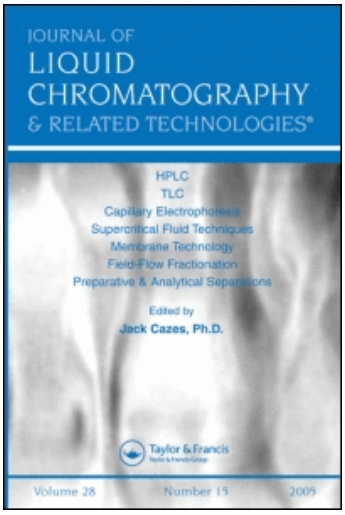

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Enantioselective HPLC Determination of R and S Trimipramine in Human Serum Using an Octyldecylsilane Column with β-Cyclodextrin as Mobile Phase Additive and Solid Phase Extraction

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ENANTIOSELECTIVE HPLC DETERMINATION OF R AND S TRIMIPRAMINE IN HUMAN SERUM USING AN OCTYLDECYLSILANE COLUMN WITH β-CYCLODEXTRIN AS MOBILE PHASE ADDITIVE AND SOLID PHASE EXTRACTION

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ABSTRACT

A stereospecific HPLC method was developed for the analysis of the enantiomers of trimipramine in human serum. The assay uses amitriptyline as the internal standard and a C_{18} solid phase extraction column for serum sample clean-up. It is free of interference from desmethyl-trimipramine, 2-hydroxy-desmethyl-trimipramine and 2-hydroxy-trimipramine, the three major metabolites of trimipramine. Recoveries of 98.8% and 97.5% were obtained for the R and S enantiomers of trimipramine, respectively. Resolution of the enantiomers was obtained using an octyldecylsilane column with β-cyclodextrin as the mobile phase additive. The composition of the mobile phase was 80:20 v/v aqueous 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)- absolute ethanol containing 20mM β-cyclodextrin at a flow rate of 0.7mL/min.

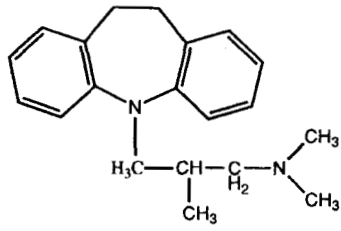
Linear calibration curves were obtained in the 25-400ng/mL range for each enantiomer in serum. The detection limit based on a S/N = 3 was 10ng/mL for each enantiomer in serum with UV detection at 220nm. The limit of quantitation for each enantiomer was 25ng/mL. Precision calculated as %RSD and accuracy calculated as % error were in the range 0.7-4.5% and 0.9-3.1%, respectively, for the R enantiomer and 0.7-5.1% and 0.4-4.4% respectively, for the S enantiomer. Separation of the three major metabolites of trimipramine was also investigated.

INTRODUCTION

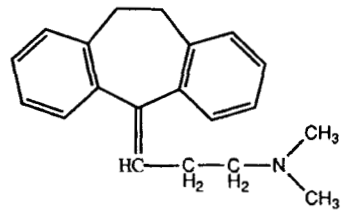
Trimipramine, 10,11-dihydro-N,N,β-trimethyl-5H-dibenz[b,f]azepine-5-propanamine is a dibenzazepine-derivative tricyclic antidepressant (TCA) commonly used for its antidepressant and anxiolytic activity (Figure 1). TCAs generally act by blocking the uptake of neuronal norepinephrine and serotonin in varying degrees.[1] Monitoring therapeutic levels of TCAs in serum as an adjunct to the clinical management of patients with depression is important since the side effects of these drugs are quite common and mainly dose related.[2]

Trimipramine and its three major metabolites, desmethyl-trimipramine, 2-hydroxy-trimipramine and 2-hydroxy-desmethyl-trimipramine, are chiral compounds with an asymmetric center at the side chain. Hydroxylation and desmethylation are the two major pathways of the metabolism of trimipramine (Figure 2). Although the chemical structure of trimipramine is closely related to other TCAs, it differs from many of them in some pharmacological aspects.[3] The most striking difference is the induction of supersensitivity of the noradrenergic system after long term treatment with trimipramine.[4] Differences in the physiological and behavioral effects induced by the two enantiomers have been demonstrated.[5] It has also been found that the enantiomers show different affinities for the D1 and D2 (dopamine), the $\alpha_{1A/B}$, α_{2A}, α_{2B} (noradrenaline) and the 5-hydroxytryptamine ($5\text{-}HT_2$) receptor subtypes.[3]

Cyclodextrins are the most commonly used chiral selectors in HPLC chiral separations. Native cyclodextrins such as α, β, and γ cyclodextrins are neutral natural cyclic oligosaccharides containing six, seven and eight glucose units, respectively. The shape of a cyclodextrin is similar to that of a truncated cone with a cavity that is hydrophobic within and hydrophilic outside. Enantiorecognition with cyclodextrins is a combination of inclusion phenomenon and additional interactions with the hydroxyl functional groups on the rim of the cyclodextrin.



TRIMIPRAMINE



AMITRIPTYLINE

Figure 1. Chemical structures of trimipramine and the internal standard amitriptyline.

Some of the earlier methods reported for the determination of racemic trimipramine in body fluids used gas chromatography[6-7] and high performance liquid chromatography.[8-11] Chiral separation of trimipramine enantiomers has also been reported.[12-16] The only method reported for the separation of trimipramine enantiomers and its major metabolites in serum used liquid-liquid extraction for sample pretreatment which was time consuming.[12]

This paper describes the separation and quantitation of trimipramine enantiomers in human serum and is free of interference from the three major metabolites, desmethyl, 2-hydroxy-trimipramine, and 2-hydroxy-desmethyl-trimipramine. The method involves solid phase extraction followed by separation on an octyldecylsilane column with β-cyclodextrin as the mobile phase additive and UV detection. The method is linear in the range 25-400ng/mL of each enantiomer.

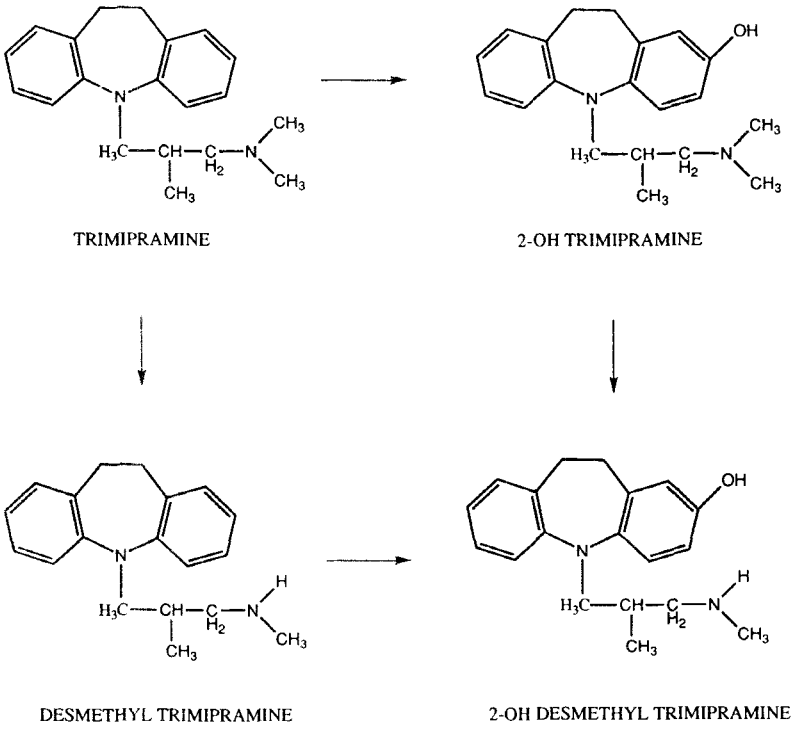


Figure 2. Major metabolic pathways of trimipramine in humans.

EXPERIMENTAL

Reagents and Chemicals

Trimipramine maleate was purchased from Sigma Chemical Co. (St. Louis, MO, USA). R and S trimipramine were a gift from Rhone-Poulenc Rorer Centre de Recherches (Vitry-Alforville, France). Desmethyl-trimipramine maleate, 2-hydroxy-desmethyl-trimipramine fumarate, and 2-hydroxy-trimipramine fumarate were kindly supplied by Dr. C. B. Eap of Hospital de Cerry (CH-1008 Prilly-Lausanne, Switzerland). Amitriptyline hydrochloride was obtained from Merck Sharp & Dohme (Rahway, NJ, USA). Absolute ethyl alcohol USP (200 proof) was purchased from Aaper Alcohol & Chemical Co. (Shelbyville, KY, USA) and ammonium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). β-cyclodextrin (β-CD) was supplied by American

Maize Products Company (Hammond, IN, USA). All solvents were HPLC grade. Drug free human serum was obtained from Biological Specialty Corporation (Colmar, PA, USA). The C_{18}, C_8 and CN solid phase extraction columns (100mg/cc) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA).

Instrumentation

The HPLC system consisted of a Beckman Model 110A pump (Beckman, San Ramon, CA, USA) and a Model 728 autosampler (Micromertics Instruments Corporation, Norcross, GA, USA) equipped with a 20μL loop. The detector was a Waters Millipore Model 481 LC Spectrophotometer (Milford, MA USA) and a Spectra-Physics Model 4270 integrator (Spectra-Physics, San Jose, CA, USA) was used to record each chromatogram and peak height responses. Separation of the analytes was achieved on a Prodigy 5μ ODS(3) 100Å column(150x3.20mm i.d., Phenomenex, Torrance, CA) equipped with a 0.2μ Opti-solv precolumn minifilter (Optimize Technologies, Portland, OR, USA).

The mobile phase consisted of 80:20 v/v aqueous 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)- absolute ethanol containing 20mM β-CD and it was delivered at a flow rate of 0.7mL/min. The mobile phase was filtered through a 0.45μm filter (Alltech Associates, Deerfield, IL USA) and sonicated prior to use. The column was operated at ambient temperature (23±1°C).

Preparation of Stock and Standard Solutions

Individual stock solutions of 100μg/mL of R and S trimipramine (maleate salts) calculated as trimipramine base and 100μg/mL of internal standard amitriptyline were prepared in 10mL volumetric flasks by adding 2mL of methanol followed by the addition of deionized water to volume. The solutions were kept refrigerated at 4°C. Stock solutions of 10μg/mL of racemic 2-hydroxy-desmethyl-trimipramine (fumarate salt), 2-hydroxy-trimipramine (fumarate salt) and desmethyl trimipramine (maleate salt) calculated as their free bases were also prepared and kept refrigerated at 4°C.

Appropriate dilutions of the R and S trimipramine stock solutions gave 5μg/mL solutions which were used for spiking blank human serum.

Preparation of Spiked Human Serum Samples

Accurately measured aliquots (10, 30, and 50μL of the 5μg/mL standard solutions) of R and S trimipramine diluted solutions were pipetted into a 1mL volumetric tube and evaporated. Then 10μL of the internal standard solution was added to the tubes and drug-free human serum added to volume and mixed well to give final concentrations of 50, 150, and 250ng/mL of each trimipramine enantiomer.

Assay Method

One milliliter of the spiked human serum samples were vortexed for 2min and then passed through a C_{18} Bond-Elut solid phase extraction (SPE) column attached to a vacuum manifold (Vac-Elut, Varian Sample Preparation Products, Harbor City, CA USA). The column was previously conditioned with 2x1mL of absolute methanol followed by 2x1mL of deionized water. After the application of the serum sample, the column was washed with 2x1mL deionized water and the analytes were eluted with 4x125μL of 10mM acetic acid in methanol and evaporated to dryness under a slow nitrogen stream. The residue was reconstituted in 1mL of mobile phase, filtered through a 0.2μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA) and 20μL injected into the liquid chromatograph.

For absolute recovery experiments, spiked samples were compared to unextracted stock solutions. Drug peak height ratios were used to calculate the recoveries. Linear regression analysis of the peak-height ratios of each enantiomer to internal standard versus concentration of each enantiomer in the range of 25-400 ng/mL produced slope and intercept data which were used to calculate concentrations of R and S trimipramine in each serum sample.

RESULTS AND DISCUSSION

When cyclodextrins are used as chiral mobile phase additives in reversed phase liquid chromatography, the separation mechanism is thought to be a combination of inclusion phenomenon and the additional interaction of the analyte with the functional groups on the rim of the cyclodextrin. Among the factors that control the enantioseparation process are (i) differences in the stability/binding constants of the CD complexes, (ii) differences in the adsorption of CD complexes on the surface of the stationary phase, and (iii) differences in the adsorption of free solute molecules on the CD layer that is adsorbed on the surface.[17] Hence the differences in inclusion complex

strengths between solutes and the CD cavity, as well as differences in the interaction with the rim functional groups, can result in improved chromatographic separations.

The enantiomers of trimipramine were successfully resolved on a nonporous octyldecylsilane column in our laboratory with β-CD as the mobile phase additive.[18] The bulky nature of trimipramine forms inclusion complexes with native β-CD and the side chains interact favorably with the CD rim. It is important to note that trimipramine enantiomers failed to separate with hydroxypropyl-β-CD because of the lack of any hydrogen bonding functional groups at or near the chiral center of the analyte with which to interact with the hydroxypropyl groups on the derivatized CD.

The nonporous column would not successfully resolve desmethyl trimipramine, one of the metabolites of trimipramine from the parent trimipramine despite modifications of the components of the mobile phase. A Prodigy ODS(3) column was, therefore, investigated because of its reported success of using the technique of β-CDs as mobile phase additives for chiral separation.[19] The Prodigy column successfully resolved the enantiomers of trimipramine and desmethyl-trimipramine. The mobile phase composition was 80:20 v/v 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)-absolute ethanol containing 20mM β-CD.

The influences of β-CD, ethanol and ammonium acetate buffer concentrations on the resolution of trimipramine and the three metabolites were investigated. Typically, the use of sodium or potassium phosphate buffers failed to resolve any of the analytes. An increase in the concentration of ethanol reduced retention times for all the analytes, but resolution was either reduced or lost completely as in the case of 2-hydroxy-desmethyl trimipramine and 2-hydroxy trimipramine.

The method is free of interference from human serum containing trimipramine and the three metabolites. Good resolution was obtained for the enantiomers of desmethyl trimipramine, partial resolution for the enantiomers of 2-hydroxy trimipramine, and no resolution for the enantiomers of 2-hydroxy-desmethyl trimipramine. Typical HPLC chromatograms for both blank human serum and serum spiked with 150ng/mL of each enantiomer of trimipramine and the three metabolites and the internal standard are shown in Fig.3.

The two more polar metabolites, 2-hydroxy-desmethyl trimipramine and 2-hydroxy trimipramine had shorter retention times and were not well resolved on the Prodigy column compared to the less polar desmethyl trimipramine.

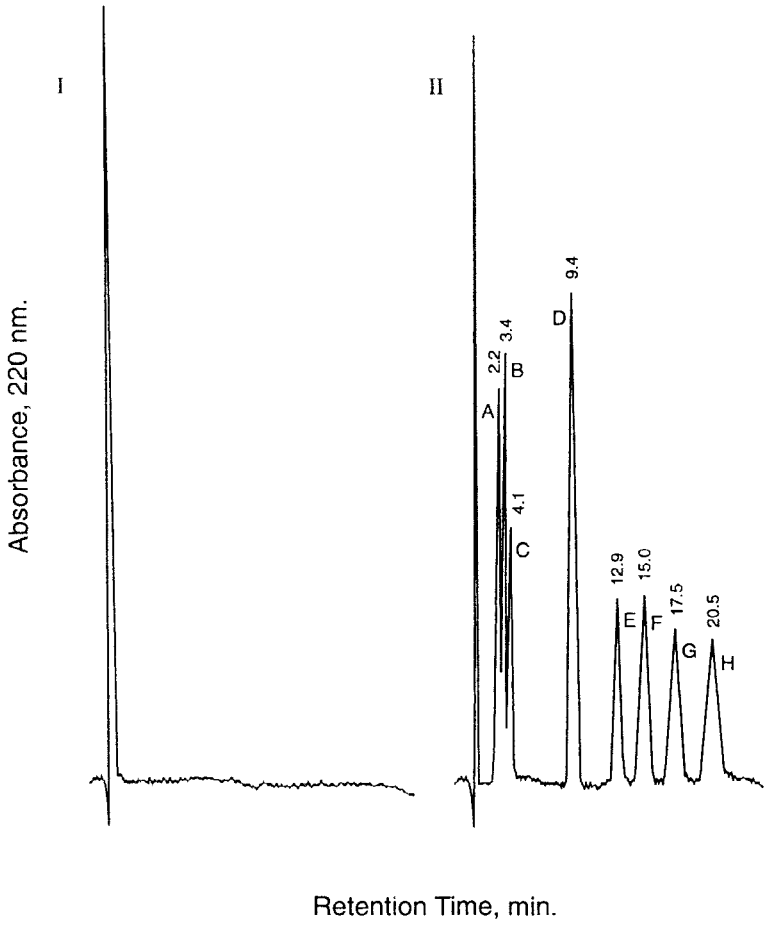


Figure 3. Typical chromatograms of (I) blank serum (left) and (II) serum spiked with 50ng/ml. of each enantiomer of trimipramine, desmethyl-trimipramine, 2-hydroxy-desmethyl-trimipramine, 2-hydroxy-trimipramine and the internal standard. Peaks: A and C = 2-hydroxy-trimipramine, B = unresolved racemic 2-hydroxy-desmethyl-trimipramine; D = internal standard; E = R-trimipramine enantiomer; F = S-trimipramine enantiomer; G and H = desmethyl-trimipramine.

Modifying the composition of the mobile phase to 95:5v/v 10mM ammonium acetate buffer pH 4 (adjusted with acetic acid)- absolute ethanol enabled 2-hydroxy-desmethyl trimipramine and 2-hydroxy trimipramine to be completely resolved (see Fig.4).

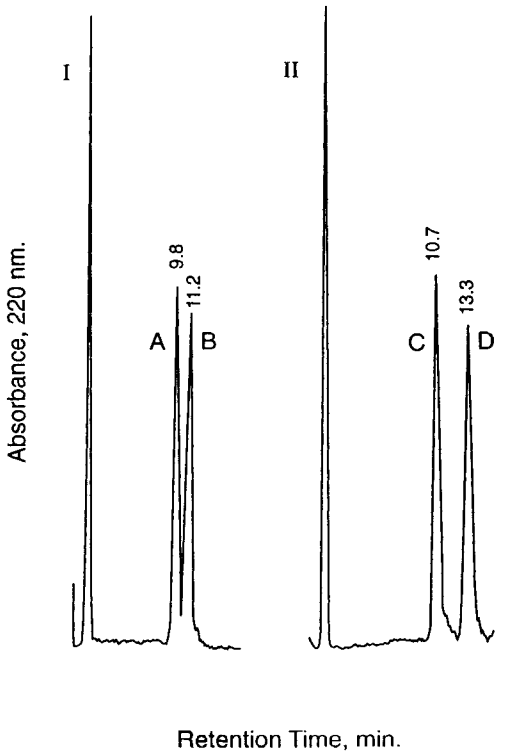


Figure 4. Separation of (I) 2-hydroxy-trimipramine enantiomers A and B and (II) 2-hydroxy-desmethyl-trimipramine C and D on the Prodigy ODS(3) column with mobile phase of 95:5v/v 10mM ammonium acetate buffer pH 4(adjusted with acetic acid)-absolute ethanol containing 20mM β-CD.

To decrease sample preparation time, a solid phase extraction (SPE) procedure was developed. Initial solid phase extraction experiments using ethylsilane, octylsilane, and cyanopropyl cartridges gave less than 70% recoveries of the two enantiomers. The octadecylsilane sorbent was selected because it allowed excellent recoveries of trimipramine enantiomers and the internal standard. Absolute recoveries of >98% (n=3) were obtained for each enantiomer with 10mM acetic acid in methanol. Addition of 10mM acetic acid to the methanol ionized the trimipramine at an acidic pH and hence reduced the affinity of the drug for the bonded phase silica. Amitriptyline was selected as the internal standard based on its structural similarity to trimipramine and also its similar extraction behavior to trimipramine (recovery of 99%).

Table 1

Accuracy and Precision Data for Trimipramine Enantiomers in Spiked Human Serum

Analyte	Conc. Added (ng/mL)	Conc. Found[a] (ng/mL)	Error (%)	RSD (%)
Intra-Day				
R trimipramine	50	49.32±1.57	1.4	3.2
	150	152.17±1.96	1.5	1.3
	250	247.63±4.08	0.9	1.6
S trimipramine	50	48.90±2.47	2.2	5.1
	150	151.27±2.92	0.9	1.9
	250	253.15±3.68	1.3	1.5
Inter-Day				
R trimipramine	50	48.45±2.16	3.1	4.5
	150	151.85±2.07	1.2	1.4
	250	252.19±1.85	0.9	0.7
S trimipramine	50	47.80±1.87	4.4	3.9
	150	148.67±2.35	0.9	1.6
	250	249.12±1.86	0.4	0.7

[a] Based on n=3 for intra-day assay and n=9 for inter-day assay.

Linear calibration curves were obtained in the 25-400ng/mL range for each trimipramine enantiomer. Standard curves were fitted to the linear regression equation $y=ax+b$, where y represents the ratio of drug/internal standard peak heights, a and b are constants, and x is the trimipramine concentration. Typical regression parameters of a (slope), b (y-intercept), and correlation coefficient were calculated to be 0.00151, 0.006525, and 0.99984, respectively, for R trimipramine and 0.00147, 0.01789, and 0.9994, respectively, for S trimipramine (n=3). The precision, calculated as %RSD and accuracy calculated as % error of the method, was ascertained using spiked samples at 50, 150, and 250ng/mL levels.

The intraday precision and accuracy as expressed by %RSD and %error were in the range 1.3-3.2% and 0.9-1.4% (n=3), respectively, for the R trimipramine and 1.5-5.1% and 0.9-2.2% (n=3), respectively, for the S enantiomer. The interday precision and accuracy were in the range 0.7-4.5% and 0.9-3.1% (n=9), respectively, for the R enantiomer and 0.7-3.9% and 0.4-4.4% (n=9), respectively, for the S enantiomer.

The detailed data is listed in Table 1. The minimum detectable concentration of each enantiomer was determined to be 10ng/mL (S/N=3). The limit of quantitation was found to be 25ng/mL for each enantiomer.

In conclusion, an HPLC method has been developed and validated for the assay of R and S trimipramine in human serum using β-CD as the mobile phase additive and a Prodigy ODS(3) column. The method uses an octadecylsilane solid phase extraction for sample clean-up and is applicable to the separation and quantitation of each trimipramine enantiomer in the 25-400ng/mL range. In addition, the method is free of interference from 2-hydroxy-desmethyl trimipramine, 2-hydroxy trimipramine, and desmethyl trimipramine, the three major metabolites of trimipramine.

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