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Integrated approach to the multidimensional analysis of complex biological samples by microseparation techniques Analysis of glycoprotein factor associated with cancer cachexia ☆

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Abstract

Microanalytical separation techniques including capillary liquid chromatography, capillary electrophoresis and capillary electrochromatography are suitable for detection of diagnostically important changes in the metabolic profiles of biological fluids. A prototype instrument was employed to serve as an integrated platform for the analysis of urine sample from patients suffering from cancer cachexia. The instrument provides for convenient, rapid and efficient multidimensional approach towards method development which would facilitate simultaneous analysis of complex biological mixtures by the above techniques. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Instrumentation; Multidimensional separation; Glycoproteins; Proteins

1. Introduction

Metabolic disorders often lead to accumulation of characteristic metabolites in serum, urine or cerebral spinal fluids [1–4]. Characterization of these biological fluids may contribute to a better understanding of the physiological processes in the organism. However, detection of diagnostically important changes in the metabolic profiles obtained by analyzing these fluids is a challenging task. Usually biomolecules of diagnostic interest are determined by immunological methods such as radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA). Immunological detection is however limited to biomolecules against which specific antibodies have been generated. Furthermore, only substances bearing certain epitopes can be detected by these methods. Even for cases where well defined immunological methods exist high-resolution microanalytical separation techniques such as capillary liquid chromatography (μLC) and capillary electrophoresis (CE) are attractive due to the low sample volumes required, excellent detection levels along with rapid and efficient separations. Beside their high reproducibility these techniques require minimum

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sample preparation and thus are being increasingly employed for clinical analysis of such disorders as adenylosuccinase deficiency, 5-oxoprolinuria, Bence–Jones proteinuria and nephrotic syndrome [5].

In this work an integrated analytical instrument was employed for the separation of urine samples collected from cancer patients exhibiting advanced muscle proteolysis, i.e., cachexia. Although these cancer patients typically have lower nutrient input, they also suffer from accelerated protein breakdown, resulting in significant muscle wasting [6]. Recently, this has been associated with a proteolysis inducing or cachectic factor, identified as a sulfated glycoprotein [7–10]. The prototype instrument enables method development by orthogonal techniques of CE, μLC, electric field assisted μLC as well as capillary electrochromatography (CEC). This ability to develop rapid multidimensional separation schemes would tremendously assist the analysis of clinically relevant biological sample.

2. Experimental

2.1. Chemicals and materials

HPLC-grade acetonitrile and boric acid were obtained from Sigma (St. Louis, MO, USA). Sodium tetraborate was obtained from J.T. Baker (Philipsburg, PA, USA). Deionized water purified by a Milli-Q (Millipore, Bedford, MA, USA) unit was used throughout. Sodium tetraborate was prepared as a 60 m*M* solution. The pH of this solution was measured as 9.2. Neat aqueous borate buffer was prepared as a 100 m*M*, pH 8.5 stock solution. The solutions were filtered through a nylon 66 membrane from Anspec (Ann Arbor, MI, USA). Eluents for μLC were prepared by appropriate dilutions of the stock solution with water and acetonitrile without further adjustment of pH such that the final concentration of borate buffer was 10 m*M*. In this text buffer compositions are stated as 10 m*M* borate buffer in a water–acetonitrile (*x*:*y*, v/v) mixture.

Polyimide coated fused-silica capillaries of 375 μm O.D.×75 μm I.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA). 3.5 μm Zorbax ODS and 3 μm Hypersil ODS stationary phases were obtained from Hewlett-Packard (HP). 75 μm fused-silica capillaries with a bubble cell for detection were obtained from Hewlett-Packard (Waldbronn, Germany) and used for performing CE.

2.2. Clinical samples

This study was approved by the Georgetown University Institutional Review Board. After providing informed consent, urine samples were collected from healthy volunteers and cancer patients with clinically diagnosed cachexia. The samples were concentrated (50×), dialyzed to remove low-molecular-mass species (M_r 18 000 cutoff) and further diluted in water. The urine samples were analyzed for the presence of cachectic factor using Western blot analysis employing antibodies raised against the factor isolated from a murine adenocarcinoma, MAC-16.

2.3. Instrumentation

A prototype HP instrument equipped with a diode array detector and HP 1100 pumps was used for all micro-analytical separations. The instrument illustrated in Fig. 1 could be operated in various modes that include CE, μLC, μLC with electric field assistance as well as CEC. On-column UV detection of analytes was employed in all four modes. The packed capillary column (1) is installed in the front compartment of a modified temperature controlled cartridge. The back compartment houses the fused-silica tubings (5, 7) that transfer the eluent from the pump to the seal vial and the excess solvent from the seal vial to the waste reservoir. The eluent is delivered by HP 1100 pumps through a hollow electrode (2) to a propriety seal vial (8). The packed capillary column (1) is inserted in the hollow portion of the electrode and held in place with the help of a tightening nut. The top of the electrode has two short stainless steel tubes (3, 4) connected to it. One stainless steel tube (3) is connected to a 1 m×100 μm fused-silica capillary (5) that connects to a metal union (6). The other stainless steel (4) tube takes the solvent to the waste through a 0.5 m×100 μm fused-silica capillary (7). The two fused-silica capillaries thus have a hydraulic resistance of 2:1. The

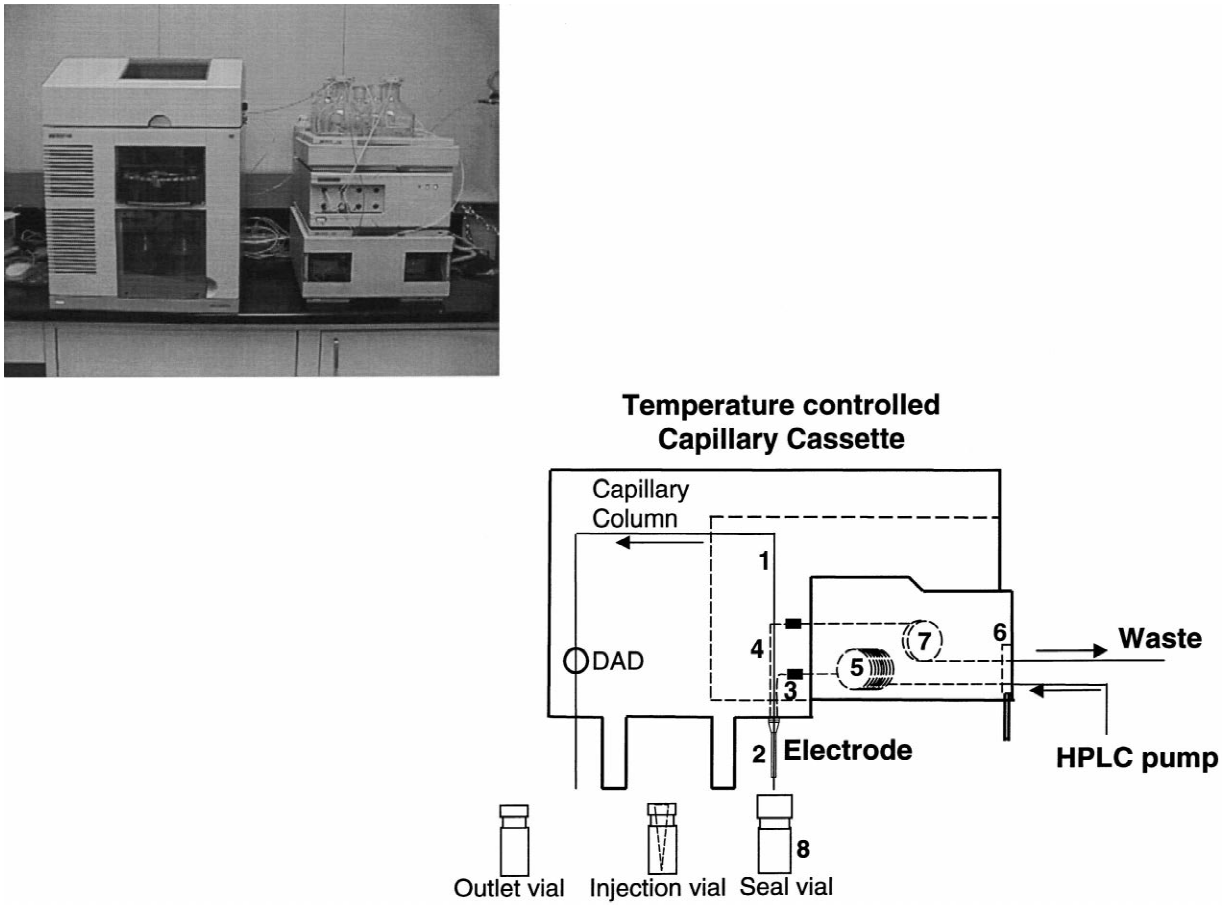

Fig. 1. Illustration of the integrated platform for micro-analytical separations. (1) Packed capillary column, (2) hollow electrode, (3, 4) stainless steel tubes, (5) 1 m×100 μm fused-silica capillary, (6) ground, (7) 0.5 m×100 μm fused-silica capillary, (8) seal vial.

metal union is grounded and prevents the current from leaking to the pump while operating under CEC and electric field assisted μLC mode. The flow going to the seal vial is split such that a part of it goes to the packed capillary column and the rest goes to waste. The split ratio depends on the permeability of the packed capillary column (1) as well as the dimensions of the fused-silica capillary (7) transferring the eluent form the seal vial to the waste. In gradient elution mode the changing mobile phase composition is delivered by the binary pump to the seal vial continuously. The mobile phase enters the packed capillary column either under hydrodynamic (μLC) or electroosmotic (CEC) forces.

2.4. Procedures

2.4.1. Packing capillary columns

Fused silica capillary columns of 75 μm I.D. with the packed and total lengths of 25 and 33.5 cm, respectively were used for μLC, electric field assisted μLC and CEC. The stationary phase was either 3.5 μm Zorbax or 3.0 μm Hypersil ODS particles. The capillaries were packed using a modified version of the procedure described by Smith and Evans [11]. A 50-mg sample of the stationary phase was suspended in a mixture of 110 μl acetone and 110 μl toluene, sonicated for 5 min and then transferred to a slurry reservoir. The capillaries were

packed with acetone as the packing solvent at a constant pressure of 500 bar.

2.4.2. Equilibration of packed capillary column

The packed capillary column is installed in the cartridge by first inserting the detection window in the detection cell. Thereafter the injection end is inserted into the hollow electrode at the injection side and tightened in place there. The injection end of the capillary is subject to pressures anywhere between 0 and 300 bar and hence it is important to ensure proper tightening of the capillary at this end. The column is pre-equilibrated with the instrument in the manual mode for μLC. The seal vial is placed at the inlet end of the capillary. The lift moves up to seal the vial. Thereafter the flow-rate of the 1100 pump is adjusted such that the pressure reading of the pump is between 200 and 250 bar. This happens when the pump is at a flow-rate of 1.0–1.5 ml/min for a 25 cm×75 μm capillary column packed with 3 μm particles. The flow-rate for a required pressure drop will also depend on the composition of the eluent used to equilibrate the column.

2.4.3. Capillary liquid chromatography mode

The instrument is set to μLC mode. The sample vial is placed at the inlet end of the capillary column. The sample can now be injected either under pressure (0–12 bar) or under voltage (0–30 kV). For the purpose of this study electrokinetic injection was performed by applying a voltage of 5 kV for 5 s. Since the column has been equilibrated with 10 m*M* borate buffer, pH 8.5, enough electroosmotic flow (EOF) is generated at this pH to allow the sample to be injected into the column. For conditions where low or no EOF is generated upon application of voltage a pressure injection would be more appropriate. After performing injection; the sample vial is replaced with the seal vial and the pump is started. The flow-rate of the 1100 pump is adjusted such that the pressure reading of the pump is between 200 and 250 bar for the case of the packed capillary column used for this study. The linear flow velocity as measured by a neutral tracer is between 1 and 1.5 mm/s. In case of gradient elution the 1100 pumps generate a suitable gradient that is continuously delivered to the seal vial and pumped to the column under pressure. The delay volume for the gradient to reach from the pump to the electrode is about 250 μl.

2.4.4. Electrically assisted capillary liquid chromatography mode

The instrument is operated in the manner similar to μLC mode. A voltage (0–30 kV) is applied across the capillary column in addition to the pressure from the pump. At an appropriate pH an EOF is generated and the net flow through the column is a combination of the electroosmotic as well as hydrodynamic flow. Charged analytes experience an electric field for the fraction of time they spend in the mobile phase [12].

2.4.5. Capillary electrochromatography mode

The instrument is set to CECgrad mode. A 12 bar pressure is applied to both the inlet as well as the seal vial. The flow-rate of the pump is set between 0.1 and 0.15 ml/min. Under gradient elution conditions the eluent of changing composition is delivered to the seal vial continuously, and the eluent is drawn into the column electroosmotically.

3. Results and discussion

In a previously reported study, the analysis of a urine sample from a cancer patient exhibiting cachexia has been demonstrated by CE [13]. The separation is illustrated in Fig. 2 and was performed on a 64.5 cm (effective length 56 cm)×75 μm I.D. fused-silica capillary column at an applied voltage of 15 kV with 60 m*M* sodium tetraborate, pH 9.2 as the buffering electrolyte. The peak eluting at 15 min could be identified as the proteolysis inducing or the cachectic factor. Micropreparative CE was performed and the fraction containing the factor was collected for subsequent off-line analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) which indicated the factor to be highly heterogeneous and having an average molecular mass of ~24 000 [13]. Structural studies have indicated that the factor consists of a short polypeptide chain (average molecular mass of ~4000) that is phosphorylated and extensively glycosylated at both Asn and Ser residues [9]. The carbohydrate chains are also sulfated and these N- and O-linked sulfated oligosaccharides

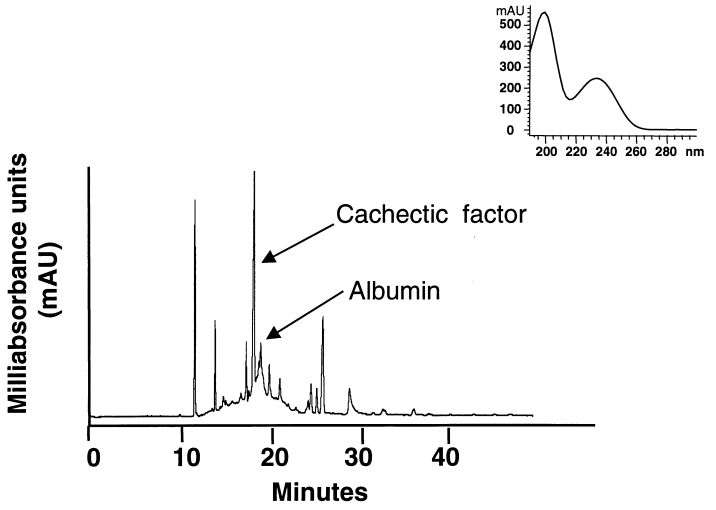

Fig. 2. Capillary electrophoretic separation of urine sample from a cancer patient suffering from cachexia. Conditions: fused-silica capillary 64.5 cm (effective length 56 cm)×75 μm; applied voltage, 15 kV; buffer, 60 m*M* sodium tetraborate, pH 9.2. (Adapted from Ref. [13].)

were found to be both antigenic and biological determinants. The high degree of glycosylation enables the separation to take place at high pH in a fused-silica capillary. The inset in the figure shows the UV spectra that is consistent with the cachectic factor. The combination of CE and MALDI-TOF-MS resulted in an improved analysis with the mass measurement providing complimentary data to the electrophoretic analysis. However, the variability of the sample from one patient to another and the analysis by CE reaching the limits of detection in certain cases, required the extension of this study to other separation methods.

Rozing et al. [14] have reported the development of a prototype instrument that can be operated to perform analysis by μLC, μLC with electric field assistance and CEC. In order to validate the identity of the factor by an orthogonal technique the separation was subsequently performed by μLC using the prototype instrument. Fig. 3 shows the separation of urine sample from a cachectic patient under isocratic and gradient elution conditions. The separation was performed on a 33.5 cm (effective length 25 cm)×75 μm column packed with 3.5 μm Zorbax ODS particles at pH 8.5. Fig. 3a shows the separation under gradient elution conditions with a rapid 5-min gradient from a 20–100% gradient former. The peak eluting at about 8.5 min was identified as the

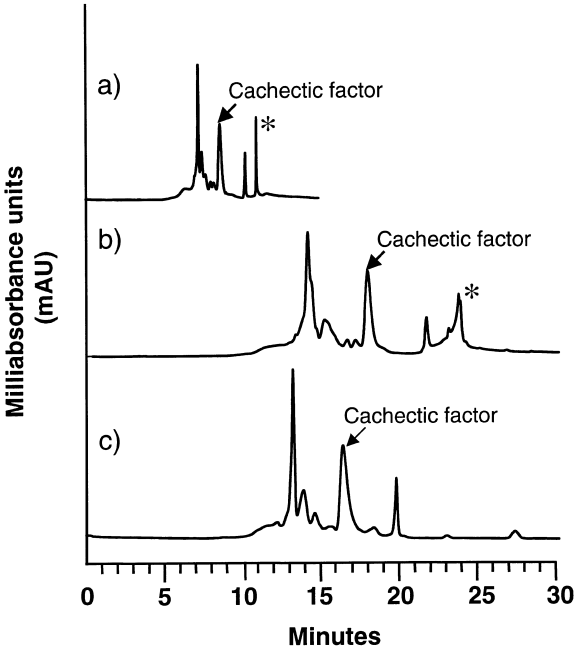

Fig. 3. μLC of urine sample from a cachectic patient. Column, 33.5 cm (effective length 25 cm)×75 μm packed with 3.5 μm Zorbax ODS particles; starting eluent (A), 10 m*M* borate buffer, pH 8.5; gradient former (B), 10 m*M* borate in a water–acetonitrile (1:4, v/v) mixture; gradient, (a) 20–100% B in 5 min, (b) 20–100% B in 15 min, (c) isocratic at 20% B; electrokinetic injection, 5 kV, 5 s.

cachectic factor by comparing the UV spectra of this peak to that obtained by CE (Fig. 2). The CE peak had earlier been identified by off-line MALDI-TOF-MS [13]. A comparison of Fig. 3a and b shows that as the gradient time is increased to 15 min, the retention time increases with the glycoprotein of interest eluting at 18 min, while the last peak (marked with an *) becomes broad. Highly glycosylated proteins and other polar polypeptide derivatives are usually not retained strongly on a reversed-phase stationary phase so the separation was tried under isocratic conditions by keeping the gradient former at a constant composition of 20%. It is seen in Fig. 3c that the cachectic factor now elutes at 16.5 min.

Relatively few applications illustrating separation of biological mixtures by CEC are available in the literature. This is partly due to the difficulty in choosing the appropriate mobile and stationary phase conditions. Fig. 4 illustrates the chromatographic separation of a urine sample from a cancer patient who is exhibiting loss of muscle on the same packed fused-silica capillary column with the prototype instrument operating in different modes. The separation in capillary liquid chromatographic mode under isocratic conditions on a Zorbax ODS column illustrated in Fig. 4a is completed in 30 min. Upon application of 20 kV voltage in addition to applied pressure, electroosmotic forces come into play and resulting separation takes only 8 min to complete. Thus, although the analysis time is reduced by a factor of 4 there is also some loss in resolution upon application of voltage. Fig. 4c shows the separation under isocratic conditions in CEC mode at an applied voltage of 20 kV. The inlet and outlet buffer vials were pressurized with 12 bar external pressure in this

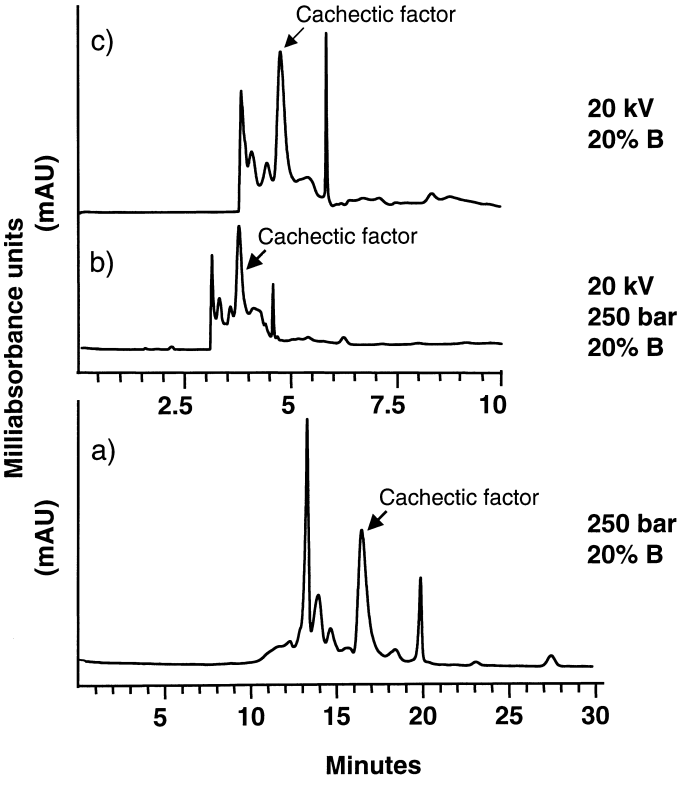

Fig. 4. Separation of urine sample from a cachectic patient by various orthogonal modes. Column, 33.5 cm (effective length 25 cm)×75 μm packed with 3.5 μm Zorbax ODS particles; starting eluent (A), 10 m*M* borate buffer, pH 8.5; gradient former (B), 10 m*M* borate in a water–acetonitrile (1:4, v/v) mixture; gradient, isocratic at 20% B; electrokinetic injection, 2 kV, 2 s. Modes, (a) μLC, 250 bar, (b) electric field assisted μLC, 20 kV, 160 bar, (c) CEC, 20 kV, 0 bar.

mode. Since both end vials are pressurized there is no pressure driven flow through the column and the mobile phase is driven though the column under strictly electroosmotic forces. The profile obtained in CEC mode shows the cachectic factor eluting at about 5 min. Thus, the above results highlight the advantage of a prototype instrument where the separation by all three modes could be readily performed on the same column under identical operating conditions. The above three modes also offer a higher loading capacity than capillary electrophoresis and hence can further aid in the analysis of several components that may go undetected by CE.

The prototype instrument enables rapid method development and optimization of various parameters influencing resolution during separation by different analytical modes. Fig. 5 provides a comparison of the μLC separation on two different stationary phases. Significant differences in the resolution and analysis time of the peaks were observed for separation on the Hypersil and Zorbax stationary phases. The cachectic factor peak that eluted at about 16.5 min on Zorbax ODS elutes at 32 min on the Hypersil stationary phase primarily due the longer retention of the glycoprotein on a more hydrophilic stationary phase. Similarly, the peak eluting at 13 min on the Zorbax stationary phase elutes at 21 min on the Hypersil ODS and appears to show higher resolution from its neighboring peaks. Fig. 6 shows the effect of applied voltage on the separation of the urine sample by CEC and electric field assisted μLC. The separation was performed on a 3 μm Hypersil stationary phase packed in a 33.5 cm (effective length 25 cm)×75 μm column. As the applied voltage is increased from 5 to 25 kV the separation efficiency increases and the total analysis time decreases. This of course is at the cost of resolution for some of the peaks. Fig. 6 indicates that the optimum voltage is 15 kV and 10 kV for CEC and field assisted μLC, respectively. Temperature is

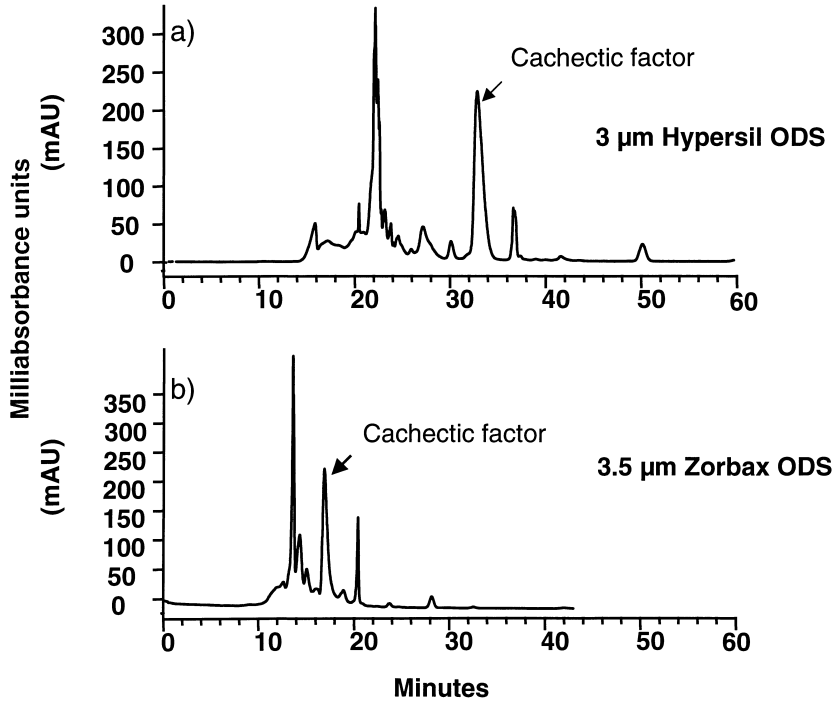

Fig. 5. Comparison of capillary liquid chromatographic separation obtained on two different stationary phases. Column, 33.5 cm (effective length 25 cm)×75 μm packed with (a) 3 μm Hypersil ODS, (b) 3.5 μm Zorbax ODS particles; starting eluent (A), 10 m*M* borate buffer, pH 8.5; gradient former (B), 10 m*M* borate in a water–acetonitrile (1:4, v/v) mixture; gradient, isocratic at 20% B; electrokinetic injection, 5 kV, 5 s.

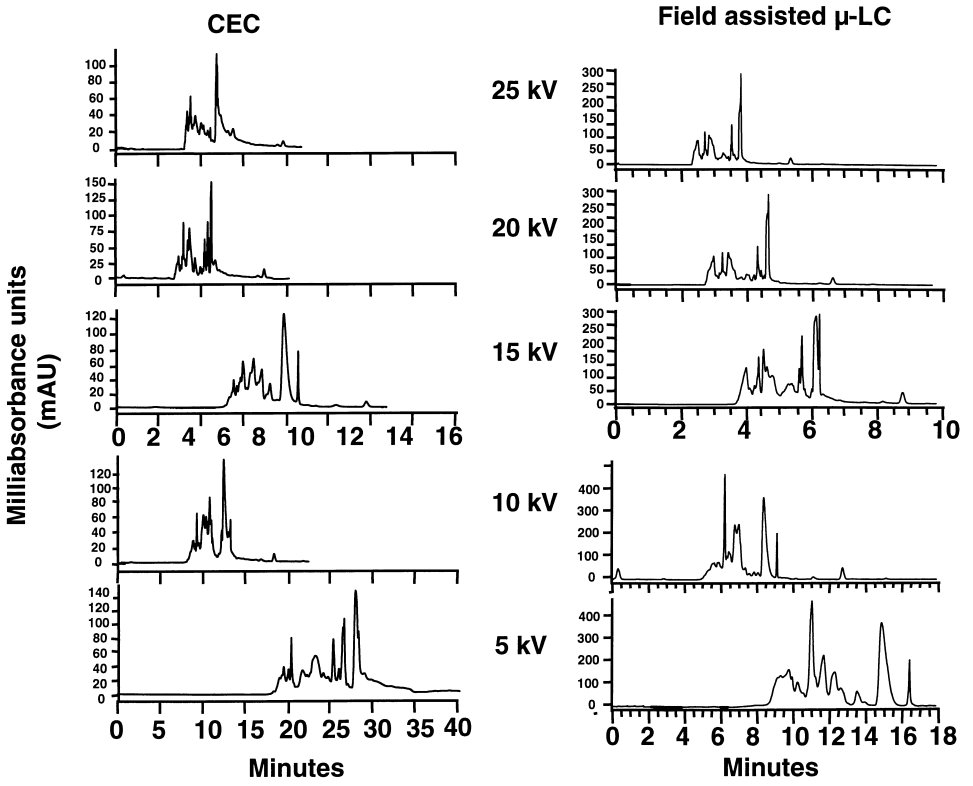

Fig. 6. Effect of voltage on separation of a urine sample from a cachectic patient by capillary electrochromatography and field assisted capillary liquid chromatography. Column, 33.5 cm (effective length 25 cm)×75 μm packed with 3 μm Hypersil ODS particles; starting eluent (A), 10 m*M* borate buffer, pH 8.5; gradient former (B), 10 m*M* borate in a water–acetonitrile (1:4, v/v) mixture; gradient, isocratic at 20% B; electrokinetic injection, 5 kV, 5 s. Modes, CEC, 0 bar, electric field assisted μLC, 160 bar.

another important operational variable that is known to cause significant changes in the retention times and selectivity of macromolecules [15–19]. Fig. 7 shows the effect of temperature on the separation of the urine sample by all three capillary separation modes for a patient who exhibits cachexia. Increasing the temperature from 25 to 60°C appears to have a detrimental effect on the separation of this particular sample by CEC. During separation by μLC, as the temperature is increased, the analysis time decreases from 50 min at 25°C to 30 min at 60°C. The efficiency of separation also increases while the peak of interest (marked with an *) is still well resolved from its neighboring peaks. Similarly, increasing the temperature for field assisted μLC results in a decreased analysis time. The peak efficiency is higher at 60°C but there is a loss in resolution of the cachectic factor peak which now is barely baseline resolved from its neighboring peak.

Although individual instrumentation exists for performing separations by μLC, CE and CEC, an integrated platform offers the ability to compare separations on the same column under similar operating conditions. Contributions to extra-column band broadening arising at the injection and detection end would be identical along with parameters like injection and gradient delay volumes. Advantages of the integrated analytical instrument that offers analysis by several orthogonal techniques is further illustrated in Fig. 8. It shows a comparison of the chromatogram for the urine sample from a cachectic patient by CEC and field assisted μLC at an applied voltage of 20 kV. Distinct differences in the resolution and efficiency of the early eluting peaks are

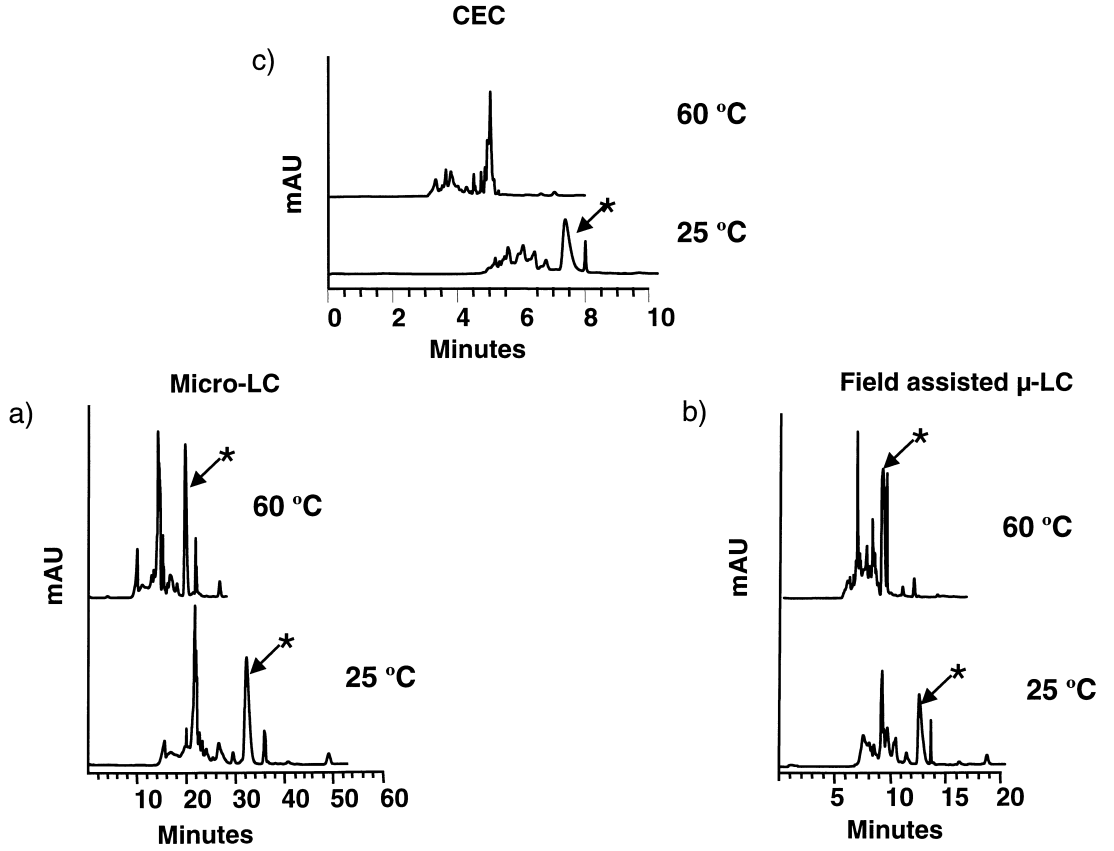

Fig. 7. Effect of temperature on the separation of a urine sample from a cachectic patient by capillary liquid chromatography, field assisted capillary liquid chromatography and capillary electrochromatography. Column, 33.5 cm (effective length 25 cm)×75 μm packed with 3 μm Hypersil ODS particles; starting eluent (A), 10 m*M* borate buffer, pH 8.5; gradient former (B), 10 m*M* borate in a water–acetonitrile (1:4, v/v) mixture; gradient, isocratic at 20% B; electrokinetic injection, 5 kV, 5 s. Modes, (a) CEC, 15 kV, 0 bar, (b) electric field assisted μLC, 5 kV, 250–180 bar, (c) μLC, 160–110 bar.

observed that assists the analysis of the complex biological mixture [12].

4. Conclusions

The present work demonstrates the potential of a novel analytical instrument in the clinical analysis of biological fluids, i.e., diagnosis of cachectic factor in the urine of cancer patients by three different techniques in a three-dimensional fashion. The instrument provides for convenient, rapid and efficient multidimensional approach towards method development and simultaneous analysis of the cachectic factor by the techniques of capillary zone electrophoresis, μLC with and without applied electric field, and CEC. Such an integrated scheme provides an added improvement over the currently practiced immunological methods for the analysis of cachectic factor by requiring minimal sample preparation and handling. To our knowledge this is the first study presenting the analysis of a complex biological fluid by the technique of CEC. Furthermore, the compatibility of each method with electrospray ionization mass spectrometry is particularly attractive and is being further explored to characterize the microheterogeneity observed in different patient samples.

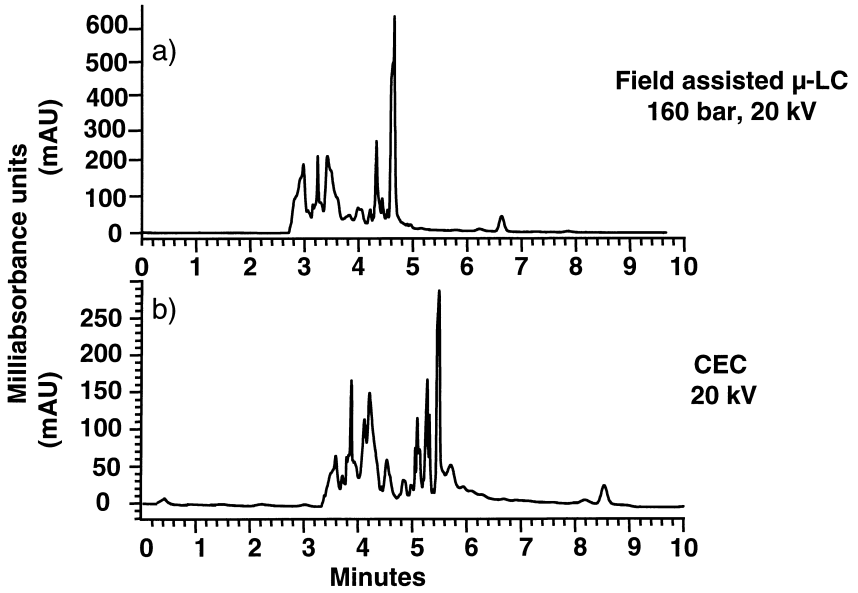

Fig. 8. Selectivity differences observed for separation by capillary electrochromatography and field assisted capillary liquid chromatography. Column, 33.5 cm (effective length 25 cm)×75 μm packed with 3 μm Hypersil ODS particles; starting eluent (A), 10 m*M* borate buffer, pH 8.5; gradient former (B), 10 m*M* borate in a water–acetonitrile (1:4, v/v) mixture; gradient, isocratic at 20% B; electrokinetic injection, 5 kV, 5 s. Modes, (a) electric field assisted μLC, 20 kV, 160 bar, (b) CEC, 20 kV, 0 bar.

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