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Determination of debrisoquine and 4-hydroxydebrisoquine in urine by high-performance liquid chromatography with fluorescence detection after solid-phase extraction

A. BOZKURT,*† N.E. BASCI,† A. ISIMER‡ and S.O. KAYAALP†

†*Department of Pharmacology, Faculty of Medicine, Hacettepe University, 06100 Ankara, Turkey*
‡*Gulhane Military Medical Academy, 06018 Ankara, Turkey*

Abstract: A simple, selective and sensitive method has been developed to determine debrisoquine and 4-hydroxydebrisoquine in human urine. Separation of the analytes was obtained using a mobile phase of 0.1 M sodium dihydrogen phosphate–acetonitrile (87:13, v/v) and a μBondapak C_{18} column. The column effluent was monitored with fluorescence detection at 210 nm (ex) and 290 nm (em). Rapid sample preparation was achieved by solid-phase extraction columns (Bond Elut CBA, 3 ml capacity) which provided excellent recovery values for both compounds. The cost per sample using this approach could be minimized by column regeneration and re-use. The within-day and the day-to-day reproducibilities were less than 7% for both components. The method was shown to be suitable for the study of the debrisoquine–sparteine type genetic polymorphism in man.

Keywords: *Debrisoquine; 4-hydroxydebrisoquine; high-performance liquid chromatography; solid-phase extraction.*

Introduction

Debrisoquine (DEB) is an antihypertensive drug which undergoes oxidative metabolism in man. Formation of the major metabolite, namely 4-hydroxydebrisoquine (4-OHDEB), displays genetic polymorphism [1]. By measuring the urinary ratio of DEB to its main 4-hydroxy metabolite (so called metabolic rate), two distinct phenotypes can be observed. Poor metabolizers are defined as subjects with metabolic rate of 12.6 and above [2]. The prevalence of poor metabolizer phenotype is reported to be between 3 and 10% of the white population [3, 4]. DEB has been used in many studies as a marker for oxidation polymorphism. Furthermore, poor metabolizer individuals also have impaired metabolism of over 20 other drugs including spartein, dextromethorpan, perhexilin, phenformin and certain tricyclic antidepressant and β-adrenergic blocking drugs [5].

Several analytical methods are currently available to measure DEB and its hydroxy metabolite. For this purpose, gas chromatography is widely used for simultaneous determination of debrisoquine and its main metabolite in human urine [6, 7]. Although this gas chromatographic method is highly sensitive and selective, it requires complicated and time-consuming sample preparation and derivatization. Recently, several liquid chromatographic methods have been developed [8–15], two of them again involved derivatization procedures similar to those used in gas chromatographic methods [8, 9]. In the liquid chromatographic techniques, extraction of DEB and its 4-hydroxy metabolite from urine using organic solvents are reported to be difficult, giving unsatisfactory low recoveries. However, Duche *et al.* [10] recently suggested that hexanol can be used for extraction of these compounds. On the other hand, Harrison *et al.* [11] obtained high and reproducible recoveries by using solid-phase extraction columns containing silica modified with carboxylic acid ion-exchange functional groups. Furthermore, Westwood *et al.* [12] were even able to obtain a good separation by direct injection of filtered urine onto a reversed-phase column.

Most of the published liquid chromatographic techniques for the analysis of DEB and its 4-hydroxy metabolite in human urine were carried out with UV detection and on a

* Author to whom correspondence should be addressed.

reversed-phase column. Recently, a direct injection technique with fluorescence detection was also described for reversed-phase liquid chromatography [13]. In our experience, a high level of background UV absorption and co-eluting peaks usually occur when using solid-phase or organic solvent extraction procedures as well as direct injection technique. This paper describes a high-performance liquid chromatographic method for the determination of DEB and 4-OHDEB in human urine, after solid-phase extraction. HPLC with fluorescence detection combines high sensitivity, specificity and rapidity for routine use.

Experimental

Chemicals and reagents

Debrisoquine sulphate and 4-hydroxydebrisoquine sulphate were kindly donated by Roche (Istanbul, Turkey). HPLC-grade acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). All other reagents were of analytical grade and purchased from E. Merck (Darmstadt, Germany). De-ionized water was used throughout. For solid-phase extraction, CBA Bond Elut extraction columns, Analytichem (Harbor City, CA, USA), were utilized.

Stock solutions containing 1 mg ml^{-1} DEB and 4-OHDEB were prepared in distilled water and stored at −20°C. Urine standard solutions containing 0.1, 0.5, 1, 2, 5, 10 and 20 μg ml^{-1} of both DEB and 4-OHDEB were prepared by appropriately diluting the stock solutions with centrifuged drug-free urine. The urine standards were extracted in the same way as the sample.

Chromatography

The chromatographic system consisted of a Waters (Model 6000 A) instrument equipped with a variable-wavelength ultraviolet (UV) detector (Model 481), a scanning fluorescence detector (Model 470), a pump (Model 510) and WISP (Model 710B). A stainless-steel column (300 mm × 4 mm i.d.) packed with μBondapak C_{18} (particle size 10 μ) and a guard column packed with Bondapak C_{18} (particle size 40 μ) were used.

The mobile phase consisted of 0.1 M sodium dihydrogen phosphate and 130 ml l^{-1} acetonitrile. The mobile phase was filtered before use under vacuum through a 0.45 μm cellulose nitrate filter. The mobile phase and the column were at room temperature (19–23°C). The mobile phase was pumped at a flow rate of 1.0 ml min^{-1} through the column. The UV detector was set at 210 nm and the fluorescence detector was set at 210 nm for excitation and 290 nm for emission. Signals were recorded and quantitated by a Waters Model 730 data module or a Waters Baseline 810 Chromatography Workstation with System Interphase Module (SIM). Under the chromatographic conditions used, 4-OHDEB and DEB had retention times of 5.4 and 13.2 min, respectively.

Extraction procedure

Extraction of compounds from urine was performed by the method of Harrison *et al.* [11]. DEB and 4-OHDEB were extracted from urine using Bond Elut columns (3 ml size) containing silica modified with carboxylic acid ion-exchange functional groups. The columns were placed in luer fittings at the top of the Sep-Pak Cartridge Rack (Waters) which has the capacity for eight columns. A vacuum of 40–50 mm Hg was applied to effect the various phases of the extraction. Prior to use, the columns were activated by washing with 1 ml of acetonitrile–0.1 M hydrochloric acid (40:60) followed by 1 ml of distilled water.

A 1 ml volume of urine samples or standard solutions was applied to an activated column. The column was then washed with 1 ml of distilled water, 1 ml of acetonitrile–distilled water (50:50), 1 ml of distilled water and 0.5 ml of 0.1 M hydrochloric acid. Finally debrisoquine and 4-hydroxydebrisoquine were eluted with 3 ml of acetonitrile–0.1 M hydrochloric acid (40:60). An aliquot (60 μl) of this extract was injected onto the HPLC column. All analyses were performed in duplicate.

Recovery

The recovery of DEB and 4-OHDEB was determined by comparison with a nonextracted standard solution containing the same concentration in distilled water. The recovery was expressed as determined from the mean of 10 replicates (mean ± SD).

In addition, the performance of a single column was tested. For this purpose a urine standard solution containing known concentration of DEB and 4-OHDEB was applied 20 times onto a single column and recovery of DEB and 4-OHDEB was determined after each extraction. During this procedure,

columns, after an application of a urine standard solution, were regenerated by washing with 1 ml of acetonitrile–0.1 M hydrochloric acid (40:60) followed by 1 ml of distilled water.

Collection of urine samples from humans

Eleven healthy volunteers (eight men and three women, age 20.6 ± 1.3 years, weight 69.3 ± 7.6 kg, height 175.6 ± 5.9 cm, mean ± SD) who had not received any medication during the previous 7 days participated in the study after signing up their informed consent. After overnight fast, DEB sulphate (Declinax tablet, 10 mg) was administered orally with 100–150 ml of water. No food was allowed until 1 h after dose. All urine was collected for the next 8 h and a 10 ml aliquot was stored at −20°C for subsequent analysis.

Results and Discussion

Figure 1(B) shows the simultaneously recorded UV and fluorescence chromatogram obtained after extraction of DEB and 4-OHDEB from a spiked urine sample. A high level of background UV absorption and many interfering peaks occurred when using a UV detector. However, with fluorescence detection, co-eluting peaks disappeared and elution of all components was achieved in 15 min. After this observation, fluorescence detection was used in the rest of the study.

The extraction efficiency was determined by comparing the peak area obtained when urine standard samples were subjected to the previously described extraction procedure and subsequently chromatographed with those obtained with injections of standard solutions. The mean ± SD recovery from urine was found to be 93.8 ± 4.2% for DEB at 2 μg ml^{-1} (n = 5) and 76.9 ± 9.4% for 4-OHDEB at 5 μg ml^{-1} (n = 5). The cost of solid-phase extraction columns may be reduced by repetitive use since regeneration of the columns can be effected easily. The effects on recovery of repetitive use of extraction columns following regeneration were assessed

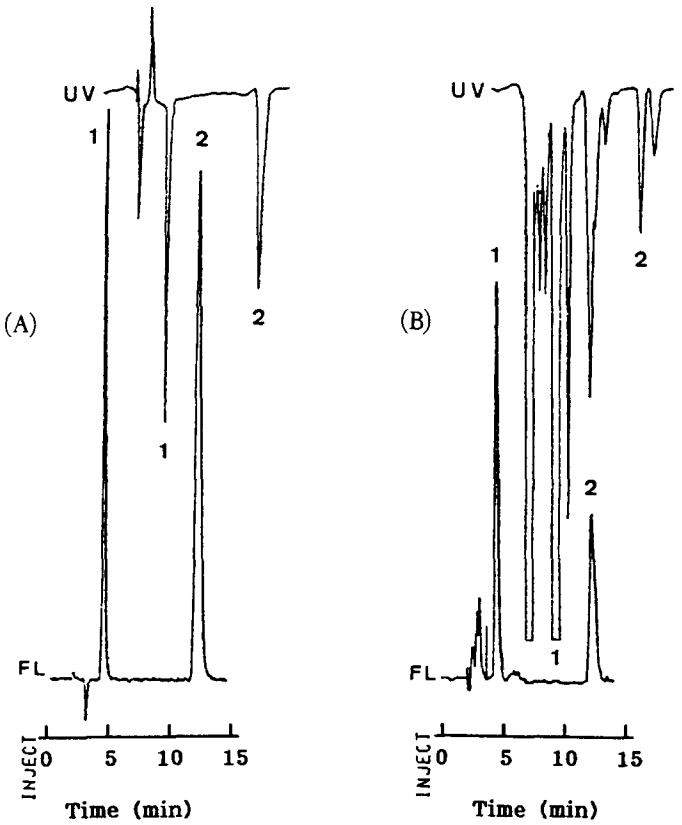

Figure 1
HPLC chromatograms of (A) a standard solution containing 5 μg ml^{-1} 4-OHDEB and 5 μg ml^{-1} DEB and (B) a urine extract spiked with 5 μg ml^{-1} 4-OHDEB and 2 μg ml^{-1} DEB, monitored at 210 nm for UV and at 210 nm (ex), 290 nm (em) for fluorescence (FL) detector. Extraction procedure and chromatographic conditions were as described in the text. Peaks: 1 = 4-OHDEB; 2 = DEB.

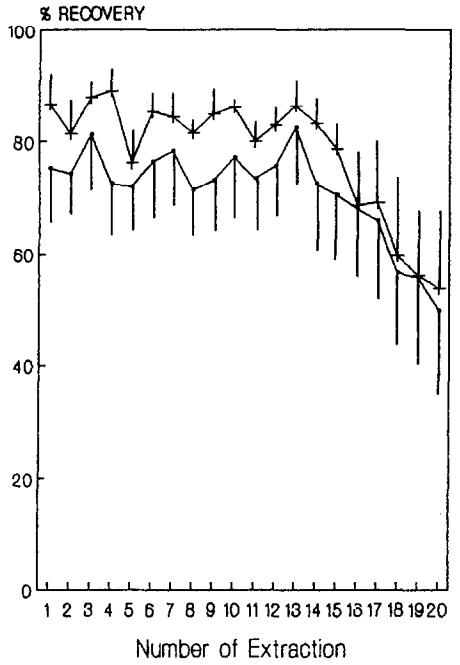

Figure 2
The effects of repetitive use of extraction columns (containing silica modified with carboxylic acid ion-exchange functional groups, Bond Elut) on the recovery of 4-OHDEB (●) and DEB (+) from human urine. Extraction procedure was as described in the text. After each extraction, a 60 μl aliquot was injected into HPLC column. Values are the mean ± SD of the three experiments.

by re-using the columns up to 20 times each. The results, presented in Fig. 2, are comparable adequately with those established following single extraction, although there is some decline in performance. It would appear, therefore, that the recovery and variability of the extraction are not seriously affected by repetitive use up to the maximum of 13 extraction tested.

The calibration curves of DEB ($y = 0.5800 + 4.3095x$) and 4-OHDEB ($y = 0.6979 + 1.9377x$) were linear over the concentration range of from 0.1 to 20 μg ml^{-1} for both DEB and 4-OHDEB. The mean correlation coefficients for DEB and 4-OHDEB obtained from six analytical runs were 0.9994 ± 0.0020 and 0.9984 ± 0.0030 (mean ± SD), respectively.

In order to assess the accuracy of the method, reproducibility for both day-to-day and within-day variations was determined by analysis of two replicate samples which were prepared in drug-free urine. These solutions were the mixture of DEB plus 4-OHDEB at two concentration levels. One of them contained 0.15 μg ml^{-1} of DEB plus 0.15 μg ml^{-1} of 4-OHDEB and the other 2 μg ml^{-1} of DEB plus 5 μg ml^{-1} of 4-OHDEB. One extraction column was used for each measurement. The results presented in Table 1 indicate that precision of the method is adequate for routine use since within-day and day-to-day relative standard deviations are less than 7% for both compounds. Storage of samples prior to analysis was at −20°C which has been found to maintain stability for approximately 3–6 months. The detection limits corresponding to a signal-to-noise ratio 2.5:1 were 25.0 ng ml^{-1} for DEB and 12.5 ng ml^{-1} for 4-OHDEB. Results suggest that the method described in this study is more sensitive than most of the published high-performance liquid chromatographic methods since HPLC with UV detection can measure DEB and 4-OHDEB concentrations of 100 and 200 ng ml^{-1}, respectively, in a 1.0 ml sample of urine [16].

Table 1
Precision and accuracy of measurement of DEB and 4-OHDEB in spiked urine samples

Compound	Concentration Given (μg ml^{-1})	Observed* (μg ml^{-1})	RSD† (%)
Within-day variation			
DEB	0.15	0.142 ± 0.003	2.5
	2.0	1.96 ± 0.03	1.42
4-OHDEB	0.15	0.133 ± 0.005	3.6
	5.0	4.89 ± 0.04	0.72
Day-to-day variation			
DEB	0.15	0.137 ± 0.007	4.8
	2.0	1.98 ± 0.06	2.86
4-OHDEB	0.15	0.125 ± 0.008	6.7
	5.0	4.96 ± 0.08	1.55

*Each value is given as the mean ± SD of five measurements.
†Relative standard deviation.

For the subjects examined, urinary concentrations of DEB and 4-OHDEB, urinary DEB/4-OHDEB ratios and urinary excretion rates of DEB plus 4-OHDEB as percentage of given doses in 8 h following oral administration of 10 mg of debrisoquine sulphate are listed in Table 2. The concentrations of DEB and 4-OHDEB were between 0.45 and 15 μg ml^{-1} and between 0.15 and 8 μg ml^{-1}, respectively, that indicates almost 30–55 fold difference between the lowest and the highest concentrations. The absolute concentration of DEB and 4-OHDEB in urine depends not only on the large inter-individual variations of the

Table 2
Urinary levels of DEB, 4-OHDEB, DEB/4-OHDEB ratios and urinary excretion rates of DEB + 4-OHDEB (as % given dose in 8 h) for 11 subjects following 10 mg oral dose of debrisoquine sulphate

Subjects	DEB ($\mu g\ ml^{-1}$)	4-OHDEB ($\mu g\ ml^{-1}$)	DEB/4-OHDEB ratio	% Dose excreted as DEB + 4-OHDEB
1	0.45	4.91	0.09	31.0
2	0.50	3.98	0.13	25.0
3	1.01	3.47	0.29	40.0
4	2.78	7.14	0.39	28.0
5	2.44	4.07	0.60	33.0
6	6.30	8.06	0.78	40.2
7	2.62	2.36	1.11	39.0
8	11.09	4.80	2.31	27.0
9	15.21	3.47	4.37	37.0
10	3.20	0.15	20.99	37.0
11	4.27	0.15	28.06	30.0

metabolism, even within a genetically defined population, but also on the urine produced within the collection period. However observed levels of the analytes in this study as well as in previous published studies are far above the lower detection limits of quantitation for DEB and 4-OHDEB. The metabolic ratios of DEB/4-OHDEB of the subjects were between 0.09 and 28.06. The separation point for poor and extensive hydroxylators is given as 12.6 in the literature [2]. According to this ratio two subjects are classified as poor hydroxylators (ratio >12.6). Urinary excretion rate of the drug (DEB + 4-OHDEB) is about 25–40% of the dose (Table 2). These values are in agreement with those previously reported after determination by gas chromatography [17, 18].

In conclusion a fast, sensitive and reliable method was described for the simultaneous measurement of DEB and 4-OHDEB in human urine and could be used for the study of the debrisoquine–sparteine type genetic polymorphism in man.

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