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Diagnostic applications of chromatography and capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) equipped with a diode-array detector, and GC–MS have been used to determine diagnostic metabolites occurring in urine of patients with various metabolic disorders. The urine samples were injected directly onto the CE instrument without any pretreament. GC–MS required extraction and derivatisation before separation. Identification of abnormal metabolites was based on migration times and characteristic diode-array spectra, or mass spectral library search when GC–MS was used. The CE method has previously been shown capable of diagnosing several metabolic diseases, and was now used on more difficult cases. CE readily diagnosed glyceric aciduria and the secondary metabolite in lysinuric protein intolerance, orotic acid. Methylmalonic aciduria required pressure elution in addition to high voltage to accomplish diagnosis. In mevalonic aciduria the characteristic metabolite had weak light absorption and the mevalonate peak also co-eluted with endogenous aromatic acids making diagnosis difficult. Both in the latter case and with the disorders glutaric aciduria I and glyceroluria, GC–MS was the method of choice. A possible role of CE in the routine system for diagnosing metabolic disorders, might be to use this method for pre-testing all urine samples. Samples with abnormal CE-profiles would subsequently be given high priority for more elaborate analysis with GC–MS and amino acid analyzer. In a different project a CE instrument designed for serum protein analysis was used to study sera from patients with myelomatosis. The method also allowed identification of the various immunoglobulins using immunosubtraction. Samples collected after diagnosis as well as many years prior to disease were available through the Janus-bank. This large serum bank comprises samples collected since 1973 at intervals from nearly 300 000 blood donors. It was found that the monoclonal immunoglobulins characteristic of the disease started to appear in serum up to 15 years before clinical diagnosis.

Keywords: Metabolic disorders; Glyceric aciduria; Methylmalonic aciduria; Lysinuric protein intolerance; Mevalonic aciduria; Myelomatosis; Diagnostic metabolites; Serum proteins; Immunoglobulins

1. Introduction

Several human diseases, in particular metabolic disorders, often lead to accumulation of characteristic metabolites in serum and urine. Multicomponent analytical techniques, including chromatography and electrophoresis are suitable to detect diagnostically important changes in the metabolic profiles obtained by analyzing these body fluids [1–4]. The profiling techniques currently used in our laboratory [5,6] include gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) with diode-array detector, automated amino acid analysis and classical paper- and thin layer chromatography. Also DNA techniques and certain

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enzyme assays have been included in our routine analytical system.

In a recent paper [7] we described the use of capillary electrophoresis with a diodearray detector for the analysis of diagnostic metabolites. The CE method as applied to urine proved to be rapid (15 min analysis time), automated, simple and required no sample preparation whatsoever. In the present paper we have elaborated further on this simple CE method and have compared it with classical GC–MS methodology.

In the paper mentioned above [7] we also used a CE instrument dedicated for automated protein analysis to study sera from the Janus-bank [8]. It was shown that the protein profile, as determined with CE, was remarkably stable even after 22 years of storage. We have now selected persons who developed multiple myelomas (myelomatosis), and have analyzed serum specimens from these patients, collected after diagnosis and many years prior to disease. Using the CE method it can now be shown that the monoclonal immunoglobulins characteristic for the disease, began to appear 10–15 years prior to clinical diagnosis of the cancer.

2. Experimental

2.1. CE-analysis of urine

The Hewlett-Packard HP^{3D} capillary electrophoresis system with built-in diode-array detector was employed for analysis of the urine samples. The capillary had a total lenght of 64.5 cm and an effective lenght of 56 cm. The I.D. was 50 μm and the effective light path was 150 μm (use of a bulb cell at the detector site). A borate buffer (300 m*M*, pH 8.5) was used and voltage applied was 30 kV. The instrument was equipped with an autoinjector. The capillary was washed for 3 min with 0.1 *M* sodium hydroxide and for 4 min with the borate buffer between each run. Urine samples were injected directly onto the CE without any sample pre-treatment.

Urinary creatinine was determined using the Hitachi 911 automatic analyzer (Boeringer Mannheim, Germany) and the classical Jaffé reaction with picric acid. The creatinine determinations were done to compensate for differant degrees of natural dilution of the urine samples. This is required because a constant volume of sample was injected onto the CE. The creatinine concentrations were transferred to the data system of the HP CE. Signals from dilute urines (low creatinine) were more amplified than signals from concentrated urines (higher creatinine) so that the various electropherograms could be directly compared. In some cases the creatinine peak on the electropherogram was used as "internal standard" and the signals were amplified to give a creatinine peak area identical to that obtained with a standard creatinine solution containing 10 mmol/l.

Patient samples were selected from our collection of deep-frozen urine specimens from controls and from patients with a known metabolic disorder. The samples were thawed and aliquots were used for creatinine determination and for CE analysis. Identification of abnormal metabolites was usually based on comparison of migration times and diode array spectra with the corresponding set of data obtained by analyzing authentic compounds.

2.2. GC–MS profiling of urine

The GC–MS instrument was a Hewlett-Packard 5970 mass selective detector coupled to a GC with an automatic sample injection system (HP 5890 GC with HP 7673A 100 sample autoinjector) and a HP 300 data system. The GC was fitted with a fused-silica capillary column (30 m) and the coatings were usually SP-1000 or SPB-5 (Supelco, Bellefonte, PA, USA). The GC peaks were automatically identified using mass spectral library search. The libraries were both commercially available data bases (Wiley, 130 000 mass spectra) as well as a "laboratory-made" library and search program, which automatically identifies about 200 urinary organic acids, many of which are known to carry specific, diagnostic information.

The urine samples were acidified, extracted with diethyl ether and the organic acids were subsequently methylated with diazomethane before injection onto the GC–MS. The capillary column was usually programmed from 80–220°C at a rate of 4°C/min [5].

2.3. CE-analysis of serum proteins

The Beckman Paragon CZE 2000 capillary electrophoresis system [9,10], designed for automated protein analysis, was used for analysis of serum. The instrument had seven capillary columns operated in parallel, and was equipped with UV detectors (214 nm). The samples were diluted 1:7 (Beckman 446300 borate buffer). The capillaries were 20 cm× 25 μm I.D. and the separation buffer was 5% sodium borate, pH 10 (Beckman buffer 446280). The applied voltage was 9 kV. The instrument was also operated in the immunosubtraction mode in order to characterize the immunoglobulin type present.

Serum-samples were selected from the Janus-serum bank in Norway [8]. This bank was initiated in 1973 and comprises serum samples consolidated during 20 years from about 300 000 donors. The specimens are stored at −25°C. From 1–13 consecutive samples are available from each donor.

In the present study blood donors who subsequently had developed myelomatosis (see Section 3.2) were selected. Serum samples from 3 patients with this condition were collected after diagnosis, and were compared to samples donated by the same persons many years prior to their disease. All specimens were analysed with CE before and after immunosubtraction [11], allowing the identification of the type of immunoglobulin occurring.

3. Results

3.1. Analysis of urine from patients with metabolic disease

Fig. 1 shows the GC–MS profile of urine from a patient with the rare disorder glyceric aciduria [12]. Glycerate was the second largest peak on the chromatogram. In normal urine this metabolite is usually not detected with our standard GC–MS procedure. In Fig. 2 (left panel, top) the same sample has been analyzed by CE. Fig. 2 (left panel, bottom) shows that authentic glyceric acid has identical retention time and peak shape as the peak in the patient urine. Fig. 2 (right) shows that also the diode array absorption spectra were similar. Analysis time using CE was 15 min total, compared to 65 min plus sample work-up time (150 min) when GC–MS was used.

Fig. 3 shows the GC–MS profile of urine from a patient with methylmalonic aciduria/acidemia [13]. Large amounts of methyl malonic acid (MMA) was seen. When the same urine was analyzed by CE and was compared with the CE profile of a normal urine, no MMA was detected. Even if the CE run was extended to over 60 min, no MMA peak appeared (not shown). In Fig. 4 the CE runs were repeated, with the exception that a 5 kPa constant pressure was applied during the CE process. All peaks eluted with

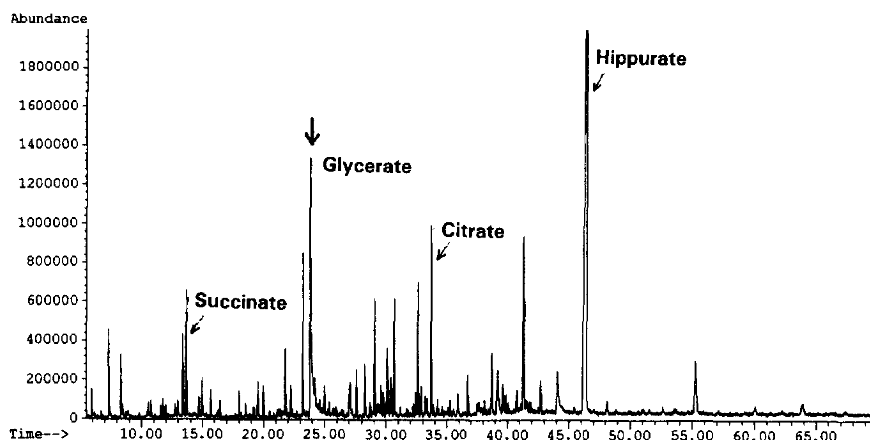

Fig. 1. GC–MS profile of urine from a patient with glyceric aciduria. The sample was acidified, extracted with diethyl ether and methylated with diazomethane before analysis. The separation was achieved in a Hewlett-Packard 5970 GC–MS system (see Section 2.2) with an automated sample injection system. The SP-1000 fused-silica capillary column was programmed from 80–220°C at a rate of 4°C/min. Note the glycerate peak, which is absent in normal urine samples.

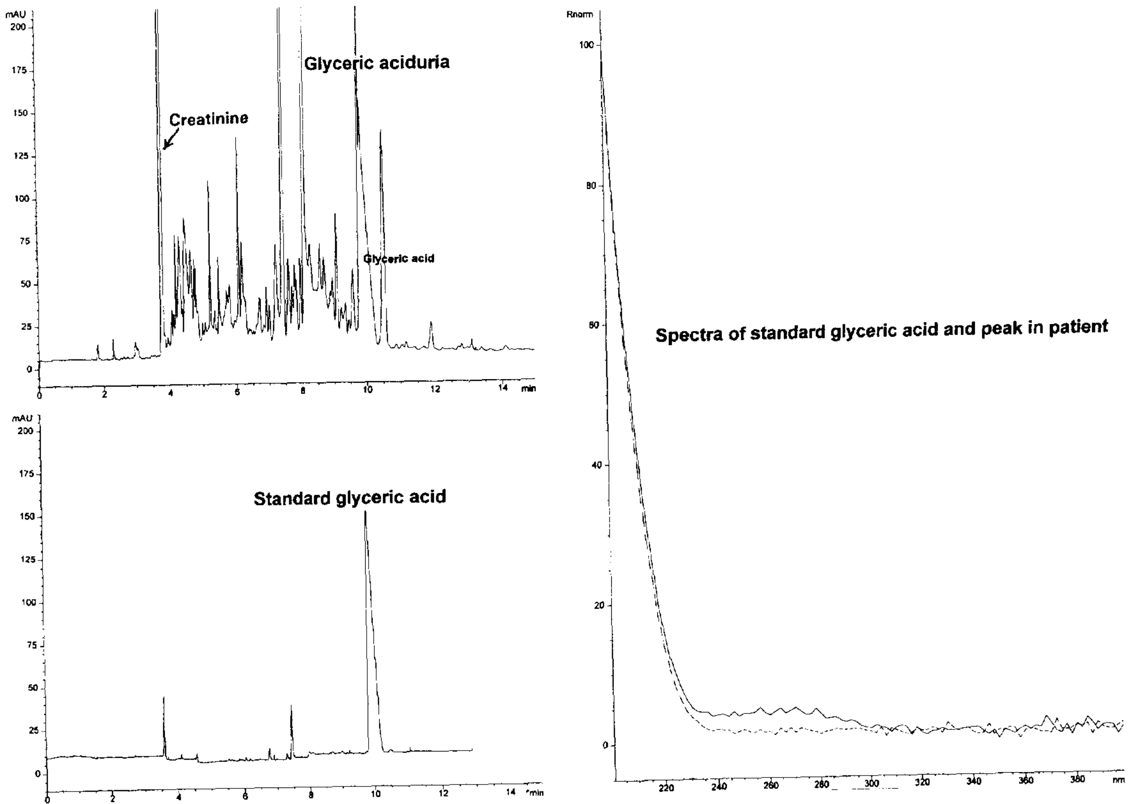

Fig. 2. CE diode array analysis of urine from a patient with glyceric aciduria. Hydrodynamic injection was used to inject the urine (7 nl) without any pre-treatment. Borate buffer (300 m*M*, pH 8.5) was used, voltage 30 kV. Capillary: effective length 56 cm, 50 μm I.D. The electropherograms were corrected for different degree of urinary dilution as determined by the creatinine values. Left panel: patient urine (top); authentic glycerate (bottom). Profiles displayed at 200 nm. Right: comparison of absorbance spectra of the glycerate peak in the patient and standard glycerate.

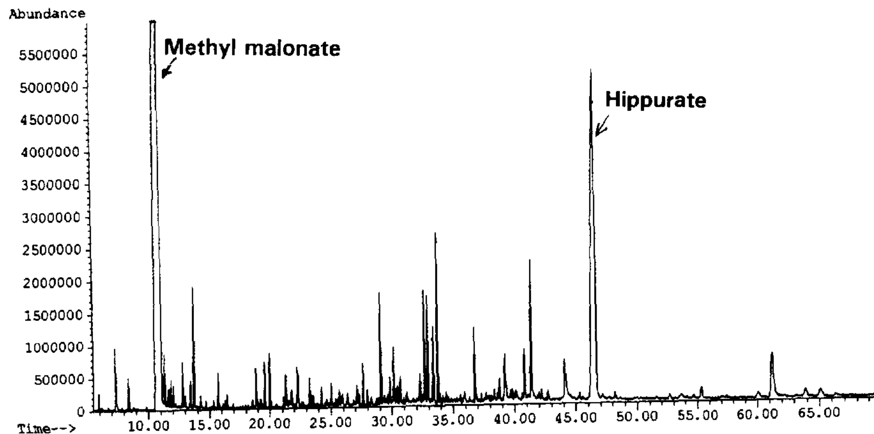

Fig. 3. GC–MS profile of urine from a patient with methyl malonic acidemia. Experimental conditions as in Fig. 1.

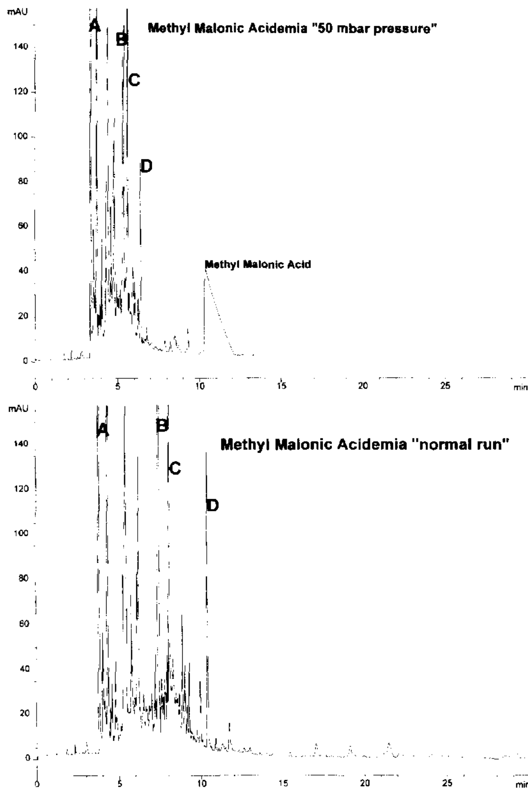

Fig. 4. CE diode array analysis of urine from a patient with methyl malonic acidemia. Top: CE conditions as in Fig. 2 and a constant pressure of 5 kPa during the run. Bottom: normal CE conditions (no pressure applied). Note compression of the peaks on the top electropherogram.

shorter migration times (compare Fig. 4 top and bottom), and under these conditions MMA appeared after 10 min as a skewed peak. Obviously, the doubly charged small anion methyl malonate is travelling so fast towards the positively charged electrode that the molecule has difficulty in reaching the detector at the negative end despite the electro-osmotic flow.

It should be mentioned, however, that methylmalonic acid readily may be analyzed by CE both in normal urine and serum after derivatization with pyrenyldiazomethane and detection by laser-induced fluorescence [14]. This method is valuable to evaluate functional cobalamine deficiency.

Mevalonic aciduria is a metabolic defect in the pathway leading to formation of cholesterol [15]. Fig. 5 shows the GC–MS profile of urine from a patient with this rare disorder. The dominating peak is not mevalonic acid (methyl ester), but rather its lactone, which is formed in the injector port of the GC–MS. Fig. 6 (left panel, top) shows the CE profile of a urine sample from the same patient, compared to a normal CE profile (bottom). The mevalonic acid peak is completely hidden under some normally occuring aromatic acids. These aromatic compounds have much stronger absorption characteristics (Fig. 6, right) than mevalonate. Thus, it is clear that CE under the presently used separation conditions, is not suitable to diagnose mevalonic aciduria.

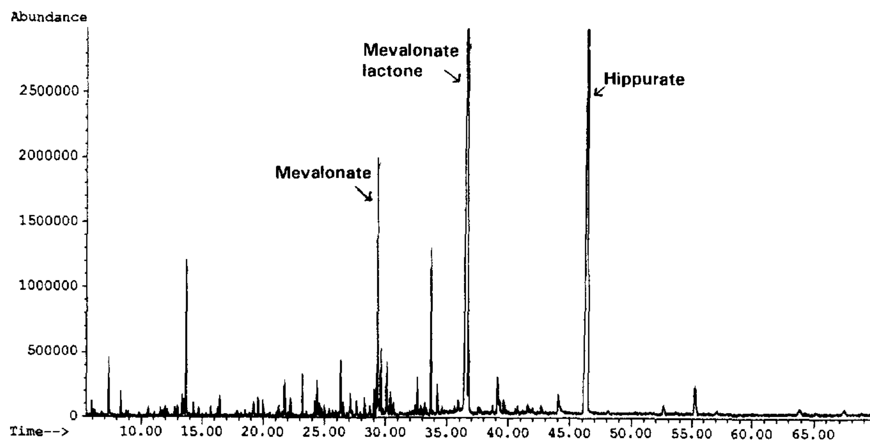

Fig. 5. GC–MS profile of urine from a patient with mevalonic aciduria. Experimental conditions as in Fig. 1.

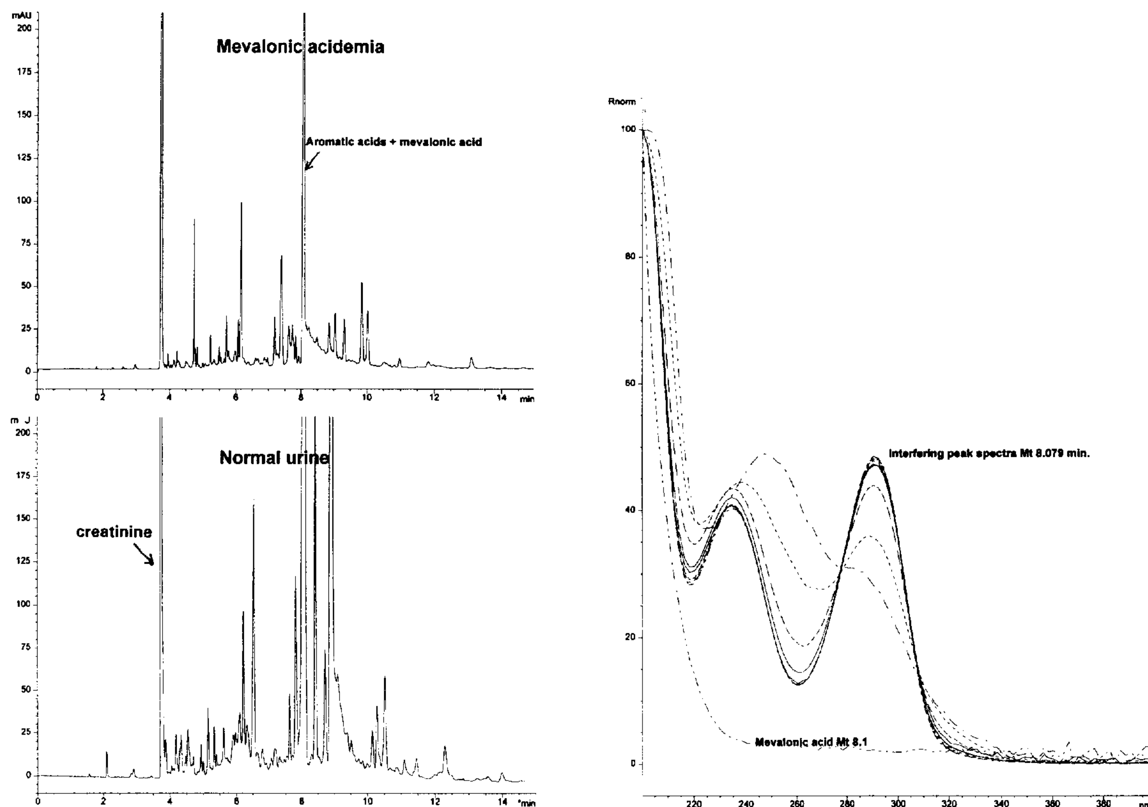

Fig. 6. CE diode array analysis of urine from a patient with mevalonic aciduria. Left panel: top=patient urine; bottom=normal urine. Right: diode array spectra of interfering peaks co-migrating with authentic mevalonate. Experimental conditions as in Fig. 2.

Lysinuric protein intolerance is a rare disorder in most countries, except in Finland where this disease is more common [16]. Patients with this disorder excrete large amounts of lysine. During episodes when the clinical condition is out of dietary balance, the ammonia level in the cells tends to increase with the secondary formation of orotic acid. Fig. 7 shows the results of CE analysis of urine from such a patient. The orotic acid peak is quite prominent, whereas the large quantities of lysine barely shows up on the electropherogram. This is due to the poor light absorption of this amino acid even at 200 nm.

In the present study we have also analyzed with CE urine samples from patients with glutaric aciduria I and glyceroluria. Due to poor light absorption of the diagnostic metabolites, it was not possible to diagnose these diseases by means of the present, simple CE method.

3.2. CE analysis of sera from patients with myelomatosis

Multiple myeloma or myelomatosis is a malignant tumor in the bone marrow. The disease occurs when B lymphocytes or their precursors multiply into clones of cancerous plasma cells that produce massive amounts of antibody protein. This antibody or myeloma protein, has no infection-fighting capability and displaces most healthy antibodies in the blood. The disease is progressive and may be lethal despite agressive treatment e.g., with chemotherapy. Laboratory diagnosis is usually agarose gel electrophoresis to demonstrate the presence of large amounts of a monoclonal myeloma protein. Subsequent typing of this immunoglobulin is usual, followed by the detection of free light chains (Bence–Jones protein) in the urine. The mere presence of a monoclonal

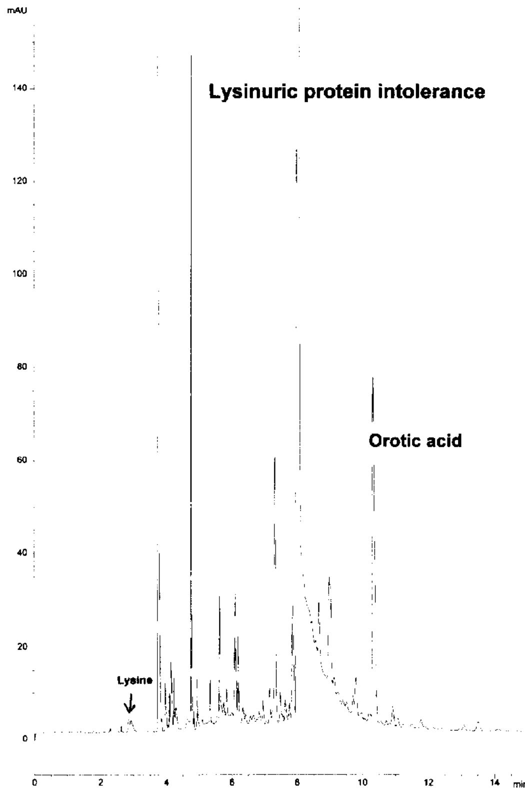

Fig. 7. CE diode array analysis of urine from a patient with lysinuric protein intolerence. Experimental conditions as in Fig. 2.

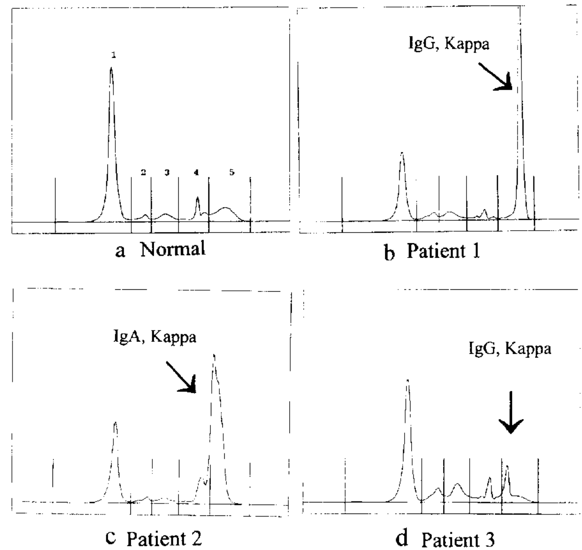

Fig. 8. CE analysis of serum from a control person (a), and from three patients with myelomatosis (b–d). The Beckman Paragon CZE 2000 capillary electrophoresis system was used. Experimental conditions as described in Section 2.3. All sera were analysed in May 1996. All persons had donated blood to the Janus serum bank [8]. The specimens had been stored at −25°C before analysis. Axes (not drawn by the computer) are: Y=absorbance at 214 nm. X=minutes, total separation time 5 min. Peaks (see panel a): 1=albumin, 2=alpha-1 zone, 3=alpha-2 zone, 4=beta zone, and 5=immunoglobulin zone (gamma zone).

component in serum, however, is not proof of myelomatosis, as many persons have a benign monoclonal gammapathy [17], which may or may not progress into malignant disease.

Fig. 8a–d shows the electrophoretic pattern of proteins in normal serum, compared to sera from three patients, two of whom had a confirmed myelomatoses. The third was suspected to have this diagnosis. Identification of the monoclonal immunoglobulins was done by immunosubtraction [11] as illustrated in Fig. 9. The serum was allowed to come in contact with immobilized antibodies directed towards the various immunoglobulins and their light chains (anti-IgG, anti-IgA, anti-IgM and anti-kappa, anti-lambda). The specific immunoglobulin with it's specific light chain was retained by the corresponding immobilized antibody. When the remaining serum was re-analyzed by CE, the bound protein was missing from the protein profile. This is clearly seen in Fig. 9a–f which shows the result when serum from patient 1 was analyzed. Fig. 9a shows the original electropherogram. Fig. 9b and Fig. 9c show the complete disapperance of the major peak (the myeloma protein) when treated with anti-IgG and anti-Kappa. Treatment with all the other antibodies did not affect the protein profile. The conclusion is that patient 1 produced large amounts of IgG with Kappa light chains. Using the same technique, it was demonstrated that the monoclonal component in patient 2 was IgA-Kappa, and in patient 3 IgG-Kappa (immunosubtraction results not shown).

Patient 1 was a male, born 1930. Myelomatosis was diagnosed in 1992. Serum samples collected in 1977, 1982 and in 1995 were available from this patient. Fig. 10a shows that the abnormal immunoglobulin (IgG, Kappa) was readily detected as early as 1977, it had increased in 1982 (Fig. 10b) and had become the dominating serum protein when disease was a fact (Fig. 10c). The figure clearly demonstrate

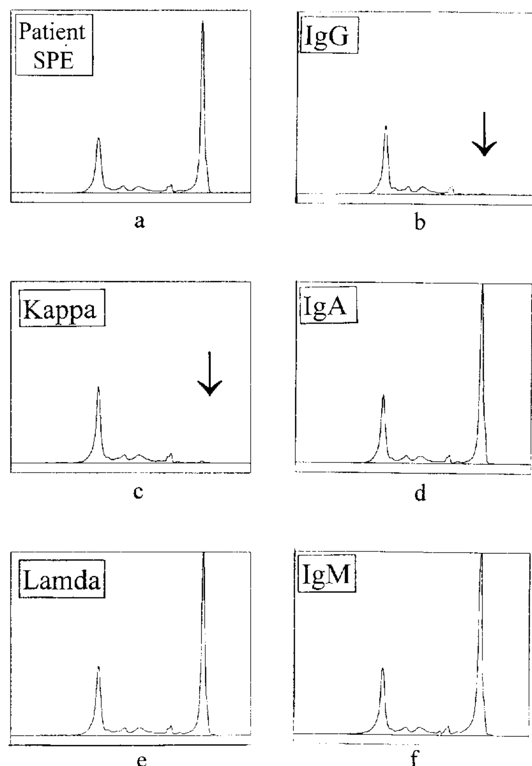

Fig. 9. CE analysis of serum from patient 1 before and after immunosubtraction: (a) before subtraction; treatment with (b) anti-IgG, (c) anti-Kappa, (d) anti-IgA, (e) anti-Lambda, and (f) anti-IgM. Note the complete removal of the major peak, the myeloma protein, with anti-IgG and anti-Kappa.

the occurrence of the pathological immunoglobulin 15 years prior to the cancer diagnosis.

Patient 2 was a female, born 1934. Myelomatosis with IgA-Kappa production was diagnosed in 1989. She had donated blood to the Janus-bank in 1977,

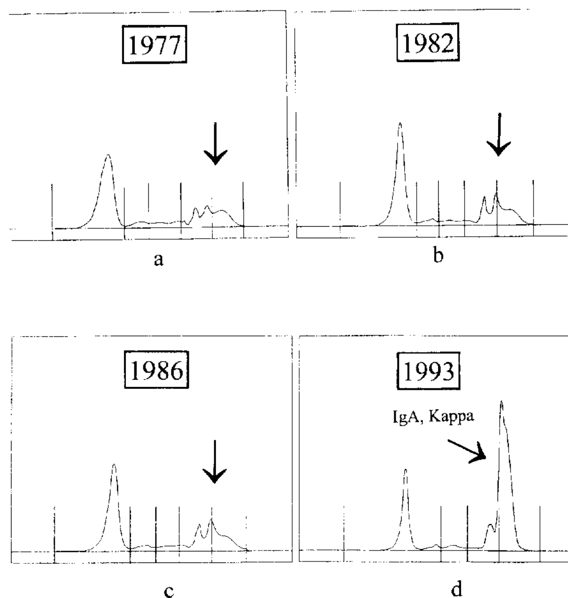

Fig. 11. CE analysis of sera from patient 2. The person had donated blood to the Janus bank in (a) 1977, (b) 1982, (c) 1986, and (d) 1993. The sera were stored at −25°C before analysis in 1996. Myelomatosis was diagnosed in 1993.

1982, 1986 and in 1993. From Fig. 11a–d it is apparent that production of the pathological protein was quite evident in 1982, and slightly more in 1986. In the years after 1986, the synthesis of the myeloma protein accellerated so that in 1993 the patient produced enormous amounts of the pathological IgA-Kappa protein (Fig. 11d). Even in 1977 the protein profile deviated slightly from normal, possibly so much that suspicion would have been raised had CE been available then and had the sample been analyzed at that time.

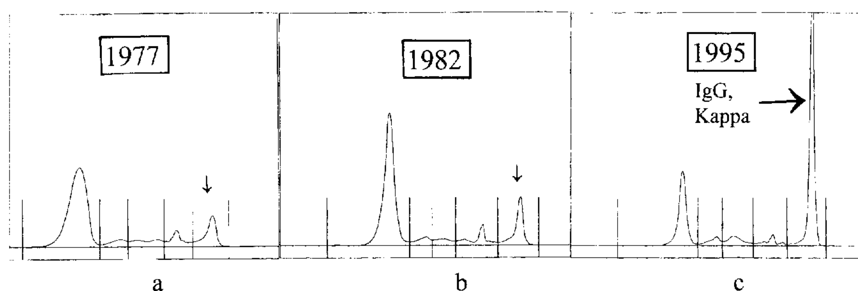

Fig. 10. CE analysis of sera from patient 1. The person had donated blood to the Janus bank in (a) 1977, (b) 1982, and (c) 1995. The sera were stored at −25°C before analysis in 1996. Myelomatosis was diagnosed in 1992.

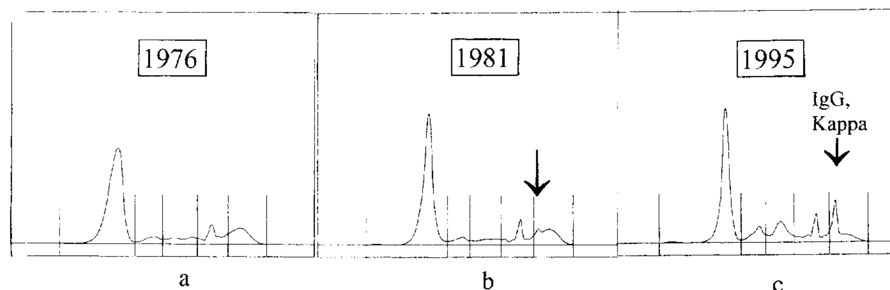

Fig. 12. CE analysis of sera from patient 3. The person had donated blood to the Janus bank in (a) 1976, (b) 1981, and (c) 1995. The sera were stored at −25°C before analysis in 1996. Myelomatosis was tentatively diagnosed in 1995.

Patient 3 (female, born 1934) was suspected, but not confirmed, to suffer from myelomatosis. This patient gave blood to the Janus bank at the time of admission to the hospital in 1995, and she had also donated serum in 1976 and in 1981. CE analysis with immunosubtraction proved that she produced moderate amounts of IgG-Kappa (Fig. 12c). This monoclonal protein was not detectable in 1976, as the protein profile from the 1976-serum appeared quite normal (Fig. 12a). In the 1981-sample, however, the pathological protein is clearly beginning to appear (Fig. 12b) and is very evident in the 1995-serum sample. Thus, in this patient there was sign of changes taking place in the blood 14 years prior to clinical suspicion of her disease.

4. Discussion

4.1. Metabolic disorders

The results confirm our previous experience [7], that urine may be injected directly onto the CE instrument without any pretreament. This is possible because of the use of a high ionic strength borate buffer (300 m*M*) of relatively high pH (8.5), which rapidly dissolves salt precipitates often occuring in a urine sample. In the previous paper [7] the following diseases were studied: HHH-syndrome, adenylosuccinase deficiency, multiple carboxylase deficiency, pyroglutamic aciduria, propionic aciduria and ketoacidosis. All these clinical conditions were readily diagnosed by means of CE.

We now selected some cases expected to be more difficult to detect by CE-diodearray. Diagnosis of glyceric aciduria was straight forward, whereas methylmalonic aciduria required a special combination of CE and pressure elution for the diagnostic metabolite to be detected. In lysinuric protein intolerence the secondary metabolite orotic acid was readily seen, whereas lysine itself was hardly detected.

In many other metabolic disorders, like mevalonic aciduria, glutaric aciduria and glyceroluria, the diagnostic metabolites have so poor light absorption properties that they cannot be detected by the diode array. GC–MS is the method of choice for recognition of these disorders, at least for the time being. The combination of CE–MS with on-line analyte preconcentration [18] may, however, alter the situation.

Our data suggest that the use of CE-diode array detector with direct injection of urine, due to its extreme simplicity, is likely to be useful in a routine screening system for diagnosis of metabolic disease. We now propose to analyse every urine sample by the direct CE-method as described. The capacity would be about 2–3 samples per hour for 24 h a day completely automated and without any sample preparation. This procedure would routinely be carried out first, as a pre-test, so that all samples giving deviations from the normal CE-profile could be given high priority for more elaborate examinations e.g., with GC–MS and quantitative amino acid analyzer. We expect that about 50 different metabolic disorders are likely to be seen on the CE-profiles in this way.

As shown in this paper, samples giving apparently normal CE-profiles may still stem from patients with a metabolic disorder. These samples will obviously be subjected to our routine multicomponent ana-

lytical system [5] as usual, simply neglecting negative and inconclusive CE results.

4.2. Serum analysis

CE analysis of serum proteins appears to be simple to carry out in practise, and several hundred samples may be run in a short time (40–60 samples per hour). A small to middle size hospital laboratory usually performs only 50–100 classical agarose gel electrophoresis of serum proteins per week, indicating that the new CE instrumentation has a large over-capacity for such a laboratory. The question may be raised whether the CE technology should be applied in a different way than the agarose method. Perhaps CE could be employed more as a screening method, and used on many more patients than is the case today. Applied in such a way, early signs (up to 15 years before clinical symptoms) of gammapathy may be detected in this manner as indicated in this report.

It is also possible that other disorders causing changes in the serum protein profile may be detected. Much more work is required, however, before CE can be recommended as a screening procedure.

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