ELSEVIER

Journal of Chromatography B, 677 (1996) 178–182

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Improved high-performance liquid chromatographic determination of debrisoquine and 4-hydroxydebrisoquine in human urine following direct injection

Reginald F. Frye*, Robert A. Branch

Department of Pharmacy and Therapeutics, 726 Salk Hall, School of Pharmacy and Center for Clinical Pharmacology, University of Pittsburgh Medical Center, University of Pittsburgh, Pittsburgh, PA 15261, USA

Received 30 May 1995; revised 6 September 1995; accepted 6 September 1995

Abstract

A sensitive and specific reversed-phase high-performance liquid chromatographic assay was developed for the determination of debrisoquine and 4-hydroxydebrisoquine in urine. The urine samples were directly injected following an ether clean-up step which eliminated interference. Separation of the analytes was achieved using a mobile phase consisting of acetonitrile–methanol–0.02 *M* heptane sulfonic acid (pH 3.0) (6:37:57) and a μBondapak C_{18} analytical column. The assay utilizes fluorescence detection at 208 nm (ex) and 562 (em). The within-day and between-day coefficients of variation were $\leq$10% for both components and accuracy was within 12%. The method is suitable for pharmacogenetic studies utilizing debrisoquine.

Keywords: Debrisoquine; 4-Hydroxydebrisoquine; Cytochrome P450

1. Introduction

Debrisoquine (DEB) is an antihypertensive agent that is metabolized to its primary metabolite, 4-hydroxydebrisoquine (HDEB), by the cytochrome P450 isozyme 2D6 (CYP2D6, Fig. 1). In addition to benzylic hydroxylation, DEB also undergoes phenolic hydroxylation to yield 5-, 6-, 7-, and 8-hydroxydebrisoquine metabolites. These phenolic metabolites are relatively minor and are also thought to be produced by CYP2D6 [1]. A number of mutations in the CYP2D6 gene result in a genetically determined polymorphism of enzyme activity in up to 10% of caucasian individuals but less than 1% of asian individuals [2,3]. Subjects with a mutation lack a functional copy of this isozyme and thus are unable to metabolize CYP2D6 substrates. DEB is commonly used as an in vivo probe of CYP2D6 activity and can be used to phenotype individuals as either extensive (EM) or poor metabolizers (PM). The

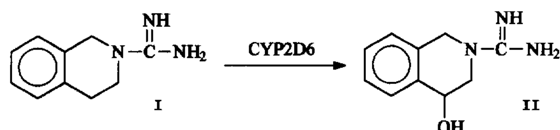

Fig. 1. The metabolic conversion of debrisoquine (I) to its primary metabolite 4-hydroxydebrisoquine (II) by CYP2D6.

*Corresponding author.

0378-4347/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(95)00380-0

estimation of in vivo CYP2D6 activity is achieved by calculation of either the DEB recovery ratio [DBRR=HDEB/(HDEB+DEB)] or the DEB metabolic ratio [DMR=DEB/HDEB] in a 6–8 h urine collection following oral administration of DEB. The DMR is frequently used as an index of CYP2D6 activity but has the disadvantage that this measure is not linearly related to activity, while the DBRR is proportional to CYP2D6 activity and more closely relates to the formation clearance of HDEB [4]. Individuals with a DBRR less than 0.12 or DMR greater than 12.5 are considered PM [4,5].

Several methods for the determination of DEB and HDEB have been reported. Initial investigations used GC or GC–MS methods but these methods involve extensive sample preparation including derivatization [6–8]. More recently, HPLC methods utilizing UV absorption have been reported [9–12], but these methods lack sufficient sensitivity and specificity for routine use and some also involve derivatization with acetylacetone [13–16]. Currently, direct injection HPLC methods utilizing fluorescence detection appear to be the preferred method of analysis [17,18]. These methods offer comparable sensitivity to GC methods and the use of direct injection minimizes sample preparation. However, our early experience with these methods demonstrated significant background interferences, especially in the diverse patient populations under study at our institution. Bozkurt et al. [19] experienced similar difficulties with direct injection methods and reported a modified urine sample preparation method that utilized solid-phase extraction. We have developed a method that uses a liquid extraction clean-up step prior to direct injection, thus eliminating the expense of the solid-phase extraction method while still providing a sensitive, specific and reliable method for the determination of DEB and HDEB in human urine.

2. Experimental

2.1. Reagents and chemicals

DEB and HDEB were provided by Roche (Basel, Switzerland). Acetonitrile, ethyl ether and methanol were of HPLC grade and obtained from Baxter Scientific (McGaw Park, IL, USA). All water used in the analysis was obtained from a four-bowl Milli-Q reagent water system (Millipore, Bedford, MA, USA). Heptane sulfonic acid, hydrochloric acid, and orthophosphoric acid were of analytical grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). Polypropylene tubes (8 ml) were obtained from Sarstedt (Newton, NC, USA).

2.2. Equipment/instrumentation

The HPLC system consisted of a Waters Model 501 solvent delivery pump, Model 712 Wisp automatic sample processor, and a Model 470 scanning fluorescence detector which was set at 208 and 562 nm for excitation and emission, respectively. Signal output was captured with Waters Maxima 820 chromatography workstation (Waters Corporation, Milford, MA, USA). Separation of DEB and HDEB was achieved with an Alltech Assoc. (Deerfield, IL, USA) direct-connect guard column (20 × 2 mm I.D.) filled with pellicular C_{18} packing connected to a Waters 300 × 3.9 mm I.D. μBondapak 10-μm C_{18} analytical column. The mobile phase used for the analysis consisted of acetonitrile–methanol–0.02 *M* heptane sulfonic acid (pH 3.0) (6:37:57) at a flow-rate of 1 ml/min (80 bar). The mixture was degassed and filtered through a 0.22-μm Nylon 66 membrane before use. Sample preparation and analysis were performed at ambient temperature.

2.3. Preparation of stock solution and spiked urines

Stock solutions containing 0.1 and 1.0 mg/ml DEB and HDEB were made in distilled water and stored at 4°C. These solutions were stable for at least 1 year when stored at 4°C. Standards and quality control samples were made by dilution of the stock solutions with drug free urine and stored in 400 μl aliquots at −20°C. Urinary standards and control samples frozen in aliquots were stable for at least 6 months.

2.4. Preparation of urine samples

To 400 μl urine [spiked standards, quality control (QC) samples, patient samples] were added 20 μl of 1.0 *M* hydrochloric acid. The samples were mixed

briefly on a vortex-mixer and then 5 ml ethyl ether was added. The samples were vortex-mixed for 2 min and then centrifuged at 2000 *g* for 10 min. The organic layer was aspirated off and discarded. The samples were placed under a stream of nitrogen to evaporate any residual ether. An aliquot of 200 μl of the sample was transferred to Wisp microinserts and 25-μl aliquots were injected onto the HPLC system.

2.5. Calibration and linearity

Calibration curves using ten different concentrations of DEB and HDEB in urine were obtained daily for three days by calculating the peak height of these compounds versus the respective concentrations of DEB and HDEB. The concentrations of standards evaluated for both HDEB and DEB were 50, 100, 250, 500, 750, 1000, 2500, 5000, 7500, and 10 000 ng/ml. All standards were run as duplicates.

2.6. Precision and accuracy

The precision and accuracy of the assay was determined through the analysis of QC urine samples spiked with both HDEB and DEB at concentrations of 200, 2000, and 8500 ng/ml. Four QC samples at each concentration were analyzed for three days and the intra- and inter-day means, standard deviations and coefficients of variation were calculated.

3. Results and discussion

Representative chromatograms of a spiked urine standard, a PM patient sample and an EM patient sample from an 8-h urine collection following an oral 10-mg DEB dose are shown in Fig. 2. The retention times of HDEB and DEB were 10.6 and 19.5 min, respectively. Calibration curves for DEB and HDEB were generated by weighted ($1/y^2$) linear regression analysis. Linear calibration curves were obtained for both compounds over the concentration range 50–10 000 ng/ml with correlation coefficients (*r*) greater than 0.999. The lower limit of detection for HDEB and DEB, at a signal-to-noise ratio of 5:1, was approximately 20 ng/ml and 30 ng/ml, respectively. The lower limit of quantitation was 50 ng/ml for both HDEB and DEB based on intra-day and inter-day C.V. of less than 15%. The intra-day C.V. at each of three control concentrations of HDEB and

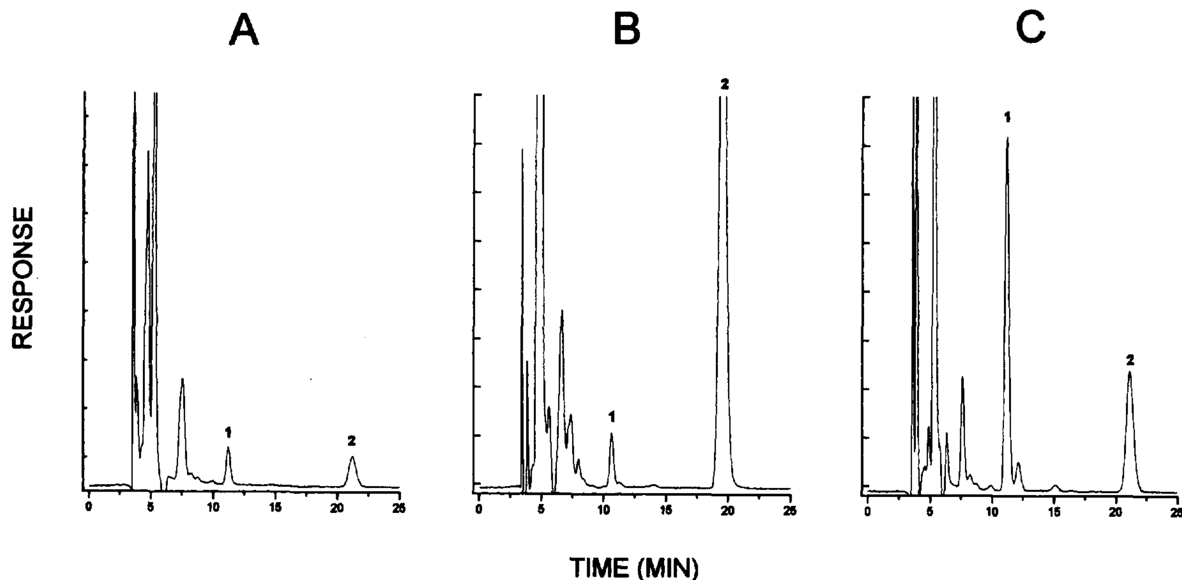

Fig. 2. HPLC chromatograms of (A) urine spiked with 500 ng/ml HDEB (1) and DEB (2); (B) urine sample of a poor metabolizer; and (C) urine sample of an extensive metabolizer. Concentrations of the samples were 10 098 ng/ml DEB and 741 ng/ml HDEB in the poor metabolizer (DBRR=0.07) and 1963 ng/ml DEB and 4936 ng/ml HDEB in the extensive metabolizer (DBRR=0.72).

Table 1
Intra- and inter-day precision and accuracy for debrisoquine (DEB) and 4-hydroxydebrisoquine (HDEB) in urine

Compound	Concentration (ng/ml)		C.V. (%)	Deviation (%)
	Added	Found (mean ± S.D.)		
Intra-assay reproducibility[a]				
DEB	200	178.9±3.4	1.9	−10.6
	2000	1770.0±29.9	1.7	−11.5
	8500	7469.9±96.2	1.3	−12.1
HDEB	200	175.0±2.3	1.3	−12.5
	2000	1832.4±33.3	1.8	−8.4
	8500	7598.3±99.0	1.3	−10.6
Inter-assay reproducibility[b]				
DEB	200	184.4±6.3	3.4	−7.8
	2000	2035.8±204.6	10.1	1.8
	8500	8483.4±794.0	9.4	−0.2
HDEB	200	179.8±14.2	7.9	−10.1
	2000	2067.3±180.3	8.7	3.4
	8500	8595.6±766.1	8.9	1.1

[a] Four samples per concentration.
[b] Four samples per day per concentration for three days.

DEB was <2% and the inter-day C.V. at each concentration was ≤10% (Table 1).

It is interesting to note the additional peak observed adjacent to HDEB. This peak was not present in any predose samples and was present in all EM patient samples. It is possible that this peak is 7-hydroxydebrisoquine, a minor DEB metabolite. This peak was only separated under the current HPLC conditions at pH 3.0; previously reported methods do not appear to separate this metabolite from HDEB. The formation of this metabolite has been shown to correlate with HDEB formation suggesting it is also formed by CYP2D6 [1,20]. This peak was present in only a minority of PM samples which is consistent with previous reports for 7-hydroxydebrisoquine [20].

The method presented here is currently being used to support several investigations of drug and disease state effects on CYP450 activity. We are using DEB in addition to caffeine, chlorzoxazone, dapsone, and mephenytoin as simultaneously administered in vivo probes of the CYP isozymes 2D6, 1A2, 2E1, 3A4, and 2C19, respectively. No analytical interferences were found from the co-administered drugs or their metabolites and no interferences have been observed in urine samples from different patient populations, including normal volunteers, patients with cancer, liver and renal disease, and transplant patients. A representative listing of concurrent medications from our study populations is included in Table 2.

Table 2
Alphabetical listing of concurrent medications in patient populations evaluated

Acetaminophen	Glipizide	Prednisone
Alprazolam	Glyburide	Ranitidine
Diazepam	Ibuprofen	Spironolactone
Diltiazem	Isosorbide	Temazepam
Enalapril	Lisinopril	Theophylline
Flurazepam	Nifedipine	*trans*-Retinoic acid
Furosemide	Phenytoin	Triamterene

In conclusion, we have presented a reliable method for the determination of DEB and HDEB in human urine. The method described employs a clean-up step and is devoid of analytical interferences seen with previously published direct injection methods without sample clean-up. This method is suitable for studies of CYP2D6 activity utilizing DEB.

Acknowledgments

This work was supported in part by USPHS NIH grant CA 59834.

References

[1] N.M. Woolhouse, B. Andoh, A. Mahgoub, T.P. Sloan, J.R. Idle and R.L. Smith, Clin. Pharmacol. Ther., 26 (1979) 584.
[2] M. Eichelbaum and A.S. Gross, Pharmacol. Ther., (1988) 243.
[3] L. Bertilsson, Y.Q. Lou, Y.L. Du, Y. Liu, T.Y. Kuang, X.M. Liao, K.Y. Wang, J. Reviriego, L. Iselius and F. Sjoqvist, Clin. Pharmacol. Ther., 51 (1992) 388.
[4] A. Kaisary, P. Smith, E. Jacqz, C.B. McAllister, G.R. Wilkinson, W.A. Ray and R.A. Branch, Cancer Res., 47 (1987) 5488.
[5] D.A.P. Evans, A. Mahgoub, T.P. Sloan, J.R. Idle and R.L. Smith, J. Med. Genet., 17 (1980) 102.
[6] M.S. Lennard, J.H. Silas, A.J. Smith and G.T. Tucker, J. Chromatogr., 133 (1977) 161.
[7] J.G. Allen, P.B. East and J.L. Haigh, Drug. Metab. Dispos., 3 (1975) 332.
[8] P. Erdtmansky and T.J. Goehl, Anal. Chem., 47 (1975) 750.

[9] J. Moncrieff, J. Chromatogr., 428 (1988) 178.
[10] D. Decolin, A. Nicolas, N. Motassim and G. Siest, J. Chromatogr., 419 (1987) 469.
[11] B.E. Westwood, P.J. Harman and M.L. Mashford, J. Chromatogr., 374 (1986) 200.
[12] P.M. Harrison, A.M. Tonkin, S.T. Dixon and A.J. McLean, J. Chromatogr., 374 (1986) 204.
[13] K. Rona, L. Vereczkey and B. Gachalyi, J. Chromatogr., 378 (1986) 509.
[14] C.O. Meese, P. Thalheimer and M. Eichelbaum, J. Chromatogr., 423 (1987) 344.
[15] K. Chan, J. Chromatogr., 425 (1988) 311.
[16] J. Kallio, Int. J. Clin. Pharmacol. Ther. Toxicol., 28 (1990) 223.
[17] J. Hempenius, B. Staal, H.J. Jeuring, C.K. Mensink and J.H.G. Jonkman, J. Liq. Chromatogr., 13 (1990) 1627.
[18] S.D. Fox, G.L. Shaw, N.E. Caporaso, S.E. Welsh, D.W. Mellini, R. Falk and H.J. Issaq, J. Liq. Chromatogr., 16 (1993) 1315.
[19] A. Bozkurt, N.E. Basci, A. Isimer and S.O. Kayaalp, J. Pharm. Biomed. Anal., 11 (1993) 745.
[20] L. Bertilsson, C.O. Meese, Q.Y. Yue, M.L. Dahl, M. Ingelman-Sundberg, I. Johansson, J. Sawe and M. Eichelbaum, Pharmacogenetics, 3 (1993) 94.