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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

007150

APR 28 1989

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Happy Jack Flealine Flea and Tick Collar

TO: Mr. Dennis Edwards, PM 12
Registration Division (H7505C)

FROM: Byron T. Backus, Toxicologist Byron T. Backus 4/27/89
Fungicide/Herbicide/Antimicrobial Toxicology Branch
HED (H7509C)

THROUGH: K. Clark Swentzel K. Clark Swentzel 4/27/89
Acting Section Head, Review Section II
Fungicide/Herbicide/Antimicrobial Toxicology Branch
HED (H7509C)

and

Marcia van Gemert, Acting Branch Chief Marcia van Gemert 4/27/89
Fungicide/Herbicide/Antimicrobial Toxicology Branch
HED (H7509C)

EPA Record No. 221153

Project No. 9-1200

EPA Reg. No. 2781-GL

Tox. Chem. 219AA

Action Requested:

Review 3 studies on a 3% chlorpyrifos-containing cat collar relating to cholinesterase (ChE) inhibition and rate of release (cat exposure to) chlorpyrifos from this collar.

Comments and Recommendations:

1. The chlorpyrifos rate of release study has been classified as acceptable. This study adequately defines a one-collar exposure to cats of approximately 0.2 to 0.5 mg/kg/day chlorpyrifos from normal collar wear. The highest exposure level (approximately 0.5 mg/kg/day) occurs in the period (first 1-2 weeks) immediately following collar application. After this initial period the exposure drops to a "maintenance" dosage of between 0.1 and 0.2 mg/kg/day.

1 25

2. The 13-week cat cholinesterase study has been classified as core minimum data. This study demonstrates that considerable (>80%) plasma ChE depression occurs in cats wearing a single 3% chlorpyrifos collar. The procedure used to measure ChE activity was that of Ellman et al; this method appears to give a considerably greater value for plasma ChE depression than the delta-pH method of Michel. No evidence for RBC ChE inhibition was observed, and there were no symptoms indicative of cholinergic poisoning, even in cats wearing five of these collars.

   The levels of plasma ChE inhibition observed were consistent with the initial dermal dosage of 0.5 mg/kg/day chlorpyrifos, and the subsequent reduction to a "maintenance" dosage of 0.1 to 0.2 mg/kg/day. The plasma ChE depression observed in this study was consistent with findings in other studies where the exposure was oral rather than dermal, particularly a two-year dog feeding study conducted by Dow in which the plasma ChE NOEL was 0.01 mg/kg/day and the LEL was 0.1 mg/kg/day, and the RBC ChE NOEL was 0.1 mg/kg/day and the LEL was 1 mg/kg/day.

3. Because of the relatively short duration (5 weeks) and the low numbers of cats used (1 per sex per dose level) the range-finding study has been classified as core supplementary data. The findings of this study are essentially the same as those of the first 3 weeks of the subsequent cat ChE study.

4. Based on the findings of these studies, Toxicology Branch 2 would have no objections to the registration of this product for the proposed use(s) with the labeling as proposed by the registrant.

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Reviewed by: Byron T. Backus Byron T. Backus 4/27/89
Section 2, HFASB (H7509C)

Secondary Reviewer: K. Clark Swentzel K. Clark Swentzel 4/27/89
Section 2, HFASB (H7509C)

DATA EVALUATION REPORT I

STUDY TYPE: Chlorpyrifos - rate of release from a cat collar TOX CHEM NO. 219AA

MRID NO: ACC. NO: 406031-04

TEST MATERIAL: Collar with 3% Chlorpyrifos

SYNONYMS: Flealine Flea and Tick Collar

STUDY NUMBER(S): no study number

SPONSOR: Happy Jack^{R} Inc.
P.O. Box 475
Highway 258 South
Snow Hill, NC 28580

TESTING FACILITY: Fred W. Knapp
2407 Vince Road
Nicholasville, Kentucky 40356

TITLE OF REPORT: 13 Week Release Rate Study

AUTHOR(S): Ott, J.

REPORT ISSUED: 4/1/88

CLASSIFICATION: Acceptable

CONCLUSIONS:

1. This study adequately defines an exposure level (chlorpyrifos release rate from this collar) of approximately 0.2 to 0.5 mg/kg/day to cats as a result of normal wear of a collar. The highest exposure level (approximately 0.5 mg/kg/day) occurs in the period (first 1-2 weeks) immediately following collar application. After this initial period the daily chlorpyrifos exposure drops to a "maintenance" dosage of between 0.1 and 0.2 mg/kg/day.

2. The chlorpyrifos release rate obtained in this study can be correlated with the plasma cholinesterase depression observed in cats wearing a single 3% chlorpyrifos collar in the 13-week cholinesterase study.

A. MATERIALS:

1. Test compound: Cat Flea Collar, lot no. 80187, identified as containing 3% Dursban (Chlorpyrifos). Individual collars weighed about 11 grams before trimming.

2. Animals used: 16-mixed breed cats divided into 4 groups.

B. STUDY DESIGN:

1. Collar application: 16 individual collars were weighed, and then were placed (after trimming) on each of 16 cats. The trimmings were weighed and saved. At 1, 2, 6 and 13 weeks 4 collars were removed and weighed. Following collar removal, the chlorpyrifos content of both the trimming and corresponding worn piece of collar were analytically determined at Missouri Analytical Laboratories, Inc.

2. There is a signed and dated Good Laboratory Practice Statement on page 3 of the report. Each Certificate of Analysis (pages 13-21) from Missouri Analytical Laboratories is signed by the Director of Quality Assurance.

C. RESULTS:

Collar # and week removed	Weight of collar (gram) before trimming	trim	worn piece	dif.	Chlorpyrifos content (%) of trim	worn piece	dif.
#2 wk 1	10.88	3.25	7.61	0.02	3.11	3.05	0.06
#8 wk 1	10.75	3.38	7.36	0.01	3.14	3.06	0.08
#9 wk 1	10.99	3.21	7.77	0.01	3.14	3.06	0.08
#15 wk 1	10.95	3.96	6.43*	0.56*	3.13	3.05	0.08
#3 wk 2	10.72	2.39	8.32	0.01	3.38	3.16	0.22
#7 wk 2	10.83	3.35	7.43	0.05	3.14	3.04	0.10
#10 wk 2	10.74	3.01	7.66	0.07	3.30	3.08	0.22
#16 wk 2	10.72	3.48	7.21	0.03	3.28	3.34	+0.06
#1 wk 6	10.98	2.63	8.29	0.01	3.10	2.92	0.18
#5 wk 6	10.75	2.08	8.60	0.07	3.30	3.05	0.25
#11 wk 6	11.10	3.61	7.44	0.05	3.06	2.95	0.11
#13 wk 6	10.83	2.62	8.12	0.09	3.10	2.78	0.22
#4 wk 13	11.08	2.42	8.55	0.11	3.17	2.83	0.34
#6 wk 13	11.11	2.81	7.58	0.72	3.16	2.80	0.36
#12 wk 13	10.99	3.25	6.88	0.86	3.10	2.76	0.34
#14 wk 13	10.65	3.34	7.20	0.11	3.37	2.78	0.59

*Collar number 15 is reported as having had a small piece missing from its end "as if it was nicked with the knife when it was trimmed."

D. RATE OF RELEASE:

The following calculations were made from the data as presented in this report.
The total amount of chlorpyrifos released was calculated by adding two components, A and B, where A = chlorpyrifos released from the plastic matrix of the collar. This value was determined by multiplying weight of collar at week n by % loss of chlorpyrifos over the period from week 0 to week n. B = chlorpyrifos released by "wear" of the collar. This was determined by multiplying [(weight of the collar at week 0 - weight of the collar at week n) - A] x 0.03 (= 3%, the amount of chlorpyrifos present in the original collar formulation).

Rate of release during the first week:

Collar	Weight after one week (gm)		% difference 0-1 week		Chlorpyrifos release (A) in gm
#2	7.61	x	0.06	=	0.004566
#8	7.36	x	0.08	=	0.005888
#9	7.77	x	0.08	=	0.006216
#15	6.43	x	0.08	=	0.005144

Chlorpyrifos release, component B:

#2	(0.02-0.004566)(0.03) = 0.000463 grams
#8	(0.01-0.005888)(0.03) = 0.0001234 grams
#9	(0.01-0.006216)(0.03) = 0.0001133 grams
#15	Estimated 0.0002 grams

Total chlorpyrifos release from each collar (A + B):

#2	0.004566 + 0.000463 = 0.0050 grams
#8	0.005888 + 0.0001234 = 0.0060 grams
#9	0.006216 + 0.0001133 = 0.0063 grams
#15	0.005144 + 0.0002 = 0.0053 grams

The total weight for these 4 cats was 6 + 3.6 + 4.25 + 3.75 lbs = 17.6 lbs = 8 kg.

A total of 22.6 mg of chlorpyrifos was released on 8 kg body weight of cats over a period of one week; this translates out to 2.825 mg/kg/wk = 0.4 mg/kg/day.

Rate of release during the first two weeks:

Collar	Weight after two weeks(gm)		Chlorpyrifos difference 0-2 week		Chlorpyrifos release (A) in gm
#3	8.32	x	0.0022	=	0.018304
#7	7.43	x	0.0010	=	0.00743
#10	7.66	x	0.0022	=	0.016852
#16	7.21	x	-0.0006 (?)		not calculated

Chlorpyrifos release, component B:

#3	(0.01-0.018304)(0.03) = negative value, not used
#7	(0.05-0.00743)(0.03) = 0.0001277 grams
#10	(0.07-0.016852)(0.03) = 0.0015944 grams
#16	not calculated

Total chlorpyrifos release from each collar (A + B):

#3	0.018304 + ? = 0.018304 grams
#7	0.00743 + 0.001277 = 0.008707 grams
#10	0.016852 + 0.0015944 = 0.0184464 grams
#16	not calculated

The total weight for the 3 cats with collars 3, 7 and 10 was 5.625 + 4.75 + 4.325 lbs = 14.7 lbs = 6.68 kgs.

A total of 45.46 mg of chlorpyrifos was released on 6.68 kg body weight of cats over a period of two weeks: this translates out to 6.805 mg/kg/2 wks = 0.486 mg/kg/day.

Rate of release over six weeks:

Collar	Weight after six weeks(gm)		Chlorpyrifos difference 0-6 week		Chlorpyrifos release (A) in gm
#1	8.29	x	0.0018	=	0.014922
#5	8.60	x	0.0025	=	0.0215
#11	7.44	x	0.0011	=	0.008184
#13	8.12	x	0.0022	=	0.017864

Chlorpyrifos release, component B:

#1	(0.01-0.014922)(0.03) = negative value, not used
#5	(0.07-0.0215) (0.03) = 0.001455 grams
#11	(0.05-0.008184)(0.03) = 0.0012545 grams
#13	(0.09-0.017864)(0.03) = 0.0021641 grams

Total chlorpyrifos release from each collar (A + B):

#1	0.014922 +	?	= 0.014922 grams
#5	0.0215 +	0.001455	= 0.022955 grams
#11	0.008184 +	0.0012545	= 0.0094385 grams
#13	0.017864 +	0.0021641	= 0.0200281 grams

The total weight for the 4 cats was 6 + 4.375 + 5.25 + 4.125 lbs = 19.75 lbs = 8.977 kg.

A total of 67.34 mg of chlorpyrifos was released on 8.977 kg of cats over a period of 6 weeks; this works out to 7.501 mg/kg/6 weeks = 0.179 mg/kg/day.

Rate of release over thirteen weeks:

Collar	Weight after 13 weeks(gm)		Chlorpyrifos difference 0-13 week		Chlorpyrifos release (A) in gm
#4	8.55	x	0.0034	=	0.02907
#6	7.58	x	0.0036	=	0.027288
#12	6.88	x	0.0034	=	0.023392
#14	7.20	x	0.0059	=	0.04248

Chlorpyrifos release, component B:

#4	(0.11-0.02907)(0.03) = 0.0024279 grams used
#6	(0.72-0.027288)(0.03) = 0.0207814 grams
#12	(0.86-0.023392)(0.03) = 0.0250982 grams
#14	(0.11-0.04248)(0.03) = 0.0020256 grams

Total chlorpyrifos release from each collar (A + B):

#4	0.02907 +	0.0024279	= 0.0314979 grams
#6	0.027288 +	0.0207814	= 0.0480694 grams
#12	0.023392 +	0.0250982	= 0.0484902 grams
#14	0.04248 +	0.0020256	= 0.0445056 grams

The total weight for the 4 cats was 5 + 5.125 + 4.5 + 4.875 lbs = 19.5 lbs = 8.864 kg.

A total of 172.56 mg chlorpyrifos was released onto 8.864 kg over a period of 13 weeks; this works out to 19.4675 mg/kg/13 weeks = 0.214 mg/kg/day.

Summary:

Interval	Cumulative chlorpyrifos exposure (mg/kg)	Mean daily chlorpyrifos (mg/kg)
week 0-week 1	2.825	0.40
week 0-week 2	6.805	0.49
week 0-week 6	7.501	0.18
week 0-week 13	19.468	0.21

E. DISCUSSION:

The study adequately defines an exposure (chlorpyrifos release) rate of approximately 0.2 to 0.5 mg/kg/day in cats as a result of normal wear of trimmed collars, with the higher level of exposure (approximately 0.5 mg/kg/day) occurring in the period (first 1-2 weeks) immediately following collar application. After this initial period the daily chlorpyrifos exposure drops to a "maintenance" dosage between 0.1 and 0.2 mg/kg/day. This exposure rate range can be correlated with the plasma cholinesterase inhibition observed in cats wearing a single collar over a period of 13 weeks.

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Section 2, HFASB (H7509C)

DATA EVALUATION REPORT II

STUDY TYPE: Cholinesterase - collar exposure, cat TOX CHEM NO. 219AA

MRID NO: ACC. NO: 406031-03

TEST MATERIAL: Collar with 3% Chlorpyrifos

SYNONYMS: Flealine Flea and Tick Collar

STUDY NUMBER(S): WIL-52013

SPONSOR: Happy Jack^{R} Inc.
P.O. Box 475
Highway 258 South
Snow Hill, NC 28580

TESTING FACILITY: Wil Research Laboratories, Inc.
Ashland, Ohio 44805-9281

TITLE OF REPORT: 3-Month Flea Collar Cholinesterase Study in Cats

AUTHOR(S): Tompkins, E. C.

REPORT ISSUED: 4/12/88

CLASSIFICATION: Core Minimum Data

CONCLUSIONS:

1. The study demonstrates that considerable (>80%) plasma ChE depression occurs in cats wearing a single 3% chlorpyrifos collar. The procedure used to measure ChE activity was that of Ellman et al; this method appears to give a considerably greater value for plasma ChE depression than the delta-pH method of Michel.

2. From the rate of release data (see DER I) the initial dermal dosage of chlorpyrifos is approximately 0.5 mg/kg/day in the 1-2 week period immediately following collar application. Subsequently, the rate dropped to a "maintenance" dosage level of something like 0.1 to 0.2 mg/kg/day. This is consistent with the initial drop in plasma ChE activity during the first weeks, and the slow subsequent upward trend.

3. There was no indication of RBC ChE inhibition at any time in this study, and there were no symptoms of cholinergic toxicity even in cats wearing five collars.

4. The plasma ChE depression observed in this study is consistent with findings in other studies where the exposure was oral rather than dermal (90-day feeding study in rats conducted by Dow; ChE NOEL 0.1 mg/kg/day with a LEL for plasma and RBC ChE depression at 1 mg/kg/day; 6-month rat feeding study with a ChE NOEL of 0.15 mg/kg/day and a LEL for plasma and RBC ChE inhibition of 0.75 mg/kg/day). In a two-year dog feeding study conducted by Dow the plasma ChE NOEL was 0.01 mg/kg/day and the LEL was 0.1 mg/kg/day. The RBC ChE NOEL was 0.1 mg/kg/day and the LEL was 1 mg/kg/day.

A. MATERIALS:

1. Test compound: Cat Flea Collar, identified as containing 3% Dursban (Chlorpyrifos). According to information received from the registrant, the individual collars weighed 0.46 oz (= 13 grams); collars were trimmed to fit the individual cats.

2. Animals used: Domestic short hair cats, approximately 8-10 months old, received from Liberty Laboratories, Liberty Corner, New Jersey. Each animal was uniquely identified with an ear tattoo. The cats were received October 8, 1987 and the collars were placed on the cats on October 28, 1987. The cats used in the study weighed from 2352 to 4667 g at study initiation.

B. STUDY DESIGN:

1. Collar application:

From p. 12: "Cats judged to be suitable for testing were assigned randomly to the study in a stratified block design using a computer-generated program. The animal numbers and corresponding body weights were entered into the WIL Computer Data Management System, and a printout was generated containing the animal numbers and group assignment... The animals were arranged into study groups according to the printout. For computer entry purposes, the males were assigned numbers 1-12 and the females were assigned numbers 13-24..."

"The following figure presents the study group assignment."

Group Number	Test Material	No. of Collars/Animal	Number of cats Males	Females
1	Placebo collar	1	4	4
2	3% Dursban collar	1	4	4
3	3% Dursban collar	5	4	4

"At the initiation of the study, each cat was fitted with the appropriate number of cat flea collar(s). Test material and placebo collars remained on the animals for 91 consecutive days. During the course of the study, damaged collars were repaired as necessary."

3. Quality assurance:

On page 19 of the report there is a signed and dated Quality Assurance Unit Statement. In addition, on page 3 of the report there is a signed and dated statement that the study was conducted in compliance with EPA Good Laboratory Practice Standards.

C. METHODS AND RESULTS:

1. Observations:

From p. 13: "The animals were observed at least twice daily (once in the morning and once in the afternoon) for mortality and overt signs of toxicity. Detailed physical examinations were conducted on all cats weekly, beginning one week prior to study initiation (week 0). Following the conclusion of the study, all animals were sacrificed and discarded."

Results: From p. 15: "All animals in the study survived to study termination. No treatment-related clinical signs was observed throughout the study period. The only observations noted in cats during the treatment period were scabbing on the dorsal neck on one day in a low dose female and scabbing and hair loss around the mouth on two days in a control group male. No other remarkable observations were noted in animals throughout the pretest, treatment and recovery periods."

2. Body weights: From p. 13: "Body weights were recorded weekly and are presented for week 0 (pretest) to week 17 (study termination). Body weight changes were calculated for each corresponding interval."

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Results: Mean body weights/group (grams):

Week	Males			Females		
	Placebo collar	one collar	five collars	Placebo collar	one collar	five collars
0	3864	3797	3795	2721	2695	2774
1	3955	3881	4036	2801	2863	2734
2	3987	3977	4023	2835	2869	2871
3	4227	4144	4173	2921	2958	3059
4	4412	4291	4322	2991	3008	3128
5	4473	4389	4399	2988	3021	3135
6	4700	4599	4580	3096	3177	3270
7	4851	4677	4594	3149	3152	3321
8	4916	4741	4584	3117	3188	3352
9	4898	4708	4544	3097	3130	3291
10	4925	4809	4554	3173	3146	3289
11	4895	4847	4564	3130	3042	3228
12	4933	4835	4534	3149	3111	3277
13	4803	4755	4410	3075	3051	3195
14	4669	4680	4301	2996	2969	3128
15	4605	4694	n.a.	2965	3003	n.a.
16	4467	4595	n.a.	2875	2874	n.a.
17	4390	4587	n.a.	2869	2883	n.a.

Males wearing five collars tended to have a lower mean weight than their controls after week 3, but there was no significant difference by Dunnett's test. The mean body weights for the five-collar males were lowered by cat #9 which had the lowest weight of any males at the initiation of the study. At 14 weeks this cat was the only male weighing less than 4 kg. No significant difference (or even an indication of possible effects involving mean body weights) was observed for female groups.

There were no significant differences between groups at any time with respect to mean weekly body weight changes. A considerable amount of individual variation occurred, but no consistent exposure-related trend was evident.

3. Food consumption:

From p. 13: "Individual food consumption was recorded daily beginning one week prior to study initiation (week 0). When animals were fasted overnight prior to blood collection, a 6-day average was reported for the week.

Results:

Among males, the placebo collar (control) group tended to have higher mean weekly food consumption (both on an absolute basis and in terms of food consumed/body weight) than

either the 1 or 5-collar groups. Mean weekly food consumption for controls was significantly ($p \leq 0.05$ by Dunnett's Test) higher (both in absolute terms and on a body weight basis) with respect to both the 1 and 5-collar groups in the period from week 3 to 4, and with respect to the 5-collar group from week 5 to 6.

For the females, no significant differences between groups were observed for any weekly interval with respect to mean weekly food consumption.

4. Cholinesterase activities:

RBC and plasma ChE activities were measured with a Boehringer-Mannheim diagnostic kit, using a colorimetric method based on the work of Ellman et al. Refer to appended pages 1 and 2. Measurements were made at weeks -2, -1, 0, 1, 2, 3, 4, 8, 13 and (for controls and 1-collar animals only) 15.

In both males and females wearing 1 or 5 collars there was a dramatic (also significant, with $p \leq 0.01$ by Dunnet's Test) drop in plasma ChE activity during the first week of the study. Substantial (but not complete) recovery had taken place in the 1-collar animals two weeks after their collars were removed. No significant differences were seen at any time between groups with respect to mean RBC ChE activities.

Results:

Mean plasma ChE activities (expressed in mU/ml or milli-units/ml).

	Males			Females		
Week	Placebo collar	one collar	five collars	Placebo collar	one collar	five collars
-2	1252	1229	1454	1275	1223	1167
-1	1328	1443	1621	1372	1264	1375
0	1384	1562	1706	1319	1372	1360
1	1469	345**	284**	1501	374**	258**
2	1506	290**	199**	1466	269**	240**
3	1463	299**	196**	1387	255**	205**
4	1404	351**	199**	1354	287**	231**
8	1313	375**	193**	1293	284**	228**
13	1044	357**	185**	1100	296**	196**
15	1112	907	n.d.	1188	892	n.d.

n.d. = not done

**Significantly different from control mean at $p \leq 0.01$.

The following table gives mean plasma ChE activities from weeks 1-15 expressed as percentages of the control value:

	Males			Females		
Week	Placebo collar	one collar	five collars	Placebo collar	one collar	five collars
1	100.0	23.5	19.3	100.0	24.9	17.2
2	100.0	19.3	13.2	100.0	18.3	16.4
3	100.0	20.4	13.4	100.0	18.4	14.6
4	100.0	25.0	14.2	100.0	21.2	17.1
8	100.0	28.6	14.7	100.0	22.0	17.6
13	100.0	34.2	17.7	100.0	26.9	17.8
15	100.0	81.6	n.d.	100.0	75.1	n.d.

The percentages above were calculated from values given in the preceding table, so no statistical significance is given (presumably it would be the same as in the preceding table).

In short, the greatest mean plasma ChE depression (in terms of control activity) occurred in one-collar cats at week 2 and in five-collar cats at weeks 2-3. At maximum, mean plasma ChE depression exceeded 80% in one-collar cats and exceeded 85% in 5-collar cats.

Mean RBC ChE activities

	Males			Females		
Week	Placebo collar	one collar	five collars	Placebo collar	one collar	five collars
-2	6662	7534	7416	7114	7005	7054
-1	2652[a]	2790[a]	2476[a]	2773[a]	2943[a]	2884[a]
0	7035	6653	7067	7084	6766	6544
1	5754	6195	5141	4445	6027	5762
2	8086	7899	7783	7096	8539	6646
3	5156	6380	5320	5121	5862	5587
4	4847	5685	5499	4620	5572*	5664*
8	6127	5292	6753	6358	8821	6417
13	5699	6112	5860	5853	6274	5779
15	5800	5828	n.d.	6043	6006	n.d.

n.d. = not done
[a]Apparent dilution error; 0.1 cc instead of 0.2 cc of whole blood used in sample preparation.
*Significantly different from control mean at $p \leq 0.05$

Mean blood ChE activities

Week	Males			Females		
	Placebo collar	one collar	five collars	Placebo collar	one collar	five collars
-2	3247	3832*	3773	3539	3510	3247
-1	1814[a]	1960[a]	1989[a]	1901[a]	1901[a]	1931[a]
0	3510	3539	3920	3335	3481	3218
1	3013	2486	2135*	2545	2457	2282
2	3832	2954*	3042	3452	3042	2516*
3	2720	2428	1931**	2662	2155*	2048**
4	2545	2194	1843**	2428	2018	2077
8	3013	2048**	2574	3188	3013	2399
13	2896	2603	2574	2779	2311**	2165**
15	2896	2896	n.d.	2837	2574	n.d.

n.d. = not done
[a]Apparent dilution error; 0.1 cc instead of 0.2 cc of whole blood used in sample preparation.
*Significantly different from control mean at $p \leq 0.05$
**Significantly different from control mean at $p \leq 0.01$.

The following table gives mean whole blood ChE activities expressed as percentages of the control value in the period from weeks 1-15:

Week	Males			Females		
	Placebo collar	one collar	five collars	Placebo collar	one collar	five collars
1	100.0	82.5	70.9	100.0	96.5	89.7
2	100.0	77.1	79.4	100.0	88.1	72.9
3	100.0	89.3	71.0	100.0	80.2	76.9
4	100.0	86.2	72.4	100.0	83.1	85.5
8	100.0	68.0	85.4	100.0	94.5	75.3
13	100.0	89.9	88.9	100.0	83.2	77.9
15	100.0	100.0	n.d.	100.0	90.7	n.d.

D. DISCUSSION:

The study demonstrates that considerable (>80%) plasma ChE depression occurs in cats wearing a single collar containing 3% chlorpyrifos. The methodology used to measure ChE depression was that of Ellman et al; this procedure appears to give a considerably greater value for plasma ChE depression than the delta-pH method of Michel.

The most important observation with respect to the margin of safety associated with exposure to this collar and that at which symptoms of cholinesterase inhibition might be expected to occur is that no significant RBC ChE depression was observed, even in cats wearing five collars (it is assumed that these cats were receiving a greater exposure to chlor-

pyrifos that those with one collar, and this is consistent with the greater degree of plasma ChE depression observed in these animals). The overall whole blood ChE depression in cats wearing five collars was no worse than 30%, and this was essentially due to plasma ChE depression.

From the rate of release data submitted by the registrant (see DER I) the initial dermal dosage of chlorpyrifos is approximately 0.5 mg/kg/day in the 1-2 weeks immediately following collar application. Subsequently, the rate dropped to a "maintenance" dosage level of something like 0.1 to 0.2 mg/kg/day. This is consistent with the initial drop in plasma ChE activity during the first week, and a slow subsequent upward trend (at 13 weeks one collar males had a mean of 34.2% of the plasma ChE activity that their controls had, while females had a mean of 26.9% of that of their controls, indicating some recovery had taken place).

The plasma ChE depression observed in this study is consistent with findings in other studies involving feeding (90-day oral feeding study in rats conducted by Dow; ChE NOEL 0.1 mg/kg/day with LEL (plasma and RBC ChE depression) at 1 mg/kg/day; ChE NOEL in a 6 month rat study at 0.15 mg/kg, LEL of 0.75 mg/kg (both plasma and RBC ChE inhibition). In the two-year dog feeding study conducted by Dow the plasma ChE NOEL was 0.01 mg/kg/day, and the LEL was 0.1 mg/kg/day. The RBC ChE NOEL was 0.1 mg/kg/day and the RBC ChE LEL was 1 mg/kg/day.

The study is classified as core minimum data.

Boehringer Mannheim Diagnostics

ReagentSet™

CHOLINESTERASE

CATALOG NO. 124117

FOR THE QUANTITATIVE DETERMINATION OF CHOLINESTERASE IN SERUM, PLASMA, OR WHOLE BLOOD

Esterases which hydrolyze cholinesters at a rate faster than other esters are designated as cholinesterases. There are two major types of cholinesterases. The first type is composed of the acetylcholinesterases (known as red cell, true or specific cholinesterases) of which two isoenzymes have been found in erythrocytes and are distinguished by their different pH optima. A second group of eleven isoenzymes has been identified in serum. These enzymes are known as the nonspecific serum cholinesterases or pseudocholinesterases and have widely differing characteristics.

There are various types of assays for determining cholinesterase activities, including manometric, electrometric or titrimetric and photometric measurements.

The method described below is a convenient photometric test based on the work of Ellman et al.[1]

PRINCIPLE OF THE PROCEDURE

Acetylthiocholine $\xrightarrow{\text{cholinesterases}}$ thiocholine + acetate

Thiocholine + dithiobisnitrobenzoic acid ⟶ thionitrobenzoic acid

Acetylthiocholine can be used as substrate for the determination of the "true" acetylcholinesterase (EC 3.1.1.7) as well as for the "nonspecific" serum cholinesterases (EC. 3.1.1.8). It is split into acetate and thiocholine which reacts with dithiobisnitrobenzoic acid (Ellman's reagent) to form the yellow-colored 2-nitro-5-mercapto benzoate. Increasing color intensity is directly proportional to the cholinesterase activity and can be measured kinetically between 400-420 nm.

Abbreviations:
EDTA — Ethylenediaminetetraacetate
M — molar
mM — millimolar
mU — milliunit
nm — nanometer
U — International Unit

REAGENTS

The Cholinesterase Reagents are intended for *in vitro* diagnostic use.

1 BUFFER/CHROMOGEN

Reactive Ingredients (after reconstitution):
50 mM Phosphate buffer, pH 7.2
0.25 mM Dithiobisnitrobenzoic acid

Precautions: Exercise the normal precautions required for the handling of all laboratory reagents.

Storage: Store unopened at 2-8°C. Each vial bears the expiration date on the label

2 SUBSTRATE

Reactive Ingredient (after reconstitution):
156 mM Acetylthiocholine iodide

Precautions: Exercise the normal precautions required for the handling of all laboratory reagents.

Storage: Store unopened at 2-8°C. Each vial bears the expiration date on the label. If there is any indication that moisture has penetrated the seal, discard the vial.

Preparation of Working Reagents

Buffer/Chromogen Reagent

Dissolve the contents of one vial of Buffer/Chromogen (bottle 1) in 100 ml distilled or deionized water. This solution is stable for 6 weeks at 2-8°C. If the solution shows evidence of bacterial contamination, it should be discarded.

Substrate Reagent

Dissolve the contents of one vial of Substrate (bottle 2) with 3.0 ml distilled or deionized water. This solution is stable for 6 weeks at 2-8°C.

When prepared as directed, with specimen added, the individual assay mixture will contain the following concentrations:

48	mM	Phosphate buffer, pH 7.2
0.24	mM	Dithiobisnitrobenzoic acid
5	mM	Acetylthiocholine iodide

SPECIMEN COLLECTION AND PRESERVATION

Serum: Cholinesterase is stable in serum for several days when stored at + 4°C.[2]

Whole blood/Plasma: No inhibition of cholinesterase activity occurs with EDTA. Use of other anticoagulants is not recommended.

Whole blood: Sample preparation for whole blood cholinesterase activity is as follows:

1. Mix whole blood thoroughly by inversion.
2. Determine hematocrit value for whole blood sample.
3. Prepare whole blood hemolysate as follows:
   a. Into a clean, dry test tube, pipette 1.8 ml of distilled water.
   b. Add 0.2 ml of the whole blood sample.
   c. Mix until hemolysis is complete.
4. Determine the cholinesterase activity in the hemolysate as per assay instructions.

PROCEDURE

Materials Provided

1 Buffer/Chromogen

2 Substrate

Materials Required (but not provided)

10 ml laboratory centrifuge tubes and test tubes
Pipetting devices for measuring 3.0, 2.0, 0.2, 0.1 and 0.02 ml
Graduated laboratory cylinder for measurement of 100 ml
Laboratory centrifuge
Spectrophotometer with a wavelength capability of 405 nm
Cuvettes, recommended by the instrument manufacturer, with known lightpath
Isotonic saline
Distilled or deionized water
Materials necessary to do a hematocrit

General Comments on the Procedure

Units of enzyme activity are an expression of the conversion of substrate per minute under defined conditions (International Union of Biochemistry).[3] The product of this reaction is formed from the substrate in equimolecular amounts. The molar absorbancy of the product can be measured at 405 nm. This factor or coefficient is an established physical property. Therefore, the formation of product can be chosen as a means of measurement.

The instrument employed to measure the molar concentration of product must be calibrated to a precision of within ±2% of theoretical response to a known product concentration.

Pooled human sera or commercial control materials may be used for quality control. Precilip is recommended. Values obtained for these pools and controls with the Cholinesterase Test should fall within the limits established by the laboratory or specified by the manufacturer respectively. If such correlation is not obtained and the repetition of assay excludes errors in technique, the following steps should be taken:

1. Check wavelength setting and light source.
2. Check cleanliness of glassware, especially cuvettes.
3. Check water. Contaminants, i.e., bacterial growth, may contribute to false results.
4. Check reaction temperature using a thermometer calibrated against a certified thermometer.
5. Check the expiration date of the reagent package and the reconstituted reagents.
6. Contact Boehringer Mannheim Diagnostics' Technical Service Department in Houston, Texas.
7. DO NOT REMOVE reagent vials from kits for storage.

Method

Please read "REAGENTS", "SPECIMEN COLLECTION AND PRESERVATION", "Materials Required", and "General Comments on the Procedure" before proceeding.

Preparation of Working Reagents

For the preparation and stability of the working reagents, see the "REAGENTS" section of this insert.

Test Procedure

Wavelength: 405 nm
Temperature: Constant, between 25°C and 37°C (See Temperature Conversion Chart)
Measure against water.

1. Pipette into test tube:

Buffer/Chromogen Reagent	3.00 ml
Substrate Reagent	0.10 ml

2. Bring mixture to reaction temperature.
3. Add:

Serum, plasma, or hemolysate	0.02 ml

4. Mix well by gentle inversion, transfer reaction mixture immediately into cuvette and measure.

Read absorbance (A) and record the change per minute. From this determine the mean ΔA/min. and use it for calculation. Recording a minimum of 3 absorbance changes is recommended to ensure linearity.

RESULTS

An international enzyme unit per liter (U/l) is defined as the activity of enzyme which converts 1 µmole/l of substrate in 1 minute at standard conditions.

Cholinesterase in serum or plasma:

A factor for calculating enzyme activity is derived by applying the following formula:

007150

$$\frac{\Delta A/\text{min.} \times \text{total assay volume (ml)} \times 1000}{\text{abs. coeff.} \times \text{lightpath (cm)} \times \text{spec. vol. (ml)}} = \text{mU/ml specimen}$$

Δ = Change
A = Absorbance
1000 = Factor for conversion of units/ml to milliunits/ml
abs. coeff. = Absorbancy coefficient = 13.3 $cm^2/\mu mole$ thionitrobenzoic acid at 405 nm

Therefore, the calculation for cholinesterase activity in serum or plasma, with a lightpath of 1 cm, becomes:

at 405 nm: $$\frac{\Delta A/\text{min.} \times 3.12 \times 1000}{13.3 \times 1\text{ cm} \times 0.02\text{ ml}} = \Delta A/\text{min.} \times 11700 = \text{mU/ml specimen}$$

Table of values for serum or plasma at 405 nm:

ΔA/min.	mU/ml	ΔA/min.	mU/ml
0.010	117	0.110	1287
0.020	234	0.120	1404
0.030	351	0.130	1521
0.040	468	0.140	1638
0.050	585	0.150	1755
0.060	702	0.160	1872
0.070	819	0.170	1989
0.080	936	0.180	2106
0.090	1053	0.190	2223
0.100	1170	0.200	2340

Cholinesterase in whole blood:
The preparation of the hemolysate constitutes a 10 fold dilution of the whole blood, therefore the calculation factor for cholinesterase activity in whole blood is 10 times the plasma factor.

Therefore, the calculation for cholinesterase activity in whole blood, with a lightpath of 1 cm, becomes:

at 405 nm: $$\frac{\Delta A/\text{min.} \times 3.12\text{ ml} \times 1000 \times 10}{13.3 \times 1\text{ cm} \times 0.02\text{ ml}} = \Delta A/\text{min.} \times 117000 = \text{mU/ml specimen}$$

Calculation of erythrocyte (RBC) activity.

After plasma (Pl) and whole blood (WB) activities have been determined, erythrocyte (RBC) activity is calculated as follows; since the compartmentilization of cholinesterase is given by the equation:

$$W.B. = (R.B.C. \times Hct.^*) + [Pl \times (1 - Hct.^*)]$$

solving this equation for RBC we obtain

$$RBC = \frac{WB - [Pl. \times (1 - Hct.^*)]}{Hct.^*}$$

*Hematocrit is expressed as decimal equivalent, e.g., 44% = 0.44.

Temperature Conversion:
To convert into values for 25°C, multiply results by the factor for the actual reaction temperature.

Temp.	Factor	Temp.	Factor
25°C	1.00	32°C	0.72
26°C	0.95	33°C	0.69
27°C	0.90	34°C	0.66
28°C	0.86	35°C	0.64
29°C	0.82	36°C	0.62
30°C	0.78	37°C	0.60
31°C	0.75		

If, while operating at 25°C, it is necessary to convert values obtained to values corresponding to another temperature, divide the 25°C values by the factor for the desired temperature.

Illustrative Calculation:
At 405 nm and 25°C, the following data were recorded:

Time	Absorbance	ΔA/min.
0 min.	0.522	—
1 min.	0.675	0.153
2 min.	0.828	0.153
3 min.	0.980	0.152
Mean ΔA/min.		0.153

Applying the factor derived from the above formula:
0.153 × 11700 = 1790 mU/ml specimen at 25°C.

Should it be necessary to convert this value to a value which would be obtained at 30°C, the following calculation, based on the temperature conversion chart, would apply:

$$\frac{1790\text{ mU/ml}}{0.78} = 2295\text{ mU/ml serum at 30°C.}$$

LIMITATIONS OF THE PROCEDURE

ΔA/min. greater than 0.400 (at 405 nm) indicates very high activity in the specimen and can result in nonlinear readings. In such cases, dilute specimen 1 + 4 with isotonic saline and repeat assay using 0.02 ml of this dilution. Multiply final result by 5.

If measurements of cholinesterase activity cannot be made at 405 nm or if the instrument has a "bandwidth" greater than 10 nm, the absorbancy coefficient differs from the theoretical value. Calculation must then be based on a calibration curve prepared with a cysteine solution. Preweighed cysteine (Cat. No. 125431) and instructions are available from Boehringer Mannheim Diagnostics.

EXPECTED VALUES

25°C

Whole blood	3600 - 5500 mU/ml
Plasma	1000 - 3500 mU/ml
Serum[4]	1900 - 3800 mU/ml

Calculated RBC activity 6,700-10,000 mU/ml

Since the expected value or "normal" ranges, in general, are affected by age, sex, diet, geographical location, and other factors, each laboratory should establish its own "normal" range based upon the specific population encountered in the daily course of laboratory operation.

SPECIFIC PERFORMANCE CHARACTERISTICS

Sensitivity: In most cases, the sensitivity of a manual enzymatic assay is limited by a change of 0.001 absorbance units/30 seconds. Under assay conditions, this corresponds to 23.5 mU/ml specimen (405 nm). It is recommended, however, that each laboratory establish its own range of sensitivity by evaluating the smallest possible differentiation of absorbance changes per time unit.

Specificity: Refer to the "SPECIMEN COLLECTION AND PRESERVATION" and "LIMITATIONS OF THE PROCEDURE" sections of this insert.

Precision:
Serum: Employing the method as described above, using precision pipettes and precision instruments, standard deviations of ± 50 at the normal and of ± 10 at the pathologic levels were found. The day-to-day repetition resulted in CV's of 2.0% at normal and of 2.0% at abnormal levels.

Whole blood:

Within Run	I	II	III
x̄	4165	7348	10,553
SD	148	164	776
CV	3.6	2.2	7.3
n	10	10	10

Day to Day	I	II	III
x̄	4226	6869	10,654
SD	286	577	780
CV	6.77	8.37	7.32
n	34	34	34

Accuracy: Recovery of purified acetylcholinesterase added to a normal specimen with known levels was 99%. Linear correlation of increasing levels of added activities was obtained.

Method Comparison: A comparison of the package reagents with the assay method described by Ellman[1] resulted in a linear regression equation of Y = 0.987X + 0.24 and a correlation coefficient of 0.995.

BIBLIOGRAPHY

1. Ellman, G. L., et al. *Biochem. Pharmacol.* 7, 88 (1961).
2. Pilz, W. *Methods of Enzymatic Analysis*, 2nd English Edition, H. U. Bergmeyer, ed., Academic Press, Inc., New York (1974), p. 845.
3. Dybkaer, R., and Jorgensen, K. *Quantities and Units in Clinical Chemistry*, Munkagaard, Copenhagen (1967).
4. Weber, H. *Dtsch. med. Wschr.* Vol. 91, (1966), p. 1927.

ITEMS

	CATALOG NO.
ReagentSet Cholinesterase (Includes: 1 Buffer/Chromogen, 750 mg; and 2 Substrate, 140 mg)	124117
Precilip, 20 x 3 ml (dried)	125067
Cysteine Calibrator, 420 mg	125431

Boehringer Mannheim Diagnostics, Inc.
7800 Westpark
Houston, Texas 77063

Revised November, 1981

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Reviewed by: Byron T. Backus Byron T. Backus 7/24/89
Section 2, HFASB (H7509C)

Secondary Reviewer: K. Clark Swentzel K. Clark Swentzel 4/27/89
Section 2, HFASB (H7509C)

DATA EVALUATION REPORT III

STUDY TYPE: Cholinesterase - collar exposure, cat TOX CHEM NO. 219AA

MRID NO: ACC. NO: 406031-02

TEST MATERIAL: Collar with 3% Chlorpyrifos

SYNONYMS: Flealine Flea and Tick Collar

STUDY NUMBER(S): WIL-52012

SPONSOR: Happy Jack^{R} Inc.
P.O. Box 475
Highway 258 South
Snow Hill, NC 28580

TESTING FACILITY: Wil Research Laboratories, Inc.
Ashland, Ohio 44805-9281

TITLE OF REPORT: Range-Finding Flea Collar Cholinesterase Study in Cats

AUTHOR(S): Tompkins, E. C.

REPORT ISSUED: 12/9/87

CLASSIFICATION: Core Supplementary Data

CONCLUSIONS:

1. The findings of this preliminary study (involving exposure of groups with one cat/sex/dose level) are essentially the same as those of the subsequent cat collar study using four cats/sex/dose level. There was substantial plasma ChE depression (generally greater than 70%) associated with exposure to one collar, and even greater plasma ChE depression (>80%) as a result of exposure to 5 collars. As in the subsequent cat collar study, there was no indication of any RBC ChE depression.

2. The procedure used to measure ChE activity was a modification of the colorimetric method of Ellman et al; this method appears to be considerably more sensitive (and gives greater

values for plasma ChE depression) than the delta-pH method of Michel.

3. For the cats wearing one collar, there was considerable (although not complete) recovery of plasma ChE activity in the one week period following removal of the collar (at week 3). Plasma ChE activity recovered to 63.7% of the week 0 level in the male and to 60.9% in the female.

A. MATERIALS:

1. Test compound: Cat Flea Collar, identified as containing 3% Dursban (Chlorpyrifos). According to information received from the registrant, the individual collars weighed 0.46 oz (= 13 grams); collars were trimmed to fit the individual cats.

2. Animals used: Domestic short hair cats, approximately 8-12 months old, received from Liberty Laboratories, Liberty Corner, New Jersey. Each animal was uniquely identified with an ear tattoo. The cats were received July 30, 1987, and the collars were placed on the cats on August 26, 1987. The cats used in this study weighed from 2528 to 4762 grams at study initiation.

B. STUDY DESIGN:

1. Collar application:

From p. 11: "Cats judged to be suitable for testing were assigned randomly to the study in a stratified block design using a computer-generated program. The animal numbers and corresponding body weights were entered into the WIL Computer Data Management System, and a printout was generated containing the animal numbers and group assignment... The animals were arranged into study groups according to the printout. For computer entry purposes, the males were assigned numbers 1-3 and the females were assigned numbers 4-6..."

"The following figure presents the study group assignment."

Group Number	Test Material	No. of Collars/Animal	Number of cats Males	Females
1	Placebo collar	1	1	1
2	3% Dursban collar	1	1	1
3	3% Dursban collar	5	1	1

"At the initiation of the study, each cat was fitted with the appropriate number of cat flea collar(s). Test material and placebo collars remained on the animals for 21 consecutive days. During the course of the study, damaged collars were repaired as necessary."

3. Quality assurance:

On page 17 of the report there is a signed and dated Quality Assurance Statement. In addition, on page 3 of the report there is a signed and dated statement that the study was conducted in compliance with EPA Good Laboratory Practice Standards.

C. METHODS AND RESULTS:

1. Observations:

From p. 12: "The animals were observed at least twice daily (once in the morning and once in the afternoon) for mortality and overt signs of toxicity. Detailed physical examinations were conducted on all cats weekly, beginning one week prior to study initiation (week 0). Following the conclusion of the study, all animals were sacrificed and discarded."

Results: From p. 14: "All animals in the study survived to study termination. The only remarkable observations noted in cats during the treatment period were mucoid feces on one day in the high dose male and scabbing around the mouth on two days in the control group female. No other remarkable observations were noted in animals throughout the treatment and recovery periods."

2. Body weights and food consumption: "Weekly body weights, body weight gains and food consumption in the treated groups were comparable to the pretest (week 0) and control group values throughout the study. Differences observed between animals were attributed to individual variation and did not indicate a relationship to treatment."

3. Cholinesterase activities:

RBC and plasma ChE activities were measured with a Boehringer-Mannheim diagnostic kit, using a colorimetric method based on the work of Ellman et al. Refer to appended pages 1 and 2. Measurements were made at weeks -1, 0, 1, 2, 3, and (for controls and 1-collar cats only) 4.

Results:

In the males and females wearing 1 or 5 collars there was a dramatic drop in plasma ChE activity during the first week of exposure to the collar. Substantial (but not complete) recovery occurred in the one collar animals one week after their collars were removed. No significant differences or indications of a possible effect were seen at any time between groups with respect to mean RBC ChE activities.

Results:

Mean plasma ChE activities (expressed in mU/ml or milli-units/ml).

	Males			Females		
Week	Placebo collar	one collar	five collars	Placebo collar	one collar	five collars
-1	1427	1977	1556	1217	1580	1463
0	1556	2129	1743	1240	1708	1474
1	1603	644	257	1088	445	304
2	1708	515	234	1240	421	316
3	1778	281	211	1240	421	281
4	1720	1357	n.d.	1123	1041	n.d.

n.d. = not done

D. **DISCUSSION:**

This study was conducted as a preliminary to the 13-week cat collar ChE study conducted at the same facility. The major findings (considerable plasma ChE inhibition, but no effect on RBC ChE, with a considerable amount of plasma ChE activity recovery in the one week period after the collar removal) in cats exposed to the 3% chlorpyrifos collar were essentially the same as those in the 13-week study.

Because of the relatively short duration of the study (5 weeks) and the low numbers of animals used (1 per sex per dose level) the study is classified as core supplementary data.

Boehringer Mannheim Diagnostics

ReagentSet™
CHOLINESTERASE
CATALOG NO. 124117

FOR THE QUANTITATIVE DETERMINATION OF CHOLINESTERASE IN SERUM, PLASMA, OR WHOLE BLOOD

Esterases which hydrolyze cholinesters at a rate faster than other esters are designated as cholinesterases. There are two major types of cholinesterases. The first type is composed of the acetylcholinesterases (known as red cell, true or specific cholinesterases) of which two isoenzymes have been found in erythrocytes and are distinguished by their different pH optima. A second group of eleven isoenzymes has been identified in serum. These enzymes are known as the nonspecific serum cholinesterases or pseudocholinesterases and have widely differing characteristics.

There are various types of assays for determining cholinesterase activities, including manometric, electrometric or titrimetric and photometric measurements.

The method described below is a convenient photometric test based on the work of Ellman et al.[1]

PRINCIPLE OF THE PROCEDURE

Acetylthiocholine $\xrightarrow{\text{cholinesterases}}$ thiocholine + acetate

Thiocholine + dithiobisnitrobenzoic acid $\longrightarrow$ thionitrobenzoic acid

Acetylthiocholine can be used as substrate for the determination of the "true" acetylcholinesterase (EC 3.1.1.7) as well as for the "nonspecific" serum cholinesterases (EC. 3.1.1.8). It is split into acetate and thiocholine which reacts with dithiobisnitrobenzoic acid (Ellman's reagent) to form the yellow-colored 2-nitro-5-mercapto benzoate. Increasing color intensity is directly proportional to the cholinesterase activity and can be measured kinetically between 400-420 nm.

Abbreviations:
EDTA — Ethylenediaminetetraacetate
M — molar
mM — millimolar
mU — milliunit
nm — nanometer
U — International Unit

REAGENTS

The Cholinesterase Reagents are intended for *in vitro* diagnostic use.

1 BUFFER/CHROMOGEN

Reactive Ingredients (after reconstitution):
50 mM Phosphate buffer, pH 7.2
0.25 mM Dithiobisnitrobenzoic acid

Precautions: Exercise the normal precautions required for the handling of all laboratory reagents.

Storage: Store unopened at 2-8°C. Each vial bears the expiration date on the label.

2 SUBSTRATE

Reactive Ingredient (after reconstitution):
156 mM Acetylthiocholine iodide

Precautions: Exercise the normal precautions required for the handling of all laboratory reagents.

Storage: Store unopened at 2-8°C. Each vial bears the expiration date on the label. If there is any indication that moisture has penetrated the seal, discard the vial.

Preparation of Working Reagents

Buffer/Chromogen Reagent

Dissolve the contents of one vial of Buffer/Chromogen (bottle 1) in 100 ml distilled or deionized water. This solution is stable for 6 weeks at 2-8°C. If the solution shows evidence of bacterial contamination, it should be discarded.

Substrate Reagent

Dissolve the contents of one vial of Substrate (bottle 2) with 3.0 ml distilled or deionized water. This solution is stable for 6 weeks at 2-8°C.

When prepared as directed, with specimen added, the individual assay mixture will contain the following concentrations:

48 mM Phosphate buffer, pH 7.2
0.24 mM Dithiobisnitrobenzoic acid
5 mM Acetylthiocholine iodide

SPECIMEN COLLECTION AND PRESERVATION

Serum: Cholinesterase is stable in serum for several days when stored at + 4°C.[2]

Whole blood/Plasma: No inhibition of cholinesterase activity occurs with EDTA. Use of other anticoagulants is not recommended.

Whole blood: Sample preparation for whole blood cholinesterase activity is as follows:

1. Mix whole blood thoroughly by inversion.
2. Determine hematocrit value for whole blood sample.
3. Prepare whole blood hemolysate as follows:
   a. Into a clean, dry test tube, pipette 1.8 ml of distilled water.
   b. Add 0.2 ml of the whole blood sample.
   c. Mix until hemolysis is complete.
4. Determine the cholinesterase activity in the hemolysate as per assay instructions.

PROCEDURE

Materials Provided

1 Buffer/Chromogen
2 Substrate

Materials Required (but not provided)

10 ml laboratory centrifuge tubes and test tubes
Pipetting devices for measuring 3.0, 2.0, 0.2, 0.1 and 0.02 ml
Graduated laboratory cylinder for measurement of 100 ml
Laboratory centrifuge
Spectrophotometer with a wavelength capability of 405 nm
Cuvettes, recommended by the instrument manufacturer, with known lightpath
Isotonic saline
Distilled or deionized water
Materials necessary to do a hematocrit

General Comments on the Procedure

Units of enzyme activity are an expression of the conversion of substrate per minute under defined conditions (International Union of Biochemistry).[3] The product of this reaction is formed from the substrate in equimolecular amounts. The molar absorbancy of the product can be measured at 405 nm. This factor or coefficient is an established physical property. Therefore, the formation of product can be chosen as a means of measurement.

The instrument employed to measure the molar concentration of product must be calibrated to a precision of within ±2% of theoretical response to a known product concentration.

Pooled human sera or commercial control materials may be used for quality control. Precilip is recommended. Values obtained for these pools and controls with the Cholinesterase Test should fall within the limits established by the laboratory or specified by the manufacturer respectively. If such correlation is not obtained and the repetition of assay excludes errors in technique, the following steps should be taken:

1. Check wavelength setting and light source.
2. Check cleanliness of glassware, especially cuvettes.
3. Check water. Contaminants, i.e., bacterial growth, may contribute to false results.
4. Check reaction temperature using a thermometer calibrated against a certified thermometer.
5. Check the expiration date of the reagent package and the reconstituted reagents.
6. Contact Boehringer Mannheim Diagnostics' Technical Service Department in Houston, Texas.
7. DO NOT REMOVE reagent vials from kits for storage.

Method

Please read "REAGENTS", "SPECIMEN COLLECTION AND PRESERVATION", "Materials Required", and "General Comments on the Procedure" before proceeding.

Preparation of Working Reagents

For the preparation and stability of the working reagents, see the "REAGENTS" section of this insert.

Test Procedure

Wavelength: 405 nm
Temperature: Constant, between 25°C and 37°C (See Temperature Conversion Chart)
Measure against water.

1. Pipette into test tube:	
Buffer/Chromogen Reagent	3.00 ml
Substrate Reagent	0.10 ml
2. Bring mixture to reaction temperature.	
3. Add:	
Serum, plasma, or hemolysate	0.02 ml
4. Mix well by gentle inversion, transfer reaction mixture immediately into cuvette and measure.	

Read absorbance (A) and record the change per minute. From this determine the mean ΔA/min. and use it for calculation. Recording a minimum of 3 absorbance changes is recommended to ensure linearity.

RESULTS

An international enzyme unit per liter (U/l) is defined as the activity of enzyme which converts 1 µmole/l of substrate in 1 minute at standard conditions.

Cholinesterase in serum or plasma:

A factor for calculating enzyme activity is derived by applying the following formula:

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$$\frac{\Delta A/\text{min.} \times \text{total assay volume (ml)} \times 1000}{\text{abs. coeff.} \times \text{lightpath (cm)} \times \text{spec. vol. (ml)}} = \text{mU/ml specimen}$$

Δ = Change
A = Absorbance
1000 = Factor for conversion of units/ml to milliunits/ml
abs. coeff. = Absorbancy coefficient = 13.3 $cm^2/\mu mole$ thionitrobenzoic acid at 405 nm

Therefore, the calculation for cholinesterase activity in serum or plasma, with a lightpath of 1 cm, becomes:

at 405 nm: $\frac{\Delta A/\text{min.} \times 3.12 \times 1000}{13.3 \times 1\text{ cm} \times 0.02\text{ ml}} = \Delta A/\text{min.} \times 11700 = \text{mU/ml specimen}$

Table of values for serum or plasma at 405 nm:

ΔA/min.	mU/ml	ΔA/min.	mU/ml
0.010	117	0.110	1287
0.020	234	0.120	1404
0.030	351	0.130	1521
0.040	468	0.140	1638
0.050	585	0.150	1755
0.060	702	0.160	1872
0.070	819	0.170	1989
0.080	936	0.180	2106
0.090	1053	0.190	2223
0.100	1170	0.200	2340

Cholinesterase in whole blood:
The dilution of the hemolysate constitutes a 10 fold dilution of the whole blood, therefore the calculation factor for cholinesterase activity in whole blood is 10 times the plasma factor.

Therefore, the calculation for cholinesterase activity in whole blood, with a lightpath of 1 cm, becomes:

at 405 nm: $\frac{\Delta A/\text{min.} \times 3.12\text{ ml} \times 1000 \times 10}{13.3 \times 1\text{ cm} \times 0.02\text{ ml}} = \Delta A/\text{min.} \times 117000 = \text{mU/ml specimen}$

Calculation of erythrocyte (RBC) activity.

After plasma (Pl) and whole blood (WB) activities have been determined, erythrocyte (RBC) activity is calculated as follows; since the compartmentilization of cholinesterase is given by the equation:

$$W.B. = (R.B.C. \times Hct.^*) + [Pl \times (1 - Hct.^*)]$$

solving this equation for RBC we obtain

$$RBC = \frac{WB - [Pl. \times (1 - Hct.^*)]}{Hct.^*}$$

*Hematocrit is expressed as decimal equivalent, e.g., 44% = 0.44.

Temperature Conversion:

To convert into values for 25°C, multiply results by the factor for the actual reaction temperature.

Temp.	Factor	Temp.	Factor
25°C	1.00	32°C	0.72
26°C	0.96	33°C	0.69
27°C	0.92	34°C	0.66
28°C	0.88	35°C	0.64
29°C	0.85	36°C	0.62
30°C	0.82	37°C	0.60
31°C	0.78		

If, while operating at 25°C, it is necessary to convert values obtained to values corresponding to another temperature, divide the 25°C values by the factor for the desired temperature.

Illustrative Calculation:

At 405 nm and 25°C, the following data were recorded:

Time	Absorbance	ΔA/min.
0 min.	0.522	—
1 min.	0.675	0.153
2 min.	0.828	0.153
3 min.	0.980	0.152
Mean ΔA/min.		0.153

Applying the factor derived from the above formula:
0.153 × 11700 = 1790 mU/ml specimen at 25°C.

Should it be necessary to convert this value to a value which would be obtained at 30°C, the following calculation, based on the temperature conversion chart, would apply:

$$\frac{1790\text{ mU/ml}}{0.78} = 2295\text{ mU/ml serum at 30°C.}$$

LIMITATIONS OF THE PROCEDURE

ΔA/min. greater than 0.400 (at 405 nm) indicates very high activity in the specimen and can result in nonlinear readings. In such cases, dilute specimen 1 + 4 with isotonic saline and repeat assay using 0.02 ml of this dilution. Multiply final result by 5.

If measurements of cholinesterase activity cannot be made at 405 nm or if the instrument has a "bandwidth" greater than 10 nm, the absorbancy coefficient differs from the theoretical value. Calculation must then be based on a calibration curve prepared with a cysteine solution. Prewelghed cysteine (Cat. No. 125431) and instructions are available from Boehringer Mannheim Diagnostics.

EXPECTED VALUES

25°C

Whole blood	3800 - 5500 mU/ml
Plasma	1000 - 3500 mU/ml
Serum[4]	1900 - 3800 mU/ml

Calculated RBC activity 6,700-10,000 mU/ml

Since the expected value or "normal" ranges, in general, are affected by age, sex, diet, geographical location, and other factors, each laboratory should establish its own "normal" range based upon the specific population encountered in the daily course of laboratory operation.

SPECIFIC PERFORMANCE CHARACTERISTICS

Sensitivity: In most cases, the sensitivity of a manual enzymatic assay is limited by a change of 0.001 absorbance units/30 seconds. Under assay conditions, this corresponds to 23.5 mU/ml specimen (405 nm). It is recommended, however, that each laboratory establish its own range of sensitivity by evaluating the smallest possible differentiation of absorbance changes per time unit.

Specificity: Refer to the "SPECIMEN COLLECTION AND PRESERVATION" and "LIMITATIONS OF THE PROCEDURE" sections of this insert.

Precision:

Serum: Employing the method as described above, using precision pipettes and precision instruments, standard deviations of ± 50 at the normal and of ± 10 at the pathologic levels were found. The day-to-day repetition resulted in CV's of 2.0% at normal and of 2.0% at abnormal levels.

Whole blood:

Within Run	I	II	III
x̄	4165	7348	10,553
SD	148	164	778
CV	3.6	2.2	7.3
n	10	10	10

Day to Day	I	II	III
x̄	4226	8889	10,654
SD	298	577	780
CV	6.77	6.37	7.32
n	34	34	34

Accuracy: Recovery of purified acetylcholinesterase added to a normal specimen with known levels was 99%. Linear correlation of increasing levels of added activities was obtained.

Method Comparison: A comparison of the package reagents with the assay method described by Ellman[1] resulted in a linear regression equation of Y = 0.987X + 0.24 and a correlation coefficient of 0.995.

BIBLIOGRAPHY

1. Ellman, G. L., et al. *Biochem. Pharmacol.* 7, 88 (1961).
2. Pilz, W. *Methods of Enzymatic Analysis*, 2nd English Edition, H. U. Bergmeyer, ed., Academic Press, Inc., New York (1974), p. 845.
3. Dybkaer, R., and Jorgensen, K. *Quantities and Units in Clinical Chemistry*, Munkegaard, Copenhagen (1987).
4. Weber, H. *Dtsch. med. Wschr.* Vol. 91, (1966), p. 1927.

ITEMS

	CATALOG NO.
ReagentSet Cholinesterase (Includes: 1 Buffer/Chromogen, 750 mg; and 2 Substrate, 140 mg)	124117
Precilip, 20 x 3 ml (dried)	125067
Cysteine Calibrator, 420 mg	125431

Boehringer Mannheim Diagnostics, Inc.
7030 Westpark
Houston, Texas 77063

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Collar rate of release; Fred W. Knapp; no number; 4/1/88	cat collar with 3% chlorpyrifos	406031-04	Mean rates of release from cat collars: 1 week: 0.4 mg/kg/day; 2 weeks: 0.486 mg/kg/day; 6 weeks: 0.179 mg/kg/day; 13 weeks: 0.214 mg/kg/day.		Acceptable
13-week cholinesterase study from collar exposure-cat; Wil Research Labs; WIL-52013; 4/12/88	cat collar with 3% chlorpyrifos	406031-03	Exposure levels: 0 (placebo), 1 and 5 collars with 4 cats/sex/level. Method of Ellman et al. used to measure ChE activities. Substantial (>80%) plasma ChE depression occurs in cat. wearing a single 3% collar; no indication of RBC ChE depression or symptoms of ChE inhibition were noted, even in cats wearing five collars. Substantial (to 60-80% normal activity) plasma ChE recovery occurred in cats which had worn one collar in the two week period after collar removal at week 13.		Core Minimum
Preliminary (3 week collar exposure) cholinesterase study-cat; Wil Research Labs; WIL-52012; 12/9/87	cat collar with 3% chlorpyrifos	406031-02	Preliminary study with 1 cat/sex/exposure level. Substantial plasma ChE depression (>70%) associated with exposure to one collar, and even greater plasma ChE depression (>80%) from exposure to five collars. No indication of RBC ChE depression or symptoms of ChE inhibition even in cats wearing five collars. Considerable (to 60-65% normal activity) recovery of plasma ChE occurred in the period from week 3 (when collars were removed) to week 4.		Core Supplementary