Statistical Approach to Evaluating Effect of Physical and Chemical Factors on Fecal Excretion of Chlorophenothane

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Abstract ☐ A study using ^{14}C-labeled chlorophenothane was statistically designed to evaluate the effect of five treatments (cold, fasting, levarterenol bitartrate, prediphenylhydantoin, and post-diphenylhydantoin) upon the fecal elimination of the pesticide from rats. To 24-hr.-fasted rats, 7 μc. of ^{14}C-chlorophenothane was administered intraperitoneally. The pesticide was allowed to distribute into the tissues of the animals for 5 days prior to initiation of the treatments which were continued for 3 days. The cold group was kept at 2-4°. The fasted rats were deprived of food but had water *ad libitum*. Levarterenol bitartrate and diphenylhydantoin were administered twice a day at 1.0 and 75.0 mg./kg. body weight, respectively. Diphenylhydantoin was administered before chlorophenothane (prediphenylhydantoin) to one group of rats and after administration of the pesticide (postdiphenylhydantoin) to a second group. The ^{14}C-activity in the feces was measured by liquid scintillation. Thirty-six rats were assigned in a randomized complete block design of three replicates with two rats per treatment, five treatments, and a control per replicate. Homogeneity of variance tests indicated that the variances between rats were homogeneous. Two-way analysis of variance tests showed that replicates could be pooled and the data reanalyzed as if a completely randomized design were run. Hence, a one-way analysis of variance and the Newman-Keuls sequential range tests were performed. The latter tested for significant differences between all pairs of treatments. The statistical design allowed for minimum expenditure of animals and radioisotope and provided reliable data. Cold treatment was the only treatment that increased the fecal elimination of chlorophenothane. Preadministration of diphenylhydantoin may have stimulated hepatic enzymes to alter deposition and increase apparent elimination of chlorophenothane.

Keyphrases ☐ ^{14}C-Chlorophenothane fecal excretion—effects of cold, fasting, and levarterenol bitartrate and diphenylhydantoin administration, statistical analysis, rats ☐ Pesticide removal from tissues—effects of cold, fasting, and levarterenol bitartrate and diphenylhydantoin administration on ^{14}C-chlorophenothane fecal excretion, rats ☐ Excretion, fecal, ^{14}C-chlorophenothane—effects of cold, fasting, and levarterenol bitartrate and diphenylhydantoin administration, rats

Although some countries and several states in the United States have banned the use of chlorophenothane[1] (1), the fact remains that the pesticide has been with us for more than 30 years and is still with us today. For most persons, residues in food represent the greatest source (90%) of exposure to persistent pesticides (2–5). Therefore, recent research activity on the accelerated removal of pesticides from animal and poultry products has increased (6). Many workers demonstrated the extensive accumulation of chlorophenothane in the fat depots of the body (7–20). The chlorophenothane concentrations found in adipose tissue were approximately 10 times those of liver and 100 times the concentration found in the kidney, gonads, and brain (21). The storage of chlorophenothane in body lipids was suggested as an effective detoxifying mechanism for

such a lipophilic insecticide (22). However, this adipose tissue storage was nonpermanent, especially during accelerated lipid mobilization induced by starvation or increased energy expenditure due to exposure to cold (17, 22–26). Mobilization of fat eliminated some lipid-soluble pesticide from animals, and the remaining residues were further diluted as depot fat was replaced. Induction of drug-metabolizing enzymes of the liver microsomes involved in the metabolic degradation of many lipoid-soluble chemicals was another approach recommended for increasing the rate of removal of persistent pesticides from animals (6). The anticonvulsant drugs, specifically diphenylhydantoin and phenobarbital, are potent inducers of drug-metabolizing enzymes in the rat as well as man (1, 27–32).

The objectives of this work were to: (*a*) study the effect of some physical (cold and fasting) and chemical (levarterenol bitartrate and diphenylhydantoin) treatments on the fecal excretion of ^{14}C-chlorophenothane, and (*b*) find a treatment that would quickly deplete the residues from rats following a measured exposure to the pesticide. Cold, fasting, and endogenous norepinephrine (and the comparable drug entity, levarterenol bitartrate) stimulate the mobilization of fatty tissues (17, 19, 23, 25, 26), the main storage site of chlorophenothane. On the other hand, diphenylhydantoin stimulates certain hepatic microsomal enzymes that are involved in the breakdown of chlorophenothane (1, 27). A simple and efficient statistical design was used to minimize expenditure of animals, radioisotopes, and human efforts and to evaluate the treatment effects, replicability, and the interaction between replicates and treatment factors. For these purposes, randomized complete block and completely randomized designs (33) were applied.

Previous results (34) regarding the respiratory excretion of ^{14}C-chlorophenothane by rats indicated negligible (0.007% of the administered activity) ^{14}CO$_2$ in the exhaled air after 24 hr. The cumulative ^{14}C urinary and fecal excretion of the same animals after 5 days were 1.8 and 12.4%, respectively, of the administered dose. Therefore, it was decided to study the fecal radioactivity as a reflector of the influence of the defined treatments on the elimination of chlorophenothane.

EXPERIMENTAL

^{14}C-Chlorophenothane Dosing Solution—The radiochemical purity of ^{14}C-chlorophenothane (*p,p'*-DDT), ring uniformly labeled[2] (specific activity 3.85 mc./mmole), was greater than 98.7%, as shown by TLC, autoradiography, and liquid scintillation counting techniques. The radiolabeled pesticide was dissolved in peanut oil.

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[1] 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT).

[2] Mallinckrodt-Nuclear, St. Louis, Mo.

Each milliliter of this solution contained 12.9 μc. of ^{14}C and 20 mg. of chlorophenothane. The administered radioactivity ranged from 6.78 to 7.32 μc./rat.

Treatments—The physical treatments investigated were cold and fasting. The cold was provided by a walk-in environmental room[3] maintained at 2–4°. Animals exposed to this treatment are referred to as the "cold group." The fasted rats were deprived of food, but water was allowed *ad libitum*. This group is referred to as the "fasted group." The chemical treatments evaluated were diphenylhydantoin[4] and levarterenol bitartrate[5]. The rats were treated with diphenylhydantoin either prior to administration of chlorophenothane (prediphenylhydantoin group) or after receiving the pesticide (postdiphenylhydantoin group). The dosage (30, 31) of diphenylhydantoin was 75 mg./kg. body weight i.p. twice a day. The animals receiving levarterenol bitartrate are referred to as the levarterenol group. The dosage (23) of levarterenol bitartrate was 1 mg./kg. body weight s.c. twice a day. The doses of diphenylhydantoin and levarterenol bitartrate were calculated as the base of the drug, and the necessary corrections were made to account for the difference due to the salts (sodium diphenylhydantoin and levarterenol bitartrate hydrate). The amount of diphenylhydantoin or levarterenol bitartrate administered to the animals was calculated individually, depending upon the rat body weight. The radioactivity in the feces was measured as a reflector of chlorophenothane excretion.

Thirty-six male Sprague–Dawley strain descendent rats[6], weighing 180–210 g. at the beginning of the experiment, were acclimated to laboratory conditions for 2 days. Each animal was kept in a 20 × 11.5-cm. stainless steel metabolism cage[7]. The rats were assigned to the six treatments in a randomized complete block design (33) of three replicates with two rats per treatment. The six treatments included control animals receiving only ^{14}C-chlorophenothane and the cold, fasted, prediphenylhydantoin, postdiphenylhydantoin and levarterenol bitartrate treatments previously defined. All groups of the first replicate, except prediphenylhydantoin, were fasted for 24 hr. prior to intraperitoneal dosing (on Day 1) with ^{14}C-chlorophenothane. The pesticide was allowed to distribute into the tissues of the animals for 5 days prior to initiation of treatments. On Day 6 following administration of the ^{14}C-chlorophenothane, each group was subjected to the assigned treatment, which was continued for 3 days. On Day 9 after dosing with the pesticide, the animals were sacrificed by decapitation using a small guillotine[8]. The prediphenylhydantoin group received diphenylhydantoin treatment for 3 days prior to the ^{14}C-chlorophenothane administration and then followed the same scheme as the other groups. The rats of the prediphenylhydantoin group were also fasted 24 hr. prior to dosing with the pesticide. The other two replicates were started on different days to allow subsequent replicate evaluation. The feces were collected daily, weighed, and stored frozen until the day of analysis.

Radioanalysis—The ^{14}C-radioactivity in all fecal samples was measured with an internal liquid scintillation spectrometer[9] equipped with bialkali phototubes. The scintillation solution consisted of 0.4% 2,5-diphenyloxazole in an equal volume of toluene and 2-ethoxyethanol. The percent counting efficiency of each sample was determined by adding ^{14}C-toluene internal standard and recounting. Whenever practical, the samples were counted with less than 5% counting error at the 95% confidence level. The feces pellets were homogenized by grinding in a glass mortar, and aliquots were digested as described by Bishara *et al.* (34). To about 20 mg. of the homogenized feces in a counting vial, 0.2 ml. of perchloric acid (70%) was added and mixed well to ensure thorough wetting. Two-tenths milliliter of hydrogen peroxide (30%) was then added and the contents were mixed. The vials were put in the oven for 15 min. at 70–75° and then cooled to room temperature. Another 0.5 ml. of hydrogen peroxide solution was added and the samples were bleached for 30 min. in the oven. The vials were allowed to

Table I—Daily Fecal ^{14}C-Activity (Chlorophenothane and/or Metabolites)

Treatment	Excreted Percentage of Administered Dose[a]			
	Day 1	Day 2	Day 3	Day 4
Control	1.22	2.04	2.60	3.19
Postdiphenylhydantoin[b]	—	—	—	—
Prediphenylhydantoin	2.38	3.44	4.21	4.64
Cold[b]	—	—	—	—
Fasted	1.40	1.74	2.52	2.98
Levarterenol bitartrate[b]	—	—	—	—
Standard error	0.27	0.28	0.39	0.54

	Excreted Percentage of Administered Dose[a]			
	Day 5	Day 6	Day 7	Day 8
Control	3.67	3.81	4.16	3.58
Postdiphenylhydantoin[b]	—	2.51	2.91	4.16
Prediphenylhydantoin	4.08	3.57	3.71	3.60
Cold[b]	—	5.29	4.85	5.07
Fasted	2.84	1.75	1.55	1.91
Levarterenol bitartrate	—	2.32	2.37	2.65
Standard error	0.51	0.45	0.48	0.79

[a] Data expressed as mean ± *SE* for six rats. [b] Fecal samples were not analyzed for Days 1–5.

cool to room temperature, and 15 ml. of the scintillation solution was added, mixed, and counted. Most fecal samples (72%) were assayed in quintet and the rest were assayed in triplicate. The results were averaged to get a mean value per sample of feces. The weights of the rats were recorded at the beginning and end of the experiment.

Statistical Analysis of Data[10]—Homogeneity of variance was tested for the 18 cells (six treatments × three replicates) using the Bartlett (35) and/or Burr–Foster (36) tests for all days. A two-way analysis of variance (33) was conducted to investigate the effects of treatments, replicates, and the interaction between replicates and treatment for each day. Since almost all of the analyses showed no significant effect at the 0.25 level (37) between replicates (no significance at the 0.17 level the 1st day) or interaction between replicates and treatments, the data were then pooled and analyzed as if the experiment were a completely randomized design (33). For the analysis of the completely randomized design, the pooled data were then subjected to a one-way analysis of variance (33), and the resultant *F*-ratio for testing the differences between the means of groups was compared with the corresponding critical *F*-value at the 0.05 probability level. Whenever a significant *F*-ratio was obtained, the data were tested by a Newman–Keuls sequential range test (38) to locate the treatment means that were significantly different by pairs.

RESULTS AND DISCUSSION

During the first 5 days of the experiment, analysis of daily fecal samples for ^{14}C-activity was conducted on the control, prediphenylhydantoin, and fasted groups. Analysis of the feces of the fasted and the control groups was to test the hypothesis that all animals could be considered as controls in the Day 1–5 period except the prediphenylhydantoin group. Analysis of the feces of the latter group allowed comparison of the effect of diphenylhydantoin on the excretion of chlorophenothane in relation to the control group since *diphenylhydantoin has been reported as a hepatic enzyme activator* (1, 27, 32, 39).

All daily fecal samples collected during Days 6 through 8 were analyzed to monitor the effect of exposing the animals to the five treatments.

Daily Excretion—The daily fecal raw data from the randomized complete block design of the groups and days defined previously were accepted to have homogeneous variances. Interpretation of the two-way analysis of variances showed that there was no effect

[3] Hotpack, Philadelphia, Pa.
[4] Dilantin (sterile sodium diphenylhydantoin USP); Parke, Davis and Co., Detroit, Mich. Solution (S–V 105) contained 50 mg. Dilantin/ml.
[5] L-Arterenol bitartrate hydrate, CNE-E-grade, Calbiochem, Los Angles, Calif. Solution in saline contained 1 mg. norepinephrine/ml.
[6] Laboratory Supply Co., Indianapolis, Ind.
[7] Acme Metal Products, Chicago, Ill.
[8] Harvard Apparatus Co., Dover, Mass.
[9] Tri-Carb model 2002, Packard Instrument Co., Downers Grove, Ill.

Table II—Newman-Keuls Sequential Range Test of Daily Fecal 14C-Activity (Chlorophenothane and/or Metabolites)[a]

Treatment	Fasted					Levarterenol Bitartrate					Postdiphenyl-hydantoin					Prediphenyl-hydantoin					Control				
	1	2	3	6	7	1	2	3	6	7	1	2	3	6	7	1	2	3	6	7	1	2	3	6	7
Cold				**	**				**	**				**	*				*					*	
Control				*	**																				
Prediphenylhydantoin	*	**	*	*	*																*	**	•		
Postdiphenylhydantoin																									
Levarterenol bitartrate																									

[a] * = significant at 5% level, and ** = significant at 1% level.

Table III—Cumulative Fecal 14C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 1

Treatment	Excreted Percentage of Administered Dose[a]			
	Day 1	Day 2	Day 3	Day 4
Control	1.22	3.26	5.87	9.06
Prediphenylhydantoin	2.38	5.82	10.04	14.68
Fasted	1.40	3.15	5.67	8.65
Standard error	0.27	0.45	0.72	1.15

Treatment	Excreted Percentage of Administered Dose[a]			
	Day 5	Day 6	Day 7	Day 8
Control	12.73	16.54	20.70	24.28
Prediphenylhydantoin	18.76	22.33	26.04	29.64
Fasted	11.49	13.25	14.79	16.70
Standard error	1.59	1.92	2.28	2.76

[a] Data expressed as mean ± SE for six rats.

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of replicates nor treatment by replicate interaction. This indicated that repeating the experiment at different time intervals had no effect on the observed results; i.e., the time interaction did not have any effect. Therefore, the data of each treatment were pooled and analyzed as if the experiment were a completely randomized design.

The pooled data were again statistically analyzed as described in the *Statistical Analysis of Data* section. Table I shows the mean daily excretion results of the pooled data for all groups from Days 6 to 8 and from Days 1 to 5 for the control, prediphenylhydantoin, and fasted groups.

Analysis of variance (ANOVA) was performed on the daily means to evaluate the differences due to the treatment effects. Significant F-ratios at the 0.05 level were obtained for Days 1, 2, 3, 6, and 7, indicating that the treatments affected the rats differently.

To locate the specific treatment effect, the data were then subjected to the Newman-Keuls sequential range test. The results are reported in Table II with the corresponding level of significance.

No significant differences were observed between the control and the fasted groups, indicating that for Days 1 3 the two groups could be considered as controls. Further elaboration on the fasted and control groups will be made later. The rats of the prediphenylhydantoin group excreted more 14C-activity (chlorophenothane and/or metabolites) than the control groups for Days 1 3. The high excretion of 14C-activity in the feces by the prediphenylhydantoin group may be the result of inducing the liver microsomal enzymes that metabolized chlorophenothane (39).

On Day 6, the cold treatment was significantly different from all other treatments. This indicated that the cold treatment has the strongest stimulating effect on the fecal excretion of chlorophenothane from the rats. There were also differences between the cold treatment and the fasted, levarterenol bitartrate, and postdiphenylhydantoin groups on Day 7. Since cold has been reported to mobilize fat (23, 25, 26), the high 14C fecal activity, which was significantly different from other treatments, may be due to the availability of more chlorophenothane to be metabolized and excreted. However, the effect of the cold treatment seemed to wear off with time, since less differences were noticed on Day 7 and none was noticed on Day 8 between the cold group and the other groups. This may be due to the adaptability of the rats to, the cold environment. Dirksen (40) reported that a cold environment affected rats most (detected by low adrenal ascorbic acid) at 6 and 18 hr. of exposure and that the animals adapted to the cold stress after this period.

The fasted group differed from the control group at the 0.05 and 0.01 levels of significance for Days 6 and 7, respectively. Although the fasted group was expected to mobilize its fat and the associated chlorophenothane. the low fecal excretion (due to no food ingestion) may explain the low activity excreted by the fasted group. There was a significant difference between the prediphenylhydantoin and the fasted groups at Days 6 and 7, which again reflected the low fecal excretion of the fasted group.

Cumulative Excretion— The daily means reported in Table I were then expressed as cumulative activity and were subjected to the same statistical tests. These were performed on data of Days 1-8 for the control, prediphenylhydantoin, and fasted groups. The mean cumulative fecal 14C-activity for these three groups are reported in Table III. The statistical evaluation of the cumulative data clearly showed a significant difference (Table IV) between the fecal excretion of the prediphenylhydantoin group and each of the control and fasted groups for the Days 1-6 period. These results favorably backed the assumption that all rats were controls until the treatments started on Day 6. Further differences were observed between the fasted and prediphenylhydantoin groups for Days 7 and 8, thus indicating low fecal excretion of chlorophenothane and/or its metabolites by the fasted group.

When comparing the control and fasted groups based upon the cumulative data (Table III), no significant differences between them were noticed (Table IV). During the first 5 days of the experiment, these results were expected since it has been previously shown that both groups could be considered as controls. Due to the effect of the treatment starting on Day 6, a difference of chlorophenothane fecal excretion between the fasted and control groups was anticipated. However, the expected increased elimination was not observed. This may be due to the masking effect of the first 5 days of cumulative excretion.

The cumulative means starting from Days 6 to 8 for all six groups are reported in Table V. The data of prediphenylhydantoin are included in the table for completeness. It has already been shown (Table IV) that by Day 6 the prediphenylhydantoin group could be considered as a control group since the effect of the drug has been

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Table IV —Newman-Keuls Sequential Range Test of Cumulative Fecal 14C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 1[a]

Treatment	Control								Fasted							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Prediphenylhydantoin Fasted	*	**	**	**	*	*			*	**	**	**	*	*	**	*

[a] * = significant at 5% level, and ** = significant at 1% level.

Table V—Cumulative Fecal ¹⁴C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 6

Treatment	Excreted Percentage of Administered Dose[a]		
	Day 6	Day 7	Day 8
Control	3.81	7.96	11.55
Postdiphenylhydantoin	2.51	5.42	9.58
Prediphenylhydantoin	3.57	7.28	10.88
Cold	5.29	10.14	15.21
Fasted	1.75	3.30	5.21
Levarterenol bitartrate	2.32	4.68	7.33
Standard error	0.45	0.84	1.48

[a] Data expressed as mean ± SE for six rats.

Table VII—Initial and Final Body Weight of the Rats[a]

Treatment	Initial Body Weight, g.[b]	Final Body Weight, g.[c]
Control	192.3	268.2
Postdiphenylhydantoin	186.2	225.0
Prediphenylhydantoin	191.7	258.7
Cold	197.7	242.6
Fasted	193.4	191.7
Levarterenol bitartrate	194.2	252.1
Standard error	3.8	5.9

[a] Data expressed as mean ± SE for six rats. [b] Body weight at 1st day of adaptation. [c] Body weight at day of sacrifice.

dissipated. The cumulative data clearly reflect that there is no statistical difference between the prediphenylhydantoin and control groups on Days 6–8 (Table VI).

The cumulative chlorophenothane fecal excretion starting from Day 6 (Table V) of the cold group was always the highest and was followed by the control, prediphenylhydantoin, postdiphenylhydantoin, levarterenol bitartrate and fasted groups in descending order. The cold group was statistically different from all other groups on Day 6. Also, significant effects (Table VI) of the cold treatment existed between this group and each of the fasted and levarterenol bitartrate groups for Days 7 and 8. On Day 7, the cold group differed from the postdiphenylhydantoin group. This difference was previously explained in the discussion of daily excretion data.

The fasted group differed from the control group on Days 6–8 while the levarterenol bitartrate group differed from the control only on Day 7. The latter case may be explained by the observation that the rats of the levarterenol group reduced their food consumption after administration of the compound, which resulted in less fecal excretion as was the case with the fasted group. Another explanation of the significant difference between the control and levarterenol bitartrate groups may be due to the fact that this drug inhibited some metabolizing enzymes (28, 41, 42). This may have resulted in low excretion of chlorophenothane and/or its metabolites.

The mean initial and final body weights of the rats used in this experiment are reported in Table VII. The final body weight means were all higher than the initial weights, except for the fasted group which was the same. The Newman–Keuls sequential range test (Table VIII) for these data showed a significant difference of the body weights between the fasted and all the other treatments at the 0.01 level. Postdiphenylhydantoin and cold groups differed from the control group at a significance level of 0.01 and 0.05, respectively. In the case of the postdiphenylhydantoin group, the animals did not consume as much food after dosing. On the other hand, the animals exposed to cold may have lost some of their fat that had been mobilized for energy during cold exposure.

In conclusion, the cold treatment was the most effective of all studied treatments in stimulating fecal excretion of ¹⁴C-chlorophenothane and/or metabolites. However, the efficiency of this treatment diminished after the first 24 hr. due to adaptation.

The prediphenylhydantoin treatment may have stimulated the hepatic microsomal enzymes that metabolize chlorophenothane. This was clearly shown in the cumulative data starting from Day 1

(Table III) and is in agreement with previously published data (1, 27, 32, 39).

The postdiphenylhydantoin treatment was not effective in enhancing the fecal excretion of chlorophenothane and/or its metabolites. Similar results have been reported in the case of humans taking daily doses of diphenylhydantoin to determine the effect on chlorophenothane residue reduction. No reduction was detectable at 3 weeks, and the study was to be continued for 3 months (43). Recently, Davies et al. (44) reported that oral sodium diphenylhydantoin (300 mg. daily for up to 9 months) reduced the chlorophenothane and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) residues in human volunteers by 75 and 61% of pretreatment levels, respectively.

Although several authors used diet restriction and fasting to mobilize fat (17, 19, 25) together with its pesticide content, the redistribution of the pesticide in other tissues was always the end result (19, 22, 24, 25, 45). In this study, fasting, which is a form of diet restriction, had the least effect on the excretion of chlorophenothane from rats.

The statistical designs (randomized complete block and completely randomized) used in this work complied with the basic principles of experimental design, namely replication, randomization, and local control (33). The designs also supplied statistically reliable data which lead to valid inferences with respect to the results obtained using minimum effort and expense.

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Table VI—Newman–Keuls Sequential Range Test of Cumulative Fecal ¹⁴C-Activity (Chlorophenothane and/or Metabolites) Starting from Day 6[a]

Treatment	Day														
	Fasted			Levarterenol Bitartrate			Postdiphenyl-hydantoin			Prediphenyl-hydantoin			Control		
	6	7	8	6	7	8	6	7	8	6	7	8	6	7	8
Cold	**	**	**	**	**	**	**	**		*			*		
Control	*	**	*		*										
Prediphenylhydantoin	*	*	*												
Postdiphenylhydantoin															
Levarterenol bitartrate															

[a] * = significant at 5% level, and ** = significant at 1% level.

Table VIII—Newman-Keuls Sequential Range Test of Final Body Weight of the Rats[a]

Stress	Fasted	Postdiphenyl-hydantoin	Cold	Levarterenol Bitartrate	Prediphenyl-hydantoin
Control	**	**	*		
Prediphenylhydantoin	**	**			
Levarterenol bitartrate	**	**			
Cold	**	*			
Postdiphenylhydantoin	**				

[a] * = significant at 5 % level, and ** = significant at 1 % level.

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