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Advantages and limitations of coupling isotachophoresis and comprehensive isotachophoresis–capillary electrophoresis to time-of-flight mass spectrometry

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Abstract

Capillary isotachophoresis (ITP) and comprehensive isotachophoresis–capillary electrophoresis (ITP–CE) were successfully coupled to electrospray ionization (ESI) orthogonal acceleration time-of-flight mass spectrometry (TOF-MS) using angiotensin peptides as model analytes. The utility of ITP–TOF-MS and ITP–CE–TOF-MS for the analysis of samples containing analyte amounts sufficient to form flat-top ITP zones (30 μ*M*) as well as for samples with trace analyte amounts (0.3 μ*M*) was studied. Separations were performed in 150 μm internal diameter (I.D.) capillaries for the ITP experiments, and in 200 μm I.D. (ITP) and 50 μm I.D. (CE) capillaries for ITP–CE experiments. The fused-silica columns were coated with poly(vinyl alcohol) to suppress electroosmotic flow that can disrupt ITP zone profiles. The sample loading capacity in both ITP and comprehensive ITP–CE was greatly enhanced (up to 10 μl) compared with typical nanoliter-sized injection volumes in CE. It was concluded that ITP–TOF-MS alone was adequate for the separation and detection of high concentration samples. The outcome was different at lower analyte concentrations where mixed zones or very sharp peaks formed. With formation of mixed zones, ion suppression and discrimination could occur, complicating quantitative determination of the analytes. This problem was effectively overcome by inserting a CE capillary between the ITP and TOF-MS. In such an arrangement, samples were preconcentrated in the high load ITP capillary and then injected into a CE capillary where they were separated into non-overlapping peaks prior to their detection by TOF-MS. The advantage of this comprehensive arrangement, which we have described previously, is that there is no need to discard portions of the sample in order to avoid overloading of the CE capillary. The whole sample is analyzed by multiple injections from ITP to CE. Thus, this method can be used for the analysis of complex samples with wide ranges of component concentrations.

Keywords: Isotachophoresis; Isotachophoresis–capillary electrophoresis, comprehensive; Interfaces, electrophoresis-MS; Capillary electrophoresis; Mass spectrometry; Angiotensins; Peptides

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1. Introduction

Capillary electrophoresis (CE) has shown wide applicability to separations demanding high resolution while requiring only very small sample volumes [1,2]. However, an often-cited drawback of CE is its concentration limit of detection (cLOD) resulting from the short path length of small internal diameter (I.D.) capillaries (20–100 μm) and the low sample capacities (10–100 nl) of these capillaries [3–7]. This cLOD limitation is particularly apparent when conventional detectors such as ultraviolet–visible (UV–Vis) absorption or electrical conductivity are used for trace analysis. This is especially a problem for many biological specimens, where analytes are often present at concentrations below the detector cLOD, thus necessitating preconcentration (preferably on-line) of the analytes before their final separation and detection [8,9].

Several approaches to on-line sample preconcentration have already been explored. Thompson et al. [10] divides these approaches into two categories: (1) sorption, including membrane preconcentration [6,9,11] and solid-phase extraction [9,12,13], and (2) electrophoretic methods, such as sample stacking [9,14–17], isotachophoresis [4,16,18,19] and capillary isoelectric focusing [20–24] (for protein and peptide analysis). The electrophoretic methods are usually simpler and more straightforward.

This study utilizes isotachophoresis (ITP) as an on-line preconcentration method as well as a separate separation technique. Similarly to CE, ITP separates the analyte bands according to differences in their electrophoretic mobilities [25]. However, ITP differs from CE in that it utilizes a discontinuous buffer system comprised of a leading electrolyte (LE) and a terminating electrolyte (TE). The LE and TE have electrophoretic mobilities that are higher and lower, respectively, than the analytes of interest in the sample. The sample is inserted between the two electrolyte solutions and the analytes partition or stack in order of their electrophoretic mobilities (from highest to lowest) immediately after the LE zone. Once the zones reach their steady state, the entire system moves at constant velocity, hence the name ''iso'' for same and ''tacho'' for speed. The analyte concentration within each separated zone is determined by the concentration of the LE, which is typically 1000-fold or more higher than the predicted analyte concentrations in the initial sample. This means that ITP can increase analyte concentrations commonly by a factor of 100 to 1000, but as high as 10^6 for trace components [7]. However, it can also dilute bulk components to levels matching that of the LE. ITP also has the capability of separating the analytes from potentially interfering substances having electrophoretic mobilities that do not lie within the range of the LE–TE pair (Fig. 5). Compounds with mobilities that are greater than the LE, or smaller than the TE, do not migrate with the ITP stack, but are diluted in the LE or TE zones.

Although ITP can serve as a stand-alone separation technique for certain applications [26–28], it has some serious limitations. For qualitative and quantitative determinations, the detection method used must recognize the front and rear boundaries of the zone of interest [29]. This may be difficult when conventional UV or conductivity detectors are used, since the ITP zones are not separated from each other as peaks in CE, but follow one another ''back-to-back'' to maintain electrical continuity. Consequently, most ITP applications involve the determination of fairly concentrated analytes in relatively simple matrices; and despite the higher loading capacity advantage of ITP over CE, the detection limits in ITP are generally not sufficient for trace analysis. As Caslavska et al. [30] found from a comparative study of capillary ITP, CE and capillary electrophoretic focusing, the ITP detection limits can approach those observed in CZE only when the ITP spike technique (in which baseline-resolved UV absorption peaks of solutes are produced by bracketing the solute with discrete, nonabsorbing spacers) is applied. These limitations can be, to a large degree, overcome by using a detection technique capable of signal deconvolution, such as mass spectrometry [31,32].

In this work, we have used time-of-flight mass spectrometry (TOF-MS) as a specific detection method. TOF-MS has several advantages over scanning mass spectrometers such as ion trap, quadrupole, and sector instruments. Instead of scanning the mass range, it acquires the signal for all m/z values simultaneously. Thus, a complete mass spec-

trum can be generated with each pulsed ion extraction. The pulsing is performed at rates as high as 5 kHz, allowing the TOF-MS to generate mass spectra at rates much faster than scanning mass spectrometers. The TOF-MS instrument used in this study can record, sum, and store complete mass spectra at rates as high as 100 spectra/s. In addition to speed, other characteristics of TOF-MS, such as high duty cycle, high ion transmission, and ease of peak deconvolution using selected-ion monitoring, make TOF-MS an ideal detector for complex samples and extremely narrow peaks produced by fast separations or when trace compounds are analyzed by ITP.

When the amounts of analytes are insufficient to form fully developed ITP zones, sharp peaks or narrow mixed zones bracketed by normal ITP zones or by LE and TE result [7,16]. Detection and identification of analytes within such zones using conventional detectors is problematic. Because of its speed of data acquisition and deconvolution capabilities, TOF-MS can provide qualitative information about the composition of such peaks or mixed zones. However, when several analytes elute from the column at the same time, signal discrimination and suppression in the electrospray ionization interface can occur. This makes accurate quantitative measurement difficult if not impossible. A complicated solution to this problem would be the use of spacers that do not interfere with the ionization process [33,34]. A more generalizable approach would be coupling the high load ITP technique with a CE separation.

Earlier, we described the on-line combination of capillary isotachophoretic preconcentration with CE separation in a comprehensive arrangement [35,36]. One of the key advantages of comprehensive ITP–CE using coupled columns is that the entire sample is analyzed in a comprehensive manner as opposed to other ITP–CE techniques, such as sample splitting or heart-cutting, that discard a major portion of the sample to avoid column overloading.

This paper reports the results of a study utilizing five angiotensin peptides as model compounds to determine advantages and limitations of coupling both ITP and comprehensive ITP–CE with TOF-MS for the analysis of samples with analyte amounts sufficient to form flat-top ITP zones and samples with trace amounts of analytes.

2. Experimental

2.1. Materials and chemicals

Fused-silica capillaries of 50 μm I.D.×187 μm O.D. and 150 and 200 μm I.D.×365 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Deionized water for buffer solutions and rinsing of the ITP and ITP–CE capillaries was produced by a Milli-Q water purification system (Millipore, Waterford, MA, USA). HPLC-grade acetonitrile, methanol, and triethylamine were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glacial acetic acid (HAC) was obtained from EM Science (Gibbstown, NJ, USA) and ammonium acetate was from Mallinckrodt (Hazelwood, MO, USA). Angiotensins, I, I (1–7), II, III and IV (AI, AI(1–7), AII, AIII and AIV), as well as poly(vinyl alcohol) (PVA, 99+%, average M_r 89,000–98,000 g/mol) were purchased from Aldrich (Milwaukee, WI, USA). All buffers were degassed under house vacuum with sonication. Compressed nitrogen was obtained from Airgas (Salt Lake City, UT, USA). Nitrogen was used as a curtain gas to help desolvate compounds emerging from the electrospray interface.

2.2. Isotachophoresis (ITP)

A Crystal CE Model 300 (UNICAM, Madison, WI, USA) capillary electrophoresis instrument with both electrokinetic and hydrodynamic injection capabilities and a voltage limit of ±30 kV was used for ITP. The capillary column was initially rinsed and filled with LE by immersing the inlet end of the capillary in the LE buffer vial and applying pressure (1000 p.s.i.; 1 p.s.i.=6894.76 Pa). The inlet of the ITP column was then inserted into a sample vial, and ~10 μl were injected onto the column hydrodynamically using pressure (1000 p.s.i.). The column and a platinum electrode were then inserted into the TE buffer vial and a voltage was applied to begin the ITP process. All of these steps were programmed to run automatically using the Crystal CE program-

mable interface. For ITP–UV, a Model 759 A UV–Vis absorption detector (Applied Biosystems, Foster City, CA, USA) was used. The data were collected using ChromPerfect 3.54 data acquisition software.

2.3. Comprehensive isotachophoresis–capillary electrophoresis

The instrumentation and methodology for ITP–CE used in this work were described in detail previously [36]. Briefly, the ITP–CE instrument consisted of a Valco (Houston, TX, USA) Model C22 four-port manually actuated sample valve (10 μl sample loop) connected to both the ITP column and the TE buffer reservoir with a platinum electrode attached to a Spellman (Hauppauge, NY, USA) 60 kV/500 μA SL60PN30 high-voltage power supply. A custom-made quartz tee (365 μm I.D., InnovaQuartz, Phoenix, AZ, USA) was used at the bifurcation point between the ITP and CE columns to minimize dead volume and residual flow effects after LE counter-flow infusion. A UV 300 UV–Vis detector (ThermoQuest, San Jose, CA, USA) was positioned just before the bifurcation point to signal when the ITP stack arrived at the junction between the ITP and CE columns prior to infusion of the LE counterflow from a Harvard Apparatus (Holliston, MA, USA) Model 22 syringe pump.

2.4. Electrospray interface

The electrospray ionization (ESI) interface using liquid sheath flow to provide electrical contact with the column effluent was built in-house [37]. The liquid sheath flow-rate was controlled by syringe pump (Model 11, Harvard Apparatus) with a 250-μl glass Gastight syringe fitted with a 2-in. 22-gauge stainless steel needle (Hamilton, Reno, NV, USA). The syringe was connected to a 50 μm I.D.×187 μm O.D. fused-silica capillary transfer line (Polymicro) using a Chemfluor PTFE straight union (Norton Performance Plastics, Akron, OH, USA). A Chemfluor tee connected the transfer line to the analytical CE column. As the liquid sheath entered the tee, it flowed between the CE column and the inside of the 27-gauge stainless steel electrospray needle to which a voltage (4 kV) was applied. The needle was tapered at the tip using fine-grit sandpaper. The end of the CE column extended ~0.5 mm beyond the edge of the stainless steel electrospray needle. The needle was then positioned ~1 cm from the TOF-MS interface plate. To produce the ESI mass spectra for the individual angiotensins and for subsequent separations, a liquid sheath solution of methanol–water (70:30) with 0.1% acetic acid was used at a flow-rate of 1.5 μl/min. The interface was mounted on an *xyz* stage (Series 462, Newport, Engelwood, CO, USA) to aid in the optimization of the electrospray needle position relative to the TOF-MS sample orifice. A microscope fitted with a 15–60× adjustable objective (Edmund Scientific, Barrington, NJ, USA) was used together with a Series 41722 fiber optic illuminator (Cole-Parmer, Arcade, NY, USA) to observe the integrity of the Taylor cone at the electrospray tip.

2.5. Time-of-flight mass spectrometry

A commercial Jaguar TOF-MS (LECO, St. Joseph, MI, USA) with orthogonal extraction was used as a detector. The Jaguar TOF-MS used a heated (80 °C) nitrogen curtain gas running counter to the flow of ions into the mass spectrometer to desolvate ions emerging from the electrospray tip. After desolvation at atmospheric pressure, ions are carried through a nozzle, skimmer and a radiofrequency (RF) quadrupole to the ion pulsing region ($\sim 2\times10^{-6}$ mbar). In this region, a portion of the ion beam receives a pulse of kinetic energy applied orthogonal to the direction of the ion beam. The ions then travel the length of the flight tube and are detected using a microchannel plate (MCP) detector. Ions separate in the flight tube according to the differences in their m/z ratios. Detection is rapid, and complete averaged mass spectra with mass range of up to 6000 m/z can be collected at a rate of up to 100 spectra/s. The electrospray voltage applied to the 27-gauge stainless steel needle, which housed the fused-silica capillary and sheath flow, was 4.0 kV, with the counter electrode (the interface plate of the Jaguar TOF-MS system) voltage held constant at +650 V. Data were collected at a rate of 3.1 spectra/s.

2.6. Preparation of PVA-coated capillaries

The ITP and CE capillaries were coated prior to use with PVA to suppress electroosmotic flow. A coating procedure similar to that of Clarke et al. [38] was used. Briefly, a solution of 6% PVA dissolved in water was degassed using sonication. A fused-silica capillary (2.5 m) was attached to a small pressure vessel containing the PVA solution. The PVA solution was passed through the capillary for 1 h at 100 p.s.i. The column was then emptied by applying 30 p.s.i. for 1 h. Finally, the column was thoroughly dried using nitrogen at 20 p.s.i. for 1 h and placed in a GC oven (HP 5890, Hewlett-Packard, Palo Alto, CA, USA) under a stream of helium (20 p.s.i.) and subjected to a temperature program of 40 °C ramped to 145 °C at 5 °C min^{-1}, followed by 5 h at 145 °C. The capillary was then reversed and the process repeated to ensure sufficient and uniform coating.

3. Results and discussion

3.1. ITP–UV

Using ITP, the disadvantages of the limited sample capacity of CE can be overcome. However, due to the rectangular, zone-like appearance of the ITP signal, its processing is not as straightforward as that of the CE signal. For both qualitative and quantitative analysis, the detection technique used must recognize the front and rear boundaries of the zone of interest [29]. As can be seen in Fig. 1, which shows a typical ITP–UV analysis of a sample containing five model angiotensin peptides at 30 μ*M* concentrations, it was not possible to distinguish the boundaries of all of the individual zones. While there was some indication of the separation of the zones of AIII, AI, and AIV, it was not clear whether AI(1–7) and AII had very similar absorption coefficients or the zones were not completely separated from each other. Similar situations arise with the use of conductivity detection. While there have been reports describing evaluation of both the conductivity and UV detection signals by combining commercial chromatographic software with a code for handling the step-like isotachopherogram [30], this approach is not common.

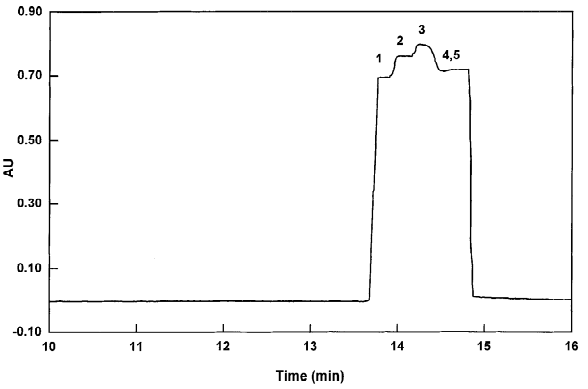

Fig. 1. ITP–UV separation of a 30 μ*M* mixture of angiotensins. Conditions: LE, 10 m*M* ammonium acetate; TE, 10 m*M* acetic acid; injection, 20 cm sample plug (50 mbar, 0.8 min); capillary, 82 cm (70 cm effective length)×150 μm I.D. PVA coated; separation voltage, +30 kV for 13 min, +15 kV for remainder of the run. Peak identifications: (1) AIII, (2) AI, (3) AIV, (4) AI(1–7), (5) AII.

3.2. ITP–TOF-MS

It is obvious from Fig. 2 that much more insight can be obtained on both the qualitative and quantitative composition of a stack of ITP zones using an

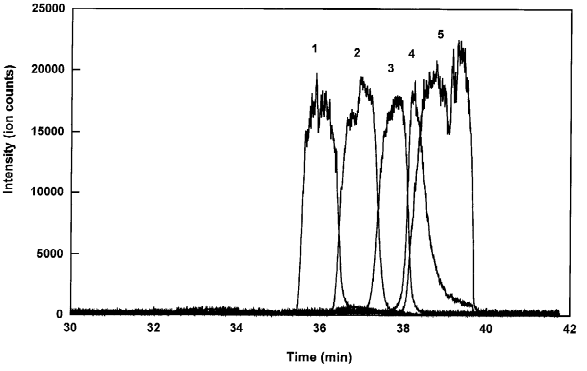

Fig. 2. ITP–TOF-MS extracted ion electropherogram (EIE) of a 30 μ*M* mixture of angiotensins, m/z=466.0, 432.7, 388.0, 450.0, and 523.6, for AIII, AI, AIV, AI(1–7), and AII, respectively. Conditions: ITP settings as in Fig. 1, except for 82 cm effective column length (electrospray positioned at the end of the capillary) and ITP voltage +30 kV for 15 min, +15 kV for remainder of the run; liquid sheath, 1.5 μl/min (MeOH–H_2O (70:30) with 0.1% HAc); electrospray voltage, 4 kV; TOF-MS interface plate temperature, 80 °C. It should be noted that the presence of the electrospray voltage lowers the effective separation voltage, thus increasing the migration times. Peak identification as in Fig. 1.

information-rich detection technique such as mass spectrometry. Each zone was clearly defined based on the unique mass-to-charge ratios of the individual angiotensins. The m/z values and ionization efficiencies (I.E.) of the angiotensin ionization products are provided in Table 1. The major ion products of each peptide were assigned I.E. = 100%. The I.E. of a minor product represents a fraction of the I.E. of a major ion of the respective angiotensin. In our experiments, the main ionization products were doubly [AII, AI(1–7), AIII, AIV] and triply (AI) charged ions. The MS spectra of AII and AIII angiotensins contained only the doubly charged ions as predicted. AI(1–7) ionized almost completely as a doubly charged ion with a minor peak of a triply charged ion (<3% I.E.). The spectrum of AI showed, in addition to a major peak of the predicted triply charged cation, a smaller peak (40% I.E.) of a doubly charged cation. In case of AIV, the predicted singly charged ion represented a minor peak in the spectrum (~20%), whereas a doubly charged ion was the dominant one. These results are (with the exception of AIV) in agreement with the charges expected on the analytes under the acidic conditions of the liquid sheath and electrolytes employed in this study. They are also consistent with the results published by Lazar et al. [39].

Unlike UV detection, TOF-MS detection made it apparent that the ITP conditions used had not allowed the system to reach steady state with complete separation of the zones. The last two zones, which represent AI(1–7) and AII, were only partially separated from each other. Thus we can see that mass spectrometry not only provides excellent means of detection and identification of the typical flat-top ITP zones but can also serve as a tool for monitoring the migration behavior and the extent of separation of the analytes in ITP.

When flat-top ITP zones were present, quantitation

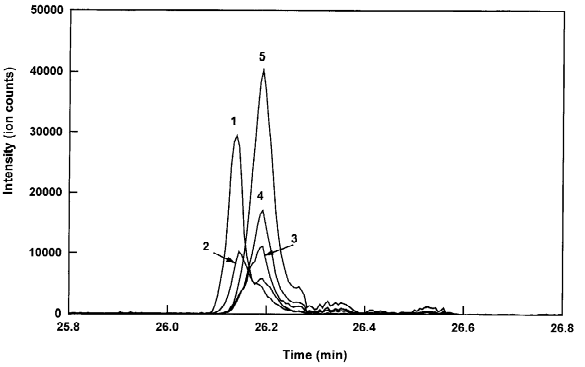

Fig. 3. ITP–TOF-MS EIE of a low concentration (3 μ*M*) mixture of angiotensins. Conditions: same as in Fig. 2 except 3 μ*M* angiotensins. Peak identifications as in Fig. 1, m/z ratios as in Fig. 2.

was relatively straightforward, since the length of each zone relates directly to the absolute quantity of a particular analyte. Such classical ITP has proven to be a valuable tool in the control of the composition and purity of drug preparations [29] and in similar applications where the analysis is not limited by the sample size and concentration and the matrix is not very complicated. It has been more difficult to use ITP for the analysis of drugs or biomarkers in biological fluids, since the sample matrix often contains high concentrations of ionic components and the amount of analyte is too low to create a fully developed, flat-top ITP zone long enough for reliable detection. The ITP separation of a mixture of 3 μ*M* angiotensin standards in Fig. 3 demonstrates that when the analyte concentrations in the analyzed volume were too low, the typical ITP zones started to collapse into peak-shaped mixed zones. It was not possible to analyze such a zone using traditional UV or conductivity detectors. TOF-MS on the other hand, identified all analyte peaks present, even if the peaks were severely overlapping. However, when

Table 1
Electrospray ionizations (I.E.), charge states, m/z ratios and ionizatin efficiencies (I.E.) of angiotensins

Charge	AIII		AI		AIV		AI(1–7)		AII	
	m/z	I.E.	m/z	I.E.	m/z	I.E.	m/z	I.E.	m/z	I.E.
1+	–	–	–	–	775.9	20%	–	–	–	–
2+	466.0	100%	648.3	40%	388.0	100%	450.0	100%	523.6	100%
3+	–	–	432.7	100%	–	–	900.0	<3%	–	–

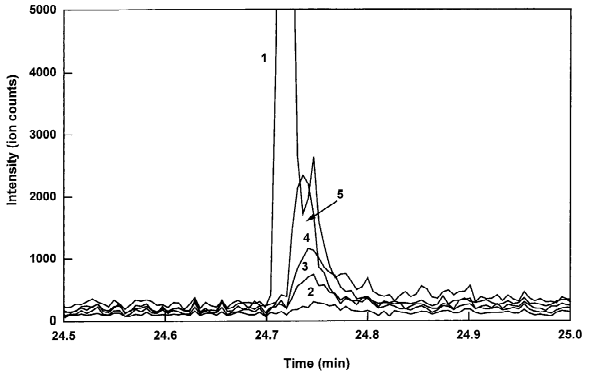

Fig. 4. ITP–TOF-MS EIE of a low concentration (0.3 μ*M*) mixture of angiotensins. Conditions: as in Fig. 2 except 0.3 μ*M* angiotensins. Peak identifications as in Fig. 1, *m*/*z* ratios as in Fig. 2.

several analytes are delivered into the electrospray at the same time, ion suppression and discrimination can occur. This phenomenon is apparent in Fig. 4, which shows an ITP separation of five angiotensins in the same injection volume as in Fig. 3, but now at a concentration of 0.3 μ*M* each. Under these conditions, ionization of other ions in the sample was greatly suppressed by angiotensin III. The extent of ion suppression and discrimination depends on the relative concentrations of the compounds within the mixed zone and the analyte ionization efficiencies under the conditions used. Consequently, while MS detection offers a qualitative description of even complicated mixed zones, its ability to quantitate the analytes within the zone may be compromised. Additionally, some components of the sample may ionize with the same *m*/*z* ratio and thus, without sufficient electrophoretic separation, the mass spectrometer may fail to identify such compounds as distinct entities [32].

3.3. Comprehensive ITP–CE–TOF-MS

The above mentioned shortcomings of ITP were eliminated to a large degree by combining the high sample capacity and concentration capability of ITP with the high separation efficiency of CE. Previously, we reported the development of comprehensive ITP–CE with UV detection [35,36]. We have further developed this technique by replacing the more general and fairly non-specific UV detector with the highly specific TOF-MS. One of the key advantages of comprehensive ITP–CE using coupled columns, compared with other ITP–CE techniques that use sample splitting or heart-cutting techniques to avoid column overloading, is that the entire sample is analyzed in a comprehensive manner.

In our arrangement, the ITP column had an internal diameter that was larger (200 μm I.D.) than the outer diameter of the CE column (187 μm I.D.). The larger I.D. of the ITP column allowed for a larger sample volume to be introduced for analysis. The injected sample volume (up to 10 μl in this study) was first concentrated and separated in the ITP column. The ITP zones were then allowed to migrate just past the inlet end of the CE column, thereby allowing some sample to enter the column. Using a counterflow of LE, the remaining analytes, which were not moved onto the CE column, were pushed back into the ITP column, where they continued to focus and migrate once again towards the CE column. This refocusing occurred at the same time as the sample introduced into the CE column was separated, since the focusing/separation voltage was on continuously. The buffer infused at the bifurcation point at the junction of the ITP and CE columns acted as both background electrolyte in CE and as LE in ITP. These multiple injections were repeated until the whole sample was analyzed. The total analysis times varied depending on the concentration of the sample. Samples with higher analyte concentrations required greater number of CE injections and subsequent refocusing steps, leading to longer analysis times. For quantitation purposes, the results of the multiple CE injections can be added together to provide a summed electropherogram as shown by Chen and Lee [35] and Bowerbank and Lee [36].

One of the differences between ITP–CE–UV and ITP–CE–TOF-MS was, that with UV detection, a window was placed on-column by removing a small portion of the polyimide coating, and a buffer reservoir was placed at the end of the column to serve as ground to complete the circuit. This buffer vial also produced a ‘‘closed’’ system whereby hydrodynamic flow was suppressed inside the CE capillary when the LE counterflow was initiated at the bifurcation point. On the other hand, using TOF-MS as the detection method, an electrospray was

established at the end of the CE column, leaving it open to the atmosphere. In all separations with ITP–CE–TOF-MS, some peak tailing was observed. This can be attributed to the presence of hydrodynamic flow induced by the LE counterflow. Although not evaluated in this study, either the coupling of an ITP column with a larger I.D. or the use of a CE column with a smaller I.D. would favor the ITP column as the path of least resistance for the LE counterflow and should decrease the flow inside the CE capillary.

3.4. Analysis of a high concentration sample by comprehensive ITP–CE–TOF-MS

Ten microlitres of an angiotensin sample (30 μ*M* each analyte) were introduced into the ITP capillary. After initial 30-min ITP focusing time, the voltage was lowered from 24 to 10 kV. The formed ITP zones were monitored via a UV detector placed at the bifurcation point. A small portion of the zones was allowed to migrate into the CE capillary and the rest of the zones were pushed back into the ITP capillary by injecting 4 μl of leading electrolyte at 60 μl/min. The zones distorted by the injection of LE were allowed to refocus for ~10 min in the ITP capillary and were again allowed to enter the CE capillary. This process was repeated until the whole ITP stack was analyzed by the CE. A representative ITP–CE–TOF-MS analysis of a 30 μ*M* angiotensin sample is shown in Fig. 5. In this case, 14 injections were made from the focused ITP stack into the CE capillary. Time zero on the time axis corresponds to the first injection from the ITP into the CE capillary. The first peak appeared in ~3 min. Peaks resulting from subsequent injections eluted from the CE capillary in ~10 min intervals. Each window represents the *m*/*z* trace of one particular angiotensin.

While the ITP electropherogram in Fig. 2 is characterized by flat-top zones, ITP–CE separation in Fig. 5 produces more recognizable CE peaks. However, some of the analyte peaks [AI(1–7) and AII] in Fig. 5 are broader than expected. Our explanation for the discrepancy is that as a consequence of manually controlling the injection time there is a possibility of the ITP stack migrating too far past the front of the CE capillary, thus allowing some TE to enter the CE capillary along with the analytes. Since LE was infused behind the sample

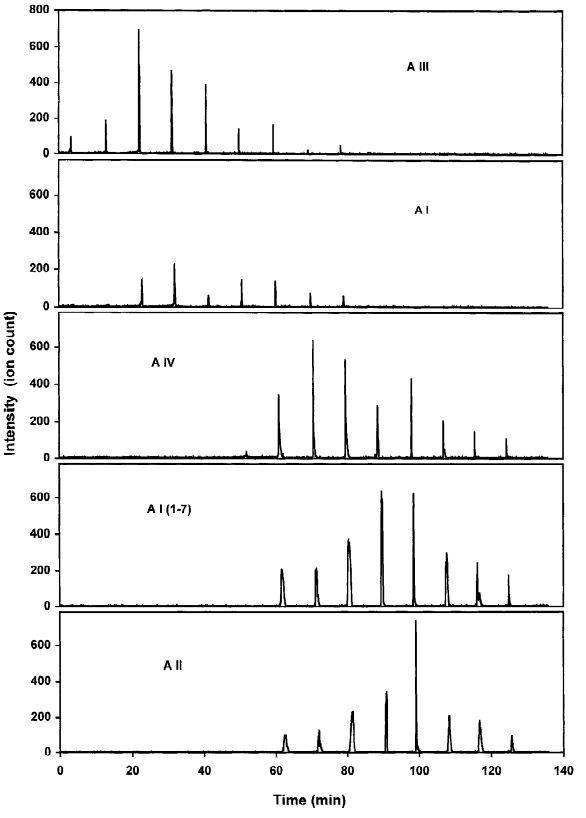

Fig. 5. Comprehensive ITP–CE–TOF-MS EIE of a high concentration (30 μ*M*) mixture of angiotensins (*m*/*z* ratios as in Fig. 2). Conditions: ITP column, 20 cm (15 cm effective separation length)×200 μm I.D., 365 μm O.D., PVA-coated, coupled with CE column, 20 cm×50 μm I.D.×187 μm O.D., PVA-coated; injection volume, 10 μl; separation voltage, +24 kV for initial ITP concentration, reduced to +10 kV for subsequent ITP and CE steps; LE, 10 m*M* triethylamine; TE, 10 m*M* acetic acid; LE counterflow, 4 μl; infusion rate, 60 μl/min. TOF-MS and liquid sheath conditions as in Fig. 2.

plug at the bifurcation point, the LE (having a higher electrophoretic mobility) raced through the sample to the front of the plug, resulting in the separation of AIII, AI and AIV (when present). However, it appears that due to the small volume of TE, higher amounts of AI(1–7) and AII at the end of the stack, and short length of the CE capillary, the transition of the last two ITP zones into CE peaks might not have been complete. This problem can be solved by increasing the length of the CE capillary and/or using computer control of the injection time based on a signal from the detector monitoring the analyte

position in the ITP capillary. Such automated design has not yet been developed.

In this experiment, when a fairly concentrated sample containing only major components was used, multiple successive injections were required to analyze the entire sample. As shown in Fig. 2, the same sample was separated in one fairly short run using ITP–TOF-MS alone. Therefore, it would seem more advantageous to use ITP–TOF-MS instead of comprehensive ITP–CE–TOF-MS for the analysis of more concentrated samples. However, many clinical specimens are complex, consisting of components differing greatly in concentration. Often, it might be desirable to measure analytes present at trace levels together with other, more abundant compounds. While ITP–TOF-MS alone would adequately detect and quantitate the major components, the signal of the trace analytes, represented by sharp peaks or mixed zones between adjacent flat-top zones, could be either suppressed or difficult to quantitate. Although not fully investigated in this study, we believe that ITP–CE–TOF-MS would be a valuable tool for such applications.

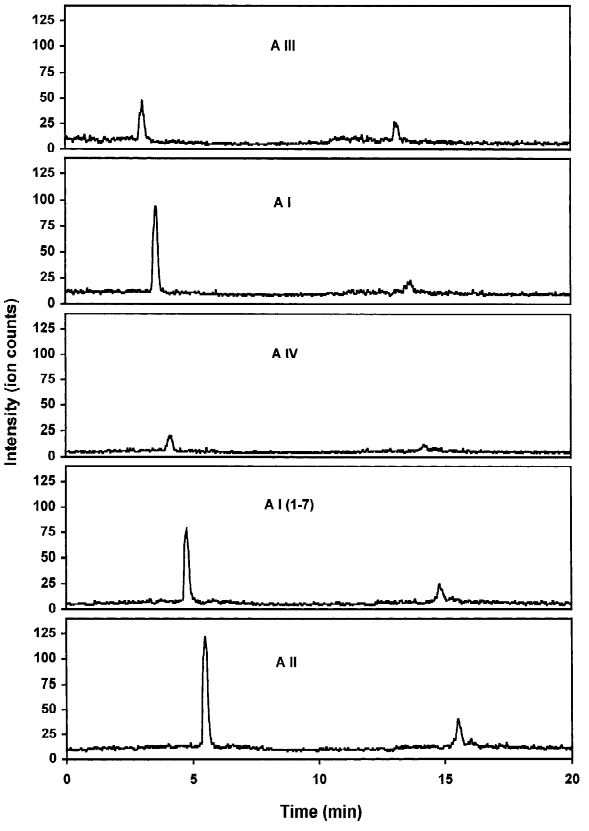

Fig. 6. Comprehensive ITP–CE–TOF-MS EIE of a low concentration mixture of angiotensins (m/z ratios as in Fig. 2). Conditions: as in Fig. 5 except 0.3 μ*M* AIV, 3 μ*M* AIII, 5 μ*M* AI and I(1–7), and 8 μ*M* AII.

3.5. Analysis of a low concentration sample by comprehensive ITP–CE–TOF-MS

Fig. 6 represents an ITP–CE–TOF-MS analysis of a low concentration angiotensin sample. The settings in Fig. 6 were identical to those for Fig. 5. The concentrations of analytes in Fig. 6 were 0.3 μ*M* for AIV, 3 μ*M* for AIII, 5 μ*M* for AI and AI(1–7), and 8 μ*M* for AII. AIV was purposely added at a concentration 10–30 fold lower compared to the rest of the analytes to demonstrate the capability of the ITP–CE technique to separate trace compounds from more concentrated analytes. The whole sample zone concentrated in the ITP capillary was analyzed using two consecutive injections into the CE capillary. The first peak in each selected-ion plot results from the first injection, the second peak from the second injection.

As stated above, detection of ITP zones in samples containing analytes present in amounts insufficient to form discrete flat-top zones was difficult due to ion suppression and discrimination that occurred within the narrow mixed zones (see Figs. 3 and 4). However, Fig. 6 demonstrates, that when CE was added as a second dimension separation, the detection problems of mixed ITP zones were solved, because each component of the sample preconcentrated by ITP was detected as a separate CE peak.

When we analyzed angiotensin standards using CE–TOF-MS with pressure injection and ESI conditions similar to those for ITP–CE–TOF-MS, we obtained detection limits of ~10 μ*M* for each angiotensin (results not shown). These results are consistent with the values reported by Lazar et al. [39]. From Fig. 6 it is apparent that the signal intensities obtained with ITP–CE–TOF-MS for this application and this instrumentation represent approximately two orders of magnitude increase in sensitivity over those achieved with simple CE–TOF-MS.

4. Conclusions

ITP and comprehensive ITP–CE were successfully coupled to electrospray ionization TOF-MS for the analysis of model mixtures of angiotensins. The suppression of electroosmotic flow inside the capillaries using PVA coating permitted the use of shorter capillaries and thus allowed for higher electric fields to be applied in order to achieve higher resolution and separation efficiency than would have been possible with uncoated capillaries. While ITP–TOF-MS was satisfactory for the analysis of samples with analyte levels sufficient for the formation of well-developed flat-top zones, at lower concentrations and with the same sample volume, the well-defined ITP zones collapsed into overlapping narrow, peak-shaped mixed zones. The detection of the sample components in such zones, otherwise very complicated or impossible with conventional UV or conductivity detectors, was made possible by the use of the selected ion monitoring capability of the TOF-MS. However, in some cases, ion suppression and discrimination may make quantitative analysis of narrow mixed ITP zones highly problematic. Coupling CE separation to an ITP preconcentration step in a comprehensive ITP–CE–TOF-MS mode allowed us to readily separate and detect all analytes in the low-concentration samples as separate CE peaks. In this two-dimensional analysis, ion suppression is avoided or at least significantly reduced, which makes quantitation much more reliable. The comprehensive ITP–CE–TOF-MS system was also capable of performing multiple-injection CE analyses of higher concentration samples. The combination of large volume injection and high resolving power of comprehensive ITP–CE together with the specificity of TOF-MS detection is very promising for the analysis of trace components next to major components in complex matrices and will be explored in greater detail in future studies.

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