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CHROMATOGRAPHIC PROFILE OF HIGH BOILING POINT ORGANIC ACIDS IN HUMAN URINE

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SUMMARY

The profile of high boiling point organic acids in urine samples from both normal subjects and patients suspected of having some form of metabolic disorder has been determined by combined gas chromatography—mass spectrometry. Fifteen different compounds eluting after hippuric acid have been identified, including two, cinnamoylglycine and acetyltributylcitrate, which have not been recognised previously. Relative retention times and abbreviated mass spectra of the identified compounds are presented.

INTRODUCTION

The diagnosis of many inborn errors of metabolism is based on the determination of organic acids in urine. In recent years the separation and identification of these acids has become greatly facilitated by the development of methods based on combined gas chromatography—mass spectrometry (GC—MS) [1—5]. With these methods, screening of samples from patients suspected of having such disorders is now possible.

Together with the development of the methodology, there has been a gradual accumulation of knowledge of the organic acid constituents of urine under a wide range of conditions [6—9]. The compilation of such data is

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necessary for the rapid assessment of chromatograms of urinary organic acids in screening situations.

Most gas chromatograms of urinary organic acids from adult subjects are characterised by a large peak of hippuric acid which forms a readily recognisable landmark. Most compounds of interest in the diagnosis of metabolic disorders elute between the solvent front and this peak. In general, the commonly occurring compounds in this region of the chromatogram have been well characterised in both normal and abnormal states.

For compounds eluting after hippuric acid, the data are much less extensive for a number of reasons. Firstly, few peaks are found in this region of the chromatogram in most samples and, so far, none has been associated with a particular metabolic disorder. Secondly, many of the compounds are eluted at temperatures close to the maximum thermal stability of the commonly used chromatographic columns (such as OV-17). This often results in considerable masking of the urinary constituents by bleeding of the stationary phase.

To extend the data on urinary organic acids excreted under a variety of conditions, we undertook a study of the compounds eluting after hippuric acid in acid diethyl ether extracts of urine samples from both normal subjects and patients suspected of having some form of metabolic disorder. The results were obtained using the type of procedure commonly employed in screening situations rather than the more time-consuming quantitative methods which are preferable when studying particular disorders. The resulting profile contains those compounds which are likely to be observed during normal screening for organic acids and should aid in the interpretation of chromatograms obtained under such conditions.

MATERIALS AND METHODS

Chemicals

Reference compounds were obtained commercially or synthesised if unavailable. Cinnamic acid, 3- and 4-hydroxybenzoic acids were obtained from Fluka (Buchs, Switzerland) and 3-hydroxybenzaldehyde from Sigma (St. Louis, Mo., U.S.A.).

Glycine conjugates of cinnamic acid and the hydroxybenzoic acids were prepared by reaction of the acid with glycine ethyl ester using N,N′-dicyclohexylcarbodiimide with subsequent transesterification to the methyl ester as previously described [10].

3-(3-Hydroxyphenyl)hydracrylic acid was prepared by a Reformatsky reaction of 3-hydroxybenzaldehyde with ethyl bromoacetate and zinc.

Acetyltributylcitrate was prepared from citric acid by acetylation with acetic acid—acetic anhydride followed by butylation with HCl-saturated 1-butanol.

Preparation of samples

Early morning urine samples were obtained from 10 normal subjects and 50 patients suspected of having some form of metabolic disorder. Three methods were used to obtain an organic acid extract of these samples. In most cases the

compounds were extracted from acidified urine into diethyl ether, either manually or with a continuous extraction apparatus [2]. These procedures were used for most samples as the aim was to determine those compounds which would be detected under normal screening conditions where a rapid answer is often required. Under these conditions, speed and ease of extraction are more important than quantitative recovery.

For quantitative recovery of the organic acids and extraction of highly polar compounds, the ion-exchange chromatography method of Horning and Horning [1] was used. Analysis of samples using this method ensured that no commonly occurring compounds had been overlooked by using the less-quantitative extraction procedures.

The organic acids were converted to their methyl esters with diazomethane prior to GC, as previously described [2]. *n*-Eicosane was added to all samples as an internal standard.

Gas chromatography

A Varian Model 2100 gas chromatograph was fitted with a 2-m glass column (4 mm I.D.) packed with 6% Dexsil 300 on Chromosorb W HP, 80—100 mesh, obtained from Analabs (North Haven, Conn., U.S.A.). The injector and detector temperatures were set at 280° and the column temperature was programmed from 150° to 350° at a rate of 6°/min. The carrier gas was nitrogen at a flow-rate of 20 ml/min. The gas chromatograph was fitted with a hydrogen flame ionisation detector.

Dexsil 300 was chosen as the stationary phase in this study as it has a particular high thermal stability. This allows analysis of compounds with a high boiling point without interference from bleeding of the column material. In addition, this material is slightly less polar than the stationary phases which are commonly used in the analysis of organic acids so that retention times are generally shorter [11].

Gas chromatography—mass spectrometry

A 2-m glass column was packed with Dexsil 300 as described above and used in a Varian Model 1440 gas chromatograph connected to a Varian CH7 mass spectrometer (Varian MAT, Bremen, G.F.R.) by means of a glass-frit type molecular separator. The GC conditions were as described above except that the carrier gas was helium at a flow-rate of 30 ml/min. The mass spectrometer was operated at an ionisation energy of 70eV.

For detection of known compounds by MS the mass spectrometer was connected on-line to a computer system (SpectroSystem 100 MS, Varian MAT, Bremen, G.F.R.). In addition, an off-line computer system was used to match peaks of unrecognised mass spectra with a library file of recorded spectra.

Compounds from the various urine samples were identified by comparison of GC retention times and mass spectra with those of reference compounds. In general, quantitation of the amounts of the different compounds was not attempted.

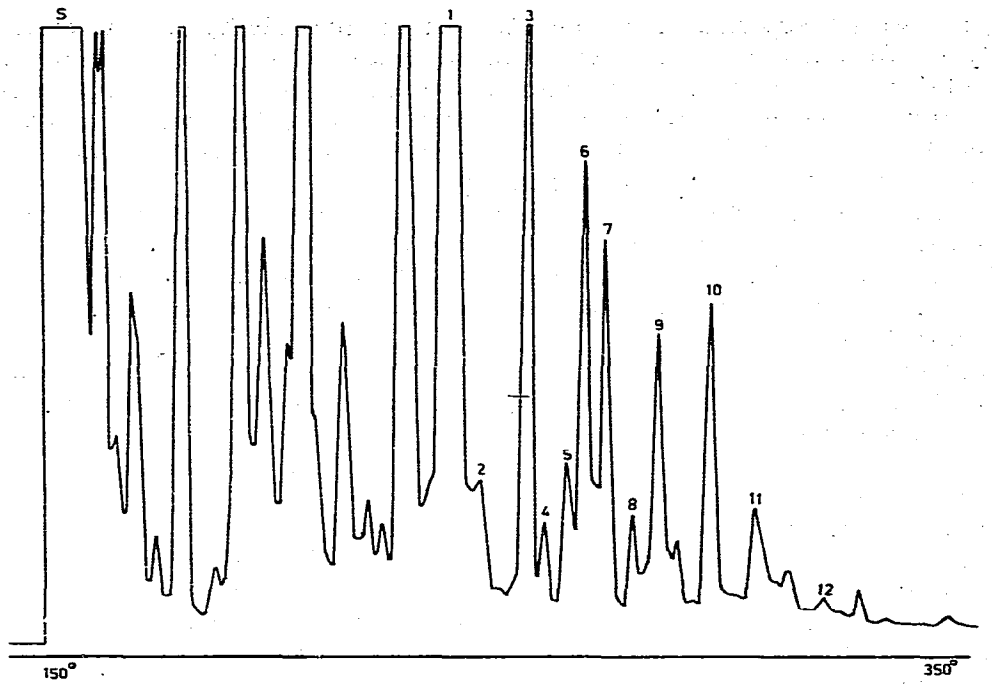

Fig. 1. Profile of high boiling point organic acids in a continuous ether extract of a normal urine sample. The extract was analysed on a column of Dexsil 300 as described in the text. The compounds identified are: 1, hippuric acid; 2, 3-(3-hydroxyphenyl)hydracrylic acid; 3, indoleacetic acid; 4, palmitic acid; 5, caffeine; 6, eicosane; 7, unknown compound of molecular weight 296; 8, 3-hydroxyhippuric acid methyl ester, methyl ether; 9, 4-hydroxyhippuric acid methyl ester, methyl ether; 10, cinnamoylglycine; 11, 4-hydroxyhippuric acid methyl ester; 12, phenylacetyl glutamate. The solvent front is indicated by S.

RESULTS

A gas chromatogram on Dexsil 300 of a continuous diethyl ether extract of a normal urine sample is shown in Fig. 1. In this particular sample, ten compounds eluting after hippuric acid have been identified. Under normal screening conditions, only three or four of these compounds would be detected in an average urine sample as the efficiency of the extraction process is much lower. Even when quantitative extraction methods are used, few urine samples contain more than five or six peaks in this region of the chromatogram. Fig. 1 shows clearly that the compounds under consideration can be readily separated under the GC conditions used in this study.

Fig. 2 shows compounds which were commonly found in both normal urine and in samples from patients with undiagnosed metabolic disease. This list of compounds was prepared from the results obtained by studying a large number of urine samples each of which contained only a few of the compounds. The compounds are located according to their relative retention times on Dexsil 300 with reference to hippuric acid.

To aid the identification of these compounds, the five major peaks in their mass spectra and their molecular weights are presented in Table I.

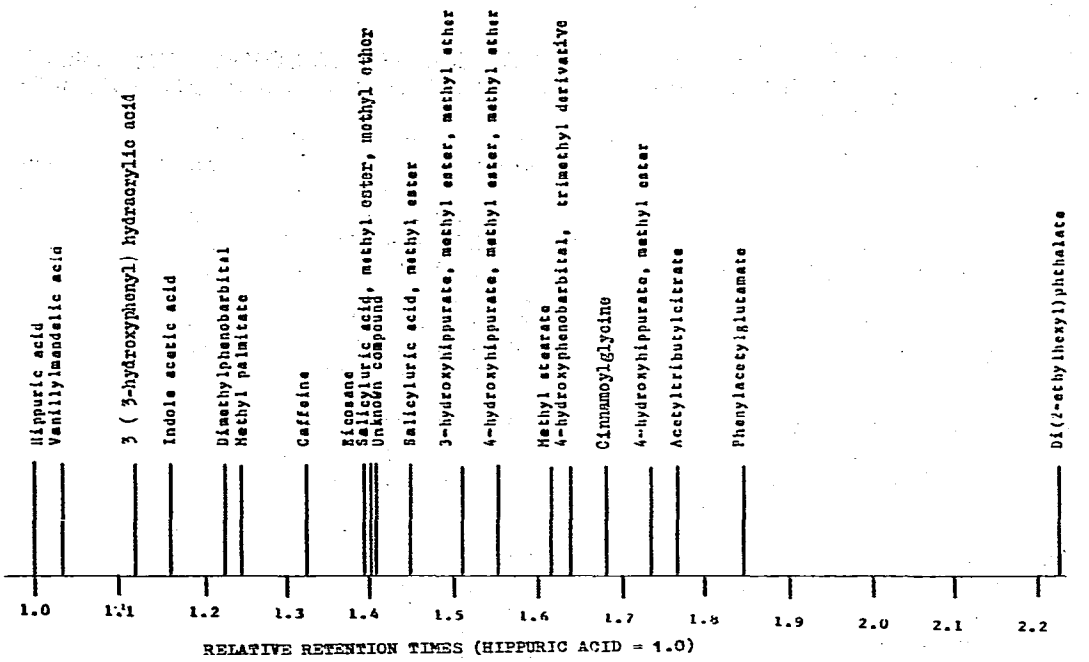

Fig. 2. Compounds commonly found in organic acid extracts of human urine both in normal subjects and patients suspected of having some metabolic disorder. The compounds are presented according to their relative retention times on Dexsil 300 with respect to hippuric acid.

DISCUSSION

The compounds shown in Fig. 2 were commonly found in urine samples and merit consideration when examining chromatograms of urinary organic acids. Of the compounds, only five are of mainly endogenous origin and these rarely occur as major peaks. These compounds are vanillylmandelic acid, indoleacetic acid, palmitic and stearic acids and phenylacetylglutamate (from phenylacetylglutamine).

Three drug metabolites are included as they are commonly found in urine samples submitted for organic acid analysis and may be present as major peaks. The presence of these drug metabolites, salicyluric acid, phenobarbital and 4-hydroxyphenobarbital, may cause some confusion if methyl esters are prepared for GC, as several derivatives can be formed from each compound. In particular, there may be up to six derivatives of phenobarbital and 4-hydroxyphenobarbital depending on the degree of methylation.

The major group of compounds eluting after hippuric acid comprises the phenolic acids and related compounds. Many of these have a high boiling point as they are excreted as glycine conjugates, for example the hydroxyhippuric acids and cinnamoylglycine. Under normal circumstances, these compounds are considered to arise from dietary sources, particularly the flavonoid constituents of plants [12]. However, in some forms of gastrointestinal disease,

TABLE I

THE FIVE MAJOR PEAKS IN THE MASS SPECTRA OF THE COMPOUNDS IDENTIFIED IN THE HIGH BOILING POINT FRACTION OF HUMAN URINE ORGANIC ACID EXTRACTS AS IN FIG. 2

Compound	*m/e* ratios	Molecular weight
Hippuric acid	105 77 134 51 193	193
Vanillylmandelic acid	167 139 226 114 108	226
3-(3-Hydroxyphenyl)hydracrylic acid	123 95 122 196 121	196
Indoleacetic acid	130 189 77 105 103	189
Dimethylphenobarbital	232 117 146 118 175	260
Methyl palmitate	74 87 43 55 270	270
Caffeine	194 109 55 67 82	194
Eicosane	43 57 71 41 85	282
Salicyluric acid methyl ester, ether	135 77 90 92 136	223
Unknown compound	179 236 222 147 207	296
Salicyluric acid methyl ester	121 209 120 65 92	209
3-Hydroxyhippuric acid methyl ester, ether	135 77 223 107 92	223
4-Hydroxyhippuric acid methyl ester, ether	135 223 77 92 107	223
Methyl stearate	74 87 43 56 75	298
4-Hydroxyphenobarbital(trimethyl derivative)	261 290 234 148 262	290
Cinnamoylglycine	131 103 77 102 218	219
4-Hydroxyhippuric acid methyl ester	121 209 65 93 150	209
Acetyltributyl citrate	185 259 129 43 57	402
Phenylacetyl glutamate	91 116 142 174 84	292
Di-(2-ethylhexyl)phthalate	149 57 167 71 70	279

they can be formed from the metabolism of phenylalanine and tyrosine by gastrointestinal microorganisms [13].

The 3- and 4-hydroxyhippuric acids were found in almost all samples and have been listed in several previous tables of urinary organic acids [6—9]. They are readily detected during organic acid screening. Cinnamoylglycine was also found in almost all samples although the amount present was usually quite small. This compound does not appear to have been reported in earlier lists of urinary organic acids.

In samples from adult subjects, caffeine was a common constituent. The excretion of this compound has been extensively studied in man [14].

Most urine samples also contained 3-(3-hydroxyphenyl)hydracrylic acid, although the amount varied from a major peak to just detectable levels, in agreement with the findings of Duncan et al. [15]. This compound was first characterised by Armstrong and Shaw [16] and is considered to be synthesised from phenylalanine by gastrointestinal microorganisms.

Two other compounds were commonly found in samples from patients which were submitted for organic acid analysis. These are the plasticisers di(2-ethylhexyl) phthalate and acetyltributyl citrate. The first of these is a common contaminant of biological materials [17] and usually arises from plastic storage containers. Acetyltributyl citrate is present in PVC transfusion tubing [18] and many other plastic materials but has not previously been described in

human urine. Whether it is excreted following exposure or accumulates in the sample during storage in plastic containers is unknown.

In addition to the compounds identified above, one other compound was found in almost all samples but has so far not been identified. This compound has a relative retention time of 1.41 with respect to hippuric acid and a molecular weight of 296 in the form of the methyl ester. The parent compound is resistant to acid hydrolysis. The major peaks in the mass spectrum of this compound are presented in Table I.

Whereas the method described above allows the identification of the most commonly occurring organic acids eluting after hippuric acid, it is not particularly suitable for the metabolites of catecholamines which are formed endogenously and which are mainly present in this region of the chromatogram. This group of compounds contains a number of hydroxy- and methoxyphenolic acids. The formation of methyl esters of these compounds for GC often also results in the formation of methyl ethers with the free phenolic hydroxyl groups and the extent of this process is quite variable. This alters the relative number and position of the hydroxyl and methoxyl groups, making identification difficult. Analysis of catecholamine metabolites is therefore best carried out using some other type of derivative (such as trimethylsilyl esters and ethers) which does not affect the number of methoxy substituents.

The GC profile of compounds with a high boiling point and their characteristic mass spectra presented in this paper include those compounds which are most likely to be found in organic acid extracts of human urine. The information should be useful in the rapid evaluation of organic acid chromatograms under normal screening conditions.

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