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Note

Liquid chromatographic method for the determination of debrisoquine and its 4-hydroxy metabolite in human urine

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It is well known in pharmacogenetics, that the metabolism of some drugs shows polymorphism. One of the most studied of these is the hydroxylation of debrisoquine (D), an antihypertensive agent (Fig. 1).

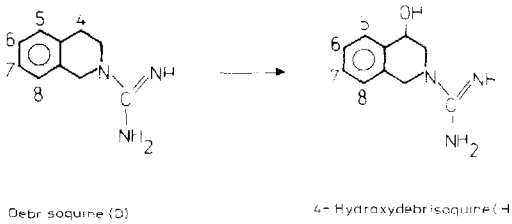

Fig. 1. The main metabolic pathway of debrisoquine.

The metabolic rate (MR), determined as the D/4-hydroxydebrisoquine (HD) ratio in urine collected between 0 and 8 h after the administration of D, is bimodal [1, 2]. Some 8% of the Caucasian population are poor metabolizers. Poor metabolizers are defined as subjects with an MR of 12.6 and above. Subjects with an MR below this value are extensive metabolizers. Since HD, the main metabolite of D, is pharmacologically inactive, the determination of the D/HD ratio plays an important role not only in the investigation of the population but also in the dosage regimen [3—5].

Since the description of hydroxylation polymorphism of D by Mahgoub et al. [1] and Tucker et al. [2] in 1977, the papers dealing with the distribution of hydroxylation phenotypes determined D and HD by gas chromatography after an in situ reaction with acetyl acetone [6—8].

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We wished to study the distribution of hydroxylation phenotypes in a Hungarian population. For this study, we have elaborated a new high-performance liquid chromatographic (HPLC) method based on the reaction of D and HD with acetyl acetone as described by Allen et al. [8].

EXPERIMENTAL

Chemicals

Debrisoquine, 3,4-dihydro-2(1*H*)-isoquinolinecarboxamidine sulphate (2:1), and 4-hydroxydebrisoquine, *rac*-3,4-dihydro-4-hydroxy-2(1*H*)-isoquinolinecarboxamidine sulphate (2:1) were generously supplied by Drs. Bernauer and Gutmann (Hoffmann-La Roche, Basle, Switzerland), and the internal standard guanoxan (G; guanidomethylbenzodioxane) by Pfizer Scientific Centre (Budapest, Hungary). Acetonitrile, methanol, diethyl ether and Extrelut were the products of Merck (Darmstadt, F.R.G.). Acetyl acetone was purchased from Reachim (Moscow, U.S.S.R.). All other reagents were from Reanal (Budapest, Hungary) and were of the best commercially available grade.

The stock solutions were prepared as follows: G: 3.1 mg of G (2.5 mg of guanoxan base) dissolved in 1.0 ml of 0.1 *M* hydrochloric acid and diluted to 25 ml with distilled water; D: 12.8 mg of D (10 mg of debrisoquine base) dissolved in 3.0 ml of 0.1 *M* hydrochloric acid and diluted to 100 ml with distilled water; HD: 12.68 mg of HD (10 mg of 4-hydroxydebrisoquine base) dissolved in 3.0 ml of 0.1 *M* hydrochloric acid and diluted to 100 ml with distilled water. The stock solutions were kept at 4°C and 10- to 100-fold dilutions were made with distilled water for the calibration curves.

Analytical procedure

To 1.0 ml of centrifuged urine, 10 μg of G (internal standard) in 100 μl of 0.001 *M* hydrochloric acid, 0.5 ml of 1.2 *M* sodium bicarbonate, 0.5 ml of methanol and 0.5 ml of acetyl acetone were added. After shaking for 5 s in a shaker (Kutesz, Hungary), the solution was incubated at 50°C for 16 h. Afterwards, the samples were poured onto a chromatographic column (250 × 5 mm I.D.) filled with 1.5 g of Extrelut and were eluted with 10 ml of diethyl ether after 15 min. The ether eluate was shaken with 350 μl of 4 *M* hydrochloric acid for 4 × 15 s. The water phase was collected, and after the addition of 450 μl of 4 *M* sodium hydroxide (vortex-mixed for 10 s) it was poured onto another column (250 × 5 mm I.D.) filled with 0.5 g of Extrelut. After 15 min, the samples were eluted with 5 ml of diethyl ether. This second purification step is important to achieve sufficient selectivity. Diethyl ether was evaporated to dryness in a nitrogen stream; the dry residue was dissolved in 100 μl of methanol—water (1:1) solution by shaking for 1 min, and 5-μl aliquots were injected onto the analytical column.

Preparation of calibration curves

D and HD were added in amounts of 0.1—40.0 μg to 1.0 ml of centrifuged human urine free of any drugs, and the samples were assayed according to the analytical procedure.

Chromatographic conditions

HPLC was performed on a Hewlett-Packard 1090 chromatograph equipped with a diode-array detector and an HP 85B system master. Area integrations, calculations and plotting of the chromatogram were carried out by a Hewlett-Packard 3390A integrator. A MOS-Hypersil RP-8 prepacked column (200 × 4.6 mm I.D.; 10 μm particle size) (Hewlett-Packard) at room temperature was used for the separation of the compounds. The flow-rate was 1.0 ml/min. The absorbance of the effluent was monitored at 232 and 248 nm according to a time-programme; G was measured at 232 nm and the others at 248 nm. The mobile phase was ternary; 75% pump A: organic solvent, which consisted of acetonitrile—methanol (1:3); 25% pump B: water.

In vivo experiments

The investigated persons fasted overnight and received 10 mg of D orally with 100 ml of tap-water. D and HD were determined in the urine collected between 0 and 8 h.

RESULTS AND DISCUSSION

The structure of the compounds resulting when D and HD react with acetyl acetone were identified by Allen et al. [8] using mass spectrometry and nuclear magnetic resonance spectroscopy. It has been established that the amidino group of D and its derivative (HD) gives a 4,6-dimethylpyrimidine ring with acetyl acetone.

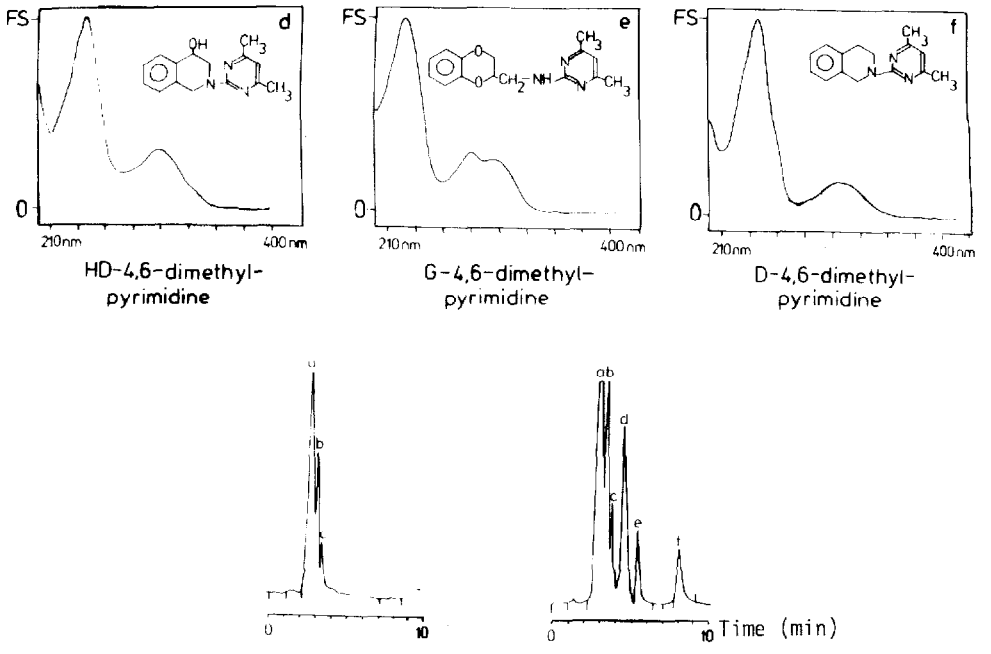

Fig. 2. Top: UV spectra of the peaks of human urine extracts. Bottom: chromatograms of a blank urine extract (left) and a urine model extract with 10 μg of D, HD and G per ml of urine, respectively (right).

The homogeneity and identity of chromatographic peaks were checked, using the diode-array detector of the HP 1090 liquid chromatograph as a peak detector. This means that UV spectra of the inflection points and apex of each peak were taken during the chromatographic elution and UV spectra were drawn after normalization in one coordinate system and analysed for differences (Fig. 2). This precise procedure seemed to be necessary as the patients also received one or more other drugs, simultaneously. The following drugs were taken: aminophylline, atropine sulphate, captopril, chlordiazepoxide, chlorthalidone, diazepam, digoxin, doxycycline, glutethimide, metoprolol, nitrazepam, oxprenolol hydrochloride, pindolol, propranolol, vinpocetin. So far, we have not detected any drug that disturbs the determination of D or HD with our method.

The retention times for HD, G and D were 4.4, 5.6 and 8.6 min, respectively. The difference in retention time was great enough to allow a change in the measurement wavelength according to a time-programme, thus each compound could be measured at its own absorption maximum (248 nm for D and HD, and 232 nm for G).

The calibration curves were linear in the concentration range of interest (0.1—40.0 μg D or HD per ml urine). The linear regression curve for the data used for calibration can be described by the equations $y_D = 0.0791x + 0.145$ ($r^2 = 0.996$) and $y_{HD} = 0.133x + 0.211$ ($r^2 = 0.998$), where y_D and y_{HD} are the D/G and HD/G peak-area ratios, respectively. The day-to-day coefficients of variation of the slope of the calibration curves were 6.59% for D and 4.56% for HD ($n = 5$). The coefficients of variation for identical samples were 12.8% for D and 10.92% for HD at 0.25 μg/ml, 11.7% for D and 9.56% for HD at 1.0 μg/ml, 8.8% for D and 3.0% for HD at 10.0 μg/ml ($n = 5$ at each concentration).

The detection limit was found to be 0.1 μg D and HD per ml of urine. It should be noted that the minimal urinary concentration of D or HD was 0.5 μg/ml after the investigation of 50 persons. Stability of the samples was as follows: the samples left at −20°C for up to 60 days showed no signs of

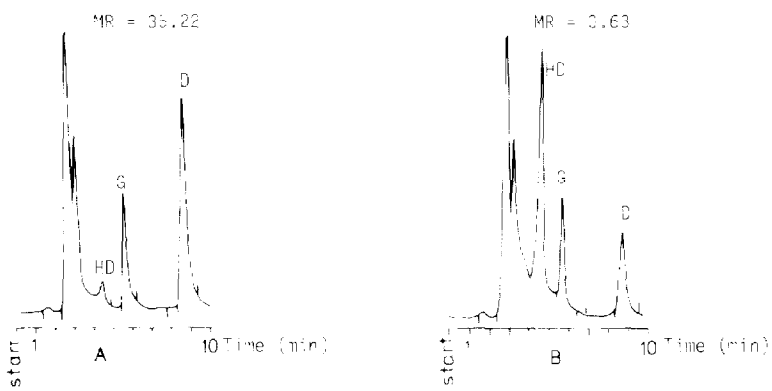

Fig. 3. Chromatograms of human urine extracts of (A) a poor metabolizer and (B) an extensive metabolizer, both after oral administration of D at a dose of 10 mg per person. Peaks: HD = 4-hydroxydebrisoquine (0.58 μg/ml for A; 11.96 μg/ml for B); G = guanoxan (internal standard); D = debrisoquine (20.43 μg/ml for A; 7.55 μg/ml for B).

decomposition and practically the same MR was obtained, suggesting that D and HD are stable under these conditions for at least two months.

In Fig. 3, two typical chromatograms are shown, one from a poor metabolizer and one from an extensive metabolizer.

The results presented demonstrate that the method is suitable for routine analysis (30–35 urine samples per day) and for pharmacogenetic studies.

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