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Journal of Chromatography A, 916 (2001) 143–153

JOURNAL OF CHROMATOGRAPHY A

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Capillary zone electrophoresis of orotic acid in urine with on-line isotachophoresis sample pretreatment and diode array detection

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Abstract

Potentialities of capillary zone electrophoresis with on-line isotachophoresis sample pretreatment and diode array detection (ITP–CZE–DAD) to the separation, detection and identification of trace analytes present in biological matrices were investigated. Urine represented a multicomponent, variable and high ionic strength matrix while orotic acid was chosen as a model analyte of a practical clinical relevance in this investigation. Using the ITP–CZE combination in the column-coupling configuration of the separation system ITP provided an enhanced sample load capacity to the separation system (a 30 μl sample injection volume), concentrated the analyte and served as an on-line sample clean up technique. On the other hand, CZE performed a final separation of the analyte from matrix constituents present in the ITP pretreated sample and provided favorable conditions for its detection and identification by DAD. Using current correction and smoothing procedures analytically relevant DAD spectra of orotic acid could be obtained also in instances when this was injected in a model sample at a $2 \cdot 10^{-7}$ mol/l concentration (an estimated limit of determination of orotic acid at a 218 nm detection wavelength). ITP–CZE separations of urine samples (based on differences in acid–base properties and host–guest complexations of the analyte and matrix anionic constituents) led to significant sample clean ups. Consequently, DAD spectra of orotic acid matching its reference spectrum, could be acquired also in instances when the acid was present in urine matrices (loaded in 30 μl injection volumes of 20-fold diluted urine samples) at $4–6 \cdot 10^{-7}$ mol/l concentrations. Here, residual trace matrix interferents prevented a closer approach to the above value attainable for model samples. Although this work was focused only on one analyte and urine matrix it implies very promising potentialities of the ITP–CZE–DAD combination in the identification and quantitation of trace analytes present in biological matrices, in general.

Keywords: Isotachophoresis-capillary zone electrophoresis; Orotic acid

1. Introduction

Sample preparation has an important role in the analysis of samples of biological origins by capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) [1,2], especially, when an identification and/or determination of a particular analyte on a trace concentration level is required. This is due to the fact that biological matrices introduce into the CE separations some general limitations:

Some samples of biological origins (e.g., serum, plasma, urine) contain inorganic constituents at very high concentrations. For example, concentration of

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0021-9673/01/$ – see front matter
PII: S0021-9673(00)01079-7

chloride in urine may be in the range of $0.5–5\cdot10^{-1}$ mol/l and its concentration in serum is about $1.5\cdot10^{-1}$ mol/l [1]. This is limiting the sample volumes that can be currently loaded onto the column as otherwise an undesired sample stacking may be the result [3].

Some biological samples (e.g., serum, plasma) contain proteins at (very) high concentrations. A negative impact of proteineous matrix on the performance of CE separation systems may be manyfold [1,2].

Although very high separation efficiencies are attainable in CZE and MEKC separations [4] these cannot overcome inherent risks of the analyte peak overlap with constituents of complex multicomponent matrices (e.g., urine). This fact is reflecting common limits of the column separation techniques [5].

Low concentration limits of detection (often essential in the determination of some analytes present in biological samples) cannot be achieved in current, low sample load capacity CZE and MEKC columns unless very sensitive detection techniques are used [6] or appropriate sample pretreatment is applied before the CE analysis [1,2,7–13].

A well-defined concentration of the analyte(s) and sample clean up is integrated into the separation carried out by CZE on-line coupled with isotachophoresis (ITP) in the column-coupling separation system [14–29]. Here, the sample pretreatment performed in the ITP stage of the combination is followed by a sensitive detection of the analyte during the final separation of the pretreated sample in the CZE stage. The use of ITP significantly enhances an overall analytical effect as this CE technique can increase the load capacity of the separation system by a factor of 10^3 or more in comparison to a current single-column CZE and MEKC. Consequently, CZE with on-line ITP sample pretreatment provide very favorable concentration limits of detection (LODs) for the analytes also with the aid of current photometric absorbance detectors.

From papers dealing with the ITP–CZE analysis of biological samples [18,22,23,26] it is apparent that this combination provide means for obtaining ''clean'' analyte peaks in the CZE stage of the combination. These capabilities make it a convenient alternative for a combination with spectral detection and identification techniques such as diode array detection (DAD). Although the use of DAD is rather current in CE (see, e.g., Refs. [30–37]), so far, no attention was paid to the ITP–CZE–DAD combination in spite of the fact that this combination offers means effective, especially, to identification and quantitation of (trace) analytes present in complex biological mixtures.

This work was aimed at investigating some basic analytical aspects of the use of the ITP–CZE–DAD combination in the analysis of biological samples. Here, urine served as a typical multicomponent, variable and high ionic strength biological matrix. Orotic acid was chosen as a model analyte since its determination in urine has practical biomedical relevance (orotic aciduria [38]). In addition, ITP–CZE was already shown [23] to provide a promising CE alternative to its determination in urine. Partial aims of our work included: (1) A search for ITP–CZE separating conditions providing besides the concentration of the analyte sample clean up conditions leading to minimum matrix disturbances to the diode-array based identification of the analyte within a spectral range of a practical importance (200–500 nm). (2) An assessment of detection capabilities of the DAD for orotic acid in urine at selected detection wavelengths under optimum ITP–CZE separating conditions. (3) An evaluation of identification capabilities of DAD in the CZE stage of the ITP–CZE separation, especially, under working conditions corresponding to analytical limits attainable by the ITP–CZE–DAD combination.

2. Experimental

2.1. Instrumentation

An ITAChrom EA-101capillary electrophoresis analyzer (J&M, Aalen, Germany), assembled in the column-coupling configuration of the separation unit, was used in this work. The sample was injected by a 30 μl internal sample loop of the injection valve of the analyzer. An ITP column was provided with a 800 μm I.D. capillary tube made of FEP (fluorinated ethylene–propylene copolymer) and an on-column conductivity sensor. Its total length was 90 mm. A CZE column was provided with a 320 μm I.D. capillary tube made of fused silica (J&W, Folsom,

CA, USA) of a 180 mm total length (140 mm to the detection cell).

A TIDAS, multiwavelength photometric absorbance diode array detector (J&M) was connected to an on-column photometric detection cell, mounted on the CZE column, via optical fibers (J&M). The detector operated under the following conditions: (1) scanned wavelength range 200–500 nm; (2) integration time 15 ms; (3) scan interval 0.75 s; (4) number of accumulations 15. The spectral data were acquired and processed by a Spectralys program (version 1.81, J&M).

2.2. Chemicals and samples

Chemicals used for the preparations of the electrolyte solutions and model samples were obtained from Fluka (Buchs, Switzerland), Serva (Heidelberg, Germany), Sigma (St. Louis, MO, USA), Lachema (Brno, Czech Republic) and Merck (Darmstadt, Germany). Methylhydroxyethylcellulose 30 000 (m-HEC) obtained from Serva was employed as a suppressor of electroosmotic flow in the separation compartment. The electrolyte solutions were prepared in demineralized water and were filtered through disposable membrane filters (a 1.2 μm pore size) before the use.

Urine samples, obtained from healthy adults with different diet habits, were diluted 10 times with deionized water immediately after the receipt and, subsequently, filtered [1.2 μm pore size filter (Millipore, Molsheim, France)]. Citrate and phosphate, serving as discrete spacers in the ITP–CZE separations, were added (each at a $5 \cdot 10^{-4}$ mol/l concentration) to aliquots of the samples. pH of the samples were adjusted to values of 3.3–3.5 by aspartic acid. The samples prepared in this way were stored in a freezer at −15°C. They were melted at a room temperature prior to the analysis and were filtered [a 0.45 μm pore size (Millipore)] before the injection into the CE equipment.

3. Results and discussion

3.1. ITP–CZE separating conditions

The choice of the electrolyte system providing favorable separating conditions for a particular analyte in the ITP–CZE combination has rational guidelines also in instances when the composition of the multicomponent matrix is unknown and variable [19]. In addition, combinations of the ITP and CZE electrolyte systems offering minimum disturbances due to the sample matrix can be predicted for the analyte with the aid of calculations based on a relatively simple model when the acid–base equilibria are effective in the separation [19,39]. However, such an explicit prediction is not possible, e.g., for the separation mechanisms based on complex equilibria or when several separation mechanisms is combined. Therefore, our search for the electrolyte system providing in the CZE stage a ''clean'' orotic acid zone also in instances when this was loaded in urine matrices followed general rules formulated for an optimum performance of the ITP–CZE combination [19]: (1) A number of sample constituents transferred into the CZE stage with the analyte after the ITP sample pretreatment is kept at a minimum; (2) The separation mechanism employed for the sample pretreatment in the ITP stage differs from that used in the CZE stage for a final separation of the analyte from matrix constituents transferred into this stage.

The compositions of the ITP electrolyte systems used in our experiments (Table 1) were chosen to meet, mainly, the first of these criteria. With respect to acid–base properties of orotic acid ($pK_a = 2.07$ [23]), the separations (sample pretreatment) at a low pH were favored. This approach reduced a number of matrix constituents transferable into the CZE stage as it prevented ITP migrations of the constituents with pK values higher than that of the terminating constituent (see, e.g., Refs. [40,41]). A further reduction of this number was achieved by transferring into the CZE stage only the constituents accompanying orotic acid in its ITP migration position between a pair of discrete spacers (Fig. 1). Such a transfer of the analyte into the CZE stage was already shown very effective in the analysis of urine samples [18]. Under our working conditions, with phosphate and citrate as discrete spacers, it prevented entrance of 60–90% of urine matrix constituents from the ITP stack into the CZE stage. α-CD used in one of the leading electrolytes employed in this work (ITP2, in Table 1) was found [42], to enhance this pH based sample clean up of urine.

Final separations of orotic acid from matrix con-

Table 1
Electrolyte systems[a]

Parameter	ITP		Parameter	CZE	
	ITP1	ITP2		CZE1	CZE2
Solvent	Water	Water	Solvent	Water	Water
Leading anion	Chloride	Chloride	Carrier anion	Asp	Asp
Concentration (mol/l)	10^{-2}	10^{-2}	Concentration (mol/l)	$3\cdot10^{-2}$	$3\cdot10^{-2}$
Counterion	β-ala	β-ala	Counterion	DETA	DETA
Concentration (mol/l)	$1.45\cdot10^{-2}$	$1.45\cdot10^{-2}$	Concentration (mol/l)	$3.9\cdot10^{-3}$	$3.9\cdot10^{-3}$
EOF suppressor	m-HEC	m-HEC	EOF suppressor	m-HEC	m-HEC
Concentration (%, w/v)	0.2	0.2	Concentration (w/v, %)	0.2	0.2
Complexing agent	–	a-CD	Complexing agent	–	β-CD
Concentration (mol/l)	–	$3\cdot10^{-2}$	Concentration (mmol/l)	–	10^{-2}
pH	3.25	3.25	pH	3.5	3.5
Terminating anion	Asp	Asp			
Concentration (mol/l)	$5\cdot10^{-3}$	$5\cdot10^{-3}$			

[a] Asp=aspartic acid; β-ala=β-alanine; a-CD=a-cyclodextrin; β-CD=β-cyclodextrin; DETA=diethylenetriamine; m-HEC=methylhydroxyethylcellulose; EOF=electrosmotic flow.

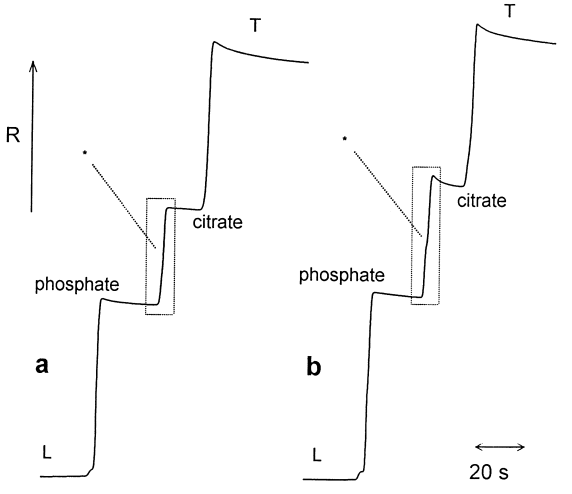

Fig. 1. Typical isotachopherograms as obtained from the pretreatment of urine samples in the ITP stage of the ITP–CZE combination. (a) A pretreatment based on differences in acid–base properties of orotic acid and matrix constituents (system ITP1, in Table 1); (b) a pretreatment combining the use of differences in acid–base properties and host–guest complexations (α-CD) of orotic acid and matrix constituents (system ITP2, in Table 1). A 20-times diluted urine sample, spiked with orotic acid at a 10^{-6} mol/l concentration, was injected (a 30 μl internal injection loop) into the ITP–CZE equipment in both instances. Dash line boxes mark orotic acid (asterisks indicate its migration position in the ITP stack) containing sample fractions transferred into the CZE stage of the ITP–CZE combination. L, T=leading and terminating zones, respectively. The driving current during the pretreatment was stabilized at 250 μA.

stituents in the CZE stage were carried out at a low pH (CZE1 and CZE2, in Table 1). Diethylenetriamine, a multiple charged constituent, serving as a pH buffering counter-ionic constituent in the carrier electrolytes implemented into the separations in this stage a differentiation of separands via their effective charge numbers [43]. From electropherograms in Fig. 2a and b it is apparent that this separation mechanism did not lead to the resolution of single charged orotate from matrix constituents in any of the both alternatives used for the ITP sample pretreatment. Nevertheless, these electropherograms show that the matrix constituents accompanying orotic acid in the transferred fractions differed in these ITP electrolyte systems. This apparently indicates different separation mechanisms effective in the ITP sample pretreatment procedures based on these electrolyte systems.

α-CD and β-CD significantly influence the effective mobilities of many urine constituents that migrate anionically at low pH values while their influences on the effective mobility of orotic acid are only marginal [42]. Therefore, β-CD was used (CZE2, in Table 1) to implement into the separation in the CZE stage the separation mechanism based on differences in the host–guest complexing properties of orotic acid and the transferred matrix constituents. An electropherogram in Fig. 2c shows that this mechanism led to a significant improvement of the separating conditions in this stage. This improvement

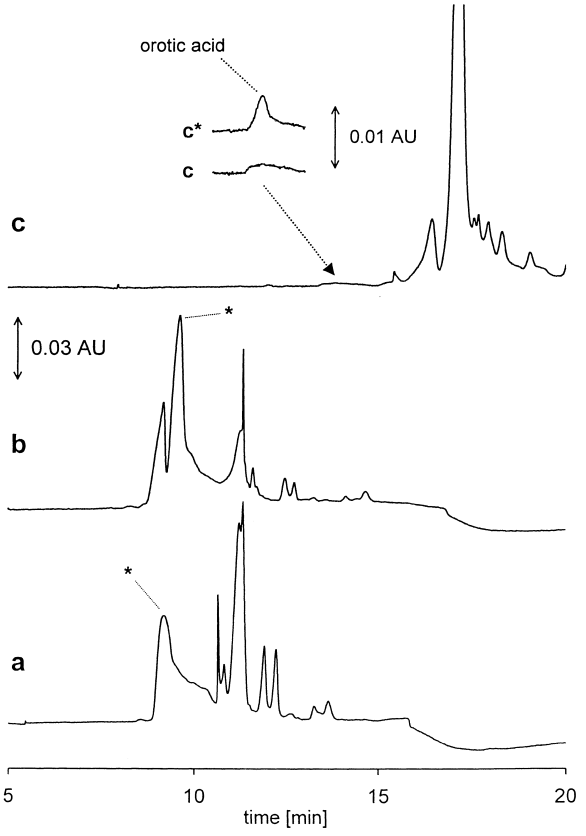

Fig. 2. Electropherograms from the separations of orotic acid fractions of urine obtained in the electrolyte systems implementing different separation mechanisms into the ITP and CZE stages of the combination. The electropherograms were plotted from the spectral data acquired by DAD at a 218 nm wavelength (the migration positions of orotic acid are indicated by asterisks). In all instances the same urine sample was injected (30 μl volumes of a 20-fold diluted urine). The transferred orotic acid fraction is marked by a dash line box on a particular isotachopherogram (Fig. 1). (a) Differences in acid–base properties (ITP) vs. differences in acid–base properties and charge numbers (CZE) [ITP1–CZE1, in Table 1]; (b) differences in acid–base and complexing (α-CD) properties (ITP) vs. differences in acid–base properties and charge numbers (CZE) [ITP2–CZE1, in Table 1]; (c) differences in acid–base and complexing (α-CD) properties (ITP) vs. differences in the charge numbers and complexing (β-CD) properties (CZE) [ITP2–CZE2 (Table 1)]. (c*) the same sample as in (c) spiked with orotic acid at a 10^{-6} mol/l concentration. The driving current during the ITP pretreatment was stabilized at 250 μA. The driving currents in the CZE stage were stabilized at 160 μA [(a) and (b)] or 120 μA (c).

is, especially, apparent when this electropherogram is compared to the one obtained, under otherwise identical separating and ITP sample pretreatment conditions, without β-CD in the carrier electrolyte (Fig. 2b).

The separation at a low pH combined with the use of α-CD and β-CD in the ITP and CZE electrolyte systems (ITP2–CZE2, in Table 1), respectively, provided a considerable selectivity in the separation of orotic acid from urine matrix constituents. For example, under these separating conditions even a complete transfer of the matrix constituents migrating in the ITP stack into the CZE stage introduced into its detection only minimum disturbances (Fig. 3). In addition, analyses of a larger series of urine samples of healthy adults by the ITP–CZE–DAD combination revealed a versatility of these separating conditions in terms of minimized interferences in the migration position of the analyte.

3.2. DAD and quantitation of orotic acid

6 and $7 \cdot 10^{-8}$ mol/l concentrations of orotic acid were estimated as its LOD at 280 and 218 nm detection wavelengths, respectively, under electrolyte conditions providing minimum interferences due to urine constituents (ITP2–CZE2, Table 1). It is apparent that such low LOD values can be attributed, besides the performance of DAD, to a 30 μl sample volume loaded onto the separation system and a 320 μm I.D. capillary tube used in the CZE stage of the separation [44]. Practical limits attainable in the detection of orotic acid at 218 and 280 nm detection wavelengths in urine samples were set by clean-up capabilities of the ITP–CZE combination under the present working conditions. These are are illustrated by typical electropherograms as obtained from the analyses of a larger series of urine samples (Fig. 4). In some of these samples we detected at 280 nm a trace constituent migrating in the neighborhood of the analyte (it corresponded to about $2–3 \cdot 10^{-7}$ mol/l concentration of orotic acid in the injected sample). On the other hand, no constituent at a comparable concentration was detected at 218 nm and, here, peak levels of potential interferents approached the detector noise at this detection wavelength (see also Fig. 5b). However, their fluctuations from sample to sample caused that only orotic acid present in urine matrices at $3–4 \cdot 10^{-7}$ mol/l concentrations (30 μl volumes of 20-fold diluted urine samples) could be detected with confidence at this wavelength. A

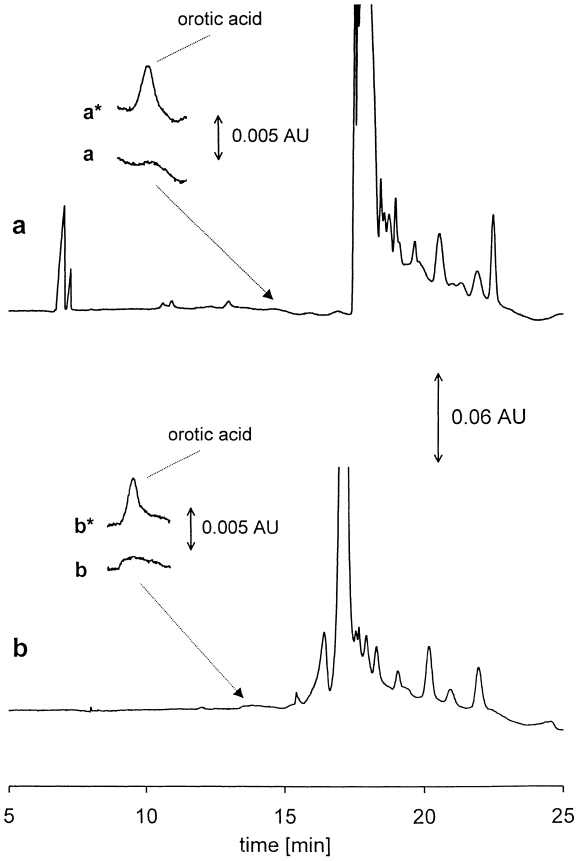

Fig. 3. An impact of the size of the pretreated orotic acid fraction of urine on CZE profiles of matrix constituents in the migration position of the analyte under optimized separating conditions [ITP2–CZE2 (Table 1)]. (a) A transfer of the complete ITP stack (defined by L and T on the isotachopherogram in Fig. 1b); (a*) the same as in (a) only the sample was spiked with orotic acid at a 10^{-6} mol/l concentration; (b) a transfer of the fraction between phosphate and citrate (the fraction is marked by a dash line box on an isotachopherogram in Fig. 1b); (b*) the same as in (b) with the sample spiked with orotic acid at a 10^{-6} mol/l concentration. In all instances the same urine sample as in Fig. 2 was used. The electropherograms were plotted from the spectral data acquired by the DAD at a 218 nm wavelength. The driving currents in the ITP and CZE stages were 250 and 120 μA, respectively.

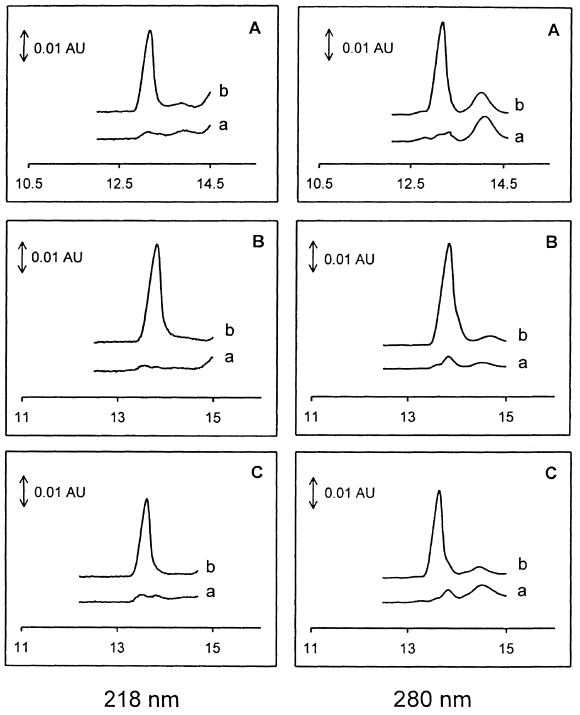

Fig. 4. Electropherograms from the analyses of urine samples and urine samples spiked with orotic acid (at $3{\cdot}10^{-6}$ mol/l concentrations) by the ITP–CZE–DAD combination. (A–C) Urine samples (a) obtained from healthy adult volunteers (20-fold dilutions) and the same samples spiked with orotic acid (b). The separations were carried out in the electrolyte system ITP2–CZE2 (Table 1) and phosphate and citrate defined the transferred analyte fraction (see Fig. 1). The driving currents in the ITP and CZE stages were 250 and 120 μA, respectively.

fragment of the electropherogram of a 20-fold diluted urine sample spiked with orotic acid at a $6{\cdot}10^{-7}$ mol/l concentration and the one for a $2{\cdot}10^{-7}$ mol/l concentration of orotic acid present in a model mixture (estimated as its limit of quantitation) illustrates detection possibilities of the present ITP–CZE–DAD procedure close to limiting conditions (Fig. 5).

Urine samples spiked with orotic acid on two fortification levels, corresponding to the concentrations of this acid in urine [23], were used in an evaluation of performance of the ITP–CZE–DAD combination in quantitation of orotic acid. The recovery data (Table 2), calculated from the determination of the acid based on an external calibration (Table 3), indicate that the present ITP–CZE procedure offers its accurate determinations in urine. Higher recovery values as found in the same ITP–

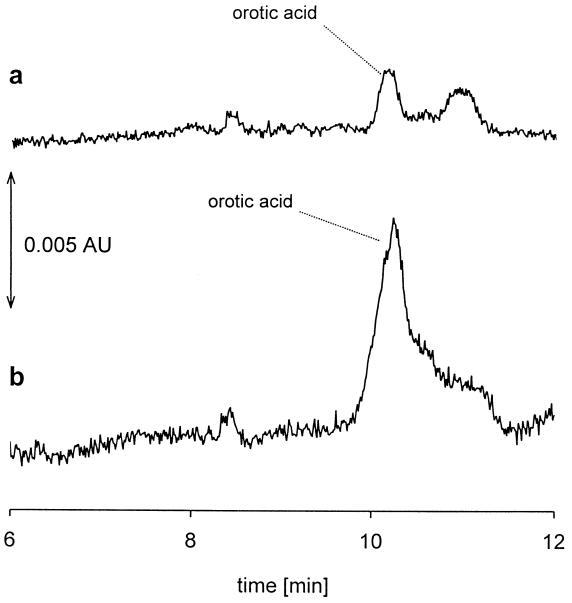

Fig. 5. Electropherograms from the separations of orotic acid present in model and urine samples at concentrations corresponding to its limit of quantitation attainable by the ITP–CZE–DAD at a 218 nm wavelength. (a) A run with a model sample containing orotic acid (at a $2 \cdot 10^{-7}$ mol/l concentration), phosphate and citrate (each at a $5 \cdot 10^{-4}$ mol/l concentration). (b) A run with a urine sample (a 20-fold diluted urine) spiked with orotic acid at a $6 \cdot 10^{-7}$ mol/l concentration. The separations were carried out in the electrolyte system ITP2–CZE2 (Table 1)]. In both instances, phosphate and citrate defined the transferred analyte fraction (see Fig. 1). The driving currents in the ITP and CZE stages were 250 and 120 μA, respectively.

CZE runs at 280 nm can be very likely ascribed to a small systematic bias due to a constituent migrating in a close vicinity of the analyte (see Fig. 4). Repeatabilities of the determination of orotic acid on both fortification levels characterized 3–5% RSD values.

3.3. Confirmation of identity of orotic acid by DAD

A spectrum of orotic acid (200–500 nm) acquired by the DAD system in the CZE stage of separation under the working conditions identical with those used in the ITP–CZE analysis of urine samples, served as a reference spectrum in the confirmation of identity of this acid in model and urine samples. This spectrum (Fig. 6) was corrected for the background in the way described below.

Pearson's correlation coefficient served as a numerical criterion (match factor) expressing a match of the spectrum of the analyte with the standard spectrum in our experiments [45].

Although the separation in the ITP stage concentrated orotic acid present in the injected sample 500–1000 times, the values of absorbance ratios at characteristic wavelengths (maxima at 212 and 278 nm and a minimum at 243 nm), plotted along its peak as registered in the CZE stage, indicate contributions of the carrier electrolyte to these values (Fig. 7). A minimum impact of the electrolyte on the analyte spectrum can be expected in the peak apex. An average spectrum calculated from the spectra acquired before the start and behind the end of the peak of the acid in an actual ITP–CZE run was used for this background correction. Pearson's correlation coefficients for corrected and uncorrected spectra of orotic acid in Table 4 show a relevance of this correction procedure. As is apparent from these data the correction procedure was needed already for a $5 \cdot 10^{-6}$ mol/l concentration of orotic acid in the injected sample. Therefore, in identity confirmations

Table 2
Recoveries of orotic acid in urine[a]

	Added (mol/l)					
	10^{-6}	10^{-6}	10^{-6}	$2 \cdot 10^{-6}$	$2 \cdot 10^{-6}$	$2 \cdot 10^{-6}$
Found (218 nm) (mol/l)	$0.87 \cdot 10^{-6}$	$0.88 \cdot 10^{-6}$	$0.90 \cdot 10^{-6}$	$1.82 \cdot 10^{-6}$	$1.83 \cdot 10^{-6}$	$1.88 \cdot 10^{-6}$
Found (280 nm) (mol/l)	$0.94 \cdot 10^{-6}$	$1.00 \cdot 10^{-6}$	$0.98 \cdot 10^{-6}$	$1.86 \cdot 10^{-6}$	$1.85 \cdot 10^{-6}$	$1.85 \cdot 10^{-6}$

[a] Three samples fortified on both concentration levels.

Table 3
Regression equations of the calibration graphs ($y = ax + b$) for the determination of orotic acid at 218 and 280 nm detection wavelengths of DAD[a]

Wavelength (nm)	Parameter				
	a	s_a	b	s_b	r
218	139.4×10^6	0.93×10^6	−13.07	3.69	0.9998
280	157.3×10^6	2.17×10^6	10.41	8.61	0.9991

[a] y = peak area (AU. min); x = concentration of orotic acid in the injected sample (mol/l); r = correlation coefficient; s_a = standard deviation of the slope (a) of the calibration line; s_b = standard deviation of the intercept (b) of the calibration line; *for 12 data points covering a $2–5\cdot10^{-7}$ mol/l concentration span of the analyte.

of orotic acid we used the spectrum acquired in the peak apex corrected in this way. Smoothing of the corrected spectra of orotic acid (by the procedure of Savitzky-Golay with a 21-point window) improved the values of the Pearson's correlation coefficient when the acid was present in the injected model sample at a $5\cdot10^{-7}$mol/l concentration or less (Table 4). We found that under these data processing conditions a $2\cdot10^{-7}$ mol/l concentration of orotic acid in the injected sample (Fig. 8) still provided in the CZE stage spectrum matching the reference with an acceptable value of the Pearson's correlation coefficient (Table 4).

In a confirmation of identity of orotic acid present in urine samples we used the same procedure as discussed above. Here, contrary to model samples, traces of comigrants present close to the migration position of the acid (see Fig. 4) made the above correction of the spectrum for the background slightly less efficient. Therefore, the values of the Pearson's correlation coefficient higher than 0.99 (as-

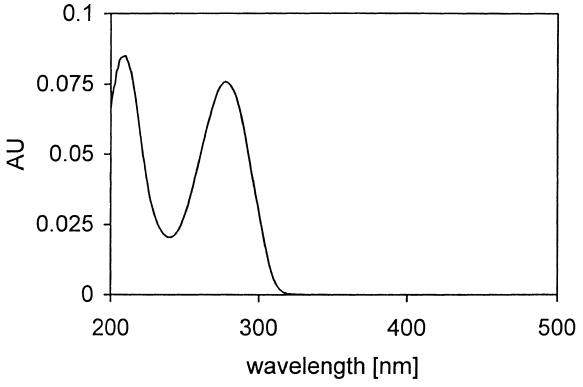

Fig. 6. A reference spectrum of orotic acid acquired by DAD in the CZE stage of the ITP–CZE combination. The spectrum was acquired from the peak apex and it was corrected in the way described in the text. The injected sample, containing orotic acid at a $8\cdot10^{-6}$ mol/l concentration, was separated in the electrolyte system ITP2–CZE2 (Table 1). Phosphate and citrate defined a transfer of the acid into the CZE stage (see Fig. 1). The driving currents in the ITP and CZE stages were 250 and 120 μA, respectively.

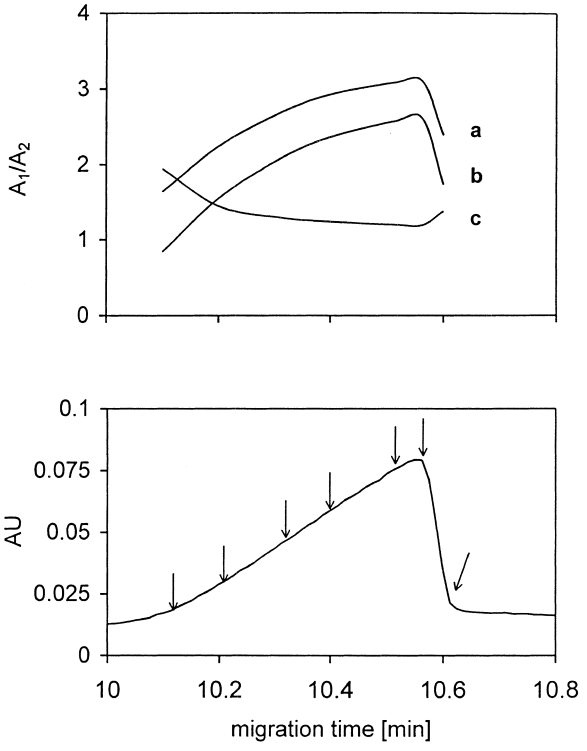

Fig. 7. Plots of ratios of absorbancies (A) at characteristic wavelengths of orotic acid along its peak in the CZE stage. The plots are based on the data calculated for the positions on the peak marked with arrows. (a) A_{212}/A_{243}; (b) A_{278}/A_{243}; (c) A_{212}/A_{278}. For further details see Fig. 6.

Table 4
Pearson's correlation coefficients for uncorreted and corrected spectra of orotic acid present at various concentrations in model and urine samples

Concentration of orotic acid in the sample (mol/l)	Raw spectrum	Spectrum corrected for the background	Spectrum corrected for the background and smoothed
Model samples			
$2\cdot10^{-7}$	0.7210	0.9918	0.9974
$5\cdot10^{-7}$	0.8037	0.9977	0.9994
$8\cdot10^{-7}$	0.8492	0.9995	–
10^{-6}	0.8507	0.9993	–
$2\cdot10^{-6}$	0.9509	0.9998	–
$5\cdot10^{-6}$	0.9881	1.0000	–
Urine samples			
10^{-6}	0.9708	0.9980	0.9984
$6\cdot10^{-7}$	0.9348	0.9967	0.9976

sumed to give an acceptable certainty in a confirmation of the identity of the analyte) were not reached when the concentration of orotic acid in the injected sample was lower than ca. $3–5\cdot10^{-7}$ mol/l. Although this was about 2–3 times higher concentration in comparison to model samples (Fig. 8), spectra acquired on this concentration level of the analyte in a urine matrix (Fig. 9), undoubtedly, illustrate benefits of the ITP–CZE combination in providing working conditions suitable for a DAD identification of orotic acid, especially, when current possibilities of DAD coupled to single-column CE separation systems are also considered [33–37].

4. Conclusions

Experiments with model and urine samples performed in the ITP–CZE equipment with the column-coupling configuration of the separation unit and monitored in the CZE stage by DAD showed that this CE combination provides very favorable conditions for DAD and identification of the analytes. Undoubtedly, $6–7\cdot10^{-8}$ mol/l concentration limits of detection at 280 and 218 nm wavelengths as estimated for our test analyte (orotic acid) from CE runs with model samples are closely linked with a 30 μl sample injection volume in which the analyte could be loaded into the equipment and concentrating capabilities of ITP.

From a practical point of view, it is important that the ITP–CZE combination provides means for an effective combination of the electrolyte systems so that besides the concentration of orotic acid we could achieve a significant sample clean up when the acid was to be detected and/or identified in urine samples. The use of α- and β-cyclodextrins in the electrolyte solutions was found very beneficial in this respect. These clean up capabilities, favorable from the point of view of the absorbance detection and/or spectral identification, made possible to detect with confidence $3–5\cdot10^{-7}$ mol/l concentration of orotic acid present in urine samples (30 μl volumes of 20-fold diluted urine samples).

Using current correction and smoothing procedures we could obtain analytically relevant DAD spectra of orotic acid also in instances when this was present in the injected model sample at a $2\cdot10^{-7}$ mol/l concentration (this concentration coincided with an estimated limit of the determination of orotic acid). The DAD spectra of orotic acid present in urine samples (30 μl injection volumes of 20-fold diluted urine samples) acquired at $4–6\cdot10^{-7}$ mol/l concentrations of the acid still matched its reference spectrum with reasonable certainties (Fig. 9). Here, apparently, trace constituents from urine matrix migrating in the neighborhood of orotic acid (see Figs. 4 and 5), prevented a closer approach to a limiting value ($2\cdot10^{-7}$ mol/l concentration) as found for the same sample volumes in the ITP–CZE runs with model samples. Nevertheless, when we consider concentration levels of orotic acid in clinically

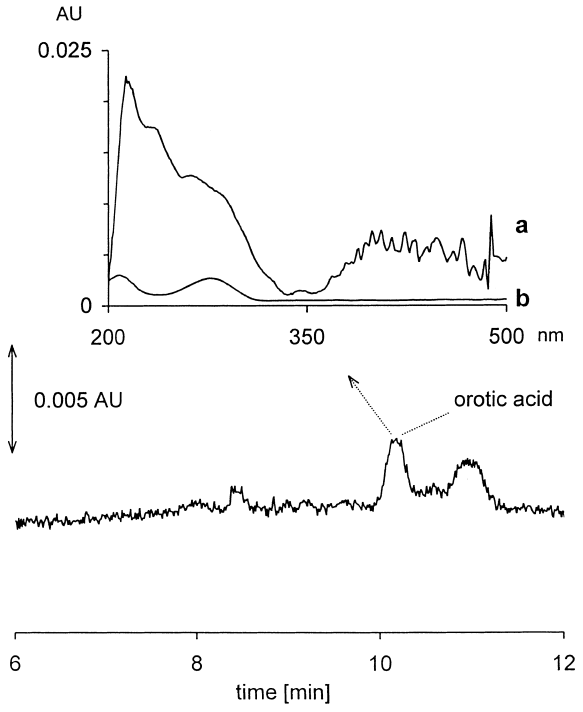

Fig. 8. A DAD spectrum acquired for a $2 \cdot 10^{-7}$ mol/l concentration of orotic acid in the CZE stage of the ITP–CZE combination for a 30 μl sample injection volume. (a) A raw spectrum acquired in the peak apex (for details describing the spectrum acquisition see Experimental); (b) a corrected and smoothed spectrum (a) using a correction and smoothing procedure described in the text. The spectrum matched the reference spectrum (Fig. 6) with a 0.9974 value of the Pearson's correlation coefficient. The separations were carried out in the electrolyte system ITP2–CZE2 (Table 1). Phosphate and citrate defined the transferred analyte fraction (see Fig. 1). The driving currents in the ITP and CZE stages were 250 and 120 μA, respectively.

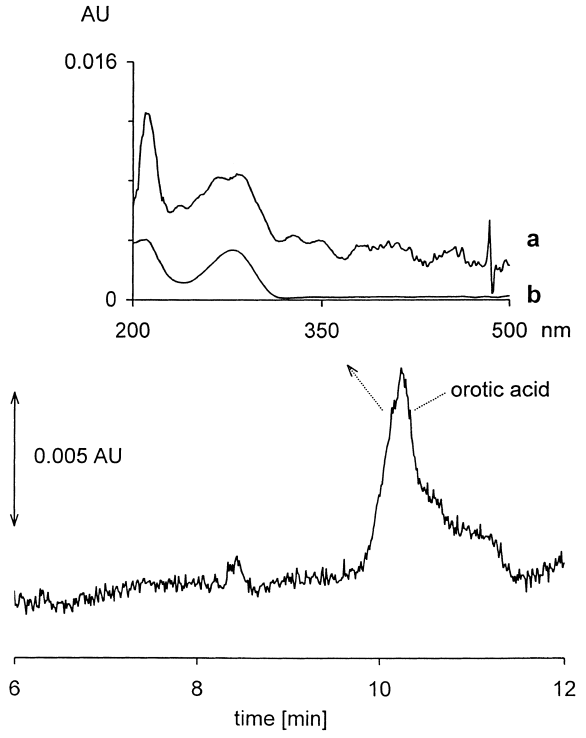

Fig. 9. A confirmation of identity of orotic acid present in the injected urine sample (30 μl) at a $6 \cdot 10^{-7}$ mol/l concentration via its DAD spectrum acquired in the CZE stage of the ITP–CZE combination. (a) A raw spectrum acquired in the peak apex (for details describing the spectrum acquisition see Experimental); (b) a corrected and smoothed spectrum (a) using a correction and smoothing procedure described in the text. The spectrum matched the reference spectrum (Fig. 6) with a 0.9978 value of the Pearson's correlation coefficient. The separations were carried out in the electrolyte system ITP2–CZE2 (Table 1). Phosphate and citrate defined the transferred analyte fraction (see Fig. 1). The driving currents in the ITP and CZE stages were 250 and 120 μA, respectively.

relevant samples [23], these results indicate that DAD coupled to the ITP–CZE combination offer means for a confirmation of identity of orotic acid in urine in such instances.

A need to dilute urine samples, to prevent losses of anionic constituents due to precipitation [18], reduced amount of urine that could be loaded onto the separation system by the injector (a 30 μl internal loop) of the analyzer. From the work by Procházková et al. [23] it can be deduced that the amount of urine ionic constituents loadable on the ITP–CZE separation system used in this work can be increased 3–4 times in comparison to what was possible in the present work. This is indicating a potential for further reduction of concentrations at which our model analyte could detected or identified in urine by the ITP–CZE–DAD combination.

Although this work was focused only on one analyte and urine as a typical multicomponent matrix it, undoubtedly, indicates very promising po-

tentialities of the ITP–CZE–DAD combination in the identification and quantitation of trace analytes present in biological matrices, in general.

Acknowledgements

This work was supported by a grant from the Slovak Grant Agency for Science under the project No. 1/7247/20. The authors thank to J&M (Aalen, Germany) for providing a TIDAS multiwavelength photometric absorbance DAD system.

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