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Capillary electrophoresis for clinical problem solving: analysis of urinary diagnostic metabolites and serum proteins

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Abstract

Many clinical laboratories employ gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) to detect abnormal compounds occurring in urine and serum due to disease. The methods, particularly GC–MS, often require laborious sample pre-treatment, and separation times may exceed an hour. We describe the use of capillary electrophoresis (CE) equipped a with a diode-array detector in an attempt to improve the efficiency of an analytical system routinely used for diagnosis of human metabolic disease. It was found that urine samples could be injected directly onto the CE instrument without any pre-treatment, and over 50 metabolites were separated in 15 min. Identification of abnormal metabolites was based on migration times and characteristic diode-array spectra. The method readily diagnosed adenolysuccinase deficiency, 5-oxoprolinuria, propionic acidemia and disorders having orotic acid as diagnostic metabolite (e.g. the HHH-syndrome). The results show that CE may become a useful additional tool for diagnosis of metabolic disease. In a different project CE was used to study sera from the Janus-bank. This large serum bank comprises samples collected at intervals from nearly 300 000 blood donors. As the sera are stored at −25°C and not at a lower temperature, a major concern has been the stability of the specimens. GC–MS, 2D-protein electrophoresis, certain immunological assays and enzyme measurements have previously been used to evaluate the stability of the sera. We can now also show that the protein profile, as determined by CE, is remarkably stable even after 22 years of storage. The results moreover confirmed that the CE-method and traditional gel electrophoresis gave almost identical results, except for small amounts of fibrinogen which did not show up on the CE-pattern.

Keywords: Proteins; Capillary electrophoresis; Metabolic disorders; Oxoprolinuria; Propionic acidemia; Adcrylosuccinase; Orotic acid

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 enzyme assays have been included in our routine analytical system. A home-built capillary electrophoresis (CE) system with laser-induced fluores-

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cence detector has also been considered for inclusion in the analytical system [7], but is not yet used on a routine basis. The same holds true for high resolution two-dimensional protein electrophoresis [8].

Most of these separation methods, particularly GC–MS, often require laborious sample pre-treatment (extraction and derivatization), and separation times typically exceed one hour. In the present report we have used automated CE with a diode-array detector in an attempt to improve the efficiency of our analytical system routinely used for diagnosis of human metabolic disease.

In a different project a CE instrument designed for automated protein analysis was used to study sera from the Janus-bank [9]. This serum bank comprises 0.5 million samples collected at intervals in the period between 1973 and 1992. As the sera are stored at −25°C and not at a lower temperature, a major concern has been the stability of the specimens. GC–MS, 2D-protein electrophoresis, certain immunological assays and enzyme measurements have previously been used to evaluate the stability of the sera [9]. We can now show that the protein profile, as determined by CE, is remarkably stable even after 22 years of storage.

2. Experimental

The Hewlett–Packard HP CE with built-in diode-array detector was employed for analysis of the urine samples. The capillary had a total length of 64.5 cm and an effective length of 56 cm. The I.D. was 50 μm and the effective light path was 150 μm. 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.

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 different degrees of natural dilution of the urine samples. This is required because a constant volume of sample was injected onto the CE system. 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.

Patient samples were selected from our collection of deep-frozen urine specimens from controls and from patients with a known metabolic disorder. The following diseases were studied: HHH-syndrome, adenylosuccinase deficiency, multiple carboxylase deficiency, pyroglutamic aciduria (glutathione synthetase deficiency), propionic aciduria and ketoacidosis (for information on these disorders see textbook [10]). 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.

The Beckman Paragon CZE™ 2000 capillary electrophoresis system [11,12], designed for automated protein analysis, was used for analysis of serum. The instrument has seven capillary columns which operate in parallel, and is equipped with UV detectors (214 nm). The samples were diluted 1:20 (Beckman buffer 446300). The capillaries were 20 cm long, 25 μm I.D., and the separation buffer was borate (Beckman buffer 446280). The applied voltage was 9 Kv.

Serum samples were selected from the Janus serum bank in Norway [9]. This bank was initiated in 1973 and now comprises about 0.5 million serum samples consolidated from 293 692 donors. The specimens are stored at −25°C. From 1–13 consecutive samples are available from each donor. In this study on the stability of the sera, 4 sequential samples from a control person and from a case (one who has developed cancer) were analyzed by CE and by classical gel electrophoresis.

3. Results

3.1. Analysis of urine from patients with metabolic disease

Initially it was expected that the urine samples had to be centrifuged or filtered before the analysis to

avoid plugging of the capillary. It was found, however, that even if many of the samples apparently had some insoluble material (salts), the urine could be injected directly onto the CE instrument without any pre-treatment. The high ionic strength borate buffer (300 m*M*) of pH 8.5 seemed to dissolve any precipitate present and the capillary remained open during all the analyses.

When the metabolic profile of a normal urine sample was displayed at 200 nm, over 50 different compounds could be seen (Fig. 1A, top). Analysis of urine from a patient with HHH-syndrome (hyperornithinemia – hyperammonemia – homocitrullinuria [10]) showed the presence of an extra compound (peak A, Fig. 1A, bottom). This peak had the same migration time (10.3 min) as authentic orotic acid Fig. 1B and the same absorption spectrum Fig. 1C as the reference substance. Orotate has an absorption maximum at 280 nm (Fig. 1B,C) and when the electropherogram is displayed at this wavelength (Fig. 1D) one can clearly see large amounts of the abnormal compound orotate in the urine from the patient (Fig. 1D, top) as compared to the control urine (also shown at 280 nm, Fig. 1D, bottom). Orotate, incidently, is a metabolite of diagnostic importance in several other diseases resulting in overproduction of ammonia. Certain disorders of the urea cycle, e.g. OCT-deficiency [10] and lysinuric protein intolerance [10] are examples where analysis of orotate is of relevance.

Fig. 2A shows the presence of large amounts of an abnormal metabolite in the urine of another patient. The compound had migration time and absorption spectrum identical to pyroglutamate (5-oxoproline) Fig. 2B. This particular patient [13] suffered from generalized glutathione deficiency and produced pyroglutamate (30–40 g/24 h) as a secondary product.

The metabolic disorder adenylosuccinase deficiency [14] leads to the formation of two diagnostic metabolites, adenylosuccinate (AS) and succinylamino-imidazole-carboxamid ribotide (SAICR). When urine from a patient with this disorder was analyzed by CE, an abnormal peak with migration time of about 12.5 min appeared ("X" in Fig. 3A). Enlargement of this peak showed that it consisted of two partially resolved compounds Fig. 3B. The absorption spectra Fig. 3C showed that AS had a slightly shorter migration time than SAICR (see [5,14] for spectra of authentic compounds).

The disorder propionic acidemia (defective propionyl CoA carboxylase) results in the excretion of a number of metabolites including methylcitrate, tiglylglycine and free propionic acid [10]. When these compounds are derivatized, e.g. methylated or silylated before GC–MS analysis, propionic acid is usually lost in the solvent front. This is avoided in CE analysis. Propionate has a migration time of about 9.8 min, and a peak due to this metabolite is readily seen in a sample from a patient with propionic acidemia Fig. 4A. Fig. 4B and C also show the occurrence in the patient's urine of 3 unknown peaks with rather special absorbance spectra. We are currently investigating the nature of these unknowns.

Some other disorders were also studied (results not shown). Thus in multiple carboxylase deficiency the diagnostic metabolite 3-methylcrotonylglycine (but not 3-hydroxyisovaleric acid) was readily recognized. Acetoacetate (but not 3-hydroxybutyrate) was clearly seen in the urine from a ketoacidotic patient. The reason why some of the diagnostic metabolites known to be present in these samples were difficult to see, is because of the poor light absorption of the compounds in the wavelength range of the diode-array detector (190 nm to 600 nm).

3.2. CE analysis of serum proteins

Fig. 5 shows the electrophoretic pattern of proteins in serum collected from the same person in 1973, 1977, 1982 and 1990. All sera were analyzed in 1995, and the oldest specimen had therefore been stored at −25°C for 22 years. No significant changes in the serum proteins could be seen.

Fig. 6 shows the electropherograms of serum collected from another person (case) in 1973, 1975, 1981 and 1986. This female was diagnosed with breast cancer in 1989, i.e. about 3 years after the last serum sample was donated. Again the CE analyses were carried out in 1995. No deterioration of the specimens were observed.

The same specimens as above were also analyzed in duplicate by classical gel electrophoresis using the Beckman Paragon SPE system. The results show that the protein patterns of the various sera were very similar, confirming the stability during long term

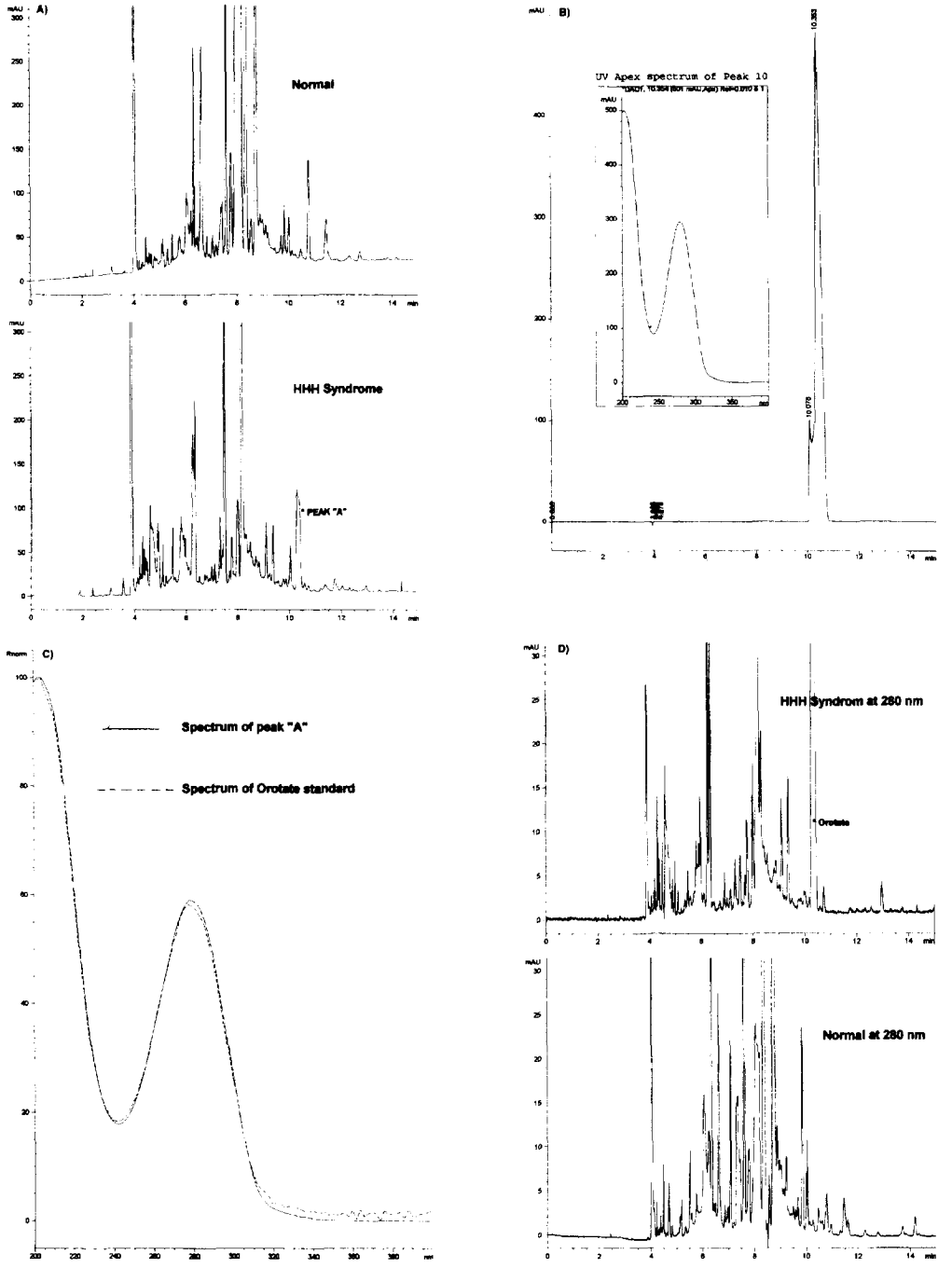

Fig. 1. CE diode-array analysis of urine from a patient with HHH-syndrome (hyperornithinemia–hyperammonemia–homocitrulluria). Hydrodynamic injection was used to inject the urine (7 nl) without any pre-treatment. Borate buffer (300 m*M*, pH 8.5) was used, voltage 30 kV. Capillary: effective length 56 cm, 50 μm I.D. The electropherograms were corrected for different degree of urinary dilution as determined by the creatinine values. (A) Control (top) and patient (bottom). Profiles display absorption at 200 nm. Note peak A. (B) Analysis of authentic orotate. (C) Comparison of absorbance spectra of peak A and orotate standard. (D) Patient (top) and control (bottom). Profiles displayed at 280 nm.

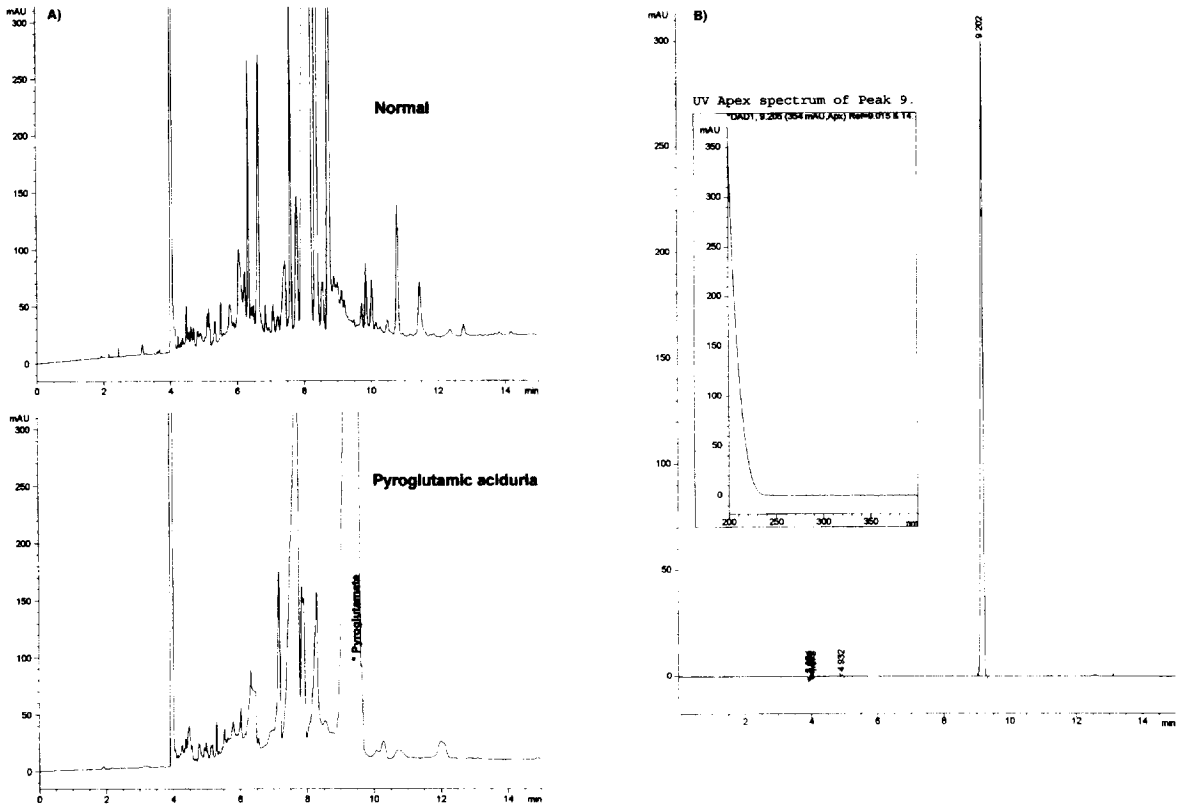

Fig. 2. CE diode-array analysis of urine from a patient with pyroglutamic aciduria (5-oxoprolinuria). Experimental conditions as in Fig. 1. (A) Control (top) and patient profile (bottom) displayed at 200 nm. Concentration of pyroglutamate in the patient's urine, 35 g/l. (B) Analysis of authentic pyroglutamate (5-oxoprolinuria).

storage. The results also confirm the findings of others [15–19] that CE as used above and classical gel electrophoresis produced very similar protein patterns. The only noticeable difference between CE analysis and gel electrophoresis is apparent from Fig. 7, top. The lane marked with an arrow shows the presence of a band due to small amounts of fibrinogen remaining in the serum. This fibrinogen band was not seen in the CE-electropherogram.

4. Discussion

One is currently witnessing a dramatically growing interest in the application of CE to biomedical and clinical problems [7,20–38]. In this paper we have described the use of automated CE with diode-array detection for the diagnosis of metabolic disease. The CE method as applied to urine is rapid (15 min analysis time), automated, simple and requires no sample preparation whatsoever. Because of this encouraging experience it is now intended to implement CE in the routine diagnostic system used in Oslo [6]. The disadvantages are that diagnostic metabolites with poor light absorption escape detection, and that the identification is based on migration times and absorption spectra only but not on MS. Advances in the on-line combination of CE with MS are, fortunately, rapidly progressing (see e.g. [38,39]). In the not-too-distant future one can therefore expect a considerable impact of CE and CE–MS in the clinical laboratory, where this technology may complement and even replace some of the classical chromatographic methods used today.

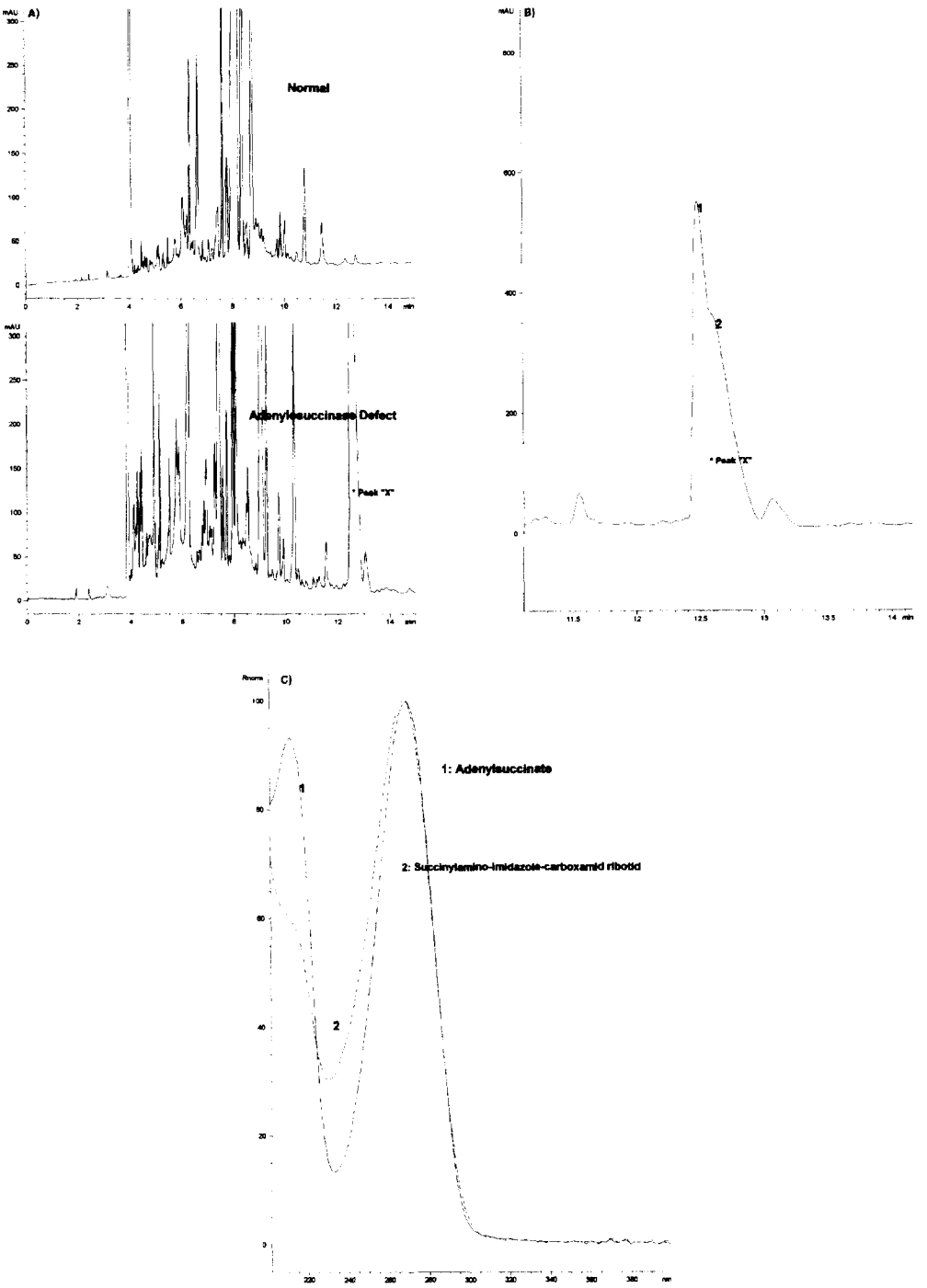

Fig. 3. CE diode-array analysis of urine from a patient with adenylisuccinase deficiency. Experimental conditions as in Fig. 1. (A) Control (top) and patient (bottom) profiles displayed at 200 nm. (B) Enlarged portion of peak "X". (C) Absorption spectra recorded at point 1 and 2 of peak "X", Fig 3b. Spectrum 1 and 2 resemble adenylosuccinate and succinylamidoimidazolecarboxamid ribotid, respectively [5,14].

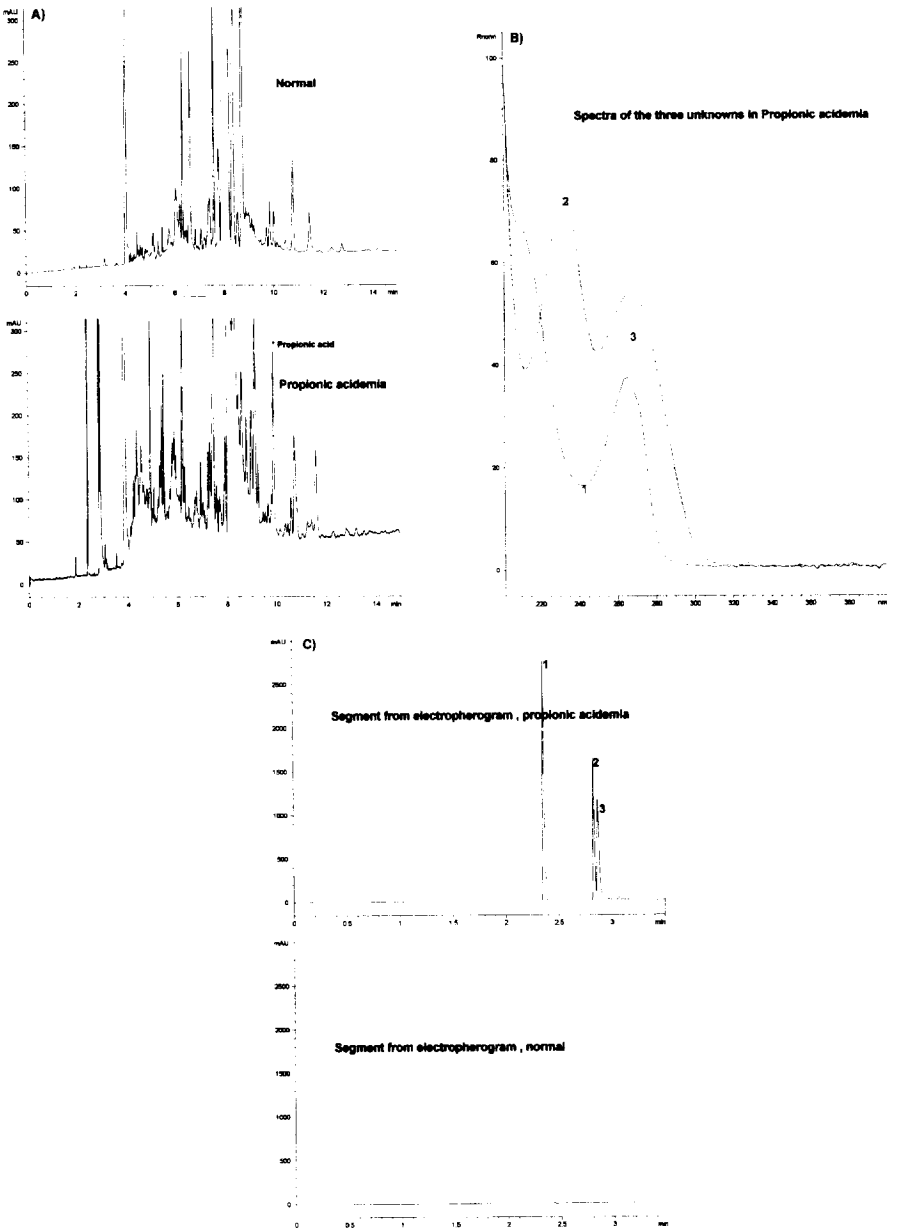

Fig. 4. CE diode-array analysis of urine from a patient with propionic acidemia (propionyl-CoA carboxylase deficiency). (A) Control (top) and patient (bottom) profiles displayed at 200 nm. (B,C) Absorption spectra of three unknown, rapidly migrating metabolites not found in normal urine.

Further discussion of clinical aspects is outside the scope of this article. The interested reader should consult e.g. [1–3].

In the field of serum protein analysis, the CE methodology has now also been refined to a considerable degree. The CE electropherograms give comparable results to classical gel electrophoresis, as shown in this report and by several others [15–19].

Use of CE to analyze sera from the large Janus serum bank [9] demonstrated a satisfactory stability

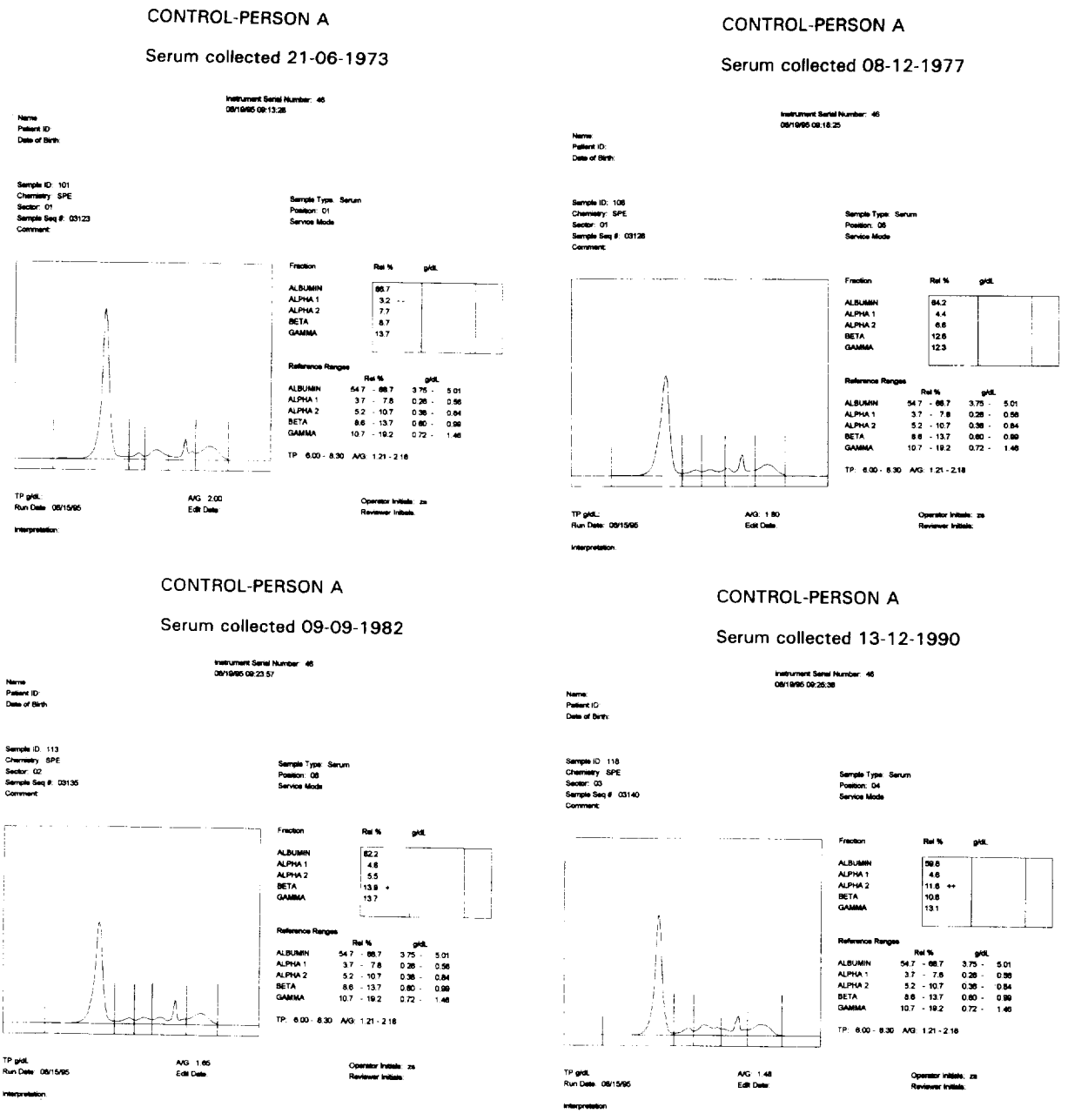

Fig. 5. CE analysis of sera from a control person. The Beckman Paragon CZE™ 2000 capillary electrophoresis system was used. Experimental conditions as described in the text. All sera were analyzed in 1995. The control person had donated blood to the Janus serum bank at various intervals, and the specimens were stored at −25°C before analysis. Top panel, left, storage time: 22 years. Right: storage time 18 years. Bottom panel, left: storage time: 13 years. Right: storage time: 5 years.

of the specimens, even after storage for 22 years. In the period from 1973 and up to 1995 about 16.000 of the nearly 300.000 originally healthy blood donors have developed some form of cancer. Frozen serum samples collected from a few months to many years prior to clinical recognition of their disease are thus

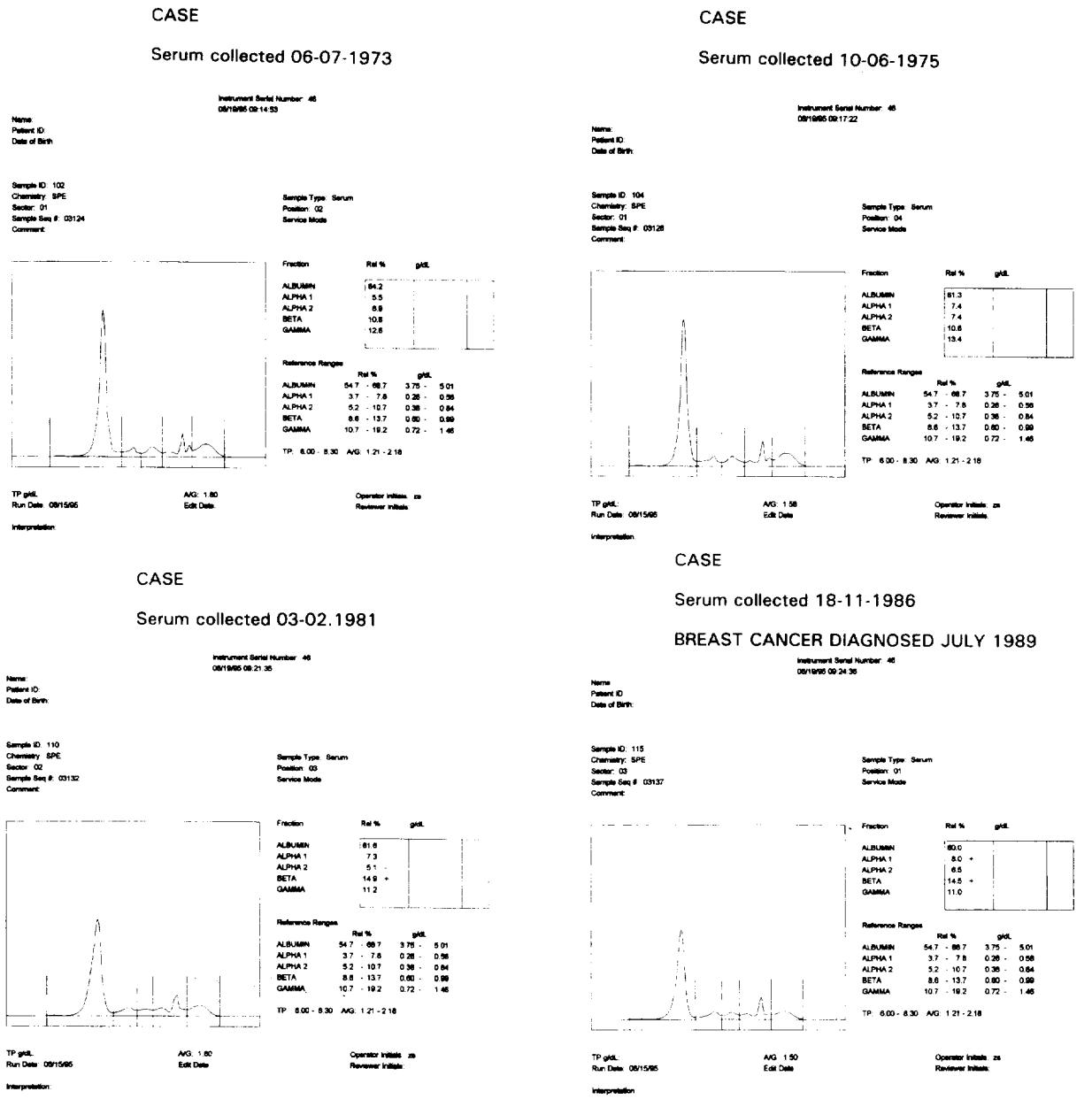

Fig. 6. CE analysis of sera from a case. Experimental conditions as in Fig. 5 This person donated blood to the Janus bank in the period 1973 to 1986. In 1989 breast cancer was diagnosed. Sera were analyzed in 1995. Top panel, left, storage time: 22 years. Right: storage time: 20 years. Bottom panel, left: Storage time: 14 years. Right: Storage time: 9 years.

available for research purposes. The principle aim of the Janus-project is to search in the pre-morbid sera for chemical, biochemical, immunological or other changes that might be indicative of early stage cancer development. The sera in the bank will continue to be monitored by CE, GC–MS and 2D-protein analysis to assure the stability, so that the collection remains useful in many aspects of cancer research. For further information on studies which have utilized the bank up to now see [40–49].

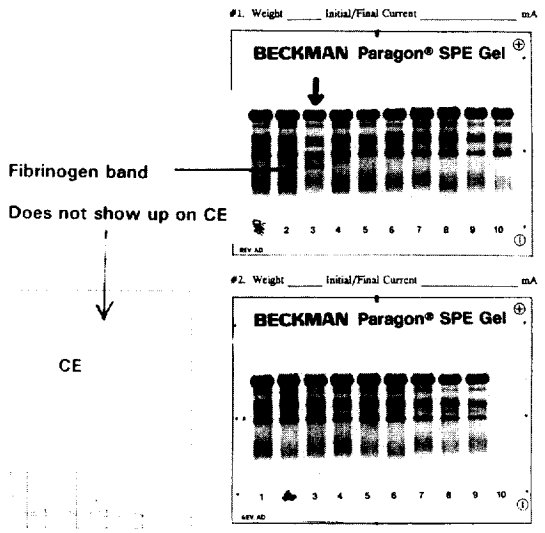

Fig. 7. Classical gel electrophoresis of sera from the control person and the case. The same sera as in Figs. 5,6 were analyzed using Beckman Paragon® SPE gels. The lane marked with an arrow shows the presence of a fibrinogen band, which is not apparent on the corresponding CE-electropherogram.

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