ELSEVIER

Journal of Chromatography A, 838 (1999) 213–221

JOURNAL OF CHROMATOGRAPHY A

Analysis of orotic acid in human urine by on-line combination of capillary isotachophoresis and zone electrophoresis

Andrea Procházková, Ludmila Křivánková*, Petr Boček

Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, 611 42 Brno, Czech Republic

Abstract

The techniques of the on-line combination of capillary isotachophoresis with zone electrophoresis in two coupled capillaries (ITP–CZE) and a single capillary zone electrophoresis (CZE) were used for the sensitive determination of orotic acid (OA) in human urine. The simple CZE system was successfully applied for fast and reliable analyses of urine of healthy adult volunteers (the detection limit $1.7 \cdot 10^{-6}$ *M* OA, the total time of analysis 6 min). However, this method failed in analyses of OA in urine of ill children due to more complex matrix of the samples. Here, the ITP preconcentration and preseparation step coupled on-line with CZE proved to serve well with an electrolyte system developed and optimized for this purpose. The maximum selectivity and resolution of OA from other sample constituents in ITP–CZE was achieved by use of an electrolyte system of very low pH 2.15 both for ITP and CZE stage. The sensitivity of detection and simplicity of OA identification were enhanced by use of an external UV scanning detector. High sensitivity of ITP–CZE combination (limit of detection $3 \cdot 10^{-7}$ *M* OA), low sample consumption (1 μl), good reproducibility of migration times (inter-day RSD$<$1.86%) and acceptable reproducibility of the determination of OA in urine samples (average RSD$=$7.27%) make this technique suitable for routine determination of trace concentration of OA especially in urine of ill children under various pathological conditions and medication.

Keywords: Isotachophoresis–capillary zone electrophoresis; Orotic acid

1. Introduction

Orotic acid (OA) is an intermediary metabolite in the biosynthesis of pyrimidine nucleotides [1]. Its concentration in urine of healthy persons is lower than 10^{-5} *M*, however, at the deficiency of enzyme uridine monophosphate synthase, which participates in the synthesis of uridylic acid from OA, the excretion of OA into urine increases (orotic aciduria) [1–3]. Orotic aciduria also occurs in the deficiencies of urea cycle enzymes, in lysinuric protein intolerance and is induced by use of some drugs [3].

To diagnose inborn errors of organisms, it is important to use a method enabling the determination of both increased and normal levels of OA in urine. A variety of methods based on different principles describe the determination of OA in different matrices, e.g., high-performance liquid chromatography (HPLC) [3–9], gas chromatography–mass spectrometry (GC–MS) [5,6,10], isotachophoresis (ITP) [11], capillary zone electrophoresis (CZE) [12], colorimetric [13,14] and enzymatic methods [15]. The majority of them, however, when applied for the analyses of complex biological samples, suffer from low sensitivity and require a time consuming and laborious sample pretreatment.

To avoid sample pretreatment and achieve sufficiently low limit of detection (LOD), the on-line

*Corresponding author. Tel: +420-5-726-8123; fax: +420-5-4121-2113.

E-mail address: krivan@iach.cz (L. Křivánková)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00114-4

combination of capillary isotachophoresis with zone electrophoresis (ITP–CZE) in a column coupling instrumentation [16–18] was chosen. The technique combines the high separation capacity of ITP with high separation efficiency and sensitivity of CZE. On condition that a suitable electrolyte system is selected for ITP step, performed in the first preseparation capillary of a higher internal diameter (I.D.), sample components create correct and stable isotachophoretic zones with sharp boundaries. The concentration in their zones is adjusted at a defined ratio according to the concentration of the leading ion, which results in preconcentration of the microcomponents. The macrocomponents are detected in the first step and driven afterwards out of the separation path. Only a well defined fraction of the sample containing the stacked analyte is transferred into the second analytical capillary and analyzed by CZE with high resolution under the optimum conditions. It was already proved that ITP–CZE performed in a column coupling instrumentation is capable to separate micro- and macrocomponents in the concentration ratio up to $1:10^6$ in complex samples and the high sensitivity and reproducibility of the CZE separation are not influenced by varying sample composition and origin [19–22].

This work presents a simple and reliable ITP–CZE method for a sensitive determination of OA in human urine. For the success, sensitivity and reproducibility of the ITP–CZE analysis, the selection of a suitable electrolyte system is of key importance. The suitable electrolyte system was developed and optimized for ITP–CZE and adopted for a single CZE analysis of OA in human urine as well. Results obtained by both techniques were compared.

2. Experimental

2.1. Instrumentation

A CS isotachophoretic analyzer EA 100 Villa Labeco (Spišská Nová Ves, Slovak Republic) equipped with the column switching system was used for the ITP–CZE measurements. The capillary used for ITP separations made from fluorinated ethylene–propylene copolymer (FEP) was of 16 cm to the bifurcation point (9 cm to the conductivity detector)×0.8 mm I.D. The CZE capillaries were both made from FEP and fused silica. The I.D. of the FEP capillaries was 0.2 mm, the total length was either 23 or 35 cm, the effective lengths to the UV detector (λ=254 and 289 nm) were 18.5 and 30.5 cm, respectively. The fused silica capillary for CZE was of 80 cm [effective length to the scanning detector (Spectra Focus, Thermo Separation Products, San Jose, CA, USA) 60 cm]×0.1 mm I.D. In the ITP step, constant current of 500 μA was used for the separation and for the detection was decreased to 250 μA. The CZE separation in the second capillary was run at constant current of 35–120 μA. The sample was injected with a 10–μl microsyringe (Hamilton, Bonaduz, Switzerland). The electrolyte chambers containing the leading electrolyte (LE) and the background electrolyte (BGE) were separated from the electrolytes in capillaries by semipermeable Cellophane membranes.

All the CZE experiments in a single capillary were performed with a P/ACE 5010 capillary electrophoretic instrument controlled by P/ACE Control Software (Beckman, Fullerton, CA, USA). The fused silica capillaries of I.D. 0.1 mm with the length either 27 cm or 47 cm [19 cm and 39 cm to the UV detector (λ=200, 280 nm), respectively] were used uncoated or with the inner surface coated with linear polyacrylamide according to the Hjerténś method [23] modified as described in Ref. [24]. The polarity of the high voltage was set with the cathode at the injection end and the anode at the detection end. The electroosmotic mobility measured in the coated capillary with mesityloxide at reversed polarity was less than $2\cdot10^{-9}$ m^2 V^{-1} s^{-1}. The samples were injected by pressure 34.4 kPa for 5–30 s, corresponding to a volume of about 39–234 nl. Separations were performed at a constant voltage of 7–15 kV and at the operating temperature 25°C.

2.2. Chemicals

All chemicals used were of the analytical grade. Orotic acid and spermine were purchased from Fluka (Buchs, Switzerland), glutamic acid from Merck (Darmstadt, Germany), 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) and orotate

reductase (EC 1.3.1.14) from Sigma (St. Louis, MO, USA), hydroxypropylcellulose (HPC) from Ega (Steinheim/Albuch, Germany). The other chemicals were supplied by Lachema (Brno, Czech Republic). The fresh untreated samples of urine were obtained from healthy volunteers and from ill children under various pathological conditions and medication (Medical Hospital, Olomouc, Czech Republic), the urinary creatinine was determined using the classical Jaffé reaction with the Hitachi 917 automatic analyzer (Boehringer, Mannheim, Germany). Deionized water prepared by mixed-bed ionex aqua purificator G7749 (Miele, Gütersloh, Germany) was used for the preparation of all solutions.

2.3. Sampling

Urine of healthy volunteers and ill children was stored frozen at −8°C, before analysis it was thermostatted at 50°C for 1 h and sonicated for 5 min with the aim to dissolve completely OA present in the sample. Urine samples were diluted with deionized water (1:4, v/v) to avoid the undesirable difference between the sample and the electrolyte density and analyzed directly without any pretreatment.

The separation capacity of ITP system used corresponds to 7 μl of undiluted urine. Usually, only 5 μl of diluted urine was injected because the separation capacity of CZE stage was limited even though the longer capillary (35 cm×0.2 mm I.D. or 80 cm×0.1 mm I.D.) was used.

3. Results and discussion

3.1. ITP–CZE

3.1.1. Selection of the electrolyte system for ITP–CZE

OA is a strong acid having low pK_a value (2.07 [25]) and ionic mobility of $33.1 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} [26]. In urine samples OA occurs in the presence of a variety of other micro- and macrocomponents, the effective mobilities of some of them are close to OA. The full ITP separation of OA from chlorides, citrates, lactates and especially from phosphates was achieved at the pH of the leading electrolyte (pH_{LE}) 6.8 (system I, Table 1). At this pH_{LE} all the macrocomponents created isotachophoretic zones migrating in front of the zone of OA and after the detection they were driven out of the capillary to the helping electrode, and thus removed from the migration path. The zone of OA was transferred quantitatively into the analytical capillary filled with BGE. For the full separation of OA from other unknown constituents of the sample, which were transferred together with OA into the analytical capillary, lower pH of BGE was used than the value adjusted in ITP step (6.95, calculated by using the procedure published in Ref. [27]). Further improvement of resolution was achieved by involving an interaction of polyamine spermine with sample components. To meet the demands on optimum pH (4–5.2) and spermine concentration (7 m*M*), the resulting concentration of glutamic acid in BGE was estimated to

Table 1
Electrolyte systems used

	ITP–CZE	
	System I	System II
Leading electrolyte	0.01 *M* HCl+BTP, pH 6.8+0.02% (w/v) HPC	0.01 *M* HCl+glycine, pH 2.15+0.02% (w/v) HPC
Terminating electrolyte	0.01 *M* glutamic acid	0.01 *M* H_3PO_4
Background electrolyte	0.03 *M* glutamic acid+0.007 *M* spermine, pH 5.2	0.03 *M* H_3PO_4+glycine, pH 2.15
	CZE	
	System III	System IV
Background electrolyte	0.1 *M* glutamic acid+0.02 *M* spermine, pH 5.2	0.03 *M* H_3PO_4+glycine, pH 2.15

be 0.03 *M*. This optimized composition of the electrolyte system I was verified in a series of samples of urine of healthy adult volunteers spiked with OA and a reliable resolution of OA from all other sample microcomponents was proved. In the analyses of urine of ill children under various pathological condition and medication, however, the resolution of OA from other absorbing microcomponents of the sample was unsatisfactory due to the more complex matrix. Reliable results were achieved by shifting pH of the separation medium to low values (system II, Table 1), which is possible due to low pK_a value of OA. At pH_{LE} 2.15 the effective mobility of majority of the macrocomponents (phosphates, lactates, citrates) is lower than that of OA. Phosphates are used both as a suitable terminating electrolyte (TE) for ITP step and a co-ion of BGE for CZE, optimum pH of BGE (pH_{BGE}) was found to be 2.15. This optimized electrolyte system II was tested on a series of children's urine and reliable results were obtained.

3.1.2. Qualitative and quantitative analysis

3.1.2.1. Urine of adult volunteers

Qualitative and quantitative analyses of urine of healthy adult volunteers were performed using the electrolyte system I under the optimum conditions. Detection was performed at both wavelengths offered by the instrumentation, i.e., 254 nm with Hg lamp or 289 nm with iodine lamp. Due to substantially lower noise of Hg lamp, better results and lower limit of detection (signal:noise=3:1) for OA determination were achieved using the detection at 254 nm in spite of the fact that OA has the absorption maximum at 284 nm.

The migration times of OA in CZE stage of ITP–CZE combination were constant regardless of the sample composition and amount of the sample injected. The intra- and inter-day relative standard deviation (RSD), the linearity and the equations of calibration curves of both peak height and area vs. OA concentration constructed in aqueous solutions and urine and achieved LODs are summarized in Table 2. The record of the ITP–CZE analysis of urine of a healthy adult person and urine spiked with $1 \cdot 10^{-4}$ *M* OA is shown in Fig. 1, the content of OA in all analyzed samples was found to be below the LOD.

3.1.2.2. Urine of ill children

The determination of OA in children's urine was performed in electrolyte system II. To increase further the sensitivity of detection and to make the identification of OA easier, the external UV Spectra Focus scanning detector was used and the absorption signal was followed at three wavelengths corresponding to two absorption maxima (λ=210 and 280

Table 2
Equations of calibration curves, linearity, RSDs and LODs for ITP–CZE

Electrolyte system		System I		System II	
Instrumentation		1st capillary: FEP 90×0.8 mm I.D. 2nd capillary: FEP 350×0.2 mm I.D. λ=254 nm (Villa Labeco)		1st capillary: FEP 90×0.8 mm I.D. 2nd capillary: silica 800×0.1 mm I.D. λ=280 nm (Spectra Focus)	
Sample		Aqueous solution	Urine of adults	Aqueous solution	Urine of children
Equation of calibration curve	Peak height (mAU) vs. c_{OA} (μM)	$y=1.0672+0.5655x$ $r^2=0.990$	$y=1.2367+0.6160x$ $r^2=0.9981$	$y=0.0653+0.8980x$ $r^2=0.9985$	$y=0.0276+0.9620x$ $r^2=0.9984$
	Peak area (μAU s) vs. c_{OA} (μM)	$y=-2146.1+19\,501.8x$ $r^2=0.9978$	$y=-11\,608.6+18\,711.1x$ $r^2=0.9996$	$y=-1626.6+30\,557.8x$ $r^2=0.9993$	$y=-2163.9+31\,957.1x$ $r^2=0.9974$
Linearity range		LOD $1 \cdot 10^{-4}$ *M* OA		LOD $2 \cdot 10^{-5}$ *M* OA	
LOD (*M*)		$3.6 \cdot 10^{-6}$	$3.0 \cdot 10^{-6}$	$3.8 \cdot 10^{-7}$	$4.0 \cdot 10^{-7}$
RSD of migration times (%)	Intra-day	1.12 (n=6)	1.54 (n=9)	1.08 (n=5)	1.10 (n=6)
	Inter-day	1.25 (n=8) within 4 days	1.86 (n=5) within 5 days	1.48 (n=6) within 4 days	1.49 (n=10) within 4 days

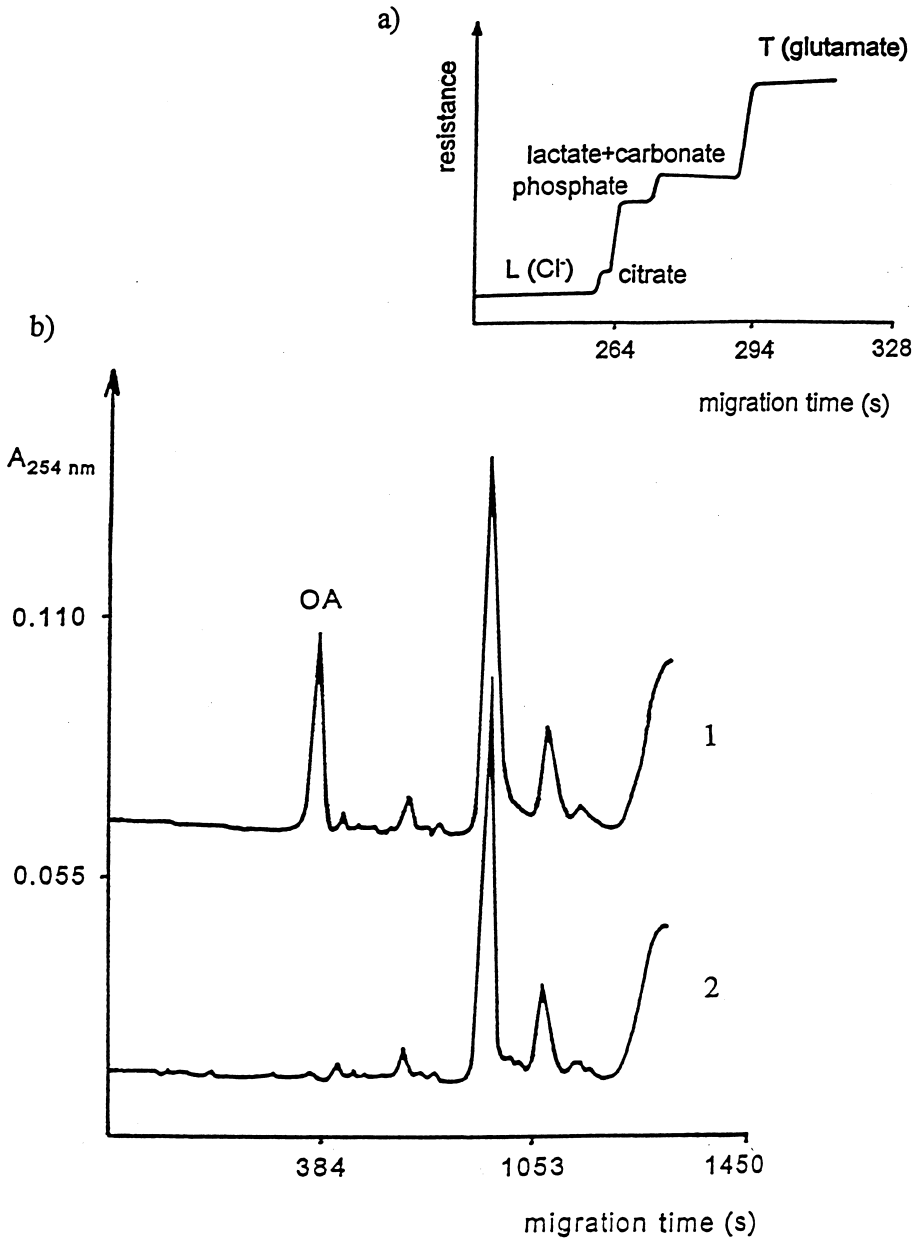

Fig. 1. Determination of OA in urine of a healthy adult person by ITP–CZE combination. (a) Trace of ITP step in the preseparation capillary; (b) trace of CZE step in the analytical capillary. Sample: 5 μl of 5× diluted urine of a healthy adult with (1) and without (2) addition of $1 \cdot 10^{-4}$ *M* OA. Electrolyte system I (Table 1). Instrumentation: CS isotachophoretic analyzer Villa Labeco, 1st capillary: FEP, 9 cm×0.8 mm I.D., conductivity detector, constant current 500 μA was used and decreased to 250 μA for the detection; 2nd capillary: FEP, 35 cm×0.2 mm I.D., UV detector (λ=254 nm), constant current 120 μA.

nm) and the minimum (λ=240 nm) of OA spectrum. The ratio of absorption signals was $A_{210}:A_{240}:A_{280}$= 10:2:9 and as no other microcomponent migrating in the neighborhood of OA showed similar ratio, it was used for the unambiguous identification of OA.

The reproducibility of migration times of OA in

CZE stage of ITP–CZE combination expressed by intra- and inter-day RSD, the equations of calibration curves constructed in aqueous solutions and children's urine, the linearity and achieved LODs are summarized in Table 2. No interferences were observed in 14 tested samples of children's urine, the concentrations of OA found are given in Table 3. To compare the results with the literature values, the concentrations of OA were related to the creatinine concentrations and it was found out that they correspond to the level of OA in urine of 1–10 years old children reported in [3,7]. The OA concentrations were evaluated from the peak area, average RSD of determination was 7.27% ($n=7$). The record of the ITP–CZE analyses of urine samples from two children is shown in Fig. 2.

3.2. Single CZE

3.2.1. Selection of the electrolyte system for CZE

For analyses of urine of adult volunteers, the composition of BGE identical with that in system I (Table 1) was applied and reliable results were obtained. By using this system for analyses of urine of ill children, however, the resolution of OA from other sample constituents was insufficient and the spermine concentration had to be increased. To maintain optimum pH_{BGE} 5.2, the concentration of glutamic acid in BGE was estimated to be 0.1 *M* (system III, Table 1). Using the electrolyte system III, however, the determined levels of OA in samples of children's urine were by one- to two-orders of magnitude higher compared to the results obtained by ITP–CZE or to values presented in literature [3,7]. Treatment of urine with orotate reductase (50 μl of urine was incubated with 0.011 U of enzyme orotate reductase for 10 min at 25°C prior to CZE analysis) revealed that the peak assigned to OA does not correspond solely to this analyte but also to other unknown absorbing sample constituents. The sufficient and reliable resolution of OA from other components of children's urine was not reached even if BGE of low pH 2.15 (system IV, Table 1) was used. Hence single CZE was used for reliable determination of OA in urine of healthy adults only.

3.2.2. Qualitative and quantitative analysis

The qualitative and quantitative analyses of urine of healthy adult volunteers were performed in the BGE of electrolyte system I (Table 1). In this system, OA was resolved from other sample constituents and reliably detected within 6 min in a capillary of the effective length 19 cm. The reproducibility of migration times, the linearity and equations of calibration curves and achieved LODs are summarized in Table 4, the record of analysis of urine of an adult volunteer is shown in Fig. 3.

4. Conclusions

From the results shown it follows that the main

Table 3
Content of OA found in samples of children's urine analyzed by ITP–CZE using electrolyte system II

Sample	c_{OA} (μ*M*)	$c_{creatinine}$ (m*M*)	$c_{OA}/c_{creatinine}$ (μmol/mmol)
1	5.73	4.1	1.4
2	<LOD	0.7	–
3	1.68	2.66	0.63
4	<LOD	0.99	–
5	<LOD	0.98	–
6	0.42	3.75	0.11
7	3.48	3.68	0.95
8	16.7	5.05	3.31
9	0.75	3.52	0.21
10	<LOD	1.36	–
11	5.79	6.5	0.89
12	<LOD	1.64	–
13	0.9	1.74	0.52
14	3.7	5.92	0.63

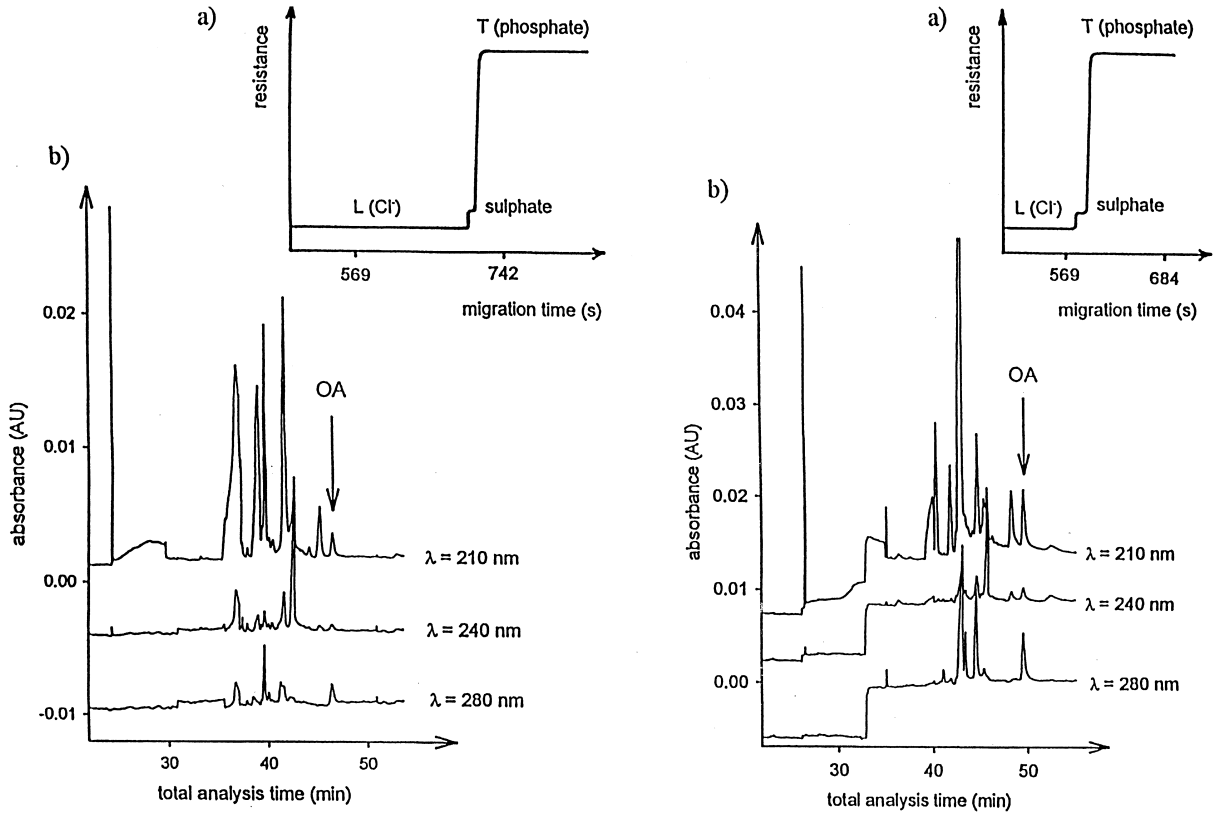

Fig. 2. Determination of OA in urine of two different children by ITP–CZE combination. (a) Trace of ITP step in the preseparation capillary; (b) trace of CZE step in the analytical capillary. Sample: 5 μl of 5× diluted urine from two different children, estimated OA concentration $5.79 \cdot 10^{-6}$ *M* and $1.67 \cdot 10^{-5}$ *M*. Electrolyte system II (Table 1). Instrumentation: CS isotachophoretic analyzer Villa Labeco, 1st capillary: FEP, 9 cm×0.8 mm I.D., conductivity detector, constant current 500 μA; 2nd capillary: silica, 80 cm (60 cm effective length)×0.1 mm I.D., UV scanning detector (λ=210, 240, 280 nm), constant current 35 μA.

difficulties of the determination of OA in human urine stem from the complexity of sample matrix which has great impact on the course of the separation process. CZE performed under optimized conditions in a single capillary arrangement gave good results when the urine samples from adults were analyzed, however, the method failed in analyses of more complex matrix of children's urine. The combination of CZE with an on-line preseparation ITP step enabled to perform both an effective clean-up of the sample resulting in high reproducibility of analyses, and to increase the analyte concentration in its zone resulting in lower LOD. Further enhancement of sensitivity and simplification of OA identification were obtained by using UV detector working at three wavelengths. Due to high sensitivity of determination (LOD $3 \cdot 10^{-7}$ *M* OA), low sample consumption (1 μl), good reproducibility of migration times (RSD<1.86%) and acceptable reproducibility of the determination (average RSD=7.27%) achieved, the technique of the ITP–CZE combination can be recommended for the routine determination of OA in urine of both adults and children.

Acknowledgements

This work was supported by the Grant Agency of the Academy of Sciences of the Czech Republic, grant No. A 4031703, by the Grant Agency of the Czech Republic, grant No. 203/96/0124, and by the Ministry of Education, grant No. VS-96021. The

Table 4
Equations of calibration curves, linearity, RSDs and LODs for single capillary CZE

Electrolyte system		BGE of system I
Instrumentation		Silica capillary: 27 cm (effective length 19 cm)×0.1 mm I.D. λ=200 nm (P/ACE 5010)
Sample		Adult urine
Equation of calibration curve	Peak height (mAU) vs. c_{OA} (μM)	$y=-0.3319+0.3186x$ $r^2=0.9995$
	Peak area (μAU s) vs. c_{OA} (μM)	$y=857.41+1571.87x$ $r^2=0.9991$
Tested range of linearity		$1.7\cdot10^{-6}$–$8\cdot10^{-5}$ M OA
LOD (M)		$1.7\cdot10^{-6}$ M OA
RSD of migration times (%)	Intra-day	1.21 (n=8)
	Inter-day	1.53 (n=23) within 5 days

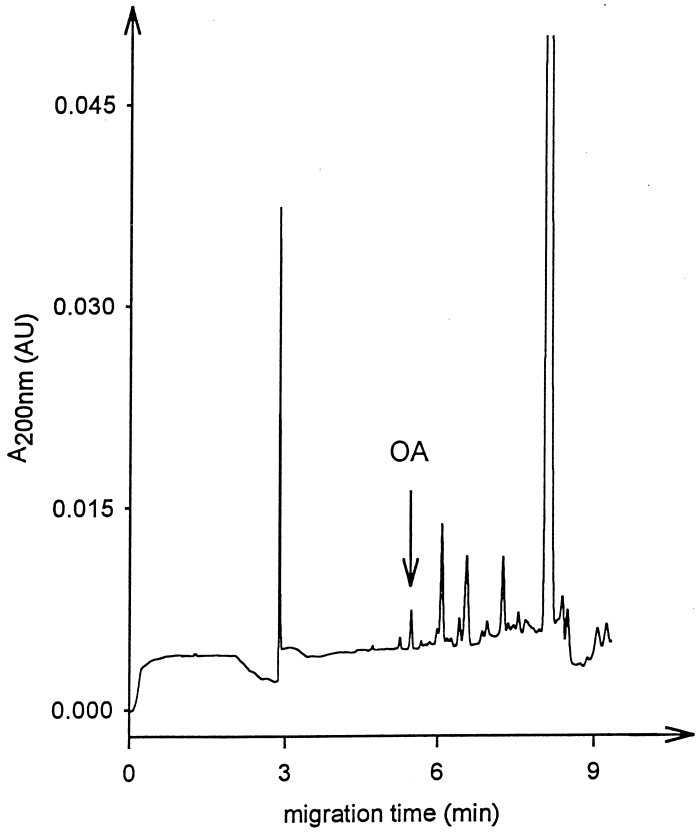

Fig. 3. Determination of OA in urine of a healthy adult by CZE. Sample: urine of a healthy adult person, 5× diluted with deionized water. BGE: identical with BGE in system I (Table 1). Instrumentation: P/ACE 5010. Capillary: silica, 27 cm (effective length 19 cm)×0.1 mm I.D., UV detector (λ=200 nm). Voltage 15 kV, temperature 25°C, high-pressure sampling 10 s.

authors thank Dr. T. Adam (Medical Hospital, Olomouc) for providing of samples of urine of ill children.

References

[1] H.A. Harper, Review of Physiological Chemistry, Lange Medical Publications, Los Altos, CA, 1975.
[2] J. Hyánek et al., Klinické a Biochemické Aspekty Vrozených Metabolických Poruch, Avicenum, Prague, 1979.
[3] S.W. Brusilow, in: F.A. Hommes (Ed.), Techniques in Diagnostic Human Biochemical Genetics – A Laboratory Manual, Wiley–Liss, New York, 1991, pp. 345–357.
[4] J.F. Bellinger, N.R.M. Buist, Clin. Chem. 17 (1971) 1132–1133.
[5] H. Miyazaki, Y. Matsunaga, K. Yoshida, S. Arakawa, M. Hashimoto, J. Chromatogr. 274 (1983) 75–85.
[6] V. Ferrari, G. Giordano, A.T. Cracco, N. Dussini, L. Chiandetti, F. Zacchello, J. Chromatogr. 497 (1989) 101–107.
[7] S. Ohba, K. Kidouchi, C. Nakamura, T. Katoh, M. Kobayashi, Y. Wada, in: R.A. Harkness (Ed.), Purine and Pyrimidine Metabolism in Man VII, Part B, Plenum Press, New York, 1991, pp. 27–30.
[8] S. Ohba, K. Kidouchi, T. Katoh, T. Kibe, M. Kobayashi, Y. Wada, J. Chromatogr. 568 (1991) 325–332.
[9] S.W. Brusilow, E. Hauser, J. Chromatogr. 493 (1989) 388–391.
[10] M.T. McCann, M.M. Thompson, I.C. Gueron, M. Tuchman, Clin. Chem. 41 (1995) 739–743.
[11] T. Takechi, H. Wakiguchi, T. Kurashige, J. Chromatogr. 532 (1990) 144–149.
[12] J. Ševčík, T. Adam, V. Sázel, Clin. Chim. Acta 259 (1997) 73–81.
[13] T. Adachi, A. Tanimura, M. Asahina, J. Vitaminol. 9 (1963) 217–226.
[14] L. Kesner, E.L. Aronson, M. Silverman, P.C. Chan, Clin. Chem. 21 (1975) 353–355.
[15] A.M. Glasgow, Am. J. Clin. Pathol. 77 (1982) 452–455.
[16] L. Křivánková, P. Boček, J. Chromatogr. B 689 (1997) 13–34.
[17] L. Křivánková, P. Gebauer, W. Thormann, R.A. Mosher, P. Boček, J. Chromatogr. 638 (1993) 119–135.
[18] L. Křivánková, P. Gebauer, P. Boček, J. Chromatogr. A 716 (1995) 35–48.
[19] D. Kaniansky, J. Marák, V. Madajová, E. Šimuničová, J. Chromatogr. 638 (1993) 137–146.
[20] D. Kaniansky, I. Zelenský, A. Hybenová, F.I. Onuska, Anal. Chem. 66 (1994) 4258–4264.
[21] L. Křivánková, A. Vraná, P. Gebauer, P. Boček, J. Chromatogr. A 772 (1997) 283–295.
[22] A. Procházková, L. Křivánková, P. Boček, Electrohoresis 19 (1998) 300–304.
[23] S. Hjertén, J. Chromatogr. 347 (1985) 191–198.
[24] S. Krásenský, S. Fanali, L. Křivánková, P. Boček, Electrophoresis 16 (1995) 968–973.
[25] L.G. Sillén, Stability Constants of Metal–Ion Complexes, Chemical Society, London, 1971.
[26] T. Hirokawa, M. Nishino, N. Aoki, Y. Kiso, Y. Sawamoto, T. Yagi, J. Akiyama, J. Chromatogr. 271 (1983) D1–D106.
[27] J.L. Beckers, F.M. Everaerts, J. Chromatogr. 68 (1972) 207–230.