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Note

Use of gas chromatography–mass spectrometry for the diagnosis and study of metabolic disorders

Screening and identification of urinary aromatic acids

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In a normal individual, the concentration of acidic metabolites in the urine is fairly constant. In cases where one or more of the biochemical processes in the metabolism may be defective from birth (phenylketonuria), or where they become defective at a later stage in life due to a diseased state (neuroblastoma), the concentration of normal acidic metabolites may change considerably and sometimes new abnormal metabolites may be present. For this reason, accurate and sensitive methods of assay for urinary acids, which allow one to distinguish between several similar structures of the same metabolic pathway, are needed[1–4].

One promising approach to the identification and measurement of aromatic acids is the application of gas–liquid chromatography (GLC) for separation and mass spectrometry (MS) for identification purposes. By using standard silylation techniques to provide the necessary volatile input into such a GC–MS system, it has been shown that urinary metabolic profiles will become a valuable aid in the diagnosis of metabolic disorders[5]. Unfortunately the required volatile trimethylsilyl esters of aromatic acids are unstable[6] and we have found it necessary to prepare the samples immediately prior to analysis, a situation which precluded their use for routine clinical application.

We now describe an analytical procedure for the GC–MS profiling or urinary aromatic acids commonly associated with diseases of amino acid metabolism which does not suffer from this disadvantage.

MATERIALS AND METHODS

Reagents

The aromatic acids were obtained from Sigma (Missouri, Mo., U.S.A.). Linde molecular sieve (Type 3A; 1/16 in.) was supplied by Matheson, Coleman and Bell (East Rutherford, N.J., U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilazane (TMCS) and trimethylanilinium hydroxide (TMAH; 0.4 *M* solution in methanol) were obtained from Pierce (Rockford, Ill., U.S.A.).

Equipment

A Becker (Delft, The Netherlands) Model 409 gas chromatograph fitted with a 8 ft. × 1/8 in. column packed with 5% Silicone OV-17 on dimethyldichlorosilane-treated Chromosorb W and a Varian (Walnut Creek, Calif., U.S.A.) Model 600D gas chromatograph interfaced via a Watson-Bieman separator to an EAI (Palo Alto, Calif., U.S.A.) Model 300D quadrupole mass spectrometer were used.

Procedure

Urine (1 ml) was acidified to pH 1 with HCl (0.1 ml, 6 *N*) and saturated NaCl (2 ml) was then added. The solution was extracted twice with ethyl acetate (2 ml) and then two times with ether (2 ml). The organic extracts were pooled and dried with magnesium sulphate and the solution was evaporated to dryness under dry nitrogen.

Trimethylsilyl derivatives. Derivatization was achieved by the addition of pyridine (0.1 ml), BSTFA (0.2 ml), TMCS (0.1 ml) and molecular sieve (Type 3A). After heating at 60° for 30 min a 2-μl sample was injected into the gas chromatograph.

Methylated derivatives. The dried residue was dissolved in a methanolic solution of TMAH (0.1 ml, 0.4 *M*) and molecular sieve (Type 3A) was added. After 10 min at room temperature a 2-μl sample was injected into the gas chromatograph.

Preparation of reference compounds. The aromatic acid (1 mg) was dissolved in methanolic TMAH (0.1 ml, 0.4 *M*) and molecular sieve (Type 3A) was added. After 10 min about 2 μl of the sample was used for analysis (Table I).

Calibration curves for phenylacetic, 4-hydroxyphenylacetic and 4-hydroxy-3-methoxyphenylacetic acid. Phenylacetic acid, 4-hydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid (0.5, 1.0, 1.5, 2.0 and 2.5 × 10^{-3} m*M*) were derivatized in turn with methanolic TMAH (0.1 ml, 0.4 *M*) (Fig. 2). A 2-μl sample was injected into the gas chromatograph and instrument parameters used were as specified in Table II.

TABLE I

GC RETENTION DATA* FOR AROMATIC ACIDS

Aromatic acid	*Structure of volatile derivative*	*Retention time (min)*	*Temperature (°C)*
Phenylacetic acid	C_6H_5–CH_2–$COOCH_3$	8.9	135
Phenyllactic acid	C_6H_5–$CH(OCH_3)$–$COOCH_3$	12.9	167
4-Hydroxyphenylacetic acid	CH_3O–C_6H_4–CH_2–$COOCH_3$	14.0	176
4-Hydroxyphenyllactic acid	CH_3O–C_6H_4–$CH(OCH_3)$–$COOCH_3$	17.2	201
2,5-Dihydroxyphenylacetic acid	2,5-$(CH_3O)_2$–C_6H_3–CH_2–$COOCH_3$	17.3	202
4-Hydroxy-3-methoxyphenylacetic acid	3,4-$(CH_3O)_2$–C_6H_3–CH_2–$COOCH_3$	17.4	203
4-Hydroxy-3-methoxymandelic acid	3,4-$(CH_3O)_2$–C_6H_3–$CH(OCH_3)$–$COOCH_3$	19.4	219
5-Hydroxyindoleacetic acid	VII (see p. 438)	23.6	252

* Chromatographed isothermally for 2 min at 80° and then programmed at 8°/min.

TABLE II

MASS SPECTRAL FRAGMENTS SUITABLE FOR THE IDENTIFICATION OF URINARY AROMATIC ACID DERIVATIVES

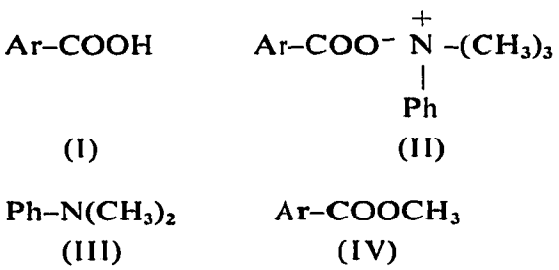
GC instrument parameters were as specified in Table I. Carrier gas, helium, 25 ml/min; Bieman separator temperature, 250°; ionizing energy, 70 eV; current, 100 μA; scan time, 6 sec between *m/e* 50 to 600.

Derivative	*m/e value of ion*		
	M^*	$(M - COOCH_3)$	*Others*
$C_6H_5-CH_2-COOCH_3$	150 (50%)	91 (100%)	
$C_6H_5-CH_2-CH(OCH_3)-COOCH_3$	—	135 (100%)	(M − CH_3OH) 162 (60%) ($C_6H_5-CH_2$) 91 (75%)
$CH_3O-C_6H_4-CH_2-COOCH_3$	180 (16%)	121 (100%)	
$CH_3O-C_6H_4-CH(OCH_3)-COOCH_3$	224 (1%)	—	($CH_3O-C_6H_4-CH_2$) 121 (100%)
$2,5-(CH_3O)_2-C_6H_3-CH_2-COOCH_3$	210 (26%)	151 (74%)	($CH_3O-C_6H_4-CH_2$) 121 (100%)
$3,4-(CH_3O)_2-C_6H_3-CH_2-COOCH_3$	210 (26%)	151 (100%)	
$3,4-(CH_3O)_2-C_6H_3-CH(OCH_3)-COOCH_3$	240 (2%)	181 (100%)	
VII (see p. 438)	233 (15%)	174 (100%)	

* Molecular ion.

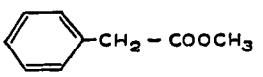
RESULTS AND DISCUSSION

The derivatization of the aromatic acids for GC involves the conversion of the acids (I) to their trimethylanilinium salts (II) (ref. 7). Pyrolysis of these salts in the injector port of the GC yields dimethylaniline (III) and the methyl ester derivatives (IV) which are separable by GC (Table I).

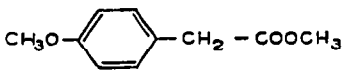


The trimethylanilinium salt (II) solutions are quite stable and can be kept for several weeks. Identification of the GC peaks as methyl ester derivatives is based on a comparison of their low-resolution mass spectra, with spectra obtained from aromatic acid methylesters prepared by classical methods using diazomethane[8] or boron trifluoride (14% w/v)–methanol[9]. The results show (Table II) that phenylacetic acid is chromatographed as its methyl ester (V), that a phenolic acid is converted to the O-methyl methyl ester derivative (VI) and the ring nitrogen of indole (VII) and the

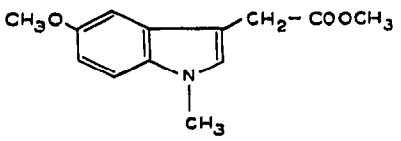
α-hydroxy groups of phenyllactic acid (VIII), 4-hydroxyphenyllactic acid (IX) and 4-hydroxy-3- methoxymandelic acid (X) are also methylated.



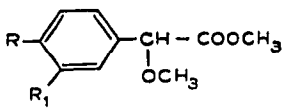
(V)



(VI)



VII



(VIII) R = H, R_1 = H
(IX) R = OCH_3, R_1 = H
(X) R = OCH_3, R_1 = OCH_3

The homogeneity of the GC peaks in the chromatograms was established with a fast scanning mass spectrometer and the linear relationship between acid concentration and peak height was established for several aromatic acids (Fig. 1). The pyrolysis methylation technique was used for the analysis of urine samples from selected patients who were suspect of metabolic disease from a clinical point of view. The resulting characteristic metabolic profiles (Figs. 2a–e) were compared with the urinary profiles obtained by the silylation method (Figs. 3a–e).

The experimental results reported here point to the effectiveness of GC–MS for the analysis of urinary aromatic acids. The on-column methylation by TMAH adds significantly to the potential of GC–MS as an identification tool in clinical studies of metabolic disorders.

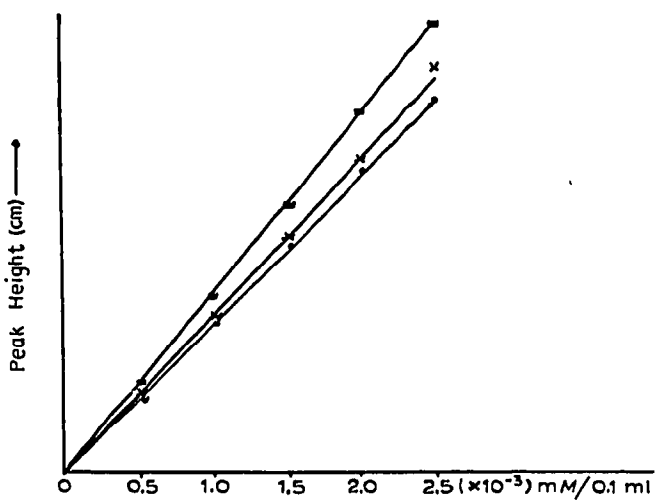


Fig. 1. Calibration curves for phenylacetic acid (●), *p*-hydroxyphenylacetic acid (×), and 4-hydroxy-3-methoxyphenylacetic acid (■) derivatives.

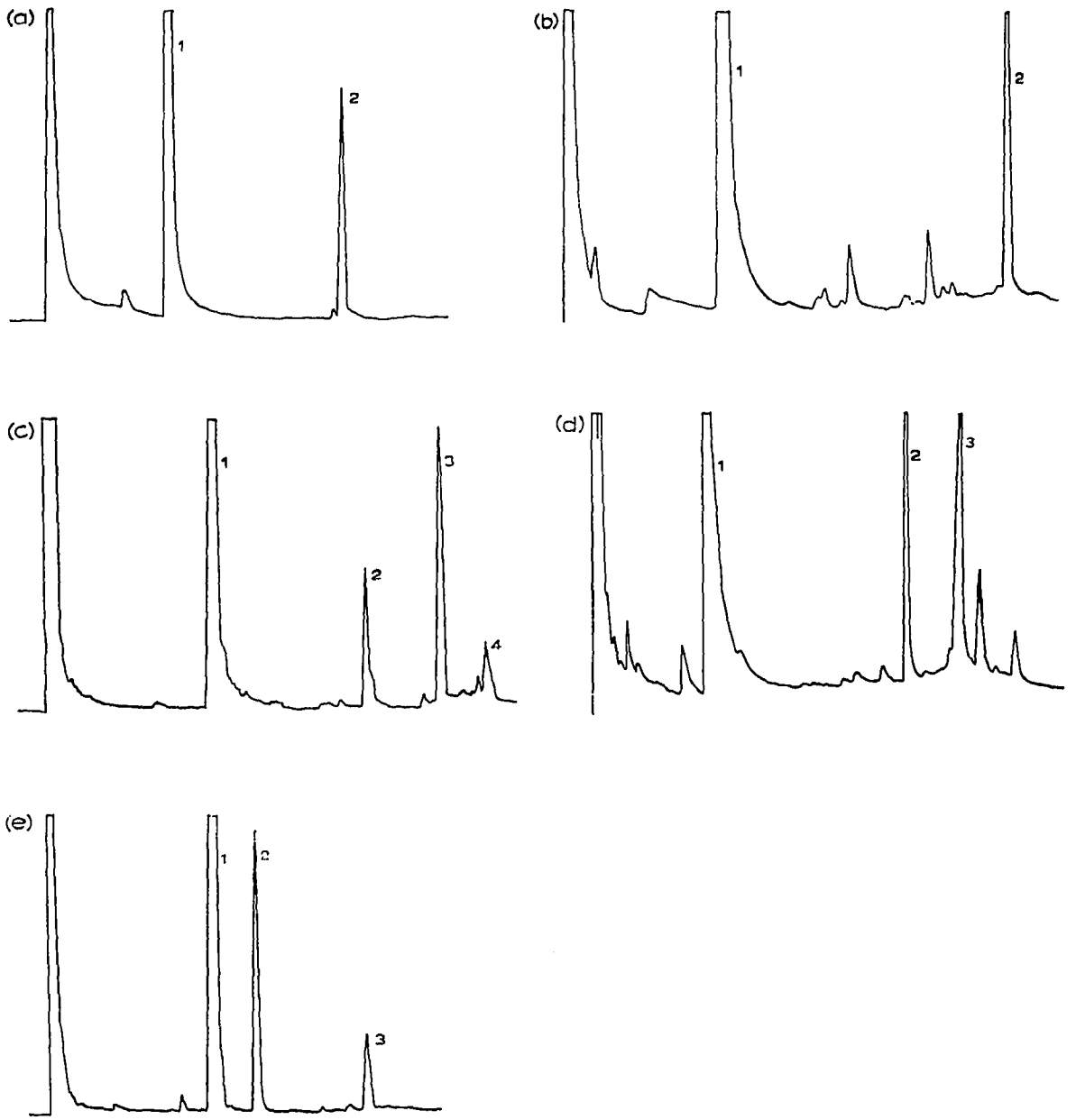


Fig. 2. (a) Urine aromatic acids from a patient with homogentisic aciduria. 1 = Dimethylaniline; 2 = 2,5-dihydroxyphenylacetic acid. (b) Urine aromatic acids from a patient with carcinoid syndrome. 1 = Dimethylaniline; 2 = 5-hydroxyindoleacetic acid. (c) Urine aromatic acids from a tyrosinosis patient. 1 = Dimethylaniline; 2 = phenyllactic acid; 3 = 4-hydroxyphenyllactic acid; 4 = hippuric acid [M^+ 207, ($M^+ - COOCH_3$) 148, $PhCO^+$ 105]. (d) Urine aromatic acids from a patient with neuroblastoma. 1 = Dimethylaniline; 2 = 4-hydroxy-3-methoxyphenylacetic acid; 3 = 4-hydroxy-3-methoxymandelic acid. (e) Urine aromatic acids from a phenylketonuric patient. 1 = Dimethylaniline; 2 = phenylacetic acid; 3 = phenyllactic acid.

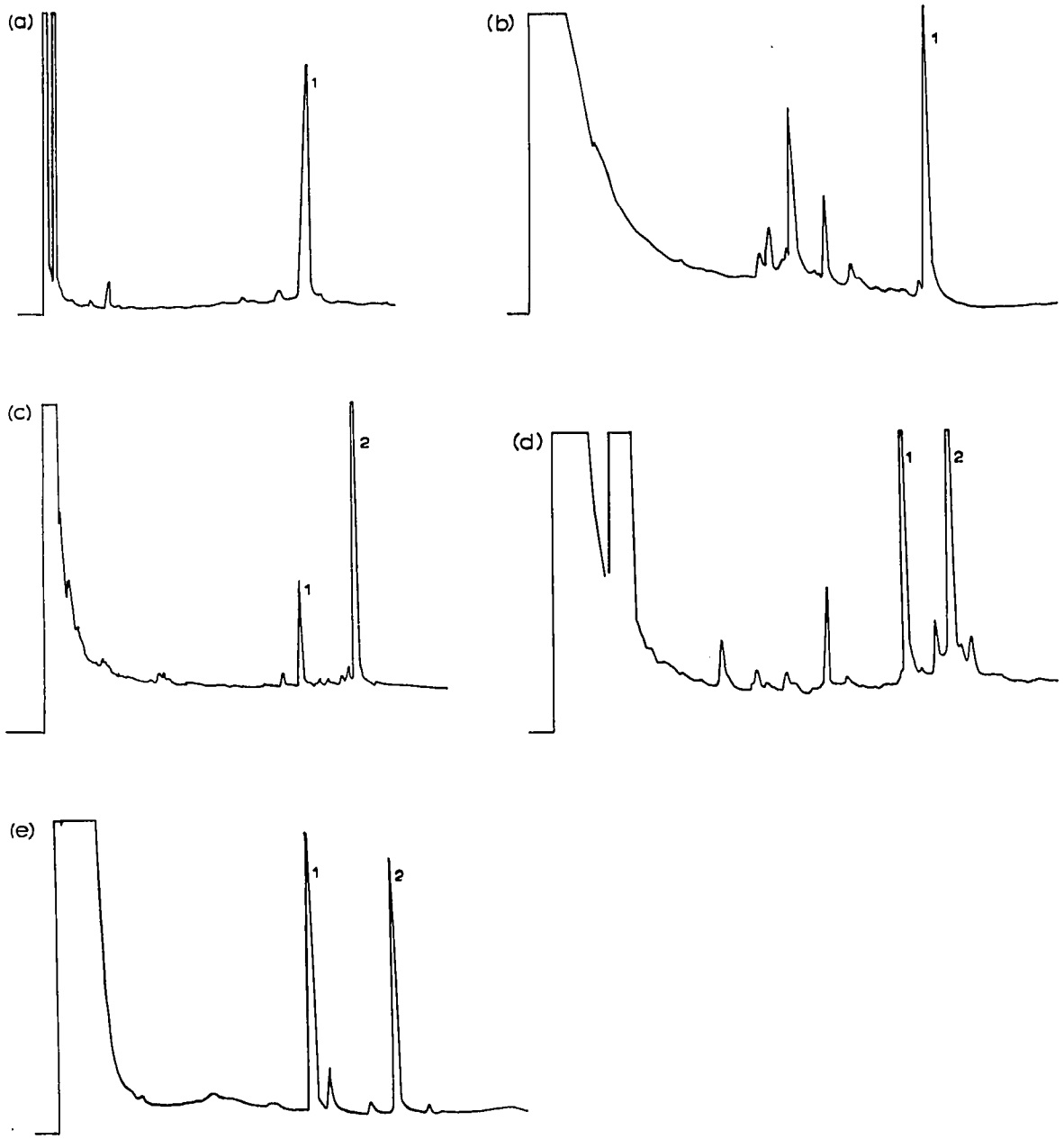


Fig. 3. (a) Urine aromatic acids from a patient with homogentisic aciduria (TMS system). 1 = 2,5-Dihydroxyphenylacetic acid. (b) Urine aromatic acids from a patient with carcinoid syndrome (TMS system). 1 = 5-Hydroxyindoleacetic acid. (c) Urine aromatic acids from a tyrosinosis patient (TMS system). 1 = Phenyllactic acid; 2 = 4-hydroxyphenyllactic acid. (d) Urine aromatic acids from a patient with neuroblastoma (TMS system). 1 = 4-Hydroxy-3-methoxyphenylacetic acid; 2 = 4-hydroxy-3-methoxymandelic acid. (e) Urine aromatic acids from a phenylketonuric patient (TMS system). 1 = Phenylacetic acid; 2 = phenyllactic acid.

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