antipyretic effect between taurinophenetidine hydrochloride and nicotinoyltaurinophenetidine hydrochloride; it is hoped that this problem will be solved in the near future.

In conclusion, taurinophenetidine and nicotinoyltaurinophenetidine are scarcely excreted in rat feces and in rat and rabbit bile. The amount of both compounds distributed in organs of mice and rats is very small. Taurinophenetidine has some analgesic and antipyretic activities, and nicotinoyltaurinophenetidine shows some analgesic and anti-inflammatory activities.

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Chromatographic Determination of Benzoic Acid Derivatives for Application to Metabolism Studies

JOSEPH E. SINSHEIMER and GEORGE O. BREAULT*

Abstract □ Thin-layer and gas chromatographic systems were developed in support of metabolism studies to detect 4-hydroxybenzoic, 2-hydroxyanisic, and 3-hydroxyanisic acids in the presence of benzoic acid. Chromatographic systems were also developed for the detection of 4-chlorobenzoic acid in the presence of benzoic and 4-hydroxybenzoic acids. Quantitative results were obtained by gas chromatography and by reverse TLC fluorimetry of the quenching of a fluorescent background.

Keyphrases □ Benzoic acid derivatives—determination as metabolites □ GLC—analysis □ TLC—analysis □ Fluorometry, quenching TLC—analysis

It was found necessary to develop procedures for the separation, identification, and assay of benzoic acid derivatives in support of studies in these laboratories of the importance of cleavage of the ethylenic bond in the metabolism of stilbenes (1). Sensitive methods applicable to rabbit urine extracts containing benzoic acid as a normal metabolite were required.

The initial stilbene under consideration was 4,4′-dimethoxystilbene. It was expected from the reported cleavage of *trans*-stilbene to benzoic acid (2, 3) that the cleavage product of 4,4′-dimethoxystilbene would be anisic acid. Moreover, since *O*-demethylation and aromatic hydroxylation are common metabolic reactions, it was felt that cleavage could also yield 4-hydroxybenzoic, 2-hydroxyanisic, and 3-hydroxyanisic acids. Therefore, it was necessary to develop analytical systems for the detection and estimation of these compounds as possible metabolites.

The major expected cleavage product of 4,4′-dichlorostilbene would be 4-chlorobenzoic acid; emphasis was placed on the analysis of this product in the presence of naturally occurring benzoic and 4-hydroxybenzoic acids.

TLC on a fluorescent background and gas chromatography of trimethylsilyl (TMS) esters met the requirements for the determination of the compounds of interest. The purpose of this paper is to describe the development of these procedures as general methods for the analyses of minor amounts of benzoic acid derivatives.

EXPERIMENTAL

Reagents—All common reagents and solvents utilized were of analytical reagent grade. The following reagents were used: silica gel G;[1] cellulose;[2] fluorescent indicator;[3] SE-52, OV-1, OV-17, and OV-25;[4] Chromosorb G, 100/120 mesh;[5] bis(trimethylsilyl)-acetamide (BSA);[4] benzoic acid;[6] 4-hydroxybenzoic acid;[7] 4-anisic acid;[8] and 2-hydroxy- and 3-hydroxyanisic acids prepared according to the literature (4–6).

TLC—Silica gel G TLC plates were prepared by coating five 20 × 20-cm. glass plates at a thickness of 250 μm. with a slurry composed of 21 g. of silica gel G, 2.1 g. of fluorescent indicator, and 50 ml. of water. Cellulose TLC plates were prepared by similarly coating five 20 × 20-cm. glass plates with a slurry composed of 10 g. of cellulose, 1.0 g. of fluorescent indicator, and 65 ml. of water. After air drying at room temperature, plates were activated at 105° for 30 min. prior to use. Spotting was performed with a 1.0-μl. pipet. Chromatograms were developed to a distance of 15 cm. by the ascending technique. Developing tanks were lined with filter paper and

[1] Warner-Chilcott Labs.
[2] Camag.
[3] Phosphor 2282, Research Specialties Co.
[4] Applied Science Labs.
[5] Johns-Manville Product Corp.
[6] J. T. Baker & Co., Phillipsburg, N. J.
[7] Eastman Organic Chemicals.
[8] Aldrich Chemical Co.

Table I—TLC of Dimethoxystilbene Reference Acids on Silica Gel G Plates

Acid	R_f Value in Solvent System[a] A	B
Benzoic	0.69	0.86
Anisic	0.56	0.86
2-Hydroxyanisic	0.49	0.86
3-Hydroxyanisic	0.17	0.76
4-Hydroxybenzoic	0.15	0.64

[a] Solvent Systems: A, petroleum ether–acetic acid (4:1); and B, chloroform–acetic acid (4:1).

were saturated with the appropriate solvent mixture. Detection of spots was by the quenching of fluorescence under a UV lamp.

Reverse TLC Fluorimetry—Silica gel G plates, scored in 0.5-cm. channels, were spotted in alternate channels with varying amounts of the compounds to be measured and developed. The developed plates were air dried and placed in the scanning apparatus[9] with the adsorbent side down. Scanning was done with the following conditions: scanner speed, 1 r.p.m.; recorder speed, 60 in./hr.; and sensitivity and meter multiplication, set to give a response of 90–100% for the background fluorescence. Anisic acid was measured with an activation wavelength of 277 nm. and emission wavelength of 530 nm.

Gas Chromatography—Column packings were prepared by coating the liquid stationary phase on the solid support in a rotary evaporator. The dry packing was added to one end of the coiled copper tubing, which was connected at the other end to a water aspirator by means of a glass tube plugged with glass wool. The copper tubing was agitated until fully packed, and the ends were plugged with glass wool.

Qualitative and quantitative gas chromatography was performed with an F&M 700 gas chromatograph equipped with dual flame-ionization detectors. The following conditions were used for all analyses: carrier gas, helium, 60 ml./min. flow; hydrogen flow, 50 ml./min. (16 p.s.i.); air flow, 380 ml./min. (16 p.s.i.); injection port temperature, 250°; detector temperature, 250°; attenuation, 5; range, 10^2; and injection size, 5.0 μl., with a Hamilton 701-N 10-μl. syringe. Other conditions were varied for the determination of different compounds as follows: A, column, 3.7 m. × 0.32 cm. o.d. (12 ft. × 0.125 in. o.d.), 3% OV-17 on Chromosorb G 100/120 mesh; column oven temperature, 190°; B, same as A but with column oven temperature 170°; and C, column, 1.8 × 0.32 cm. o.d. (6 ft. × 0.125 in. o.d.), 3% OV-1 on Chromosorb G 100/120 mesh; column oven temperature, 150°. TMS derivatives were prepared by dissolving the samples in 0.3 ml. tetrahydrofuran and 0.2 ml. BSA at least 15 min. prior to analysis.

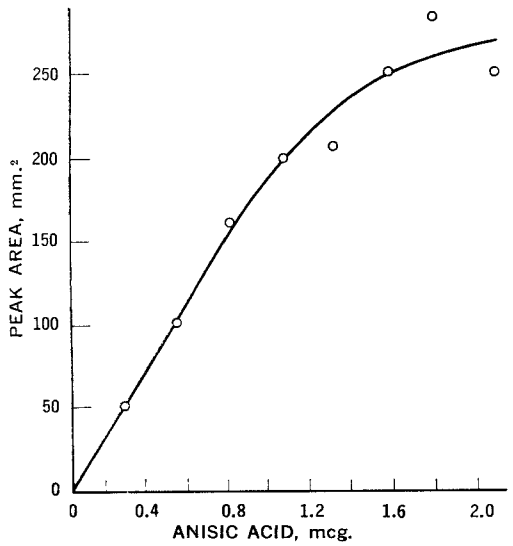

Figure 1—*Reverse TLC fluorimetry of anisic acid.*

[9] Aminco thin-film scanner (No. 4-8221A) connected to an Aminco-Bowman spectrophotofluorometer (SPF 4-8202) and a 100-mv. Beckman recorder (93500).

Table II—TLC of Benzoic, 4-Chlorobenzoic, and 4-Hydroxybenzoic Acids

Acid	R_f Value in Solvent System[a] C	D
4-Chlorobenzoic	0.84	0.75
Benzoic	0.75	0.61
4-Hydroxybenzoic	0.35	0.12

[a] Solvent Systems: C, 2-propanol–ethanol–water–ammonium hydroxide (50:20:10:5); and D, *n*-butanol saturated with ammonium hydroxide.

RESULTS AND DISCUSSION

TLC—Table I lists the R_f values of benzoic and the four reference acids on TLC plates of silica gel G in the solvent systems indicated. While System A yields the better separation of these acids, System B enhances the separation of 3-hydroxyanisic and 4-hydroxybenzoic acids. 4-Chlorobenzoic acid in reference to the naturally occurring urinary metabolites, benzoic and 4-hydroxybenzoic acids, was best analyzed by TLC on cellulose plates in the solvent systems listed in Table II.

Detection of these acids could be performed with bromcresol green solution (7) at a limit of sensitivity of from 10 to 20 mcg. of acid. Except for benzoic acid, this limit of sensitivity could be improved 10–100 times by observing the quenching of a fluorescent indicator added to the TLC plates. The comparatively poor sensitivity of this method for benzoic acid was actually an advantage in these studies since benzoic acid was expected to be present in control urine extracts.

Reverse TLC Fluorimetry—Since fluorescent compounds can be easily analyzed on TLC plates, quantitation of the quenching of background fluorescence was investigated for reference acids by a similar procedure used for direct TLC fluorimetry (8). Plates were scanned perpendicular to the solvent front. Plates were scored in 0.5-cm. channels before development to limit lateral diffusion to the same length as the activation wavelength slit of the fluorescent scanner. Spotting was done in alternate channels. Maximum fluorescence was obtained with activation wavelength 277 nm. and emission wavelength 530 nm. The fluorescence baseline was most stable when the 10% fluorescent indicator was used. Results obtained for

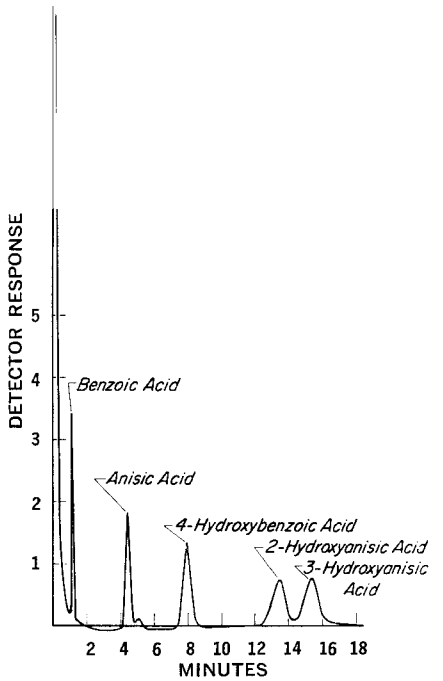

Figure 2—*Gas chromatographic separation of reference acids as their TMS esters.*

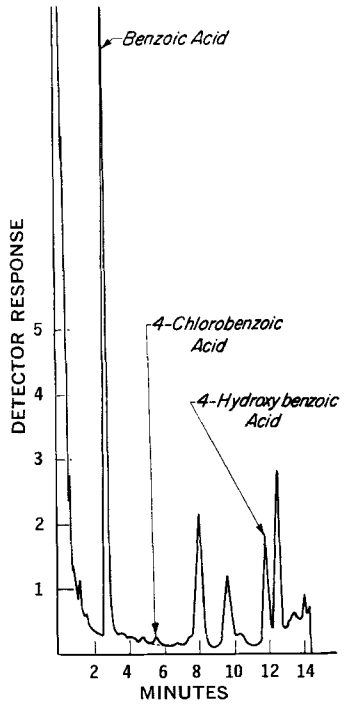

Figure 3—*Gas chromatographic detection (as TMS esters) of 4-chlorobenzoic acid added to rabbit urinary acids.*

anisic acid after development in Solvent System A are shown in Fig. 1. This graph reveals that the method can be used for the quantitative determination of anisic acid below 1 mcg. However, in contrast to direct TLC scanning, baseline stability can be a problem in the reverse technique in a manner analogous to densitometry scanning.

Gas Chromatography—The separation of TMS derivatives of dimethoxystilbene reference acids by gas chromatography was investigated. Good separation was obtained on 3% SE-52, OV-1, OV-17, and OV-25 on Chromosorb G 100/120 mesh columns. The results obtained using Condition C (3% OV-1) are reproduced in Fig. 2. Acid fractions of rabbit urine showed a number of peaks when gas chromatographed, and anisic acid in urine extracts was best analyzed with Condition A (3% OV-17). For quantitative determination of anisic acid, a graph of height or area of the peak *versus* equivalent weight of acid injected was plotted. It was found that using peak height gave a linear relationship only to 15 mcg., whereas peak area gave a linear plot to 150 mcg. The peak area was measured by either a Disc integrator (model 201-B) or by the product of the peak height times the width at half height. The two methods of measuring peak area gave approximately the same results. When anisic acid was added to urine, 90–95% was recovered by extraction and gas chromatographic analysis. The extraction scheme consisted of continuous extraction with ether of acidified urine, back extraction with 5% sodium bicarbonate, acidification of the bicarbonate extract, and final recovery of acids by ether extraction. While this scheme results also in the isolation of a series of naturally occurring acids, they did not interfere with the determination of anisic, 2-hydroxyanisic, or 3-hydroxyanisic acids.

Gas chromatographic analysis of 4-chlorobenzoic acid was performed with Condition B (3% OV-17). 4-Chlorobenzoic acid, when added to a control rabbit urine acid fraction, had a retention time of 5.5 min. and a detection limit of 1 mcg., as shown in Fig. 3. Note also in Fig. 3 that benzoic and 4-hydroxybenzoic acids are indicated on the basis of cochromatography with reference acids. It was attempted to increase the sensitivity by the use of an electron-capture detector (^{3}H). However, the presence of electron-capturing impurities required that the solutions be diluted, which resulted in approximately the same overall sensitivity obtained with flame-ionization detection.

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