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High-performance liquid chromatographic determination of urinary 4′-hydroxymephenytoin, a metabolic marker for the hepatic enzyme CYP2C19, in humans

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Abstract

The preferential hydroxylation of (*S*)-mephenytoin to 4′-hydroxymephenytoin (4′-OH-M) displays a genetic polymorphism of drug metabolism in humans. Thus the excreted 4′-OH-M is considered to be an important marker for the hepatic (*S*)-mephenytoin 4′-hydroxylase. Accordingly, a mixture of urine containing total 4′-OH-M after enzymatic deconjugation and phenobarbital as internal standard (I.S.) was extracted with absolute diethyl ether. The residue remaining after evaporation was dissolved in 50 μl of eluate and 20 μl were injected into the chromatographic system. All components were separated isocratically on a reversed-phase column using acetonitrile–water (24:76, v/v) as the mobile phase at a flow-rate of 1.2 ml/min. The effluent was monitored at 204 nm. The retention times for 4′-OH-M and the I.S. were within 6 min. The absolute recovery was in the range 84–89% for 4′-OH-M and that of the I.S. was $75.9 \pm 4.2\%$. Quantification was performed by measuring the peak-height ratio compared with the ratio of the amount of 4′-OH-M divided by that of the I.S. The intra- and inter-day variations were less than 8% and 10%, respectively. The proposed method is simpler and more convenient than those reported previously. Its practical applicability was assessed by phenotyping the efficient and deficient hydroxylators among the Chinese minorities and Han Chinese populations.

1. Introduction

The anticonvulsant mephenytoin is administered clinically as a racemic mixture. However, the stereoselective 4′-hydroxylation of (*S*)-mephenytoin exhibits a genetic polymorphism of drug oxidation in humans [1,2], with the *S*-enantiomer being almost completely hydroxylated in the *para* position of phenyl ring and rapidly excreted largely as 4′-OH-M glucuronide [3,4]. The 4′-hydroxylation of (*S*)-mephenytoin in human liver is under the control of a specific hepatic cytochrome P450 enzyme that was recently identified as CYP2C19 [5]. The deficient hydroxylation of (*S*)-mephenytoin appears to be very important in Oriental populations since the prevalence of the deficient phenotype is observed in 2–5% of Caucasians, but at a higher incidence (12–23%) in Orientals [1,2,6–9]. Further, this genetic defect affects the metabolism of a number of other commonly used drugs [7,8]. If this polymorphism affects rational drug selection and use, such a deficiency might be of more clinical concern in Orientals than in Caucasians.

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Although only trace or undetectable amounts of mephenytoin are excreted as the unchanged form [1] and the recovery of 4′-OH-M from 8-h urine of subjects with impaired hydroxylation is very low [2], the catalytic activity of CYP2C19 was expressed as either the percentage excretion of 4′-OH-M [6,9–11] and hydroxylation index [1,2,6,9] derived from the 8-h urinary recovery of 4′-OH-M, or the enantiomeric (*S*/*R*)-mephenytoin ratio [2,12] in 0–8-h urine collection. Thus individuals can be phenotyped as either extensive (EM) or poor (PM) metabolizers simply based on the urinary metabolic profiles after an oral test dose of racemic mephenytoin. HPLC determination of the 0–8-h urinary recovery of 4′-OH-M [1,2,6,9–11] as a result of human liver microsomal (*S*)-mephenytoin 4′-hydroxylase activities [13–17] is a simpler approach. Accordingly, this paper describes an improved reversed-phase HPLC assay that we have recently developed for the investigation of phenotype distribution among the different Chinese minorities and Han Chinese populations.

2. Experimental

2.1. Chemicals and reagents

Dry powder of 4′-OH-M as a standard was kindly donated by Dr. G.R. Wilkinson (Vanderbilt University School of Medicine, Nashville, TN, USA). The internal standard (I.S.), sodium phenobarbital, was obtained from Xinya Pharmaceutical (Shanghai, China). Mephenytoin tablets (Mesantoin, 100 mg each tablet) were purchased from Sandoz Pharmaceutical (East Hanover, NJ, USA). β-Glucuronidase (EC 3.2.1.31) was Type VII from *Escherichia coli* (Sigma). Acetonitrile (Huangyan, Zhejiang, China) of HPLC grade and doubly distilled water were required for HPLC and UV detection. All other chemicals were of analytical-reagent grade unless specified otherwise.

2.2. Instrumentation

The HPLC system consisted of an HP Series 1050 solvent-delivery system, an on-line degasser, a manual injector, a reversed-phase column (HP Spherisorb ODS-2, 125 mm × 4 mm I.D., 5-μm particle size) and a variable-wavelength detector. The data system consisted of an HP Vectra DOS ChemStation Controller and an HP DeskJet 500 printer. All the above apparatus was purchased from Hewlett-Packard (Palo Alto, CA, USA).

2.3. Standard solutions

A stock standard solution (500 μg/ml) was prepared by dissolving 4′-OH-M in HPLC-grade acetonitrile and was stored frozen at −20°C. A stock standard solution (500 μg/ml) of the I.S. was prepared by dissolution in HPLC-grade methanol, and was diluted fivefold to obtain a working standard solution (100 μg/ml). All the standard solutions were also kept frozen at −20°C until used.

2.4. Chromatographic conditions

The mobile phase was acetonitrile–doubly distilled water (24:76, v/v) at a flow-rate of 1.2 ml/min. The UV signal of each analyte was monitored at 204 nm [13–16]. Chromatograms and data were recorded with an HP ChemStation (HP Vectra 486/33VL).

2.5. Sample preparation

Aliquots of urine (0.1 ml) were pipetted into 10-ml screw-capped polyethylene centrifuge tubes which contained 0.9 ml of water. After addition of 50 μl of β-glucuronidase solution (5000 Fishman U/ml) prepared with 0.5 *M* sodium acetate buffer (pH 5), the mixture was incubated for 12 h at 37°C in a water-bath for deconjugation of 4′-OH-M glucuronide. The hydrolysate was spiked for 50 μl of I.S. working standard solution and was extracted with 4 ml of diethyl ether. Tubes were capped and shaken vigorously for 3 min. Following centrifugation at 1500 *g* for 5 min, 3 ml of the ether layer were transferred into a clean 10-ml conical glass tube and evaporated to dryness in a water-bath at 40°C. The residue was dissolved in 50 μl of

eluent and vortex mixed and 20 μl were injected into the HPLC system.

2.6. Method validation

Selectivity

Five blank urine samples from different healthy medication-free volunteers were prepared as described in Section 2.5 and analysed by comparing the response differences between the pre- and postdose urine.

Precision and accuracy

Spiked replicated urine samples containing 2.5, 25 and 100 μg/ml of 4'-OH-M were prepared in the linear response range for intra- ($n = 5$) and inter-day ($n = 10$) reproducibility and accuracy. These samples were extracted as described in Section 2.5 and analysed on the same day and on different days, respectively.

Extraction recovery

The spiked sample (0.1 ml) was extracted as described in Section 2.5, but the residue was reconstituted in 150 μl of mobile phase and vortex mixed and 20 μl were injected on to the column. The preparation of unextracted urine was performed by the following procedure. First, three levels (2.5, 25.0 and 100.0 μg/ml) of 4'-OH-M working standard solution were prepared by diluting the stock standard solution (500 μg/ml) with mobile phase, then 0.1 ml drug-free urine (not spiked with I.S.) was extracted as described in Section 2.5 but the residue was reconstituted in 100 μl of the different 4'-OH-M working standard solution and 50 μl of I.S. working standard solution and vortex mixed and 20 μl were injected on to the column. The extraction recovery was determined by comparing the peak-height counts of extracted and unextracted spiked urine containing 2.5, 25 and 100 μg/ml of 4'-OH-M and 100 μg/ml of I.S., respectively.

Linearity, limit of detection and quantification

A standard response curve was constructed by plotting peak-height ratio against the ratio of the amount of 4'-OH-M to that of the I.S. with eleven different known levels of 4'-OH-M in the range 0.05–250 μg/ml and the I.S. at 100 μg/ml in the spiked urine. Weighted linear regression analysis was used to determine slopes, intercepts and correlation coefficients of the calibration graphs. The limit of detection of this method was defined at a signal-to-noise ratio of 3:1. Concentrations of 4'-OH-M in the unknown urinary samples were determined from the peak-height ratio and response factors of the calibration samples at three different levels within the linear range.

2.7. Applications

Each fasting subject volunteered to be given a single 100-mg tablet of racemic mephenytoin (Mesantoin, Sandoz) immediately after voiding. Urine samples were collected during the subsequent 8-h period, the urine volume was measured and aliquots (10 ml) were stored frozen at −20°C until analysed.

3. Results

3.1. Selectivity

Fig. 1A and B show representative chromatograms obtained from human blank urine and blank urine spiked with 4'-OH-M and I.S., respectively. No endogenous interfering peaks were found in the blank urine samples. 4'-OH-M and the I.S. gave rapidly eluting, fully resolved and sharp symmetrical peaks; the total elution time per run was within 6 min.

3.2. Intra-day reproducibility

The results are summarized in Table 1. The coefficients of variation (C.V.) for the intra-day reproducibility ranged from 4.1 to 7.7%.

3.3. Inter-day reproducibility

The results obtained from identical extraction and assay on the different days are summarized in Table 1. The C.V.s for the inter-day reproducibility were in the range 4.2–9.1%.

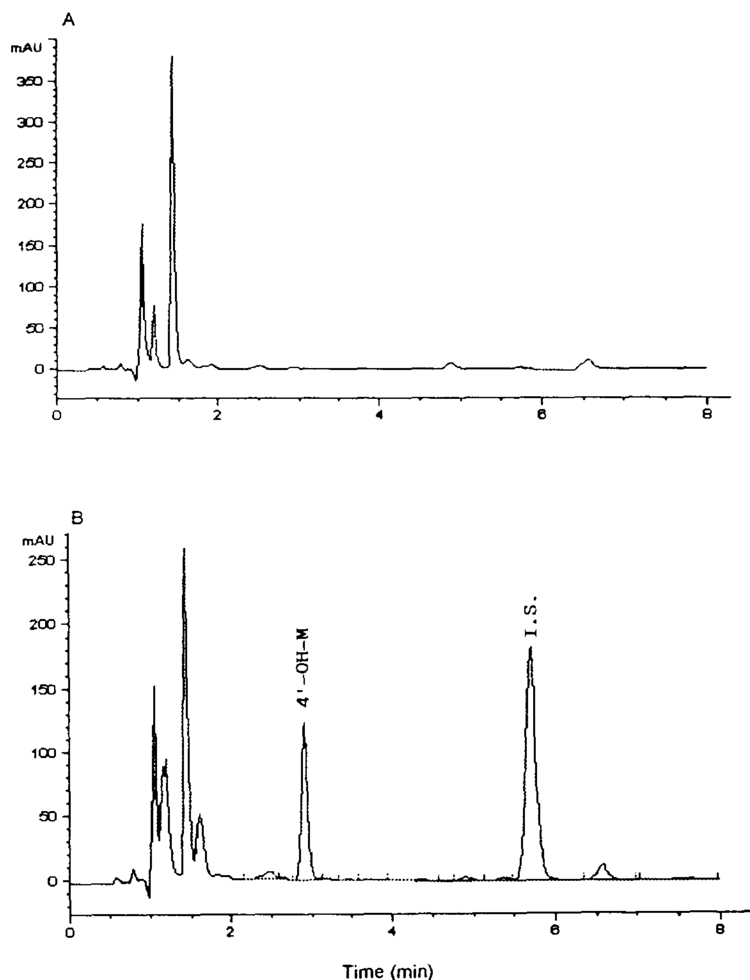

Fig. 1. Representative chromatograms of (A) extracted human urine blank and (B) human urine spiked with 4'-OH-M (25 μg/ml) and I.S. (100 μg/ml).

3.4. Accuracy

The results from the accuracy studies are given in Table 2. The relative recovery derived from the found and spiked levels ranged from 95 to 105%.

3.5. Extraction recovery

The extraction recovery is given in Table 3. The absolute recovery of 4'-OH-M at the three levels of 2.5, 25 and 100 μg/ml ranged from 83.5 to 89.3%, and that of the I.S. was $75.9 \pm 4.2\%$ ($n = 5$).

3.6. Linear range and detection limit

A linear calibration graph was obtained for 4'-OH-M in urine over the range 0.5–100 μg/ml

Table 1
Precision of the determination of 4'-OH-M in human urine

Spiked level (μg/ml)	C.V. (%)	
	Intra-day (n = 5)	Inter-day (n = 10)
2.5	7.7	9.1
25.0	4.1	4.2
100.0	5.2	4.8

Table 2
Accuracy of the HPLC–UV assay of 4′-OH-M in human urine (mean ± S.D., $n = 5$)

Spiked concentration (μg/ml)	Found concentration (μg/ml)	Relative recovery (%)
2.5	2.46 ± 0.23	98.5 ± 9.4
25.0	26.06 ± 1.06	104.7 ± 4.9
100.0	95.43 ± 5.56	95.4 ± 5.6

with a correlation coefficient (r) of 0.9994; another wider linear range also existed in the range 0.1–250 μg/ml ($r = 0.9984$). This method has a limit of detection of ca. 0.05 μg/ml.

3.7. Applications

The formation of 4′-OH-M is impaired in PM of CYP2C19 enzyme defect. The hydroxylation index is calculated based on the ratio of a half racemic dose (50 mg) divided by the excreted amounts of 4′-OH-M in 8-h urine [1]. A representative chromatogram from a PM of Chinese Dong minority is shown in Fig. 2A and one from an EM in Fig. 2B. The phenotypic data of the different populations will be reported in detail elsewhere.

4. Discussion

The urinary (S/R)-mephenytoin ratio and the excreted 4′-OH-M have been chosen as the two specific phenotypic traits for further study [7,8]. The S/R ratio is an important characteristic of the polymorphic 4′-hydroxylation of (S)-mephenytoin in vitro and in vivo [7,16], and is commonly measured by a modification [18] of the chiral capillary GC methods described as Wedlund and co-workers [2,19] and by a chiral HPLC assay [20]. This approach has several advantages over the determination of the excreted 4′-OH-M [2,7], including the absence of a requirement for absolute quantification of concentrations, and less dependence on complete urine collection and the R-enantiomer as an index of compliance. However, rapid processing of urine shortly after its collection is required in order to avoid inaccurate phenotype assignments based on the urinary S/R ratio [21]. This is because an acid-labile metabolite of mephenytoin concurrently excreted in the urine can be converted into (S)-mephenytoin by strong acid and then alter the S/R ratio [21]. The formation of 4′-OH-M is a direct reflection of hepatic drug-metabolizing enzyme CYP2C19 activities [1,5]. Hence the determination of 4′-OH-M has recently been considered to be additional accurate and practical phenotyping approach used in 8-h urine collection after dosing [1,2,6,9–11] and in human liver microsomes [13–17].

Assay methods for 4′-OH-M include GC [1–3,6,11] and HPLC [2,13–17]. Based on the previous reports, HPLC is an easier and more rapid procedure. Accordingly, we recently developed a simple and rapid HPLC assay for the investigation of CYP2C19 enzyme defect among the Chinese population. In the present assay, an aliquot of 8-h urine was extracted with diethyl ether after β-glucuronidase hydrolysis [10,11]. The time required for complete hydrolysis of the 4′-OH-M glucuronide was determined for 12 h based on a recent report [22] and our preliminary hydrolysis data. Phenobarbital was chosen as an appropriate I.S. [13–16] because it showed the same properties as 4′-OH-M in the extraction procedure and also a comparable retention time [15]. UV monitoring at 204 nm was selected on the basis of previous reports [13–16] and UV scanning of 4′-OH-M in the eluate. The time of analysis by HPLC was within 6 min. This assay is considered to be simpler and more convenient than those reported previously [13–15] and has

Table 3
Extraction recovery of 4′-OH-M from human urine (mean ± S.D., $n = 5$)

4′-OH-M (μg/ml)	Peak-height counts		Recovery (%)
	Unextracted	Extracted	
2.5	22.9 ± 2.3	19.1 ± 1.6	83.9 ± 9.4
25.0	227.5 ± 12.1	189.5 ± 5.7	83.5 ± 6.3
100.0	861.0 ± 21.6	768.6 ± 43.3	89.3 ± 4.2

Recovery of I.S. at 100 μg/ml was 75.9 ± 4.2% ($n = 5$).

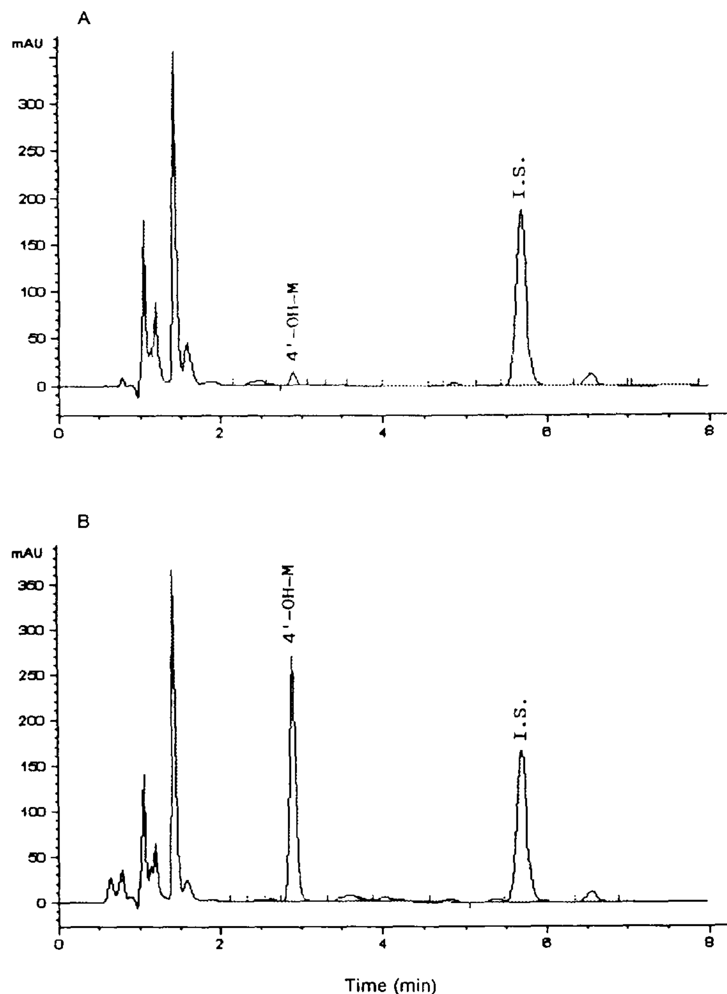

Fig. 2. Typical chromatograms of extracted urine from (A) a Dongzu Chinese (PM) with 4′-OH-M at 2.57 μg/ml and (B) a Dongzu Chinese (EM) with 4′-OH-M at 67.56 μg/ml.

been successfully utilized in the measurement of phenotypic traits of CYP2C19 among the Chinese minorities and Han Chinese populations.

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