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Enantioselectivity of debrisoquine 4-hydroxylation in Brazilian Caucasian hypertensive patients phenotyped as extensive metabolizers

Paula Macedo Cerqueira, Fabiano Henrique Mateus, Evandro José Cesarino, Pierina Sueli Bonato, Vera Lucia Lanchote*

Universidade de São Paulo, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Avenida do Café s/n, Campus da USP, 14040-903 Ribeirão Preto, SP, Brazil

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Abstract

Debrisoquine (D), an antihypertensive drug metabolized to 4-hydroxydebrisoquine (4-OHD) by CYP2D6, is commonly used as an in vivo probe of CYP2D6 activity and can be used to phenotype individuals as either extensive (EMs) or poor metabolizers (PMs) of such drugs as β-adrenergic blockers, tricyclic antidepressants, and class 1C antiarrhythmics. This report describes reversed-phase HPLC systems by which D and 4-OHD or *S*-(+) and *R*-(−)-4-OHD in urine are more selectively quantified without the need for derivatization techniques. We also studied the urinary excretion of *R*-(−)- and *S*-(+)-4-hydroxydebrisoquine in EM hypertensive patients in order to determine weather 4-OHD formation exhibits enantioselectivity. Twelve patients with mild to severe essential hypertension were admitted to the study. They received a single tablet of Declinax containing 10 mg debrisoquine sulfate. All the urine excreted during the following 8 h was collected. The debrisoquine metabolic ratio (DMR) was calculated as % of dose excreted as D/% of dose excreted as 4-OHD and the debrisoquine recovery ratio (DRR) was calculated as % of dose excreted as 4-OHD/% of dose excreted as D+4-OHD. Debrisoquine and its metabolite were determined in urine by HPLC using a reversed-phase Select B LiChrospher column, a mobile phase of 0.25 N acetate buffer, pH 5–acetonitrile (9:1, v/v) and a fluorescence detector. The limit of quantitation was determined to be 25.0 ng/ml for D and 18.75 ng/ml for 4-OHD. Intra- and inter-day relative standard deviations (RSDs) were less than 10%. All hypertensive patients studied showed a DMR of less than 12.6 or a DRR higher than 0.12 and were classified as EMs. Direct enantioselective separation on chiral stationary phase involved resolution of *S*-(+)-4-OHD and *R*-(−)-4-OHD on a Chiralcel OD-R column with a mobile phase of 0.125 N sodium perchlorate, pH 5–acetonitrile–methanol (85:12:3, v/v/v). The quantitation limit of each enantiomer was 3.75 ng/ml of urine. Intra- and inter-day RSDs were less than 10% for each enantiomer. A high degree of enantioselectivity in the 4-hydroxylation of D favouring the *S*-(+) enantiomer was observed, resulting in *R*-(−)-4-OHD not detected in the urine of the EM hypertensive patients studied. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Extensive metabolizers; Debrisoquine; 4-Hydroxydebrisoquine

*Corresponding author. Tel.: +55-16-6024-159; fax: +55-16-6331-936.
E-mail address: lanchote@fcfrp.usp.br (V.L. Lanchote).

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1. Introduction

Genetic polymorphism represents a crucial factor in the pharmacokinetic variability of many drugs. Oxidative polymorphism type debrisoquine/sparteine (CYP2D6 substrates) or *S*-mephenytoin (CYP2C19 substrate) differentiates the individuals of a given population into extensive metabolizers (EMs) or poor metabolizers (PMs) [1].

Debrisoquine (D) (Fig. 1) is extensively eliminated by metabolism. Hydroxylation at position 4, catalysed by CYP2D6, results in the formation of 4-hydroxydebrisoquine (4-OHD), which is completely excreted into urine. Other metabolites detected in urine include phenolic compounds (5-, 6-, 7- and 8-hydroxydebrisoquine), which are relatively minor and are probably produced by CYP2D6, and acid compounds. There is no evidence of conjugates with glucuronic acids or with sulfates [2].

The in vivo activity of CYP2D6 can be estimated as D recovery ratio (DRR=4-OHD/D+4-OHD) or D metabolic ratio (DMR=D/4-OHD) in urine collected 6–8 h after the administration of a single per oral dose of debrisoquine. The DMR parameter, although frequently employed in clinic studies, does not represent a linear measurement of in vivo CYP2D6 activity. The recovery ratio (DRR) is proportional to CYP2D6 activity and is more related to 4-OHD formation. DMR varies in the population from ≈0.01 to >100, and individuals who express DMR lower than 12.6 are classified as extensive metabolizers or even as ultrarapid metabolizers (DMR<0.2). The DRR greater than 0.12 also phenotypes as extensive metabolizers [3,4]. Approximately 7% of Caucasians and a higher percentage of Black people have been reported to be ultrarapid metabolizers. The frequency of the PM phenotype varies among populations, being high among Caucasians (5–10%) and low among other populations, such as the Chinese and Japanese ones (<1%) [5,6].

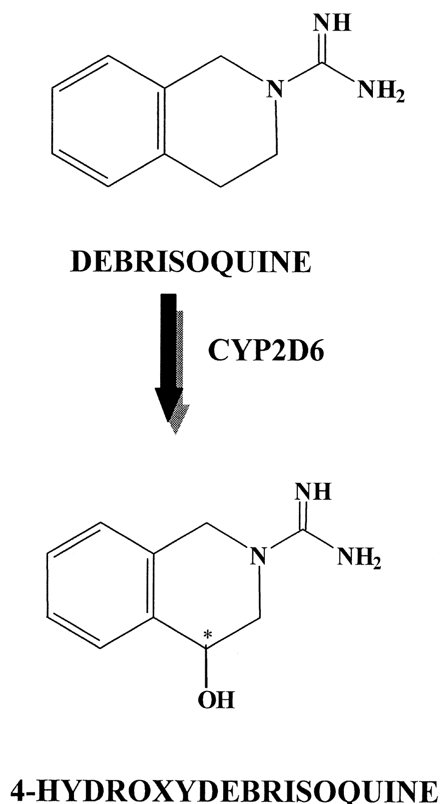

Fig. 1. Chemical structures of D and its metabolite 4-OHD.

Debrisoquine has been extensively described in the literature as a marker drug of the oxidative phenotype of patients included in protocols for the investigation of drug pharmacokinetics, with elimination depending on the polymorphic isoform CYP2D6. Drugs metabolized by CYP2D6 contain a basic nitrogen atom and a flat hydrophobic region coplanar to the oxidation site, which is 5–7 Å away from the basic nitrogen [2,7]. Oxidative metabolism of the debrisoquine/sparteine type is determinant in terms of the metabolic rate of a growing number of drugs such as β-blockers, antiarrhythmics and opiates, although the clinical relevance of this phenomenon is mainly expressed for tricyclic antidepressants, some neuroleptics and a few other non-psychotropic drugs [1]. The association of this oxidative polymorphism with diseases such as cancer [8], diabetes [9], Parkinson's disease [10], epilepsy, and tardive dyskinesia in schizoprenia [11,12], among others, should also be emphasised [13].

Debrisoquine hydroxylation at position 4 by CYP2D6 creates a chiral centre in the molecule, with the possible formation of the *S*-(+)-4-hydroxydebrisoquine and *R*-(−)-4-hydroxydebrisoquine metabolites [14]. Eichelbaum et al. [14] investigated enantioselectivity in the urinary excretion of 4-hydroxydebrisoquine in healthy volunteers phenotyped as EMs or PMs and observed loss of enantioselectivity in PM volunteers and almost exclusive urinary

excretion of the enantiomer *S*-(+)-4-hydroxydebrisoquine in EM volunteers. This result permits the exploration of enantioselectivity in the urinary excretion of 4-hydroxydebrisoquine as a complementary method or even as an additional criterion for the evaluation of the debrisoquine polymorphism [2,14–16].

Sequential analysis of debrisoquine and 4-hydroxydebrisoquine in urine can be performed using gas chromatography–flame ionisation detection (GC–FID) [17], GC–nitrogen–phosphorus detection (NPD) [17] or GC–mass spectrometry (MS) [18] after derivatization with acetylacetone. Other procedures involve high-performance liquid chromatography (HPLC) with detection by UV [19,20] or by fluorescence [3,21], LC–MS [22], or even the use of capillary zone electrophoresis (CZE) [23]. Enantioselective analysis of 4-hydroxydebrisoquine in urine by HPLC was only described by Meese et al. [15]. These investigators used derivatization with acetylacetone for 72 h, followed by separation of pyrimidine derivatives on a ''Pirkle'' chiral stationary phase column [(*R*)-*N*-3,5-dinitrobenzoylphenylglycine] and detection by fluorescence. Lanz et al. [23] used CZE in the separation of 4-hydroxydebrisoquine enantiomers via addition of a β-cyclodextrin derivative to the CZE buffer.

The present report describes reversed-phase HPLC systems by which D and 4-OHD or *S*-(+)- and *R*-(−)-4-OHD in urine are more selectively quantified without the need of a derivatization technique, and presents the study of urinary excretion of D and 4-OHD and *R*-(−)- and *S*-(+)-4-hydroxydebrisoquine in Brazilian Caucasian hypertensive patients in order to determine the oxidative phenotype and whether 4-OHD formation exhibits enantioselectivity.

2. Materials and methods

2.1. Analytical methods

2.1.1. Chemicals and reagents

Debrisoquine sulfate was obtained from Sigma (St. Louis, MO, USA) and 4-hydroxydebrisoquine was kindly provided by Professor Sompson Wanwimolruk (University of Otago, Otago, New Zealand). Sodium chloride, sodium hydroxide and sodium perchlorate monohydrate were reagent grade and were purchased from Merck (Darmstadt, Germany). A 1.0 *M* NaOH solution was washed with the extraction solvent dichloromethane–isopropanol (6:4, v/v). The solvents used for the preparation of standard solutions, urine extraction and chromatographic analysis were obtained from Merck as HPLC grade.

A stock solution of debrisoquine was prepared in 0.4 mg/ml methanol and diluted in order to obtain solutions at concentrations of 80, 40, 20 and 8 μg/ml. The stock solution of racemic 4-hydroxydebrisoquine was prepared in methanol–water (1:1, v/v) and diluted in methanol in order to obtain solutions at concentrations of 160, 60, 30 and 12 μg/ml. The solutions were stored at −20°C and remained stable for 3 months.

2.1.2. Preparation of urine samples

A 1.0-ml aliquot of urine was alkalinized with 50 μl of 1.0 *M* NaOH, supplemented with 200 mg NaCl and 10.0 ml of dichloromethane–isopropanol (6:4, v/v). The tube was vortex-mixed for 3 min followed by centrifugation at 2000 *g* for 5 min. The organic layer (9 ml) was removed and evaporated under a nitrogen stream and the residue was reconstituted with 100 μl of the mobile phase immediately prior to analysis. A 20-μl aliquot was assayed by HPLC.

2.1.3. High-performance liquid chromatography procedures

2.1.3.1. Debrisoquine and 4-hydroxydebrisoquine. Debrisoquine and its metabolite were analysed on a 25×4.0 mm Select B LiChrospher column 60 with particle size 5 μm (Merck). The mobile phase was acetonitrile–0.25 N acetate buffer, pH 5.0 (1:9, v/v) at a flow-rate of 1.0 ml/min (pressure 139 kg/cm^2). The peaks were detected by fluorescence monitoring (RF-551, Shimadzu, Kyoto, Japan) with excitation and emission wavelengths of 210 and 290 nm, respectively.

2.1.3.2. R-(−)- and S-(+)-4-hydroxydebrisoquine. The enantiomers *R*-(−)- and *S*-(+)-4-hydroxydebrisoquine were analysed on a 250×4.6 mm Chiralcel OD-R column [cellulose tris(3,5-dimethylphenyl carbamate) on a 10 μm silica-gel

substrate; Daicel, Los Angeles, CA, USA]. The mobile phase was methanol–acetonitrile–0.125 N sodium perchlorate, pH 5.0 (3:12:85, v/v/v) at a flow-rate of 0.8 ml/min (pressure 24 kg/cm^2). The peaks were detected by fluorescence monitoring (RF-551, Shimadzu) with excitation and emission wavelengths of 210 and 290 nm, respectively.

2.1.4. Calibration curves

The calibration curves were constructed using drug-free urine samples (1.0 ml) enriched with 25 μl of each standard solution. Concentrations of 0.2–2.0 μg debrisoquine/ml urine and of 0.3–4.0 μg racemic 4-hydroxydebrisoquine/ml urine were evaluated in duplicate. The calibration curves for enantioselective analysis of 4-hydroxydebrisoquine were constructed in a similar manner at concentrations of 0.15–2.0 μg of each enantiomer/ml urine.

2.1.5. Validation

Absolute recovery of debrisoquine and its metabolite was determined at concentrations of 0.2 and 2.0 μg/ml of each drug (n=3). Drug-free urine samples were spiked with standard drug solutions and submitted to extraction procedures. The urine concentrations of drugs were calculated from the calibration curves obtained by the direct injection of the drugs.

The quantification limit, defined as the lowest concentration with a relative standard deviation (RSD) of <10%, was obtained by analysis of urine samples spiked with decreasing drug concentrations compared to those used in the calibration curves.

Linearity was determined by the analysis of drug-free urine samples spiked with drugs in the range of 0.2–20.0 μg of D/ml, 0.3–15.0 μg of 4-OHD/ml and 0.15–15.0 μg of each enantiomer of 4-OHD/ml.

Inter- and intra-day precision and accuracy were assessed by replicate analysis of spiked samples at high (2.0 μg/ml of D and 7.5 μg/ml of 4-OHD or 9.6 μg/ml of each enantiomer of 4-OHD) and low (0.2 μg/ml of D and 0.3 μg/ml of 4-OHD or 0.32 μg/ml of each enantiomer of 4-OHD) concentrations in urine against calibration curves. Intra-day precision was determined by analysing spiked samples in replicate (n=10) on the same day. The procedure was repeated on 5 different days with the same spiked samples to determine inter-day precision.

Interference from commonly used drugs was evaluated by injecting solutions of the drugs prepared in the mobile phase into the chromatographic system and comparing their retention times with those of the D and 4-OHD or 4-OHD enantiomers. The following drugs were checked: acetaminophen, aminopyrine, amiodarone, atenolol, benzydamine, captopril, carbamazepine, clobazam, chlorimipramine, chlorpromazine, dapsone, digoxin, ethidocaine, lidocaine, metochlopramide, nitrazepam, propaphenone, quinidine, theophylline, trimipramine and sotalol.

2.2. Clinical study

The study was conducted on Brazilian Caucasian patients of both sexes (five men and seven women) aged 28 to 76 years and weighing 49 to 103 kg, with arterial hypertension (>140×90 mmHg as determined on three different occasions) and with normal hepatic and renal function determined by clinical examination and laboratory tests (Table 1). The patients gave informed written consent to participate in the study, which was approved by the Ethics Committee of the local hospital.

The selected patients received a single oral dose of 10 mg debrisoquine (Declinax; Roche, Welwyn Garden City, UK) in the morning after complete bladder emptying and a 12 h fast. Urine was collected until 8 h after the administration of the marker drug. The total volume of urine was homogenized and measured and an aliquot of approximately 10 ml was stored at −20°C.

3. Results

The use of debrisoquine as a marker drug of the in vivo activity of CYP2D6 required the development of simultaneous analysis of D and its metabolite 4-OHD or of the S-(+)- and R-(−)-4-OHD enantiomers in urine. The data presented in Tables 2–4 demonstrate the high sensitivity, precision and selectivity of the analytical methods. The data obtained in the study of linearity correspond to the highest D and 4-OHD concentrations tested that presented a linear relation with the detector response. The chromato-

Table 1
Demographic, clinical and laboratory data of the hypertensive patients

Patient No. (sex)	Age (years)	Weight (kg)	Height (cm)	Cl_{CR} [a] ($ml\ min^{-1}\ 1.73\ m^{-2}$)	Concurrent medications
1 (M)	32	60	160	110.8	
2 (M)	45	78	174	141.6	
3 (F)	76	67	166	56.9	
4 (F)	75	49	155	69.0	
5 (F)	63	67	169	131.2	Estradiol benzoate
6 (M)	55	103	168	90.3	Acethylsalicilic acid, propathylnitrate
7 (F)	43	72	147	104.6	
8 (M)	49	84	170	101.0	Bromazepam
9 (F)	28	76	169	65.6	*N*-Buthylscopolamine
10 (F)	61	91	160	– [b]	
11 (M)	43	95	185	124.4	Carbamazepine, lithium, imipramine
12 (F)	67	79	168	67.5	

[a] Cl_{CR}, Creatinine clearance.
[b] –, Not determined.

grams presented in Figs. 2 and 3 show that the endogenous components of urine do not interfere with the analytical methods.

The results of the investigation of the oxidative phenotype of D are presented in Table 5. The urinary excretion D/4-OHD (DMR) ratios lower than 12.6

Table 2
Confidence limits of the analysis of D and 4-OHD in urine

	D		4-OHD	
Absolute recovery (%)	90.5		81.6	
Quantitation limit (ng/ml)	25.0		18.75	
RSD (%)	2.7		5.2	
Linearity				
Range (μg/ml)	0.2–20.0		0.3–15.0	
Slope	10.71		14.13	
Intercept	16 632.4		10 404.7	
Correlation coefficient	0.9888		0.9954	
Accuracy intra-day				
Concentration (μg/ml)	0.2	2.0	0.3	7.5
n	10	10	10	10
% Bias	−5.0	−4.8	−0.6	−2.0
Precision intra-day				
Concentration (μg/ml)	0.2	2.0	0.3	7.5
n	10	10	10	10
RSD (%)	4.08	2.78	7.2	3.5
Precision inter-day				
Concentration (μg/ml)	0.2	2.0	0.3	7.5
n	5	5	5	5
RSD (%)	5.7	5.2	7.6	7.7

RSD=relative standard deviation.

Table 3
Confidence limits of the analysis of 4-OHD enantiomers in urine

	R-(−)-4-OHD		S-(+)-4-OHD	
Absolute recovery (%)	81.0		76.6	
Quantitation limit (ng/ml)	3.75		3.75	
RSD (%)	6.23		6.24	
Linearity				
Range (μg/ml)	0.15–15.0		0.15–15.0	
Slope	21.07		26.48	
Intercept	10 451.5		9890.7	
Correlation coefficient	0.9922		0.9907	
Accuracy intra-day				
Concentration (μg/ml)	0.32		0.32	
n	10		10	
% Bias	−10.91		−12.50	
Accuracy inter-day				
Concentration (μg/ml)	0.32		0.32	
n	5		5	
% Bias	−7.38		−8.88	
Precision intra-day				
Concentration (μg/ml)	0.32	9.6	0.32	9.6
n	10	10	10	10
RSD (%)	1.46	2.95	1.12	2.24
Precision inter-day				
Concentration	0.32	9.6	0.32	9.6
n	5	5	5	5
RSD (%)	5.21	4.50	5.93	4.58

RSD=relative standard deviation.

and the 4-OHD/D+4-OHD (DRR) ratios higher than 0.12 designate the EM oxidative phenotype for all patients investigated. A high degree of enantioselectivity was detected in the analysis of 4-OHD enantiomers in urine, with the detection of only the *S*-(+)-4-OHD enantiomer in the 12 patients investigated.

Table 4
Selectivity study: analysis of D and 4-OHD in urine

Drug	Concentration	Retention time (min)	
	(μg/ml)	RP-Select B column	Chiralcel OD-R column
D	1.0	25.3	ND[a]
4-OHD	0.75	8.2	–
(*S*)-(−)-4-OHD	1.0	–	8.7
(*R*)-(+)-4-OHD	1.0	–	9.2
Atenolol	0.4	7.1	6.2
Sotalol	6.0	7.3	6.4

[a] ND, Not detected in 0 to 45 min interval: acetaminophen, aminopyrine, amiodarone, benzydamine, captopril, carbamazepine, clobazam, chlorimipramine, chlorpromazine, dapsone, digoxin, ethidocaine, lidocaine, metochlopramide, nitrazepam, propaphenone, quinidine, theophylline, trimipramine, verapamil.

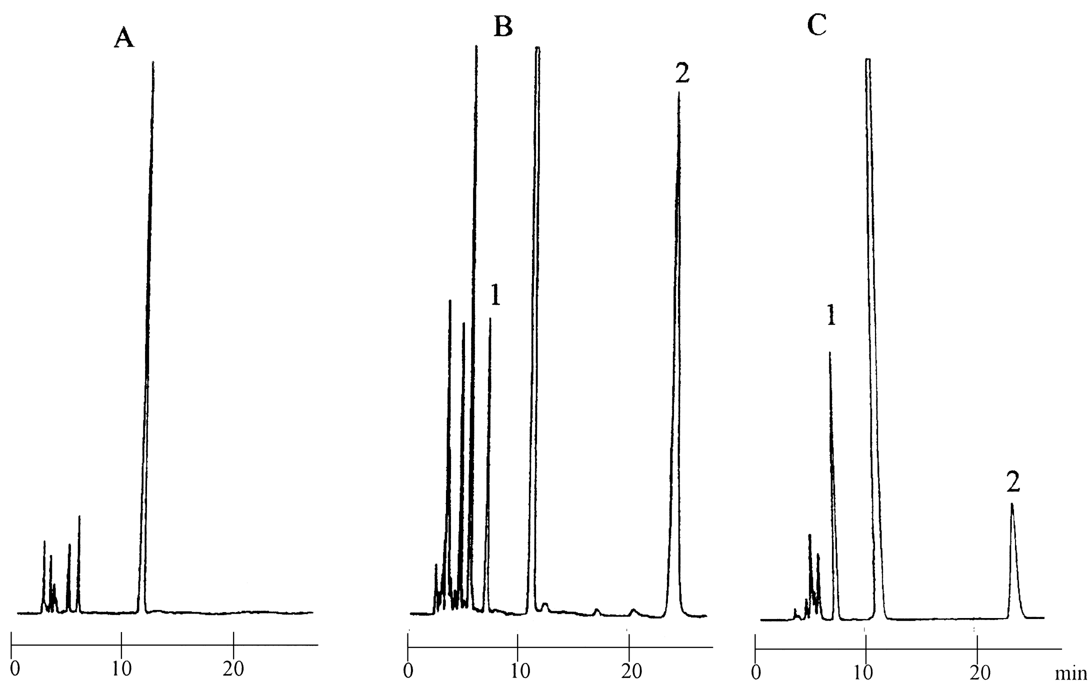

Fig. 2. Assay for D and its 4-OHD metabolite: chromatograms of (A) blank human urine, (B) urine sample spiked with 4-OHD (1) and D (2) and (C) 8 h human urine after oral ingestion of 10 mg of debrisoquine.

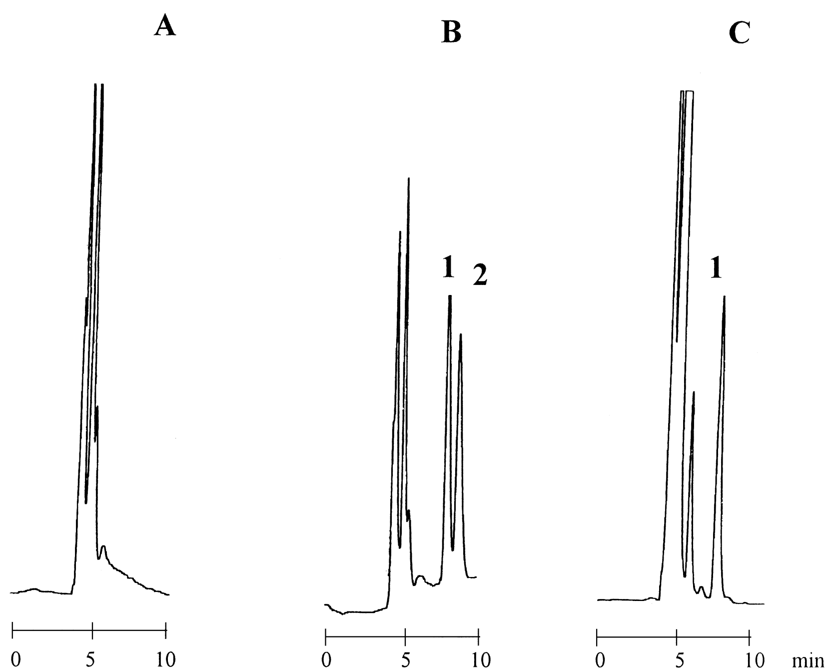

Fig. 3. Assay for (+)-(*S*)-4-OHD and (−)-(*R*)-4-OHD: chromatograms of (A) blank human urine, (B) urine sample spiked with racemic 4-OHD and (C) 8 h human urine after oral ingestion of 10 mg of debrisoquine. Peaks: 1=(+)-(*S*)-4-OHD; 2=(−)-(*R*)-4-OHD.

Table 5
Investigation of oxidative phenotype of debrisoquine[a]

Patient	DMR	DRR	(+)-(*S*)-4-OHD (ng/ml)
1	0.30	0.77	3573.5
2	0.40	0.72	3303.0
3	0.43	0.70	730.5
4	0.46	0.69	117.9
5	0.95	0.51	1454.5
6	0.98	0.51	1848.5
7	0.42	0.70	3181.8
8	0.56	0.64	1685.8
9	0.61	0.60	303.0
10	0.30	0.77	3870.7
11	0.18	0.85	1902.6
12	0.23	0.81	1923.0

[a] DMR, Debrisoquine metabolic ratio; DRR, debrisoquine recovery ratio. (−)-(*R*)-4-OHD was not detected in concentrations ≥3.75 ng/ml of urine.

4. Discussion

The analytical method developed with the use of the RP-Select-B column and a fluorescence detector does not require derivatization procedures and permits the simultaneous analysis of D and 4-OHD, with confidence limits compatible with application in studies of metabolism (Tables 2–4). Most of the methods previously reported in the literature use derivatization procedures that require up to 16 h for sample preparation [17,18]. Daumas et al. [18] reported low sensitivity in the analysis of D and 4-OHD after derivatization with acetylacetone and detection by GC–MS, with detection limits of the order of 200 ng/ml. Johnson et al. [19] used solid-phase extraction (CN) and HPLC with UV detection in the analysis of D and 4-OHD at concentrations of 0.4 to 1.8 μg/ml urine. Wanwimolruk and Ferry [20] developed a direct method for D and 4-OHD analysis in urine by liquid–liquid extraction (1-hexanol–ether, 6:4) and by the use of a UV detector and reported detection limits of 100 ng/ml for D and 50 ng/ml for 4-OHD. Frye and Branch [3] reported a quantitation limit of 50 ng/ml for D and 4-OHD by direct injection of urine samples previously washed with ethyl ether in acid medium. These values represent concentrations higher than the quantitation limits obtained in the present study, i.e., 25 ng/ml for D and 18.75 ng/ml for 4-OHD. Similar results were reported by Pereira et al. [21] using the fluorescence detector, i.e., quantitation limits of 12 ng/ml for D and 23 ng/ml for 4-OHD. The use of capillary electrophoresis reported by LANZ et al. [23] for the analysis of D and of the 4-OHD enantiomers resulted in detection limits of 150 ng of each drug per ml urine. This result may prevent the quantitation of D in the urine of extensive metabolizers. The D concentrations obtained in the urine of the 12 hypertensive patients studied here ranged from 0.1 to 2.0 μg/ml.

The enantioselective analysis of 4-OHD in urine by HPLC was previously reported by Meese et al. [15] with the use of derivatization with acetylacetone. The 4,6-dimethyl-2-pyrimidyl derivatives separated on a Pirkle-type chiral phase column were eluted with retention times of 2 and 23 min. The method described by them presented a detection limit of 25 pmol/ml urine for the two 4-OHD enantiomers. The analytical method developed in the present study, without derivatization procedure and with the use of a Chiralcel OD-R column, permitted the elution of the *S*-(+)- and *R*-(−)-4-OHD enantiomers within approximately 9 min. The quantitation limits obtained for both 4-OHD enantiomers were 3.75 ng/ml urine. The concentrations of the *S*-(+) enantiomer ranged from 0.1 to 3.9 μg/ml, while the *R*-(−) enantiomer was not detected in the urine of the hypertensive patients investigated.

Both methods reported in our study presented high precision based on intra- and inter-day RSDs of less than 10% (Tables 2 and 3). No analytical interferences were found from the co-administered drugs (Table 4) or their metabolites and no interferences were observed in urine samples from any of the patients.

We used a DMR of less than 12.6 and a DRR higher than 0.12 to classify extensive metabolizers [3]. In our study the debrisoquine metabolic (DMR) and the debrisoquine recovery ratios (DRR) varied among the hypertensive patients, ranging from DMR=0.18 to 0.97 and DRR=0.51 to 0.85, so all patients were phenotyped as EMs (Table 5). Without any exception, in all the patients studied, a high degree of enantioselectivity in the 4-hydroxylation of D favouring the *S*-(+)-4-OHD was observed, since patients formed *S*-(+)-4-OHD almost exclusively. The enantioselectivity in 4-OHD isomer formation

might provide an additional phenotyping criterion which might be useful for definite phenotype assignment of subjects whose DMR is close to 12.6. Eichelbaum et al. [14] classified as PM individuals that had a *S*-(+)-4-OHD excess of 90% or less.

In conclusion, we have presented rapid and selective methods for the simultaneous analysis of D and 4-OHD and for the analysis of *R*-(−)- and *S*-(+)-4-OHD in human urine. These methods are suitable for studies of CYP2D6 activity utilising D as a probe drug. With no exception, all patients were phenotyped as EMs of D, and presented a high degree of enantioselectivity in the 4-hydroxylation of D, favouring the formation of *S*-(+)-4-OHD.

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