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On-line isotachophoresis–capillary zone electrophoresis versus sample self stacking capillary zone electrophoresis Analysis of hippurate in serum

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Abstract

This paper is aimed at the problem of sensitive analysis of a microcomponent in a sample with a bulk macrocomponent and complex matrix by capillary electrophoresis. In such samples, the simple way to increase the sensitivity by increasing the injected sample volume is counterworked by the stacking–destacking processes due to the presence of transient isotachophoresis (ITP) and a certain maximum sample load exists which gives useful results. The important analytical task of the analysis of hippuric acid (benzoyl glycine) was selected as the model problem. The aim was to reach the highest possible sensitivity in the analysis of hippuric acid in serum of healthy persons where hippuric acid represented a microcomponent and serum was a representative of a sample matrix containing varying amounts of macrocomponents. In order to find the maximum allowable sample load (volume) that enabled maximum sensitivity, various stacking techniques in two types of instrumentation for capillary zone electrophoresis (CZE) and a commercially available instrumentation for the on-line ITP–CZE combination were compared. The systems for CZE differed in detectors and in using an open or closed capillary of different diameter and material, which resulted in different sensitivity of the analyses of model samples. In analyses of serum samples, however, comparable limits of detection were obtained using both a single ITP and CZE with the open or closed capillary ($6 \cdot 10^{-5}$ *M* hippurate). A limit of detection two-orders of magnitude lower ($7 \cdot 10^{-7}$ *M* hippurate) was reached in the instrumentation for the ITP–CZE combination even though the parameters of the capillary and the detector that were available for the CZE step were substantially worse than those of the open CZE system that was used.

Keywords: Sample stacking; Isotachophoresis–capillary zone electrophoresis; Hippuric acid

1. Introduction

Capillary zone electrophoresis (CZE) with a UV absorbance detector is a fast and efficient separation method used for analyses of a large number of species [1,2]. Its main limitation is the relatively low sample concentration detection limit so that many trace components in real samples cannot be analysed by this technique directly.

The limit of detection (LOD) can be improved by several orders of magnitude when more sensitive detectors such as laser-induced fluorescence are used; as only a small number of analytes exhibit native fluorescence, derivatization has to be carried out in most cases. This may, however, cause other problems such as incomplete derivatization of highly diluted samples, instability of fluorescent products or possible formation of more than one defined product [3], for the elimination of which various other techniques and procedures [4,5] have to be used.

Another way to increase the separation capability, sensitivity and detectability of CZE is the use of

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on-line pre-concentration techniques. Here, the simplest one is stacking of the sample with a low conductivity matrix by using a high conductive background electrolyte (BGE) [6–17]. At the rear BGE boundary, the concentration of analytes is adjusted to the level corresponding to the value of the Kohlrausch regulating function of BGE, which results in short concentrated sample zones that provide high detector responses. However, samples of biological origin are very seldom free from ionic contaminants that increase their conductivity; in such cases this type of sample stacking cannot be applied. In addition, some samples, as e.g. proteins, are frequently unstable in low ionic strength media [18].

When analysing samples containing a macrocomponent differing in mobility from the co-ion of the BGE, self stacking may occur. The macrocomponent acts here either as the leading or as the terminating ion (stacker) while the co-ion of the BGE possesses the function of the terminating or leading ion, respectively. The result is transient isotachophoretic migration. Analytes with mobilities between those of the stacker and the BGE co-ion are stacked into narrow isotachophoretic zones with sharp boundaries. Their concentrations are adjusted to fit the Kohlrausch regulating function. During the analysis, the transient isotachophoretic mode of the migration is gradually transformed in the zone electrophoretic one and the separated components are detected [8,18–28].

A transient isotachophoretic stack in a CZE system may also be established on purpose by filling the capillary with appropriate electrolytes [18,19,29–37]; the problem here is the decrease of the separation efficiency due to the change of electrolytes in the electrode chamber during the analysis.

The transition from isotachophoresis (ITP) into CZE migration is a rather complicated process and has been theoretically described [24,25,38,39]. The possible modes of electrolyte combinations and the transition process from isotachophoretic to zone electrophoretic migration have been described [38,39] and experimentally verified [39]. The duration and characteristics of the transient isotachophoretic step influence the CZE zone parameters such as the detection time and variance and resolution [25]. To obtain useful results, destacking from ITP prior to the detection has to be ensured, which can be a problem especially with complex samples of varying composition and macrocomponent concentration. These difficulties can be overcome by performing the ITP–CZE combination in two coupled capillaries [19,38–51].

The coupled column arrangement is characterized by isotachophoresis in the first capillary, serving as an efficient pre-separation and concentration stage, followed by the on-line transfer of the sample cut into the second capillary where analytical zone electrophoresis proceeds as the second stage. Besides the pre-concentration of analytes, the ITP step has several other specific features that are advantageous for CZE, such as the high sample load, transfer of a well-defined fraction of the sample into CZE, and an ideal sample injection for CZE. The load capacity of the pre-separation ITP step can be increased by employing the first capillary with a wider diameter compared to the second capillary [39]. Here the major components are detected also in the ITP capillary and subsequently driven out of the system to the helping electrode, whereas the minor components in the form of a short stack of sharp zones are separated by CZE and detected in the second capillary. By using this technique, bulk and trace sample components can be determined simultaneously in a concentration ratio up to 10^4:1 [39,41].

The aim of this work was to compare the on-line ITP–CZE combination in two coupled capillaries with the sample-induced transient ITP in CZE performed in a single capillary. Results obtained with separate ITP and CZE methods are shown as well. Hippuric acid (HA) in blood serum was chosen as the analyte in a complex matrix.

Hippuric acid is a physiological component of the serum and is created in liver from benzoic acid and glycine. Benzoic acid originates from the diet (fruits and vegetables, food preservatives), from mitochondrial β-oxidation of phenyl-fatty acids and from oxidative breakdown of phenylalanine through bacterial action in the intestines [20]. The concentration of HA in serum of healthy people was estimated to be below 10^{-5} M [52]. In the case of chronic renal failure, however, HA and other metabolic end products are retained in the organism and are responsible for the uremic syndrome [53] by affecting metabolic pathways due to the modification of enzymatic reactions [54]. The increased concentration of HA (10^{-4} M) is an important parameter for the investigation of the renal excretion function in kidney

diseases [55] or for the comparison of different dialysis procedures for uremic patients [56].

Several methods, including high-performance liquid chromatography (HPLC) [20,53,55–58], gas chromatography (GC) [52,55], GC coupled with mass spectrometry (GC–MS) [52,55] and micellar electrokinetic chromatography (MEKC) [59] were used to quantitate the levels of HA in serum, plasma, ultrafiltrate and urine. Comparison of several methods is given in [55]. Electrophoretic methods were used both for the pre-concentration of hippurate in uremic serum prior to HPLC [60] and for the determination of HA in body fluids either after extraction or directly in the blood [20,21,61], urine [62–64] or other complex matrices [58].

CZE analyses of HA in biological samples were carried out in a polytetrafluorethylene capillary with suppressed electroosmotic flow [20,21] or in an untreated silica capillary with high electroosmotic counterflow at pH 9–10 [58,61] and in the presence of a cationic surfactant as the electroosmotic flow modifier [64]. Interfering effects of proteins and other particles from the biological matrices were eliminated by ultrafiltration [20,21,61] and by dilution of the sample prior to the analysis [20], changes in migration times and peak heights resulting from the varying contents of small ions (namely chlorides) in the matrix could however still be observed [20,58], which made the direct evaluation of the record impossible. As these matrix effects can be explained by the sample-induced transient ITP stacking, we show here some practical approaches to minimize or to control this type of stacking if the analysis is performed in one capillary by CZE. We demonstrate that the on-line combination of ITP and CZE performed in two coupled capillaries results in an analytical method which is robust to changes in the composition of the sample in a very wide range. With this system, correct results are obtained and can easily be evaluated.

2. Experimental

2.1. Instrumentation

The ITP experiments were performed on a CS Isotachophoretic Analyser EA 100 Villa Labeco (Spišská Nová Ves, Slovak Republic) equipped with a 9-cm or 16-cm long capillary of 0.3 mm I.D. made from fluorinated ethylene–propylene copolymer (FEP). For detection, a conductivity detector was used. The sample was injected via the sampling valve (25 μl) or by a 10-μl microsyringe (Hamilton, Bonaduz, Switzerland). The electric current was 90 μA or 65 μA, respectively.

The CZE experiments in a closed system were carried out on the same instrument as mentioned above. A 35-cm long (effective length of 30 cm) FEP capillary of 0.2 mm I.D. was equipped with a UV detector (254 nm). The sample was injected via a ceramic valve (300 nl), the current was 40 μA (10 m*M* BGE) or 180 μA (50 m*M* BGE).

For the CZE experiments in an open system, a laboratory-made instrument was used. It was assembled from a CZE 1000R high voltage power supply from Spellman (Plainview, NY, USA), and a Spectra Focus optically scanning detector from Thermo Separation Products (San Jose, CA, USA) coupled to a PC computer with Spectra System Software PC 1000 operating under OS 2 system. Electrode chambers were made from Plexiglas. A 72-cm long (effective length of 56 cm) polyimide-coated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 100-μm I.D., with the inner surface coated with linear polyacrylamide according to Hjertén's method [65] modified as described in [66], was used. The polarity of the high voltage was set so that the cathode was at the injection end and the anode at the detection end. The electroosmotic mobility measured with mesityloxide at reversed polarity, was less than $2.5 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$. The sample was injected hydrodynamically, using $\Delta h = 5$ or 16 cm, the time of injection was 0.25–14 min. Separations were performed at a constant voltage of 15 kV or at a constant current of 20 μA at ambient laboratory temperature of about 22°C.

The ITP–CZE experiments were carried out using the CS Isotachophoretic Analyser EA 100 Villa Labeco equipped with the column switching system. Both pre-separation and analytical capillaries were made from FEP. The pre-separation capillary was of 0.8-mm I.D. and 9 or 16 cm long and was equipped with a conductivity detector positioned 3.8 cm from the bifurcation point. Experiments were performed at a constant current of 275 μA. The 35-cm long (effective length of 30 cm) analytical capillary of 0.2 mm I.D. was equipped with UV (254 nm) detector.

Current was 40 μA (10 m*M* BGE) or 140 μA (50 m*M* BGE). The sample was injected via a sampling valve (25 μl) or with a 10-μl microsyringe. The electrolyte chambers containing the leading electrolyte (LE) and the BGE, were separated from the electrolytes in capillaries by semipermeable Cellophane membranes.

In the first step, current is switched only through the first capillary between the terminating and leading electrode. The sample components with mobilities between that of leading and terminating ions, are separated and create zones with common sharp boundaries. They pass along the detector, detectable zones can be evaluated and, by proper switching the current between the terminating and leading electrodes and terminating and BGE electrodes, any zones that are not to migrate into the second capillary are driven to the leading electrode. The cut containing HA is transferred into the second capillary by switching the current through the whole capillary system. The first capillary is now filled with the adjusted terminator and so is the second capillary. The separation continues in the CZE mode until the separated zones pass the detector.

2.2. Chemicals

All chemicals used for the preparation of model mixtures were of the analytical reagent grade and were supplied by Lachema (Brno, Czech Republic). 2-Morpholinoethanesulfonic acid (MES) and histidine (His) used for the preparation of electrolyte solutions were from Fluka (Buchs, Switzerland) and Reanal (Budapest, Hungary), respectively. A standard lyophilised ultrafiltrate of human serum was from Sigma (USA) and a pooled fresh untreated serum both from healthy children and children suffering from chronic renal failure was from Faculty Children Hospital (Brno, Czech Republic). Deionized water prepared from distilled water by trapping ions in a mixed-bed ion exchanger (Ostion AD+KS, Spolchemie, Ústí nad Labem, Czech Republic) was used for the preparation of all solutions. For electrolyte systems used, see Table 1.

Table 1
Electrolyte systems used

ITP	
Leading electrolyte	0.01 *M* HCl+His, pH 5.5
Terminating electrolyte	0.01 *M* MES
CZE	
Background electrolyte	0.01 *M* (0.05 *M*) MES+His, pH 6.2
ITP–CZE	
Leading electrolyte	0.01 *M* HCl+His, pH 5.5
Terminating electrolyte	0.01 *M* MES
Background electrolyte	0.01 *M* (0.05 *M*) MES+His, pH 6.2

3. Results and discussion

The migration behaviour of HA in the presence of macrocomponents was studied both in serum and in a model mixture composed of several serum components at concentrations corresponding to the average serum levels in healthy people. The composition of the model mixture and values of p*K* and ionic mobilities of studied compounds are given in Table 2.

3.1. Isotachophoresis

In the commercial instrument used for isotachophoretic experiments (see Section 2) the separation FEP capillary of 0.3-mm I.D. is combined with an injection valve of 1-mm I.D. which increases the load capacity of the system. The maximum volume of serum or model mixture that can be completely separated before the zone of hippurate reaches the detector was calculated according to [72] and confirmed experimentally to be 7 μl for the 9-cm long capillary (Fig. 1) or 10 μl for the capillary length of 16 cm. This resulted in a comparable detection limit of HA both in serum and in the model mixture which was $6 \cdot 10^{-5}$ *M* and $4 \cdot 10^{-5}$ *M* hippurate, respectively. The calibration curve was linear in the investigated concentration range from 0 to $6 \cdot 10^{-3}$ *M* hippurate with a correlation coefficient $r = 0.999$.

Isotachophoresis is, due to the high separation efficiency, a suitable method for the analysis of blood serum and could be used for the determination of increased concentrations of HA, e.g., in uremic serum, the detection limit is however not low enough to detect hippurate in both standard and normal pooled serum.

Table 2
Some parameters of the studied systems

Compound	pK	$u \cdot 10^9$ ($m^2 V^{-1} s^{-1}$)	Concentration (mM)	
			In serum	In model mixture
Chloride	−2	−79.1	98–106[f]	100
Phosphate	2.12[a]	−35.1[a]	0.65–1.6[f]	1.6
	7.47[a]	−61.5[a]		
	12.36[a]	−71.5[a]		
Hippurate	3.7[b]	−29.0[c]	0–0.05 (health)[h]	2×10^{-4}–0.7
		−26.7[d]	0.18–0.91 (renal failure)[h]	
		−26.1[e]		
Urate	5.7[b]	−27.9[d]	0.18–0.45[g]	0.33–0.45

[a] From [67].
[b] From [68].
[c] Calculated using molar conductivity of sodium hippurate at 5.10^{-4} M (25°C)[b].
[d] Calculated according to [71].
[e] Calculation based on ITP measurements, related to picrate.
[f] From [69].
[g] From [70].
[h] From [52].

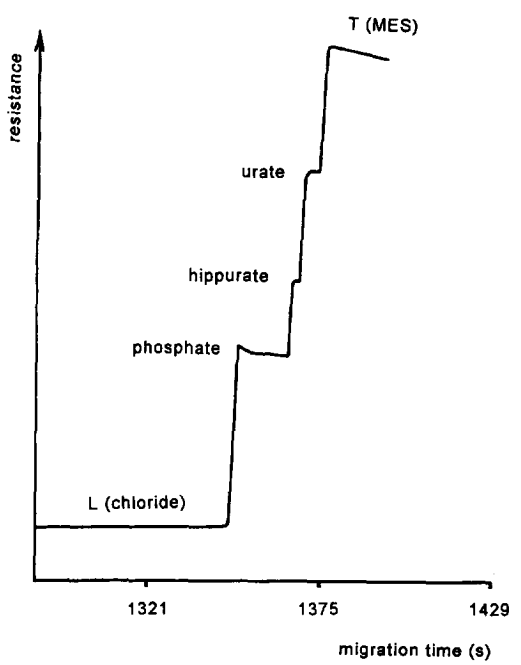

Fig. 1. ITP: Record illustrating HA separation from macrocomponent present in the serum. Sample: uremic serum with addition of HA $6\cdot10^{-4}$ M. Capillary: FEP, 90×0.3 mm; applied current: 90 μA; injection by microsyringe: 7 μl; LE: 10 mM HCl+His, pH 5.5; TE: 10 mM MES. Conductivity detection.

3.2. Capillary zone electrophoresis in a closed system

Experiments were performed in the commercial apparatus (see Section 2) equipped with a FEP capillary of a relatively wide I.D. (0.2 mm). A ceramic valve was used for the reproducible injection of a constant sample volume (300 nl). The only wavelength that could be used with the UV detector that was available with the instrument was 254 nm, which is not the optimum wavelength for HA determination (the molar extinction coefficient is higher towards the lower wavelength). Using this arrangement, the LOD (signal-to-noise ratio 3:1) was found to be $5\cdot10^{-5}$ M and $6\cdot10^{-5}$ M of HA in the model mixture and in serum, respectively. A linear dependence was found when both peak height and area were plotted vs. hippurate concentration in the investigated concentration range 0–$5\cdot10^{-4}$ M with correlation coefficients being r=0.999. Reproducible results were obtained both in the model mixture and in pooled serum samples spiked with HA (Fig. 2), however, the sensitivity of the analysis was too low to be used for the determination of HA in the normal serum.

Chlorides and phosphates in serum are responsible for the stacking effects of the leading type with respect to HA [25]. Their concentration varies in a

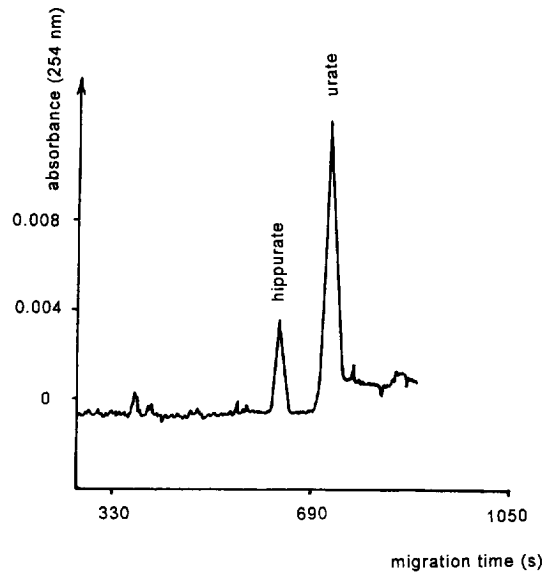

Fig. 2. CZE in closed system: Record illustrating the position of HA peak. Sample: serum with addition of HA $1.5 \cdot 10^{-4}$ *M*. Capillary: FEP, 350 mm (300 mm to detector)×0.2 mm; applied current: 180 μA; injection by ceramic valve: 300 nl; BGE: 50 m*M* MES+His, pH 6.2. Detection at 254 nm.

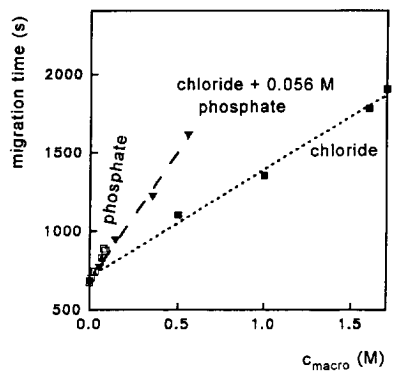

Fig. 3. CZE in closed system: Dependence of the migration time of HA on the concentration of macrocomponents in the sample. Sample: $7 \cdot 10^{-4}$ *M* HA, $3 \cdot 10^{-4}$ *M* urate, 0–1.7 *M* chloride, $5.6 \cdot 10^{-2}$ *M* phosphate. Capillary: FEP, 350 mm (300 mm to detector)×0.2 mm; applied current: 40 μA; injection by ceramic valve: 300 nl; BGE: 10 m*M* MES+His, pH 6.2. Detection at 254 nm.

certain range (see Table 2) and, as a result, the effect of stacking is not constant either. It was verified experimentally that the increasing concentrations of chlorides or phosphates or both of them increased linearly the migration time of hippurate. The closer the mobility of the macrocomponent to the mobility of microcomponent, the more pronounced was the effect that could be observed, i.e. the effect of phosphates was higher than that of chlorides (Fig. 3). Nevertheless, with the experimental arrangement used, hippurate succeeded in leaving the transient ITP stack prior to detection and passed along the detector as a separated CZE zone even when the concentrations of stacking ions (chlorides and phosphates) were by 1.5 order of magnitude higher than their physiological concentrations. Within the physiological shifts of chloride and phosphate concentrations, only a minor effect of stacking on the separation can be expected; nevertheless, for the identification of a microcomponent it is not recommended to rely only on migration times and an internal standard should be used as well. A high amount of a stacker added deliberately to the sample in order to enhance the sensitivity is accompanied by an enormous increase in the time of analysis and cannot be recommended here.

3.3. Capillary zone electrophoresis in an open system

The most often used system in CZE is an open one where the ends of the capillary are connected directly with the electrolyte in electrode chambers without any membrane between. In such a system, a different time of injection results in variations of the sample volume that replaces the BGE in the capillary. With stacking involved, sharp peaks of analytes can be obtained with relatively large volumes injected, i.e. a pronounced decrease of the LOD can be reached. The volume of the sample cannot be increased arbitrarily. The analyte leaves the stack in dependence on its mobility, on the mobility of the stacker and also on the concentrations of the co-ion and stacker [73]. There must be a minimum path for the analyte to leave the stack and to separate from the preceding analyte ion with at least unity resolution. Otherwise, the minor analyte is detected still unresolved from the other micro or macrocomponents of the stack and can hardly be both identified and quantified.

The transition from the ITP stack into CZE migration can be experimentally followed for a given

analyte by the peak width. A sharp narrow peak corresponds to the migration in the ITP stack, the diffuse peak is characteristic of CZE. As can be deduced from Fig. 4, up to a 29-cm long sample zone could be injected into the capillary of 0.1-mm I.D. and 56 cm of effective length to allow hippurate sufficient time to leave the stack.

Another possibility for finding the critical sample volume is to follow the peak height or area. In the case that a neighbouring zone absorbs as well, a radical change can be observed in the calibration curve plotted as the dependence of the peak height or area on the sample volume. Such a dependence is shown in Fig. 5 where the HA zone in the ITP stack coincided with some faster unknown impurity behind the large non-absorbing zone of phosphate. As can be seen, a good correlation was obtained using both approaches. Fig. 6 shows an analysis record of the maximum volume of the sample that could be separated in this experimental arrangement (2.2 μl corresponds to a 29-cm long sample zone, which is 52% of the effective capillary length). The LOD reached under these conditions was $4 \cdot 10^{-8}$ *M* HA.

A more detailed view of the situation in the capillary can be obtained from the record of the scanning detector (Fig. 7) and used for differentiation between transient ITP migration and CZE migration. Depending on the amount and mobility of a compound, various stackers can appear to influence components of the sample with the mobilities slightly different from that of the stacker. Sharp boundaries of the stacking and stacked zones distinguish them clearly from the diffused boundaries of compounds migrating freely in the BGE. Here, some minor impurities that were reproducibly found in the record are detected still stacked behind the faster stackers while HA is detected after destacking and its zone has the typical Gaussian shape.

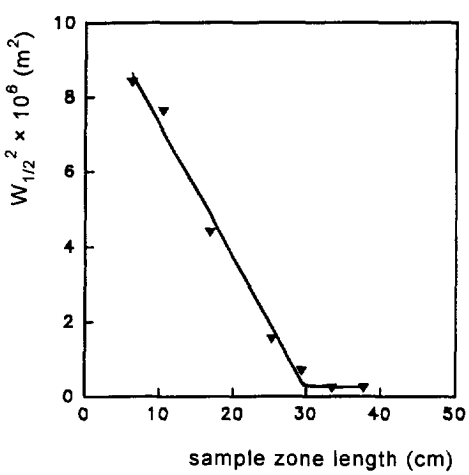

Fig. 4. CZE in open system involving sample-stacking: Change of zone width during passage from ITP to CZE migration mode. Sample: $5 \cdot 10^{-7}$ *M* HA, $4.8 \cdot 10^{-4}$ *M* urate, $1 \cdot 10^{-1}$ *M* chloride, $1.6 \cdot 10^{-3}$ *M* phosphate. Capillary: fused-silica capillary coated with polyacrylamide, 72 cm (56 cm to the detector)×0.1 mm; voltage 15 kV; hydrodynamic injection: Δh=5 and 16 cm; time of injection: 0.25–14 min. BGE: 50 m*M* MES+His, pH 6.2. Detection at 254 nm.

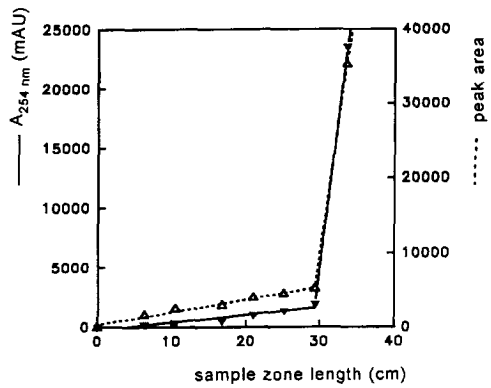

Fig. 5. CZE in open system involving sample-stacking: Change of peak height and area during passage from ITP to CZE migration mode. Sample and conditions as in Fig. 4.

An interesting course was observed in the case of the dependence of the migration time on the sample volume. It is known that the higher the concentration of the leading-type stacker [24], the longer the migration time. However, when the concentration of the stacker is constant and its amount is changed due to the sample volume, hardly any changes in the migration times of hippurate could be observed when the measurements were performed at a constant electric field strength. The migration times of faster ions of the permanent minor impurities in the sample increased while slower ones reached the detector faster when longer sample zones (and thus amounts of stackers) were injected (Fig. 8a). This could be explained by the substantial conductivity difference between the sample and BGE of comparable zone lengths, which causes substantial changes in the electric field strength in both zones and thus in

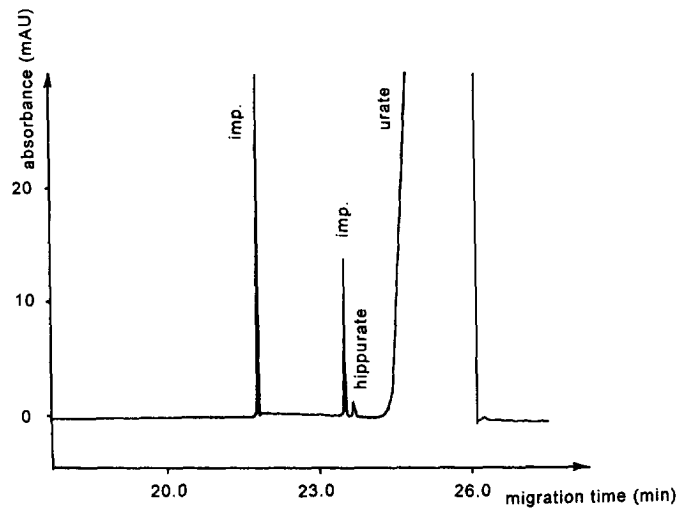

Fig. 6. CZE in open system involving sample stacking: Determination of HA in a model mixture. Sample: $5 \cdot 10^{-7}$ *M* HA, $4.8 \cdot 10^{-4}$ *M* urate, $1 \cdot 10^{-1}$ *M* chloride, $1.6 \cdot 10^{-3}$ *M* phosphate, sample volume 2 μl. Conditions as in Fig. 4 except for $\Delta h = 16$ cm and injection time of 7 min. Detection limit: $4 \cdot 10^{-8}$ *M* HA.

migration times under the constant voltage regime. When the same measurement was repeated at a constant current, a linear increase of migration time was observed (Fig. 8b) which is in correspondence with the above considerations.

From the results it follows that very sensitive analyses can be obtained in the open system due to the possibility to inject and separate large volumes of a sample which does not have a too complex composition; the method, however, fails in the case of complex biological matrices where results comparable to ITP or CZE in the closed system were obtained. The sample amount that could be injected to obtain a record with HA fully separated from other components and to obtain reproducible results differed with different serum batches and was substantially lower than in model mixture analyses. Usually, instead of 29 cm, only a 0.27-cm long serum zone (corresponding to a volume of 21 nl) of serum diluted 1:1 with water could be injected. The limit of detection ($1 \cdot 10^{-5}$) for HA in serum deteriorated by 250 times compared with the model mixture. The calibration curve for the sample volume of 21 nl (peak height vs. amount of HA) was linear in the investigated concentration range from 0 to $0.5 \cdot 10^{-5}$ *M* HA with a correlation coefficient $r = 0.994$.

Because of the deteriorated LOD, the procedure is not suitable for the determination of HA in normal serum (Fig. 9a,b). In uremic serum, the peak of hippurate can be observed and confirmed by co-migration with a standard of HA, however, the presence of another substance with a similar mobility cannot be excluded (Fig. 9c).

3.4. ITP–CZE in two coupled capillaries

The on-line combination ITP–CZE enables one to increase the injection volume of the sample and to eliminate the majority of interfering components present in serum. For this purpose, a commercial apparatus equipped with the column-switching system was used (see Section 2). The analytical capillary used for the CZE step was connected to the electrode chamber behind the detection cell via a semipermeable membrane (closed system). Before the analysis started, the system was filled with electrolytes – the first capillary with LE up to the injection point and with the terminator behind it, the analytical capillary with the terminator serving as the BGE for CZE step. The pH value of the BGE corresponded to the adjusted pH value of TE according to the Kohlrausch regulating function.

In such an electrolyte combination (T–S–T) (see

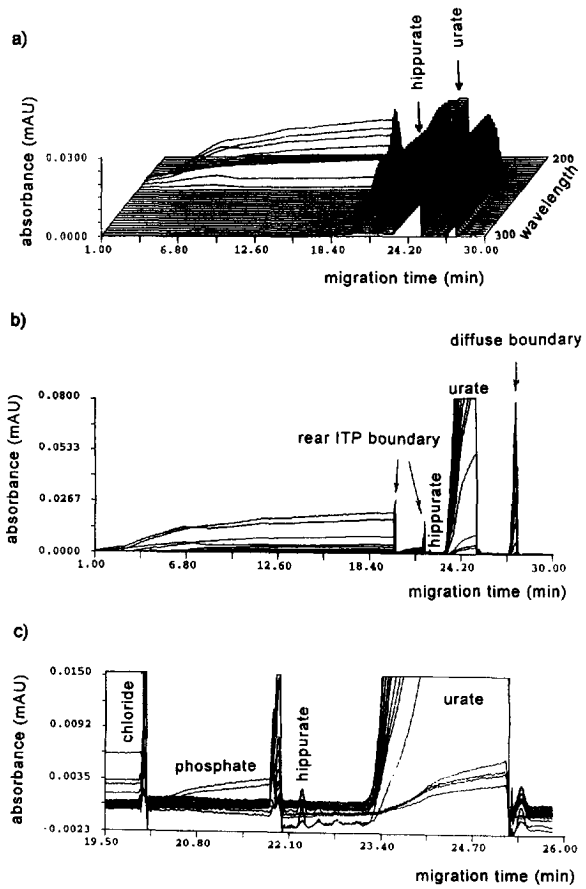

Fig. 7. CZE in open system involving sample-stacking: View of the situation in capillary obtained from the scanning detector used for differentiation between ITP and CZE migration mode. (a) Three-dimensional electropherogram of a model mixture; (b) two-dimensional electropherogram; (c) the detailed view of (b) containing hippurate. Sample and conditions as in Fig. 4 except of detection performed at 200–300 nm, Δh=16 cm and injection time of 7 min. Sample volume 2 μl.

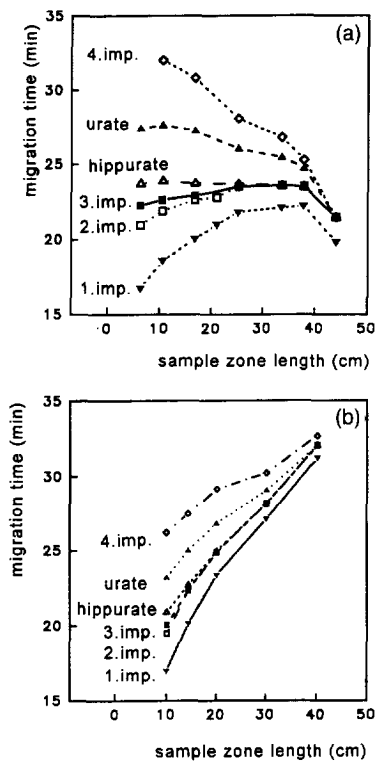

Fig. 8. CZE in open system involving sample stacking: Dependence of the migration time on the sample volume (a) at the constant voltage 15 kV, (b) at the constant current 20 μA. Sample and conditions as in Fig. 4. Unknown impurities reproducibly accompanying the sample are labelled 1–4 imp.

[38]), the reproducibility of migration times and peak heights (areas) is dependent on the size of the leading segment (or a macrocomponent possessing the stacking ability) accompanying the sample cut into the second capillary, because the ITP stack that entered the second capillary decays gradually in dependence on the amount of the stacker accompanying the analyte and on characteristics of both the analyte and the stacker [38,39].

The detector in the first capillary ensures that the time of current switching can be set precisely with respect to the actual composition of the separated sample so that always the same size of the stacking segment can be transferred into the second capillary irrespective of how many and how long were the zones of macrocomponents preceding the analyte. This results in a reproducibility of migration time better than 1%. Calibration curves of HA were plotted for model mixtures of varying concentrations of chlorides, phosphates and urates in the range of $0–9\cdot10^{-2}$ M, $2.6\cdot10^{-5}–1.6\cdot10^{-4}$ M and $1\cdot10^{-6}–4.5\cdot10^{-5}$ M, respectively. For the concentration range of 0 to $6\cdot10^{-5}$ M HA, the curves were linear with correlation coefficients r=0.999.

Due to the pre-concentration and cleaning-up effect of the ITP step, similar LODs were obtained both for the analysis of HA in the model mixture ($4\cdot10^{-7}$ M) and in serum ($7\cdot10^{-7}$ M). The maximum volume of untreated serum that could be separated was 7 μl. Two analyses of different

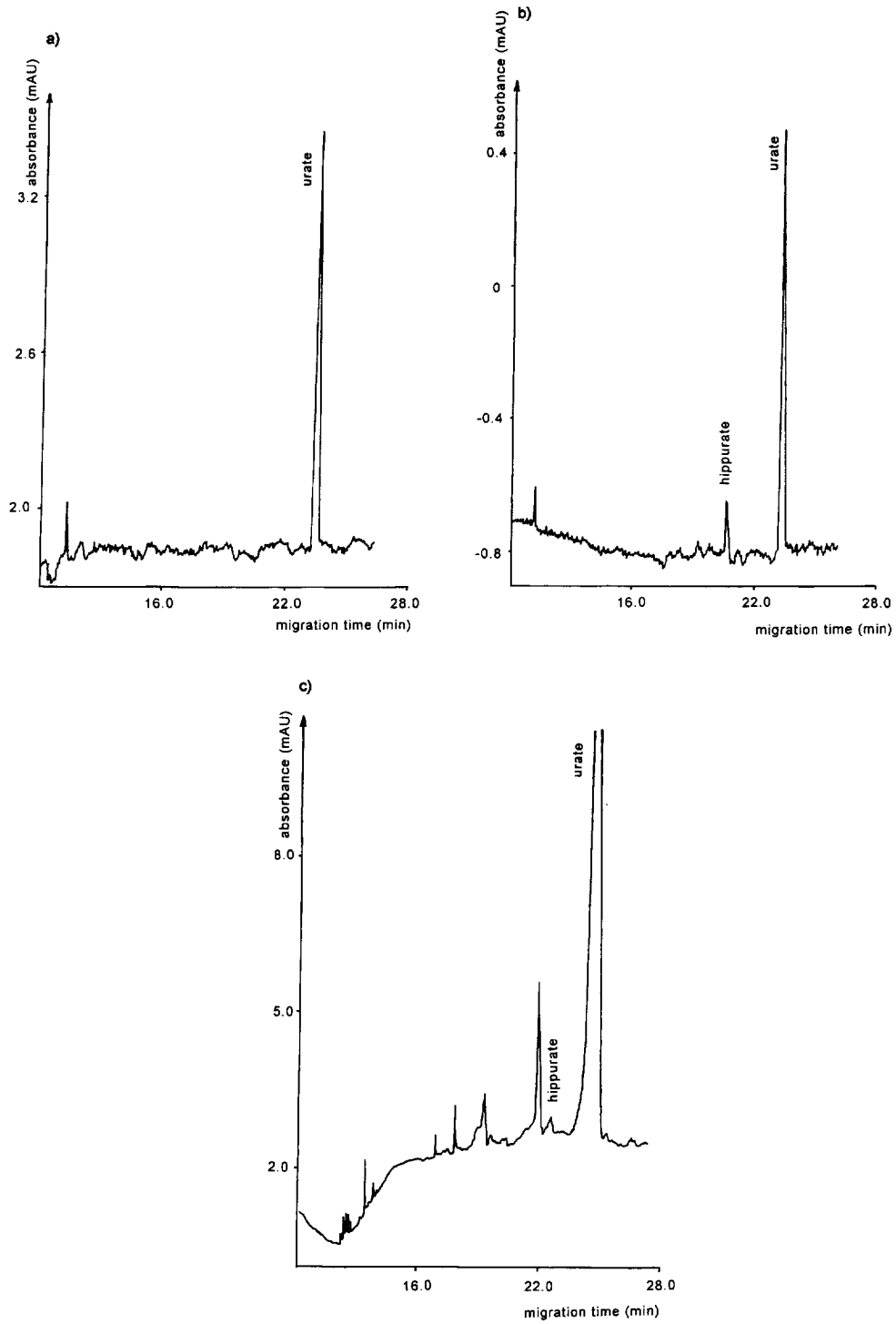

Fig. 9. CZE in open system: Determination of HA in serum. Sample: (a) standard serum sample; (b) $2.5 \cdot 10^{-5}$ M HA added to the standard serum; (c) uremic serum. All serum samples diluted 1:1 with distilled water. Conditions as in Fig. 4 except for $\Delta h = 5$ cm and injection time of 0.25 min (a,b) and 2.4 min (c). Sample volume 21 nl (a,b) and 230 nl (c).

combined serum samples of healthy children carried out by the described procedure are shown in Fig. 10. The records demonstrate the simplicity of the sample cut and reproducibility of the migration time for different serum batches.

3.5. Comparison of the used techniques

Results obtained by using ITP and CZE in open and closed systems and by the ITP–CZE on-line combination are summarized in Table 3. It is evident that for a model sample containing macro and microcomponents in a concentration ratio up to 10^6:1 where stacking effects of macrocomponents could be utilized, the highest sensitivity of the analysis was reached in the single capillary CZE without membranes between the capillary and electrode chambers (the open system). The maximum injected sample volume enabling sufficient separation and resolution of HA from neighbouring zones was as large as one half of the capillary effective length and resulted in a LOD of $4 \cdot 10^{-8}$ *M* HA. Results obtained in other single-capillary methods (ITP and CZE in the closed system) were comparable and were limited both by the sensitivity of the detector used and by the load capacity of the system. LOD can be improved by on-line combination with ITP. In the ITP–ITP combination, when a wider and longer capillary is used for the pre-separation step and a narrower one for the detection step, the load capacity can increase by up to one order of magnitude, however, the migration of the short zone of HA in the ITP stack makes the detection and identification of HA still rather complicated. On the other hand, the combination of ITP with CZE makes it possible to separate HA from the neighbouring zones so that unequivocal identification is possible. In comparison with separate ITP or CZE in the closed system, sensitivity is improved by two orders of magnitude.

In the analyses of both untreated and ultrafiltered serum, no changes in sensitivity were observed in the single ITP, CZE in the closed system and ITP–CZE combination. CZE in the open system was found to be the most sensitive to changes in the character of the matrix. Sensitivity of analyses decreased by three orders of magnitude due to the fact that in dependence on the serum batch less then 1% of the effective capillary length could be filled with the sample in order to avoid interference effects of the matrix.

Table 3
Detection limits of hippuric acid in different techniques used

Method	Capillary	LOD (*M*)	
		Model sample	Serum
ITP	FEP 90×0.3 mm I.D.	$4 \cdot 10^{-5}$	$6 \cdot 10^{-5}$
CZE	FEP closed 300×0.2 mm I.D.	$5 \cdot 10^{-5}$	$6 \cdot 10^{-5}$
CZE	silica open 720×0.1 mm I.D.	$4 \cdot 10^{-8}$	$1 \cdot 10^{-5}$
ITP–CZE	FEP (1) 160×0.8 mm I.D. (2) 300×0.2 mm I.D.	$4 \cdot 10^{-7}$	$7 \cdot 10^{-7}$

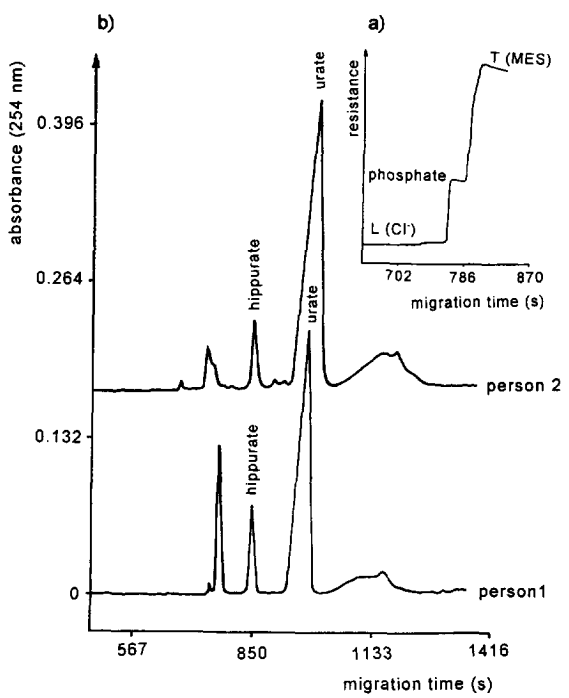

Fig. 10. ITP–CZE: Determination of HA in serum. (a) ITP step record; (b) CZE step record. Sample: 7 μl of untreated serum from two different person, estimated HA concentration $3.1 \cdot 10^{-5}$ *M* and $3.6 \cdot 10^{-5}$ *M*. Instrumentation: first capillary: FEP, 160 mm×0.8 mm, conductivity detection, current 275 μA; second capillary: FEP, 350 mm (300 mm to detector)×0.2 mm, UV detection at 254 nm, current 140 μA. LE: 10 m*M* HCl+His, pH 6.2; TE: 10 m*M* MES; BGE: 50 m*M* MES+His, pH 6.2.

The expectation that the ITP–CZE technique would be efficient for the analyses of trace components in complicated matrices was thus experimentally confirmed. We would like to point out that not only was LOD unaffected by the varying composition of the sample but that the reproducibility of migration times was also excellent (R.S.D.<1%). In addition to this, a proper switching of the current and the selection of suitable electrolyte systems results in the CZE analysis of a very simplified cut of the sample, which further eliminates interfering effects of the matrix.

4. Conclusions

From the results shown it follows that the combination of ITP–CZE is a technique especially suitable for the analysis of microcomponents in complicated matrices of varying composition. The robust ITP step provides a reproducible sampling of the sample cut into the CZE step that ensures sensitive detection. The sample volumes that can be injected using this combined technique are up to 10^3 orders of magnitude higher in the case of natural biological samples than those that can be analyzed in a single-capillary CZE technique. Excellent reproducibility of migration times (R.S.D. less than 1%) and resistance to changes in the matrix composition make the results reliable, and a LOD of $7 \cdot 10^{-7}$ *M* HA enables the determination of HA in serum not only for patients suffering from renal diseases but also for healthy individuals.

In a single capillary CZE, in the commonly used open system, when the sample-induced transient ITP was involved and a sample pulse as large as 52% of the effective capillary length could be injected, the detection limit for a model mixture, with a composition similar to serum with respect to the chloride and phosphate concentrations, was $4 \cdot 10^{-8}$ *M* hippurate. However, a substantially worse LOD was reached in real serum samples when it did not differ from the LOD obtained in the single-capillary ITP and CZE in the closed system.

Generally, the best detection limit, reliability and reproducibility for analyses were obtained with the ITP–CZE combination. It is evident that further improvement of sensitivity could be reached with a CZE capillary of a smaller I.D., more sensitive detector and detection performed at the absorption maximum of HA.

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