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Quantitation of trimipramine enantiomers in human serum by enantioselective high-performance liquid chromatography and mixed-mode disc solid-phase extraction

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Abstract

A sensitive and stereospecific method for the quantitation of trimipramine enantiomers in human serum was developed. The assay involves the use of a novel mixed-mode disc solid-phase extraction for serum sample clean-up prior to HPLC analysis and is also free of interference from the enantiomers of desmethyltrimipramine, 2-hydroxytrimipramine, and 2-hydroxydesmethyltrimipramine, the three major metabolites of trimipramine. Chromatographic resolution of trimipramine enantiomers was performed on a reversed-phase cellulose-based chiral column (Chiralcel OD-R) under isocratic conditions using a mobile phase consisting of 0.3 *M* aqueous sodium perchlorate–acetonitrile (58:42, v/v) at a flow-rate of 0.5 ml/min. Recoveries for *R*- and *S*-trimipramine enantiomers were in the range of 93–96% at 25–185 ng/ml levels. Intra-day and inter-day precisions calculated as R.S.D. were in the ranges of 0.30–8.00% and 1.60–10.20% for both enantiomers, respectively. Intra-day and inter-day accuracies calculated as percent error were in the 0.01–2.10% and 1.00–3.00% ranges for both enantiomers, respectively. Linear calibration curves were in the concentration range 15–250 ng/ml for each enantiomer in serum. The limit of quantification of each enantiomer was 15 ng/ml. The detection limit for each enantiomer in serum using a UV detector set at 210 nm was 10 ng/ml ($S/N=2$). In addition, separation of the enantiomers of desmethyltrimipramine, 2-hydroxytrimipramine, and 2-hydroxydesmethyltrimipramine were investigated. The desmethyltrimipramine enantiomers could be resolved on the Chiralcel OD-R column under the same chromatographic conditions as the trimipramine enantiomers, but the other two metabolite enantiomers required different mobile phases on the Chiralcel OD-R column to achieve satisfactory resolution with R_s values of 1.00.

Keywords: Enantiomer separation; Solid-phase extraction; Trimipramine

1. Introduction

Trimipramine, 10,11-dihydro-*N*,*N*,β-trimethyl-5H-dibenz[*b*,*f*]azepine-5-propanamine, is a dibenzazepine derivative tricyclic antidepressant (Fig. 1). Trimipramine shares the pharmacological actions and toxic potentials of other tricyclic antidepressants and is used primarily in the treatment of endogenous depression [1,2]. Studies on rabbits and dogs have shown that trimipramine is extensively metabolized and the metabolism of trimipramine in humans appeared to be similar [3,4]. Hydroxylation and desmethylation are the two major pathways of the metabolism of trimipramine in humans (Fig. 2).

Trimipramine and its three major metabolites, desmethyltrimipramine, 2-hydroxytrimipramine, and

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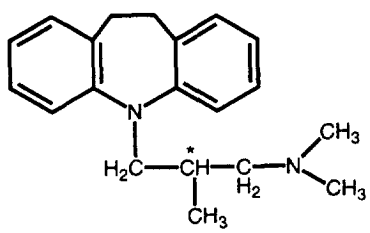
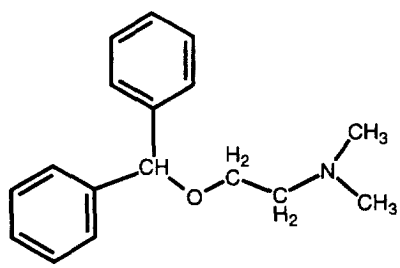


Trimipramine



Diphenhydramine (IS)

Fig. 1. Chemical structures of trimipramine and diphenhydramine (I.S.)

2-hydroxydesmethyltrimipramine, are chiral compounds with an asymmetric center at the side chain. Although trimipramine is administered as a racemate, it was reported that there are differences in the physiological and behavioral effects of trimipramine enantiomers [5–7].

The determination of antidepressant drugs remains one of the most commonly requested drug assays in clinical laboratories due to their widespread use and high toxicity [8]. Several methods have been reported for the determination of racemic trimipramine and/or its major metabolites using HPLC and GC [9–14]. Chiral resolution of trimipramine and/or its major metabolites was investigated on protein and Pirkle-type chiral columns such as the Chiral AGP and Sumichiral OA-4700 columns [15,16]. Only one paper reported the chiral separation of trimipramine and determination of the two hydroxyl metabolite enantiomers [16]. No study has yet determined the levels of each trimipramine enantiomer in human serum.

In this paper, a sensitive and stereospecific assay for the quantitation of trimipramine enantiomers in human serum is described using a reversed-phase cellulose-based chiral stationary phase (Chiralcel OD-R) and mixed-mode disc solid-phase extraction. The method is linear over the range 15–250 ng/ml using a UV detector set at 210 nm. The detection limit of the method for each trimipramine enantiomer is 10 ng/ml ($S/N=2$). The procedure is free of interference from the three major metabolites of trimipramine. In addition, the desmethyltrimipramine enantiomers could be resolved on the Chiralcel OD-R column under the same chromatographic conditions as the trimipramine enantiomers, but the other two metabolite enantiomers required different mobile phases on the Chiralcel OD-R column to achieve satisfactory resolution with R_s values of 1.00.

2. Experimental

2.1. Reagents and chemicals

Racemic trimipramine maleate was purchased from Sigma (St. Louis, MO, USA). The *R*- and *S*-trimipramine enantiomers were obtained from Rhône-Poulenc Rorer Centre de Recherches (Vitry-Alfortville, France). Desmethyltrimipramine maleate, 2-hydroxytrimipramine fumarate, and 2-hydroxydesmethyltrimipramine fumarate were kindly supplied by Dr. C.B. Eap of Hôspital de Cery (Prilly-Lausanne, Switzerland). The internal standard diphenhydramine hydrochloride was obtained from Parke-Davis (Detroit, MI, USA). Reagent-grade monobasic potassium phosphate, HPLC-grade acetonitrile and methanol were all purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade sodium perchlorate and triethylamine were obtained from Fisher Scientific (Pittsburgh, PA, USA). Drug-free human serum was obtained from Biological Specialty Corporation (Colmar, PA, USA). The PLUS™·MP1 solid-phase extraction discs (15 mg/3 cc size) were obtained from Ansys (Irvine, CA, USA). The Vac-Elut vacuum manifold was obtained from Analytichem International (Sunnyvale, CA, USA).

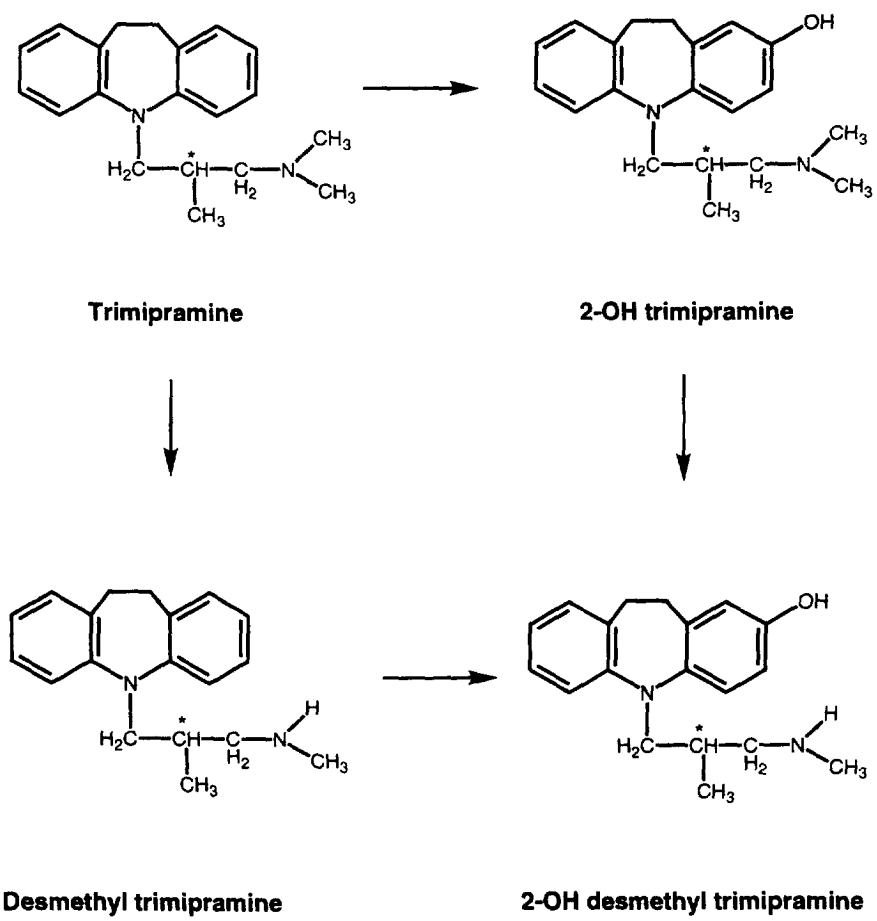


Fig. 2. Major metabolic pathways of trimipramine in humans

2.2. Chromatographic system

The HPLC system consisted of an Alcott Model 760 HPLC pump (Norcross, GA, USA), an Alcott Model 728 autosampler (Norcross, GA, USA) equipped with a 100-μl loop, a Kratos Model 757 variable-wavelength UV–Vis detector (Ramsey, NJ, USA) set at 210 nm and a Hewlett-Packard Model 3395 integrator (Avondale, PA, USA). The Chiralcel OD-R column (10 μm, 250 mm×4.6 mm I.D.) equipped with an opti-guard guard column RP C_8 (1.5 cm×1 mm I.D., Optimize Technologies, Portland, OR, USA) was obtained from Chiral Technologies (Exton, PA, USA), and operated at ambient temperature. The mobile phase consisted of 0.3 *M* aqueous sodium perchlorate–acetonitrile (58:42, v/v) and was delivered at a flow-rate of 0.5 ml/min. The solution was filtered through a 0.45-μm nylon membrane obtained from Alltech Associates (Deerfield, IL, USA) and sonicated prior to use.

2.3. Preparation of standard solutions

Stock solutions of 0.1 mg/ml of *R*- and *S*-trimipramine (maleate salts) calculated as trimipramine base were prepared in 10-ml volumetric flasks by adding 2 ml of acetonitrile followed by the addition of deionized water to volume and stored protected from light at 4°C. Stock solutions of 10 μg/ml of racemic desmethyltrimipramine (maleate salt), 2-hy-

droxytrimipramine (fumarate salt), and 2-hydroxydesmethyltrimipramine (fumarate salt) calculated as their free bases were also prepared in the same manner as stock solutions of trimipramine enantiomers and stored protected from light at 4°C. A stock solution of 10 μg/ml of the internal standard diphenhydramine (hydrochloride salt) calculated as the free base was prepared in double-distilled deionized water and stored at 4°C. The stock solutions of trimipramine enantiomers and the metabolites were prepared every 2 days. Appropriate dilutions of the *R*- and *S*-trimipramine stock solutions with deionized water gave 5 μg/ml solutions which were used for spiking blank human serum.

2.4. Preparation of spiked human serum samples

Accurately measured aliquots (3, 10, 24, 40, and 50 μl of the 5-μg/ml standard solution) of *R*- and *S*-trimipramine were each added into 1-ml volumetric tubes followed by the addition of 30 μl of 10 μg/ml of internal standard solution. Drug-free human serum was added to volume and mixed well to give final concentrations of 15, 50, 120, 200 and 250 ng/ml of each trimipramine enantiomer.

2.5. Assay method

PLUS™·MP1 solid-phase extraction (SPE) discs were attached to a vacuum manifold and conditioned with 0.2 ml of absolute methanol followed by 0.2 ml of 0.1 *M* potassium phosphate monobasic (pH 6.0) (note: do not allow sorbent to dry). The spiked human serum samples (1 ml) were diluted with 1.0 ml of 0.1 *M* potassium phosphate monobasic (pH 6.0) and mixed well. Into the discs were transferred blank and diluted spiked human serum samples and a vacuum (about 2 kPa) was applied. After the entire serum sample had been aspirated through the disc, the disc was washed with 500 μl of 1 *M* acetic acid followed by 500 μl of methanol and then dried under full vacuum for about 5 min. Then, the vacuum manifold was opened and collection tips were wiped. The trimipramine enantiomers and internal standard were eluted with 2×300 μl of acetonitrile–triethylamine (100:2, v/v). The eluent was evaporated to dryness under a nitrogen stream at ambient temperature. The residue was redissolved in 800 μl of the mobile phase and triplicate 100-μl injections were made into the liquid chromatograph. The peak heights of each trimipramine enantiomer and the internal standard were recorded for each sample. Five concentrations in the range 15–250 ng/ml of the *R*- and *S*-trimipramine enantiomers were used to construct the calibration curves. Linear regression analysis of the concentration of each trimipramine enantiomer versus the ratio of drug to internal standard peak heights (D/I.S.) produced slope and intercept data. The concentration of each trimipramine enantiomer in a given serum sample was calculated from the equation: concentration= (slope)(D/I.S.)+intercept.

3. Results and discussion

Trimipramine enantiomers have been resolved on Chiral AGP and Sumichiral OA 4700 columns [15,16]. The Chiral AGP column is a protein-type column with the α_1 acid glycoprotein as a chiral selector. Although the Chiral AGP column provides a considerable range of selectivities for the resolution of racemic drugs, the application of the Chiral AGP column to the analysis of chiral drugs is limited by the low stability and high price of the column. The Sumichiral OA 4700 column is a Pirkle-type column with the (*S*)-*tert.*-leucine-(*R*)-1-[α-naphthyl]ethylamine as a chiral selector and is operated in the normal-phase mode. Hexane is the usual principal component of the mobile phases, but isopropanol, dichloroethane, chloroform, and small amounts of triethylamine are also used as the mobile-phase modifiers. The Chiralcel OD-R column is a reversed-phase cellulose-base column which employs the cellulose tris(3,5-dimethylphenyl carbamate) as a chiral selector. The chiral selectivity in the cellulose-base columns involves interactions such as hydrogen bonding, dipole–dipole interactions, π–π interactions, and the formation of inclusion complexes. Compared with the normal-phase mode, the reversed-phase mode is more widely used since less organic waste is produced and harmful organic solvents such as hexane, chloroform, and methylene chloride are not used in mobile phases.

Initial baseline separation (R_s=2.40) of *R*- and *S*-trimipramine enantiomers on the Chiralcel OD-R

column in our laboratories was obtained using a mobile phase containing 0.5 *M* aqueous sodium perchlorate–acetonitrile (60:40, v/v) with retention times of 25–30 min. The influences of sodium perchlorate and acetonitrile concentrations in mobile phases on separation of trimipramine and its three major metabolite enantiomers were investigated. The fact that the three metabolites are also chiral made the chromatographic separation much more complicated especially with changing concentrations of sodium perchlorate. Generally, an increase in acetonitrile concentration in the mobile phase reduced the retention times at the expense of loss of resolution. However, an increase in the sodium perchlorate concentration in the mobile phase enhanced the resolution between each pair of trimipramine, desmethyltrimipramine, and 2-hydroxytrimipramine enantiomers, but reduced the resolution between the second eluting desmethyltrimipramine enantiomer and the first eluting *R*-trimipramine enantiomer. The final composition of the mobile phase was 0.3 *M* aqueous sodium perchlorate–acetonitrile (58:42, v/v). The authors examined two Chiracel OD-R columns and received comparable resolution of the analytes. This mobile phase provided good resolution (R_s=1.80) of the two trimipramine enantiomer peaks (G and H) and reasonable retention times (19–23 min) and sensitivity in the desired ng/ml range. Under the chromatographic conditions used for the determination of trimipramine enantiomers, the metabolites had shorter retention times (8–18 min) due to their higher polarity compared with the parent drug. Resolution (R_s) of the first eluting *R*-trimipramine enantiomer (G) and the second eluting desmethyltrimipramine enantiomer (F) was 1.70. Good separation (R_s=1.40) was obtained for the desmethyltrimipramine enantiomers (E and F), partial resolution (R_s<0.50) for the 2-hydroxytrimipramine enantiomers (B and C), and no separation for the 2-hydroxydesmethyltrimipramine enantiomers (A). Typical HPLC chromatograms for blank human serum and serum spiked with 50 ng/ml of each enantiomer of trimipramine and its three major metabolites and 300 ng/ml of internal standard are shown in Fig. 3. No interferences were observed in blank human serum and human serum containing the metabolites. Therefore, this method will allow an analyst, if desired and if pure enantiomers of desmethyltrimipramine are available, to simultaneously determine trimipramine and desmethyltrimipramine enantiomers in human serum.

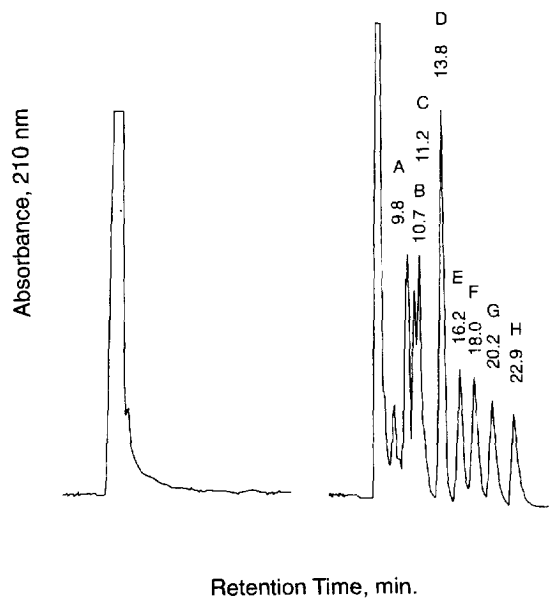


Fig. 3. Typical chromatograms of (left) blank serum and (right) serum spiked with 50 ng/ml of each enantiomer of trimipramine, desmethyltrimipramine, 2-hydroxytrimipramine and 2-hydroxydesmethyltrimipramine and 300 ng/ml of internal standard. Peaks: A, unresolved racemic 2-hydroxydesmethyltrimipramine; B and C, partially resolved 2-hydroxytrimipramine enantiomers; D, internal standard; E and F, desmethyltrimipramine enantiomers; G, *R*-trimipramine enantiomer; H, *S*-trimipramine enantiomer.

The separation of the three major trimipramine metabolites was also investigated on the Chiralcel OD-R column by modifying components of the mobile phases. The enantiomers of desmethyltrimipramine were well separated (R_s=1.40) with the mobile phase used for the determination of trimipramine enantiomers. Resolution of the 2-hydroxytrimipramine and the 2-hydroxydesmethyltrimipramine enantiomers could be obtained by an increase in the sodium perchlorate concentration and a decrease in the acetonitrile concentration in the mobile phase. In this manner, the 2-hydroxytrimipramine and 2-hydroxydesmethyltrimipramine enantiomers were partially resolved with R_s values of 1.00 using mobile phases of 1.0 *M* aqueous sodium perchlorate–acetonitrile (65:35, v/v) and 1.0 *M*

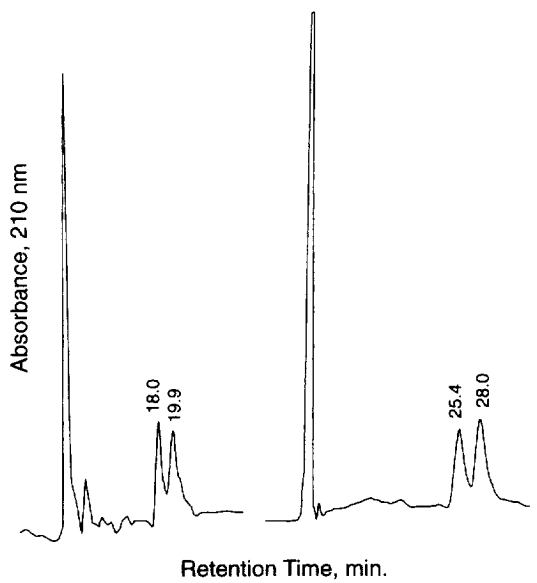


Fig. 4. Separation of (left) 2-hydroxytrimipramine enantiomers and (right) 2-hydroxydesmethyltrimipramine enantiomers on the Chiralcel OD-R column with mobile phases of 1.0 *M* aqueous sodium perchlorate–acetonitrile (65:35, v/v) and 1.0 *M* aqueous sodium perchlorate–acetonitrile (70:30, v/v), respectively.

aqueous sodium perchlorate–acetonitrile (70:30, v/v) mobile phases, respectively (Fig. 4).

The selection of diphenhydramine as internal standard was based on its suitable retention time (13.8 min) and resolutions between diphenhydramine (D) and the second eluting 2-hydroxytrimipramine enantiomer (C) (R_s 1.80) and between diphenhydramine (D) and the first eluting desmethyltrimipramine enantiomer (E) (R_s 1.80). Quantitation was based on the plot of the concentration of each trimipramine enantiomer versus peak-height ratio of each trimipramine enantiomer to internal standard.

Three solid-phase extraction cartridges (C_{18}, C_8 and cyanopropyl) and four solid-phase extraction discs (C_{18} AR, C_8, PLUS™·MP3 and PLUS™·MP1) were investigated for serum sample clean-up prior to the HPLC assay. The C_8 cartridge and C_8 disc showed no recoveries of *R*- and *S*-trimipramine. The C_{18} cartridge and C_{18} disc were also found to be unacceptable due to co-elution of endogenous serum components with trimipramine enantiomer peaks. The cyanopropyl cartridge provided good clean-up, but gives very low recoveries of trimipramine enantiomers. Although both PLUS™·MP3 and PLUS™·MP1 discs provided similar recoveries for the analytes of interest, the PLUS™·MP1 disc produced better results in terms of clean-up and recoveries of *R*- and *S*-trimipramine enantiomers and internal standard. The PLUS™·MP1 and PLUS™·MP3 are mixed-mode solid-phase extraction discs. The difference between them is that the PLUS™·MP3 contains slightly polar and strong cation-exchange phases, while the PLUS™·MP1 discs consists of nonpolar and strong cation-exchange phases. The presence of triethylamine in the elution solvent was essential to the extraction since it interfered with the ion-exchange interaction between the analytes and the cation phase of the mixed-mode disc such that trimipramine enantiomers and internal standard were more easily eluted. The analytes extracted by the disc method remained stable throughout these studies.

The suitability of the chiral HPLC system for the separation of the trimipramine enantiomers is shown in Table 1. The retention times of *R*- and *S*-trimipramine enantiomers and internal standard were 19.60±0.58, 22.25±0.70, and 13.45±0.33 min, respectively (n=5). Retention factors (k') for *R*- and *S*-trimipramine enantiomers and internal standard were 2.41±0.13, 2.87±0.16, and 1.34±0.08, respectively (n=5). The calculated theoretical plates for *R*- and *S*-trimipramine enantiomers were 2965±57 and

Table 1
Chromatographic parameter data (mean±S.D., n=5) for trimipramine enantiomers and internal standard

Analyte	R_s	α[a]	k'	t_R (min)	n[b]
Diphenhydramine (I.S.)	4.40	1.79	1.34±0.08	13.45±0.33	—[c]
R-Trimipramine	1.80	1.19	2.41±0.13	19.60±0.58	2965±57
S-Trimipramine			2.87±0.16	22.25±0.7	3345±47

[a] Separation factor, calculated as k'_2/k'_1.
[b] Theoretical plates, calculated as $n=16\,(t_R/w)^2$.
[c] Not calculated.

Table 2
Absolute recovery data for trimipramine enantiomers and internal standard in human serum

Analyte	Concentration level (ng/ml)	Recovery[a] (mean±S.D., n=5) (%)	R.S.D. (%)
R-Trimipramine	25	95.67±7.05	7.3
	105	93.33±9.19	9.8
	185	94.97±5.16	5.5
S-Trimipramine	25	93.84±4.86	5.2
	105	95.67±3.92	4.1
	185	95.34±3.96	4.2
Diphenhydramine (I.S.)	300	98.99±2.97	3.0

[a] Recoveries were calculated by a comparison of the extracted analyte peak area to the unextracted analyte peak area.

3345±47 per 25 cm column (n=5). Relative retention of the *R*- and *S*-trimipramine enantiomers was expressed by the separation factor α, calculated to be 1.19. Resolution (R_s) of the internal standard (D) and first eluting desmethyltrimipramine enantiomer (E) peaks and of the internal standard (D) and the second eluting 2-hydroxytrimipramine (C) peaks and of the second eluting desmethyltrimipramine (F) and the first eluting *R*-trimipramine enantiomer (G) and of the *R*- and *S*-trimipramine enantiomer peaks (G and H) were 1.80, 1.80, 1.70 and 1.80, respectively.

The recoveries of *R*- and *S*-trimipramine enantiomers from human serum were assessed by using spiked samples at three concentration levels. The absolute recoveries of *R*- and *S*-trimipramine were determined by a comparison of the extracted analyte peak area with the unextracted analyte peak area. The results are shown in Table 2.

Linear calibration curves were obtained in the 15–250 ng/ml range for each trimipramine enantiomer. Standard curves were fitted to a first degree polynomial, $y=ax+b$, where y is the concentration of trimipramine enantiomer, x is the ratio of drug/internal standard peak heights, and a and b are constants. Typical values for the regression parameters a (slope), b (y-intercept), and correlation coefficient were calculated to be 348.18, 0.43 and 0.9999 for *R*-trimipramine enantiomer, and 366.70, 4.87 and 0.9997 for *S*-trimipramine enantiomer, respectively (n=10). The precision and accuracy (percent error) of the method were determined by using human serum samples spiked at 25, 105, and 185 ng/ml levels (Table 3). The data indicates that intra-day

Table 3
Accuracy and precision data for trimipramine enantiomers in human serum

Analyte	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Error (%)	R.S.D. (%)
Intra-day				
R-Trimipramine	25	25.12±2.01	0.48	8.0
	105	105.02±1.15	0.02	1.1
	185	185.02±4.08	0.01	2.2
S-Trimipramine	25	25.51±0.49	2.04	1.9
	105	104.62±0.89	0.85	0.3
	185	185.89±4.31	0.48	2.3
Inter-day				
R-Trimipramine	25	24.63±0.40	1.48	1.6
	105	103.66±2.54	1.28	2.5
	185	187.44±4.01	1.32	2.1
S-Trimipramine	25	24.33±2.47	2.68	10.2
	105	103.83±1.72	1.11	1.7
	185	87.46±3.69	1.33	2.0

[a] Based on n=3 for the intra-day study and n=8 for the inter-day study.

precision was in the 0.30–8.00% range ($n=3$) and intra-day accuracy in the 0.01–2.10% range ($n=3$) for both trimipramine enantiomers and that inter-day precision was in the 1.60–10.20% range ($n=8$) and inter-day accuracy in the 1.00–3.00% range ($n=8$) for both trimipramine enantiomers.

The minimum detectable concentration of each trimipramine enantiomer was determined to be 10 ng/ml ($S/N=2$). The lowest quantifiable level was found to be 15 ng/ml for each trimipramine enantiomer: *R*-enantiomer, 4.30% R.S.D., 3.10% error; *S*-enantiomer, 4.60% R.S.D., 4.20% error.

In conclusion, a stereoselective HPLC method has been developed and validated for the assay of *R*- and *S*-trimipramine enantiomers in human serum using a Chiralcel OD-R column operated in the reversed-phase mode. The method utilizes a mixed-mode PLUS™·MP1 disc solid-phase extraction for sample clean-up. The procedure is free of interference from the enantiomers of desmethyltrimipramine, 2-hydroxytrimipramine and 2-hydroxydesmethyltrimipramine, the three major metabolites of trimipramine, and is suitable for the separation and quantification of each trimipramine enantiomer in the 15–250 ng/ml range. The method will also allow an analyst, if desired, to simultaneously determine both trimipramine and desmethyltrimipramine enantiomers in human serum since these enantiomer pairs were essentially baseline separated and recoveries of trimipramine and desmethyltrimipramine enantiomers from human serum were more than 90%. In addition, the enantiomers of the three major trimipramine metabolites were separated on the same column with the R_s values in a range of 1–1.4 using different mobile phases.

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