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GAS CHROMATOGRAPHIC DETERMINATION OF LOW CONCENTRATIONS OF BENZOIC ACID IN HUMAN PLASMA AND URINE

ANTOINE SIOUFI* and FRANÇOISE POMMIER

Ciba-Geigy, Centre de Recherche Biopharmaceutique, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

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SUMMARY

A method for the determination of benzoic acid down to concentrations of 10 ng/ml in plasma or urine is described. After addition of an internal standard, benzoic acid is extracted at acid pH into diethyl ether. Both compounds are derivatized with pentafluorobenzyl bromide. The derivatives are determined by gas chromatography using a ^{63}Ni electron-capture detector. Hippuric acid is hydrolysed in plasma and urine and total benzoic acid is determined by the same technique.

INTRODUCTION

Numerous methods have been already proposed for the quantitative assay of carboxylic acids and particularly benzoic acid in biological fluids. Rowland and Riegelman [1] described a gas—liquid chromatographic (GLC) method using acid pH and diethyl ether for the extraction from plasma, and carbon disulphide as solvent for the preparation of the trimethylsilyl derivatives of submicrogram amounts of carboxylic acids. Coward and Smith [2] reported the gas chromatography of aromatic acids as their trimethylsilyl derivatives, including applications to urine analysis. Sinsheimer and Breault [3] made determinations of various benzoic acid derivatives to test their suitability for metabolism studies. Quantitative determinations of microgram amounts of these derivatives were obtained by gas chromatography, and by reverse thin-layer chromatography—fluorimetry using the quenching of a fluorescent background. Gossele [4] described a gas chromatographic determination of aromatic acids used as preservatives in food. After extraction, these acids were derivatized with N,O-bis(trimethylsilyl)acetamide and detected with a flame ionization

*To whom correspondence should be addressed.

detector. Benzoic acid was quantitatively determined in this way. Fransson et al. [5] used a liquid–liquid chromatographic system based on ion-pair partition, with silica microparticles as support for the stationary phase, to separate anionic compounds of biochemical and pharmacological interest, including benzoic acid. Amsel and Levy [6] described a pharmacokinetic study of the simultaneous conjugation of benzoic and salicylic acids with glycine, giving hippuric and salicyluric acid, respectively.

All these methods suffer from a lack of sensitivity, as none of them is capable of detecting benzoic acid down to 1 μg/ml. This paper describes the GLC determination of benzoic acid in human plasma and urine at concentrations down to 10 ng/ml using 3-phenylpropionic acid as internal standard. This technique permits the quantitative assay of free benzoic acid in plasma and urine. Acid hydrolysis is used for the determination of total benzoic acid in plasma and urine, thus giving the conjugated benzoic acid (hippuric acid) by difference.

MATERIALS AND METHOD

Reagents

All reagents and solvents are of analytical grade: diethyl ether, sulphuric acid, acetone (Merck, Darmstadt, G.F.R.) and benzene (Mallinckrodt, St. Louis, Mo., U.S.A.). Pentafluorobenzyl bromide (Regis, Morton Grove, Ill., U.S.A.) is stored at 4° in glass containers.

The two methanolic internal standard solutions contain, respectively, 500 ng per 100 μl and 2000 ng per 100 μl of 3-phenylpropionic acid.

Apparatus

Glass tubes are washed with 5 ml of diethyl ether by shaking them mechanically (Infors shaker) for 15 min.

A Hewlett-Packard Model 5710A gas chromatograph equipped with a Hewlett-Packard Model 18713A electron-capture detector is used. The peak areas are given by a Hewlett-Packard Model 3380A electronic integrator. The column is operated at 148°, the injector at 250° and the detector at 300°, with an argon–methane (90:10) flow-rate of 60 ml/min. Glass columns are washed with 1 *M* hydrochloric acid, distilled water, acetone and benzene, and then silanized with a 1% (v/v) solution of hexamethyldisilazane in benzene. After the treatment, the columns are washed again with benzene and dried at 100°.

The column packing is 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The filled columns are flushed with the carrier gas at a flow-rate of 40 ml/min and heated to 300° at a rate of 1°/min. The column temperature is held overnight at 300° and throughout the next day at 270°. The temperature is then repeatedly increased from 150 to 250° for 24 h. During these programmed cycles, the columns are further conditioned by injecting a total of 100 μl of Silyl 8 (Pierce, Rockford, Ill., U.S.A.) by fractions between 150 and 220°. After this treatment, the columns are ready for use.

Acid hydrolysis in plasma and urine

Aliquots (1 ml) of plasma or urine (diluted with water if necessary) and 1 ml of concentrated hydrochloric acid are heated for 16 h at 100° [6]. The extraction is then performed as described below.

Extraction

One hundred microlitres of the internal standard solution are measured into a glass tube, to which 1 ml of the sample, 1 ml of 0.5 *M* sulphuric acid and 5 ml of diethyl ether are then added. The tube is stoppered and shaken mechanically (Infors shaker) for 15 min at 300 rpm and centrifuged at 3900 *g* for 10 min.

An aliquot of the ether phase is transferred to another tube and taken to dryness under vacuum in a rotary evaporator (Büchi) in a water-bath at room temperature.

Derivatization and chromatography

To the dry residue are added 100 mg of anhydrous potassium carbonate and 1 ml of acetone containing 5 mg of pentafluorobenzyl bromide. Each tube is stoppered tightly and put in a dry heating block (Grant Instruments) at 50° for 2 h. After this time, the contents of the tube are evaporated to dryness under a nitrogen stream in a water-bath at room temperature. To the dry residue, 2 ml of water and 1 ml of benzene (4 ml after acid hydrolysis of urine) are added, and the tube is shaken mechanically for 10 min at 300 rpm and centrifuged for 3 min at 2450 *g*.

A 2-μl portion of the benzene layer is injected into the gas chromatograph by the solvent-flush technique.

The benzoic acid content is calculated from the ratio of the peak areas by reference to a calibration curve prepared from a series of methanolic benzoic acid solutions added to water to yield concentrations between 10 and 1000 ng/ml (with 500 ng internal standard), and between 1000 and 6000 ng/ml (with 2000 ng internal standard). This latter range is used after acid hydrolysis of urine.

Collection of samples

Plasma and urine were obtained from three healthy male subjects, who had been instructed not to take any drugs from eight days before and until the end of the study.

Blood samples were drawn at the beginning of the experiment (about 8 a.m.) and at 2, 4, 6, 8, 24, 72 and 120 h thereafter; the blood was transferred to heparinized tubes and centrifuged immediately. Plasma was removed and stored at −20° until analysis.

Urine was collected at the following intervals: 0–4, 4–8, 8–12, 12–24, 24–32, 32–48, 72–80, 80–96, 120–128 and 128–144 h. The volume was measured and an aliquot was stored at −20° until analysis.

RESULTS AND DISCUSSION

Reaction time

The duration of the derivatization reaction was varied from between 30 min and 3 h. A maximum yield of derivative was obtained after 2 h.

Evaporation

After the extraction of benzoic acid with diethyl ether, this solvent is evaporated under vacuum. No reproducible results were obtained when it was taken to dryness under a nitrogen stream at room temperature.

Plasma or urine interference

The chromatograms of blank human plasma and urine extracts showed a peak with the same retention time as the derivative of benzoic acid. Gas chromatographic—mass spectrometric analysis showed the compound corresponding to this peak to be identical with the derivative of benzoic acid: it is known that benzoic acid is normally present in human plasma and urine. For this reason, the calibration curve is obtained using aqueous solutions of benzoic acid. Some chromatograms of urine extracts showed a peak with the same retention time as the internal standard (3-phenylpropionic acid). In these cases, phenylacetic acid was used as internal standard. After ten consecutive injections, a 90-min interval is allowed to wash out non-hydrolysed plasma and urine residues from the column.

Fig. 1 shows the chromatograms of a reagent blank extract and of water containing 400 ng of benzoic acid, 500 ng of 3-phenylpropionic acid and 500 ng of phenylacetic acid. Fig. 2 shows the chromatogram of a blank plasma extract (500 µl of plasma). Fig. 3 shows the chromatogram of a blank urine extract (1 ml from urine diluted 1/100) after acid hydrolysis.

Sensitivity and reproducibility

Table I gives the results obtained when the method is applied to aqueous

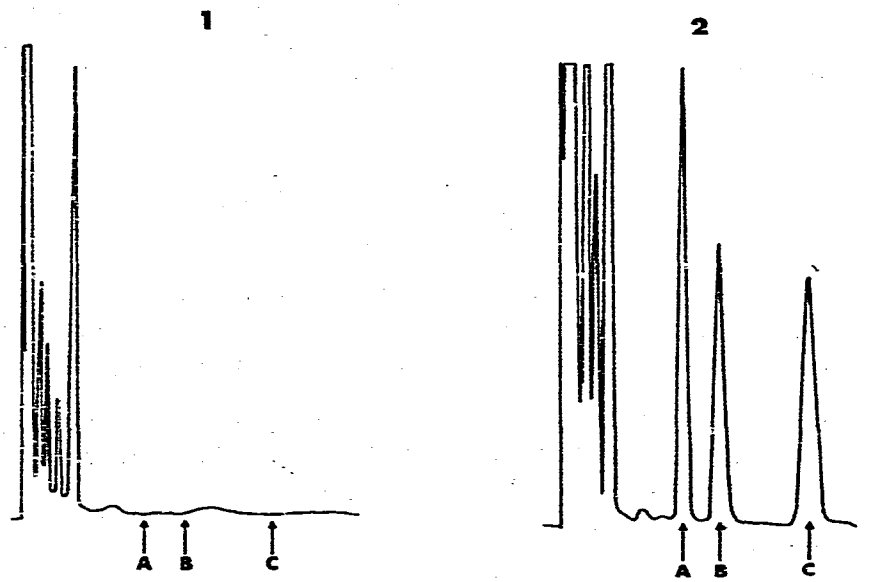

Fig. 1. Examples of chromatograms: (1) reagent blank extract; (2) water containing benzoic acid at 400 ng/ml (A), phenylacetic acid at 500 ng/ml (B) and 3-phenylpropionic acid at 500 ng/ml (C).

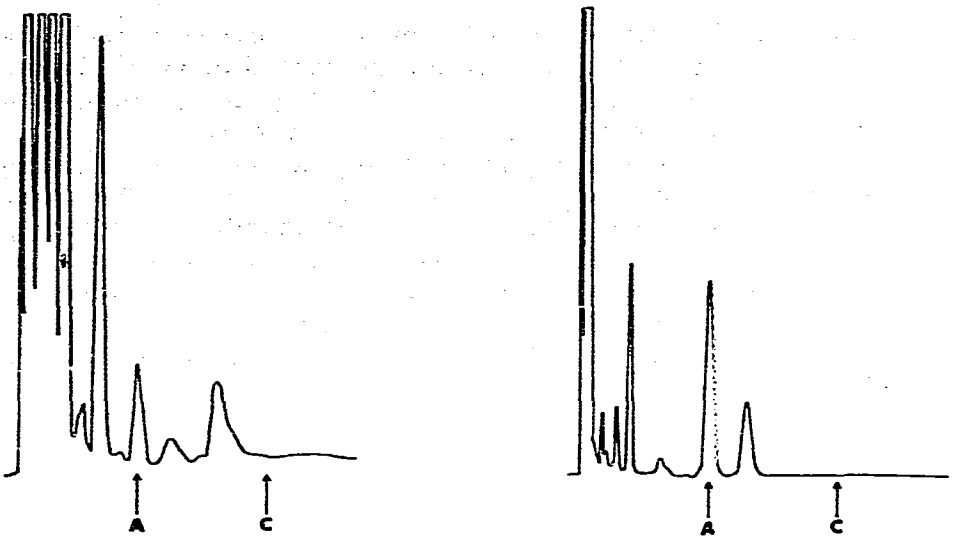

Fig. 2. Blank plasma extract (500 μl of plasma) containing free benzoic acid (A) without any interference at the location of 3-phenylpropionic acid (C).

Fig. 3. Blank urine extract (1 ml from urine diluted 1/100) after acid hydrolysis containing total benzoic acid (A) without any interference at the location of 3-phenylpropionic acid (C).

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF BENZOIC ACID APPLIED TO AQUEOUS SOLUTIONS

Amount added (ng/ml)	Amount found: average of six assays (ng/ml)	95% confidence interval	Coefficient of variation (S.D. %)
10	11.1	9.5—12.6	13.3
20	21.1	19.0—23.3	9.8
50	48	45—50	4.6
200	197	190—204	3.4
400	394	373—416	5.2
1000	1029	999—1058	2.8
2000	2004	1942—2066	2.9

solutions. The 95% confidence intervals and the coefficients of variation were calculated on the basis of six replicate analyses of each sample. The lower concentration (10 ng/ml) may be taken as the sensitivity limit of the assay, although even lower concentrations could be detected.

Application

The technique was applied to measure the elimination of free and total benzoic acid in non-medicated subjects down to very low concentrations. The plasma and urine concentrations of free and total benzoic acid are given in Tables

II and III, respectively. The differences between total and free benzoic acid were expressed as hippuric acid; they are within the normal range of hippuric acid excretion in human urine, 0.1—1.0 g per 24 h [7]. In addition, small amounts of free benzoic acid were found in both plasma and urine.

Hippuric acid is normally present in human urine as a metabolite of dietary components containing food additives. Lehninger [8] reported that fatty acids with an uneven number of carbon atoms are partly metabolized into benzoic acid, but these fatty acids do not exist in terrestrial animals. The formation of hippuric acid from small doses of benzoic acid is extremely rapid in man, and the renal excretion of hippuric acid is not rate limited by the capacity of the renal tubular transport system, even at the highest excretion rates obtained after administration of benzoic acid [6].

TABLE II

FREE AND TOTAL BENZOIC ACID IN THE PLASMA OF THREE HEALTHY SUBJECTS DURING SIX DAYS, THE DIFFERENCE BETWEEN TOTAL AND FREE BENZOIC ACID BEING EXPRESSED AS HIPPURIC ACID

Subject	Hours	Free benzoic acid (ng/ml)	Total benzoic acid (ng/ml)	Hippuric acid (ng/ml)
1	0	40	1040	1467
	2	26	860	1224
	4	48	790	1089
	6	14	1310	1901
	8	36	680	945
	24	18	1240	1793
	72	36	950	1341
	120	62	1130	1567
2	0	30	1540	2215
	2	52	1160	1626
	4	66	1150	1590
	6	46	1390	1972
	8	52	1380	1948
	24	28	1640	2365
	72	50	2000	2861
	120	36	1990	2867
3	0	164	960	1168
	2	64	1220	1696
	4	136	820	1004
	6	92	1280	1743
	8	82	1160	1582
	24	34	880	1153
	72	86	850	1121
	120	224	930	1036

TABLE III

FREE AND TOTAL BENZOIC ACID IN THE URINE OF THREE HEALTHY SUBJECTS DURING SIX DAYS, THE DIFFERENCE BETWEEN TOTAL AND FREE BENZOIC ACID BEING EXPRESSED AS HIPPURIC ACID

Subject	Hours	Urine volume (ml)	Free benzoic acid (μg)	Total benzoic acid (mg)	Hippuric acid (mg)	Hippuric acid (mg per 24 h)
1	0—4	0	—	—	—	
	4—8	440	510	66	95	
	8—12	0	—	—	—	
	12—24	570	730	450	659	754
	24—32	165	426	166	242	
	32—48	725	1073	435	637	879
	72—80	475	1739	148	214	
	80—96	570	1277	213	311	525
	120—128	266	1229	87	126	
	128—144	580	1032	373	546	672
2	0—4	140	511	125	182	
	4—8	0	—	—	—	
	8—12	560	1764	195	283	
	12—24	300	930	315	461	926
	24—32	420	1638	318	464	
	32—48	320	1024	316	463	926
	72—80	770	2695	323	469	
	80—96	885	1726	279	406	875
	120—128	235	693	175	255	
	128—144	1020	1989	395	577	832
3	0—4	177	404	45	66	
	4—8	168	349	48	70	
	8—12	210	336	85	125	
	12—24	450	765	217	317	578
	24—32	430	568	104	151	
	32—48	500	820	250	365	516
	72—80	165	198	18	26	
	80—96	525	536	190	277	303
	120—128	490	431	102	150	
	128—144	525	567	207	302	452

CONCLUSION

The gas—liquid chromatographic technique described permits the quantitative assay of free benzoic acid and hippuric acid at low concentrations in plasma and urine.

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