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DETERMINATION OF BENZOIC ACID AND HIPPURIC ACID IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

For patients with inborn errors of urea synthesis, oral administration of sodium benzoate is the usual treatment to increase the nitrogen excretion. Thus, monitoring hippuric acid and benzoic acid simultaneously in human biological fluids is considered to be clinically important. We developed a simple and accurate high-performance liquid chromatographic method for the simultaneous determination of hippuric acid and benzoic acid in human plasma and urine. This method requires no extraction step. Aliquots of urine and plasma are added to a solution of internal standard (*o*-chlorobenzoic acid) in acetonitrile and directly injected onto a reversed-phase column using an acidic (pH 2.7) eluent and ultraviolet detection at 235 nm. The preliminary plasma concentration–time and urinary excretion rate–time profiles of hippuric acid and benzoic acid from a healthy subject receiving small, medium and large doses of sodium benzoate are reported.

INTRODUCTION

The use of sodium benzoate in combination with restriction of the dietary consumption of nitrogen appears to control hyperammonemia, prolong life and improve clinical outcome. Large doses of sodium benzoate are known to increase urinary nitrogen excretion in the form of hippurate [1,2]. Activation of this alternate pathway of waste nitrogen excretion has offered therapeutic options for patients with a variety of inborn errors of urea synthesis [1,2]. The biotransformation of benzoic acid to its glycine conjugate, hippuric acid, has been known to be a saturable process [3]. Benzoic acid is apparently eliminated following a Michaelis–Menten kinetic behavior [4]. Therefore, monitoring of the serum concentration is needed to avoid potentially toxic benzoate levels. For this reason we developed a rapid, simple and reliable high-performance liquid chromatographic (HPLC) assay for hippuric acid and benzoic acid in human plasma and

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urine. A preliminary study on the determination of benzoic acid and hippuric acid in plasma and urine samples of a healthy subject receiving three different doses of sodium benzoate demonstrated the clinical applicability of the assay for therapeutic monitoring as well as for pharmacokinetic studies.

EXPERIMENTAL

Chemicals and reagents

Benzoic acid, hippuric acid, *o*-chlorobenzoic acid (internal standard), sodium bicarbonate, sodium acetate and acetic acid were of reagent grade and acetonitrile was of HPLC grade. All these chemicals were purchased from Wako (Osaka, Japan). Saccharo-1,4-lactone was purchased from Sigma (St. Louis, MO, U.S.A.) and β-glucuronidase/aryl sulfatase (*Helix pomatia*) was purchased from Calbiochem (San Diego, CA, U.S.A.).

A standard aqueous stock solution containing 2000 μg/ml benzoic acid and hippuric acid and 20 mg/ml sodium bicarbonate was prepared. An internal standard stock solution (*o*-chlorobenzoic acid, 625 μg/ml) was prepared in acetonitrile. Both solutions were diluted to the required concentration for each compound. All solutions were stored at 4 °C until used.

Instrumentation and chromatographic conditions

The HPLC apparatus consisted of a Hitachi pump (Model 635A, Hitachi, Tokyo, Japan), a Waters autosampler system (WISP 710B, Waters Assoc., Milford, MA, U.S.A.), a UVILOG 5 IV variable-wavelength UV detector (Oyobunko Kiki, Tokyo, Japan), and a Shimadzu C-RIB Chromatopac integrator (Shimadzu, Kyoto, Japan). Reversed-phase HPLC separations were carried out on a Yanapak ODS-A column (250 mm × 4.6 mm I.D., 7-μm particles, Yanagimoto, Kyoto, Japan). The detection wavelength was set at 235 nm with a sensitivity setting of 0.16–1.28 a.u.f.s. The mobile phase used was acetonitrile–water–acetic acid (35:63:2, v/v/v) at a flow-rate of 1.0 ml/min at 30 °C.

Sample preparation

A 100-μl plasma sample and 200 μl of acetonitrile containing the internal standard (125 μg/ml) were combined in a 1.5-ml centrifuge tube, which was then stoppered, vortexed for 20 s and centrifuged at 9500 *g* for 1 min. A 5–10 μl aliquot was directly injected onto the chromatograph. Urine samples were diluted 10 times (or 20 or 40 times if necessary) with distilled water. The 200-μl diluted urine sample and 400 μl of acetonitrile containing the internal standard (625 μg/ml) were combined in a 1.5-ml tube and then treated in the same manner as the plasma samples.

Clinical pharmacokinetic study

A healthy, male volunteer, 33 years old, participated in the preliminary pharmacokinetic study of sodium benzoate after giving a written informed consent. Three single doses of sodium benzoate (2.7, 5.4 and 10.8 g or 0.04, 0.08 and 0.16 g/kg, respectively) dissolved in 200 ml of water were orally administered after an

overnight fast. Blood samples were drawn at 0 (predose), 0.5, 1, 1.5, 2, 3, 4.5, 6, 9 and 12 h after the administration. Urine samples were collected −1.5–0, 0–1.5, 1.5–3, 3–4.5, 4.5–6, 6–9, 9–12 and 12–24 h postdose. Blood samples were immediately centrifuged to obtain plasma. Plasma and urine samples were stored at −20 °C until analysed.

RESULTS

Representative chromatograms obtained for plasma and urine samples are shown in Fig. 1. The retention times for hippuric acid, benzoic acid and *o*-chlorobenzoic acid were 4.1, 7.2 and 8.3 min, respectively. Detectable concentrations of hippuric acid were observed in almost all urine samples obtained from subjects who did not receive sodium benzoate. As shown in Fig. 1g, benzoic acid could not be detected in urine samples after the administration of any dose of sodium benzoate. A few unknown peaks were found in several of the plasma samples and in most of the urine samples from a healthy subject receiving sodium benzoate. However, the compounds of interest were well separated and the analyses performed were not affected by the unknowns.

The absolute recoveries of the three analytes from plasma and urine were assessed by comparing peak heights obtained for the standard stock solutions of the analytes and for analyte-free plasma or urine spiked with the respective analytes. An exception was made for urinary hippuric acid, in which case the value was estimated by subtracting the peak height observed before spiking from the one found for the spiked urine sample. As expected from the simple sample prep-

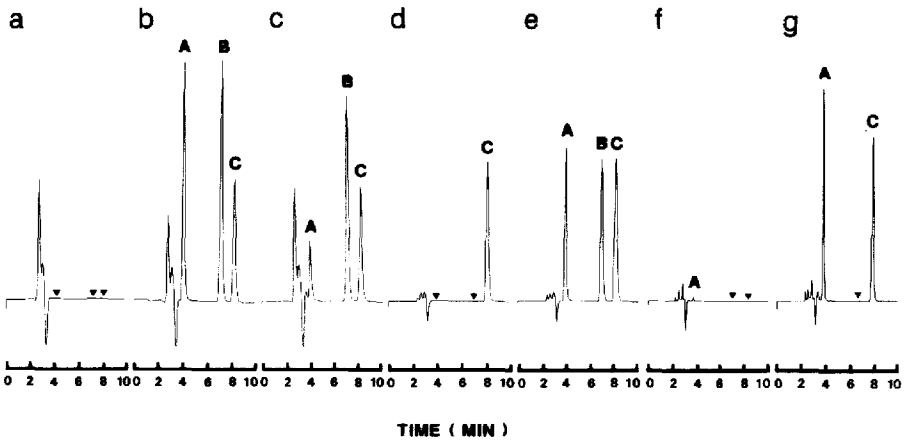

Fig. 1. Representative chromatograms from (a) blank plasma, (b) 100 μl of plasma spiked with 200 μg of hippuric acid (A) and benzoic acid (B) and 25 μg of internal standard (C), (c) plasma obtained from a volunteer after administration of 0.08 g/kg sodium benzoate, (d) blank urine with internal standard, (e) 200 μl of the same urine as in (d) spiked with 100 μg of hippuric acid and 250 μg of internal standard, (f) ten times diluted urine obtained from a volunteer before taking sodium benzoate and (g) ten times diluted urine obtained from a volunteer after administration of 0.08 g/kg sodium benzoate. ▼ indicates the anticipated peak appearance.

aration in which no extraction was involved, the recoveries from plasma were good (97.7–102.0%) for hippuric acid and benzoic acid at concentrations of 20 and 100 μg/ml and for *o*-chlorobenzoic acid at a concentration of 125 μg/ml. The recoveries from urine ranged from 96.9 to 100.6% for hippuric acid and benzoic acid at concentrations of 100 and 500 μg/ml and for *o*-chlorobenzoic acid at a concentration of 625 μg/ml.

The calibration curves were made by plotting the peak-height ratios of hippuric acid and benzoic acid to *o*-chlorobenzoic acid for five different concentrations in plasma and urine covering the range expected to result from the sodium benzoate dosages used for the treatment of patients with inborn errors of urea synthesis. The regression lines were linear over the concentration range examined (10–200 μg/ml for both hippuric acid and benzoic acid in plasma and 50–1000 μg/ml for the acids in diluted urine). The respective correlation coefficients of the calibration curves ranged between 0.9992 and 0.9997.

To assess the precision of the analytical procedure, reproducibilities for both within-day and day-to-day variations were determined (Table I). The coefficients of variation (C.V.) for five different concentrations in the within-day study

TABLE I

PRECISION AND ACCURACY IN THE DETERMINATION OF BENZOIC ACID AND HIPPURIC ACID IN PLASMA AND URINE

Benzoic acid				Hippuric acid			
Concentration given (μg/ml)	Concentration determined (mean ± S.D.) (μg/ml)	C.V. (%)	Relative error	Concentration given (μg/ml)	Concentration determined (mean ± S.D.) (μg/ml)	C.V. (%)	Relative error
Plasma: within-day variation (n = 7)							
10	10.09 ± 0.79	7.8	0.9	10	9.96 ± 0.94	9.5	−0.5
20	19.50 ± 0.57	2.9	−2.5	20	19.67 ± 0.88	4.5	−1.6
50	50.26 ± 1.68	3.3	0.5	50	50.64 ± 2.20	4.3	1.3
100	97.84 ± 2.79	2.9	−2.2	100	98.67 ± 4.12	4.2	−1.3
200	200.97 ± 1.31	0.7	0.5	200	200.51 ± 2.20	1.0	0.3
Plasma: day-to-day variation (n = 7)							
20	20.85 ± 2.0	9.7	4.2	20	21.09 ± 0.94	4.5	5.4
100	98.89 ± 6.2	6.2	−1.1	100	100.82 ± 1.37	1.4	0.8
Urine: within-day variation (n = 7)							
50	46.95 ± 2.06	4.4	−6.1	50	47.75 ± 3.60	7.5	−4.5
100	98.65 ± 0.92	0.9	−1.3	100	96.00 ± 2.29	2.4	−4.0
200	210.74 ± 3.17	1.5	5.4	200	205.50 ± 3.54	1.7	2.7
500	523.01 ± 4.82	0.9	4.6	500	516.87 ± 8.42	1.6	3.4
1000	986.22 ± 2.63	0.3	−1.4	1000	990.40 ± 4.77	0.5	−1.0
Urine: day-to-day variation (n = 7)							
100	103.55 ± 1.50	1.4	3.6	100	101.59 ± 2.17	2.1	1.6
500	495.68 ± 1.51	0.3	−0.9	500	496.69 ± 1.29	0.3	−0.7

varied between 0.7 and 9.5% for plasma and between 0.3 and 7.5% for urine samples, whereas those in the day-to-day study ranged from 1.4 to 9.7% for plasma and from 0.3 to 2.1% for urine samples.

The accuracy was assessed by analysing known amounts of analytes. The observed concentrations were in good agreement with the actual concentrations. The relative error ranged from −2.5 to 5.4% for plasma and from −6.1 to 5.4% for urine samples (Table I).

Detection limits were determined using diluted stock solutions. Both hippuric acid and benzoic acid could be detected in concentrations down to 1 μg/ml (signal-to-noise ratio > 5) in plasma or diluted urine when a 5-μl aliquot of prepared sample was injected onto the chromatograph.

To investigate the nature of the unknown peak which eluted at 3.5 min before the peak of hippuric acid (Fig. 2a), enzymatic hydrolysis was applied to the urine sample obtained during the time interval 3–4.5 h after the administration of sodium benzoate (0.08 g/kg). To 20-μl urine samples, 10 μl of β-glucuronidase/aryl sulfatase (0.055 and 0.015 I.U., respectively) and 170 μl of 1.0 *M* acetate buffer (pH 5.2) containing varying concentrations of saccharo-1,4-lactone were added. By adding β-glucuronidase/aryl sulfatase, the peak height of the unknown decreased and the peak height corresponding to benzoic acid increased (Fig. 2b). This effect was suppressed by adding 1 m*M* saccharo-1,4-lactone (final concentration) (Fig. 2c). When > 10 m*M* saccharo-1,4-lactone was added, the increase in the peak height for benzoic acid was almost completely inhibited (Fig. 2d). Thus, the unknown peak which eluted at 3.5 min before the peak of hippuric acid

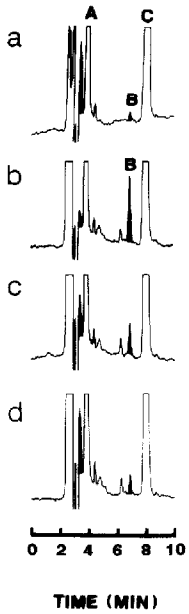

Fig. 2. Chromatograms of the same urine sample as in Fig. 1g (a) without and (b) with β-glucuronidase/sulfatase (0.055 and 0.015 I.U., respectively). Peak height of the unknown (dark shaded, left) eluting just before hippuric acid (A) decreased and that of benzoic acid (B) (dark shaded, right) increased. The above change observed in (b) is suppressed by (c) 1 m*M* and (d) 10 m*M* saccharo-1,4-lactone. Peak C indicates internal standard.

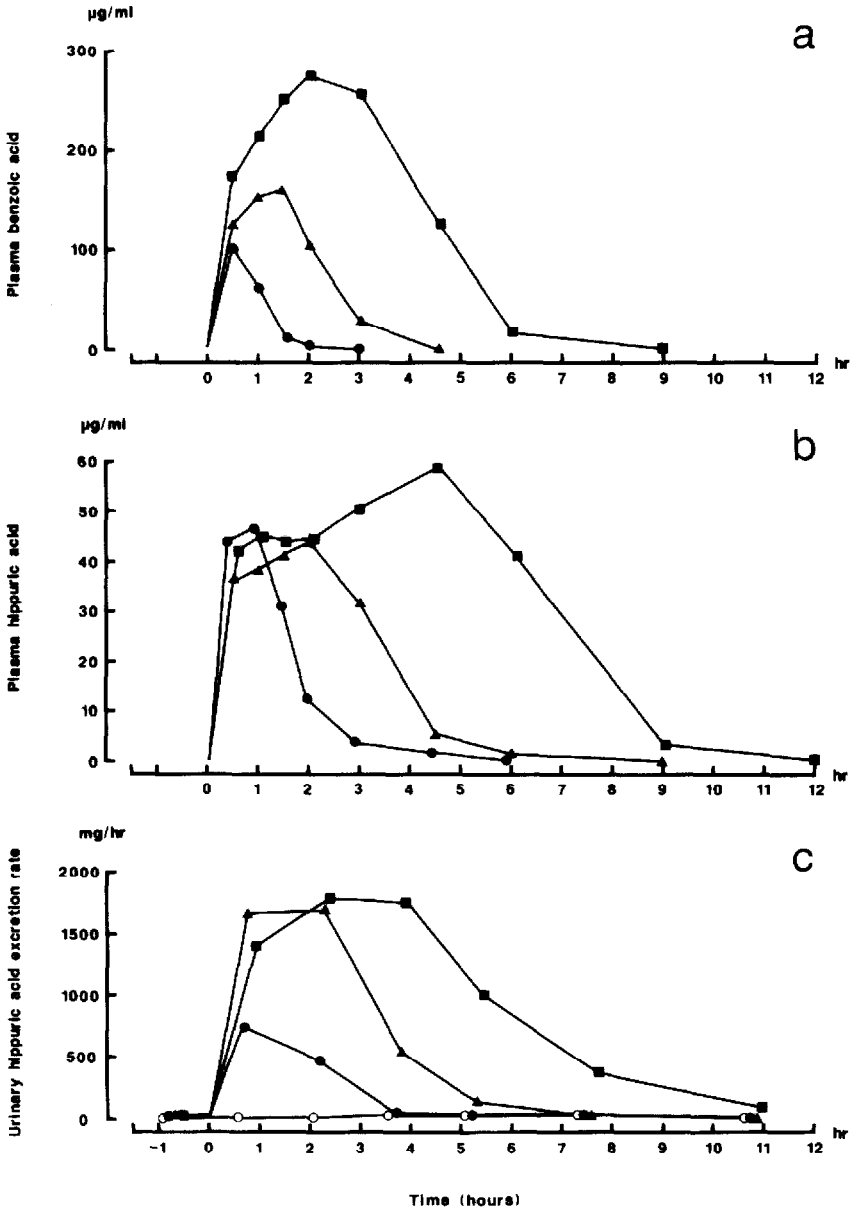

Fig. 3. Plasma concentration-time profiles of (a) benzoic acid and (b) hippuric acid, and (c) urinary excretion rate-time profiles of hippuric acid following the three different doses of sodium benzoate. ●, ▲ and ■ indicate the data observed after the administration of 0.04, 0.08 and 0.16 g/kg sodium benzoate, respectively. The data on urinary hippuric acid (○) without dosing sodium benzoate are plotted, since endogenously derived hippuric acid, although negligible, is excreted in the blank (pre-dose) urine.

TABLE II

PHARMACOKINETIC PARAMETERS DETERMINED FROM A HEALTHY SUBJECT TAKING THREE SINGLE ORAL ADMINISTRATIONS OF 0.04, 0.08 AND 0.16 g/kg SODIUM BENZOATE

Abbreviations: C_{max} = maximum plasma concentration; t_{max} = time to reach C_{max}; AUC_0^∞ = area under the plasma concentration–time curve from zero to infinity; X_{u0}^∞ = cumulative urinary excretion of hippuric acid; Cl_r = renal clearance calculated as $X_{u0}^\infty / AUC_0^\infty$.

Parameter		Value		
		0.04 g/kg	0.08 g/kg	0.16 g/kg
Benzoic acid				
C_{max}	(μg/ml)	100.8	159.4	277.5
t_{max}	(h)	0.5	1.5	2
AUC_0^∞	(μg/ml·h)	92.9	331.6	1075.4
Hippuric acid				
C_{max}	(μg/ml)	46.9	43.8	58.5
t_{max}	(h)	1	2	4.5
AUC_0^∞	(μg/ml·h)	78.1	139.8	350.7
X_{u0}^∞	(mg)	1949	6018	10388
	(% dose)	58.5	90.3	77.9
Cl_r	(ml/min/kg)	6.21	10.71	7.37

was concluded to consist at least in part of benzoyl glucuronide. The concentration of this conjugate was calculated to be approximately 0.1 mg/ml in the undiluted urine sample when expressed as benzoic acid. Roughly, 1.5 (when 0.04 g/kg was given) to 3.5% (when 0.08 and 0.16 g/kg were given) of the administered sodium benzoate was estimated to be converted to the glucuronide.

The preliminary data on the clinical applicability of the proposed HPLC method for hippuric acid and benzoic acid after the oral administration of three different oral doses of sodium benzoate to a healthy normal volunteer are shown in Fig. 3. The pharmacokinetic data for the two analytes after the three different doses are summarized in Table II. The area under the curve (AUC) for plasma benzoic acid does not increase proportionally to the increasing doses administered, whereas the AUC values for hippuric acid were roughly proportional to the doses (Table II). In addition, the maximum plasma concentrations and the urinary excretion rates of hippuric acid were roughly independent of the dose size. These profiles indicate that the biotransformation of benzoic acid to hippuric acid occurs in a saturable or non-linear fashion in the human body.

DISCUSSION

The present paper describes a rapid, sensitive and reliable method for the determination of benzoic acid and hippuric acid. Brief, general descriptions of the determination of hippuric acid and benzoic acid by HPLC in clinical investiga-

tions have occasionally been reported [1,4]. This paper comprehensively describes specific procedures required for research-oriented studies. The precision and accuracy obtained in the present report prove the method to be reproducible and reliable (Table I). In addition, an excellent linearity was observed for the calibration curves for plasma and urine samples ($r > 0.9992$). However, when the assay is applied for measuring hippuric acid in urine samples, some caution is required. Occasionally, following the administration of sodium benzoate, the hippuric acid concentration in urine samples was higher than 10 mg/ml. Because the linearity of calibration curve tended to be lost when hippuric acid concentrations in the samples exceeded 1000 μg/ml, urine should be diluted more than ten times when urine samples with more than 10 mg/ml hippuric acid are to be analysed.

The AUC value for benzoic acid in plasma after administering the largest dose of sodium benzoate (0.16 g/kg) was more than ten times that after the smallest dose (0.04 g/kg), whereas the increases in the AUC values for hippuric acid in plasma were roughly proportional to the dose size (Table II). In addition, the maximum concentrations of hippuric acid in plasma and the maximum urinary excretion rates of hippuric acid were independent of the dose size. Moreover, the concentrations of hippuric acid in plasma and the urinary excretion rates of hippuric acid seemed to remain fairly constant during 0.5–3 h after the concentration of benzoic acid in plasma began to decline. The above findings indicate that the elimination of hippuric acid from the human body occurs at a first-order rate or in a non-saturable manner. Because no benzoic acid was detectable in the urine samples (Fig. 3a), its elimination by the kidneys is assumed to be negligible.

The cumulative urinary excretion of hippuric acid after the administration of sodium benzoate can be estimated by subtracting the amount of hippuric acid excreted during the period (-1.5 to 0 h or predose period) on the day of the sodium benzoate administration. This yielded 58.5–90.3% of the administered dose of sodium benzoate, when hippuric acid was expressed as the corresponding amount (equivalent) of sodium benzoate. To the best of our knowledge, the complete metabolic disposition of sodium benzoate or its metabolite(s) other than hippurate in the human body is not yet known. However, glucuronide, which is a major metabolite of benzoate in dogs [5], is known to be one of the minor metabolites in man [3,6]. Indeed, a peak eluted at 3.5 min, i.e. before that of hippuric acid in the chromatograms of plasma and urine samples, was concluded to consist at least in part of a glucuronidated form of benzoate, assumed to be derived from the other possible metabolite(s). The results of enzyme hydrolysis showed that part of this peak corresponds to benzoyl glucuronide.

In conclusion, our findings (Tables I and II and Fig. 3) indicate that the present assay may be useful for therapeutic monitoring of benzoic and hippuric acids during treatment with sodium benzoate in patients with inborn errors of urea synthesis, as well as for pharmacokinetic studies of these analytes. Such a study is being conducted in our laboratory, and the results will be reported elsewhere.

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