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DETERMINATION OF HOMOCYSTEINE IN URINE AND SALIVA BY MICROCHIP ELECTROPHORESIS

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An online combination of isotachophoresis (ITP) and capillary zone electrophoresis (CZE) was employed on a microchip with a column-coupling technology for the determination of homocysteine in various body fluids. ITP with its high concentration ability was used as a sample pretreatment and injection technique for CZE, which facilitated the rapid and sensitive determination of homocysteine. The resolution of the analyte from other constituents present in real complex samples was enhanced by discrete spacers, which were added to the injected sample. A solid-phase microextraction (SPME) pretreatment technique based on silver- and barium-form resins was used prior to the ITP-CZE analysis to remove high concentrations of chloride and sulfate naturally present in the analyzed samples. The combination of the micro-pretreatment and microelectrophoresis techniques facilitated the determination of trace concentrations of homocysteine in samples of urine and saliva.

Keywords: homocysteine, body fluids, microchip electrophoresis, solid-phase microextraction

1. Introduction

Homocysteine (Hcy) is a non-proteinogenic sulfur-containing amino acid whose metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration. In remethylation, Hcy acquires a methyl group from N5-methyltetrahydrofolate or from betaine to form methionine. The reaction with N5-methyltetrahydrofolate occurs in all tissues and is vitamin B12-dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B12-independent. A considerable proportion of methionine is then activated by ATP to form S-adenosylmethionine [1].

Increased concentration levels of Hcy in body fluids are considered to be an important risk factor or marker of various diseases, particularly cardiovascular ones [2]. In review papers [3–5], individual methods of determining Hcy in biological samples are summarized. Analytical methods using high-performance liquid chromatography (HPLC) to separate Hcy are some of the most commonly used. HPLC methods for the quantification of Hcy utilize derivatization procedures as well as procedures with non-derivatized Hcy by electrochemical detection (ECD). The growing interest in clinical analyses has generated increased attention due to the rapid determination of Hcy using automated methods. For this reason, the immunoassay of Hcy in plasma has become a preferred analytical approach [5].

Recently several procedures have been published for the determination of Hcy by capillary electrophoresis (CE). CE compared to HPLC is more advantageous in terms of the need for a very small sample volume, good resolution, short analysis time, simplicity of automation and elimination of various (toxic) solvents. This is evidenced by many of the works that have dealt with the determination of Hcy in body fluids, e.g. in plasma [6–18], serum [13] or urine [16, 19]. Most of them dealt with the use of laser-induced fluorescence (LIF) as a detection technique, however, UV detection was also used.

A miniaturized form of CE, microchip electrophoresis (MCE), was used for the separation and detection of Hcy and glutathione. The analysis time on the glass microchip using amperometric detection was less than 80 s [6]. A polydimethylsiloxane (PDMS) microchip produced by a simple photolithographic technique facilitated the rapid separation of HCy and cysteine [20]. MCE analyses are more favorable than CE because they are considerably shorter, achieve higher degrees of separation efficiency and, in particular, reduce overall costs associated with chemical consumption and waste production.

This paper deals with the development of a new method for the determination of Hcy in various body fluids on a microchip with coupled-channels (CC). The

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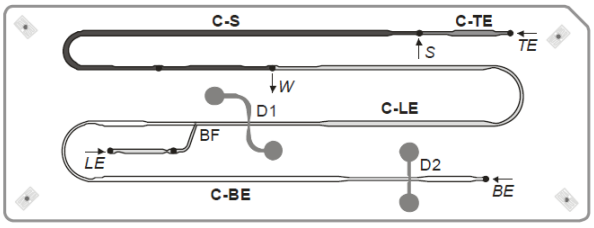

Figure 1: A scheme of the CC microchip with conductivity detection. C-LE = the first (ITP) separation channel (volume of 4.5 μL) filled with a leading electrolyte; C-BE = the second (CZE) separation channel (volume of 4.3 μL) filled with a background electrolyte; C-TE = the third channel (volume of 0.8 μL) filled with a terminating electrolyte; C-S = sample injection channel (volume of 9.9 μL); D1, D2 = Pt conductivity sensors; BF = bifurcation section; BE, LE, TE, S = inlets for the background, leading, terminating and sample solutions to the microchip channels, respectively; W = an outlet channel for the waste container.

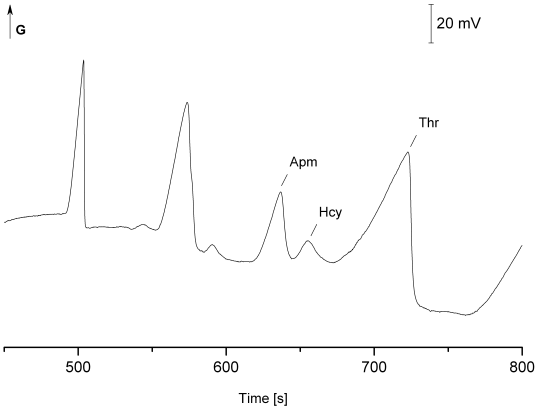

Figure 2: Electropherogram from the ITP-CZE separation of Hcy in the presence of discrete spacers. The sample injected onto the microchip: 100 μmol/L Apm, 15 μmol/L Hcy, 150 μmol/L Thr in a 50 % terminating electrolyte. Apm = aminopimelate; Hcy = homocysteine; Thr = threonine; G = conductivity.

CC technology employed on the microchip with contact conductivity detection enables online capillary zone electrophoresis (CZE) to be coupled with the isotachophoresis (ITP) sample pretreatment. Samples of urine and saliva were simplified by solid-phase microextraction (SPME) prior to the ITP-CZE separations on the microchip. SPME based on silver- and barium-form resins provided a high degree of compatibility with MCE and a high level of selectivity, whilst simultaneously removing a huge amount of chloride and sulfate in the analyzed samples of body fluids.

2. Experimental

ITP-CZE separations were carried out on a poly(methyl methacrylate) microchip (*Fig.1*) with the CC technique and integrated conductivity detection sensors (IonChip^{TM} 3.0, Merck, Darmstadt, Germany).

Chemicals used for the preparation of electrolyte solutions and model samples were obtained from Sigma-Aldrich (Bratislava, Slovakia), Fluka Chemika-Biochemika (Buchs, Switzerland), Serva Electrophoresis GmbH (Heidelberg, Germany) and Erba Lachema s.r.o. (Brno, Czech Republic). Samples of urine and saliva were collected from volunteers. Before the analysis, the samples were homogenized and analyzed after being diluted appropriately with deionized water, and pretreated by SPME to remove chloride and sulfate. Solid-phase extraction (SPE) microcolumns of 0.5 mL in volume containing silver- and barium-form resins (Alltech, Grace Davison Discovery Sciences, Deerfield, Illinois, USA) were used for this purpose.

3. Results and Discussion

ITP-CZE separations were performed in a hydrodynamically closed separation system with suppressed electroosmotic flow. These working conditions effectively reduce the fluctuations in the total migration velocity of the analytes which is crucial to obtain reproducible results, especially on the microchip with short separation paths [21]. In the ITP separations, a leading electrolyte with a pH of 9.1 and glycine as a terminating electrolyte were used. In the CZE step of the ITP-CZE technique, the pH of the background electrolyte was 9.8.

The ITP realized in the first separation channel on the microchip (Fig. 1) preconcentrates the analyte and other constituents of the sample for the CZE separation step. On the other hand, the close migration configuration of the constituents in the ITP stage of the ITP-CZE technique can be a limiting factor for achieving the required resolution of the analyte from matrix constituents, especially on the microchip with short separation channels. In such a case, discrete spacers (DSs) are effectively used to define the fraction of the sample transferred to the second CZE channel when the CC technique is employed. Then, the constituents of the sample migrating outside of the mobility interval defined by the DSs are removed from the separation system. In this way, ITP works as an online sample clean-up technique prior to the CZE. In our case aminopimelate and threonine were used as the front and rear DSs, and the undesirable constituents of the sample were electrophoretically removed from the separation system by column switching prior to the CZE separation realized in the second channel (Fig. 2).

A 1.4 μmol/L limit of detection (LOD) for Hcy was obtained by the ITP-CZE technique on the microchip. A relatively large volume of the sample that was injected onto the microchip (9.9 μl) contributed to achieving the low value of LOD when a universal and relatively low-sensitive conductivity detector was used. Under the working and separation conditions employed, the reproducible migration velocities (the relative standard devi-

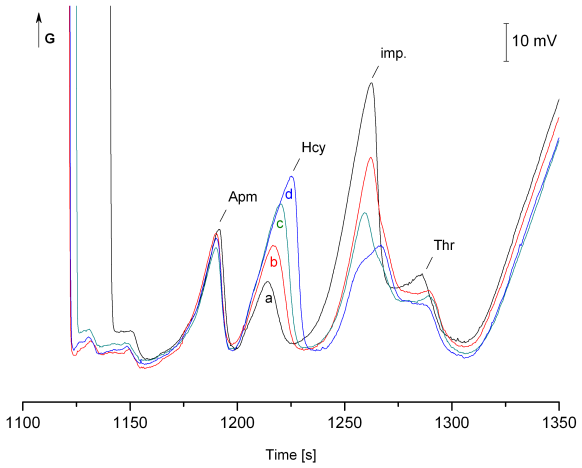

Figure 3: Electropherograms from ITP-CZE analyses of urine samples after SPME pretreatment with various additions of Hcy. Sample injected on the microchip: urine diluted twice, 400 µmol/L Apm, 200 µmol/L Thr in 50 % terminating electrolyte with (a) 0; (b) 10; (c) 20; (d) 40 µmol/L Hcy. Apm = aminopimelate; Hcy = homocysteine; Thr = threonine; imp. = impurity; G = conductivity.

ations (RSDs) of migration times were between 0.5 and 1.2 %) and determinations of trace concentrations of Hcy (RSDs of peak areas were 1.2 %) were achieved.

Considering the high concentration levels of chloride and sulfate in urine and saliva samples, these interfering anions were removed before ITP-CZE analysis from real samples. For this purpose, SPME using silver- and barium-form resins was conducted. The content of Hcy in samples of body fluid was evaluated by the method of standard addition using ITP-CZE analysis after SPME pretreatment. The Hcy concentrations in the tested samples of urine and saliva were approximately 22 µmol/L and 6 µmol/L (Fig. 3), respectively.

4. Conclusion

The SPME-ITP-CZE method performed on the microchip with conductivity detection and the CC technique enabled the fast (total analysis time of approximately 20 mins.) and reliable determination of the trace concentrations of Hcy in biological samples such as urine and saliva.

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