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Note

High-performance liquid chromatographic method for the analysis of debrisoquine and its *S*-(+)- and *R*-(−)-hydroxy metabolites in urine

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Since the discovery of polymorphic drug oxidation of the sparteine/debrisoquine type [1–3], considerable interest has focused on the development of analytical methods for the determination of the urinary metabolic ratio (MR), i.e. the ratio between unchanged excreted debrisoquine (**1**) and its hydroxylated major metabolite [4] 4-hydroxydebrisoquine (**2**) [5–14]. Because enzymic hydroxylation in position 4 of achiral **1** creates a new chiral centre, two enantiomers might be formed (Fig. 1). However, none of the published analytical procedures is suitable for the simultaneous determination of the stereochemistry of the 4-hydroxy metabolite **2**. Using gas chromatography–mass spectrometry [15], we have recently observed that benzylic 4-hydroxylation of achiral **1** in extensive metabolizers (EMs) proceeds with high (e.e.* > 90% *S*-(+)-**2**) product stereoselectivity [16], which is greatly reduced in poor metabolizers (PMs) [17,18]. However, this method [15] suffered from the occurrence of partial racemization during a derivatization step.

In order to obtain quantitative data both on the MR and on the stereoselectivity of metabolite formation, a simplified and reproducible analytical procedure was required. We report here a novel and convenient method for the analysis of **1** and of the enantiomeric metabolites *S*-(+)-**2** and *R*-(−)-**2** in human urine.

*Enantiomeric excess (e.e.) $= \frac{(S\text{-}(+)\text{-}\mathbf{2}) - (R\text{-}(-)\text{-}\mathbf{2})}{(S\text{-}(+)\text{-}\mathbf{2}) + (R\text{-}(-)\text{-}\mathbf{2})} \times 100\ (\%)$.

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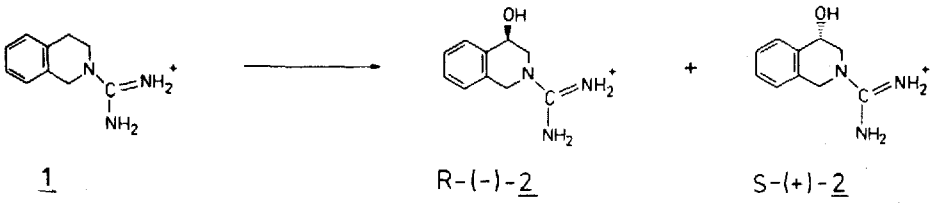

Fig. 1. Hydroxylation of achiral debrisoquine (**1**) forming the *R*-(−) and *S*-(+) enantiomers of 4-hydroxydebrisoquine (**2**).

EXPERIMENTAL

Chemicals

All solvents and reagents were of highest purity grade commercially available. 2,4-Pentanedione (AcAc, acetylacetone, Fluka puriss., Neu-Ulm, F.R.G.) was distilled in vacuo (b.p. 34–35 °C, 17 mm), and aliquots were stored under refrigeration. The synthesis of enantiomerically pure (e.e. 99%) hemisulphates *S*-(+)-**2** and *R*-(−)-**2** (MW 240.3) [16] and racemic **2** [19] have been described elsewhere.

*Synthesis of internal standard, 4-hydroxy-4-phenylpiperidine-1-carboxamidinium hemisulphate hemihydrate (**3**)*

A mixture of 4-hydroxy-4-phenylpiperidine (1.77 g, 10 mmol; Aldrich, Steinheim, F.R.G.), *S*-methyl isothiouronium sulphate (1.67 g, 6 mmol) and water (40 ml) was stirred at 40–50 °C until the development of methylmercaptane had ceased (4 h). The mixture was then refluxed for 15 min, and water was added to produce a homogeneous solution. After cooling to 4 °C for 18 h the precipitated salt **3** was collected and recrystallized twice from a minimum of boiling water: yield, 221 mg; m.p., 299–303 °C (dec.); calculated for $C_{12}H_{18}N_3O \cdot 0.5SO_4 \cdot 0.5H_2O$ (MW 277.3): C 51.97%, H 6.91%, N 15.15%, S 5.78%; found C: 51.77%, H 7.14%, N 15.20%, S 5.72%.

Extraction and derivatization

A heterogeneous mixture of urine (1.0 ml), 2.5 µl of a $2 \cdot 10^{-4}$ *M* aqueous solution of internal standard (0.5 nmol), saturated aqueous sodium hydrogencarbonate (400 µl) and distilled AcAc (200 µl) was rapidly stirred in a tightly closed reacti-vial in the dark at room temperature. After 72 h the sample was extracted with *n*-hexane (2×2 ml) and the organic phase was evaporated under nitrogen at room temperature. The residue was dissolved in 100 µl (PM urine) or 500 µl (EM urine) of *n*-hexane and ca. 40–80 µl were injected onto the high-performance liquid chromatographic (HPLC) column.

High-performance liquid chromatography

Chromatography was performed at room temperature with a Beckman Model 110 A pump, a Shimadzu Model 8400 fluorescence detector, a Shimadzu CR3A integration unit and a commercially available (Baker No. 7113-0, Gross-Gerau,

TABLE I

ANALYSIS OF **1**, *S*-(+)-**2** AND *R*-(−)-**2** IN HUMAN BLANK URINE

Amount added (nmol)			e.e. (%)	MR	Amount found (nmol)			e.e. (%)	MR
1	*S*-(+)-**2**	*R*-(−)-**2**			**1**	*S*-(+)-**2**	*R*-(−)-**2**		
1.5	5.0	0.12	95	0.29	1.2	5.6	0.19	93	0.21
2.0	5.0	0.25	91	0.38	1.8	5.5	0.24	92	0.31
2.5	5.0	0.40	85	0.46	2.2	5.5	0.46	85	0.37
3.0	5.0	0.50	82	0.55	2.4	4.9	0.44	84	0.45
3.5	5.0	1.00	67	0.58	3.7	5.7	1.03	70	0.55
4.0	5.0	1.50	54	0.61	4.4	5.8	1.66	56	0.59
5.0	5.0	2.50	33	0.67	4.9	4.5	2.53	28	0.69

F.R.G.) chiral "Pirkle" column [250 mm×4.6 mm I.D., (*R*)-N-3,5-dinitrobenzoylphenylglycine covalently coupled to 5-μm aminopropylsilica]. The mobile phase was *n*-hexane–ethanol–*tert.*-butyl methyl ether (98:1:1; v/v, %), and the flow-rate was 1 ml min^{-1} at ca. 6.8 bar. The fluorescence detector was set at $\lambda_{ex}=265$ nm and $\lambda_{em}=380$ nm. Depending on the quality of the column, the separation of **2** enantiomers was achieved with a separation factor (α) of 1.03–1.05 and a peak resolution (R) of 0.79–1.15.

Accuracy

The hemisulphates of debrisoquine (**1**) and *R*-(−)-**2** were added in increasing amounts to 1.0 ml of human blank urine containing constant amounts of *S*-(+)-**2** (5 nmol) and internal standard (0.5 nmol). Work-up, separation on the Pirkle column, and quantification by fluorescence detection of the corresponding pyrimidine derivatives by measuring the peak heights gave the results presented in Table I.

Precision of the analysis of the enantiomeric excess

Eight mixtures of synthetic hemisulphates *S*-(+)-**2** and *R*-(−)-**2** (in total 5 nmol) with calculated e.e. values of 0, 5, 11, 33, 54, 67, 82 and 90% were prepared in 1 ml of distilled water and subjected to analysis of the enantiomeric composition of **2**. Corresponding e.e. values of 0, 3, 9, 33, 53, 67, 85 and 90%, respectively ($r=0.999$), were obtained. Because the *R* enantiomer has a shorter retention time, as little as 0.5% of *R*-(−)-**2** (according to 100 pmol ml^{-1}) can be detected in the presence of excess *S*-(+)-**2** (ca. 20 nmol ml^{-1}).

Studies on racemization and autoxidation

Debrisoquine (1 nmol and 10 nmol) was added to 1 ml of blank urine and the AcAc derivatization was carried out for 72 and 48 h at both room temperature and 50 °C, respectively. HPLC analysis proved that even at elevated temperature no hydroxy metabolite, which would have been racemic, had been formed during this derivatization step. Even under prolonged derivatization times no racemi-

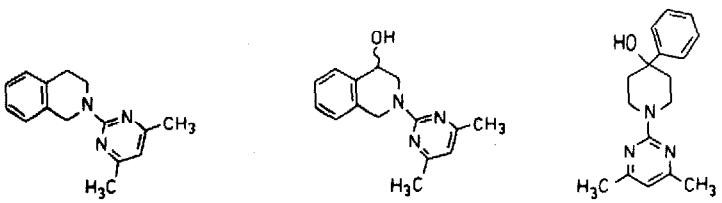

Fig. 2. Structures of derivatives of debrisoquine (D), 4-hydroxydebrisoquine (4-OH-D) and the internal standard (IS).

zation occurred in native and diluted (1:20 and 1:200) EM urine samples that contained only *S*-(+)-**2**. To aliquots of previously analysed PM urine was added a 100-, 200- and 300-fold excess of synthetic *S*-(+)-**2**. After derivatization the calculated *R*/*S* ratios were verified by chromatography since the absolute amount of *R*-(−)-**2** was kept constant in all samples.

Reproducibility and detection limit of the assay

Twenty aliquots of the urine (225 ml per 8 h) of one EM subject who had taken 10 mg of debrisoquine were stored at −20°C and analysed every two weeks to give mean (± standard error of the mean, S.E.M.) concentrations (nmol ml^{-1}) of 87.5±16.4, 18.7±4.9, and 0.22±0.11 for **1**, *S*-(+)-**2** and *R*-(−)-**2**, respectively, and a metabolic ratio of 4.8±0.9. Analysis of 1 ml of urine permits detection of ca. 5 pmol of **1** and 25 pmol of each of the enantiomers of **2**.

Collection of urine samples from humans

Ten subjects (five EMs, five PMs) each took tablets of debrisoquine (12.7 mg of the hemisulphate equivalent to 10 mg of free base). All urine was collected for the next 8 h, and a 10-ml aliquot was stored at −20°C until analysis. At this temperature **1** and **2** are stable for an unlimited period [20].

RESULTS AND DISCUSSION

Derivatization and chromatography

Because all known metabolites of debrisoquine contain the guanidine moiety, they are readily derivatized with AcAc to the corresponding 4,6-dimethyl-2-pyrimidinyl derivatives (Fig. 2), which are extractable and, as shown in other assays [21], permit extremely sensitive fluorescence detection. However, it is essential to use distilled AcAc because non-polar fluorescent impurities present in the commercial reagent interfere in the assay. The same impurities were formed when the mixtures were heated in order to shorten the time of derivatization. Best results (ca. 70% yield) were obtained after incubation at room temperature for 72 h.

Independent experiments proved that the conversion rates for **1**,**2** and **3** were identical and that subsequent extraction from the aqueous phase was quantita-

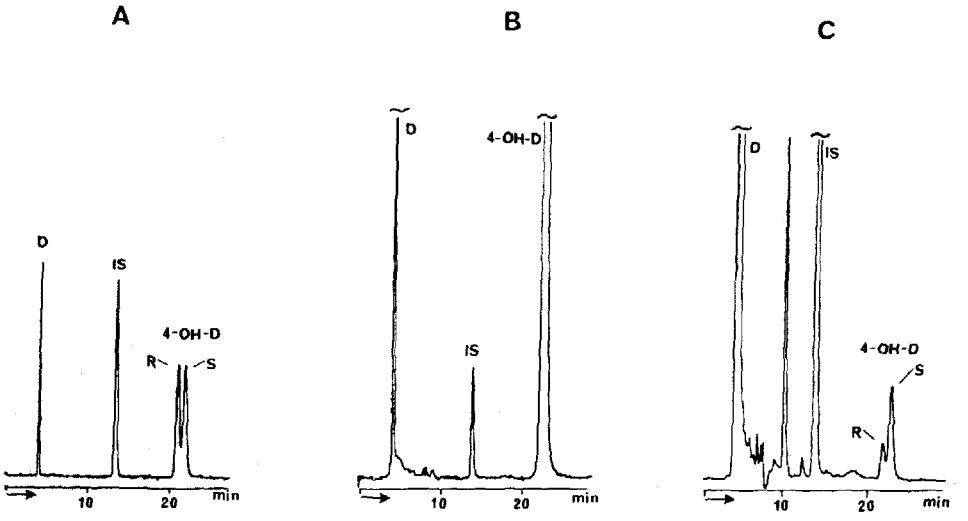

Fig. 3. Chromatographic separation of the pyrimidine derivatives (Fig. 2) of debrisoquine (D), internal standard (IS), *R*-(−)- and *S*-(+)-4-hydroxydebrisoquine (4-OH-D) on the chiral Pirkle column. (A) Mixture of pure reference samples, 20 pmol each of D and IS and 100 pmol of each enantiomer of 4-OH-D; (B) extract of EM urine, MR 0.2, 120 pmol of D, 5 pmol of IS and 750 pmol of *S*-(+)-4-OH-D, enhanced in order to demonstrate the absence of the *R*-(−) enantiomer; (C) extract of PM urine, MR 105, 41% e.e., 19.8 nmol of D, 150 pmol of IS, 56 pmol of *R*-(−)-4-OH-D and 133 pmol of *S*-(+)-4-OH-D.

tive. In order to effect chromatographic separation of the enantiomers of **2**, several chiral stationary phases were tested but only the commercially available Pirkle column gave a satisfactory resolution for the pure pyrimidine derivatives of a racemic mixture of **2** (4-OH-D; 100 pmol *S*-(+), t_R 23 min, and 100 pmol *R*-(−), t_R 22 min), internal standard **3** (I.S., 20 pmol, t_R 14 min), and **1** (D, 20 pmol, t_R 5 min) (Fig. 3A). In contrast to a recent report on the separation of a chiral drug [22], sub-ambient temperature chromatography (from 15 to −10°C) did not improve the resolution. Probably because of a distinct twisted intermediate charge transfer (TICT) effect [23], peaks of D and I.S. are five-fold more intense than the *R* and *S* enantiomers of 4-OH-D. Thus, the detection limits for D and 4-OH-D enantiomers in blank urine were 5 and 25 pmol, respectively, per injection.

Analysis of urine samples

A typical chromatogram of an extract of a urine sample from an EM subject is shown in Fig. 3B. Besides the large amounts of hydroxy metabolite, characteristic of this phenotype, a unique enantiomeric purity of this metabolite of ⩾99% e.e. *S*-(+)-**2** is apparent. Similarly, all other EM urines listed in Table II exhibit a high degree of product stereoselective metabolite formation.

In contrast, urine samples taken from subjects who had been phenotyped as PMs on the basis of an MR > 12 show a completely different peak pattern in the chromatogram (Fig. 3C). Not only was the absolute amount of excreted 4-hy-

TABLE II

URINARY EXCRETION OF DEBRISOQUINE AND THE ENANTIOMERIC 4-HYDROXY METABOLITES IN EM AND PM SUBJECTS

Values in μmol per 8 h; 56 μmol of debrisoquine administered.

EM				PM					
No.	**1**	*S*-(+)-**2***	MR	No.	**1**	*R*-(−)-**2**	*S*-(+)-**2**	e.e. (%)	MR
1	2.4	14.3	0.2	6	19.7	0.03	0.15	67	109
2	3.8	11.4	0.3	7	13.5	0.05	0.13	44	75
3	3.5	11.0	0.3	8	18.4	0.04	0.07	28	167
4	2.8	10.7	0.3	9	19.2	0.06	0.45	76	38
5	4.1	11.8	0.3	10	12.9	0.03	0.28	80	42

*Greater than 99.5%.

droxy metabolite **2** reduced, but also the enantiomeric excess, and up to 36% of total **2** [*R*-(−) and *S*-(+)] consisted of *R*-(−)-**2**-hydroxydebrisoquine (Table II).

In comparison with other analytical HPLC methods [11–14] the present procedure is not only ca. 10–100 fold more sensitive but also allows the stereochemistry of the 4-hydroxylation of **1** to be monitored in EM and PM phenotypes.

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