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Determination of debrisoquine and metabolites in human urine by gas chromatography–mass spectrometry

LUC DAUMAS, JEAN-FRANÇOIS SABOT*, ELIANE VERMEULEN, PASCALE CLAPOT, FRANÇOISE ALLEGRE and HENRI PINATEL

Laboratoire de Chimie Analytique II, Faculté de Pharmacie, 8 Avenue Rockefeller, 69373 Lyon Cedex 08 (France)

MICHEL BOUCHERAT

Laboratoire Central de Biochimie, Hôpital de l'Antiquaille, 69321 Lyon Cedex 05 (France)

and

BERNARD FRANCOIS

Service de Nephrologie, Hôpital Jules Courmont, C.H. Lyon-Sud, 69310 Lyon (France)

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ABSTRACT

A gas chromatographic–mass spectrometric analysis has been developed for the determination of debrisoquine and its metabolites in the urine of healthy individuals (controls) and patients with chronic renal faillure. The sensitive and specific assay comprises selected-ion monitoring of the drug and the metabolites 4-hydroxydebrisoquine and 8-hydroxydebrisoquine using guanoxan as the internal standard. The limit of detection is *ca.* 0.2 μg/ml. The clinical study shows that the healthy individuals and patients with chronic renal failure can be divided in two groups of extensive metabolizers and poor metabolizers, respectively. The extensive metabolizers excreted large amounts of 4-hydroxydebrisoquine and minor amounts of 8-hydroxydebrisoquine. The poor metabolizers excreted small amounts of 4-hydroxy metabolite, and no 8-hydroxydebrisoquine was detected in the urine.

INTRODUCTION

The antihypertensive drug, debrisoquine, undergoes metabolic hydroxylation in humans [1]. The hydroxylation status of a subject is determined by measuring the concentrations of debrisoquine (DEB), 4-hydroxydebrisoquine (4-OH-DEB) and 8-hydroxydebrisoquine (8-OH-DEB) in urine collected over an 8-h period following an oral dose of DEB [2]. Since 4-OH-DEB, the main metabolite of DEB, is pharmacologically inactive, the determination of ratio of DEB to 4-OH-DEB plays an important part, not only in the investigation of the population, but also in the dosage regimen [3,4]. The clinical importance of the polymorphism of debrisoquine has been stressed by Sloan *et al.* [5].

Extensive hydroxylation has been shown to be genetically controlled, with the

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existence of the two phenotypes [6]. The first is characterized by persons who 4-hydroxylated the majority of the drug and are thus designated extensive metabolizers (EM). The metabolic ratio (MR) is defined as the percentage of dose as DEB/the percentage of dose as 4-OH-DEB excreted in 0–8 h in urine. In the second phenotype (poor metabolizers, PM), MR values are higher than those in EM, and thus these individuals show a relative inability to excrete 4-OH-DEB. This group represents 10% of the population [7,8]. Poor hydroxylators may be at risk of concentration-dependent side-effects.

The oxidative metabolism of other drugs appears to be under the control of the same alleles as for DEB hydroxylation. These drugs include guanoxan and phenacetin [5], phenytoin [9], metiamide [10], 4-methoxyamphetamine [11], sparteine [12–14], certain β-blockers [15–17] and antidepressants [18,19].

High-performance liquid chromatographic (HPLC) methods have been developed for the simultaneous determination of DEB and 4-OH-DEB in human urine [20–26]. The available method to measure DEB and its hydroxy metabolites uses gas chromatography [27–29] or gas chromatography–mass spectrometry (GC–MS) [2,28,30–32]. Because of their high specificity, determinations by GC–MS are increasingly used as reference methods for drugs.

This paper describes the application of the procedure to the specific determination of DEB, 4-OH-DEB and 8-OH-DEB in human urine. We have used a derivatization procedure based on the reaction of DEB and its metabolites with acetylacetone [27,28]. This method has been used to emphasize the hydroxylation phenotype in patients suffering from chronic renal insufficiency.

EXPERIMENTAL

Reagents

Debrisoquine sulphate (Declinax), 4-OH-DEB and 8-OH-DEB were kindly supplied by Hoffmann la Roche (Basel, Switzerland) and guanoxan by Pfizer (Central Research, Sandwich, UK). Methanol, chloroform, benzene and acetylacetone (Merck, Darmstadt, Germany) were of analytical-reagent grade.

Apparatus

GC–MS analyses were carried out on a Hewlett-Packard 5790-A gas chromatograph coupled with a Hewlett-Packard 5970-A mass detector (quadrupole). A fused-silica column (25 m × 0.32 mm I.D.) coated with the non-polar phase R.S.L. 200 (0.3 μm film thickness) (Alltech-France, Templeuve, France) was used.

The temperature of the column was adjusted to obtain the maximum response from a standard injection of DEB derivative. It was programmed from 195 to 250°C at 5°C/min and was maintained at 250°C for 2 min. The first part of the programme allowed the chromatographic separation, and the second part, with the higher temperature, was used to clean the column before a new separation. The carrier gas was helium at a flow-rate of 0.9 ml/min.

DEB, 4-OH-DEB, 8-OH-DEB and guanoxan were measured by selected-ion monitoring (SIM) MS. The mass spectrometer was operated under the following conditions: nominal electron energy, 70 eV; coupling line temperature, 245°C. With this technique, the four major ions of the mass spectra (*m/z* 162 for guanoxan, *m/z* 239 for DEB ($M^{+\cdot}$), *m/z* 236 for 4-OH-DEB ($M^{+\cdot} - 19$), *m/z* 255 for 8-OH-DEB ($M^{+\cdot}$)) were recorded after elution from the column. A blank sample, consisting of an injection of solvent, was run first in order to establish the lack of contamination.

Sample processing

An aliquot (5 ml) of urine was mixed with the internal standard (20 μg of guanoxan). Guanoxan was chosen because it is structurally similar to DEB, *i.e.* both possess the guanidino group. Derivatization of the guanidino group by condensation with acetylacetone gave the corresponding pyrimidines, which possessed good GC properties and enhanced solubility in organic solvents [24].

After the addition of 0.8 ml of methanol, 1 ml of 1 *M* Na_2CO_3, 80 μl of $NaHCO_3$ (50 g/100 ml) and 0.8 ml of acetylacetone, the urine sample was incubated in a water-bath at 100°C for 24 h. The pH of all samples was adjusted to 8.5 with 3 *M* NaOH, and 14 ml of chloroform were added. The mixture was mixed on a rotary mixer for 10 min. After centrifugation at 2000 *g* for 10 min, the aqueous layer was discarded. Finally, an aliquot (5 ml) of the organic phase was transferred to a 7-ml conical tube and evaporated to dryness under a stream of nitrogen at 40°C. Prior to injection, the residue was dissolved in 100 μl of benzene, and 2 μl were injected into the column. We used the technique of splitless injection.

Calibration curve

Stock solutions containing 1 mg/ml each of DEB, 4-OH-DEB and 8-OH-DEB in distilled water were stored at −20°C. The working solutions were prepared by adding measured amounts of drug and metabolites (2–20 μg/ml) to control urine containing guanoxan (20 μg). The controls were hydrolysed, extracted and analysed by GC–MS in the same way as the urine samples.

The mass spectrometer was focused on ions *m/z* 239 ($M^{+\cdot}$ of DEB), 236 ($M^{+\cdot} - 19$ of 4-OH-DEB), 255 ($M^{+\cdot}$ of 8-OH-DEB) and 162 (fragment of guanoxan). These ions were monitored by a four-channel automatic peak selector, with a channel width of 0.3 a.m.u. and a sampling time of 50 ms per channel.

Subjects and protocol

A single dose of a 10-mg Declinax tablet, equivalent to 12.8 mg of debrisoquine sulphate, was administered to 85 healthy people and to patients with chronic renal failure, in the morning after the subjects had voided their bladders. Urine was collected for up to 8 h; the tested subjects were allowed unlimited food and drink during this period. Other drugs and alcohol were forbidden. The urine volume was recorded, and an aliquot was stored at −20°C to await analysis.

RESULTS AND DISCUSSION

The success of derivatization by acetylacetone and extraction with chloroform of DEB, 4-OH-DEB and 8-OH-DEB was confirmed by GC–MS (Fig. 1). Mass spectra, obtained in the electron-impact mode, show that DEB, 4-OH-DEB, 8-OH-DEB and guanoxan give molecular ions, with m/z 239, 255, 255 and 271, respectively.

Fig. 2 shows a typical trace, obtained by SIM, after extraction of a urine sample containing DEB and its metabolites. The integrated signals were output onto the multi-channel recorder. The selectivity of this methods was confirmed by characteristic retention times: 3 min for DEB, 4.35 min for guanoxan, 4.60 min for 4-OH-DEB and 5.50 min for 8-OH-DEB. Baseline separation of all peaks was achieved.

Calibration curves

Calibration curves for extracted DEB, 4-OH-DEB and 8-OH-DEB were plotted as the area relative to guanoxan internal standard *versus* the concentration in urine (μg/ml). The different curves were rectilinear in the concentration range of interest, 0–20 μg/ml for DEB, 4-OH-DEB and 8-OH-DEB.

The linear regression curves are described by the equations $y = 0.087x + 0.035$ ($r^2 = 0.997$) for DEB, $y = 0.203x + 0.040$ ($r^2 = 0.999$) for 4-OH-DEB and $y = 0.051x + 0.030$ ($r^2 = 0.996$) for 8-OH-DEB, where y is the relative area and x is the concentration. These three curves have negligible y-intercepts.

Precision

The intra-day precision of the method was evaluated by repeated analysis ($n = 5$) of samples of urine with known concentrations of DEB, 4-OH-DEB, 8-OH-DEB and guanoxan. The obtained coefficients of variation (C.V.) of DEB, 4-OH-DEB and 8-OH-DEB to internal standard are 7.6, 5.6 and 8%, respectively, at 2 μg/ml and 5, 4.3 and 4.4% at 10 μg/ml.

Reproducibility

The reproducibility was determined on five replicate analyses of urine, ten times a month, by the same analyst (Table I). The reproducibility was less than 12%. The best reproducibility was obtained at high concentrations.

Accuracy

One way of evaluating accuracy is to compare experimental data with theoretical values. When the results obtained for a number of samples are plotted against real concentrations, a straight regression line is obtained. In the absence of error or bias, this line has a slope of exactly unity and a y-intercept of zero.

For the three studied compounds, calculations gave y-intercepts of zero and slopes near unity (in the range 0.96–1.05 for DEB, 0.97–1.03 for 4-OH-DEB and 0.94–1.05 for 8-OH-DEB).

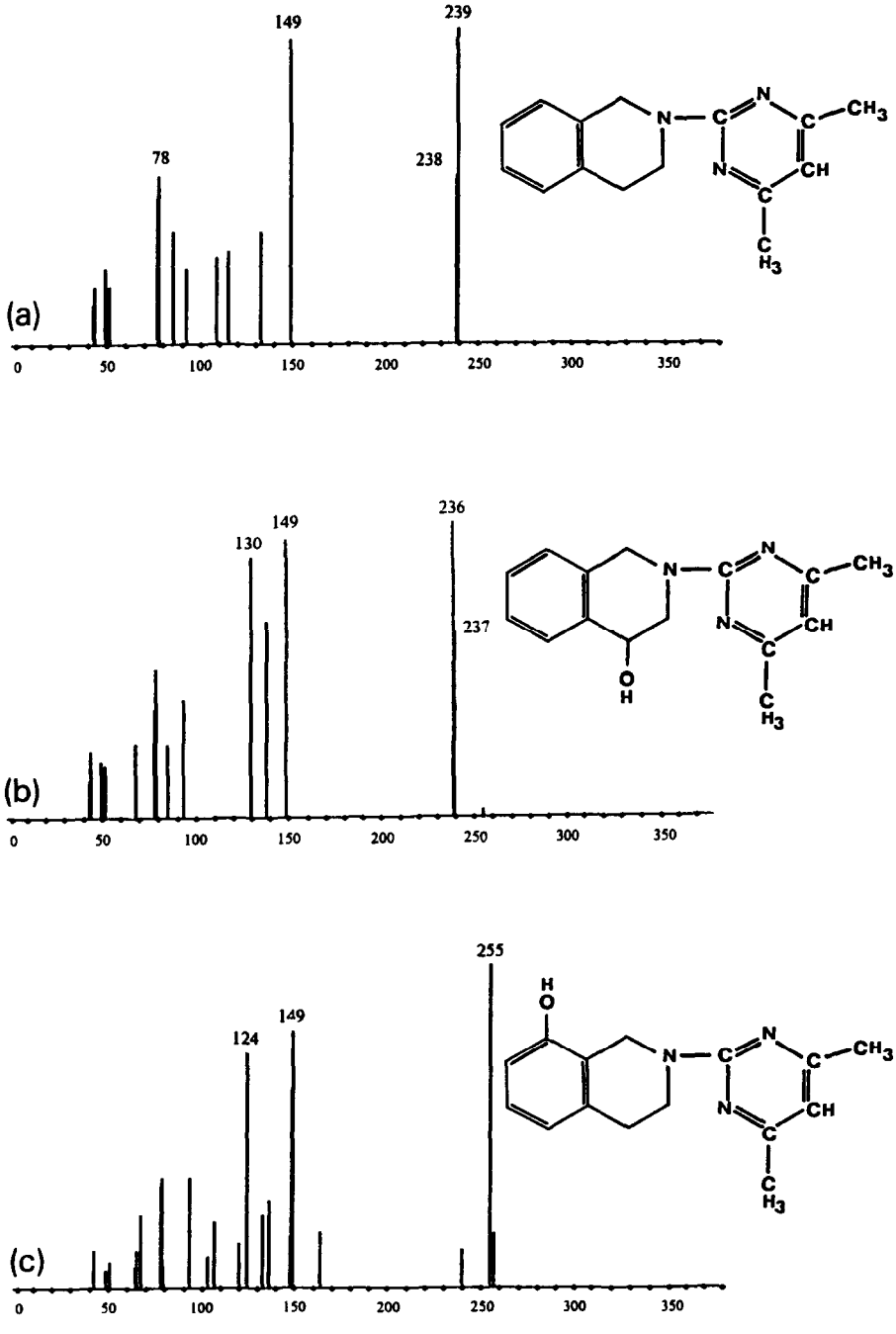

Fig. 1. Mass spectra of the acetylacetone derivatives of (a) DEB, (b) 4-OH-DEB and (c) 8-OH-DEB.

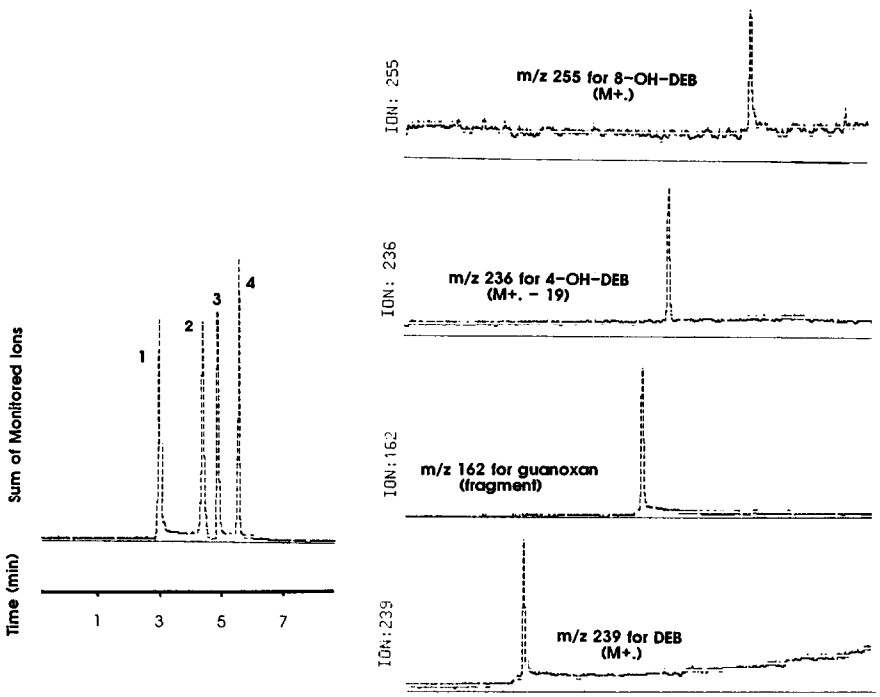

Fig. 2. Chromatogram of a urine sample spiked with 10 μg/ml DEB, 4-OH-DEB and 8-OH-DEB and selected-ion current profiles. Peaks: 1 = DEB; 2 = internal standard (guanoxan); 3 = 4-OH-DEB; 4 = 8-OH-DEB.

Sensitivity

The calculated limit of detection for a signal-to-noise ratio of 3 for DEB, 4-OH-DEB and 8-OH-DEB is 1 ng injected.

The minimum detectable amount per ml of urine is 0.2 μg. Major contributing factors to the favourable sensitivity are the lack of interferences from other urinary constituents and the very low baseline signal of the selected ions.

TABLE I

REPRODUCIBILITY OF THE METHOD

		Drug level		
		2.5 mg/l	5 mg/l	10 mg/l
DEB	Mean	2.36	5.21	9.95
	C.V. (%)	11.4	8.1	5.3
4-OH-DEB	Mean	2.46	5.07	10.02
	C.V. (%)	6.60	6.0	2.9
8-OH-DEB	Mean	2.53	4.94	9.97
	C.V. (%)	9.9	7.3	5.3

Interferences

Urine samples may contain considerable amounts of caffeine. Usually, most interference from this compound or other drugs that may be present in clinical samples can be eliminated. Here, the extraction procedure is certainly the most lengthy and cumbersome part of the analysis. Of all the current drugs examined, none interfered with DEB or its metabolites, in the SIM mode.

Clinical study

Because there is a good separation of 4-OH-DEB, 8-OH-DEB and DEB, it is possible to assay these compounds. It is interesting to calculate the metabolic ratio (MR) from the following expression [8]: MR = percentage of dose as DEB/ percentage of dose as 4-OH-DEB excreted in the 0–8 h urine.

Table II shows that the urinary excretion of the metabolites expressed as the MR is dependent on drug metabolism by the liver. In poor metabolizers (n = 5), the concentrations of metabolites are very low compared with those in extensive metabolizers. Literature data for humans reveal that the pharmacokinetics of DEB change with the genetic polymorphism of the drug oxidation [33]. Two DEB oxidation phenotypes were characterized [8], and the MR was bimorphically distributed. Mahgoub *et al.* [7] and Evans *et al.* [8] found that the PM phenotype corresponded to a metabolic ratio greater than 12.8. Westwood *et al.* [21] proposed a cut-off between extensive (MR < 12.6) and poor (MR > 12.6) hydroxylators.

Data obtained in our study are in total agreement with those in the literature, and we have calculated that a high MR value (>12.6) is an indicator of a poor metabolizer.

TABLE II

EXCRETION OF DEB AND ITS METABOLITES IN HUMAN URINE

Means and C.V. of the dose excreted as DEB, 4-OH-DEB and 8-OH-DEB in 0–8 h urine and MR values in all the subjects (n = 85), in extensive metabolizers (EM, n = 80) and poor metabolizers (PM, n = 5). Means have been calculated for each population. The means of MR were obtained by calculation, using individual MR values, for each population.

	DEB	4-OH-DEB	8-OH-DEB	MR
Total				
Mean	14.6	21.3	2.9	1.86
C.V. (%)	113	90	176	230
EM				
Mean	12.2	24	1.65	0.91
C.V. (%)	95	79	86	95
PM				
Mean	51.4	3.38	–	19.2
C.V. (%)	58	81	–	35

It is possible, therefore, that renal failure might have influenced the oxidation and/or the renal elimination of DEB metabolites [34].

CONCLUSION

The procedure described in this paper is suitable for detailed studies of DEB metabolism in humans. This GC–MS technique is sensitive and specific. It was found that no naturally occurring substances interfered with the assay.

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