytoplasm of the hepatocytes of the liver where it appeared more pink and more evenly stained than in the Control animals.

Statistical Evaluation: Body weight and organ weight data were statistically analysed for homogeneity of variance using the "F-max" test followed by a parametric ANOVA and pairwise comparisons made via Student's t-test using Fisher's F-protected LSD.

Organ weights were also analysed conditional on body weights (i.e. analysis of covariance).

Histopathology data were analysed using Fisher's Exact Probability test.

Results:

Achieved dosage:

Table 6.26 – Test article achieved intake (mg/kg bw/day) in mice

	Group 2 100/1250* ppm		Group 3 250 ppm	Group 4 625 ppm
	Week 1-3	Week 4-8	Week 1-8	Week 1-8
Males	30.3	232.2	49.4	109.4
Females	34	323.6	61.3	150.4

* After 3 weeks of dosing the 100 ppm dose level was increased to 1250 ppm

Mortality: There was one female premature decedent in the 100/1250 ppm dose group, which died one day after increasing the dose level to 1250 ppm. This death was not thought to be attributable to dosing with dodine.

Clinical signs: There were no clinical signs thought to be attributable to dosing with dodine.

Body weights: After increasing the 100 ppm dose level to 1250 ppm at the beginning of Week 4, all animals receiving this concentration of dodine showed an arrest of body weight gain that was evident until the end of Week 7. Over the last week of dosing there was a slight recovery, but there were still notable reductions in overall body weight gain relative to Controls in both sexes (18% in the males, 34% in the females) (see Figures 6.2 and 6.3). There was a reduction (14%) in body weight gain relative to Controls seen in the female mice receiving 250 ppm dodine. This decrease was not considered to be treatment-related because of the lack of a dose response.

Figure 6.2 - 8-week feeding study in mice: mean body weights-males

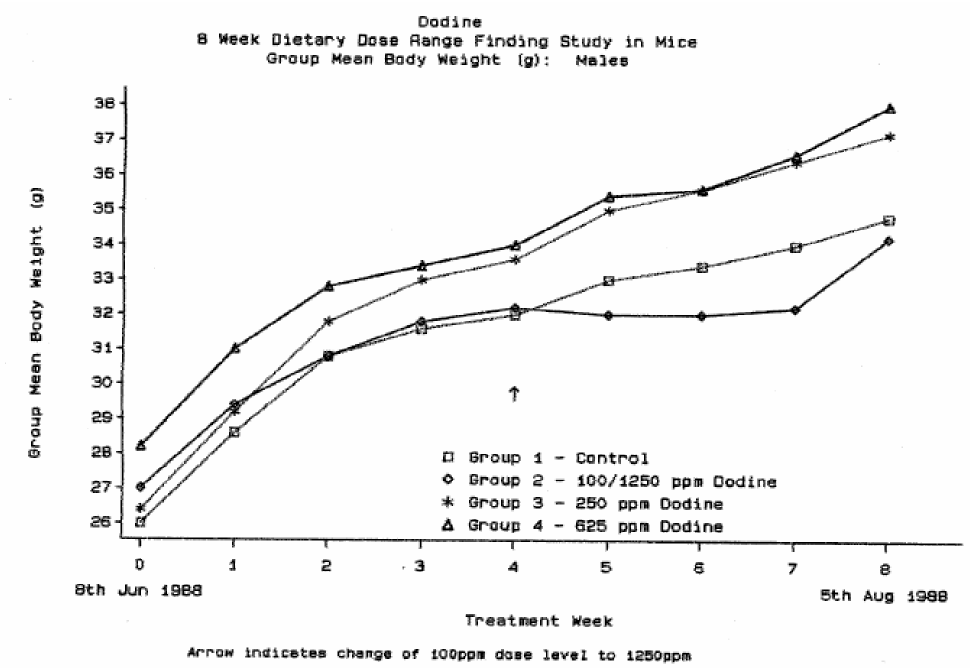
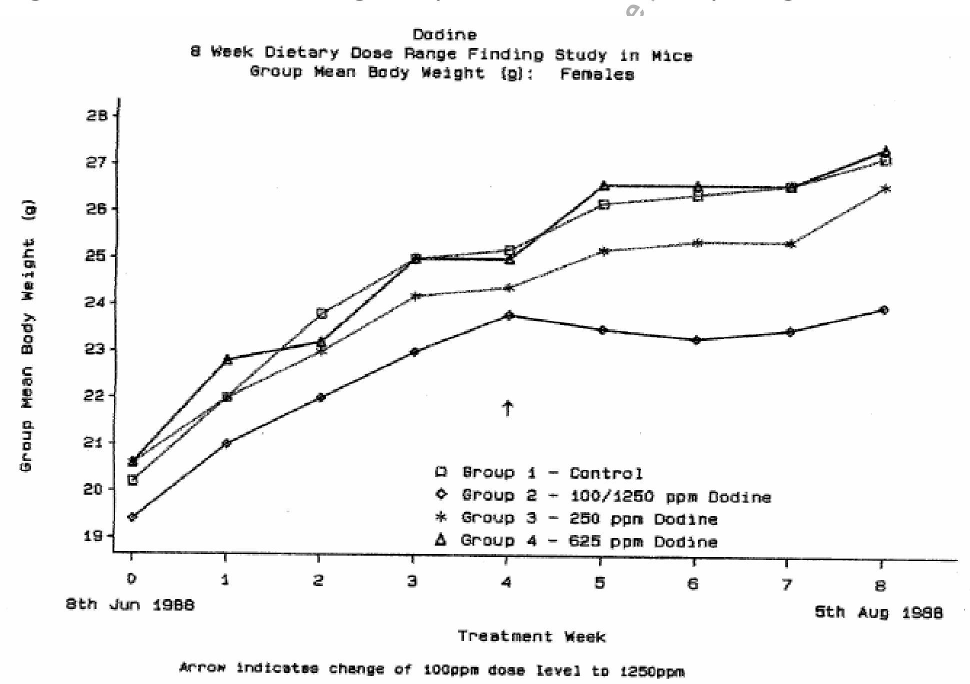


Figure 6.3 - 8-week feeding study in mice: mean body weights-females



Food and water consumption: There were no notable intergroup differences in either sex.

Organ weights: Absolute spleen weight was reduced (30%, P<0.01) in females receiving 100/1250 ppm dodine compared to Controls (see Table 6.27). This statistical significance was no longer evident after correction for final body weight. Therefore it is likely that this reduction was a reflection of the difference in body weight between the 2 groups. There were no notable intergroup differences in males.

Gross Pathology: There were no notable intergroup differences evident in either sex.

Histopathology: The only notable histopathological finding was in the liver. All males and the majority of females (3/5 animals) receiving 100/1250 ppm dodine showed mild eosinophilia (see Table 6.27. This change was considered by the author of the study to be possibly a result of an early enzyme induction, indicative of an adaptive response to metabolism of the test material.

Special stains demonstrated that there were no notable differences in the hepatocyte cytoplasmic contents of fat or glycogen between Controls and animals receiving 100/1250 ppm dodine.

Table 6.27 – 8-week feeding study in mice: absolute organ weights and histopathology (significant findings at week 8)

Parameters	males				females			
	control	250 ppm	625 ppm	100/1250 ppm	control	250 ppm	625 ppm	100/1250 ppm
Absolute spleen weight (g)	5	5	5	5	5	5	5	4**
Eosinophilia in liver	0/5	0/5	0/5	5/5**	0/5	0/5	0/5	3/5

**: Significantly different from control (Group 1) value : $p<0.01$

Conclusion:

Dosing CD-1 mice with dodine at dose levels of up to 1250 ppm for up to 8 weeks produced signs of slight toxicity at the 100/1250 ppm dose level (reduced body weight gain and mild eosinophilia in the liver, in both sexes).

The NOAEL was 625 ppm dose level corresponding to 109.4 and 150.4 mg/kg bw/day for males and females, respectively.

B.6.3.1.3 Oral in Dogs

An Oral Capsule Range-Finding Study of Dodecylguanidine Acetate (Dodine) in the Beagle Dog

Smith, S.Y. (January 14, 1994) - Report No. 84796, performed by Bio-Research Laboratories Ltd., Senneville, Quebec, Canada.

Guidelines and GLP

The study was conducted according to GLP and in house method to determine the dose levels of dodine suitable for use in the subsequent 1 year toxicity study in dogs. Deviations from OECD Guideline 409 (subchronic oral toxicity – non rodent) included that only 2 animals/sex were used instead of at least 4/sex; variations in dose/time of dosing between groups (see Table 6.28), feeding/dosing regime had to be changed from treatment week 3/4 due to in creased incidence of regurgitated capsules that could lead to partial dosing (the incidence of partial doses was low and considered not to have affected the outcome of the study according to the author). During week 3, 2 males of Group 3 were in the wrong cages causing an over and underdosing by 19% respectively (due to differences in dog's body weights) during 4 days. No determinations were made on ornithine decarboxylase, gamma glutamyl transpeptidase, total bilirubin, calcium, phosphorus or chloride in clinical biochemistry. The study is considered as additional information.

Materials and methods

Test substance: dodine technical (dodecylguanidine acetate), fine white powder, APA batch 303/90, purity: 94.07% (w/w).

Test animals: a total of 9 male and 9 female purebred, registered beagle dogs (*Canis familiaris*) were received from Hazleton Research Products, Kalamazoo, MI, 49009,U.S.A. Body weights ranged from 6.5 to 9.0 kg for males and from 6.3 to 8.1 kg for females one day prior to treatment initiation. Dogs were approximately 7 months of age at treatment initiation:

Dodine was administered in gelatin capsules to groups of 2 dogs/sex at dose levels of 12.5/50 mg/kg bw/day (Group 1, dose level was increased effective start of treatment week 2), 25 mg/kg bw/day (Group 2), 6.25/60 mg/kg bw/day (Group 3, dose level was increased effective start of treatment week 4) and 1.25 mg/kg bw/day (Group 4). Capsules were administered orally once daily, 7 days a week, for a minimum of 6 weeks (Groups l and 2) and 5 weeks (Groups 3 and 4). The duration of dosing at each dose level is summarized as follows:

Table 6.28 – Dosing schedule

Dose Level (mg/kg bw/day)	Duration (Weeks)	Study Week	Group No.
1.25	5	1 to 5	4
6.25	3	1 to 3	3
12.5	1	1	1
25	6	1 to 6	2
50	5	2 to 6	1
60	2	4 to 5	3

The stability of the test article when packed into gelatin capsules and stored for 7 days was determined before commencement of treatment. Capsule preparation was monitored and verification of capsule content was performed during the treatment period.

All animals were examined twice daily for mortality and clinical signs. In addition, each animal underwent a general physical examination once weekly. Body weights were recorded individually weekly starting at pre-treatment. Individual food intake over an approximate 4-hour daily feeding period was recorded daily presented weekly. Ophthalmoscopy was performed prior to the start of treatment and during week 3/4. Prior to commencement of treatment and during weeks 3/4 and 5/6, laboratory investigations (haematology and clinical chemistry) were performed on all animals. During week 5/6, urinalysis was performed on all animals.

During the last week of treatment, barium contrast radiography was performed on one dog treated with dodine at 1.25 mg/kg bw/day and one dog treated with dodine at 50 mg/kg bw/day to evaluate the effect of the test article on gastric emptying.

A complete necropsy was conducted immediately on one dog killed during the study and all dogs killed at the end of the treatment period. Selected organs (adrenal glands, ovaries, brain, testes, kidneys, liver, thyroid lobes and parathyroids) were weighted. Selected tissues were preserved and histopathological examinations were performed on the following ones: abnormal tissues, lung, adrenal glands, testes, kidneys, liver, thyroid lobes (and parathyroids).

Results:

Mortality: One male treated with dodine at 12.5 mg/kg bw/day for 7 days and at 50 mg/kg bw/day for 4 weeks was sacrificed due to excessive weight loss and lack of food intake. Clinical signs observed following the increase in dose level to 50 mg/kg bw/day at week 2 included vomiting, excessive salivation, soft/liquid faeces, dehydration, thin, weak body condition, reduced activity

and pale gums. Findings on clinical pathology measurements and gross necropsy were consistent with dehydration and a catabolic condition. Although the animal had poor food consumption, the stomach contained a large amount of undigested food.

Clinical signs: Excessive salivation and vomiting were observed in most dogs treated with dodine at levels of 25 mg/kg bw/day and higher. Vomiting was also noted for females treated at 12.5 mg/kg bw/day during week 1; the dose in these animals was then increased to 50 mg/kg bw/day so the findings for one week are difficult to interpret. Liquid faeces were observed on occasion in dogs treated at 25 mg/kg bw/day and higher, and 1 dog at each 1.25 and 12.5 mg/kg bw/day.

Body weights: Treatment with dodine at 50 or 60 mg/kg bw/day resulted in body weight losses in all animals; 1 dog at the 25 mg/kg bw/day treatment presented weight loss mainly during weeks 5 and 6 of treatment.

Food consumption: Reduced food consumption consistent with body weight loss was also observed in dogs treated with dodine at 50 and 60 mg/kg bw/day and one dog treated at 25 mg/kg bw/day (weeks 5 and 6). Food consumption values were slightly decreased for females only treated with dodine at 12.5 mg/kg bw/day for 1 week.

Ophthalmoscopy: No treatment related findings observed.

Haematology and urinalysis: No treatment-related changes were observed.

Clinical chemistry: At the two highest dose levels and 1 dog treated with 25 mg/kg bw/day, a decrease of total protein values was noted, due to a decrease in albumin and globulin values. Albumin/globulin ratios were within the expected physiological range. Therefore, the observed changes in total protein values were considered to be related to the nutritional status of these dogs. Elevated BUN values were seen in dogs treated with dodine at 25, 50 or 60 mg/kg bw/day. These changes were possibly related to body weight loss.

Barium contrast radiography showed a normal gastric emptying time (clearance of opaque material in 2 hours) for the low dose dog, whereas opaque material was still present after two hours in the stomach of the dog administered 50 mg/kg bw/day, no opaque material was present in the stomach of this dog after 4 hours, although there was evidence of food contents.

Organ weights: No effects on organ weights were observed.

Gross pathology: Gross pathology examinations were performed on dogs treated with dodine at 1.25, 25, 50 or 60 mg/kg bw/day. At necropsy, undigested food was found in the stomach of all dogs treated with dodine at 50 or 60 mg/kg bw/day and one dog at 25 mg/kg bw/day. In these animals dark areas/foci and/or discoloration of the stomach and/or duodenum were observed.

Histopathological evaluation revealed no treatment-related findings.

Conclusion:

The results of this study indicate that treatment of dogs with dodine at 50 or 60 mg/kg bw/day produced adverse effects on body weight and food consumption which necessitated the premature sacrifice of 1 male at 50 mg/kg bw/day. Undigested food in the stomachs of these dogs at necropsy and an abnormal clearance time of contrast material from the stomach of one dog treated at 50 mg/kg bw/day were suggestive of a treatment-related effect of dodine on gastric emptying. These dose levels were therefore not considered appropriate for use on a long-term study. Similar necropsy findings were observed for 1/4 dogs treated at 25 mg/kg bw/day, therefore, the suitability of this dose level for a long-term study is uncertain.

No consistent adverse effects were observed following treatment of dogs with dodine up to 12.5 mg/kg bw/day, although complete evaluation of the effects of treatment at 12.5 mg/kg bw/day for more than 1 week is precluded by the change in dose levels. The dose level of 1.25 mg/kg bw/day was the only dose that could be considered as a NOAEL.

B.6.3.2 Oral 90-day toxicity (Annex IIA 5.3.2)

B.6.3.2.1 Oral 90-day toxicity (rat & mice)

Sub-Chronic (90-day) Oral Toxicity Study with Dodine in Rats

Lina, B.A.R., Til, H.P. *et al.* (June, 1984) - Report No. V83.130/220623, performed by TNO, Division for nutrition and food research tno, 3700 AJ Zeist, Netherlands. Dates of Experimental Work: July 1, 1982 to October 1, 1982

Guidelines and GLP

The study was conducted according to GLP; in-house protocol followed in general recommendations of guidelines (OECD no. 408/EC method B.26). Deviations included that no determinations were made on clotting potential or platelet count in haematology, and ornithine decarboxylase and gamma glutamyl transpeptidase were not determined in clinical biochemistry. The study is acceptable.

Materials and methods

Test substance: dodine technical (dodecylguanidine acetate - DGA), white crystals, Lot no. 196.53, purity: 95%.

Test animals: Four groups of 10 males and 10 females, weanling, SPF-bred rats (Cpb:WU; Wistar random) were used in this study, obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, the Netherlands. On arrival, they were 3.5 weeks of age and weighed 35 to 50 g.

Dodine was administered in the diet to groups of 10 rats/sex at dose levels of 0 (untreated basal diet), 50, 200 or 800 ppm for 13 weeks. In the first week of the study, the top-dose diet contained 400 ppm of the test substance. The dodine content of the top-dose diet was raised to 800 ppm from day 7, because no clear growth depression was observed at 400 ppm. The stability (upon storage at room temperature), homogeneity and concentration of the test article in the diet were verified at each dose levels, immediately after preparation of each batch of diets.

The general condition and behaviour of all animals was checked daily. The eyes of all rats were examined prior to the administration of the test substance and in week 12 of the study. The individual body weights of all rats were recorded initially and then weekly. Food intake was measured per cage (5 animals) weekly and the efficiency of food utilization was calculated and expressed as gram weight gain per gram food consumed. Water consumption was estimated by visual inspection of the water bottles, especially during the first two weeks of the study.

Samples of blood were collected from the tip of the tail of all male rats on day 84 and of all female rats on day 85, and examined for the following parameters: haemoglobin, haematocrit, red blood cells, thrombocytes, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells, differential white blood cell count.

On day 89, blood was collected for glucose determinations; at autopsy, blood samples were collected and the following measurements were made: glucose, total protein, albumin, alkaline phosphatase, GOT (AST), GPT (ALT), bilirubin (total), urea, creatinine, inorganic phosphate, chloride (Cl-), calcium (Ca), potassium (K) and sodium (Na).

On day 89, urine was collected from individual animals during the last 16 hours of the deprivation (of food and water) period; volume and density were determined individually; appearance pH, protein, glucose, occult blood, ketones, urobilinogen, bilirubin and sediment were observed semi-quantitatively in pooled samples (one sample/sex/group).

In week 14, the animals were killed and then examined grossly for pathological changes. The following organs were weighed: adrenals, ovaries, brain, spleen, heart, testes, kidneys, thymus, liver and thyroid with parathyroid. Samples of tissues and organs of all animals were preserved. Detailed microscopic examinations were carried out on all animals of the top-dose and control groups on the following tissues and organs: adrenals, aorta, prostate, axillary lymph nodes, rectum, brain (brain stem, cerebrum and cerebellum), seminal vesicles, caecum, skeletal muscle (thigh), colon, epididymides, small intestines (3 levels), spleen, heart, stomach (cardia, fundus and pylorus), kidneys, liver, lungs, testes, mesenteric lymph nodes, thymus, oesophagus, thyroid with parathyroid, ovaries, trachea, pancreas, urinary bladder and uterus (with cervix). Other organs were preserved but not processed for histopathological examination (pituitary, sciatic nerve, skin (flank), exorbital lachrymal glands, spinal cord, eyes, femur with joint, sternum (with bone marrow), sublingual salivary glands, submaxillary salivary glands, and parotid salivary glands).

Statistical analysis: Data on body weights, organ weights, red blood cells, clinical chemistry and volume and density of the urine were evaluated by one-way analysis of (co-)variance, followed by Dunnett's multiple comparison test. Data on food intake and food efficiency were evaluated by analysis of variance followed by the L.S.D. test. Total and differential white blood cell counts were analysed by the Mann-Whitney U-test. The histopathological findings were examined by the Fisher exact probability test.

Results:

The stability, homogeneity and concentrations of test article in the diet were satisfactory.

Achieved dosages: The mean achieved dose levels expressed in mg/kg bw/day of dodine throughout the treatment period were as follows:

Table 6.29 – Test article achieved intake (mg/kg bw/day)

	Mean achieved dietary intake of dodine		
	50 ppm	**200 ppm**	**800 ppm**
Males (mg/kg bw/day)	3.6	14.1	55.8
Females (mg/kg bw/day)	3.9	14.9	60.4

Mortality and clinical signs: No death or abnormalities of condition or behaviour were observed in any of the groups.

Ophthalmoscopic examination did not reveal any treatment-related change.

Body weights: Mean body weights were slightly decreased in the top-dose group in both sexes throughout the study (see Table 6.30); the differences with the controls were statistically significant at several weightings. Overall (Days 0-91), body weight gain was decreased 10% and 11% in the high-dose males and females, respectively.

Food consumption: Food intake was diminished in the top-dose group in both sexes and was relatively low in females of the mid-dose group. The differences with the controls were statistically significant throughout the study in females of the top-dose group only. Food conversion efficiency was slightly decreased in the top-dose group in both sexes in the first two weeks of the study.

Water intake: Visual inspection of the water bottles showed no notable differences in water intake between the test groups and the controls.

Haematology: A statistically significant decrease in mean corpuscular haemoglobin concentration in males of the top-dose group was not accompanied by significant changes in haemoglobin concentration, packed cell volume or red blood cell count. Therefore, no toxicological significance

was attached to this finding. The increased number of neutrophils, accompanied by a shift in the lymphocyte/neutrophil ratio in males of the top-dose group, might be attributed to the feeding of dodine (see Table 6.30).

Clinical biochemistry: The slight decrease in plasma calcium levels in males of all dose groups showed no dose-response relationship. Since, moreover, all values were within the normal range, this finding is considered to be of no toxicological significance. In females, plasma alanine aminotransferase (glutamic-pyruvic transaminase) activity was statistically significantly decreased in the top-dose group.

Urinalysis: There were no changes of any significance in the composition of the urine with respect to appearance, pH, protein, glucose, ketones, occult blood, urobilinogen, bilirubin or microscopy of the sediment.

Organ weight: In males, statistically significant increases were seen in the relative weight of the heart and testes in the top-dose group. The increase in the relative heart weight was slight and within the range of historical control values; the increase in relative weight of the testes was not considered a toxic effect, but rather the reflection of the lower body weights.

The relative weight of the kidneys showed slight, and not dose-related, increases in males of the mid- and top-dose group. The absolute weight of the kidneys was slightly decreased in females of the top-dose group (Table 6.30). The increase in the relative weight of the kidneys in males of the mid- and top-dose group was not considered to be of toxicological importance, because:

- this finding was not accompanied by significant microscopical changes,
- there was no distinct dose-response relationship,
- the changes were only slight and, moreover, the absolute weight of the kidneys was comparable in males of all dose groups,
- none of the urinary parameters measured showed any significant change.

Gross pathology: There were no treatment-related gross changes.

Microscopic pathology: Upon microscopical examination no histopathological changes were observed that were considered to be treatment-related. The increased heart and kidney weights were not accompanied by an increase in number or severity of any morphological lesion.

The abnormalities observed in the various organs are common findings in the strain of rats used. Moreover, they were about equally distributed among the control and top-dose group, or they occurred only in a single animal. Therefore, no toxicological significance was attached to these minor lesions.

Table 6.30 – Body weights, food intake, haematology, clinical chemistry and organ weights findings in rats treated with dodine for 13 weeks in diet

Parameters	males				females			
	Group 1 control	Group 2 50 ppm	Group 3 200 ppm	Group 4 800ppm	Group 1 control	Group 2 50 ppm	Group 3 200 ppm	Group 4 800ppm
Mean body weights (g)	273.1	263.0	267.8	251.5	170.6	169.5	168.3	157.5
Mean food intake (g/rat/week)	131.6	132.2	131.7	122.7	93.7	91.9	88.0	83.3
Haematology								
MCHC (mmol/l)	20.6	20.5	20.1	19.9 ↓*	20.4	20.4	20.2	20.2
Neutrophils (%)	8.3	8.8	8.1	13.9 ↑*	9.3	8.8	9.2	10.5
Lymphocytes (%)	90.7	89.7	90.4	84.8 ↓*	89.4	90.0	89.5	88.7
Clinical chemistry								
Ca^{+} (mmol/l)	2.82	2.65 ↓*	2.61 ↓**	2.62 ↓**	2.70	2.72	2.74	2.73
ALT (U/l) -GPT	31.4	32.2	34.0	32.1	30.3	30.7	29.7	24.1 ↓**
Mean organ weights								
Absolute kidneys weights (g)	2.13	2.15	2.24	2.13	1.42	1.35	1.40	1.27 ↓*
Relative kidneys weights (g/kg)	5.65	5.80	6.13 ↑**	6.11 ↑*	6.59	6.29	6.62	6.40
Relative heart weights (g/kg)	3.00	2.98	3.02	3.27 ↑*	3.70	3.55	3.63	3.72
Relative testes weights (g/kg)	8.55	5.44	8.87	9.64 ↑*	-	-	-	-

↑↓: Significantly different from control (Group 1) value : * : $p<0.05$; ** : $p<0.01$

Conclusion:

The slight growth retardation observed in the top-dose group might be attributed to the decrease in food consumption, possibly as a result of unpalatability of the top-dose diet. However, a toxic action of the test substance cannot be entirely excluded.

The NOAEL was 200 ppm (14.1 and 14.9 mg/kg bw/day in males and females, respectively), based on decreased body weight and body weight gain in both sexes and decreased food consumption in females.

A 13-Week Dietary Toxicity Study of Dodecylguanidine Acetate (Dodine) in the Albino Mouse

Kangas, L. (January 14, 1994) - Report No. 84582, performed by Bio-Research Laboratories, Ltd., Senneville, Quebec, Canada.

Guidelines and GLP

The study was conducted according to GLP and to EPA Guidelines for Toxicity Testing (40 CFR Part 158 and Subdivision F). Deviations from OECD guideline 408 or its correspondent EU guideline B.26 included that no determinations were made on ornithine decarboxylase and gamma glutamyl transpeptidase were not determined in clinical biochemistry.

The study is acceptable.

Materials and methods

Test substance: dodine technical (dodecylguanidine acetate), fine white powder, APA Batch No. 303/90, purity: 94.07% (w/w).

Test animals: Six groups of 10 males and 10 females Swiss CRl:CD®-1 (ICR)BR mice (28 days old) were received from Charles River Canada; St. Constant, Quebec, Canada. The weight ranges were 23.2 to 30.1 g for males and 17.3 to 23.9 g for females one day prior to treatment initiation.

Dodine was administered in the diet to groups of 10 rats/sex at dose levels of 0 (untreated basal diet), 150, 300, 600, 1250 or 2500 ppm for 13 weeks. The stability (upon storage at room temperature for 7 and 14 days), homogeneity and concentration of the test article in the diet preparation for administration in weeks 1, 6 and 13 were verified. Fresh diets were prepared weekly and stored protected from light in labelled air-tight plastic containers until the period of use.

Prior to assignment to dosage groups, 10 male and 10 female mice were randomly selected from the total population and subjected to necropsy for health screen purposes.

During the pre-treatment and treatment periods, each animal was examined twice daily for mortality and for general health status and/or for signs of reaction to treatment; in addition, detailed clinical observations (involving the removal of each animal from its cage) was performed once weekly. Body weights and food consumption were recorded weekly commencing 1 week prior to treatment initiation. Funduscopic (indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations were performed on all animals during the pre-treatment period and on all surviving animals during week 13 of treatment.

At study termination, laboratory investigations (haematology and clinical chemistry) were performed, where possible, on each surviving animal.

For each animal found dead; sacrificed in extremis or sacrificed at study termination, necropsy consisted of an external examination including identification of all clinically recorded lesions as well as a detailed internal examination (including retention of tissues). For each animal sacrificed at termination, a fasted body weight was obtained and the following organs were dissected free of fat and weighed: adrenals, kidneys, pituitary, brain, liver, spleen, heart, ovaries and testes. Selected tissues were preserved. For each animal sacrificed, femoral bone marrow smears were prepared and stained but were not examined. Histopathological examination was performed on all selected tissues for animals found dead or sacrificed in extremis during the treatment period, and for all animals in the control (Group 1) and high dose (Group 6) groups. Furthermore, histopathological examination was also performed on all gross lesions observed in animals from all dose groups.

Statistical analysis: Individual data including body weights, body weight gains, food consumption, haematology, clinical chemistry and organ weights were subjected to calculation of group mean values with standard deviations. The data were analyzed for homogeneity of variances using Bartlett's test. Homogeneous data were analyzed using analysis of variance and the significance of intergroup differences was assessed using Dunnett's 't' test. Heterogeneous data were analyzed using Kruskal-Wallis test and the significance of intergroup differences was assessed using Dunn's test.

Results:

Analyses of the dosed feed during week 1, 6 and 13 revealed that the test article concentrations in the dosed feed were acceptable as they were ± 15% of the nominal concentrations.

Overall mean average achieved intakes of dodine throughout the treatment period were as follows:

Table 6.31 – Test article achieved intake (mg/kg bw/day)

	Mean achieved dietary intake of dodine				
	150 ppm	**300 ppm**	**600 ppm**	**1250 ppm**	**2500 ppm**
Males (mg/kg bw/day)	24	48	94	181	350
Females (mg/kg bw/day)	31	60	116	223	305

Mortality: Treatment-related deaths were observed in the high-dose female group (4/10) during the first 2 weeks of treatment. A generalized deterioration in body condition was noted prior to death consisting mainly of a reduction in activity, respiratory rate and body temperature, tremors, generalized paleness or bluish skin coloration, partly closed eyes, dehydration, weakness and stiffening of the tail. Gross pathological examination did not reveal any abnormal findings except in 1 female where dilatation of the urinary bladder and a small spleen were noted. Significant histopathological findings consisted of lymphoid atrophy of the spleen and/or lymphoid atrophy and/or necrosis of the thymus. These findings were probably stress-related rather than treatment-related based on the condition of the animals prior to death.

Clinical signs: An abnormal clinical sign observed only in females receiving 2500 ppm and considered to be related to treatment was an apparent-stiffening of the tail.

Body weights: Significantly reduced growth was noted for animals treated with 2500 ppm of dodine. This was reflected in the weekly mean body weights which were significantly lower than those of the control animals throughout the treatment period for the males and during weeks 1 and 2 and weeks 6 to 10 inclusive for females. The mean body weight gains for animals treated at 1250 ppm were slightly lower than the corresponding control animals, however, the difference (12% and 7% for males and females respectively) was not statistically significant.

Table 6.32 – Total mean body weight gain (g) over 13-week treatment period in mice

	0 (control)	**150 ppm**	**300 ppm**	**600 ppm**	**1250 ppm**	**2500 ppm**
Males	8.77	8.15 (93%)	8.65 (99%)	9.03 (103%)	7.76 (88%)	2.85 ^{B} (32%)
Females	6.03	6.17 (102%)	6.61 (110%)	7.02 (116%)	5.62 (93%)	3.40 ^{A} (56%)

Significantly different from control (Group 1) value : ^{A} : $p<0.05$; ^{B} : $p<0.01$
( ) percent of control value

Food consumption: Lower mean food consumption values were noted for high dose animals receiving 2500 ppm throughout treatment and for animals receiving 1250 ppm during the first weeks of treatment. The values were intermittently statistically significant when compared to the control values for high dose animals. The most significant effect was seen in high dose females, where mean total food consumption was approximately half that of the control value.

Table 6.33 – Total mean food intake (g) calculated from the group mean values for the 13-week treatment period in mice

	0 (control)	**150 ppm**	**300 ppm**	**600 ppm**	**1250 ppm**	**2500 ppm**
Males	472.2	465.5 (99%)	478.2 (101%)	466.8 (99%)	419.5 (89%)	330.3 (70%)
Females	460.3	482.1 (105%)	457.4 (99%)	458.0 (100%)	404.6 (88%)	250.5 (54%)

( ) percent of control value

The food consumption ratios were variable across all groups. There were no trends to indicate a consistent impairment in feed efficiency.

Ophthalmoscopy: There were no ocular changes considered to be related to treatment with dodine.

Haematology: The only treatment-related changes observed in haematological parameters was a significant increase in mean segmented neutrophil values and a decrease in mean eosinophil values in the Group 6 (2500 ppm) males when compared to control values.

Clinical chemistry: Significantly increased levels of BUN were observed in animals which received 2500 ppm of dodine in the diet when compared with controls. Slight but not statistically significant increased levels of total bilirubin and AST were noted in some of the males which received dodine at 2500 ppm. These changes were most likely related to the nutritional status of the animals. Slightly increased levels of AST were noted in some of the females in groups 2 (150 ppm), 5 (1250 ppm) and 6 (2500 ppm) when compared to control values. Histopathological examination of the kidney and liver did not reveal any abnormal findings therefore these changes were not considered to be of toxicological significance.

Table 6.34 – Haematology and clinical chemistry analysis in mice

	0 (control)	150 ppm	300 ppm	600 ppm	1250 ppm	2500 ppm
Males						
Neut Seg (%)	28.2	24.4	20.7	16.4 ↓ [A]	20.8	40.9 ↑ [A]
Eosinophil (%)	1.6	2.1	2.6	2.0	1.7	0.1
BUN (mg/dl)	28.0	25.3	28.7	23.6	27.6	36.7 ↑ [C]
Total bilirubin (mg/dl)	0.13	0.15	0.15	0.14	0.12	0.17
AST (U/l)	105.6	123.8	115.6	79.8	107.3	252.7
Females						
BUN (mg/dl)	23.4	31.9	28.3	21.5	33.5	61.5 ↑ [D]
AST (U/l)	123.4	315.1	151.7	119.4	168.9	223.5

↑↓ : Significantly different from control (Group 1) value :
[A] : $p<0.05$; [B] : $p<0.01$ (Dunnett's)
[C] : $p<0.05$; [D] : $p<0.01$ (Dunn's)

Organ weights: In Group 6 males significantly lower absolute spleen, kidneys, brain and pituitary weights were noted when compared to control values. Significantly higher relative to body weight liver, heart, adrenals, testes and brain weights were observed. These differences were most likely due to the lower body weights of these animals and therefore were not directly related to treatment with dodine.

In females, significantly lower absolute spleen weights and higher kidney weights and significantly higher liver and kidney weights (relative to body weight and brain weight) and significantly lower spleen weights (relative to body weight) were observed in Group 6. Significantly higher relative (to body weight) kidney weights were also observed in Group 5. These changes were not considered to be biologically significant since no histopathological changes were noted in any of the organs described above.

Table 6.35 – Organ weights (absolute and relative to body weight: significant findings

	0 (control)	150 ppm	300 ppm	600 ppm	1250 ppm	2500 ppm
Males						
Total bw	31.3	29.9	30.3	30.6	29.6	24.4 [B]
Absolute spleen	0.084	0.088	0.075	0.076	0.071	0.061 [B]
Abs. kidneys	0.518	0.488	0.515	0.472	0.485	0.424 [B]
Abs. brain	0.513	0.521	0.510	0.494	0.486	0.468 [B]
Abs. pituitary	0.003	0.002	0.002	0.002 [B]	0.002 [B]	0.002 [B]
Relative liver	4.230	4.246	4.251	4.319	4.436	5.080 [B]
Rel. heart	0.613	0.636	0.654	0.607	0.648	0.706 [A]
Rel. adrenals	0.015	0.015	0.016	0.013	0.015	0.020 [B]
Rel. testes	0.817	0.867	0.818	0.849	0.827	1.000 [B]
Rel. brain	1.650	1.755	1.694	1.619	1.651	1.923 [B]
Females						
Abs. spleen	0.083	0.080	0.076	0.078	0.064	0.056 [A]
Abs. kidneys	0.350	0.340	0.347	0.362	0.373	0.407 [B]
Rel. liver	4.489	4.502	4.412	4.630	4.757	5.493 [B]
Rel. spleen	0.370	0.361	0.336	0.325	0.295	0.262 [A]
Rel. kidneys	1.557	1.541	1.543	1.539	1.728 [C]	1.928 [D]

Significantly different from control (Group 1) value :
[A] : $p<0.05$; [B] : $p<0.01$ (Dunnett's)
[C] : $p<0.05$; [D] : $p<0.01$ (Dunn's)

Gross and histopathological findings: There were no gross or histopathological findings which could be attributed to treatment with dodine.

Conclusion:

Treatment with dodine during dietary administration for 13 weeks at a concentration of 2500 ppm caused decreased body weight and food consumption, neutrophilia and eosinopenia in males and resulted in 4 deaths in females.

Treatment at 1250 ppm resulted in reduced food intake and lower weight gains of 12% and 7% in males and females respectively. No treatment-related effects were noted at dietary levels of 150, 300 or 600 ppm. The NOAEL was the dose level of 600 ppm corresponding to 94 and 116 mg/kg bw/day in males and females respectively.

B.6.3.2.2 Oral 1-year toxicity (dog)

52-Week Toxicity Study in Dogs with Dodine

Trutter, J.A. (December 9, 1996) - Report No. CHV 656-192, performed by Corning Hazleton Inc., Vienna, Virginia, USA

Guidelines and GLP

The study was conducted according to GLP and US EPA FIFRA 40 CFR Part 158 Guideline Subdivision F, no. 83-1. Deviations from OECD guideline 452/E.U. method B.30 included that: no haematological and urinalysis examinations were performed after 3 months treatment with dodine, in clinical biochemistry, no determinations were made on alkaline phosphatase activity, gamma glutamyl transpeptidase, or ornithine decarboxylase. The study is acceptable.

Materials and methods

Test substance: dodine technical (dodecylguanidine acetate), white powder, batch No. 1174, purity: 98.6% (w/w).

Test animals: a total of 16 male and 16 female purebred beagle dogs were received from HRP, Inc.; Cumberland, Virginia, USA. At initiation of dosing, dogs were approximately 6 months of age and body weights ranged from 6.2 to 9.4 kg for males and from 5.4 to 8.8 kg for females.

Dodine was administered in gelatin capsules to groups of 4 dogs/sex at dose levels of 0, 2, 10 or 20 mg/kg bw/day. Capsules were administered orally once daily, 7 days a week, for at least 52 weeks (dogs dosed for 367 or 368 days). The control dogs received a single empty gelatin capsule on a daily basis according to the same schedule. Treatment continued until the day prior to necropsy. Stability of dodine, prepared in a ½ ounce capsule and stored at room temperature, was determined at Days 0 and 10 for representative low- and high-dose capsule preparations. Capsules were prepared at least once weekly, with the daily amount of test material for each animal based on the most recently recorded body weight times the dose level.

All dogs were observed twice daily for mortality and moribundity, and once daily for clinical signs. Detailed clinical observations were conducted weekly. As well, cageside observations were performed approximately 1 to 2 hours after dosing during weeks 3-11 of the study to evaluate the timing of occurrences of emesis and ensure absorption of dodine. These observations were terminated following review of findings which indicated that adequate absorption of dodine was occurring. Body weights and food consumption were recorded weekly, ophthalmoscopic examinations were performed once prior to treatment and during Week 52. Clinical pathology tests (haematology, serum chemistry and urinalysis) were performed once prior to treatment (Week -2) and during Weeks 26 and 52. After 52 weeks of treatment, full necropsies were performed on all animals. Brain with brainstem, ovaries, kidneys, testes with epididymides, liver/drained gallbladder were weighted and organ-to-terminal-body-weight and organ-to-brain-weight ratios were calculated. All organs and tissues were preserved and examined microscopically.

Statistical analysis: Mean body weight, body weight gain, food consumption, clinical pathology data (except cellular morphology gradings and routine urinalysis data), and organ weight data of the treated groups were compared statistically to the data from the same sex of the control group. If variances of untransformed data were heterogeneous, a rank transformation of the data was performed to achieve variance homogeneity. If the transformation did not achieve variance homogeneity, the analyses was still performed on the rank-transformed data. Analysis of Variance, Bartlett's Test, Dunnett's t-Test for Control vs. Treatment Comparisons, and Levene's Test were used for clinical pathology/pathology.

Results:

Results of stability analyses indicated that dodine was stable in a gelatin capsule for up to 10 days at room temperature, indicating that the test material was stable throughout the dosing period.

Mortality: All animals survived until the terminal sacrifice.

Clinical observations: There were no clinical signs in the dodine-treated groups indicative of toxicity. Remarkable clinical differences from control were few in the test group animals and principally consisted of a high incidence of slight to severe salivation in the Group 3 and 4 dogs. Salivation, seen most often prior to dosing, was a persistent finding during the course of the study and was first noted in the Group 3 males during Week 10, Group 3 females and Group 4 males during Week 3, and Group 4 females during Week 2. Salivation generally was more frequent and pronounced in the Group 3 and 4 females compared to males in these groups. The observed salivation was considered to be a conditioned reflex and not an indication of toxicity.

Occasional test group animals showed higher frequencies of various other findings (soft faeces and emesis) when compared to incidences seen in individual control animals. However, individual frequencies for the other dogs in these groups were within the range of, or generally similar to, the incidences seen among the control dogs. Thus, no meaningful differences from control were evident.

Body weights: Mean body weight and body weight change values of the test groups were generally similar when compared to the concurrent control values. No statistically significant differences from control were found in mean body weights at any interval or in the total body weight gains of the test groups.

Body weights (and food consumption) were monitored closely throughout the study due to anticipated problems in adjustment of the test dogs to the capsule dosing of dodine. There were 3 dogs (1 female of Group 3, and 1 male and 1 female of Group 4) that exhibited notably marked body weight losses starting during the first few weeks of compound administration that prompted supplemental feeding (with basal diet mixed with water and/or canned dog food) of these animals to preclude mortality. Two of the three dogs were successfully returned to basal diet by Week 8 or 15 and the third (the 20 mg/kg bw/day female) was maintained on supplemental feeding throughout the majority of the study, continuing through study termination. In other animals, body weight fluctuations did not clearly demonstrate any apparent difficulty in adaptation to dosing and were considered to reflect normal variability for this parameter.

Table 6.36 – 52-week toxicity study in dog: mean body weight and body weight change

Week	Dose Level (mg/kg bw/day)							
	Males				females			
	0	2	10	20	0	2	10	20
1	7.3	7.8	7.9	9.0	7.4	7.3	7.3	7.3
4	7.9	8.0	8.4	8.9	7.5	7.4	7.3	7.2
13	9.0	8.8	9.2	9.9	8.0	7.9	8.0	7.8
26	9.3	9.1	9.2	10.0	8.3	8.1	7.8	8.1
52	9.5	9.5	9.5	10.5	8.7	8.3	8.4	8.5
Weight change - week 1-13 (% of control)	1.7	1.0 (59)	1.3 (76)	0.9 (53)	0.6	0.6 (100)	0.7 (117)	0.5 (83)
Weight change - week 1-53 (% of control)	2.5	1.7 (68)	1.4 (56)	1.2 (48)	1.2	0.8 (67)	1.0 (83)	1.1 (92)

Food consumption: Besides the previous notes on supplemental feeding, mean food consumption of the test groups was generally similar to concurrent control values. Only sporadic instances of statistical differences from control were found in mean weekly food consumption values. These statistical differences were considered incidental and unrelated to compound administration. Mean food efficiency values were generally similar among the groups.

Ophthalmology: Dogs examined prior to initiation of dosing that exhibited ophthalmoscopic lesions were excluded from study consideration. At week 52, dog's eyes were normal or had variation considered as normal findings.

Haematology: Mean haematology values of the treated groups were generally similar when compared to concurrent control values. The mean total leukocyte count and the mean segmented neutrophil count were higher in the Group 4 females at Weeks 26 and/or 52, when compared to the concurrent control value, primarily due to two females. There was no evidence of inflammation in

the clinical observation, gross pathology, or remaining clinical pathology data. All findings were considered incidental to administration of the test material.

Serum chemistry: Mean serum chemistry values of the treated groups were generally similar when compared to the concurrent control values. The mean aspartate aminotransferase value of the Group 4 males at Week 26 was significantly increased, but this finding was not attributed to compound administration due to the low magnitude and lack of biologic importance of the change and the fact that the mean and all individual values were within reference ranges for age- and sex-matched beagle dogs at this laboratory. Individual alanine aminotransferase activities for 1 Group 3 male and 2 Group 4 males at Week 26 and/or 52 were above the reference ranges established for age-matched male beagle dogs at this laboratory. However, there were too few affected dogs and no histomorphologic correlate to allow a definitive association with treatment.

Urinalysis: The urinalysis findings were generally unremarkable and comparable between the groups with the exception that slightly higher incidences were observed for epithelial cells in the Group 4 females and for sperm in the Group 4 males at Week 26. Specific gravity readings were also somewhat higher in the Group 4 females at Week 26. These findings are mild and of little biologic importance.

Gross pathology: There were no findings at necropsy that were attributed to administration of the test compound.

Organ Weights: Mean organ weight values for the treated groups were generally similar when compared to the concurrent control group. No statistically significant differences from control or test article organ weight variations were evident.

Histopathology: There were no microscopic findings attributed to test article administration.

Conclusion:

Three animals (one 10 mg/kg bw/day female, one 20 mg/kg bw/day male, and one 20 mg/kg bw/day female) exhibited notably marked body weight losses and low feed intake during the first few weeks of compound administration, indicating adaptation problems to dosing. Supplemental feeding regimens were instituted for the three dogs to preclude mortality; two of the three dogs were successfully returned to basal diet by Week 8 or 15 and the third (the 20 mg/kg bw/day female) was maintained on supplemental feeding throughout the majority of the study, continuing through study termination. This finding was found by the author to indicate that the maximum tolerated dose in dogs was closely approximated in this evaluation.

No definitive evidence of toxicity was seen in any of the other parameters evaluated in this study: The only clear pattern indicative of a treatment-related difference was the occurrence of dose-related salivation, which was most frequently noted in anticipation of dosing in the 10 and 20 mg/kg bw/day dogs. This finding was considered most likely to be a conditioned reflex or secondary effect, rather than a direct treatment-related effect.

No subchronic toxic effects were evident in the male and female low- and mid-dose dogs and 10 mg/kg bw/day was the NOAEL. A supplemental feeding regimen was required for a high-dose female throughout the majority of the study and was considered necessary to prevent mortality. Thus, the LOAEL was 20 mg/kg bw/day.

B.6.3.3 Percutaneous 28-day toxicity (rat) (Annex IIA 5.3.3)

A 28-Day Dermal Toxicity Study of Dodine Technical Material in Rats

Kern, T.G. (July 21, 1999) - Report No. WIL 21140, performed by WIL Research Laboratories, Inc., Ashland, OH 44805-9281, USA

Guidelines and GLP

The study was conducted according to GLP and OPPTS Guideline 870.3200. Deviations from OECD guideline 410/E.U. method B.9 or study protocol consisted of:

- Based on the physical state of the test article, its severe irritation potential, and the necessity of slightly shifting the site of test article application, less than 10% coverage of the total body surface was unavoidable. This coverage was acceptable and did not impact the quality or integrity of the study because it minimized factors affecting the assessment of dermal absorption and toxicity of the test article, such as: severe irritation, compromising the dermis, and thickening of the skin in the shaved treatment area.
- On day 15; five females in the 200 mg/kg bw/day group were inadvertently not unwrapped after the 6-hour exposure period. They were unwrapped on day 16 (approximately 18 hours past the scheduled time for removal of wrappings). This protocol deviation had no adverse impact on the outcome of the study, based upon clinical signs, food consumption and body weights.
- Food jars were inadvertently not weighed for one-half of the animals at week 4. The result was that no week 4 food consumption values were obtained for an equal number of animals from each group.
- Ornithine decarboxylase was not determined.

These deviations did not impact the outcome of the study; the study is acceptable.

Materials and methods

Test substance: Dodine technical, off-white powder, Lot No. OP750142, purity: 98.0% (w/w).

Test animals: A total of 46 male and 46 female Crl:CD®IGS(SD)BR rats were received from Charles River Laboratories, Inc., Raleigh, North Carolina, USA. At initiation of dosing, rats were approximately 8 weeks old and body weights ranged from 271 to 313 g for males and from 180 to 224 g for females.

A non-GLP pilot study was conducted in advance for the study to evaluate any potential cumulative irritation from test article application. One male and one female each were dosed at 200, 350 and 500 mg/kg bw/day for five consecutive days (days 0 to 4). Clinical observations and body weights were recorded for these animals. Dermal scores were recorded at the time of .dose administration and prior to euthanasia (day 6). There were no remarkable clinical signs or effects on body weights at any dose level. Test article-related dermal irritation was generally similar at all three dose levels and included very slight to severe erythema, very slight oedema, desquamation, eschar and blanching. Atonia was limited to the 200 mg/kg bw/day group female. Based on the results of this pilot study, dose levels of 50, 125 and 200 mg/kg bw/day were selected for the main study.

Definitive 28-day study: Test article was moistened with deionised water (to form a paste) and applied to 4 groups of 10 rats/sex, 5 days/week, for 4 consecutive weeks to the shaved intact dorsal skin of each rat, for a total 20 applications, at dosage levels of 0 (control), 50, 125 and 200 mg/kg bw/day. The application sites were covered with gauze, occluded with plastic wrap and secured with Dermiform® tape for a period of six hours per exposure. Following each six-hour exposure

period, the test sites were washed with tepid tap water and wiped with disposable paper towels to remove residual test article.

Due to the irritating nature of the test article, within the shaved treatment area of each animal (20-25% of the total body surface), the application site was moved as necessary to minimize induction of severe irritation upon repeated dosing. The area of the test article application was measured and recorded once per week for a representative animal of each sex in each group.

The animals were examined twice daily for signs of mortality and moribundity. Clinical examinations were performed once daily prior to dosing or at approximately the same time on non-dosing days. Detailed physical examinations were performed weekly, beginning one week prior to the initiation of the test article administration. The application sites were examined weekly for signs of dermal irritation. Individual body weights and food consumption were recorded weekly. Haematology, serum chemistry and urinalysis were evaluated prior to necropsy. Complete necropsies were performed on all animals. Selected organs were weighed and microscopic examinations were conducted on selected tissues from all animals. Ophthalmological examinations were performed prior to the initiation of dosing (week -1) and during study week 3.

Statistical methods: All analyses were conducted using two-tailed tests for significance levels of 5% and 1% comparing the test article-treated groups to the control group by sex. Statistical tests were not conducted if the number of animals was two or less. Body weight, body weight change, food consumption, clinical laboratory and absolute and relative organ weight data were subjected to a one-way analysis of variance (ANOVA) followed by Dunnett's tests if the results of the ANOVA were statistically significant ($p<0:05$). Clinical laboratory values for cell types that occur at a low incidence (i.e. monocytes, eosinophils and basophils) were not subjected to statistical analysis.

Results:

No test article-related effects were observed related to survival, clinical condition, food consumption, haematology, serum chemistry, urinalysis, ophthalmological and organ weight parameters.

Mean body weight gains were significantly ($p<0.05$ or $p<0.01$) reduced when compared to the control group for the 125 and 200 mg/kg bw/day group males for week 0 to 1 resulting in 13% and 22% lower cumulative (weeks 0 to 4) body weight gains when compared to the control group. However, these changes were not considered by the study author to be test article-related as the gains in these males after the initial week of dosing were comparable to the control group gains. Furthermore, there was no trend present in the females. In the 200 mg/kg bw/day group females there was a significantly ($p<0.01$) decreased mean weight gain for week 2 to 3 followed by a significantly ($p<0.05$) increased gain for week 3 to 4. No other remarkable differences from the control group were observed.

According to the Notifier, the effects may be considered treatment-related and toxicologically significant for the following reasons: 1) the effect during week 0-1 was severe, i.e. decreases of 26% and 37% in comparison to control for the mid- and high-dose males, respectively; 2) there was no effect on food consumption, indicating that the animals lost weight, despite eating normally; 3) body weight gains were comparable to controls for weeks 1-2 and 2-3, but then were decreased for week 3-4, resulting in an overall decrease of 13% and 22% for the mid- and high-dose males, respectively, and indicating that animals did not recover from the initial week 0-1 effect. In the high-dose females, there was a significant decrease in body weight gain (71%) for weeks 2-3; however, there was a significant increase for weeks 3-4. Overall (weeks 0-4), there was a 17% decrease that was not significant.

Overall RMS agrees with Notifier that mean body weight gain (week 0-4) in the mid- and high dose groups males can be considered as adverse although observation of up and down body weight gain

values relative to control group in male rats seems more stress-related (due to severe dermal irritation) than actual systemic signs of toxicity.

Table 6.37 – 28-day dermal toxicity study in rats: mean body weights (g)

Week	Dose Level (mg/kg bw/day)							
	Males				females			
	0	50	125	200	0	50	125	200
-1	227	224	225	226	171	176	171	173
0	291	290	290	291	203	204	203	203
1	326	320	316	313	219	218	218	218
2	351	349	345	337	234	231	233	228
3	372	375	365	357	248	240	246	232
4	380	378	367	359	244	238	240	236

None significantly different from control group

Table 6.38 – 28-day dermal toxicity study in rats: mean body weight gains (g)

Week	Dose Level (mg/kg bw/day)							
	Males				females			
	0	50	125	200	0	50	125	200
-1 – 0	64	67	65	65	33	29	32	31
0 – 1	35	30	26*	22**	16	14	15	14
1 – 2	26	29	29	24	14	13	15	11
2 – 3	20	26	20	19	14	9	13	4**
3 – 4	9	4	2	3	-5	-3	-6	4*
0 –4	89	88	77	69	40	33	37	33

* : Significantly different from the control group at 0.05 using Dunnett's test
** : Significantly different from the control group at 0.01 using Dunnett's test

Test article-related dermal findings were noted in all treated groups during the study. Overall, the females were more affected than the males. Dermal irritation was more prevalent in the 125 and 200 mg/kg bw/day groups. Very slight to severe erythema and very slight to slight oedema was observed for all or almost all of the animals in these groups. Other dermal findings observed at a high incidence in the 125 and 200 mg/kg bw/day groups included desquamation, focal eschar/eschar and encrustation. Additional dermal findings in these groups consisted of blanching, exfoliation and fissuring at the application sites. Dermal irritation was also noted in the 50 mg/kg bw/day group: mainly erythema (mostly very slight to slight) and desquamation, but other dermal findings at lower incidences including very slight oedema, focal eschar/eschar, blanching, encrustation, exfoliation and fissuring were also present in this group. The only dermal finding observed in the control group was a single occurrence of focal eschar for one female at the end of the study, most likely due to shaving/tape abrasion.

Table 6.39 – 28-day dermal toxicity study in rats: dermal findings at week 4

	Dose Level (mg/kg bw/day)							
	Males				females			
	0	50	125	200	0	50	125	200
Erythema	0/10	5/10	10/10	9/10	0/10	10/10	10/10	10/10
Oedema	0/10	2/10	9/10	8/10	0/10	3/10	10/10	10/10
Focal eschar / eschar	0/10	2/10	8/10	9/10	1/10	3/10	9/10	9/10
Encrustation	0/10	3/10	7/10	6/10	0/10	1/10	9/10	8/10
Blanching	0/10	4/10	2/10	4/10	0/10	4/10	4/10	0/10
Exfoliation	0/10	0/10	1/10	5/10	0/10	1/10	3/10	5/10
Fissuring	0/10	0/10	1/10	0/10	0/10	1/10	1/10	1/10
Occurrence of desquamation / no. animals affected	0/0	18/9	29/10	30/10	0/0	25/10	32/10	33/10

Test article-related macroscopic findings were limited to the treated skin. Scabbing of the treated skin was noted at all dose levels; most of these animals had encrustation, eschar, exfoliation and/or fissuring at the last dermal observation.

The only test article effect observed microscopically was localized at the treated skin site. In most 125 and 200 mg/kg bw/day group males and females, focal or multifocal lesions indicative of mild irritation were observed. These included ulcers, suppurative inflammation, epidermal hyperplasia, hyperkeratosis, subacute inflammation, inflammatory exudate and parakeratosis. One 50 mg/kg bw/day group female had some of these findings (minimal exudate and parakeratosis) suggestive of slight irritation that appeared treatment-related.

Conclusion:

In conclusion, dermal administration of dodine at 50, 125 and 200 mg/kg bw/day to male and female rats 6h/day, 5 days/week for 4 weeks resulted in local dermal irritation at all dose levels. Signs of systemic toxicity could not be ruled out due to overall decrease body weight gain in male rats at the two highest dose levels.

The study author concluded that no signs of systemic toxicity were observed at any dose level and thus the NOAEL for systemic toxicity of dodine was the highest dose level tested of 200 mg/kg bw/day. The Applicant considered that there was some evidence that dermal application at mid- and high-dose group caused systemic toxicity (decreased bw and bw gain in males), and that the severe dermal irritation may have contributed to these findings. Based on the above, RMS agrees with Applicant to consider the systemic NOAEL as the dose level of 50 mg/kg bw/day.

There was no NOAEL for local skin irritation.

A 21-Day Dermal Toxicity Study in Rats with CT-334-87

Auletta C.S. (July 7, 1989) - Report No. 4932-88, performed by Bio/dynamics Inc., East Millstone, New Jersey, USA.

Guidelines and GLP

The study was conducted according to GLP and US EPA FIFRA guideline 82-2 corresponding to OECD 410. Deviations from OECD guideline 410/E.U. method B.9 or study protocol consisted of:

- Because of excessive ambient humidity, humidity values throughout most of the equilibration and treatment periods exceeded the desired range. However, temperature was

generally maintained within the desired range, animals appeared to be in good health throughout the study and control values were comparable to historical control data for this laboratory. Therefore, although the humidity deviations were significant, they are not considered to have affected the validity of the study or the ability to make meaningful conclusions based on the data generated.

- Ornithine decarboxylase and gama-glutamyl transpeptidase were not determined.

A 35% SL formulation was used based on dodine hydrochloride instead of dodine acetate. These deviations did not impact the outcome of the study, however, as no information on the total composition of the test substance is available, the study is considered as additional information.

Materials and methods

Test substance: CT-334-87: dodine HCl, (1-dodecylguanidinium hydrochloride), clear peach-coloured liquid with 35% SL, "biocide" formulation. This study was not performed with dodine under its acetate salt form but under its hydrochloride form. The active dodecyl moiety is exactly the same (cation) under both forms, dodine purity: 35%. The total composition of this preparation was not stated.

Test animals: A total of 20 male and 20 female Sprague-Dawley (CD®) rats were received from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts, USA. At initiation of dosing, rats (age not stated) body weights ranged from 260 to 327 g for males and from 234 to 262 g for females.

An 8-day pilot study was performed with 1 male and 1 female rat/dose levels of 20, 100 and 200 mg/kg bw/day in a dose volume of 2.0 ml/kg bw in distilled water. Low dose level resulted in very slight erythema in both animals 3 to 4 days post dosing and desquamation on day 8. Well-defined to moderate erythema and desquamation were observed at the mid-dose level of 100 mg/kg bw/day in both sexes, the male presented also atonia and necrosis. Well-defined erythema was also observed in the animals of the top dose group leading to the impossibility of scoring on day 8 due to severity of response, necrosis and eschar formation.

Definitive 28-day study: Test article was administered to the clipped dorsal skin of 4 groups of 5 rats/sex, 5 days/week, for 21 consecutive days for a total of 15 applications over a 3-week period. Dosage levels were 0 (control), 12.5, 25 and 50 mg/kg bw/day in a 2 ml/kg bw volume in distilled water. The test sites were covered by a polyethylene patch (approximately 3x3 cm) and the patch was covered by an adhesive bandage (Elastoplast®) wrapped around the trunk. Following approximately 6 hours of exposure, the wrappings were removed. Prior to each application, residual material was gently wiped from the exposure site with a soft clean paper towel moistened with water.

Dosing Mixtures (aqueous dilutions in distilled water) were prepared fresh daily through Day 6. Based on confirmation of one-week stability by the sponsor, fresh mixtures were prepared weekly beginning on Day 7. Analyses of dosing mixtures were performed by the sponsor.

Viability check was performed twice daily (7 days/week), detailed physical examinations were conducted daily. Qualitative evaluation of dermal irritation was performed once daily; prior to administration of the test material (no scores were assigned) and dermal irritation scores were evaluated at pre-test and weekly during the study. Body weights and food consumption were recorded pre-test and weekly during the study. Clinical Laboratory investigations (haematology and clinical chemistry) were performed on all animals at termination of the study. No urinalysis was conducted. At the end of the treatment period, all animals were subjected to a complete gross post-mortem examinations; kidneys, liver and testes with epididymides were weighed; liver (2 sections), both kidneys, skin (normal and treated) and target organs were preserved and examined microscopically for all animals in the control and high-dose groups.

Statistical analysis was performed on the following: body weights, food consumption, haematology, clinical chemistry, organ weights and organ/body weight ratios. Statistical evaluation of equality of means was made by the appropriate one way analysis of variance technique, followed by a multiple comparison procedure if needed. First Bartlett's test was performed to determine if groups had equal variance. If the variances were equal, parametric procedures were used; if not, nonparametric procedures were used. The parametric procedures were the standard one way ANOVA using the F distribution to assess significance. If significant differences among the means were indicated, Dunnett's test was used to determine which means were significantly different from the control. If a nonparametric procedure for testing equality of means was needed, the Kruskal-Wallis test was used, and if differences were indicated a summed rank test (Dunn) was used to determine which treatments differed from control.

A statistical test for trend in the dose level was also performed. In the parametric case (i.e., equal variance) standard regression techniques with a test for trend and lack of fit were used. In the nonparametric case Jonckheere's test for monotonic trend was used.

Results:

Mortality: There was no death related to treatment with dodine.

Clinical signs: No abnormalities were observed throughout the study.

Body weights: The majority of animals gained weight throughout the study. Weight gains (Weeks 0-3) in high-dose females were slightly lower than gains in control females (mean control gain: 35 grams; mean high-dose gain: 22 grams). However, differences in weight gains were not dose related (mean gains for low- and mid-dose females were 24 and 29 grams, respectively), mean weekly body weights for control and treated groups were considered comparable, no statistically significant differences were seen in weekly weights, and the mean body weight of high-dose females was within 4% of the control weight at study termination (Week 3).

Food consumption: Food consumption values for control and treated groups of males were comparable throughout the study. Mean values for high-dose females were generally slightly lower than values for control females, with the difference at Week 2 being statistically significant ($p \leq 0.05$). This is consistent with the slightly reduced body weight gain in this group.

Dermal observations: Dermal irritation was observed in all treated groups. Time of onset, incidence and severity were generally dose-related. Irritation in the low- and mid-dose groups was considered minimal, while irritation in the high-dose group was generally moderate (see Table 6.40).

In the low-dose group (12,5 mg/kg bw/day), scoring of the dose sites on Days 7, 14 and 21 revealed only very slight or slight erythema, with more females than males exhibiting this observation. Isolated observations of fissuring or oedema were seen (in single animals) and a small area of superficial necrosis was seen in a single animal on Days 18, 19 and 20. No severe irritation or tissue destruction was evident in this group.

In the mid-dose group (25 mg/kg bw/day), scoring of the dose site on Days 7, 14 and 21 revealed very slight to moderate erythema on Day 7, with females more severely affected than males and very slight or slight erythema on Days l4 and 21, with no marked differences between males and females. Occasional observations of atonia, fissuring and/or superficial necrosis were also noted in one to three animals.

All animals in the high-dose group (50 mg/kg bw/day) exhibited erythema by Day 5 or 6 (after 2 or 3 application) and all developed desquamation by Day 13. Several also exhibited atonia beginning on Day 11 and a few were noted to have oedema and/or fissuring. Areas of necrosis and/or superficial necrosis were seen in some animals as early as Day 7. Eschar formation and exfoliation of the eschar tissue generally occurred subsequently. Most high-dose animals exhibited some evidence of tissue damage (necrosis, eschar formation) at one or more intervals during the study.

Scoring of the dose sites on Days 7, 14 and 21 revealed generally slight erythema with little or no oedema in most animals.

Table 6.40 – 21-day dermal toxicity study in rats with CT-334-87: summary incidence of dermal irritation (in 10 animals/group) – observations on dosing days only

Group (mg/kg bw/day)	Dermal observation	Days														
		1*	4	5	6	7	8	11	12	13	14	15	18	19	20	21
I: Control	Desquamation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
II: 12.5	Erythema	0	0	0	1	5	5	3	1	3	3	3	1	1	1	2
	Desquamation	0	0	0	0	0	0	4	6	6	6	4	3	6	5	5
	Fissuring	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
	Superficial N.	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
III: 25	Erythema	0	0	2	5	6	8	5	8	8	8	8	2	3	2	7
	Atonia	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0
	Desquamation	0	0	0	0	0	2	10	10	10	9	9	9	9	10	8
	Fissuring	0	0	0	0	0	0	2	2	3	3	1	1	1	1	2
	Superficial N.	0	0	0	0	0	0	2	2	2	0	0	1	1	0	0
IV: 50	Erythema	0	0	8	10	8	9	10	9	10	10	10	7	8	7	10
	Oedema	0	0	0	1	1	0	2	2	1	1	2	1	1	1	2
	Atonia	0	0	0	0	0	0	5	6	6	6	6	2	2	3	3
	Desquamation	0	0	1	3	1	1	7	8	10	10	10	10	10	10	10
	Fissuring	0	0	0	0	0	1	4	4	3	3	3	3	3	3	3
	Eschar	0	0	0	0	0	0	2	1	0	0	0	4	5	3	3
	Exfoliation	0	0	0	0	0	0	2	2	1	0	0	0	3	5	2
	Superficial N.	0	0	0	0	2	0	3	3	3	2	1	0	0	0	3
	Necrosis	0	0	0	0	1	2	2	1	0	1	1	4	4	3	1

*day 1 = predose
N. : necrosis

Haematology: The only haematological parameter affected was total white blood cells count that was slightly increased (not statistically significant) in high-dose males and females. For the males, the elevated mean value resulted from a marked elevation in one animal.

Clinical chemistry: Mean total protein and albumin values for high-dose males were slightly lower than mean control values at study termination. The difference in albumin values was statistically significant. Mean terminal ALT was statistically significantly increased in the high-dose males. Similar differences were not apparent for high-dose females or for low- and mid-dose animals of either sex.

Organ weights: The mean liver weight and liver/body weight ratio for high-dose females were slightly higher than values for control females: However, the differences were not statistically significant and microscopic examination of livers revealed no evidence of hepatic pathology. Liver weights for low- and mid-dose females and for all treated groups of males were comparable to control values.

Macroscopic examination: Morphologic abnormalities considered to be related to the test material were limited to the treated skin. Gross examination confirmed the dermal abnormalities seen during the antemortem phase of the study, i.e., the presence of erythema, eschar, desquamation, oedema, atonia, fissures and/or eschar (scabs/scores) which occurred in a dose-related pattern. Dermal abnormalities were noted at necropsy in the majority of high-dose animals, some mid-dose animals and one low-dose animal. No dermal abnormalities were seen grossly in the control group, nor in untreated skin.

Microscopic examination: Microscopic examination of samples of treated and comparable untreated skin was limited to males and females from the control and high-dose groups. The treated skin from high-dose animals showed the presence of surface accumulations of inflammatory cells/cell debris, hyperkeratosis and parakeratosis, squamous cell hyperplasia and subacute (chronic active)/chronic inflammation for almost all males and females; these findings were considered to be minimal to moderate in severity. Also, erosions/ulcers (essentially of minimal severity) were seen in several males and females and focal epithelial necrosis (minimal severity) was seen in one male. The aforementioned findings were not seen in control animals nor were they seen in the comparable samples of untreated skin from any animals in either group.

Conclusion:

Dermal applications of CT-334-87 to rats at doses of 12.5, 25 and 50 mg/kg bw/day for 3 weeks produced dermal irritation at all dose levels; time of onset, incidence and severity were dose-related. No clear, consistent, systemic effects of test material administration were seen. The NOAEL for systemic toxicity resulting from dermal administration of CT-334-87 to rats under conditions of this study was 50 mg/kg bw/day, the highest dose tested.

No NOAEL could be established for dermal irritation effects.

B.6.4 Genotoxicity (Annex IIA 5.4)

B.6.4.1 *In vitro* genotoxicity testing (Annex IIA 5.4.1)

B.6.4.1.1 Bacterial assay for gene mutation

Evaluation of Dodine Tech. 95% for Mutagenic Activity in the Ames Test

Willems, M.I. (March 1981) - report No. V81.102/210064-7 (project # B81-0064-7), performed by TNO, Division for Nutrition and Food Research tno, Zeist, The Netherlands;

Guidelines and GLP

The study was conducted according to GLP and following the Ames *et al.* (1975) method with no significant deviations from the B.13/14 EU method.

The study is acceptable.

Materials and methods

Test substance: dodine, (1-dodecylguanidinium acetate), a white powder, Lot No. 51-24-3, purity 95%. Vehicle: methanol.

Test system: histidine requiring *Salmonella typhimurium* mutants TA 1535, TA 1537; TA 1538, TA 98 and TA 100 and a liver microsome fraction of Aroclor-induced rats for metabolic activation (tested with and without S9 mix). The following test solutions in methanol were prepared: 0, 0.06, 0.19, 0.56, 1.67 and 5.0 µg/plate based on a preliminary test. The solvent and vehicle control used was methanol. Each concentration was cultured in triplicates (except positive mutagens that were tested in duplicate) with $4x10^8$ to $1x10^9$ cells/ml incubated at 37ºC. The results were not confirmed in an independent assay.

The reference mutagens used as positive controls were:

- sodium azide for strains TA 1535 and TA 100 without S-9 mix;

- hycanthone methanesulphonate for strains TA 1537, TA 1538 and TA 98 without S-9 mix;

- 2-aminoanthracene for all strains in the presence of S-9 mix.

Results:

The results of the preliminary test are shown in Table 6.41, it appeared that 10 µg of the test substance/plate was still toxic whereas 1 µg/plate did not show a growth-inhibiting effect. Therefore 5 µg/plate was chosen as the highest dosage level for the mutagenicity test.

Table 6.41 – Chemical toxicity testing of dodine

µg test product/0.1 ml/plate	Background growth	n.º of revertant colonies
10000	0 [1]	0
1000	0	0
100	0	0
10	LD [2]	50
1	NG [3]	36
0	NG	42

[1] 0 = no growth
[2] LD = less dense background lawn of bacterial growth
[3] NG = normal background lawn of bacterial growth

It appears that incorporation of the test substance with the bacteria did not increase the numbers of his+ revertants with any of the five tester strains, either in the absence or in the presence of the S-9 mix (see Table 6.42).

The background lawn of bacterial growth in control and test plates was comparable, indicating that the chemical toxicity of the test substance did not interfere with the mutagenicity testing.

Table 6.42 - Evaluation of dodine in the Salmonella/microsome mutagenicity test

Test product (µg/plate)	S9-mix added	Numbers of his+ revertants (mean of 3 plates ± SD) with				
		TA 1535	TA 1537	TA 1538	TA 98	TA 100
Control (0)	No	61 ± 13	12 ± 5	14 ± 3	28 ± 11	107 ± 30
Dodine 0.06	No	52 ± 7	15 ± 2	9 ± 4	37 ± 9	123 ± 18
Dodine 0.19	No	48 ± 13	15 ± 3	11 ± 5	24 ± 4	115 ± 14
Dodine 0.56	No	47 ± 13	15 ± 2	13 ± 3	30 ± 6	128 ± 12
Dodine 1.67	No	49 ± 10	16 ± 8	11 ± 3	33 ± 6	122 ± 35
Dodine 5.0	No	58 ± 20	15 ± 1	14 ± 2	27 ± 9	133 ± 29
Control (0)	Yes	38 ± 15	22 ± 6	40 ± 10	45 ± 6	129 ± 19
Dodine 0.06	Yes	45 ± 6	20 ± 6	30 ± 6	46 ± 7	132 ± 38
Dodine 0.19	Yes	44 ± 6	21 ± 6	27 ± 5	39 ± 11	145 ± 54
Dodine 0.56	Yes	66 ± 22	18 ± 1	23 ± 4	54 ± 14	129 ± 21
Dodine 1.67	Yes	50 ± 8	16 ± 7	32 ± 13	48 ± 10	137 ± 34
Dodine 5.0	Yes	56 ± 11	19 ± 1	30 ± 4	57 ± 6	153 ± 12
Positive controls	No	257 ± 49	153 ± 40	182 ± 12	148 ± 8	369 ± 6
	Yes	159 ± 13	49 ± 0	373 ± 23	324 ± 68	413 ± 84
No. of bacteria/ml		$1x10^9$	$4x10^8$	$8x10^8$	$7x10^8$	$6x10^8$

[1] Reference mutagens were tested in duplicate

Conclusion:

It was concluded that dodine did not show any mutagenic activity in the Salmonella/mammalian microsome mutagenicity test under the conditions employed in this evaluation.

Evaluation of the Mutagenic Activity of Dodine Technical in the *Escherichia Coli* Reverse Mutation Assay (With Independent Repeat)

Verspeek-Rip C.M. (November 4, 2003)- report No. 394482, performed by Notox B. V., Hambakenwetering 7, 5203 DD's-Hertogenbosch, Netherlands.

Guidelines and GLP

The study was conducted according to GLP and followed OECD Guideline 471 (1997), EEC Directive 2000/32/EC, B.13/14 (2000), with no significant deviations.

The study is acceptable.

Materials and methods

Test substance: dodine technical, a white powder, Batch No. S01L01, purity 98.5% was dissolved in ethanol.

Test system: tryptophan-requiring *Escherichia coli* bacterial strain WP_2uvrA, which is capable of detecting base-pair substitution mutagens. The assay was conducted in the absence and presence of a metabolizing system (S9-mix – rat liver microsomal enzymes prepared from Aroclor 1254-induced male Wistar rats). The solvent and vehicle control used was ethanol.

The reference mutagens used as positive controls were:

- 4-nitroquinoline N-oxide (4-NQO), 10 µg/plate in DMSO (without S-9 mix);

- 2-aminoanthracene (2AA), 10 µg/plate in DMSO (with S-9 mix);

- solvent for reference substances was DMSO (dimethyl sulfoxide)

Table 6.43 - Study design

	With S9-mix	Without S9-mix
Dose range-finding test	10, 33, 100, 333, 1000, 3330 and 5000 µg/plate (in triplicate)	
First experiment	0.3, 1, 3, 10, 33, 66 and 100 µg/plate	0.1, 0.3, 1, 3, 10, 24 and 33 µg/plate
Second experiment	1, 3, 10, 33, 100 and 200 µg/plate	0.3, 1, 3, 10, 33 and 66 µg/plate

First experiment

Since severe toxicity was observed in almost all dose levels tested in the dose range-finding test, the first experiment test was performed with the following concentrations: 0.1, 0.3, 1, 3, 10, 24 and 33 µg/plate in the absence of S9-mix and 0.3, 1, 3, 10, 33, 66 and 100 µg/plate in the presence of S9-mix.

Second experiment

The highest concentration of dodine used in the subsequent mutation assay was the level at which the test substance inhibited bacterial growth. Six different doses, 0.3, 1, 3, 10, 33 and 66 µg/plate in the absence of S9-mix and 1, 3, 10, 33, 100 and 200 µg/plate in the presence of S9-mix of the test substance were tested in triplicate.

The plates were incubated in the dark at 37 ± 1°C for 48 h. After this period revertant colonies (tryptophan independent (Trp+)) were counted automatically with a Protos model 50000 colony counter or manually, if less than 40 colonies per plate were present. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Interpretation

Acceptability of the assay: An *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory background historical range.

Table 6.44 - Negative control historical range

Strain	Minimum value	Maximum value	Mean ± 3 x S.D.
WP_2uvrA – S9-mix	4	32	14 ± 15
WP_2uvrA + S9-mix	4	31	14 ± 14

b) The positive control chemicals should produce responses, which is within the laboratory historical range documented for each positive control substance. Furthermore, the mean plate count should be at least two times the concurrent vehicle control group mean.

Table 6.45 - Positive control historical range

Strain	Minimum value	Maximum value	Mean ± 3 x S.D.
WP_2uvrA – S9-mix	64	1399	675 ± 841
WP_2uvrA + S9-mix	56	1053	223 ± 446

c) The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate.

Data evaluation: A test substance is considered negative (not mutagenic) in the test if:

a) The total number of revertants at any concentration is not greater than two times the solvent control value, with or without metabolic activation.

b) The negative response should be reproducible in at least one independently repeated experiment.

A test substance is considered positive (mutagenic) in the test if:

a) It induces a number of revertant colonies, dose related, greater than two-times the number of revertants induced by the solvent control, either with or without metabolic activation. However, any mean plate count of less than 20 is considered to be not significant.

b) The positive response should be reproducible in at least one independently repeated experiment.

Results:

Dose range-finding test

Precipitate: The test substance precipitated in the top agar at concentrations of 1000 µg/plate and upwards.

Toxicity: An extreme reduction of the bacterial background lawn and an increase in the size of the microcolonies compared to the solvent control plate was observed at the test substance concentration of 33 µg/plate (without S9-mix) and 100 µg/plate (with S9-mix). A complete lack of any microcolony background lawn was observed at 100 µg/plate and upwards (without S9-mix) and at 333 µg/plate and upwards (with S9-mix).

Mutagenicity: No increase in the number of revertants was observed upon treatment with dodine under all conditions tested.

First mutation experiment

Precipitate: The test substance did not precipitate in the top agar or on the plates at the start and at the end of the incubation period.

Toxicity: In the absence of S9-mix, a slight reduction of the bacterial background lawn was observed at the test substance concentration of 24 µg/plate and a moderate reduction at 33 µg/plate. In the presence of S9-mix, a moderate reduction of the bacterial background lawn was observed at test substance concentrations of 66 and 100 µg/plate. No biologically relevant decrease in the number of revertants was observed at any of the concentrations-tested.

Mutagenicity: No increase in the number of revertants was observed upon treatment with dodine under all conditions tested.

Second mutation experiment

Precipitate: Dodine did not precipitate in the top agar or on the plates at the start and at the end of the incubation period.

Toxicity: In the absence of S9-mix, a moderate reduction of the bacterial background lawn was observed at the test substance concentration of 33 µg/plate. An extreme reduction of the bacterial background lawn and an increase in the size of the microcolonies compared to the solvent control plate was observed at the test substance concentration of 66 µg/plate. In the presence of S9-mix, a moderate reduction of the bacterial background lawn was observed at the test substance concentration of 100 µg/plate. An extreme reduction of the bacterial background lawn and an increase in the size of the microcolonies compared to the solvent control plate was observed at the test substance concentration of 200 µg/plate.

Mutagenicity: No increase in the number of revertants was observed upon treatment with dodine under all conditions tested.

Discussion and conclusion:

The bacterial strain WP_2uvrA showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in independently repeated experiments.

The negative and strain-specific positive control values were within the performing laboratory background historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that dodine technical was not mutagenic in the *Escherichia coli* reverse mutation assay.

B.6.4.1.2 Test for clastogenicity in mammalian cells (Annex IIA 5.4.1)

Chromosome Analysis of Cultured Human Lymphocytes Treated *in Vitro* with Dodine

Wilmer, J.W.G.M. (April 1985) - Report no. V85.164/250209, performed by TNO, Division for Nutrition and Food Research tno, 3700 AJ Zeist, Netherlands.

Guidelines and GLP

The study was conducted according to GLP and followed OECD Guideline 473, and E.U. method B.10; main deviation was that only a single sampling time was used for all doses, which has a reflex

on the evaluation of the results as specified in the text below, a clear dose-response relationship may be absent in the case of a positive response. The study is acceptable.

Materials and methods

Test substance: dodine technical, a white powder, Batch No. KG 8507, purity 98% was dissolved in ethanol.

Test system: The experiments were carried out with freshly withdrawn blood (10-20 ml) of a healthy male donor (not smoking, not receiving medication or suffering from viral infection) with a previously established low incidence of chromosome damage. S9-fraction was prepared from Wistar rats previously exposed (i.p.) to 20% w/v of Aroclor 1254 (= 500 mg/kg bw).

Controls: vehicle (ethanol) was used as negative control, because of the toxicity of the vehicle for the cells the experimental procedure was slightly modified by adding 50 µl of the appropriate test solution to the culture medium instead of 100 µl.

The reference mutagens used as positive controls were:

- methylmethanesulphonate (MMS), 30.0 µg/ml medium (3.0×10^{-4} M) without S-9 mix;

- cyclophosphamide (CP), 20 µg/ml medium (7.2×10^{-5} M) with S-9 mix;

- solvent for reference substances was DMSO (dimethyl sulfoxide)

Table 6.46 - Study design

	With S9-mix	Without S9-mix
Toxicity test	1.65, 4.94, 14.81, 44.44, 133.33 and 400.0 µg/ml	
Chromosome aberration test	0, 0.56, 1.67, 5.0 and 15.0 µg/ml	0, 0.37, 1.11, 3.33 and 10.0 µg/ml

Toxicity test

A preliminary test with 6 concentrations of the test substance and the vehicle (both in the absence and in the presence of the S9-mix) was carried out to assess the toxicity of the test substance for the cells. Cultures were incubated at 37°C and approximately 90% humidity in air containing 5% CO_2 for 48 hours. After this period the dividing cells were exposed to different doses of the test substance for 24 hours in the absence of S-9 mix. In the presence of the S-9 mix, the exposure of the cells to the test substance was reduced to 2 hours, because of the toxicity of the S9-mix for the cells. Two hours before the end of the total incubation period (72 hours) the cells were arrested in the metaphase stage of the mitosis by the addition of colcemid (final concentration: 0.1 µg/ml medium). The cells were harvested by centrifugation, treated for 12-15 minutes with a hypotonic solution, fixed, transferred to clean microscope slides and stained. Four slides were prepared from each culture.

At least 1000 nuclei per culture were analysed (250 from each slide) to determine the mitotic index (percentage cells in mitosis).

Chromosome aberration test

The lymphocytes were treated with 4 concentrations of the test substance ranging from 0.37 to 10.0 µg/ml in the absence of the S9-mix and from 0.56 to 15.0 µg/ml in the presence of S9-mix. The doses to be used were established on the basis of the results of the toxicity test: the highest dose of the test substance should reduce the mitotic index by approximately 50%. Lymphocyte cultures were set up as for the toxicity test.

Duplicate cultures were used for each dose of the test substance and for the negative and positive controls.

If feasible, in each culture 100 well-spread metaphases (25 from each slide, 200 per dose level), each containing 46 centromeres were analysed for a wide range of structural chromosomal aberrations (gaps, breaks, fragments, dicentrics, exchanges) according to the criteria recommended by Savage (1975). Vernier-readings of all metaphases scored were recorded.

Data processing: Different types of aberrations (chromatid-type and chromosome-type) were listed with their numbers and frequencies for all test and control cultures. Gaps were recorded separately and were not included in the final assessment of clastogenic activity. The number of cells scored, the number of aberrant metaphases (including and excluding gaps) and the mitotic index were given for test and control cultures. Data were evaluated, when appropriate, by Fischer's Exact test or the chi-square test using the number of cells with aberrations, to determine significant differences between test and control cultures.

Evaluation of results: The major criterion to designate the results of a chromosome aberration test as positive is a statistically significant dose-related increase in the number of structural chromosomal aberrations. However, when a single sampling time is used for all doses, a clear dose-response relationship can be absent because the yield of chromosomal aberrations can vary markedly with post-treatment sampling time of an asynchronous population and because increasing doses of clastogens induce increasing degrees of mitotic delay. A test substance producing neither a statistically significant dose-related increase in the number of structural chromosomal aberrations nor a statistically significant and reproducible positive response at any of the doses is considered non-clastogenic in this system.

Results:

Toxicity test

The results showed that - both in the absence and in the presence of the S9-mix - the test substance was clearly toxic for the cells at the 3 highest dose levels used. At dose levels of 4.94 and 14.81 µg/ml, the test substance reduced the number of mitoses as compared with the control to 48% and 32% in the absence of S9-mix and to 59% and 30% in the presence of S9-mix.

In view of these observations 10.0 µg and 15.0 µg per ml culture medium were chosen as the highest dose levels for the chromosome aberration test in the absence and in the presence of the S9-mix respectively.

Table 6.47 - Results of the toxicity test with cultured human lymphocytes treated *in vitro* with dodine

Treatment	Concentration (µg/ml)	S9-mix -/+	Mitotic index (%)[1]
Control (ethanol)	-	-	5.0[2]
Dodine	1.65	-	3.6
	4.94	-	2.4
	14.81	-	1.6
	44.44	-	0.3
	133.33	-	-
	400.0	-	-
Control (ethanol)	-	+	9.0[2]
Dodine	1.65	+	8.5
	4.94	+	5.3
	14.81	+	2.7
	44.44	+	0.2
	133.33	+	-
	400.0	+	-

[1] percentage of cells in mitosis, determined in 1000 nuclei
[2] mean value of duplicate cultures

Chromosome aberration test

From the results obtained, it appeared that treatment of the human lymphocytes with the test substance did not increase in a statistically significant way the number of cells with gaps, breaks or exchanges, either in the absence or in the presence of the S9-mix.

At the two highest dose levels used, the test substance reduced the number of mitoses by 50% or more as compared with the control.

The positive control substances, methylmethanesulphonate and cyclophosphamide, gave the expected increase in the number of cells with structural chromosomal aberrations.

Table 6.48 - Chromosome aberration test with cultured human lymphocytes treated *in vitro* with dodine in the absence of S9-mix

Treatment dose (µg/ml)	N[1]	Number of cells with aberrations			% cells with aberrations		Mitotic [2] index (%)
		gaps[4]	breaks[5]	exchanges[6]	Incl. gaps	Excl. gaps	
Control	200	17	1	0	9.0	0.5	3.9
Dodine 0.37	200	11	1	0	6.0	0.5	3.8
Dodine 1.11	200	10	0	0	5.0	0.0	3.4
Dodine 3.33	200	21	1	0	11.0	0.5	2.2
Dodine 10.0	200	20	0	0	10.0	0.0	2.3
MMS[3] 30.0	200	39**	23***	17***	34.0***	16.5****	4.5

[1] N - number of metaphases analysed
[2] percentage of metaphases determined in 1000 nuclei (mean value of duplicate cultures)
[3] positive control substance: methylmethanesulphonate
[4] gaps include chromatid and isochromatid (chromosome) gaps
[5] breaks include chromatid and isochromatid (chromosome) breaks, interstitial deletions (minutes) and acentric fragments not associated with any obvious exchange process
[6] exchanges include chromatid and chromosome inter- and intrachanges
Statistics: Fisher's exact probability test (two-sided) *: $p<0.05$; **: $p<0.01$ *** : $p<0.001$

Table 6.49 - Chromosome aberration test with cultured human lymphocytes treated *in vitro* with dodine in the presence of S9-mix

Treatment dose (µg/ml)	N[1]	Number of cells with aberrations			% cells with aberrations		Mitotic [2] index (%)
		gaps[4]	breaks[5]	exchanges[6]	Incl. gaps	Excl. gaps	
Control	200	9	1	0	5.0	0.5	9.8
Dodine 0.57	200	9	2	0	5.5	1.0	8.3
Dodine 1.67	200	14	2	0	8.0	1.0	8.1
Dodine 5.0	200	18	1	0	9.5	0.5	4.6
Dodine 15.0	200	7	0	0	3.5	0.0	0.9
CP[3] 20.0	200	62***	51***	17***	48.5***	28.5***	2.4

[1] N - number of metaphases analysed
[2] percentage of metaphases determined in 1000 nuclei (mean value of duplicate cultures)
[3] positive control substance: cyclophosphamide
[4] gaps include chromatid and isochromatid (chromosome) gaps
[5] breaks include chromatid and isochromatid (chromosome) breaks, interstitial deletions (minutes) and acentric fragments not associated with any obvious exchange process
[6] exchanges include chromatid and chromosome inter- and intrachanges
Statistics: Fisher's exact probability test (two-sided); *: $p<0.05$; **: $p<0.01$ *** : $p<0.001$

Conclusion:

From the above findings it was concluded that dodine did not show any clastogenic activity in cultured human lymphocytes under the conditions employed in this evaluation.

B.6.4.1.3 Test for gene mutation in mammalian cells (Annex IIA 5.4.1)

An Investigation into the Possible Induction of Point Mutation at the HGPRT Locus of Chinese Hamster Ovary Cells by Dodine

Davis, P.B. (May 10, 1985) - Report no. R 85/105, performed by TNO, Division of Technology for society TNO, 2600 AE Delft, Netherlands.

Guidelines and GLP

The study was conducted according to GLP; no statement of compliance with recognised guideline was included in the report, the method was based on the "subculture method" of 0'Neill and Hsie, deviations from OECD Guideline 476 (1984) corresponding to E.U. guideline B.17, were that the results were not confirmed by an independent experiment.

The study is acceptable.

Materials and methods

Test substance: dodine technical, a white powder, Batch No. KG 8507, purity 98% was dissolved in ethanol (test substance was not soluble in DMSO). The concentration of the active test substance in the test solutions was not determined. The concentrations quoted in this report refer to the test substance as received and are nominal concentrations.

Cell culture: Chinese hamster ovary (CHO) fibroblast cells (cell line CHO-K1, ATTC no. CCL 61), maintained in Ham's F10 medium supplemented with newborn calf serum (15%) and glutamine (2 mM) were originally obtained from Flow Laboratories, Irvine, Scotland. Streptomycin (50 mg/l) and penicillin G (50 mg/l) were additionally added to the medium used in the toxicity and point mutation tests. The cells were passed once in medium containing aminopterin to reduce the

background frequency of 6-thioguanine resistant cells prior to carrying out the toxicity and point mutation tests.

A rat liver homogenate (S9) derived from rats previously exposed to Aroclor 1254 (500 mg/kg bw) was added to the medium when metabolic activation was required.

Principle of the method: Subsequently to exposure with dodine (with or without S9-mix), the cells were subcultured in growth medium to determine cytotoxicity of the test substance and to allow phenotypic expression prior to mutant selection. At the end of the expression period cells were subcultured in growth medium to determine viability of the cells and in selective medium to determine the number of mutants. From the results obtained, the mutant frequency (the number of mutants per 1'000'000 clonable cells) was calculated.

The following selective agent was used:

- 6-thioguanine (6TG)

The reference mutagens used as positive controls were:

- ethyl methanesulphonate (EMS), 0.4 µg/ml without S-9 mix, exposure period of 5h;

- dimethylnitrosamine (DMN), 2 and 5 µg/ml medium with S-9 mix, exposure period of 5h;

Table 6.50 - Study design

	With S9-mix	Without S9-mix
Toxicity test (exposure period of 5h)	0, 2.5, 5.0, 10.0, 15.0, 20.0 µg/ml (in triplicate)	
Point mutation test (exposure period of 5h)	0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 µg/ml	0, 2.5, 5.0, 10.0, 15.0 and 20.0 µg/ml

Procedure: In a preliminary solubility test, test substance remained soluble at concentrations up to 35 µg/ml, the highest concentration tested. A toxicity test (with and without metabolic activation) was carried out with concentrations of dodine between 0 and 20 µg/ml, and with an exposure duration of 5 h.

In view of the results obtained in the toxicity test (see Table 6.49) the point mutation test was carried out with test substance concentrations of 0 – 20 µg/ml (without metabolic activation) and 0 - 35 µg/ml (with metabolic activation) and an exposure duration of 5 h.

The density of cell suspension was adjusted to 175'000 cells/ml and portions of 20 ml (= 3'500'000 cells) were added to culture flasks and incubated for about 24 h. At least 4 concentrations of the test substance were then added to separate cell cultures in a small volume of solvent. Additional flasks receive the solvent alone, or a positive control compound. When metabolic activation was included, S9-mix was added just before addition of the test substance. After incubation for 5 h (with or without metabolic activation or 24 h (without metabolic activation only), the medium containing the test substance was removed, the cells washed, fresh growth medium was then added and incubation continued.

About 27 hours after the addition of the test substance, (on day 1 of the study) the cultures were trypsinized and counted. One 175 cm^2 plastic tissue culture flask, containing 3'000'000 cells and about 25 ml growth medium, or two flasks containing 1'500'000 cells and about 25 ml growth medium, were prepared from the cell suspensions of each concentration level. The cells were passaged anew at two- or three-day intervals during the expression period.

a) The initial survival of the cells was measured as follows: the cell suspensions obtained on day 1 were further diluted to a density of about 1000 cells/10 ml. Ten ml portions of each dilution so

obtained were transferred to each of five plastic Petri dishes. The Petri dishes were incubated for about 7 or 8 days. After this time the clones were clearly visible to the naked eye and were stained.

b) The frequency of 6-thioguanine (6TG) resistant mutants was measured as follows: On day 8 or 9 the cell cultures were trypsinized and the cells suspended in growth medium. Each cell suspension was diluted with medium lacking hypoxanthine and thymidine and containing 6TG (final concentration: 10 µM) to a cell density of 20'000 cells/ml. Ten ml portions of each dilution so obtained were transferred to each of 10 Petri dishes. The Petri dishes were then incubated for about 7 or 8 days and then the clones were stained.

c) The final survival of the cells (necessary for calculation of the absolute mutant frequency) was measured as follows: The cell suspensions (from b) above) were diluted with growth medium to a density of about 200 cells/10 ml. Ten ml portions of each of the dilutions were transferred to each of three Petri dishes. The Petri dishes were then further treated as described in a).

The clones in the initial survival, the mutant selection and the final survival dishes were fixed and stained. The clones on the initial survival plates were counted with an automatic colony counter; the clones on the mutation and final survival plates were counted by hand.

Treatment of results: The mean number of initial and final survivors and mutants was calculated. The mutant frequency (the number of mutants per 1'000'000 clonable cells), was then calculated from the number of mutants and final survivors.

Results:

Toxicity test

Significant decreases of survival cells due to toxicity were seen at 5, 10, 15, 20 µg/ml without metabolic activation and only at 20 µg/ml with metabolic activation.

Table 6.51 - Results of the toxicity test with dodine in CHO cells cultured *in vitro*

Dodine (µg/ml, nominal)	S9-mix -/+	Clonable cells (mean in 3 plates)	Survival (%)
0.0	-	590	100
2.5	-	587	99
5.0	-	430	73
10.0	-	334	57
15.0	-	88	15
20.0	-	#	-
0.0	+	444	100
2.5	+	466	105
5.0	+	470	106
10.0	+	522	118
15.0	+	451	102
20.0	+	245	55

#: survival too low

Point mutation test

Concentration-dependent increases in the number of mutants at the HGPRT-locus were not observed either with or without metabolic activation. Exposure to 15 µg/ml (without metabolic activation) and 30 µg/ml (with metabolic activation) resulted in significant (initial) toxicity for the CHO cells; this indicates that the test was carried out with appropriate concentrations of the test substance. Exposure to higher concentration (20 µg/ml without metabolic activation; and 35 µg/ml with metabolic activation) resulted in virtually complete mortality.

Exposure to EMS gave the expected increases in the mutation frequency. The mutagenicity of DMN vas somewhat lower than normal (2 µl/ml generally yields a mutant frequency of about $200/10^6$ survivors) indicating that the ethanol used as solvent may have reduced the efficiency of the metabolic activation system.

Table 6.52 - Results of the point mutation test with dodine in CHO cells cultured *in vitro*

Test substance (µg/ml, nominal)	S9-mix -/+	Mean relative initial survival (%)	Mean relative final survival (%)	Mutation frequency (per 1'000'000 clonable cells)
0.0	-	100	86	16
2.5	-	100	77	24
5.0	-	64	76	22
10.0	-	67	67	26
15.0	-	27	73	9
20.0	-	5	#	-
EMS (0.4 µl/ml)	-	40	53	811
0.0	+	100	94	32
5.0	+	103	93	15
10.0	+	83	90	18
15.0	+	69	122	17
20.0	+	54	95	7
25.0	+	34	99	6
30.0	+	17	101	9
35.0	+	#	#	-
DMN (2 µl/ml)	+	71	89	56
DMN (5 µl/ml)	+	17	72	149

#: survival too low

Conclusion:

It can be concluded from the results of this study that dodine does not induce mutations at the HGPRT-locus in CHO Chinese hamster ovary fibroblast cells under the conditions of the test.

B.6.4.2 *In vivo* genotoxicity testing (somatic cells) (Annex IIA 5.4.2)

B.6.4.2.1 Micronucleus test in rodents

Examination of Dodine in the Micronucleus Test

Willems, M.I. (May 22, 1985) - Report no. V 85.198/250208, performed by TNO, Division for nutrition and food research tno, 3700 AJ Zeist, Netherlands.

Guidelines and GLP

The study was conducted according to GLP; no statement of compliance with recognised guideline was included in the report, deviations from OECD Guideline 474 (1983) corresponding to E.U. guideline B.12, were that no signs of toxicity were apparent, which gives no indication whether bone marrow was actually exposed to a.s.

The study is considered as additional information.

Materials and methods

Test substance: dodine technical, a white powder, Batch No. KG 8507, purity 98% was dissolved in propylene glycol.

Animals: Thirty-six male and 36 female young, adult, albino mice (Swiss random) were obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, the Netherlands. The initial mean body weight of the males was about 28-29 g and that of the females about 21-23 g.

From the results obtained in the acute oral toxicity test (limit test) in mice, where the oral LD_{50} of dodine for mice was found to exceed 500 mg/kg bw, this latter dose was chosen for the present study.

Two groups of 15 males and 15 females each were treated once orally by gavage with either 5 ml/kg bw of a 10% suspension of the test material in propylene glycol (test group) or with 5 ml/kg bw propylene glycol (negative control group). A positive control group of 6 males and 6 females was treated once intraperitoneally with 1.5 mg/kg bw in 20 ml saline of the mutagen Mitomycin C.

The animals were weighed on days 0, 1, 2 and on day 3, just prior to sacrifice.

At 24, 48 and 72 h after treatment 10 negative controls (5/sex), 10 test animals (5/sex) and 4 positive controls (2/sex) were killed. Samples of bone marrow were collected from the femurs and bone marrow smears were prepared for microscopic examination. Five slides were prepared from each animal. The smears were air dried, fixed in methanol and stained according to May-Grunwald Giemsa.

The stained smears were examined by light microscopy to determine the incidence of micronucleated erythrocytes and the ratio of polychromatic to normochromatic erythrocytes.

The ratio of poly- and normochromatic erythrocytes was determined by counting the numbers of these cells in a total of 400 erythrocytes per animal.

The incidences of micronucleated cells were determined in a total of 1000 erythrocytes per animal.

Results:

Treatment with the dodine did not induce any apparent signs of intoxication. Mean body weights of test and control animals were fully comparable both in males and in females.

The incidence of micronucleated erythrocytes and the percentage of polychromatic erythrocytes in control and in test animals were fully comparable.

Positive control animals, killed at 24, 48 or 72 h after treatment with Mitomycin C, showed a considerably increased incidence of micronucleated erythrocytes. The percentage of polychromatic erythrocytes in bone marrow of positive control animals was decreased the lowest incidence occurring 48 h after treatment with Mytomycin C (see Table 6.51).

Table 6.53 - Mean numbers of micronucleated erythrocytes and percentage of polychromatic erythrocytes in bone marrow of mice treated with dodine

Time of sacrifice	Controls		dodine		Mitomycin C	
	% PCE[1]	MNC/1000 E (range)	% PCE[1]	MNC/1000 E (range)	% PCE[1]	MNC/1000 E (range)
Males						
24 h	51	1.4 (0-4)	51	1.2 (1-2)	55	28 (27-28)
48 h	59	0.6 (0-1)	47	1.6 (0-4)	17	10 (6-14)
72 h	59	0.4 (0-1)	52	0.8 (0-2)	28	7 (4-10)
Females						
24 h	53	1.4 (0-3)	49	0.4 (0-2)	39	14 (3-25)
48 h	61	0.6 (0-2)	50	1.2 (0-2)	23	15 (14-15)
72 h	59	1.0 (0-3)	63	1.0 (0-2)	40	4 (2-5)

[1]) % PCE = PCE x 100/(PCE + NCE)
MNC = Micronucleated cells
E = Erythrocytes (poly- and normochromatic)
NCE = Normochromatic erythrocytes
PCE = Polychromatic erythocytes

Conclusion:

The results of the present micronucleus test did not provide any evidence of chromosomal damage and/or damage to the mitotic apparatus caused by oral administration of 500 mg dodine/kg bw.

Mutagenicity Test on Dodecylguanidine Acetate, Technical *in Vivo* Mammalian Micronucleus Assay

Hemalatha Murli, (April 14, 1992) - Report no. 14710-0-455, performed by Hazleton Washington, Inc., Kensington, MD 20898, USA.

Guidelines and GLP

The study was conducted according to GLP and U.S. EPA FIFRA Guideline 84-2, with no significant deviations from OECD Guideline 474 (1983).

The study is acceptable.

Materials and methods

Test substance: dodine technical, a white powder, APA Batch No. 303/90, purity 94% (by HPLC), titration: 97.6% in certificate of analysis.

Animals: Adult male and female mice, strain ICR, were purchased from Harlan Sprague-Dawley, Inc., Frederick, MD. The weight range of the animals used in the micronucleus assay was 24.9 - 38.8 g and 23.6 - 31.5 g for the males and females, respectively. The age of the animals at the time of dosing was about 8 weeks.

Dose range-finding study

(Hemalatha Murli, Report No. 14710-0-459PO)

A dose range-finding study was conducted in 3 mice/sex/dose group. The weight range of the animals used was 31.0 - 38.6 g and 24.4 - 30.6 g for the males and females, respectively; the age of the animals was about 8 weeks at the time of dosing. Since the test article did not form suitable

solutions/suspensions in sterile deionised water or carboxymethylcellulose, corn oil was selected as the vehicle. The test article was suspended in corn oil and dosed by oral gavage at 50.0, 162.5, 275, 387.5, and 500 mg/kg bw in a dosing volume of 10 ml/kg bw. All animals were examined after dosing and daily throughout the duration of the study (three days) for toxic effects and/or mortalities.

Micronucleus assay

The test article was suspended in corn oil and dosed by oral gavage at 100, 200 and 400 mg/kg bw in a dosing volume of 10 ml/kg bw, based upon the results of the previously conducted dose range-finding assay. Ten animals (5/sex) were randomly assigned to each dose/harvest time group. A second group of animals (designated Secondary Dose Group) was also assigned to the study and was dosed with the high dose of dodine; these animals were only used in the assay as replacement for any which died in the primary dose group. All animals were observed immediately after dosing and periodically throughout the duration of the assay for toxic symptoms and/or mortalities.

Vehicle (corn oil, 10 ml/kg bw) and positive control (cyclophosphamide, CP, 80 mg/kg bw in sterile deionised water) groups euthanized approximately 24 h after dosing were included in the assay. The animals were dosed with dodine and were euthanized approximately 24, 48 and 72 hours after dosing for extraction of the bone marrow from (both) tibiae.

The marrow was aspirated from the bone and mixed in a syringe with 0.1 ml foetal calf serum to form a suspension. The cells were then placed on slides and air-dried, fixed in methanol, and stained in May-Grünwald solution.

The coded slides were scored for micronuclei and the polychromatic (PCE) to normochromatic (NCE) cell ratio. 1000 PCEs per animal were scored. The frequency of micronucleated cells was expressed as percent micronucleated cells based on the total PCEs present in the scored optic field. The normal frequency of micronuclei in this mouse strain is about 0.0-0.4%.

The frequency of PCEs versus NCEs was determined by scoring the number of PCEs and NCEs observed in the optic fields while scoring the first 1000 erythrocytes.

Evaluation criteria: The analysis of the data was performed using an Analysis of Variance on the square root arcsine transformation which was performed on the proportion of cells with micronuclei per animal (square root arcsine proportion). Once the Analysis of Variance had been performed, Tukey's Studentized range test (HSD) with adjustment for multiple comparisons (Sokal and Rohlf, 1981) was used at each harvest time to determine which dose groups, if any, were significantly different ($p<0.05$) from the vehicle control. Analyses were performed separately for each harvest time and sex combination, and also at each harvest time for the sexes combined.

The criteria for determining a positive response involved a statistically significant dose-related increase in micronucleated PCEs, or the detection of a reproducible and statistically significant positive response for at least one dose level. A test article that induced neither a statistically significant dose-response nor a statistically significant and reproducible increase at one dose level was considered negative.

Results:

Dose range-finding study

No toxic effects were noted in any of the animals observed immediately after dosing. Approximately 41 hours after dosing, one male at the 387.5 mg/kg bw dose-group, and one male from the 500 mg/kg bw dose-group were found dead: All remaining animals in the 387.5 and 500 mg/kg bw dose groups had rough hair coats. All animals in the 50.0, 162.5, and 275 mg/kg bw dose groups appeared normal and healthy. Prior to euthanasia at the completion of the dose range-finding study, all remaining animals in the 387.5 and 500 mg/kg bw dose groups appeared languid, with rough hair coats and all animals in the 50.0, 162.5, and 275 mg/kg bw dose groups appeared normal

and healthy. Based upon the mortality data and toxic symptoms in the animals in this study, the MTD was estimated to be 500 mg/kg bw (the LD_{50} could not be calculated accurately).

Micronucleus assay

All animals in the vehicle and positive control groups appeared normal after dosing and remained healthy until the appropriate harvest times. All test article dosed groups appeared normal immediately after dosing. Approximately 6 hours after dosing, one male dosed with 400 mg/kg bw was found dead. Prior to the 24 hour harvest, one male from the secondary high dose group was found dead. Prior to the 48 hour harvest, one male from the 200 mg/kg bw dose group, and one male from the 400 mg/kg bw dose group had distended abdomens. Prior to the 72 hour harvest, one male from the 400 mg/kg bw dose group had a distended abdomen. All remaining animals from the 100, 200, and 400 mg/kg bw dose groups appeared normal and remained healthy until the appropriate harvest times.

Dodine induced no significant increases in micronucleated polychromatic erythrocytes over the levels observed in the vehicle controls in either sex or at any of the harvest times. The positive control, CP, induced significant increases in micronucleated PCEs in both sexes, with means and standard errors of 1.20% ± 0.25% and 2.12% ± 0.40% for the males and females, respectively.

Table 6.54 - Micronucleus data summary table

Treatment / dose	Harvest time (h)	% Micronucleated PCEs mean of 1000 per animal ± S.E.			Ratio PCE:NCE mean ± S.E.	
		Males	Females	Total	Males	Females
vehicle control, corn oil / 10 ml/kg bw	24	0.04 ± 0.04	0.04 ± 0.04	0.04 ± 0.03	0.25 ± 0.04	0.59 ± 0.09
Positive control, CP / 80 mg/kg bw	24	1.20 ± 0.25*	2.12 ± 0.40*	1.66 ± 0.27*	0.30 ± 0.07	0.67 ± 0.21
Dodine / 100 mg/kg bw	24	0.06 ± 0.02	0.06 ± 0.04	0.06 ± 0.02	0.35 ± 0.07	0.51 ± 0.12
	48	0.06 ± 0.04	0.02 ± 0.02	0.04 ± 0.02	0.67 ± 0.11	0.37 ± 0.08
	72	0.02 ± 0.02	0.00 ± 0.00	0.01 ± 0.01	0.53 ± 0.15	0.48 ± 0.15
Dodine / 200 mg/kg bw	24	0.00 ± 0.00	0.06 ± 0.02	0.03 ± 0.02	0.45 ± 0.13	0.63 ± 0.15
	48	0.00 ± 0.00	0.04 ± 0.02	0.02 ± 0.01	0.53 ± 0.15	0.66 ± 0.20
	72	0.00 ± 0.00	0.04 ± 0.04	0.02 ± 0.02	0.59 ± 0.10	0.30 ± 0.07
Dodine / 400 mg/kg bw	24	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.23 ± 0.05	0.63 ± 0.15
	48	0.06 ± 0.04	0.06 ± 0.02	0.06 ± 0.02	0.64 ± 0.12	0.64 ± 0.09
	72	0.08 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.36 ± 0.06	0.29 ± 0.06

* : Significantly greater than the corresponding vehicle control, $p<0.05$.

Conclusion:

The test material, dodine technical, did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes under the conditions of this assay and was considered negative in the mouse bone marrow micronucleus test.

B.6.5 Long-term toxicity and carcinogenicity (Annex IIA 5.5)

Chronic toxicity and carcinogenicity study of dodecylguanidine acetate (dodine) in the Sprague-Dawley rat by dietary administration

Dange, M. (September 1998) – Report No.SA 95083, 10/09/1998. Performed by Rhone Poulenc Agro, Sophia, Antipolis, France; dates of experimental work: 01 March 1995 to 23 March 1998.

Guidelines and GLP:

The study was conducted in compliance with FIFRA 83-5 (1984), and OECD 453 (1981). The sudy is GLP compliant.

The study is acceptable.

Material and methods:

The objectives of this study were to determine the potential chronic toxicity and carcinogenicity of dodine in rats following continuous dietary administration for one year (chronic toxicity) and a minimum of 2 years (carcinogenicity).

Experimental design: Dodine (batch DA717: 98.6%) was administered in the diet to 60 male and 60 female Sprague-Dawley rats per group at concentrations of 0, 200, 400 or 800 ppm (equivalent to approximately 0, 10.17, 20.34 or 41.93 mg/kg bw/day for males and 0, 13.19, 26.5 or 53.50 mg/kg bw/day for females, respectively) for 106 weeks. In addition, 10 male and 10 female rats per group administered the same concentrations were terminated at 53 weeks. Dose selection for the high dose was based on previous toxicity studies in which doses greater than 800 ppm caused decreased body weight gain.

Table 6.55 – Details of group size and treatment

Group	Diet concentration of dodine	Number of animals per sex	
	(ppm)	Interim sacrifice[1]	Terminal sacrifice[2]
1	0	10	60
2	200	10	60
3	400	10	60
4	800	10	60

1: Corresponding to chronic toxicity phase of study with sacrifice during week 53
2: Corresponding to carcinogenicity phase of study with sacrifice during weeks 105 and 106

Daily observations: Animals were checked for moribundity and mortality twice daily (once daily on week-ends or public holidays). Observed clinical signs were recorded at least once daily for all animals. The nature, onset, severity, duration and reversibility of clinical -signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces. Detailed physical examinations including palpation for masses were performed twice monthly during the first 13 weeks of the study and weekly thereafter. The appearance, progression, duration, location and dimension of the masses were recorded. Debilitated animals were observed carefully and were eventually isolated.

Body weights: Each animal was weighed twice during the acclimatization period then weekly for the first 13 weeks of study and once every 4 weeks thereafter.

Food consumption: The weight of food supplied and of that remaining at the end of the food consumption period was recorded for each cage. Food consumption was recorded weekly during the first 13 weeks of treatment and once every 4 weeks thereafter.

The weekly mean achieved dosage intake in mg/kg/day was calculated based on food consumption intervals.

Ophthalmological examination: Funduscopic (indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations were performed on al1 animals during the acclimatization phase and on all surviving animals after one and two years. Each eye was first examined by direct ophthalmoscopy,

and then after instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme), each eye was re-examined by means of a slit lamp and an indirect ophthalmoscope.

Clinical pathology: Prior to the start of treatment, haematology, clinical chemistry and urinalysis were performed on 10 male and 10 female health-screen animals.

Laboratory investigations: Haematology and clinical chemistry were performed on the animals of the interim sacrifice group at Weeks 26 and 52 ; urinalysis was performed on the same animals at Weeks 25 and 51. Haematology and clinical chemistry was performed for the first ten surviving animals of the terminal sacrifice groups at Weeks 26, 52, 78 and 104; urinalysis was performed on the same animals at Weeks 25, 51, 79 and 103.

Blood was sampled from ether (Weeks 26, 52) or isoflurane (Weeks 78, 104) anesthetized animals by puncture of the retro-orbital venous plexus after overnight fasting. Blood was collected in tubes containing EDTA for hematology (0.5 m1), lithium heparin (for plasma) for clinical chemistry (2.5 m1) and sodium citrate for coagulation (0.9 m1). Urine samples were collected overnight. Animals had diet and water withdrawn during the overnight (approximately 16 hours) collection period. When possible, a blood smear was prepared from moribund animals, just before necropsy.

Hematology: Red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell total and differential counts and platelet count were assayed using a Technicon H1 (Byer Diagnostics, Puteaux, France).

A blood smear was prepared and stained with Wright stain. It was examined only when the results of Technicon H1 determinations were abnormal.

Reticulocytes were stained with brilliant cresyl blue. A smear was prepared but not examined since no significant red blood cell changes were observed. For moribund animals, the blood smears were stained with Wright stain but not examined (no request of the study pathologist).

Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).

Clinical chemistry: Any significant change in the general appearance of the plasma was recorded. Tota1 bilirubin, glucose, urea, total protein, albumin, total cholesterol, triglycerides and inorganic phosphate concentrations, and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using a Cobas Fara (Roche, Neuilly sur-Seine, France).

Chloride, sodium, potassium, calcium and creatinine concentrations were measured using an Astra 4 (Beckman, Gagny, France).

Urinalysis: Any significant change in the general appearance of the urine was recorded.

Post Mortem examinations

Necropsy procedure: All animals, either found dead or moribund or killed by design, were necropsied. Any animal found dead during the study was necropsied as soon as possible but within 24 hours of the time of discovery. Animals for interim and terminal necropsies and animals in extremis were killed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 50 or 60 mg/kg body weight).

For scheduled necropsies in study Weeks 53 (chronic toxicity phase) and 105 - 106 (carcinogenicity phase), an approximately equal number of animals randomly distributed amongst a11 groups were killed on each day. Animals were fasted overnight prior to scheduled sacrifice. The terminal body weight of all animals at scheduled sacrifice was recorded. Necropsy included the macroscopic examination of external surfaces, all orifices, all major organs, tissues and body cavities.

Organ weight: Adrenal gland, brain, epididymis, heart, kidney, liver, ovary, pituitary gland, prostate, spleen, testis, thymus, thyroid gland (with parathyroid) and uterus were weighed fresh at scheduled sacrifices only. Paired organs were weighed together.

Tissue collection: The following organs or tissues were sampled:

Adrenal gland	Mammary gland
Aorta	Ovary
Articular surface (femoro-tibial joint)	Pancreas
Bone (sternum)	Pituitary gland
Bone marrow (sternum)	Prostate Sciatic nerve
Brain	Seminal vesicle
Epididymis	Skeletal muscle
Esophagus	Skin
Eye and optic nerve	Spinal cord (cervical, thoracic, lumbar)
Harderian (lachrymal) gland	Spleen
Heart	Stomach
Intestine (duodenum, jejunum, ileum, testis cecum, colon, rectum)	Sub maxillary (salivary) gland
	Thymus
Kidney	Thyroid gland (with parathyroid)
Larynx	Tongue
Liver Trachea	Urinary bladder
Lung	Uterus (with cervix)
Lymph nodes (submaxillary, mesenteric)	Vagina

For each sacrificed animal, two bone marrow smears were prepared from one femur, one of which was stained with May-Grunwald Giemsa and the other one was kept unstained. These smears were not examined.

In addition to the above mentioned organs and tissues, macroscopic abnormalities and masses (including regional lymph nodes, if possible) were collected. Samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, harderian gland, epididymis and testis which were fixed in Davidson's fixative.

Histopathology: All the above mentioned samples were embedded in paraffin wax and histological sections, stained with hematoxylin and eosin, were prepared from all organs and tissue samples from all animals in all groups.

For animals assigned to the chronic toxicity phase of the study, histopathological evaluations were performed on all tissues of all animals found dead or moribund, all animals in the control and high dose groups, on the liver, lung, kidney and spleen of all the animals from the low and intermediate dose groups and on macroscopic findings.

For animals assigned to the carcinogenicity phase of the study, histopathological evaluations were performed on all tissues of all animals.

All suspected tumors were diagnosed using special stains when necessary and the incidences of benign and malignant tumors of different types in the various treatment-groups were tabulated. The pathologist decided whether or not a tumour was the cause of death.

Statistical analysis: Means and standard deviations (STD) were calculated for each sex separately for each group at each time period.

Average food consumption (per rat and per day) was calculated per cage as follows:

$$\frac{\text{Total food consumed}}{\quad}$$

Sum of number of surviving days of each rat

For food consumption the statistical unit was the cage.

Results of body weight, body weight gains and food consumption were intercompared for the exposed groups and the control group by use of BARTLETT's test for homogeneity of variances, analysis of variance (ANOVA).

- If BARTLETT'S test indicated homogeneous variances and the ANOVA was significant, the group means were inter-compared to the control mean using the DUNNET's test.

- If BARTLETT's test indicated heterogeneous variances, non-parametric statistical procedure was performed using the KRUSKAL-WALLIS non-parametric one-way analysis of variance by ranks. If the KRUSKAL-WALLIS test was significant, MANN-WHITNEY was used to compare each group to the control.

Statistical analyses were performed using SAS programs.

Results of hematology (except percentages and absolute values for eosinophils, basophils, monocytes and large unstained cells), clinical chemistry, urinalysis variables (pH, volume and refractive index) and organ weights were inter-compared for the exposed groups and the control group with PathTox computer system by use of DUNNET's test if BARTLETT's test indicated homogeneous variances. If BARTLETT's test indicated non-homogeneous variances, variables were analyzed using SAS programs and a non-parametric statistical procedure was performed using the KRUSKAL-WALLIS non-parametric one-way analysis of variance by ranks. If the KRUSKAL-WALLIS test was significant, MANN-WHITNEY's test was used to compare each group to the control.

Statistical analysis of animals with convulsions was performed using Fisher's Exact Test (one-tail).

Results:

Mortality and survival:

In both sexes, there was no significant trend or group differences based on the reported mortality rates. Statistical analysis showed no differential mortality between the treated groups and the control group in females and in males, supporting the conclusion that the mortality rates were comparable among all groups (see Table 6.56).

Table 6.56 – Mortality of the rats

	After 1 year chronic and carcinogenicity phase		After 2 years carcinogenicity phase	
	male	female	male	female
Control	2/70	1/70	30/60	23/60
200 ppm	4/70	3/70	37/61	28/60
400 ppm	3/70	2/70	32/61	24/60
800 ppm	2/70	1/70	23/60	24/60

Clinical signs:

There was a dose-responsive increase in the number of treated males (control, low-, mid- and high-dose) with several clinical signs of toxicity, including no righting reflex (0, 2, 3, 4 respectively), absent traction reflex (1, 0, 1 and 5, respectively) absent grasping reflex (2, 4, 6 and 8, respectively) and hunched posture (1, 2, 4 and 7, respectively). However, a review of the individual animal data reveals that the signs are agonal and not evidence of neurotoxicity (Semino, G (17 January 2000). Dodine: Evaluation of clinical signs in the rat chronic study). No statistical analyses were done on these data. Other clinical signs seen in control and treated males and females but not in a dose-

responsive incidence included clonic/tonic convulsions, reduced motor activity, hind limb edema/sore/induration/limping, hind limb paralysis, prostration, appears thin and fur piloerection. The selected clinical signs are presented below (Table 6.57).

Table 6.57 – Selected clinical signs in rats dosed with dodine for up to 106 weeks

	Dose (ppm)							
	Males (n=70)				Females (n=70)			
	0	200	400	800	0	200	400	800
Tonic/clonic convulsions	1	5	2	2	4	6	3	2
Reduced motor activity	8	11	12	12	8	13	15	9
Ind limb edema/sore/induration/limping	19	16	12	6	1	3	2	0
Hind limb paralysis	4	1	3	1	0	1	1	0
Prostation	1	6	3	1	5	8	5	8
Appears thin	16	14	16	12	12	15	15	12
No righting reflex	0	2	3	4	2	1	3	3
Traction reflex absent	1	0	1	5	1	4	0	0
Grasping reflex absent[a]	2	4	6	8	3	8	2	5
Posture hunched	1	2	4	7	6	4	9	9
Piloerection	5	11	10	10	10	12	11	13

[a] most of the animals showing grasping absent, also show no righting reflex and/or traction absent

Body weight:

The body weight evolution of the animals treated at 800 ppm was reduced up to 10% in males and to 15% in females. Mean body weights for high dose females were statistically lower than the control values beginning at Week 1 and continuing throughout the study. For high dose males, statistically lower mean body weights were noted for Weeks 1 to 37 and Weeks 85 and 89.

Food consumption:

Food consumption was occasionally decreased in animals treated at the high dose level.

Achieved dosages:

The mean achieved dosage intake per group was as follows:

	Mean achieved test material intake (weeks 1 – 49) Interim sacrifice animals	
Diet concentrations ppm	Males mg/kg bw/day	Females mg/kg bw/day
200	11.712	15.549
400	23.244	29.928
800	47.003	61.806

	Mean achieved test material intake (weeks 1 – 101) Interim and terminal sacrifice animals	
Diet concentrations ppm	Males mg/kg bw/day	Females mg/kg bw/day
200	10.171	13.191
400	20.336	26.459
800	41.928	53.504

Ophthalmological examinations:

Ophthalmoscopic examination of controls and treated animals after 49 and 100 weeks did not reveal any treatment-related findings.

Clinical pathology

Haematology:

When compared with control group, mean total white blood cell count was lower by 24% (P≤0.05) in males at 800 ppm on Week 26. This variation was accompanied by a lower mean absolute lymphocyte count (-26%, P≤0.05). The same trend, non statistically significant, was noted on Week 52, mean total white blood cell count was lower by 22% and mean absolute lymphocyte count by 20%.

In females, statistically significantly higher mean prothrombin times were observed on Week 26 at 800 ppm (+4%, P≤0.01) and on Week 52 at 800, 400 and 200 ppm (+8%, +6% and +7% respectively, P≤0.01). These changes were considered to be of no biological or toxicological significance in the absence of a clear dose-effect relationship and considering the low amplitude of change relative to controls.

On Week 104, in males at 400 ppm, mean corpuscular haemoglobin was statistically significantly higher (+12%, P ≤0.01), when compared with the control group. This difference appeared to be attributable primarily to one rat which had high mean corpuscular haemoglobin, as a result of a slightly lower red blood cell count and normal haemoglobin concentration; mean corpuscular volume was also higher for this animal. In addition, on Week 104, at 400 ppm, one female rat had very low red blood cell count, haemoglobin concentration and hematocrit, with higher mean corpuscular volume. At 800 ppm one female rat had higher total white blood cell count, with higher neutrophil count. These findings in individual animals are considered to be spontaneous in occurrence and unrelated to treatment.

Clinical chemistry:

No significant treatment-related variations were noted at any of the scheduled blood sampling periods for any of the parameters assayed. Statistically significant differences were observed in some treated groups, when compared with the controls but as described below were not judged to be treatment-related.

A statistically significant lower mean alkaline phosphatase activity (-18%, P ≤0.01) was seen, on Week 26, in males at 800 ppm. However this difference was mainly attributed to unusually high activities in two rats in the control group. On Week 26, in females, at 800 and 200 ppm, and on Week 78, in males, at 800 and 400 ppm, a tendency towards lower triglycerides concentrations, when compared to control values, was seen. However these changes were transient and no clear dose-effect relationship was noted. On Week 78, mean potassium concentration was higher by 9% (P ≤0.05) in females treated at 800 ppm, however as all the individual values remained within the control range, this difference was not considered biologically or toxicologically significant.

On Week 104, mean alkaline phosphatase activities were higher at 800 and 400 ppm in females (+310% and +150% respectively; P ≤0.01). However, these changes were mainly due to 1/10 and 2/10 high activities, respectively, in each treated group. The statistical changes in cholesterol concentration in 200 ppm females at Week 26 and in ALAT for 200 ppm males at Week 104 were considered to be spurious findings in the absence of dose responses.

Urinalysis:

A tendency towards higher protein content was noted, when compared with control groups, on Week 25 in all male treated groups and on Week 25 and 51 in females treated at 800 ppm. This finding was considered to be of minimal importance and of no toxicological significance.

When compared with controls, statistically significantly lower mean urinary volumes were noted at 800 ppm, in males on Week 25 and 51 (-47%, P ≤0.01 and -29%, P ≤0.05 respectively) and in females on Week 51 (-46%, P ≤0.05). In view of the scattering of the individual values, these changes were considered to be of no toxicological importance. At 800 ppm, other isolated statistically significant changes were seen on one sampling period and therefore not considered to be important, for example mean refractive index was slightly higher in males on Week 25 (P ≤0.01) and in females on Week 79 (P ≤0.05), mean pH was slightly lower in males on Week 25 (-7%, P ≤0.05) and in females on Week 51 (-8%, P ≤0.05). Therefore no meaningful changes were noted in any of the parameters assayed.

Post Mortem examinations:

Terminal body weight and organ weights

Animals killed at interim sacrifice: At interim sacrifice on Week 53, up to 800 ppm, there was no statistically significant difference in mean terminal body weight of treated rats when compared to control group.

A few statistically significant mean organ weight changes were noted at interim sacrifice. However, in the absence of a dose-effect relationship, these changes (higher mean pituitary to brain weight ratio in males at 200 ppm only, lower mean uterus weight and uterus to body weight ratio at 200 ppm only, lower mean brain to body weight ratio in females at 200 ppm only and higher mean ovary weight and ovary to body weight ratio at 400 ppm only) were considered incidental.

Animals killed at final sacrifice: At final sacrifice after at least 104 weeks of treatment, up to 800 ppm, there was no statistically significant difference in mean terminal body weight of treated rats when compared to control group. However, a tendency towards lower mean terminal body weight was noted in males (-5%) and females (-11%) from the 800 ppm group consistent with the lower body weight gain of this group during the study. A statistically significant minimally lower (-10%, P ≤0.05) liver to brain weight ratio was noted in males at 800 ppm. However, in the absence of a similar change in the absolute weight or the ratio to body weight, this change was not considered to be biologically or toxicologically significant. At 400 and 800 ppm, there was a higher mean epididymis weight (absolute weight and ratio to body weight) in males and a higher brain to body weight ratio in females. However, in the absence of a dose-effect relationship, these changes were not considered to be treatment-related. In the absence of a similar effect at higher dietary concentrations, the statistically significant lower heart weight (absolute weight and ratio to brain weight) noted in females at 400 ppm only was considered incidental.

Table 6.58 – Mean absolute organ weights and terminal body weight (g) at final sacrifice in male and female rats

		0 ppm		200 ppm		400 ppm		800 ppm	
		M	F	M	F	M	F	M	F
Terminal body weight	N	30	37	24	32	29	36	37	36
	MEAN	634.5	493.4	629.2	481.2	647.6	448.9	600.5	439.4
	SD	110.16	84.18	100.76	110.00	113.16	105.31	80.30	93.93
Brain	N	30	37	24	32	29	36	37	36
	MEAN	2.237	2.076	2.235	2.050	2.281	2.085	2.293	2.051
	SD	0.0876	0.1001	0.0916	0.0685	0.1277	0.1216	0.1139	0.0935
Heart	N	30	37	24	32	29	36	37	36
	MEAN	2.260	1.688	2.303	1.590	2.316	1.511**	2.141	1.571
	SD	0.3103	0.2562	0.4203	0.2390	0.4629	0.2207	0.3515	0.2203
Liver	N	30	37	24	31	29	36	37	36
	MEAN	15.89	13.83	15.29	12.28	15.23	12.73	14.62	12.64
	SD	2.630	4.031	2.754	3.388	2.562	4.835	1.963	4.609
Pituitary gland	N	30	37	24	32	29	36	37	36
	MEAN	0.033	0.056	0.030	0.036	0.035	0.064	0.024	0.052
	SD	0.0618	0.0620	0.0324	0.0366	0.0590	0.0716	0.0261	0.0689
Spleen	N	30	37	24	32	29	36	37	36
	MEAN	1.185	0.890	1.233	0.858	1.282	1.227	1.135	0.898
	SD	0.3241	0.2085	0.2474	0.1538	0.3617	2.1487	0.3289	0.2949
Kidney	N	30	37	24	32	29	36	36	36
	MEAN	5.192	2.999	4.993	2.969	4.790	2.867	4.525	2.905
	SD	2.1403	0.5344	1.2861	0.5334	0.9116	0.4392	1.1779	0.5302
Adrenal	N	30	37	24	32	29	36	37	36
	MEAN	0.142	0.113	0.091	0.099	0.097	0.091	0.087	0.105
	SD	0.2718	0.0700	0.0316	0.0565	0.0504	0.0339	0.0291	0.0461
Thymus	N	30	33	21	30	28	34	36	36
	MEAN	0.268	0.219	0.257	0.291	0.352	0.302	0303	0.267
	SD	0.1676	0.1604	0.1599	0.1540	0.2673	0.2218	0.1878	0.2141
Thyroid	N	30	37	24	32	29	36	37	36
	MEAN	0.053	0.070	0.046	0.044	0.056	0.046	0.063	0.039
	SD	0.0250	0.1913	0.0142	0.0328	0.0221	0.0397	0.0698	0.0265
Epididymides	N	30		24		29		37	
	MEAN	1.425		1.447		1.578**		1.509*	
	SD	0.2369		0.2283		0.1979		0.3380	
Prostate	N	30		24		29		37	
	MEAN	1.205		1.294		1.196		1.044	
	SD	0.3004		1.0538		0.4155		0.2533	
Testis	N	30		24		29		37	
	MEAN	3.441		3.388		3.671		3.375	
	SD	0.6832		0.7191		0.6720		0.9303	
Ovary	N		37		32		35		36
	MEAN		0.268		0.147		0.127		0.135
	SD		0.5074		0.1267		0.0966		0.1167
Uterus	N		37		32		36		36
	MEAN		0.778		0.886		0.729		0.893
	SD		0.4688		0.6238		0.2919		0.5161

mean value of group was significantly different from control with Dunnett`s test or Mann-Whitney`s test of significance

* alpha = 0.05

** alpha = 0.01

N : number of animals

Gross pathology

Animals killed at interim sacrifice: At interim sacrifice on Study Week 53, up to 800 ppm, there was no evidence of treatment-related macroscopic changes.

Animals killed or dying during the treatment period*:* A total of 221 animals were either found dead or killed on humane grounds during the entire study period. At necropsy, there was no evidence of specific changes considered to be attributable to dodine administration. The main factors having contributed to death were those usually encountered in aging Sprague-Dawley rats, i.e. severe nephropathy (end-stage renal disease), large pituitary gland tumors, or large, frequently ulcerated and/or hemorrhagic subcutaneous/mammary gland tumors.

Animals killed at final sacrifice: At final sacrifice after at least 104 weeks of treatment, a lower incidence of dark spots on the liver and of ovarian cysts was noted in female rats from the 400 and 800 ppm groups when compared to control group. At 800 ppm, there was a slight numerical increase of female rats presenting enlarged and/or white mottled adrenal glands when compared to control group; however, in the absence of a clear dose-effect relationship, this change was considered incidental.

Microscopic pathology

Non-neoplastic findings

Animals killed at interim sacrifice*:* At interim sacrifice on Week 53, up to 800 ppm, there was no evidence of a treatment-related effect. All the microscopic changes observed were recognized as ones that occur spontaneously in rats of this age and strain. There was an increase in incidence of progressive cardiomyopathy noted in females from the 800 ppm group (6/10 or 60%) when compared to control group (2/10 or 20%). However, a similar effect was not evidenced in the decedent animals nor in animals sacrificed at the end of the treatment period. In addition, progressive cardiomyopathy is recognized as a spontaneous cardiac change occurring in aging rats, the amplitude of the change remained minimal and within the range usually observed in untreated control rats in this type of study; therefore, it was considered incidental.

Animals killed or dying during the treatment period: There was no evidence of treatment-related changes in the incidence of non-neoplastic microscopic changes in rats that died or were killed moribund during the study. Some numerical increases or decreases in incidence of various non-neoplastic microscopic findings were observed but most of these changes were either not confirmed in the final sacrifice animals, not accompanied by changes in organ weights or within the control range for rats. All the changes were recognized as ones that do occur spontaneously in aging rats of this strain. Some changes which were also evidenced in the final sacrifice animals will be discussed in the following section of the report. All the other changes were considered non significant.

Animals killed at final sacrifice*:* There was no evidence of any treatment-related non-neoplastic change in the animals killed after at least 104-weeks of continuous exposure to dodine by dietary administration. The following apparent numerical increases in incidence were noted in treated animals when compared to control group:

- ovary granulosa/theca cell hyperplasia, characterized by focal increase in number of granulosa/theca cells. This change was generally unilateral and present as a single small focus of minimal severity. There was an apparent increase in incidence of this change at 800 ppm in final sacrifice females when compared to control group (Table 6.59). This change was not accompanied by a correlative change in ovarian weight and no ovarian granulosa/theca cell tumors were observed after two years of treatment. There was no statistically significant difference when compared to control group. Therefore, this change was not attributed to test compound administration.

Table 6.59 - Group-related incidence of ovary granulosa/theca cell hyperplasia in female rats

	0 ppm	200 ppm	400 ppm	800 ppm
Interim sacrifice	0/10 (0%)	-	-	0/10 (0%)
Decedents	1/22 (5%)	1/28 (4%)	4/24 (17%)	2/24 (8%)
Final sacrifice	3/37 (8%)	1/32 (3%)	0/36 (0%)	8/36 (22%)
total	4/69 (6%)	2/60 (3%)	4/60 (7%)	10/70 (14%)
Prevalence tumor tests[a]	p= 0.0264*	p = 0.4017	p = 0.4083	p = 0.0672

* p<0.05
** p<0.01
*** p<0.001

[a]: Beneath the control incidence is the p-value associated with the trend test. Beneath each treated group's incidence is the p-value corresponding to the pair wise comparison between the treated and control groups. All statistical tests conducted were one-sided tests.

- Numerical increases in the incidence of prostate and seminal vesicle atrophy characterized by a flattened epithelium lining the glands and a decreased luminal colloid content were observed for the decedent males from the 800 ppm group when compared to controls. However, in the absence of a dose-response at terminal sacrifice, changes in organ weights or gross abnormalities, these increases were considered incidental.

All of these changes were recognized as the ones that do occur spontaneously in rats of this strain and age, and their overall incidence was generally within the expected range for untreated control animals.

The following numerical decreases in incidence were noted in treated animals when compared to control group:

- kidney pelvic mineralization and transitional cell hyperplasia: There was a dose-related lower incidence of pelvic mineralization in male rats killed at final sacrifice; the incidence was 11/30 (37%), 7/24 (29%), 4/29 (14%) and 6/36 (17%) at 0, 200, 400 and 800 ppm, respectively. There was a dose-related lower incidence of transitional cell hyperplasia of the renal pelvis in females killed at final sacrifice; the incidence was 16/37 (43%), 14/31 (45%), 13/36 (36%) and 8136 (22%) at 0, 200, 400 and 800 ppm, respectively.
- liver bile duct hyperplasia: there was a lower incidence of this change at 400 and 800 ppm in males and at all dose levels in females killed at the end of the treatment period. The incidence was 15/30 (50%), 12/24 (50%), 10/29 (34%) and 10/37 (27%) in males and 22/37 (59%), l0/32 (31%), 13/36 (36%) and 7/36 (19%) in females at 0, 200, 400 and 800 ppm, respectively.
- liver angiectasis: there was a lower incidence at all dose levels in females killed at the end of the treatment period. The incidence was 21/37 (57%), 12/32 (38%), 14/36 (39%) and 8/36 (22%) at 0, 200, 400 and 800 ppm, respectively. This change did correlate with the lower incidence of dark spots noted at necropsy in these animals.

Lower incidence of spontaneous age-related degenerative changes such as the ones described above are frequently observed in this type of study in association with a minimal body weight effect and/or reduced food consumption, are not considered to reflect a direct effect of the test substance and are not considered to represent an adverse effect.

The incidence of all other non-neoplastic changes observed in rats of either sex killed at the end of the treatment period did not suggest any treatment effect relationship.

Neoplastic findings:

There was no evidence of a treatment-related change in the total number of tumor-bearing animals.

Animals killed at interim sacrifice*:* At interim sacrifice on Week 53, up to 800 ppm, there was no evidence of a treatment-related change in the incidence of neoplastic lesions. The tumors observed in a very few rats in the control and treated groups with no evidence of a treatment-related effect were recognized as ones that do occur spontaneously in Sprague-Dawley rats of this age and strain and therefore considered incidental.

Animals killed or dying during the treatment period*:* There was no evidence of a treatment-related change in the incidence of neoplastic lesions in the treated rats of either sex when compared to control group. The major tumor types which contributed to the death of the majority of the rats were the ones usually found in aging Sprague-Dawley rats i.e. pituitary gland adenomas or carcinomas, and mammary gland fibro adenomas and/or adenocarcinomas.

Animals killed at final sacrifice*:* At final sacrifice, there was no evidence of a treatment-related change in the incidence of neoplastic lesions in treated rats of either sex when compared to control group. Benign ovary cystadenomas were noted in 0/37 (0%), 3/32 (9%), 5/36 (14%) and 2/36 (6%) of the females from the 0, 200, 400 and 800 ppm groups, respectively. Considering the absence of a dose-effect relationship and the fact that the only malignant cystadenocarcinoma was noted in 1/137 (3%) final sacrifice female from the control group, this change was not considered to be related to treatment. There was a minimally lower incidence of benign endometrial stromal polyp in the uterus of the final sacrifice females at 400 ppm (6/36 or 17%) and 800 ppm (5/36 or 14%) when compared to control group (8/37 or 22%).

All tumors, all animals*:*

There was no convincing (according the authors of the study) evidence of a treatment-related effect on any of the tumor types observed in this study when considering all animals from the chronic, and carcinogenicity phase of the study which were examined histologically.

In females, at 800 ppm, there was a numerical decrease in the incidence of benign endometrial stromal polyp of the uterus when compared to control group. The overall incidence of this tumor was 12/70 (17%), 11/60 (18%), 10/60 (17%) and 6/70 (9%) at 0, 200, 400 and 800 ppm, respectively.

There was a numerical increase in benign and malignant C-cell tumors of the thyroid gland in male rats only at 800 ppm (Table 6.60). The differential diagnosis between focal C-cell hyperplasia and C-cell adenoma is based on a size criterion (if the lesion is larger than five average follicles, it is classified as an adenoma), and there is a continuous spectrum of changes from focal hyperplasia to adenoma and subsequently carcinoma (diagnosed on the basis of invasion of the thyroid capsule or parathyroid gland). Therefore it is usual for this type of lesions to consider the combined incidence of hyperplasia, adenoma and carcinoma together to evaluate treatments.

Table 6.60 - Group-related incidence of Thyroid C-Cell focal hyperplasia, adenoma and carcinoma in male rats in this study

Findings	0 ppm	200 ppm	400 ppm	800 ppm	Historical control data (three studies, ≈ 223 animals)
Thyroid focal C-cell hyperplasia	6/66 (9%)	5/52 (10%)	3/60 (5%)	7/62 (11%)	46/168 (24.47%)
Prevalence tumor tests [a]	p = 0.4942	p = 0.4209	p = 0.2819	p = 0.4942	-
Thyroid C-cell adenoma	19/66 (29%)	20/52 (38%)	20/60 (33%)	26/52 (42%)	28.70%
Prevalence tumor tests [a]	p = 0.1237	p = 0.2195	p = 0.4347	p = 0.1057	-
Thyroid C-cell carcinoma	4/66 (6%)	1/52 (2%)	7/60 (12%)	7/62 (11%)	6.73%
Prevalence tumor tests [a]	p = 0.0762	p = 0.2212	p = 0.2534	p = 0.2971	-
Combined thyroid adenomas and carcinomas	23/66 (35%)	21/52 (40%)	27/60 (45%)	33/62 (53%)	32.74%
Combined thyroid hyperplasia adenoma and/or carcinoma	27/66 (41%)	26/52 (50%)	27/60(45%)	31/62 (50%)	49.74%
Prevalence tumor tests [a]	p = 0.2682	p = 0.2546	p = 0.4879	p = 0.2695	-

Values listed parenthetically are % of incidences
* p<0.05
** p<0.01
*** p<0.001
- no data
[a]: Beneath the control incidence is the p-value associated with the trend test. Beneath each treated group's incidence is the p-value corresponding to the pair wise comparison between the treated and control groups. All statistical tests conducted were one-sided tests.

In males, at 800 ppm, there was an apparent numerical increase in the incidence of benign prostate adenoma. These benign tumors, detected microscopically, represent an amplification of focal epithelial hyperplasia and the differential diagnosis between a large focus of hyperplasia and a small adenoma is somewhat arbitrary. These two lesion types should therefore be considered together since they represent a continuum of changes from hyperplasia to adenoma and to carcinoma. In the present study, no malignant tumors of the prostate were observed. When considering the combined incidence of epithelial hyperplasia and adenoma (Table 6.61), there was no evidence of a treatment-related effect. Statistical analysis showed no differences between control and treated groups. Therefore, the numerical increase in prostate adenoma observed at 800 ppm was considered incidental.

Table 6.61 – Group-related incidence of prostate epithelial hyperplasia and adenoma in male rats

	0 ppm	200 ppm	400 ppm	800 ppm
Hyperplasia	29/69 (42%)	32/60 (53%)	25/61 (41%)	30/69 (43%)
B-adenoma	3/69 (4%)	0/60 (0%)	0/61 (0%)	6/69 (9%)
Hyperplasia and/or adenoma	30/69 (43%)	32/60 (53%)	25/61 (41%)	30/69 (43%)
Prevalence tumor tests [a]	p = 0.1717	p = 0.1652	p = 0.1396	p = 0.4837

* p<0.05
** p<0.01
*** p<0.001
- no data
[a]: Beneath the control incidence is the p-value associated with the trend test. Beneath each treated group's incidence is the p-value corresponding to the pair wise comparison between the treated and control groups. All statistical tests conducted were one-sided tests.

Conclusion:

Under the conditions of the study, the No Observed Adverse Effect Level for lifetime exposure of rats to dodine was 400 ppm approximately 20 and 26 mg/kg bw/day in males and females respectively, based on decreased body weight, body weight gain and food consumption. There was a statistically non-significant increase in the incidence of combined thyroid C-cell adenomas and carcinomas in males at 800 ppm, and the incidence in all treated males exceeded the mean and upper limit of the range for historical controls.

Dodine: Evaluation of clinical signs in the rat chronic study.

Dodine: Clinical signs in the rat study: spreadsheet of incidence in males rats sacrificed moribund or found dead.

Semino, G. (17 January 2000). Performed by Aventis CropScience BP, Sophia Antipolis, France

Introduction:

Dodine is a foliar fungicide widely used to control diseases of fruit trees since 1957.

Recently, a concern on possible neurotoxicity effects after exposure to dodine was raised by the US EPA reviewer during the evaluation of the rat two year carcinogenicity study, because of a dose-related increased incidence of absence of the following reflexes grasping, righting and traction (Table 6.62). This paper provides a reasoned review and statistical analyses of the above clinical signs in the chronic rat study.

The re-evaluation clearly demonstrates that the observed neuromuscular impairment was associated with a general reduced motor activity occurring in moribund animals. Animals had no reflexes because they were dying and not because dodine affected the nervous system.

Clinical signs observed in the long term carcinogenicity study

In the two year chronic/carcinogenicity study rats were administered dodine in the diet at 0, 200, 400 and 800 ppm. As required by the study guidelines, animals were routinely monitored during the study to assess their general health and to define the effects of test article. In chronic studies daily observations are critical in tracking tumor development and for determining animals in extremis which should be euthanized to minimize suffering, autolysis, and tissue loss. Detailed clinical

observations for toxic signs were performed in a systematic fashion. First, each animal was observed in its home cage prior to disturbance for overt signs, such as unusual posture, tremors, gross muscle fasciculations, activity level, and unusual behaviour. Next the animal was gently grasped around the trunk and retrieved from its cage. During this phase the grasping and traction reflexes were evaluated by observing if the rat grabbed at the wire-mesh of its stainless steel cage with its forelimbs while being suspended by the tail and gently pulled away from the cage. It was then inspected for general appearance, abnormalities and level of activity. During handling, it was noted whether the rat felt cool or hot to touch, had respiratory abnormalities or abnormalities in muscle tone or muscle mass, and/or or had evidence of injury. The righting reflex was then evaluated on a flat surface: the rat was placed on its back and it was observed how easily it regained stance on all four feet. Finally, the rat was returned to its cage and also observed for signs including clonic or tonic movement, response to stimuli (by snapping fingers), gait abnormality, and level of activity.

All clinical signs were carefully recorded. Global evaluation of clinical signs did not raise any indication of a potential neurotoxic effect. In fact, even if an increased absence of grasping, traction and righting reflexes in the males top dose group was observed, these signs were not correlated to other variations in behavioural and neurological manifestations which could indicate evidence of neurotoxicity, nor to structural or neuropathological findings. Therefore, the observed findings were determined to be general signs of systemic toxicity usually observed in these type of studies in old animals exposed to overt toxic doses.

Table 6.62 – Incidence of absence of reflex in the rat carcinogenicity study

	Males				Females			
Dose level (ppm)	**0**	**200**	**400**	**800**	**0**	**200**	**400**	**800**
No righting reflex	0	2	3	4	2	1	3	3
Traction absent	1	0	1	5	1	4	0	0
Grasping absent	2	4	6	8	3	8	2	5

Statistical Analysis

Nevertheless, a statistical evaluation of the grasping, traction and righting reflexes data was carried out to evaluate any effect of the treatment on the above clinical signs. There is less persuasive evidence of direct neurotoxic effect, even if some neurological signs are affected, but only at the high dose and in conjunction with other over signs of toxicity, including systemic toxicity, large decreases in body weight, decreases in body temperature or debilitation. Firstly, comparisons of the incidences of clinical signs of the treated groups with respect to the control group were performed for both sexes, using the Fisher's exact test (one sided). Results are summarised in tables 6.63 and 6.64.

In males, the absence of grasping reflex was statistically significant for the top dose animals only, whereas no significant difference was observed for the absence of traction and righting reflex. The trend test was positive for all the three clinical signs when the incidences at the top dose were considered and negative when excluded.

Results from the trend test indicate that an effect on the absence of grasping, traction and righting reflex occurs at the top dose only.

In females, the incidence of animals with absence of grasping, traction and righting reflex in treated groups was not significantly different from controls. Also results of the trend tests (one sided) were negative, confirming the absence of any direct or indirect effect of dodine on the neuromuscular function.

Table 6.63 – Incidences of absence of grasping, traction and righting reflex in males

	0 ppm	200 pp	400 ppm	800 ppm	Trend test	Trend test excluding top dose
Animals with absence of grasping / Total	20/70	4/70 NS	6/70 NS	8/70*	*	NS
Animals with absence of traction / Total	1/70	0/70 NS	1/70 NS	5/70 NS	*	NS
Animals with absence of righting /Total	0/70	2/70 NS	3/70 NS	4/70 NS	*	NS

NS : not significant
* : significant (p = 0.05)

Table 6.64 – Incidences of absence of grasping, traction and righting reflex in females

	0 ppm	200 ppm	400 ppm	800 ppm	Trend test
Animals with absence of grasping / Total	3/70	8/70 NS	2/70 NS	5/70 NS	NS
Animals with absence of traction / Total	1/70	4/70 NS	0/70 NS	0/70 NS	NS
Animals with absence of righting /Total	2/70	1/70 NS	3/70 NS	3/70 NS	NS

NS : not significant

Since statistical analyses showed an effect at the top dose in males only, further statistical analyses were carried out in data from males to clarify the relationship among the different clinical signs and the correlation between the absence of reflexes with the health status of the animals. Since the absence of grasping was noted in most of the males, the relationship between the absence of grasping and the other two reflexes was checked using Fisher's exact test (2 sided).

A relationship between absence of grasping and absence of righting was observed in all groups. The relationship between the absence of grasping and traction was more difficult to establish since there were less animals not showing this reflex. No statistical test was performed for animals dosed at 200 ppm because there was no absence of traction in any animals. At 400 ppm the relationship was not significant, even if the only animal with absence of traction had also absence of grasping. Finally the relationship was significant in control and top dose groups. Because statistical tests showed that absence of righting or traction reflexes were highly correlated to absence of grasping, the absence of the above reflexes may be indicative of possible impairment of the motor function. In particular, the absence of grasping and traction is indicative of weakness and the absence of righting is indicative of weakness and difficulty in coordination. To check whether dodine had a direct effect on the motor function or whether the animals were weak due to toxicity, the incidence of animals with reduced motor activity was also considered. In fact, reduced motor activity is another parameter routinely used to evaluate the motor function impairment. The incidences are summarized in the first row of table 6.65. In this table it is also reported that a large proportion of animals with absence of grasping and/or traction and/or righting reflexes showed also motor reduced activity.

Table 6.65 – Relationship between absence of reflex and reduced motor activity

Number of males with:	**0 ppm**	**200 ppm**	**400 ppm**	**800 ppm**
Reduced motor activity	8	11	12	12
Absence of traction and/or absence of righting and/or absence of grasping	2	4	7	8
Absence of traction and/or absence of righting and/or absence of grasping and reduced motor activity	2	2	5	7

This means that the observed neuromuscular impairment was limited to a rather restricted number of animals, in particular in males administered dodine at toxic dose levels. To confirm this suggestion the health status of the animals with absence of neuromuscular effect and other behavioral and neurological indicators of neurotoxicity was further examined. As indicated in the following table (table 6.66), impaired neuromuscular function occurred in most of the animals sacrificed moribund.

Table 6.66 – Moribund animals with absence of reflexes and/or reduced motor activity

	0 ppm	**200 ppm**	**400 ppm**	**800 ppm**
Number of males sacrificed moribund	6	9	9	9
Percentage of males moribund with absence of traction and/or absence of righting and/or absence of grasping and/or reduced motor activity	4/6 (67%)	6/9 (67%)	8/9 (89%)	8/9 (89%)

Thus statistical analysis has demonstrated that:

- there is an increase in the absence of grasping, traction and righting reflex in males only and only at the top toxic dose
- absence of grasping, righting or traction reflexes were highly correlated signs and were related to reduced motor activity.
- these findings described males which were sacrificed moribund.

Therefore the apparent increase in absence of grasping, traction and righting reflexes in the chronic study is attributable to decreased motor activity in aged, moribund animals.
Further evidence that dodine is not a neurotoxic compound is provided by the absence of any pathological change affecting the nervous system. In fact, alterations in the structure of the nervous system like neuropathy, axonopathy, myelinopathy, terminal degenerations are regarded as evidence of a neurotoxic effect. As indicated in the report of the two year rat carcinogenicity study, there were no significant histological changes affecting the CNS or PNS.

Conclusion:

An accurate investigation of a possible effect of dodine on neuromuscular function has been performed, by reviewing data from the two year carcinogenicity study in rats.

Analysis of the data shows that the absence of traction, and/or righting and/or grasping reflex and/or the reduced motor activity occurred in most of animals sacrificed moribund. The absence of the above clinical signs were recorded in affected animals during the last days of their life, when the general health status of these aged animals was severely impaired by illness not affecting the nervous system.

The extensive toxicological database of dodine indicates that structural, neuropathological, behavioral and neurological end-points were not affected after exposure to this fungicide. Therefore there is adequate information to conclude that dodine is devoid of neurotoxicological potential.

78-week dietary oncogenicity study with dodine in mice

Kevin D. Williams (October 1998) – Report No. 6224-220, 9 October 1998. Performed by Covance Laboratories, Madison, Wisconsin, USA; dates of experimental work: 1 June 1995 to 9 October 1998.

Guidelines and GLP:

The study was conducted in compliance with FIFRA 83-2, and OECD 451. The study is GLP compliant.

The study is acceptable.

Material and methods:

The objectives of this study was to asses the toxicity of the test material, dodine, when fed to mice for at least 52 weeks and to assess the oncogenicity potential of the test material when fed to mice for at least 78 weeks.

Dodine (batch DA717, purity 98.6%) was administered in the diet (*ad libitum*) to 60 males and 60 females Crl:CD-1_(ICR)BR mice per group at concentrations of 0, 200, 750 or 1500 ppm (equivalent to 0, 29, 110 or 225 mg/kg bw per day for males and 0, 28, 136 or 277 mg/kg bw per day for females calculated as a weighted average) for 78 weeks. An additional ten males and ten females per group were sacrificed at 53 weeks. Dose selection was based on a 13-week dietary study in the mouse.

Table 6.67 – Group size and dosage levels.

Group	Diet concentration of dodine (ppm)	Number of animal/sex	
		Interim sacrifice[1]	Terminal sacrifice[2]
1	0	10	60
2	200	10	60
3	750	10	60
4	2000	10	60

[1] sacrifice at week 53 (chronic phase of the study)
[2] sacrifice at week 78 (carcinogenicity phase)

Antemortem Observations

The animals were observed twice daily (a.m. and p.m.) for mortality and moribundity. Signs of poor health or abnormal behaviour were recorded as they were observed. At least once each week, each animal was removed from its cage and examined. Any unusual or abnormal findings were recorded. In addition, special attention was paid to mass development and the onset, location, size, appearance, and progression of each grossly visible or palpable mass were recorded.

Body Weights

Individual body weight data were recorded on the first day of treatment, weekly for 13 weeks, and at least every 4 weeks thereafter through 77 weeks of testing. Body weights were also recorded for animals sacrificed at unscheduled intervals.

Food Consumption

Individual food consumption data were recorded weekly for 13 weeks and at least every 4 weeks thereafter through 77 weeks of testing.

Clinical Pathology

Blood smears were made and held for possible future examination from each animal during Week 53 and at the terminal sacrifice. When possible, blood smears were also made and held for possible future examination from animals sacrificed at unscheduled intervals. Examination of the blood films was considered unnecessary because histopathologic examination of appropriate tissues indicated the test material did not induce haematopoietic neoplasia. Blood sample collections were done at the necropsy of mice at unscheduled and the scheduled interim (10/sex/group during Week 53) and terminal (all survivors after Week 78) sacrifices; for those animals sampled at Week 53 but not terminated, blood was collected from the retro-orbital plexus.

Anatomical Pathology

A necropsy was done on each animal that died or was sacrificed at unscheduled intervals. During Weeks 53 (10/sex/group; interim sacrifice) and 79 and 80 (all survivors; terminal sacrifice), animals that were fasted for at least 4 hours before sacrifice were anesthetized with sodium pentobarbital and weighed, blood smears were prepared, and the animals were exsanguinated and necropsied. Animals were necropsied in random order; terminal necropsies were done over an 11-day period.

The necropsy included a macroscopic examination of the external surface of the body; all orifices; the cranial cavity; the brain and spinal cord; the nasal cavity and paranasal sinuses; and the thoracic, abdominal, and pelvic cavities and viscera.

At each scheduled sacrifice, the following organs (when present) were weighed; paired organs were weighed separately:

Adrenals	Liver with gallbladder (drained)
Brain	Ovaries
Kidneys	Testes

Organ-to-body weight percentages and organ-to-brain weight ratios were calculated.

The following tissues (when present) or representative samples were collected and preserved in 10% phosphate-buffered formalin:

Adrenals	Ileum
Aorta	Jejunum
Brain	Kidneys
Cecum	Lesions
Cervix	Liver
Colon	Lungs
Duodenum	Lymph nodes (superficial cervical and mesenteric)
Epididymides	Mammary gland (females only)
Esophagus	Masses and associated tissues
Eyes	Salivary gland (submaxilar)
Femur with bone marrow (articular surface of the distal end)	Sciatic nerve
Gallblader	Seminal vesicles
Harderian gland	Skeletal muscle (thigh)
Heart	Stomach
Spinal cord (cervical, mid-thoracic, and lumbar)	Testes
Spleen	Thymus
Sternum with bone marrow	Thyroids with parathyroid
Ovaries	Trachea
Pancreas	Urinary bladder
Pituitary	Uterus with cervix
Prostate	Vagina
Rectum	

Statistical Analyses

Only data collected on or after the first day of treatment were analyzed statistically.

Levene's test (Levene, 1960) was done to test for variance homogeneity. In the case of heterogeneity of variance at $p < 0.05$, transformations were used to stabilize the variance.

Analysis of variance [ANOVA (Winer, 1971)] was done on the homogeneous or transformed data. If the ANOVA was significant, Dunnett's t-test (Dunnett, 1964) was used for pairwise comparisons between treated and control groups.

One-way ANOVA was used to analyze body weights, cumulative body weight gains, food consumption, organ weights, organ-to-body weight percentages, and organ-to-brain weight ratios.

Survival and Tumor Analyses

Survival was analyzed by life table techniques consisting of Kaplan-Meier product limit estimates, Cox-Tarone binary regression on life tables, and Gehan-Breslow nonparametric methods (Thomas, Breslow, and Gart, 1977).

Neoplastic lesions were chosen for statistical analyses if the incidence in at least one treated group was increased or decreased by at least two over the control. The incidences of hemangiosarcoma and lymphoma were evaluated by animal, not by site. Initially, the selected neoplastic lesions were analyzed by Cochran-Armitage method for trend and Fisher-Irwin exact test for control versus treatment comparisons (Thakur, Berry, and Mielke, 1985). Further, survival adjusted tumor analyses, considering any possible intercurrent mortality differences due to competing toxicity among the treated groups, were performed on various lesions as follows: The incidental (occult or nonpalpable) tumors were analyzed by interval based exact survival adjusted prevalence test (Ali, 1990) or logistic regression of tumor prevalences (Dinse and Lagakos, 1983) if at least one group incidence in an analysis was greater than or equal to 10. The cut-off points for the interval based method were Weeks 0-52, 53-before terminal sacrifice, and terminal sacrifice. The lethal tumors and palpable tumors of the mammary gland were analyzed by Cox-Tarone binary regression method as in the case of survival, using the first palpation time (if applicable) as the surrogate for onset time. Trend probability is reported when there is existence of any monotone response in the incidence data. Continuity corrected test statistics for asymptotic tests were used for evaluation of

all the incidence tables, where appropriate. The benign and malignant neoplastic incidences were evaluated separately and combined, where appropriate. The criteria for combination were based on the work of McConnell et *al.* (1986).

Results:

Dose Analyses

Homogeneity evaluations conducted before initiation of the study on pretest diets mixed to contain 200 and 1,500 ppm dodine indicated the mixing procedure (a premix in approximately 9 kg of diet followed by a finish-mix of 48 kg of total diet) produced a homogenous distribution of the test material. Mean values from the four locations sampled ranged from 95.5 to 97.0 % of theoretical for the 200-ppm level, and from 89.3 to 100% for the 1,500-ppm level.

After 9 and 20 days at room temperature, 30 days refrigerated, 18 days, room temperature and 10 weeks refrigerated, results of stability analyses indicated that the mean concentrations ranged from 131.0 to 142.0% and 91.3 to 101.0% of the initial theoretical concentrations for the diets containing 200 and 1,500 ppm, respectively.

A special set of analyses was done on various diet samples from the diets prepared for Weeks 41 through 44. This was done because it was suspected, based on animal health and food scattering behaviour during the first day the 1,500-ppm diet was offered to the Group 4 animals during Week 44, that these animals had received an elevated concentration of test material. This elevated level for Group 4 was confirmed by various analyses of remaining feed and feed in animal dishes of Group 4. The elevated level was believed to be the result of a mechanical error in diet preparation. This elevated dietary level of dodine was fed for only the first day of Week 44, after which it was replaced with freshly prepared diet; this diet was analyzed, found to contain the appropriate concentration of dodine, and was used during Weeks 44 through 47. Retention samples taken from the finished mixes of the 0-, 200-, and 750-ppm concentrations prepared for Weeks 41 through 44 were also analyzed and found to contain less than the limit of quantification, 231 and 911 ppm, respectively. Overall, these results indicate that only the 1,500 ppm diet was mixed inappropriately. This resulted in the death or sacrifice of 6 females.

The mean concentrations of the routine dose preparation analyses for all levels ranged from 89% to 108% of the theoretical concentrations. These data indicate that the levels of dodine in the dose preparations were acceptable.

Survival

Survival analysis indicated that survival was increased in males offered 1,500 ppm dodine (Group 4) compared to the control males, as evidenced by results of the Cox-Tarone and Gehan-Breslow tests which are more sensitive for late and early mortality, respectively.

For females these tests did not reveal a statistical difference in survival regardless of whether the females that died during Week 44 (the six 1,500 ppm group females deaths attributed to the diet preparation error and one 200 ppm group female death unrelated to the diet preparation error) were included or censored from the survival analyses.

Table 6.68 – Mortality of the mice of both sexes

Dose level (ppm)	After 78 weeks	
	males	females
0	16/70	13/70
200	14/70	9/70
750	9/70	11/70
1500	3/70	15/70*

* including 6 dead females due to preparation error

Antemortem Observations

The incidence of whole body tremors was approximately 40% of control males and 20% of control females. The incidence of whole body tremors was similar to those of the control population in males and females fed 200 ppm dodine, but was numerically higher than those of controls (approximately 11 and 14% higher for males and females, respectively) in mice that were fed the 750- or 1,500-ppm dodine diets. Excessive salivation was frequently observed in the animals displaying whole body tremors, and although these occurrences were not identical, the animals displaying excessive salivation were generally those exhibiting whole body tremors. Due to the high background incidence, this is not considered to be a primary response to the test material.

On the first day of Week 44, Group 4 animals were fed diets that were subsequently found to contain a level of dodine well in excess of the targeted 1,500-ppm concentration; the death of 6 females (Animal Nos. A57464, A57469, A57470, A57487, A57492 and A57510) were considered to be attributable with this occurrence. Other than animals Nos. A57464 and A57510, no noteworthy clinical signs were detected in the 1,500-ppm animals that were judged to be associated with the one day exposure to these diets; these two animals were noted as being cold to touch, with hunched and/or recumbent posture, and irregular breathing (one was also seen as pale in appearance) before being sacrificed in moribund condition on Days 303 and 304 (the second and third days of Week 44). The other four affected 1,500 ppm group females were found dead on Day 303 and had no antemortem observations recorded after the one day exposure to the erroneous test diet and before the deaths were noted.

Other antemortem observations were similar in nature and in the total number of affected animals in all groups, and there were no observations noted that were considered to be uncommon to this strain of mouse. Similarly, there were no differences in the number of animals with masses detected in-life that were attributable to dietary exposure to dodine.

Body Weights and Body Weight Gains

Animals fed the 1,500 ppm level had lower mean body weights than those of controls for most of the study. For males at 1,500 ppm statistical difference were apparent beginning at Week 2, and for females statistically lower body weights were first noted at Week 7. Mean body weight gains of the 1,500-ppm group were also significantly reduced for males and females over Weeks 1 to 14, and for females over Weeks 14 to 54 and 54 to 78. The overall mean body weight gain for males and females fed the 1,500-ppm concentration were significantly reduced compared with that of the controls (25.5 and 34.6% for males and females, respectively). At 750-ppm body weights for both sexes tended to be lower than controls, and statistically significant differences were noted frequently for both sexes, but were consistently observed in the females, and intermittently observed for the males. The overall mean body weight gain for females fed the 750-ppm concentration was significantly reduced compared with that of the controls (20.1 % lower). No differences in mean body weight of males or females at 200-ppm were considered treatment related.

Food Consumption

Mean food consumption for males and females fed the 1,500-ppm diets as well as for females fed the 750-ppm diet was were generally reduced compared with that of the control group, and these reduced values were frequently found to be significantly different from control values. In other treated groups, sporadic differences in mean food consumption were noted; these occurrences were not considered to be treatment-related.

Test Material Consumption

Calculated test material consumption (mg dodine consumed/kg of body weight/day, based on the targeted dietary ppm concentrations of dodine and for the intervals of measured body weight and weekly food consumption) ranged from 24 to 38, 94 to 139, and 191 to 281 mg/kg/day for males and from 31 to 47, 116 to 158, and 231 to 332 mg/kg/day for females fed the 200-, 750-, and 1,500-ppm dodine diets, respectively.

Anatomical Pathology

Interim Sacrifice**:** Terminal body weights were decreased in males and females given 1,500 ppm. Although not statistically significant, female body weight decreased 10% at high dose-level. With the exception of the absolute weight for the left kidney, absolute and relative kidney weights were increased in the females given 1,500 ppm. The remaining statistically significant organ weight changes were thought to be incidental due to the absence of an effect in the contra lateral organ or the lack of a significant difference in the absolute weight.

There were no macroscopic findings that suggested a relationship to the administration of the test material.

Terminal Sacrifice: Terminal body weights were significantly lower than those of controls for males given 1,500 ppm and for females given 200, 750, or 1,500 ppm. For the females given 200 ppm, the lower terminal body weight was not considered to be an adverse change because the magnitude of the difference was small, and significantly lower body weight was not a consistent finding during the in-life portion of the study for this group. Absolute and relative kidney weights were significantly increased in the females given 1,500 ppm. The remaining statistically significant differences were thought to be incidental and were secondary to the observed body weight loss.

The incidence of light areas or masses in the liver was slightly increased in the animals given 1,500 ppm. There were no other macroscopic findings that suggested a relationship to the administration of the test material.

The incidence of non-neoplastic changes was generally similar between control and treated groups. There were no microscopic correlates to the kidney weight increase observed in females.

In females given 1,500 ppm, the incidence of adenomas and combined hepatocellular adenomas and carcinomas, but not carcinomas alone, had a significant trend test. The only significant group comparison difference was for combined adenomas and carcinomas in females given 1,500 ppm. The group comparison for adenomas alone at 1,500 ppm vs. control resulted in a p value slightly greater that 0.05 (i.e., was not significant). The next table (Table 6.69) shows the incidence of neoplastic findings in liver in relation to historical data in 78-week duration studies with CD-1 mice. The data for the high-dose group and the three other groups (control, low, and mid dose combined) are shown separately. These data show that the incidence of carcinoma alone was comparable to the historical data for both males and females. Additionally, the incidence of adenoma across the control, low, and mid-dose groups (24/180 for males) was higher than the historical data for each sex indicating that this population of animals had a slightly higher background rate of hepatocellular adenoma. Group comparisons for combined adenoma and carcinoma showed a test material-related increase only at the high dose, and was statistically

significant only for females. The overall conclusion is there is some evidence indicating that the high dose of dodine (1,500 ppm) caused an increased incidence of benign hepatocellular neoplasia. However, the incidence of basophilic foci was similar across all groups. No evidence indicating that the high dose was associated with a further progression to carcinoma was apparent in the data from this study.

Table 6.69 – Incidence of hepatocellular tumours in mice treated with dodine.

Hepatocellular neoplasm	Males		Females	
	Covance 6224-220	Historical control data	Covance 6224-220	Historical control data
Adenoma	1500 ppm 14/60 (23.3%)	27/398 (6.8%)	1500 ppm 4/60 (6.7%)	3/395 (0.8%)
	All other groups 24/180 (13.3%)	[range 2/50 to 10/50 (4% to 20%)]	All other groups 2/177 (1.1%)	[range 0/50 to 2/60 (0% to 3%)]
Carcinoma	1500 pp 1/60 (1.7%)	10/398 (2.5%)	1500 ppm 1/60 (1.7%)	3/395 (0.8%)
	All other groups 5/180 (2.8%)	[range 0/50 to 4/50 (0% to 8%)]	All other groups 1/177 (0.6%)	[range 0/50 to 1/50 (0% to 2%)]
Combined adenoma/carcinoma	1500 ppm 15/60 (25.0%)	37/398 (9.3%)	1500 ppm 5/60 (8.3%)	6/395 (1.6%)
	All other groups 29/180 (16.1%)	[range 0/50 to 11/50 (0% to 22%)]	All other groups 3/177 (1.7%)	[range 0/50 to 2/60 (0% to 3%)]

These data show that the incidence of carcinoma alone was comparable to the historical data for both males and females. Additionally, the incidence of adenoma across the control, low, and mid-dose groups (24/180 for males) was higher than the historical data for each sex indicating that this population of animals had a slightly higher background rate of hepatocellular adenoma. Group comparisons for combined adenoma and carcinoma showed a test material- related increase only at the high dose, and was statistically significant only for females. The overall conclusion is there is some evidence indicating that the high dose of dodine (1,500 ppm) caused an increased incidence of benign hepatocellular neoplasia. However, the incidence of basophilic foci was similar across all groups. No evidence indicating that the high dose was associated with a further progression to carcinoma was apparent in the data from this study.

Unscheduled Deaths

No test material-related decrease in survival was noted in either sex. In the males, considerably fewer animals died or were sacrificed moribund in the groups given 750 or 1,500 ppm. There was no test material-related increase in incidence of neoplasms in animals that died or were sacrificed moribund. The incidence of non-neoplastic changes was generally similar between control and treated groups.

Statistical analyses report on survival and neoplastic lesions.

Survival:

In the males, there was a significant negative trend in mortality ($p < 0.01$ for both Cox-Tarone and Gehan-Breslow tests) associated with a significantly decreased mortality in the high-dose group compared with that of the control (3/70 vs. 16/70, $p < 0.01$ for both tests).

In the females, there was no significant trend or group comparisons based on the reported mortality rates, although the trend was in an increasing fashion. However, when the animals died at Week 44

(Animal Nos. 57330, 57464, 57469, 57470, 57487, 57492, and 57510) were treated as censored animals, the high-dose group mortality was decreased over that of the control, although still not significantly.

Neoplastic lesions:

In the males, there was no significant dose-response in any of the selected tumor incidences nor were there any significant group comparisons observed.

In the females, based on the results of adjusted analyses there was a significant positive trend of the liver hepatocellular adenoma, but no significant group comparisons were observed. There was also a significant positive trend (p = 0.0136) in the combined incidences (0/60, 2/58, 1/59, and 5/60 for Groups 1, 2, 3, and 4, respectively) of hepatocellular adenoma, carcinoma, or both, in the liver of females, which was mostly due to a significant increase in the high-dose incidence compared with that of the control (p = 0.0248). No other significant findings were observed in the females.

Table 6.69a – Results of statistical analyses of neoplastic lesions for males and females mouse

Group	Males				Females			
	1	2	3	4	1	2	3	4
Liver Hepatocellular Adenoma								
Incidence rate	8/60a	7/60	9/60	14/60	0/60	1/58	1/59	4/60
Unadjusted p	0.669+	0.5000-	0.5000+	0.1189+	0.0219+*	0.4915+	0.4958+	0.0594+
Adjusted p	0.1396+!	0.4687-	0.5967+	0.2493+!	0.0159+*	0.5155+	0.5104+	0.0533+
Liver Hepatocellular Carcinoma								
Incidence rate	2/60	0/60	3/60	1/60	0/60	1/58	0/59	1/60
Unadjusted p		0.2479-	0.5000+	0.5000-	#	#	#	#
Adjusted p		0.2362-	0.5703+	0.4095-	#	#	#	#
Liver Hepatocellular Adenoma/Carcinoma								
Incidence rate	10/60a	7/60	12/60	15/60	0/60	2/58	1/59	5/60
Unadjusted p	0.0786+	0.3008-	0.4070+	0.1844+	0.0192+*	0.2395+	0.4958+	0.0287+*
Adjusted p	0.1829+!	0.2797-!	0.4188+!	0.3859+!	0.0136+*	0.2631+	0.5104+	0.0248+*

a: because only one animal died of tumour, it was treated as incidental for the purpose of analysis; !: logistic regression p

+ : effect in the positive direction; -: effect in the negative direction;*: significant at $0.01 \leq p \leq 0.05$; **: significant at $p \leq 0.01$

p value under group 1 is for linear trend of Group 1 vs 2 through 4 and the p-values under other groups are for comparisons of those versus Group 1 (control)

#: No statistical analysis was done due to lack of tumor bearing animals

Conclusions:

The primary purpose of this study was to evaluate the carcinogenicity of dodine when offered to CD-1 mice by dietary admixture for 78 weeks at concentrations of 0, 200, 750, and 1,500 ppm. The no-observed-adverse-effect level for findings other than carcinogenicity was 200 ppm. No differences in mean body weight of males or females at 200-ppm were considered treatment related.

The NOAEL was 200 ppm (29 and 38 mg/kg bw per day in males and females, respectively) based on decreased body weight gains and food consumption. A positive trend in the incidence of hepatocellular adenomas was observed in females with 1,500 ppm.

B.6.6 Reproductive toxicity (Annex IIA 5.6)

B.6.6.1 Two generation reproductive toxicity (Annex IIA 5.6.1)

Two generation reproduction study with dodine in rats

Susan M. Henwood (1996) – Report No. HWI 6224-218 of 17 December 1996; Performed by Chimac-Agriphar, Corning Hazleton, 3301, Kinsman Boulevard, Madison, Wisconsin 53704, USA; dates of experimental work: 10 July 1995 to 17 December 1996.

Guidelines and GLP

The study was conducted in compliance with FIFRA 83-4, and OECD 416. The study is GLP compliant.

The study is acceptable.

Materials and methods

The purpose of this study was to provide information on the effects on male and female reproductive performance an on the growth and development of the offspring when dodine was fed to rats for two generations. Dodine (batch DA717; purity 98.6%) was administered continuously to 30 male and 30 female rats Crl:CD®(SD)BR VAF/Plus® per group at concentrations of 0, 200, 400 or 800 ppm (0, 13.14, 26.20 and 52.61 mg/kg bw/day and 0, 18.06, 35.34 and 67.58 mg/kg bw/day for F0 males and females respectively; 0, 14.91, 30.20 and 63.03 mg/kg bw/day and 0, 19.18, 38.77 and 76.63 mg/kg bw/day in F1 males and females respectively, ingestion levels have been calculated in an addendum, "Calculation of the actual test material intake", Chimac-Agriphar, Corman C. (2002), Unpublished statement).

Dose administration

Males and females of the F0 generation received the test material in the diet *ad libitum* for 10 weeks before mating and throughout mating, gestation, and lactation of the F1 litters until necropsy. Males and females of the F1 generation had access to the test material in the diet *ad libitum* at the same levels as their respective parents as nursing pups; and for at least 10 weeks following weaning before mating and throughout mating, gestation, and lactation of the F2 litters until necropsy.

Clinical observations

The animals were observed twice daily (a.m. and p.m.) for mortality and moribundity. Signs of poor health or abnormal behaviour were recorded as they were observed. Mated females were observed for signs of abortion, premature delivery, or difficult or prolonged parturition. Animals were removed from their cages and examined when body weights were recorded.

Body weights

Individual body weight data were recorded for males on the first day of treatment and weekly thereafter. Females were weighed on the first day of treatment; weekly during premating and mating; on Days 0, 7, 14, and 20 of gestation; and on Days 0, 4, 7, 14, and 21 of lactation. Females that did not have positive evidence of mating or deliver were weighed weekly.

Food consumption

Individual food consumption data were recorded for males and females weekly during premating. Food consumption was measured for mated females for Days 0-7, 7-14, and 14-20 of gestation and for females that delivered litters for Days 0-4, 4-7, 7-10, and 10-14 of lactation.

Oestrus cycle monitoring

Vaginal smears were done daily for 3 weeks before mating until mating was confirmed or until the completion of the mating period.

Reproductive procedures

Each female was paired with one male from the same group for a maximum of 14 days. Vaginal examinations were done daily during cohabitation, and the presence of sperm in the vaginal smear or copulatory plugs was considered positive evidence of mating. The day when such evidence was found was considered Day 0 of gestation, and the female was then housed individually. Pregnancy was confirmed during gestation by the presence of a vascularized membrane in the vagina or palpation of uterine contents.

Litter observations

Litters were observed daily for evidence of abnormal behaviour or poor health. In addition, daily records of mortalities and changes in litter size were maintained.

Whenever possible, offspring found dead were sexed and examined externally and internally for abnormalities and possible cause of death; then discarded. Pups found cannibalized were recorded as such.

Birth (Day 0 of lactation)

As soon as possible after birth, the litter size (total number of pups born live or dead) was recorded. Each live pup was sexed, examined for external abnormalities, and weighed.

Day 4

The number of live pups was recorded. Live pups were sexed, examined for external abnormalities, and weighed individually before culling. Litters with more than eight pups were culled to produce, as nearly as possible, litters that contained four males - and four females. Culled pups were sacrificed and examined for cervical; thoracic, or abdominal visceral abnormalities. A mid-coronal slice was made to examine the brain. Tissues were discarded.

Days 7, 14 and 21

The number of live pups was recorded. Live pups were examined for external abnormalities and weighed individually by sex.

Weanling selections

After weaning, one male and one female, when possible, were selected at random from each F1 litter for assignment as F1 adults. Additional weanlings were selected from the remaining weanlings to provide 30 F1 generation animals/sex/group. On Lactation Day 21, 10 pups/sex/group from the F1 and F2 litters were selected at random from the remaining pups and necropsied. Pups not selected for F1 generation animals or for necropsy were sacrificed with carbon dioxide and discarded.

Anatomical pathology

A necropsy was done on all adults that were found dead or were sacrificed at scheduled or unscheduled intervals.

After mating for the parental males or the litters were weaned for the parental females, the FO and F1 adults were anesthetized with sodium pentobarbital, weighed, exsanguinated, and necropsied. Females that did not deliver a litter were necropsied after Gestation Day 26. On Lactation Day 21, 10 pups/sex/group from the F1 and F2 litters were anesthetized with sodium pentobarbital, weighed, exsanguinated, and necropsied.

The necropsy included a macroscopic examination of the external surface of the body; all orifices; the cranial cavity; the brain and spinal cord; and the thoracic, abdominal, and pelvic cavities and viscera. Uteri and ovaries were examined for implantations and corpora lutea, respectively, from adult females that cohabited with a male but did not deliver a litter. Uteri with no visible implantations were stained with a 10% ammonium sulfide solution to confirm pregnancy status. These uteri were not examined microscopically.

The following (when present) were weighed from all FO and F 1 adults at scheduled sacrifice; the underlined tissues only were weighed for selected F1. and F2 weanlings at scheduled sacrifice. Paired organs were weighed separately, unless otherwise specified:

Adrenals
Brain
Epididymides
Kidneys
Liver
Ovaries/oviductus
Prostate
Seminal vesicle with coagulating gland (weighed together)

Uterus with cervix (except for uteri stained with ammonium sulfide)

Spleen

Testes

Thymus

Organ-to-body weight percentages and organ-to-brain weight ratios were calculated.

At the scheduled necropsy of the FO and F 1 adult animals and selected F 1 and F2 weanlings, the following (when present) or representative samples were preserved in 10% phosphate-buffered formalin, unless otherwise specified:

Adrenals	Testes (preserved in Bouin's fixative)
Pituitary	Liver
Brain	Uterus with cervix (except for uteri stained with ammonium sulfide)
Prostate	
Coagulating gland	Lymph nodes (sub maxillary and mesenteric)
Seminal vesicles	Vagina
Epididymides	Ovaries with oviducts
Spleen	
Kidneys	

The above (as appropriate) were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically from all FO and F 1 adults in the control and high-dose groups. Examination of the ovaries included evaluation of a minimum of 10 sections from the completely sectioned left ovary for each female in the control and high-dose groups.

Reproductive organs of females in the low- and mid-dose groups that failed to mate or that mated but failed to deliver a litter and males that failed to sire progeny were examined microscopically.

Lesions of the FO and F1 adults were examined microscopically from low- and mid-dose groups.

Semen evaluation

The total number of sperm (concentration), percent sperm motility, and sperm morphology were determined for each FO and F1 adult male at scheduled sacrifice. The left epididymis was used to determine concentration. Motility and morphology assessments were made from sperm obtained from the left vas deferens.

Statistical analyses

Levene's test (Levene, 1960) was done to test for variance homogeneity. In the case of heterogeneity of variance at $p \leq 0.05$, rank transformation was used to stabilize the variance.

Analysis of variance [ANOVA (Winer, 1971 a)] was done on the homogeneous or transformed data. If the ANOVA was significant, Dunnett's multiple comparison t-test (Dunnett, 1964) was used for pairwise comparisons between groups. When transformation did not establish variance homogeneity at $p \leq 0.05$, the data were also examined by nonparametric techniques using the Wilcoxon-Mann-Whitney (Hollander and Wolfe,1973) two-sample rank test.

One-way ANOVA was used to analyze continuous data such as body weights, body weight changes, food consumption, litter data, days to mate, length of gestation, semen evaluation data (where appropriate), and organ weight data. Reproduction indices were analyzed by the Fisher-Irwin exact test (Christensen, 1990).

One-way analysis of covariance [ANCOVA (Winer, 1971b)] was used to analyze the pup body weights (male and female), with the number of pups in the litter as the covariate.

Groups 2 through 4 were compared with Group 1 (control). Group comparisons found to be statistically significant at the 5.0% and 1.0% two-tailed probability levels are indicated "*" and "**," respectively. As appropriate, for values calculated to analyze litter data or mean pup weight data., values were first derived within the litter, and the group mean values were derived as a mean of individual litter values.

Results:

All differences cited are based on comparisons with the control group, unless otherwise specified.

Clinical observations

F0 Generation: One female in the control group and one male in the low-dose group were sacrificed during Weeks 9 and 7, respectively. The female was sacrificed after an apparent mechanical injury. Observations of hypoactive, red fluid on pan paper, dry brown material around the nasal area and red stained haircoat were noted before the sacrifice of the male. The condition of these animals was unrelated to treatment with dodine.

There were no test material-related clinical observations noted for the F0 adults or F1 pups.

F1 Generation*:* Survival was 100% in the control, low-, and high-dose groups. One male in the mid-dose group was found dead during Week 11. There were no clinical observations for this animal before death.

There were no test material-related clinical observations noted during premating, gestation, or lactation for F 1 adults or F2 offspring.

Body weights:

F0 Generation*:* Mean body weights for the 800-ppm animals were consistently lower throughout the FO generation (premating, gestation, and lactation) after the initiation of treatment. Significant differences were noted at 800 ppm for the males for Weeks 1 through 12 and for the females for Weeks 3, 5 through 8, and 10 of premating, throughout gestation; and on Lactation Days 0, 4, 7, and 14. Mean body weights were also significantly lower for females given 400 ppm on Lactation

Day 4; however, this difference is considered to be incidental. There were no significant differences in the other group offered dodine.

F1 Generation: Mean body weights were lower for the 800-ppm males during the F1 generation, with significant differences noted for Weeks 0 through 12. Mean body weights were significantly lower during premating for females given 400 and 800 ppm; significant differences were noted at Weeks 0 through 8 and 0 through 10, respectively.

During gestation, significantly lower body weights were noted for Days 7, 14, and 20 for females given 400 ppm and for Days 0, 7, 14, and 20 for females given 800 ppm. The mean body weights were significantly lower for Lactation Days 4, 7, and 14 for females given 400 ppm and for Lactation Days 0, 4, 7, 14, and 21 for females given 800 ppm.

There were no consistent significant differences in mean body weights for the 200-ppm group.

The lower body weights in the 400- and 800-ppm groups were test material-related.

Body weight changes:

F0 Generation: Mean cumulative body weight changes were significantly lower for the 800-ppm males at Week 1 and continued throughout the FO generation. Mean cumulative body weight changes were significantly lower for the 800-ppm females during premating and significantly higher for Lactation Days 14 to 21 and 0 to 21. There were no other significant differences in the mean cumulative body weight changes for the animals given 200 or 400 ppm.

The differences in body weight changes for the 800-ppm animals during the FO generation were test material-related.

F1 Generation: Mean cumulative body weight changes were significantly lower for the 800-ppm males throughout the F1 generation. The females given 800 ppm had significantly lower mean body weight changes during Weeks 2- 8 and 10 of premating, gained significantly less weight at Gestation Days 0-7, 7-14, and 0-20; and gained significantly more weight at Lactation Days 14 - 21 and 0 - 21. There were no test material-related, significant differences in the mean cumulative body weight changes for the animals given 200 or 400 ppm.

The differences in body weight changes for the 800-ppm F1 animals were test material-related.

Food consumption:

F0 Generation: Mean food consumption was significantly reduced for males given diets at 800 ppm during Weeks 0-1, 1-2, and 2-3 and for males given 400 ppm at Week 0 to 1, but the decrease in food consumption at 400 ppm during a single interval is not considered to be toxicologically important. There were no other significant differences in food consumption for the 800-ppm males at subsequent intervals or for males in the remaining test material-treated groups during the F0 generation, indicating that early decreases in food consumption were probably associated with decreased palatability.

Mean food consumption was significantly reduced at Weeks 0-1, 1-2, 2-3, 3-4, and 4-5 and Lactation Days 4-7, 7-10, and 10-14 for females given diets at 800 ppm. Mean food consumption was significantly lower during Lactation Days 7 to 10 for females given dodine at 400 ppm; this difference is not considered to be toxicologically important.

The reductions in food consumption for animals given diets at 800 ppm were test material-related.

F1 Generation: Mean food consumption was significantly lower for the 800-ppm males throughout the F1 generation. There were no other significant differences in food consumption for the males given 200 or 400 ppm.

For females at 800 ppm, food consumption was significantly decreased throughout premating, gestation, and lactation (except for Days 0 to 4 of lactation). Sporadic significant differences in

mean food consumption were noted at Week 4 to 5 for females given 200 ppm and at Weeks 3 to 4 and Lactation Days 7 to 10 for females given 400 ppm; these differences are not considered to be toxicologically important.

The decreased food consumption for the F 1 animals given diets at 800 ppm was test material-related.

Oestrus data:

Although there were irregular cycles and differences noted in the length of the estrous cycles, these differences were noted in all groups, not related to ingestion of Dodine, and did not affect mating performance.

Reproduction data:

F0 Generation (see Tables 6.70 and 6.71)

Male data: Based on the percentage of inseminated and pregnant females, there were no test material-related differences in mating performance or male fertility.

Female data: There were no test material-related differences in mating performance, mean number of days to mating, or mean length of gestation. The fertility index was reduced for the 400- and 800-ppm groups; however because this was not repeated in the F1 generation and is not dose related, it is not considered to be test material-related.

Litter data: There were no test material-related significant differences in gestation, live birth, viability, or weaning indices or mean number of pups delivered. There were no notable differences observed for the sex ratio. Covariate-adjusted mean body weights were significantly decreased for the male and female pups in the 800-ppm group from Lactation Days 4 through 21 and on Days 4 (precull and postcull, females only), 14 (females only), and 21 (males and females) for the pups in the 400-ppm group. The reductions in pup body weights at 400 and 800 ppm were test material-related.

There were no test material-related findings noted during the necropsy of dead pups or pups culled on Lactation Day 4.

F1 Generation (see Tables 6.72 and 6.73)

Male data: Based on the percentage of inseminated and pregnant females, there were no test material-related differences for mating performance or male fertility.

Female data: There were no test material-related differences in fertility, mating performance, or mean number of days to mating. Litter Data. There were no test material-related significant differences in the gestation index, live birth, viability, or weaning indices or in the mean number of pups delivered.

There were no notable differences observed for the sex ratio. Covariate-adjusted mean body weights were significantly lower on Days 4 (precull and postcull, males only), 7, 14, and 21 for pups in the 800-ppm group and on Days 14 (males only) and 21 for pups in the 400-ppm group. The reduced pup body weights in the 400- and 800-ppm groups were test material-related.

There were no test material-related findings noted during the necropsy of dead pups or pups culled on Lactation Day 4.

Table 6.70 –Summary of reproductive performance (FO Generation)

Dose level		0 ppm	200 ppm	400 ppm	800 ppm
Number of paired females	N	29	30	30	30
Total number inseminated	N	28	30	29	30
	%	97	100	97	100
Total number pregnant	N	28	28	26	27
	%	100	93	90	90
Fertility index	%	97	93	87	90
N: number of females					

Table 6.71 –Summary of delivery and litter data (FO Generation)

Dose level			0 ppm	200 ppm	400 ppm	800 ppm
Females	Mated	N	28	30	29	30
	Pregnant	N	28	28	26	27
		%	100	93	90	90
	Delivering	N	28	28	26	27
		%	100	93	90	90
Duration of gestation		MEAN	22.1	22.1	21.9	22.1
		SD	0.4	0.5	0.5	0.4
		N	28	28	26	27
Females with live born pups		N	28	28	26	27
Gestation index		%	100	100	100	100
With stillborn pups		N	7	5	1	5
		%	25	18	3.8	19
Females with no live born pups		N	0	0	0	0
		%	0.0	0.0	0.0	0.0
Females with no pups delivered		N	0	0	0	0
		%	0.0	0.0	0.0	0.0
Pups delivered		TOTAL	380	385	324	356
		MEAN	13.25	13.75	12.46	13.19
		SD	3.24	2.17	3.00	2.13
		N	28	28	26	27
Live born		TOTAL	365	379	323	347
Stillborn		TOTAL	15	6	1	9
Uncertain		TOTAL	0	0	0	0
Pup survival indices						
Live birth index		MEAN %	96	98*	100**	97
Viability index		MEAN %	98	99	97	98
Weaning index		MEAN %	100	100	98	99
Live pups/Litter with live pups						
Day 0		MEAN	13.04	13.54	12.42	12.85
		SD	3.24	2.27	2.94	2.46
		N	28	28	26	27
Day 4 Precull		MEAN	12.75	13.39	12.00	12.67
		SD	3.26	2.31	2.83	2.53
		N	28	28	26	27
Day 4 Postcull		MEAN	7.64	7.93	7.69	7.96
		SD	1.19	0.38	0.79	0.19
		N	28	28	26	27
Day 7		MEAN	7.64	7.93	7.58	7.93
		SD	1.19	0.38	0.95	0.27
		N	28	28	26	27
Day 14		MEAN	7.64	7.93	7.58	7.89
		SD	1.19	0.38	0.95	0.32
		N	28	28	26	27

Dose level		0 ppm	200 ppm	400 ppm	800 ppm
Day 21	MEAN	7.64	7.89	7.58	7.89
	SD	1.19	0.42	0.95	0.32
	N	28	28	26	27
Pup weight/Litter (grams)					
Day 0 Males	MEAN	6.30	6.29	6.30	6.47
	SD	0.54	0.52	0.64	0.52
	N	27	27	26	27
	Covariate adjusted MEAN	6.36	6.35	6.21	6.45
Day 0 Females	MEAN	6.08	6.05	5.93	6.11
	SD	0.64	0.64	0.56	0.46
	N	28	28	26	27
	Covariate adjusted MEAN	6.12	6.11	5.84	6.10
Day 4 Males Precull	MEAN	9.85	9.87	9.52	9.26
	SD	1.04	0.74	1.67	1.19
	N	27	27	26	27
	Covariate adjusted MEAN	9.92	10.09	9.29	9.21*
Day 4 Females Precull	MEAN	9.56	9.63	9.06	8.74
	SD	1.31	1.19	1.45	1.04
	N	28	28.	26	27
	Covariate adjusted MEAN	9.57	9.81	8.88*	8.73**
Day 4 Males Postcull	MEAN	9.85	9.90	9.55	9.29
	SD	1.13	0.81	1.61	1.20
	N	27	27	26	27
	Covariate adjusted MEAN	9.92	10.14	9.31	9.23*
Day 4 Females Postcull	MEAN	9.60	9.66	9.08	8.75
	SD	1.28	1.21	1.43	1.03
	N	28	28	26	27
	Covariate adjusted MEAN	9.61	9.83	8.91*	8.74**
Day 7 Males	MEAN	16.01	16.50	15.28	14.32
	SD	1.60	1.09	2.44	1.41
	N	27	27	26	27
	Covariate adjusted MEAN	16.01	16.48	15.31	14.31**
Day 7 Females	MEAN	15.53	15.97	14.65	13.71
	SD	2.00	1.42	2.08	1.26
	N	28	28	26	27
	Covariate adjusted MEAN	15.48	16.04	14.57	13.77**
Day 14 Males	MEAN	33.86	34.61	31.86	28.09
	SD	2.86	2.08	4.60	1.49
	N	27	27	26	27
	Covariate adjusted MEAN	33.86	34.56	31.93	28.07**
Day 14 Females	MEAN	32.76	33.52	30.76	27.11
	SD	3.41	2.23	3.76	1.39
	N	28	28	26	27
	Covariate adjusted MEAN	32.71	33.60	30.69*	27.17**
Day 21 Males	MEAN	55.08	55.97	51.05	46.05
	SD	5.06	3.42	7.11	2.72
	N	26	27	26	27
	Covariate adjusted MEAN	55.08	55.94	51.09*	46.04**
Day 21 Females	MEAN	52.63	53.45	48.51	44.21
	SD	5.49	3.63	5.46	2.55
	N	27	28	26	27
	Covariate adjusted MEAN	52.56	53.56	48.41**	44.29**

Significant different from control: * p< 0.05; ** p< 0.01
TOTAL: number of pups or implants
N: number of females

Table 6.72 –Summary of reproductive performance (F1 Generation)

Dose level		0 ppm	200 ppm	400 ppm	800 ppm
Number of paired females	N	30	30	30	30
Total number inseminated	N	30	30	29	30
	%	100	100	97	100
Total number pregnant	N	30	29	27	30
	%	100	97	93	100
Fertility index	%	100	97	90	100
N: number of females					

Table 6.73 –Summary of delivery and litter data (F1 Generation)

Dose level			0 ppm	200 ppm	400 ppm	800 ppm
Females	Mated	N	30	30	29	30
	Pregnant	N	30	29	27	30
		%	100	97	93	100
	Delivering	N	30	29	27	29
		%	100	97	93	97
Duration of gestation		MEAN	22.2	22.1	22.1	22.0
		SD	0.4	0.4	0.4	0.3
		N	30	29	27	28
Females with live born pups		N	30	29	27	29
Gestation index		%	100	100	100	97
With stillborn pups		N	9	8	7	5
		%	30	28	26	17
Females with no live born pups		N	0	0	0	0
		%	0.0	0.0	0.0	0.0
Females with no pups delivered		N	0	0	0	0
		%	0.0	0.0	0.0	0.0
Pups delivered		TOTAL	430	421	346	385
		MEAN	14.33	14.52	12.81	13.28
		SD	2.23	2.68	2.87	1.79
		N	30	29	27	29
Live born		TOTAL	419	404	334	377
Stillborn		TOTAL	11	17	12	7
Uncertain		TOTAL	0	0	0	1
Pup survival indices						
Live birth index		MEAN %	97	96	97	98
Viability index		MEAN %	98	96	98	99
Weaning index		MEAN %	100	97	100	98
Live pups/Litter with live pups						
Day 0		MEAN	13.97	13.93	12.37	13.00
		SD	2.39	3.07	2.75	1.93
		N	30	29	27	29
Day 4 Precull		MEAN	13.73	13.59	12.04	12.86
		SD	2.39	3.57	2.72	1.92
		N	30	29	27	29
Day 4 Postcull		MEAN	7.97	7.59	7.78	8.00
		SD	0.18	1.57	0.97	0.00
		N	30	29	27	29
Day 7		MEAN	7.97	7.82	7.78	7.97
		SD	0.18	0.94	0.97	0.19
		N	30	28	27	29
Day 14		MEAN	7.97	7.82	7.78	7.90
		SD	0.18	0.94	0.97	0.41
		N	30	28	27	29

Dose level		0 ppm	200 ppm	400 ppm	800 ppm
Day 21	MEAN	7.97	7.82	7.78	7.83
	SD	0.18	0.94	0.97	0.54
	N	30	28	27	2*9
Pup weight/Litter (grams)					
Day 0 Males	MEAN	6.36	6.26	6.61	6.35
	SD	0.50	0.65	0.76	0.51
	N	30	29	27	29
Covariate adjusted MEAN		6.44	6.37	6.48	6.29
Day 0 Females	MEAN	5.99	5.83	6.15	6.06
	SD	0.49	0.57	0.54	0.49
	N	30	29	26	29
Covariate adjusted MEAN		6.03	5.88	6.10	6.02
Day 4 Males Precull	MEAN	10.17	10.13	10.34	9.54
	SD	1.03	0.90	1.76	1.00
	N	30	28	27	29
Covariate adjusted MEAN		10.34	10.39	9.99	9.45**
Day 4 Females Precull	MEAN	9.68	9.41	9.77	9.21
	SD	1.06	1.34	1.45	1.00
	N	30	29	26	29
Covariate adjusted MEAN		9.74	9.46	9.69	9.18
Day 4 Males Postcull	MEAN	10.17	10.13	10.36	9.57
	SD	0.99	0.94	1.76	102
	N	30	28	27	29
Covariate adjusted MEAN		10.34	10.40	10.02	9.48**
Day 4 Females Postcull	MEAN	9.72	9.51	9.75	9.22
	SD	1.05	1.36	1.45	0.98
	N	30	29	26	29
Covariate adjusted MEAN		9.78	9.5	9.68	9.19
Day 7 Males	MEAN	16.60	16.63	16.15	15.04
	SD	1.50	1.28	2.48	1.46
	N	30	28	27	29
Covariate adjusted MEAN		16.66	16.59	16.07	15.10**
Day 7 Females	MEAN	15.93	15.89	15.34	14.42
	SD	1.56	1.37	2.09	1.36
	N	30	28	26	29
Covariate adjusted MEAN		15.94	15.86	15.34	14.43**
Day 14 Males	MEAN	34.47	34.96	32.83	29.00
	SD	2.41	1.76	3.88	2.26
	N	30	28	27	29
Covariate adjusted MEAN		34.50	34.94	32.80*	29.01**
Day 14 Females	MEAN	33.17	33.29	31.75	27.82
	SD	2.34	2.14	30.5	2.24
	N	30	28	26	29
Covariate adjusted MEAN		33.14	33.33	31.72	27.82**
Day 21 Males	MEAN	56.67	56.66	53.02	46.90
	SD	3.73	3.12	5.74	3.43
	N	30	28	27	29
Covariate adjusted MEAN		56.79	56.63	52.94**	46.88**
Day 21 Females	MEAN	54.16	53.39	50.27	44.55
	SD	3.78	4.12	4.26	3.39
	N	30	28	26	29
Covariate adjusted MEAN		54.16	53.39	50.27**	44.55**

Significant different from control: * p< 0.05; ** p< 0.01
TOTAL: number of pups or implants
N: number of females

Semen evaluation

There were no significant differences in the percent motility, mean sperm concentration, of mean percent normal sperm for the FO or F1 males.

Anatomical pathology:

Dietary administration of the test material at 800 ppm to adult rats caused lower terminal body weights and correspondingly lower absolute organ weights, lower organ-to-brain weight ratios, and higher organ-to-body weight percentages for some organs. There were few macroscopic and microscopic findings and no major differences between control and treated animals in the incidence of any finding. All findings were considered incidental and unrelated to the test material. Similar terminal body weight and organ weight changes were observed in the F 1 and F2 weanlings at 800 ppm, and the few macroscopic observations in these animals were also considered incidental and unrelated to the test material.

Conclusions:

Based on the results of this study, the no-observable-adverse-effect level (NOAEL) of Dodine for systemic toxicity was 200 ppm (equivalent to 13.14 mg/kg bw/day) when administered continuously in the diet through two generations of CrI:CD®BR VAF/Plus® rats. The NOAEL for reproductive performance was 800 ppm (equivalent to 52.61 mg/kg bw/day). The NOAEL for offspring growth and development was 200 ppm (equivalent to 13.14 mg/kg bw/day) based on decreased pup weights at 400 and 800 ppm.

B.6.6.2 Developmental toxicity studies (Annex IIA 5.6.2)

Dose range finding study in rats preliminary to teratogenicity study

K.P. Hazelden, J.A. Wilson (1989a) – Report No. 5596, project No. 437750 of 09 February 1989; Performed by Inveresk Research International, Tranent, EH33 2NE, Scotland; dates of experimental work: 12 January 1989 to 09 February 1989.

Guidelines and GLP:

The study was conducted in compliance with an in house method.

The study is acceptable.

Materials and methods:

The purpose of this study was to estimate a maximum tolerated dose which may be used as the high dose level in the main rat teratogenicity study.

Mated female Sprague-Dawley rats were randomised into 4 treatment groups, each containing l0 animals. These animals were dosed dodine (batch 92/88/2; purity 95%) orally by gavage once daily over Days 6-16 inclusive of gestation, where Day 0 was the day of detection of a copulatory plug in situ and/or sperm in a vaginal lavage. Dose levels of test material were as follows, expressed as mg per kilogram of body weight:

	mg dodine/kg bw/day
Control	0
Low dose	50
Intermediate dose	70
High dose	100

Observations

Clinical examinations once daily for mortality and signs of ill health or behavioural changes, body weights (days 0, 6, 9, 13, 17, 20 of gestation), food consumption (recorded daily).

At termination on day 20 of gestation: examinations of thoracic and abdominal cavities, reproductive tract (weight), uterus, number, location and classification of all implantations, number of corpora lutea in each ovary, examination of each foetus, total weight of all the live foetuses.

Statistical analyses

ANOVA using normal linear model for one way classification, Fisher's F-protected least significant difference (LSD), Kruskal-Wallis non parametric analysis, Dun's procedure, Contigency tables with either the Chi-squared test or Fisher's exact probably test.

Findings:

There was maternal toxicity at 100 mg/kg bw/day, indicated by one mortality and by reduction in body weight gain and food consumption during the treatment period.

Some maternal toxicity at 70 mg /kg bw/day was indicated by a smaller reduction in body weight gain and food consumption during the treatment period, than was observed for the animals receiving 100 mg/kg bw/day.

There was only marginal toxicity at 50 mg /kg bw/day, indicated by a marginal reduction in food consumption during the treatment period.

Conclusions:

Based on these results, 90 mg dodine/kg bw/day was considered to be an appropriate upper dose level for use in a teratogenicity study.

Suitable dose levels for the Intermediate dose and Low dose levels in such a study might then be 45 and 10 mg dodine/kg bw/day, respectively.

Dodine: Teratogenicity study in rats

K.P. Hazelden, J.A. Wilson (1989b) – Report No. 5965, project No. 437766 of 10 July 1989; Performed by Inveresk Research International, Tranent, EH33 2NE, Scotland; dates of experimental work: 23 March 1989 to 10 July 1989.

Guidelines and GLP:

The study was conducted in compliance with FIFRA 83-3, and OECD 414. The study is GLP compliant.

The study is acceptable.

Materials and methods:

Dodine (batch 92/88/2; purity 95%) was administrated by oral gavage to 25 mated Sprague-Dawley rats at doses of 0, 10, 45 or 90 mg/kg bw/day on gestation days 6 through 16, where Day 0 was the detection of a copulatory plug in situ and/or sperm in a vaginal lavage.

The test material was formulated as a suspension, the vehicle being distilled dose, and dose levels were as follows:

	mg dodine/kg bw/day
Control	0
Low dose	10
Intermediate dose	45
High dose	90

Clinical observations

All the animals were checked for viability at the beginning of each day, and again as late as possible on each day.

All the animals were examined for reaction to treatment on each day. The onset, duration and intensity of any signs were recorded, particular attention being paid to the period 1 – $1^{1/2}$ h after dosing.

Body weight

Individual body weights were recorded on Days 0, 6, 9, I3, 17 and 20 of gestation.

Food Consumption

The weight of food consumed by each animal was recorded daily, commencing on Day 4 of gestation.

Terminal Studies

On Day 20 of gestation, the animals were killed by carbon dioxide asphyxiation.

The contents of the thoracic and abdominal cavities were inspected. Any lesions seen were described and representative samples of abnormal tissue were preserved, where appropriate; in neutral buffered 10% formalin.

The reproductive tract was removed and weighed intact, then opened and the contents were examined. The number of corpora lutea graviditatis in each ovary and the number and position of all implant sites in the uterus were recorded. Each implant was classified as being live, a foetal death (death judged to have occurred after ca Day 16 of gestation), a late embryonic death (death judged to have occurred in the period ca Day 12-Day 16) or an early embryonic death (death judged to have occurred prior to ca Day 12).

Each live foetus was individually identified within the litter and its weight was recorded. The foetuses were examined for externally visible abnormalities. Approximately one half of the foetuses were fixed in methylated ethyl alcohol and the remaining half in Bouin's fluid.

Those foetuses fixed in alcohol were subsequently examined for visceral abnormalities by open dissection. The eviscerated carcasses were then cleared in potassium hydroxide and glycerol, and the skeletons were stained with Alizarin Red S. Skeletal structures in these foetuses were examined for abnormalities and variants; including state of ossification. The sex of each foetus was determined during the dissection procedures.

Statistical Analysis of Results

Where considered appropriate, analyses were performed to determine the statistical significance of observed differences between treatment groups.

Maternal weight gain from Day 6-9 of gestation was analysed using the nonparametric Kruskal-Wallis test, treatments being compared using Chi-squared protected z-tests based on mean rank scores.

All other maternal weight gains were subjected to analysis of variance (ANOVA) using the Normal linear model for a one-way classification. Treatments were compared using Fisher's F-protected least significant difference procedure

Results:

Clinical Observations and Necropsy Findings

Three animals receiving 90 mg /kg bw/day were observed to have excessive salivation for one or 2 days during the treatment period.

In all 3 cases this was coincidental with some reduction in food consumption. There was a very low incidence of animals with red/brown stained fur around the mouth at 45 and 90 mg/kg bw/day dose groups. This finding can be associated with stress in the rat and could have been an adverse individual response to the dosing procedure itself and/or to treatment with dodine.

The above clinical findings were recorded during the mid to late dosing period.

Necropsy findings in the lungs of 2 animals receiving 90 mg/kg bw/day were considered by the pathologist most likely to have been agonal, and therefore of no toxicological significance.

Body Weight Performance

At 90 mg/kg bw/day 11 out of 23 animals lost weight over Days 6-9 of gestation, while mean weight gain for the remaining pregnant animals in this group was reduced, as compared with Control. Weight gain from Days 9-17 of gestation was then similar to the controls, so that the deficit occurring at the beginning of the treatment period was not recovered. This resulted in the statistically significant reduction in weight gain over the treatment period:

At 45 mg/kg bw/day, mean weight gain from Day 6-9 of gestation was reduced as compared to the controls, with 4 animals actually losing weight. However, over the remainder of the treatment period, mean weight gain was slightly increased, compensating for the earlier deficit. Thus, the weight gain calculated over the treatment period as a whole was not significantly lower than for the controls.

Body weight performance of animals receiving 10 mg/kg bw/day was considered to have been unaffected by treatment with dodine.

Food Consumption

The majority of animals receiving 90 mg/kg bw/day showed some reduction in food consumption during the treatment period. The effect occurred principally at the beginning of the treatment period, between Days 6 and 10 of gestation:

Food consumption was also reduced in 4 animals at 45 mg/kg bw/day, again principally during the first few days of treatment.

At 10 mg/kg bw/day dose group the food consumption was unaffected by treatment.

Pregnancy Performance and Foetal Weight

None of the pregnancy performance parameters, nor foetal weight, were considered to have been affected by treatment with dodine.

Table 6.74 – Pregnancy performance and foetal weight

	Groups/Dose levels (mg/kg bw/day)			
	1 (0)	**2 (10)**	**3 (45)**	**4 (90)**
Number of animals mated	25	25	25	25
Number pregnant	22	21	23	24
Number pregnant at day 20 necropsy	22	21	23	24
Number of premature decedents	0	0	0	0
Pregnancy frequency as %	88	84	92	96
Total corpora lutea	320	295	348	361
total implants	303	254	324	347
Pre-implantation loss as %	5	14	7	4
Total live implants (%of total implants)	290 (96	248 (98)	316 (98)	336 (97)
Total dead implants (%of total implants)	13 (4)	6 (2)	8 (2)	11 (3)
Total early deaths (%of total implants)	13 (4)	5 (2)	8 (2)	9 (2)
Total late deaths (%of total implants)	0	1 (0.4)	0	2 (1)
Total foetal deaths (%of total implants)	0	0	0	0
Mean corpora lutea	14.5 ± 1.7	14.0 ± 2.6	15.1 ± 2.0	15.0 ± 1.8
Mean implants	13.8 ± 2.7	12.1 ± 4.9	14.1 ± 3.3	14.5 ± 2.3
Mean live implants	13.2 ± 2.6	11.8 ± 4.8	13.7 ± 3.3	14.0 ± 2.2
Mean dead implants	0.6 ± 0.7	0.3 ± 0.7	0.3 ± 0.6	0.5 ± 0.6
Mean early deaths	0.6 ± 0.7	0.2 ± 0.5	0.3 ±0.6	0.4 ± 0.6
Mean late deaths	0	0.05 ± 0.2	0	0.1 ± 0.3
Mean foetal deaths	0	0	0	0
Total live male foetuses (% of total live implants)	148 (51)	119 (48)	152 (48)	153 (46)
Total live female foetuses (% of total live implants)	142 (49)	129 (52)	164 (52)	183 (54)
Live foetal sex ratio, m:f	1 : 0.96	1 : 1.08	1 :1.08	1 :1.20
Mean total uterus weight (g)	79.4 ± 13.9	72.0 ± 26.7	83.5 ± 19.3	85.3 ± 10.0
Mean litter mean foetal weight (g)	3.84 ± 0.22	3.94 ± 0.33	3.86 ± 0.27	3.85 ± 0.27
Means are given ± Standard Deviation				

Foetal Abnormalities and Variants

There were sporadic occurrences of both major and minor abnormalities in all groups, including the Controls. None of these occurrences were considered to have been related to treatment with dodine.

There was no indication that treatment with dodine had any effect on skeletal ossification in the developing foetus.

Conclusions

There was maternal toxicity at 45 and 90 mg/kg bw/day, indicated by reduced body weight gain and reduction in food consumption during the treatment period, principally during the first few days of treatment.

Under the conditions of this study, the no adverse effect level (NOAEL) for the maternal toxicity was the dose level of 10 mg/kg bw/day, and for developmental toxicity was the dose level of 90 mg/kg bw/day. Dodine is not teratogen in rats up to 90 mg/kg bw/day.

Dodine: Dose range finding study in rabbits preliminary to teratogenicity study

C.Mc Cay, K.P. Hazelden (1989a) – Report No. 5687, project No. 437724 of 15 February 1989; Performed by Inveresk Research International, Tranent, EH33 2NE, Scotland; dates of experimental work: 23 January 1989 to 15 February 1989.

Guidelines and GLP:

The study was conducted in compliance with an in house method.

The study is acceptable.

Materials and methods:

The purpose of this study was to estimate a maximum tolerated dose which may be used as the high dose level in the main rabbit teratogenicity study.

Mated New Zealand White rabbits were randomised into 3 treatment groups, each containing l0 animals. These animals were dosed orally by gavage once daily over Days 6-18 inclusive of gestation, where Day 0 of gestation was the day of mating. The dose levels applied were 0, 70 and 100 mg dodine/kg bw/day.

Observations

Clinical examinations twice daily for mortality and signs of ill health or behavioural changes, body weights (days 0, 6, 9, 12, 15, 19, 22 of gestation), food consumption (recorded daily). At termination on day 29 of gestation: examinations of thoracic and abdominal cavities, reproductive tract (+weight), uterus, number, location and classification of all implantations, number of corpora lutea in each ovary, examination of each foetus, total weight of all the live foetuses.

Statistical analyses

ANOVA using normal linear model for one way classification, Fisher's F-protected least significant difference (LSD), Kruskal-Wallis non parametric analysis, Dun's procedure, Contingency tables with either the Chi-squared test or Fisher's exact probability test.

Results:

Clinical Observations and Necropsy Findings

At 100 mg/kg bw/day, there were 5 premature decedents, which had shown weight loss and reduced food consumption. Hyperplasia of the stomach mucosa, indicative of irritation, was found in these animals. Other findings in the group also indicated gastro-intestinal disturbance: gaseous distension of the caecum with softened/liquid contents, and reduced faecal output. All these occurrences were considered to be related to treatment with dodine.

At 70 mg/kg bw/day, one animal was killed prematurely, owing to its poor condition, following marked weight loss and reduced food consumption. Hyperplasia of the stomach mucosa was found in this animal. Another animal in the group had liquid caecal contents. These occurrences again were attributed to treatment with test material.

The incidences of other clinical signs and necropsy findings were not considered to be related to treatment with dodine.

Body Weight Performance and Food Consumption

At l00 mg dodine/kg bw/day, there was weight loss and a marked reduction in food consumption during the dosing period, the effect being most severe over Days 6-9 of gestation.

At 70 mg dodine/kg bw/day, for the majority of animals, there was either no effect on body weight performance and food consumption, or only a slight and transitory effect on food consumption early in the dosing period. By the end of the dosing period these animals had accumulated no overall deficit in weight gain or food consumed, as compared with Control. Animal 20 in this group, however, showed weight loss and a marked reduction in food consumption throughout the dosing period.

Pregnancy Performance

At 100 mg dodine/kg bw/day, only 4 animals reached Day 29 of gestation and therefore only individual values have been presented. From the limited data available in this group, there was no obvious effect of dodine on the pregnancies.

At 70 mg dodine/kg bw/day, the pregnancy performance parameters were considered unaffected by treatment with dodine.

Discussion and conclusions

At 100 mg dodine/kg bw/day, the marked maternal toxicity demonstrated the unsuitability of this dose level for further use.

At 70 mg dodine/kg bw/day, one animal was killed prematurely, showing signs of excessive toxicity, but the other animals in this group were either only marginally affected, or were unaffected by treatment. The marked toxicity shown by-one animal at this level may have been associated with the fact that it was the last animal in the group to be dosed on each day of treatment, and the analytical result for the sample of dosing suspension taken on one day, just after this animal was dosed, showed that the achieved concentration was as high as for animals in the higher dose group. The results from this animal in particular were obliged to be considered separately from those of the rest of the group.

Based on the results of this study, it was considered that 80 mg dodine/kg bw/day would be suitable as an upper dose level for use in a teratogenicity study. Suitable dose levels for the Intermediate and Low dose groups in such a study could then be 40 and 10 mg dodine/kg bw/day, respectively.

Dodine: Teratogenicity study in rabbits

C.Mc Cay, K.P. Hazelden (1989b) – Report No. 5861, project No. 4377456 of 19 July 1989; Performed by Inveresk Research International, Tranent, EH33 2NE, Scotland; dates of experimental work: 10 March 1989 to 19 July 1989.

Guidelines and GLP:

The study was conducted in compliance with FIFRA 83-3, and OECD 414. The study is GLP compliant.

The study is acceptable.

Materials and methods:

Mated female New Zealand White rabbits were randomised into 4 treatment groups, with Control; Low and Intermediate dose groups containing 16 animals and the High dose group containing 20. These animals were dosed once daily by oral gavage dodine (batch 92/88/2; purity 95%) over Days 6-18 inclusive of gestation, where Day 0 was the day of mating. The test material was formulated as a suspension, the vehicle being distilled water, and dose levels were as follows:

	mg dodine/kg bw/day
Control	0
Low dose	10
Intermediate dose	40
High dose	80

Clinical observations

All the animals were checked for viability at the beginning of each day, and again as late as possible on each day. Any animal showing signs of severe debility were killed. Any animal showing signs of

abortion were killed, except where it was considered that the animal might have retained some live implants. All the animals were examined for reaction to treatment on each day. The onset; duration and intensity of any signs were recorded, particular attention being paid to the period 1-2 h after dosing.

Body Weight

Individual body weights were recorded on Days 6, 9, 12, 15, 19, 22, 26 and 29 of gestation.

Food Consumption

The weight of food consumed by each animal, from the 250 g offered each day, was recorded daily, commencing on Day 4 of gestation (weighed quantity first offered on Day 3).

Terminal Studies

Killing was by intravenous injection, via the marginal ear vein, of sodium pentobarbitone at a dose of approximately 150-200 mg/kg.

Premature Decedents and Abortions of Pregnancy

Those animals that were killed because of debility, or died during the study, were examined at necropsy with a view to diagnosis of the cause of the animal's condition. The 4 animals that aborted their pregnancies were similarly examined at necropsy for any condition which may have predisposed to the abortion, while their reproductive tracts were examined, where practicable, as described below for necropsies conducted on Day 29 of gestation.

Termination on Day 29 of Gestation

The contents of the thoracic and abdominal cavities were inspected. Any lesions seen were described and representative samples of abnormal tissue were preserved in neutral buffered 10% formalin.

The reproductive tract was removed and weighed intact, then opened and the contents were examined. The number of corpora lutea graviditatis in each ovary and the number and position of all implant sites in the uterus were recorded. Each implant was classified as being live, a foetal death (death judged to have occurred after ca Day 18 of gestation), a late embryonic death (embryonic remains were visible) or an early embryonic death (only placental remains were visible). The borderline between early and late embryonic deaths in this classification lies at ca Day 12 of gestation.

The weights of individual viable foetuses were recorded and the foetuses (including foetal deaths) were examined for externally visible abnormalities. All viable foetuses were examined for gross ocular abnormalities and individually identified prior to fixation. Two thirds of the viable foetuses from each uterus were fixed in methylated ethyl alcohol and the remaining third in Bouin's fluid. The foetuses fixed in alcohol were subsequently examined for visceral abnormalities by open dissection and the cranium was sectioned once through the coronal suture to allow inspection of the brain in that region. The eviscerated carcasses were then cleared in potassium hydroxide and glycerol, and the skeletons were stained with Alizarin Red S. Skeletal structures in these foetuses were examined for abnormalities .and variants, including state of ossification: The foetuses fixed in Bouin's fluid were examined for soft tissue abnormalities by whole-body dissection, the internal head structures being examined by means of a free- hand razor blade sectioning technique. All foetuses were sexed during dissection procedures.

Statistical Analysis of Results

Formal statistical analyses were not considered necessary for the results of this study. Interpretation was facilitated by inspection of individual and group mean values.

Results:

Clinical Observations and Necropsy Findings

At 80 mg dodine/kg bw/day, 2 animals aborted their pregnancies and another was killed owing to its poor condition. These animals had shown weight loss and reduced food consumption, and softened/liquid intestinal and/or stomach contents were found at necropsy. One of these animals also had mucus-like material and several irregular white areas covering the pyloric mucosa of the stomach. These findings were considered to have been related to treatment with dodine.

There were 2 other premature decedents at the High dose level. These had exhibited breathing difficulties, and at necropsy reddened/dark areas in the lung lobes were found. Lung changes of this type were also found in 2 other animals in this group. The animals in the High dose group struggled during the dosing procedure and the breathing problems and lung findings were considered to have been a result of accidental damage during dosing.

At 40 mg dodine/kg bw/day, accidental damage during dosing was considered to have resulted in the death of one animal and to have been the cause of noisy breathing and lung changes in a second animal. No animal restraint problems were reported during the dosing of this group.

At necropsy, one animal had liquid caecal contents; in view of the gut disturbances seen in premature decedents at the High dose level, it is possible that this finding also was related to treatment, but such a conclusion would remain questionable.

The other clinical signs and necropsy findings, including the single incidental abortion which occurred in each of the Control and Low dose groups, were not considered to indicate any effect of treatment with dodine.

Body Weight Performance

At 80 mg dodine/kg bw/day, over Days 6-9 of gestation, there was generally a minor loss in weight. Subsequently 3 animals (Nos. 51, 62 and 67) continued to lose weight, one of which had to be killed before the end of treatment, while the other 2 later aborted their pregnancies. For the remaining animals in the group, treatment period weight gain subsequent to Day 9 was essentially similar to Control.

At 40 and 10 mg dodine/kg bw/day, body weight performance was essentially similar to control.

Food Consumption

At 80 mg dodine/kg bw/day, there was generally a period of reduced food consumption during the first few days of treatment. Three animals (Nos. 51, 62 and 67) which subsequently showed a particularly severe effect were killed before scheduled termination. Transient reductions in consumption were observed sporadically among the other animals during the mid to late treatment period, but comparable events also occurred among control animals at various times during treatment. The continued reduction in group mean food consumption after the first few days of treatment was a result of inclusion in the means of the 3 severely affected animals mentioned previously. Thus, overall, the effect of 80 mg /kg bw/day was to reduce food consumption transiently during the early part of the treatment period, although for a minority of animals the effect was, or became, so severe that early termination was eventually required for them.

Food consumption was not considered to have been significantly affected by treatment with either 40 or 10 mg /kg bw/day.

Pregnancy Performance and Foetal Weight

The pregnancy performance parameters, including foetal weight, were unaffected by treatment with dodine (Table 6.75).

Table 6.75 – Pregnancy performance and foetal weight

	Groups/Dose levels (mg/kg bw/day)			
	1 (0)	**2 (10)**	**3 (40)**	**4 (80)**
Number of animals mated	16	16	16	20
Number pregnant	15	15	16	17
Number pregnant at day 29 necropsy	14	14	15	12
Number of premature decedents	1	1	1	5
Number of decedents pregnant	1	1	1	5
Pregnancy frequency as %	94	94	100	85
Total corpora lutea	154	167	175	145
total implants	128	139	150	118
Pre-implantation loss as %	17	17	14	19
Total live implants (%of total implants)	115 (90)	124 (89)	121 (81)	98 (83)
Total dead implants (%of total implants)	13 (10)	14 (10)	29 (19)	20 (17)
Total early deaths (%of total implants)	7 (5)	10 (7)	15 (10)	10 (8)
Total late deaths (%of total implants)	2 (2)	1 (14)	9 (6)	5 (4)
Total foetal deaths (%of total implants)	4 (3)	4 (3)	5 (3)	5 (4)
Mean corpora lutea	11.0 ± 2.5	11.9 ± 3.2	11.7 ± 1.9	12.1 ± 1.9
Mean implants	9.1 ± 2.5	9.9 ± 2.0	10.0 ± 2.3	9.8 ± 3.5
Mean live implants	8.2 ± 2.2	8.9 ± 1.9	8.1 ± 2.3	8.2 ± 2.9
Mean dead implants	0.9 ± 1.2	1.1 ± 1.4	1.9 ± 1.8	1.7 ± 1.6
Mean early deaths	0.5 ± 0.9	0.7 ± 1.1	1.0 ±1.1	0.8 ± 1.1
Mean late deaths	0.1 ± 0.4	0	0.6 ± 1.1	0.4 ± 0.9
Mean foetal deaths	0.3 ± 0.5	0.3 ± 0.7	0.3 ± 0.6	0.4 ± 0.9
Total live male foetuses (%of total live implants)	56 (49)	57 (46)	68 (56)	49 (50)
Total live female foetuses (% of total live implants)	59 (51)	67 (54)	53 (44)	49 (50)
Live foetal sex ratio, m:f	1.0 : 1.1	1.0 : 1.2	1.0 : 0.8	1.0 :1.0
Mean total uterus weight (g)	542 ±101	573 ± 104	539 ± 124	539 ± 156
Mean litter mean foetal weight (g)	44.8 ± 6.0	43.3 ± 3.3	43.6 ± 5.0	44.5 ± 5.8
Means are given ± Standard Deviation				

Foetal Abnormalities and Variants

The incidences of the various abnormalities and variants did not indicate an adverse effect of treatment with dodine. Also, many of the major abnormalities seen in this study are known to occur as background findings in this strain of animals.

None of the skeletal ossification parameters were considered to have been affected by treatment with dodine.

The incidences of the various abnormalities and variants did not indicate an adverse effect of treatment with dodine. Also, many of the major abnormalities seen in this study are known to occur as background findings in this strain of animals.

None of the skeletal ossification parameters were considered to have been affected by treatment with dodine.

Conclusions:

Maternal toxicity was manifest at 80 mg dodine/kg bw/day, generally as reduced food consumption and a minor loss in weight over the first few days of treatment. For a minority of animals, however, the effect was severe, resulting in necessary early euthanasia or in abortion of pregnancy. There was no significant effect on the dams at either 40 or 10 mg dodine/kg bw/day.

The maternal NOAEL was 40 mg/kg bw/day based on decreased mean food consumption during the dosing period.

The developmental NOAEL was the dose of 80 mg/kg bw/day. Dodine is not teratogen up to 80 mg/kg bw/day for rabbits.

B.6.7 Delayed neurotoxicity (Annex IIA 5.7)

Chimac-Agriphar SA did not carry out neurotoxicity studies with the fungicide dodine since it was not deemed necessary.

In none of the toxicological studies there was indication of a mode of toxicity involving the central or peripheral nervous system (please see Dodine: Evaluation of clinical signs in the rat chronic study, and Clinical signs observed in the long term carcinogenicity study, Semino, G. January 200)Annex B.6, point B.6.5). No functional or neuropathological effects on central or peripheral nervous system were observed in toxicity studies carried out with pups, young adults or old animals. Moreover, taking in account dodine chemical structure, no delayed neurotoxic effects are expected.

Available information on dodine toxicological database and chemical structure of dodine, do not suggest any potential neurotoxic effect. Consequently, Chimac-Agriphar SA feels that there is no justification for performing additional studies involving the sacrifice of further animals.

B.6.8 Further toxicological studies (Annex IIA 5.8)

Not relevant in the light of the results of the existing toxicity data.

B.6.8.1 Toxicity studies on metabolites (Annex IIA 5.8.1)

The Notifier did send the next justification.

Extensive degradation of dodine and rapid excretion of the metabolites through urine and faeces occurred in the rat. In the fruit metabolism studies, no significant metabolites were found and the residue consisted mainly in the parent compound. Thus, separate toxicity studies on metabolites are not necessary.

B.6.9 Medical data and information (Annex IIA 5.9)

B.6.9.1 Report on medical surveillance on manufacturing plant personnel (Annex IIA 5.9.1)

Notifier did send the next information:

There is one report only on the surveillance and health monitoring of personnel directly involved since 15 years in the manufacture of dodine by the Doctor of the plant at a contract synthesis plant of Chimac-Agriphar (the report was confidential). During this time, substantial quantities of this material were manufactured. The monitoring did not result in any significant findings which can be attributed to the active substance.

The doctor states that clinical examination of the workers takes place bi-annually. What is complicating the allocation of specific effects to the exposure to specific substances must be seen in the simple fact that the manufacturing personnel has to handle a whole series of different chemicals and primary materials during work time. Up to 13 workers are involved in the manufacture of

dodine which last approx. 6 months per year. The routine, bi- annual investigation into haematology, urinalysis and clinical chemistry did not establish any pathological finding which could be related to the exposure to dodine.

The JMPR toxicological review dated 1975 summarizes an unpublished medical survey from the initial manufacturer of dodine, the company American Cyanamid: workers involved in the production of dodine in the United States from 1958-1964, during which time substantial quantities of this material were manufactured, have been monitored. It has been shown that the occupational exposure to dodine produces minor superficial effects. Acute dermatitis and acute eye irritation accompanied by conjunctival and corneal burn were readily reversible and are of no long lasting significance (Hartmann, 1964).

B.6.9.2 Report on clinical cases and poisoning incidents (Annex IIA 5.9.2)

To the best knowledge of the notifier and summary dossier compilers, information on clinical cases and poisoning incidents related to the intake of, and/or exposure to dodine is relatively limited. A comprehensive data base research was performed in the following data banks:

- Belgian poisoning center / Brussels
- Extoxnet
- RTECS
- HSDB
- Toxline
- Medline.

One recent report from the University of Milan / Italy (Med. Lav. 1994 (Jul-Aug) 85(4):321-6) reported one person suffering from oculo-rhinitis due to sensitization reaction to Dodine (paper not send to the RMS).

One case of fatal poisoning was reported (Toxicol. Eur. Res 1978 May; 1(3):181-4). However, the plant protection product contained a mixture of monocrotophos, dodine and dinocap and the real toxicological impact of dodine in the mixture is questionable when compared to the high toxicity of monocrotophos. Monocrotophos could be measured in all tissues and in blood. Dodine and dinocap were not detected in these materials above the LOD. The gastric contents contained 52 mg of monocrotophos, 7.5 mg of dodine and 20 mg of dinocap.

The Belgian poisoning center informed s that they received 7 calls related to dodine between 01/01/1991 and 19/12/2001. 5 of them related to the use of dodine in mixture with other pesticides and 2 to the use of dodine alone. Symptoms in the 7 cases were as follows:

1. after inhalation:

- headache and epistaxis by a professional worker exposed to dodine in mixture with an organophosphate (a.s. not mentioned)

- pyrosis 2 hours after inhalation of dodine in mixture with 2 others pesticides (a.s. not mentioned)

- abdominal cramps 3 days after exposition during one day to dodine in mixture with another fungicide (a.s. not mentioned)

- weakness, dizziness, vomiting 10 hours after exposure to dodine alone

2. after contact with eye:

- ocular irritation (red eye, irritation, tears) after exposure to dodine alone (concentration unknown)

3. after oral ingestion:

- no symptoms 4hours after ingestion (unknown concentration)

4. after dermal exposure:

- irritation, skin burn 4hours after exposure to dodine in a mixture with organochloride (a.s. not mentioned)

Due to the fact that the number of calls is quite limited and that the calls often concern dodine in mixture with other compounds, it is difficult to estimate the real toxicological impact of dodine.

In conclusion, taking into account that dodine is manufactured since nearly 50 years and used in significant amount around the world, it can be concluded that risk to humans is relatively limited.

B.6.9.3 Observations on exposure of the general population and epidemiological studies (Annex IIA 5.9.3)

Not available.

B.6.9.4 Clinical signs and symptoms of poisoning and details of clinical tests (Annex IIA 5.9.4)

By skin contact, skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering. Inhalation or ingestion may cause nausea, vomiting, abdominal pains. By eye contact, symptoms of irritation occur.

B.6.9.5 First aid measures - Therapeutic regimes (Annex IIA 5.9.5)

If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a doctor. Loosen tight clothing such as collar, tie, belt or waistband. In case of skin contact, immediately flush skin with plenty of water. Cover the irritated skin with an emollient. Remove contaminated clothing and shoes. Wash clothing before re-use. Thoroughly clean shoes before reuse. Get medical attention. If in contact with eye, check for and remove any contact lenses. Immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Therapeutic regimes: No specific treatment, treat symptomatically.

B.6.9.6 Expected effects and duration of poisoning as a function of the type, level and duration of exposure or ingestion (Annex IIA 5.9.6)

The actual cases of intoxication with dodine are not well documented. Therefore, this point cannot be properly addressed.

The long existence of this product (over 50 years) without major known intoxication events rather suggests accidental or occupational poisoning to be hardly possible.

B.6.10 Summary of mammalian toxicology and proposed ADI, AOEL, ARfD and drinking water limit (Annex IIA 5.10)

Kinetics and metabolism:

Absorption, distribution, metabolism and excretion of dodine were tested in 2 studies in Sprague-Dawley rats using a single dose (low), a second single dose (high) and a repeated dose (low) by the oral route.

The first study from 1985 used also a single intravenous administration at the low dose level of 5 mg/kg bw. Plasma levels of radioactivity after single oral administration were low reaching a maximum at 4 h post dosing and decreasing rapidly during the following 8 hours. When administered intravenously, the elimination from plasma was rapid, 1% of the administered dose was recorded 5 minutes post dosing. Repeated oral administration of dodine for 7 days indicated that the peak plasma levels (4 hours post dosing) increased slightly until the day 6, after the 5th dose however, the peak plasma levels remained approximately constant and the overall profile of plasma radioactivity after the 7th dose was similar to that observed after a single oral dose. Oral administration of a high dose (50 mg/kg bw) resulted also to a maximum level of radioactivity 4 hours post dosing, the peak plasma level of radioactivity was maintained from 4 h until 12 hours decreasing thereafter slowly to 24 hours.

Following oral administration, dodine was eliminated almost equally via the urine and faeces (approx 45% each), whereas the majority of radioactivity was eliminated in the urine (approx 70%) in the case of intravenous administration. This indicates either low absorption of the compound or increased biliary elimination following oral administration. Expired $^{14}CO_2$ was a minor route of elimination (ca. 0.5% on both types of administration), suggesting that complete degradation is limited. Radioactivity remaining in the residual carcass was higher by intravenous injection (8%) compared to oral administration (0.6%), in both cases excretion was rapid; urine and faeces accounted for more than 80% of radioactivity eliminated in 96 hours.

Following single oral administration of ^{14}C-dodine, radioactivity was distributed unevenly to the tissues; whilst radioactivity in most tissues rapidly declined over 96 h post dose, the levels of fat tissues declined only slowly. The ovaries, thyroid and skin showed some degree of retention similar to that of the fat tissue. Multiple dose administration resulted in a slower decrease of radioactivity than after single dosing, showing the same pattern of elimination.

The plasma protein binding study indicates that dodine is rapidly metabolised to more water soluble metabolites with less affinity to plasma proteins. Analysis of the metabolites indicates that ^{14}C-dodine was rapidly metabolised to a number of unidentified polar components, which were chromatographically dissimilar to dodine dodecylamine and dodecylurea.

The second study (1992), guideline compliant, was conducted with oral dose levels of 40 (single and multiple administration) and 400 mg/kg bw (single administration). The major portion (>90% of the total urinary dose) of the low (single or multiple) oral dose was eliminated by 48 hours in urine and faeces of both sexes, but in the high dose group, elimination took 120 h to be complete (only c.a. 50% of the total urinary dose was excreted in 48 h). The study author presumed that inhibition of GI tract motility (peristaltic movements) may have resulted in the prolonged excretion of dodine in the high dose group following first-order absorption process.

In this study, faecal elimination (47.6-59.7%) was higher than urine excretion (40.5-45.3%) in all dose groups. Very low amounts of radioactivity were recovered from tissues and carcass at the 120 h post dosing (all together ranged from 0.62 to 3.34% of the administered dose), the overall distribution pattern was similar in all dose groups and in both sexes. Analysis of the ^{14}C-content of expired air during the preliminary study indicated that less than 1% of the initial dose was recovered as either $^{14}CO_2$ or volatiles throughout the 72-hours period. There was no evidence of accumulation of dodine or its metabolites in tissues after single or multiple exposures.

Analysis of the faecal extracts using mass spectrometry indicated that dodine-derived radioactivity excreted in faeces was mainly the parent compound (M1), representing 42-63% of the dose, but most of the dodine-derived radioactivity in urine was eliminated as metabolites; no significant amounts of the parent compound were detected. Urine analysis using HPLC indicated 4 major peaks: M2, M3, M4 and M5, which together accounted for 33 to 48% of the dose.

M2 was identified as an alcohol of dodine, dodecylanolguanidine (DOLG), an omega-oxidation product; this was the major peak in urine samples, accounting for 11-23% of the cumulative percent of dose excreted in urine up to 120 hours post dosing. M5 was identified as urea and accounted for 3-5% of the cumulative percent of dose excreted in the urine samples. The second and third major peaks in the chromatogram (M3 and M4) were not identified; these 2 regions typically comprised 4-13% of the cumulative percent of dose excreted in urine sample. M4 was however tentatively identified as a mixture of acidic products produced by beta-oxidation of the alkane side chain of dodine. Overall, the 4 major urine peaks typically accounted for 33-48% of the dose. No glucuronide or sulphate conjugates were found.

Because of the presence of the hydroxy dodecylguanidine (M2) and the other tentatively identified acids in the M4 peak, the author of the study postulated that, the metabolism of dodine follows a beta oxidation pathway similar to that of medium- or long-chain fatty acids. Upon entering the liver cell, dodine may be activated by formation of a CoA derivative. With the help of a carrier (similar to carnitine) it may be entering the mitochondrial matrix, and being oxidized by a sequence of reactions in which the alkyl chain of dodine is shortened by two carbon atoms at a time (beta oxidation). This series of reactions may also be catalyzed by a monooxygenase that requires NADPH, O_2, and cytochrome P450.

The absorbed dodine probably enters the liver through the portal circulation and is metabolized to hydroxydodecylguanidine and other intermediate products with shorter chain lengths which are then eliminated through the urine. Urea may also be formed in the liver as a result of the action of arginase on dodine and/or one or more of its metabolites and eliminated through the urine.

A Summary of kinetic and metabolism studies is presented in Table 6.76.

Table 6.76 – Summary of kinetic and metabolism of dodine

Study type	Species / strain	Vehicle	Results	Comments	Reference
Single dose (p.o. & i.v.), second single dose and repeated dose, oral route	Sprague Dawley CD rat (3 rats/sex/ group)	Oral: 0.5% CMC aqueous sol. i.v: ethanol	After oral dosing, ~45% of ^{14}C-dodine was eliminated via urine and ~45% via faeces; after i.v. dosing, elimination via urine was more important: up to 70% in 96h; Expired $^{14}CO_2$ was a minor route of elimination; Residual radioactivity in carcass was higher after i.v. dosing (8%) than after oral dosing (0.6%)	Oral absorption was considered to be about 45%; No potential for bioaccumulation was observed, although higher levels of radioactivity remained in fat (mainly), ovaries, thyroid and skin; Multiple dosing caused a slower elimination than single dosing	Cameron, B.D., Milner, N.P. & Dunsire., J.P., 1985

Study type	Species / strain	Vehicle	Results	Comments	Reference
Single dose, second single dose and repeated dose, oral route	Sprague Dawley Crl: CD (BR) rat (5 rats/sex/ group)	Corn oil	Major portion of low dose (single or multiple was eliminated in 48h. in urine & faeces; elimination of high dose was complete only after 120h. Elimination via urine was between 40 and 45% of dose. Recovery of radioactivity was low in all tissues (0.62 to 3.34% of dose. In urine, no significant amount of parent compound was found and metabolites M2, M3, M4 and M5; in faeces, major compound (M1) was the parent compound	M1: parent compound 40-55% of dose in faeces M2: hydroxy dodecylguanidine 11-23% (major) in urine M3 unidentified metabolite, 7-11% in urine M4: tentatively identified as a mixture of acidic products of β-oxidation, 8-13% of dose in urine M5: urea, 3-5% of dose in urine	Reddy, V., Litle, L. and Murrill, E., 1992

Mammalian toxicity:

Acute toxicity

Dodine technical is harmful by ingestion with a LD_{50} oral of 851 mg/kg bw in rat, both sexes combined. Most frequent observations were abnormal defecation, various discoloured areas due to discharges/excretions and hypoactivity; with exception of discoloured areas and hair loss, all surviving animals appeared normal by day 12. Necropsy findings on animals which died prematurely, showed gastrointestinal abnormalities, emaciated abdominal cavity with thick white material in it, which were correlated to the severely irritating properties of test material. In mice, dodine tested orally at the limit dose of 500 mg/kg bw did not show any signs of toxicity.

By the dermal route, dodine presented low systemic toxicity in rats (LD_{50} dermal > 5000 mg/kg bw), showing, however, sign of severe erythema and slight oedema on the site of application.

If inhaled as an aerosol (nose only during 4 hours), dodine was found to be toxic in rats (LC_{50} = 0.45 mg/l air, both sexes combined). Abnormal breathing, swollen abdomen, pilo-erection, and/or staining around snout and/or jaws, and wet, matted fur were observed during the post exposure period. Macroscopical findings included severe congestion of the lungs and intestines, and distension of the gastro intestinal tract, mainly at the doses causing death.

In rabbits, dodine was found to be irritating to the skin and to cause serious damage to the eye. In a guinea pigs Magnussen & Kligman test, test material did not show sensitizing potential.

Table 6.77 summarises the results of acute toxicity testing conducted with dodine. It was concluded that dodine has to be classified as toxic by inhalation, harmful if swallowed, irritating to skin and with risk of serious damage to eyes derived from the acute toxicity studies.

Table 6.77 - Summary of acute toxicity of dodine including irritancy and skin sensitization

Test/Species (purity of a.s.)	Dose levels / vehicle	Results	Comments / Classification	References
Acute oral, rat (96.7%)	450, 761 and 1285 mg/kg bw in 0.5% methylcellulose	LD_{50} = 851 mg/kg bw	R22 – harmful if swallowed	Kern, T.G., 1999
Acute oral, mouse (98%)	250 and 500 mg/kg bw 10% in propylene glycol	LD_{50} > 500 mg/kg bw	No death and no signs of intoxication were observed in either dose levels. **Additional information**	Spanjers, M.Th.; Til, H.P., 1985
Acute dermal, rat (96.7%)	5000 mg/kg bw in deionised water	LD_{50} > 5000 mg/kg bw	No classification is required	Kern, T.G., 1999
Acute inhalation, rat (96.7%)	0, 0.25, 0.34 and 0.51 mg/l air/4h Nose only, aerosol	LC_{50} = 0.45 mg/l air	R23 – toxic by inhalation	Kenny, T., 1999
Skin irritation, rabbit (96.7%)	3 rabbits, 0.5 g in deionised water	erythema: 2/1.3/2 oedema: 0/0/0.3 almost completely reversible in days 14	R38 – Irritating to skin	Kern, T.G., 1999
Eye irritation, rabbit (96.7%)	1 animal 47 mg/0.1 ml termination at day 7	Cornea: 4; iris: 2; redness conj: 2.7; chemosis: 4; not reversible at day 7	R41 – Risk of serious damage to eyes	Kern, T.G., 1999
Skin sensitization – M&K, guinea pig (96.7%)	40% in corn oil (challenge)	All scores were 0 after challenge	No classification is required	Manciaux, X., 1999

Short-term toxicity

The short-term oral toxicity of dodine was investigated through 6 subacute/dose range-finding studies and 3 subchronic studies in rat, mouse and dog. Two dermal 21-28-day studies in rat were also presented.

Oral route

Dose range-finding studies in rats were conducted by gavage and by dietary administration. 28-day gavage administration of doses from 75 to 200 mg/kg bw/day resulted in excessive toxicity as demonstrated by a dose related increased incidence of mortality at all dose levels. Treatment-related increases incidences of deterioration of health status (reduced activity, hunched posture, partly closed eyes, blue skin tone, decreased body temperature), of respiratory distress, firm abdomens and abnormal faeces were observed at 100 mg/kg bw/day and higher dose levels. Body weight, body weight gain and food consumption were decreased at all dose levels, and microscopical examination of gastrointestinal tract showed oedema, cell infiltration, hyperplasia of the squamous mucosa of the stomach also in all groups.

Dietary administration of dodine for the same period of time in the same strain of rats at dose levels of 0, 500, 750 and 1000 ppm [corresponding to 0, 47, 71 and 87 mg/kg bw/day in males and 0, 50, 72 and 92 mg/kg bw/day in females] resulted also in decreased body weight/body weight gain and food consumption at all dose levels.

A subsequent 28-day dietary toxicity study tested only dose levels of 0, 200 and 800 ppm [corresponding to 0, 17.7 and 67.7 mg/kg bw/day in males and 0, 19.2 and 76.7 mg/kg bw/day in females] and reached the same kind of effects at the 800 ppm dose level as the ones referred in the previous study, as well as a statistically significantly decrease in absolute and relative to body

weight liver weight without correlated histopathological findings. The NOAEL was the dose level of 200 ppm corresponding to 17.7 and 19.2 mg/kg bw/day in males and females respectively.

A further dietary study in rats conducted at the same dose levels of 0, 200 and 800 ppm (conducted in parallel with the previous study) revealed no abnormal gut motility neither after a 7-day nor 28-day treatment period with dodine.

Another range-finding study in mouse was conducted at dose levels of 0, 100/1250, 250 and 650 ppm [corresponding to 0, 30.3/232.2, 49.4 and 109.4 mg/kg bw/day in males and 0, 34/323.6, 61.3 and 150.4 mg/kg bw/day in females] for a period of 8 weeks. After 3 weeks dosing, the low concentration of 100 ppm was increased to 1250 ppm due to no obvious toxic effects being observed. Toxic effects were limited to this latter higher dose level (decreased body weight/body weight gain and mild eosinophilia in the liver), however no haematological or biochemical exams were performed. The NOAEL was considered to be the 625 ppm dose level corresponding to 109.4 to 150.4 mg/kg bw/day only as additional information.

Dose-range finding study in dogs was carried out for a period of up to 6 weeks, 2 low dose levels were increased after 1 week (12.5 mg/kg bw/day → 50 mg/kg bw/day) and 3 weeks (6.25 mg/kg bw/day → 60 mg/kg bw/day) of dosing, using 2 dogs/group according to the following dosing schedule:

Table 6.78 – Dosing schedule

Dose Level (mg/kg bw/day)	Duration (Weeks)	Study Week	Group No.
1.25	5	1 to 5	4
6.25	3	1 to 3	3
12.5	1	1	1
25	6	1 to 6	2
50	5	2 to 6	1
60	2	4 to 5	3

Vomiting (at 12.5 mg/kg bw/day during 1 week and up) and excessive salivation were observed in most dogs treated with dodine at levels of 25 mg/kg bw/day and higher. Weight losses along with decreased food consumption were observed in all 3 highest doses tested and it was necessary to sacrifice prematurely 1 male at 50 mg/kg bw/day due to its poor health condition. Undigested food in the stomach was noted at necropsy in the same 3 highest dose animals and abnormal clearance time of contrast material from the stomach of the one dog tested at 50 mg/kg bw/day, while the low dose (1.25 mg/kg bw/day) animal showed a normal emptying time. Although no consistent adverse effects were observed at 12.5 mg/kg bw/day, no conclusion could be derived on this dose level (if it can be considered as a NOAEL or not) because treatment at this dose level lasted only for 7 days, females had episodes of vomiting and decreased food consumption, one animal presented soft faeces and no information is available at necropsy due to change in dose level.

Oral subchronic toxicity was investigated in rat, mouse and dog; in dog, no 90-day toxicity study is available, but a 1-year toxicity study with observations at 6 months treatment was consider adequate to assess this end-point (which is in line with the revised version of Annex II to Council Directive 91/414/EEC), although an important deviation in this study was that no determination on haematological and urinalysis parameters were performed after 3 months treatment with dodine as recommended by guideline (B.30 of EU).

In a 1984 dietary study in rats, dose levels of 0, 50, 200 and 400 ppm were used initially, after the first week of treatment (day 7), dose level of 400 ppm was increased to 800 ppm because no growth depression was observed at 400 ppm. Achieved dosages were (0), 3.6, 14.1 and 55.8 mg/kg bw/day

in males and (0), 3.9, 14.9 and 60.4 mg/kg bw/day in females for the 0, 50, 200 and 800 ppm dose levels respectively for the 90-day treatment period. Body weights and food consumption were decreased in both sexes of the top dose group throughout the study. In the top dose group, increased number of neutrophils in males, and a decreased plasma alanine aminotransferase activity in females were the only findings in clinical pathology and the NOAEL was the dose level of 200 ppm corresponding to 14.1 and 14.9 mg/kg bw/day in males and females respectively.

A 90-day dietary toxicity study in mouse was conducted in 1994, dose levels used were 0, 150, 300, 600, 1250 and 2500 ppm corresponding to 0, 24, 48, 94, 181 and 350 mg/kg bw/day in males and 0, 31, 60, 116, 223 and 305 mg/kg bw/day in females. Deaths (4/10 females), apparent stiffening of the tail in females were observed at the top dose level of 2500 ppm; reduced growth accompanied with lower food consumption was observed at dose levels of 2500 ppm and 1250 ppm. Significant increase in mean segmented neutrophil and decrease in mean eosinophils values were noted in 2500 ppm males group; increased levels of BUN, bilirubin and aspartate aminotransferase (AST) values at the same dose level were considered related to nutritional status of the animals, AST was also increased at lower dose levels in females, but without association with histopathological changes in the liver or kidneys. Some difference in organ weights relative to control animals at 1250 and 2500 ppm were not considered biologically significant as no histopathological changes were noted in any of the organs considered. The NOAEL was the dose level of 600 ppm corresponding to 94 and 116 mg/kg bw/day in males and females respectively, based on the reduced body weight gain at the higher level of 1250 ppm.

Oral 1-year toxicity in dogs (1996) was performed at dose levels of 0, 2, 10 and 20 mg/kg bw/day administered in gelatine capsules. Three animals (one 10 mg/kg bw/day female, one 20 mg/kg bw/day male, and one 20 mg/kg bw/day female) exhibited notably marked body weight losses and low feed intake during the first few weeks of compound administration, indicating adaptation problems to dosing. Supplemental feeding regimens were instituted for the three dogs to preclude mortality; two of the three dogs were successfully returned to basal diet by Week 8 or 15 and the third (the 20 mg/kg bw/day female) was maintained on supplemental feeding throughout the majority of the study, continuing through study termination. These findings indicate that the maximum tolerated dose in dogs was closely approximated in this evaluation. No definitive evidence of toxicity was seen in any of the other parameters evaluated in this study: The only clear pattern indicative of a treatment-related difference was the occurrence of dose-related salivation, which was most frequently noted in anticipation of dosing in the 10 and 20 mg/kg bw/day dogs. This finding was considered most likely to be a conditioned reflex or secondary effect, rather than a direct treatment-related effect. The NOAEL was the dose level of 10 mg/kg bw/day.

Dermal route

A 28-day dermal toxicity study of dodine was conducted in rats in 1999. Dose levels of 0, 50, 125 and 200 mg/kg bw/day were applied on the shave skin and occluded 6 hours/day, 5 days/week for 4 weeks resulted in a dose related local dermal irritation at all dose level. Decreased body weight gain was observed in male rats at the 2 highest dose levels. Although the study author considered this finding as not related to dodine administration because, the gains in these males after the initial week of dosing were comparable to the control group gains, there was no trend present in the females, in the 200 mg/kg bw/day group females there was a significantly decreased mean weight gain for week 2 to 3 followed by a significantly increased gain for week 3 to 4. The overall decrease in bodyweight was 13% and 22% at 125 and 200 mg/kg bw/day respectively, RMS agreed with Applicant that severe dermal irritation may have contributed to these findings, but systemic toxicity could not be ruled out and the NOAEL was considered to be the 50 mg/kg bw/day dose level; no NOAEL could be established for local dermal irritation effects.

A 21-day dermal study was conducted with a 35% SL formulation of dodine hydrochloride salt, which produced the same kind of effects as the previous study performed with dodine (acetate

form) technical. Dose levels of 12.5, 25 and 50 mg/kg bw/day presented dose-related dermal irritation, but no clear, consistent systemic effects. The systemic NOAEL was considered to be the highest dose level used of 50 mg/kg bw/day and no NOAEL could be established for dermal irritation effects.

Conclusion

It was concluded from the short-term toxicity studies in rat, mouse and dog, that the most consistently observed effects were decreased body weight and body-weight gain, which were frequently accompanied by decreased food consumption. The NOAELs for these parameters were relatively similar in the short- and longer term studies and between species. Mice showed to be less sensitive by a factor of 10 than rats or dogs. Other toxic effects were reported only rarely in these studies. Delayed gastric emptying, as measured by barium contrast radiography, was observed in one dog at 50 mg/kg bw/day.

In a mechanistic study, rats administered up to 800 ppm of dodine in the diet for 7 or 28 days and then a charcoal suspension had no evidence of altered gastrointestinal motility.

The short-term dermal studies of 21 of 28 days in duration demonstrated that dodine is a severe irritant at doses as low as 12.5 mg/kg bw/day. There was some evidence that systemic toxicity resulted from dermal application at a dose as low as 50 mg/kg bw/day, however the contribution of the severe dermal irritation could not be dismissed.

Lowest NOAEL from in the oral studies were found in the 90-day oral toxicity study in rat (14.1 mg/kg bw/day) and in the 1-year toxicity in dog (10 mg/kg bw/day); **the overall short-term NOAEL was the dose level of 10 mg/kg bw/day from the 1-year toxicity study in dog.**

Table 6.79 summarises the results of short-term toxicity studies conducted with dodine.

Table 6.79 - Summary of short term-toxicity of dodine

Test / Species (purity of test substance)	Dose levels	Results			References
		NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Critical endpoints	
Oral, 28-day / rat (94.07%), range-finding by gavage	0, 75, 100 and 200 mg/kg bw/d	-	MTD < 75 mg/kg bw	75 mg/kg: 1/10 rat died, respiratory distress, salivation and/or staining of the fur; ↓ body weight, changes in clinical pathology, and histopathology	Batham, P., 1994a
Oral, 28-day / rat (94.07%), range-finding dietary	0, 500, 750 and 1000 ppm <> 0, 47, 71 and 87 (M) and 0, 50, 72 and 92 (F) mg/kg bw/d	< 500 ppm <> 47 -50 mg/kg bw/d	500 ppm <> 47 – 50 mg/kg bw/d	500 ppm: ↓ body weight gain (M + F)	Batham, P., 1994b
Oral, 28-day / rat (98.6%), range-finding dietary	0, 200 and 800 ppm <> 0, 17.7 and 67.7 (M) and 0, 19.2 and 76.7 (F) mg/kg bw/d	200 ppm <> 17.7 – 19.2 mg/kg bw/d	800 ppm <> 67.7 – 76.7 mg/kg bw/d	800 ppm: ↓ body weight /bw gain and food consumption (M); ↓ absolute and relative liver weight (F)	Dange, M., 1997
Assessment of gut motility / rat (98.6%), dietary for 7 – 28 days	0, 200 and 800 ppm			Normal gut motility was seen after administration of dodine for 7 and 28 days at both dose levels	Dange, M., 1996

Test / Species (purity of test substance)	Dose levels	Results			References
		NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Critical endpoints	
Oral, 8-weeks / mice (95%), range-finding dietary	0, 100/1250, 250 and 625 ppm <> 0, 30.3/232.2, 49.4 and 109.4 (M) and 0, 34/323.6, 61.3 and 150.4 (F) mg/kg bw/d	625 ppm <> 109.4-150.4 mg/kg bw/d	1250 ppm <> 232.2-323.6 mg/kg bw/d	Group 2: 100 ppm: week 1-3; 1250 ppm: week 4-8 (dose level was increased due to no obvious toxic effects observed; 1250 ppm: ↓ body weight gain and mild eosinophilia in the liver **Additional information**	Mulhern, M., Perry C.J., Snodgrass E., 1988
Oral, 6-week / dog (94.07%), range-finding by capsules	1.25, 6.25/60, 12.5/50 and 25 mg/kg bw/d only 2 dogs/group	Due to varying doses/time of dosing, it was not possible to determine NOAEL/LOAEL		Undigested food in the stomach at necropsy and abnormal clearance time of contrast material from the stomach of 1 dog (50 mg/kg bw/d) suggest an effect of a.i. on gastric emptying **Additional information**	Smith, S.Y., 1994
Oral, 90-day / rat (95%), dietary	0, 50, 200 and 800 ppm <> 0, 3.6, 14.1 and 55.8 (M) and 0, 3.9, 14.9 and 60.4 (F) mg/kg bw/d	200 ppm <> 14.1-14.9 mg/kg bw/d	800 ppm <> 55.8-60.4 mg/kg bw/d	800 ppm: ↓ body weight gain (M + F), ↑ heart & kidney weights without histopathological correlates, ↓ Ca, ALT	Lina, B.A.R, Til, H.P. *et al.*, 1984
Oral, 90-day / mice (94.07%), dietary	0, 150, 300, 600, 1250 and 2500 ppm <> 0, 24, 48, 94, 181 and 350 (M) and 0, 31, 60, 116, 223 and 305 (F) mg/kg bw/d	600 ppm <> 94-116 mg/kg bw/d	1250 ppm <> 181-223 mg/kg bw/d	1250 ppm: ↓ body weight gain and food intake	Kangas, L., 1994
Oral, 1-year / dog (98.6%), by capsules	0, 2, 10 and 20 mg/kg bw/d	10 mg/kg bw/d	20 mg/kg bw/d	20 mg/kg bw/d: a supplemental feeding regimen was considered necessary (in 1 female) to prevent mortality	Trutter, J.A., 1996
Dermal, 28-day / rat (98%)	0, 50, 125 and 200 mg/kg bw/d	Systemic: 50 mg/kg bw/d No dermal NOAEL	Systemic: 125 mg/kg bw/d Dermal: 50 mg/kg bw/d	Dermal irritation at all dose levels; 125 mg/kg bw/d: ↓ body weight /bw gain (M)	Kern, T.G., 1999
Dermal, 21-day / rat (35% SL formulation)	0, 12.5, 25 and 50 mg/kg bw/d	Systemic: 50 mg/kg bw/d No dermal NOAEL		Dermal irritation at all dose levels; no consistent systemic effects **Additional information**	Auletta, C.S., 1989

(M): males; (F): females; bw: body weight

Genotoxicity

No potential for mutagenicity was observed with dodine tested *in vitro* in bacteria (Ames test in *Salmonella typhimurium* and *Escherichia coli*), and in mammalian cells (test for clastogenicity in cultured human lymphocytes and gene mutation at the HGPRT locus of Chinese hamster ovary cells). Two *in vivo* micronucleus tests in mice confirmed the negative results obtained *in vitro*. Table 6.80 summarises the results of genotoxicity studies.

Table 6.80 – Summary of genotoxicity studies

Test / Species	Purity (%)	Conditions	Results	Comments	References
***In vitro* genotoxicity testing**					
Reverse mutation assay in bacteria / *S. typhimurium* (TA 98, 100, 1535, 1537, and 1538)	95	Concentrations of 0.06-5.0 µg/plate, vehicle: methanol + and – S9-mix	Negative	Cytotoxicity was noted at 10 µg/plate; no growth inhibition at 1 µg/plate no confirmatory experiment	Willems M.I., 1981
Reverse mutation assay in bacteria / *E. coli* (strain WP_2uvrA	98.5	1st experiment: 0.3-100 µg/plate +S9-mix 0.1-33 µg/plate –S9-mix 2nd experiment: 1-200 µg/plate +S9-mix 0.3-66 µg/plate –S9-mix vehicle: ethanol	Negative	Some toxicity was observed at 24 µg/plate and up without S9-mix, and at 66 µg/plate and up with S9-mix	Verspeek-Rip C.M., 2003
In vitro mammalian chromosome aberration test / Cultured human lymphocytes	98	+S9-mix: 0.56-15.0 µg/ml, exposure period of 2h – S9-mix: 0.37-10.0 µg/ml, exposure period of 24h vehicle: ethanol	Negative	One sampling time used for all doses, 2 highest dose showed ↓ mitotic index of 50% or more	Wilmer J.W.G.M., 1985
In vitro mammalian cell gene mutation test / Chinese hamster ovary fibroblast cells (at HGPRT locus)	98	+ S9-mix: 5.0-35.0 µg/ml - S9-mix: 2.5-20.0 µg/ml vehicle: ethanol	Negative	No independent experiment was performed	Davis P.B., 1985
***In vivo* genotoxicity testing in somatic cells**					
Micronucleus test / Swiss mouse	98	5 mice/sex/group: 0 and 500 mg/kg bw by gavage; 2 mice/sex/positive control group, i.p. sacrifice at 24, 48 and 72h vehicle: propylene glycol	Negative	No signs of toxicity were evident **Additional information**	Willems M.I., 1985
Micronucleus test / ICR mouse	94	5 mice/sex/group treated by gavage at dose levels of 100, 200 and 400 mg/kg bw; sampling time: 24, 48 and 72h (dodine); positive and negative controls: 24h vehicle: corn oil	Negative	Signs of toxicity noted at 200 and 400 mg/kg bw	Hemalatha Murli, 1992

Long-term toxicity and carcinogenicity

In the mouse chronic toxicity/carcinogenicity study, the only evidence of chronic toxicity was decreased body weight gain and food consumption at 750 ppm, the mid dose. At the high dose, there was increased severity of the same effects. There was a positive trend for hepatocellular adenomas and combined adenomas/carcinomas. There was also a statistically significant pair wise increase in combined hepatocellular adenomas/carcinomas in females. The incidence of hepatocellular adenomas and combined adenomas/carcinomas in females also exceeded the historical control values for these tumors in 1500 ppm. The high dose was considered adequate for testing the carcinogenic potential of dodine in mice.

In the chronic toxicity/carcinogenicity study in rats, the only evidence of chronic toxicity was decreased body weight, body weight gain and food consumption at 800 ppm. There was a dose-dependent increase in the incidence of combined thyroid C-cell adenomas/carcinomas in the treated males. The incidence in all treated males exceeded the mean and upper limit of the historical control range. However, the incidence in the concurrent control group also exceeded the mean of the historical control data.

Table 6.81 - Summary of long-term toxicity and carcinogenicity of dodine

Test / Specie (purity of test substance)	Dose levels	Results			References
		NOAEL (mg/kg bw/day)	**LOAEL (mg/kg bw/day)**	**Critical endpoints**	
2-years, rat (98.6%)	0, 200, 400, 800 ppm (equivalent to 0, 10, 20 or 40 mg/kg bw/day for males and 0, 13, 26.5 or 53.5 mg/kg bw/day for females)	400 ppm (equivalent to 20 – 26.5 mg/kg bw/day)	800 ppm	800 ppm body weights: ↓ up to 10% males and ↓ 15% in female; mean total white blood cell count: ↓ 24% in males dose dependent increase in the combined thyroid adenomas/carcinomas: 23/66 (35%), 21/52 (40%), 27/60 (45%) and 33/62 (53%) in the control, low –mid and – high dose males respectively **but statistically with no significance**.	Dange, 1998
78 week, mice (98.6%)	0, 200, 750 , 1500 ppm (equivalent to 0, 29, 110 or 225 mg/kg bw/day in males and 0, 36, 136 or 277 mg/kg bw/day in females	200 ppm (equivalent to 29 – 36 mg/kg bw/day)	750 ppm	1500 ppm mean body weight gains: ↓ 25.1% males and ↓ 34.6% in females. females: mean food consumption ↓ significantly. Incidence of combined hepatocellular adenomas and carcinomas significantly increased in the high-dose females (5/60, 8.3% in treated vs. 0/60 in controls) 750 ppm overall mean body weight gain for females was ↓ when compared to controls (20.1% lower); mean food consumption ↓ significantly **positive trend (no statistically significant) in the incidence of hepatocellular adenomas in females**	Williams, 1998

Reproductive toxicity

There was no evidence that dodine is a reproductive or developmental toxicant. In the multigeneration reproduction study in the rat, decreased body weight, body weight gain and food consumption were observed in both the P and F1 generations at 400 ppm and 800 ppm. There was no evidence of a treatment-related effect on reproduction parameters. Offspring of both the F1 and F2 generations had decreased mean body weights at postnatal day (PND) 4 and through PND 21 at 400 and 800 ppm.

In the rat developmental study, decreased body weight gain and food consumption was observed in maternal animals at 45 mg/kg bw per day, the mid dose. At the high dose of 90 mg/kg bw per day, the same effects, with increased severity, were observed. There was no evidence of developmental toxicity at 90 mg/kg bw per day.

In the rabbit developmental study, the evidence of maternal toxicity was decreased food consumption at 80 mg/kg bw per day, the highest dose tested. There was no evidence of developmental toxicity at 80 mg/kg bw per day.

Table 6.82 - Summary of reproductive toxicity of dodine

Type of test Test species Test substance purity	Dose levels	Results			References
		NOAEL (mg/kg b w/ day)	LOAEL (mg/kg bw /day)	Critical endpoints	
rat, 2 generation study 98.6%	0, 200, 400, 800 ppm (equiv. to 0, 13.14, 26.20 and 52.61 mg/kg bw/day for F0 males and 0,18, 35.34 and 67.58 mg/kg bw/day for F0 females; 0, 14.91, 30.20 and 63.03 mg/kg bw/day for F1 males and 0, 19.18, 38.77 and 76.03 mg/kg bw/day for F1 females	200 ppm for maternal toxicity (equivalent to 13.14 mg/kg bw/day) 800 ppm for reproductive performance (equivalent to 52.61 mg/kg bw/day) 200 ppm for pup development (equivalent to 13.14 mg/kg bw/day)	400 ppm for maternal toxicity 400 ppm for pup development	800 ppm: ↓ body weight gain, body weight and food consumption for F0 and F1 males and females. 400 ppm: ↓ body weight gain in F1 females Offspring: 800 and 400 ppm ↓ body weight No effects in reproductive parameters	Henwood, 1996
rat, developmental 95%	0, 10, 45 and 90 mg/kg bw/day	10 mg/kg bw/day for maternal toxicity 90 mg/kg bw/day for developmental toxicity	45 mg/kg bw/day for maternal toxicity	↓ body weight gain and food consumption at 45 and 90 mg/kg bw/day No developmental toxicity – no teratogenic effects	Hazelden, Wilson, 1989
Rabbit, developmental 95%	0, 10, 40 and 80 mg/kg bw/day	40 mg/kg bw/day for maternal toxicity 80 mg/kg bw/day for developmental toxicity	80 mg/kg bw/day for maternal toxicity	↓ food consumption at 80 mg/kg bw/day No developmental toxicity – no teratogenic effects	Hazelden, Mc Cay, 1989

ADI

The calculation of an acceptable daily intake (ADI) is established on the basis of the highest dose at which no adverse effect is observed in the most appropriate study in the most sensitive species.

With a conclusive data package dodine was found to cause no specific concern after repeated exposure: main critical effects were reduced body weight gain, eventually associated with decreased food consumption.

The most critical effects were observed in the combined chronic/carcinogenicity study in rats, where a non-statistically significant dose dependent increase in combined thyroid adenomas/carcinomas was observed in males [23/66 (35%), 21/52 (40%), 27/60 (45%) and 33/62

(53%) in the control, low –mid and –high dose males respectively] and in the carcinogenicity study in mice, where a positive trend (also not statistically significant) in the incidence of hepatocellular adenomas was observed in females. These observations were not considered to reflect a carcinogenic potential of dodine.

Dodine is unlikely to be genotoxic, no effects on reproductive or developmental parameters were observed and no concern on neurotoxicity was raised.

The more relevant NOAEL for this purpose is considered to be the dose level of 10 mg/kg bw/day derived from the 1-year, dog study. A safety factor of 100 is proposed on the basis of the low concern on the toxicological endpoints, resulting in an ADI of 0.1 mg/kg bw/day.

$$\text{ADI dodine} = \frac{\text{NOAEL}}{\text{S.F.}} = \frac{10}{100}\ \text{mg/kg bw/day} = 0.1\ \text{mg/kg bw/day}$$

ARfD

Dodine was found to be harmful if swallowed and toxic by inhalation in the acute toxicity studies; it was also irritating to skin and with risk of serious damage to eyes. As referred before, a low degree of concern was raised from the whole toxicological data package submitted.

As the main concern from the acute toxicity studies refers to the inhalative route, no acute reference dose is proposed for dodine.

AOEL

According to the principles of Annex VI to Directive 91/414 EEC, the proposed AOEL should be established on the basis of the highest dose at which no adverse effect is observed in relevant studies in the most sensitive species.

Considering that short-term and long-term studies gave similar results, as shown in the following table, the same NOAEL was considered appropriate for the AOEL as the one used for the ADI:

Study type	Species	NOAEL (mg/kg bw/day)
28-day oral	rat	17.7
90-day oral	rat	14.1
90-day oral	mice	94
1-year oral	dog	10
2-year	rat	20
78-week	mice	29
2 generations reproduction	rat	13.1
teratogenicity oral	rat	10
teratogenicity oral	rabbit	40

Considering the NOAEL of 10 mg/kg bw/day from the 1-year dog study, which is the same as the NOAEL obtained for maternal toxicity in the rat teratogenicity study, a safety factor of 100 as no specific concern on toxicological endpoints was raised, and an oral absorption of 45% based on urinary excretion within 24 h (see B.6.1.5.), the following systemic AOEL is proposed:

AOEL $_{systemic}$ dodine =	$\frac{\text{NOAEL}}{\text{S.F.}}$	**x oral abs.**	**=**	$\frac{10}{100}$	**x 0.45**	**mg/kg bw/day**	**=**	**0.045 mg/kg bw/day**

Drinking water limit

Assuming average consumption of 2 litres of water per person per day and body weight of 70 Kg and one tenth of the ADI allocated to drinking water, according to the WHO approach, the maximum allowable concentration (MAC) in water results:

MAC =	$\frac{\text{0.1 x 70 x 0.1}}{\text{2}}$	**=**	**0.35 mg/l**

B.6.11 Acute toxicity including irritancy and skin sensitization of preparations (Annex IIIA 7.1)

B.6.11.1 Acute oral toxicity

EXP 10343: Acute oral toxicity (Limit test) in the rat

Dreher D.M. (1991) – Project No. 282/141 of 19 November 1991; Performed by Safepharm Labs., UK; dates of experimental work: 24 September 1991 to 16 October 1991.

Guidelines and GLP:

The study was conducted in compliance with OECD # 401; Method B1 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods:

Based on the results of the range-finding study a further group of animals (5 males and 5 females Sprague-Dawley strain rats) was treated withl: Syllit® (400 SC, EXP10343) with active substance, ISO common name: dodine; IUPAC name: 1-dodecylguanidinium acetate at nominal concentration of 400g/l, batch 910600, purity: 387 g/l.

All animals were dosed only by oral gavage (dosing of 2000 mg/kg. Dose volume: 1.98 ml/kg) using a metal cannula attached to a graduated syringe. The volume administered to each animal was calculated according to its fasted bodyweight at the time of dosing.

Deaths and overt signs of toxicity were recorded 1/2, 1, 2 and 4 hours after dosing and subsequently once daily for 14 days.

Individual bodyweights were recorded on the day of treatment (day 0) prior to dosing and on days 7 and 14, or at death.

At the end of the study the surviving animals were killed by cervical dislocation. All animals were subjected to gross pathological examination. This consisted of an external examination and opening of the abdominal and thoracic cavities. The appearance of any macroscopic abnormalities was recorded. No tissues were retained.

Results:

Mortality Data

Two animals (one male and one female) were found dead three days after dosing.

Clinical Observations

Signs of systemic toxicity noted in two males and one female were hunched posture and lethargy. The female also showed pilo-erection, tiptoe gait, diarrhoea, emaciation, ataxia and red/brown stains around the mouth. These animals appeared normal seven or nine days after dosing. All other surviving animals appeared normal throughout the study.

Bodyweight

Surviving animals showed expected bodyweight gain during the study, except for three animals which showed reduced bodyweight gain or bodyweight loss during the first week.

Necropsy

Abnormalities noted at necropsy of animals that died during the study were haemorrhagic lungs, dark liver, dark kidneys, haemorrhage of the non-glandular epithelium of the stomach, gaseous distension of the small and large intestines and haemorrhage of the small intestine.

Sloughing or white thickened areas of the non-glandular epithelium of the stomach was noted at necropsy of five animals that died during the study or were killed at the end of the study. No abnormalities were noted at necropsy of four animals that were killed at the end of the study.

Table 6.83 - Acute oral toxicity of Syllit 400 SC

Dose	Males mortality	Time of death	Females mortality	Time of death
2000 mg/kg bw/day	1/5	3 days	1/5	3 days

Conclusion:

The acute oral median lethal dose (LD_{50}) of the test material, EXP 10343, in the Sprague-Dawley strain rat was found to be greater than 2000 mg/kg bodyweight. In accordance with the provisions of Council Directive 93/21/EEC and 99/45/EC, classification is not required.

B.6.11.2 Acute percutaneous toxicity

Acute dermal toxicity (limit test) in the rat

Dreher D.M. (1991) – Project No. 282/142 of 19 November 1991; Performed by Safepharm Labs., UK; dates of experimental work: 01 October to 15 October 1991.

Guidelines and GLP

The study was conducted in compliance with OECD # 402; Method B3 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods

SyIlitR (400 SC, EXP10343) with active substance: ISO common name: dodine ; IUPAC name: 1-dodecylguanidinium acetate at nominal concentration of 400g/l, batch 910600, purity: 387 g/l; dosing of 2000 mg/kg as single semi-occluded dermal application (dose volume 1.98 ml/kg) to intact skin (5 males and 5 females Sprague-Dawley strain rats) for a period of 24 hours.

Shortly after dosing the dressings were examined to ensure that they were securely in place. After the 24-hour contact period the bandage was carefully removed and the treated skin and surrounding hair wiped with cotton wool moistened with distilled water to remove any residual test material. The animals were returned to group housing for the rest of the study.

Deaths, overt signs of toxicity and dermal reactions were recorded 1/2, 1, 2 and 4 hours after dosing and subsequently once daily for 14 days.

Individual bodyweights were recorded on the day of treatment (day O) prior to application of the test substance and on days 7 and 14: At the end of the study the animals were killed by cervical dislocation and subjected to gross pathological examination. This consisted of an external examination and opening of the abdominal and thoracic cavities. The appearance of any macroscopic abnormalities was recorded. No tissues were retained.

Results:

Mortality Data

There were no deaths.

Clinical Observations

No signs of systemic toxicity were noted during the study. Signs of skin irritation noted were green-coloured possible necrosis, well- defined erythema, scattered mild redness, small superficial scattered scabs, hardened light brown-coloured scab, light brown discolouration of the epidermis, superficial cracking of the epidermis and well-defined erythema surrounding other skin reactions.

Bodyweight

No toxicologically significant effects on bodyweight were noted during the study.

Necropsy

No abnormalities were noted at necropsy.

Table 6.84 - Acute dermal toxicity of Syllit 400 SC

Dose	Males mortality	Time of death	Females mortality	Time of death
2000 mg/kg bw/day	0/5	--	0/5	--

Conclusion:

The acute dermal median lethal dose (LD_{50}) of the test material, EXP 10343, in the Sprague-Dawley strain rat was found to be greater than - 2000 mg/kg bodyweight. No signs of systemic toxicity and no abnormalities at necropsy were noted. In accordance with the provisions of Council Directive 93/21/EEC and 99/45/EC, classification is not required.

B.6.11.3 Acute inhalation toxicity

Acute inhalation toxicity study four-hour exposure (nose only) in the rat

Blagden S.M. (1992) – Project No. 282/146 of 24 March 1992; Performed by Safepharm Labs., UK; dates of experimental work: 29 October to 18 February 1992.

Guidelines and GLP:

The study was conducted in compliance with OECD # 403; Method B2 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods:

SyIlitR (400 SC, EXP10343) with active substance: ISO common name: dodine ; IUPAC name: 1-dodecylguanidinium acetate at nominal purity of 400g, batch 910600, purity: 392 g/l; 5 female and 5 male rats per dose group were exposed for 4 hours in a nose-only system to the test material. The main exposure parameters were as follows:

Parameter	Low dose group3	Mid dose group 1	High dose group 2
Nomimal concentration (mg/l)	13.8	26.5	34.8
Mean achieved concentration (mg/l)	0.38	0.59	0.93
Mean mass median aerodynamic diameter	2.9 µm	2.6 µm	3.0 µm
Respirable fraction (4 < µm)	70.7%	69.3%	66.9%
Geometric standard deviation (µm)	0.54	0.41	0.52

Findings:

Mortality

All animals exposed to 0.93 mg/litre were killed in extremis due to respiratory distress 10 minutes after completion of exposure. Two males and one female exposed to 0.59 mg/litre were also killed in extremis following clinical observations on day one. There were no deaths in animals treated with 0.38 mg/litre.

Table 6.85 – Mortality data

Group number	Mean achieved atmosphere concentration mg/l	Deaths		Total
		Male	Female	
2	0.93	5/5	5/5	10/10
1	0.59	2/5	1/5	3/10
3	0.38	0/5	0/5	0/10

Clinical observations

Animals in all dose groups showed wet fur during exposure and there were incidents of decreased respiratory rate particularly in animals exposed to 0.93 mg/litre.

On removal from the chamber common abnormalities additionally noted were hunched posture and pilo-erection.

Animals exposed to 0.93 mg/litre also showed lethargy, ptosis and gasping respiration. Incidents of noisy respiration, ataxia and red/brown stains around the eyes were also evident.

Ataxia was noted in all animals exposed to 0.59 mg/litre on removal from the chamber and the males showed ptosis. One male also showed lethargy, gasping and noisy respiration and red/brown stains around the eyes. One female also showed noisy respiration and decreased respiratory rate. One hour after completion of exposure to 0.59 mg/litre the males showed lethargy and there were signs of decreased respiratory rate. On day one following exposure similar signs were noted though the condition of two males and one female deteriorated to include gasping and noisy respiration and pallor of the extremities. Decreased respiratory rate was more common and a few animals showed red/brown staining on the head or around the eyes. Three females developed frequent sneezing on day two. Signs of toxicity gradually regressed in surviving animals but two males showed hunched posture throughout most of the study appearing normal on days eleven and twelve.

Animals exposed to 0.38 mg/litre showed few signs of toxicity on the day of exposure, all showed hunched posture and pilo-erection and there were incidents of decreased respiratory rate, noisy respiration, ptosis, ataxia and red/brown stains around the eyes. On day one following exposure, however, the conditions of the males and one female appeared to worsen and included signs of decreased respiratory rate, gasping and noisy respiration and there were signs of red/brown stains on the head and around the eyes or snout. Four females recovered quickly but others, particularly three males recovered slowly showing frequent sneezing, hunched posture and pilo-erection up to day seven. All animals in this dose group appeared normal on day eight and for the rest of the study.

Bodyweight

During week one following exposure to 0.59 mg/litre slightly decreased bodyweight gain was noted in surviving animals. One female showed- bodyweight loss and one female showed normal body-weight gain.

During week two surviving animals showed expected bodyweight development except for one female in which a decrease in bodyweight gain was still evident.

Some animals exposed to 0.38 mg/litre showed reduced bodyweight gain- and bodyweight loss particularly the males during week one following exposure. Normal bodyweight development was noted in all animals by the end of the study.

Necropsy

Animals exposed to 0.93 mg/litre showed lungs that were swollen and pale and many showed dark areas. Abnormal redness of the lungs was noted in one female. Opacity over the cornea of the eye was observed in three animals following exposure.

The three animals killed in extremis after exposure to 0.59 mg/litre showed changes to the lungs including swollen, pale, grey, discolouration, slight redness and dark foci. (One surviving female also showed a dark area on the lungs). These three decedents also showed an accentuated lobular pattern on the liver and gaseous distension in the small and large intestines, one male also showed congestion in the intestines. No abnormalities were detected in six of the surviving animals in this group.

One female exposed to 0.38 mg/litre showed a dark area and a dark focus on the lungs at necropsy. No other abnormalities were detected in this dose group.

Conclusion

The acute inhalation median lethal concentration (LC_{50}) and 95% confidence limits of the test material EXP 10343, in the Sprague-Dawley strain rat, were calculated to be:

Males only: 0.62 (0.50 - 0.77) mg/litre

Females only: 0.68 (0.57 - 0.81) mg/litre

All animals: 0.65 (0.57 - 0.75) mg/litre

In accordance with the provisions of Council Directive 93/21/EEC and 99/45/EC, labelling with the risk phrase R23: "Toxic by inhalation" is required.

B.6.11.4 Skin irritation

Acute dermal irritation test in the rabbit

Dreher D.M. (1991) – Project No. 282/143 of 19 November 1991; Performed by Safepharm Labs., UK; dates of experimental work: 03 October to 10 October1991.

Guidelines and GLP:

The study was conducted in compliance with OECD # 404; Method B4 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods:

SyllitR (400 SC, EXP10343) with active substance: ISO common name: dodine ; IUPAC name: 1-dodecylguanidinium acetate at nominal concentration of 400g/l, batch 910600, purity: 387 g/l; 0.5 ml of undiluted test item were applied to a 2.5 c m x 2.5 cm area (gauze patch, semi-occluded) on the back (intact skin) of each of 3 New Zealand White rabbits (2 females and 1 male). The exposure period was 4 hours.

Results:

Well-defined erythema was noted at all treated skin sites one hour after patch removal and at the 24, 48 and 72-hour observations. Loss of skin elasticity was also noted at one treated skin site at the 72-hour observation. Crust formation was noted at all treated skin sites seven days after treatment.

Moderate to severe oedema was noted at all treated skin sites one and 24 hours after patch removal, with slight to severe oedema at the 48-hour observation and very slight to moderate oedema at the 72-hour observation. No signs of systemic toxicity were noted.

Table 6.86 – Individual skin irritation scores

Animal number	Erythema			Oedema		
	53 female	67 male	68 male	53 female	67 male	68 male
After 1 hr	2	2	2	3	3	4
After 24 hr	2	2	2	3	3	4
After 48 hr3	2	2	2	2	2	4
After 72 hr	2	2	2	1	2	3

Conclusion

In accordance with the provisions of Council Directive 93/21/EEC and 99/45/EC, classification with the symbol "C" and the indication of "Corrosive" and labelling with the risk phrase R34: "Causes burns", will be required.

Acute dermal irritation in rabbits

Manciaux X. (1999) – Unpublished report No. 18689TAL of 27 July 1999; Performed by CIT-Centre international de toxicologie, France; dates of experimental work: 01 June to 05 June 1999.

Guidelines and GLP

The study was conducted in compliance with OECD # 404; Method B4 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods

SyllitR (400 SC, EXP10343) with active substance: ISO common name: dodine ; IUPAC name: 1-dodecylguanidinium acetate at nominal concentration of 400g/l, batch OP980255, purity: 404 g/l and diluted in water to a concentration of 0.225% (2.25 ml/l) reflecting the normal condition of use of the diluted spray; 0.5 ml of test item (at 2.25 ml/l) were applied to the right clipped flank (gauze patch, semi-occluded) of each of 3 males New Zealand White. The exposure period was 4 hours; the treated skin area was 6 cm^2.

Results:

A very slight erythema (grade 1) was noted in two animals on day 1; it persisted up to day 4 in one of them.

No other cutaneous reactions were observed during the study.

Mean scores over 24, 48 and 72 hours for each animal were 1.0, 0.0 and 0.0 for erythema and 0.0, 0.0 and 0.0 for oedema.

Table 6.87 – Individual skin irritation scores

Animal number	Erythema			Oedema		
	841 male	842 male	843 male	841 male	842 male	843 male
After 1 hr	1	0	1	0	0	0
After 24 hr	1	0	0	0	0	0
After 48 hr3	1	0	0	0	0	0
After 72 hr	1	0	0	0	0	0

Conclusion:

Under our experimental conditions, the test substance EXP 10343 A is non-irritant when applied topically to rabbits at the concentration of 2:25 ml/1.

B.6.11.5 Eye irritation

Acute eye irritation test in the rabbit

Dreher D.M. (1991) – Unpublished report No. 282/144 of 19 November 1991; Performed by Safepharm Labs., UK; dates of experimental work: 16 October of 1991.

Guidelines and GLP:

The study was conducted in compliance with OECD # 405; Method B5 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods:

SyIlitR (400 SC, EXP10343) with active substance: ISO common name: dodine ; IUPAC name: 1-dodecylguanidinium acetate at nominal concentration of 400g/l, batch 910600, purity: 387 g/l; 0.1 ml of undiluted test item was instilled into the conjunctival sac of the right eye of one New Zealand White rabbits(male). Observation took place immediately after instillation and one hour *post applicationem*.

Results:

Areas of opalescent corneal opacity, iridial inflammation and severe conjunctival irritation were noted in the treated eye one hour after treatment. Sloughing of the cornea and haemorrhage of the nictitating membrane were also noted at this time. The animal was killed for humane reasons immediately after the one hour observation. No further animals were treated to avoid unnecessary suffering.

Table 6.88 – Individual ocular irritation scores

Animal 236 (male)	Cornea		Iris	Conjunctivae	
1 hr	Degree of opacity: 3 (sloughing observed)	Area of opacity: 4	1	Redness: 2 (haemorrhage)	Chemosis: 4

Conclusion:

In accordance with the provisions of Council Directive 93/21/EEC and 99/45/EC, labelling with the R41: "Risk of serious damage to the eyes", will be required; the formulation is already classified with R34, so R41 was implicit.

Acute eye irritation in rabbits

Manciaux X. (1999) - unpublished report No. 18690TAL of 27 July 1999CIT; Performed by CIT - Centre international de toxicologie, France; dates of experimental work: 9 June 1999 to 12 June 1999.

Guidelines and GLP:

The study was conducted in compliance with OECD # 405; Method B5 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods:

SyllitR (400 SC, EXP10343A) with active substance: ISO common name: dodine; IUPAC name: 1-dodecylguanidinium acetate at nominal purity of 400g/I, batch OP980255, purity: 404 g/I and diluted in water to a concentration of 0.225% (2.25-ml/L) reflecting the normal condition of use of the diluted spray; 0.1 ml of test item (at 2.25 ml/I) was instilled into the conjunctival sac of the left eye of three New Zealand White rabbits (all males). Observation took place 1, 24, 48 and 72 hours *post applicationem*.

Results

On day 1, a very slight chemosis (grade 1) was recorded in two animals and a very slight redness of the conjunctiva (grade 1) was observed in one animal. No other ocular reactions were observed

during the study. The mean scores calculated for each animal over 24, 48 and 72 hours were 0.0, 0.0 and 0.0 for chemosis, 0.0, 0.0 and 0.0 for redness of the conjunctiva, 0.0, 0.0 and 0.0 for iris lesions and 0.0, 0.0 and 0.0 for corneal opacity. The results obtained are summarised below:

Table 6.89 – Individual ocular irritation scores

	Cornea			Iris			Conjunctivae					
							chemosis			redness		
Animal	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1 hr	0	0	0	0	0	0	0	1	1	0	1	0
24 hr	0	0	0	0	0	0	0	0	0	0	0	0
48 hr	0	0	0	0	0	0	0	0	0	0	0	0
72 hr	0	0	0	0	0	0	0	0	0	0	0	0

Conclusion:

The scores obtained prove the test item (diluted spray at 0.225% (2.25 ml Syllit 400 SC/ l water) to be non irritant to the eye.

B.6.11.6 Skin sensitization

Buehler delayed contact hypersensitivity study in guinea pig

Dreher D. M. (1992) - unpublished report No. 282/145 of 17 January 1992; Performed by Safepharm Labs, UK; dates of experimental work: 15 October 1991 to 02 January 1992.

Guidelines and GLP:

The study was conducted in compliance with OECD # 406; Method B6 of Directive 84/449/EEC. The study is GLP compliant.

The study is acceptable.

Materials and methods:

SyIlitR (400 SC, EXP10343) with active substance: ISO common name: dodine; IUPAC name: 1-dodecylguanidinium acetate at nominal purity of 400g/I, batch 910600, purity: 392 g/I.

A modified nine-induction Buehler Delayed Contact Hypersensitivity study was requested to assess the skin contact sensitisation potential of the test material. Due to the severity of the dermal reaction noted after two inductions of the undiluted test material the study was terminated. A Buehler Delayed Contact Hypersensitivity Study using three inductions of the test material was then performed.

Selection of Concentrations for main study (Sighting tests)

The test material formulation (0.5 ml) were occlusively applied to the clipped guinea pig flanks on absorbent lint patches (approximate size 15 mm x 35 mm). The exposure period will be approximately 6 hours. The procedures were as follows:

a) Selection of concentration for topical induction

Two previously untreated guinea pigs were treated with 0.5 ml of the undiluted test material and three concentrations of the test material in distilled water (75%, 50%, and 25% v/v). The highest

concentration of the test material which did not produce excessive irritation, 24 or 48 hours after a 6-hour occlusive dermal exposure, was selected for the topical induction stage of the main study.

b) Selection of concentration for topical challenge

Two guinea pigs were treated with 0.5 ml of each of two concentrations of the test material in distilled water (10%, 5%, 2% and 1% v/v). These animals had been treated identically to the control animals of the main study on days 0 and 14. The highest concentration of the test material which produced no evidence of dermal irritation 24 or 48 hours after a 6-hour occlusive dermal exposure, was selected for the topical challenge stage of the main study.

Initial topical induction

A group of thirty guinea pigs was used: twenty test and ten control animals.

Topical applications (0.5 ml) of the undiluted test material were applied. Severe dermal reactions were noted in all test animals on day 3 of the study following two inductions. These were necrosis, loss of skin elasticity and flexibility and oedema with an isolated incident of grade 3 erythema (intense redness and swelling) and sunken, hardened, dark brown/black coloured scab.

At the request of the study sponsor the study was terminated and repeated using a 50% v/v concentration of the test material for the topical induction.

Main Study

A total of thirty guinea pigs were used for the study: twenty test and ten control animals.

The bodyweight of each animal was recorded at the start and end of the study.

Two main procedures were involved in the Buehler study; a) an induction of a response and b) a challenge of that response.

a) Induction

Induction of the test animals: A topical application (0.5 ml) of the test material at a concentration of 50% v/v in distilled water was applied on absorbent lint. This occlusive dressing was kept in place for 6 hours. The induction procedure was repeated on days 7 and 14. Due to the severity of the reactions obtained after the first induction, the concentration of the test material was reduced to 10% v/v for the subsequent inductions. The application sites were decontaminated after each induction with distilled water.

Approximately 24 hours after each induction application any erythematous reactions were quantified.

b) Induction of the control animals: The topical applications followed the same procedure as for the test animals except that the vehicle alone was applied.

c) Challenge

Shortly before treatment on day 28, an area approximately 50 mm x \70 mm on the right flank of each animal was clipped free of hair with veterinary clippers.

A quantity of 0.5 m1 of the test material at a concentration of 5 % v/v in distilled water was applied to the shorn right flank of each animal on absorbent lint (approximate size 15 mm x 30 mm) which was held in place by a strip of surgical adhesive tape (BLENDERM: approximate size 40 mm x 50 mm). To ensure that the maximum non-irritant concentration was used at challenge, the test material at a concentration of 2% (v/v) in distilled water was also similarly applied to a separate skin site on the right shorn flank.

After six hours, the dressing was carefully cut using blunt- tipped scissors, removed and discarded.

Approximately 24 and 4e hours after dressing removal, any erythematous reactions were quantified.

Results:

Topical sighting tests

Based on the results, the following concentrations were selected for the main study:

Topical induction: 50% or 10% (v/v) in distilled water

Topical challenge: 2% and 5fo (v/v) in distilled water

Main study

a) Skin reactions observed after topical induction

One test animal was found dead on day 13. The absence of this animal did not affect the purpose or integrity of the study.

Scattered mild redness and moderate and diffuse redness were elicited by the test material. Oedema was also noted following topical induction. Dermal necrosis was noted at two treatment sites following the first induction.

b) Skin reactions observed after topical challenge

Table 6.90 – Summary of skin reactions observed after topical challenge with 2%

Group	Observation time (hours)	Concentration of test material	Skin response (scale 0 -3)			
			0	1	2	3
Test	24	2 % (v/v) in distilled water	19	0	0	0
	48		19	0	0	0
	24		10	0	0	0
Control	48		10	0	0	0

Table 6.91 – Summary of skin reactions observed after topical challenge with 5%

Group	Observation time (hours)	Concentration of test material	Skin response (scale 0 -3)			
			0	1	2	3
Test	24	5 % (v/v) in distilled water	19	0	0	0
	48		19	0	0	0
	24		10	0	0	0
Control	48		10	0	0	0

2% (v/v) in distilled water

No adverse reactions were noted at the test sites of test and control animals at the 24 and 48-hour observations.

5% (v/v) in distilled water

No adverse reactions were noted at the test sites of test and control animals at the 24 and 48-hour observations.

Bodyweights

Bodyweight gains of guinea pigs in the test group, between day 0 and day 30, were comparable to those observed in the control group animals over the same period.

Conclusion:

In accordance with the provisions of Council Directive 93/21/EEC and 99/45/EC, Syllit 400 SC is not a skin sensitizer, classification is not required.

B.6.12 Dermal absorption (Annex IIIA 7.3)

Dodine formulation: Absorption study in the male rat after topical application

Bounds S. V. J. (1995) - unpublished report No. 95/RHA552/0850 of 18 September 1995; Performed by Pharmaco LSR, Toxicology Services, Eye, Suffolk IP23 7PX, England, UK; dates of experimental work: 14 June 1995 to 31 July 1995.

Guidelines and GLP:

The Guideline was not mentioned in the study. The study is GLP compliant.

The study is acceptable.

Materials and methods:

Radiolabelled dodine: batch 950214, radiochemical purity ≥ 99%, formulated as 400 g/l SC (pure formulation) and a diluted 0.6 g/l formulation.

Sprague Dawley CD rats (Source: Charles River, UK), were housed individually in a metabolism cage, an area of dorsal skin was shaved on the day prior to dosing. Immediately prior to dosing a silicone rubber saddle was attached to the shaved area using Superglue. At dosing the formulation was applied via a pipette (calibrated to dispense 120 µl) to an area of shaved skin approximately 12 cm2. The dose was then evenly distributed over the selected area using a dose spreader which was retained, rinsed with methanol and analysed to determine the residual dose.

Following administration the application site was semi-occluded with a stainless steel gauze held in place over the silicone rubber saddle by surgical tape.

At eight hours after dosing the gauzes were removed and retained for analysis. The application site was then swabbed with water containing 1 % Tween 80 in order to remove and retain any unabsorbed dose. The gauze and swabs were retained for analysis.

Animals which were required to provide samples beyond 8 hours received clean gauzes and were replaced in their cages.

Treatment groups

Study Section	Group Number	Number of animals	Doses (g/l)
Preliminary	1	3	0.6
	2	3	400
Mass Balance	3	4	0.6
(low dose)	4	4	0.6
	5	4	0.6 l
Mass Balance	6	4	400
(high dose)	7	4	400
	8	4	400

Preliminary study

The objective of the preliminary study was to obtain an indication of the proportion of the [14C]-formulation excreted as 14C02 or other volatile organic material following topical administration of the dose formulations.

Group 1 low dose formulation (0.6 g/1)

Group 2 high dose formulation (400 g/1)

The rats in each group were given a topical application of the test formulation and were placed in metabolism cages in order to collect urine, faeces, expired carbon dioxide and volatile organic material.

Urine, faeces and cage wash and debris were collected during 0-24 and 24-48 hours after administration.

Expired carbon dioxide and volatile organic material were collected during 0-6, 6-24 and 24-48 hours after administration.

Mass Balance study (low dose)

Three groups of rats, each consisting of four males, were selected to follow the excretion and residual radioactivity after the topical application of the [14C)-low dose formulation (0.6 g/l).

The study design was as follows:

Group number	Time of kill (hours after dose)
3	8
4	24
5	72

Immediately after application of the radioactive dose, the rats were housed individually in metabolism cages in order to collect urine and faeces.

Mass Balance study (high dose)

Three groups of rats; each consisting of four males, were selected to follow the excretion and residual radioactivity after the topical application of the [14C]-high dose formulation (400 g/1).

The study design was as follows:

Group number	Time of kill (hours after dose)
6	8
7	24
8	72

Immediately after application of the radioactive dose, the rats were housed individually in metabolism cages in order to collect urine and Sampling and storage

Removal of non-absorbed dose

At the end of the exposure period (8 hours), the gauze was removed and was retained for analysis. The application site was swabbed with water containing 1 % Tween 80 and the swabs were retained for analysis. During this whole operation the animal was held over the metabolism cage in order to ensure collection of any urine.

Urine and faeces

Urine and faeces were collected separately from the metabolism cages. The urine and faeces collection flasks were surrounded by ice to minimise degradation of excreted material. At the end of each collection period faecal pellets were removed from the metabolism cages which were carefully washed with distilled water. The faecal pellets were added to the, faeces collection. The washings were retained for analysis.

Expired carbon dioxide

Expired air was drawn through two traps in series containing 200 ml of 2 M NaOH to trap the carbon dioxide. The traps were sampled at specific times during the study. The trapping fluid was replaced after each sampling period.

Volatile organic material

Expired air was drawn through two traps in series containing 200 ml of 2-ethoxyethanol to trap the volatile organic material. The traps were sampled at specific times during the study. The trapping fluid was replaced after each sampling period.

Carcass

Animals (in groups 3-8) were anaesthetised with halothane anaesthetic and a sample of blood withdrawn from a retro-orbital sinus. The blood was collected in vials containing lithium heparin. The animals were then killed by an overdose of halothane. All other animals were killed by an overdose of halothane.

The gauze and surgical tape were removed and kept for analysis. The area of treated skin plus approximately 1 cm of surrounding skin was carefully dissected away and retained for analysis. All carcasses were retained for analysis.

Storage

All samples when not analysed immediately after collection were placed in a freezer at about -20°C or lower as soon as possible after collection. The trapping fluids for expired carbon dioxide and volatile organic material were stored at about +4°C.

Results:

Preliminary study

Low dose formulation 0.6 g/l

The mean total recovery of radioactivity (at 48 hours) (as a percentage of the radiochemical dose) was 0.33 ± 0.17% in the urine, 0.04 ± 0.00% in the faeces, 0.03 ± 0.04% in the cage washes and 45.3 ± 3.87% in the tissues, of which 45.2 ± 3.80% was found in the treated skin. Of the applied radioactivity 59.9 ± 14.1 % was recovered in the washings of the application site 8 hours after dosing and 2.07 ± 0.47% in the washings of the protective cover. No radioactivity was detected in the traps for expired carbon dioxide and volatile organic material. The overall total mean recovery of radioactivity was 108 ± 15.1 %.

High dose formulation 400 g/l

The mean total recovery of radioactivity (at 48 hours) (as a percentage of the radiochemical dose) was 0.22 ± 0.12% in the urine, 0.03 ± 0.02% in the faeces, 0.05 ± 0.05% in the cage washes and 4.50 ± 1.14% in the tissues, of which 4.34 ± 1.04% was found in the treated skin. Of the applied radioactivity 91.6 ± 4.30% was recovered in the washings of the application site and 1.05 ± 0.71 % in the washings of the protective cover. No radioactivity was detected in the traps for expired carbon dioxide and volatile organic material. The overall total recovery of radioactivity was 97.5 ± 3.54%.

Main study

Low dose formulation (0.6 g/l) (Terminal kill at 8 hours)

The mean total recovery of radioactivity (as a percentage of the radiochemical dose) was 0.12 ± 0.07% in the urine, 0.03 ± 0.04% in the faeces, 0.02 ± 0.01 % in the cage washes and 37.0 ± 4.86% in the tissues, of which 36.6 ± 4.73% was found in the treated skin. Of the applied radioactivity 67.9 ± 6.06% was recovered in the washings of the application site and 4.80 ± 4.49% in the washings of the protective cover. No radioactivity was detected in blood 8 hours following application. The overall total recovery of radioactivity was 110 ± 6.55%.

Terminal kill at 24 hours

The total recovery of radioactivity (as a percentage of the radiochemical dose) for animals 11 and 14 were greater than 125%. These data are not included in the mean calculations for this group. The mean total recovery of radioactivity (as a percentage of the radiochemical dose) was 0.14 ± 0.06% in the urine, 0.01 ± 0.01% in the faeces, 0.04 ± 0.05% in the cage washes and 49.9 ± 5.21 % in the tissues, of which 49.5 ± 5.54% was found in the treated skin. Of the applied radioactivity 53.6 ± 7.20% was recovered in the washings of the application site and 2.15 ± 1.79% in the washings of the protective cover. No radioactivity was detected in blood 24 hours following application. The overall total mean recovery of radioactivity was 106 ± 10.8%.

Terminal kill at 72 hours

Animal 18 ingested some of the applied topical dose by chewing though the protective saddle. Thus the total recovery of radiochemical (as a percentage of the radiochemical dose) for animal 18 is not included in the mean calculations for this group.

The mean total recovery of radioactivity (as a percentage of the radiochemical dose) was 0.36 ± 0.20% in the urine, 0.06 ± 0.03% in the faeces, 0.11 ± 0.11% in the cage washes and 40.6 ± 8.82% in the tissues, of which 40.3 ± 8.81 % was found in the treated skin. Of the applied radioactivity 51.4 ± 3.97% was recovered in the washings of the application site and 7.18 ± 7.13% in the washings of the protective cover. No radioactivity was detected in blood 72 hours following application. The overall total mean recovery of radioactivity was 99.6 ± 6.85%.

High dose formulation 400 g/1 (Terminal kill at 8 hours)

The mean total recovery of radioactivity (as a percentage of the radiochemical dose) was 0.05 ± 0.04% in the urine, 0.00 ± 0.00% in the faeces, 0.01 ± 0.01 % in the cages washes and 4.22 ± 0.51 % in the tissues, of which 3.87 ± 0.43% was found in the treated skin. Of the applied radioactivity 85.3 ± 6.72% was recovered in the washings of the application site and 9.05 ± 6.57% in the washings of the protective cover. No radioactivity was detected in blood 8 hours following application. The overall total mean recovery of radioactivity was 98.7 ± 2.81 %.

Terminal kill at 24 hours

The mean total recovery of radioactivity (as a percentage of the radiochemical dose) was 0.14 ± 0.06% in the urine, 0.01 ± 0.01% in the faeces, 0.09 ± 0.05% in the cage washes and 3.97 ± 1.04% in the tissues, of which 3.65 ± 0.77% was found in the treated skin. Of the applied radioactivity 93.8 ± 1.95% was recovered in the washings of the application site and l .50 ± 1.13% in the washings of the protective cover. No radioactivity was detected in blood 24 hours following application. The overall total mean recovery of radioactivity was 99.5 ± 1.30%.

Terminal kill at 72 hours

The total recovery of radioactivity (as a percentage of the radiochemical dose) for animal 27 was 87.5%. This data is not included in the mean calculations for this group.

The mean total recovery of radioactivity (as a percentage of the radiochemical dose) was 0.16 ± 0.06% in the urine, 0.02 ± 0.02% in the faeces, 0.03 ± 0.02% in the cage washes and 5.26 ± 0.26% in the tissues, of which 5.17 ± 0.26% was found in the treated skin. Of the applied radioactivity 90.2 ± 3.35% was recovered in the washings of the application site and 3.15 ± 2.32% in the washings of the protective cover. No radioactivity was detected in blood 72 hours following application. The overall total mean recovery of radioactivity was 98.9 t 1.44%.

Discussion and Conclusions:

Following a single topical application of [^{14}C]-Dodine at two concentrations to the shaved dorsal area of the male rat, the absorption of radioactive material was minimal and rapid, with the majority of the absorbed material being absorbed in the first 8 hours.

The majority (group means >90%) of the applied radioactivity was recovered in the skin swabs and gauze washes for all groups dosed with the high dose formulation (400 g/1). Only a small proportion (group means 3 to 5%) of the applied radioactivity remained in the treated skin, with a very small proportion of applied radioactivity being absorbed and found in other tissues and excreta (group means 0.3 to 0.6%). No radioactivity was found in the blood at any time point. These results are summarised in Table 6.92 below.

Table 6.92 - Percentage of radiochemical material and mg equivalents of Dodine absorbed remaining in the treated skin and removed from the site of application following a single topical application of [^{14}C]-Dodine formulation (approximately 400 g/l)

Time	Absorbed**		Treated skin		Skin swab/gauze wash		Total
(h)	%	mg	%	mg	%	mg	%
8	0.42	0.20	3.87	1.81	94.4	44.2	98.7
24	0.56	0.26	3.65	1.70	95.3	44.4	99.5
48*	0.46	0.21	4.34	1.97	92.7	42.1	97.5
72	0.30	0.14	5.17	2.45	93.4	44.3	98.9
* data from preliminary study; **= urine, faeces, cage wash, carcass and untreated skin							

The majority (group means 55 to 73%) of the applied radioactivity was recovered in the skin swabs and gauze washes for all groups dosed with the low dose formulation (0.6 g/1). The proportion of applied radioactivity remaining in the treated skin ranged between 36 and 50% (group means) with a very small proportion of applied radioactivity being absorbed and found in other tissues and excreta (group means 0.5 to 0.8%). No radioactivity was found in blood at any time point. These results are summarised in Table 6.93 below:

Table 6.93 - Percentage of radiochemical material and µg equivalents of Dodine absorbed remaining in the treated skin and removed from the site of application following a single topical application of [^{14}C]-Dodine formulation (approximately 0.6 g/l)

Time	Absorbed**		Treated skin		Skin swab/gauze wash		Total
(h)	%	µg	%	µg	%	µg	%
8	0.59	0.31	36.6	19.4	72.7	38.5	110
24	0.56	0.22	49.5	19.8	55.8	22.3	106
48*	0.53	0.37	45.2	31.3	62.0	42.9	108
72	0.77	0.30	40.3	15.7	58.6	22.9	99.6
* data from preliminary study; **= urine, faeces, cage wash, carcass and untreated skin							

The absence of any radioactivity detected in the blood, and the very small proportions of radioactivity detected in the remaining carcass and excreta at both dose levels, indicate that ^{14}C-Dodine is very poorly absorbed, distributed and excreted by the circulating system following topical application.

Despite the much higher proportion of dosed radioactivity found in the treated skin of the low dose formulation (group mean range 36.6 to 49.5%) compared to the high dose formulation (group mean range 3.65 to 5.17%), the proportion of dosed radioactivity found in the non-treated skin was very similar (group mean range 0.10 to 0.32% low dose, 0.09 to 0.26% high dose). This is a further indication of the low potential for [14C]-Dodine to be distributed to areas of the body other than the application site.

The achieved balance of radioactivity with recoveries ranging from 98.7% to 108% renders the study acceptable and valid.

The maximum amount of absorption was rapidly reached already after 8 hours. The amount of dodine absorbed did not increase significantly afterwards. There was no apparent relationship between dose absorbed and time of exposure. Both at the low and at the high dose, dodine was poorly absorbed at a maximum of 0.77% of the dose administered. At the low dose, a significant amount (40% of the applied quantity) remained in the treated skin even after 72h.

In vivo absorption and skin distribution study of Dodine formulation after topical exposure in the rat

Esdaile D. (1999) - unpublished report No. SA 99254 of 27 August 1999; Performed by Rhône Poulenc Agro, B.P. 153, Sophia Antipolis, France; dates of experimental work: 01 June1999 to 25 June 1999.

Guidelines and GLP

The Guideline was not mentioned in the study. The study is GLP compliant.

The study is acceptable.

Materials and methods

In a previous study, more than 40% of the applied radiolabel remained in the skin residue following rinsing. It was considered probable that much of the skin residue was in the form of a deposit on the skin surface which was not removed by the washing procedure used (wetted cotton wool held in the fingers of the operator). In the study reported here, the washing method involved wetted natural sponge held in forceps which gave a better rinsing of the whole application site, more representative of the use of soap and water by a worker. Differences between the two studies are attributed to the washing procedure used.

Radiolabelled dodine: batch ?, radiochemical purity ?; the test formulation was prepared to represent a typical 0.6 g/l aqueous spray concentration formulation of dodine with a specific activity of the [^{14}C]-Dodine approximately 2.78 MBq/ml.

Three groups of 3 male rats Sprague Dawley CD rats (Source: Charles River, UK), were housed individually in a metabolism cage. The back of each rat was clipped approximately 24 hours before dosing. Two circular surfaces of exposure site were marked on the skin (3 cm^2 each). At dosing, 30 µl of the formulation was applied to each area by use of a calibrated pipette (i.e. 0.67 MBq/kg was applied at 10 µl/cm^2 over 6 cm^2). The liquid was allowed to dry and the area protected by a raised rectangular stainless steel mesh supported by a silicone border, held in place with an elastic bandage.

Group 1: animals were kept in metabolism cage for 3 days after treatment.

Group 2: animals were kept in metabolism cage for 5 days after treatment.

Group 3: animals were kept in metabolism cage for 10 days after treatment.

At 8 hours after application, the treated sites were swabbed with 1 % Tween 80 in physiological saline on biological sponge swabs, approximately 10 times (when no more radiolabel was removed as detected by a Geiger-Muller monitor). The swabs were retained for analysis. After swabbing, and daily thereafter, animals received a fresh semi-occlusive dressing to prevent [^{14}C] in desquamated skin falling into the collection bowl of the metabolism cage.

Urine and faeces

The urine and faeces were collected during and at the end of the experiment. Collection was made at the following intervals 0-8h, 8-24h, 24-48h, 48-72h, 72-96h, 96-120h, 120-144h, 144-168h, 168-192h, 192-216h and 216-240 hours after dosing.

Collected faeces were homogenised with the appropriate volume of water using a top drive homogeniser (Ultra-Turrax). Three samples of various weights, after drying, were combusted in a sample oxidiser (Packard model 387 Tri-carb), following addition of a small quantity of cellulose powder (approx 0.05 g to 0.1 g). The carbon dioxide generated by combustion was absorbed in a trapping agent (Carbo-Sorb, 10 ml) which was then mixed with an appropriate scintillation cocktail (Permafluor, 12 ml) prior to radioassay.

Metabowl cage washes

Metabowl cage washes (with distilled water) were performed at the end of the each 24 hour time period, just after the collection of urine and faeces samples, up to 240 hours post dosing.

Expired carbon dioxide

The collection of expired air was not performed because existing data showed that there was no was significant excretion of dodine as CO2 (<1%).

Treated areas

At the end of the experiment of the two application areas were separately treated. Where hair growth was found, the hair was clipped and collected, and then treated as for the skin, fur and carcass.

Upper site

When the animals were terminated, the upper application site was carefully excised and solubilised in 10 ml of Soluene 356 (Packard, France) in order to quantify the radiolabel in the full thickness of the skin.

Lower site

The lower site of application was subject to tape stripping with D.Squam tape (3M, France) to remove the stratum corneum. Each D.Squam disc was placed on the treated skin area. A standard pressure was applied (100 g/cm2) for five seconds, then the tape was carefully removed. Each tape strip was numbered and counted separately. Tape stripping continued until the skin appeared to be "shiny" indicating that stratum corneum had been removed (approximately 10 tape strips). The remaining skin, epidermis plus dermis, was excised and separated after heating at 60°C for 1 to 2 minutes. It was not possible to tape strip the entire treated site; so the stripped area of skin and the unstripped area of skin were weighed separately, and the ratio used to correct the radiolabel count to the whole treated site.

Skin fractionation was performed on one of the two application sites. It has been assumed that the radiolabel distribution was the same in both sites, hence where results have been expressed as percentage of applied radiolabel in a skin fraction per animal, the percentage in the single site has been doubled to represent the total residue in the two sites.

Blood

Blood samples were combusted directly after drying of three weighed aliquots on Combusto-pads (Packard, France) contained in Combusto-cones (Packard, France).

Skin adjacent to treated site skin and fur, and carcass

The remaining skin and fur were removed from the carcass. The skin, fur and carcass samples were solubilized (in alcoholic 2M potassium hydroxide) for a period of 24 hours at 50°C, before addition of aliquots of the solubilized material to Hionic Fluor liquid scintillation cocktail (10 ml) prior to radioassay.

Results

Formulation purity, stability and homogeneity

The homogeneity of the formulation was evaluated by examination of the variability between the radiolabel counted in the six 30 µl dose check samples. The mean value (after adjustment for background) was 4204817 dpm with a standard deviation of 2.6%. The low variability between samples indicates a good homogeneity.

Three day group

The mean total recovery of radiolabel (as a percentage of radiochemical dose) was 1.65 ± 0.85% absorbed (of which about half was found in the carcass and half in the urine & faeces), 93.12 ± 1.97% was removed from the skin at the end of the 8 hour exposure period (67.22 ± 19.15% was rinsed from the skin and 25.90 ± 20.62% was associated with the application system), 7.28 ± 1.57% was sloughed from the application site after the rinsing procedure or associated with the fur & skin away from the treated site, 4.69 ± 0.24% was within the skin at the treated site (of which over 90% was in the stratum corneum). The total recovery of radiolabel was 109.0±2.43% (see Table 6.94).

Five day group

The mean total recovery of radiolabel (as a percentage of radiochemical dose) was 0.86 ± 0.09% absorbed (of which about two thirds was found in the carcass and one third in the urine & faeces), 89.49 ± 0.84% was removed from the skin at the end of the 8 hour exposure period (61.76 ± 7.23% was rinsed from the skin and 27.73 ± 7:63% was associated with the application system), 7.46 ± 0.91 % was sloughed from the application site after the rinsing procedure or associated with the fur & skin away from the treated site, 4.05 ± 0.53% was within the skin at the treated site (of which over 90% was in the stratum corneum). The total recovery of radiolabel was 103.1 ± 0.17% (see Table 6.94).

Ten day group

One animal regularly escaped from the bandage system (with both the application system and the daily dressings) and sometimes consumed parts of the dressing. It was evident from the data that this animal had consumed a significant amount of test material with the dressing, and hence the data from this animal has been excluded from the means.

The mean total recovery of radiolabel (as a percentage of radiochemical dose) was 1.67 ± 0.16% absorbed (of which about 40% was found in the carcass and about 60% in the urine & faeces), 86.62 ± 1.21 % was removed from the skin at the end of the 8 hour exposure period (73.10 ± 8.57% was rinsed from the skin and 13.52 ± 7.37% was associated with the application system), 12.73 ± 0.07% was sloughed from the application site after the rinsing procedure or associated with the fur & skin away from the treated site, 0.67 ± 0.08% was within the skin at the treated site (of which about 90% was in the stratum corneum). The total recovery of radiolabel was 103.4 ± 1.36% (see Table 6.94).

Table 6.94 – Percentage of radiochemical material absorbed, removed from the site of application, sloughed from skin or remaining in skin at 3, 5 or 10 days after a single topical application of [^{14}C]-Dodine formulation at 0.6 g/l

Group		1 (3 days)		2 (5 days)		3 (10 days)	
		Mean (n=3)	SD	Mean (n=3)	SD	Mean (n=2)	SD
Urine		0.366	0.354	0.232	0.046	0.46	0.078
Faeces		0.352	0.462	0.038	0.034	0.40	0.018
Cage wash		0.07	0.121	0	0	0.15	0.108
Cardiac blood		0	0	0	0	0.00	0.000
Carcass		0.86	0.243	0.593	0.044	0.66	0.007
Total		**1.65**	**0.85**	**0.863**	**0.09**	**1.67**	**0.16**
Skin washing (8 h)		67.22	19.15	61.76	7.23	73.10	8.57
Application system rinsing (8 h)		25.90	20.62	27.73	7.63	13.52	7.37
Total "on skin"		**93.12**	**1.97**	**89.49**	**0.84**	**86.62**	**1.21**
Sloughed skin 24-72 h (skin dressing + fur and skin from the non-treated site)		6.92	1.50	7.21	0.93	12.51	0.04
Skin total		0.36	0.11	0.25	0.02	0.22	0.03
Total "in sloughed skin"		**7.28**	**1.57**	**7.46**	**0.91**	**12.73**	**0.07**
Treated skin	Stratum corneum	3.89	0.96	4.09	0.70	0.67	0.16
	epidermis	0.12	0.05	0.06	0.02	0.01	0.00
	Dermis	0.23	0.07	0.16	0.14	0.07	0.04
Total in skin and stratum corneum		**4.24**	**1.05**	**4.31**	**0.57**	**0.75**	**0.11**

Discussion and conclusion

Following topical administration of [^{14}C]-Dodine at 0.6 g/1 to the shaved dorsal area of male rats, the absorption was minimal. The majority of the applied radiolabel was removed by washing at 8 hours, most of the remaining residue was associated with the stratum corneum.

At the end of the application period approximately 90% of the applied radiolabel was removed by rinsing the application site. Following application less than 2% of the applied radiolabel was absorbed. At 3 to 5 days after application, approximately 7% of the applied radiolabel had been lost from the skin surface by desquamation, at 10 days this amount increased to almost 13% (skin lost by desquamation was collected in dressings placed on the animals daily, some of this will have spread under the dressing to the fur away from the treated site, both the dressings and the fur & skin were included in the estimate of total desquamation). The applied radiolabel associated with the skin at the treated site was mainly found in the stratum corneum, at 3 to 5 days approximately 4% of the applied radiolabel was within this layer but by Day 10 the level had fallen to about 0.7% (these values were extrapolated from disc 2 where the radiolabel in the various skin compartments were measured). These results are summarised Table 6.94.

The changes in distribution of the applied radiolabel over the duration of the study demonstrated that the [^{14}C]-Dodine was poorly absorbed through the skin (<2%) and that the residue within the skin was mainly associated with the stratum corneum. The data show that during the post application period, there were increasing amounts recovered from skin dressings (i.e. skin lost by desquamation) and decreasing amounts remaining in the stratum corneum, but there was no increase in the total amounts found in the urine, faeces, blood or carcass.

For risk assessment was considered that dermal absorption was 2.75% (considering the amount in the stratum corneum at 10 days) for both concentrate and the dilution.

B.6.13 Toxicological data on non active substances (Annex IIIA 7.4 and point 4 of the introduction)

According to Directive 99/45/EC it is not necessary to refer any formulant on the labels of the packages for the formulation Syllit 400 SC, due to its toxicological characteristics and its concentrations in the preparation.

B.6.14 Exposure data (Annex IIIA 7.2)

B.6.14.1 Operator exposure according to UK POEM model

Worst-case scenarios of application of Syllit 400 SC were considered with the following assumptions:

- Syllit 400 SC is a suspension concentrate containing 400 g/l of dodine;
- Container sizes are 5 and 10 litres;
- Duration of exposure is 6 hours/day;
- The value of 2.75 % is considered (see B.6.12) for dermal absorption when handling both the concentrate and the dilution.

The product is to be applied, by tractor-mounted/trailed broadcast air-assisted sprayer: 500 l/ha.

1. For the application with tractor-mounted/trailed broadcast air-assisted sprayer the work rate is 15 ha/day, the maximum rate of application will be of 1.7- 2.25 litre product/ha, 0.9 kg a.s./ha (maximum 5 applications) in an application volume of 500 - 1500 l/ha (apples/pears and peachs).
2. Calculations were done with no PPE (disregarding the recommendations on the label) – no personal protective equipment is used when handling the undiluted product and during application.
3. Calculations were done with PPE – gloves during mixing and loading and application.

Predicted exposure is presented in the tables bellow, and being also expressed as **percentage of systemic AOEL 0.045 mg/kg bw/day** (see B.6.10).

Table 6.95 - Predicted exposures after application of Syllit 400 SC (1.7 l Syllit 400SC/ha in an application volume of 500 l/ha) and respective percentages of systemic AOEL

Application method	Container size	Predicted exposure (mg/kg bw/day)		Percentage of AOEL (%)	
		no Gloves	Gloves during mix/loading & application	no Gloves	Gloves during mix/loading & application
Tractor-mounted/trailed broadcast air-assisted sprayer	5 l	**0.093**	**0.06**	**206.6**	**133.3**
	10 l	**0.11**	**0.062**	**244.4**	**137.7**

Table 6.96 - Predicted exposures after application of Syllit 400 SC (2.25 l Syllit 400SC/ha in an application volume of 1500 l/ha) and respective percentages of systemic AOEL

Application method	Container size	Predicted exposure (mg/kg bw/day)		Percentage of AOEL (%)	
		no Gloves	Gloves during mix/loading & application	no Gloves	Gloves during mix/loading & application
Tractor-mounted/trailed broadcast air-assisted sprayer	5 l	**0.492**	**0.0270**	**1093.3**	**60**
	10 l	**0.073**	**0.0283**	**162.2**	**63**

It was concluded that, using the UK POEM, exposure is acceptable when PPE [like gloves during mixing/loading and application, but based on the corrosivity potential of the formulation, R 34 "Causes burns" PPE are already recommended (safety phrases S36/37/39: Wear suitable protective clothing, gloves and eye/face protection, see B.6.4.2)] are used during mixing and loading and application of 1500 l of spray, therefore **the intended uses are acceptable.** With the application of 500 l of spray the exposure even when PPE are used is always higher than the AOEL.

B.6.14.1.1 Estimation of operator exposure using the German Model

Syllit 400SC is applied using tractor-mounted sprayers for high crops (apple/pears, peaches and cherries) and using hand held sprayers.

Operator exposure estimates were calculated using the "Uniform Principles for Safeguarding the Health of Applicators of Plant Protection Product (Uniform Principles for Operator Protection); Mitteilungen aus der Biologischen Bundesanstalt für Land-und Forstwirtschaft; Berlin-Dahlem n.º 277, 1992" ("German Model").

The following assumptions have been used in calculating operator exposure:

The treated area in one day is:

8 ha/d for high crops tractor mounted

1 ha/d for high crops hand held

The worst-case scenario will be the highest application rate for each of the recommended application technique.

The application doses are:

0.9 kg a.s/ha high crops tractor mounted (apples/ pears and peaches)

0.9 kg a.s/ha high crops hand held (apples/ pears and peaches)

The calculation of estimated operator exposure was made for two assumptions regarding the personal protective equipment (PPE):

no PPE (disregarding the recommendations on the label) - no personal protective equipment is used when handling the undiluted product and during application.

with PPE – like gloves during mixing/loading and application

Table 6.97 - Estimated operator exposure (mg/person/d)

	HCTM		HCHH	
	No PPE	**Gloves during mix/loading & application**	**No PPE**	**Gloves during mix/loading & application**
Dermal exposure (D)				
mixing/loading	17.28	0.1728	45	0.45
application	82.8	77.8104	36.36	26.91
Total	**100.8**	**77.9832**	**81.36**	**27.36**
Inhalation exposure (I)				
mixing/loading	0.00432	0.00432	0.72	0.72
application	0.1296	0.1296	0.27	0.27
Total	**0.13392**	**0.13392**	**0.99**	**0.99**
HCTM = High crop tractor mounted; HCHH = High crop hand held				

Determination of the tolerable exposure (Laboratory animal data)

For the determination of the tolerable exposure levels all relevant studies performed with dodine are taken into account.

On the basis of the relevant studies (see B.6.10) a NOAEL of 10 mg/kg bw/day should be used to calculate the tolerable dermal exposure, assuming the values of 2.75% for dermal absorption when handling the concentrate and the dilution (see B.6.12).

For estimation of inhalation exposure, the NOAEL of 10 mg/kg bw/day should be used, assuming 100% absorption.

Assuming a body weight of 70 kg and a safety factor of 100, the tolerable dermal (D_{tol}) and inhalation (I_{tol}) exposure are calculated to be:

D_{tol} = (163.63 mg/kg bw/day X 70) **:** 100 = 114.54 mg/person/day

I_{tol} = (100 mg/kg bw/day X 70) **:** 100 = 3.15 mg/person/d.

Comparison of estimated and tolerable exposure

Using the following equation the total degree of exposure (E) is calculated for the two different application methods and for the two conditions of operator protection respectively.

The value of E < 1 indicates that regarding systemic toxicity no risk for the applicators is to be assumed.

$$\mathbf{D : D_{tol} + I : I_{tol} = E}$$

Table 6.98 - Comparison of estimated and tolerable exposure

		D	D_{tol}	I	I_{tol}	E
HCTM	no PPE	1008	114.54	0.13392	3.15	0.9225
	with PPE	77.9832	114.54	0.13392	3.153	0.7225
HCHH	no PPE	81.36	114.54	0.99	3.15	1.024
	with PPE	27.36	114.54	0.99	3.15	0.552

To show the risk for man on the basis of the estimates for dermal and inhalative exposure the Total Systemic Exposure (TSE) as percentage of the usual AOEL (oral exposure) can be calculated. Using the AOEL oral/systemic of 0.045 mg/kg bw/day, assuming 2.75 % dermal absorption when handling the concentrate product (mixing and loading) and the diluted product (application) (see B.6.12), and applying a body weight of 70 kg the results for TSE and respective percentage of the AOEL are referred in the Table 6.99.

Table 6.99 - Comparison of the total systemic exposure and the acceptable systemic exposure level

Application method	Total Systemic Exposure (mg/kg bw/day)		TSE as % of AOEL	
	no PPE	with PPE	no PPE	with PPE
HCTM	0.0412	0.0325	**91.6**	**72.3**
HCHH	0.04612	0.0248	**102.4**	**55.3**

One may conclude that the TSE for the estimated worst-case operator exposure was always not more than 72.3 % of the AOEL systemic, even when no PPE are used, but based on the corrosivity potential of the formulation, R 34 "Causes burns" PPE are already recommended (safety phrases S36/37/39: Wear suitable protective clothing, gloves and eye/face protection, see B.6.4.2). Therefore, with the German Model safes uses can be derived.

B.6.14.2 Bystander exposure

B.6.14.2.1 Estimation of bystander exposure

For calculation of bystander exposure no general official model is available. Therefore, the following definitions and assumptions are considered:

- a bystander could be any person whose presence is quite incidental and unrelated to work
- exposure to bystanders can only occur during application of pesticides via drift

- it is assumed that the bystander will leave the area of a potential exposure after a very short period of time
- it is assumed that machine driven applications in high crops with full foliage represents the worst case for bystander exposure
- repeated exposure is unlike
- it is assumed that only ordinary clothing is worn; the total uncovered area amounts to 0.4225 m^2 (e.g. head, back and front of neck, forearms, $^1/_2$ upper arms and hands)

Based on the assumption that dermal exposure for bystanders is directly correlated to (1) the amount of active substance (a.s.) applied per area (use rate), (2) the size of the uncovered body surface contaminated and (3) the drift distance (distance between bystander and application machinery) the RMS has predicted potential exposure as follows:

- it takes one minute for the tractor to pass a bystander, the exposure time is only the 360th part of the exposure time of the applicator (spraying 6 hours a day)
- Calculation of 100% deposition high crops (fruit crops): 0.9 kg a.s./ha = 900 000 mg a.s/10 000 m^2 = 90 mg a.s/m^2
- Average spray drift deposition in 10 m distance apple and pears = 3.60 % (Ganzelmeier *et al*., 1995).

Dermal exposure of a bystander from High crops is:

D = 100% deposition x drift deposition x exposed area (m^2)

D = 90 mg a.s./m^2 x 0.0036 x 0.4225 m^2/person/day

D = 0.13689 mg a.s./person/day

The inhalation exposure of a bystander is as predicted for the operator (German model):

I = $I^*_{A\ (tractor\ mounted)}$ x WR x AR

$I^*_{A\ (tractor\ mounted)}$ = Spec. Exposure application, $_{HCTM}$ = 0.018 mg a.s./person x ha/kg a.s.

WR = Work rate $_{(HCTM)}$: 8[ha/day] = 6 h spraying/day

AR = Application rate $_{(HCTM)}$: 0.9 kg a.s./ha

I = 0.018 x 8 x 0.9

I = = 0.1296 mg a.s./person/day

Adjusted for 1 minute exposure (instead of 6 hours for operator)

I = 0.1296 mg a.s./person/day x 1 min/360 min

I = 0.00036 mg a.s./person/day

Total systemic exposure of a bystander is:

Considering 2.75 % (dilution in use) dermal and 100 % inhalation absorption and assuming a 70 kg person, the total systemic exposure (E) is calculated to be.

E = (0.13689 x 0.0275) + (0.00036 x 1)

E = 0.004124 mg a.s./person/day

E = 0.0000589 mg/kg bw/day

Assessment

Predicted systemic exposure for a bystander (assuming a 70 kg body weight and 2.75 % dermal absorption) from a treatment in high crops (orchards) is **0.0000589 mg/kg bw/day** which is equivalent to 0.131 % of the systemic AOEL.

So we can conclude that the calculated amount of dodine which might reach a bystander has no toxicological relevance, and for the proposed uses is acceptable taking into account the proposed AOEL.

B.6.14.3 Worker exposure

B.6.14.3.1 Estimation of worker exposure

RMS has predicted exposure to workers entering treated areas and performing work tasks using an exposure model proposed by Krebs *et al* (1996).

As an estimate it is taken into consideration that workers re-enter the treated crop after the spray has dried.

For the transfer of residues from foliage to the clothes or skin of a worker, Krebs *et al* (1996) propose a transfer coefficient of 30000 (cm2/person/h) be used in initial estimates of exposure, in the case of workers harvesting top fruit (search/reach/pick).

For Foliar Dislodgeable Residue (FDR) the values from degradation studies (see B.6.14.4), for HIGH CROPS as soon as spray dries after the fourth application is 4.43 µg/ cm2 per kg a.s./ha applied (in peach leaves, California Site).

HIGH CROPS

Worker exposure for entering high crops is estimated according to the following formula:

D = FDR x TC x WT x AR x P

Where:

D = Dermal exposure [µg a.s./person/day]

FDR = Foliar Dislodgeable Residues in µg a.s./cm^2 x ha/kg a.s.

= 4.43 µg a.s./cm^2 x ha/kg a.s. (as soon as spray dries after the fourth application, see B.6.14.4)

TF = Transfer factor (worst case, double sided) [cm^2/person h]
= 30000 cm^2/person h
WT = Work time/day = 8 h/day

AR = Application rate [Kg a.s./ha]
= 0.9 Kg a.s./ha

P = Penetration factor for clothing %

D = 4.43 x 30 000 x 8 x 0.9 x P

P = 1 (when no PPE such gloves, long sleeved shirt and long trousers are taken into account)

P = 0.1 (when PPE like gloves, long sleeved shirt and long trousers are taken into account)

Dermal exposure unprotected = 956.880 mg/person/day

Dermal exposure protected = 95.688 mg/person/day

Assessment

Predicted exposure, for high crops were compared with the AOEL (0.045 mg/kg bw/day) for dodine in Table 6.100. Systemic exposure values assume 2.75% dermal absorption and a 70 Kg worker.

Table 6.100 - Predicted worker exposure, arising from high crops use Diazol 60 EC, and comparison with systemic AOEL's.

Use	Transfer coefficient assumed	Systemic exposure (mg/kg bw/day)	Systemic exposure as % of AOEL (human data)
High crops - no PPE	30 000	0.3759	**835.33**
High crops - with PPE	30 000	0.03759	**83.53**

Predicted exposure for workers entering Fruit crops as soon as spray is dried after the fourth application is within the AOEL when PPE (gloves, long sleeved shirt and long trousers) are worn.

Conclusions

Application of Syllit 400SC represents an acceptable level of risk for operators, when PPE are worn (gloves during mixing/loading and application) and for bystanders. Re-entry exposure as soon as spray was dried and during 35 days after the last of 4 applications in high crops, treated with Syllit 400SC represents an acceptable level of risk for workers when PPE like gloves, long sleeved shirt and long trousers are worn.

B.6.14.4 Measurement of worker exposure

Dodine: Dissipation of dislodgeable foliar dodine residues from peaches treated with Syllit 65W

Macy L.J. (2000) - unpublished report # 99X17415 of 07 July 2000; Performed by ABC Laboratories California, USA + Southeast Ag Research / Georgia USA + Horizon Laboratories Columbia USA; dates of experimental work: 30 July 1999 to 08 February 2000.

Guidelines and GLP

Guidelines: US EPA OPPTS 875.2100. The study is GLP compliant.

The study is acceptable.

Materials and methods:

Syllit® (65%WP) with active substance: ISO common name: dodine; IUPAC name: 1-dodecylguanidinium acetate at nominal concentration of 650g/kg, batch 09JK0076-6, concentration: 65.07 g/kg.

Test system

Two field trials were established: one in California (warm and arid climate) and one in Georgia (warm and humid climate). One plot at each site was untreated and one plot was treated. Treated plots sizes were approx. 1000 m^2 and comprised 5 rows of trees. Syllit 65WP was applied four times at a rate of each 2.24 ka a.s. /ha (highest registered dose in the US, and about 2 times higher than the proposed dose in UE) at 7-day intervals during August (last application 15 days before harvest) using a water volume of approx. 950 L/ha.

Samples comprised 400 cm^2 of leaf material and were collected using a leaf punch sampler (approx 5 cm^2 maximum per leaf).

Samples were collected prior to and shortly after each application, and at 0.5, 1, 3, 5, 7, 10, 14, 21, 28 and 35 days after the last application. Within three hours after sampling, the leaf punches were dislodged by using 2 x 100 ml of 0.01 % aqueous surfactant solution (bis-2-ethylhexyl-sodium sulfosuccinate). The dislodged leaf punches and the dislodging solutions were placed in the freezer. Untreated dislodging solutions were fortified at the field sites to verify stability during storage. Samples were shipped frozen to the laboratory and analyzed by using a fully validated HPLC/UV method.

Results:

The HPLC/UV analytical method used to quantitate Dodine residues in dislodging solutions was validated and was acceptable over the full range of residues detected in actual samples. Fortified and unfortified untreated duplicate samples were analysed at three levels (LOQ = 0.1 µg/ml, 10XLOQ, 20XLOQ). Recoveries were all acceptable and ranged from 78.8% to 98.8%. The recoveries in daily sample sets ranged from 81.7% to 110.3%. Recoveries of the field fortification sample ranged from 78.7% to 100.2% and demonstrated that the field samples were stable throughout sample collection, shipping and storage.

Residues in control plot rinsates were either not detected or were less than LOQ. The residue values were generally higher at the California site than at the Georgia site. In both cases, the residues were less than about 5% of what theoretically would have been deposited onto the leaves at each application.

Residue Levels

Dodine residues from all untreated control samples were either not detected or were less than the LOQ (< 0.1 µg/ml). Residues from the California trial ranged from an average high of 11.7 µg /ml

(5.84 µg a.s./cm^2) 10 days after the fourth application, remained constant, then dropped slightly to an average of 8.8 µg/ml after 35 days. Since the California results did not show a significant dissipation, no dissipation curve could be calculated (see Table 6.101).

Table 6.101 - Average Dislodgeable residues found compared with expected surface residues at the California site

Sample timing	Average residues (µg/ml)	Average residues (µg a.s./cm^2)	Expected residues* (µg a.s./cm^2)
0 – 1 day pre-application 1	0.00	0.00	0
As soon as spray dries post-application 1	3.09	1.54	22.4
0 – 1 day pre-application 2	2.88	1.44	22.4
As soon as spray dries post-application 2	3.80	1.90	44.8
0 – 1 day pre-application 3	3.60	1.80	44.8
As soon as spray dries post-application 3	9.86	4.93	75.1
0 – 1 pre-application 4	7.98	3.99	75.1
As soon as spray dries post-application 4	8.86	4.43	97.5
12 hr post-application 4	8.78	4.39	97.5
1 day post-application 4	10.7	5.35	97.5
3 days post-application 4	11.3	5.65	97.5
5 days post-application 4	9.37	4.68	97.5
7 days post-application 4	10.5	5.24	97.5
10 days post-application 4	11.7	5.84	97.5
14 days post-application 4	10.8	5.40	97.5
21 days post-application 4	11.1	5.53	97.5
28 days post-applicatio 4	11.2	5.59	97.5
35 days post-application 4	8.88	4.44	97.5
* The expected residues are based on the application rate in lb as/A converted to µg a.s./cm^2			

The residues found in Georgia samples did not increase with each application but showed that the residues may be additive with multiple applications and ranged from an average high of 5.33 µg /ml (2.68 µg a.s./cm^2) immediately after the second application. At 35 days after the fourth application, residues had dropped to an average value of 0.48 µg/ml. The half-life at the Georgia site was determined to be about 10 days using exponential dissipation curve. This shows that some factors at the Georgia site (like higher rainfall and humidity) caused dissipation of the residue when compared to the California site (see Table 6.102).

Table 6.102 – Average Dislodgeable residues found compared with expected surface residues at the Georgia site

Sample timing	Average residues (µg/ml)	Average residues (µg a.s./cm^2)	Expected residues* (µg a.s./cm^2)
0 – 1 day pre-application 1	<LOQ	<LOQ	0
As soon as spray dries post-application 1	3.24	1.62	22.4
0 – 1 day pre-application 2	1.94	0.970	22.4
As soon as spray dries post-application 2	5.36	2.68	44.8
0 – 1 day pre-application 3	1.20	0.60	44.8
As soon as spray dries post-application 3	3.33	1.67	67.2
0 – 1 pre-application 4	1.58	0.791	67.2
As soon as spray dries post-application 4	3.90	1.95	89.6
12 hr post-application 4	4.06	2.03	89.6
1 day post-application 4	4.18	2.09	89.6
3 days post-application 4	3.19	1.60	89.6
5 days post-application 4	2.64	1.32	89.6
7 days post-application 4	2.42	1.21	89.6
10 days post-application 4	1.96	0.981	89.6
14 days post-application 4	2.08	1.04	89.6
21 days post-application 4	0.765	0.383	89.6
28 days post-application 4	0.521	0.261	89.6
35 days post-application 4	0.484	0.242	89.6
* The expected residues are based on the application rate in lb as/A converted to µg a.s./cm^2			

Conclusion:

The maximum dislodgeable residue of dodine from leaves of peach trees treated 4 times at 2.2 kg a.s./ha was found to be 11.7 µg a.s./ml (5.84 µg a.s./cm^2) 10 days after the last application and 5.36 µg/ml (2.68 µg a.s./cm^2) immediately after the second application for the California and the Georgia site respectively.

At the California site, the dislodgeable foliar residues increased with each application, reaching their peak after 10 days after the fourth application. This shows that dodine residues did increase with multiple applications of Syllit® 65W the residues then remained constant throughout the remainder of the study, showing that the residues dissipated slightly over the next 25 days.

At the Georgia site, the dislodgeable residues were highest immediately after the second application; however, residues found immediately after the fourth application were higher than those found immediately after the third application, showing the residues may be additive with multiple applications. The residues after the fourth (last) application did dissipate with time, giving a half-live of about 10 days. This shows that some factor or factors at the Georgia site caused dissipation of the dodine residues. There was significant rainfall at the Georgia (compared to no rainfall at the California site). This high humidity at the Georgia site could have an impact of the dissipation of the residues; the dodine would have been more likely to stay in solution, allowing the chemical to be more readily absorbed or adsorbed by the leaves. These factors, plus other undeterminated factors, could have had an effect on the dissipation of the dodine residues.

The dislodgeable foliar residue data generated in this study along with the dissipation curve and half-live can be used with transfer coefficients to determine worker exposure. The California data represent a hot, arid climate, while the Georgia dissipation curve and half-life calculation represent a hot, humid climate.

After the last application, the dislodgeable residue decreased slowly in California (warm and dry) but more rapidly in Georgia (warm and humid) with a half-life of 10 days for the Georgia site.

Dislodgeable residue showed a potential to accumulate following several applications with 7 days intervals.

B.6.15 References relied on

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection claimed Y/N	Owner
Annex IIA					
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IIA, 5.2.1/01	Kern T.G.	1999	Acute oral toxicity study of dodine technical material in albino rats WIL Research Laboratories, USA Report no. WIL-21130 GLP Unpublished	Yes	CAG
IIA, 5.2.2	Kern T.G.	1999	Acute dermal toxicity study of dodine technical material in albino rats WIL Research Laboratories, USA Report no. WIL-21131 GLP Unpublished	Yes	CAG
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IIA, 5.2.6	Manciaux X.	1999	Dodine technical : skin sensitization test in guinea-pigs (Maximization method of Magnusson, B. and Kligman, A.M.) Centre International de Toxicologie, France Report no. 17473 TSG GLP Unpublished	Yes	CAG

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IIA, 5.3.1/02	Batham P.	1994b	A 4 week oral (diet) toxicity study of dodecylguanidine acetate (dodine) in the albino rat Bio-Research Laboratories, Canada Report no. 84569 GLP Unpublished	Yes	CAG
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IIA, 5.3.3/01	Kern T.G.	1999	A 28-day dermal toxicity study of dodine technical material in rats WIL Research Laboratories, USA Report no. WIL-21140 GLP Unpublished	Yes	CAG

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IIA, 5.4.1/02	Verspeek-Rip C.M.	2003	Evaluation of the mutagenic activity of dodine technical in the *Escherichia Coli* reverse mutation assay (with independent repeat) Notox, Netherlands Report no. 394482 GLP Unpublished	Yes	CAG
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IIA, 5.4.2/02	Hemalatha Murli	1992	Mutagenicity test on dodecylguanidine acetate technical *in vivo* mammalian micronucleus assay Hazleton Washington, USA Report no. 14710-0-455 GLP Unpublished	Yes	CAG
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IIA, 5.5/01 *bis*	Semino G.	2000	Dodine : Evaluation of clinical signs in the rat chronic study Aventis CropScience, France Unpublished Statement	Yes	CAG
IIA, 5.5/02	Williams K.D.	1998	78-week dietary oncogenicity study with dodine in mice Covance Laboratories, USA Report no. 6224-220 GLP Unpublished	Yes	CAG

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IIIA, 7.1.1	Dreher D.M.	1991	EXP 10343: Acute oral toxicity (limit test) in the rat Safepharm Laboratories, UK Report no. 282/141 GLP Unpublished	Yes	CAG
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IIIA, 7.2.3.2	Macy L.J.	2000	Dodine: Dissipation of dislodgeable foliar dodine residue from peaches treated with Syllit 65 W ABC Laboratories California, USA + Southeast Agresearch/Georgia USA + Horizon Laboratories Columbia USA Report no. 99X17415 GLP Unpublished	Yes	CAG
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