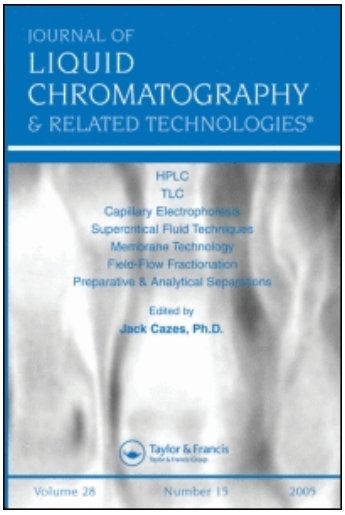

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Simultaneous Ion-Pairing Liquid Chromatographic Determination of the Major Metabolites of Styrene and Carbamazepine, and of Unchanged Carbamazepine in Urine

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SIMULTANEOUS ION-PAIRING LIQUID CHROMATOGRAPHIC DETERMINATION OF THE MAJOR METABOLITES OF STYRENE AND CARBAMEZEPINE AND OF UNCHANGED CARBAMAZEPINE IN URINE

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ABSTRACT

A high-performance liquid chromatographic method for a simultaneous quantitative determination of carbamazepine (CBZ) and of the major metabolites of CBZ (trans-10,11-dihydroxy-10,11-dihydrocarbamazepine, TDC ; carbamazepine-10,11-epoxide, CBZ-E) and those of styrene (S) (hippuric acid, HA; mandelic acid, MA; phenylglyoxylic acid, PA) in the rat urine is described. Separation is achieved on a Nova-Pak reverse-phase column by isocratic elution. Excellent resolution was obtained by adding to the acetonitrile-water mobile phase, tetrabutylammonium chloride (0.005 M) and methanol (1%). Detection is effected by UV absorption at 230 nm with a total analysis time of less than 18 min. An aliquot of diluted urine is injected directly onto the liquid chromatographic column. The limits of sensitivity of CBZ, CBZ-E, TDC, HA, MA, and PA are 3.3, 2.0, 1.8, 3.1, 1.2, and 3.1 μg/ml of diluted rat urine, respectively.

Precision and accuracy of the method are found to be acceptable. The method can be used for studying the interaction between these two xenobiotics. Preliminary studies have shown its potential application to human investigations.

INTRODUCTION

Styrene (S), which is widely used in the manufacture of synthetic plastics (1), is absorbed into the bloodstream by the percutaneous and pulmonary routes, to produce hepatorenal toxicity through the formation of a reactive and unstable intermediate metabolite, styrene oxide (2,3). When the workers exposed to S in plastic industries, have to take carbamazepine (CBZ), an anticonvulsant drug (4), several questions may be raised on the consequences of such a mixed exposure on the toxicity and pharmacokinetics of these two xenobiotics, and on the pharmacological effects of CBZ. This is due to the fact that both the compounds are known to alter the activities of some microsomal enzymes responsible for their metabolism (5-10).

The two xenobiotics are mainly metabolized in the liver by the microsomal cytochrome P-450 dependent mixed-function oxidase system to an electrophilic intermediate, carbamazepine-10,11-epoxide (CBZ-E) and styrene oxide, which may be hydrated as well as conjugated with glutathione (1,3,11,12). Hydration of the stable CBZ-E leads mainly to trans-10,11-dihydroxy-10, 11-dihydrocarbamazepine (TDC), and both metabolites are found in the urine (11,12). The end-metabolites of unstable styrene oxide following its hydration are mandelic (MA), phenylglyoxylic (PA), and hippuric (HA) acids, and represent the major metabolites of S. The biologic monitoring of S exposure can be effected by quantitating the amounts of these three metabolites excreted in the urine (13,14).

In order to study such interaction at the level of pharmacokinetics, an analytical method is certainly needed to determine simultaneously the xenobiotics and/or their major metabolites in biological fluids. Previously reported liquid chromatographic techniques (15-17) for studying the metabolism of CBZ or S were found to give inadequate resolution of the peaks. This report describes a rapid and simple isocratic ion-pairing high-performance liquid chromatographic (HPLC) method for the simultaneous determination of the major metabolites of S and CBZ, and of the unchanged form of CBZ in the rat urine.

MATERIALS AND METHODS

Chemicals

Carbamazepine, carbamazepine-10,11-epoxide and trans-10,11-dihydroxy-10,11-dihydrocarbamazepine were gifts of Ciba-Geigy Limited (Basle,Switzerland). Hippuric, mandelic and phenylglyoxylic acids were purchased from Sigma Chemical Company (St-Louis, MO). Tetrabutylammonium chloride (TBA^+Cl^-) and m-dinitrobenzene (MDNB) were provided by Aldrich Chemical Co. (Montreal, QC). HPLC grade acetonitrile and methanol were supplied by BDH Chemicals (Toronto, Ont.).

Apparatus

Analysis was performed on a Varian Model 5010 liquid chromatograph (Varian Instruments, Palo Alto, CA, USA) equipped with a Varichrom variable wavelength ultraviolet detector (Varian) and a Varian recorder Model G-2500. The

column used was a Nova-Pak C18 column (15 cm x 0.4 cm ID) prepacked with an octadecylsilica phase (5 µm mean particle size) supplied by Waters Chromatography Division (Milford, MA, USA). A Rheodyne® 1 µm filter (Rheodyne, Cotati, CA, USA) was installed between the Valco loop injector (Varian) of the chromatograph and the analytical column.

Preparation of urine samples and of standards curves

Urine was collected from male Sprague-Dawley rats, weighing approximately 250 g (Charles River Canada Inc., St-Constant, QC), placed in metabolism cages, and centrifuged at 500 g for 15 min to precipitate suspended particulates. The urine was then filtered on a 0.22 µm cellulose filter and diluted 15-fold (or more when the concentrations were high in samples) in distilled water.

To prepare the standard curves, standard solutions were made up in methanol in a range of concentrations from 3.4 to 31.2 µg/ml for CBZ, 6.3 to 31.7 µg/ml for CBZ-E and 1.9 to 31.9 µg/ml for TDC, or in distilled water in a range of concentrations from 2.6 to 20.8 µg/ml for HA, 2.0 to 26.0 µg/ml for MA and 3.2 to 26.0 µg/ml for PA. A standard solution of the internal standard (IS), m-dinitro benzene (MDNB), in methanol, was also prepared at a concentration of 200 µg/ml. For each CBZ metabolite or CBZ concentration, 100 µl of standard solution of CBZ, CBZ-E and TDC along with 100 µl of IS, were added to conical tubes, and the resulting mixture was evaporated to dryness. The residue was dissolved in 100 µl of mobile phase. To the resulting solution, were added 100 µl of diluted urine, and for each S metabolite concentration, 100 µl of standard solutions of HA, MA and PA were also added. The final mixture was vortex-mixed and 10 µl were injected into the chromatograph.

The quantification of CBZ and the major metabolites of CBZ and S were achieved by regression analysis of the ratio of the peak heights of each analyte to the peak height of internal standard plotted against concentration of the corresponding analyte.

Chromatographic procedure

To a 15 ml conical tube, were added, 100 µl of the internal standard solution which were then evaporated to dryness. The residue was then dissolved in 100 µl of mobile phase, 100 µl of diluted urine and 300 µl of distilled water. After vortexing this mixture, 10 µl were injected into the chromatograph.

The mobile phase consisted of a mixture of an aqueous TBA^+Cl^- 0.005 M solution and acetonitrile (80:20). After mixing, the pH was adjusted to an apparent value of 6.1 with 0.2 M HCl and NaOH aqueous solutions, and one percent of methanol was added. Detector was set at 230 nm and usually at an attenuation of 0.2 to 0.02 AUFS. The flow rate was set at 1.2 ml/min and the column was operated at ambient temperatures. Chart speed on the recorder was fixed at 0.1 in/min.

RESULTS AND DISCUSSION

The adjustment of the mobile phase pH to 6.1 was found to give optimal ionization of the analytes to allow their complexation with the ion-pairing agent TBA^+ in order to get resolution of all the peaks. The addition of methanol to the mobile phase rectified some peak broadening encountered. The chromatograms resulting from the injection of diluted

blank urine of human and rat, as well as diluted human and rat urine spiked with CBZ, CBZ-E, TDC, HA, MA and PA are presented in Figure 1. The retention times for TDC, MA, HA, CBZ-E, PA, IS and CBZ were approximately 2.8, 4.3, 5.3, 6.4, 7.1, 14.0 and 17.7 min, respectively. The chromatograms of the diluted urine blanks (Figure 1, I and III) have demonstrated that besides HA which is normally present in rat and human urine, there was no other endogenous urinary component that could interfere with the peaks of the analytes. Endogenous creatinine was found to eluate at 1.2 min. When a diluted urine control showed a HA peak, this background level was substracted from the measured values of HA in the diluted urine of treated rats. The time of analysis was less than 18 min.

The calibration plots in the rat urine were linear over the concentration ranges used for the six compounds. Within these ranges, standard curves passed close to the origin and the correlation coefficients (R) were nearly ideal (R = or > 0.98). Regression lines were not forced to pass through the origin. The average slopes and y-intercepts (± standard error) of the calibration curve equations (n = 3) were as follows:

Compound	Equation	R^2	N
TDC	$y = (0.059\pm0.003)x + (0.009\pm0.047)$	0.99	6
MA	$y = (0.015\pm0.001)x + (0.005\pm0.019)$	0.97	7
HA	$y = (0.149\pm0.011)x - (0.095\pm0.113)$	0.97	7
CBZ-E	$y = (0.078\pm0.007)x - (0.183\pm0.123)$	0.97	6
PA	$y = (0.067\pm0.002)x - (0.004\pm0.032)$	0.99	8
CBZ	$y = (0.057\pm0.002)x + (0.054\pm0.039)$	0.99	8

(N = number of concentrations in the curve)

The limits of detection, allowing a signal-to-noise ratio of 2:1 were 0.92, 0.63, 0.52, 0.50, 0.78 and 1.25 μg/ml of fifteen-fold diluted urine, for TDC, MA, HA, CBZ-E, PA and

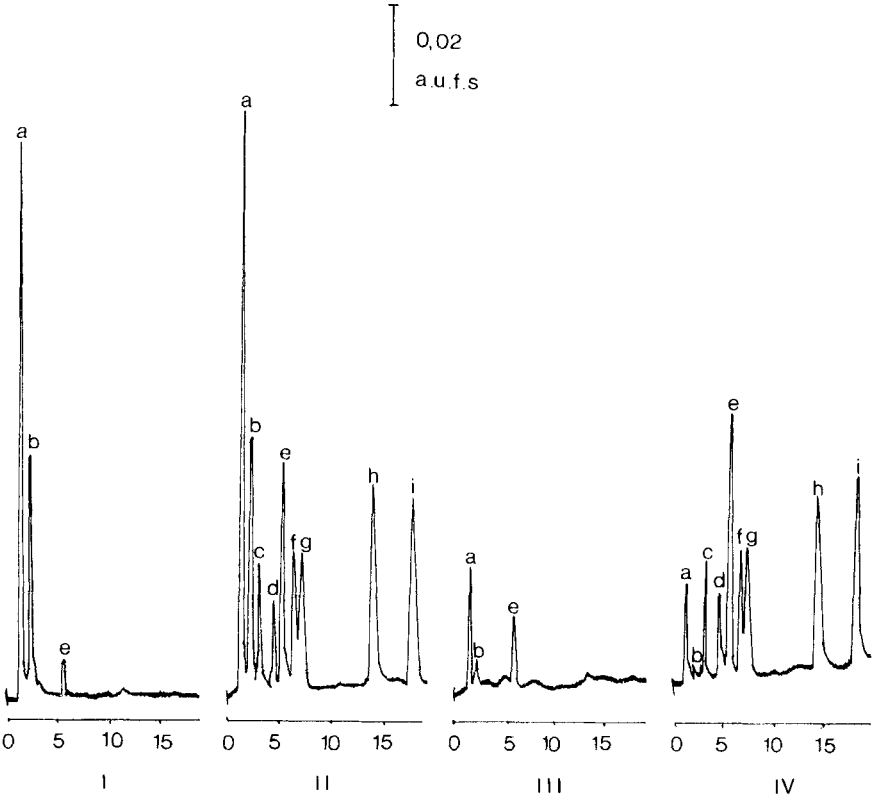

Figure 1. (I) Chromatogram of a five-fold diluted blank human urine. (II) Chromatographic elution of CBZ (200 μg/ml), CBZ-E (108.5 μg/ml), HA (100 μg/ml), MA (200 μg/ml), MDNB (200 μg/ml), PA (200 μg/ml), and TDC (105.8 μg/ml) spiked in a five-fold diluted blank human urine. (III) Chromatogram of a fifteen-fold diluted blank urine of rat. (IV) Chromatographic elution of CBZ (200 μg/ml), CBZ-E (108.5 μg/ml), HA (100 μg/ml), MA (200 μg/ml), MDNB (200 μg/ml), PA (200 μg/ml), and TDC (105.8 μg/ml) spiked in a fifteen-fold diluted urine of rat. (Peaks: a=endogenous creatinine; b=unknown endogenous compound; c=TDC; d=MA; e=HA; f=CBZ-E; g=PA; h=MDNB; i=CBZ)

CBZ, respectively. The sensitivity allowed for quantitation of at least 1.84, 1.25, 3.13, 2.00, 3.13 and 3.33 µg/ml of 15-fold diluted urine, for TDC, MA, HA, CBZ-E, PA and CBZ, respectively, using 100 µl of urine and a signal-to-noise ratio of 5:1. These latter concentrations were included in the calibration curves.

Intraday reproducibility for each compound was assessed by analysis of 5 replicates of diluted urine samples prepared from a given specimen and spiked at 3 concentrations. For each analyte, interday precision was determined by assaying, on 3 occasions, diluted urine samples spiked at 3 concentrations. In all instances, the coefficient of variation of peak height ratios was found to be acceptable (Table 1).

TABLE 1

Reproducibility of the method

Analyte	Concentration[1] (Coefficient of variation)[2]		
INTRADAY (n=5)			
TDC	4.63 (13.70)	14.23 (7.95)	18.50 (5.56)
MA	5.21 (12.65)	17.36 (6.05)	20.03 (4.90)
HA	2.98 (13.11)	13.88 (7.35)	16.02 (3.54)
CBZ-E	5.82 (11.00)	21.47 (5.10)	27.91 (5.64)
PA	5.21 (11.50)	16.67 (6.31)	20.83 (5.81)
CBZ	6.25 (11.85)	12.50 (6.73)	16.67 (5.23)
INTERDAY (n=3)			
TDC	4.63 (8.22)	14.23 (7.11)	18.50 (3.96)
MA	5.21 (10.06)	17.36 (8.08)	20.03 (3.80)
HA	2.98 (9.37)	13.88 (6.88)	16.02 (1.37)
CBZ-E	5.82 (9.51)	21.47 (5.01)	27.97 (1.93)
PA	5.21 (9.40)	16.67 (7.61)	20.83 (6.25)
CBZ	6.25 (6.94)	12.50 (3.30)	16.67 (3.73)

(1) Concentration, µg/ml of 15-fold diluted rat urine
(2) Coefficient of variation of peak height ratios

To assess the accuracy of the method for each analyte, diluted rat urine samples were spiked, in duplicates, at 3 concentrations that were different from those used for the calibration curve, and analyzed. Deviations, in %, between the mean concentration value determined by interpolation following linear regression of the appropriate standard curve, and the theoretical concentration, were calculated. The deviation (Δ) obtained for each compound, was found to be, in general, low and satisfactory (Table 2).

The successful use of the present method was demonstrated by the results obtained from Sprague-Dawley rats treated with intraperitoneal injection of carbamazepine (30 mg/kg at 8:30 AM and 5:00 PM for four days) and/or styrene (800 mg/kg on the fourth day at 9:30 AM). The rats were sacrificed on the fifth day at 9:30 AM.

TABLE 2

Accuracy of the method

Analyte	Conc.[1]	Δ[2]	Analyte	Conc.[1]	Δ[2]
	5.03	6.43		5.00	2.89
TDC	15.10	4.70	MA	15.00	14.81
	20.14	1.53		20.00	1.04
	5.03	3.28		5.00	3.00
CBZ-E	15.09	10.93	HA	15.00	3.54
	20.12	2.85		20.00	5.20
	5.00	6.11		5.00	1.49
CBZ	15.00	4.41	PA	15.00	2.90
	20.00	3.23		20.00	1.75

(1) Theoretical concentration, µg/ml of diluted rat urine.
(2) Difference, in %, between the value of the estimated concentration and the theoretical concentration in 15-fold diluted rat urine.

The 24-h urines collected on the fourth day were analysed to quantitate the urinary excretion of the styrene and carbamazepine metabolites, and of the unchanged carbamazepine. Representative chromatograms are shown in Figures 2 and 3. For each xenobiotic, the elution pattern was equivalent to the one obtained above with spiked rat urine (Figure 1, IV). Comparisons of the chromatograms of the urine of rats treated with styrene or carbamazepine alone did not show any presence of peaks that could interfere with the peaks of the 6 analytes measured in coexposure experiments. Other chromatograms of diluted urine, not containing the internal standard and obtained from rats treated with carbamazepine or styrene alone and with carbamazepine and styrene in the coexposure experiment, revealed that there was no peak interference with the MDNB. The amounts measured in a 24-h urine sample of a rat treated with styrene and carbamazepine (Figures 2, I and 3, III) were 0.0565, 3.6148, 17.8616, 11.0479, 29.6020, and 2.3994 μmol per 100 g of body weight for CBZ, CBZ-E, HA, MA, PA, and TDC, respectively. For the doses of xenobiotics used, the urinary concentrations of CBZ, CBZ-E, and TDC being much lower than those of styrene metabolites, a three-fold dilution of urine was required for the assay of CBZ and its metabolites. The detailed results of this study on the metabolic and toxicologic interactions between styrene and carbamazepine in rats will be published elsewhere (18).

In summary, this simple assay provides a procedure for simultaneous analysis of CBZ and of the major metabolites of CBZ and S from small volumes of rat urine. Preliminary studies have also indicated that the method could be potentially useful for human investigations, as human urine did not reveal interference peaks of endogenous impurities.

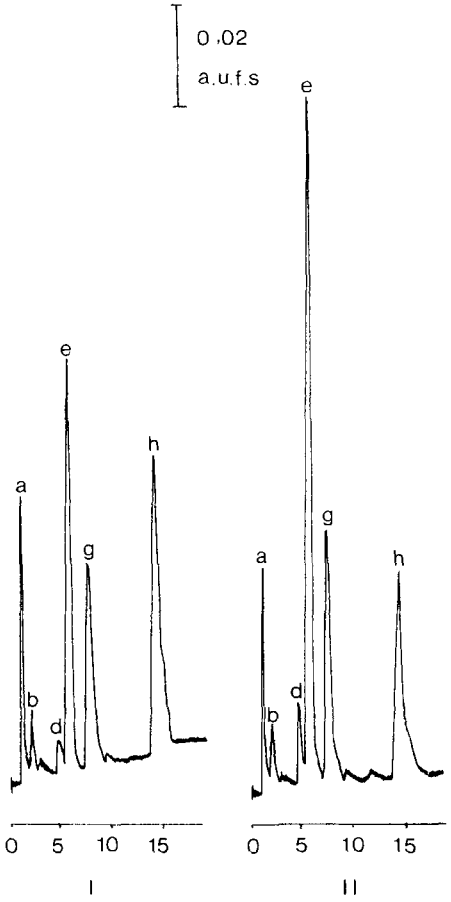

Figure 2. (I) Chromatogram of a rat fifteen-fold diluted urine sample following intraperitoneal administration of a single dose of styrene (800 mg/kg). (II) Chromatogram of a rat fifteen-fold diluted urine sample following intraperitoneal co-administration of carbamazepine (30 mg/kg) and styrene (800 mg/kg). (The symbols used for peaks identification are the same as given in Fig.1)

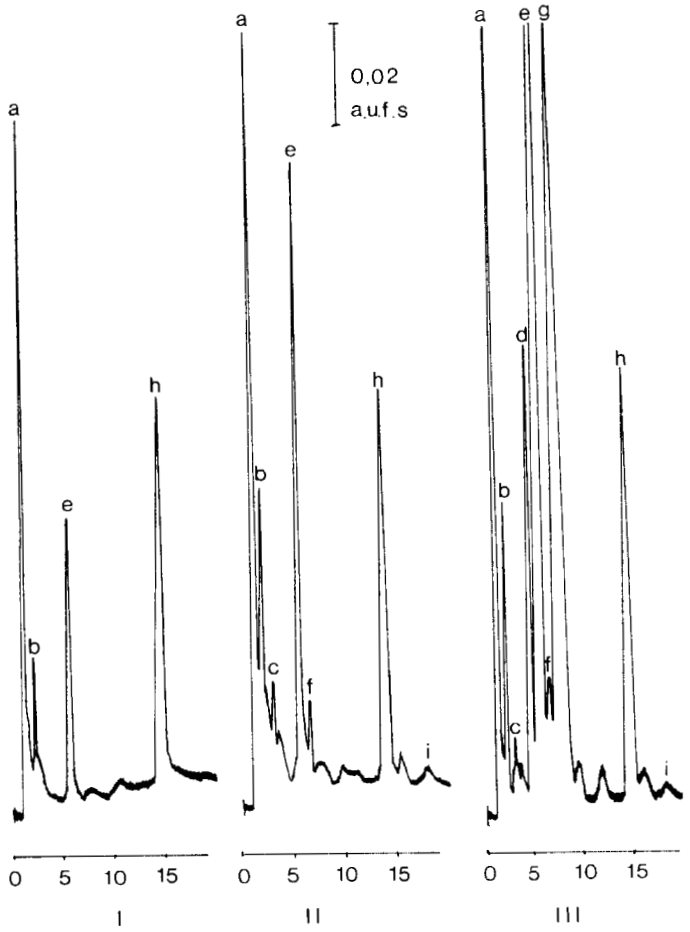

Figure 3. (I) Chromatogram of a three-fold diluted blank urine of rat. (II) Chromatogram of a rat three-fold diluted urine sample following intraperitoneal administration of carbamazepine (30 mg/kg). (III) Chromatogram of a rat three-fold diluted urine sample following intraperitoneal co-administration of carbamazepine (30 mg/kg) and styrene (800 mg/kg). (The symbols used for peaks identification are the same as given in Fig.1)

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