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Comparative Assays for Doxepin and Desmethyldoxepin Using High-Performance Liquid Chromatography and High-Performance Thin-Layer Chromatography

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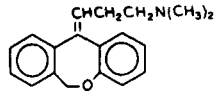
Received July 12, 1982, from the *Department of Pharmaceutics, College of Pharmacy, University of Houston, Houston TX 77030.* Accepted for publication September 1, 1982.

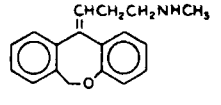
Abstract □ Two chromatographic methods, high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) were compared for sensitivity and reproducibility in the analysis of the tricyclic antidepressant doxepin and its metabolite, desmethyldoxepin, in plasma. The HPLC procedure yielded a better reproducibility, as reflected by the coefficient of variation, and a higher sensitivity, as reflected by the minimum detectable quantity. The application of these methods for therapeutic and subtherapeutic monitoring of plasma levels of the drug is described.

Keyphrases □ Doxepin—high-performance thin-layer and liquid chromatographic methods, comparison, human plasma, desmethyldoxepin metabolite □ High-performance liquid chromatography—doxepin and desmethyldoxepin in human plasma, comparison with high-performance thin-layer chromatography □ Thin-layer chromatography—high-performance, doxepin and desmethyldoxepin in human plasma, comparison with high-performance liquid chromatography

Doxepin (I) is a tricyclic antidepressant commonly prescribed for the treatment of endogenous depression. Increasing evidence of a correlation between the total tricyclic plasma level [doxepin plus desmethyldoxepin (II)] and the antidepressant effect suggests that monitoring tricyclic plasma levels may be beneficial in the clinical management of depression.



I



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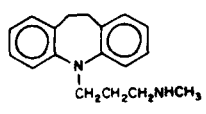

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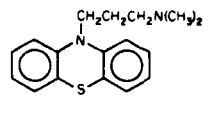

IV

Several assay methods for I and II have been reported, including GLC (1–3), radioimmunoassay (4), GC–mass fragmentography (5), and high-performance liquid chromatography (HPLC) (6–9). Most of these methods either require extensive sample preparation or lack the necessary specificity and sensitivity for pharmacokinetic studies. This paper compares two chromatographic procedures, HPLC and high-performance thin-layer chromatography (HPTLC), for sensitivity and reproducibility in the analysis of I and II in plasma. Desipramine (III) and promazine (IV) were used as internal standards in HPLC and HPTLC, respectively.

EXPERIMENTAL

Materials—A high-performance liquid chromatograph[1] equipped with a variable-wavelength UV detector[2], a sample loop injection valve[3], and a 5-μm octadecylsilane column[4] (150 mm × 4.6-mm i.d.) was used for HPLC analyses. A scanning spectrophotometer–densitometer[5] was used for HPTLC measurements.

Doxepin[6], desmethyldoxepin[6], desipramine[7], and promazine[8] were obtained as hydrochloride salts. Toluene[9], chloroform[9], pentane[9], and 2-propanol[9] were reagent grade and were glass-distilled before use. HPLC-grade acetonitrile[10] and reagent-grade *N*-nonylamine[11] were used as supplied. All glassware used for samples or extracts were silylated with hexamethyldisilazine at elevated temperature and reduced pressure. Working standard solutions were prepared in the following strengths: I, 1 μg/ml; II, 1 μg/ml; III, 2μg/ml; IV, 1 μg/ml.

HPLC Procedures—Analyses were performed using a modified procedure of Kabra *et al.* (10). Plasma standards were prepared by spiking known quantities of I and II in 1 ml of plasma containing 0.1 μg of desipramine internal standard (III). To the spiked plasma samples were added 0.25 ml of saturated sodium carbonate and 4.5 ml of pentane. The sample mixture was rotated for 45 min and then centrifuged. The pentane phase was transferred into a silylated conical vial[12] and back-extracted with 0.1 ml of 0.1 *N* HCl. The organic layer was discarded, and aliquots of the aqueous phase were injected into the liquid chromatograph. The mobile phase was 45% acetonitrile in 0.01 *M* phosphate buffer containing 600 ppm of *N*-nonylamine (pH 3.1). The deaerated and fil-

[1] ConstaMetric IIG; Laboratory Data Control, Riviera Beach, Fla.
[2] Spectromonitor III; Laboratory Data Control, Riviera Beach, Fla.
[3] Model SV-7; Glenco Scientific Inc., Houston, Tex.
[4] Spherisorb; Custom LC Inc., Houston, Tex.
[5] Zeiss Instruments, New York, N.Y.
[6] Pennwalt Corp., Rochester, N.Y.
[7] USV Pharmaceutical Corp., Tuckahoe, N.Y.
[8] SKF Laboratories, Philadelphia, Pa.
[9] Fisher Scientific, Fair Lawn, N.J.
[10] MCB Mfg. Chemicals Inc., Cincinnati, Ohio.
[11] Aldrich Chemical Co., Milwaukee, Wis.
[12] Reacti-Vial; Pierce Chemical Co., Rockford, Ill.

0022-3549/83/1000-1165$01.00/0
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Table I—HPLC Determinations of Peak Height Ratios in Plasma [a]

Amount, ng	Ratio of I/III			Ratio of II/III		
	Mean[b]	SD	CV[c]	Mean[b]	SD	CV[c]
10	0.182	0.018	9.9	0.227	0.025	10.9
20	0.338	0.019	5.9	0.394	0.038	9.6
40	0.653	0.035	5.5	0.733	0.041	5.6
60	1.012	0.061	6.0	1.152	0.066	5.7
100	1.572	0.065	4.1	1.800	0.073	4.0

[a] Absorbance measured at 200 nm. [b] Mean of six determinations. [c] Coefficient of variation.

tered mobile phase was pumped through the column at 2.5 ml/min, and the effluents were detected at 200 and 240 nm.

HPTLC Procedures—Analyses were performed following a modified procedure of Fenimore *et al.* (11). To plasma standards containing various amounts of I and II were added 50 ng of promazine internal standard (IV), 0.25 ml of saturated sodium carbonate, and 4.5 ml of pentane. The mixture was gently shaken for 45 min and then centrifuged. The aqueous layer was frozen in an acetone–dry ice bath, and the organic phase was decanted into a silylated conical vial. After complete evaporation of the organic phase, the residue was dissolved in 60 μl of heptane–ethanol (3:1), and 50 μl of the reconstituted sample was spotted onto the HPTLC plate[13]. Development of the plate was carried out in a twin-trough chamber[14] using two solvent systems: toluene–chloroform–2-propanol (20:20:10) and toluene–chloroform–2-propanol–ammonium hydroxide (20:20:10:1). The developed plates were air dried prior to UV scanning.

RESULTS AND DISCUSSION

Figure 1 shows representative liquid chromatograms of a control plasma blank, plasma spiked with III, and plasma to which I, II, and III were added. The retention times were 4.6, 3.3, and 6.3 min for I, II, and III, respectively. As indicated in Fig. 1a, no interfering peaks were found in the control plasma.

The peak height ratios obtained following HPLC analyses of I and II in control plasma are summarized in Table I. The calibration curves were characterized by linear regression equations of $y = 0.0156x + 0.0345$, $r^2 = 0.9981$ for I, and $y = 0.0177x + 0.0480$, $r^2 = 0.9983$ for II. The reproducibility in concentration measurements, as reflected by the coefficient of variation, was 6.3 ± 2.2% for I and 7.2 ± 2.9% for II. The lower detection limit was 5 ng for both I and II.

Figure 2 depicts the thin-layer chromatograms of a control plasma blank, plasma to which promazine internal standard (IV) was added, and

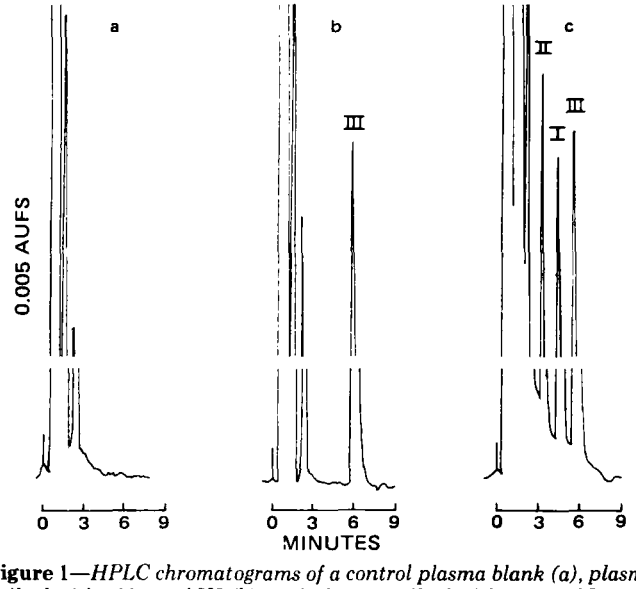

Figure 1—*HPLC chromatograms of a control plasma blank (a), plasma spiked with 100 ng of III (b), and plasma spiked with 60 ng of I, 60 ng of II, and 100 ng of III (c).*

[13] Silica Gel 60; E. Merck, Darmstadt, GFR.
[14] Camag Inc., New Berlin, Wis.

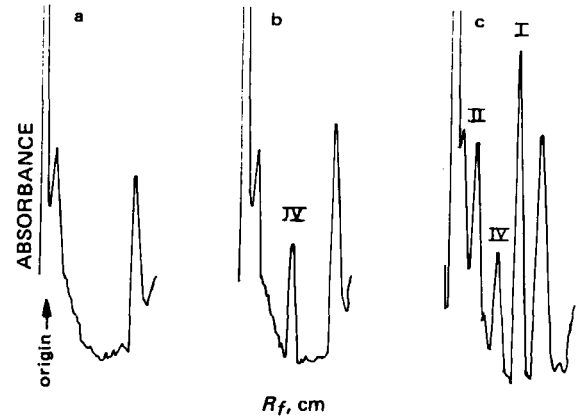

Figure 2—*HPTLC chromatograms of a control plasma blank (a), plasma spiked with 50 ng of IV (b), plasma spiked with 60 ng of I, 60 ng of II, and 50 ng of IV (c).*

Table II—HPTLC Determinations of Peak Height Ratios in Plasma [a]

Amount, ng	Ratio of I/IV			Ratio of II/IV		
	Mean[b]	SD	CV[c]	Mean[b]	SD	CV[c]
10	0.142	0.015	10.6	—[d]	—	—
20	0.232	0.012	5.2	0.097	0.013	12.8
40	0.513	0.027	5.3	0.198	0.020	10.2
60	0.627	0.031	4.9	0.342	0.027	7.8
100	1.040	0.065	6.3	0.550	0.047	8.5

[a] Absorbance measured at 240 nm. [b] Mean of four determinations. [c] Coefficient of variation. [d] II not distinguishable from baseline at 10 ng.

plasma with added I, II, and IV. No interfering signals were detected in the blank plasma at R_f values of 1.6 cm for I, 0.6 cm for II, and 1.0 cm for IV.

The peak height ratios derived at 240 nm following HPTLC analyses of various amounts of I and II in control human plasma are presented in Table II. In the 10 to 100-ng range, the assay reproducibility was 6.5 ± 2.4% for I and 9.8 ± 2.2% for II. Typical calibration curves were characterized by $y = 0.0099x + 0.0551$, $r^2 = 0.9951$ for I, and $y = 0.0057x - 0.0190$, $r^2 = 0.9980$ for II. The sensitivity of measurements was 10 ng for I and 20 ng for II at 200 nm, which decreased to 20 ng for I and 30 ng for II at 240 nm.

Since a common internal standard was not available for both chromatographic procedures, each method requires its own internal standard.

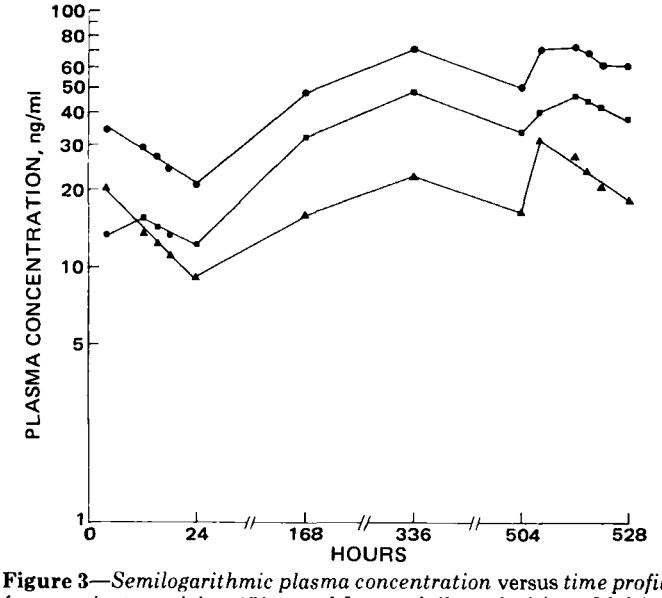

Figure 3—*Semilogarithmic plasma concentration* versus *time profile for a patient receiving 150 mg of I once daily at bedtime. Multiple samples were collected for the 24-hr periods following the 1st and 21st doses. Key: (▲) I; (■) II; (●) I and II.*

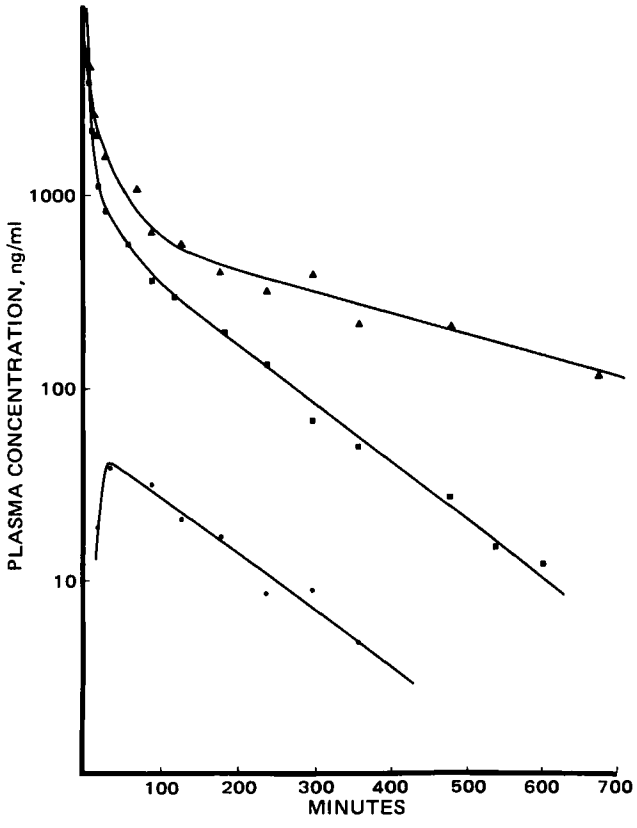

Figure 4—*Semilogarithmic plasma concentration* versus *time plots for I and II in a normal dog following intravenous administrations of 50 mg of I and 50 mg of II on two occasions separated by 2 weeks. Key: (▲) I; (●) II generated from I; (■) II given as a separate dose.*

Promazine was selected as the internal standard for HPTLC method because of the proximity of its R_f value to that of I and II. Similarly, desipramine was used in the HPLC procedure because of its relatively short retention time: 6 min as opposed to 13 min for promazine.

When both chromatographic procedures were compared for detection sensitivity at 200 nm on an equivalent weight basis, the HPLC method appeared to be superior. Since back-extraction was carried out in the HPLC procedure, which could conceivably reduce the extraction recovery, the lower sensitivity of the HPTLC method could not be attributed to the extraction process. One possibility is that the HPLC mobile phase favors the UV absorbance for I and II compared with the silica gel of the HPTLC plate.

In an attempt to compare the reproducibility of the HPTLC procedure at two different wavelengths (200 and 240 nm), coefficients of variation were determined: 10.3 ± 5.0% for I and 12.4 ± 5.6% for II at 200 nm as opposed to 6.5 ± 2.4% for I and 9.8 ± 2.2% for II at 240 nm. The reproducibility was less impressive at 200 nm; however, it was comparable with HPLC at 240 nm. The detecting sensitivity, on the other hand, notably decreased at the longer wavelength.

Figure 3 shows the plasma concentration *versus* time plot for a patient receiving 150 mg of I once daily at bedtime. Multiple samples were collected for 24-hr following the 1st and the 21st doses. Half-lives of I and II were 17.3 and 35.5 hr, respectively, after the first dose, and 23.8 and 40.6 hr following the last dose. It is not clear, based on this patient study, whether multiple dosing of I alters its metabolism.

Figure 4 presents plasma concentration *versus* time plots for a normal dog intravenously administered a 50-mg dose of I and a 50-mg dose of II on two occasions separated by 2 weeks. The β-phase half-life of I in the dog, 2.5 hr, was considerably shorter than that of the depressed human, 17.2 hr. In contrast to the human, the dog exhibited a shorter half-life for II than for I [17.3/35.5 (I/II) in the human *versus* 3.3/2.1 in the dog]. In addition, the dog presented a substantially lower fraction of II in the plasma. These observations suggest an interspecies difference in the metabolic and excretory patterns of I between dogs and humans.

In summary, for measurements of therapeutic levels of I and II in plasma, both HPLC and HPTLC provide adequate sensitivity and reproducibility. The HPLC method offers a higher detection sensitivity and is suggested for pharmacokinetic studies in which subtherapeutic concentrations are to be measured. On the other hand, the HPTLC procedure offers a greater processing capacity and a shorter turnaround time, which are preferred in a clinical setting for the routine monitoring of tricyclic plasma levels.

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ACKNOWLEDGMENTS

This work was presented at the APhA Academy of Pharmaceutical Sciences meeting in Las Vegas, April 1982.

The authors wish to thank Dr. C. M. Davis for granting us permission to use the HPTLC equipment at the Texas Research Institute of Mental Sciences.