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CHROMATOGRAPHY FOR DIAGNOSIS OF METABOLIC DISEASES

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SUMMARY

Chromatographic techniques, including computerized capillary gas chromatography–mass spectrometry and high-performance liquid chromatography with rapid scanning detection are important parts of a multicomponent analytical system designed for the diagnosis of human metabolic disorders. The usefulness of such a system is exemplified by the finding of a new case with the very rare disease hypersarcosinuria and the detection of unusual metabolites in a case of phenylketonuria.

INTRODUCTION

During the past 20 years a number of centres for the diagnosis of metabolic diseases have been established in various parts of the world[1–3]. Various chromatographic methods, including thin-layer chromatography, gas chromatography, gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) and HPLC with a computerized diode array detector, are suitable to detect and identify metabolites that are characteristic of the various diseases. The chromatography system currently used in our laboratory[4] uses all these techniques and is capable of diagnosing over 100 different metabolic diseases.

In the present report the value of chromatography is exemplified by some recent diagnostic applications of our analytical system.

MATERIALS AND METHODS

Sarcosine (N-methylglycine), pyroglutamic acid (5-oxoproline) and BSTFA were products of Sigma (St. Louis, MO, U.S.A.). N-Nitrosomethylurea, for diazomethane production, was obtained from K&K Labs. (New York, NY, U.S.A.). N-Acetylphenylalanine was prepared from L-phenylalanine by acetylation with a mixture of acetic acid and acetic anhydride at 80°C for 1 h. All other chemicals and solvents used were commercially available products of analytical grade.

Amino acid analysis

A Kontron automatic amino acid analyser, Liquamat III (Labotron Instrumente, Zürich, Switzerland) was used for quantitative amino acid analysis, using ion-exchange chromatography and post-column ninhydrin reaction.

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GC–MS analysis

The instrument used was a Hewlett-Packard 5970 mass selective detector coupled to a gas chromatograph with an automatic sample injection system (HP 5890 GC with HP 7673A 100 sample injector) and a HP 300 data system. The GC column was a 30 m fused-silica capillary column coated with SP-1000 (Supelco, Bellefonte, PA, U.S.A.). Nearly all GC peaks were identified using the mass spectral library search program supplied by Hewlett-Packard and modified in our laboratory to automatically identify *ca.* 100 organic acids known to carry specific, diagnostic information[4]. Urine samples are usually acidified, extracted with diethyl ether and methylated with diazomethane before injection into the system[4].

RESULTS AND DISCUSSION

Unusual metabolites in a case of phenylketonuria (PKU)

Routine screening for PKU (measurement of phenylalanine in blood) recently resulted in the recognition of a newborn boy suspected of carrying this disease. As the PKU diagnosis was not certain in this case, a urine sample was submitted to our laboratory for confirmatory analysis using advanced chromatographic techniques. Amino acid analysis revealed increased urinary phenylalanine (30 times above the normal excretion) and also increased amounts of some other amino acids (not shown). Fig. 1 (top) shows the complete urinary organic acid profile as determined by GC–MS. Fig. 1 (bottom) shows the middle portion of this profile on an expanded scale. Large amounts of the typical phenylalanine metabolite, phenyllactate, were found and also considerable amounts of its oxidized form, phenylpyruvate (the latter metabolite decomposes partly at the high temperature in the GC–MS system resulting in a false low peak on the GC trace). In addition, unusually high amounts of N-acetylphenylalanine was present, indicating that the large intracellular excess of phenylalanine had undergone extensive N-acetylation in this patient. N-Acetylation is usually only a minor pathway in amino acid metabolism[5,6]. One of the peaks (see Fig. 1) was furthermore identified as pyroglutamic acid, which was increased some 20-fold above the usual level. Pyroglutamate (5-oxoproline), a metabolite of the γ-glutamyl cycle involved in amino acid transport[7], has previously not been reported to be elevated in PKU. From Fig. 1 (top) it is also evident that the urine contained considerable amounts of *p*-hydroxyphenyllactate, indicating reduced liver function.

These results confirmed the PKU diagnosis. The patient, however, has an atypical urinary metabolite pattern with considerable amounts of N-acetylphenylalanine and pyroglutamate in addition to the typical PKU metabolites, phenylpyruvate and phenyllactate.

Diagnosis of a case with hypersarcosinuria

Urine from a 2-year-old girl, mentally retarded and of small stature, contained large amounts of an usual peak in the amino acid chromatogram (Fig. 2). From the retention time and comparison with reference compounds it was inferred that the unknown compound might be sarcosine (N-methylglycine). The profile of urinary organic acids as determined by GC–MS of a methylated diethyl ether extract was normal (not shown). This was not surprising in view of the insolubility of sarcosine in diethyl ether. To identify the unknown compound with certainty, an aliquot (200

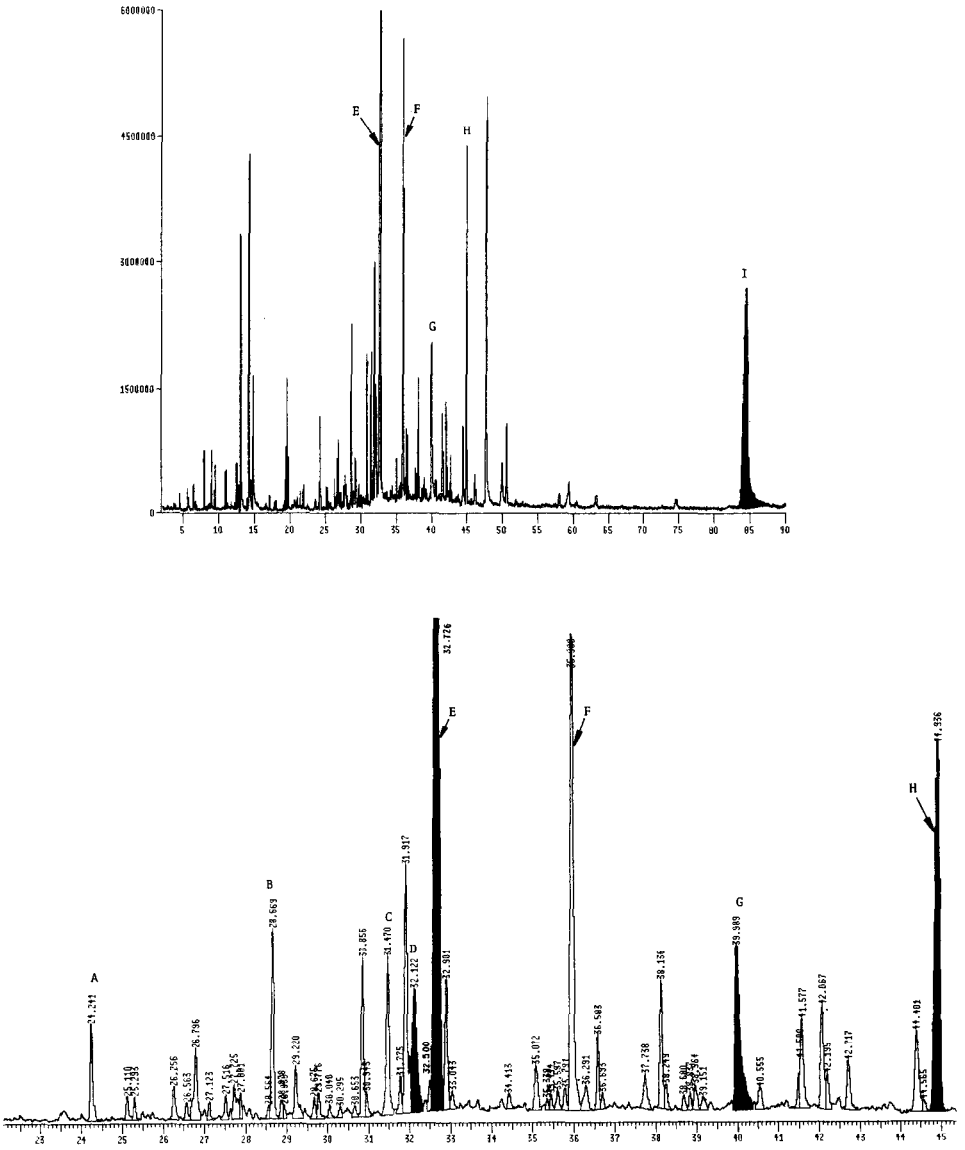

Fig. 1. Organic acid profile of urine from a patient suspected of having phenylketonuria (PKU). The sample was treated as described in the text. The fused-silica capillary column (30 m, SP-1000) was programmed from 50°C to 250°C. (Top) Complete chromatogram; (bottom) middle portion of the chromatogram on an expanded scale. Peaks: A = 3-hydroxy-3-methylglutarate; B = α-ketoglutarate; C = mandelate; D = phenylpyruvate; E = phenyllactate; F = citrate; G = pyroglutamate (5-oxoproline); H = N-acetylphenylalanine; I = *p*-hydroxyphenyllactate. Note the unusual metabolites G and H in this PKU urine.

μl) of urine from the patient was lyophilized and the dry residue was silylated with BSTFA. This method of derivatization had to be used instead of methylation, as the glycine that is always present in urine may be methylated by diazomethane to produce

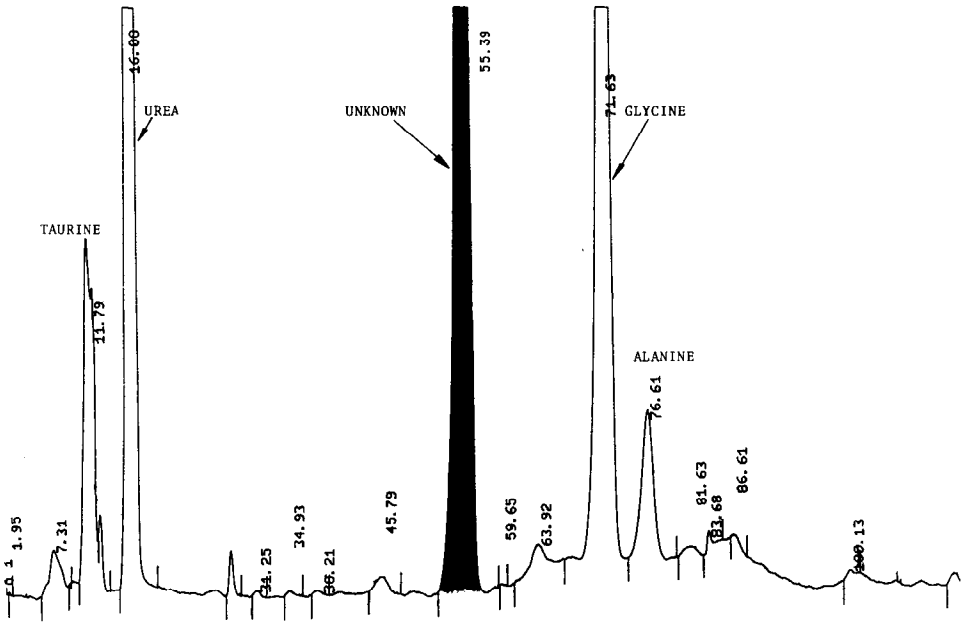

Fig. 2. Initial part of the amino acid chromatogram of urine from a two-year-old patient. Traditional ion-exchange chromatography with ninhydrin post-column reaction. Note the large amounts of an unknown compound.

sarcosine artificially. The mass spectra of the trimethylsilyl (TMS) derivatives of sarcosine are furthermore rather similar to the spectra of the TMS derivatives of alanine (not shown). Fortunately the GC retention times differ considerably, making the differentiation between sarcosine and alanine easy.

Fig. 3 (top) shows a portion of the chromatographic profile of urine from the patient. The two major peaks have identical retention times with authentic sarcosine, mono- and di-TMS derivatives, respectively (Fig. 3, bottom). Moreover, the mass spectra of the two sarcosine peaks in the patient sample were identical with the spectra of the reference compound shown in Fig. 4. It can thus be concluded with certainty that the patient excreted large amounts of sarcosine in her urine.

Hypersarcosinuria is a very rare condition[8]. Only about a dozen cases have been reported since it was first identified in 1965. The enzyme defect is located to the so-called one-carbon cycle[8], but it is unclear whether this deficiency is responsible for the clinical condition, which appears to vary considerably in the few cases known.

CONCLUSIONS

The discovery of new metabolic disorders has often been preceded by the availability of new laboratory tests and instrumental techniques. During the first part of this century the analytical possibilities were simple chemical methods, *e.g.* the ferric chloride test, the nitroprusside reaction and other spot tests. Only a handful of metabolic diseases were recognized at that time, including PKU. A dramatic change occurred with the advent of the ninhydrin reaction and amino acid analysis by paper

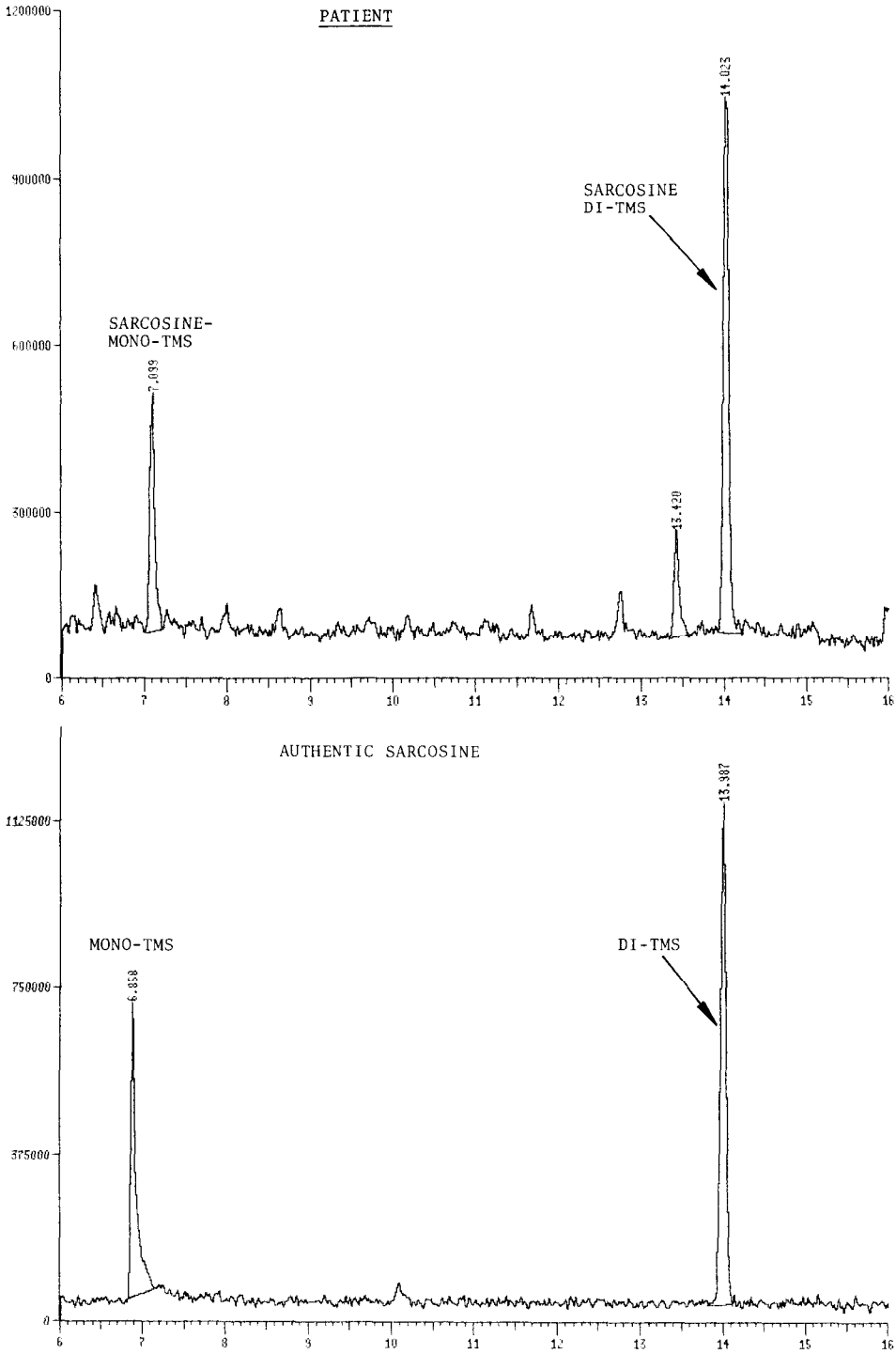

Fig. 3. (Top) Early part of the chromatographic profile of urine from the patient in Fig. 2; lyophilized urine (200 μl) was derivatized with BSTFA before GC–MS. (Bottom) Chromatogram from GC–MS analysis of authentic sarcosine. The two peaks are the mono- and di-TMS derivatives, respectively.

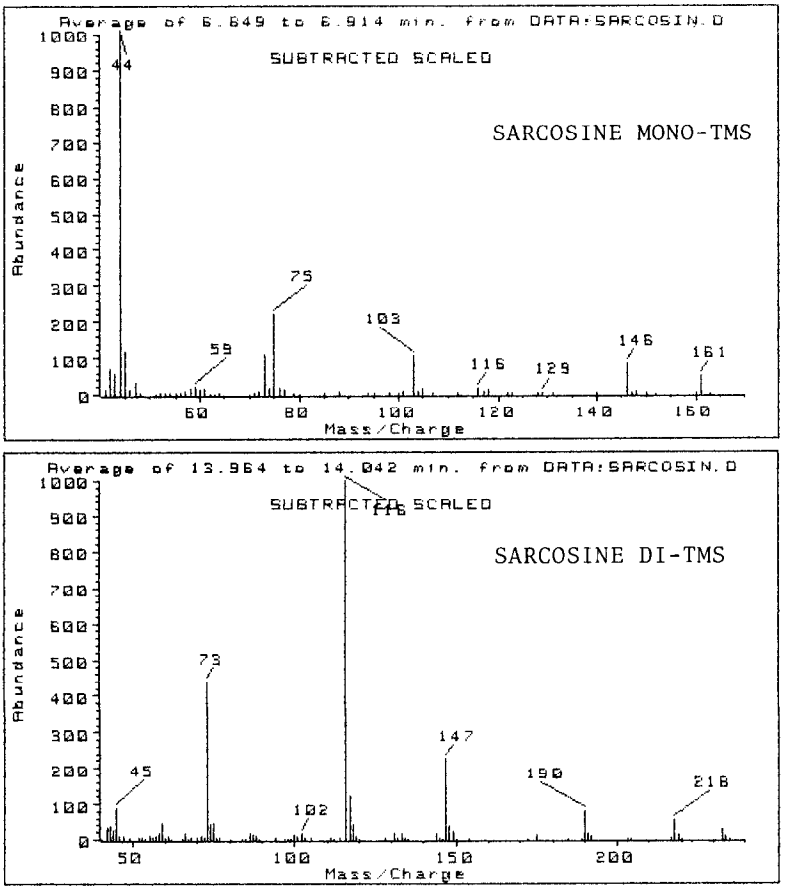

Fig. 4. Mass spectra of mono- and di-TMS derivatives of sarcosine.

and ion-exchange chromatography. Many disorders related to amino acid metabolism were soon found. During the past two decades we have witnessed the progress of GC, MS, and HPLC, and have seen how these techniques can lead to diagnosis of many new diseases.

Although modern DNA technology has been introduced for diagnostic purposes, this technique is as yet only suitable in the diagnosis of cases in which a particular disease is suspected due to inheritance. The DNA methods are therefore particularly appropriate for prenatal diagnosis in situations where the family already has a child with a known disease. The chromatographic methods, in contrast, can be used to diagnose close to one-half of all the 250–300 metabolic diseases recognized today, without knowing what to look for. It can be postulated, therefore, that chromatography, for many years to come, will continue to be a most helpful tool for diagnosing and learning more about human metabolic diseases.

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