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Stereoselective and simultaneous measurement of *cis*- and *trans*-isomers of doxepin and N-desmethyldoxepin in plasma or urine by high-performance liquid chromatography

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Abstract

Doxepin is a tricyclic antidepressant marketed as an irrational mixture of *cis*- and *trans*-geometric isomers in the ratio of 15:85. A convenient high-performance liquid chromatographic (HPLC) procedure for simultaneous quantitation of geometric isomers of doxepin and N-desmethyldoxepin in plasma and urine is described. The HPLC procedure employed a normal phase system with a silica column and a mobile phase consisting of hexane–methanol–nonylamine (95:5:0.3, v/v/v), a UV detector and nortriptyline as the internal standard. The liquid–liquid extraction solvent was a mixture of *n*-pentane–isopropanol (95:5, v/v). The limit of quantitation was 1 ng/ml for each isomer. The calibration curves were linear over the ranges 1–200 ng/ml (plasma) and 1–400 ng/ml (urine). In plasma, the accuracy (mean±S.D.) (97.53±1.67%) and precision (3.89±1.65%) data for *trans*-doxepin were similar to corresponding values for urine, i.e., 97.10±2.40 and 3.82±1.14%. Accuracy and precision data for *trans*-N-desmethyldoxepin in plasma were 97.57±2.06 and 4.38±3.24%, and in urine were 97.64±3.32 and 5.26±1.83%, respectively. Stability tests under three different conditions of storage indicated no evidence of degradation. The recovery of doxepin was 61–64% from plasma and 63–68% from urine. The method has been applied to analyses of plasma and urine samples from human volunteers and animals dosed with doxepin.

Keywords: Enantiomer separation; Doxepin; N-Desmethyldoxepin

1. Introduction

The tricyclic antidepressant doxepin is marketed as a mixture of geometric isomers in a *cis–trans* ratio of 15:85. In most in vivo and in vitro tests, the *cis*-isomer is the more potent of the two geometric forms [1]. Doxepin (Fig. 1) is well absorbed after oral administration and measurable amounts of doxepin and N-desmethyldoxepin (Fig. 1) rapidly appear in the blood stream.

There have been a number of reports that the ratio of *cis*- to *trans*-N-desmethyldoxepin equals or surpasses unity in plasma and urine of patients and healthy volunteers after oral administration of doxepin, while this phenomenon of "enrichment" was not evident for the parent drug [2–7]. N-Desmethyldoxepin is known to possess potent antidepressant activity. Hence, any "enrichment" of the *cis*-isomer may have therapeutic significance. Moreover, stereoselective analyses will be essential if mechanis-

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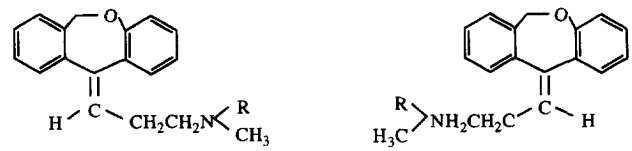

Fig. 1. Structures of *cis*- and *trans*-doxepin (R=CH_3) and N-desmethyldoxepin (R=H).

tic aspects of any "enrichment" process are to be explored.

Several publications have described stereoselective analytical procedures for *cis*- and *trans*-isomers of doxepin and/or N-desmethyldoxepin based on gas–liquid chromatography (GLC) [2,5,6], GC–MS [8] and HPLC [6,9,10]. Recently, the first HPLC procedure that permitted simultaneous, stereoselective quantitation of both doxepin and N-desmethyldoxepin geometric isomers was reported [7], although the method lacked the sensitivity required for single dose pharmacokinetic studies with the lower limits of quantification of 10 ng/ml for doxepin and 5 ng/ml for N-desmethyldoxepin in a reversed-phase system. This paper presents a sensitive and specific normal phase HPLC method that separates and quantitates *cis*- and *trans*-isomers of both doxepin and N-desmethyldoxepin in a single run.

2. Experimental

2.1. Materials

Doxepin hydrochloride and nortriptyline hydrochloride were purchased from Sigma (St. Louis, MO, USA). N-Desmethyldoxepin was prepared in the laboratory according to a published method [11]. *trans*-N-Desmethyldoxepin hydrochloride, and *cis*- and *trans*-doxepin hydrochloride were kindly supplied by Pfizer (Groton, CT, USA). All solvents and chemicals used in the HPLC system were of analytical grade and those used in synthesis and extraction were of reagent grade. Standard solutions of doxepin, *cis*-, *trans*-doxepin, *trans*-N-desmethyldoxepin and nortriptyline hydrochlorides were prepared in double distilled water at a concentration of 100 μg/ml and stored at 4°C.

2.2. Instrumentation

The HPLC system consisted of a Waters WISP Model 712 intelligent sample processor, a Waters Lambda-Max Model 480 UV detector, a Waters Model 501 HPLC pump and either a Shimadzu (Kyoto, Japan) Chromatopac C-R3A integrator or a Waters Maxima 820 data system. A 150×4.5 mm I.D. column packed with 3 μm Spherisorb silica was used for the separation. Chromatographic conditions were as follows: the mobile phase is an organic solution (filtered and degassed in situ) and consisted of hexane, methanol and nonylamine in the ratio of 95:5:0.3 (v/v/v). The flow-rate was 1.0 ml/min, the detector was operated at 254 nm and the injection volume was 60 μl. The chromatography was performed under temperature-controlled conditions (23°C).

2.3. Preparation of standard curves and quality control (QC) samples

Standard stock solutions were diluted to 0.1–10 μg/ml for spiking control biofluids to achieve final concentrations (in duplicate) of 1, 10, 50, 100, 200 and 400 ng/ml of *trans*-doxepin and *trans*-N-desmethyldoxepin (urine) or 1, 3, 10, 50, 100 and 200 ng/ml (plasma). The concentration of the internal standard, nortriptyline, was 400 ng/ml (urine) or 200 ng/ml (plasma). Three concentrations across the range of the standard curve (lower, middle and upper regions) were used for the preparation of QC samples (duplicates, operator blind). A 15% deviation from the quotient of the determined value and the nominal value was the criterion of acceptance, except at the lowest concentration, where 20% deviation was allowed. No more than two out of six QC samples were allowed to fail to meet the acceptance criteria (no more than one at any given

concentration). Standard calibration curves and QC samples were all made fresh each day of the assay.

2.4. Sample preparation procedures for various tests in the method validation

In order to compare detector response to the *cis*- and *trans*-isomers, standard aqueous solutions (2.5 μg/ml) of *cis*- and *trans*-doxepin were added to control plasma or urine (2.0 ml) to give different *cis*-isomer percentages [*cis*/(*cis*+*trans*)×100%] (Table 1). The total concentration of the two isomers in each sample was 125 ng/ml, while the lowest concentration of an individual isomer was 12.5 ng/ml. The solutions were analyzed in replicates of three or five on each of three consecutive days. QC samples (operator blind) were used to assure the validity of each day's results.

Stability under three different conditions was studied in both urine and plasma: (i) stability after two freeze–thaw cycles (−20°C), (ii) stability after two days of storage at 4°C (day 1 and day 2); and (iii) stability of extracts reconstituted in mobile phase and stored at room temperature (for up to 16.5 h). Experiments to assess the stability under conditions (i) and (ii) above were carried out at two concentrations (10 and 100 ng/ml, in triplicate), whereas those for condition (iii) were carried out at three concentrations (10, 50 and 100 ng/ml, in duplicate).

In the recovery study, an aqueous solution of the doxepin hydrochloride salt was neutralized with sodium hydroxide and the free base of doxepin was extracted with chloroform. Evaporation of the organic solvent left the oily free base. This free base was weighed and redissolved in mobile phase for direct injection. Three concentrations (20, 80 and 400 ng/ml) were selected in the study.

2.5. Extraction procedure

To samples of spiked or unknown plasma or urine (2.0 ml), a 3 *M* ammonia solution (solution A; 0.5 ml) and a mixture of *n*-pentane–isopropanol (95:5, v/v; solution B; 7 ml) were added. The tube was capped and shaken in an overhead shaker for 20 min, and then left standing for 10 min to allow separation to occur. The upper organic layer was transferred to a clean test tube containing 0.1 *M* hydrochloric acid (1.0 ml). The mixture was shaken for 20 min and then allowed to separate for 5 min. The organic phase was aspirated to waste. Pentane (3.0 ml) was used to wash the remaining aqueous layer (shaken for 10 min) and then discarded after separation. To the washed aqueous residue, solution A (0.5 ml) and solution B (6 ml) were added and the mixture was shaken for 20 min. After separation of the layers, the organic phase was transferred to a clean test tube and dried at 65°C under a flow of nitrogen. The resulting dry residue was reconstituted with 160 μl of the mobile phase and samples (60 μl) were injected on to the column (autosampler).

3. Results and discussion

Standard curves were based on the *trans*-isomers of the parent drug and its metabolite, since only trace quantities of the *cis*-isomers were available. The method was therefore based on the assumption that the *cis*- and *trans*-isomers should have the same peak area if their UV detector responses were

Table 1
Equivalence of detector response to *cis*- and *trans*-isomers

Nominal *cis*-doxepin (%)	10	20	40	60	80
Number of samples	15	9	15	9	15
Average back calculated *cis*-doxepin (urine) (%)	10.28	19.63	40.03	60.09	79.98
Average back calculated *cis*-doxepin (plasma) (%)	10.47	19.68	39.86	59.67	80.39
C.V. (urine) (%)	7.53	3.12	2.76	2.46	2.54
C.V. (plasma) (%)	8.84	2.77	3.09	3.53	2.37
Accuracy (urine) (%)	97.20	98.15	99.93	99.85	99.98
Accuracy (plasma) (%)	95.30	98.40	99.65	99.45	99.51

equivalent, notwithstanding any difference in retention times and peak sharpness. Accordingly, peak area was used throughout as the basis of quantitation.

For equivalent detector response tests, the original peak area data of *cis*- and *trans*-doxepin were used directly in the calculation of *cis*-isomer percentage [*cis*/(*cis*+*trans*)×100%]. Averages of observed ratios were tested against nominal ratios by linear regression (no weighting factor). The corresponding calibration curves were used in back calculation. Intra- and inter-assay C.V.s (%) and percent accuracy were based on back-calculated values. Coefficients of determination (r^2) ranged from 0.999 to unity. Table 1 gives the average back-calculated values obtained on three consecutive days.

Tests on intra- and inter-assay variability were carried out on three consecutive days with five or three samples at each concentration. After linear regression (peak response weighting: 1/concentration), the back-calculated concentrations were compared to nominal ones using the same criterion described previously for QCs. A typical linear regression equation for *trans*-doxepin was $y=0.003x-0.002$ ($r^2=0.9997$), whereas it was $y=0.003x-0.001$ ($r^2=0.9997$) for *trans*-N-desmethyldoxepin in validation studies with plasma. For the studies with urine, they were $y=0.008x-0.028$ ($r^2=0.9994$) for *trans*-doxepin and $y=0.006x-0.001$ ($r^2=0.9996$) for *trans*-N-desmethyldoxepin. Intra- and inter-assay C.V.s were both <15%. Table 2 lists the data on back-calculated concentrations, accuracy and precision. The mean values of accuracy and precision (mean±SD%) for *trans*-doxepin were 97.53±1.67 and 3.89±1.65 (plasma), 97.10±2.40 and 3.82±1.14 (urine), while those for *trans*-N-desmethyldoxepin were 97.57±2.06 and 4.38±3.24 (plasma), 97.64±3.32 and 5.26±1.83 (urine), respectively.

The stability data showed no evidence of degra-

Table 2
Accuracy and precision

Matrix	*n*	Added (ng/ml)	Found (ng/ml)	Accuracy (%)	Precision (%)
trans-Doxepin					
Urine	13	1	1.07	93.00	5.15
	9	10	10.44	95.60	3.41
	15	50	48.68	97.36	2.84
	9	100	98.84	98.84	5.27
	9	200	197.32	98.66	3.63
	15	400	403.41	99.15	2.62
Plasma	15	1	1.05	95.00	5.75
	9	3	2.90	96.67	6.04
	15	10	9.71	97.10	3.79
	9	50	48.90	97.80	2.97
	15	100	101.11	98.89	2.66
	9	200	199.41	99.71	2.11
trans-N-Desmethyldoxepin					
Urine	14	1	1.09	91.00	6.79
	9	10	10.14	98.60	2.91
	15	50	48.96	97.92	7.44
	9	100	99.55	99.55	3.33
	9	200	198.00	99.00	5.86
	15	400	400.81	99.80	5.23
Plasma	13	1	1.06	94.00	10.01
	8	3	2.97	99.00	5.62
	15	10	9.74	97.40	4.26
	9	50	48.33	96.66	1.07
	15	100	99.55	99.55	3.73
	9	200	202.37	98.82	1.59

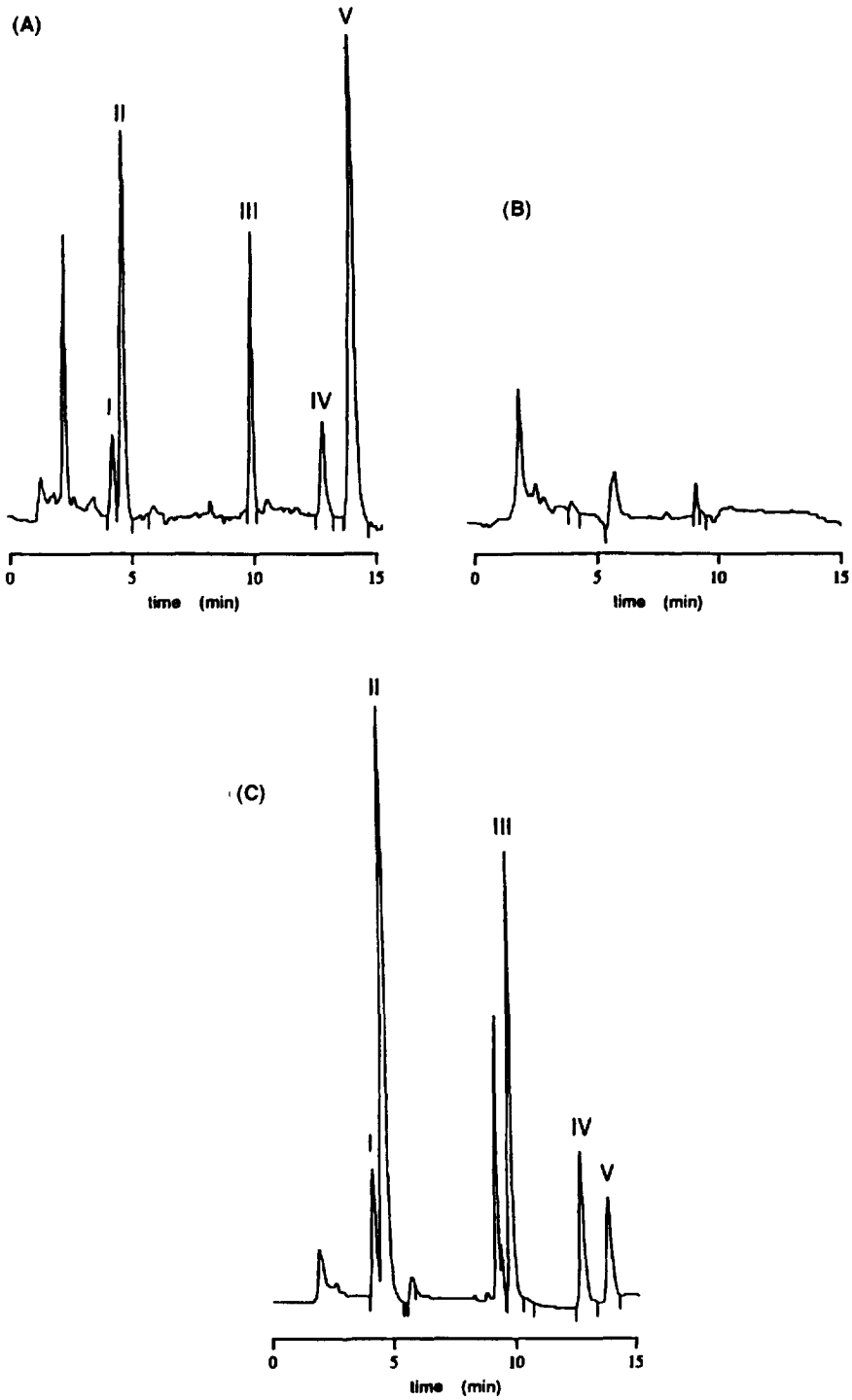

Fig. 2. HPLC chromatograms: (A) Standard reference compounds (I, *cis*-doxepin; II, *trans*-doxepin; III, nortriptyline; IV, *cis*-N-desmethyldoxepin; V, *trans*-N-desmethyldoxepin); (B) blank urine from the human volunteer; (C) 24 h urine from the human volunteer after a single oral dose of doxepin hydrochloride (75 mg). Spectra were obtained using a C-R3A integrator.

dation of *trans*-doxepin and *trans*-N-desmethyldoxepin under the three storage conditions described in Section 2.4. Recovery was investigated with commercially available doxepin because supplies of the pure isomers were limited. The recovery was calculated by comparing the peak height (sum of *cis*- and *trans*-doxepin) of extracted samples ($n=6$) with those of directly injected ones ($n=2$). Recoveries were 63–68% from urine and 61–64% from plasma.

This HPLC method was applied successfully to pilot experiments in humans and dogs. Fig. 2 shows spectra (C-R3A integrator) of extracts of control urine and cumulative 24 h urine from a healthy human volunteer following an oral single dose of 75 mg. Fig. 3 shows similar spectra (data system) of extracts from control dog urine and urine from a dog dosed orally (20 mg/kg). Fig. 4 shows plasma concentration versus time profiles of *cis*- and *trans*-isomers of doxepin and N-desmethyldoxepin in one healthy human subject after a single oral dose (75 mg).

The method presented proved to be a very useful tool for a direct, sensitive, stereoselective and also reliable quantitative assay of doxepin and its pharma-

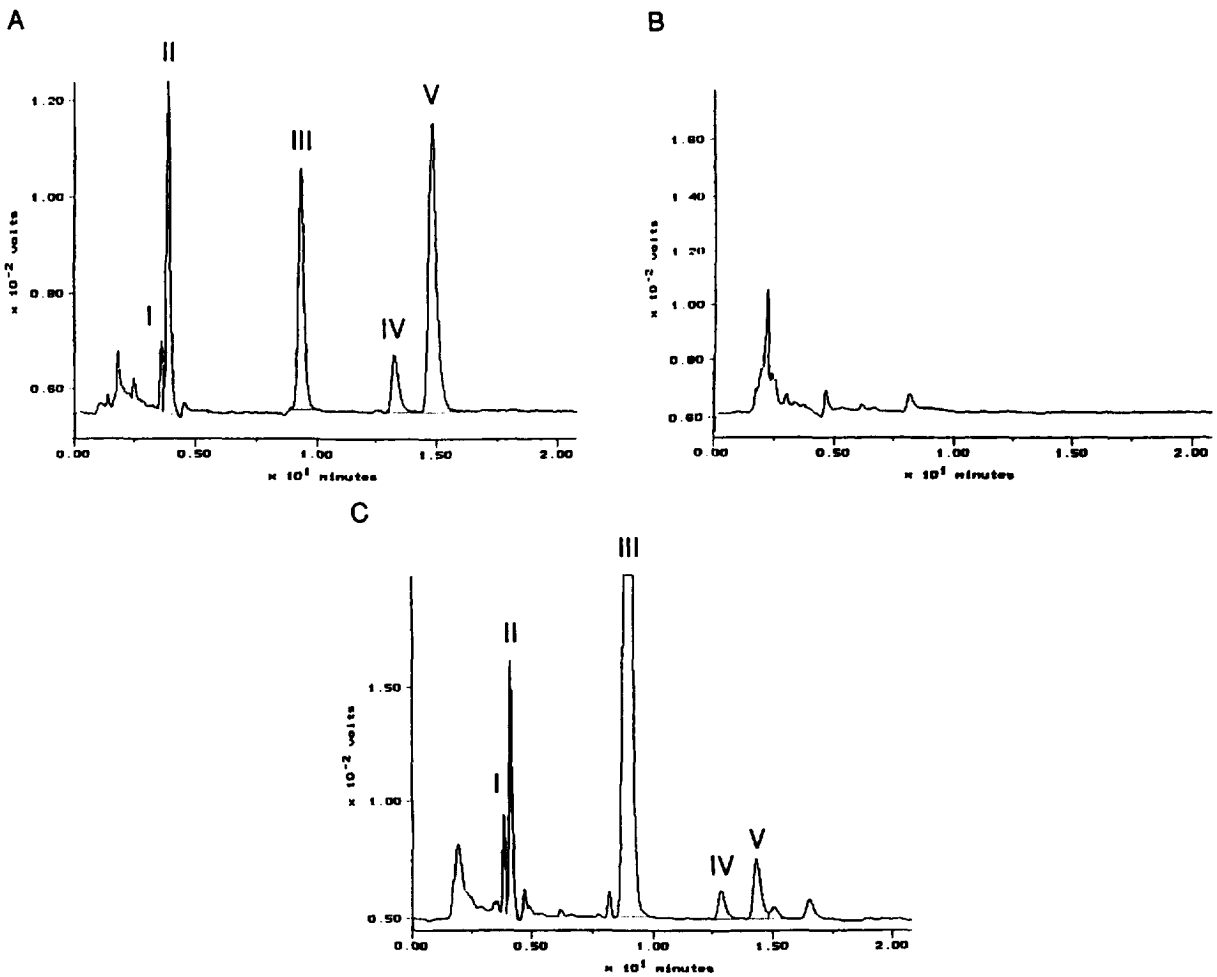

Fig. 3. HPLC chromatograms: (A) Standard reference compounds (I, *cis*-doxepin; II, *trans*-doxepin; III, nortriptyline; IV, *cis*-N-desmethyldoxepin; V, *trans*-N-desmethyldoxepin); (B) blank plasma from the dog; (C) plasma taken from the dog 6 h after administration of a single oral dose of doxepin hydrochloride (20 mg/kg). Spectra were obtained using the data system.

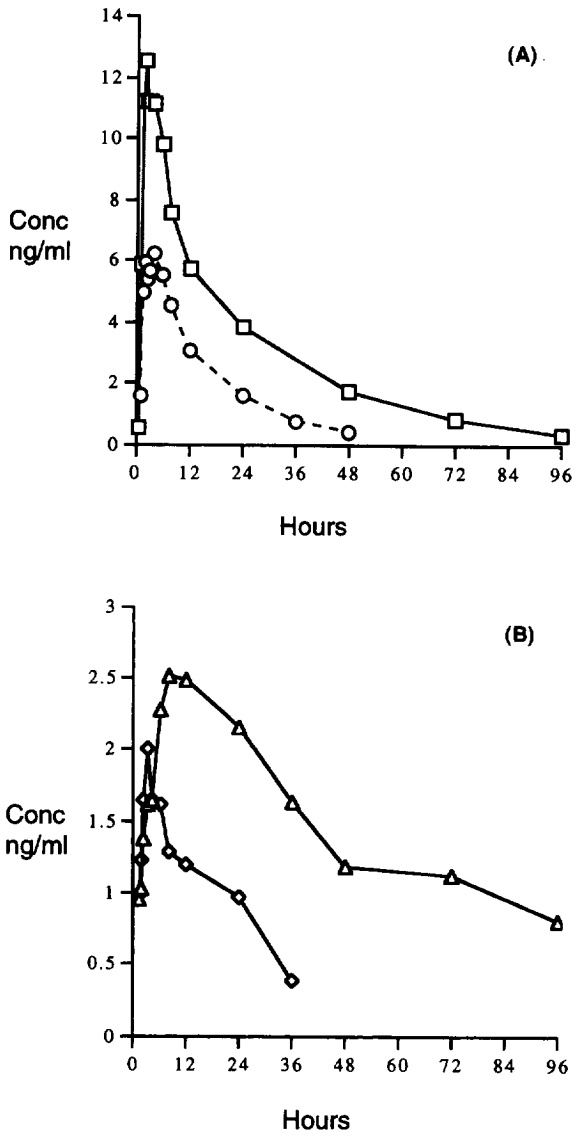

Fig. 4. Plasma concentration versus time profiles in a human subject following a single oral dose of doxepin (75 mg): (A) *cis*- (○) and *trans*- (□) isomers of doxepin; (B) *cis*- (△) and *trans*- (◇) isomers of N-desmethyldoxepin.

cologically active, major metabolite, N-desmethyldoxepin. The selection of a silica column under normal phase chromatographic conditions was based on an earlier stereoselective method [6], which was less sensitive and measured only the parent drug. Optimization of the chromatographic conditions, including solvent composition and the nature of the competing base, required extensive investigative work because the system was very sensitive to even a minor alteration in the ratio of mobile phase components. The ratio reported herein provides both adequate resolution of isomers and a reasonable retention time. For example, decreasing the polarity of the mobile phase by reducing the ratio of methanol to hexane led to greater isomeric resolution but also to prolongation of the retention time of N-desmethyldoxepin, rendering the method impractical for clinical and laboratory use when a large number of samples are to be run. Similarly, competing base content also played a critical role in achieving optimal chromatographic results. By comparison with the method of Adamczyk et al. [7], the present procedure for sample preparation is time saving and gives higher sensitivity. Although a back extraction step was employed in both methods, the time needed for extraction (20 min) and separation (5–10 min) in the present study was less compared with values of 60 and 30 min, respectively, for the earlier method. Moreover, concentrated extracts were reconstituted with mobile phase in a single step, in contrast with the two-step procedure used by Adamczyk et al. [7]. Furthermore, the column required only 1–2 h of equilibration time as opposed to 12 h or more in the earlier method. The method gives satisfactory separation of the *cis*- and *trans*-isomers of the parent drug and the N-desmethyl metabolite (Figs. 2 and 3) and good accuracy and precision.

Some clinical investigators have implied [3,5,12] that stereoselective data on doxepin and its active N-desmethyl metabolite are essential for studies on correlations between plasma levels and therapeutic/toxic effects of doxepin. Hence, for future therapeutic drug monitoring, a convenient, sensitive, simultaneous, stereoselective and inexpensive assay, such as this method, is required.

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