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Note

High-performance liquid chromatographic quantitation of 4-hydroxydebrisoquine in microsomal incubates by use of silica columns, aqueous mobile phase and automated column switching

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The antihypertensive drug debrisoquine (D) is metabolized to 4-hydroxydebrisoquine (4-OH-D) [1]. It has been shown that this oxidation is performed by a cytochrome P450 isozyme [2] and is under monogenic control [3]. The in vivo capacity for hydroxylation of D measured as the urinary ratio of D to 4-OH-D correlates with the oxidation of several other drugs [4–9]. In order to characterize the isozyme responsible for this oxidation, analytical methods for determination of the in vitro 4-hydroxylation of D by liver microsomes were developed. These methods were based on selective extraction [2] or high-performance liquid chromatographic (HPLC) separation [10] prior to derivatization and gas chromatography–electron-impact mass spectrometry (GC–MS) [2]. The aim of the present study was to develop a more rapid quantitative HPLC method. A column-switching system was used to eliminate extraction and derivatization procedures, making direct UV quantitation possible.

EXPERIMENTAL

Microsomal preparation

Human adult liver was obtained shortly after circulatory arrest from kidney transplant donors with total cerebral infarction, and microsomes were prepared [11]. The project was ethically approved by the Swedish Board of Health and Welfare.

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Chemicals

Debrisoquine sulphate and 4-hydroxydebrisoquine sulphate, obtained from Roche Products (Basel, Switzerland), were dissolved in glass-distilled water and stored at −20°C. Reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) tetrasodium salt, type 1, was from Sigma (St. Louis, MO, U.S.A.). Sodium octanesulphonate (für Tensiduntersuchungen) and acetonitrile (LiChrosolv) were obtained from E. Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade and used as received from the manufacturers. Glass-distilled water was used for the mobile phase.

Apparatus

The chromatographic system consisted of two Constametric pumps and a Spectromonitor III UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Two sample injection valves (Model 7020, Rheodyne, CA, U.S.A.) were used, one equipped with a valve position sensing switch (Model 7161-020) that signals a control unit for the operation of, via solenoid valves (Model 7163), a pneumatic actuator (Model 7001) on the second injector. The programmable control unit was constructed in our laboratory.

Microsomal incubation

Maximum velocity studies were performed with a substrate concentration of 1 m*M*. The incubation mixture contained 6 m*M* magnesium chloride, 50 m*M* Tris–HCl buffer (pH 7.4), 0.5 mg of microsomal protein and 1.2 m*M* NADPH in a final volume of 0.5 ml [2]. After preincubation at 37°C for 30 s the reaction was started by the addition of NADPH and performed at 37°C in air for 30 min. The reaction was stopped by the addition of 200 μl of ice-cold sodium phosphate buffer (pH 2.7, $I=0.1$) and thorough mixing for 10 s. The samples were heated at 95°C for 5 min and centrifuged for 10 min at 1750 *g*. Blank (without substrate or cofactor) and zero time (with inactivated microsomes) samples were analysed at each incubation experiment.

Chromatography

Chromatographic system. The chromatographic system is outlined in Fig. 1. For equilibration of the mobile phase, columns E_1 and E_2 (45×4.6 mm I.D.) were dry-packed with silica gel (LiChrospher, Merck, 20 μm) and inserted between the pumps and the injectors. Two concentration columns (C_1 and C_2) were placed in the injectors instead of the injection loop. These columns (27×2 mm I.D.) were slurry-packed (according to the manufacturer) manually with 5-μm styrene–divinylbenzene (PRP-1, Hamilton). The separation columns (S_1 and S_2, 150×4.6 mm I.D.) were 5-μm silica gel columns (Ultrasphere-Si, Beckman Instruments, Irvine, CA, U.S.A.).

The mobile phase consisted of 5 m*M* sodium octanesulphonate in sodium phosphate buffer (pH 2.7, $I=0.01$)–acetonitrile (88:12, v/v). The mobile phase was degassed by vacuum filtration (Millipore HF, 45 μm). The flow-rates used throughout the experiments were 0.7 ml/min for C_1–S_1 and 1.0 ml/min for C_2–S_2. The chromatographic system was operated at room temperature.

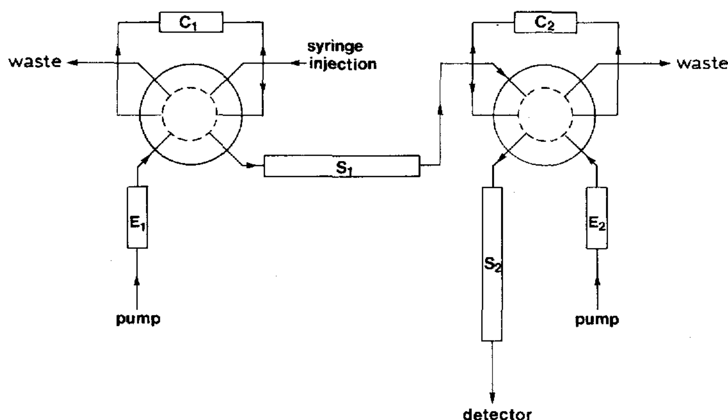

Fig. 1. Schematic drawing of the chromatographic system. E_1 and E_2 are silica gel equilibration columns, C_1 and C_2 are PRP-1 concentration columns, and S_1 and S_2 are silica gel separation columns. For further details see the text.

Procedure. The supernatant (100 μl) was injected onto the first concentration column (C_1) followed by 1 ml of water. The sample was introduced onto the preseparation column (S_1) by back-flushing C_1. Switching of the injection valve initiates the control unit to rotate the second valve at preprogrammed times. The eluate from S_1 was directed to waste except for the fraction containing 4-OH-D, which was directed onto the second concentration column (C_2) where 4-OH-D was retained for the preprogrammed time interval (100 s). Back-flushing of C_2 introduced 4-OH-D onto the second separation column (S_2), which was connected to the detector.

The retention time for 4-OH-D was established before each analysis by injection of reference substance without initiating the switching unit and with the detector connected to the outlet of the first column (S_1).

Peak heights were measured and concentrations were calculated from standard curves consisting of samples of composition identical with the incubation samples, spiked with known amounts of 4-OH-D (0.2–20 μM). The standards were processed in the same way as the incubates after inactivation of the microsomes before incubation.

RESULTS AND DISCUSSION

The column system was developed for on-line separation of D and microsomal disturbances from 4-OH-D. A concentration column was inserted between two silica separation columns to collect the fraction from the first separation column containing 4-OH-D. Concentration was obtained by retention of 4-OH-D as the octanesulphonate ion-pair on a reversed-phase column (PRP-1). A wide range of capacity factors could be obtained by varying the counter-ion and organic solvent concentrations. A different retention mechanism was obtained by using silica eluted with the same aqueous mobile phase [12, 13], and retention and separation could be adjusted by varying the content of organic modifier, although

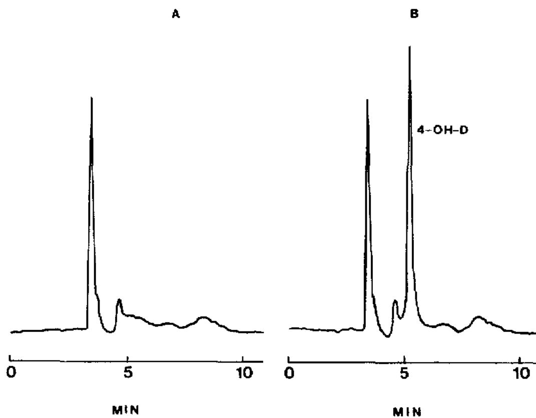

Fig. 2. Chromatograms of (A) a blank incubation sample and (B) an incubation sample of 1 m*M* debrisoquine with human liver microsomes processed as described in Experimental. 4-Hydroxydebrisoquine is eluted at 5 min.

the counter-ion concentration had no effect [13]. To further reduce interference from the crude sample and disturbance of the column equilibrium, a column (PRP-1) was inserted instead of injection loop to desalt the sample and to reduce the injected volume.

A chromatogram of an incubated sample on the column-switching system is shown in Fig. 2. Without this system 4-OH-D cannot be quantitated at concentrations present after the microsomal incubation of 1 m*M* D. 4-OH-D could be easily detected at a concentration of 200 n*M* when the switching system was used. Linear regression coefficients for the standard curves were 0.998 ± 0.001 (mean $\pm$ S.D.; $n=4$). The coefficient of variation for identical incubations was 7.8% (805 n*M* $\pm$ 63; mean $\pm$ S.D.; $n=10$). The method was successfully applied to measure 4-OH-D formed on incubation of D with human liver and rat brain microsomes [7, 14, 15].

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