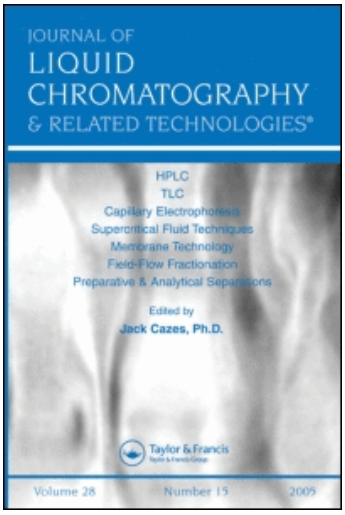

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DETERMINATION OF DEBRISOQUINE AND 4-HYDROXYDEBRISOQUINE IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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ABSTRACT:

A rapid, simple and selective method for the determination of debrisoquine and 4-hydroxydebrisoquine in human urine is described.

The sample preparation was done by direct injection of urine after dilution with water.

Separation and quantitation was performed by reversed phase high performance liquid chromatography with fluorescense detection.

The lower limits of quantitation for debrisoquine and 4-hydroxydebrisoquine were 0.25 $mg.L^{-1}$ using 200 μL of urine sample. The intra-day reproducibility was about 3.5 % or better for both components. The inter-day reproducibility was 4.5 % or better for both compounds.

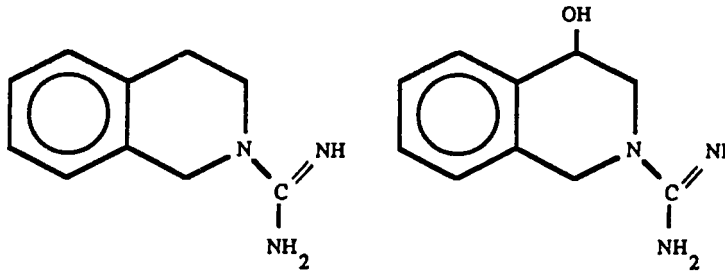
INTRODUCTION:

Debrisoquine, an antihypertensive agent, is being converted into a number of metabolites in man. The most important metabolite is the hydroxy metabolite 4-hydroxydebrisoquine (Fig.1). The ability to form 4-hydroxydebrisoquine varies greatly among individuals and is genetically determined. About 8 to 10 percent of the caucasian population can be classified as poor metabolizer with a metabolic ratio (= amount of parent drug divided by amount of metabolite) of 12.6 or higher. The metabolic ratio is generally determined by measurement of the amounts of debrisoquine and 4-hydroxydebrisoquine in urine which has been collected over a period of 8 hours after an oral dose of 10 mg of debrisoquine.

Many drugs like the tricyclic antidepressants nortriptyline, imipramine and desipramine, and the β-blocking drugs metoprolol, timolol and alprenolol seem to be metabolized through the same or a similar metabolism as debrisoquine [1]. The same dose of these drugs given to individuals results in disparate plasma concentrations. Persons who are poor metabolizers are at a higher risk for side effects or toxic effects of these drugs. Therefore it can be important to use debrisoquine as a marker to determine the phenotype of individuals with respect to aromatic hydroxylation.

For the determination of debrisoquine and 4-hydroxydebrisoquine in urine several methods have been developed. These methods use either high performance liquid chromatography [2,3,4,5] or gas - chromatography [6,7,8]. The gas chromatographic methods involve laborious sample preparations with time consuming extractions and derivatizations. One of the liquid chromatographic methods [2] describes a direct injection technique with UV detection at 209 nm. This method could be ideal for the fast screening of urine samples, but it has the disadvantage that it is sensitive to interferences e.g. due to bacterial growth in the samples.

In this paper a method is described that also uses a direct injection technique. However,because of a more specific fluorescence detection method has been used, this method is less sensitive to interferences.



Debrisoquine 4-hydroxydebrisoquine

Figure 1 Structural formula of debrisoquine and 4-hydroxydebrisoquine.

MATERIALS AND METHODS :

Chemicals and reagents

Debrisoquine and 4-hydroxydebrisoquine were kindly donated by Hoffmann-La Roche (Hoffmann-La Roche & Co Ltd, Basel, Switzerland). Acetonitrile was of HPLC-grade (Bio-Lab, Jerusalem 91160, Israel). Triethylamine and phosphoric acid were of analytical grade (Merck, D-6100 Darmstadt, F.R.G.).
Water was purified by a Milli-Q system (Millipore Corp.,Bedford, MA 01730, U.S.A.).

The mobile phase was prepared by adding 500 µL of triethylamine to about 800 mL of water. The pH was adjusted to 3.5 with phosphoric acid, 100 mL of acetonitrile was added and the volume was adjusted to 1000 mL with water. After mixing, this solution was filtered and degassed through a 0.45 µm HVLP filter (Millipore, Milford, MA 01757, USA).

Stock solutions of debrisoquine and 4-hydroxydebrisoquine were prepared by dissolving 64.0 mg of debrisoquine sulfate and 62.8 mg of 4-hydroxydebrisoquine sulfate in 50.0 ml of water, which was acidified with 100 µL of 5 N HCl. Stock solutions were stored at 4 °C. Calibration standards were made by properly diluting stock solutions with drug free urine and stored at - 20 °C.

Instruments and instrumental conditions

The assay was performed on a liquid chromatograph consisting of the following components :

A Waters M 590 solvent delivery system (Waters Assoc., Milford, MA 01757, U.S.A.) used at a flow rate of 0.6 $mL.min^{-1}$.

A Perkin Elmer LS-4 spectrofluorescense detector (Perkin Elmer Ltd., Beaconsfield, Bucks HP9 1QA, England). The excitation wavelength was 220 nm, the emission wavelength was 562 nm.

The 100 x 4.6 mm ID cartridge glass column was packed with Chromsphere CN 5 μm (Chrompack,4330 EA Middelburg, The Netherlands).

Injections were made by means of a Waters Wisp Model 712 (Waters, see above). Data analysis was performed with a Spectra Physics SP 4270 computing integrator (Spectra Physics, San Jose, CA 95134, U.S.A.), by measurement of peak heights.

Sample preparation

An aliquot of 200 μL of the sample to be analysed was pipetted into a 1 mL autosampler vial and 500 μL of water was added. The vial was capped and mixed thoroughly by vortexing. Injections of 20 μL were made.

RESULTS AND DISCUSSION :

Selectivity :

Fig. 2 shows chromatograms obtained after the analysis of (A) drug-free urine sample and (B) an urine sample spiked with known amounts of debrisoquine and 4-hydroxydebrisoquine. No interfering peaks were observed at the retention times of the components of interest.

Sensitivity :

The lower limits of quantitation were 0.25 $mg.L^{-1}$, the detection limits (defined as three times the baseline noise) were about 0.10 $mg.L^{-1}$ for both compounds.

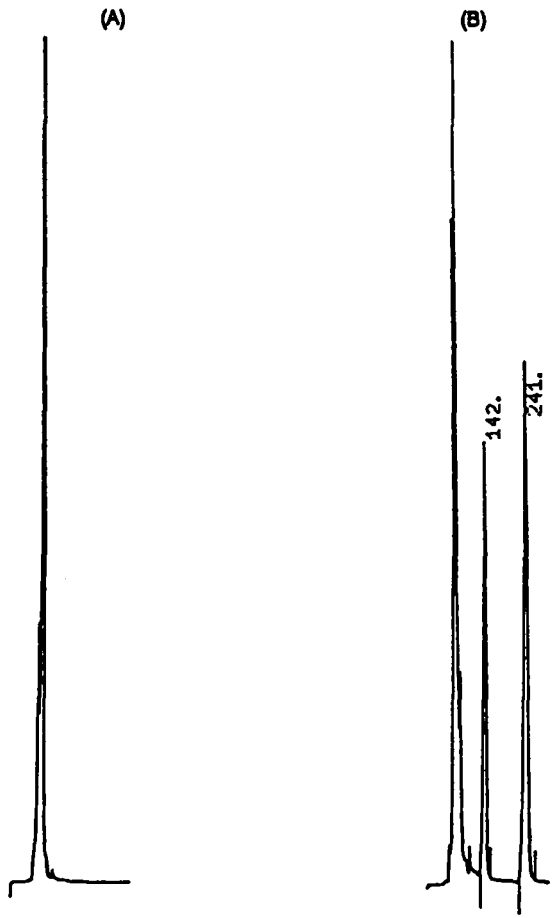


Figure 2 Chromatograms obtained after the analysis of (A) a drug-free urine sample and (B) an urine sample spiked with debrisoquine and 4-hydroxydebrisoquine.

Retention times	:	142 s	: 4-hydroxydebrisoquine.
	:	241 s	: debrisoquine.
Concentrations	:	10.0 $mg.L^{-1}$	: 4-hydroxydebrisoquine.
	:	10.0 $mg.L^{-1}$	: debrisoquine.

TABLE 1 : Intra-day reproducibility

concentration: added ($mg.L^{-1}$)	observed concentration : ($mg.L^{-1}$) mean:	s.d.:	c.v.: (%)	bias: (%)	n:
debrisoquine					
0.25	0.25	0.009	3.47	+ 0.0	7
0.50	0.51	0.005	0.92	+ 2.6	7
1.00	1.00	0.005	0.53	- 0.2	7
2.50	2.48	0.063	2.53	- 0.2	7
5.00	5.03	0.073	1.45	+ 0.5	7
10.0	10.1	0.103	1.02	+ 0.6	7
15.0	15.3	0.347	2.27	+ 2.1	7
20.0	20.0	0.233	1.17	- 0.3	7
4-hydroxydebrisoquine:					
0.25	0.25	0.009	3.43	+ 1.4	7
0.50	0.51	0.009	1.85	- 1.6	7
1.00	0.99	0.009	0.88	- 1.5	7
2.50	2.50	0.041	1.63	- 0.1	7
5.00	5.06	0.093	1.84	+ 1.2	7
10.0	10.0	0.158	1.58	- 0.3	7
15.0	15.5	0.249	1.61	+ 3.4	7
20.0	20.0	0.367	1.83	- 0.1	7

Linearity :

The calibration curves (see Fig.4) were calculated using weighted linear regression (W=1/Y). For both compounds the linearity of the method was significant ($p<0.05$) over the calibration range of 0.25 to 20.0 $mg.L^{-1}$. In general, correlation coefficients of 0.9990 or higher were found.

Intra-day reproducibility :

The intra-day reproducibility of the method was determined by replicate analyses (n=7) of drug-free urine spiked with known concentrations of debrisoquine and 4-hydroxydebrisoquine (see Table 1).

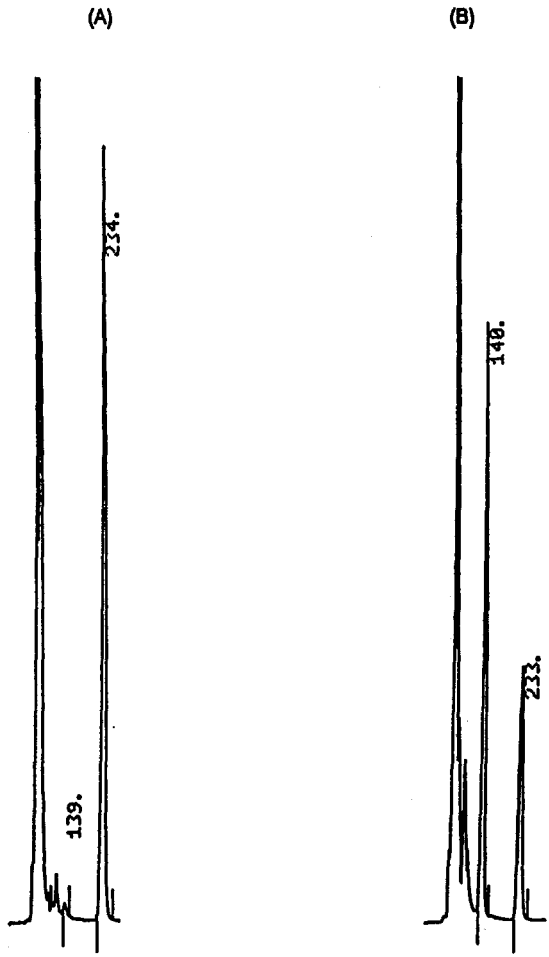


Figure 3 Chromatogram obtained after the analysis of :
(A) - a urine sample of a poor metabolizer, Metabolic Ratio = 52
(B) - a urine sample of a extensive metabolizer, Metabolic Ratio

Retention times : 140 s : 4-hydroxydebrisoquine.
: 233 s : debrisoquine.

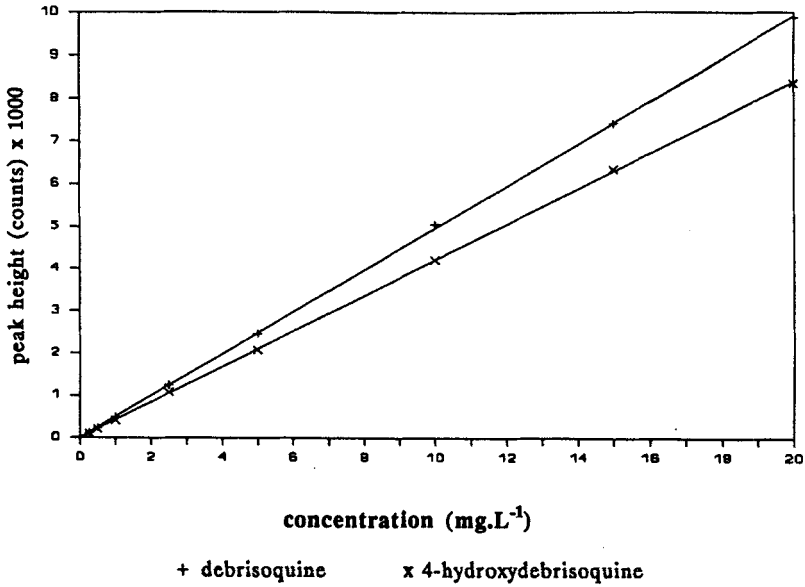


Figure 4 : Example of a calibration curve of debrisoquine and 4-hydroxydebrisoquine

Inter-day reproducibility :

Inter-day reproducibility was determined from the calibration data over a period of seven subsequent days of analysis. The concentrations of the calibration samples were determined by means of peak response on the calibration curve of that specific day. These data (see Table 2) show the good precision and accuracy of the method. The precision of the method is for both compounds 4.5 % or better and the bias is less than 3.5 % for all concentrations of both compounds.

Application of the method :

The method was used to phenotype individuals with respect to aromatic hydroxylation. Chromatograms obtained after the analysis of urine sample of (A) a poor metabolizer and (B) an extensive metabolizer after ingestion of 10 mg of debrisoquine are shown in Fig.3.

TABLE 2 : Inter-day reproducibility

concentration: added ($mg.L^{-1}$)	observed concentration : ($mg.L^{-1}$)				
	mean:	s.d.:	c.v.: (%)	bias: (%)	n:
debrisoquine					
0.25	0.26	0.009	3.64	+ 2.8	7
0.50	0.50	0.011	2.25	+ 0.1	7
1.00	0.99	0.013	1.32	- 1.4	7
2.50	2.49	0.061	2.43	- 0.2	7
5.00	4.89	0.093	1.90	- 2.1	7
10.0	10.2	0.298	2.93	+ 1.5	7
15.0	14.9	0.129	2.86	- 0.5	7
20.0	20.1	0.265	1.32	+ 0.4	7
4-hydroxydebrisoquine:					
0.25	0.26	0.012	4.46	+ 3.5	7
0.50	0.50	0.008	1.66	- 0.1	7
1.00	0.99	0.014	1.41	- 1.4	7
2.50	2.50	0.050	2.01	+ 0.1	7
5.00	4.90	0.096	1.95	- 2.1	7
10.0	10.2	0.188	1.89	- 0.3	7
15.0	15.1	0.170	1.12	+ 0.5	7
20.0	20.1	0.186	0.92	+ 0.4	7

CONCLUSIONS :

A very simple, rapid and accurate method for the simultaneous determination of debrisoquine and 4-hydroxydebrisoquine in human urine is described. The method has shown to be very suitable for metabolic phenotyping procedures.

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