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Note

Assay of debrisoquine and 4-hydroxydebrisoquine in urine by reversed-phase high-performance liquid chromatography using on-line sample clean-up on a standard isocratic chromatograph

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Inter-individual variability in the rate of oxidative drug metabolism has been noted for some time [1]. Mahgoub et al. [2] showed that poor metabolism of the anti-hypertensive agent, debrisoquine (D), was an inherited, autosomal, recessive trait dividing the population into extensive (fast) metabolizers and poor metabolizers for this type of oxidation. Since then, D has been used as a probe drug in the determination of individual hydroxylation ability. D undergoes P450 cytochrome-assisted oxidation to 4-hydroxydebrisoquine (OHD) and the hydroxylation status (metabolic rate, MR) of an individual is the ratio of the amount of D compared to the amount of OHD found in the total 0–8 h urine after dosing with D. In Caucasians ca. 8% of the population are poor oxidative metabolizers (PM) of D with MR > 12 [3], whilst in Japanese no poor metabolizers with MR > 12 are found [4].

To determine the metabolic rate [D]/[OHD], reliable assay methods for D and OHD are necessary. Gas chromatographic (GC) [5–7] and gas chromatographic–mass spectrometric (GC–MS) methods [8,9] have been developed for this assay, but involve extensive sample preparation and derivatization. Methods using high-performance liquid chromatography (HPLC) have recently appeared [10–14]. The method used by Róna et al. [10] requires extensive sample preparation followed by separation on an RP-8 column. Harrison et al. [11] simplify sample preparation to solid-phase extraction, collecting the final effluent (ca. 1 ml) and injecting 20 μl of it onto an RP-8 column. The simplest method is that of Westwood et al. [12], where filtered urine is directly injected onto an RP-8 column. We have found that a high background of UV absorption and co-eluting

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peaks occur when using this rapid and direct method, though this can be overcome by using fluorescence detection [13].

Recently Decolin et al. [14] have used a pre-column switching device for the on-line clean-up of directly injected urine. The method is very similar to the one we have been using to investigate the polymorphism of D oxidation in various African communities, but requires the use of a sophisticated chromatograph with an additional switching valve and two solvent delivery pumps. The method described below uses a standard isocratic liquid chromatograph, such as is found in even the most modest liquid chromatographic laboratory in the third world. Online solid-phase sample clean-up is used for a rapid, direct and precise method for the assay of D and OHD in urine.

EXPERIMENTAL

Reagents and standards

All solvents used were spectroscopic grade and all water was purified by the Milli-Q system (Millipore, Milford, MA, U.S.A.). All other reagents were analytical-reagent grade; 0.02 *M* diammonium phosphate (pH 7.6) was used as sample buffer.

D and OHD were donated by Hoffmann-La Roche (Basle, Switzerland). Practolol was donated by ICI South Africa. Practolol was found to be satisfactory as an internal standard in the elution and detection conditions used, 20 μg/ml practolol in sample buffer was used as the internal standard solution.

Sample collection

D (10 mg Declinax) was administered orally to each fasting subject and the total urine was collected for the 0–8 h period. Sodium metabisulphite (ca. 4 mg/ml) was added as a preservative to 20-ml aliquots of the measured 8-h collection before freezing the samples in liquid nitrogen for storage at $-18°C$ until assay.

Sample preparation

A 200-μl volume of urine was added to 100 μl sample buffer plus 100 μl internal standard solution (20 μg/ml practolol in sample buffer) and vortexed for 5 s, then 350 μl were injected onto the pre-column.

Preparation of standards

A 100-μl volume of standard in sample buffer (range 1–20 μg/ml) was added to 200 μl blank urine with 100 μl internal standard solution, vortexed and injected, as for the sample urines.

Chromatography

HPLC separation was performed on a Spectra Physics 8100 liquid chromatograph with a Valco loop valve. The sample loop was replaced by a self-packed 20 mm × 4.6 mm pre-column containing Supelco 30-μm LC-18 pellicular packing (Fig. 1). The analytical column was a 250 mm × 4.6 mm Spherisorb S5 nitrile, preceded by a 20 mm × 4.6 mm ODS guard column. Isocratic binary elution was

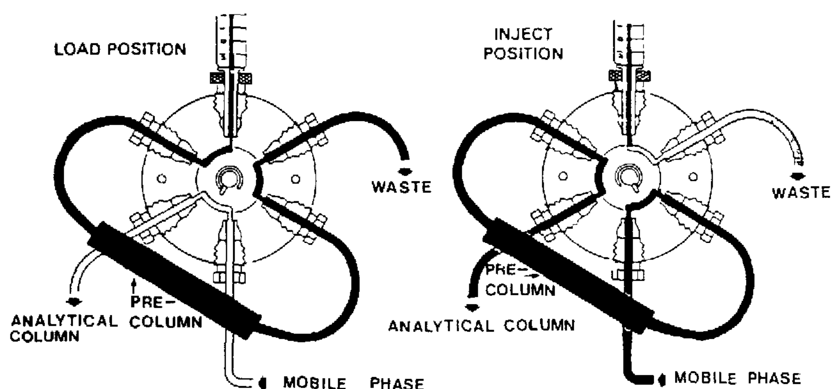

Fig. 1. Pre-column and injector valve arrangement

performed with a mobile phase of acetonitrile–0.1 *M* diammonium phosphate (pH 2.5) buffer (6:94). The flow-rate was 2 ml/min and the column temperature was 36°C. UV detection was performed at 208 nm and was recorded simultaneously on a strip-chart recorder and a Spectra Physics SP4200 integrator.

Analytical procedure

With the injector valve in the load position, 350 μl of prepared urine sample were injected onto the pre-column using a gas-tight syringe. This was immediately followed by the slow injection of 1 ml of 20% methanol in sample buffer over 30 s to flush the very polar components to waste. The valve was then turned to the inject position to back-wash the remaining materials onto the analytical column with mobile phase. After 3 min the injector valve was returned to the load position and the pre-column regenerated by flushing with 2 ml water, ready for the following sample to be loaded.

RESULTS

The high background found for blank samples after straight injection is shown in Fig. 2A. The arrows indicate the times at which the required analytes elute. Much of this polar tail is removed by the on-line clean-up procedure, Fig. 2B, which allows the analytes to be satisfactorily separated and detected as seen in Fig. 2C and D.

The amounts of D and OHD were estimated on the basis of peak-height ratios from the standard calibration curves produced during the assay procedures.

Using the above technique good separation and detectability of the analytes were obtained. The standard calibration curves were linear from 0.2 to 100 μg/ml and the inter-sample percentage standard deviation was 1.84 and 1.78% at 5 μg/ml and 2.64 and 4.58% at 0.5 μg/ml for D and OHD, respectively, over five samples. The on-column detectabilities were 50 and 20 ng for D and OHD, respectively. The pre-column was repacked after ca. 50 samples, i.e. daily, and the analytical column was still in good condition after ca. 500 assays.

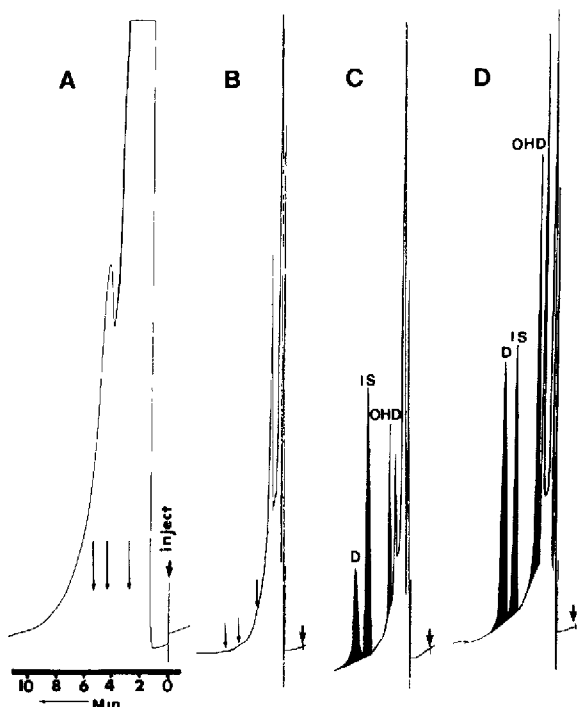

Fig. 2. Chromatograms of urine and standard samples. (A) Straight injection of 350 μl prepared blank urine; (B) 350 μl same blank urine after on-line clean-up, (C) 350 μl spiked 1 μg/ml standard in same blank urine after on-line clean-up; (D) 350 μl prepared 0–8 h urine after 10 mg debrisoquine administered orally and after on-line clean-up. Peaks: D = debrisoquine, IS = practolol, internal standard; OHD = 4-hydroxydebrisoquine.

DISCUSSION

The large differences in polarity between moderately lipophilic D and its hydrophilic metabolites indicated using a bonded-phase nitrile (cyanopropyl) column in the reversed-phase mode for the assay. This allowed the analytes to elute in a usable time span. The resolution and detectability were further improved by optimizing the pH, temperature and $\lambda_{detection}$.

Direct injection of urine resulted in a high background from polar urine components. Acid/base extraction to eliminate this background showed a high loss of metabolite. Previous experience [15] had shown that on-line clean-up of urine samples was easily achieved, so solid-phase clean-up of the samples was investigated. For compatibility with reversed-phase sample elution an ODS extraction column was used in the investigation. The successful conditions, using the mobile phase as final eluent, were then applied to on-line clean-up with the extraction column mounted at the loop position on a standard Valco injector valve (Fig. 1). Sample preparation prior to loading was thus reduced to simple addition of internal standard and dilution with buffer.

Because of slight variability in the volume of sample injected, the use of an internal standard gave improved assay reproducibility.

The above method for sample clean-up, with the analytical elution conditions used, eliminates the need for extensive sample preparation and allows resolution of D and OHD from interfering urine components. This results in highly reproducible assays of the analytes and an elution time of ca. 6 min.

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