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Quantitation of promethazine enantiomers in human serum using a chiralcel OJ-R column and mixed-mode disc solid-phase extraction

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Abstract

A liquid chromatographic method was developed for the assay of $R(+)$- and $S(-)$-promethazine enantiomers from human serum. The method involves the use of the mixed mode disc solid-phase extraction technique for sample clean-up. Chromatographic resolution of the enantiomers was performed on a reversed-phase cellulose-based chiral column (Chiralcel OJ-R) under isocratic conditions using a mobile phase consisting of 0.5 M aqueous sodium perchlorate/acetonitrile (63:37, v/v) at a flow rate of 0.5 ml min^{-1}. Recoveries in the range of 97–99% at 20 ng ml^{-1} levels were obtained for both promethazine enantiomers. Intra-day and inter-day precision calculated as R.S.D.% was in the 3–8% ranges for both enantiomers. Intra-day and inter-day accuracy calculated as percent error was in the 0–10 and 1–7% ranges for both enantiomers, respectively. Linear calibration curves were obtained for each enantiomer in serum in the concentration range 5–90 ng ml^{-1}. The limit of quantitation of each enantiomer was 10 ng ml^{-1}. The detection limit for each enantiomer in serum using UV detection at 249 nm was 2 ng ml^{-1} (S/N = 2).

Keywords: Promethazine; Enantiomers; Reversed-phase LC; Serum

1. Introduction

Promethazine, 10-[2-(dimethylamino)propyl]-phenothiazine, possesses a moderately potent antihistaminic action, an antiemetic effect, a tranquilizing action, and a potentiating action on analgesic and sedative drugs. The drug possesses one asymmetric center and has been developed as the racemate (Fig. 1). It is clinically used as an antihistamine or as a sedative and antiemetic. Plasma concentrations of promethazine in human range from 2–20 ng ml^{-1} [1–3].

Racemic promethazine in biological fluids has been assayed by reversed-phase high performance liquid chromatography with ultraviolet or electrochemical detection [1–6]. Almost all of the methods involve time-consuming liquid–liquid extraction steps. Only one method has been devel-

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oped for the determination of $R(+)$- and $S(-)$-promethazine enantiomers in human serum and urine [7]. It involved the use of a brush-type chiral column KK-CARNU operated in the normal-phase mode, standard addition method for the analysis of promethazine enantiomers, and relatively tedious mixed-mode cartridge solid-phase extraction procedures.

This paper describes a simple and sensitive assay for the determination of promethazine enantiomers in human serum using a Chiralcel OJ-R column and mixed-mode disc solid-phase extraction procedures. The method is linear over the range 5–90 ng ml^{-1} using UV detection at 249 nm. The detection limit of the procedure for each enantiomer was 2 ng ml^{-1} (S/N = 2).

2. Experimental

2.1. Reagents and chemicals

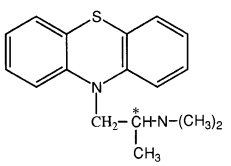
Racemic promethazine hydrochloride and the internal standard verapamil hydrochloride were supplied by Sigma (St. Louis, MO). The $R(+)$- and $S(-)$-promethazine enantiomers were synthesized in our laboratories by Dr Garratt W. Ponder through crystallization using D- and L-dibenzoylated tartaric acid, and analyzed for purity using liquid chromatography and polarimetry [8]. The purity of the individual promethazine enantiomers was determined to be more than 99% (w/w). HPLC-grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ). HPLC-grade triethylamine and sodium perchlorate were obtained from Fisher Scientific (Pittsburgh, PA). Drug-free human serum was obtained from Biological Specialty Corporation (Colmar, PA).

2.2. Apparatus

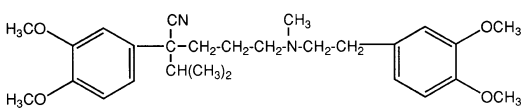
The HPLC system consisted of an Alcott Model 760 HPLC pump (Norcross, GA), an Alcott Model 728 auto-sampler (Norcross, GA, USA) equipped with a 100 μl loop, a Kratos Model 757 variable wavelength UV-Vis detector (Ramsey, NJ) set at 249 nm and a Hewlett-Packard Model 3395 integrator (Avondale, PA). The Chiralcel OJ-R column (5 μm, 150 mm × 4.6 mm I.D.) equipped with an opti-guard guard column RP C8 (1.5 cm × 1 mm I.D., Optimize Technologies, Portland, OR) was obtained from Chiral Technologies, (Exton, PA) and operated at ambient temperature. C18, C8, MP3, and PLUS™•MP3 solid-phase extraction discs (15 mg/3 cc size) were obtained from Ansys. Inc (Irvine, CA). C18 and C8 solid-phase extraction cartridges (100 mg/1 cc size) were purchased from Varian Sample Preparation Products (Harbor City, CA). The Vac-Elut vacuum manifold was obtained from Analytichem (Sunnydale, CA).

2.3. Preparation of the mobile phase

The mobile phase consisted of 0.5 M aqueous sodium perchlorate/acetonitrile (63:37, 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) and sonicated prior to use.



Promethazine



Verapamil (IS)

Fig. 1. Chemical structures of promethazine and verapamil (IS).

2.4. Preparation of standard solutions

Stock solutions of 0.1 mg ml^{-1} of $R(+)$- and $S(-)$-promethazine enantiomers (hydrochloride salts) calculated as promethazine base were prepared in double distilled deionized water and stored protected from light at 4°C. A stock solution of 0.3 mg ml^{-1} of internal standard verapamil (hydrochloride salt) calculated as the free base was also prepared in the same manner as stock solutions of promethazine enantiomers and stored protected from light at 4°C. Both solutions were weekly prepared. Appropriate dilutions of the $R(+)$- and $S(-)$-promethazine enantiomer stock solutions with deionized water gave 0.5 and 5 μg ml^{-1} solutions which were used for spiking blank human serum.

2.5. Preparation of spiked human serum samples

Accurately measured aliquots (10, 30, and 50 μl of the 0.5 μg ml^{-1} standard solutions, 15 and 18 μl of the 5 μg ml^{-1} standard solutions) of $R(+)$- and $S(-)$-promethazine enantiomers were each added into 1 ml volumetric tubes followed by the addition of 20 μl of 0.3 mg ml^{-1} of internal standard solution. Drug-free human serum was added to volume and mixed well to give final concentrations of 5, 15, 25, 75, and 90 ng ml^{-1} of each enantiomer.

2.6. Assay method

PLUS™•MP3 solid-phase extraction (SPE) discs were attached to a vacuum manifold and conditioned with 0.2 ml of absolute methanol followed by 0.4 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 1.5 ml of 0.1 M potassium phosphate monobasic pH 6.0 methanol (100:40, v/v) and mixed well. Into the discs were transferred blank and diluted spiked human serum samples and a small vacuum (about 2 kPa) was applied. After the entire serum sample had been aspirated through the disc, the disc was washed with 2×300 μl of water/methanol (2:1, v/v), and then dried under full vacuum for a minimum of 5 min. Then, the vacuum manifold was opened and collection tips were wiped. The promethazine enantiomers and internal standard were eluted with 4×300 ml of acetonitrile/triethylamine (100:2, v/v) (freshly prepared). 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.

2.7. Calculations

The peak areas of each promethazine enantiomer and the internal standard were recorded for each sample. Five concentrations in the range 5–90 ng ml^{-1} of the enantiomers were used to construct the calibration curves. Linear regression analysis of the concentration of each promethazine enantiomer versus the ratio of drug to internal standard peak areas (D/IS) produced slope and intercept data. The concentration of each promethazine enantiomer in a given serum sample was calculated from the equation: promethazine enantiomer conc. = (slope)(D/IS) + intercept.

3. Results and discussion

Previous work in our laboratories showed that promethazine enantiomers were resolved on several normal phase chiral stationary phases such as Chiralcel OJ, KK-CARNU, and Sumichiral OA 4700 [9]. The Chiralcel OJ-R column employs the same chiral selector [cellulose tris(4-methylbenzoate)] as its normal phase counterpart (Chiralcel OJ), but is designed for analytical applications using reversed-phase chromatography. The chiral selectivity in both columns involves similar interactions such as hydrogen bonding, dipole–dipole interactions, π-π interactions, and the formation of inclusion complexes. However, the Chiralcel OJ-R column is more ideal than Chiralcel OJ for the analysis of chiral drugs since many chiral drugs exist as salt forms and are more water-soluble. In addition, compared with the normal-phase mode, the reversed-phase mode is more friendly

to the environment since less organic waste is produced and harmful organic solvents such as hexane, chloroform, and methylene chloride are not used in the mobile phases.

Initial baseline separation (Rs 2.3) of $R(+)$- and $S(-)$-promethazine enantiomers on the Chiralcel OJ-R column was obtained using a mobile phase containing 0.5 M aqueous sodium perchlorate/acetonitrile (70:30, v/v) with retention times in the range of 22–30 min. The influences of sodium perchlorate and acetonitrile concentrations in mobile phase on separation were investigated. A decrease in sodium perchlorate concentration or an increase in acetonitrile concentration in mobile phase reduced the retention times with some loss of resolution of promethazine enantiomers. The effect of buffer salt type (phosphate vs. perchlorate) was also investigated. There was no resolution between promethazine enantiomers using aqueous monobasic potassium phosphate in the mobile phase. The final composition of the mobile phase was selected to be 0.5 M sodium perchlorate/acetonitrile (63:37, v/v) since it provided good resolution of the two enantiomer peaks (Rs 1.8) and gave suitable retention times (12–15 min) with sensitivity in the desired ng ml^{-1} range. Typical HPLC chromatograms for blank human serum and serum spiked with 50 ng ml^{-1} of each enantiomer and 6 μg ml^{-1} of internal standard are shown in Fig. 2. No interferences were observed in blank human serum at the retention times of $R(+)$- and $S(-)$-promethazine peaks.

The selection of verapamil as internal standard was based on its suitable retention time (9.5 min), a separation factor (α) of 1.50 between verapamil and the first eluting $R(+)$-promethazine enantiomer and reproducible recovery (97%). Quantitation was based on a plot of the concentration of each enantiomer versus peak-area ratio of each promethazine enantiomer to internal standard.

The suitability of the chromatographic system for the separation of the promethazine enantiomers is shown in Table 1. The retention times of $R(+)$- and $S(-)$-promethazine enantiomers and internal standard verapamil were 12.23 ± 0.006, 14.12 ± 0.013, and 9.52 ± 0.004 min, respectively ($n = 6$). Retention factors (k) for

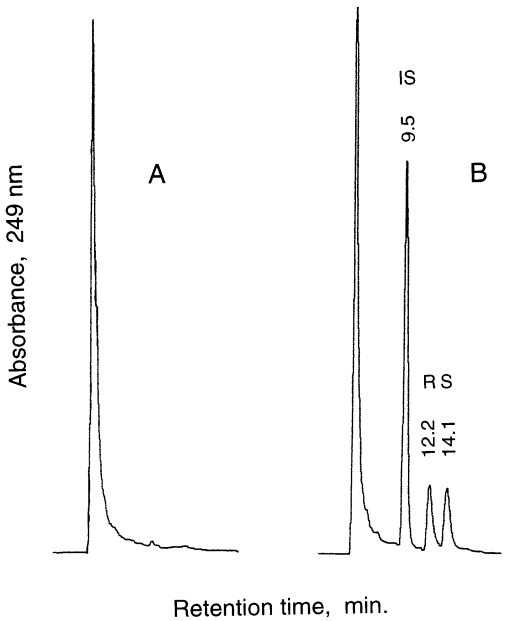


Fig. 2. Typical chromatograms of (A) blank serum and (B) serum spiked with 50 ng ml^{-1} of each enantiomer and 6 μg ml^{-1} of internal standard. Peaks: R, $R(+)$-promethazine; S, $S(-)$-promethazine; and IS, internal standard, verapamil.

$R(+)$- and $S(-)$-promethazine enantiomers and internal standard were 2.06 ± 0.011, 2.53 ± 0.014, and 1.38 ± 0.008, respectively ($n = 6$). The calculated theoretical plates for $R(+)$- and $S(-)$-promethazine enantiomers were 2856 ± 135 and 2600 ± 90 per 15 cm column ($n = 6$). Relative retention of the $R(+)$- and $S(-)$-promethazine enantiomers was expressed by the separation factor α, calculated to be 1.23. Resolution (Rs) of the internal standard and first eluting $R(+)$- enantiomer peaks and of the $R(+)$- and $S(-)$-promethazine enantiomer peaks were 2.9 and 1.8, respectively.

Two solid-phase extraction cartridges (C18 and C8) and four solid-phase extraction discs (C18, C8, MP3, and PLUS™•MP3) were investigated for serum sample clean-up prior to the HPLC assay. The C8 cartridge and C8 disc showed no recoveries for $R(+)$- and $S(-)$- promethazine peaks. The C18 cartridge and C18 disc were found to be unacceptable due to co-elution of endogenous serum components with

Table 1
Chromatographic parameter data for promethazine enantiomers and internal standard in spiked human serum samples

Analyte	Rs	α	Mean ± S.D. ($n = 6$)		
			k	t_R (min)	N
Verapamil (IS)			1.38 ± 0.008	9.52 ± 0.004	—
	2.9	1.50			
$R(+)$-Promethazine			2.06 ± 0.011	12.23 ± 0.006	2856 ± 135
	1.8	1.23			
$S(-)$-Promethazine			2.53 ± 0.014	14.12 ± 0.013	2600 ± 90

α, Separation factor, calculated as k_2/k_1.
N, Theoretical plates, calculated as $N = 16\ (t_R/w)^2$
—, Not calculated.

promethazine enantiomer peaks. The PLUS™•MP3 disc provided the best results in terms of clean-up and recoveries of $R(+)$- and $S(-)$- promethazine enantiomers. The MP3 and PLUS™•MP3 discs are mixed-mode solid-phase extraction discs with slightly polar and strong cation-exchange phases. The discs were designed for the extraction of trace analytes from biological fluids. To condition a mixed-mode disc, it is necessary to perform reversed-phase conditioning with solvents such as methanol followed by ion exchange conditioning with a buffer. The MP3 and PLUS™•MP3 discs consist of the same sorbent. The only difference between them is that the PLUS™•MP3 disc has a filter on the top of the sorbent. Without the filter, the MP3 disc easily clogged although it provided a similar clean-up result as the PLUS™•MP3 disc. 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 promethazine enantiomers and internal standard were more easily eluted.

The recoveries of $R(+)$- and $S(-)$-promethazine enantiomers from human serum were assessed by using spiked samples at several different concentration levels. The absolute recoveries of $R(+)$- and $S(-)$-promethazine 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 5–90 ng ml^{-1} range for each enantiomer. Standard curves were fitted to a first degree polynomial, $y = ax + b$, where y is the concentration of promethazine enantiomer, x is the ratio of drug/internal standard peak areas, and a and b are constants. Typical values for the regression parameters a (slope), b (y-intercept), and correlation coefficient were calculated to be 194.6183, 2.307942, and 0.9988 for $R(+)$-enantiomer, and 177.2424, 0.380608, and 0.9998 for $S(-)$-enantiomer, respectively ($n = 10$). The precision and accuracy (percent error) of the method were determined by using human serum samples spiked at 10, 20, and 50 ng ml^{-1} levels (Table 3). The data indicates that intra-day precision was in the 3–8% range ($n = 3$) and intra-day accuracy in the 0–10% range ($n = 3$) for both promethazine enantiomers and that inter-day precision was in the 3–8% range ($n = 4$) and inter-day accuracy in the 1–7% range ($n = 4$) for both promethazine enantiomers.

The minimum detectable concentration of each enantiomer was determined to be 2 ng ml^{-1} (S/N = 2). The limits of quantitation were found to be 10 ng ml^{-1} for each enantiomer: $R(+)$, 7.7% R.S.D., 10% error; $S(-)$, 5.0% R.S.D., 4.6% error.

Table 2
Recovery data for promethazine enantiomers and internal standard in spiked human serum samples

Analyte	Concentration added (ng ml^{-1})	Recovery[a] (%) (mean ± S.D., $n = 6$)	R.S.D. (%)
R(+)-Promethazine	10	92.91 ± 4.77	5.1
	20	97.24 ± 3.68	3.8
	50	97.70 ± 3.35	3.4
S(−)-Promethazine	10	89.56 ± 1.65	1.8
	20	99.63 ± 3.85	3.9
	50	95.56 ± 4.03	4.2
Verapamil (IS)	6000	97.46 ± 2.41	2.5

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

Table 3
Accuracy and precision data for promethazine enantiomers in spiked human serum samples

Analyte	Concentration added (ng ml^{-1})	Concentration found[a] (ng ml^{-1})	Error (%)	R.S.D. (%)
Intra-day				
R(+)-Promethazine	10	11.00 ± 0.85	10.0	7.7
	20	20.00 ± 1.30	0.0	6.5
	50	49.46 ± 1.61	1.1	3.3
S(−)-Promethazine	10	9.54 ± 0.48	4.6	5.0
	20	20.51 ± 0.92	2.6	4.5
	50	48.07 ± 1.60	3.9	3.3
Inter-day				
R(+)-Promethazine	10	10.40 ± 0.64	4.0	6.2
	20	20.89 ± 0.81	4.5	3.9
	50	51.22 ± 1.84	2.4	3.6
S(−)-Promethazine	10	9.76 ± 0.62	2.4	6.4
	20	21.38 ± 1.10	6.9	5.1
	50	50.53 ± 3.57	1.1	7.1

[a] Based on $n = 3$ for the intra-day study and $n = 4$ for the inter-day study.

4. Conclusions

An HPLC method has been developed and validated for the assay of *R*(+)- and *S*(−)-promethazine enantiomers in human serum using a Chiralcel OJ-R column operated in the reversed mode and a mixed-mode disc solid-phase extraction procedure for sample clean-up prior to HPLC analysis. The method is suitable for the separation and quantification of each enantiomer in 5–90 ng ml^{-1} range. If additional analyte sensitivity is needed due to the drug dosage schedule or mode of administration, it is recommended that a 3 ml serum sample be extracted and the residue redissolved in 300 μl of the mobile phase prior to HPLC analysis. This modification will provide a linear concentration range of 0.8–15 ng ml^{-1}.

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