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# A micromethod for quantitation of debrisoquine and 4-hydroxydebrisoquine in urine by liquid chromatography

V.A. Pereira[3], J.O. Auler Jr.[1], M.J. Carmona[1], F.H. Mateus[2], V.L. Lanchote[2], D.D. Breimer[4] and S.R.C.J. Santos[1]

[1]Instituto do Coração, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil
[2]Laboratório de Toxicologia, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[3]Laboratório de Farmacologia Terapêutica, Unidade 2, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brasil
[4]Leiden-Amsterdam Center for Drug Research, Leiden, Holland

## Abstract

We describe a new simple, selective and sensitive micromethod based on HPLC and fluorescence detection to measure debrisoquine (D) and 4-hydroxydebrisoquine (4-OHD) in urine for the investigation of xenobiotic metabolism by debrisoquine hydroxylase (CYP2D6). Four hundred µl of urine was required for the analysis of D and 4-OHD. Peaks were eluted at 8.3 min (4-OHD), 14.0 min (D) and 16.6 min for the internal standard, metoprolol (20 µg/ml). The 5-µm CN-reverse-phase column (Shimpack, 250 x 4.6 mm) was eluted with a mobile phase consisting of 0.25 M acetate buffer, pH 5.0, and acetonitrile (9:1, v/v) at 0.7 ml/min with detection at $\lambda_{excitation}$ = 210 nm and $\lambda_{emission}$ = 290 nm. The method, validated on the basis of measurements of spiked urine, presented 3 ng/ml (D) and 6 ng/ml (4-OHD) sensitivity, 390-6240 ng/ml (D) and 750-12000 ng/ml (4-OHD) linearity, and 5.7/8.2% (D) and 5.3/8.2% (4-OHD) intra/interassay precision. The method was validated using urine of a healthy Caucasian volunteer who received one 10-mg tablet of Declinax®, *po*, in the morning after an overnight fast. Urine samples (diuresis of 4 or 6 h) were collected from zero to 24 h. The urinary excretion of D and 4-OHD, Fel (0-24 h), i.e., fraction of dose administered and excreted into urine, was 6.4% and 31.9%, respectively. The hydroxylation capacity index reported as metabolic ratio was 0.18 (D/4-OHD) for the person investigated and can be compared to reference limits of >12.5 for poor metabolizers (PM) and <12.5 for extensive metabolizers (EM). In parallel, the recovery ratio (RR), another hydroxylation capacity index, was 0.85 (4-OHD: ΣD + 4-OHD) versus reference limits of RR <0.12 for PM and RR >0.12 for EM. The healthy volunteer was considered to be an extensive metabolizer on the basis of the debrisoquine test.

**Correspondence**
S.R.C.J. Santos
Laboratório de Pesquisa
Instituto do Coração, HC, FM, USP
Av. Dr. Eneas C. de Aguiar, 44
05403-000 São Paulo, SP
Brasil

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- Debrisoquine test validation
- CYP2D6

A series of drugs commonly prescribed for the treatment of cardiac patients have been identified as substrates of the CYP superfamilies I, II or III. Debrisoquine (D) hydroxylase, CYP2D6, an enzyme of super-family II, is responsible for alicyclic acid hydroxylation in the oxidative metabolism of many drugs used for cardiovascular therapy. The *in vivo* activity of CYP2D6 can be estimated by the debrisoquine test (1)

after a single *po* dose of Declinax®, a known antihypertensive agent commercialized by Roche (Produtos Roche Químicos e Farmacêuticos S.A., São Paulo, SP, Brazil) which contains 10 mg debrisoquine sulfate per tablet. The measurement of CYP2D6 activity involves urine collection and simultaneous analysis and determination of D and 4-hydroxydebrisoquine (4-OHD). The high sensitivity required, of the order of nanograms per milliliter of urine, requires the use of specific and sensitive methods.

Several sophisticated and high cost chromatographic methods involving gas chromatography with a flame ionization detector (2), a nitrogen-phosphorous flame ionization detector (2,3) and a mass spectrometer detector (4) have been proposed but a series of difficulties were detected in their application to routine phenotyping. Liquid chromatography techniques using an ultraviolet detector have been reported (3,5-8). However, the low sensitivity of UV detection requires pre- or post-column derivatization procedures.

Since only a fluorescence detector can provide sufficient sensitivity without requiring derivatization, the objective of the present study was to develop a simple, rapid and sensitive fluorimetric method for the simultaneous determination of D and 4-OHD in urine for routine population phenotyping.

All reagents and organic solvents, analytical or chromatography grade, were purchased from Sigma Chemical Company (St. Louis, MO, USA), EM Science (Gibbstown, NJ, USA), Merck (Darmstadt, Germany) and Grupo Química (Penha, RJ, Brazil). Sodium chloride, sodium hydroxide and sodium acetate (Sigma), acetonitrile and AXO142-1 (EM Science), dichlormethane and LiChrosolv 6044 (Merck), and isopropanol (UV-HPLC 03008) (Grupo Química) were necessary for the procedure. The nitrogen (ONU1066) used for solvent evaporation in the organic extracts and the helium (ONU1046) used to degas the mobile phase of chromatography, 99.99% purity, were purchased from IBG Indústria Brasileira de Gases Ltda. (Jundiaí, SP, Brazil). A type HA 45 membrane was used for buffer filtration and a type FHLP45 membrane was used for filtration of the mobile phase and of the organic extracts (Millipore Corporation, Bedford, MA, USA).

The reference standard, debrisoquine sulfate, was donated by Roche and the 4-hydroxydebrisoquine standard was kindly provided by Professor Sompon Wanwimolruk (Department of Pharmacy and MRC Toxicology Research Unit, University of Otago Medical School, Dunedin, New Zealand).

Ultrapure water was obtained using the MILLIQ® MILLIRO® systems (Millipore Corporation) and was used to prepare the buffer solution of the mobile phase of chromatography and to clean and regenerate the liquid chromatography apparatus (Shimadzu Corporation, Tokyo, Japan).

Debrisoquine was determined in urine samples by high performance liquid chromatography using a Shimadzu LC-10AS apparatus equipped with an RF-10AXL fluorescence detector and connected to a model 7125 Rheodyne® injector with a 50 µl loop, a Nova-Pak® CN Guard-Pak™ HPLC precolumn insert (Waters Corporation, Milford, CT, USA) and a Shimpack® CLC-CN Shimadzu column, 250 x 4.6 mm ID, 5 µm. The mobile phase consisted of 0.25 M acetate buffer, pH 5.0, and acetonitrile (9:1, v/v, final pH = 5.4) in an isocratic elution system at a flow rate of 0.7 ml/min. The column effluent was monitored at 210 nm ($\lambda_{excitation}$) and 290 nm ($\lambda_{emission}$). The chromatograms were obtained with a Shimadzu C-R6A Chromatopac® printer-plotter, which provided the elution diagrams and peak integration.

Fifty µl metoprolol in methanol (20 µg/ml), the internal standard, was added to a dry clean extraction tube, followed by evaporation in a water bath at 37°C under a nitrogen flow. Four hundred-µl urine aliquots were

used in duplicate both for calibration with reference standards and for the samples. The biological sample was purified by the addition of 80 mg sodium chloride/assay, urine was alkalinized to pH 9.0 by the addition of 50 µl 0.4 M sodium hydroxide (20 µmol) and extraction was performed with a mixture of dichloromethane:isopropanol (6:4, v/v) in a vortex type tube shaker for 1 min. After extraction and centrifugation at 3000 rpm for 10 min, the aqueous phase was separated from the organic phase by aspiration and discarded. The tube containing the remaining organic phase was then immersed in liquid nitrogen for 10 s. The organic extract was then transferred to a clean and dry conical tube. The extracts were concentrated to dryness in a water bath at 37°C under a nitrogen flow. The dried extract was then dissolved in 100 µl of the mobile phase and 50 µl was injected into the liquid chromatography apparatus.

Calibration curves were constructed by the addition of 100 µl of D (150 µg/ml stock methanol solution) and 100 µl of 4-OHD (300 µg/ml stock aqueous solution) to a volumetric flask. Volume was completed with drug-free human urine up to 5 ml. The spiked urine was diluted serially to obtain concentrations of D of 390 to 3120 ng/ml and of 4-OHD in the 750- to 6000-ng/ml range. The standard calibration curve was prepared in duplicate and contained the internal standard (metoprolol, 1 µg/assay).

We determined the absolute recovery of D (85 ± 8%) and 4-OHD (80 ± 7%) by comparing the injection of purified urine extracts with direct injection of standards (N = 8 replicates for D and 4-OHD). Relative recoveries calculated on the basis of the internal standard were 83 ± 5% (D) and 95 ± 4% (4-OHD). The detection limit was 3 ng/ml (7.9%) and 6 ng/ml (8.0%) for D and 4-OHD, respectively. The quantification limits determined on the basis of the analysis of urine aliquots (10 replicates) were 12 ng/ml D (7.7%) and 23 ng/ml 4-OHD (7.6%). The precision of the analytical procedure measured by quantitative analysis of the two compounds under study in urine aliquots (5 replicates) on the same day (intraday precision) was 5.7/5.3% for D/4-OHD, and 8.2% for both D and 4-OHD in 5 replicates on five consecutive days (interday precision) (Table 1).

We evaluated the debrisoquine activity of a healthy adult volunteer, a 50-year-old Caucasian female, height 172 cm, weight 60 kg, and body surface area 1.70 $m^2$, with normal hepatic, renal, endocrine and cardiac functions. The volunteer received detailed information about the procedures to be performed and gave written informed consent to participate in the study. The study protocol was approved by the Ethics Committee of the School Hospital under number 1414/98/109. The volunteer then received a single *po* dose of Declinax®, one tablet containing 10 mg debrisoquine sulfate, in the morning after an overnight fast. Urine was then collected from 0 to 4 h during the 0-24 h interval after administration of the CYP2D6 marker drug for validation of the analytical method described earlier.

The chromatographic pattern of the purified urine extracts is illustrated in Figure 1A. Chromatography was carried out for about 20 min.

The high selectivity of the chromatographic system utilized, together with the use of the fluorescence detector (Figure 1A), provided high sensitivity despite the small urine volume used. The linearity obtained for D and 4-OHD (Figure 1B) guaranteed good precision, with high reproducibility of the results. The confidence limits of the analytical method of simultaneous determination of D and of its 4-OHD in urine are listed in Table 1.

Since the micromethod for the determination of D and 4-OHD by liquid chromatography proved to be selective, sensitive and precise, we validated it by the debrisoquine test applied to a healthy adult volunteer.

The exponential decay of D and 4-OHD forms in urine is illustrated in Figure 1C by plotting the fraction of dose administered and excreted into urine (Fel) versus time of midpoint (Tmp) (9-11). It is important to emphasize that D and 4-OHD were excreted at a 1:4 molar ratio during the 24-h period of investigation. The elimination rate constant for D in urine (Ku), formation rate constant for 4-OHD (Kf) and their respective half-lives (t(1/2)ß) were estimated by Fel versus Tmp, using a semilogarithmic plot, as follows: 0.133/h (Ku), 0.119/h (Kf) and 5.2 h (D), 5.8 h (4-OHD) for the biological half-lives.

On the basis of the data for accumulated

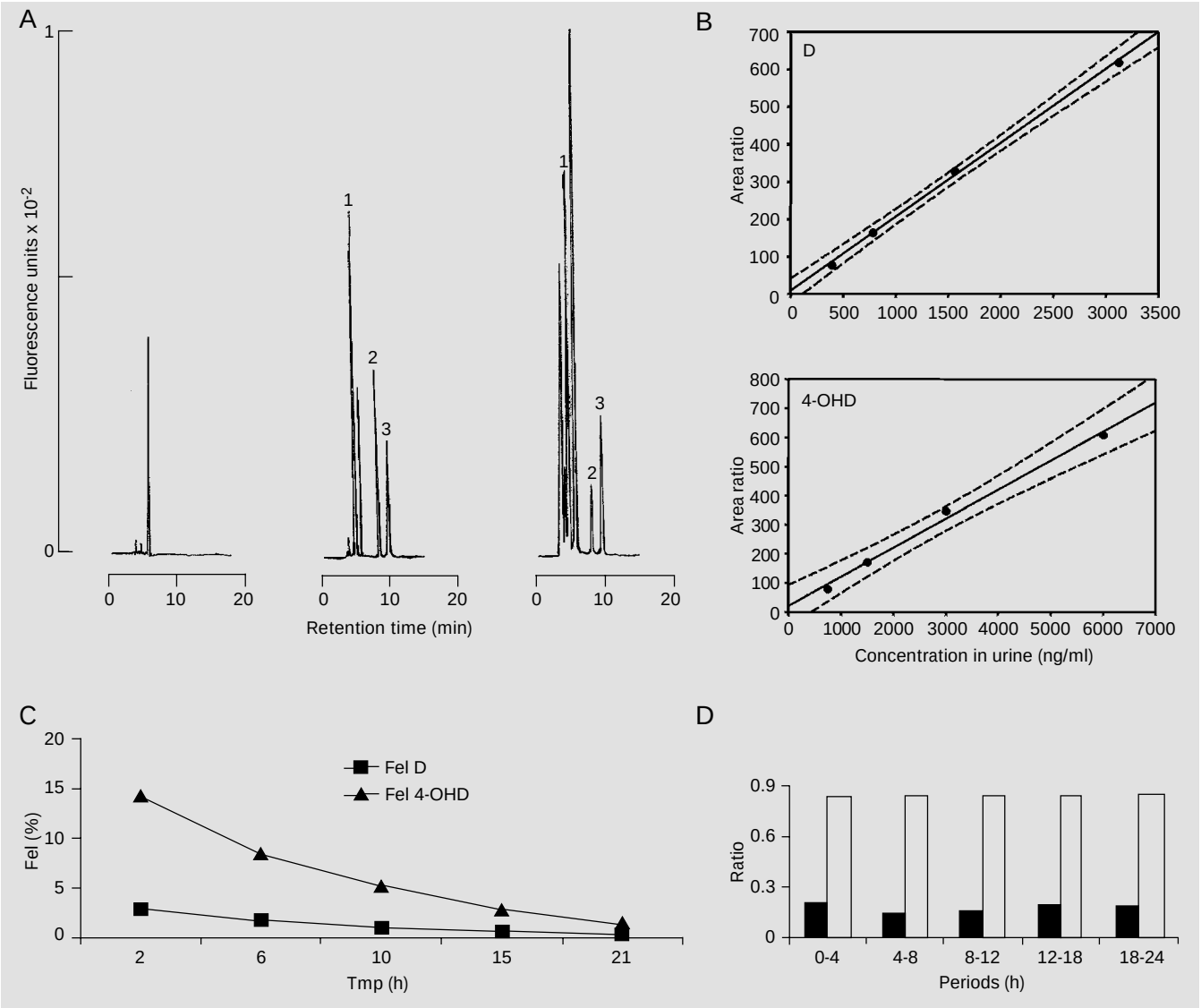


Figure 1 - *A*, Chromatographic pattern for the simultaneous determination of D and 4-OHD in urine by HPLC-F. Peaks: 1 - 4-OHD: 8.3 min, 300 ng; 2 - D: 14.0 min, 160 ng; 3 - internal standard (metoprolol): 16.6 min, 8 µg. *B*, Calibration curves for D (upper) and 4-OHD (lower) peak area ratio versus concentration in urine. *C*, Exponential curve decay. Urinary excretion of debrisoquine (Fel D, squares) and of 4-hydroxydebrisoquine (Fel 4-OHD, triangles) versus time of midpoint (Tmp) after administration of the CYP2D6 marker drug to a healthy Caucasian volunteer. *D*, Ability to hydroxylate debrisoquine up to 24 h: metabolic ratio (closed bars) and recovery ratio (open bars) of diuresis (4- or 6-h intervals) for the healthy Caucasian volunteer investigated.

fraction of dose administered and excreted into urine versus time, the following percentages were obtained for the D:4-OHD ratio: 2.9:14.2% (0-4 h), 4.6:22.6% (0-8 h), 5.6:27.8% (0-12 h), 6.2:30.6% (0-18 h), and 6.4:31.9% (0-24 h). It is interesting to observe that the total eliminated fraction (D + 4-OHD) ranged from 20 to 40% during the study period, as follows: 17.1% (0-4 h), 27.2% (0-8 h), 33.4% (0-12 h), 36.8% (0-18 h), and 38.3% (0-24 h).

The capacity for D hydroxylation measured on the basis of the recovery ratio (RR) that corresponds to the 4-OHD excretion in relation to the total eliminated (D + 4-OHD) yielded a value of 0.85 for the recovery ratio versus reference values of RR >0.12 and RR <0.12 described previously for extensive metabolizers (EM) and poor metabolizers, (PM), respectively, for the 0-8-h period (12). The patient studied is an EM since the value obtained was 0.85>0.12. It should also be pointed out that the ratios were constant at all intervals from zero to 24 h as illustrated in Figure 1D.

When the capacity for D hydroxylation was measured on the basis of the metabolic ratio (MR) which corresponds to the excretion of D in relation to the metabolite eliminated, 4-OHD (1), the ratio obtained for the subject investigated was 0.18 versus reference values of MR >12.5 for PM and MR <12.5 for EM (Figure 1D). Therefore, this calculation confirmed the result obtained previously, since this was an EM.

The debrisoquine test based on 24-h urine collection (4 to 6 h diuresis) designed for this study protocol indicates that MR and RR, indexes of hydroxylation capacity, remained unchanged throughout the investigation. These findings indicate that urine collection can be simplified by reducing it to 8 h (0-4, 4-8 h diuresis), allowing not only population phenotyping of hospitalized patients and outpatients, but also estimation of the kinetic parameters such as half-life and rate constants.

Table 1 - Confidence limits of the analytical method for simultaneous determination of debrisoquine and its hydroxylated metabolite by HPLC-F of urine.

(D:4-OHD) spiked blank of urine: low standard (390:750 ng/ml), high standard (3120:6000 ng/ml). CV: Coefficient of variation; r: coefficient of linear correlation.

| Parameter | Debrisoquine | 4-Hydroxydebrisoquine |
|---|---|---|
| Linearity (ng/ml) | 390-6240 | 750-12000 |
| (CV%) | (5.2%) | (7.1%) |
| Regression | r = 0.998 | r = 0.998 |
| Detection limit (ng/ml) | 3 | 6 |
| (CV%) | (7.9%) | (8.0%) |
| Regression | r = 0.903 | r = 0.954 |
| Quantification limit (ng/ml) | 12 | 23 |
| (CV%) | (7.7%) | (7.6%) |
| Regression | r = 0.993 | r = 0.992 |
| Recovery | | |
| absolute (%) | 85 ± 8 | 80 ± 7 |
| relative (%) | 83 ± 5 | 95 ± 4 |
| Systematic error (%) | 0.5 | 1.1 |
| Interday precision (%) | 8.2 | 8.2 |
| High standard (ng/ml) | 3260 ± 256 | 6027 ± 444 |
| (CV%) | (7.8%) | (7.4%) |
| Low standard (ng/ml) | 383 ± 33 | 745 ± 67 |
| (CV%) | (8.6%) | (8.9%) |
| Intraday precision (%) | 5.7 | 5.3 |
| High standard (ng/ml) | 3204 ± 177 | 6170 ± 352 |
| (CV%) | (5.5%) | (5.7%) |
| Low standard (ng/ml) | 391 ± 23 | 749 ± 36 |
| (CV%) | (5.9%) | (4.8%) |

On the basis of the confidence intervals obtained in the present study and their validation by the application of the debrisoquine test to a healthy Caucasian volunteer (explained in detail above), we consider the proposed micromethod for simultaneous analysis to be sufficient for application to routine population phenotyping as an index of the capacity for hydroxylation through the measurement of CYP2D6 activity. This methodology has permitted the application of this test in studies currently carried out on high-risk surgical patients, guaranteeing the dose adjustment and the success of pharmacological therapy. Finally, MR and RR proved to be good indexes of the hydroxylation capacity of the enzyme, CYP2D6 for population phenotyping purposes.

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