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Analyte focusing in capillary electrophoresis using on-line isotachophoresis

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ABSTRACT

The on-line coupling of isotachophoresis with capillary electrophoresis as presented earlier was improved with respect to recovery, reproducibility and ease of operation by developing a new system. Owing to the isotachophoretic sample pretreatment resulting in analyte focusing, the corresponding determination limits in capillary electrophoresis could be enhanced by three orders of magnitude. It is shown that both the reproducibility and selectivity are improved considerably owing to the two-dimensional electrophoretic approach. The potential of one-line coupled isotachophoresis–electrophoresis was demonstrated using a fluorescein isothiocyanate derivative of the peptide angiotensin III as test compound.

INTRODUCTION

In the last year, a number of papers [1–7] have been published dealing with sample pretreatment methods which are compatible with a highly efficient, miniaturized technique such as capillary electrophoresis (CE). Major goals are the improvement of both the loadability of the CE system and the applicability of CE in bioanalysis. The combination of two orthogonal separation methods such as high-performance liquid chromatography (HPLC) and CE has already been applied in various configurations [8,9]. The major advantage of this combination is an enhanced resolution and peak capacity, although the effective loadability is still limited.

Another approach is to use preconcentrating sample pretreatment techniques [10]. Similarly to the use of precolumns in HPLC, they can be applied in combination with CE. Micro-scale precolumns can be installed in series with the CE capillary. A disadvantage of these systems is that they are very easily plugged by particles or denaturating macromolecular compounds, making this method less suitable for routine analysis of biological samples. A similar method with promising results is the use of conventional HPLC off-line precolumn cartridges (1–0.1 ml), which are commercially available and can be used instead of micro-scale columns. This method led to an increased detectability by a factor of 15 [11].

The application of coating of the fused-silica wall capable of binding the analyte has also been described [12,13]. Micellar electrokinetic capillary chromatography (MECC) is an example combining the mechanisms of electrophoresis and chromatography, making CE more suitable for the analysis of biological fluids [14]. These electrochromatographic techniques increase the selectivity of the electrophoretic system but give little or no improvement in the loadability.

Instead of combining a chromatographic method with CE for a two-dimensional separation, it is also possible to couple two electrophoretic methods. Similarly to two-dimensional chromatography, two-dimensional electrophoresis can be applied using different electrophoretic separation mechanisms to increase the loadability and selectivity. The most common example of such an approach is in fact the injection of diluted samples, which induces a higher field strength than the electrophoresis buffer in the

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CE system. This principle is also called sample stacking [15–17]. In this instance a discontinuous electrophoresis system is created, resulting in isotachophoretic zone sharpening and concentration. Sample stacking has been used successfully in gel electrophoresis but can also be used in free solution electrophoresis [18]. However, in free solution electrophoresis the isotachophoretic effect may be decreased by the electroosmotic flow (EOF). This EOF can be reduced by using capillaries that are deactivated by a special coating minimizing the zeta potential. Such capillaries are already supplied by various manufacturers. Concentration factors of about 25 can be obtained by applying these isotachophoretic phenomena in capillary electrophoresis [12]. Everaerts and co-workers [19–21] reported the use of ITP and CE effects in both ITP and CE, demonstrating the good compatibility of both electrophoretic methods.

Recently several papers have been published that describe the use of a complete isotachophoretic system coupled on-line to a capillary electrophoresis system. Kaniansky and Marak [22] used an isotachophoretic system with a sample preconcentration part. Both the ITP and CE parts of their system had an I.D. of 300 μm. Dolnik *et al.* [23] developed an ITP system consisting of compartments with decreasing diameters, permitting the introduction of larger volumes. Stegehuis and co-workers [1,24–26] presented a system in which a separate ITP system was coupled to a separate CE system. The ITP system had an I.D. of 500 μm and the CE an I.D. of 50 μm. The dimensions were chosen to ensure that both systems could operate at maximum performance. An increase in detectability by a factor of 100 was shown. Foret *et al.* [27] described a similar system using dyes to determine the moment of injection from ITP to CE.

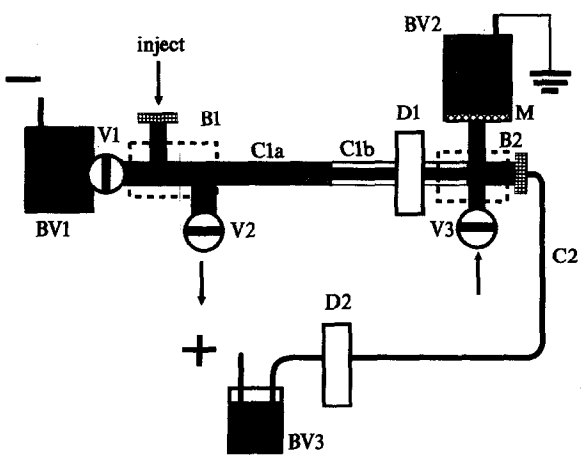

Fig. 1. Schematic diagram of complete ITP–CE system. V1 = valve 1; V2 = valve 2; V3 = valve 3; D1 = ITP UV detector; D2 = CE LIF detector; M = membrane; BV1 = terminating buffer vial; BV2 = leading buffer vial; BV3 = electrophoresis buffer vial; B1 = injection block; B2 = ITP–CE interface block; C1a = separation part of the ITP capillary; C1b = detection part of the ITP capillary; C2 = CE capillary.

This paper describes an improved ITP–CE system derived from the system presented earlier [1]. With the developed system improvements were achieved with respect to recovery, reproducibility and ease of operation. The detectability was improved by three orders of magnitude compared with off-line CE. A fluorescein isothiocyanate derivative of angiotensin III was used as a test compound.

EXPERIMENTAL

Materials

Hydrochloric acid (HCl) and acetonitrile (ACN) was purchased from Baker Chemicals (Deventer, Netherlands), tris(hydroxymethyl)aminomethane (Tris), barium hydroxide and sodium borate from Merck (Darmstadt, Germany), hydroxypropylmethylcellulose (HPMC) from Sigma (St. Louis, MO, USA), β-alanine (β-ala) and fluorescein isothiocyanate (FITC) from Aldrich (Steinheim, Germany) and angiotensin III (A III) from Bachem Feinchemicalien (Bubendorf, Switzerland).

The leading buffer (LB) was 0.01 *M* HCl adjusted to pH 9.2 with 0.5 *M* Tris containing 0.05% (w/w) HPMC. After preparation the buffer can be used for about 2 weeks. The same buffer was used for CE. The terminating buffer (TB) was 0.01 *M* β-ala adjusted to pH 10.4 with a saturated solution of $Ba(OH)_2$. This buffer was prepared freshly every day. The borate buffer used for the FITC derivatization reaction was 0.2 *M* sodium borate. Demineralized water was used throughout.

Equipment

Isotachophoresis. A schematic diagram of the coupled ITP–CE system is given in Fig. 1. The buffer vials and the injection block were laboratory-

made and constructed from Plexiglas. The injection block of the ITP system was constructed as described [28]. The leading buffer vial and the capillary were connected by a Plexiglas block which also formed the interface to the CE system. Between this interface block and the leading buffer compartment a semi-permeable membrane was placed in order to prevent the LB from the buffer vial from flowing into the capillary either electrokinetically or hydrodynamically. The ITP capillary consisted of two parts, a separation part made of PTFE (250 mm × 450 μm I.D. × 700 μm O.D.) and a detection part made of fused silica (50 mm × 320 μm I.D. × 450 μm O.D.). A few millimetres of coating were burned off for on-column detection, for which a modified liquid chromatographic UV detector was used (Model 440, Waters Assoc., Milford, MA, USA). The conventional flow cell was replaced with a messing block positioning the capillary in-line with a 300-μm pinhole through which light was guided. The signal in the reference channel was reduced to the same extent.

Injection into the ITP system was done with a 100-μl syringe. For the ITP system a power supply with reversible polarity (Model RR 100-1.5R Gamma, High Voltage Research, Coimex, Hattem, Netherlands) was used in the negative mode.

Capillary electrophoresis

The CE system was a modular laboratory-made system using capillaries of 50 μm I.D. and 220 μm O.D. (SGE, North Melbourne, Australia). The total length of the capillary was *ca.* 500 mm with a length of *ca.* 300 mm to the detector. In order to obtain a reproducible performance, the dynamic coating with HPMC in the CE capillary was renewed and saturated between every run by flushing it with LB for 10–15 min.

Detection was based on laser-induced fluorescence (LIF) using an air-cooled argon ion laser (Model 161 C, Spectra-Physics, San Jose, CA, USA) lasing at 488 nm. On-capillary detection was performed by focusing the laser beam on the capillary, from which the coating on a small part had been removed, placed in a laboratory-made holder.

The fluorescence was detected with a blue–green-sensitive photomultiplier tube (PMT) (Model 635B, Thorn EMI, Ruislip, Middlesex, UK) operating at 600 V generated by a high-voltage power supply (Model 244, Keithley Instruments, Cleveland, OH, USA). The signal was processed by a current amplifier (Model 427, Keithley Instruments). For CE a 40-kV positive high-voltage power supply (Model RR 40-1.5P Gamma) was used. Electropherograms of both ITP and CE were registered on a flat-bed recorder (Model 40, Kipp & Zonen, Delft, Netherlands).

Coupled isotachophoresis–capillary electrophoresis. To couple the ITP and the CE system the CE capillary was inserted into the ITP capillary through the Plexiglas interface block (Fig. 1). The CE capillary was positioned just "downstream" of the location where UV detection takes place on the ITP capillary. The exact position of the capillary was determined by inserting the CE capillary just before blocking the light beam of the UV source. The levels of the terminating buffer vial and the buffer vial at the outlet of the CE capillary were adjusted carefully to prevent hydrodynamic flow.

Procedures

Derivatization of angiotensin III. To 50 μl of a 1 m*M* A-III solution (1 mg/ml), 50 μl of 2.5 m*M* FITC solution in borate buffer (0.2 *M*, pH 9.5) were added. The derivatization mixture was heated at 60°C for 30 min in order to complete the reaction. The samples for the calibration graphs were prepared by appropriate dilutions of A-III–FITC in demineralized water.

Pretreatment of plasma samples. Before injection into the ITP system, the plasma samples were deproteinated by adding 2 ml of ACN, vortexing for 10 s and centrifugating for 10 min at 2000 *g*. To the decanted supernatant either FITC (blank plasma samples) or A-III + FITC (spiked plasma samples) was added.

RESULTS AND DISCUSSION

In order to make optimum use of the focusing effect of the ITP system and the efficiency of the CE system, the coupled system was developed such that the mutual influence of the two systems was minimal. Further, the system was optimized with respect to reproducibility and ease of operation.

Isotachophoresis

In the system used, the leading and terminating

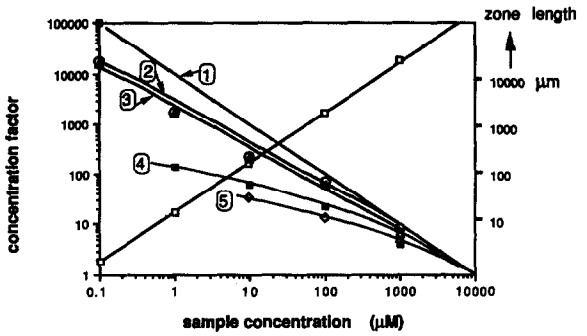

Fig. 2. Concentrating capacity of the ITP system as a function of the sample concentrations at different percentages of HPMC in the leading buffer. 1 = Theoretical concentration factor; 2 = 0.1% HPMC; 3 = 0.05% HPMC; 4 = 0.025% HPMC; 5 = 0.01% HPMC. On the right-hand ordinate the zone length (□) at different concentrations is given at a concentration of 0.05% HPMC in the leading buffer.

buffers have relatively high pH values, resulting in a very high EOF. Usually in ITP the effect of EOF can be limited but not eliminated by using PTFE capillaries and using a membrane between the leading electrolyte buffer vial and the capillary. However, in the developed system a piece of fused silica was installed in order to perform on-capillary detection. At pH 9.2 this piece of fused silica induces such a high EOF that the ITP process is extremely hampered. The EOF can be decreased by adding a non-ionic surfactant such as HPMC [29–31]. This non-ionic surfactant increases the viscosity of the buffer and forms a dynamic coating on the capillary wall. Fig. 2 shows the influence of different percentages of HPMC on the concentrating capacity of the ITP system. Without HPMC an EOF exists in the direction of the terminating buffer vial, while the ITP process takes place in the opposite direction. In this way the concentrating capacity and the zone-sharpening effect are almost completely destroyed. By adding HPMC the ITP process functions again according to Kohlrausch's law [32], implying that the sample zone will acquire a concentration related to that of the leading buffer and the respective mobilities. Despite the addition of HPMC it must be noted that below a certain zone length the observed signal starts to deviate from the theoretical value. This can be explained by the fact that below a critical zone length the sample zone starts to become shorter than the size of the detection window (300 μm). Consequently, the intensity of the signal is no longer representative of the concentration factor.

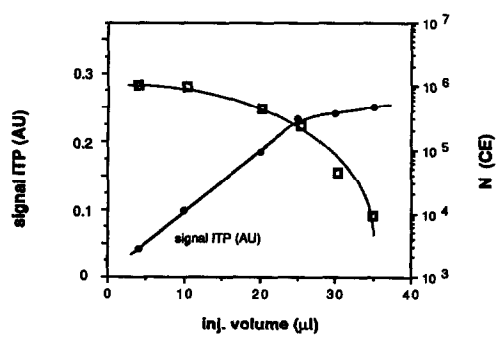

Fig. 3. Influence of injection volume on the detector signal measured in the ITP system [left-hand ordinate (●)] and the performance of the CE system [right-hand ordinate (□)].

The optimum concentration of HPMC was 0.05% w/w, this being a compromise between reduction of the EOF and an unacceptable increase in viscosity. At HPMC concentrations of 0.1% (w/w) and higher, flushing of the 50-μm I.D. CE capillary became a major problem and blockage of the CE capillary repeatedly occurred.

Injection into the ITP system took place using a 100-μl injection syringe. Possible memory effects can be avoided by flushing the needle between injections with a buffer solution containing HPMC. Fig. 3 shows the performance of the ITP system using different injection volumes. Injection volumes above 20 μl resulted in a decreased signal and reproducibility. The internal volume of the Plexiglas block in which the sample is injected is about 50 μl, whereas the internal volume of the capillary is only 35 μl. The capacity of the ITP system is related to the volume of the ITP capillary. Consequently, the capacity can be increased by using longer capillaries, but doubling the length would raise the loadability only by a factor of two but would increase the analysis time considerably. Taking into consideration that by using the concentration capacity of the ITP system an improvement in detection limit of three orders of magnitude can be obtained, the factor of two resulting from doubling the length of the capillary can be neglected.

Capillary electrophoresis

The leading buffer of the ITP system containing HPMC was also used as the electrophoresis buffer in the CE system. The addition of HPMC to the CE system had major consequences for the EOF. Although the EOF was not completely stopped it was

lowered considerably. The EOF was slowed so much that the negatively charged FITC derivatives migrated in the direction of the anode. As a consequence of this strongly reduced EOF, relatively short capillaries (300 mm to the detector) could be used for CE separation.

Coupled isotachophoresis–capillary electrophoresis

Injection into the CE system is possible electrokinetically, hydrodynamically or as a combination of the two. Because the CE capillary is exactly positioned "downstream" of the UV detector in the ITP system, it should be possible to time the moment of injection accurately. This injection method leads to the highest selectivity because a perfect heart cut could be made out of a large number of zones. Nevertheless, making a reproducible electrokinetic injection of a zone a few micrometres in length, migrating with a velocity of *ca.* 250 μm/s, would still be very difficult.

A less selective but more reproducible injection strategy would be to choose ITP conditions so that as little as possible of the zones comigrate with the analyte zone, and all zones migrating between the leading and terminating buffers are injected hydrodynamically into the CE system. Such a hydrodynamic injection has to be performed very carefully in order not to disturb either the ITP or CE process, which can be done by syphoning in the direction of the CE capillary during the ITP process. Because of the large differences in dimensions between the ITP and CE systems, this can be achieved by applying a small difference in level (*ca.* 4 cm) between the terminating buffer vial and the electrophoresis buffer vial, generating a small hydrodynamic flow. After starting the CE process, valve 1 (Fig. 1) is closed and valves 2 and 3 are opened. Now all vessels are on the same level and the CE process will take place without disturbance caused by hydrodynamic flow. During the hydrodynamic injection a radial split with a ratio of 1:10 is made. This means that 10% of the analyte ions injected into the ITP system are introduced into the CE system.

The injection into the CE system was optimized with respect to the moment of stopping the ITP process and starting the CE process, and the time between starting the CE and flushing the ITP system. The optimum moment of injection can be determined by plotting the signal obtained in CE *versus* the moment of injection. The moment of injection can be defined by t_i, the time between the appearance of the signal in the ITP system and actually stopping the ITP process. A negative value of t_i means that the ITP is stopped before appearance of the signal. The results are shown in Fig. 4. Although in principle a recovery of 100% can be obtained, the reproducibility is very poor and the efficiency is also affected. In that case not only an axial but also a radial migration is needed, leading to considerable entrance functions. The poor reproducibility is caused by the fact that the moment of injection is very critical, as discussed above. A recovery of 100% can only be obtained if the zone is exactly and completely in front of the capillary. At the moment the high voltage is switched on the zones are injected electrokinetically. Zones with a length in the micrometres or even nanometres range are therefore difficult to inject. Also, matrix compounds that migrate in front of the analyte zone might influence the retention time of the analyte, making reproducible time-based injection with 100% recovery impossible.

After $t_i \geqslant 2$ min (Fig. 4), a very reproducible split of 1:10 is made. This injection is exclusively made by hydrodynamic flow and not electrokinetically. Although only a recovery of 10% is obtained in this way, this moment of injection is preferred because of the high reproducibility and the possibility of time-based injection. Within a time window of at least 5 min the injections can still be performed with a relative standard deviation (R.S.D.) of $\leqslant 5\%$.

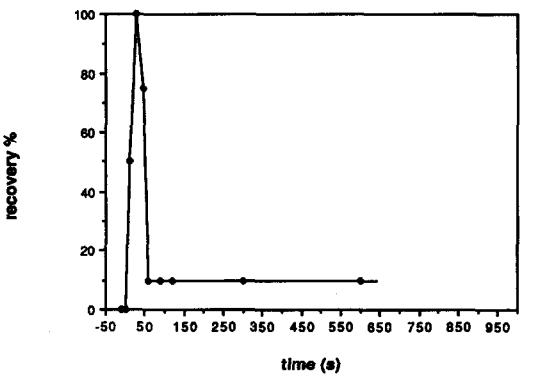

Fig. 4. Recovery in the CE system as a function of the moment of injection from the ITP into the CE system ($t = 0$ is the time that the signal has been detected in the ITP detector).

This gives the ITP process sufficient space for retention time variations caused by differences in sample composition.

After the CE process has been started, the ITP system must be backflushed, which is done after closing valve 1 (Fig. 1) and opening valves 2 and 3, in order to prevent the terminating buffer from entering the CE system and disturbing the continuous buffer system. The time between starting the CE system and flushing the ITP system is only critical if injections are made at $t_i \leqslant 2$ min. In these situations the analyte is still at the initial part of the CE capillary. The hydrodynamic velocity in the CE capillary is much smaller than the migration velocity during electrophoresis, so with the sample still being at the initial part of the CE capillary the possibility exists that the sample is flushed partly or completely out of the CE capillary during the backflushing of the ITP system, which can cause irregularities. If $t_i \geqslant 2$ min and the time before flushing is between 5 and 10 s, no sample will be flushed out and the TB will not influence the CE process.

Quantitative aspects

In order to investigate the quantitative potential of the combined ITP–CE system, calibration graphs of an FITC derivative of the peptide angiotensin III were made. For comparison with CE also some graphs for this derivative were obtained with off-line CE, using the optimized conditions as given in Table I. The results for both systems are given in Table II.

From these results from Fig. 5a and b (representing an injection of a 5 μl/ml and a 5 ng/ml solution in an off-line CE and an ITP-CE system, respectively) it can be seen that a factor of 1000 in detectability has been achieved. The absolute minimum detectable amount is the same in both configurations because detection takes place in both systems in the same CE-LIF combination. The minimum detectable concentration could be lowered a factor of 1000 by applying analyte focusing using a two-dimensional electrophoresis system. The precision of CE determinations can also be improved from an R.S.D. of the peak area of 9–7% to 5–3% by coupling it on-line to an ITP pretreatment unit.

Influence of biological matrix

To establish whether this two-dimensional electrophoretic system also results in an increase in selectivity, some experiments were done with plasma samples. Plasma was spiked with FITC-derivatized A-III. There was no intention of determining endogenous A-III because the derivatization procedure was not optimized for this application. These experiments were carried out only to observe the performance of the ITP-CE system when plasma samples were injected repeatedly. In off-line CE often a rapid decrease in performance is observed with repeated injections of biological samples. Fig. 6a shows plasma samples containing blank deprotei-

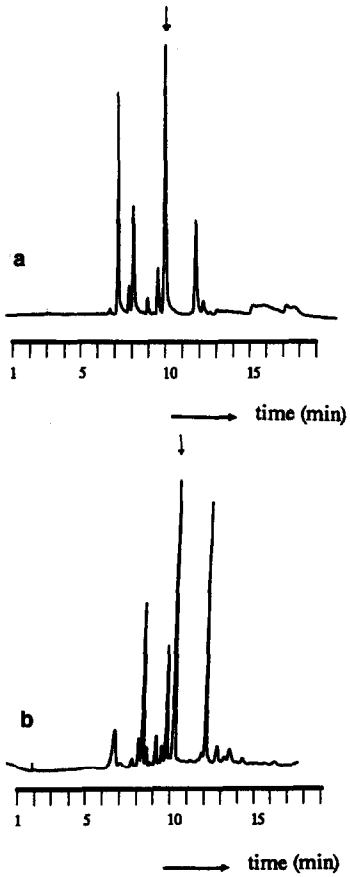

Fig. 5. (a) Electropherogram of off-line CE. An equivalent of about 1 nl of a solution of A-III–FITC derivative with a concentration of 5 μg/ml was injected hydrostatically. For other conditions, see Table I. The arrow indicates the A-III–FITC derivative peak. (b) Electropherogram of on-line ITP-CE. 10 μl of a solution of A-III–FITC derivative with a concentration of 5 ng/ml were injected into the ITP system. For other conditions, see Table I. The arrow indicates the A-III–FITC derivative peak.

TABLE I

OPTIMIZED CONDITIONS FOR THE ITP AND CE SYSTEMS

Parameter	ITP	CE
Buffers	0.01 *M* β-ala	
TB	pH = 10.4	
	$Ba(OH)_2$	
LB	0.01 *M* Cl^-–Tris	0.01 *M* Cl^-–Tris
	pH = 9.2	pH = 9.2
HPMC	0.05% (w/w)	0.05% (w/w)
Voltage (kV)	5	25
Capillary:		
Total length (cm)	25	50
Length to detector (cm)	22.5	30
Material	20 cm PTFE +	50 cm fused silica
	5 cm fused silica	
Detection	UV (254 nm)	LIF ($\lambda_{ex}/\lambda_{em}$ 488/514 nm)
	on-capillary	on-capillary
Injection	10 μl (syringe)	10% equivalent of
		ITP injection

TABLE II

VALIDATION AND COMPARISON OF THE PERFORMANCE OF THE CE SYSTEM AND THE COUPLED ITP–CE SYSTEM

Conditions as in Table I.

Parameter	CE		ITP–CE	
	A-III–FITC concentration (μg/ml)	R.S.D. (%)	A-III–FITC concentration (ng/ml)	R.S.D. (%)
	1	8.5	0.5	3.7
	5	7.0	5	2.9
	20	7.0	10	4.5
Day to day ($n = 3$)	5	7.7	5	3.5
Calibration graph	$y = (0.51 \pm 0.02)x + (0.1 \pm 0.4)$		$y = (1.43 \pm 0.01)x + (0.04 \pm 0.04)$	
Correlation coefficient	0.9982		0.999	
Minimum detectable concentration				
(signal-to-noise = 3)	100 ng/ml		100 pg/ml	
	100 nmol/l		100 pmol/l	
Absolute detection limit	100 fg		100 fg	
	100 amol		100 amol	

nized plasma spiked with an overload of FITC and Fig. 6b shows a deproteinized plasma sample spiked with A-III and an overload of FITC. Even with such complex samples reproducible results are obtained on applying ITP–CE, making CE suitable for the routine analysis of biological samples. About 10% of the whole zone that migrates in the ITP system is injected into the CE system. Therefore, the selectivity has to be tuned by the choice of the combination of LB and TB. If this buffer system forms a very small mobility window around the analyte of interest, the selectivity will be high. If several components have to be monitored by CE, a relatively large mobility window can be chosen in the ITP buffer system, making a more universal analysis method possible.

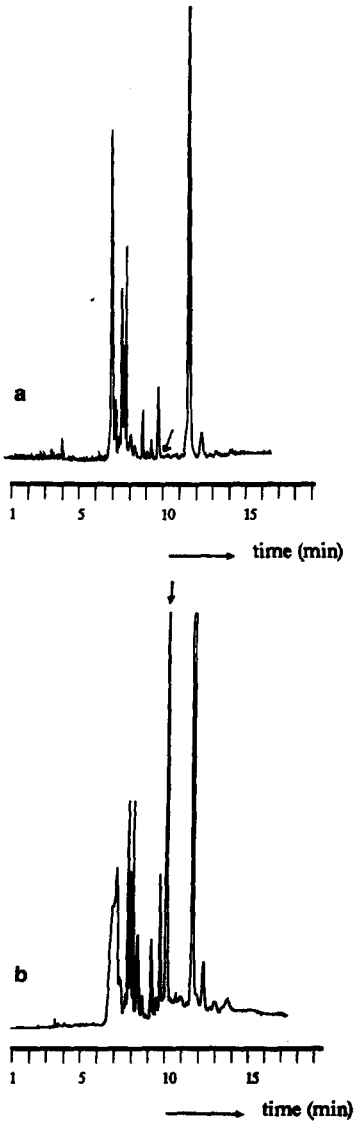

Fig. 6. (a) Electropherogram of deproteinized plasma derivatized with FITC. 10 μl were injected into the ITP system. For other conditions, see Table I. The arrow indicates the time at which the A-III–FITC derivative would migrate. (b) Electropherogram of deproteinated plasma derivatized with FITC and spiked with A-III–FITC (50 ng/ml). A 10-μl sample was injected into the ITP system. For other conditions, see Table I. The arrow indicates the A-III–FITC derivative peak.

CONCLUSIONS

Analyte focusing in CE using on-line ITP has been performed in a two-dimensional electrophoresis system which has been developed and optimized. Analogously to multi-dimensional chromatography, this resulted in increases in detectability, selectivity and reproducibility. In comparison with CE an improvement in the minimum detectable concentration of a factor of 1000 is achieved. The reproducibility of the peak area is improved from R.S.D. *ca.* 7% to *ca.* 3.5% without the use of an automated system. The ease of operation is improved in comparison with off-line CE because injection can be performed by syringe.

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