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Preliminary investigations of preconcentration–capillary electrophoresis–mass spectrometry

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Abstract

Analyte preconcentration on-line with capillary electrophoresis–mass spectrometry (PC–CE–MS) is described. Preconcentration cartridges were fabricated from PTFE tubing filled with ca. 1–2 mm bed of reversed-phase C_{18} HPLC packing or polymeric reversed-phase beads. The particle size of the stationary phase was of larger dimension than the internal diameter of the CE capillary. Therefore, PC–CE capillaries were assembled without frit material and held together by friction. The wide applicability of on-line PC–CE–MS is demonstrated by the analysis of solutions containing peptides, proteins, and synthetic analogues of putative metabolites of the neuroleptic agent haloperidol.

1. Introduction

The relatively poor concentration limits of detection (CLOD) of capillary electrophoresis (CE), due to limitations of focusing large sample volumes, has warranted the development of several on-line analyte concentration techniques. These have included coupled capillaries for combination of capillary isotachophoresis (CITP) with CE [1], transient isotachophoresis (tITP) in a single capillary [2–4], analyte stacking [5,6], and field amplification [7]. Such electrophoretic techniques have extended the applicability of CE for analyzing dilute analyte solutions. However, it is becoming increasingly important, particularly in the biomedical research environment, to develop methods that are capable of analyzing compounds that are present at concentrations below those that can be effectively focused and preconcentrated by electrophoretic techniques. In these instances, analysis of several microliters of sample solution is often required to enable detection of analytes of interest. Furthermore, off-line sample concentration of dilute analyte solutions may be precluded since analyte losses to surfaces (pipette tips, vials, etc.) can be substantial. Such losses are particularly pronounced for peptides and proteins. Also protein denaturation caused by sample handling can result in poor solubility, hence it is desirable to minimize sample handling. Therefore, on-line preconcentration–CE (PC–

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CE) and analyte concentrator capillaries have been developed and described [8–13]. These techniques utilize a small bed of HPLC material (e.g., reversed-phase C_{18}) [9–12], hydrophobic membranes [13] or materials that exhibit specific immunological properties [8] at the inlet of the CE capillary. Analytes that are "trapped" by this phase can be subsequently eluted in a small volume of solvent, hence, concentrated before separation by capillary electrophoresis. Furthermore, sample contaminants (e.g., matrix components) can be eluted from the phase and washed from the capillary prior to elution of the analytes of interest to effect on-line sample clean-up.

The mass spectrometer is a unique detector for CE, affording both high sensitivity and structural characterization of a wide range of biologically important molecules. On-line CE–MS, pioneered by Smith et al. [14,15] and Henion et al. [16,17], is emerging as a powerful and practical technique, particularly in the biomedical sciences, for identification of biologically active agents. However, while the mass spectrometer is an inherently sensitive detector, CE–MS also suffers relatively poor CLOD caused by the poor focusing capacity of the CE capillary upon injection of large sample volumes [18–20]. Therefore, we have been investigating applications of on-line PC–CE–MS and have recently published, to our knowledge, the first reported use of this technique [21].

In the present study, we demonstrate the wide applicability of PC–CE–MS for the analysis of small molecules, peptides, and proteins.

2. Experimental

2.1. Chemicals

Acetic acid (99.9% + grade) was obtained from Aldrich Chemical Company (Milwaukee, WI, USA) and HPLC grade methanol and water were obtained from Baxter (Minneapolis, MN, USA) and silica-based C_{18} (irregular particles) from Waters (Milford, MA, USA). PLRP-S (300 Å) a styrene–divinyl benzene copolymer stationary phase was obtained from Polymer Laboratories (Amherst, MA, USA). Horse skeletal muscle apomyoglobin, bradykinin, bombesin, luteinizing hormone–releasing hormone (LH–RH), α-melanocyte-stimulating hormone (α-MSH) and angiotensin II were obtained from Sigma (St. Louis, MO, USA). Polybrene was obtained from Applied Biosystems (San Jose, CA, USA). Haloperidol and the synthetic analogs were a gift from Professor J.W. Gorrod (University of London, UK).

2.2. Apomyoglobin digestion protocol

A solution of apomyoglobin (100 μl, 1 mg/ml) was reduced to dryness under vacuum and reconstituted in 50 m*M* triethanolamine-formate pH 8.0 (100 μl). The protein was reduced and carboxymethylated according to the method of Edelman et al. [22] prior to digestion with lysine-C endopeptidase (10 μl, 10 μg/ml enzyme solution in 50 m*M* triethanolamine-formate pH 8.0). Further enzyme solution was added at 2 and 4 h and the digestion was continued overnight (total time 24 h) at 37°C. Protein digestion was abated by acidification (50 μl of a 1.0% formic acid solution). The sample was then taken to dryness under vacuum and reconstituted in a 0.1% formic acid solution (50 μl) to give an equivalent protein concentration of 1 mg/ml. A final solution equivalent of 10 μg/ml of protein was produced by serial dilution. This sample was analyzed without further manipulation by PC–CE–MS.

2.3. PC–CE–MS

The preconcentration capillary used in these experiments was prepared from uncoated fused-silica tubing (50 μm I.D.) pretreated with sodium ethoxide, methanol, and CE separation buffer. The capillary that was used for the analysis of intact apomyoglobin was coated with polybrene according to the manufacturer's instructions, then washed with CE separation buffer. A 1–2 mm packed bed of dry end-capped silica C_{18}

material (HAL studies) or PLRP-S (300 Å) polymeric packing (peptide and protein studies) was packed in PTFE tubing (ca. 400 μm I.D.) and connected to the treated silica capillary 1.5 cm from the end of the inlet. The inlet of the CE capillary was extended to full length by the addition of 1.5 cm of treated fused-silica tubing. The final dimensions of the capillary were 77 cm × 50 μm I.D. The entire PC capillary was then conditioned under high pressure (1.34 bar) for 10 min each with methanol, water, and separation buffer. All subsequent capillary treatments and sample loading, washing, and elution were also carried out under high pressure (1.34 bar). It is noted that while the use of a PC cartridge reduces the rigidity of a CE capillary, this has caused no practical CE instrumentation problems in our hands.

The method of analysis included a cleaning regime of methanol (0.2 min) and separation buffer (5 min), followed by a high-pressure injection of the respective mixture (0.5–2 min injections). The capillary was then washed with separation buffer for 5 min and analytes were eluted from the packing material with methanol (haloperidol mixture) or methanol–water–trifluoroacetic acid (80:20:0.1, v/v/v) (peptide mixture) followed by a plug of CE separation buffer. CE separations were performed using a Beckman P/ACE 2100 instrument (Fullerton, CA, USA) modified with a Beckman-MS adapter kit for use with a mass spectrometer and coupled to a Reason Technology 486 PC (Rochester, MN, USA) with system control and data capture by System Gold software (Beckman). The CE separation medium used to afford optimum separation of the mixtures is described in the figure legends. Analyses of HAL analogs and apomyoglobin were carried out on a Finnigan MAT 900 mass spectrometer (Bremen, Germany) of EB configuration (where E is an electric sector and B is a magnet) with a PATRIC (position and time resolved ion counter) focal plane detector. Peptide analyses were performed on a Finnigan MAT 95Q (Bremen, Germany) of EBQ_1Q_2 configuration (where Q_1 is an rf-only octapole collision cell and Q_2 is a quadrupole mass filter). A Finnigan MAT electrospray ion source was used in positive ion mode. This source employs a spray needle that is floated to voltage (typically 6–8 kV) and a heated metal capillary (200°C) as the first stage of separation of the atmospheric pressure spray region from the vacuum of the mass spectrometer. With the exception of the analysis of apomyoglobin, neither auxiliary nor sheath gas were used with the Finnigan MAT source. All experiments were conducted at a resolution of ca. 1000 using an 8% PATRIC window or a secondary electron multiplier, scanning at 2 or 3 s/decade. The mass range scanned for the haloperidol analysis was 125–450 Da, for the synthetic peptide analysis/apomyoglobin digest 300–1300 Da and for the apomyoglobin analysis 200–2000 Da.

3. Results and discussion

The efficacy of PC–CE–MS for the structural characterization of small molecules is demonstrated by the analysis of a mixture containing the neuroleptic agent haloperidol (HAL) and six structurally similar analogs. In this example, ca. 350 nl of a predominantly aqueous solution, containing ca. 4 fmol/nl of each component was loaded onto a cartridge of solid-phase C_{18} packing material by hydrostatic pressure (ca. 1.4 pmol of each component was loaded onto the PC cartridge). The PC cartridge and CE capillary were washed with the CE separation buffer in preparation for electrophoresis. Subsequently, analytes were eluted into the CE capillary with a small volume of methanol and separated by electrophoresis. This resulted in the detection of all seven components, as demonstrated in the PC–CE–MS ion electropherograms shown in Fig. 1. Furthermore, although these compounds are structurally very similar, all were resolved. However, the peak widths of individual responses were broader (ca. 1 min wide) than observed by conventional CZE–MS [23]. Substantial component tailing was also evident. This loss of CE performance is related to the analysis of relatively large amounts of each component increasing both analyte–analyte and analyte–

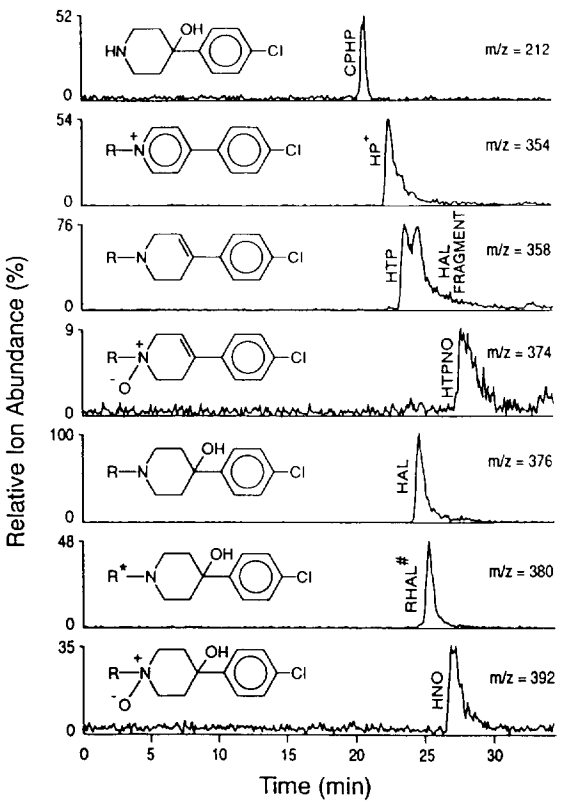
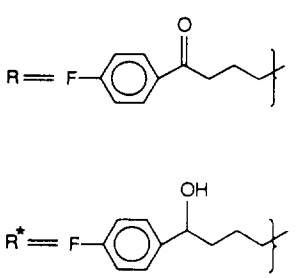


Fig. 1. PC–CE–MS analysis of ca. 350 nl of a mixture containing ca. 4 fmol/nl of the neuroleptic agent haloperidol and 6 synthetic analogs CPHP, HP^+, HTP, HTPNO, RHAL, and HNO. PC–CE capillary 70 cm × 50 μm I.D. containing a 2-mm bed of end-capped silica C_{18} packing; separation buffer 50 m*M* NH_4OAc–10% methanol and 1% acetic acid. Components were eluted from the solid phase with 200 nl of methanol. Applied separation voltage was 30 kV. Actual voltage difference across the capillary was 21.4 kV (17.2 μA). The capillary was maintained at ca. 25°C. A sheath liquid of 2-propanol–water–acetic acid (60:40:1, v/v/v) was delivered at a rate of 3 μl/min. Voltage applied to the electrospray needle was 8.5 kV. Scan range was 125–450 Da at a scan speed of 2 s/decade. Analyte detection was with PATRIC focal plane detector 8% window. RHAL#, denotes detection of the ^{37}Cl isotope contribution of $MH^+ = 378$.



wall interactions. In addition, analyte migration times were also longer by PC–CE–MS than observed in previous CE–MS studies of these components [23]. This can be attributed, at least in part, to the recently reported reduction of EOF brought about by the solid phase [11], as well as the presence of a relatively large volume of organic phase used for analyte elution into the CE capillary [2].

Another important area of biomedical research is the characterization of peptides and proteins. Determination of the physical properties (e.g., molecular mass and amino acid sequence) of these biopolymers significantly contributes to the understanding of their physiological properties. However, biological systems of most interest are invariably complex mixtures of analytes that are at concentrations below their limits of detection. Furthermore, a limited amount of sample often precludes multiple stages of purification. These factors may preclude the use of CE–MS for the analysis of peptides and proteins. Therefore, we have evaluated the applicability of PC–CE–MS for such analyses.

To demonstrate the efficacy of PC–CE–MS for peptide analysis, a mixture of five peptides (angiotensin II, bradykinin, α-MSH, LH–RH, and bombesin) were dissolved in CE separation buffer (2 m*M* NH_4OAc–1% AcOH) to produce a concentration of 1 fmol/nl of each peptide. A volume of ca. 1 μl (ca. 1 pmol of each peptide) was applied to a PC cartridge containing PLRP-S polymeric phase. The PC cartridge and CE capillary were washed with CE separation buffer and peptides were eluted from the PC cartridge with a small volume of organic phase (methanol–water–TFA, 80:20:0.1, v/v/v) followed by a plug of CE separation buffer. Subsequent electrophoresis afforded the PC–CE–MS ion electropherogram shown in Fig. 2. Here, all five peptides were baseline resolved, with four producing reasonable peak shapes. This demonstrates the potential of PC–CE–MS for the analysis of dilute solutions of peptides.

The versatility of PC–CE–MS is further demonstrated by the analysis of a solution (ca. 1 fmol/nl) of the globular protein apomyoglobin

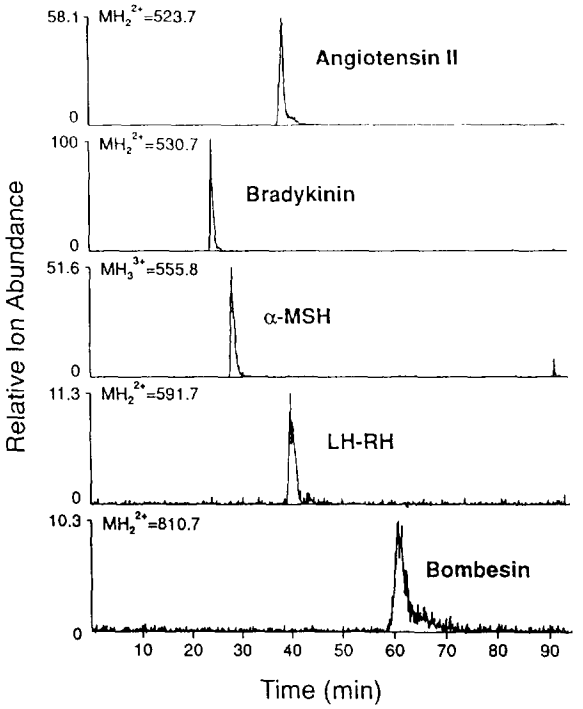


Fig. 2. PC–CE–MS analysis of ca. 1 μl of a dilute mixture of angiotensin II, bradykinin, α-MSH, LH–RH and bombesin. PC–CE capillary 77 cm × 50 μm I.D. containing a 1-mm bed of PLRP-S polymeric phase. Separation buffer was 2 m*M* NH_4OAc–1% acetic acid. Peptides were eluted from the solid phase with 200 nl of a solution of methanol–water–TFA (80:20:0.1, v/v/v). Applied separation voltage was 30 kV. Actual voltage difference across the capillary was 21.4 kV (1.3 μA). The capillary was maintained at ca. 25°C. A sheath liquid of 2-propanol–water–acetic acid (60:40:1, v/v/v) was delivered at a rate of 3 μl/min. Voltage applied to the electrospray needle was 8.5 kV. Scan range was 300–1300 Da at 3 s/decade and detection was with a secondary electron multiplier.

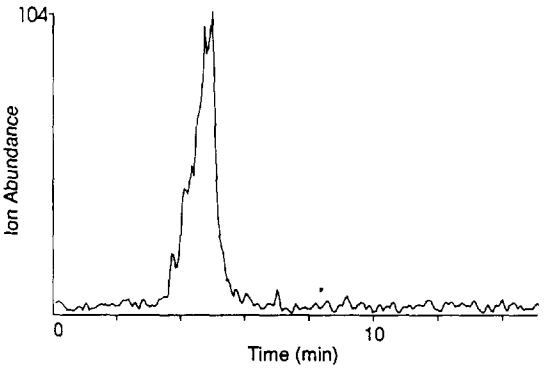


Fig. 3. PC–CE–MS analysis of a solution of apomyoglobin (1 fmol/nl). PC–CE capillary 77 cm × 50 μm I.D. polybrene-coated capillary containing a 1-mm bed of PLRP-S polymeric phase. Separation buffer was 1% formic acid in water. The apomyoglobin was eluted from the solid phase with a solution of methanol–water–TFA (80:20:0.1, v/v/v). Applied separation voltage was −30 kV. Actual voltage difference across the capillary was −38.5 kV (9.5 μA) which was maintained at ca. 25°C. A sheath liquid of methanol–water–acetic acid (25:75:1, v/v/v) was delivered at a rate of 3 μl/min and a N_2 sheath gas was used (ca. 2 bar). Voltage applied to the electrospray needle was 8.5 kV. Scan range was 200–2000 Da at 2 s/decade and detection was with a PATRIC focal plane detector, 8% window.

(see Fig. 3). In this example, ca. 1 pmol of protein (ca. 1 μl of protein solution) was applied under hydrostatic pressure to a PC cartridge containing PLRP-S polymeric phase which was installed at the inlet of a polybrene-coated capillary. This modified CE capillary was used for the PC–CE–MS analysis of apomyoglobin, since it is well known that proteins strongly adsorb to the silica walls of an untreated capillary [24]. The effects of these strong electrostatic interactions between the positively charged protein and anionic capillary wall include substantial peak broadening and loss of CE separation efficiency [24]. In addition, protein recovery from the CE capillary is reduced. However, coating the silanol groups of the CE capillary with an amine (such as polybrene) reverses the charge on the capillary wall. This reduces protein–wall interaction (by charge repulsion), which diminishes associated detrimental effects on CE performance [25]. In this example, the analysis of apomyoglobin by PC–CE–MS (Fig. 3) established the applicability of this technology for the analysis of proteins. Furthermore, the use of a polybrene-modified capillary demonstrated the compatibility of PC–CE–MS with coated CE capillary technology.

Often one of the goals of protein analysis is sequence confirmation. Therefore, an aliquot of the apomyoglobin sample analyzed by PC–CE–MS was digested with lysine-C endopeptidase (LEP). The resultant peptide mixture was analyzed with minimal off-line pretreatment by PC–CE–MS (conditions as described for the PC–

CE–MS analysis of the standard peptides). In this example, the CE separation buffer wash of PC cartridge and CE capillary affects both sample clean-up and prepares the CE capillary for subsequent electrophoresis. The results of this study, demonstrated by the PC–CE–MS ion electropherograms in Fig. 4 led to the clear characterization of ca. 70% of the sequence of this protein. Also both the N-terminus (GLSDGEWQQVLNVWGK, $MH_2^{2+} = 909.0$) and C-terminus (ELGQG, $MH^+ = 650.5$) of apomyoglobin were observed but only upon a high-pressure CE-buffer rinse of the PC–CE capillary. We believe this is due to the highly polar nature of these peptides, and we are currently evaluating the use of coated CE capillaries and other stationary phases to improve the analysis of such peptides by PC–CE–MS.

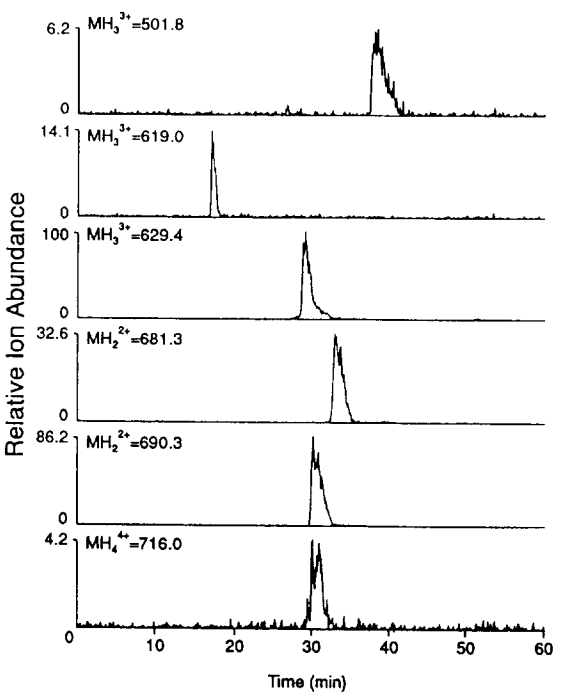


Fig. 4. PC–CE–MS analysis of ca. 2 μl of a LEP digest of apomyoglobin (ca. 1 pmol of total protein was applied to the capillary). Conditions as Fig. 2. Peptide identity was as follows: *m/z* 501.8, HPGFGADAQGAMTK; *m/z* 619.0, GHHEAELKPLAQSHATK; *m/z* 629.4, YLEFISDAIIHVLHSK; *m/z* 681.3, ALELFRNDIAAK; *m/z* 690.3, HGTVVLTALGGILK; and *m/z* 716.0, VEADIAGHGQEVLIRLFTGHPETLEK.

4. Conclusions

In the present study, we have shown that a small bed of solid phase (e.g., C_{18} HPLC material) can be installed at the inlet of a CE capillary for analyte preconcentration prior to CE–MS. Indeed, in our hands, the solid phase at the inlet of the CE capillary has exhibited a high analyte capacity permitting the preconcentration of several picomoles of material from dilute analyte mixtures without breakthrough due to overloading. Therefore, while we continue to develop this methodology, it exhibits potential for application to studies of biological relevance. This is further demonstrated by the results of this study which include PC–CE–MS analysis of mixtures containing small organic molecules, peptides, and proteins. Finally, we note that, while a possible limitation of PC–CE–MS is a reduction of CE efficiency upon incorporation of the solid phase, component resolution can still be effected.

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