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Note

Direct determination of debrisoquine and 4-hydroxydebrisoquine using a high-performance liquid chromatographic switching technique

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Debrisoquine [3,4-dihydro-2-(1*H*)-isoquinoline carboxamidine] is an antihypertensive drug, which is essentially oxidized to 4-hydroxydebrisoquine. Mahgoub et al. [1] have reported that 4-hydroxylation of this drug is under monogenic control and two phenotypes are present: extensive and poor metabolizers. The determination of the metabolic status of subjects consists of measuring the ratio of debrisoquine to 4-hydroxydebrisoquine in urine, after an oral dose of debrisoquine. For this purpose, a gas chromatographic (GC) method for the measurement of debrisoquine and its metabolite has been proposed by Lennard et al. [2]. This technique is based on time-consuming derivatization and extraction steps, which limit the number of treated samples per day. Recently, Westwood et al. [3], Harrison et al. [4] and Rona et al. [5] have published high-performance liquid chromatographic (HPLC) methods for the assay of these molecules in urine. The procedures described by Westwood et al. [3] and Harrison et al. [4] require an extraction or a filtration step of urine prior to analysis, and the method of Rona et al. [5] involves reaction of debrisoquine and 4-hydroxydebrisoquine with acetylacetone and two purification steps.

In this paper, we propose a new process based on direct injection of urine samples onto the HPLC system using a precolumn switching method. The aim of this study was to develop a rapid and precise method for the simultaneous determi-

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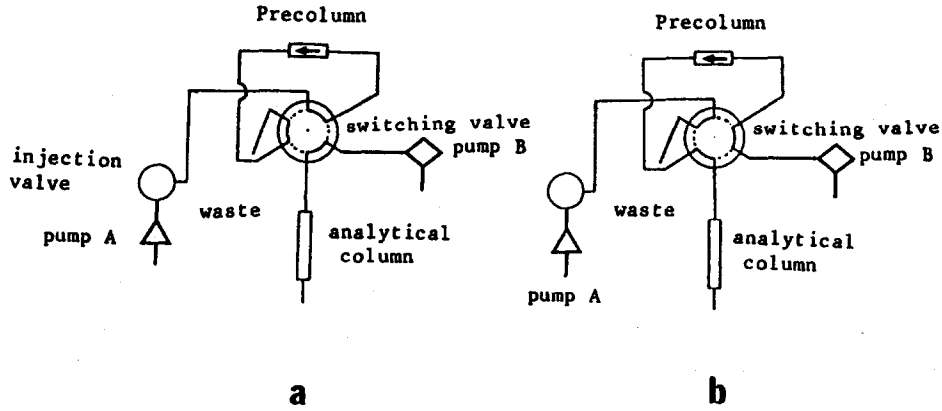

Fig. 1. Schematic representation of the column-switching system used for the analysis of debrisoquine and 4-hydroxydebrisoquine in urine. (a) Sample clean-up; (b) sample elution.

nation of debrisoquine and 4-hydroxydebrisoquine in urine, which could be adapted to large-scale analysis and easily automated.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents used were of analytical-reagent grade. Debrisoquine and 4-hydroxydebrisoquine were kindly supplied by Hoffman-La Roche (Basle, Switzerland). Human urine samples free of any drugs were obtained from healthy volunteers.

Analytical procedure

The analytical system (Fig. 1) consisted of two solvent-delivery pumps (Waters 6000 A) (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne 7125 injection valve with a 20-μl sample loop and a Rheodyne 7010 six-way valve (Rheodyne, Cotati, CA, U.S.A.). A UV–visible detector was operated at 208 nm, 0.08 a.u.f.s. (Merck LMC system SM 25) (E. Merck, Darmstadt, F.R.G.).

Urine samples (20 μl) were directly injected onto a precolumn dry-packed with LiChrosorb CN (Hibar RT, 30×4 mm I.D., 10 μm particle size, E. Merck) and flushed at a flow-rate of 0.5 ml/min with 8 m*M* phosphate buffer (pH 5) containing 5% (v/v) acetonitrile (sample clean-up step). Then, 3 min after injection, the valve was switched over and the sample was eluted onto the analytical column (LiChrosorb CN-HIBART RT, 250×4 mm I.D., 5 μm particle size, E. Merck) with the same buffer containing 60% (v/v) acetonitrile at a flow-rate of 2 ml/min (pressure 17 MPa) (sample elution step). The system was operated at room temperature.

Preparation of calibration curves

Stock solutions of debrisoquine (0.2 mg/ml in methanol) and 4-hydroxydebrisoquine (0.5 mg/ml in methanol) were diluted ten-fold with methanol, and

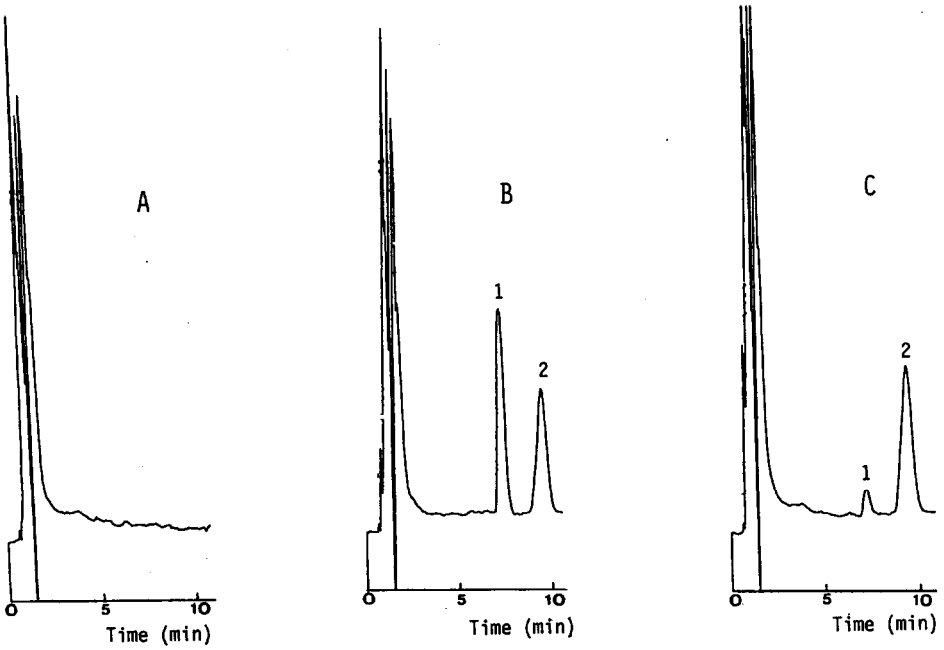

Fig. 2. Chromatographic profiles obtained with HPLC column switching of (A) drug-free urine, (B) urine spiked with 8 μg/ml debrisoquine and 20 μg/ml 4-hydroxydebrisoquine and (C) urine from a poor metabolizer receiving 10 mg of debrisoquine (debrisoquine 9.8 μg/ml, 4-hydroxydebrisoquine 2.3 μg/ml). The elution times are given as a function of the time of column switching. Peaks: 1 = 4-hydroxydebrisoquine; 2 = debrisoquine.

aliquots from 100 μl to 1 ml for debrisoquine and from 50 μl to 1 ml for 4-hydroxydebrisoquine were poured into conical tubes. Methanol was then evaporated at 40°C in a nitrogen stream, and 1 ml of urine was added to the dry residue. After mixing, 20 μl were analysed as previously described.

GC and HPLC correlation study

For this study, nineteen healthy volunteers received 10 mg of debrisoquine orally (Declinax). Urine was collected for 8 h and frozen at −20°C before analysis. Debrisoquine and 4-hydroxydebrisoquine levels were determined both by the GC method proposed by Lennard et al. [2] and by our HPLC method. If a precipitate appeared during the conservation period, the urine was centrifuged before analysis.

RESULTS

Fig. 2 shows a typical chromatogram obtained from the analysis of debrisoquine and 4-hydroxydebrisoquine in urine. No endogenous sources of interference were observed, and the resolution between debrisoquine and 4-hydroxydebrisoquine was very satisfactory ($R_s > 2$).

Standard curves were constructed by plotting peak areas versus drug and metabolite concentrations. Instrument response and concentrations were line-

TABLE I

PRECISION OF THE HPLC ASSAY FOR DEBRISOQUINE AND 4-HYDROXYDEBRISOQUINE IN URINE

Compound	Concentration of drug added (μg/ml)	Concentration found (mean ± S.D., $n=3$) (μg/ml)
Debrisoquine	3	2.70 ± 0.09
	7	6.90 ± 0.15
4-Hydroxydebrisoquine	7.5	7.33 ± 0.14
	17.5	17.41 ± 0.57

arly related for both debrisoquine and 4-hydroxydebrisoquine over ranges 2–20 and 2.5–50 μg/ml, respectively. The linear regression curves for the data used for calibration can be described by the equations: $y=1.56x-0.35$ ($r=0.997$) for debrisoquine and $y=0.34x+0.10$ ($r=0.998$) for 4-hydroxydebrisoquine.

The precision of the method was evaluated for two concentrations of debrisoquine (3 and 7 μg/ml) and 4-hydroxydebrisoquine (7.5 and 17.5 μg/ml) by analysing each one three times on the same day. The data shown in Table I demonstrate the high degree of precision of the method.

The intra- and inter-assay coefficients of variation (C.V.) for replicate analysis of debrisoquine and 4-hydroxydebrisoquine in urine are shown in Table II. The C.V. did not exceed 3.3 and 3.8% for intra- and inter-assay, respectively.

The limit of detection for both debrisoquine and 4-hydroxydebrisoquine was lower than 0.1 μg/ml using a 20-μl sample loop (signal-to-noise ratio 4.5 for debrisoquine and 4 for 4-hydroxydebrisoquine).

The comparison between the GC and HPLC switching techniques for the determination of the metabolic status of nineteen healthy volunteers showed a high correlation between the two methods ($r=0.999$) (Fig. 3).

TABLE II

INTRA- AND INTER-ASSAYS COEFFICIENTS OF VARIATION FOR DEBRISOQUINE AND 4-HYDROXYDEBRISOQUINE IN URINE

Compound		Concentration (μg/ml)	C.V. (%)
Debrisoquine	Intra-assay ($n=6$)	2	2.9
		10	1.1
	Inter-assay ($n=6$)	2	3.8
		10	2.2
4-Hydroxydebrisoquine	Intra-assay ($n=6$)	2.5	3.3
		20	2.8
	Inter-assay ($n=6$)	2.5	3.8
		20	3.1

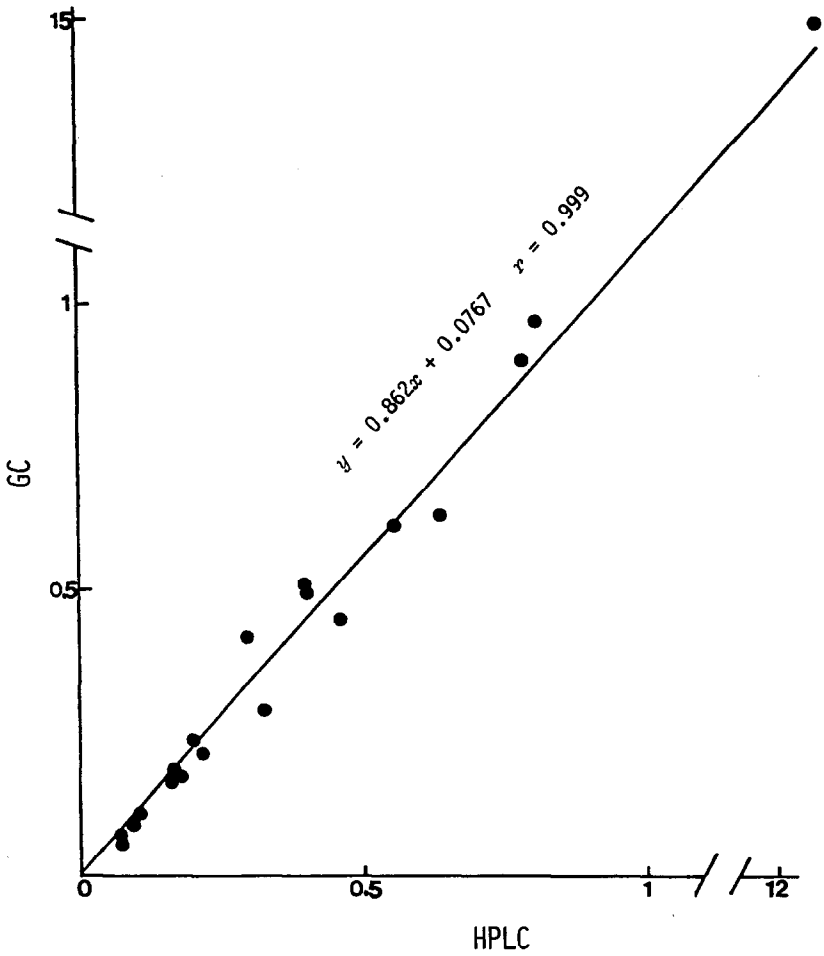

Fig. 3. Correlation between GC and HPLC analysis of debrisoquine and 4-hydroxydebrisoquine in urine. The units correspond to the metabolic ratio debrisoquine/4-hydroxydebrisoquine.

DISCUSSION

The linearity and the precision of our method, the intra- and inter-assay variations and the correlation with the GC method can be judged as very satisfactory and permit the validation of the proposed method. The essential points of this method are its rapidity and its simplicity. Direct injection of the urine sample avoids lengthy sample preparation procedures and derivatization of debrisoquine and 4-hydroxydebrisoquine. Moreover, since there are no preparation steps during which analytes can be lost, the use of an internal standard is not needed, the C.V. being lower than those reported by Westwood et al. [3].

Direct injection of urine onto the analytical column (without a sample clean-up step) means that debrisoquine and 4-hydroxydebrisoquine are well isolated from endogenous impurities. Despite that, the use of a precolumn clean-up step

by a switching technique avoids contamination of the analytical column and prolongs its lifetime. More than 100 samples can be injected before the precolumn has to be changed owing to increasing pressure in the analytical system.

A wash-time of 3 min with the mobile phase containing only 5% (v/v) acetonitrile means that all the impurities are discarded, and debrisoquine and 4-hydroxydebrisoquine are retained on the precolumn. They are then eluted by modification of the mobile phase composition by a switching technique. The different parameters (precolumn length, wash-time, mobile phase composition, flow-rate) were chosen according to two criteria: (i) to discard the impurities and retain the compounds to be analysed on the precolumn in a first step and to elute these molecules in a second step; (ii) to avoid peak-widening and a loss of resolution. This technique allowed us to test the urine of nineteen volunteers in order to establish their metabolic status. One of them turned out to be a poor metabolizer and the eighteen others were extensive metabolizers. This is in accord with the results of other pharmacogenetic studies [1,6]. The results presented demonstrate that the method is very suitable for easy routine analysis.

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