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Quantitation of promethazine and metabolites in urine samples using on-line solid-phase extraction and column-switching

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Abstract

A chromatographic method for the quantitation of promethazine (PMZ) and its three metabolites in urine employing on-line solid-phase extraction and column-switching has been developed. The column-switching system described here uses an extraction column for the purification of PMZ and its metabolites from a urine matrix. The extraneous matrix interference was removed by flushing the extraction column with a gradient elution. The analytes of interest were then eluted onto an analytical column for further chromatographic separation using a mobile phase of greater solvent strength. This method is specific and sensitive with a range of 3.75–1400 ng/ml for PMZ and 2.5–1400 ng/ml for the metabolites promethazine sulfoxide, monodesmethyl promethazine sulfoxide and monodesmethyl promethazine. The lower limits of quantitation (LLOQ) were 3.75 ng/ml with less than 6.2% C.V. for PMZ and 2.50 ng/ml with less than 11.5% C.V. for metabolites based on a signal-to-noise ratio of 10:1 or greater. The accuracy and precision were within ±11.8% in bias and not greater than 5.5% C.V. in intra- and inter-assay precision for PMZ and metabolites. Method robustness was investigated using a Plackett–Burman experimental design. The applicability of the analytical method for pharmacokinetic studies in humans is illustrated.

Keywords: Column switching; Promethazine; Promethazine sulfoxide; Monodesmethylpromethazine sulfoxide; Monodesmethyl promethazine

1. Introduction

Neurovestibular adaptation during space flight influences several physiological and biochemical systems in microgravity including disruption of gastrointestinal function, fluid and electrolyte balance, circulatory dynamics and organ blood flow as well as hormonal and metabolic perturbations [1]. One expression of this influence during early flight days is space motion sickness, which is currently treated with promethazine (PMZ) [2]. PMZ has a reported 75% efficacy via the intramuscular route [2]. It is expected that perturbations in the gastrointestinal tract, liver, or kidney function would influence the absorption, distribution, metabolism, or excretion of nutrients and pharmaceuticals. These physiologic changes might alter its bioavailability when PMZ is administered during space flight. Therefore, it is very important to characterize the induced changes and associated pharmacotherapeutic

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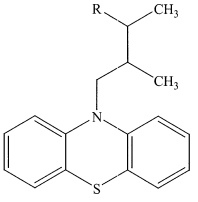
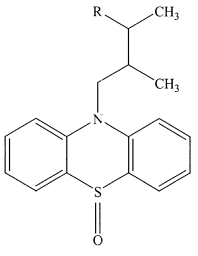
consequences in order to develop effective treatments for in-flight pathologic incidents.

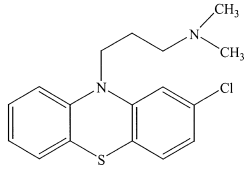
Promethazine is cleared from the body after administration by hepatic metabolism, yielding two major metabolites, promethazine sulfoxide (PMZSO) and monodesmethyl promethazine sulfoxide (Nor_1PMZSO), and one minor metabolite, monodesmethyl promethazine (Nor_1PMZ) (Fig. 1). We proposed to characterize the bioavailability and evaluate the pharmacokinetics of PMZ along with the assessment of the drug's pharmacodynamics in normal human subjects and in astronauts during space flight. We developed a sensitive, robust and reproducible bioanalytical method for monitoring the concentrations of PMZ and metabolites in urine, plasma, and saliva, which is essential for conducting such studies.

Although a number of analytical methods for the determination of PMZ in human biological fluids have been reported, a procedure to determine PMZ and its three metabolites simultaneously with an automated sample preparation is not available. Existing approaches involved pre-treatment of the sample with off-line methods, typically using liquid–liquid extraction (LLE) [3–7]. Off-line solid-phase extraction (SPE) is also reported [8]. LLE and off-line SPE without a robotic system set up are labor intensive. Other methods involving electrochemical detection (ECD) also require time-consuming system set-up and stabilization and are limited to isocratic separation [3,4]. A gas chromatography coupled with mass spectrometry method needs an extra derivatization step [5]. In addition, the majority of the reported liquid chromatographic assays were only for the determination of PMZ except for the ones published by Allender and Taylor separately that described assays to measure PMZ, PMZSO, Nor_1PMZ and didesmethyl PMZ [9,10].



1. Promethazine R= CH_3 (PMZ)
2. Monodesmethyl promethazine R= H (Nor_1PMZ)





5. Chlorpromazine (IS)

3. Promethazine sulfoxide R= CH_3 (PMZSO)
4. Monodesmethyl promethazine sulfoxide R= H (Nor_1PMZSO)

Fig. 1. Structures of PMZ and its three metabolites and the internal standard.

In contrast to these methods, the on-line SPE representing a fast, reliable and simple approach to trace analysis is the choice of our approach [11–19]. This on-line sample preparation can be performed with an advanced automated sample processor (AASP) or by column-switching system. The AASP utilizes disposable cartridges or disks and is far more expensive and complicated in operation compared to the use of column-switching techniques. The column-switching system, a form of on-line SPE, is a reliable and less expensive alternative. With the introduction of the column-switching valve, the existing laboratory high-performance liquid chromatography (HPLC) instruments can be easily transformed into an automated on-line purification system. This type of system is very flexible and can be applied to automated analyses of a wide range of drugs from different biological sample types with only a minor alteration in sample preparation. In addition, on-line SPE operates automatically without further handling of the samples between the trace-enrichment and the separation step. This technique dramatically reduces labor time for sample preparation, the risks of sample contamination and degradation from exposure to light [16,17,20]. It also improves recovery by reducing analyte loss [21]. Furthermore, the precision of a highly automated method is often better than similar manual methods [12,13,17]. For the first time, the method reported here combines on-line SPE and HPLC for the simultaneous determination of PMZ and its three metabolites, Nor_1PMZSO, PMZSO, and Nor_1PMZ,

in human urine. The procedure was accurate, precise, and applicable for clinical studies.

2. Experimental

2.1. Chemicals

PMZ hydrochloride and chlorpromazine hydrochlorides (internal standard, I.S.; Fig. 1) were purchased from USP (Rockville, MD, USA). PMZSO hydrochloride, Nor_1PMZSO hydrochloride, and Nor_1PMZ hydrochloride were custom manufactured and supplied by Dr. David Bourne (University of Oklahoma, Okalahoma City, USA; Fig. 1). Alpha-Q purified water (Millipore, Bedford, MA, USA) was used for all HPLC analysis. All solvents used for the mobile phases were of HPLC-grade and were obtained commercially (Fischer Scientific, Rochester, NY, USA). Ammonium acetate (Aldrich, Milwaukee, WI, USA) and ammonium hydroxide (Sigma, St. Louis, MO, USA) were ACS-quality reagents.

2.2. Coupled high-performance liquid chromatography apparatus and operating conditions

Automated on-line sample clean-up and enrichment was performed using a column switching system. To perform the complete process of on-line SPE and sample enrichment for HPLC analysis, the HPLC system was configured to incorporate two pump systems. This system was equipped with a high pressure six-port switching valve (Model LabPRO 2-position, Rheodyne, Rohnert Park, CA, USA), an autosampler (717 plus Series, Waters Corp., Milford, MA, USA), and a quaternary gradient pumping system (600-MS Series, Waters Corp.) for on-line SPE. In addition, an external binary pump (510 Series, Waters Corp.) was incorporated into the system through a pump control module to deliver the mobile phase for the analytical separation. A programmable multi-wavelength UV detector (490E Series, Waters Corp.) was used for detection at 236 nm. The Millenium^{32} chromatography manager (version 3.05.01) was used to control the HPLC system and to perform data acquisition and manipulation.

Two different columns were used in the column switching system. Oasis HLB extraction column (1× 50 mm, 30 μm particle size) (Waters Corp.) was employed for the on-line SPE. The analytical column was Zorbax SB-CN (4.6×250 mm, 5 μm particle size) (MAC-MOD Analytical, Chadds Ford, PA, USA), preceded by a Sentry guard column with Symmetry C_{18} packing (3.9×20 mm, 5 μm particle size) (Waters Corp.) with the flow-rate maintained at 1.2 ml/min. Two 2 μm PEEK in-line filters (Upchurch Scientific, Oak Harbor, WA, USA) were placed one each in front of the Oasis and the analytical columns. The analytical column was kept inside a column heater (Model CHM, Waters Corp.) at 30°C controlled through a temperature control module (Model TCM, Waters Corp.).

The sample analysis process was divided into steps: purification, elution, and separation. On-line SPE steps are detailed in Table 1. An appropriate volume (1–2 ml) of the urine sample was directly loaded onto the preconditioned extraction column by an autosampler. By pumping a gradient of water–methanol–30 m*M* ammonium acetate containing 2% ammonium hydroxide, the polar components of the urinary matrices were directly washed out into waste. The flow-rate was 4 ml/min during this step. Concurrently, the analytical column was equilibrated with the HPLC mobile phase.

During the following step at $t=2.5$ min, the valve was switched to in-line with the analytical column. The trapped analytes were eluted from the extraction column into the analytical column in back-flushing mode with the HPLC mobile phase D (Table 1). The flow-rate was set at 1.2 ml/min. After a 6-min running time, the analytes were completely eluted to the analytical column, the valve was turned back to the original position. While the analytes were separated in the analytical column, the extraction column was washed with methanol and then equilibrated with water for the next injection concurrently.

2.3. Preparation of mobile phase, standards and samples

The mobile phase for analytical separation consisted of mixtures of acetonitrile and 30 m*M* ammonium acetate buffer (60:40, v/v, pH 5.5–5.7)

Table 1
On-line SPE chromatographic process

Time	Flow-rate (ml/min)	Base/organic wash gradient (%)			Analytical separation	Column-switching configuration	Function
		Pump A	Pump B	Pump C	Pump D (ml/min)		
0.00	4.0	100	0	0	1.2	A (On)	(1) Inject sample
0.55	4.0	90	10	0	1.2		(2) Extraction and divert to waste
1.00	4.0	0	25	75	1.2		
2.50	1.0	100	0	0	1.2	B (Off)	(1) Elution and divert to LC
6.00	4.0	100	0	0	1.2	A (On)	(1) Divert to waste and equilibration to
7.00	4.0	0	100	0	1.2		recondition the
12.00	4.0	100	0	0	1.2		extraction column
15.00	4.0	100	0	0	1.2		
25.00	0.0	0	100	0	0.0		(2) End of program

Pump A = H_2O (mobile phase A); pump B = methanol (mobile phase B); pump C = 2% ammonium hydroxide in 30 m*M* ammonium acetate, pH 10.0 (mobile phase C); pump D: acetonitrile in 30 m*M* ammonium acetate (60/40, v/v) pH 5.5 (mobile phase D).

filtered through a nylon membrane (0.45 μm) and degassed with helium prior to use. The solvents for the on-line SPE procedure were water, methanol, and 30 m*M* ammonium acetate containing 2% ammonium hydroxide (pH 10).

Duplicate stock solutions (1 mg/ml) for PMZ (free bases) and metabolites were prepared in methanol–water (50:50, v/v) and stored in the dark at −20°C. One was used as a control solution to prepare a quality control (QC) standard, the other as a calibration standard solution. Working solutions (10 and 1 μg/ml) were prepared with methanol–water (10:90, v/v) for each run from stock solutions.

The internal standard stock solution (1 mg/ml) and working solution (10 μg/ml) of chlorpromazine (free base) were prepared in methanol–water (50:50) and methanol–water (10:90), respectively, and stored at −20°C.

The calibration standards and QC samples were prepared by diluting the working solutions of PMZ and the metabolites with analyte-free blank urine. For calibration, 8–9 urine pools were prepared, containing 2.5, 10, 40, 80, 120, 250, 750, 1000, or 1400 ng/ml of PMZ or metabolites. For assay validation, four additional QC pools were prepared with PMZ or metabolites in concentrations of 20, 80, 250, and 1000 ng/ml. For in-study control, three additional QC pools were prepared at low, medium, and high concentrations of 10, 120, and 750 ng/ml.

All samples, including calibration and QC samples, were thawed 2 h prior to analysis. After vortexing, 20 μl of internal standard were added to each sample and then 10% methanol was added to sufficient volume to yield 4 ml of total sample. Subsequently, the samples were again vortexed and centrifuged for 15 min at 3500 *g* prior to loading onto the autosampler.

2.4. Quantitative analysis

Calibration curves for four analytes were constructed in the range of 2.5–1400 ng/ml by analyzing control urine spiked with the standards of PMZ or its metabolites. Peak height ratios (analyte/internal standard) versus the nominal concentrations were used to generate the curves by a $1/x$ weighted linear least squares regression. The concentrations of urine analytes were determined from the calibration curves. All calculations concerning the quantitative analysis and regression were automatically performed with Millennium[32] chromatography manager software. The consistency of the coupled column chromatographic system was determined prior to, during and at the end of the analysis by analyzing QC samples. Typically, there was less than 5% deviation as determined from the measurements of QC samples prepared at low, medium, and high concentrations within the range of the calibration curves.

2.5. Validation experiments

The linearity of the method was assessed by measuring the peak height ratios versus the concentrations of QC validation samples in triplicate preparations by the weighted linear least squares regression. The goodness of fit and the lack of fit tests were performed.

The selectivity was assessed by analyzing the control blank urine and incurred samples from subjects after administration of PMZ. Analysis of the authentic reference standards of PMZ and the metabolites was conducted to identify the retention times of the analytes. The lack of interfering peaks at the same analyte retention times was considered as acceptable selectivity.

Intra- and inter-assay accuracy and precision variability or bias were determined by analyzing QC samples at concentration levels of 20, 80, 250, and 1000 ng/ml in five replicates during four validation runs. The mean concentration, standard deviation and the coefficient of variation (C.V.) were calculated from QC replicates of each concentration and used to determine the within- and between-run precision, respectively. Accuracy was assessed as percent bias.

The stability of PMZ and its metabolites was assessed after approximately 40 h at room temperature and through three freeze–thaw cycles (−20°C to room temperature). The pooled QC samples at low, medium, and high concentrations of 10, 120, and 750 ng/ml were injected every 6 h for a total period of 40h. During this period, the samples were kept in the autosampler and protected from light. The peak heights of PMZ and the metabolites were used to evaluate their stability over time. The stability through three freeze–thaw cycles was assessed by the determinations of the mean values of the nominal concentration at four concentration levels for each analyte when fresh and after three freeze–thaw cycles.

3. Results and discussion

3.1. LC method development

On-line SPE with Oasis HLB packed with a hydrophilic–lipophilic sorbent was employed due to the characteristics of this column for the retention of polar compounds [11,21–23]. To develop a procedure to remove all endogenous components within a limited time, a generic approach was initially adapted and further optimized with the organic concentration and buffer pH (see Table 1). In the sample-loading step, water was intentionally applied without any organic solvent in order to retain the analytes. The endogenous component profile evaluation indicated that a 2.5-min cleaning step is long enough to remove all urine matrix interference. Methanol of concentrations 15, 20, 30 and up to 45% was incorporated into the extraction step in order to achieve cleaner samples. The organic profile indicated that 45% methanol resulted in a loss of analytes and up to 30% methanol was safe enough to retain all analytes. In Table 1, 25% methanol was used in the actual extraction procedure. In addition, 2% ammonium hydroxide was added into the buffer to keep the solution basic (pH$<$12.0). Finally, a 5-min wash with methanol minimized the carry-over of the Oasis HLB extraction column. The use of an in-line peek filter prior to Oasis column extended the usage of the extraction column by removing all particulates in the system remaining in the sample after centrifugation. In addition, a 4 ml/min flow-rate was applied to the Oasis HLB extraction column containing a large particle size stationary phase, which resulted in a turbulent flow [24,25]. The turbulent flow chromatography was adapted into the extraction step in order to achieve a faster and more rugged extraction with reduced carryover. It has been demonstrated that the backpressure of the extraction column operated under a turbulent flow condition remain unchanged after more injections compared to the laminar flow [24,25]. The unique feature of turbulent flow demonstrated consistent analyte recovery and good peak shape along with virtually no retaining carryover for a minimum of 150 injections without significant increase of backpressure.

Polar column Zorbax SB-CN was employed for chromatographic separation due to the high polarity of PMZSO and Nor_1PMZSO. The eluting mobile phase is optimized for an isocratic separation that provided sufficient selectivity to attain adequate resolution between all analytes and internal standard. The pH of the mobile phase, controlled at 5.5–5.7, is essential to achieving a good resolution between

Table 2
Factors and levels in the method robustness evaluation

Factor	Target value	Test level I (low) (–)	Test level II (high) (+)
HPLC system	A	B	A
HPLC column	Zorbax SB-CN 250×4.6 mm	Y	X
Column temp (°C)	30	Ambient	32
pH of mobile phase	5.50	5.3	5.7
%ACN in mobile phase	60	58	62
Flow-rate	1.2	1.0	1.4
Ionic strength (m*M*)	30 NH_4Ac	25	35

PMZSO and Nor_1PMZSO. The final method combined the optimized extraction procedure and the optimized analytical separation step through the column-switching valve. The retention times of Nor_1PMZSO, PMZSO, Nor_1PMZ, PMZ, and I.S. were approximately 8.6, 9.3, 11.9, 13.3, and 15.9 min, respectively. The total run time was 20 min.

It was noticed during the method development that the method was sensitive to the pH and composition of the mobile phase and buffer concentration. Therefore, the robustness of the method performance was tested in order to evaluate its capacity and reliability capable of remaining unaffected with small but deliberate variations in method parameters. Solvent composition, pH, temperature, buffer ionic strength, flow-rate, column, and HPLC system are the expected variations during the normal usage. Experimental design with these seven experimental factors simulating the method variations was used to approach the evaluation. These factors were tested at two levels in order to compare the outcomes. Instead of 2^7 or 128 experiments, eight experiments were performed in triplicate injections using a fractional factorial design (the so-called Plackett–Burman design) [26,27]. Table 2 lists the values of the seven factors used in the eight determinations. The fractional factorial experimental design is shown in Table 3. The impact of each factor to the responses was calculated according to the Youden technique [28], which states that the effect of a factor on a response can be determined by taking the average of the response at the high level (+) minus the average of the response at the low level (−). For example, the effect of factor no. 5 (%ACN in mobile phase) was estimated using the formula of $(d_2 + d_4 + d_5 + d_7)/4 - (d_1 + d_3 + d_6 + d_8)/4$, where d represents the experimental response (resolution, theoretical plates, tailing factor), and the subscript number indicates the experiment number performed. Therefore, the effect of a small change in %ACN in the mobile phase on the resolution of the Nor_1PMZSO–PMZSO pair, −0.020 (Table 4), was calculated using the formula $(R_{s2} + R_{s4} + R_{s5} + R_{s7})/4 - (R_{s1} + R_{s3} + R_{s6} + R_{s8})/4$. The calculated results using Youden technique are summarized in Table 4.

The method's overall robustness was then evaluated statistically using the following approach. The effects of each factor in terms of the magnitudes on

Table 3
Fractional factorial experimental design in the method robustness evaluation

Factors	Experiment number							
	1	2	3	4	5	6	7	8
HPLC system	A	A	A	A	B	B	B	B
HPLC column	X	X	Y	Y	X	X	Y	Y
Column temp (°C)	Ambient	32	Ambient	32	Ambient	32	Ambient	32
pH of mobile phase	5.25	5.25	5.75	5.75	5.75	5.75	5.25	5.25
%ACN in mobile phase	58	62	58	62	62	58	62	58
Flow-rate (ml/min)	1.0	1.4	1.4	1.0	1.0	1.4	1.4	1.0
Ionic strength (m*M*)	25	35	35	25	35	25	25	35

Table 4
Effects of each factor on each response[a]

	Factor	HPLC system	Column	Temperature mobile phase	pH of mobile phase	% ACN in mobile phase	Flow-rate	Ionic strength
Resolution	Pair 1 Nor_1PMZSO–PMZSO	0.165	0.060	−0.405	−0.385	−0.020	−0.405	0.140
	Pair 2 Nor_1PMZ–PMZ	0.025	0.085	−0.295	0.215	−0.315	−0.235	0.205
Theoretical plates	Nor_1PMZSO	156	2507	2133	−10 554	53	2362	120
	PMZSO	1209	822	1291	−4706	−288	963	101
	Nor_1PMZ	381	1772	1685	−2722	1128	212	577
	PMZ	552	1731	2101	−4490	557	73	177
Tailing factor	Nor_1PMZSO	−0.147	0.00275	−0.0528	−0.253	−0.00275	0.0472	0.0972
	PMZSO	0.0687	0.119	−0.119	0.131	0.131	0.0813	−0.169
	Nor_1PMZ	−0.0045	−0.0045	0.0045	0.0040	0.0045	0.0045	0.0045
	PMZ	−0.0920	−0.042	−0.108	0.192	0.042	−0.0080	0.042

[a] All the data were generated by Waters Millennium[32] software.

each response were then rearranged from the lowest to the highest. For instance, the factors against resolution between pair Nor_1PMZSO–PMZSO and pair Nor_1PMZ–PMZ were ranked in Table 5. The normal probability values, M (y-axis) [29] versus the ranked factors on resolutions (x-axis) are plotted in Fig. 2. The points form two fairly straight lines for the pair of Nor_1PMZSO and PMZSO and the pair of Nor_1PMZ and PMZ with correlation coefficients $r =$ 0.92 and 0.95, respectively. The linear correlation in Fig. 2 indicates that the method is robust for the peak resolutions between pair Nor_1PMZSO–PMZSO and pair Nor_1PMZ–PMZ. For the theoretical plate and tailing factor, fairly linear responses for PMZ and three metabolites with an r-value between 0.80 and 0.97 were observed. Therefore, it is expected that

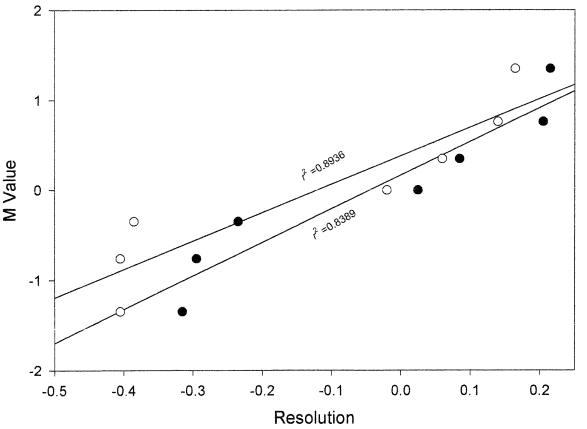


Fig. 2. Ranked effects for the resolution of pair 1 between Nor_1PMZSO and PMZSO (close circle) and pair 2 between Nor_1PMZ and PMZ (open circle).

Table 5
Ranked effects: resolutions

Pair 1 Nor_1PMZSO–PMZSO			Pair 2 Nor_1PMZ–PMZ		
Factor	Resolution	M-value	Factor	Resolution	M-value
Temperature (°C)	−0.405	−1.35	%ACN in mobile phase	−0.315	−1.35
Flow-rate (ml/min)	−0.405	−0.76	Temperature (°C)	−0.295	−0.76
pH of mobile phase	−0.385	−0.35	Flow-rate	−0.235	−0.35
%ACN in mobile phase	−0.02	0	HPLC system	0.025	0
Column	0.06	0.35	Column	0.085	0.35
Ionic strength (mM)	0.14	0.76	Ionic strength (mM)	0.205	0.76
HPLC system	0.165	1.35	pH of mobile phase	0.215	1.35

theoretical plates and the tailing factor should remain unaffected with the small variations in method parameters. All chromatograms generated from the eight experiments were overlaid in Fig. 3. A visual inspection in Fig. 3 supports the conclusion that the assay was robust within the tolerance limits (test levels) of the parameters tested because all chromatograms exhibited acceptable resolution and peak shapes.

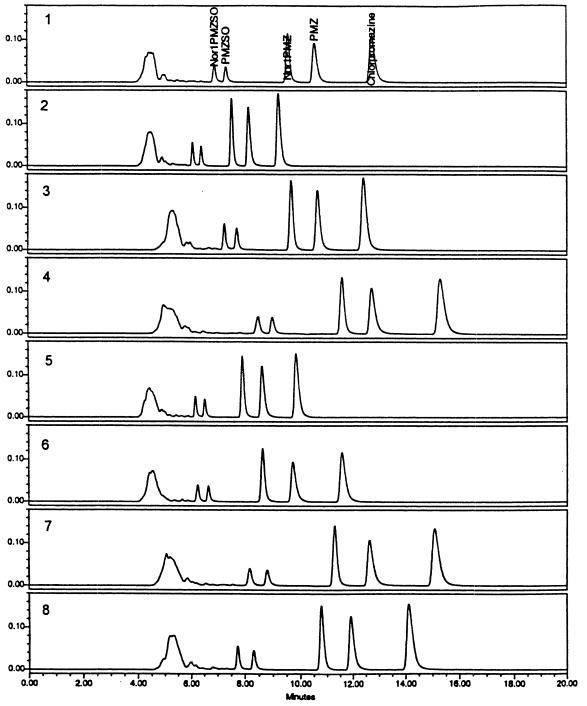


Fig. 3. Stack chromatograms resulted from eight runs in the robustness experiments. Chromatographic peaks in order: Nor_1PMZSO, PMZSO, Nor_1PMZ, PMZ, and Chloropromazine (I.S.). The experimental conditions are listed: (1) ACN–25 m*M* NH_4Ac (58/42, v/v), pH 5.75, flow-rate = 1.4 ml/min, T = 32°C, column X; (2) ACN–35 m*M* NH_4Ac (62/38, v/v), pH 5.25, flow-rate = 1.4 ml/min, T = 32°C, column X; (3) ACN–35 m*M* NH_4Ac (58/42, v/v), pH 5.25, flow-rate = 1.0 ml/min, T = 32°C, column Y; (4) ACN–25 m*M* NH_4Ac (62/38, v/v), pH 5.75, flow-rate = 1.0 ml/min, T = 32°C, column Y; (5) ACN–25 m*M* NH_4Ac (62/38, v/v), pH 5.25, flow-rate = 1.4 ml/min, T = ambient, column Y; (6) ACN–35 m*M* NH_4Ac (58/42, v/v), pH 5.75, flow-rate = 1.4 ml/min, T = ambient, column Y; (7) ACN–35 m*M* NH_4Ac (62/38, v/v), pH 5.75, flow-rate = 1.0 ml/min, T = ambient, column X; (8) ACN–25 m*M* NH_4Ac (58/42, v/v), pH 5.25, flow-rate = 1.0 ml/min, T = ambient, column X.

3.2. Method validation

3.2.1. Sensitivity

The proposed lower limit of quantitations (LLOQ) for PMZ and metabolites were validated by the analysis of eleven concentration levels in triplicate preparations. A signal-to-noise ratio of ≥ 10 was used as the criterion for a significant response in quantitative analyses. The LLOQs were 3.7 ng/ml for PMZ and 2.5 ng/ml for Nor_1PMZSO, PMZSO, and Nor_1PMZ, respectively for 2-ml aliquots of urine. The %C.V. at the LLOQ levels was less than 11.5% for Nor_1PMZSO, 1.3% for PMZSO and Nor_1PMZ, and 6.2% for PMZ. The bias was within 18.5% for PMZ and the metabolites. It was sensitive enough to support urinary excretion evaluation.

An alternative approach of LLOQ determination according to ICH guidelines is based on the calibration curve approaching the limit of detection [30]. The LLOQ is estimated based on the standard deviation of the peak response and the slope with the expression of the equation: $\text{LLOQ} = 10\sigma/S$ where σ = the standard deviation of the response and S = the slope of the calibration curve.

The standard deviation of the y-intercept by a $1/x$ weighted linear least squares regression, where x is the concentration of each analyte, was used as the standard deviation. Details of the statistical calculation can be found in Refs. [26,31–33]. The LLOQs calculated were 15.5 ng/ml for PMZ, 12.9 for Nor_1PMZSO, 16.3 for PMZSO, and 16.2 for Nor_1PMZ. The precision and accuracy at this level were within acceptable limits (C.V. and bias ±20%).

3.2.2. Linearity

Linearity was observed for PMZ and metabolites in the range of 2.5–1400 ng/ml. Best-fit calibration lines of the chromatographic peak response versus concentration were determined by a $1/x$ weighted linear least squares regression. A test for lack of fit showed that the first-order model ($y = ax + b$), with a weighting factor of $1/x$, was appropriate for establishing a relationship between concentration and response ($r^2 \geq 0.9999$). For PMZ, the goodness of fit using the F-test was highly significant [$F_{\text{calc.}} = 39\,765.7$, F_{critical} ($\alpha = 0.05$) = 4.23, $F_{\text{calc}} \gg F_{\text{critical}}$] [34] with a risk of less than 5% of being error. No

Table 6
Testing the significance of a weighted least squares regression

	PMZ	Nor_1PMZSO	PMZSO	Nor_1PMZ
Weighting factor	$1/x$	$1/x$	$1/x$	$1/x$
r^2	0.9999	0.9999	0.9999	0.9999
Goodness of fit				
$F_{calc.}$	39 765.7	65 366.2	67 860.5	45 146.5
$F_{critical}$ ($\alpha=0.05$)	4.23	4.23	4.23	4.23
Lack of fit				
$F_{calc.}$	0.0037	0.0024	0.0010	0.0032
$F_{critical}$ ($\alpha=0.05$)	2.54	2.54	2.54	2.54

significant lack of fit was observed [$F_{calc.}=0.0037$, $F_{critical}$ ($\alpha=0.05$)$=2.54$, $F_{calc.} \ll F_{critical}$] with residuals being the result of experimental error rather than a consequence of model deviations. The F-test results of lack of fit and goodness of fit for PMZ metabolites are shown in Table 6.

3.2.3. Selectivity

No major interferences (<0.2 times the response of the LLOQ for Nor_1PMZSO and PMZSO) from endogenous components in the urine matrix were observed at the retention times of PMZ, metabolites, and internal standard based on the analysis of blank control urines and incurred urine samples of in-study validation.

3.2.4. Precision and accuracy

The intra- and inter-assay precision were determined from the relative standard deviation of the quality control samples. In each validation run, six replicates of the QC sample pool were assayed. A summary of the results of the intra- and inter-assay precision for PMZ and the metabolites of the QC replicates in four concentration levels over four validation runs is given in Table 7. The intra-assay precision expressed as average within-run precision

Table 7
Precision and accuracy data

Compound		Nominal concentration (ng/ml)			
		20	80	250	100
Nor_1PMZSO	Average within-run precision (C.V.)	2.2	2.6	2.0	2.4
($N=24$)	Between-run precision (C.V.)	2.3	4.4	4.6	3.6
	Accuracy (%bias)	−8.7	−1.6	4.1	1.5
PMZSO	Average within-run precision (C.V.)	2.5	2.9	2.0	2.4
($N=24$)	Between-run precision (C.V.)	4.7	4.9	5.2	4.3
	Accuracy (%bias)	−11.5	0.3	8.0	1.2
Nor_1PMZ	Average within-run precision (C.V.)	2.9	1.5	1.2	2.4
($N=24$)	Between-run precision (C.V.)	4.1	3.9	3.6	4.5
	Accuracy (%bias)	−8.6	5.4	10.7	−4.1
PMZ	Average within-run precision (C.V.)	2.7	1.2	1.2	1.9
($N=24$)	Between-run precision (C.V.)	4.8	5.5	3.3	4.6
	Accuracy (%bias)	−10.1	7.0	11.8	−0.1

was not greater than 2.9% at all concentration levels. The inter-assay precision expressed as between-run precision was not greater than 5.5%. The inter-assay accuracy was determined by comparing the mean measured concentrations of the quality control sample with their nominal concentrations over four validation runs. As shown in Table 7, the bias of PMZ and the metabolites varied between −11.5% and +11.8% at all concentration levels. These results indicate that the assay is precise and accurate according to the criteria recommended in the FDA Draft Guidance on Bioanalytical Methods Validation.

3.2.5. Stability

The issue of stability during storage and sample handling was addressed by several experiments. A sequential analyses of QC samples at the concentrations of 10, 120, and 750 mg/ml in duplicates was used to monitor and confirm the stability of the analytes under actual conditions of the assay. The autosampler stability is determined by comparing the mean measured concentrations of QC samples stored in the autosampler for up to 40 h at room temperature to the freshly prepared urine QC samples. The data shown in Table 8 only varied between 99 and 104%. For the stability of the freeze–thaw cycle, the percent differences of the mean calculated concentration of the samples after three cycles were within ±11.8% for both metabolites and PMZ. During the study, it was found that the photolytic stability of PMZ and its metabolites appeared to be consistent with the reported results [35,36]. Necessary precautions were taken during sample preparation and analysis to assure that there was no noticeable degradation of any of the analytes during the performance of the analytical procedure.

3.2.6. Application of the assay for pharmacokinetics

The described assay was successfully applied to measure urinary levels of PMZ and metabolites after intramuscular administration of a 50-mg dose to human subjects. Results indicated that a significant percentage of the administered dose was metabolized into Nor_1PMZSO and PMZSO and excreted in the urine during the 48-h ensuing period. The concentrations of Nor_1PMZ, a third metabolite in the urine were consistently low and could not be quantitated. Fig. 4 depicts representative cumulative excretion profiles in the urine of PMZ and the two major metabolites in the 48 h after drug administration. Preliminary evaluation of these results indicate that less than 10% of the administered dose was excreted in the urine during the first 48 h after intramuscular administration of the drug. Results from the pharmacokinetic analysis of these data will be published elsewhere.

4. Conclusion

A bioanalytical method based on on-line SPE using turbulent flow chromatography has been developed for the simultaneous determination of PMZ and its three metabolites in plasma, saliva, and urine and validated for application in clinical research with PMZ. The system utilizes an extraction column for on-line purification and an analytical column for

Table 8
Stability data of promethazine in human urine within 40 h at room temperature[a]

Time duration (h)	PMZ concentration (ng/ml)			DPMZS concentration (ng/ml)			PMZS concentration (ng/ml)			DPMZ concentration (ng/ml)		
	10	120	750	10	120	750	10	120	750	10	120	750
0	100	100	100	100	100	100	100	100	100	100	100	100
10	101	101	100	100	100	100	100	100	99	101	100	100
18	101	101	101	100	100	100	104	101	100	101	100	100
40	100	101	100	100	99	99	104	100	99	100	100	99
Precision (%RSD)	0.43	0.34	0.71	0.33	0.56	0.87	2.17	0.35	0.43	0.49	0.37	0.50

[a] Concentration at 0 h is set as 100% recovery.

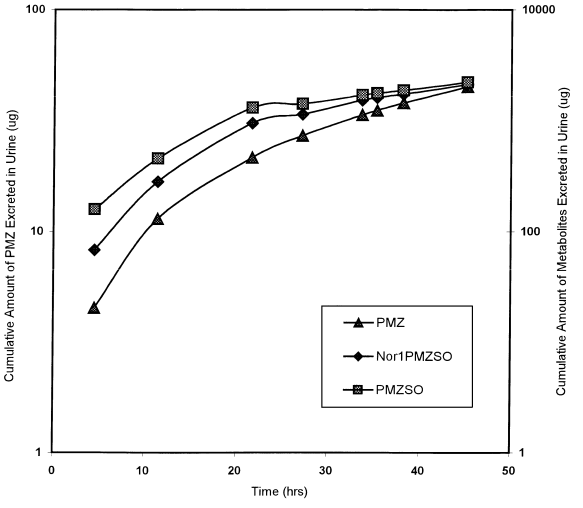


Fig. 4. Cumulative excretion of PMZ and metabolites in urine after a 50-mg intramuscular administration of a 50-mg dose to a human subject.

quantitation. The versatile on-line purification system is simple and can be easily set up using existing autosamplers and pumps based on the availability of instrumentation in the laboratory. This setup allows for large amounts of samples to be injected directly onto the extraction column for concentration and cleanup and an analytical column for chromatographic selectivity. On-line processing of the sample ensures minimal sample loss during the sample preparation and preserves the sample integrity by eliminating exposure to light since PMZ and its metabolites exhibit a light sensitive degradation. The method possesses adequate sensitivity, selectivity, accuracy, and precision. Our results demonstrated that this on-line SPE coupled with HPLC by column-switching is an ideal technique for the quantitative analysis of PMZ and its metabolites and is suitable for clinical research applications.

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References

[1] A.E. Nicogossian, J.F. Parker Jr., Space Physiology and Medicine, US Government Printing Office, Washington, DC, 1982, NASA SP-447.
[2] J.R. Davis, R.T. Jennings, B.G. Beck, J.P. Bagian, Aviat. Space Environ. Med. 64 (1993) 230.
[3] A.R. Fox, D.A. Mclouglin, J. Chromatogr. 631 (1993) 255.
[4] M. Bagli, M.L. Rao, G. Hoflich, J. Chromatogr. A 657 (1994) 144.
[5] D.E. Leelavathi, D.E. Dressler, E.F. Soffer, S.D. Yachetti, J.A. Knowles, J. Chromatogr. 339 (1985) 105.
[6] A. Tracqui, P. Kintz, P. Mangin, J. Forensic Sci. 40 (1995) 254.
[7] I.M. McIntyre, C.V. King, S. Skafidis, O.H. Drummer, J. Chromatogr. 621 (1993) 215.
[8] G.W. Ponder, J.T. Steward, J. Pharm. Biomed. Anal. 13 (1995) 1161.
[9] W.J. Allender, A.W. Archer, J. Forensic Sci. 29 (1984) 515.
[10] G. Taylor, J.B. Houston, J. Chromatogr. 230 (1982) 194.
[11] M.C. Hennion, J. Chromatogr. A 856 (1999) 3.
[12] H. Lindenblatt, E. Kramer, P. Holzmann-Erens, E. Gouzoulis-Mayfrank, K.-A. Kovar, J. Chromatogr. B 709 (1998) 255.
[13] B. Malavasi, M. Locatelli, M. Ripamonti, V. Ascalone, J. Chromatogr. B 676 (1996) 107.
[14] R.A. Coe, L.S. DeCesare, J.W. Lee, J. Chromatogr. B 730 (1999) 239.
[15] A. Marchese, C. McHugh, K. Kehler, H. Bi, J. Mass Spectrom. 33 (1998) 1071.
[16] T.E. Gundersen, E. Lundanes, R. Blomhoff, J. Chromatogr. B 691 (1997) 43.
[17] T.E. Gundersen, R. Blomhoff, Methods Enzymol. 299 (1999) 430.
[18] H.S. Lee, C.K. Jeong, H.M. Lee, S.J. Choi, K.S. Do, K. Kim, Y.H. Kim, J. Chromatogr. A 858 (1999) 179.
[19] J. Ding, U.D. Neue, Rapid Commun. Mass Spectrom. 13 (1999) 2151.
[20] V. Pichon, M.-C. Hennion, J. Chromatogr. A 665 (1994) 269.
[21] M.-C. Hennion, V. Coquart, J. Chromatogr. 642 (1993) 211.
[22] Y.F. Cheng, U.D. Neue, L. Bean, J. Chromatogr. A 828 (1998) 273.
[23] Y.F. Cheng, U.D. Neue, L.L. Woods, J. Chromatogr. B 729 (1999) 19.
[24] J.-T. Wu, H. Zeng, M. Qian, B.L. Brogdon, S.E. Unger, Anal. Chem. 72 (2000) 61.
[25] M. Jemal, O. Zheng, Y.-Q. Xia, M.L. Powell, Rapid Commun. Mass Spectrom. 13 (1999) 1462.
[26] J.C. Miller, J.N. Miller, in: Statistics For Analytical Chemistry, 3rd Ed., Ellis Horwood PTR Prentice Hall Publisher, Eaglewood Cliffs, NJ, 1993, p. 87, Chapter 4.
[27] R.L. Plackett, J.P. Burman, Biometrika 33 (1946) 305.
[28] W.J. Youden, in: Statistical Techniques for Collaborative Tests, Association of Official Analytical Chemists (AOAC), Arlington, VA, 1987, p. 33, Chapter 6.
[29] P.A. Peters, T.C. Paino, Pharm. Technol. (January 1999) 8, Analytical Validation (Suppl.).

[30] (a) ICH harmonised tripartite guideline, Q2a and Q2b, in: Validation of Analytical Procedures: Definitions and Terminology, Methodology. Federal Register, Vol. 60, p. 11260, 1 March 1995;
(b) ICH harmonised tripartite guideline, Q2A and Q2B, in: Validation of Analytical Procedures: Definitions and Terminology, Methodology. Federal Register, Vol. 62, p. 27463, 19 May 1997.
[31] I. Krull, M. Swartz, LC·GC 16 (1998) 922.
[32] J. Vial, A. Jardy, Anal. Chem. 71 (1999) 2672.
[33] N.R. Draper, H. Smith, in: Applied Regression Analysis, 3rd ed., Wiley Series in Probability and Statistics, Wiley, New York, 1998, p. 29, Chapter 1.
[34] J.H. Zar, in: Biostatistical Analysis, 3rd ed., Prentice-Hall, Upper Saddle River, NJ, 1996, p. 326, Chapter 16.
[35] S. Stavchansky, J. Wallace, M. Chue, J. Newburger, J. Liq. Chromatogr. 6 (1983) 1333.
[36] S. Stavchansky, J. Wallace, P. Wu, J. Pharm. Sci. 72 (1983) 546.