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SIMULTANEOUS DETERMINATION OF THE MAJOR METABOLITES OF STYRENE AND ACETAMINOPHEN, AND OF UNCHANGED ACETAMINOPHEN IN URINE BY ION-PAIRING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An improved isocratic high-performance liquid chromatographic method for the quantitative determination of the major urinary metabolites of styrene in urine is described. The high separation efficiency of an ion-pairing system using tetrabutyl ammonium chloride allows also the simultaneous determination of acetaminophen and its three major metabolites with those of styrene in rat urine. Simplicity, reproducibility and a short analysis time provide a useful tool for the toxicokinetic studies of these two hepatorenotoxic xenobiotics after their co-administration. An aliquot of the diluted urine is directly injected into the liquid chromatograph. The limits of sensitivity and detection of the metabolites of styrene are better than those reported before. Preliminary works indicate that the method would also be applicable for analysis of these metabolites in human urine and would therefore be useful in monitoring styrene exposure to workers especially when they take acetaminophen.

INTRODUCTION

Styrene, an aromatic compound used in the plastic industry, is metabolized in many animal species and in man by oxidation to mandelic (MA), phenyl-

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glyoxylic (PA) and hippuric (HA) acids, which are finally excreted in the urine [1]. The degree of styrene exposure can be estimated by determination of the quantity of its urinary metabolites [2]. Prior to the formation of these latter substances, styrene is biotransformed into a reactive epoxide intermediate having hepato- and renotoxic properties, which is detoxified by epoxide hydrase to styrene glycol and by conjugation with liver or kidney glutathion (GSH) [3] to form GSH conjugates. An almost similar pathway of detoxification is followed by acetaminophen, a widely used analgesic drug [4–6]. Since the GSH pool is limited in man and the mammalians, a competition for detoxification during co-exposure of acetaminophen with styrene can result in faster depletion of GSH and hence manifestation of early toxic signs. Acetaminophen, being an over-the-counter drug, is readily available to workers being exposed to styrene. In order to study the toxicokinetic interaction between these two xenobiotics, the development of a selective analytical method is required for the simultaneous quantitative assay in urine of the major metabolites of styrene (mentioned above) and acetaminophen [namely acetaminophen glucuronide (AG), acetaminophen sulfate (AS) and acetaminophen mercapturate (AM)] and the unchanged form of acetaminophen.

In this paper, we describe a simple and rapid isocratic ion-pairing high-performance liquid chromatographic (HPLC) procedure for the simultaneous quantitative determination of the major metabolites of styrene and acetaminophen, and of the unchanged form of acetaminophen in rat urine. With this technique, the time of analysis for all these compounds is less than 10 min, a single aliquot of diluted urine is injected directly into a Nova-Pak® C_{18} reversed-phase column and the limits of sensitivity for the determination of styrene metabolites is improved.

EXPERIMENTAL

Materials

HPLC-grade (LiChrosolv) acetonitrile was supplied by BDH (Toronto, Canada). Styrene metabolites, namely HA, MA and PA, were from Sigma (St. Louis, MO, U.S.A.) and were used as received. Acetaminophen and its cysteine, glucuronide, mercapturate and sulfate conjugates were generous gifts from Dr. J.N. Tam of McNeil (Fort Washington, PA, U.S.A.). Tetrabutyl ammonium (TBA) chloride 85% and *p*-hydroxybenzoic acid (PHBA) were purchased from Aldrich (Milwaukee, WI, U.S.A.).

Apparatus

Analysis was performed at ambient temperature on a Varian Model 5010 liquid chromatograph (Varian Instruments, Palo Alto, CA, U.S.A.) equipped with a Varichrom variable-wavelength ultraviolet detector (Varian) and a Varian Model G-2500 recorder. The column used was a Nova-Pak C_{18} column (150 mm × 4.0 mm I.D.) prepacked with an octadecylsilica phase (5 μm mean particle size) supplied by Waters Assoc. (Milford, MA, U.S.A.). A Rheodyne® 1-μm filter (Rheodyne, Cotati, CA, U.S.A.) was installed between the Valco loop injector (Varian) of the chromatograph and the analytical column.

Preparation of urine samples and of standard curves

Urine was collected from male Sprague—Dawley rats, weighing approx. 250 g (Charles River Canada, St. Constant, Canada), through the use of a metabolism cage, and centrifuged at 500 *g* for 10 min to precipitate suspended particulates. A 10- to 100-fold dilution was made with distilled water to obtain an appropriate medium.

In order to prepare the standard curves, six diluted urine samples were then spiked with increasing concentrations of HA (0.2–15 μg/ml), MA (0.2–15 μg/ml), PA (0.2—15 μg/ml), acetaminophen (0.1–10.5 μg/ml), AG (0.3–11.5 μg/ml), AM (0.5—9.5 μg/ml) and AS (0.5—11.4 μg/ml). To conical glass tubes, 1 ml of spiked diluted urine and 100 μl of a 6.25 mg/100 ml acetonitrile solution of PHBA, the internal standard, were added. The resulting mixture was then vortexed for 10 s and 10 μl were injected directly into the chromatograph.

Standard curves were constructed by plotting the HA/PHBA, MA/PHBA, PA/PHBA, acetaminophen/PHBA, AG/PHBA, AM/PHBA and SA/PHBA peak-height ratios against the concentration of the appropriate analyte.

Treatment of experimental urine samples was similar to the one used for the construction of standard curves.

HPLC procedure

The mobile phase was a mixture of aqueous 0.01 *M* TBA chloride–acetonitrile (80:20). After mixing, the pH was adjusted to an apparent value of 6.1 with 0.2 *M* hydrochloric acid and sodium hydroxide solutions using a pH meter (Ionalyzer, Model 801/digital pH, Orion Research, Cambridge, MA, U.S.A.). This value was found to give optimal ionization of the compounds to be chromatographically resolved, in order to enhance their complexation with the ion-pairing agent TBA^+. Flow-rate was maintained at 1.0 ml/min, producing a low 8.6-MPa pressure in the column. UV measurement was performed at two different wavelengths: 245 nm for acetaminophen, AG, AM, AS and PA, and 215 nm for HA and MA. Varying the wavelength allowed optimization in absorbance, and, consequently, in sensitivity also. Usually, sensitivity of detection was fixed at 0.1 a.u.f.s. which gives a good response without a too noisy background. Chart speed on the recorder was tuned at 0.254 cm/min to produce the best peak shape.

Precision

Within-day precision for each compound was determined by analysis of six diluted urine samples at three concentrations from the same spiked specimen. Between-day precision was evaluated by assaying urine standards at three concentrations for each compound on three occasions. Coefficient of variation was calculated from the standard deviation of the arithmetic mean value of peak-height ratio at each concentration.

The procedure was also validated for each compound by spiking diluted urine samples, in duplicate, with five known amounts of the appropriate analyte, in order to obtain five concentrations that are different from the one used in the standard curve. Deviations between the mean concentration value determined from the appropriate standard curve equation and the theoretical concentration are expressed in percent.

Quantitation

Linearity of standard curves was tested by analysis of variance using the coefficient of correlation, r, and the standard error of the slope and of the y-intercept, as the criterions of adequacy. The concentration of each compound in urine samples was determined by interpolation following linear regression of the appropriate standard curve. All results are expressed in terms of arithmetic mean, $\bar{x}$, and standard error, S.E.

RESULTS AND DISCUSSION

HPLC methods for determination of styrene metabolites or of acetaminophen and its metabolites

When we tried to apply the HPLC methods reported in the literature for the quantitative determination of styrene metabolites [7, 8] in urine, we found that acetaminophen and its metabolites were interfering, with the peaks corresponding to styrene metabolites. We also found that the existing HPLC methods, for determination of major styrene metabolites, presented some disadvantages. The method of Ogata and Sugihara [7] requires urine extraction of metabolites and has a poor sensitivity of 250—500 μg/ml of urine. The procedure of Poggi et al. [8] also needs an urine extraction of metabolites, but its sensitivity is 1—25 μg/ml of urine. With the technique of Gaetani et al. [9], the acid constituents of urine are separated on a Sep-Pak C_{18} column before injection into the liquid chromatograph, the time of analysis is long (20 min), the column is heated at 40°C and the limits of sensitivity are 25—50 μg/ml of urine.

The use of isocratic HPLC methods [10—13] developed for the quantitative determination of acetaminophen and its major metabolites posed the same interference problem.

Modifications of ion-suppression procedures [7, 8, 10—13] did not solve our problem. The principles of the ion-pairing technique was then considered [9, 14, 15], and application, at room temperature, of the method of Gaetani et al. [9] for the major metabolites of styrene did not resolve all the peaks to be separated. On the other hand, the method of Hart et al. [14] for the determination of acetaminophen and its metabolites requires an elution gradient of methanol in water and was not considered because of this technical disadvantage. Different ion-pairing conditions were then investigated which lead to the development of a selective method.

Chromatograms of blank and spiked urine

Representative chromatograms of blank and standard urines are shown in Fig. 1. The chromatogram of a standard solution of the major metabolites of styrene and acetaminophen, of the unchanged form of acetaminophen and of the internal standard in ten-fold diluted rat urine is presented in Fig. 1, IV. The results indicate that there was good separation with good peak shape among endogenous creatinine, the analytes and the internal standard. The retention times of creatinine, acetaminophen, AG, PHBA, AM, MA, HA, AS and PA were ca. 1.2, 1.8, 2.8, 3.4, 4.4, 5.8, 6.5, 8.8 and 9.5 min, respectively. Analysis of the ten-fold diluted urine blank (Fig. 1, II) has demonstrated that there were

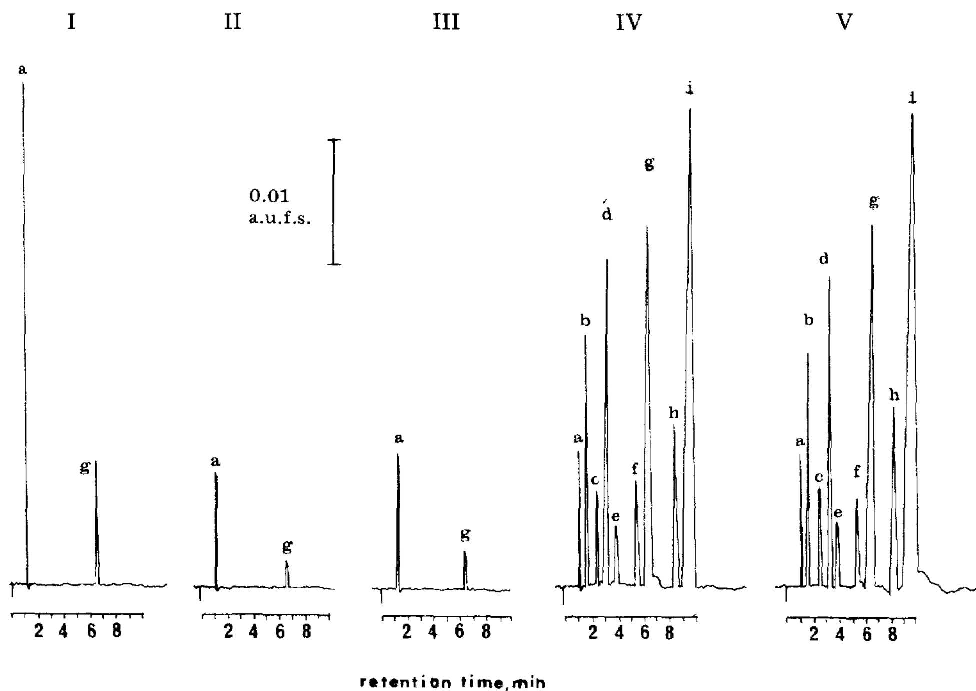

Fig. 1. (I) Chromatogram of undiluted blank urine of rat. (II) Chromatogram of a ten-fold diluted blank urine of rat. (III) Chromatogram of a ten-fold diluted blank human urine. (IV) Chromatographic elution of acetaminophen (7.35 μg/ml), AG (7.98 μg/ml), AM (6.68 μg/ml), AS (11.41 μg/ml), HA (5.0 μg/ml), MA (15.0 μg/ml), PA (5.0 μg/ml) and PHBA (5.6 μg/ml) spiked in ten-fold diluted rat urine. (V) Chromatographic elution of the compounds found in chromatogram IV which were spiked at the same concentration in ten-fold diluted human urine. Peaks: a = creatinine; b = acetaminophen; c = AG; d = PHBA; e = AM; f = MA; g = HA; h = AS; i = PA.

no peaks of normal urine components that interfere with the peaks of the monitored substance. Creatinine is fully resolved from other peaks and elutes with the solvent front. HA, which is normally present in urine, is not detectable when normal urine is 100-fold diluted. When the urine control shows an HA peak, this background level must be subtracted from the measured values of HA. The cysteine conjugate of acetaminophen which has not been quantitated in this work, because it is usually present in a negligible amount in urine, had a retention time of 4.6 min.

Calibration curves

Calibration curves for the seven compounds were linear, with minimum y-intercepts and with correlation coefficients, r, of 0.99 or better. Equations for the calibration curves were as follows: acetaminophen: $y = 0.204\ (\pm\ 0.040)x - 0.059\ (\pm\ 0.022)$; AG: $y = 0.081\ (\pm\ 0.002)\ x + 0.009\ (\pm\ 0.012)$; AM: $y = 0.073\ (\pm\ 0.003)\ x - 0.022\ (\pm\ 0.015)$; AS: $y = 0.069\ (\pm\ 0.003)\ x - 0.016\ (\pm\ 0.013)$; HA: $y = 0.052\ (\pm\ 0.002)\ x + 0.050\ (\pm\ 0.014)$; MA: $y = 0.017\ (\pm\ 0.001)\ x - 0.014\ (\pm\ 0.009)$; PA: $y = 0.066\ (\pm\ 0.006)\ x + 0.123\ (\pm\ 0.069)$. For all equations $n = 7$ and $r = 0.99$. The values in parentheses represent the standard error of the slopes and the y-intercepts.

TABLE I

LIMITS OF SENSITIVITY AND OF DETECTION OF THE HPLC METHOD

Sensitivity and detection limits were determined using ten-fold diluted urine of rat.

Compound	Sensitivity limit (μg/ml)	Detection limit (μg/ml)
Acetaminophen	0.1	0.03
AG	0.3	0.1
AM	0.5	0.1
AS	0.5	0.1
HA	0.2	0.04
MA	0.2	0.04
PA	0.2	0.04

Limits of sensitivity and detection

Sensitivity was evaluated by decreasing the concentration of the analytes which were assayed by the HPLC procedure until a good detector response could be made based on a signal-to-noise ratio of 5. This minimum peak-height ratio was automatically included in each standard curve. The limits of sensitivity range from 0.1 to 0.5 μg/ml of diluted urine, depending on the compound being studied, as shown in Table I. The limits of detection which were determined on the basis of a signal-to-noise ratio of 2 are presented in Table I and are ranging from 0.03 to 0.1 μg/ml for the different analytes. A blank of undiluted urine of rat was injected into the chromatograph and showed no

TABLE II

REPRODUCIBILITY OF THE HPLC METHOD

Concentrations were determined in ten-fold diluted urine of rat; coefficients of variation (C.V.) were calculated from the standard deviation of the arithmetic mean value of peak-height ratio at each concentration.

Compound	Concn. (μg/ml)	C.V. (%)	Concn. (μg/ml)	C.V. (%)	Concn. (μg/ml)	C.V. (%)
Within-day (n = 5)						
Acetaminophen	1.05	(5.6)	5.25	(4.2)	7.35	(2.8)
AG	1.14	(5.3)	5.70	(3.3)	7.97	(4.3)
AM	0.95	(5.1)	4.77	(4.1)	6.68	(5.1)
AS	1.14	(4.5)	5.70	(2.5)	7.98	(3.5)
HA	2.50	(2.7)	7.50	(1.8)	15.00	(3.6)
MA	2.50	(2.9)	7.50	(3.7)	15.00	(2.1)
PA	2.50	(3.7)	7.50	(3.3)	15.00	(2.9)
Between-day (n = 3)						
Acetaminophen	1.05	(6.1)	5.25	(5.2)	7.35	(3.8)
AG	1.14	(5.8)	5.70	(4.3)	7.98	(4.8)
AM	0.95	(6.1)	4.77	(5.1)	6.68	(6.1)
AS	1.14	(5.0)	5.70	(3.5)	7.98	(4.0)
HA	2.50	(4.6)	7.50	(4.1)	15.00	(3.2)
MA	2.50	(4.0)	7.50	(4.5)	15.00	(3.5)
PA	2.50	(2.8)	7.50	(2.5)	15.00	(1.7)

response besides the creatinine and the HA peaks (Fig. 1, I) indicating that the limits of sensitivity and detection found are true values and not the result of an interference phenomenon.

Reproducibility

Data showing the within-day and between-day precision of the assay are summarized in Table II. For any given concentration, the determination of every analyte was highly reproducible as shown by the small coefficients of variation of the mean peak-height ratios.

The low deviation values obtained for each analyte between theoretical and experimentally measured concentrations are presented in Table III and gave additional support on the validity of the method.

TABLE III

DEVIATIONS FROM THEORETICAL CONCENTRATION

Values are difference between the average value of the estimated concentrations and of the corresponding theoretical concentration in ten-fold diluted urine of rat.

Compound	Difference (%)					
	Theoretical concentration (μg/ml)					
	0.5	2.0	4.0	6.0	8.0	12.0
Acetaminophen	2.5	6.0	5.0	1.7	2.5	–
AG	5.0	2.0	7.5	3.3	1.3	—
AM	2.5	6.0	5.0	1.7	1.3	–
AS	5.0	4.0	5.0	3.3	2.5	—
HA	—	5.0	5.0	3.3	1.3	1.7
MA	—	5.0	2.5	3.3	2.5	2.5
PA	—	5.0	2.5	1.7	1.3	8.3

Advantages of the method for quantitation of styrene metabolites

When applied to the quantitative determination of the major styrene metabolites only, the proposed method presents some advantages over those previously reported [7–9]: a shorter analysis time (10 min), direct injection of an aliquot of diluted urine (not requiring extraction), avoidance of an elution gradient or of a column heater, improvement of the limits of sensitivity and detection. The method is also more convenient than the gas chromatographic methods [16–18], which require derivatization of extracted urine metabolites prior to injection into the chromatograph and do not offer better limits of sensitivity.

Application and interference

The successful use of the assay was demonstrated by the results obtained in toxicokinetic studies conducted in our laboratory where one group of male Sprague–Dawley rats was treated with simultaneous intraperitoneal administration of styrene (800 mg/kg) and acetaminophen (750 mg/kg). The 24-h urines, following the administration of xenobiotics, were assayed to quantitate the

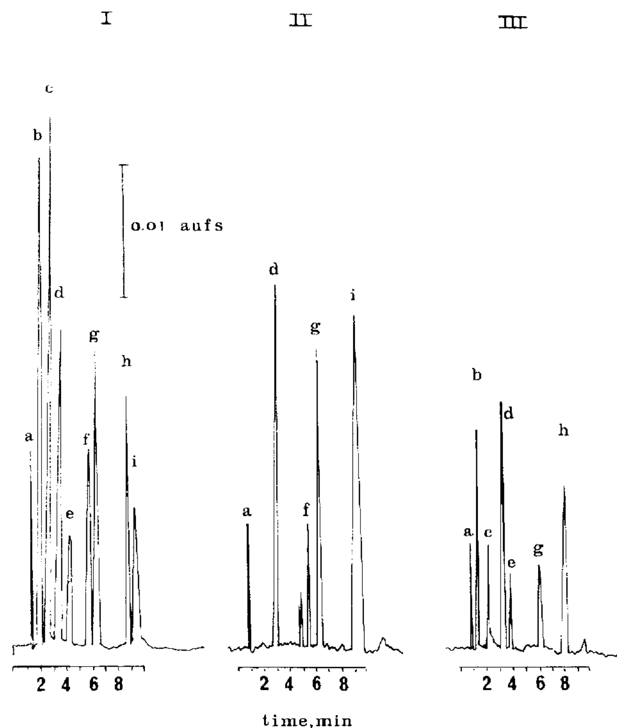

Fig. 2. (I) Chromatogram of a rat 100-fold diluted urine sample following intraperitoneal co-administration of single doses of styrene (800 mg/kg) and acetaminophen (750 mg/kg). (II) Chromatogram of a rat 100-fold diluted urine sample following intraperitoneal administration of a single dose of styrene (800 mg/kg). (III) Chromatogram of a rat 100-fold diluted urine sample following intraperitoneal administration of a single dose of acetaminophen (750 mg/kg). The symbols used for peak identification are the same as given in Fig. 1.

urinary excretion of the styrene and acetaminophen metabolites and of the unchanged form of acetaminophen. A representative chromatogram which was thus obtained is given in Fig. 2. The elution pattern was equivalent to the one obtained above with spiked rat urine. The amounts measured in a 24-h urine sample were 6.54, 14.06, 1.78, 10.22, 2.29, 4.58 and 5.06 mg per 100 g of body weight for acetaminophen, AG, AM, AS, HA, MA and PA, respectively. The detailed results of this investigation on the metabolic and toxicologic interactions between styrene and acetaminophen in rats will be published elsewhere [19].

In these toxicokinetic studies, the comparison of the chromatograms of the urine of rats treated with styrene (800 mg/kg) alone and those of the urine of rats treated with acetominophen (750 mg/kg) alone did not show the appearance of peaks that could interfere with the peaks of the seven analytes measured in coexposure experiments (Fig. 2, II and III).

However, when styrene is administered alone, it gives a small peak with a retention time of 5 min, which is not present when both xenobiotics are given. The identification of this urinary metabolite remains to be elucidated.

To show the applicability of the present method to human urine, fresh urine

samples from a healthy volunteer were spiked with known amounts of the eight compounds studied. Representative chromatograms of a blank and spiked urine sample are given in Fig. 1 (III and V). The elution pattern is found to be very similar to the one obtained with rat urine (Fig. 1, I, II and IV). The potential use of this HPLC procedure for the quantitative determination of the styrene and acetaminophen metabolites and of the unchanged form of acetaminophen thus appears to be evident for clinical monitoring.

CONCLUSION

The present HPLC method is capable of fully separating eight compounds in less than 10 min, when a diluted urine sample is directly injected into the chromatograph, to quantitate with a good sensitivity acetaminophen and its major metabolites, and with an improved sensitivity the styrene major metabolites, reaching the 200 ng/ml level. The procedure is practical from the standpoint of simplicity and reproducibility. Preliminary work indicates that the method would also be applicable in human urine and would be useful in the monitoring of styrene exposure, especially when acetaminophen is taken by the workers.

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