Journal of Chromatography, 573 (1992) 173–177
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6115

Short Communication

Determination of mephenytoin stereoselective oxidative metabolism in urine by chiral liquid chromatography employing β-cyclodextrin as a mobile phase additive

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(First received May 2nd, 1991; revised manuscript received August 14th, 1991)

ABSTRACT

A direct high-performance liquid chromatographic assay was developed for the separation and determination of *S*- and *R*-mephenytoin enantiomers in human urine. Separation was achieved on a Supelcosil LC-8 column with methanol–0.1 *M* acetate buffer as the mobile phase and β-cyclodextrin as a mobile phase additive. The urinary *S*/*R* enantiomeric ratio of mephenytoin was measured in a 32-h urine sample (as phenotypic traits) for determination of the ability of normal subjects to hydroxylate racemic mephenytoin after oral administration of the drug.

INTRODUCTION

Mephenytoin (M), an antiepileptic drug, is used as a racemate. During metabolization only *S*-M undergoes hydroxylation to 4-hydroxymephenytoin (4-OH-M), followed by glucuronidation. However, both enantiomers can be demethylated to phenylethylhydantoin (PEH), as shown in Fig. 1 [1–4].

In case of extensive metabolizers, the *S*-enantiomer is rapidly hydroxylated to 4-OH-M with a half-life of *ca.* 3–5 h, whereas the *R*-enantiomer is transformed to *R*-PEH with a half-life of five to six days. Being under autosomal genetic control, 4-OH-M is practically not formed in case of the poor metabolizers, so this metabolizing step is polymorphically regulated. Owing to the lack of hydroxylation of *S*-M in poor metabolizers, both enantiomers are trans-

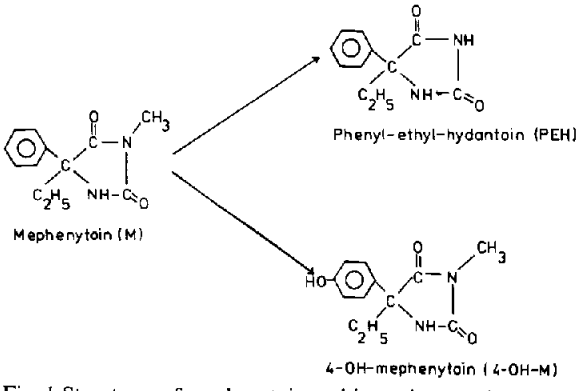

Fig. 1 Structures of mephenytoin and its major metabolites.

0378-4347/92/$05.00

formed to PEH, a biologically active compound, which accumulates in human organs thus leading to drug intoxication [4,5]. The incidence of poor metabolizers is *ca.* 3–8% in Caucasians, but as high as 17–23% in Far Eastern populations [6–10].

Two methods may be used to determine the phenotype. The first, introduced by Küpfer and co-workers [2,3], is based on the measurement of the amount of 4-OH-M excreted in the urine, mainly as the glucuronide conjugate, within 8 h after the oral administration of 100 mg of racemic M. The two phenotypes can be sharply differentiated by using the so-called hydroxylation index (HI), which is the coefficient in micromoles of the *S*-M dose and of 4-OH-M excreted into the urine during 8 h [2,3]. For assessing 4-OH-M in the urine several gas chromatographic (GC) [1,2,7], gas chromatographic–mass spectrometric (GC–MS) [6] and high-performance liquid chromatographic (HPLC) methods [1,9,11] are used. They all have the common problems of needing 4-OH-M, which is not available commercially, as a prerequisite for the assessment, and requiring a long time for sample preparation for 4-OH-M release from the conjugate.

The second form of phenotyping is based on the direct determination of the *S*/*R*-mephenytoin ratio in the urine. Wedlund and co-workers [12–15] separated the *R*- and *S*-M enantiomers in urine samples using a chiral capillary GC column (Chirasil-Val). Their method was developed further by Sanz *et al.* [8] who showed that when the *S*/*R* enantiomeric ratio was used for phenotyping an easier and more distinctive differentiation can be made, since instead of collecting the urine for 0–8 h a 24–32 h urine sample is processed, and *S*-M has virtually disappeared from the urine of extensive metabolizers after 24 h.

The mephenytoin 4-hydroxylase activity of liver microsomes was measured by Yasumori *et al.* [16] by determining the *S*/*R* enantiomeric ratio on a Chiralcel OJ column with HPLC. Enantiomers were eluted from the cellulose triacetate column with ethanol.

Racemic hydantoin derivatives, such as M, were used by Enquist and Hermansson [20] for studying the applicability of the chiral α_1-acid glycoprotein column. The mobile phase was 0.01 *M* phosphate buffer with 1–40% 2-propanol or acetonitrile.

It is known that cyclodextrins (CD) (cyclic oligosaccharides) can be successfully used for the separation of enantiomers of chiral compounds, either as stationary phases or as mobile phase additives. Armstrong *et al.* [17] by applying β-CD in the mobile phase separated racemic M into two enantiomers with thin-layer chromatography (TLC). The same authors [18] also separated racemic M on a β-CD bonded-phase column with methanol–1% triethylamine acetate (pH 4.1) (15:85) as the mobile phase. A similar study was carried out by Maguire [19], who also used covalently linked β-CD chiral stationary phases for the separation of the enantiomers of, among others compounds, M. A buffer containing 10–20% methanol and 10% acetonitrile was used as the eluent.

Our aim was to achieve the phenotyping of M metabolism, using the *S*/*R* enantiomeric ratio in the urine, on a commercially available reversed-phase HPLC column. We drew on the work of Sybilska *et al.* [21], who separated M and barbiturate enantiomers on a reversed-phase C_{18} column by using β-CD in the mobile phase: a 30 m*M* concentration of β-CD buffered in ethanol–acetate with a flow-rate of 0.6 ml/min on a 25.0-cm LiChrosorb RP C_{18} column resulted in a retention time of *ca.* 40 min.

Gazdag and co-workers [22–25] studied in detail the applicability of the various types of CD in mobile phases, as well as the effect of mobile phase modifiers (pH, ion strength, CD concentration, etc.) on the retention and separation parameters.

The above-mentioned studies did not deal with the assessment of M from a biological medium, because they examined its chromatographic properties arising from the cavital structure of CD. Thus we aimed at designing an HPLC method, using a simple reversed-phase column and with CD in the mobile phase, for separating and determining *S*- and *R*-M in the urine.

EXPERIMENTAL

Chemicals

R-M and *S*-M (3-methyl-5-phenyl-5-ethylhy-

dantoin) were generously supplied by Professors Branch and Wilkinson (Vanderbilt University, Nashville, TN, USA), and the racemic mephenytoin (1:1) by Alkaloida (Tiszavasvári, Hungary). Cyclodextrin was kindly donated by Chinoin (Budapest, Hungary). Methanol and chloroform were the products of Merck (Darmstadt, Germany).

Sep-Pak silica cartridges were purchased from Waters (Milford, MA, USA). All the other reagents were from Reanal (Budapest, Hungary) and were of the best commercially available quality and grade.

The stock solution of M was 1 mg/ml in methanol. Working solutions were made by appropriate dilutions with methanol.

Sample collection

After emptying the bladder for drug-free sample collection, the volunteers of the study were given 100 mg of racemic mephenytoin (Alkaloida) orally. Urine was collected between 24 to 32 h after administration. Aliquots of *ca.* 20 ml were stored at −20°C until analysis.

Sample preparation

To 10.0 ml of centrifuged urine, 15 ml of chloroform were added (the pH of urine samples was adjusted with 0.1 *M* HCl or 0.1 *M* NaOH, as required). The mixture was shaken for 30 s in a shaker (Kutesz, Budapest, Hungary) and then filtered through a Whatman IPS phase separator (Balston, Maidstone, UK) and poured onto a Sep-Pak silica cartridge, which was primed with 2 ml of chloroform. A 2-ml volume of methanol was used to elute the compounds. The eluate was collected in a vial and evaporated to dryness under nitrogen, and the dry residue was redissolved in 50 μl of methanol by shaking for 30 s. Finally a 20-μl aliquot was injected into the analytical column.

Chromatographic conditions

HPLC separation was performed on a Shimadzu LC-6A liquid chromatograph (LC-6A pump unit, SPD-6A UV detector and C-R6A integrator) fitted with a Beckman Model 210 injector with a 20-μl loop. Separation was achieved isocratically on a 75 mm × 4.5 mm I.D. Supelcosil LC-8 (3 μm particle size) column (Supelco, Bellefonte, PA, USA) at room temperature with methanol–0.1 *M* acetate buffer (pH 5.0, with 10 m*M* β-CD) (20:80, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. The absorbance was monitored at 230 nm. Air was removed from the eluent with helium, prior to its use.

TABLE I

EFFECT OF SOLVENT AND pH ON RECOVERY

Solvent	Recovery (%)		
	pH 4	pH 6	pH 8
Chloroform	66.4	72.8	54.7
Ethylene chloride	59.0	61.3	50.8
Ethyl acetate	64.9	69.2	52.8

RESULTS AND DISCUSSION

Under the chromatographic conditions used the retention times for *S*- and *R*-M were 8.3 and 9.8 min, respectively. This, as shown in Fig. 2, results in a good baseline separation ($k'_1 = 6.80$, $k'_2 = 8.21$, $\alpha = 1.2$, $R_s = 3.26$).

In designing this chromatographic separation we tested C_{18} reversed phases; however, owing to the 50-min retention, these columns are not prac-

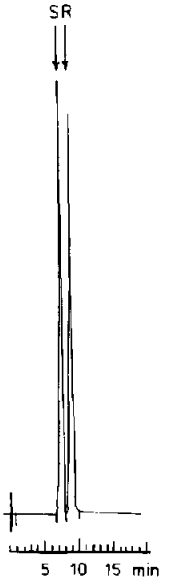

Fig. 2. Separation of enantiomers of mephenytoin (chromatographic conditions are given in the text).

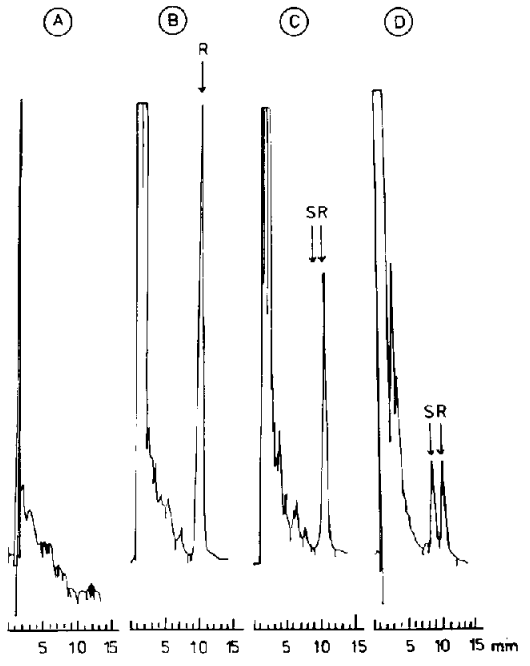

Fig. 3. Chromatograms of human urine extracts. (A) Drug-free urine; (B) standard sample containing 20.0 μg/ml *R*-mephenytoin; chromatograms from (C) an extensive metabolizer and (D) a poor metabolizer. Chromatographic conditions are given in the text. The sample in C contained 15.20 μg of *R*-M and 0.68 μg of *S*-M, and that in D contained 8.54 μg of *R*-M and 9.74 μg of *S*-M in 1 ml of urine.

tical for pharmacokinetic studies requiring a large number of measurements. We also tested α- and γ-CD in the mobile phase, but this resulted in poorer separations than when β-CD was used.

In the urine extraction step, three solvents were tried at various pH values. The best extraction was achieved with chloroform, which was used later at a pH of 6 (Table I).

The endogenous polar contaminants in the urine matrix were retained by Sep-Pak silica cartridges. However, no trace of M was found in the extract containing chloroform passed through the cartridge. Methanol elutes M from the silica cartridge with a recovery rate of almost 100%.

To illustrate the applicability of the method, Fig. 3 shows chromatograms of a drug-free urine extract, a model sample and two typical urine extracts from an extensive metaboliser and a poor metaboliser. The chromatogram of the blank sample shows no components that would interfere with M.

The detection limit was found to be 0.1 μg/ml *S*- or *R*-M in urine.

Since, as outlined in the introduction, we wished to apply our method only for assessing the *S*/*R*-M concentration ratio, no internal standard was used.

Standard response curves were linear over the range 0.1–20 μg/ml *S*- or *R*-M in urine. The day-to-day coefficient of variation (C.V.) in the slope of the calibration curves was 4.59% for both enantiomers ($n = 5$). The intra-day C.V. in the assay was better than 3% for M at 20 μg/ml and 4.5% at 5 μg/ml.

ACKNOWLEDGEMENTS

We thank Professors Branch and Wilkinson for their generous gift of the enantiomers and Professor G. Gübitz (Graz, Austria) for his valuable advice. Thanks are also due to Mrs. Zs. Orosz and Miss Á. Orosz for their skillful technical assistance.

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