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Simultaneous enantiospecific separation and quantitation of mephenytoin and its metabolites nirvanol and 4′-hydroxymephenytoin in human plasma by liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the enantiospecific quantitation of *S*- and *R*-mephenytoin and its metabolites *S*- and *R*-nirvanol and *S*- and *R*-4′-hydroxymephenytoin in plasma is described. The compounds were separated using a reversed-phase C_2 column in tandem with a chiral α_1-acid glycoprotein column and were detected using ultraviolet detection at 205 nm. The lower limit of quantification was 10 ng/ml for all compounds using 0.5 ml human plasma (intra-day coefficient of variation <13%, accuracy <±20%). The method was validated for human plasma in the concentration range 10–2000 ng/ml for each of the six compounds. The method allows for the simultaneous characterisation of the metabolic capacity of two human drug-metabolising enzymes, CYP2C19 and CYP2B6, and may be used when investigating polymorphisms or changes in activity of these two enzymes.

Keywords: Mephenytoin; Nirvanol; 4′-Hydroxymephenytoin

1. Introduction

Knowledge of enzyme-specific drug–drug interactions, including inhibition, down-regulation or induction of enzymes, as well as prediction of clinically relevant genetic polymorphisms, is an important concern in drug development. Model test compounds may be used as probes to determine the capacity of individual enzymes, e.g. cytochrome P450 (CYP) enzymes, in vivo [1]. Commonly, the ratio between the concentrations of the probe compound and its metabolites in blood plasma or in urine is calculated as an index of the metabolic activity of a particular enzyme.

CYP2B6 is a liver enzyme which, over the years, has been thought to play a very minor role in human drug metabolism. However, there are an increasing number of examples of drugs found to be substrates of this enzyme [2–6]. The amount of CYP2B6 expressed in human liver is small relative to other drug-metabolising CYP450s and considerable variability in the expression of CYP2B6 has been observed [7,8]. Moreover, several mutations, exten-

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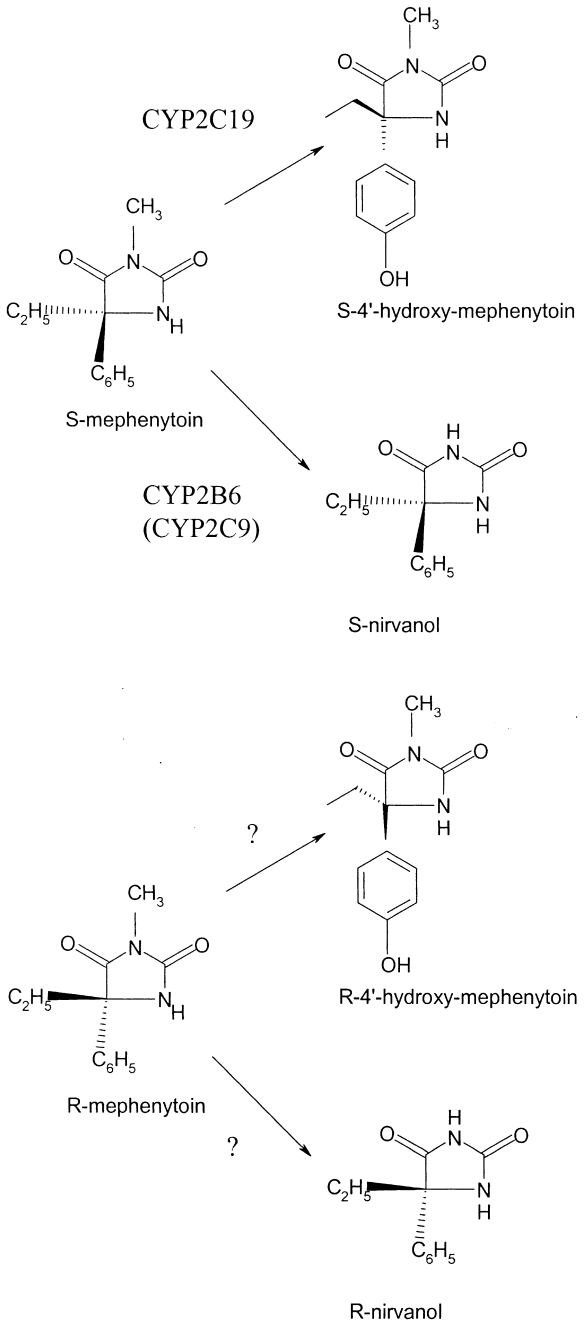

Fig. 1. *S*-Mephenytoin and *R*-mephenytoin [molecular weight (MW) 218.25 Da] and their principal metabolites nirvanol (MW 204.23 Da) and 4′-hydroxymephenytoin (MW 234.25 Da) shown with the main enzymatic pathways of metabolism in humans. In CYP2C19-poor metabolisers, *S*-4′-hydroxymephenytoin is not formed due to polymorphism.

sively affecting the inter-individual variability of CYP2B6 expression and function, have been described [9].

S-Mephenytoin *N*-demethylation is used as an enzyme reaction to characterise CYP2B6 activity in vitro [8,10,11]. Mephenytoin (Fig. 1) is a 5,5-disubstituted hydantoin which has been used clinically as an antiepileptic agent [12]. The drug has a center of asymmetry at the 5 position of the hydantoin ring, and its metabolism in man is highly stereoselective. The center of asymmetry is unaffected by metabolism and all known metabolites retain the same configuration as the parent enantiomer. In man, two routes of metabolism of mephenytoin have been described [13,14]. One route involves aromatic hydroxylation at the *para* position of the phenyl ring of 4′-hydroxymephenytoin (Fig. 1), which is subsequently conjugated with glucuronic acid. The second pathway is via *N*-demethylation to 5-phenyl-5-ethylhydantoin (nirvanol) [15,16]. Nirvanol (Fig. 1) is metabolised further by direct conjugation with glucuronic acid [13]. The *S*-enantiomer of mephenytoin, preferentially hydroxylated primarily by CYP2C19, may be used as an in vitro probe for this enzyme [17,18]. The enantiomer ratio, based on urinary concentrations, has been used as a phenotyping in vivo probe for CYP2C19 since the (*S*)-enantiomer is rapidly metabolised in extensive metabolisers but poor metabolisers are unable to carry out this 4′-hydroxylation [19,20]. In CYP2C19 EMs, the dispositions of *S*- and *R*-mephenytoin are markedly different with a mean oral clearance of 4.7 versus 0.027 l/min after a 300 mg oral dose. However, the stereoselective elimination of mephenytoin is almost absent in the CYP2C19 PM phenotype. The mean oral clearance of *S*-mephenytoin and *R*-mephenytoin, after a 200 mg racemic mephenytoin dose to CYP2C19 PMs, was reported to be 0.029 and 0.0195 l/min, respectively. Almost comparable plasma levels of *S*- and *R*-nirvanol are seen in CYP2C19 PMs, whereas lower concentrations of *S*-nirvanol compared to *R*-nirvanol are seen in CYP2C19 EMs [20]. The *N*-demethylation of *S*-mephenytoin is catalysed primarily by CYP2B6 with some contribution from CYP2C9 [21–23]. *R*-Mephenytoin is mostly demethylated by an as-yet unidentified enzyme.

Until now, no HPLC method has been published where the enantiomers of mephenytoin as well as the

enantiomers of the two metabolites, nirvanol and 4′-hydroxymephenytoin, have been separated simultaneously and detected from a biological sample.

In the past, enantiomers were separated by preparation of a diastereomeric derivative and separated on a non-chiral column [24,25]. However, the direct separation technique has advantages compared to the indirect diastereomeric technique [26]. The plasma protein α_1-acid glycoprotein (AGP) is a very stable chiral selector which can be used over a fairly wide pH range without being denatured, also tolerating organic solvents and high temperatures. There are many possibilities for regulating the enantioselectivity and the retention, such as changing the eluent pH, type and concentration of organic modifier and charged modifier [27].

We present a HPLC method based on separation on an AGP column permitting the simultaneous quantification of the enantiomers of mephenytoin, nirvanol and 4′-hydroxymephenytoin. The method is particularly useful for monitoring CYP2B6 and CYP2C19 activities with mephenytoin as a probe drug, since it allows for the separation and quantitation of *S*-mephenytoin, *S*-nirvanol and *S*-4′-hydroxymephenytoin after administration of racemic mephenytoin to man.

2. Experimental

2.1. Materials

Racemic (±)-4′-hydroxymephenytoin, *S*-(+)-mephenytoin, *R*-(−)-mephenytoin, *S*-(+)-nirvanol and *R*-(−)-nirvanol were purchased from Ultrafine (Salford Ultrafine Chemicals and Research, Manchester, UK). Acetonitrile Lichrosolve, methanol Lichrosolve, 2-propanol Lichrosolve, 1,2-dichloroethane Uvasol, sodium hydroxide pellets and 85% orthophosphoric acid of analytical grade were purchased from Merck (Darmstadt, Germany). The water was purified by a Milli-Q Academic system (Millipore, Bedford, MA, USA). Blank human plasma was obtained from the University Hospital Blood Bank, Uppsala, Sweden.

Separation was carried out on a Nucleosil 100 C_2 column, 33×3 mm I.D., 7 μm particle size, combined sequentially with a Chiral-AGP column, 150×4.0 mm I.D., 5 μm particle size (ChromTech, Hägersten, Sweden). A silica saturator, 10×4.0 mm I.D., (ChromTech) was placed before the injector and a 0.5 μm A-431 in-line filter (Upchurch Scientific, WA, USA) was placed after the injector.

2.2. Chromatographic instrumentation

The HPLC system consisted of a LC-10AD pump (Shimadzu, Kyoto, Japan), a CMA/200 refrigerated autosampler equipped with a 100 μl loop (CMA Microdialysis, Solna, Sweden) and a SPD-10A UV detector (Shimadzu). The signals were stored and processed using Chromatography Station for Windows (DataApex, Prague, Czech Republic).

2.3. Chromatographic conditions and instrument settings

The mobile phase, 2.5% acetonitrile in phosphate buffer, pH 7.0, $\mu = 0.1$, was filtered through a GH Polypro Pall 0.45 μm filter (Gelman, MI, USA) and degassed with helium before use. The flow-rate of the mobile phase was set to 0.9 ml/min, resulting in an operating pressure of 90 bar. The samples were kept at 8 °C in the autosampler. The injection volume was 100 μl. The two analytical columns were maintained at 30±0.1 °C in a MS water bath (LAUDA, Königshofen, Germany). The detector wavelength was set to 205 nm.

2.4. Preparation of standards and quality control samples

Separate methanolic stock solutions of *S*-mephenytoin, *R*-mephenytoin, *S*-nirvanol, *R*-nirvanol (each 1 mg/ml) and racemic 4′-hydroxymephenytoin (2 mg/ml) were prepared. From these stock solutions, two mixtures were made, one for the calibration curve with a concentration of 20 μg/ml of each compound, and one for the quality control samples with a concentration of 15 μg/ml of each compound. Pooled human blank plasma was spiked to 10, 20, 100, 250, 400, 1000, 1250, 1750 and 2000 ng/ml for the calibration curve and to 40, 500 and 1500 ng/ml for the quality controls. A blank, drug-free plasma sample was also included in the standard

curve. The stock solutions, standards and controls were stored at −20 °C.

2.5. Extraction procedure

The samples were thawed, gently agitated, centrifuged (10 min at 1000 *g*) and 0.5 ml plasma was transferred to glass extraction tubes. Exactly 7 ml of 1,2-dichloroethane was added and, after capping, the tubes were agitated for 15 min at 420 rpm using a KL 2 horizontal shaker (Edmund Bühler, Tübingen, Germany). After centrifugation for 10 min at 1000 *g*, the upper layer (water phase) was removed. Exactly 6 ml of the organic phase was transferred to new glass test tubes and evaporated under nitrogen at 40 °C. The residues were dissolved in 250 μl mobile phase by Vortex mixing (15 s) and sonication for 5 min. The solutions were then transferred to the auto-injector vials.

2.6. Quantification

On each day of analysis, two standard curves were constructed, one in the range 0–250 ng/ml and the other from 250 to 2000 ng/ml by linear un-weighted least squares regression of peak heights versus nominal plasma concentrations. The curves were not forced through the origin. The concentrations of the quality controls and unknown samples were calculated from the regression equations. The lower curve was used when the sample peak heights were less than or equal to the peak height corresponding to the standard sample of 250 ng/ml. The upper curve was used when the sample peak heights were greater than the peak height corresponding to the standard sample of 250 ng/ml.

2.7. Method validation

The intra-day precision, accuracy and recovery were determined in one validation run. This run included a calibration curve in single quality controls ($n=8$), and an unprocessed calibration curve in mobile phase. To determine the lower limit of quantification the 10 ng/ml standard ($n=8$) was included. The inter-day precision and accuracy were determined over nine runs. Each run included a calibration curve in single quality controls in duplicate interspersed with unknown samples. The precision of the method at each concentration was expressed as the coefficient of variation (C.V.) by calculating the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the assay was determined by expressing the mean calculated concentration as a percentage of the added concentration. The lower limit of quantification was defined as the lowest concentration with an intra-day C.V. of $<20\%$ and an accuracy of $<\pm 20\%$. The percentage extraction recoveries of the substances were determined by comparing the peak heights from the direct injection of standard solutions in mobile phase with the peak heights obtained for samples subjected to the complete extraction procedure.

2.8. Human samples

The presented analytical method was used in the routine analysis of human plasma samples from 14 subjects who had received a single oral dose of 200 mg racemic mephenytoin before (day −28) and during multiple artemisinin administration (250 mg/day orally for 9 days and 500 mg on the tenth day). A single 500 mg dose of artemisinin was also administered on day −28. The CYP2C9 in vivo marker tolbutamide was administered on day −28 and on days 7, 12 and 17 to monitor the minor involvement of CYP2C9 in *S*-mephenytoin *N*-demethylation. Venous blood samples for the quantitation of *S*-mephenytoin, *R*-mephenytoin, *S*-nirvanol, *R*-nirvanol, *S*-4-OH-mephenytoin and *R*-4-OH-mephenytoin were collected by vein puncture into heparinised Vacutainer vials (5 ml) 5 min before drug intake and after 10 h, and then one sample in the morning 1, 2, 3, 5, 9 and 13 days after mephenytoin administration. Each sample was centrifuged and the plasma harvested and transferred to a cryotube (3.6 ml, Nunc). All samples were kept at −80 °C until analysis.

To be able to distinguish the *S*-4′-hydroxymephenytoin chromatographic peak from the *R*-4′-hydroxymephenytoin peak, a single oral dose of racemic mephenytoin (200 mg) was given to CYP2C19-poor and -extensive metabolisers and repeated venous blood samples taken over time to obtain the pharmacokinetics of the enantiomers. The

subjects had been phenotyped with the use of the metabolic ratio of hydroxyomeprazole and omeprazole [28]. Three hours after intake of 20 mg of omeprazole, a venous blood sample was obtained, immediately centrifuged and thereafter plasma harvested and frozen at −80 °C. The plasma omeprazole/hydroxyomeprazole concentration ratio at 3 h was determined and used as a measure of CYP2C19 activity and indexed for CYP2C19 phenotype.

3. Results and discussion

3.1. Selection of mobile phase and columns

The chiral column was chosen from an earlier application showing separation between *S*- and *R*-mephenytoin (User's Guide, ChromTech). Using the suggested mobile phase (0.3% 1-propanol in sodium phosphate buffer, $\mu = 0.1$, pH 7.0), enantioselective separation was obtained for all compounds (*S*-mephenytoin was separated from *R*-mephenytoin, *S*-nirvanol from *R*-nirvanol, and *S*-4′-hydroxymephenytoin from *R*-4′-hydroxymephenytoin). However, in a mixture of all six enantiomers, *R*-nirvanol and *S*-mephenytoin were co-eluted on the AGP column. Alternative organic modifiers such as methanol and acetonitrile, also in combination with tetrahydrofuran, did not result in the separation of *R*-nirvanol from *S*-mephenytoin. Acetonitrile in the mobile phase gave slightly better peak performance (more narrow peaks) and was therefore chosen for further investigation. Changing the ionic strength

Table 1
Intra-day precision, accuracy and absolute recovery of *S*-4′-hydroxymephenytoin, *R*-4′-hydroxymephenytoin, *S*-nirvanol, *R*-nirvanol, *S*-mephenytoin and *R*-mephenytoin in spiked human plasma samples (quality controls and the lower limit of quantification). The compounds were separated using a reversed-phase C_2 column in tandem with a chiral AGP column prior to ultraviolet detection at 205 nm

Compound	Nominal conc. (ng/ml)	Calculated conc. (ng/ml)	C.V. (%)	Accuracy (%)	Absolute recovery (%)	*n*
S-4′-Hydroxy-mephenytoin	1580	1612	2.6	102.0	81.9	8
	505.4	517.4	1.6	102.4	81.7	8
	40.44	44.02	1.6	108.8	87.9	8
	10.53	12.61	5.4	119.7	105.7	8
R-4′-Hydroxy-mephenytoin	1580	1614	2.8	102.2	82.2	8
	505.4	514.5	1.7	101.8	82.6	8
	40.44	43.01	2.9	106.4	106.5	8
	10.53	10.03	7.0	95.3	161.2	8
S-Nirvanol	1565	1594	2.0	101.9	89.7	8
	500.6	513.1	1.7	102.5	90.5	8
	40.05	44.29	6.3	110.6	102.8	8
	10.43	10.28	13.3	98.6	118.0	8
R-Nirvanol	1607	1639	1.8	102.0	88.1	8
	514.1	522.0	2.3	101.5	88.8	8
	41.13	41.92	3.0	101.9	87.5	8
	10.71	9.646	10.8	90.1	83.3	8
S-Mephenytoin	1532	1628	3.4	106.3	94.9	8
	490.1	522.6	4.3	106.6	94.6	8
	39.21	43.84	5.4	111.8	99.8	8
	10.21	11.45	13.1	112.1	118.8	8
R-Mephenytoin	1539	1619	2.5	105.2	94.4	8
	492.5	516.0	2.5	104.8	97.2	8
	39.40	42.29	2.7	107.3	96.9	8
	10.26	10.21	11.4	99.5	91.2	8

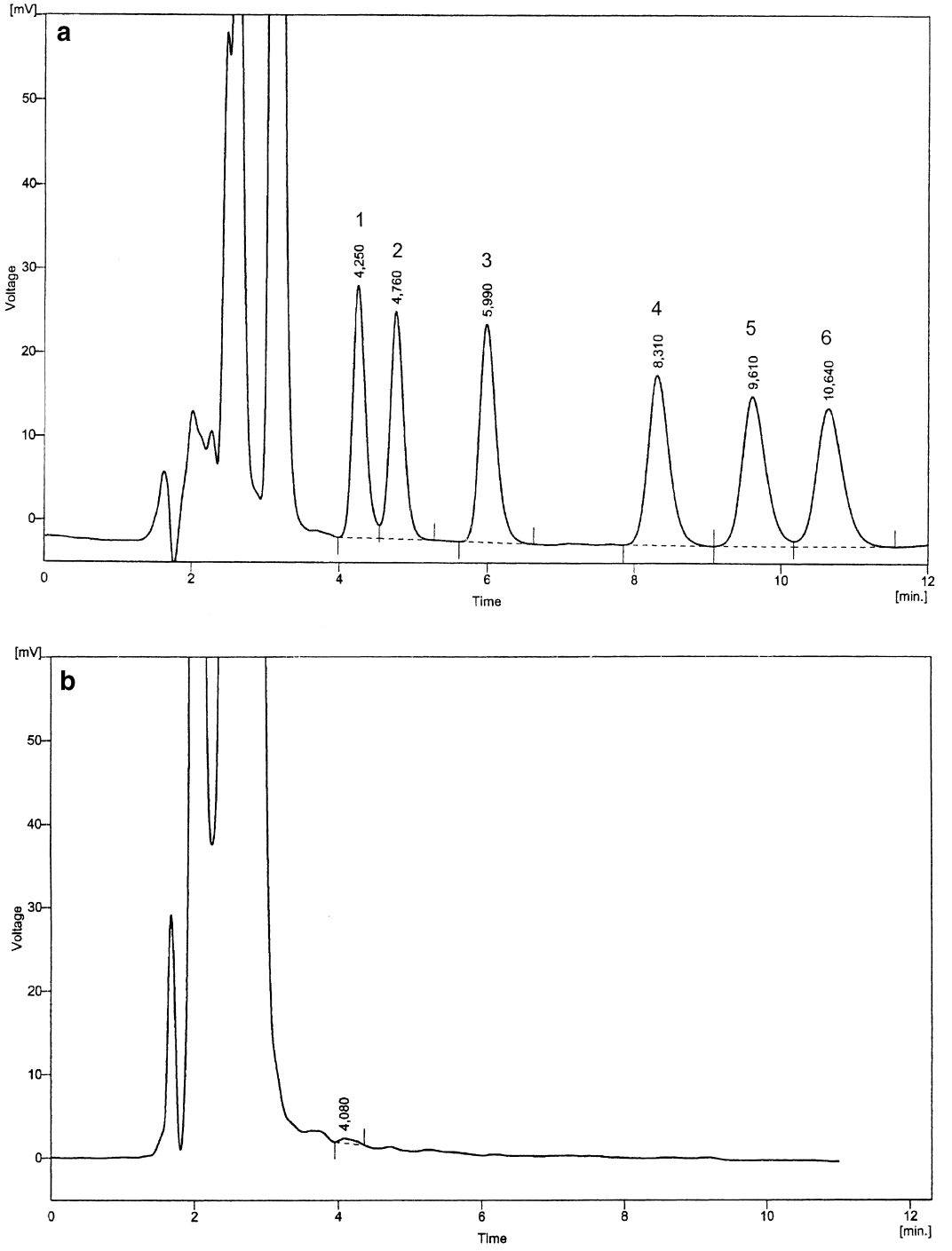

Fig. 2. Chromatograms of (a) a spiked plasma sample after extraction {400 ng/ml of each enantiomer [*R*-4′-hydroxymephenytoin (1), *S*-4′-hydroxymephenytoin (2), *S*-nirvanol (3), *R*-nirvanol (4), *S*-mephenytoin (5) and *R*-mephenytoin (6)]}; (b) a blank plasma sample; (c) a water solution containing 2500 ng/ml of each enantiomer [*R*-4′-hydroxymephenytoin (1), *S*-4′-hydroxymephenytoin (2), *S*-nirvanol (3), *R*-nirvanol (4), *S*-mephenytoin (5) and *R*-mephenytoin (6)].

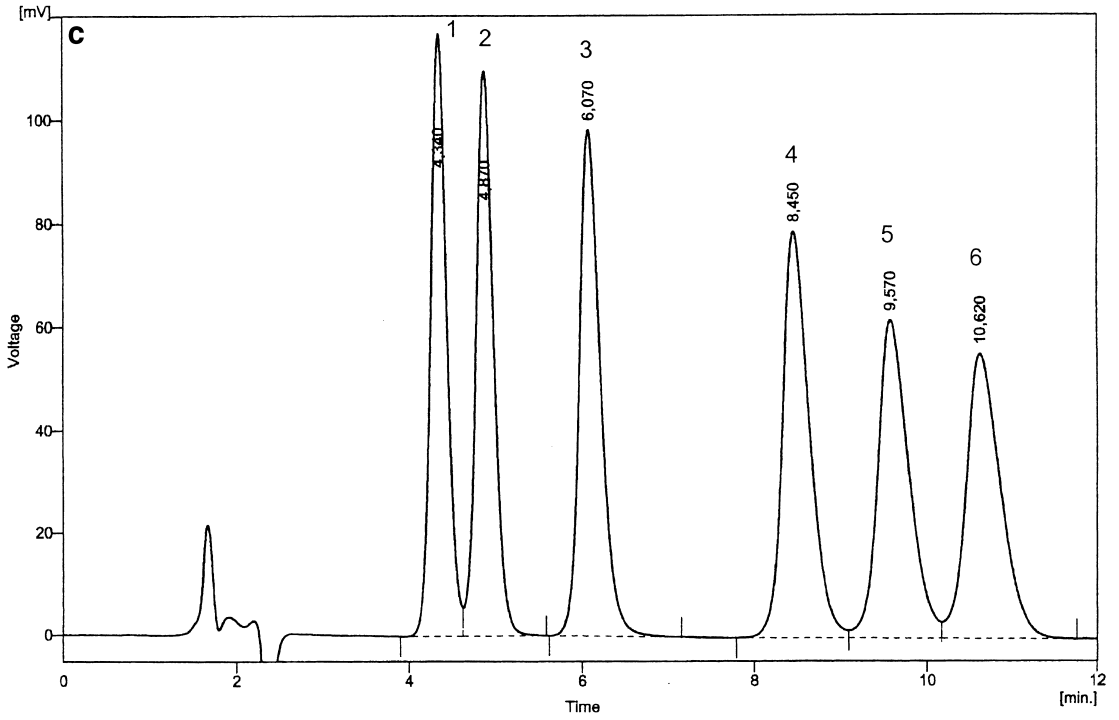

Fig. 2. (*continued*)

from 0.1 to 0.075, 0.05, or 0.01 showed decreasing resolution between *S*- and *R*-mephenytoin, but increasing resolution between *R*-nirvanol and *S*-mephenytoin. Reducing the pH to 6.8 or 6.0 resulted in a poorer resolution between *S*- and *R*-mephenytoin. Therefore, a sodium phosphate buffer ($\mu = 0.1$, pH 7.0, containing 2–3% acetonitrile) was used as the final mobile phase.

Different combinations of a reversed-phase column (CT-sil C1, CT-sil hydroxy and Zorbax SB-CN C3) coupled in series to the chiral AGP column were tested for the possibility to separate *R*-nirvanol and *S*-mephenytoin. Columns packed with CT-sil hydroxy, 10×3.0 mm (ChromTech), and Zorbax SB-CN C3, 10×3.0 mm (ChromTech), gave broad mephenytoin peaks. The guard column CT-sil C1, 10×3.0 mm (ChromTech), provided satisfactory separation of all enantiomers, but degraded quickly due to the high pH and the high ionic strength, even with a silica saturator placed before the injector. A Nucleosil 100 C_2 column, 33×3.0 mm (ChromTech), was shown to be more stable. Some deterioration was seen over time, resulting in shorter retention times and a decreasing resolution factor (*R*) between *R*-nirvanol and *S*-mephenytoin. This did not affect the response (peak height) if the Nucleosil column was exchanged when the resolution factor (*R*) between *R*-nirvanol and *S*-mephenytoin became less than 1, which occurred after approximately 24 h run time, corresponding to the analysis of about 100 samples. *R* was determined according to

$$R = \frac{2\Delta t}{W_1 + W_2}$$

where Δt is the difference between the retention times of the two peaks and *W* is the width of peaks 1 and 2 at their bases.

The presented method includes a reversed-phase C_2 column in tandem with a chiral AGP column. The selection of an AGP column is of advantage since it is a very stable chiral selector and relatively short retention times were obtained during the analysis. However, with this method, the life-span of the

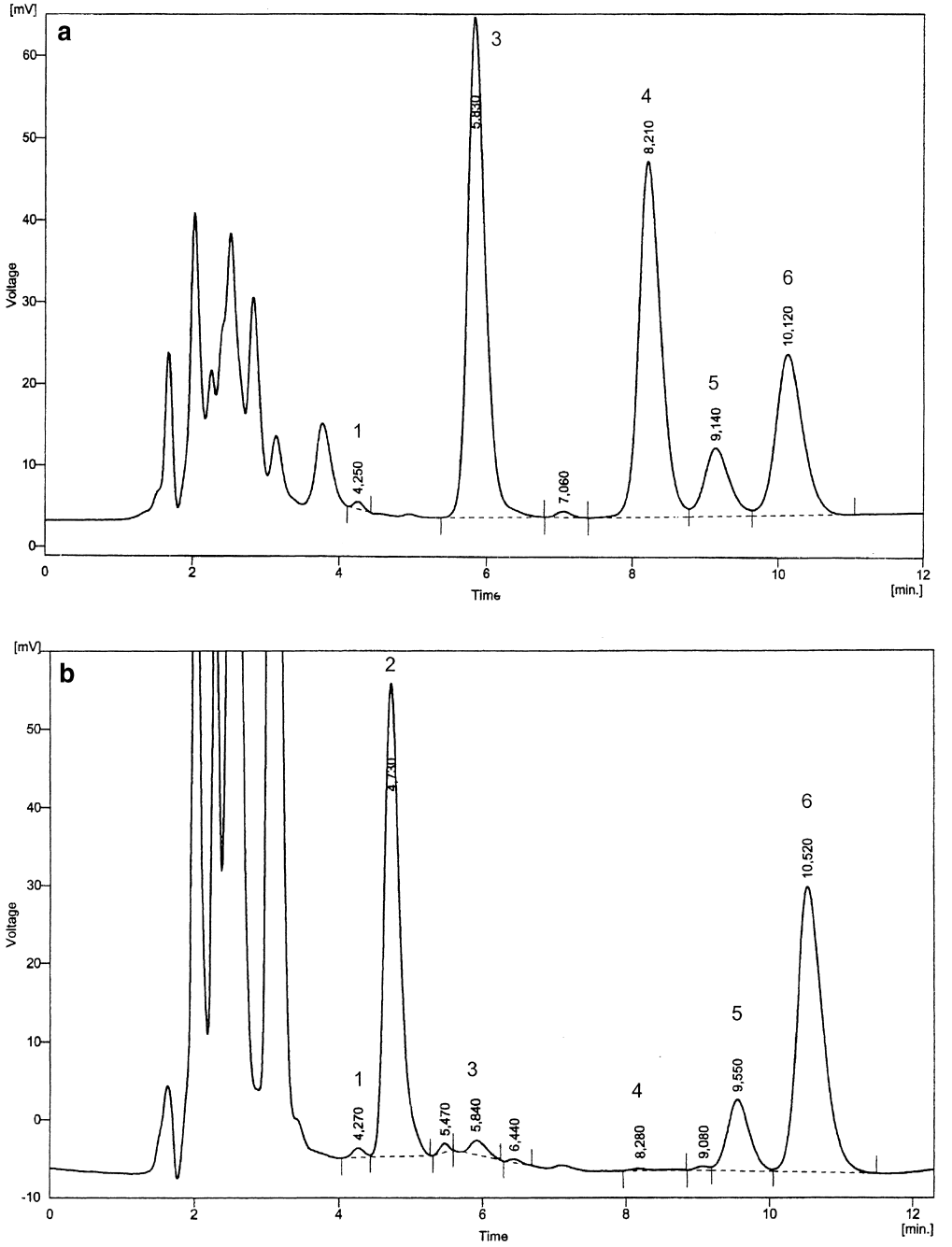

Fig. 3. Chromatograms of (a) a plasma sample obtained 72 h after ingestion of 200 mg racemic mephenytoin by a healthy CYP2C19-poor metaboliser in which the following concentrations were determined: <10 ng/ml *R*-4′-hydroxymephenytoin (1), <10 ng/ml *S*-4′-hydroxymephenytoin (2), 970 ng/ml *S*-nirvanol (3), 900 ng/ml *R*-nirvanol (4), 203 ng/ml *S*-mephenytoin (5) and 490 ng/ml *R*-mephenytoin (6); (b) a plasma sample obtained 1 h after ingestion of 200 mg racemic mephenytoin by a healthy CYP2C19-extensive metaboliser with the following concentrations: <10 ng/ml *R*-4′-hydroxymephenytoin (1), 870 ng/ml *S*-4′-hydroxymephenytoin (2), 16 ng/ml *S*-nirvanol (3), <10 ng/ml *R*-nirvanol (4), 199 ng/ml *S*-mephenytoin (5) and 897 ng/ml *R*-mephenytoin (6).

C_2 column was short, only 24 h run time, which is a disadvantage.

3.2. Column condition

Variation in room temperature made it impossible to achieve a stable baseline. By using a water bath or a column oven with a small variation in temperature (±0.1 °C), the baseline became less noisy with no noticeable drift. Setting the temperature to 30 °C resulted in shorter retention times without negatively affecting the resolution. After each experimental run, the AGP column was rinsed with water and 15% 2-propanol. No degradation of the chiral column could be observed after 1300 injections.

3.3. Linearity

When only one calibration curve (0–2000 ng/ml) was used, the intercepts differed from zero such that the quantitation of low concentrations was negatively affected. Therefore, the calibration curve was divided into two concentration ranges. A linear range of 0–250 and 250–2000 ng/ml with a correlation coefficient of at least 0.999 was obtained for each enantiomer. No optimal internal standard was found that was applicable for all enantiomers. Since the calibration curves were linear for all enantiomers, the method was considered to be reliable, even though no internal standard(s) was included in the extraction procedure.

3.4. Sensitivity

Using the described method, the lower limit of quantification (LLOQ) was taken to be 10 ng/ml for all enantiomers. At this concentration, the intra-day coefficient of variation (C.V.), determined from eight replicate samples, was about 13% or less and the accuracy was $<\pm 20\%$ for all compounds (Table 1).

Table 2
Inter-day assay precision and accuracy for *S*-4′-hydroxymephenytoin, *R*-4′-hydroxymephenytoin, *S*-nirvanol, *R*-nirvanol, *S*-mephenytoin and *R*-mephenytoin in spiked human plasma samples (quality controls). The compounds were separated using a reversed-phase C_2 column in tandem with a chiral AGP column prior to ultraviolet detection at 205 nm. Results are based on the analysis of duplicate samples at each concentration determined on nine separate occasions during the routine analyses of a larger number of clinical samples

Compound	Nominal conc. (ng/ml)	Calculated conc. (ng/ml)	C.V. (%)	Accuracy (%)	*n*
S-4′-Hydroxymephenytoin	1580	1619	5.2	102.5	18
	505.4	517.2	3.0	102.3	18
	40.44	40.35	11.2	99.8	17[a]
R-4′-Hydroxymephenytoin	1580	1613	4.7	102.1	18
	505.4	515.2	2.8	101.9	18
	40.44	39.25	7.0	97.1	17[a]
S-Nirvanol	1565	1586	4.8	101.3	18
	500.6	510.4	3.0	102.0	18
	40.05	42.14	7.6	105.2	16[b]
R-Nirvanol	1607	1628	3.5	101.3	18
	514.1	514.8	4.8	100.1	18
	41.13	39.31	6.4	95.6	16[b]
S-Mephenytoin	1532	1566	5.2	102.2	18
	490.1	504.0	4.8	102.8	18
	39.21	39.12	9.5	99.8	16[b]
R-Mephenytoin	1539	1571	5.0	102.1	18
	492.5	508.0	4.1	103.1	18
	39.40	39.01	5.0	99.0	16[b]

[a] One sample lost during the extraction procedure.
[b] Two samples lost during the extraction procedure.

3.5. Selectivity

Only racemic 4′-hydroxymephenytoin was used for the preparation of the standard curve since the enantiomers of 4′-hydroxymephenytoin were not available. No peaks corresponding to the two enantiomers of 4′-hydroxymephenytoin were detected in the samples from the CYP2C19-poor metaboliser, whereas one of the two peaks, corresponding to the second-eluted enantiomer, was detected in samples from the CYP2C19-extensive metabolisers (Fig. 3). The CYP2C19-poor metabolisers are deficient in the CYP2C19 enzyme and are therefore not capable of forming the hydroxylated metabolite to any significant extent. *R*-Mephenytoin is predominately metabolised via *N*-demethylation [20] and insignificant levels of hydroxylated *R*-mephenytoin are to be expected in both phenotypes. The absence of detectable concentrations of hydroxylated metabolites in the CYP2C19-poor metaboliser and the presence of one hydroxylated mephenytoin metabolite (peak number 2 in Fig. 3b) in the extensive metaboliser leads to the interpretation that the first- and second-eluted 4′-hydroxymephenytoin peaks are the *R*- and *S*-enantiomer, respectively. No measurable *R*-4′-hydroxymephenytoin concentrations were detected in any sample. Representative chromatograms for *S*-mephenytoin, *R*-mephenytoin, *S*-nirvanol, *R*-nirvanol, *S*-4′-hydroxymephenytoin and *R*-4′-hydroxymephenytoin in (i) a spiked human plasma sample, (ii) blank human plasma, (iii) water solution, (iv) a plasma sample obtained 72 h after ingestion of 200 mg racemic mephenytoin by a healthy CYP2C19-poor metaboliser and (v) a plasma sample obtained 1 h after ingestion of 200 mg racemic mephenytoin in a healthy CYP2C19-extensive metaboliser are presented in Figs. 2 and 3. All six compounds were well separated and exhibited chromatographic peaks

Table 3
Inter-day assay precision and accuracy for *S*-4′-hydroxymephenytoin and *R*-4′-hydroxymephenytoin in spiked human plasma samples (standards). The compounds were separated using a reversed-phase C_2 column in tandem with a chiral AGP column prior to ultraviolet detection at 205 nm. Results are based on the analysis of single samples at each concentration determined on nine separate occasions during the routine analyses of a larger number of clinical samples

Compound	Nominal conc. (ng/ml)	Calculated conc. (ng/ml)	C.V. (%)	Accuracy (%)	*n*
S-4′-Hydroxy-mephenytoin	2106	2079	3.6	98.7	9
	1769	1750	3.2	99.0	9
	1348	1346	1.8	99.9	9
	1053	1049	1.5	99.7	9
	421.2	422.6	2.2	100.3	9
	261.1[a]	264.1	3.9	101.1	9
	261.1[b]	260.9	0.2	99.9	9
	105.3	105.5	1.5	100.2	9
	21.06	20.80	11.8	98.7	9
	10.53	11.54	6.7	109.6	8[c]
R-4′-Hydroxy-mephenytoin	2106	2087	3.7	99.1	9
	1769	1753	3.1	99.1	9
	1348	1344	2.0	99.7	9
	1053	1047	1.9	99.4	9
	421.2	422.4	2.4	100.3	9
	261.1[a]	268.4	3.8	102.8	9
	261.1[c]	261.6	0.8	100.2	9
	105.3	105.7	1.9	100.4	9
	21.06	20.26	11.7	96.2	9
	10.53	9.386	14.8	89.1	8[c]

[a] Calculation from the high curve.
[b] Calculation from the low curve.
[c] One sample lost during the extraction procedure.

of high quality. For plasma samples obtained from subjects receiving tolbutamide in addition to racemic mephenytoin, the tolbutamide peak interfered with the *R*-nirvanol peak.

3.6. Intra-day precision and accuracy

Intra-day precision (C.V.) was <10% and accuracy <±12% for all compounds in the studied concentration range of approximately 40–1550 ng/ml. At a concentration of 10 ng/ml, the C.V. was <13% and the accuracy <±20% for all compounds (Table 1).

3.7. Inter-day precision and accuracy

The inter-day precision and accuracy was determined by analysing, on separate occasions, duplicate quality control samples interspersed with unknown samples from a clinical study. The results suggest a stable and reliable method of analysis (Table 2). The inter-day precision and accuracy for all concentrations in both standard curves (low and high range) are presented in Tables 3–5.

3.8. Recovery

The absolute recovery was obtained by comparing the peak heights of quality control samples with the peak heights of an unprocessed calibration curve in mobile phase analysed on the same occasion. Recoveries for *S*-mephenytoin, *R*-mephenytoin, *S*-nirvanol, *R*-nirvanol, *S*-4′-hydroxymephenytoin and *R*-4′-hydroxymephenytoin at different concentrations are shown in Table 1. For *S*-mephenytoin, *S*-nirvanol and *S*-4′-hydroxymephenytoin the recovery was

Table 4
Inter-day assay precision and accuracy for *S*-nirvanol and *R*-nirvanol in spiked human plasma samples (standards). The compounds were separated using a reversed-phase C_2 column in tandem with a chiral AGP column prior to ultraviolet detection at 205 nm. Results are based on the analysis of single samples at each concentration determined on nine separate occasions during the routine analyses of a larger number of clinical samples

Compound	Nominal Conc. (ng/ml)	Calculated Conc. (ng/ml)	C.V. (%)	Accuracy (%)	*n*
S-Nirvanol	2086	2075	1.1	99.5	9
	1752	1732	3.8	98.9	9
	1335	1332	2.5	99.8	9
	1043	1043	2.2	100.0	9
	417.2	418.7	2.1	100.4	9
	258.7[a]	256.8	4.7	99.3	9
	258.7[b]	258.3	1.6	99.8	9
	104.3	107.3	2.4	102.9	9
	20.86	20.58	5.3	98.6	9
	10.43	10.02	9.3	96.0	8[c]
R-Nirvanol	2142	2116	2.1	98.8	9
	1799	1774	3.7	98.6	9
	1371	1373	1.8	100.2	9
	1071	1076	1.7	100.4	9
	428.4	429.4	1.8	100.2	9
	265.6[a]	261.2	3.2	98.3	9
	265.6[b]	264.2	1.0	99.5	9
	107.1	108.3	2.4	101.2	9
	21.43	20.91	5.8	97.6	9
	10.71	10.00	4.9	93.4	8[c]

[a] Calculation from the high curve.
[b] Calculation from the low curve.
[c] One sample lost during the extraction procedure.

94.6, 90.5 and 82.6%, respectively, at 500 ng/ml. A high absolute recovery of 161.2% was obtained for *R*-4′-hydroxymephenytoin at 10 ng/ml due to an interfering peak from blank plasma (Fig. 2b). However, the precision and accuracy were within the criteria of ±20% since the regression line was not forced through the origin.

3.9. Clinical application

The described procedure was used for the stereoselective quantification of mephenytoin and its metabolites in plasma collected over time after administration of a single 200 mg oral dose of racemic mephenytoin to two healthy subjects, one with a CYP2C19-extensive metaboliser phenotype and one with a CYP2C19-poor metaboliser phenotype. The pharmacokinetic profiles for each component in the two phenotypes are shown in Fig. 4.

In conclusion, a novel method for the enantiospecific quantitation of *S*- and *R*-mephenytoin, and the metabolites *S*- and *R*-nirvanol and *S*- and *R*-4′-hydroxymephenytoin, respectively, in plasma after administration of racemic mephenytoin in humans has been validated by employing a coupled column HPLC system with an AGP column placed in sequence with a non-chiral separation column followed by UV detection. The presented work provides a method for the simultaneous characterisation of the pharmacokinetics of *R*-mephenytoin, *S*-mephenytoin, *R*-nirvanol, *S*-nirvanol, *R*-4′-hydroxymephenytoin and *S*-4′-hydroxymephenytoin, which may be useful for estimating basal as well as changes in both CYP2B6 and CYP2C19 activities in humans.

Table 5
Inter-day assay precision and accuracy for *S*-mephenytoin and *R*-mephenytoin in spiked human plasma samples (standards). The compounds were separated using a reversed-phase C_2 column in tandem with a chiral AGP column prior to ultraviolet detection at 205 nm. Results are based on the analysis of single samples at each concentration determined on nine separate occasions during the routine analyses of a larger number of clinical samples

Compound	Nominal Conc. (ng/ml)	Calculated Conc. (ng/ml)	C.V. (%)	Accuracy (%)	*n*
S-Mephenytoin	2042	2059	1.5	100.8	9
	1715	1695	2.0	98.8	9
	1307	1290	2.7	98.7	9
	1021	1012	2.9	99.1	9
	408.4	412.2	2.6	100.9	9
	253.2[a]	261.8	4.7	103.4	9
	253.2[b]	253.4	0.4	100.1	9
	102.1	101.8	2.6	99.7	9
	20.42	19.67	5.6	96.3	9
	10.21	10.91	6.0	106.9	8[c]
R-Mephenytoin	2052	2049	1.2	99.8	9
	1724	1709	1.8	99.2	9
	1313	1313	2.5	100.0	9
	1026	1033	2.9	100.7	9
	410.4	411.5	3.3	100.3	9
	254.4[a]	251.5	4.5	98.9	9
	254.4[b]	254.1	0.4	99.9	9
	102.6	103.4	3.3	100.7	9
	20.52	20.39	4.1	99.4	9
	10.26	10.36	6.8	101.0	8[c]

[a] Calculation from the high curve.
[b] Calculation from the low curve.
[c] One sample lost during the extraction procedure.

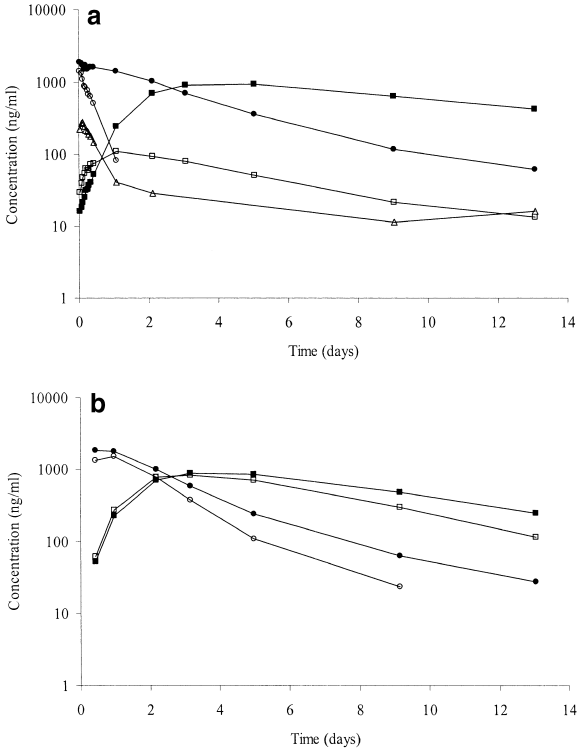

Fig. 4. Plasma concentration–time profiles (semi-log scale) of *S*-mephenytoin (○), *R*-mephenytoin (●), *S*-4′-hydroxymephenytoin (△), *S*-nirvanol (□) and *R*-nirvanol (■) in (a) a healthy CYP2C19-extensive metaboliser and (b) a healthy CYP2C19-poor metaboliser after oral administration of 200 mg racemic mephenytoin. The absence of *S*-4′-hydroxymephenytoin in the poor metaboliser is due the deficiency of the CYP2C19 enzyme in this genotype. No measurable *R*-4′-hydroxymephenytoin was detected in any sample regardless of phenotype.

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