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Direct quantitative determination of amlodipine enantiomers in urine samples for pharmacokinetic study using on-line coupled isotachophoresis-capillary zone electrophoresis separation method with diode array detection☆

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ABSTRACT

The present work illustrates possibilities of column-coupling capillary electrophoresis (CE-CE) combined with chiral selector (2-hydroxypropyl-β-cyclodextrin, HP-β-CD) and fiber-based diode array detection (DAD) for the direct quantitative enantioselective determination of trace drug (amlodipine, AML) in biological multicomponent ionic matrices (human urine). Capillary isotachophoresis (ITP) served as an ideal injection technique in CE-CE. Moreover, the ITP provided an effective on-line sample pretreatment prior to the capillary zone electrophoresis (CZE) separation. Enhanced separation selectivity due to the combination of different separation mechanisms (ITP vs. CZE–HP-β-CD) enabled to obtain pure zones of the analytes, suitable for their detection and quantitation. The DAD, unlike single wavelength UV detection, enabled to characterize the purity (i.e. spectral homogeneity) of the analytes zones. A processing of the raw DAD spectra (the background correction and smoothing procedure) was essential when a trace analyte signal was evaluated. Obtained results indicated pure (i.e. spectrally homogeneous) zones of interest confirming effective ITP–CZE separation process. The proposed ITP–CZE–DAD method was characterized by favorable performance parameters (sensitivity, linearity, precision, recovery, accuracy, robustness, selectivity) and successfully applied to an enantioselective pharmacokinetic study of AML.

1. Introduction

Calcium ions are needed for electrical activity for the contraction of cardiac and smooth muscle and conduction of nerve cell. Calcium channel blocker is a drug which inhibits the entry of excess calcium into cells and/or prevents calcium from the mobilization from intracellular stores, resulting in relaxation of blood vessel walls and cardiac muscle for blood to flow more freely, lowering blood pressure thereby reducing oxygen demand in the heart and relieving anginal pain. Amlodipine besylate (AML) is used in the treatment of hypertension and chronic stable and vasospastic angina. The chemical name is (RS)-3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate benzenesulphonate [1].

Enantioselective drug absorption, distribution, metabolism, elimination, or liberation studies are included among the most advanced problems being solved in pharmaceutical and clinical research [2–5]. It is due to a multicomponent character of biological matrices, a very low concentration of the analyte(s) among the matrix constituents, and identical physicochemical properties of enantiomers in an achiral environment. Among high performance separation techniques, capillary electromigration methods provide the best solution for the analytical enantioseparations of ionic compounds [6–8]. Here, a high-resolution power is given by (i) an extremely high peak efficiency and (ii) wide scale of various electromigration effects producing/enhancing (enantio)selectivity.

Capillary isotachophoresis (ITP) coupled on-line with capillary zone electrophoresis (CZE) provides very significant CE tool applicable for a complex ionic matrices [9,10]. Main benefits of ITP–CZE combination are: (i) compatible separation mechanisms providing different selectivity, (ii) ITP sample treatment (preconcentration, clean-up) and (iii) ITP enhancing a sample load capacity [11–14]. These factors considerably reduce (i) the concentration limits of detection (cLOD) when compared to current (single column) CZE, and (ii) external sample preparation.

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Spectral detection is useful when analyzed complex ionic mixtures. In many cases, diode array detection (DAD) has appeared to be a simple solution in a preliminary characterization of electropherograms in *z* axis (absorbance vs. migration time vs. wave length, *x*–*y*–*z*), see e.g. analysis of drugs in body fluids [15,16]. In this way DAD is expected to enhance reliability of results reflected in validation parameters (the ICH guideline [17]).

Some general concepts for CE enantioseparations of dihydropyridine derivatives by means of cyclodextrins (CDs) have been reported in refs. [18,19]. AML enantiomers have been successfully separated using CE and HPLC techniques with charged and uncharged CDs as chiral selectors or chiral stationary phases [20–23]. AML was enatiomerically resolved in ca. 22 min using phosphate buffer with pH 3 and 5 mmol/l hydroxypropyl-β-cyclodextrin (HP-β-CD), 50 mmol/l tetraethylammonium-chloride and 2 mol/l urea as buffer additives [20]. Another work [21] compared CE enantioseparation of AML using 20 mmol/l HP-β-CD and the anionic CDs, sulphobutylether-β-CD (1 mmol/l) and carboxymethyl-β-CD (2.5 mmol/l). The anionic CDs were shown to offer an enhanced enantioselectivity over the neutral CD in CE systems with electroosmotic flow (EOF). α-CD was shown to be enantioselective towards AML too [22]. However, there is only one paper dealing with an enantioselective CE determination of AML in physiological samples (human serum) [23]. The AML enantiomers were separated in ca. 10 min using phosphate buffer (75 mmol/l, pH 2.5) containing 15 mmol/l HP-β-CD. The range of quantitation for both enantiomers was 2.0–16.0 μg/ml. Intra-day and inter-day relative standard deviation (RSD; $n = 5$) was <10%. The limits of detection (LOD) and quantification (LOQ) of the AML enantiomers, at 214 nm, were approximately 0.5 and 0.7 μg/ml, respectively (S/N = 3 and 10, respectively; 5-s injection). Recovery was always >85%. The samples have been analyzed after their pretreatment (extraction procedure).

The aim of the present work was to develop an enantioselective ITP–CZE–DAD method for a highly sensitive determination of trace AML enantiomers in complex ionic matrices (human urine), useful for the enantioselective pharmacokinetic study. One of the main benefits of the ITP–CZE–DAD method should also be a possibility to perform direct analyses of samples avoiding any sample preparation procedure and, by that, enhancing reliability of analyses.

2. Experimental

2.1. Instrumentation

A capillary electrophoresis analyzer EA-101 (Villa-Labeco, Spišská Nová Ves, Slovakia), assembled in the column-coupling configuration of the separation unit, was used in this work for performing the ITP–CZE runs. The samples were injected by a 30 μl internal sample loop of the injection valve of the analyzer. An ITP column was provided with an 800 μm I.D. fused silica capillary tube of a 90 mm total length and a contactless conductivity detector. A CZE column was the same as the ITP one except for a 320 μm I.D. and a 160 mm total length.

A multiwavelength photometric absorbance diode array detector Smartline PDA Detector 2800 (Knauer, Germany) was connected to an on-column photometric detection cell, mounted on the CZE column, via optical fibers. The detector operated under the following conditions: (1) scanned wavelength range 200–800 nm; (2) integration time 6 ms; (3) scan interval 0.2 s; (4) number of accumulations 1.

Prior to the use, the capillaries were not treated by any rinsing procedures to suppress an electroosmotic flow. A dynamic coating of the capillary wall by means of hydroxyethylcellulose (HEC 30 000; Serva, Heidelberg, Germany) in leading and background electrolyte solutions served for this purpose. The separating electrolytes in the capillaries were replaced by the fresh ones between each run. ITP and CZE analyses were carried out in the cationic regime of the separation (i.e. cathodic movement of the analytes) with direct injections of the samples. The experiments were performed in constant current mode at 20 °C. The driving currents applied were 250 μA (ITP) and 150 μA (CZE).

2.2. Data evaluation and performance parameters

The absorption maximum wavelength (238 nm) of AML was used for the evaluation of analytical parameters of the optimized method. Performance parameters of the method were evaluated according to the ICH guideline [17]. Peak area of AML was corrected for the migration time [24].

Parameters of calibration lines for AML enantiomers were calculated by using QCExpert ver.2.5 statistical software (Trilobyte, Prague, Czech Republic).

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as the ratio of standard deviation of y-intercept of regression line (s_a) and the slope of the regression line (b) multiplied by factor 3.3 (LOD) or 10 (LOQ). Concentrations of AML taken for the calibration lines are given in Section 2.5.1.

Precision was evaluated as the repeatability which is expressed via relative standard deviation of (i) peak areas measured within the concentration range of calibration line and (ii) migration times of AML.

Recovery was evaluated by spiking of blank urine and water samples with AML at three different concentration levels (see Section 2.5.1) and comparing peak areas of AML obtained in different matrices. Accuracy (expressed via relative error, RE) was evaluated through the recovery test.

Robustness test examined the effect that deliberate variations in operational parameters (concentration of the complexing agent (48–52 mg/ml), leading (19–21 mmol/l) and carrier (48–52 mmol/l) cation, pH ± 0.1) had on the analysis results (enantioresolution, *R*).

2.3. Processing and comparing of DAD spectra

The migration and spectral data were acquired and processed by a EuroChrom program (version 3.05, Knauer).

The background correction (subtraction of background spectrum from the raw spectrum of the analyte) [25] was carried out to minimize the impact of the electrolyte system on AML spectrum. Such corrected spectrum was further smoothed by the procedure of Savitzky–Golay [26] (implemented in EuroChrom software) with a 5-point window.

Homogeneity of spectra of AML enantiomers was expressed via Pearson's correlation coefficients (PCCs) [27]. The value of PCC higher than 0.99 is assumed to provide an acceptable certainty in a confirmation of the identity of the analyte [25], i.e. a match of the tested (AML in urine) and reference (AML in water) spectrum.

2.4. Chemicals and samples

The electrolyte solutions were prepared from chemicals obtained from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), and Fluka (Buchs, Switzerland) in water demineralized by a Rowapure-Ultrapure water purification system (Premier, Phoenix, Arizona, U.S.A.). All chemicals used were of analytical grade or additionally purified by the usual methods. The solutions of the electrolytes were filtered before use through disposable membrane filters of a 1.2 μm pore size (Millipore, Molsheim, France).

Native β-cyclodextrin (β-CD) and (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), with the extent of labeling equal to 1.0 molar substitution dextrin, were obtained from Aldrich. Carboxyethyl-β-cyclodextrin (CE-β-CD), with the number of substituents on a CD ring (DS) 3 and CE purity, was obtained from Cyclolab (Budapest, Hungary).

Amlodipine mesilate monohydrate (AML) was obtained as a racemic reference substance from Zentiva (Hlohovec, Slovakia). Pharmaceutical tablets, Agen®, produced by Zentiva, were obtained from the local pharmacy. The declared content of AML in one tablet was 10 mg.

2.5. Procedures for sample and standard solution preparations

2.5.1. Standard solutions

The stock solution of AML reference substance was prepared by dissolving 10 mg of the powder in 10 ml of demineralized water. Working solutions were made by an appropriate dilution of the stock solution with demineralized water or by spiking AML from the stock solution into blank urine.

The effect of sample matrix on (enantio)resolution and peak area of AML was investigated using urine samples with different urine:water ratio (w/w, 1:4, 1:9, 1:19) and a 500 ng/ml concentration of AML.

The concentration levels of AML in the injected model calibration solutions (prepared in demineralized water or 10-times diluted urine) were in the range of 20–500 ng/ml (20, 50, 100, 250, 500), and each calibration point was measured twice.

For the recovery experiments, AML was spiked from its stock solution into real matrices (10-times diluted urine). The samples with three concentration levels (40, 200 and 400 ng/ml) of AML standard were prepared.

2.5.2. Urine sample preparation

Two tablets of Agen (equivalent to 20 mg of AML) were administered orally to a healthy young male volunteer (at 8.30 a.m.). The urine samples were taken in different time periods after Agen was administered (time/volume: 3 h/200 ml, 4 h/260 ml, 10 h/690 ml, 20 h/563 ml, 28 h/172 ml, 32 h/195 ml, 35 h/297 ml, 39 h/340 ml, 47 h/262 ml, 51 h/191 ml, 54 h/160 ml) to investigate the elimination of AML and its potential metabolites in urine. Each urine sample was frozen (−18 °C) immediately after the sampling and kept in the freezer until its use. The sample was thawed out just before the manipulation and preparation of the sample. Each sample was diluted 10 times with demineralized water and immediately injected into the sampling loop of the electrophoretic analyzer.

3. Results and discussion

3.1. ITP–CZE separation conditions

In the present work, the buffer constituents (inorganic: HCl, organic: glycine, acetic acid, ε-aminocaproic acid, morpholinoethanesulphonic acid), concentration of leading and carrier cations (leading cation: 10–50 mmol/l, carrier cation: 20–100 mmol/l), pH (2.5–5.5), and driving currents (ITP: 100–500 μA, CZE: 50–200 μA) in the ITP–CZE separations were tested in order to find an experimental optimum considering (i) rapid analysis (lower pH was favorable for better protonization of AML, higher driving current proportionally decreased migration velocity of AML), (ii) minimization of thermal, adsorption and electromigration dispersion effects (low conductivity organic buffers, i.e. lower concentration and mobility, mediate pH, i.e. less protons, and lower current were favorable for elimination of Joule heating; adsorption of AML on inner capillary wall was reduced using higher ionic strength of electrolyte, i.e. higher concentration of buffer constituents; electromigration dispersion was minimized choosing carrier cation with the electrophoretic mobility closest to that one of AML), (iii) sufficient (enantio)resolution of the analyte in on-line pretreated matrices (type and concentration of chiral selector, see Section 3.2), and (iv) good compatibility of the on-line coupled separation systems (optimal interface between terminating and carrier electrolyte with minimum gradient effects). The optimized electrolyte systems, considering all above mentioned aspects, are presented in Table 1.

Table 1
Electrolyte systems[a]

Parameter	ITP	Parameter	CZE
Solvent	Water	Solvent	Water
Leading cation	K^+	Carrier cation	Glycine
Concentration (mmol/l)	20	Concentration (mmol/l)	50
Counter ion	Acetate	Counter ion	Acetate
Concentration (mmol/l)	20	Concentration (mmol/l)	168
pH	4.75	pH	3.2
EOF suppressor	HEC	EOF suppressor	HEC
Concentration (%, w/v)	0.2	Concentration (%, w/v)	0.2
Terminating cation	Glycine	Complexing agent	HP-β-CD
Concentration (mmol/l)	5	Concentration (mg/ml)	50
Counter ion	Acetate		
Concentration (mmol/l)	17		
pH	3.2		

EOF: electroosmotic flow; HEC: hydroxyethylcellulose; HP-β-CD: (2-hydroxypropyl)-β-cyclodextrin.

[a] Leading electrolyte was prepared by titrating of potassium acetate in presence of HEC by isothermally distilled acetic acid to reach pH 4.75. Terminating electrolyte was prepared by titrating of glycine by isothermally distilled acetic acid to reach pH 3.2. Carrier electrolyte was prepared by titrating of glycine in presence of HEC by isothermally distilled acetic acid to reach pH 3.2. The appropriate amount of HP-β-CD was then dissolved in this solution in order to obtain the final separating buffer for the CZE stage.

The increased dimensionality of separations in comparison with a single column CE provided favorable conditions in terms of (i) separation efficiency (see N and H (height equivalent to one theoretical plate) in Table 2), (ii) separation selectivity (complete resolution of AML from sample matrix constituents and suitable homogeneity of its spectrum, see Section 3.4), (iii) sample loadability and, consecutively, sensitivity (see LOD and LOQ values in Table 2), (iv) sample handling and reliability (minimized sample preparation).

Electropherograms from the ITP–CZE separations of AML enantiomers present in model urine sample, using conductivity detection in ITP stage and UV absorbance detection at a 238 nm wave length in CZE stage, are shown in Fig. 1. These electropherograms clearly illustrate a possibility to perform separations of trace (ng/ml) AML enantiomers in complex ionic matrices (representative urine sample) with a direct injection of unpretreated sample.

3.2. Separation selectivity with chiral selector

It was found out that lower polarity and deeper (extended) cavity of CD were favorable in term of AML enantioresolution. Hence, HP-β-CD was much more effective in AML enantioresolution than CE-β-CD or native β-CD. The enantioresolution of rather hydrophobic AML molecule increased with concentration of HP-β-CD and the baseline enantioseparation was achieved at a 50 mg/ml concentration of HP-β-CD in the background electrolyte. Moreover, a sufficient resolution of AML from the sample matrix constituents migrating in CZE stage was achieved as well (Fig. 1b).

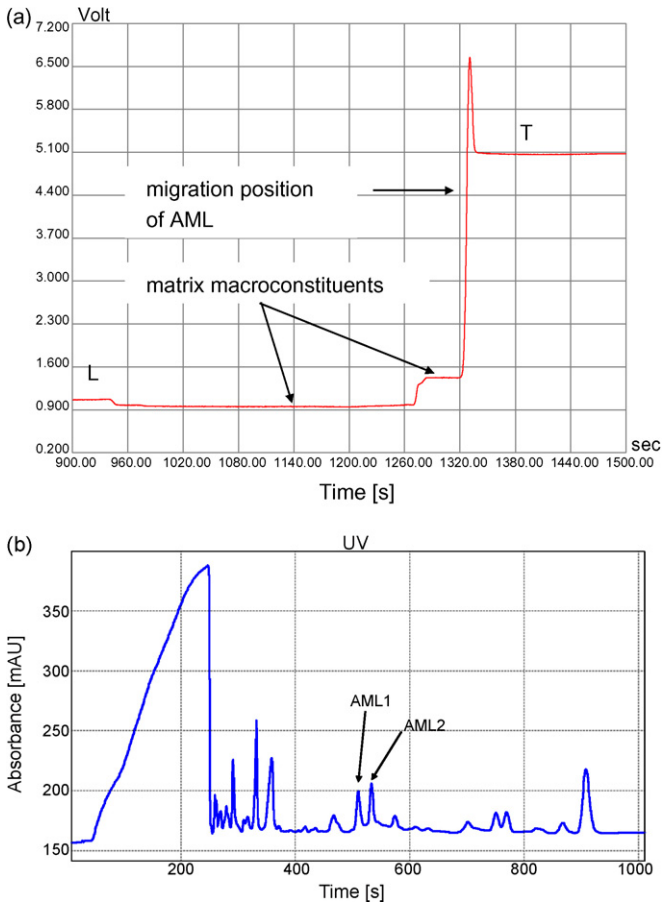

Fig. 1. Direct determination of AML enantiomers in model urine sample. Electropherograms were obtained in isotachophoretic (a) and zone electrophoretic (b) stages of on-line coupled ITP–CZE runs. Electrolyte systems are given in Table 1 and other working conditions in Section 2. A 10-times diluted blank urine spiked with a 500 ng/ml concentration of AML served as a model sample. L: leading cation; T: terminating cation; AML, AML1, AML2: migration positions of amlodipine, the first migrating enantiomer of amlodipine, the second migrating enantiomer of amlodipine, respectively.

It was also found out that the proposed chiral separation system was robust enough towards changes of concentration of the matrix constituents in the sample. Changing the urine:water ratio (as given in Section 2.5.1), the AML enantioresolution was not decreased, but it was even somewhat increased (up to 20%). Therefore, it can be supposed that variability of matrix should not influence determination of AML enantiomers in real samples significantly.

3.3. Validation of ITP–CZE method

The method with optimized separation parameters, as given in Table 1, was validated. All statistical data and performance parameters of the method are given in Table 2.

The obtained values of LOD and LOQ clearly favor the use of the column-coupled ITP–CZE method (comparing to single column one) in ultra trace quantitative determination of AML.

Good linearity of the calibration lines is indicated by the values of correlation coefficient (r) and coefficient of determination (r^2).

Evaluated repeatability is acceptable which is clearly visible from the values of standard deviations of migration times of AML enantiomers, intercept and slope of the calibration lines of AML enantiomers, s_t, s_a, s_b, respectively, and residual sum of squares, RSS.

The recovery values were slightly below 100%. Obtained REs values indicated very good accuracy of the proposed ITP–CZE method.

Altering operational parameters in the robustness test, fluctuations of R were less than 6.5% of the value obtained under the standard conditions. Therefore, it can be supposed that little changes in operational parameters should not influence R significantly.

3.4. Proofing the separation selectivity by DAD spectra

DAD spectra were applied to prove purity (i.e. spectral homogeneity) of zones of AML enantiomers (based on differences between analyte and interferent spectra). This investigation was motivated by a high probability of interferences in multicomponent mixtures (urine). The processed spectra provided relevant spectral information for AML enantiomers present in (i) model water sample (reference spectra of AML enantiomers) and (ii) model urine sample, see Fig. 2.

Table 2
Performance parameters of ITP–CZE–DAD method and parameters of calibration lines of AML enantiomers measured with water and urine model samples[a]

Parameter	Water		Urine	
	Enantiomer 1	Enantiomer 2	Enantiomer 1	Enantiomer 2
t_m (min)	8.381	8.778	8.500	8.884
s_{tm} (min)	0.047	0.053	0.087	0.095
a (mAU)	−2.310	−2.471	28.036	29.720
s_a (mAU)	1.981	2.191	2.9734	3.1557
b (mAU ng^{-1} ml)	0.75948	0.81253	1.0536	1.1172
s_b (mAU ng^{-1} ml)	0.0075	0.0080	0.01127	0.01195
QC	1.903	1.892	1.753	1.754
RSS	17.99	20.59	40.54	45.66
r	0.99990	0.99990	0.99989	0.99989
r^2	0.99980	0.99980	0.99977	0.99977
LOD (ng ml^{-1})	8.6	8.9	9.3	10.4
LOQ (ng ml^{-1})	26.1	27.0	28.2	31.5
N	21000	18500	26500	34100
H (μm)	7.1	8.1	5.7	4.4
Recovery (%)			98.9	99.5
Accuracy (RE) (%)			−1.1	−0.5
Robustness (ΔR) (%)	<6.5	<5.8		
Enantioresolution, R	2.76	3.25		

[a] Separating conditions as in Table 1 and other working conditions as in Section 2. The samples: demineralized water and 10-times diluted urine. The concentration of AML was 500 ng/ml unless otherwise stated.

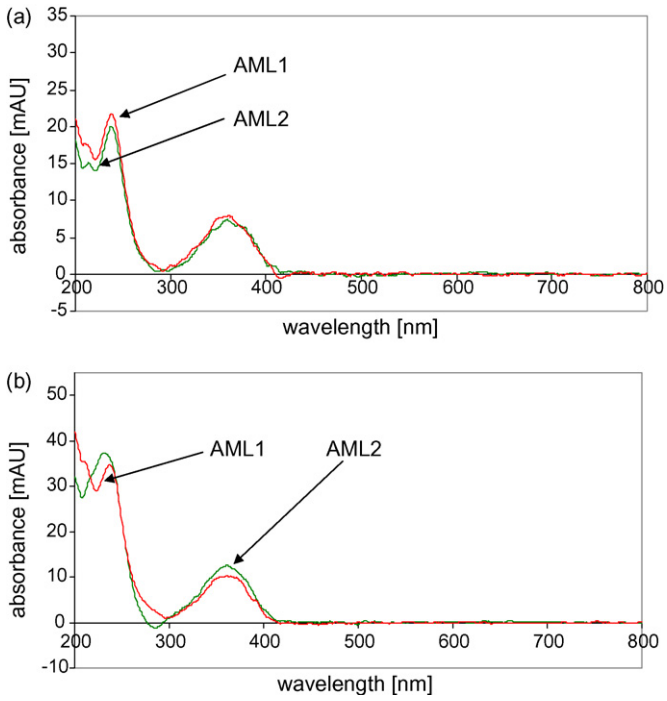

Fig. 2. Processed UV–vis spectra of AML in different matrices. (a) AML present in demineralized water, serving as a reference spectrum, (b) AML present in 10-times diluted model urine, at a 500 ng/ml concentration level of the drug in the samples. Separating and other working conditions as in Fig. 1. For the processing procedure of the spectra see Sections 2.3 and 3.4.

Homogeneity of spectra of AML enantiomers obtained from the model urine sample was expressed via PCCs. Here, the matching factors for the first and the second AML enantiomer (according to their migration order) were 0.9895 and 0.9901, respectively. This indicated, with a high probability, pure analytes zones (without interfering compounds), i.e. sufficient selectivity of the ITP–CZE separation. PCC values also indicated an importance of the spectral processing procedure, especially when the enantiomers present at concentrations near to their LOQs (e.g. 0.788913 and 0.9879 (the first enantiomer) and 0.76855 and 0.9888 (the second enantiomer) values for the raw and processed spectrum, respectively, at a 50 ng/ml concentration of AML).

3.5. Application and enantioselective pharmacokinetic study

The proposed and proved enantioselective ITP–CZE–DAD method was applied in a metabolic study aimed at the investigation of mass-time profiles of AML enantiomers in human urine after the oral administration of the drug. Representative electropherogram, shown in Fig. 3, illustrates urine CZE profile characteristic for the real urine sample taken 10 h after oral administration of 20 mg of AML.

The migration positions of AML enantiomers in the electropherograms were confirmed by spiking of real samples with the AML standard, while purities of the AML zones in particular experiments (reflecting achieved separation selectivity) were confirmed by statistical evaluation of corresponding processed spectra. PCCs were used for decision on relevance of detection signal appeared in AML migration position. PCC values of processed AML spectra for particular times of sampling (3–54 h) ranging in interval 0.9950–0.7489.

It was indicated a high purity of AML zones in the first part of the sampling interval (3–39 h, PCC values were 0.9950–0.9862, average PCC was 0.9887), and hence, reliable quantitative determination of AML enantiomers in the real urine samples (Table 3). PCCs equal and higher than 0.99 confirmed a suitability of corresponding electrophoretic peaks for calculation of AML concentration for pharmacokinetic profile (only spectrally homogeneous zones were taken for the calculations regardless a peak area in the AML migration position). Therefore, electrophoretic peaks in 39 h of the sampling were also included although corresponding concentrations were slightly below LOQ. On the other hand, AML zones of a lower purity were detected in the second part of the sampling interval (47–54 h, PCC values were 0.9430–0.7489, average PCC was 0.8235). Mixed zones in AML migration position (the dominant role of matrix interferents, metabolites or background constituents can be expected), expressed by corresponding PCCs lower than 0.99, could not be considered for calculation of AML concentration even though peak area was over LOQ of AML (in our work it was in 51 h of the sampling). In such case, it is not possible to determine a share of analyte and interfering compound(s) on the peak area. Obviously,

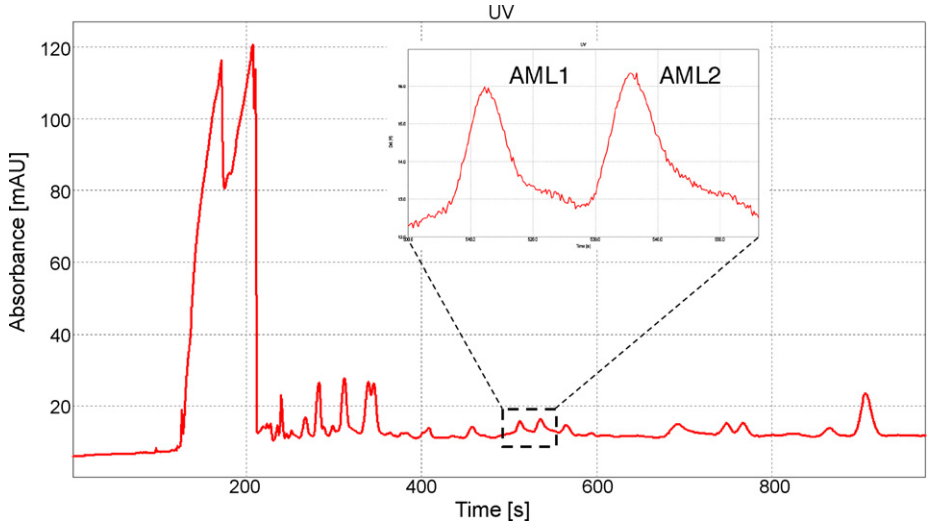

Fig. 3. Electropherogram from CZE step of the ITP–CZE–DAD combination illustrating the electrophoretic profile of AML enantiomers in a real urine sample. The sample was taken 10 h after oral administration of 20 mg of AML to a male volunteer. Migration position of AML enantiomers (AML1, AML2) is shown in detail. The urine sample was diluted 10 times. Separating and other working conditions as in Fig. 1.

Table 3
Concentration and mass levels of AML enantiomers in real urine samples[a]

Time (h)	Concentration (ng/ml)		Mass (μg)		Enantiomeric ratio (%)	
	AML1	AML2	AML1	AML2	AML1	AML2
3	56.0	63.2	14.6	16.4	47	53
4	197.0	213.4	39.4	42.7	48	52
10	134.3	139.8	92.7	96.4	49	51
20	165.3	179.0	93.1	100.8	48	52
28	376.8	408.1	64.8	70.2	48	52
32	184.4	216.4	36.0	42.2	46	54
35	64.1	66.8	19.1	19.8	49	51
39	22.4	25.3	7.6	8.6	47	53
47	≪LOQ	≪LOQ	≪LOQ	≪LOQ	–	–
51	>LOQ[b]	>LOQ[b]	>LOQ[b]	>LOQ[b]	–	–
54	≪LOQ	≪LOQ	≪LOQ	≪LOQ	–	–

[a] Samples were taken in different time spans after oral administration of 20 mg of AML (Agen tablets) to a healthy young male volunteer. The samples were diluted 10 times. Separating conditions as in Table 1 and other working conditions as in Section 2.
[b] Inhomogeneous DAD spectrum for the detection signal higher than a signal equivalent to the LOQ of AML in urine.

it does not mean that concentration of AML must be zero but for the calculations it is useless.

The human urine mass-time profiles (elimination and cumulative dependences) of AML enantiomers, obtained with the young healthy volunteer, are shown in Fig. 4. The maximal mass levels of released AML (see data in Table 3) were observed in the interval of sampling times of 10–20 h. The total amount of released AML enantiomers, monitored in 54 h, was 367.3 μg (AML1) and 397.1 μg (AML2), i.e. ~4% of the administered dose of AML. It is in a good agreement with ref. [28] demonstrating extensive metabolization of absorbed AML, with only ~5% of the dose being excreted unchanged in human urine. However, it should be realized that the pharmacokinetic profiles as well as total amount of released AML can vary with the tested individuals depending on the type of their metabolism (poor and extensive metabolizers), diet habits, etc. The elimination of AML in urine was slightly enantioselective as it can be seen from the scatter of AML1 and AML2 points in Fig. 4, and data in Table 3.

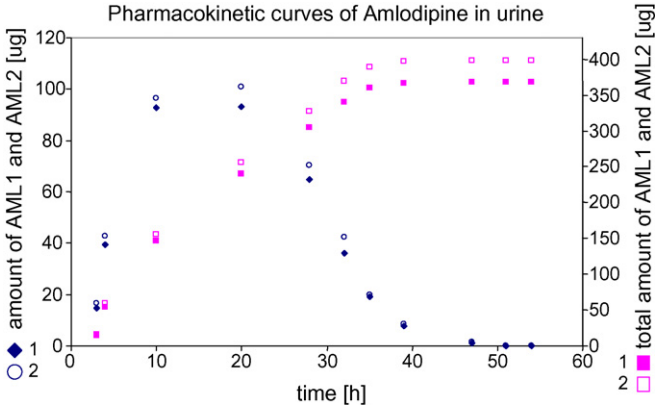

Fig. 4. Typical mass-time profiles of AML enantiomers in human urine. (a) Elimination (blue circles/rhombuses) and (b) cumulative (pink squares) dependences were obtained measuring samples taken after oral administration of 20 mg AML. The mass levels (μg) of AML enantiomers in urine in different time spans after administration of the drug are given in Table 3. The mass values for the *y* axis on left sight (for the elimination curve) were calculated from concentrations (given in Table 3) and volumes (given in Section 2.5.2) of individual urine samples taken in different time periods after oral administration of AML. The data for the *y* axis on right sight (for the cumulative curve) were calculated as the sums of the consecutive mass values (from the Table 3) in increased time periods after the administration of the drug. Separating and other working conditions as in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.6. Spectral characterization of unknown CZE zones

Metabolism of AML in humans primarily involves oxidation to the pyridine derivatives with subsequent oxidative deamination of the 2-aminoethyoxymethyl side chain or deesterification at the 5-methoxycarbonyl group [28]. The majority (<95%) of the metabolites excreted in the 0–72 h post-dose period were identified in ref. [29]. The major metabolite was 2-([4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-2-pyridyl]methoxy) acetic acid and this represented 33% of urinary radioactivity.

In our work, the peaks in the electrophoretic urine profiles were investigated in detail through their spectra. We found out that any processed spectrum was not similar to the spectrum of AML (PCCs were ≪0.99). This indicated an absence of structurally closely related compounds to AML (potential metabolites) in the electropherograms. Therefore, it can be supposed that AML metabolites (ca. 60% in urine [30]) could rather be present in urine as conjugates or acids, i.e. compounds that did not migrate under the present separating ITP–CZE conditions. It is in accordance with the results given in ref. [29], as stated above.

4. Conclusion

The benefits of the on-line coupled ITP–CZE method for the direct enantioselective separations in multicomponent ionic matrices were presented in this work. The substantial decrease in concentration LOD, increase in separation selectivity, and the elimination of sample preparation led to the performance of highly reliable ultratrace analyses and to the simplification of overall analytical procedure. In order to control a reliability of the results, we utilized spectral data from DAD (evaluation of purity of separated analytes zones; confirmation of basic structural identity of the analytes). Chemometric methods like (i) background correction and smoothing of spectrum, and (ii) comparison of (processed) spectra through their Pearson's correlation coefficients, played substantial role in evaluation of detection signals obtained for concentration level of the analytes near to their LOQ.

Successful validation and application of the proposed ITP–CZE–DAD method in enantioselective pharmacokinetic study of AML highlighted potential of this analytical approach to solve effectively advanced problems in clinical research. From this point of view, ITP–CZE–DAD can be advantageously used as a reference as well as routine method. Great advantage is a possibility to characterize electrophoretic profiles of unpretreated (unchanged)

biological samples and, by that, to investigate compounds of interest with a higher reliability.

The ITP–CZE–DAD approach is a pragmatic solution in enantioselective clinical research for its simplicity, cost and appropriate information value. Obviously, when the exact structure information is required, such results have to be supplemented by the more selective (specific) detection, e.g., mass spectrometry or nuclear magnetic resonance.

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