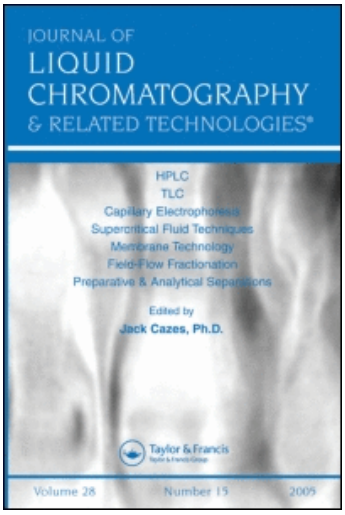

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

The Determination of Debrisoquine and its 4-Hydroxy Metabolite in Urine by Capillary Gas Chromatography and High Performance Liquid Chromatography

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To cite this Article Fox, S. D. , Shaw, G. L. , Caporaso, N. E. , Welsh, S. E. , Mellini, D. W. , Falk, R. and Issaq, H. J.(1993) 'The Determination of Debrisoquine and its 4-Hydroxy Metabolite in Urine by Capillary Gas Chromatography and High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 16: 6, 1315 — 1327
To link to this Article: DOI: 10.1080/10826079308020955
URL: http://dx.doi.org/10.1080/10826079308020955

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(6), 1315-1327 (1993)

THE DETERMINATION OF DEBRISOQUINE AND ITS 4-HYDROXY METABOLITE IN URINE BY CAPILLARY GAS CHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A comparative evaluation of capillary gas chromatography and high performance liquid chromatography for the determination of debrisoquine (D) and 4-hydroxydebrisoquine (4HOD) in human urine was undertaken to determine which is the better method to use for routine analysis with emphasis on reproducibility, selectivity and sensitivity. The results show that although GC is more sensitive than HPLC with UV detection it is comparable to HPLC with fluorescence detection. HPLC is simpler and requires very little sample clean-up, while GC requires sample clean-up, derivatization and extraction which is tedious and time consuming. The chromatographic HPLC analysis is completed in less than 10 minutes while the GC procedure takes about twice that time.

Overall, the HPLC procedure using fluorescence detection, is selective, simple, fast and gives reproducible results. The HPLC sensitivity for both D and 4HOD, using fluorescence detection is 25 µg/L which is adequate for studies involving phenotyping of human populations.

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INTRODUCTION

Debrisoquine, 3,4-dihydro-2-(1H)-isoquinoline-carboxamide, is an oral antihypertensive agent which acts as an adrenergic antagonist (1). The occurrence of profound hypotension in some patients treated with the drug led to the discovery that inherited polymorphisms of drug metabolism determine an individual's metabolic capability. The metabolic ratio has been established as a reproducible measure of this trait. The metabolic ratio is the molar ratio of excreted debrisoquine to hydroxydebrisoquine in an eight hour urine collection following an oral dose of the drug. Determination of an individual's metabolic capability is useful in identifying persons who may be subject to an increased frequency of adverse drug effects as well as in identifying the distribution of this inherited trait in the population (2,3). Several reports have suggested that individuals who are extensive metabolizers of debrisoquine may be subject to increased risks of developing cancer (4,5).

The methodology for determining excreted debrisoquine and hydroxydebrisoquine in a study of a large number of subjects raises issues of both laboratory reproducibility as well as cost and efficiency. Debrisoquine and hydroxydebrisoquine were first quantitated by gas chromatography (6-8), but most studies since 1979 have used high performance liquid chromatography with either UV or fluorescence detection (9-18). A comparison of GC and HPLC showed that GC is more sensitive than HPLC with UV detection (14). In both procedures guanoxan was used as an internal standard and D and 4HOD required derivatization and subsequent extraction and clean-up steps before chromatographic analysis (14). Hempenius et al. (13) recently published a simple HPLC procedure whereby the urine samples were diluted with water and then injected into the column and D and 4HOD were detected by fluorescence. The use of fluorescence detection eliminates some of the interferring compounds in the urine and makes the method more selective and specific than HPLC with UV detection. The detection limit reported for D and 4HOD was about 25 µg/L, at an inter- and intra-day variability of less than 5%. The advantages of the HPLC procedure combined with fluorescence

detection are (a) direct injection of urine which eliminates any clean-up steps; (b) no derivatization is required; (c) less time consuming; (d) HPLC is much simpler and less prone to interferences than the GC method; and (e) of comparable sensitivity.

In this study we compare the measurement of debrisoquine, hydroxydebrisoquine as well as the metabolic ratios determined by both GC and HPLC fluorescent detection. We also report on the modifications introduced to these two techniques (3,13) and discuss their advantages and limitations.

EXPERIMENTAL

Materials

Debrisoquine hemisulphate and 4-hydroxydebrisoquine hemisulphate were gifts from Hoffman LaRoche and Guanoxan Hemisulphate from Pfizer (Groton, CT, USA). Toluene (GC grade) was obtained from Burdick and Jackson (Muskegon, MI, USA). 1,1,1,5,5,5-Hexufluoro-2,4-pentanedione (HFAA), 99%, was obtained from Aldrich Chemical Co. (Mikwaukee, WI, USA). Sodium Hydroxide and Sodium carbonate (reagent grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Borosilicate culture tubes (15 ml) fitted with teflon-faced rubber lined screw caps were used.

Gas Chromatography

The GC determination of Debrisoquine and its 4-hydroxy metabolite in urine was accomplished using the method of Idle et al. (3), with the addition of a water wash step for the toluene extract after derivatization. This allowed the analysis to be performed on a capillary column without degrading column performance. The original procedure used packed GC column.

Analysis was performed on a Varian Model 6000 capillary gas chromatograph fitted with a Ni^{63} electron capture detector and a splitting capillary injector with a split ratio of 12:1. The column used was a 30 meter DB-1 fused silica capillary column (J and W Scientific, Folsom, CA, USA) with an inside diameter

of 0.32 millimeter and a film thickness of 0.25 micron. The carrier gas used was helium at a linear velocity of 24 centimeters per second. The oven temperature was held at 150°C for 1 minute then increased at 5°C per minute to 250°C and held for 1 minute. The injector and detector temperatures were set at 250°C and 275°C, respectively. The sample size injected was 1 micro-liter.

Quantitation was performed on a Chromatopac Model CR4A data system (Shimadzu, Columbia, MD, USA) using internal standardization and single point calibration. The retention times determined for Debrisoquine, 4-hydroxy debrisoquine, and 7-methoxy guanoxan (internal standard) were 8.0, 10.7, 15.2 minutes, respectively.

Sample Preparation: To 100 µl of urine in a 15 ml screw cap test tube, add 200 µl of 5.00 µg/ml 7-methoxy Guanoxan in 1 M sodium carbonate as an internal standard. To this solution add 1 ml toluene and 50 µl of HFAA as a derivatization reagent. Mix then heat at 100°C for one hour in a heating block, let cool, add 5 ml of 3 N sodium hydroxide to hydrolize excess HFAA and an additional 2 ml toluene and vortex. Transfer the toluene layer to a centrifuge tube, add an equal amount of water, vortex, and centrifuge for 10 minutes to allow for the phases to separate. The organic phase is then analyzed by electron capture-gas chromatography.

HPLC Procedure

Sample Preparation: Approximately 1 ml urine was passed through a 0.2 µm Nylon Acrodisc 13 (Gelman Sciences, Ann Arbor, MI, USA) directly into an autosampler vial. The sample was then ready to be injected into the HPLC system.

HPLC Conditions: The analyses were carried out on a Hewlett-Packard Model 1090 HPLC (Rockville, MD, USA) equipped with an autosampler/autoinjector and a Hewlett-Packard Model 1046A fluorescence detector. After completing scans of the excitation and emission spectra of both debrisoquine and hydroxydebrisoquine, it was determined that maximum sensitivity would be achieved using an excitation wavelength of 194 nm while monitoring emission at 572 nm. The separation was

carried out on a SPHERI-5 CYANO (5 micron, 100 x 4.6 mm) cartridge column equipped with a 1 cm CYANO guard cartridge (Applied Biosystems, Brownlee columns). The mobile phase was prepared by adding 500 µl of triethylamine (Aldrich Chemical Company) to approximately 850 ml of water. Phosporic acid (Fisher Scientific) was used to adjust the pH of the solution to 3.5. 100 ml of HPLC grade acetonitrile (ACN) (Burdick & Jackson, Muskegon, MI, USA) was added and the solution was brought to 1 L with water. This solution was applied using Pump A and HPLC grade methanol (MeOH) (Burdick & Jackson, Muskegon, MI, USA) was delivered by Pump B in the proportion 90:10 (Pump A: Pump B) at a flow rate of 1 ml/minute. The resulting mobile phase composition was 81:9:10 aqueous buffer:ACN:MeOH. All mobile phase components were filtered and degassed through a 0.45 µm Nylon filter (Alltech Associates, Inc.). 25 µl of sample or standard was injected. The method was shown to be linear between 25 µg/L and 20 mg/L.

RESULTS AND DISCUSSION

A comparison of the GC procedure with the HPLC procedure shows that the GC procedure used in our laboratory, which is an adaptation of a published one (3), is time consuming and requires the use of a centrifuge and a heater. The procedure also requires the use of a derivatizing agent and an internal standard (see Experimental section for details). Conversely, the HPLC procedure requires only the filtration of the urine specimen prior to chromatographic analysis and the use of a pre-column, which is a normal step that does not add significant time to the procedure. In order to achieve the highest sensitivity the human urine samples were not diluted with water as previously reported (13) unless the concentration of D and 4HOD was found to be high, then a 1:4 dilution was done. The HPLC analysis can be completed in half the time (10 min) required for GC analysis (20 min). As mentioned earlier the GC sensitivity was found to be higher than the HPLC with UV detection (14), however, the use of fluorescence detection allows the determination of D and 4HOD in urine at detection limits

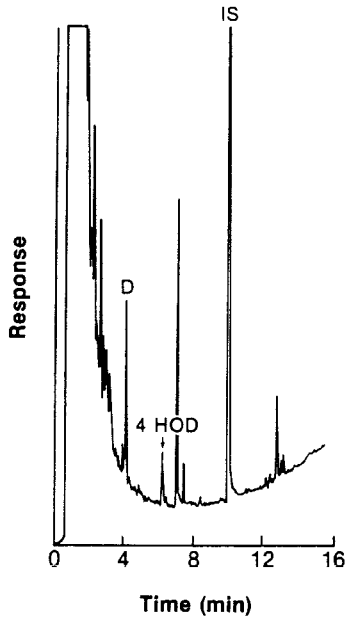

FIGURE 1. GC chromatogram of 25 µg/L D and 4HOD in spiked urine. Conditions are in the text.

comparable to those by GC (figure 1) without derivatization. Also, it was found that HPLC with fluorescence detection is more sensitive and free of interferences than HPLC with UV detection at 214 nm (compare figure 2a and 2b). The detection limit using fluorescence detection was found to be 25 µg/L (figure 3b).

In this study the HPLC procedure used by Hempenius et al. (13) was found to be sensitive and gave good resolution of D and 4HOD except in a few cases where an interferring peak (t_R 3.2 min) eluted close to the 4HOD peak (t_R 3.4 min) (figure 4). This was corrected for by changing the mobile phase from (10:90) acetonitrile:buffer to (10:9:81) methanol:acetonitrile:buffer. The addition of methanol to the mobile phase did not appreciably affect the analysis time, but allowed the quantitation of the 4HOD with more confidence. The concentration of 4HOD is a very important parameter which determines the

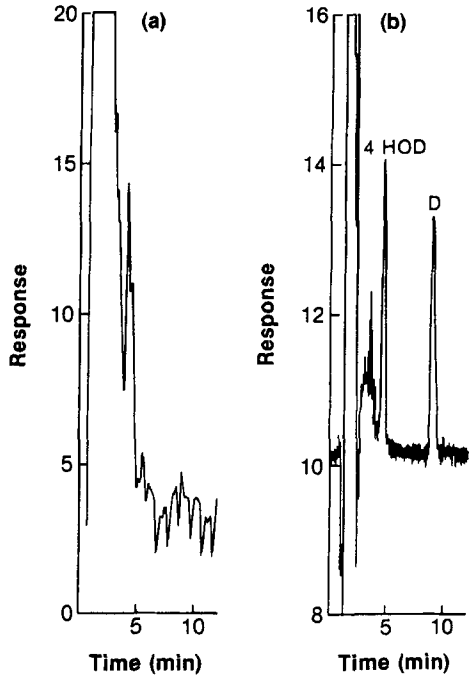

FIGURE 2. HPLC chromatograms of 500 µg/l 4HOD and D in spiked urine using (a) UV detection and (b) fluorescent detection. Other experimental conditions are in the text.

metabolic ratio. An error in quantitation may have a significant impact on the value of the metabolic ratio. Therefore, care in measurement and confidence in the results are very important when the objective of the study is the phenotyping of populations.

The GC procedure used in this study is that of Idle et al. (3) with one modification. The packed GC column was replaced by a capillary column which gave a better resolution of D from 4HOD. In our laboratory the assaying for D and 4HOD in spiked urine by gas chromatography gave reproducible results. The day-to-day and sample-to-sample variability was less than 5%. However, when physiological human urine (urine collected after administering 10 mg D orally) was analyzed for D and 4HOD, the coefficients of variation in the day-to-day

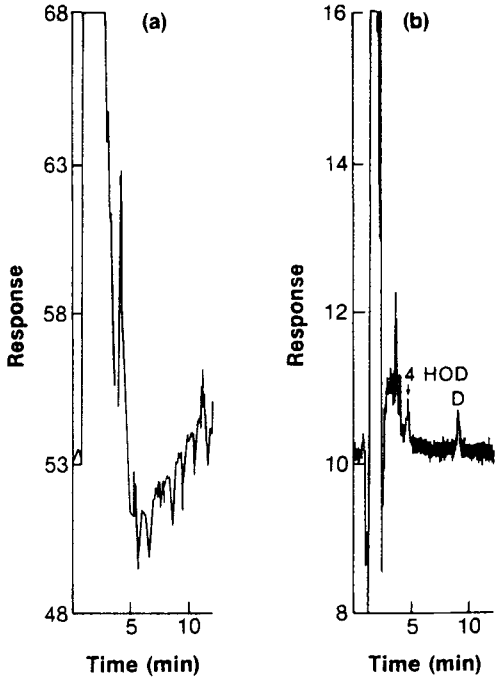

FIGURE 3. Same as figure 2 but 25 µg/l 4HOD and D in spiked urine.

reproduc-ibility was more than 12% in most cases (Tables 1 and 2). This prompted us to undertake a systematic evaluation of the GC procedure (3) to find out the source of error. A new bottle of the derivatizing agent was used daily. Also, a new standard was prepared every day. The buffer was adjusted to keep the pH of the solution after the reaction with the derivatizing agent above pH 7. It was found that when bicarbonate was used as a buffering agent the pH of the solution after completion of the derivatization reaction was in the acidic range, below pH 5. The reaction of the derivatizing agent with D and 4HOD proceeds best in a basic medium. The use of a carbonate buffer allowed the reaction to proceed to completion in a basic medium. Also, we studied the relationship between the organic (hexane) and aqueous layer volumes. Although this ratio was optimized

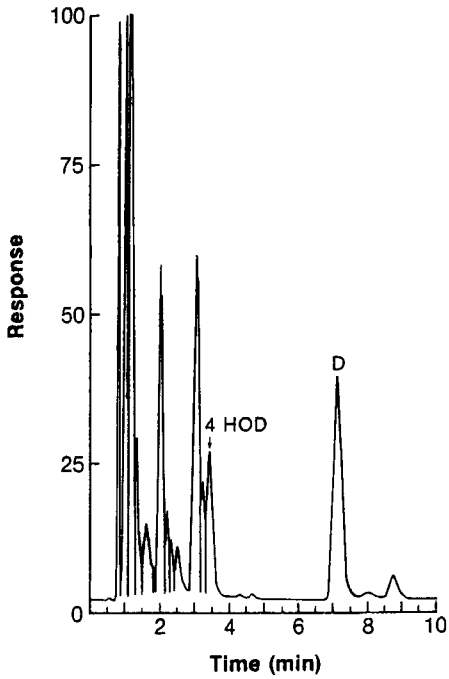

FIGURE 4. HPLC chromatogram of spiked urine using a cyanocolumn (5 µm, 100 x 4.6 mm), fluorescence detection and a mobile phase of (10:90) acetonitrile:buffer.

(7) the reproducibility problems remained at an unacceptable level. After all these modifications and adjustments to the sample clean-up and derivatization, we realized that the GC determination of D and 4HOD in physiological urine is possible but not reproducible at an acceptable level due to factors which are beyond our control. At this point it was decided to evaluate the recently published HPLC procedure using fluorescence detection (13).

Statistical Methods

Repeated measures analysis of variance (ANOVA) was performed to simultaneously test for variability within sample (day-to-day reproducibility) and between methods (GC vs. HPLC). All statistical analyses were performed using

TABLE 1. Comparison of GC and HPLC reproducibility of D from physiological urine of 14 subjects measured on three consecutive days.

A. GC Method

ID	Day 1	2	3	Mean	S.D.	% Dev
1397	1.46	0.87	1.22	1.18	0.30	25.07
1820	0.55	0.43	0.64	0.54	0.12	19.51
1822	1.26	1.00	1.22	1.16	0.14	12.07
1823	5.42	6.50	6.36	6.09	0.59	9.64
1824	0.31	0.23	0.31	0.28	0.05	16.30
1826	2.17	1.90	3.37	2.48	0.78	31.55
1836	2.24	1.44	2.29	1.99	0.48	23.97
1854	1.57	0.89	1.26	1.24	0.34	27.46
1858	1.95	1.60	1.96	1.84	0.21	11.16
1863	4.24	2.69	4.94	3.96	1.15	29.10
1864	4.57	2.96	4.09	3.87	0.83	21.34
1865	1.4o	0.88	1.49	1.26	0.33	26.20
1867	0.38	0.30	0.46	0.38	0.08	21.05
2232	2.52	2.25	2.62	2.46	0.19	7.77

B. HPLC Method

ID	Day 1	2	3	Mean	S.D.	% Dev
1397	0.94	0.96	1.09	0.10	0.08	8.17
1820	0.51	0.47	0.50	0.49	0.02	4.22
1822	0.98	0.93	1.11	1.01	0.09	9.23
1823	4.49	5.02	5.17	4.89	0.36	7.30
1824	0.24	0.26	0.32	0.27	0.04	15.23
1826	1.89	2.01	2.02	1.97	0.07	3.67
1836	1.36	1.43	1.53	1.44	0.09	5.93
1854	1.01	0.95	0.80	0.92	0.11	11.76
1858	1.70	1.82	1.66	1.73	0.08	4.82
1863	3.59	3.90	4.38	3.96	0.40	10.06
1864	2.43	2.39	2.71	2.51	0.17	6.95
1865	0.71	0.77	0.90	0.79	0.10	12.24
1867	0.27	0.28	0.29	0.28	0.01	3.57
2232	1.14	1.17	1.21	1.17	0.04	2.99

TABLE 2. Comparison of GC and HPLC reproducibility of 4HOD from physiological urine of 14 subjects measured on three consecutive days.

A. GC Method

ID	Day 1	2	3	Mean	S.D.	% Dev
1397	1.73	1.23	1.25	1.40	0.28	20.17
1820	1.20	0.98	1.16	1.11	0.12	10.53
1822	2.40	2.47	2.28	2.38	0.10	4.03
1823	1.05	1.42	0.82	1.10	0.30	27.60
1824	1.72	1.54	1.49	1.58	0.12	7.64
1826	2.08	2.50	2.44	2.34	0.23	9.71
1836	4.10	3.59	3.55	3.75	0.31	8.19
1854	2.38	1.60	1.61	1.86	0.45	24.02
1858	2.63	2.40	2.60	2.54	0.13	4.92
1863	1.45	1.22	1.28	1.32	0.12	9.06
1864	2.54	1.96	1.66	2.05	0.45	21.79
1865	6.02	4.58	5.01	5.20	0.74	14.21
1867	2.34	2.30	2.21	2.28	0.07	2.92
2232	1.74	1.67	1.32	1.58	0.23	14.27

B. HPLC Method

ID	Day 1	2	3	Mean	S.D.	% Dev
1397	1.14	1.15	1.29	1.19	0.08	7.03
1820	1.08	1.20	1.14	1.14	0.06	5.26
1822	1.87	2.06	2.26	2.06	0.20	9.45
1823	0.76	0.84	0.88	0.83	0.06	7.39
1824	1.47	1.50	1.74	1.57	0.15	9.43
1826	1.97	2.14	2.10	2.07	0.09	4.29
1836	2.33	2.52	3.00	2.62	0.35	13.20
1854	1.55	1.58	1.34	1.49	0.13	8.78
1858	2.30	2.58	2.30	2.39	0.16	6.76
1863	1.07	1.18	1.36	1.20	0.15	12.17
1864	1.28	1.34	1.61	1.41	0.18	12.47
1865	2.87	3.12	3.42	3.14	0.28	8.78
1867	1.86	2.01	2.06	1.98	0.10	5.27
2232	0.75	0.80	0.82	0.79	0.04	4.56

SYSTAT (19). The percent deviation was calculated as the standard deviation divided by the mean of the 3 measurements, and used to summarize the ANOVA results.

Levels of debrisoquine and hydroxydebrisoquine were detected from 14 samples, each analyzed on 3 separate days. Table 1 presents debrisoquine values obtained using both gas chromatography and high performance liquid chromatography methods; data for hydroxydebrisoquine are presented on Table 2. GC results appear to be more variable than HPLC; for debrisoquine, the mean % deviation is 20.2 using GC and 7.6 using HPLC, while for hydroxydebrisoquine, these figures are 12.8 and 8.2 for GC and HPLC, respectively. ANOVA results show that overall there is no statistically significant difference between HPLC and GC for either debrisoquine or hydroxydebrisoquine detection. However, debrisoquine values do vary significantly from one day to the next, with GC showing greater variability over time than HPLC ($p=0.004$). Overall, hydroxydebrisoquine values tend to be more consistent, but again, values obtained using GC are significantly more variable than those using HPLC ($p<0.001$).

CONCLUSIONS

The comparison of analysis of urine specimens for debrisoquine and hydroxydebrisoquine by GC and HPLC reveals that HPLC yields less variation in repeated measurments with the added benefits of being a simpler, less expensive and more rapidly performed procedure. The elimination of the sample derivatization and extraction steps is consistent with our finding of less variability in the repeated measurements by HPLC. Given that there is no statistically significant difference between the methods, the cost and time savings favor HPLC as the method of choice for the determination of the concentrations of debrisoquine and hydroxydebrisoquine in human urine.

ACKNOWLEDGEMENT

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Received: November 6, 1992
Accepted: November 18, 1992