THE USE OF GAS CHROMATOGRAPHY–MASS SPECTROMETRY FOR THE DIAGNOSIS AND STUDY OF METABOLIC DISORDERS

I. THE SCREENING AND IDENTIFICATION OF URINARY AND SERUM AMINO ACIDS

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Abstract

The common urinary and serum amino acids have been separated and identified by gas chromatography and mass spectrometry of their neopentylidene alkyl ester derivatives. The analysis of samples from mentally retarded children and other selected patients showed that the method can be used to detect 15 of the known inborn errors of amino acid metabolism.

I. INTRODUCTION

An increasing number of human diseases are now regarded as metabolic disorders, which are known to be due to defects of some biochemical process. Not all of these inborn errors of metabolism are pathologic. For example, some may lead to early neonatal death or to gross mental retardation, while others are known to be compatible with almost normal mental and physical development. The symptomatology of such diseases is usually quite unspecific and may manifest itself as failure to thrive, seizures starting in early infancy, developmental or psychomotor retardation, convulsion, irritability, or acidosis. The severity of the symptoms is usually related to the toxicity or the rate of accumulation of certain metabolites in the blood and tissues or both, and an early diagnosis usually depends on the detection and identification of these abnormal excretions. Since death or mental retardation can often be prevented by an early implementation of a dietary regimen, the analysis and identification of the chemical constituents in blood and urine becomes a valuable tool in the diagnosis and subsequent treatment of metabolic disorders. The present clinical practice is to rely on a whole range of chemical screening tests which may depend on chromatography, electrophoresis, colorimetric or spectrofluorometric measurements, and microbiological tests. The disadvantages of the current clinical methods lie in their non-specific nature and the total reliance of the analyst on the recognition of characteristic patterns associated with some of the more common metabolic disorders. Since many frequently prescribed therapeutic agents give colour reactions and may appear at the same R_F value as common metabolites, the clinical interpretation of positive findings usually needs further, more detailed analytical examination for confirmation. It is in this area that gas chromatography–mass

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spectrometry offers the best available answer to the need for unequivocal identification of metabolites and drugs extracted from biological samples. The successful application of such a system depends on the availability of a suitable volatile input for the gas chromatograph. There is an extensive literature (Weinstein 1966; Blau 1968) on the use of gas chromatography for detecting, separating, and quantifying amino acids. Whilst trifluoroacetylamino acid butyl esters (Gehrke *et al.* 1965; Gehrke and Shahrokhi 1966) are the derivatives of choice for protein hydrolysates, their use with crude biological samples often results in substantial contamination by acylated metabolites. The presence of these extraneous peaks in the gas chromatographic output makes the interpretation of the amino acid profile unduly difficult.

We now describe an analytical procedure for the gas-chromatographic profiling of serum and urinary amino acids commonly associated with diseases of amino acid metabolism. The technique does not suffer from the disadvantages of previously described methods.

II. Materials and Methods

(*a*) *Reagents*

L-Amino acids were obtained from Mann Research Laboratories (N.Y.). Pivaldehyde (2,2-dimethylpropanal) was supplied by K and K Laboratories Inc. (Plainview, N.Y.). Linde molecular sieve (3A, $\frac{1}{16}$-in. pellets) was obtained from Matheson, Coleman and Bell (N.J.) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce Chemical Company (Ill.).

(*b*) *Equipment*

The instruments used were a Becker model 409 gas chromatograph fitted with an 8 ft by $\frac{1}{8}$ in. column packed with Silicone OV-17 on dimethyldichlorosilane-treated Chromosorb W and an Aerograph HiFy model 600D gas chromatograph interfaced via a Watson–Bieman separator to an EAI model 300D quadrupole mass spectrometer.

(*c*) *Procedure*

(i) *Urine Samples*

Urine (5 ml) was filtered and then acidified to pH *c.* 2·0–2·5 with acetic acid (0·5 ml, 8N). The urine was passed through an ion-exchange column (0·9 cm diam. by 2·5 cm, packed with Dowex 50W-X8, H^+) (Harris *et al.* 1961) followed by dilute acetic acid (5 ml, 0·5N) and distilled water (15 ml). The adsorbed amino acids were eluted with a triethylamine solution (30 ml, 2N in 20% acetone–water). The eluate was evaporated to dryness *in vacuo* and the residue esterified under reflux (20 min; 10% thionyl chloride–dry ethanol reagent, 1 ml). After evaporation, derivatization was completed by the addition of acetonitrile (or pyridine) (300 μl), triethylamine (100 μl), pivaldehyde (40 μl), and molecular sieve (type 3A). BSTFA (0·2 ml) was added 10 min later and after standing a further 20–30 min a 2-μl sample was injected into the gas chromatograph.

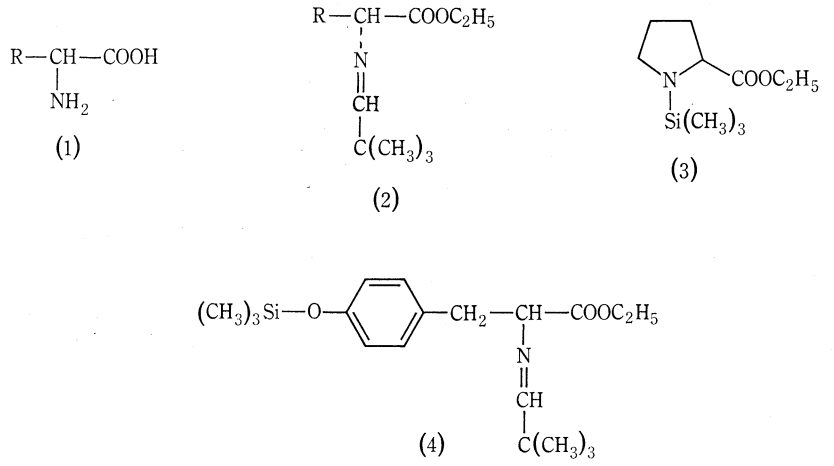
(ii) *Recovery of Reference Amino Acid Mixture from the Ion-exchange Column*

A mixture of 2-amino-n-octanoic acid, glutamic acid, lysine, and tyrosine (10^{-3} mmole of each) in water (5 ml) was chromatographed through Dowex 50W. The concentrations of the individual amino acids relative to 2-amino-n-octanoic acid, as determined by g.l.c., were compared with those of a reference mixture which had not been passed through an ion-exchange column. The recoveries of the individual amino acids were found to be glutamic acid 75%, lysine 100%, and tyrosine 98%.

(iii) *Serum Samples*

Serum (100 μl) was transferred to a centrifuge tube and a standard solution (10 μl) of 2-amino-n-octanoic acid (1 mg/ml aqueous solution) was pipetted in, followed by alcohol (400 μl).

The solution was shaken and then centrifuged (5 min, 2600 rev/min). The supernatant was removed to a 2-ml vial and evaporated to dryness under dry nitrogen. The residue was esterified (thionyl chloride–dry ethanol reagent, 1 ml) by heating the closed vial at 80°C for 30 min. After evaporation, derivatization was completed by adding pyridine (30 μl), triethylamine (20 μl), pivaldehyde (20 μl), and molecular sieve. Finally BSTFA (0·1 ml) was added and after standing approximately 20 min a sample (2 μl) was injected into the gas chromatograph.



(iv) *Preparation of Reference Compounds*

To the amino acid ethyl ester hydrochloride (1 mg) was added pyridine (300 μl), triethylamine (100 μl), pivaldehyde (40 μl), and molecular sieve. After standing at room temperature for 10 min, BSTFA (0·1 ml) was added and after a further 30 min the sample was injected into the gas chromatography–mass spectrometry system (Table 1).

TABLE 1

GAS–LIQUID CHROMATOGRAPHY RETENTION DATA* FOR AMINO ACIDS

Amino acid	Retention time (min)	Temp. (°C)	Amino acid	Retention time (min)	Temp. (°C)
Alanine	11·0	160	Aspartic acid	19·7	229
Glycine	11·6	165	Cysteine	20·2	233
Proline	12·6	173	Methionine	20·7	237
Valine	13·1	177	Glutamic acid	21·7	245
Leucine	14·8	190	Ornithine	22·4	251
Isoleucine	14·8	190	Phenylalanine	22·9	255
Serine	16·7	206	Lysine	24·0	264
Threonine	16·7	206	Tyrosine	27·3	290
2-Amino-n-octanoic acid	19·2	225	Dopa	29·2	306
			Tryptophan	32·4	310

*Chromatographed isothermally for 1 min at 80°C and then programmed from 80 to 310°C at 8 degC/min.

(v) *Linearity of Response to Lysine and Tyrosine Additions*

Tyrosine (4·68, 9·36, 14·04 mg) and lysine (4·82, 9·64, 14·46 mg) were derivatized in the presence of internal standards (*p*-chlorophenylalanine and 2-amino-n-octanoic acid). Programmed g.l.c. analyses were carried out on the OV-17 column (Fig. 1).

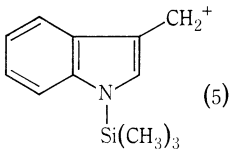
III. Results and Discussion

The derivatization of the amino acids for gas chromatography involves the esterification of the amino acid (1) followed by treatment with pivaldehyde and BSTFA in pyridine at room temperature. The resulting silylated neopentylidene

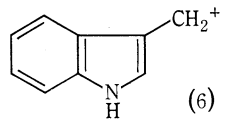
Table 2

MASS SPECTRAL FRAGMENTATION OF AMINO ACID DERIVATIVES

Amino acid	*m/e* value of ion:				
	M*	M−CH_3	M−CMe_3	M−COOEt	Others
Glycine	171	156	114	98	
Alanine	185	170	128	112	
Proline	215	200	—	142	
Valine	—	198	156	140	173 (M−$CHMe_2$)
Leucine	—	212	170	154	
Isoleucine	—	212	170	154	
Serine	—	258	216	200	170 (M−CH_2OSiMe_3)
Threonine	—	—	—	214	117 ($CH_3CHOSiMe_3^+$)
2-Amino-n-octanoic acid	—	240	198	182	
Aspartic acid	257	242	200	184	
Cysteine	217	202	160	144	
Methionine	—	230	188	172	
Glutamic acid	271	256	214	198	
Ornithine	296	281	239	—	
Lysine	310	295	253	—	
Phenylalanine	—	246	204	188	170 (M−$C_6H_5CH_2$), 91 ($C_6H_5CH_2^+$)
Tyrosine	349	—	292	276	170 (M−$CH_2.C_6H_5.OSiMe_3$), 179 ($CH_2.C_6H_5.OSiMe_3^+$)
Dopa	437	422	380	364	170 [M−$CH_2.C_6H_5(OSiMe_3)_2$], 267 [$CH_2.C_6H_5(OSiMe_3)_2^+$], 179 ($CH_2.C_6H_5.OSiMe_3^+$)
Tryptophan	372	—	—	299	202 (formula 5), 130 (formula 6)
4-Hydroxyproline	—	—	—	230	
Sarcosine	189	174	—	106	
Pipecolic acid	229	214	—	156	



(5)



(6)

* Molecular ion.

derivatives (2–4) are readily separable on a temperature-programmed, heavily loaded, silicone-type column (Table 1). The structural assignment for all derivatives was made on the basis of low-resolution mass spectral fragmentation patterns (Table 2)

and the retention behaviour of distilled reference compounds (Badr *et al.* 1965, 1966). The correct interpretation of the mass spectral fragmentation was established by

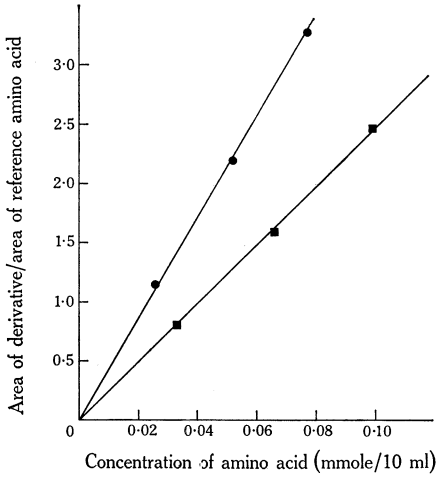


Fig. 1.—Calibration curves for tyrosine (●) and lysine (■) derivatives.

comparing several alkyl ester derivatives of each amino acid. The results show that the neutral, dibasic, and acidic amino acids are chromatographed as neopentylidene

TABLE 3

INBORN ERRORS OF AMINO ACID METABOLISM DETECTABLE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF SERUM AND URINARY AMINO ACIDS

Disease	Increased concn. of detectable amino acid* in blood and urine
Cystinuria	Cystine
Cystine–lysinurea	Lysine, ornithine
Hartnup disease	Neutral amino acids
Homocystinurea	Methionine
Hyper-β-alaninemia	β-Alanine
Hyperlysinemia	Lysine
Hypermethioninemia	Methionine
Hypertryptophanemia	Trytophan
Hypervalinemia	Valine
Maple syrup urine disease	Valine, leucine, isoleucine
Non-ketotic hyperglycinemia	Glycine
Ornithinemia	Ornithine
Phenylketonuria	Phenylalanine
Pyroglutamic aciduria	Glutamic acid
Tyrosinosis	Tyrosine

* 10–20 times normal concentration.

ethyl esters (2); that serine, threonine, tyrosine, and dopa are converted to *O*-trimethylsilyl neopentylidene derivatives (e.g. 4); and that the ring nitrogens of tryptophan, proline, and pipecolic acid are also trimethylsilylated (e.g. 3). A

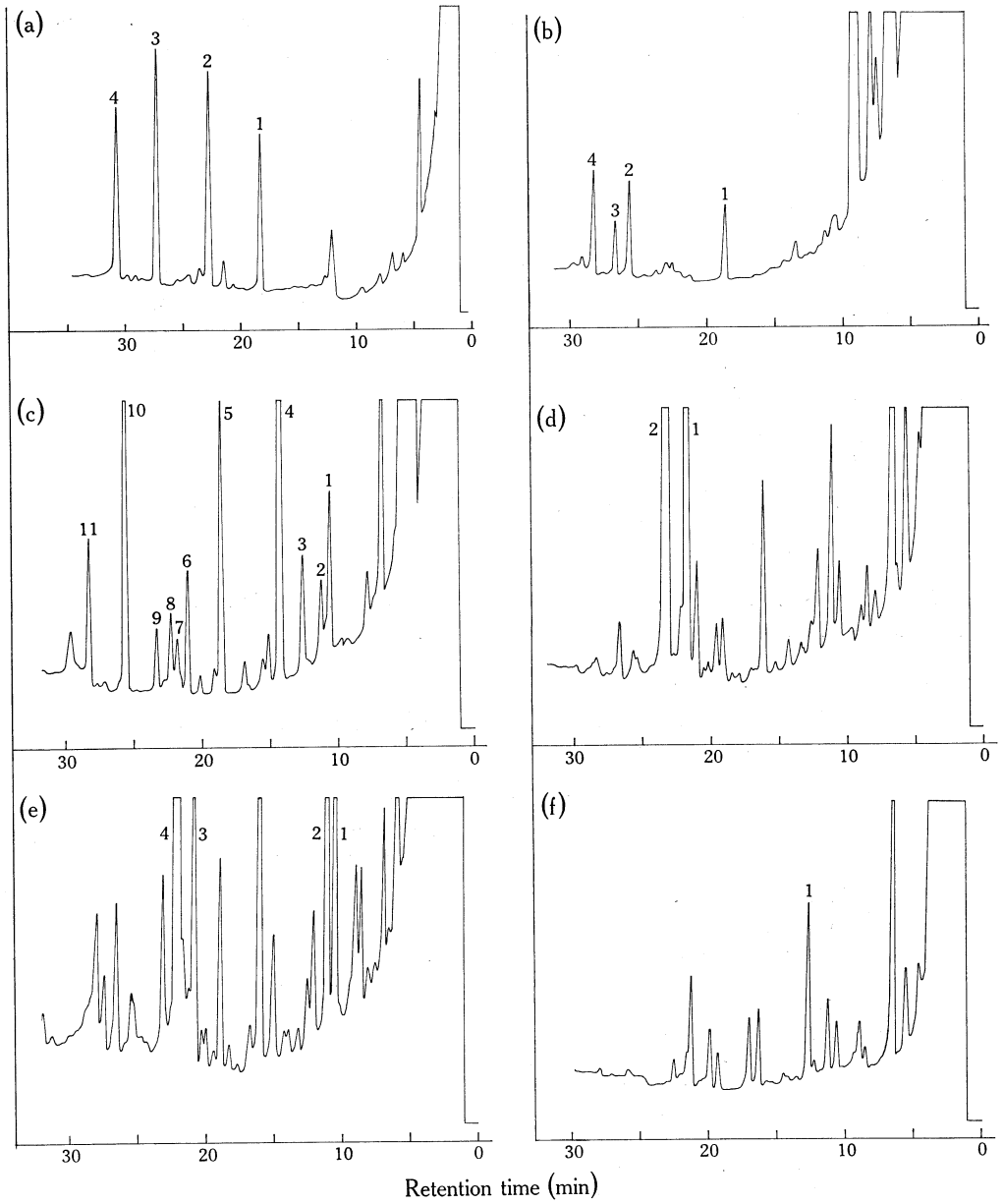


Fig. 2.—(*a*) Serum amino acids from a phenylketonuric patient. *1*, 2-Amino-n-octanoic acid (internal standard); *2*, phenylalanine; *3*, palmitic acid; *4*, stearic and oleic acids. (*b*) Serum amino acids from a tyrosinosis patient. *1*, 2-Amino-n-octanoic acid (internal standard); *2*, palmitic acid; *3*, tyrosine; *4*, stearic and oleic acids. (*c*) Serum amino acids from a case of maple syrup urine disease. *1*, Alanine; *2*, glycine; *3*, valine; *4*, leucine and isoleucine; *5*, 2-amino-n-octanoic acid (internal standard); *6*, glutamic acid; *7*, ornithine; *8*, phenylalanine; *9*, lysine; *10*, palmitic acid; *11*, stearic and oleic acids. (*d*) Urine amino acids from a patient suffering from cystine–lysinuria. *1*, Ornithine; *2*, lysine; internal standard was not added to this sample. (*e*) Urine amino acids from a case of phenylketonuria. *1*, Alanine; *2*, glycine; *3*, glutamic acid; *4*, phenylalanine; internal standard not added. (*f*) Urine amino acids from the patient with the "unknown" amino acid. *1*, β-Aminoisobutyric acid; internal standard not added.

quantitative study with several of the pure derivatives showed that 10^{-12}M of an amino acid can be detected with the flame-ionization detector [3 cm peak height at 4×10^{-11} A for full scale deflection on maximum sensitivity (range × 1, attenuator 1)].

The homogeneity of the g.l.c. peaks in the chromatograms was checked with a continuously scanning mass spectrometer and the linear relationship between amino acid concentration and peak area was established for several amino acids (Fig. 1). One of the most promising applications of the above technique is for the detection and study of disorders of amino acid metabolism (Table 3). A systematic screening of mentally retarded children and the analysis of urine and serum samples from selected patients, who were particularly suspect of metabolic disease from a clinical point of view, have resulted in the characteristic metabolic profiles illustrated in this paper [Figs. 2(*a*)–2(*f*)].

One of the most promising applications of the described technique is for the identification of "unknown" and possibly new metabolites. This is exemplified by the identification of an "unknown" amino acid detected during the routine t.l.c. screening of a urine sample at the Royal Children's Hospital (Parkville, Vic.). After our processing [Fig. 2(*f*)] the mass spectral fragmentation pattern of the unknown was found to be consistent with that of an aminobutyric acid [199, M^+; 184, $M-CH_3$; 142, $M-C(CH_3)_3$; 126, $M-COOC_2H_5$]. Comparison of the unknown spectra with that of the three possible isomeric aminobutyric acid reference spectra showed the compound to be β-aminoisobutyric acid. This urinary amino acid, although not commonly excreted, has been reported previously (Dent *et al.* 1951). In this particular case is does not appear to be associated with any abnormality or disease.

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