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Rapid, sensitive high-performance liquid chromatographic method for the quantification of promethazine in human serum with electrochemical detection

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ABSTRACT

A method of analysis has been developed to quantify promethazine in human serum with a sensitivity that was suitable for bioavailability studies following a 50.0-mg rectal dose. The limit of quantification from 1.0 ml of serum for promethazine using electrochemical detection was 0.200 ng/ml. At this concentration, the total coefficient of variation obtained from seven replicates over the course of three days of validation was 7.53%. The amount of serum required, the ease of sample preparation and the precision of the method at the limit of quantification demonstrated an improvement over previous assays. A validation study was completed that included an evaluation of recovery, ruggedness, linearity of response, accuracy, precision, sensitivity, stability and selectivity. The method was then used to determine promethazine serum levels in a 36-subject bioavailability study following a 50.0-mg suppository dose.

INTRODUCTION

The objective of this work was to develop and validate an HPLC procedure for the determination of promethazine in human serum for use in the bioavailability testing of formulations of promethazine. Due to the anticipated levels and limited sample volume, it was important to improve the precision at the limit of quantification of the assay. A 1-ml volume of serum was required in order to achieve a limit of quantification of 0.200 ng/ml.

The amount of serum required, the ease of sample preparation and the precision of the method at the limit of quantification demonstrated an improvement over previous assays [1,2].

A three-day validation included an evaluation of recovery, ruggedness, linearity of response, accuracy, precision, sensitivity, stability and selectivity. The validated method was subsequently used for the determination of promethazine in human serum following a 50.0-mg suppository dose. The precision of the method following the analysis of 36 subjects was 6.98% at 0.200 ng/ml.

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EXPERIMENTAL

Instrumentation and reagents

All reagents were analytical grade. The following reagents were used during the validation and the analysis of the subject samples: ethyl acetate, pentane, methanol, acetonitrile, isopropanol, water, glacial acetic acid, ammonium hydroxide and sodium carbonate. Human serum was obtained from Biological Specialities (Landsdale, PA, USA). Reference standards for promethazine and chlorpromazine hydrochloride (internal standard) were obtained from the USP.

The instrumentation used in this study included a Spectroflow 400 isocratic pump (ABI Analytical), a Model ISS-100 autosampler (Perkin Elmer), a Model 5100A Coulochem detector (ESA) and a Model 5011 analytical cell (ESA). Analog signals from the detector were converted to a digital output and stored in a VAX computer. Subsequently, these

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signals were integrated using Waters 860 (Version 2.2) software.

Chromatographic conditions

A Burdick & Jackson 15 cm × 4.6 mm I.D., 5 μm CN column was used for the analysis. The mobile phase consisted of acetonitrile–methanol–isopropanol–HPLC-grade water–1.0 *M* ammonium acetate, pH 7.2 (83:5:5:6.65:0.35) at a flow-rate of 1.5 ml/min. The potential for detector 1 was +0.50 V and the potential for detector 2 was +0.70 V. The gain on detector 2 was set at 10 × 4.

Sample preparation

A 1-ml sample was pipetted into a 125 mm × 16 mm culture tube. A 100-μl volume of chlorpromazine hydrochloride (100 ng base per ml) was added to each sample. The samples were then vortexed for 10 s at low speed. A 1-ml volume of 0.65 *M* sodium carbonate solution was added to each sample followed by vortex-mixing. A 7-ml volume of the extraction solvent (pentane–ethyl acetate, 50:50) was added to each sample. The tubes were capped with polyethylene stoppers and shaken vigorously for 15 min. The samples were then centrifuged for 10 min at 1110 *g*. The organic layer was tranferred to a 100 mm × 16 mm culture tube and evaporated to dryness at 65°C in a dry heating block with a nitrogen purge. The samples were then reconstituted in 300 μl of a solution containing acetonitrile–methanol–isopropanol–HPLC-grade water–1.0 *M* ammonium acetate, pH 5.0 (83:5:5:6.65:0.35), sonicated for 5 min, vortexed for 30 s, and 100-μl samples were injected. The run time was 15 min. The retention time for promethazine was between 7.0 and 8.5 min and between 9.5 and 11.0 min for chlorpromazine hydrochloride (internal standard).

RESULTS AND DISCUSSION

The combination of the extraction technique and chromatography provided an assay which was free from interfering endogenous serum peaks. Typical chromatograms for spiked human serum standards and a 7-h post-dose subject sample are shown in Fig. 1. The calibration standard line obtained for promethazine added to serum in amounts ranging from 0.200 to 15.0 ng/ml had a coefficient of determination of greater than 0.99. Best-fit calibration lines of chromatographic response *versus* concentration were determined by weighted least-squares regression analysis with a weighting factor of 1/concentration.

The intra-day precision at the limit of quantifica-

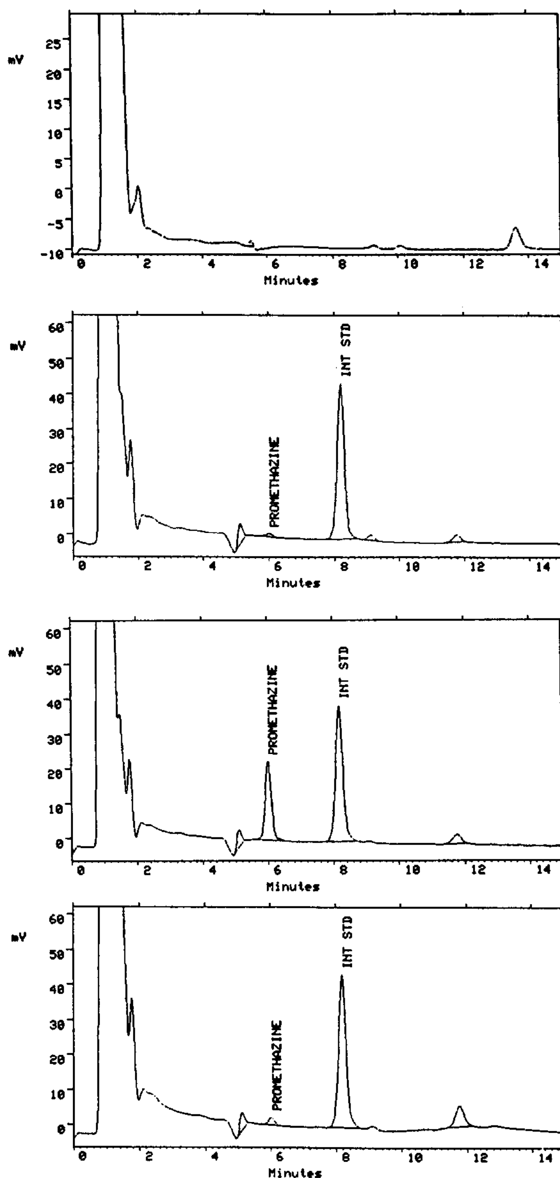

Fig. 1. Representative chromatograms of promethazine in human serum containing 0 (no internal standard), 0.500 and 10.0 ng/ml, respectively. The final chromatogram is a 7-hour post-dose subject sample.

TABLE I

INTER-DAY PRECISION AND ACCURACY DATA FOR A THREE-DAY VALIDATION FOR PROMETHAZINE IN HUMAN SERUM BY HPLC

Theoretical concentration (ng/ml)	Concentration measured (mean ± S.D.) (ng/ml)	Precision R.S.D. (%)	Accuracy (%)	n
0.200	0.220 ± 0.02	7.53	110	27
0.500	0.493 ± 0.04	9.04	98.6	8
1.00	0.982 ± 0.09	9.35	98.2	9
2.00	1.91 ± 0.11	5.88	95.5	29
5.00	4.73 ± 0.31	6.57	94.6	9
10.0	10.1 ± 0.38	3.78	101	30
15.0	15.5 ± 0.78	5.02	103	9

tion ranged from 3.98 to 5.95% R.S.D. during a three-day validation. Inter-day precision was 7.53% R.S.D. During analysis of a 36-subject study, the precision for the 0.200 ng/ml standard was 6.98% R.S.D. This sensitivity was achieved through the use of a mobile phase composition that maintained a low background for electrochemical detection. This low background resulted in an acceptable signal-to-noise ratio (6:1). It was important to achieve a sensitivity of 0.200 ng/ml in human serum because of limited sample volume available.

The inter-day precision of the method for promethazine ranged from 3.78 to 9.35%. The inter-day accuracy of the method ranged from 94.6 to 110% (see Table I).

TABLE II

RECOVERY OF PROMETHAZINE AND CHLORPROMAZINE (INTERNAL STANDARD) FROM HUMAN SERUM

Values in parentheses are R.S.D.s (%).

Concentration (ng/ml)	Mean recovery (%)	
	Promethazine (n = 7)	Chlorpromazine (n = 21)
10.0	102 (5.50)	N.A.[a]
2.00	98.6 (6.30)	N.A.[a]
0.200	112 (6.3)	N.A.[a]
33.3	N.A.[a]	74.3 (6.70)

[a] N.A. = not applicable.

The percentage recovery was determined by measuring the absolute peak heights of promethazine and chlorpromazine (the internal standard), respectively, from prepared serum validation samples at concentrations of 10.0, 2.00 and 0.200 ng/ml. The peak heights of the serum validation sample were compared to the absolute peak heights obtained by direct injection of aqueous standards in the reconstituting solution the same concentrations of promethazine and internal standard. The recovery for promethazine was 104%. The recovery for chlorpromazine (internal standard) was 74.3% (see Table II).

The issue of stability during storage and sample handling was addressed by several experiments. Stability was determined by measuring the concentration after two freeze–thaw cycles and after 24 h at room temperature (see Tables III and IV). During the course of the study, stability samples were analyzed. After two months at −20°C, 90.5% of the drug was remaining in the serum. The solution used to reconstitute the sample had a pH of 5.0 to ensure maximum autosampler stability. During the study, duplicate quality control samples containing 10.0, 2.00 and 0.500 ng/ml promethazine were determined after approximately 50 and 100% of the unknown samples. This sequence of sample analysis was used to monitor and confirm the stability of the analytes under actual conditions of the assay. There was no noticeable degradation of any of the ana-

TABLE III

STABILITY DATA FOR PROMETHAZINE IN HUMAN SERUM FOLLOWING A FREEZE–THAW CYCLE

Replicate No.	Promethazine concentration (ng/ml)	
	9.99 ng/ml[a]	0.458 ng/ml[a]
1	9.26	0.465
2	9.41	0.514
3	9.51	0.532
Mean	9.39	0.504
Precision (% R.S.D.)	1.34	6.88
Stability (%)	94.0	110
n	3	3

[a] Concentration at 0 h.

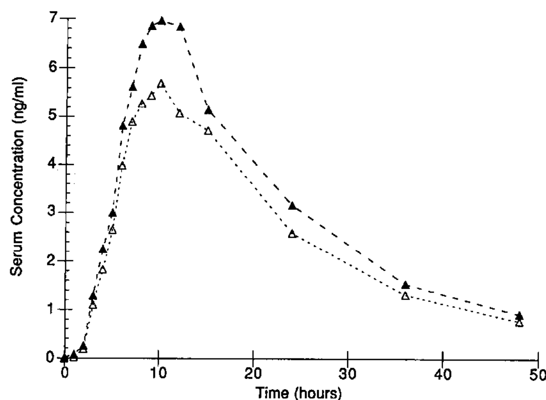

Fig. 2. Mean concentrations of promethazine measured in serum after administration of 50-mg suppositories from two different lots to 24 volunteers. ▲ = Lot 1; △ = lot 2.

lytes during the performance of the analytical procedure as demonstrated by the control values. The mean value for control samples ranged in accuracy from 104 to 109% with a precision ranging from 7.68 to 11.3%.

Ruggedness was evaluated by validating the method on two different columns. There was column-to-column variation in the tailing factor for both promethazine and chlorpromazine. This factor became the critical system suitability check for the assay. During the validation, the tailing factor ranged from 1.05 to 1.35 for promethazine and from 1.16 to 1.20 for chlorpromazine.

TABLE IV

STABILITY DATA FOR PROMETHAZINE IN HUMAN SERUM FOLLOWING 24 h AT ROOM TEMPERATURE

Replicate No.	Promethazine concentration (ng/ml)	
	9.99 ng/ml[a]	0.458 ng/ml[a]
1	11.5	0.540
2	9.80	0.486
3	10.0	0.526
Mean	10.4	0.517
		0.517
Precision (% R.S.D.)	8.93	5.42
Stability (%)	104	113
n	3	3

[a] Concentration at 0 h.

To ensure ruggedness of the extraction procedure, three lots of serum were tested. The mean values of the duplicate analysis of three spiked (10 ng/ml) lots of serum had an R.S.D. of 4.59%.

TABLE V

CONCENTRATIONS OF PROMETHAZINE MEASURED IN SERUM AFTER ADMINISTRATION OF 50-mg SUPPOSITORIES FROM TWO DIFFERENT LOTS TO 24 VOLUNTEERS

Time (h)	Concentration (mean ± S.D.) (ng/ml)	
	Lot 1	Lot 2
0	0.020 ± 0.097	0.000 ± 0.000
1	0.076 ± 0.165	0.024 ± 0.081
2	0.254 ± 0.308	0.193 ± 0.254
3	1.29 ± 1.57	1.10 ± 0.855
4	2.25 ± 2.45	1.83 ± 1.21
5	3.00 ± 3.40	2.66 ± 1.70
6	4.81 ± 5.53	3.99 ± 2.34
7	5.63 ± 5.83	4.90 ± 3.05
8	6.49 ± 7.02	5.28 ± 2.91
9	6.86 ± 7.46	5.45 ± 3.44
10	6.96 ± 7.58	5.70 ± 4.02
12	6.84 ± 7.71	5.07 ± 3.96
15	5.15 ± 6.25	4.71 ± 3.75
24	3.16 ± 3.24	2.59 ± 2.44
36	1.54 ± 1.55	1.32 ± 1.18
48	0.921 ± 0.903	0.772 ± 0.723

CONCLUSION

This assay method for promethazine in plasma is rapid, sensitive and precise. The sensitivity has been improved at the limit of quantification so that 0.200 ng/ml can be quantified from 1 ml of serum with good precision and accuracy. This method was successfully used to determine concentrations of promethazine in subjects samples following a 50-mg suppository dose (see Fig. 2). Fig. 2. is a graph of the 24 subjects that did not have a bowel movement within 4 h of dosing. Table V provides the mean serum concentrations with the standard deviation for these subjects.

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