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# Metabolic analysis of body fluids by capillary electrophoresis using noncovalently coated capillaries☆

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ABSTRACT

The potential of capillaries noncovalently coated with charged polymers for the metabolic analysis of body fluids by CE is illustrated. Firstly, the usefulness of a coating consisting of a triple layer of polybrene–dextran sulfate–polybrene for the fast analysis of organic acids is described. The CE system allowed direct injections of CSF, plasma and urine samples, yielding good separation efficiencies. RSDs for migration times and peak areas of organic acids in plasma were <3% and <5%, respectively. The usefulness of the system is illustrated by the profiling of organic acids in plasma and urine samples. Secondly, a CE system comprising a bilayer coating of polybrene–poly(vinylsulfonate), which provides a considerable EOF at low pH is described. This system was combined with TOF-MS and used for the fast analysis of amino acids in cerebrospinal fluid (CSF) and urine with minimal sample pretreatment. RSDs for migration times and peak areas of amino acids in CSF and urine were <2% and <10%, respectively. The applicability of the system is demonstrated by the profiling of endogenous low-molecular weight metabolites in CSF from a healthy individual and a patient with complex regional pain syndrome.



## 1. Introduction

Metabolic profiling of body fluids, such as urine and cerebrospinal fluid (CSF), provides an insight into the metabolic state of an organism and specific biochemical processes. Hence, metabolic profiling has become important for the screening of potential diagnostic and prognostic markers of various diseases or for the detection of pharmacological or toxicological effects obtained following dosing of compounds [1]. This novel approach has a potential to replace more classical methods in clinical laboratories which are often focused on the measurement of a specific endogenous metabolite or a class of endogenous metabolites. It provides more information about the physiological state of an organism [2].

To date, metabolic profiling has been mostly performed with GC–MS and NMR spectroscopy [3–5]. Applications of GC–MS for large-scale metabolite analysis are mainly found in the field of plant metabolomics [6]. GC–MS is limited to volatile metabolites and derivatization is often needed to yield volatile and thermostable compounds. NMR spectroscopy has commonly been used for the metabolic profiling of body fluids, such as serum and urine [7]. NMR is rapid, non-destructive and requires minimal sample preparation. Nevertheless, the sensitivity of NMR is limited and analyte amounts of several micrograms are required. The widespread use of LC–MS for global metabolic profiling is relatively new [8]. LC–MS can supply information on the chemical structure and the quantity of low-abundance metabolites. More advanced LC systems, i.e. monolithic capillary LC or UPLC offering improved separation efficiencies, are gaining more attention for the metabolic profiling of body fluids [9]. However, LC–MS also has some limitations, especially when applied to highly polar compounds. As many components in body fluids are highly polar and ionic, separation of these components using common reversed-phase LC can be problematic [10].

Capillary electrophoresis (CE) is an analytical technique capable of high-resolution separation of a diverse range of chemical compounds. It is particularly suitable for the separation of polar and charged compounds [11]. Therefore, CE is very attractive for the separation of endogenous metabolites in body fluids. Furthermore, the costs for accessories are less than in LC, due to very low organic solvent consumption, the small amount of reagents and the use of inexpensive fused-silica capillaries. Another attractive feature of CE is its small sample requirement, making it particularly well-suited for samples, such as CSF, that are volume-limited.

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Capillary zone electrophoresis (CZE) has commonly been used for the analysis of a subset of endogenous metabolites in urine in order to study metabolic disorders [10–14]. In order to improve selectivity, micellar electrokinetic chromatography (MEKC), which employs micelles as pseudo-stationary phases in the BGE, has been used for metabolic profiling of urine [15,16]. However, the coupling of MEKC to MS is problematic and often provides limited sensitivity. Variability of migration time is an important issue in CE. Procedures to correct for migration time shifts and for aligning electropherograms have been developed [10,16]. Still, for metabolic profiling studies of body fluids, reproducibility of migration times and peak areas is of utmost importance for a reliable comparison of profiles and to observe small changes in sample composition. Using CE with bare fused silica capillaries, the analysis of body fluids with minimal sample pretreatment is often not possible due to adsorption of proteins or other matrix components to the capillary wall causing irreproducible electro-osmotic flows and migration times. A promising approach to minimize this problem is the use of noncovalently coated capillaries, i.e. dynamically coating of the bare fused silica capillaries with charged polymers. Recently, we have described the methodological aspects of noncovalent coatings in CE–UV and CE–MS for metabolite profiling [17,18]. In the present study we describe the applicability of two noncovalently coated capillaries in CE-based metabolic analysis of body fluids with minimal or no sample pretreatment.

## 2. Experimental

### 2.1. Chemicals

The background electrolyte (BGE) constituents sodium dihydrogen phosphate and disodium hydrogen phosphate were from Merck (Darmstadt, Germany). Sodium or potassium salts of lactic, citric, 3-hydroxybutyric, and pyroglutamic acids were of analytical standard-grade and purchased from Sigma–Aldrich (Steinheim, Germany). Organic acid stock solutions (10 mg/mL) in water were prepared weekly. Test mixtures of organic acids (133.3 μg/mL each, unless otherwise stated) were made by adding an aliquot of the organic acid stock solutions to the appropriate volume of water or plasma, CSF or urine samples. For electro-osmotic flow (EOF) determinations, formamide was added to test samples to a final concentration of 0.033% (v/v). Polybrene (hexadimethrine bromide, PB), dextran sulfate sodium salt (DS), and 25% m/v aqueous solution of poly(vinylsulfonate) (PVS) sodium salt were purchased from Sigma–Aldrich (Steinheim, Germany). Ammonium hydroxide (25%) was from Merck (Darmstadt, Germany). Amino acids (L-alanine, L-arginine, L-glutamic acid, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-tyrosine, L-valine) with a concentration of 1 mM of each in 0.1 M hydrochloric acid were purchased from Sigma–Aldrich. HPLC-grade methanol was supplied by Biosolve BV (Valkenswaard, the Netherlands). Standard solutions of amino acids were prepared with water taken from a Milli-Q water purification system (Millipore, Bedford, MA, USA). BGE solution for CE–MS was prepared by dissolving formic acid in Milli-Q water (1 M, pH 1.8). Human CSF and plasma samples were provided by the Leiden University Medical Centre (Leiden, The Netherlands) and stored at −80 °C until analysis. Citric acid was used as an anticoagulant agent for human plasma samples. For CSF and plasma samples, no sample preparation was carried out prior to injection. Urine samples from a healthy control group were obtained from the department of Infectious Diseases of the Leiden University Medical Centre (Leiden, The Netherlands). After collection, urine samples were stored immediately at −80 °C. Prior to CE–MS analysis, the urine samples were thawed to room temperature, mixed with BGE (1:1, v/v) and centrifuged (13,200 rpm) for 5 min.

### 2.2. Capillary coating procedures

#### 2.2.1. PB–DS–PB coating

New fused-silica capillaries were rinsed with deionized water for 5 min at 1380 mbar followed by 1 M NaOH for 30 min at 1380 mbar, and deionized water for 15 min at 1380 mbar. The triple layer coating was prepared by subsequently rinsing the capillary with 10% (m/v) PB solution for 15 min at 350 mbar, deionized water for 5 min at 1380 mbar, 3% (m/v) DS solution for 15 min at 350 mbar, deionized water for 5 min at 1380 mbar, and, finally with a 10% (m/v) PB solution for 15 min at 350 mbar. The capillary was then ready for use with the BGE of choice. Before analysis, capillaries were flushed with BGE for 5 min at 1380 mbar. Between runs, the coated capillaries were flushed with a 0.1% (m/v) PB solution and BGE, each for 2 min at 1380 mbar. During the analyses of CSF or plasma samples, an additional rinse with 100 mM hydrochloric acid (10 min at 1380 mbar) was incorporated between the runs, unless otherwise stated.

#### 2.2.2. PB–PVS coating

New bare fused-silica capillaries were rinsed with deionized water for 5 min at 1380 mbar followed by 1 M sodium hydroxide for 15 min at 1380 mbar, and deionized water for 5 min at 1380 mbar. Coating was performed by rinsing for 30 min at 350 mbar with 10% (m/v) PB and successively with water for 5 min at 1380 mbar. Subsequently, the capillary was flushed with 5% (v/v) PVS for 30 min at 350 mbar, and again water for 5 min at 1380 mbar. At the start of the day, coated capillaries were flushed with deionized water for 1 min at 1380 mbar and with BGE for 2 min at 1380 mbar. Between runs, the coated capillaries were flushed with 5% (v/v) PVS for 2 min at 1380 mbar and with BGE for 1 min at 1380 mbar.

### 2.3. Instrumentation and CE conditions

CE was performed on a P/ACE ProteomeLab PA 800 (Beckman Coulter, Fullerton, CA, USA) equipped with a diode-array detector. Electropherograms of organic acids were monitored at 200 nm. Fused-silica capillaries with an internal diameter of 75 μm (Composite Metal Services Ltd., UK) had a total length of 60 cm and an effective length of 50 cm. Injections were performed hydrodynamically for 10 s at 35 mbar. The separation voltage was −10 kV and the capillary was thermostated at 25 °C. Sodium phosphate BGE (200 mM) was made by mixing equimolar solutions of sodium dihydrogen phosphate and disodium hydrogen phosphate in the appropriate ratio to reach a pH of 6.0. Electropherograms were analyzed using 32 Karat Software, version 7.0 (Beckman Coulter). For CE–MS, the CE experiments were also carried out on a P/ACE ProteomeLab PA 800 instrument. Capillaries had a total length of 130 cm and an internal diameter of 50 μm. Formic acid (1 M, pH 1.8) was used as BGE. Sample injections were performed hydrodynamically for 90 s at 90 mbar (1.3 psi). Prior to sample injection a small plug (50 mbar, 9 s) of 12.5% ammonium hydroxide was injected for pH-mediated stacking. The separation voltage was 30 kV and the capillary temperature was 25 °C. MS was performed using a micrOTOF orthogonal-accelerated time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany). Transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany). Spectra were collected with a time resolution of 1 s. Post-run internal mass calibration was performed using sodium formate cluster ions $Na^+(HCOONa)_{1-9}$ ranging from 90.9766 to 430.9137 *m/z*, which are detected in the first part of the electropherogram after urine sam-

**Table 1**
Migration time repeatability (RSD, %) of noncovalently coated capillaries ($n = 5$)

| | Coating | | | |
|---|---|---|---|---|
| | PB–DS–PB | | PB–PVS | |
| | Citric acid[a] | Lactic acid[a] | Valine[b] | Phenylalanine[b] |
| Intraday | 0.3 | 0.4 | 0.4 | 0.4 |
| Interday | 0.7 | 0.6 | 0.8 | 1.0 |
| Capillary-to-capillary | 1.0 | 1.0 | 1.7 | 2.2 |

[a] Compound (100 μM) dissolved in deionized water; data obtained by CE–UV.
[b] Compound (50 μM) dissolved in 0.5 M formic acid (pH 1.8); data obtained by CE–MS.

ple injection. CE–MS coupling was realized by a co-axial sheath liquid interface (Agilent Technologies, Waldbronn, Germany) with methanol–water–formic acid (50:50:0.1, v/v/v) as sheath liquid. The following spray conditions were used: sheath liquid flow, 4 μL/min; dry gas temperature, 180 °C; nitrogen flow, 4 L/min; nebulizer pressure, 0.5 bar. Electrospray in positive ionization mode was achieved and ESI voltage was −4.5 kV.

### 2.4. Quantification studies

Peak areas, corrected for migration times, of organic acids in plasma, urine and CSF samples were used for determination of the concentration using calibration curves constructed in 150 mM NaCl solutions. The migration time and peak area repeatability of the CE–UV method with the PB–DS–PB coating was determined for the compounds citric acid, lactic acid and pyroglutamic acid using their endogenous concentration levels by ten consecutive analyses of plasma, urine and CSF. The identity of organic acids was verified by standard addition experiments. The migration time and peak area repeatability of the CE–MS method with the PB–PVS coating was determined by 10 consecutive analyses of a pooled human urine sample spiked with 50 μM of eight amino acids.

## 3. Results and discussion

### 3.1. CE–UV system for the analysis of organic acids in body fluids

The analysis of organic acids in body fluids plays an important role in the screening, diagnosis and monitoring of a variety of metabolic disorders. Using CE, reversed polarity is required for the fast analysis of organic acids as their electrophoretic mobility towards the anode is usually higher than the electro-osmotic flow (EOF) towards the cathode. Recently, we have developed a CE–UV method based on a triple layer capillary coating for the quantitative determination of organic acids in CSF [17]. The triple layer capillary coating was prepared by successively rinsing a bare fused-silica capillary with solutions of PB, DS, and PB, yielding a positively charged coating with an pH-independent EOF directed towards the anode [19]. As the EOF was reversed, the organic acids migrated before the EOF enabling relatively fast analysis times. The repeatability of the triple layer capillary coating was evaluated on the basis of the RSD values for migration times of two organic acids. The results are summarized in Table 1. Good repeatability of migration times was obtained for intraday, interday and capillary-to-capillary analyses indicating a high coating stability. It was possible to separate organic acids in CSF without any sample pretreatment as adverse effects of proteins could be effectively minimized by the triple layer coating in combination with rinses of 0.1 M hydrochloric acid. The organic acids were analyzed with high separation efficiency, i.e. plate numbers were above the 100,000 and albumin did not interfere with the analysis of the organic acids. The CE–UV method based on the PB–DS–PB coating was used for the profiling of organic acids in CSF samples from patients with bacterial meningitis.

**Table 2**
Precision data (RSD, %) for migration times and peak areas of a few organic acids in different body fluids obtained by PB–DS–PB CE–UV ($n = 10$)

| Organic acid | Plasma | Cerebrospinal fluid | Urine |
|---|---|---|---|
| Citric acid | | | |
| Migration time | 1.8 | 1.6 | 1.9 |
| Peak area | 3.9 | 4.6 | 3.6 |
| Lactic acid | | | |
| Migration time | 2.5 | 2.9 | 1.5 |
| Peak area | 3.7 | 6.8 | 2.9 |
| Pyroglutamic acid | | | |
| Migration time | 2.4 | 1.7 | 1.8 |
| Peak area | 4.5 | 3.8 | 4.8 |

In the present study, we have evaluated the potential of this method for the profiling of organic acids in human plasma, which is also clinically very important. For instance, the simultaneous analysis of lactic acid and 3-hydroxybutyric acid is used for the monitoring of lactic acidosis in human plasma [20]. However, plasma is a much more complex matrix than CSF containing more proteins at higher levels. For example, the average concentration of albumin in plasma is ca. 60 mg/ml, while in CSF this is approximately 0.5 mg/ml. Nonetheless, Table 2 shows high repeatability for migration times and peak areas of three organic acids after direct injection of plasma. The constant migration times of the analytes demonstrate that the EOF is stable, indicating that no or minimal protein adsorption to the capillary wall occurs. It shows the high stability of the CE–UV method with the PB–DS–PB coating. Fig. 1 shows that the organic acids and other endogenous metabolites in plasma can be analyzed within 15 min and without interference from albumin. Plate numbers for most organic acids were above the 100,000 which is comparable with plate numbers for organic acids obtained in CSF. The electropherogram in Fig. 1 shows a limited number of compounds, while plasma contains more organic acids, such as acetoacetic acid and pyruvic acid. These compounds co-migrated with a system band which appears between peak 1 and peak 2 (Fig. 1). Other organic acids, such as fumaric acid and glutaric acid, were not detected in plasma which was in agreement with the results obtained by a GC–MS study [21]. Another reason for the limited number of compounds shown in Fig. 1 might be the relatively poor concentration sensitivity of CE–UV. The concentrations found for lactic acid, 3-hydroxybutyric acid and pyroglutamic

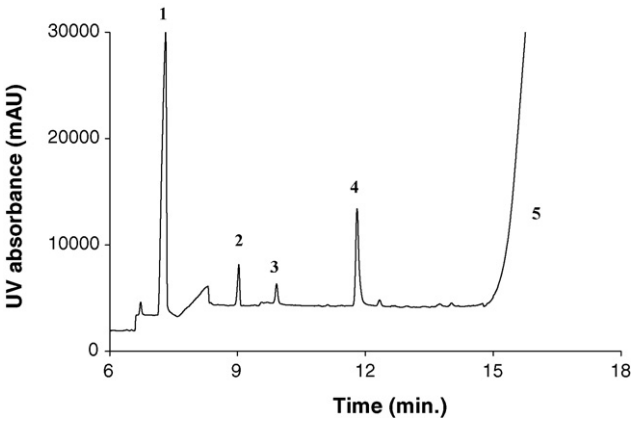


**Fig. 1.** CE–UV of a human plasma sample. Experimental conditions: BGE, 200 mM sodium phosphate (pH 6.0); injection, 35 mbar for 10 s; detection, UV at 200 nm; capillary, PB–DS–PB coated. Peaks: 1, citric acid; 2, lactic acid; 3, 3-hydroxybutyric acid; 4, pyroglutamic acid; 5, albumin.

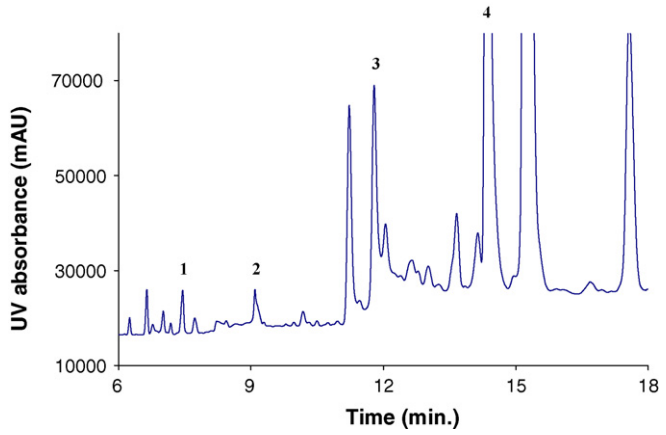


**Fig. 2.** CE–UV of a human urine sample. Experimental conditions: BGE, 200 mM sodium phosphate (pH 6.0); injection, 35 mbar for 10 s; detection, UV at 200 nm; capillary, PB–DS–PB coated. Peaks: 1, citric acid; 2, lactic acid; 3, pyroglutamic acid; 4, hippuric acid.

acid were 1540, 165, 180 μM, respectively (for details on quantification studies see Section 2.4). These values fall within the range of reference values of organic acids in human plasma determined by GC–MS including derivatization. Citric acid was used as an anticoagulant agent and, therefore, it was not relevant to determine the concentration of this compound.

The potential of the CE–UV system with the PB–DS–PB coating has also been investigated for the direct analysis of human urine. An electropherogram of a urine sample of a healthy human subject is shown in Fig. 2. Many negatively charged compounds can be observed within 20 min ($EOF_{marker} \approx 20$ min) requiring no sample pretreatment. The separation could be improved by slowing down the EOF via the addition of organic solvents, like methanol, to the BGE but at the expense of analysis time [19]. Improved selectivity can also be obtained using MEKC methods [16], but the coupling of MEKC to MS is not straightforward. The concentrations found for citric acid, lactic acid and pyroglutamic acid were 211, 37 and 39 μM, respectively and they correspond with normal values [21]. The average concentration of 3-hydroxybutyric acid is below the 1 μM in urine and therefore, this compound could not be detected as the concentration was below the detection limit of the CE–UV method [21]. An overview of repeatability data for migration times and peak areas of organic acids in different body fluids is given in Table 2. In summary, the CE–UV method with a triple-layer coated capillary shows high potential for the fast and repeatable profiling of organic acids in plasma, CSF and urine without or minimal sample pretreatment.

### 3.2. CE–MS system for metabolic profiling of body fluids

Profiling of endogenous low-molecular weight metabolites in body fluids is important for the screening of deficiencies in amino acid metabolism in routine clinical analysis [22]. So far, CE–MS methods mostly used bare fused silica capillaries for amino acids analysis in body fluids [23,24]. Analysis times were relatively long due to the slow EOF resulting from the low-pH background electrolyte (BGE) in combination with bare fused silica capillaries. Obviously, this is not very suitable for clinical studies where high-throughput analyses are required. Moreover, using bare fused silica capillaries, separation efficiency and reproducibility may be compromised. Changes in the surface of the capillary wall due to adsorption of matrix components may cause irreproducible EOFs leading to a poor migration-time repeatability.

Recently, capillaries noncovalently coated with a bilayer of oppositely charged polymers have been used for the fast and highly reproducible analysis of peptides [25]. These coated capillaries were produced by first rinsing the capillary with a solution of the positively charged polymer PB and subsequently with a solution of the negatively charged polymer PVS. The resulting PB–PVS coating provides a considerable EOF at low pH, thereby facilitating the fast separation of positively charged peptides using a BGE of formic acid (pH 2.5). In addition, the PB–PVS coating is fully compatible with MS detection causing no ionization suppression [25]. The repeatability of the PB–PVS coating was evaluated on the basis of the RSD values for migration times of two amino acids. The results are summarized in Table 1. The good repeatability of migration times for intraday, interday and capillary-to-capillary analyses indicates a high coating stability.

Using a CE–MS system with a PB–PVS coated capillary, all amino acids were analyzed within 13 min [18]. Plate numbers for the amino acids in CSF and urine varied from 50,000 to 300,000. The limits of detection for the amino acids were improved by in-capillary preconcentration based on pH-mediated stacking allowing 100-nL sample injection (i.e., ca. 4% of capillary volume). As a result, detection limits for amino acids were down to 20 nM. An important advantage of this method is the online regeneration of the capillary coating by flushing with a solution of PVS between the runs. During regeneration the ion source settings of the MS were adjusted (e.g., capillary voltage was set a 0 kV) to prevent contamination with PVS. Table 3 shows that the migration times of several amino acids were quite constant for ten successive analyses of the matrix spiked with 50 μM of each analyte. The RSD values for peak areas were below the 10%, which is acceptable for biological samples using ESI-MS. The potential of the PB–PVS coated capillary for the profiling of low-molecular weight metabolites in human CSF is shown in Fig. 3(upper trace). A metabolic profile of positively charged compounds was obtained in less than 20 min. The lower trace of Fig. 3 shows that the metabolic profile of CSF from a patient with complex regional pain syndrome (CRPS) is different from the metabolic profile of CSF from a healthy individual (upper trace). However, a large set of CSF samples from both groups should be analyzed to compare metabolic profiles of the two groups using biostatistical techniques. The analytical separation window for low-molecular weight metabolites, e.g. amino acids and small peptides, is larger using bare fused silica capillaries, however, the PB–PVS CE system is combined with a time-of-flight (TOF) MS and, therefore, the high mass accuracy provides the required extra selectivity. Therefore, the PB–PVS CE–TOF-MS method provides a very interesting tool for the fast and highly repeatable profiling of low-molecular weight metabolites in CSF and urine.

**Table 3**
Precision data (RSD, %) for migration times and peak areas of some amino acids in CSF and urine obtained by PB–PVS CE–MS ($n = 10$)

| Amino acid | Matrix | | | |
|---|---|---|---|---|
| | Cerebrospinal fluid[a] | | Urine[a] | |
| | Migration time | Peak area | Migration time | Peak area |
| Lysine | 1.4 | 7.7 | 1.2 | 8.6 |
| Arginine | 1.7 | 6.8 | 1.5 | 5.9 |
| Alanine | 1.5 | 7.8 | 1.2 | 8.9 |
| Valine | 0.8 | 8.4 | 1.1 | 8.9 |
| Methionine | 1.5 | 9.8 | 1.1 | 7.5 |
| Glutamic acid | 1.4 | 8.5 | 1.2 | 7.9 |
| Phenylalanine | 1.6 | 7.6 | 0.8 | 7.9 |
| Tyrosine | 1.6 | 7.4 | 0.9 | 8.3 |

[a] Spiked with 50 μM of each amino acid.

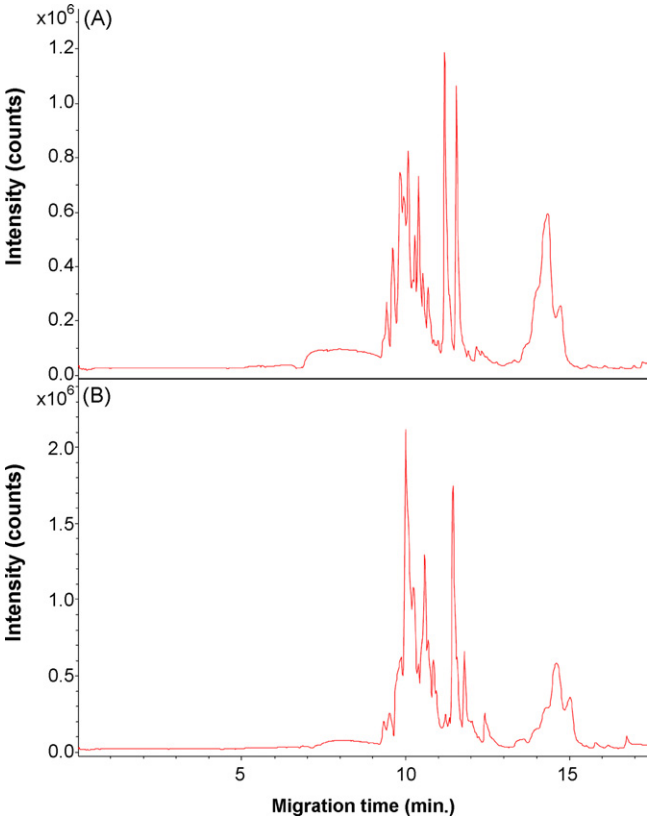


**Fig. 3.** Metabolic profiling of CSF by CE–TOF-MS. Base peak electropherogram of (A) a CSF sample from a healthy individual and (B) a CSF sample from a patient with CRPS. Experimental conditions: BGE, 1 M Formic acid (pH 1.8); sample injection, 90 mbar for 90 s; pre-injection, ammonium hydroxide (12.5%) at 50 mbar for 9 s; capillary, PB–PVS coated; scan range, 50–450 *m/z*.

## 4. Conclusion

In the present study, the potential of two noncovalently coated capillaries for the metabolic analysis of body fluids by CE(–MS) was illustrated. The high stability of the present systems provides good potential for the profiling of endogenous metabolites in biological samples with minimal sample pretreatment. Currently, we are investigating the possibility to use the CE system with the triple layer coated capillary in combination with mass spectrometric detection for the sensitive and selective profiling of negatively charged compounds in biological samples using direct sample injection. The CE system with the bilayer coated capillary will be used for the metabolic profiling of urine and CSF samples from patients with CRPS. Global metabolic profiling of body fluids can be achieved by combining the results obtained with CE–MS systems with double and triple layer capillary coatings for the analysis of positively charged and negatively charged compounds. Moreover, the selectivity of MS is very helpful for the characterization of co-migrating compounds and for the ultimate identification of compounds that are responsible for the differences between control and patient samples.

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