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Capillary electrophoresis for diagnosis and studies of human disease, particularly metabolic disorders

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ABSTRACT

High-performance capillary electrophoresis (HPCE) has been used in a multicomponent analytical system designed to diagnose and study human diseases, particularly metabolic disorders. Comparative analyses, using HPCE, high-performance liquid chromatography (HPLC) and an automated amino acid analyser, were carried out on urine and blood samples from patients with homocystinuria, cystinuria, glutathione synthetase deficiency and adenylosuccinase deficiency. HPCE of the sulphur-containing amino compounds, derivatized with monobromobimane and detected by fluorescence spectroscopy, was a quick and simple alternative to classical amino acid analysis. The detection of the characteristic succinylpurines associated with adenylosuccinase defect was equally well achieved with HPLC and HPCE (absorbance detector). Owing to the possible connection between deficiency of taurine (2-amino-1-ethanesulphonic acid) in the heart and the development of cardiomyopathy and heart failure, a simple HPCE method was developed for the determination of taurine in sub-milligram samples of biopsies of the myocardium. The homologue 3-amino-1-propanesulphonic acid was the internal standard, and derivatives of 9-fluorenylmethyl chloroformate and fluorescence detection were used. It is suggested that the potential of HPCE to analyse small volumes should be exploited in biomedicine and clinical diagnosis to analyse sub-milligram samples of tissue biopsies and cells.

INTRODUCTION

Several human diseases, in particular metabolic disorders, often lead to the accumulation of characteristic metabolites in serum, urine and cells. Multicomponent analytical techniques, including chromatography and electrophoresis, are suitable to detect diagnostically important changes in the biochemical "profiles" thus obtained. The profiling techniques currently used in this laboratory include gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) with a computerized diode-array detector, automated amino acid analysis and high-resolution two dimensional electrophoresis. DNA techniques (Southern blotting and polymerase chain reaction) and certain enzyme assays have also been included in the analytical system. The techniques currently in use [1,2] can diagnose more than 100 different disorders.

The latest addition to the analytical repertoire in this laboratory is high-performance capillary electrophoresis (HPCE) [3]. Comparative studies have been carried out to learn more about the potential of HPCE in the clinical laboratory. Some

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disorders related to the metabolism of amino acids, nucleotides and the tripeptide glutathione have been selected for this purpose, and HPCE has been used to determine taurine (2-amino-1-ethanesulphonic acid) in sub-milligram samples of biopsies of heart muscle.

EXPERIMENTAL

Chemicals

Monobromobimane (MB; Thiolyte) was from Calbiochem. (La Jolla, CA, USA). L-Cysteine, L-cystine, D,L-homocysteine, DL-homocystine, reduced glutathione (GSH), dithiothreitol (DTT, Clelands's reagent), taurine and 3-amino-1-propanesulphonic acid were from Sigma (St. Louis, MO, USA). 9-Fluorenylmethyl chloroformate (FMOC) was from Fluka (Buchs, Switzerland). All other chemicals were commercial products of analytical-reagent grade.

Instruments

The HPCE equipment used for most of the analysis was made by SpectroVision (Chelmsford, MA, USA) and consisted of a DA-30 power supply (maximum 30 kV), an FD-300 dual monochromator fluorescence detector and a DS-4 sample delivery system. Manual injection was performed using the electrokinetic technique. The capillary was 40 cm × 0.075 mm I.D. in most of the experiments, except in Fig. 4, where a 0.10 mm I.D. capillary was used. The applied voltage was about 24 kV, giving a current of approximately 150 μA using 0.05 mol/l sodium phosphate buffer at pH 7.5. The fluorescence detector was set at 375 nm (excitation) and 480 nm (emission) for measuring the MB-labelled compounds, and the corresponding settings were 265 and 305 nm for the FMOC-labelled compounds.

In some experiments (see Fig. 6) a Beckman P/ACE Systems 2000 HPCE instrument, version 1.50 (Beckman Instruments, Palo Alto, CA, USA) was used (kindly made accessible by Knut Sletten and Vidar Syversen, Biotechnology Center, University of Oslo, Oslo, Norway). The absorbance detector was set at 214 nm, the capillary was 0.075 mm I.D. and a constant voltage of 20 kV gave a current of about 90 μA using a 0.05 mol/l sodium phosphate buffer, pH 2.5. Pressure injection for 3.8 s, giving a volume of about 20 nl, was used.

The HPLC instrument was an LDC liquid chromatograph with two Consta-Metric pumps (Laboratory Data Control, FL, USA) and the diode-array detector was a 2140 rapid spectral detector (LKB, Bromma, Sweden) with an IBM-XT personal computer. The column was of the reversed-phase LC-18 type. Details of the experimental procedures used for the HPLC analysis of urine have been described elsewhere [1,2].

The GC–MS instrument was a Hewlett-Packard 5970 mass-selective detector coupled to a gas chromatograph with an automated sample injection system (HP 5890 GC with HP 7673A 100 sample injector) and an HP 300 data system. A 30 m fused-silica capillary column coated with SP-1000 (Supelco, Bellefonte, PA, USA) was used. The experimental procedures have been described elsewhere [1,2].

A Biotronic automatic amino acid analyser (Biotronic, Maintal, Germany) with an ion-exchange chromatography column and classical post-column ninhydrin detection was used. All samples, including urine, were deproteinized with sulphosal-

icylic acid prior to analysis [1,2]. The total elution time was about 130 min; the total cycle time, including the regeneration of the ion-exchange column, was about 180 min.

Standards

The standard solution of thiol-containing compounds contained 0.2 mmol/l each of cysteine, homocysteine, GSH and the drug penicillamine, in 0.05 mol/l phosphate buffer, pH 7.5. To prevent oxidation of the thiol groups, DTT was added to a final concentration of 0.8 mmol/l. For MB derivatization, 15 μl of Thiolyte MB in acetonitrile (50 mmol/l) were added to 250 μl of standard solution and injected into the HPCE instrument after 10 min.

Standard solutions (5 mmol/l) of taurine and the internal standard (3-amino-1-propanesulphonic acid), in 0.05 mol/l phosphate buffer, pH 7.5, were used. For derivatization 10 μl of each standard were mixed with 50 μl of the FMOC reagent (5 mmol/l, dissolved in acetonitrile) and 50 μl of 0.05 mol/l phosphate buffer, pH 7.5. After 20 min at room temperature the derivatization was complete.

Urine analysis: thiol compounds

Urine samples were reduced with DTT (2.5 mmol/l of urine) for 15 min, diluted with equal parts of the running buffer (sodium phosphate buffer, 0.05 mol/l), adjusted to pH 7.5, and filtered through a 0.22-μm filter. To an aliquot (250 μl) of each filtrate, 50 μl of Thiolyte MB in acetonitrile (50 mmol/l) were added. Injection was performed after 10 min.

Analysis of red blood cells: thiol compounds

Red cells from centrifuged citrate blood (15 min at 2500 rpm, 750 *g*) were diluted with equal parts of water and further lysed by freezing and thawing. Proteins were precipitated by the addition of a perchloric acid solution (1 mol/l) and removed by centrifugation. The supernatant was neutralized to pH 7.5, reduced with DTT, filtered and labelled with MB as described for urine.

Direct analysis of urine: UV-absorbing compounds

Urine from healthy controls and from patients with various metabolic disorders was analysed directly after filtration through a 0.22-μm filter and centrifugation to remove air bubbles.

Determination of taurine in heart muscle biopsies

Human heart biopsies, 1–3 mg, sufficient for several analyses, were obtained by standard surgical procedures routinely performed in this hospital. Rat heart biopsies were also used during method development. The sample size generally used for taurine determination was about 0.5–1 mg of tissue. After weighing the biopsies, the internal standard (3-amino-1-propanesulphonic acid) was added and the mixture was homogenized in 50 μl of phosphate buffer (0.05 mol/l, pH 7.5) in a specially designed glass micro-homogenizer with a ground glass, manually operated pestle. To the homogenate was added perchloric acid (20 μl of a 1 mol/l solution) and the precipitated proteins were removed by centrifugation (Beckman Microfuge). The supernatant was neutralized to pH 7.5 with a few microlitres of 1 mol/l aqueous trisodium phosphate

solution and mixed with a five-to-ten-fold excess on a molar basis of the FMOC reagent (5 mmol/l in acetonitrile). After 20 min at room temperature the derivatized amino compounds were separated by HPCE and detected by fluorescence spectroscopy as described.

RESULTS

Diagnosis of some disorders related to thiol-containing amino compounds

Separation of standards. HPCE gives a baseline separation of MB-labelled homocysteine, cysteine, the drug penicillamine and the tripeptide GSH (elution order) in less than 10 min, as shown in Fig. 1. The reagents, MB and DTT, are eluted together in the first peak, well separated from the other four compounds.

Homocystinuria. This is a disorder with various causes, all resulting in the accumulation of homocyst(e)ine in serum and urine. Clinical symptoms include disloca-

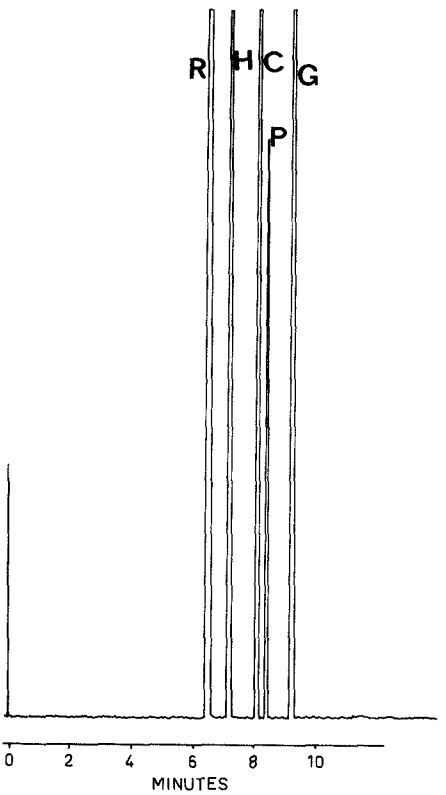

Fig. 1. Separation of thiol-containing compounds by HPCE. The standard compounds were derivatized with MB as described in the text. A SpectroVision HPCE instrument with a fluorescence detector was used, and the capillary was 40 cm × 0.075 mm I.D. A voltage of 25 kV gave a current of about 150 μA (0.05 mol/l phosphate buffer, pH 7.5). Peaks: R = reagent peak; H = homocysteine; C = cysteine; P = penicillamine; G = glutathione.

tion of the eye-lens, osteoporosis, lengthening of the bones and increased tendency to thrombosis [4]. The underlying biochemical defect may be cystathionine β-synthetase deficiency (the most frequent), or other mechanisms including failure to remethylate homocysteine to methionine [4]. Fig. 2A shows the diagnosis of homocystinuria (remethylation defect) using traditional amino acid analysis with ion-exchange chromatography and post-column ninhydrin detection. The control urine did not contain detectable amounts of homocystine, in contrast to that of the patient. The HPCE electropherogram of DTT-reduced and MB-labelled urine from the same patient is shown in Fig. 2B. Only three peaks are seen, corresponding to the reagent peak, homocysteine and cysteine. The increased cysteine peak, compared to controls, is secondary and is usually seen in homocystinuria.

Cystinuria. This is due to a defect in the cellular transport mechanism of the dibasic amino acids cystine, lysine, ornithine and arginine [5]. The defect leads to a high concentration of these four amino acids in urine because their normal reabsorption mechanism in the kidneys does not function. Cystine is only sparingly solu-

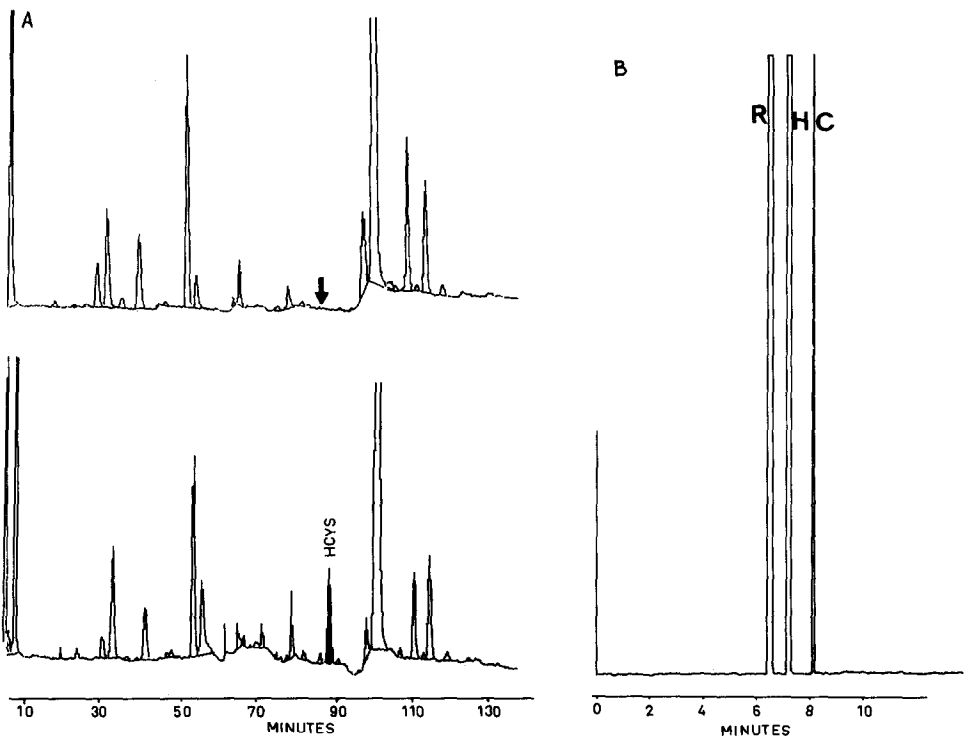

Fig. 2. (A) Amino acid analysis by classical ion-exchange chromatography of urine from a control (upper panel) and a patient with homocystinuria (lower panel). (B) HPCE analysis of urine from the patient. The sample was reduced with DTT and derivatized with MB before separation. Experimental conditions and symbols as described in the text and in Fig. 1.

ble in water, and when the concentration in urine exceeds about 300 mg/l at pH 6, crystallization may begin. Kidney-stone formation is therefore the usual clinical symptom in classical cystinuria. Variant forms of this disorder are also known [5].

The laboratory diagnosis of cystinuria is usually carried out in two steps. First, a simple colour test is performed using sodium nitroprusside after the addition of a reducing agent to the urine. Following a positive test (red colour), quantitative amino acid analysis is required to differentiate between cystinuria and homocystinuria (the latter also gives a positive nitroprusside test). Fig. 3A shows a typical amino acid chromatogram of urine from a patient with cystinuria, with large amounts of the four dibasic amino acids being excreted. The mixed disulphide homocysteine–cysteine is usually a secondary finding, as seen from the figure. Fig. 3B shows the separation by HPCE of the DTT-reduced and MB-labelled compounds. Large amounts of cysteine and some homocysteine are seen, and as only thiol groups react with the MB-reagent, no other amino acids show up on the electropherogram. Note the difference in elution time; this is about 130 min in the classical amino acid analysis and less than 10 min for the HPCE separation.

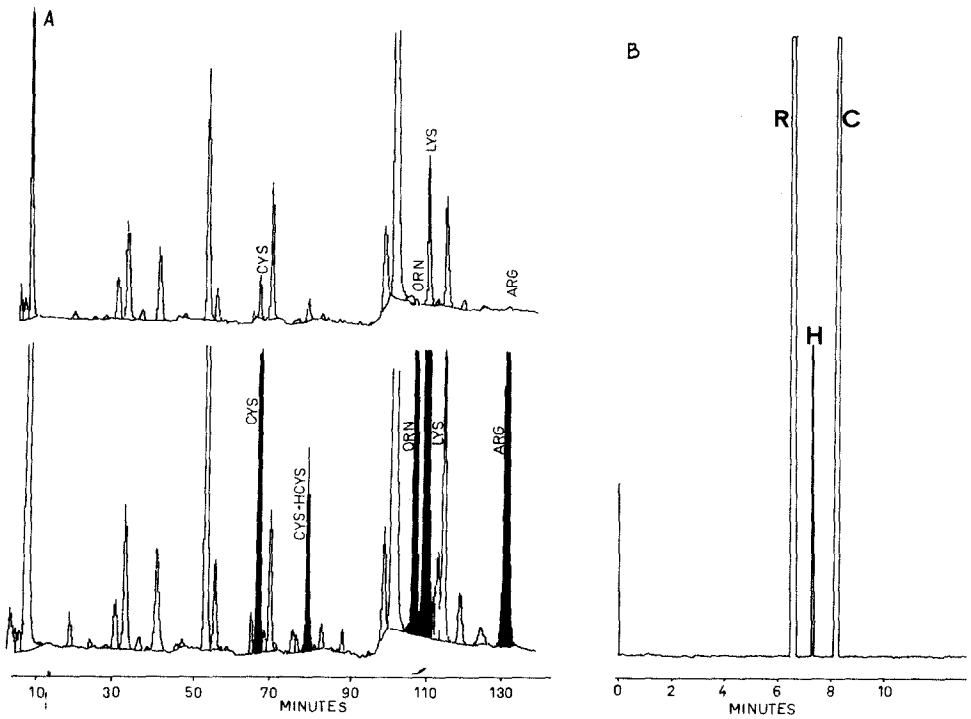

Fig. 3 (A) Amino acid analysis by classical ion-exchange chromatography of urine from a control (upper panel) and a patient with cystinuria (lower). (B) HPCE analysis of urine from the patient. The sample was reduced with DTT and derivatized with monobromobimane before separation. Experimental conditions and symbols as in Fig. 1.

Glutathione synthetase deficiency. Glutathione synthetase deficiency [6] or "pyroglutamic aciduria" as the disease was called previously [7], is associated with the massive urinary excretion of 5-oxoproline (pyroglutamate), severe metabolic acidosis and defective central nervous system function, sometimes of fatal consequence. The enzyme defect causes a deficiency of the tripeptide GSH (GSH = τ–Glu–Cys–Gly) in all cells, with overproduction of 5-oxoproline in a modified τ-glutamyl cycle as a secondary consequence [6]. GSH is believed to exert anti-oxidant properties and a lack of this protectant may lead to the oxidative damage of cells, particularly in the brain.

Laboratory diagnosis of this disorder includes the identification of large amounts (up to 30 g/l) of 5-oxoproline in urine using GC–MS [7]. GSH is normally present at a high concentration (2–3 mmol/l) in all cells [6], but is normally absent in serum and urine. The second step in the laboratory diagnosis, following the finding of 5-oxoproline in the urine, is therefore to confirm the lack of GSH in cells. Red blood cells are often the material of choice, and classical amino acid analysis gives the confirmatory diagnosis, *i.e.* lack of GSH (and its oxidized form). The same result can be achieved in a simpler and much faster way using HPCE, as shown in Fig. 4. The lack of GSH in the red cells of the patient compared with control is readily seen. Note that because MB-labelling which only reacts with thiol groups is used, and as GSH

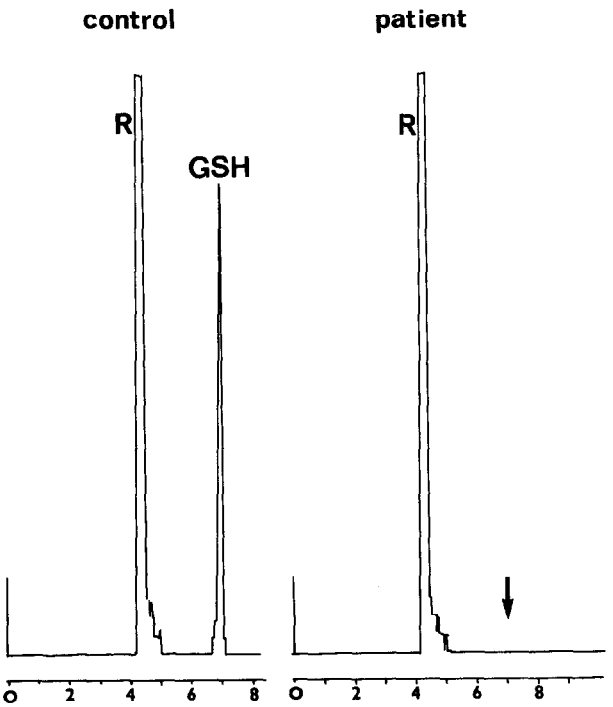

Fig. 4. HPCE analysis of GSH in red blood cells from a control (left) and a patient with GSH synthetase deficiency (right). Experimental procedures as described in the text. The capillary was 40 cm × 0.10 mm I.D., which explains the retention times being slightly different from those shown in Fig. 1. Note the absence of GSH in the cells of the patient.

normally is present at very high concentrations compared with most other cellular constituents, the only peak that is clearly visible at the attenuation level used is the GSH peak.

Adenylosuccinase deficiency. This is a rare disorder in the biosynthesis of purine nucleotides and gives rise to severe psychomotor delay and autism [8]. The diagnostic metabolites present in increased amounts in urine are the succinylpurines adenylosuccinate and succinylamino-imidazole-carboxamid ribotide. These compounds are readily detected by HPLC [1,8], but not by GC–MS. Urine from a patient with this disorder was first analysed in this laboratory by a routine HPLC system with diode-array detection, and then by HPCE using UV absorbance detection as described earlier. (The urine sample was kindly provided by Dr. Jaeken, University of Leuven, Leuven, Belgium.)

Figs. 5 and 6 show that the separation time (13–16 min) and efficiency of the HPLC and HPCE techniques are comparable, the former giving a more reliable identification due to the use of diode-array detection and spectral comparison. Sample pre-treatment included clean-up on a Bond Elute cartridge before HPLC [1], but filtration only (0.22-μm filter) before HPCE.

HPCE determination of taurine in biopsies of heart muscle

Recent reports [9–11] have caused an increased interest in the aminosulphonic acid taurine [NH_2–CH_2–CH_2–SO_3H], because of its possible role in the development of cardiomyopathy. It now seems clear that this compound is an essential amino acid in cats. Taurine-depleted cats develop retinal degeneration, cardiomyopathy, altered white-cell function and abnormal growth and development [11]. Evidence for a taurine-deficiency cardiomyopathy in humans is also beginning to appear [10], and there seems to be a direct link between decreased taurine concentration in the myocardium and decreased myocardial mechanical function, at least in the cat [9].

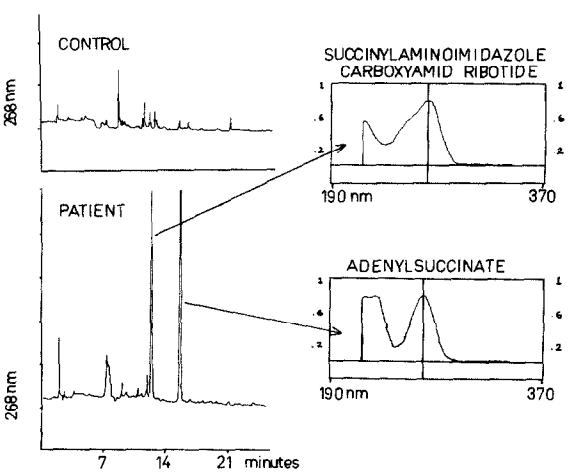

Fig. 5. Left: HPLC profiles of urine from a control (upper panel) and a patient with adenylosuccinase deficiency (lower panel). Right: Characteristic absorption spectra of the excreted succinyl purines as recorded by the LKB 2140 rapid spectral detector. The LC-18 reversed-phase column (Supelcosil) was eluted with a gradient system as follows: start solution 5 mmol/l sulphuric acid, end solution acetonitrile–water (4:6, v/v), flow-rate 1.0 ml/min.

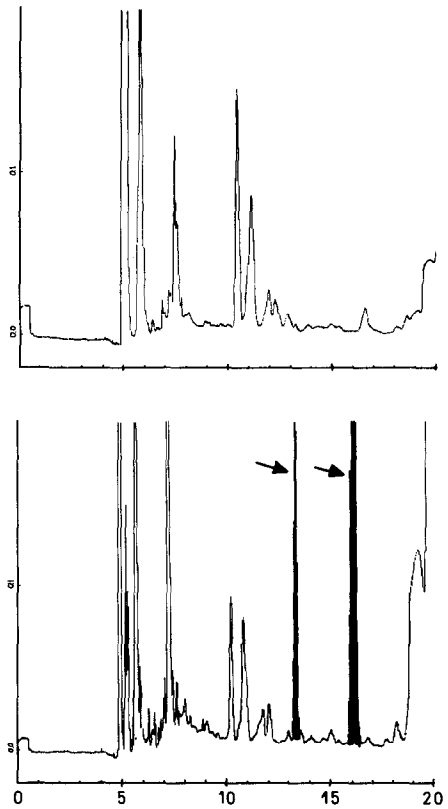

Fig. 6. HPCE profiles of urine from a control (upper panel) and from a patient with adenylosuccinase deficiency (lower panel). Same patient as in Fig. 5. A Beckman P/ACE Systems 2000 HPCE instrument with an absorbance detector set at 214 nm was used (see text). The succinyl purines are marked with arrows (see also Fig. 5).

The treatment of advanced cardiomyopathy in humans may involve heart transplantation, which is carried out routinely in this hospital. In view of the possible link between cardiomyopathy and lack of taurine in the heart muscle, there is a need for a method to determine taurine in small samples of biopsies from the myocardium. HPCE may be a suitable method for this purpose, as indicated in Fig. 7. The electropherogram in Fig. 7A shows baseline separation of equal amounts of the FMOC derivatives of taurine and the very closely related internal standard that was chosen, [NH_2–CH_2–CH_2–CH_2–SO_3H]. Fig. 7B shows the determination of taurine in sub-milligram sample of biopsy of human heart muscle to which the internal standard had been added. As the heart contains a millimolar concentration of taurine (about 5 mmol/l), the attenuation of the HPCE instrument could be kept low. At this level of sensitivity only one other major peak appears, tentatively identified as GSH (present at 2–3 mmol/l in many cells).

These initial results are now being pursued in a collaborative study, and a quantitative HPCE method which require only sub-milligram amounts of tissue will be used to study patients with cardiomyopathy and other myocardial failures.

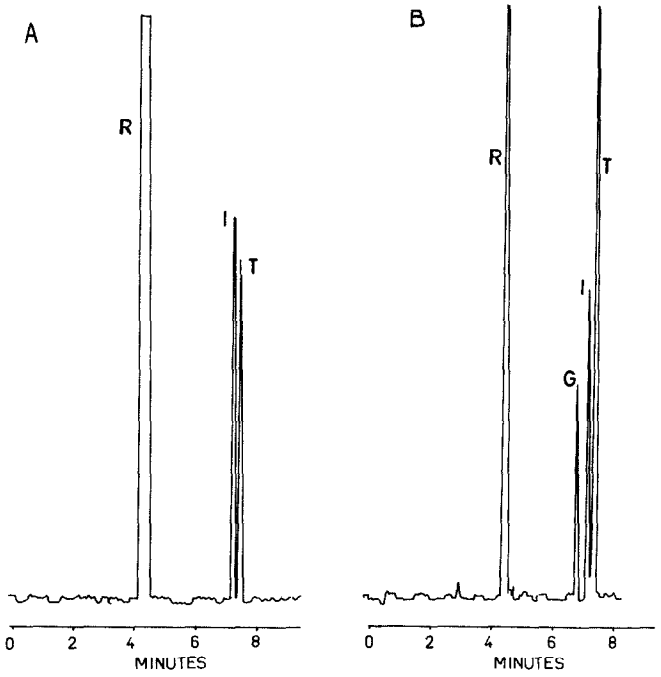

Fig. 7. (A) HPCE separation of taurine and the internal standard. (B) HPCE analysis of a sub-milligram biopsy of human heart muscle, to which the internal standard was added before derivatization with FMOC and electrophoresis. Peaks: R = reagent peak; I = internal standard; T = taurine; G = tentatively identified as GSH. The taurine concentration was calculated to be about 5 mmol/kg wet weight of tissue.

DISCUSSION

HPCE has been used to analyse urine and blood samples from some selected cases with different metabolic disorders, and to determine the aminosulphonic acid taurine in sub-milligram tissue samples (myocardial biopsies). The results have been compared with traditional automated amino acid analysis (ion-exchange chromatography), and with the HPLC (reversed-phase LC-18) and GC–MS methods routinely used in this laboratory for the diagnosis of metabolic diseases.

The selected diseases included homocystinuria, cystinuria and GSH synthetase deficiency. The diagnostic metabolites in these cases are sulphur-containing amino acids and the tripeptide GSH. MB-labelling (after reduction of the disulphides) of the thiol groups yielded derivatives that were quickly formed, baseline-separated and readily detected by HPCE with fluorescence detection. The conclusion to these experiments is that if clinical symptoms and preliminary spot tests raise the suspicion that a patient may suffer from any one of the above-mentioned disorders, confirmatory diagnosis may be made in less than 30 min by HPCE (including sample preparation) compared with 3 h by traditional amino acid analysis (including regeneration of the column). HPCE would in these cases perhaps be the method of choice.

Comparative analyses on samples from a patient with the rare disorder adenylosuccinase deficiency were carried out using HPLC with diode-array detection and

HPCE with absorbance detection. The diagnostic metabolites in this disorder are two different succinylpurines which have strong UV absorbance. Derivatization is therefore not required. In this instance little is gained by using HPCE, as both methods require about 20–30 min analysis time, including sample pretreatment. It should be noted, however, that none of the procedures has been optimized. As the HPLC diode-array detector allowed spectral comparison, in contrast to the HPCE equipment, HPLC is preferred over HPCE in this instance.

In these examples the diagnostic goals can easily be achieved with methods other than HPCE. The advantage of HPCE is, however, the small sample volume required, *i.e.* 1–10 nl. This opens up the possibility of analysing small tissue biopsies, small numbers of cells and even perhaps further down to the analysis of a single cell [12]. However, when dealing with cells and body fluids, the concentration of an interesting metabolite is often low, *e.g.* of the order of μmol/l to nmol/l. If 1 nl of unconcentrated sample is injected into the capillary column of an HPCE instrument, the detector must then be able to detect $10^{-6}/10^{-9} = 10^{-15}$ mol and $10^{-9}/10^{-9} = 10^{-18}$ mol, respectively. At present, the way to achieve such a sensitivity is to use a laser-induced fluorescence dtector [12,13]. Unfortunately, the ordinary lamp-based fluorescence detector currently available in this laboratory is not nearly as sensitive, and so far these studies have been limited to clinical problems with metabolites that are present at much higher concentrations. Such an example is the study of the role of taurine in the development of cardiomyopathy. The preliminary results shown in this paper clearly indicate that HPCE may become the method of choice for this purpose.

In conclusion, at these early stages of the application of HPCE to biomedical problems, it has been shown that the method can offer a quick and simple alternative to existing methods such as amino acid analysis. We believe, however, that it is at the cellular level that the full potential of HPCE will become apparent. Thus, the use of HPCE to analyse, for example, amniotic cells directly and without cultivation for prenatal diagnosis, can be foreseen, and it seems likely that the use of HPCE to analyse tissue biopsies will expand, particularly when laser-induced fluorescence becomes more generally available.

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