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Capillary isotachophoresis of cystine in urine with on-line isotachophoresis sample pretreatment

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Capillary isotachophoresis (ITP) with on-line isotachophoresis sample pretreatment and conductivity detection has been proposed as a method for the separation, detection and determination of cystine present in urine. Sample pretreatment was achieved using a 90 mm FEP capillary tube with a 800 μm internal diameter. ITP separations and conductivity detection were carried out in a 160 mm capillary tube with a 300 μm internal diameter, filled with a hydrochloric acid leading electrolyte and glycine terminating electrolyte, at 50 μA and 25 °C. The results show that the proposed method is suitable for resolution of cystine from matrix interferents present in the urine samples and is able to detect it at 10^{-4} mol/l concentrations by a universal detection technique after removal of a significant part of the anionic constituents of the sample migrating in the on-line coupled capillary isotachophoresis stack. This simple ITP procedure for monitoring cystine in urine samples requires no sample preparation. This makes it an alternative to the currently used procedures.

1. Introduction

Information about the amino acid content of biological samples is very important in biochemistry and clinical chemistry. These analyses are considered to play an important part in the attempt to find a correlation between amino acids and certain pathologies [1]. The determination of cystine in urine is linked mainly with the diagnosis of cystinuria [2]. Monitoring of cystine in urine samples may be carried out by various separation techniques (HPLC, CE) and currently requires sample preparation (e.g., ionex-cartridge extraction, evaporation of the solvent, derivatization of the analyte) [3–5]. The treatment of the sample before analysis, resulting in elimination of interferents and preconcentration of the analyte, is a prerequisite for enhancing the separability and the detection of the analyte. However, the sample handling brings about an increase in errors in the determinations and it is also time consuming. A promising alternative to the above mentioned analytical procedures seems to be the column-coupling CE separation system. It has recently been applied to the separation and determination of analytes present in multicomponent ionic matrices and in matrices additionally containing inorganic electrolytes at very high concentrations [6].

The aim of the present work was to take advantage of capillary isotachophoresis with on-line isotachophoresis sample pretreatment (ITP-ITP) and conductivity detection for the separation, detection and determination of cystine in urine. Using the ITP-ITP combination in the column-coupling configuration of the separation system we expected the first ITP stage to provide an enhanced sample capacity for the separation system (a 30 μl sample injection volume), to concentrate the analyte and to serve as an on-line sample clean-up technique (for the theoretical aspects see ref. [7]). On the other hand, the second ITP stage should perform a final separation of the analyte from matrix constituents present in the ITP pretreated sample and provide enhanced conditions for its detection by the conductivity detector.

2. Investigations, results and discussion

Urine represents a multicomponent, variable and high ionic strength matrix and the main emphasis of this research was to obtain a good resolution between cystine and its most disturbing urine matrix interferents. We expected that the single column separations would not be sufficient in themselves to resolve cystine from some matrix constituents (Fig. 1) and cystine would migrate in a mixed zone with urine matrix interferent(s) [trace (b) in Fig. 1]. In view of the intrinsic composition of urine the separation capacity was insufficient to obtain a pure cystine zone for the given samples. Therefore, the first ITP stage was employed to remove the most abundant sample constituents (chloride, carbonate) possessing higher mobilities than cystine as well as a great number of microconstituents with lower mobilities than cystine and to concentrate the rest of those migrating between the leading and terminating zones for injection into the second ITP stage, as shown in Fig. 2. Consequently, only analyte with a relatively small amount of matrix constituents was transferred for the final separation in the second ITP stage [compare electropherograms (c), (d) and (b) in Fig. 2]. In this way, the first ITP stage served as a sample clean-up technique which pre-

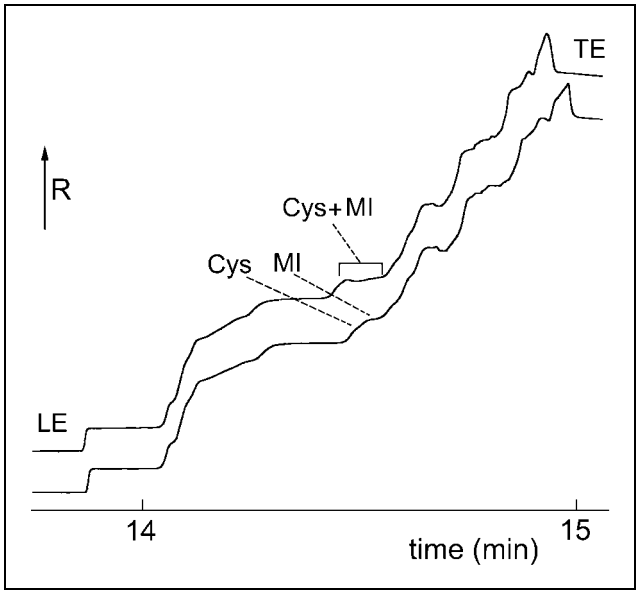

Fig. 1: ITP separation and conductivity detection of cystine in a urine sample without on-line ITP sample pretreatment. (a) Reference trace obtained from separation of urine without cystine addition. (b) Cystine present at 2×10^{-4} mol/l concentration in the injected mixture (30 µl). The urine samples were diluted 1 : 10 (v/v) with demineralized water and analyzed with no further sample preparation. The separations were carried out in electrolyte system No. 2 (Table 1). The driving current was stabilized at 200 µA. Cys: cystine, MI: matrix interferent(s), LE: leading electrolyte, TE: terminating electrolyte

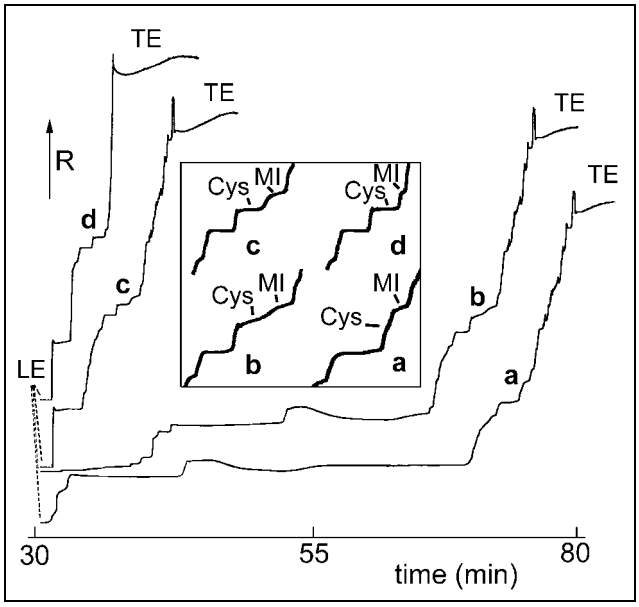

Fig. 2: Electropherograms from separations of cystine present in a urine sample illustrating importance of the post-column ITP sample clean-up in the ITP-ITP combination. (a) Reference, obtained from separation of urine without cystine addition, (b) Same sample with 4×10^{-4} mol/l concentration of cystine added (30 µl sample load) without ITP sample pretreatment, (c) Sample after removing the most abundant sample matrix constituents migrated to the front of the electropherogram, (d) Sample after removing both the front and back of the cystine migrating sample matrix constituents. The details (from individual traces) show a progress in zone resolutions of cystine from urine matrix interferent(s). For the zone assignments see legend to Fig. 1. The urine samples were diluted 1 : 4 (v/v) with demineralized water and analyzed with no further sample preparation. The separations were carried out in electrolyte system No. 2 (Table 1). The driving currents were 200 and 50 µA in the first and second ITP stages, respectively

vented the existence of mixed zones with cystine. As a consequence of this highly efficient sample clean-up [e.g., more than 95% of sample matrix constituents were removed in electropherogram (d)], resolution of the cystine zone from the matrix constituent(s) zone in the second ITP stage was achieved [see electropherograms (c) and (d)], even in spite of using identical migration regimes in both stages (separation according to ionic mobilities). Moreover, the preseparation step facilitated a significant shortening of analysis time (it was ca. 2 times shorter than an analysis without on-line sample pretreatment depending naturally on the amount of matrix components removed, see the full traces in Fig. 2).

As for the second ITP stage, where the final separation and detection of cystine were performed (after removing a crucial part of the anionic constituents of the sample), the investigation included the examination of different electrolyte systems and pH (Table 1), as well as driving current. The best results were achieved with electrolyte system No. 2 and a driving current of 50 µA. The standard curve was linear over the tested range of 1×10^{-4} to 6×10^{-4} mol/l ($n = 7$) while the mean correlation coefficient (R^2) for the calibration curve was 0.9978 (detailed analysis was based on sample IV). The limit of detection for cystine was 7×10^{-5} mol/l for the conductivity detector employed in the second ITP stage and for a 30 µl sample load. A practical use of the proposed method is obvious from the results in Table 2 where the repeatabilities of zone lengths and RSH of cystine in the ITP-ITP runs with urine samples are shown. Here, the relative standard deviation of the cystine RSH for the different urine samples (I–IV) was 1.48% while for the cystine zone lengths it was 2.99% (when cystine was present at 2×10^{-4} mol/l concentration in the urine). A combination of electrolyte systems of higher pH (8.9–9.0) with those of lower pH (8.1) in the preseparation and analytical steps, respectively, also provided good results in term of an effective sample clean-up and this can be useful when separation of cystine from the matrix interferents according to pK values is essential for zone resolution.

Table 1: Electrolyte systems

Parameter	Electrolyte system No.					
	1		2		3	
	Leading	Terminating	Leading	Terminating	Leading	Terminating
Solvent	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
Anion	Cl^-	Glycine	Cl^-	Glycine	Cl^-	GABA
Concentration (mmol/l)	10	10	10	5	10	10
Counterion	Tris	Tris	BTP	BTP	Amediol	$Ba(OH)_2$
pH	8.1	8.9	8.9	8.9	9.0	10.9
Additive	m-HEC	–	m-HEC	–	m-HEC	–
Concentration (%, w/v)	0.2	–	0.2	–	0.2	–

Table 2: Repeatabilities of the zone lengths and RSH of cystine in the ITP-ITP runs with urine samples from different adults*

Sample	Zone length [s]**	RSD [%], n = 7	RSH**	RSD [%], n = 7***
I	24.67	1.0	0.484	0.8
II	23.95	1.2	0.488	0.8
III	25.06	0.7	0.477	0.6
IV	25.73	0.9	0.472	0.4

* the separations were carried out in electrolyte system No. 2 (Table 1) and the concentration of cystine added in each urine sample was 2×10^{-4} mol/l
** average values that reflect results obtained after removing more than 95% of the sample anionic constituents by means of an on-line sample clean up
*** relative standard deviation (RSD) for the seven consecutive runs

It can be said in conclusion that this two-dimensional approach, depending on removing a required amount of the sample anionic constituents migrating in the on-line coupled ITP stack and detectable in the second ITP stage, provided a universal alternative for the separation, detection and quantitation of the model analyte, cystine, in urine. It allowed us to take urine samples for analysis with no sample preparation. A high sample load capacity of the on-line coupled ITP stage was effective in separating the samples corresponding to a ca. 8 µl volume of undiluted urine. It was possible to use a universal detection technique, thus avoiding a derivatization procedure. For similar applications of pharmaceutical and clinical interest the proposed ITP separating conditions could be modified easily if the ITP clean-up is based on separation according to ionic mobilities while the constituents present in the transferred fraction are finally separated via a different separation mechanism (e.g. according to stability constants with an appropriate complexing agent).

3. Experimental

3.1. Samples and reagents

The pure cystine for standard solutions was purchased from Aldrich (Steinheim, Germany). Sodium hydroxides used to increase solubility of cystine in the stock solutions, barium hydroxide and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Hydrochloric acid was used for preparation of the leading electrolyte solution after isothermal distillation. Glycine, 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP), tris(hydroxymethyl)aminomethane (Tris), γ-amino-n-butyric acid (GABA) and 2-amino-2-methyl-1,3-propanediol (Amediol) for preparation of the other electrolyte solutions were obtained from Sigma (St. Louis, MO, U.S.A.). Methyl-hydroxyethylcellulose 30000 (m-HEC), obtained from Serva (Heidelberg, Germany), served as an electroosmotic flow (EOF) suppressor in the leading electrolyte solutions [8]. The electrolyte and standard solutions were prepared in water demineralized by a Rowapure-Ultrapure water purification system (Premier, Phoenix, Arizona, U.S.A.).
The stock solution of cystine was prepared by dissolving the appropriate amount of cystine in sodium hydroxide solution (10^{-2} mol/l) in a volumetric flask to a 10^{-3} mol/l final concentration.

3.2. Instrumentation and ITP conditions

A CS isotachophoretic analyzer (Villa-Labeco, Spišská Nová Ves, Slovak Republic) that permitted operation in the column-coupling mode was used. Its separation unit consisted of the following subunits: (i) An ITP valve injector (a 30 µl internal sample loop); (ii) an ITP preseparation column [provided with a 800 µm I.D. capillary tube made of FEP (fluorinated ethylene-propylene copolymer) with an on-column conductivity sensor] 90 mm in length; (iii) a bifurcation block for on-line coupling of the ITP and ITP columns; (iv) counter-electrode compartments for both ITP columns. A 300 µm I.D. capillary tube 160 mm in length made of FEP was used for the ITP separations in the second stage of the ITP-ITP combination. It was provided with an on-column conductivity sensor for conductivity detection. A high voltage power supply delivered the stabilized driving current. The data from the conductivity detectors were acquired and processed by ITPPro32 software (version 1.0) obtained from KasComp (Bratislava, Slovakia). Prior to use, the capillaries were given no particular treatment to suppress EOF. Dynamic coating of the capillary walls by means of 0.2% m-HEC in the leading electrolyte solutions served for this purpose. ITP-ITP analyses were carried out in the anionic regime of the separation with direct injection of the samples. The driving currents were 200 and 50 µA in the first (preseparation) and the second (analytical) stage, respectively. The temperature was 25 °C.

3.3. Sample and standard solution preparations

Urine samples were obtained from healthy adults with different diet habits (samples I–IV). For the purpose of calibration they were spiked with cystine from the stock solution. The calibration standards were prepared at concentrations of $(1, 2, 3, 4, 5, 6) \times 10^{-4}$ mol/l standard cystine in urine. The urine samples were finally diluted 1 : 4 (v/v) with demineralized water. The samples prepared in this way were stored in a freezer at −10 °C. They were melted at room temperature prior to the analysis.

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