the blood, whereas net half-life and net clearance have been used to describe the permanent elimination of the drug from the body.

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Quantitative Determination of *N*-(*trans*-2-Dimethylaminocyclopentyl)-*N*-(3′,4′-dichlorophenyl)propanamide and Its *N*-Demethyl Metabolite in Dog Serum by Gas Chromatography

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Received July 23, 1982, from the *Pharmaceutical Research and Development, The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication February 17, 1983.

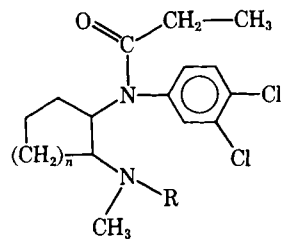
Abstract □ A gas chromatographic–electron capture (GC–EC) method has been developed for the determination of *N*-(*trans*-2-dimethylaminocyclopentyl)-*N*-(3′,4′-dichlorophenyl)propanamide, a potential antidepressant drug, and its *N*-demethyl metabolite in serum. The GC–EC system employed a 3% OV-17 on 100/120 mesh Supelcoport, 2-m × 2-mm i.d. glass column and an isothermal temperature of 195°C. The parent drug and metabolite were extracted from alkalinized serum (pH ~ 13) with toluene, back-extracted into an acidic solution (pH ~ 1), and finally, after adjusting to pH 13, extracted again with toluene. The extensive sample cleanup was necessary to remove serum components which interfered with the analysis. The analytical method was shown to give quantitative recovery of the drug and metabolite, to be linear over a 100-fold concentration range, and to have the necessary precision and sensitivity to detect and quantify as little as 1 ng/mL of the drug or its metabolite. The method has been employed to determine the serum level of drug and metabolite in dogs receiving a single oral dose and to determine the possible correlation between the administered dose and serum levels.

Keyphrases □ *N*-(*trans*-2-Dimethylaminocyclopentyl)-*N*-(3′,4′-dichlorophenyl)propanamide—quantitative determination, *N*-demethyl metabolite in dog serum, gas chromatography–electron capture □ Gas chromatography—electron capture, quantitative determination of *N*-(*trans*-2-dimethylaminocyclopentyl) -*N*- (3′,4′-dichlorophenyl) propanamide and its *N*-demethyl metabolite, dog serum

Some tricyclic antidepressants (*e.g.*, imipramine) used in the treatment of endogenous depression require an induction period of several days before improvement is noted and have been reported to have frequent side effects (1, 2). A potential antidepressant drug, *N*-(*trans*-2-dimethylaminocyclopentyl)-*N*-(3′,4′ - dichlorophenyl)propanamide (I) which may have a more rapid onset of therapeutic activity and fewer side effects was recently reported (3).

For definition of the pharmacokinetic and metabolic profiles of this potential antidepressant drug, a sensitive, precise, and specific analytical method is required. The presence of two chlorine moieties in the aromatic ring provides sufficient electronegativity for electron-capture (EC) detection after gas chromatographic (GC) separation. Initial studies[1] had shown that the drug was quickly demethylated at the dimethylamine functional group to give II. Also, a homologue of I with a cyclohexyl ring in place of the cyclopentyl ring was available (III) and was an ideal internal standard.

This report describes the GC–EC method for the quantitative determination of I, its *N*-demethyl metabolite, and the internal standard and the sample preparation procedure necessary to isolate the compounds from serum and to provide a sample amenable to GC–EC determination. Application of the methodology was demonstrated by the determination of serum levels of I and II in dogs



I: R = CH_3, n = 1
II: R = H, n = 1
III: R = CH_3, n = 2

[1] Unpublished data.

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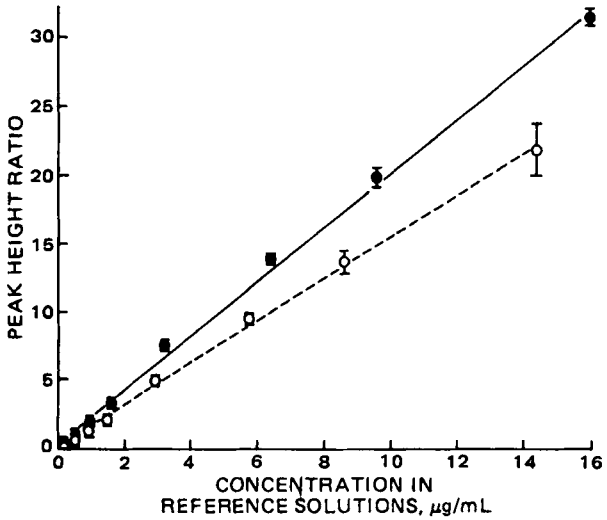

Figure 1—*Mean peak height ratio (n = 3) versus concentrations of I (●) and II (○) in the reference standard solutions. Concentrations ranged from 160 to 16,000 ng/mL for I and from 140 to 14,400 ng/mL for II. Bars represent ±2SD. Linear regression equations: (—) y = 1.97x + 0.42, r = 0.999; (- - -) y = 1.54x + 0.16, r = 0.996.*

administered a single oral dose of I and in dogs receiving different dose levels of I to determine the correlation between administered dose and serum levels.

EXPERIMENTAL

Apparatus and Reagents—The gas chromatograph[2] was equipped with a ^{63}Ni electron-capture detector. The toluene and methanol were distilled in glass[3]. Reference standards of the parent compound (I), the

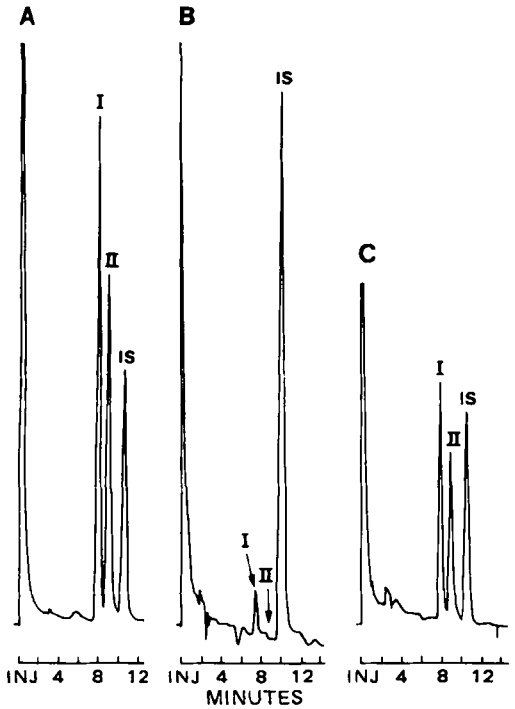

Figure 2—*Determination of I and II by GC-EC. Key: (A) reference standard solution containing 96 ng of I, 86 ng of II, and 61 ng of the internal standard; (B) extracted blank serum containing 60 ng of the internal standard (arrows indicate elution positions for I and II); (C) serum sample fortified with 64 ng/mL of I, 58 ng/mL of II, and 60 ng of the internal standard.*

[2] Model 222, Tracor Instruments, Austin, Tex.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.

Table I—Recovery of I and II Added to Serum

Amount Added, ng/mL	Amount Found,[a] ng/mL	SD ±	RSD, %	Recovery, %
	I			
1602	1530	25	1.6	95.2
1202	1100	46	4.2	91.1
961	920	26	2.9	94.9
801	790	15	1.9	98.0
641	640	10	1.6	99.1
481	480	20	4.2	98.8
320	350	6	1.7	107.8
160[b]	175	22	12.4	106.2
128	117	15	10.2	87.5
96	81	14	17.3	79.2
80	76	2	2.7	88.8
64	61	8	13.9	87.5
48	45	5	11.0	83.3
32	34	2	4.5	90.6
16	21	2	7.3	100.0
8[c]	12	1	11.8	87.5
0	5	2	34.6	—
	II			
1438	1450	76	5.3	100.8
1079	1010	35	3.4	93.6
863	830	42	5.0	96.2
719	690	35	5.0	96.0
575	570	6	1.0	99.1
431	440	15	3.5	102.1
288	310	6	1.9	107.6
144[b]	151	18	11.9	104.9
115	96	21	2.2	83.5
86	74	2	3.1	86.0
72	76	6	8.0	105.6
58	49	8	14.1	84.5
43	40	3	7.5	93.0
29	28	4	14.4	96.6
14	14	1	4.1	100.0
7[c]	8	1	8.8	114.3
0	1	0	0	—

[a] Average value; $n = 3$ unless otherwise stated. [b] $n = 5$. [c] $n = 2$.

N-demethyl metabolite (II), and the internal standard (III) were used as obtained[4]. All other reagents and chemicals were of the highest quality available.

Chromatographic Conditions—A 3% (w/w) OV-17 on 100/120 mesh Supelcoport[5] in a 2-m × 2-mm i.d. glass column was used throughout these studies. The column was conditioned at 270°C for 24 h and deactivated by injecting 20 × 5 μL of bis(trimethylsilyl)trifluoroacetamide[6]. The conditioning and deactivation were necessary to prevent tailing by the compounds. The injector and detector temperatures were maintained at 300°C, and the column was isothermal at 195°C. The argon–methane (95:5, v/v) carrier gas flow rate was 25 mL/min, and the purge flow was 40 mL/min. The ^{63}Ni EC detector was operated in the pulse mode. All injections were made with a 10-μL syringe[7], and injection volumes varied from 2 to 5 μL.

Stock Solution Preparation—Stock solutions of I and II were prepared by accurately weighing 10.0 mg of I and II each into 100 mL of toluene–methanol (80:20, v/v), with concentrations of I and II at 100 μg/mL. Aliquots from this stock solution were diluted to give a working stock containing 1000 ng/mL of each compound. The working stock solution was used to prepare reference standard solutions and to fortify serum samples at various levels of I and II. An internal standard stock solution was prepared in a similar manner. The working internal standard stock solution concentration was 250 ng/mL.

Sample Preparation Procedure—The analysis of low levels, *i.e.*, nanograms per milliliter, of compounds in physiological fluids by GC–EC techniques requires that the compounds of interest be isolated from most components present in the sample and be dissolved in an organic solvent amenable to EC detection. The following sample preparation procedure employed liquid–liquid extraction to isolate I and II from serum components.

One milliliter of serum was transferred into a culture tube with a pol-

[4] Pharmaceutical Research and Development Laboratories, The Upjohn Co., Kalamazoo, Mich.
[5] Supelco, Inc., Bellefonte, Pa.
[6] Regis Chemical Co., Morton Grove, Ill.
[7] Hamilton, Reno, Nev.

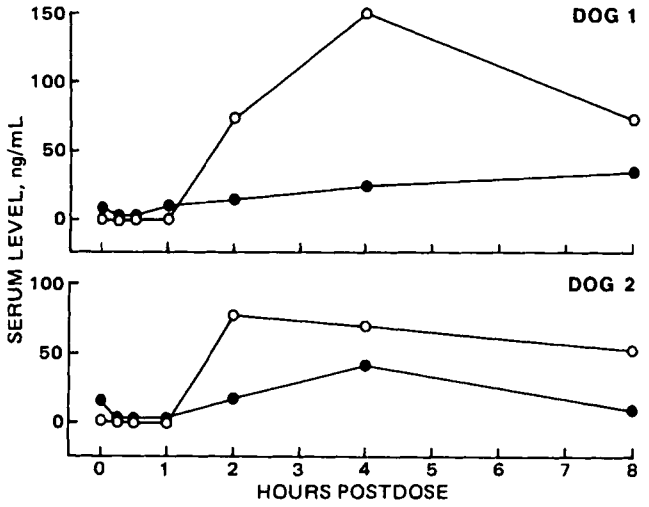

Figure 3—*Serum levels of I (●) and II (○) in dogs administered 10 mg/kg of I.*

ytef-lined screw cap. An equal volume of 1 M NaOH containing 100 mg/mL of NaCl was added, and the solution was mixed thoroughly. A 250-μL aliquot of the 250-ng/mL internal standard stock solution was added, followed by the addition of 4 mL of toluene–methanol (80:20, v/v). The solution was capped, vortexed for 45 s, and centrifuged at 2000 rpm for 10 min. The organic layer was transferred to a clean culture tube, and the extraction was repeated with 2 × 3 mL of toluene.

The organic phase was combined with the previous extract, and 2 mL of 1 M HCl was added. The solution was vortexed for 30 s, the organic phase was discarded, and the aqueous phase was washed by vortexing with 3 mL of toluene. The toluene was discarded, and the aqueous phase was made alkaline by adding 1 mL of 4 M NaOH containing 100 mg/mL of NaCl. This solution was extracted with 3 × 3 mL of toluene by vortexing for 30 s and centrifuged if necessary.

The toluene extracts were combined in a clean culture tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 μL of toluene–methanol (80:20, v/v); a 4-μL aliquot was injected into the chromatographic system.

Samples and Sample Handling—Serum levels of I and II were determined in two dogs, one male and one female, following oral administration of I. Blood samples were obtained prior to dosing and at 0.25, 0.5, 1, 2, 4, and 8 h after dosing. The blood was allowed to clot, and the serum was obtained by centrifugation. The serum was immediately frozen and maintained at −20 °C until sample preparation and analysis.

In addition, serum samples were obtained from a group of dogs being used in the evaluation of I. The dogs were a number of days into the evaluation, and thus, the samples that were collected were considered to be at steady state in relation to I. Four dogs, two male and two female, were selected from the 1-, 3-, and 10-mg/kg dosage levels and controls. Serum samples were obtained at 1 and 4 h after a daily dose. These sampling times were selected to provide samples at the expected highest serum concentration for I (1 h) and II (4 h). The serum samples were maintained at −20°C until sample preparation and analysis.

Calculations—The validation of the analytical method and the determination of I and II levels in dog serum were accomplished using the relative weight response (RWR) to an internal standard calculation method:

$$RWR_{Ref.\ Sol.} = \frac{PH_{cpd}}{PH_{IS}} \times \frac{ng_{IS}}{ng_{cpd}} \qquad \text{(Eq. 1)}$$

The average RWR of reference standard solutions of I and II analyzed with a sample set was employed to calculate the level of I and II in the samples:

$$ng_{cpd/sample} = \frac{PH_{cpd\ sample}}{PH_{IS}} \times \frac{ng_{IS\ sample}}{Avg.\ RWR_{Ref.\ Sol.}} \qquad \text{(Eq. 2)}$$

where PH_{cpd} and PH_{IS} are the peak heights of either I or II and the internal standard, and ng_{IS} and ng_{cpd} are the nanograms of internal standard and I or II in the reference solution or sample. For the serum samples, 1.0 mL of serum was used; thus, $ng_{cpd/sample}$ was equivalent to ng_{cpd}/mL of serum.

The reference standard solutions for each sample set were prepared over a concentration range equal to 15–300 ng/mL of I and II in serum. A minimum of five levels were selected for the reference standard solutions for each sample set. The recovery of I or II added to serum samples to validate the sample preparation procedure and to determine the accuracy of serum sample analyses was calculated as:

$$\%R = \frac{\text{ng of cpd in the fortified sample}}{\text{ng of cpd added + ng of cpd in the neat sample}} \times 100 \qquad \text{(Eq. 3)}$$

where %R is the percent recovery and the calculated amounts of I or II in the fortified and neat (unfortified) serum samples are in nanograms.

Table II—Serum Levels of I and II in Dogs Receiving Different Doses of I

Dose I, mg/kg	Compound	Time After Dose, h	Amount in Serum, ng/mL, Dog Group[a] A	B	C	D
10	I	1	17	13	4	11
		4	14	10	14	18
	II	1	226	53	19	60
		4	332	126	130	102
3	I	1	4	17	4	16
		4	4	5	3	4
	II	1	13	92	13	61
		4	24	64	17	21
1	I	1	2	2	5	3
		4	1	2	2	<1[b]
	II	1	7	3	6	<1
		4	2	4	6	<1
0	I	1	2	<1	~1	~1[c]
		4	<1	<1	<1	<1
	II	1	<1	<1	<1	<1
		4	<1	<1	<1	<1

[a] Dog groups A and B are female and groups C and D are male. [b] Below the detection limit (1 ng/mL) of the analytical method for I and II. [c] Small peak detected and calculated to be ~1 ng/mL.

RESULTS AND DISCUSSION

The GC–EC analytical technique was evaluated for linearity, precision, and sensitivity by analyzing triplicate reference solutions of I and II at eight concentration levels. The concentration levels ranged from 160 to 16,000 ng/mL for I and from 140 to 14,400 ng/mL for II. Figure 1 presents graphically the peak height ratio of I and II to the internal standard *versus* the concentrations of I and II. Linear regression equations and correlation coefficients for these data were $y = 1.97x + 0.42$ ($r = 0.999$) and $y = 1.54x + 0.16$ ($r = 0.996$) for I and II, respectively. The figure also shows two standard deviations around the average peak height ratio at each concentration. These data indicate the precision of the analytical

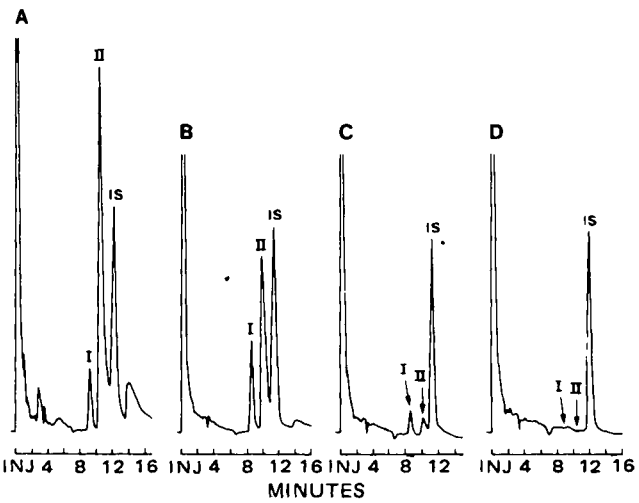

Figure 4—*GC–EC determination of I and II in serum from dogs receiving different doses of I. Key: (A) dose of 10 mg/kg; (B) dose of 3 mg/kg; (C) dose of 1 mg/kg; (D) control.*

technique. The sensitivity of the GC–EC (signal to noise ratio >5:1) was demonstrated to be <10 ng/mL for I and II. Assuming an initial serum volume of 1.0 mL and a final sample volume of 0.1 mL, the analytical technique can detect and quantify 1 ng/mL of serum for I and II.

The sample preparation procedure for the extraction of I and II from serum was developed through a series of experiments. The choice of toluene as the extraction solvent was made because of its low EC response and the high solubility of the compounds in this solvent. The serum samples were made alkaline to ensure a un-ionized species for extraction, and the addition of the salt to increase the ionic strength improved the extractability of the compounds. Initial studies indicated that stable emulsions formed when serum was extracted with toluene alone. By adding a small amount of methanol to the first toluene extraction, the emulsion problem was eliminated and the extraction efficiency was not affected. When only the first toluene extractions were employed to remove I and II from the serum, acceptable results were obtained for high levels, *i.e.*, >150 ng/mL, of I or II. However, interfering serum components at the GC elution positions of I and II prevented the single extraction technique from providing acceptable results below 150-ng/mL levels. Acidification of the toluene extracts from the serum removed I and II from the organic phase. The aqueous phase was alkalinized, and I and II were extracted with toluene. The double extraction gave very clean GC–EC chromatograms, and the recovery of I and II from the serum was high considering the extensive cleanup procedure employed. Figure 2 presents representative chromatograms of a reference solution, a blank serum sample, and a serum sample fortified with 64 ng/mL of I and 58 ng/mL of II.

The final sample preparation procedure was validated by preparing and analyzing replicate serum samples with added I and II at 16 concentration levels. The concentration ranges were 8–1600 and 7–1440 ng/mL for I and II, respectively. The results for these analyses are summarized in Table I. The average amount found for I and II at each concentration level and the standard deviation and relative standard deviation of the average are presented. Linear regression of the average amount found *versus* the amount added gave the following equations and correlation coefficients: $y = 0.947x + 8.5$, $r = 0.999$ for I and $y = 0.981x - 0.6$, $r = 0.999$ for II.

A small interfering serum component was observed at the elution position of I and gave a y-intercept of 8.5 ng/mL for I. The level of this unknown component in the blank serum was uniform and, thus, the level of I can be obtained by substracting the amount of the interfering component in blank serum from the level of I in the samples. However, serum levels of I <10 ng/mL must be considered questionable due to the possible presence of the unknown serum component. The percent recovery (%R) of I and II added to the serum gave average recoveries of 93.5 ± 7.9 for I and 97.7 ± 8.5 for II, indicating that the extraction efficiencies for I, II, and the internal standard were very similar and that no corrections for recovery of I and II from serum were necessary. These data demonstrate that the sample preparation procedure and analytical technique can provide reliable quantitative data over a large concentration range and that the recovery of I and II extracted from serum is quantitative.

The analytical method was utilized to determine the serum levels of I and II in two dogs administered a single oral 10-mg/kg dose of I. Blood samples were obtained before dosing and at 0.25, 0.5, 1, 2, 4, and 8 h after dosing. The serum from each dog was assayed for I and II, and the results are presented graphically in Fig. 3. Little or no compound was detected until 2 h after dosing. The level of the parent drug (I) in the serum was considerably lower than the *N*-demethyl metabolite (II), indicating possible extensive first-pass metabolism in the liver. Since both I and II have been reported to have similar therapeutic effects (3), the combination of the serum levels of I and II must be used to determine the concentration of active drug substance.

The method was also employed to assess if the serum levels of I and II correlated with the oral dose for dogs considered to be under steady-state conditions. Four dogs at each of three dose levels (10, 3, and 1 mg/kg) and four control dogs were evaluated; blood samples were obtained at 1 and 4 h after dosing. The data indicate that the serum levels of the parent drug and its *N*-demethyl metabolite may increase with increasing dose for the 10- and 3-mg/kg levels (Table II). At the 1-mg/kg dose, the levels of I and II in the serum approached the sensitivity limit of the method. Figure 4 presents representative chromatograms of serum samples from dogs at each dose level. The limited number of serum samples analyzed precludes statistical evaluation of the correlation of serum levels of I and II to the administered dose of I.

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Effect of pH on Theophylline Transfer Across the Everted Rat Jejunum

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Received July 7, 1980, from the *Departments of *Pharmacology and Toxicology, ‡Pediatrics, and §Pharmaceutical Sciences, Colleges of Pharmacy and Medicine, University of Arizona, Tucson, AZ 85721.* Accepted for publication January 10, 1983. ¶Present address: Departments of Pediatrics and Pharmacology, Columbus Children's Hospital, Columbus, OH 43205.

Abstract □ The effect of pH on the cumulative transfer of theophylline across the everted rat jejunum *in vitro* was ihvestigated. Intestinal integrity was assessed by light and scanning electron microscopy, while the biochemical viability of the intestine was evaluated using glucose transfer measurements. The initial (0–30 min) clearance of theophylline was directly proportional to the fraction un-ionized at pH 5.5, 7.4, 8.0, and 10.0. Plots of cumulative theophylline transfer *versus* time over 60 min were nonlinear, but could be subdivided into two linear segments of 30-min duration. Due to this nonlinearity, differences in theophylline transfer with pH were significant only over the first 30 min of the experiment. Intestinal tissue integrity and viability correlated with the time at which the clearance (slope) increased, while the magnitude of the increase in clearance was proportional to the degree of ionization of theophylline.

Keyphrases □ Theophylline—transfer across the everted-jejunum, rats, effect of pH □ Jejunum—everted, effect of pH on theophylline transfer, rats, □ pH—effect on the theophylline transfer across the everted jejunum, rats

Theophylline, a bronchodilator widely used for treating chronic obstructive pulmonary diseases such as asthma or emphysema, may be given orally or parenterally in the form of various salts. Although theophylline is usually given orally, its absorption from the GI tract can be erratic. Nausea and gastric irritation often accompany the oral

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