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ADVANCES IN THE USE OF COMPUTERIZED GAS CHROMATOGRAPHY–MASS SPECTROMETRY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH RAPID SCANNING DETECTION FOR CLINICAL DIAGNOSIS

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SUMMARY

A multi-component analytical system designed for the diagnosis of metabolic disorders is described. The urinary components are separated by a variety of chromatographic techniques, including automated amino acid analysis, high-performance liquid chromatography with diode-array detection and gas chromatography–mass spectrometry with a computerized mass spectral library search for identification of organic acids. The complete system can be used to diagnose over 100 different metabolic diseases. The usefulness of the chromatographic system is exemplified by the pre- and postnatal diagnosis of glutaric aciduria type I, the diagnosis of lysinuric protein intolerance and of alkaptonuria. Drugs and diet may cause interfering metabolites, as exemplified by glycofurol, used as a solvent for intravenous drugs, and saccharin. It is predicted that chromatography and mass spectrometry will continue to be important diagnostic tools for many years ahead.

INTRODUCTION

Nearly 25 years ago, we began to use gas chromatography (GC) to study the metabolic disorder Refsum's disease (phytanic acid storage disease). The potential of using GC, particularly in combination with mass spectrometry (MS), to diagnose different metabolic diseases was soon realized. In 1969, the first GC–MS instrument was installed in our Institute at the National University Hospital of Norway. Prior to this, we had on several occasions used the GC–MS equipment at the Karolinska Institute in Stockholm, Sweden, *e.g.*, in 1967 to diagnose the first case of methylmalonic acidaemia[1]. GC analysis, including various extraction steps, hydrolysis and different methods of derivatization and separation on various packed GC columns, was put into systematic operation in 1970[2]. The identification of metabolites present in increased amounts in urine and serum because of an enzyme deficiency was carried out by mass spectrometry, followed by an off-line computer search against a mass spectral library of reference spectra, which we have programmed[2]. This GC–MS–computer system made it possible to detect and identify many unknown compounds within a short time, and proved to be well suited to the diagnosis and studies of a number of

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different metabolic diseases. Both we[3,4] and others[5–9] soon discovered several new diseases with the aid of GC–MS.

Our analytical system for detecting metabolic diseases has undergone considerable changes over the years, *e.g.*, to include the latest advances in chromatography and electrophoresis. In this paper, our current routine system used for diagnosis is described, followed by some recent applications.

EXPERIMENTAL

Patient selection and sample preparation

The patients selected for multi-component analyses usually have one or more of the following clinical "warning signals": progressive disease, recurrent disease, similar cases in the family, failure to thrive, metabolic acidosis, peculiar smells of the body and urine, recurrent vomiting, liver pathology, sopor/coma, convulsions and other neurological symptoms of unknown etiology. In addition to the clinical information, we require that every sample submitted for analyses be accompanied by information about drug intake. Interfering drug metabolites are a serious problem, which over the years has led to much loss of time and resources.

Usually 5 ml of urine (depending on the creatinine concentration) is acidified and extracted with diethyl ether and the organic acids are subsequently converted into the corresponding methyl esters with diazomethane. Other laboratories prefer to use, *e.g.*, ion exchange for the isolation of the acids and silylation to make them volatile before GC–MS analysis[6,7].

Sample treatment prior to automatic amino acid analysis includes addition of sulphosalicylic acid to remove small amounts of urinary proteins. HPLC analysis is carried out directly on urine (diluted about 10-fold, depending on the creatinine concentration). When pathological HPLC isograms and chromatograms are seen, sample clean-up with Bond-Elut cartridges (Analytichem, Harbor City, CA, U.S.A.) is carried out before HPLC analysis[10].

GC–MS analyses

Two GC–MS instruments are in daily use with a third as back-up. The first is a Hewlett-Packard (Avondale, PA, U.S.A.) 5970 mass-selective detector coupled to a gas chromatograph (HP 5890 GC) with an automatic sample injection system (HP 7673A 100) and an HP 300 data system. The second instrument is a Finnigan (Sunnyvale, CA, U.S.A.) Model 4021 C GC–MS system with an Incos/Nova 4 data system. The back-up instrument is a Varian-Mat (Bremen, F.R.G.) 112, which is operated when required and used manually. The GC–MS instruments are fitted with fused-silica capillary columns (30 m) and the coatings are usually SP-1000 or SPB-5 (Supelco, Bellefonte, PA, U.S.A.). Nearly all GC peaks are automatically identified by a mass spectral library search. The libraries now in use contain the mass spectra of about 40 000 different compounds, including many drug metabolites and metabolites of biological interest, and the collection of 230 mass spectra of urinary organic acids published by Spiteller and Spiteller[11]. In addition to the computer search routines supplied commercially (Finnigan and Hewlett-Packard), we have developed a simple program which automatically identifies about 200 organic acids known to carry specific, diagnostic information.

HPLC with rapid scanning detection

The HPLC instrument is an LDC liquid chromatograph with two ConstaMetric pumps (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The diode-array detector is a Model 2140 rapid spectral detector (LKB, Bromma, Sweden) with an IBM-XT personal computer. The LC-18 reversed-phase column (Supelcosil) was obtained from Supelco, the Asahipak GS 320 H porous polymer column from Gasukuro Kogyo (Tokyo, Japan) and the Bond-Elut strong cation exchanger from Analytichem. An Aminex HPX-87H ion-exchange column (Bio-Rad Labs., Richmond, CA, U.S.A.) is used for the HPLC of lactate and pyruvate in cerebrospinal fluid in cases of suspected cerebral lactic acidosis[12].

RESULTS AND DISCUSSION

The complete multi-component analytical system-currently used in our laboratory is summarized below (for more details, see ref. 10). Before embarking on analyses, information on clinical condition and drug intake is an essential requirement.

Investigations begin with simple dip-stick tests for, *e.g.*, glucose, blood ketone bodies and reducing substances. Creatinine concentration is determined. Thin-layer and paper chromatography are used for the detection of mucopolysaccharides and carbohydrates, respectively. Quantitative amino acid analysis is performed with automated ion-exchange chromatography and ninhydrin detection. Organic acids are identified by computerized GC–MS with automated sample injection and an automated mass spectral library search. HPLC diode-array with a computer determines purines, pyrimidines, orotic acid, carbamylphosphate, succinylpyrines and other non-volatile compounds. Lactate/pyruvate in cerebrospinal fluid is also measured. Finally, assay of the suspected defective enzyme is carried out in leukocytes, biopsies, fibroblasts or amniotic fluid cells when required.

The complexity of this system, despite considerable automation, makes it impossible to carry out a large number on analyses on samples from patients. Fortunately, this is not required, as metabolic diseases are rare. Our current capacity is around 1000 complete patient evaluations per year.

Urine and blood samples from cases suspected of having metabolic disease are sent to our laboratory from hospitals in Norway. In many instances, the outcome of the analyses results in a correct diagnosis. Follow-up studies, *e.g.*, monitoring the effect of treatment and, in a few instances, prenatal diagnosis, are also carried out by chromatographic methods in our laboratory. Below we report some typical recent results, which illustrate how the various chromatographic techniques play important roles in diagnosis.

Glutaric aciduria type I

A 2-year-old girl had involuntary movements, epilepsy and hydrocephalus, and had been shunted when she was 1 year old. The urine analyses showed a normal amino acid chromatogram, but the organic acid profile, as determined by GC–MS, showed the presence of large amounts of glutaric acid and also some 3-hydroxyglutarate (Fig. 1). These diagnostic metabolites were recognized by the computerized mass spectral library search. It was clear that the patient suffered from glutaric aciduria type I, which is due to an enzyme defect (glutaryl-CoA dehydrogenase) in the degradation of the

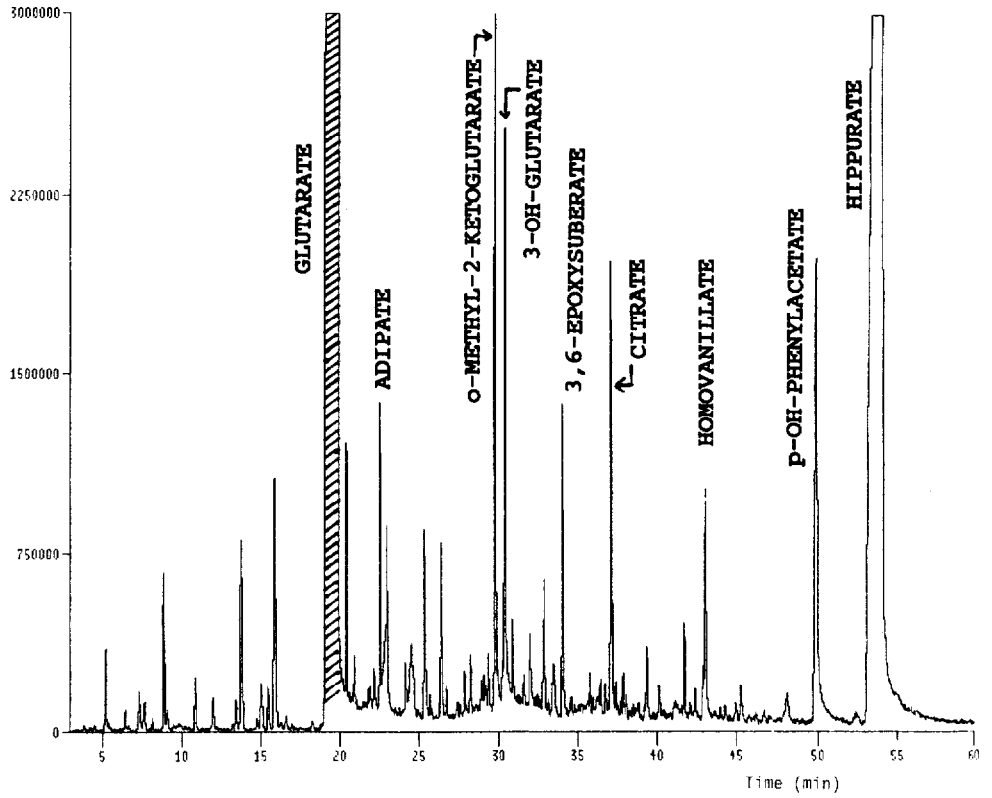

Fig. 1. Organic acid profile (total ion current *vs.* time) of urine from a 2-year-old girl suffering from glutaric aciduria type I. An SP-1000 fused-silica capillary column (30 m) was programmed from 80 to 220°C at 4°C/min, in a Hewlett-Packard 5970 GC–MS system. Note large amounts of glutarate and smaller amounts of the secondary metabolite 3-hydroxyglutarate.

amino acids lysine and tryptophan[13]. The patient is now given a diet low in these amino acids and low in proteins. The excretion of glutaric acid is frequently checked by quantitative GC–MS, using 2-methylglutarate as internal standard.

The following example illustrates the use of GC–MS in the prenatal diagnosis of glutaric aciduria type I after the discovery of two cases in the same family: the first child is a healthy boy; the second child (boy) was admitted to our hospital at the age of 2.5 years with symptoms similar to those of the patient mentioned above. Glutaric aciduria type I was diagnosed. Unfortunately, he died shortly afterwards. When a third boy was born, prenatal diagnosis had not been carried out, but his urine was analysed during his first days of life. Glutaric aciduria I was also detected in this child. Dietary treatment was immediately installed, and now at the age of 4.5 years his development is satisfactory. During the fourth pregnancy, amniotic fluid was obtained in the 16th week. After removal of the cells for culturing, the supernatant fluid was analysed by GC–MS in the selected-ion monitoring mode (the fragment of m/z 129 was used). It was evident that the glutaric acid content was not elevated compared with control amniotic fluid (Fig. 2). If the foetus had been afflicted, the glutaric acid

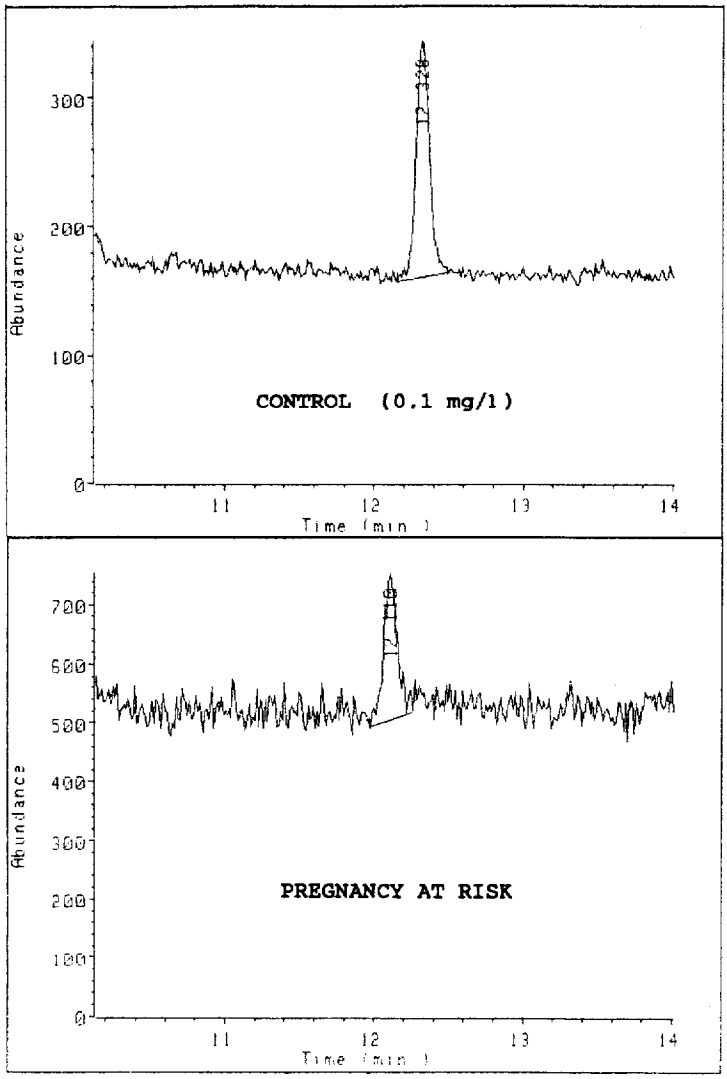

Fig. 2. Determination of glutaric acid in amniotic fluid from a control and from a pregnancy at risk by selected-ion monitoring. Dimethyl glutarate was monitored at $m/z = 129$. 2-Methylglutaric acid was used as an internal standard (not shown). The control sample contained 0.1 mg/l of glutaric acid in amniotic fluid, the pregnancy at risk sample even less, indicating that the foetus was not afflicted with glutaric aciduria type I.

concentration would have been about 50 times higher than that actually found[14]. This information could be passed on to the mother a few hours after amniocentesis, whereas the result of the enzyme studies became available only 3 weeks later and confirmed normal enzyme activity. Although GC–MS technology is very rapid and useful for the prenatal diagnosis of glutaric aciduria type I and 10–20 other diseases, *e.g.*, tyrosinaemia (determination of succinylacetone in the amniotic fluid) and isovaleric

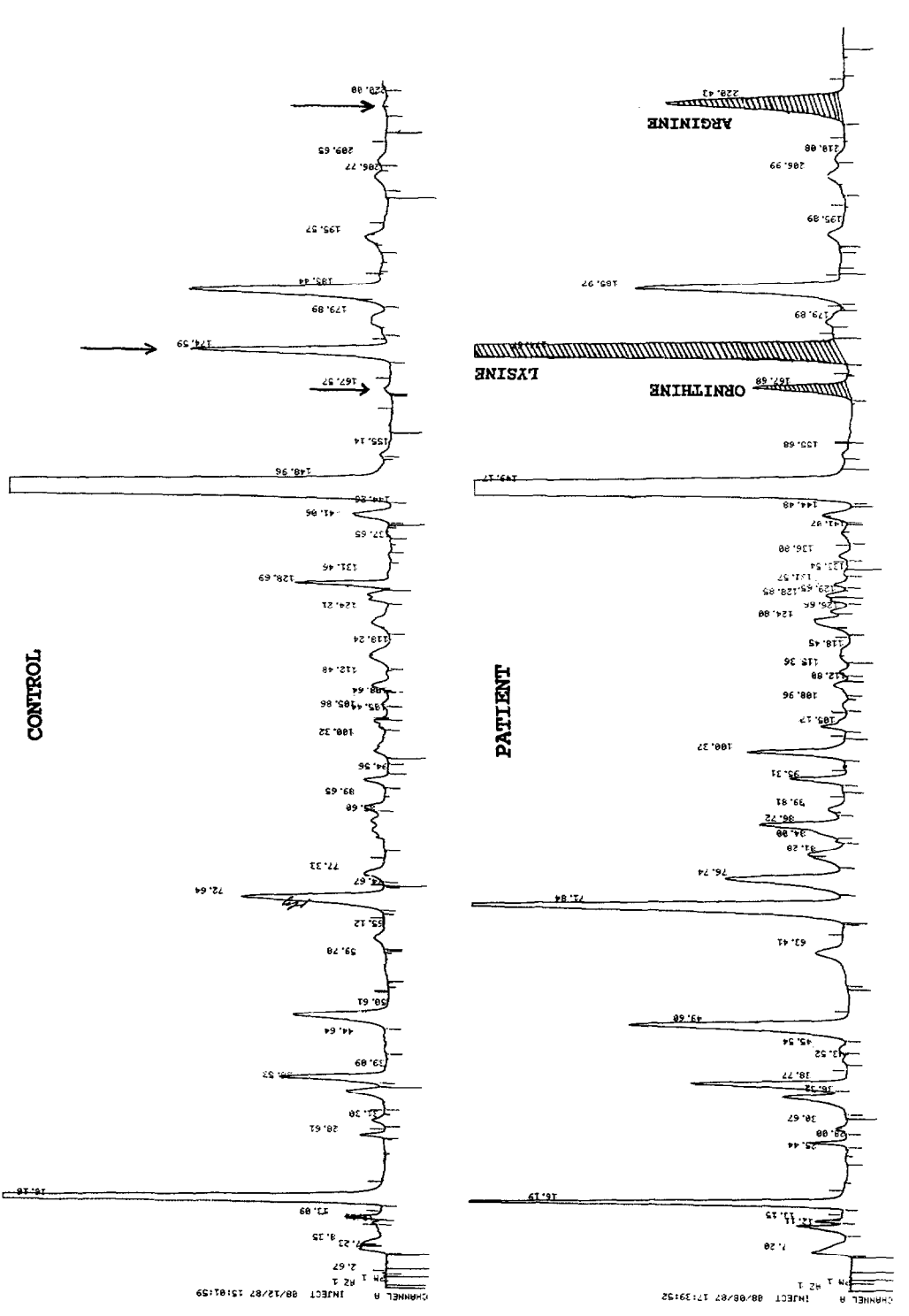

Fig. 3. Amino acid chromatograms of urine from a control (top) and from a patient (bottom) with lysinuric protein intolerance. Note the increased excretion of lysine, arginine and ornitine in the sample from the patient.

acidaemia (determination of isovalerylglycine in the amniotic fluid), it should be emphasized that, as a rule, enzyme studies and/or analyses at the DNA level are by far the most widely used techniques for the prenatal diagnosis of metabolic diseases.

Lysinuric protein intolerance

A 10-year-old girl had suffered for several years from frequent vomiting, particularly after intake of protein-rich food. There was a marked growth retardation. A urine sample was analysed with our standard chromatographic system. The organic acid pattern was normal, but the amino acid chromatogram (Fig. 3) and HPLC profile (Fig. 4) were pathological. The lysine excretion, in particular, was greatly increased, and increased amounts of arginine and ornithine were also found (Fig. 3). The HPLC trace, recorded at 278 nm (Fig. 4), showed the presence of large amounts of a compound that eluted at the same position as orotic acid. The absorption spectrum of this peak (Fig. 4, inset) as recorded by the diode-array detector was also identical with that of authentic orotic acid. Increased urinary excretion of lysine, arginine, ornithine (but not cystine) and orotic acid is characteristic of lysinuric protein intolerance (LPI)[15]. This amino acid transport disorder is very rare in most countries (the present case was the first in Norway), except in Finland, where more than 100 cases have been reported.

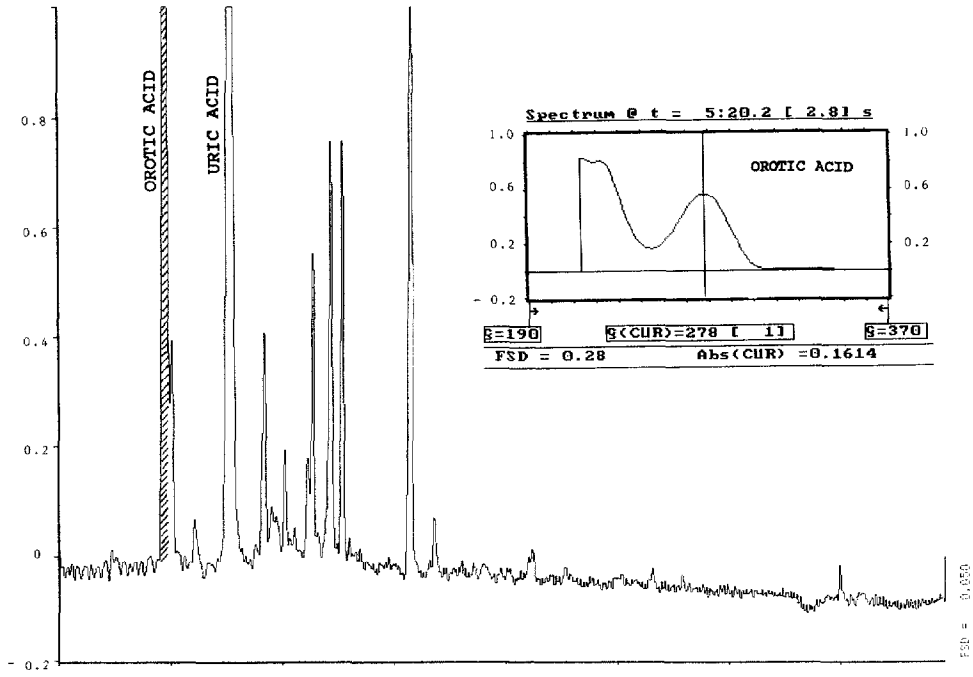

Fig. 4. HPLC profile of urine from a patient with lysinuric protein intolerance. The Supelcosil LC-18 column was eluted with a gradient system: starting eluent, 5 mmol/l H_2SO_4; end solution, 40% (v/v) aqueous acetonitrile; flow-rate, 1.0 ml/min; detection, 278 nm. The peak designated as orotic acid had a retention time and absorption spectrum identical with those of authentic orotic acid (inset).

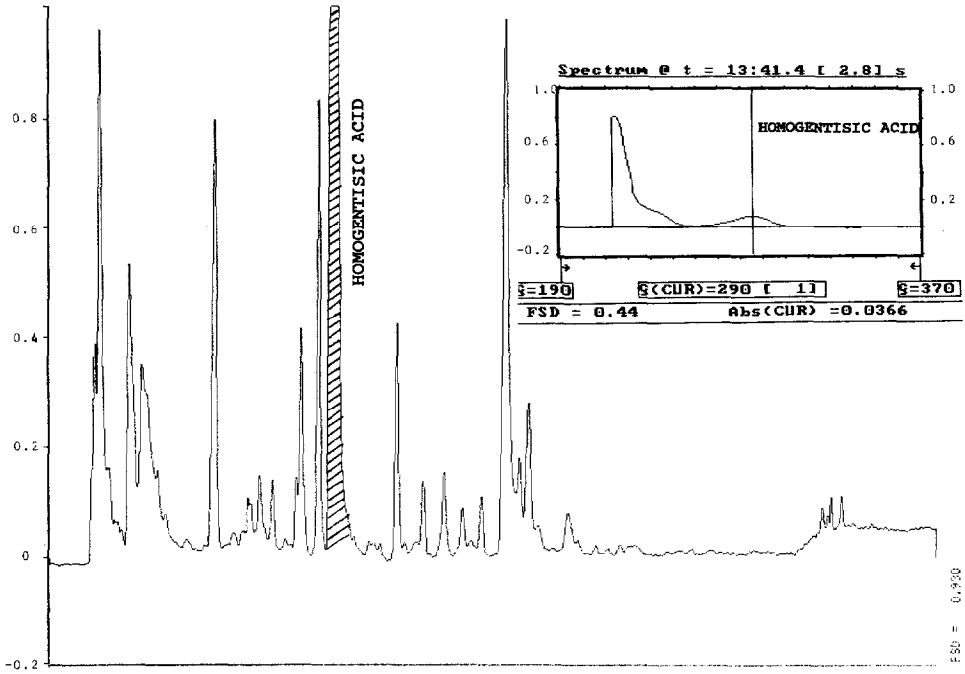

Fig. 5. HPLC profile of urine from a patient with alkaptonuria. Experimental conditions as in Fig. 4.

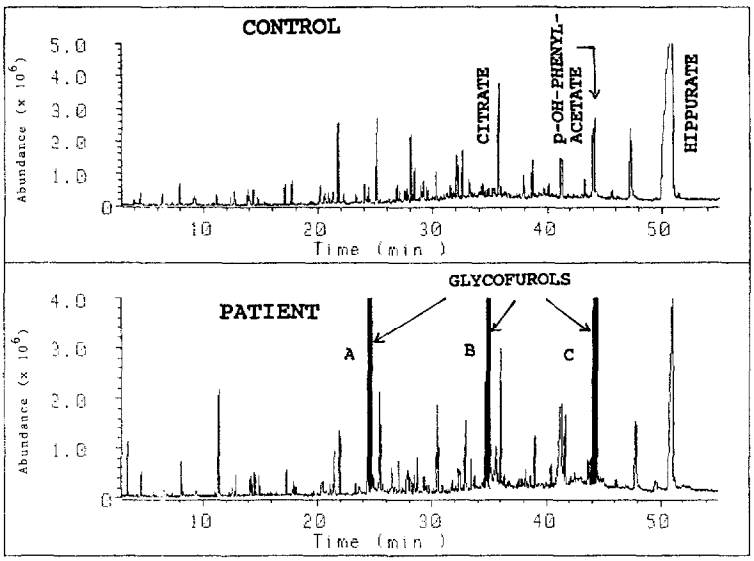

Fig. 6. Organic acid profile of urine from a control and from a patient who had received diazepam intravenously. Experimental conditions as described in the text and in Fig. 1. Peaks A, B and C are due to metabolites of glycofurol, used as a solvent in intravenous pharmaceutical preparations.

Alkaptonuria

Alkaptonuria (homogentisic acid oxidase deficiency) is one of the first inborn errors of metabolism ever recognized[16]. Children and young adults usually have no symptoms, but with age pigmentation of the sclera, cartillage or fibrous tissue occurs. Later, the patients develop arthritis. Because of the enzyme defect, homogentisic acid accumulates and is excreted in gram amounts in the urine. Homogentisic acid is oxidized by air on standing and alkalinization to a black pigment. This darkening of urine was easily spotted in former days, when chamber pots were in more frequent use. Today's patients, however, may never recognize the slow darkening of their own urine, as it has a normal, clear appearance when passed. We have come across several patients suspected of alkaptonuria. Obviously, the first and easiest test is to add alkali to the urine and observe eventual darkening. Confirmation of the presence of homogentisic acid can be obtained by GC–MS or, more conveniently, by HPLC with rapid scanning detection, as illustrated in Fig. 5.

Drugs and dietary problems

Laboratories involved in metabolic profiling of body fluids are very aware of the problems encountered with drugs and their metabolites. Much time and unnecessary work have been spent on unknown GC peaks thought to carry diagnostic information, only to discover that the compounds were drug metabolites. The present-day mass spectral library contains a number of drugs and drug metabolites and this, together with years of experience and knowledge of drugs in current use, has minimized the problem. However, occasionally we are still confronted with problems due to drugs, as exemplified below.

During the last 2 years, we have analysed some samples that contained three unusual peaks in large amounts (Fig. 6). The mass spectra of these compounds were very similar, all containing repetitive units of $m/z = 44$. Close examination of the clinical record showed that the patients had received drugs, but a variety of different ones. Further investigations (to be published separately) by high-resolution mass spectrometry and *in vivo* experiments showed that the three large peaks in Fig. 6 were metabolites of glycofurol. This is a new inert, non-toxic solvent for a variety of drugs, including antibiotics, diazepam (valium) and antiepileptics, and is particularly well suited for intravenous administration. The hydroxyl group of glycofurol [RCH_2-$(OCH_2CH_2)_nOH$] (R = 2-furyl; $n = 1$–3) is oxidized *in vivo* to give a corresponding set of monocarboxylic acids, and this accounts for the metabolites seen in Fig. 6. Whenever these are present in urine, the inference is that the patient has received some drug intravenously in which glycofurol was used as a solvent.

Another metabolite more and more frequently seen in specimens from patients is saccharin, in our case in the N-methylated form due to diazomethane derivatization. Occasionally, this artificial sweetener may be the dominating urinary metabolite, present in even larger amounts than hippurate (Fig. 7).

CONCLUSION

The past 25 years have demonstrated that chromatographic and mass spectrometric techniques are well suited for the detection of abnormal compounds in blood and urine. These methods are therefore valuable for the diagnosis of a number of

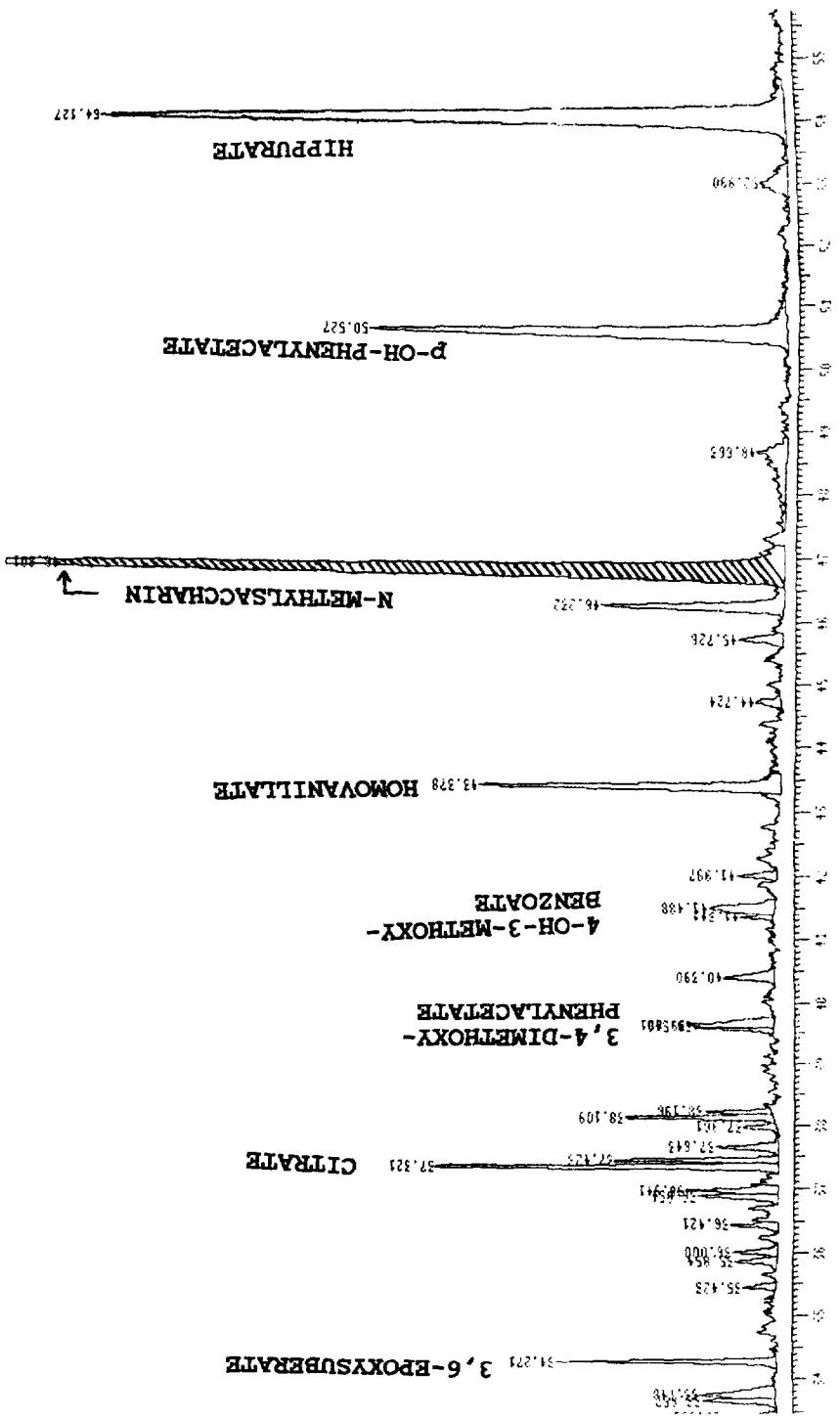

Fig. 7. Organic acid profile (second half) of urine from a person with a large intake of saccharin. Experimental conditions as described in the text and in Fig. 1.

metabolic diseases. A centre for studies of such diseases should, however, also include cell culturing in addition to biochemical techniques, such as enzyme assays, in its analytical repertoire. The question often raised is whether modern DNA technology will make chromatography obsolete for diagnostic purposes. One should realize, however, that DNA technology is as yet suitable for diagnosis only in situations where the family already has had a child with a known disease. The DNA methods are therefore particularly appropriate for prenatal diagnosis. The chromatographic methods, in contrast, can diagnose close to half of all 250–300 metabolic diseases recognized today without a prior knowledge of what to look for. It can therefore be predicted that chromatography will continue to be a most helpful tool for diagnosis and for increasing our knowledge about human metabolic diseases.

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