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Stereoselective determination of the CYP2C19 probe drug mephenytoin in human urine by gas chromatography–mass spectrometry

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Abstract

A sensitive, specific and reproducible gas chromatographic assay utilizing mass-selective detection has been developed for the stereoselective determination of mephenytoin (MP) in human urine. Following extraction of urine samples using methyl *tert*.-butyl ether, separation of *R*- and *S*-MP was achieved with a chiral capillary column; detection and quantitation were accomplished by mass spectrometry in the single ion monitoring mode (m/z 104 and 189). Excellent linearity was observed for both enantiomers over the concentration range of 5–1000 ng/ml with corresponding correlation coefficients (r)>0.99. The intra- and inter-day precision and accuracy were within ±5%. This method employs a simplified processing procedure, demonstrates improved extraction recovery, and provides at least 5-fold greater sensitivity than previously reported assays. This method is well suited for the phenotypic evaluation of CYP2C19 activity using mephenytoin.
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1. Introduction

Mephenytoin (MP) was originally introduced as an anticonvulsant agent in the US over 50 years ago [1]. It is a racemic mixture of optical enantiomers, *R*-mephenytoin (*R*-MP) and *S*-mephenytoin (*S*-MP), that exhibit stereoselective metabolism [2]. Namely, *S*-MP undergoes rapid hydroxylation to form 4′-hydroxymephenytoin (HMP), a reaction catalyzed by CYP2C19 [3], while the *R*-enantiomer is slowly *N*-demethylated (Fig. 1) [4]. Although MP is currently of limited use therapeutically, the stereoselective manner in which it is metabolized has fostered a unique niche for the compound in the area of pharmacogenetics, specifically as a phenotypic probe of the cytochrome P450 (CYP) enzyme 2C19 (CYP2C19) [2,5–7].

CYP2C19 exhibits genetic polymorphism and individuals are typically classified functionally (phenotype) as an extensive metabolizer (EM) or poor metabolizer (PM). The latter phenotype is associated with a discernible interethnic difference, evidenced by a prevalence of 1–3% in Caucasians and 13–23%

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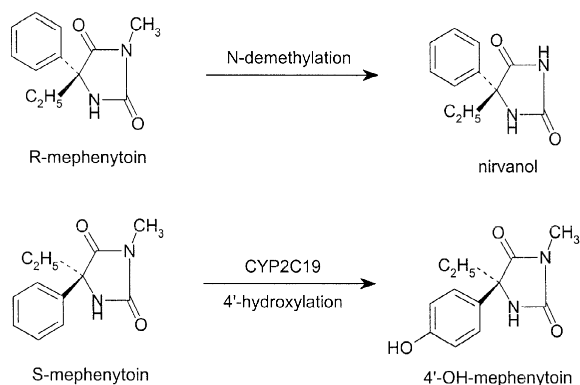

Fig. 1. Stereoselective metabolism of mephenytoin.

in Asians [8,9]. The most common CYP2C19 allelic variants contain a splicing defect (CYP2C19*2) or premature stop codon (CYP2C19*3) that result in an inability to 4′-hydroxylate *S*-MP and thus the PM phenotype. The CYP2C19 phenotype can be determined by measuring the *S*-MP to *R*-MP (S:R) ratio in urine collected for 8 h following the administration of racemic MP [5,8]. Individuals having S:R ratio values <0.9 or ≥ 0.9 are classified as EM or PM, respectively [8].

Although gas chromatography (GC) and GC–mass spectrometry (GC–MS) have been utilized for the determination of *R*- and *S*-MP in urine, the methods reported to date are cumbersome, requiring large sample volumes [10–12] and/or additional chromatographic steps (e.g. HPLC) to separate MP enantiomers prior to analysis [11]. We present a sensitive, specific and reproducible GC assay utilizing MS detection for the determination of *R*- and *S*-MP in human urine. The method described employs simplified processing, improved extraction efficiency, and is more sensitive than previously reported assays.

2. Experimental

2.1. Chemicals

Racemic MP reference standard was purchased from the US Pharmacopeia (USP, Rockville, MD, USA). Phensuximide (internal standard, I.S.) was obtained from Parke-Davis (Morris Plains, NJ, USA). Methyl *tert.*-butyl ether was purchased from Sigma–Aldrich (St. Louis, MO, USA). Dichloromethane was purchased from Burdick & Jackson (Muskegon, MI, USA). Methanol was obtained from Fisher (Pittsburgh, PA, USA). Drug-free human urine was obtained from in-house laboratory personnel.

2.2. Equipment

The GC–MS system consisted of a Hewlett-Packard (HP; Palo Alto, CA, USA) 5890A capillary gas chromatograph equipped with a HP6890 autoinjector and HP5972A mass selective detector. An Alltech Associates (Deerfield, IL, USA) Chirasil-VAL® chiral capillary column (25 m×0.32 mm I.D., film thickness, 0.2 μm) was used for analysis. Signal output was captured with HP CHEMSTATION software version B.01.00.

2.3. Sample preparation

2.3.1. Stock solutions and spiked standards

Mephenytoin stock solution (1 mg/ml) was prepared in methanol. From this primary stock solution, dilutions were made to prepare 10, 1 and 0.1 μg/ml stock solutions for preparation of the calibration standards and quality control (QC) samples. Phensuximide internal standard was dissolved in methanol to prepare a 2.5 μg/ml stock solution. All stock solutions were stored at −20 °C. Standards and QC samples were prepared at the beginning of the validation experiment by appropriate dilution of the stock solutions with urine and were stored at −20 °C to simulate the storage conditions of the study samples.

2.3.2. Urine samples

The I.S. phensuximide (10 μl) was added to urine (200 μl) in microcentrifuge tubes and briefly vortex-mixed. Methyl-*tert.*-butyl ether (1 ml) was added to each sample, tubes were capped and then vortex-mixed vigorously for 2 min. Following centrifugation at 2000 *g* for 5 min, the upper ether layer was transferred to a disposable glass tube, then placed under a stream of nitrogen and evaporated to dryness at room temperature. Sample residues were then reconstituted in dichloromethane by adding 200 μl to the glass tubes and vigorously vortex-mixing for 2

min. Lastly, samples were transferred to micro-volume GC vials and securely capped.

2.4. GC–MS procedure

A 4-μl aliquot of sample was injected onto the GC–MS system for analysis in the splitless injection mode. The injection port and detector transfer line temperatures were set at 250 and 290 °C, respectively. The oven temperature gradient was programmed at 140 °C for 2 min, then increased at a rate of 2 °C/min to 190 °C and maintained there for 2 min, resulting in an analytical run-time of 29 min. Helium was used as the carrier gas (constant flow 3.49 ml/min). A solvent delay of 3 min was used and ions were generated by electron impact ionization. All mass spectra were recorded at 70 eV. Analysis was carried out in single ion monitoring (SIM) mode (*m*/*z* 104 and 189), making *m*/*z* and retention time the primary resolving parameters. The full-scan electron-impact mass spectrum of MP depicting the two major ions produced and selectively monitored is shown in Fig. 2A; the I.S. is depicted in Fig. 2B.

2.5. Calibration and linearity

Calibration curves were constructed using six standard concentrations of MP in urine. Curves were run in duplicate and obtained daily for 3 days. Standard concentrations in urine ranged from 5 to 1000 ng/ml racemic MP. Individual standard concentrations in urine are shown in Table 1. Duplicate standard curves were analyzed for three runs (lowest standard was assayed in triplicate). For each curve, the absolute peak-area ratios of the enantiomer to the I.S. were calculated and plotted against the nominal concentration of racemic MP. Calibration curves for *R*-MP and *S*-MP were generated by weighted ($1/y^2$) linear regression analysis.

2.6. Precision and accuracy

Precision and accuracy were determined by the analysis of MP QC samples spiked at concentrations of 15, 75 and 750 ng/ml. Replicate QC samples ($n=6$) at each concentration were analyzed daily for 2 days, followed by analysis of replicate QC samples ($n=12$) at each concentration on the third day,

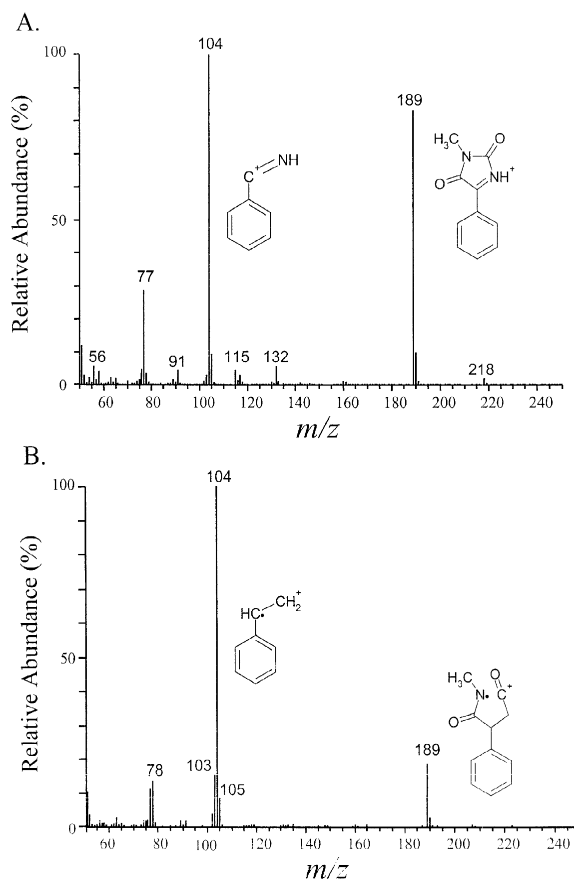

Fig. 2. Electron impact full scan mass spectrums of (A) racemic mephenytoin depicting the two major ions produced (*m*/*z* 104 and 189) and selectively monitored; (B) phensuximide internal standard.

yielding a total of $n=24$ QC samples at each concentration value. The calculated mean concentration relative to the spiked concentration was used to express accuracy (bias). Means, standard deviations and relative standard deviations (RSD) were calculated from the QC values and used to estimate the intra- and inter-day precision.

2.7. Selectivity and stability

Selectivity was evaluated by processing and analyzing blank urine obtained from six sources. The following samples were processed in duplicate and compared to test substances in dichloromethane:

Table 1
Intra- and inter-day precision and accuracy for *R*- and *S*-MP in urine

	Concentration (ng/ml)		RSD (%)	Bias (%)
	Added	Observed (mean±S.D.)		
Intra-assay reproducibility[a]				
Quality controls				
R-Mephenytoin	15	14.3±0.5	3.4	−4.5
	75	76.9±2.1	2.7	2.5
	750	776.0±16.6	2.1	3.5
S-Mephenytoin	15	14.4±0.5	3.7	−4.0
	75	77.4±1.3	1.7	3.2
	750	776.9±18.2	2.3	3.6
Inter-assay reproducibility[b]				
Quality controls				
R-Mephenytoin	15	14.2±0.6	4.1	−5.0
	75	75.1±3.4	4.5	0.2
	750	766.0±25.1	3.3	2.1
S-Mephenytoin	15	14.3±0.6	4.3	−4.6
	75	75.2±3.6	4.8	0.3
	750	765.1±27.5	3.6	2.2
Standards				
R-Mephenytoin	5	5.0±0.1	2.4	0
	25	25.3±0.8	3.3	1.3
	50	50.2±2.1	4.1	0.4
	100	99.0±4.3	4.3	−1.0
	500	504.1±24.1	4.8	0.8
	1000	1000.1±28.8	2.9	0
S-Mephenytoin	5	5.0±0.2	4.3	0
	25	25.5±0.7	2.9	1.9
	50	50.3±1.8	3.6	0.5
	100	98.6±4.1	4.2	−1.4
	500	504.3±25.4	5.0	0.9
	1000	997.7±27.3	2.7	−0.2

[a] Twelve quality control samples per concentration.
[b] Six to twelve quality control samples or two standards per day per concentration for 3 days.

blank urine and urine spiked with the analytes. Carryover was also evaluated by placing vials of blank dichloromethane and processed blank urine samples at several locations in the analysis set. Triplicate high and low QC samples were subjected to three freeze–thaw cycles (−80 °C to room temperature) prior to processing and analysis to evaluate sample stability. In addition, processed sample stability was assessed by repeated analysis of high and low QC samples for up to 26 h postprocessing.

2.8. Extraction recovery

Extraction recoveries of *R*- and *S*-MP from urine were determined by comparing the response obtained from extracted samples with the response observed after direct injection of unextracted dichloromethane reference samples. Responses observed for the reference sample were defined as 100%.

2.9. Application to CYP2C19 phenotyping

CYP2C19 phenotyping was carried out after receiving protocol approval by the University of Pittsburgh Institutional Review Board and after obtaining written informed consent from each subject. MP 100 mg (Mesantoin, Sandoz, Hanover, NJ, USA) was administered orally with eight ounces of water the morning after an overnight fast. Urine was collected and pooled from 0 to 8 h after administration of racemic MP, and aliquots of the collection were stored at −20 °C until analyzed. The concentrations of *R*- and *S*-MP were determined as described above, followed by calculation of the S:R ratio [5,8]. Individuals having S:R ratio values <0.9 or ≥0.9 are classified as EM and PM, respectively [8].

3. Results

3.1. Chromatographic separation

Representative chromatograms of urine samples recorded by SIM mass spectrometric detection are shown in Fig. 3. Retention times for phensuximide (I.S.), *S*- and *R*-MP were approximately 9.3, 26.8, and 27.0 min, respectively. The peaks of the compounds of interest were well separated and there was no interference from endogenous compounds during the elution windows for the analytes and I.S. The resolution factor for the MP enantiomers was 1.45. We used a solvent delay of only 3 min, but the delay time could be increased, since the retention time of the I.S. is >9 min, which would help maximize the service life of the mass spectral analyzer.

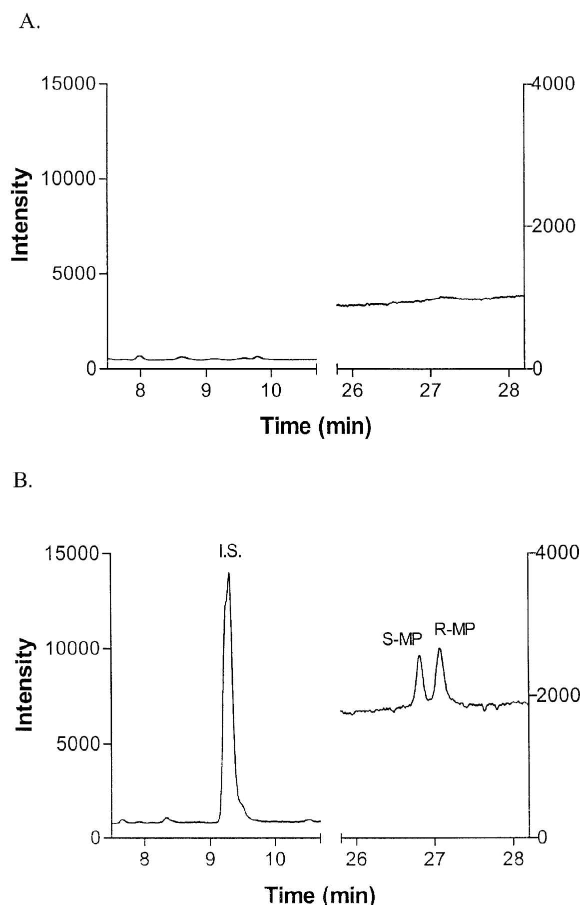

Fig. 3. Representative SIM chromatograms of urine samples; (A) blank human urine; (B) spiked human urine containing internal standard and *R*- and *S*-MP at the LOQ (5 ng/ml).

3.2. Linearity, precision and accuracy

Linear calibration curves were obtained for both enantiomers over the concentration range of 5–1000 ng/ml. The mean regression equations (±SD) were as follows

$$R\text{-MP}: y = [0.0044\ (\pm 0.0001)]x + 0.0043\ (\pm 0.0035)$$

$$S\text{-MP}: y = [0.0044\ (\pm 0.0002)]x + 0.0054\ (\pm 0.0036)$$

Correlation coefficients (*r*) were ≥0.999 for both. The lower limit of quantitation (LOQ) for both enantiomers (5 ng/ml or 20 pg "on column") demonstrated acceptable reproducibility (RSD<5%) and a signal-to-noise ratio of 18:1. The intra- and inter-day precision and accuracy were within ±5% for *R*- and *S*-MP (Table 1).

3.3. Selectivity and stability

No endogenous interfering peaks were present in six sources of blank human urine, and there was no carryover evident in any of the blank reagent samples; therefore, the assay can be regarded as selective against possible matrix constituents. Three freeze–thaw cycles had no effect on the stability of analytes in urine. Processed samples were stable at room temperature for at least 26 h.

3.4. Extraction recovery

The mean extraction recoveries of *R*- and *S*-MP from urine at the low QC concentration (15 ng/ml) were 104.4±1.7 and 110±4.5%, respectively. Recoveries at the high QC concentration (750 ng/ml) were 98±2.4% for *R*-MP and 98.6±2.5% for *S*-MP.

4. Discussion

Mephenytoin is widely used as a CYP2C19 phenotyping probe in humans [6,7,13]. A preliminary consensus on the conduct of in vitro and in vivo drug metabolism studies recently recommended using MP as a CYP2C19 probe [14], further supporting its use in this regard and providing an impetus for the development of better analytical assays. The conventional method for determining *R*- and *S*-MP has been chiral GC with nitrogen phosphorus detection as reported by Wedlund et al. [10], but other GC and combined GC–MS methods have recently been published [11,12]. Our method provides advantages over each of the above methods. For example, our processing method requires the use of greatly reduced volumes of sample and extraction solvents; 200 μl urine samples are extracted with 1 ml of solvent, compared with 1 ml and 6 ml [10], 1 ml and 7 ml [11], and 4 ml and 5 ml of sample and solvent [12], respectively. Interestingly, we observed essentially complete recovery for both *R*- and *S*-MP with

these reduced volumes, compared with the recovery reported by Yao et al. of only 74% [12]. In addition, our method has a wider dynamic range (200× the LOQ), and the LOQ of 5 ng/ml provides 5–20-fold greater sensitivity than previously reported [10–12]. Increased sensitivity is important because *S*-MP concentrations can be low (~5–50 ng/ml) in very extensive metabolizers. Britzi et al. used HPLC prior to GC–MS analysis in order to separate the MP enantiomers, which complicates the analytical procedure [11]. Our method achieves direct and excellent separation of enantiomers using a chiral capillary column.

We are currently using MP as an in vivo probe of CYP2C19 activity in humans. CYP2C19 phenotype is characterized by administering racemic MP 100 mg orally, then determining the S:R ratio in urine collected in the subsequent 8-h [5,8]. Individuals having S:R ratio values <0.9 and ≥0.9 are classified as EM and PM, respectively [8]. Representative chromatograms of an EM and PM subject are shown in Fig. 4A and B, respectively.

In conclusion, the method described here is a sensitive, specific and reproducible GC assay utilizing mass-selective detection for the stereoselective determination of MP in human urine. This method employs a simplified processing procedure, demonstrates improved extraction recovery, and provides at least 5-fold greater sensitivity than previously reported assays. This assay is being used to support pharmacogenetic studies that include phenotypic evaluation of CYP2C19 activity.

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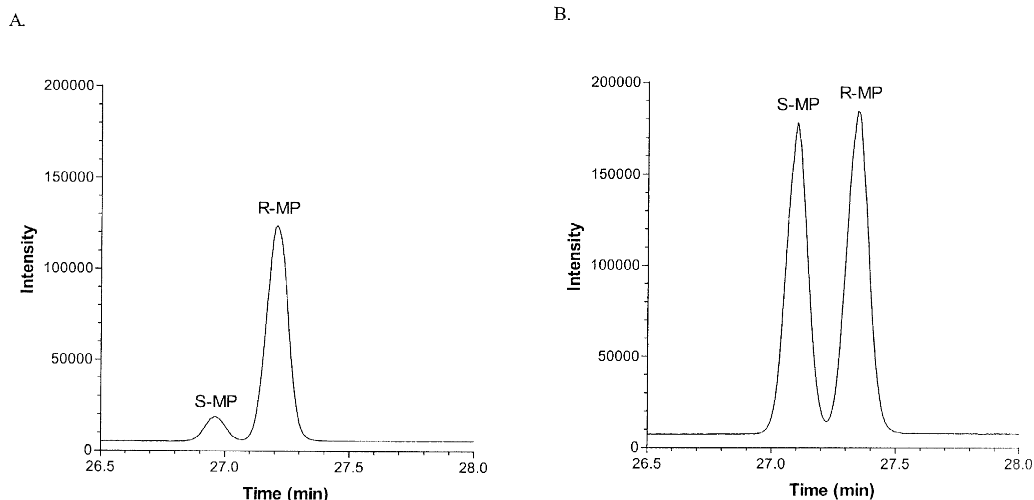

Fig. 4. Representative SIM chromatograms of samples obtained in the 8-h urine collection following a 100 mg dose of oral racemic MP; (A) urine sample with measured concentrations of 63.6 ng/ml (*S*-MP) and 592.0 ng/ml (*R*-MP) [S:R ratio=0.11; phenotypic EM subject]; (B) urine sample with measured concentrations of 565.1 ng/ml (*S*-MP) and 609.0 ng/ml (*R*-MP) [S:R ratio=0.93; phenotypic PM subject].

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