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Two-dimensional high performance liquid chromatography for determination of homocysteine, methionine and cysteine enantiomers in human serum

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ABSTRACT

A two-dimensional HPLC system with electrochemical detection was used for determination of homocysteine, methionine and cysteine enantiomers in biological samples. The amino acid separations were not possible only by using a chiral column. The compounds were separated from each other on an achiral column (Purospher RP-18 endcapped 250–4 mm, 5 μm) and their enantiomers were separated on Chirobiotic TAG (250–4.6 mm, 5 μm) column in an on-line system. The mobile phase composition and a choice of electrode potentials for detection were investigated. The L-enantiomers always eluted before the D-enantiomers. The proposed method was applied to the analysis of human serum of healthy volunteers and patients with multiple sclerosis. The limit of detection (LOD) and quantitation (LOQ) were defined as the concentration that produced a signal-to-noise ratio (S/N) of 3 and 10. The method LOD values were found to be between 0.05 and 0.50 μg mL^{-1}. The range of LOQ values were between 0.17 and 1.67 μg mL^{-1}, respectively.

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1. Introduction

Homocysteine (HCSH) is a thiol-containing amino acid that is formed during methionine metabolism. It can be converted back to methionine (Met) in remethylation pathway or to cysteine (CSH) in transsulfuration pathway. The biological association between HCSH, Met and CSH evokes the need for their simultaneous determination in biological samples. Normal ranges for total HCSH, Met and CSH have been specified in serum and urine by Stabler et al. [1] using capillary gas chromatography–mass spectrometry. The determined concentrations of HCSH, Met and CSH in human serum were about 13, 25.5 and 261 μmol L^{-1} and in urine they were about 7.2, 5.9 and 260 μmol L^{-1}. Their concentrations in human plasma were similar (11.5 μmol L^{-1} for HCSH, 6.1 μmol L^{-1} for Met and 269 μmol L^{-1} for CSH) [2]. Elevated plasma HCSH levels are associated with a higher risk of cardiovascular and cerebrovascular diseases [3]. There is assumption that HCSH might cause neuronal damage by triggering oxidative injury and DNA damage. A connection of multiple sclerosis (MS) and homocysteine metabolism has been already investigated [4]. MS is a chronic autoimmune, inflammatory neurological disease of the central nervous system in which immune system attacks the myelin that covers nerves. Myelin damage disrupts communication between the brain and the rest of the body [5]. Some studies demonstrated elevated plasma HCSH in MS patients [6–8], but in others there were no differences [9,10]. Since these amino acids are chiral, enantiomeric analysis of human samples may be desirable for a better understanding the role of metabolism and dynamics of HCSH in humans.

The presence of some D-amino acids has been reported in human samples. For instance, Armstrong et al. [11] found out that D-amino acids exist in all physiological fluids tested (plasma, urine, cerebrospinal fluid, and amniotic fluid), but their level varied considerably. The concentration of D-amino acids like aspartate [12–17], serine [12,13,17–26], alanine [17–19,21,27], proline [17–19,21], leucine [17,21,28], phenylalanine, tryptophan [19,21,28], lysine [19,21], tyrosine [19,28], aspartic acid, glutamic acid, glutamine [27], asparagine, arginine, valine and Met [21] has been already determined.

In biological samples, amino acid enantiomers have been usually determined by using high performance liquid chromatography [12,21], gas chromatography [17,19], capillary electrophoresis [29] or capillary micellar electrokinetic chromatography [27]. Amino acid enantiomeric separations can be realized for instance with

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crown-ethers [30–32], cyclodextrins [33], polysacharides [34] or macrocyclic glycopeptides [35].

The macrocyclic antibiotics were introduced by Armstrong et al. [36] as a new class of chiral selector in 1994. They are complex molecules with many different interactive sites. Therefore, functional groups can interact with enantiomers through π–π and electrostatic interactions, hydrogen bonding as well as hydrophobic interactions, steric (repulsive) hindrance or forming inclusion complexes [36,37]. There are four chiral stationary phases for chromatography (ristocetin, teicoplanin, vancomycin and teicoplanin aglycone) [38]. Mainly, teicoplanin and ristocetin columns have unique enantioselectivity for amino acids [39].

The aim of this work was to develop a HPLC method for simultaneous determination of HCSH, Met and CSH enantiomers in biological samples by using two columns in an on-line system. Enantiomers were detected by using an electrochemical detector, which belongs to a nonchiral detector and therefore the response factor of L- and D-enantiomers must be the same because of the physical (except optical activity) and chemical properties of the enantiomers are identical in an achiral environment [40].

2. Material and methods

2.1. Chemicals

Acetonitrile (ACN), sodium phosphate monobasic monohydrate, perchloric acid, sodium borohydride, L-HCSH, DL-HCSH, L-Met, D-Met, DL-Met, L-CSH, D-CSH and DL-CSH were purchased from Sigma–Aldrich (USA). Ortho-phosphoric acid and 1-octanesulfonic acid sodium salt (OSA) was purchased from Fluka Biochemika (Switzerland). Sodium hydroxide and methanol (MeOH) were obtained from Merck (Germany).

2.2. Preparation of working solutions

All standard solutions were prepared in water. Doubly deionized water (≥18 MΩ cm) was produced on the apparatus Rodem 6 (Rodem Water s.r.o., Slovakia). Working solutions of these amino acids were obtained by mixing the stock solutions. All solutions were stored at −80 °C until use.

2.3. Blood collection and sample preparation

Blood was obtained from three healthy young volunteers (two men and one woman age 21, 39 and 29) and three patients with MS (three women age 21, 39 and 29). MS patients had relapsing-remitting form and were out of relapse at the time. According to our knowledge, patients were not presented with any other serious illnesses [41]. Venous blood samples were collected after 12-h overnight fast. Within 1 h of collection, blood was centrifuged at 2200 × *g* for 15 min at 4 °C. Obtained serum was storaged at −80 °C until analysis. Serum samples were pre-treated according to Garaiova et al. [42] and measured in duplicate. Shortly, serum HCSH, Met and CSH total levels were determined on a HPLC system. 40 μL sodium borohydride (1 mol L^{-1} in 0.1 mol L^{-1} NaOH) were added to 100 μL serum, mixed and incubated at 50 °C for 30 min. 100 μL perchloric acid (0.6 mol L^{-1} in water) was added and samples centrifuged at 14,000 × *g* for 15 min. Non-diluted supernatant (20 μL) was injected into the HPLC system.

2.4. Instrumentation and chromatographic conditions

The chromatographic system consisted of the isocratic pump (DeltaChrom SDS 030, Watrex, Praha, Czech Republic) and an electrochemical detector (Coulochem II, ESA, Chelmsford, UK). The detector was composed of guard cell Model 5020 and analytical cell Model 5010A (ESA, Chelmsford, UK) with porous graphite electrodes. Analytical cells had potential +0.7 V (E1) and +0.9 V (E2), while the potential of guard cell was +1.4 V. Achiral separations were performed on Purospher RP-18 endcapped 250-4 mm (5 μm) (Merck, Darmstadt, Germany) used together with a pre-column Purospher STAR RP-18e (5 μm) (Merck, Darmstadt, Germany). Chiral separations were achieved on Chirobiotic TAG 250-4.6 mm (5 μm) column (ASTEC, USA). The mobile phase contained 25 mmol L^{-1} phosphate buffer, 1 mmol L^{-1} OSA, pH 2.7, ACN and MeOH with ratio 94:3:3 (v/v/v). The flow rate was 0.4 mL min^{-1}. A nylon filter (47 mm in diameter) was used for the mobile phase filtering. The columns were thermostated with JET STREAM II Plus HPLC Column Thermostat (WO Industrial Electronics, Austria) at 20 °C. The accuracy of temperature adjustment was ±0.1 °C.

Amino acids (DL-form) with concentration 0.01 mg mL^{-1} were injected into HPLC system individually or as the mixture. L-Forms were used for elution order control. All measurements were carried out in triplicate, except hydrodynamic voltammogram. Data were analyzed using StatsDirect statistical software (http://www.statsdirect.com, England: StatsDirect Ltd. 2008). Comparison of amino acid concentrations between healthy patients and patients with MS were performed using unpaired *t*-test. The level of significance was taken $p < 0.05$.

2.5. Calibration curves, limits of detection, limit of quantitation

Three calibration curves were constructed because of different concentrations of HCSH, Met and CSH in serum. Linear calibration curves consisted of 5 points for each compound (peak area vs. concentration). Calibration points at the range of 0.7–4.07 μg mL^{-1}, 0.7–29.9 μg mL^{-1} and 3.0–60.8 μg mL^{-1} for HCSH, Met and CSH were measured in duplicate. The limit of detection (LOD) was measured as the lowest amount of the analyte that may be detected to produce a response that is different from that of a blank (signal-to-noise ratio, S/N = 3). The limit of quantitation (LOQ) was measured as the lowest amount of analyte that can be reproducibly quantified above the baseline noise (S/N = 10).

2.6. Standard stability

Stability of standard solution (calibration standard) stored at +4, −20 and −80 °C for 1 month were carried out according to Khan et al. [43]. The standard was injected in triplicate and the percentage stability was calculated by the following equation:

$$\%\ \text{stability} = A_t/A_0 * 100 \quad (1)$$

where A_t is amino acid peak area at time *t* and A_0 is same amino acid peak area at initial time.

3. Results and discussion

In this work, we focused on the separation of HCSH, Met and CSH enantiomers in a single run. The amino acid separations were not possible only by using a chiral column. These amino acids have very similar chemical structures, which do not contain any chromophore or fluorophore (Fig. S1). Therefore, HPLC amino acid separation and enantiomeric separation was achieved by using two columns. Amino acids were separated on achiral column and their enantiomers on a chiral column in an on-line system. The selection of chromatographic conditions was crucial for their enantiomeric separations. Compatibility of mobile phases was required for the achiral–chiral system. The mobile phase composition and a choice of electrode potentials for detection were investigated. Possible

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