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GAS CHROMATOGRAPHY–MASS SPECTROMETRY STUDIES OF HIPPURIC ACID DERIVATIVES

I. EVALUATION OF METHODS FOR QUANTITATIVE DETERMINATION OF HIPPURIC ACID. A SEARCH FOR BENZOYLSARCOSINE EXCRETION IN NORMAL URINE

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SUMMARY

Hippuric acid can be determined, after conversion to its methyl ester with diazomethane, by gas chromatography on cyclohexane dimethanol succinate. Constant response factors are obtained with the methyl esters of either stearic or palmitic acid as internal standards. Benzoic acid can also be determined as its methyl ester in this system.

By on-column methylation with trimethylphenylammonium hydroxide, hippuric acid yields hippuric acid methyl ester and benzoylsarcosine methyl ester. Benzoylsarcosine was not detected in normal urine after ingestion of benzoic acid.

With trimethylsilyl as well as with the methyl derivatives of hippuric acid, the rule is confirmed that a higher methyl content leads to decreased elution times on silicon phases.

INTRODUCTION

Hippuric acid (benzoylglycine) is the major metabolite of benzoic acid in man[1]. Benzoic acid itself is formed from phenylalanine[2], toluene and other alkylbenzenes[3], the major part of benzoic acid, however, being of dietary origin.

Increased amounts of hippuric acid are excreted in patients with gastrointestinal disorders and malabsorption[4]. Quantitative aspects of hippuric acid formation are of interest in the evaluation of liver function (Quick test)[5], in the study of pharmacokinetics of salicylic acid[6,7] and in industrial medicine[8,9]. Of considerable biochemical–genetic interest has been the demonstration of diminished excretion of hippuric acid and of excretion of benzoic acid in a patient with Lesch–Nyhan syndrome[10,11], of

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the missing incorporation of radioactive glycine into hippuric acid in a patient with isovaleric acidemia[12], and of excretion of large amounts of benzoic acid in 12 cases of undefined mental retardation[13]. At standard diet, 3.86 mg of hippuric acid are excreted per kilogram of body weight per 24 h (ref. 14). Despite its large abundance in urine and despite its easy qualitative demonstration by gas–liquid chromatography (GLC), only a few papers have reported on the quantitative determination of hippuric acid by GLC[15,16].

In this paper, our attempts to develop a reliable quantitative GLC method for the determination of hippuric acid are described. In addition, we report on a search for benzoylsarcosine excretion in urine. In a subsequent paper[17], the mass spectra of trimethylsilyl and methyl derivatives of hippuric acid will be given.

MATERIALS AND METHODS

The studies were undertaken with a Varian 2100 instrument (Varian Aerograph, Walnut Creek, Calif. 94598) equipped with a flame ionization detector (FID) and a Texas Instruments recorder (Texas Instruments, Houston, Texas 77001), and with a Nuclear Chicago 5000 instrument (Nuclear Chicago, Des Plaines, Ill. 60018) equipped with an FID and a Sargent-Welch dual-pen recorder (Sargent-Welch Scientific Company, Skokie, Ill. 60076). In the first instrument, nitrogen was used as carrier gas at 25 ml/min, and in the second instrument argon at 60 ml/min. U-shaped glass columns, 6 ft. long and with I.D. 3 or 6 mm, were used.

In order to minimize the destruction of compounds by contact with metal surfaces, we exchanged the metal tubing between the column outlet and the FID in the Nuclear Chicago instrument with a segment of PTFE tubing. It was found to be stable up to 240°. Mass spectra were obtained with an LKB 9000 gas–liquid chromatographic–mass spectrometric (GLC–MS) instrument. Details of the methods and results will be given in a subsequent paper[17].

The column packings, BF_3–methanol (14%, w/v), the fatty acid and alkane standards, as well as bis(trimethylsilyl)acetamide (BSA), trimethylchlorosilane (TMCS) and bis(trimethylsilyl)trifluoroacetamide (BSTFA), were obtained from Applied Science Laboratories Inc., State College, Pa. 16801. Pyridine (silylation grade) and reaction vials with PTFE-lined screw-caps were obtained from Pierce Chemical Co., Rockford, Ill. 61105. Dexsil-300 on 100–120 mesh Supelcoport was obtained from Supelco Inc., Bellafonte, Pa. 16823. Trimethylphenylammonium hydroxide (TMPAH) as a 0.1 *M* solution in methanol was obtained from Eastman Organic Chemicals, Rochester, N.Y. 14650.

For the generation of diazomethane, Diazald was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wisc. 53233, and carbitol from Matheson, Coleman and Bell, Norwood, Ohio 45212. Hippuric acid was obtained from Sigma Chemical Co., St. Louis, Mo. 63178, benzoic acid from J. T. Baker Chemical Co., Phillipsburg, N.J. 08865, and all solvents from Mallinckrodt, Chemical Works, St. Louis, Mo. 63160.

DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. 08854. The neutral Amberlites XAD-2, -4 and -7 were obtained from Rohm and Haas, Philadelphia, Pa. 19105. [^{14}C]Hippuric acid was obtained from American Radiochemical Corp., Sanford, Fl. 32771, and [carboxyl-^{14}C]benzoic acid from International Chemical and Nuclear Corp., Irvine, Calif. 92664.

Urine extraction with ethyl acetate and ether was performed as described by Dalgliesh *et al.*[18]. The isolation of benzoic acid and hippuric acid by DEAE-Sephadex chromatography was carried out essentially by the procedure of Jaakonmaki *et al.*[19]; 20-ml plastic syringes were used as columns.

The XAD resins were washed thoroughly before use. Glass columns of 1 cm I.D. were filled to a height of 9 cm and 3 ml of urine with added radioactive acids were applied to the resin bed. Elution was carried out with 20 ml of water and 25 ml of ether at a flow-rate of about 10 drops/min.

Silylation was carried out in 100 μl of pyridine with either 100 μl of BSA plus 50 μl of TMCS or with 100 μl of BSTFA alone. For on-column methylation with TMPAH[20], hippuric acid was dissolved in the reagent and the solution injected with the injection port maintained at 270°.

Methylation with BF_3–methanol was performed in a boiling water-bath. The reaction was stopped by adding an equal volume of water and extracting twice with 5 volumes of chloroform. Methylation with diazomethane was carried out essentially by the procedure of Schlenk and Gellerman[21].

The response factors (R.F.) were calculated from the equation

$$\text{R.F.} = \frac{\text{weight of internal standard} \times \text{area of substance}}{\text{weight of substance} \times \text{area of internal standard}}$$

The methylene units were determined with linear temperature programming as described by Dalgliesh *et al.*[18]

RESULTS

Trimethylsilyl derivatives

A double peak was obtained by chromatography of silylated hippuric acid on 3% OV-17 and Dexsil-300. Mass spectra[17] showed the presence of a monosilylated and a disilylated hippuric acid, the disilylated product being eluted first on both columns. No condition could be found that led to the formation of a single derivative. With a 6-ft. column of 3 mm I.D. packed with SE-30 or OV-1, both derivatives eluted as a single peak.

Quantitative studies with TMS-palmitic acid as internal standard showed a loss of part of the TMS-hippuric acid, as shown in Fig. 1. As a consequence, the response factor for hippuric acid is dependent on the amount of substance injected. No loss was observed in the same system for TMS-benzoic acid.

On-column methylation with TMPAH

This procedure yielded two derivatives that were partly resolved on 5% OV-1 in a 6-ft. column of 6 mm I.D. Excessive tailing was observed for both components, as shown in Fig. 2. MS[17] showed the first eluting component to be N-methylhippuric acid methyl ester (benzoylsarcosine methyl ester). The second eluting component was hippuric acid methyl ester.

Methylation with BF_3–methanol

Hippuric acid methyl ester was obtained with this procedure. However, after derivatization for 30 min at 100°, 10% of the hippuric acid appeared on the chromato-

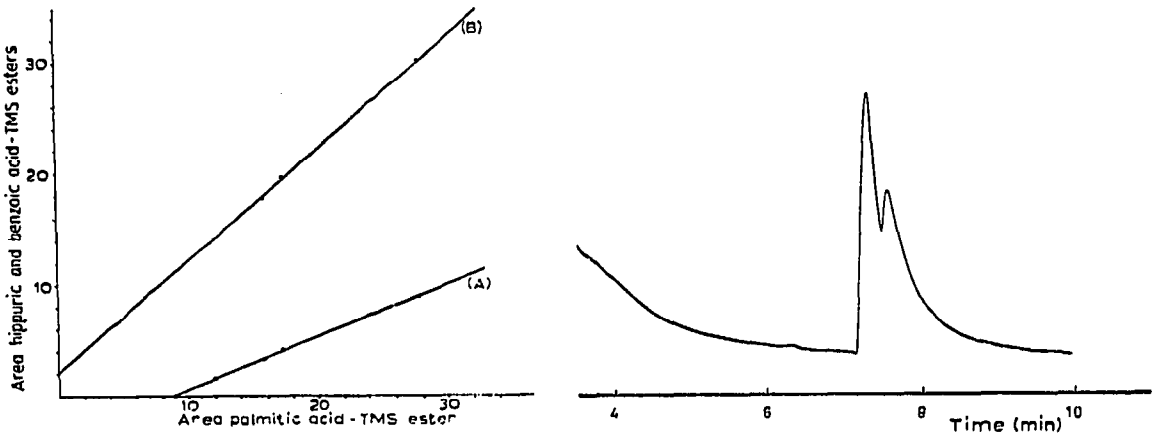

Fig. 1. Test for component loss of TMS esters of hippuric acid (A) and benzoic acid (B) with palmitic acid as internal standard. Trimethylsilylation with BSA and TMCS in pyridine. Varian Model 2100 gas chromatograph with FID. Injection port and detector maintained at 250°. Column: 6 ft., 3% SE-30 on Chromosorb Q, programmed from 100 to 200° at 6°/min, with a nitrogen flow-rate of 25 ml/min. Increasing amounts of a sample containing all three esters were injected. Sensitivity of electrometer, -9×4 and -9×8. Areas were determined by weighing the peaks cut from Xerox copies of the recordings. Large losses of TMS-hippuric acid were observed, which results in response factors being dependent on the amount of TMS-hippuric acid injected. Under the given conditions, TMS-benzoic acid appears to be more stable than TMS-palmitic acid.

Fig. 2. On-column methylation of hippuric acid with trimethylphenylammonium hydroxide. Nuclear Chicago Model 5000 gas chromatograph with FID, sensitivity -10×8. Injection port maintained at 270°. Column: 6 ft. × 6 mm I.D., 5% OV-1, programmed from 150 to 250° at 5°/min, with an argon flow-rate of 120 ml/min. Mass spectra[17] showed the first peak to be N-methylhippuric acid methyl ester and the second peak to be hippuric acid methyl ester.

gram as benzoic acid methyl ester, and after 60 min it amounted to 20%. Determinations were made on 3% EGSS-X with linear temperature programming from 70 to 185° at a rate of 5°/min. Methylation of benzoic acid at room temperature was found to be incomplete, even after incubation for 25 h.

Methylation with diazomethane

This reaction yielded hippuric acid methyl ester as a single derivative. Methyl esters of both benzoic and hippuric acid showed excessive tailing on the silicone phases 3% SE-30 and 3% OV-17, both on Gas-Chrom Q. An ideal peak shape was found for benzoic acid methyl ester on ethylene glycol succinate, 15% HI-EFF 2 BP on Gas-Chrom P, with temperature programming from 70 to 160° at 5°/min. Hippuric acid methyl ester was not eluted during the temperature program. On 3% EGSS-X on Gas-Chrom Q, with temperature programming from 70 to 185° at 5°/min, benzoic acid methyl ester showed some tailing and hippuric acid methyl ester eluted shortly after the end of the temperature program. Finally, the column packing cyclohexane dimethanol succinate (3% HI-EFF 8 BP on 100–120 mesh Gas-Chrom Q), with temperature programming from 70 to 215° at 5°/min, was found to be convenient for the determination of the methyl esters of both benzoic and hippuric acid.

In Table I, the response factors of these derivatives *versus* palmitic acid methyl

TABLE I

PROPERTIES OF METHYL BENZOATE AND METHYL HIPPURATE ON 3% HI-EFF 8BP

Conditions as given in legend to Fig. 4.

Ester	*Response factor vs. methyl palmitate*	*Methylene unit**
Methyl benzoate	1.09	14.83
Methyl hippurate	0.74	27.09

* The methylene unit of methyl hippurate was obtained by linear interpolation between the elution times of the C_{24} and C_{28} alkanes.

ester as internal standard as well as the methylene units under these particular conditions are given.

In Fig. 3, it is shown that with stearic acid methyl ester as internal standard, no indication of significant loss of components can be found for either benzoic acid methyl ester or hippuric acid methyl ester.

In Fig. 4a and 4b, two examples of the separation system are given.

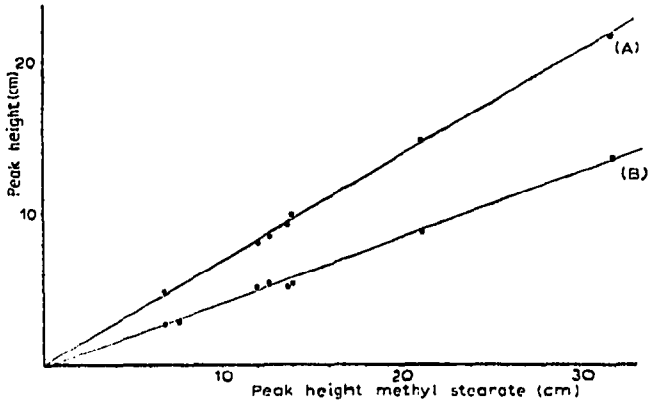

Fig. 3. Test for component loss of methyl esters of hippuric acid (A) and benzoic acid (B) with stearic acid as internal standard. Methylation with diazomethane. Increasing amounts of a sample containing all three esters were injected and the peak heights measured. Proportionality was observed for both hippuric acid and benzoic acid. Conditions as given in legend to Fig. 4. Sensitivity of electrometer, -11×4 and -11×8.

Isolation of organic acids from urine

The yields from the ethyl acetate–ether extraction method have not been determined.

The recovery of [^{14}C]hippuric acid in the buffer eluate after DEAE-Sephadex column chromatography was found to be 100%. The yield for [^{14}C]benzoic acid was found to be 90%, owing to some adsorption on the plastic column used. The recovery of [^{14}C]benzoic acid was 100% with ether elution from Amberlite XAD-2. No radioactivity was found in the water eluate. [^{14}C]hippuric acid was eluted in part by water and the remainder by ether from Amberlites XAD-2, -4 and -7.

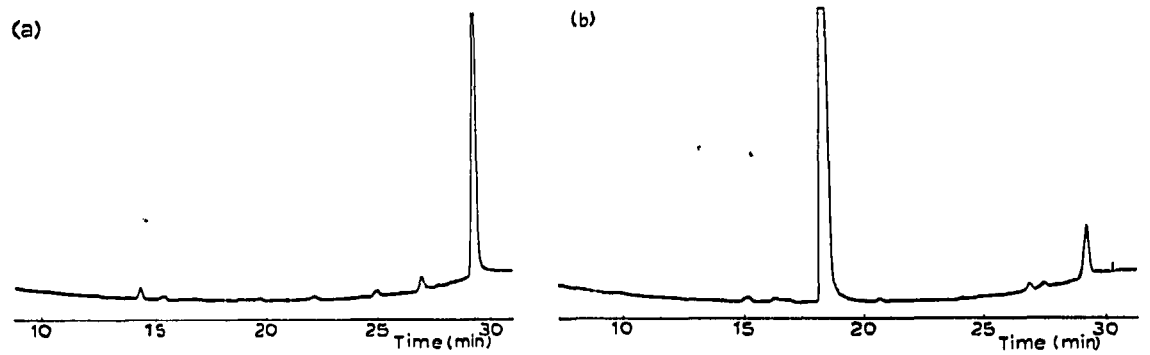

Fig. 4. (a) Separation of methyl esters of urinary organic acids from a normal adult control. Isolation of organic acids by ethyl acetate–ether extraction. Methylation was performed with diazomethane. Nuclear Chicago Model 5000 gas chromatograph with FID, sensitivity -10×4. Column: 6 ft. $\times$ 3 mm I.D., 3% HI-EFF 8 BP, programmed from 70 to 215° at 5°/min, with an argon flow-rate of 60 ml/min. The largest peak was hippuric acid methyl ester. (b) Separation of methyl esters of urinary organic acids from a patient with isovaleric acidemia (same patient as described in ref. 12). Methods and conditions as in (a). The largest peak was isovalerylglycine methyl ester (confirmed by MS), and the smaller peak was hippuric acid methyl ester (confirmed by elution time).

Search for benzoylsarcosine in normal urine

After oral ingestion of 5 g of sodium benzoate by one of us (U.L.), an aliquot of the urine obtained in the first 5 h was extracted with ethyl acetate and ether and the extract methylated with diazomethane as described above. Multiple mass scans were made of the hippuric acid methyl ester peak. The fragments at *m/e* 102, 206 and 207, characteristic of benzoylsarcosine methyl ester[17], could not be detected.

DISCUSSION

By GLC–MS studies, we confirmed the prediction of Butts[22] that the disilylated hippuric acid elutes earlier on polar silicone phases than the monosilylated derivatives. With the introduction of new methyl groups, polar interactions with the liquid phase are reduced. We found the same rule to apply to the methyl derivatives of hippuric acid: benzoylsarcosine methyl ester eluted earlier than hippuric acid methyl ester. Even though the TMS derivative of hippuric acid is more easily prepared, its instability excludes this method for use in the quantitation of hippuric acid excretion.

The time-saving method of methylation with BF_3–methanol, proposed by Alcock[23] to be carried out on lyophilized urine samples, proved to be impractical for hippuric acid because of acid hydrolysis of this compound by the reagent.

Through strong interaction with the stationary phase, the methyl esters of benzoic acid and hippuric acid showed extensive tailing on the silicone phases OV-1 and OV-17, and to a lesser degree on the mixed silicone–polyester phase EGSS-X. Tailing of hippuric acid methyl ester has also been observed by Dalgliesh *et al.*[18] on the silicone phase F-60.

Satisfactory results were obtained in the chromatography of the methyl esters (prepared from diazomethane) on cyclohexane dimethanol succinate (3% HI-EFF 8 BP on Gas-Chrom Q).

In an attempt to find a faster method for determination of benzoic acid and

hippuric acid, we modified the photometric method of Amsel and Levy[6] for possible application in GLC. Benzoic acid is extracted with carbon tetrachloride before and after acid hydrolysis of the urine and after incubation of urine with β-glucuronidase. The extraction gave a recovery of 90%. Because of the stability of TMS-benzoic acid (*cf.* Fig. 1), silylation can be used for determination by GLC. Carbon tetrachloride extracts of normal hydrolyzed urines showed only one additional peak in the chromatogram, well separated on Dexsil-300 from TMS-benzoic acid. This high selectivity of carbon tetrachloride for benzoic acid was disadvantageous: obviously, by azeotropic distillation, large losses of benzoic acid occurred during evaporation of the solvent, also after addition of piperidine as a base. This method was therefore abandoned.

Van der Heiden *et al.*[24] described a method of alkaline hydrolysis of urine and subsequent extraction with ethyl acetate. The recovery of benzoic acid was reported to be quantitative[4].

After our work was completed, we obtained the communication of Sedivec and Flek[16]. These authors reported on the determination of hippuric acid as the methyl ester prepared from diazomethane on 3% neopentyl glycol succinate in isothermal runs at 190°. Extraction of the acidified urine with ethyl acetate was found to be easier with addition of ammonium sulphate instead of sodium chloride.

The methyl ester methods described in this paper, by Williams and Sweeley[15] and Sedivec and Flek[16], although time-consuming, appear to be more suitable than the photometric[6] and thin-layer chromatographic methods[25] because of higher selectivity, greater resolution and the opportunity to study benzoic and hippuric acid in a single analysis. Another method for the simultaneous determination of benzoic and hippuric acid using column chromatography on Sephadex G-10 (ref. 26) is very simple, but, owing to a smaller degree of resolution in comparison with GLC, UV-absorbing substances (drugs, etc.) may sometimes interfere in the analysis. On silicic acid columns, hippuric acid partially overlaps with other UV-absorbing compounds[27].

The most promising method, high-pressure ion-exchange chromatography, in the present state of the art needs 24 h or, by using two columns, at least 12 h per analysis of a single urine sample[28]. Until high-pressure liquid chromatography becomes applicable to routine work, GLC remains the method of choice for the study of urinary organic acids[13].

The identification by MS of N-methylhippuric acid methyl ester after on-column methylation with TMPAH led to a search for the presence of N-methylhippuric acid (benzoylsarcosine) in urine, with negative results. It is of interest to look for this compound in the urine of patients with hypersarcosinemia[29]. To the best of our knowledge, sarcosine has not been tested as a substrate for glycine-N-acylase. This enzyme was found to be inhibited by *p*-aminohippuric acid but not by *p*-aminobenzoylsarcosine[30].

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