Clinical application of capillary electrophoresis – determination of free amino acids in body fluids

Markéta Janotová¹, Kristýna Šmerková¹, Markéta Vaculovičová¹, Markéta Vaculovičová², René Kizek²

¹ Department of Chemistry and Biochemistry, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic - European Union
² Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic - European Union

Accepted: 3. 12. 2014
Keywords: amino acids, body fluids, capillary electrophoresis

1. Introduction

Determination of biomarkers in body fluids provides information about the overall state of the organism¹. Among commonly determined markers in clinical laboratories belong AAs. The AAs (Fig. 1), basic structural units of peptides, as well as their derivatives occupy many metabolic and biochemical roles in human body ²,³. The AAs belong to zwitterions containing acidic and basic functional groups (carboxyl groups and amino, respectively). Zwitterions have neutral character at a certain pH known as the isoelectric point due the dissociation of acidic and basic groups. Therefore, the charge of AAs depend on the number of functional groups and pH environment ⁴.

Determination of AAs levels in the body fluids such as blood and/or plasma ⁵,⁶, cerebrospinal fluid ⁶,⁷, urine ⁶,⁷, saliva ⁶,⁷ or amniotic fluid ⁹ represents the significant clinical indicator not only for control of the nutritional state of the organism, but for a number of metabolic disorders¹⁰. Examples can be inherited metabolic disorders such as phenylketonuria (PKU) (an enzyme deficiency in phenylalanine hydroxylase (PAH) (Fig. 2))¹¹ or maple syrup urine disease (an enzyme deficiency in branched-chain α-ketoacid dehydrogenase (BCKD) (Fig. 3))¹².

There are many methods for AAs determination employing various techniques such as high pressure liquid chromatography (HPLC)¹³ or gas chromatography (GC) ¹⁴. During the 80’s of the 20th century CE became a routine laboratory technique¹⁵. Recently, this method has ranked among very useful tools for separation and determination of AAs ²,⁹,¹⁶,¹⁷, biogenic amines¹⁸ or peptides ¹⁹ and is often used as an alternative to HPLC.
or GC due to its electrophoretic separation mechanism. In addition, a main advantage is very low sample and solvent consumption, speed of analysis and excellent separation efficiency. Besides, due to the high resolving power is CE a promising method for the analysis of biological fluids (complex mixtures of many metabolites). The essential part of the CE instrumentation is a detector. In photometric and fluorescence detection is problem with a limited presence of chromophores and fluorophores. Because of that, the derivatization procedures prior CE are usually used, which is the one of the limiting factors. Therefore, there are attempts to analyze AAs without derivatization employing various strategies such as indirect detection, electrochemical detection (conductometric detection, amperometric detection) or CE in combination with mass spectrometry (MS).

2. Determination of AAs in body fluids
2.1 Blood and/or plasma

The plasma concentration of AAs represents not only an important indicator of the intermediary metabolism, but plays a very important role in many diseases such as diagnostic biomarker, evaluating the disease progression, and monitoring response to the drug therapy. Recent clinical studies have shown an association between the high level...
of homocysteine (hCy) and Cys and vascular diseases. hCy and Cys belong to the low-molecular-weight biothiols, which play an important role in metabolism. To this group belongs also significant tripeptide, the glutathione (GSH), composed of the glutamic acid, cysteine and glycine. GSH occurs in two forms - reduced triol (GSH) and oxidized disulfide (GSSH). The antioxidant function and detoxification processes belong between the important roles of GSH. Zunic and Spasic focused their study on determination of GSH as reflect of oxidative status and aromatic and sulfur containing AAs as good indicators of protein metabolism. They analyzed human capillary blood using CE with direct photometric detection.

As it was mentioned, the plasma concentration of AAs also plays an important role as a biomarker for a number of diseases. The association with a number of neuropsychiatric diseases was demonstrated. For example, the association between plasma level of Glu and Gly and psychotic symptomatology in bipolar disorder (BD) or the correlation between plasma levels of Glu, Ala and Ser with severity of depression and schizophrenia was observed. Lorenzo and co-workers focused their attention on determination the AAs concentration in human plasma from patients with BD. They developed a sensitive CE coupled to laser-induced fluorescence (LIF) detection. The method permitted to quantify fourteen AAs and the obtain results were consistent with published values for patients with BD. This method is suitable for studies that use the AAs as biomarkers in the monitoring of diseases. In addition, chiral CE-LIF method permits separation of L- and D- AAs, which cannot be detected with other general methods based on GC and HPLC.

In the introduction, the inherited metabolic disorders characterized by an enzyme defect were mentioned. An important role plays detection and diagnosis of these diseases in the
neonatal period as part of newborn screening, because the enzyme defect may cause an accumulation of toxic metabolites that leads to serious and irreversible complications. Jeong and co-workers focused on diagnosis of PKU by determination of Phe and Tyr from dried blood spots using capillary CE-MS with electrospray ionization. A routine clinical laboratory detection can determine semi-quantitatively the Phe using a bacterial inhibition assay and enzymatic colorimetric assay in the form of commercial kits. But these kits may lead to relatively high rates of false-positive results. Between the instrumental methods as the conventional PKU screening method belongs HPLC-MS. Jeong and co-workers examined CE-MS as an alternative for routine analytical methods for clinical applications and applied the optimized CE-MS method to real samples of Korean newborns. After comparison with the above common techniques the results showed potential of method as a routine analytical methods for clinical applications due to its different separation mechanism, speed, high separation efficiency, and extremely low sample consumption.

Attractive alternative to detection without the interaction of electromagnetic radiation with the analyte is electrochemical detection in the form of conductometry, amperometry or potentiometry. Samcova and Tuma focused their attention on CE using contactless conductivity detection (CCD) method. This alternative brings analysis of AAs without a presence of chromophore and without derivatization step prior CE. The optimized method determined 18 AAs from 20 proteinogenic AAs, 3 nonproteinogenic AAs and creatinine from plasma samples prepared from the arterial blood of 9 healthy humans and the ranges for the AAs levels in human plasma samples agreed well with those determined by the liquid chromatographic method in two other laboratories. LODs for individual AAs vary in an acceptable range from 4.3 mM for Arg to 42.9 mM for Cys.

2.2 Cerebrospinal fluid (CSF)

As a plasma concentration of AAs also a concentration of AAs in CSF plays an important role in a number of physiological and pathophysiological processes. CSF surrounds the brain and the spinal cord and it is produced from the choroid plexuses. CSF circulates in the central nervous system (CNS) and provides a constant chemical environment for brain cells. This body fluid can be used most likely to detection disturbances of the CNS due to the close relationship between CSF and the CNS. Significant indicators for the control of various functions in the central and peripheral nervous system are the AAs neurotransmitters. Among the most studied AA neurotransmitters include excitatory AAs such as Glu or Asp playing a role in learning and memory, the inhibitory neurotransmitters such as γ-aminobutyric acid (GABA), Gly or taurine (Tau) and AA Gln closely linked to the metabolic turnover of Glu and GABA. In recent studies correlation between the changes in AAs level and a number of neurological diseases was found. For example Alzheimer's disease, where anatomical and biochemical evidence suggests the dysfunction of excitatory AAs pathways. Another diseases which has been confirmed by the association between changes in AAs level and diseases was Parkinson's disease, stroke or epilepsy.

2.3 Saliva

Saliva represents one of the other significant informative body fluids primarily due to easy and noninvasive approach. This fluid permits the early gathering information about the human health. Many studies have confirmed the presence of free AAs in saliva. Association between levels of Lys and Arg and their cariostatic effect and caries was observed. By caries-free adults have been showed elevated
levels of these AAs in the saliva, as compared with caries-susceptible adults\textsuperscript{49}. An involvement of Gly in the pathogenesis of parodontitis was suggested due to its stimulatory effects of production interleukin-1beta-induced prostaglandin E2 \textsuperscript{50}. In addition, this AA is together with Glu involved in the metabolism of microorganism. Besides, the presence of Pro indicates putrefaction\textsuperscript{16,51}.

Deng and co-workers employed the saliva for practical utility their developed CE-LIF method for determination of AA neurotransmitters\textsuperscript{7}. This method has been mentioned by determination of AA neurotransmitters in CSF. Due to this method have been detected main AAs occurring in saliva from two volunteers (Pro, Ser, Gly and Glu) with the LODs in the range from 0.1 to 2.4 nM. The promising results of AAs quantification was in good agreement with data in earlier studies. For the elimination of derivatization step by determination of AAs, Coufal and co-workers employed CE-CCD \textsuperscript{21}. The detection limits of individual AAs ranged within a concentration interval from 9.1 µM for Lys to 29 µM for Asp. This method was tested on more natural samples as beer, yeast or urine and in the saliva was successfully detected Gly and Pro.

2.4 Urine

Between the other important biological fluids belongs the urine. This complex mixture presents an essential part of clinical routine for the screening of the intermediary metabolism in the body. As well as in saliva the big advantage of urine is its non-invasive approach\textsuperscript{52}. In the previous section was mentioned Coufal and co-workers CE-CCD method with elimination of the derivatization procedure. One of the tested samples was urine where managed to identify nine amino acids and creatinine. Five years later, Chen and co-workers employed for determination of AAs in urine cyclodextrin-modified CE-LIF \textsuperscript{22}. This method permitted the successful determination of seven amino acids (Phe, Glu, Pro, Gly, Ser, Ala, Val) with the LODs in range 160~330 nM and without complicated pretreatment procedures. Another method for AAs determination introduced Ramautar and co-workers. They present improved CE-TOF-MS method achieving the LODs down to 20 nM and permitting the selective detection of all commonly occurring amino acids, except for the isobaric amino acids such as leucine and isoleucine. This method is suitable for the metabolic profiling of urine and other complex fluids.

2.5 Others

The detection and determination of biomarkers play also the important role in prenatal diagnostic. For these purposes amniotic fluid (AF) serves and the monitoring of this fluid brings information about the fetus health and maturity. AF forms a sac around the fetus and creates a protective environment from mechanical and thermal shock \textsuperscript{9,53,54}. In addition to this role, is AF involved in development of the lungs, kidneys, and gastrointestinal tract \textsuperscript{9}. The volume of AF varies according week of pregnancy and is in the correlation with the growth of fetus. The volume ranges from about 50 mL at 12 weeks to 1 L at 38 weeks. After this week, the volume decreases to a volume of about 600 mL\textsuperscript{9,54}. The biochemical investigation of the AF is done in the case of the semblance of the abnormal fetal development or by the pregnant woman age over 35 years. Because of the AAs monitoring various metabolic disorders can be detected, for example mentioned PKU associated with abnormal metabolism of AAs \textsuperscript{9}. Tuma and co-workers employed CE-CCD method and applied it on the AF from 20 pregnant women aged over 35 years and 24 pregnant women with abnormal fetal development for the whole profile of AAs \textsuperscript{9}. This method permitted the sensitive determination of 20 proteinogenic AAs and 12 other biogenic compounds with LODs between 1.5~6.7 µM. The obtained results showed systematically higher mean concentration of majority of AAs for women suspect of abnormal fetal development.

Summary of CE conditions for analysis of AAs in various body fluids is given in Table 1.
<table>
<thead>
<tr>
<th>DETECTION METHOD</th>
<th>SAMPLE</th>
<th>DERIVATIZATION STEP</th>
<th>BGE</th>
<th>CAPILLARY</th>
<th>VOLTAGE</th>
<th>REF</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>Human capillary blood</td>
<td>No</td>
<td>10 mmol/l phosphate buffer (pH 2.8)</td>
<td>47 cm, 75 µm ID</td>
<td>15 kV</td>
<td>24</td>
</tr>
<tr>
<td>Indirect-UV</td>
<td>Human plasma, supernatant of macrophage cultures</td>
<td>No</td>
<td>p-Aminosalicylic acid buffered with sodium carbonate (pH 10.2 ± 0.1)</td>
<td>87 cm, 75 µm ID</td>
<td>15 kV</td>
<td>3</td>
</tr>
<tr>
<td>Indirect-UV</td>
<td>Human urine</td>
<td>No</td>
<td>5.0 mM carbonate, 2.0 mM salicylate, 0.15 mM myristyltrimethyl-ammonium bromide (pH 10.7)</td>
<td>Unknown length, 100 µm ID</td>
<td>15 kV</td>
<td>55</td>
</tr>
<tr>
<td>LIF</td>
<td>Human plasma</td>
<td>NBD-F</td>
<td>175 mM borate buffer, 12.5 mM β-cyclodextrin (pH 10.25)</td>
<td>60 cm, 75 µm ID</td>
<td>21 kV</td>
<td>10</td>
</tr>
<tr>
<td>LEDIF</td>
<td>Human cerebrospinal fluid</td>
<td>NDA</td>
<td>10 mM tetraborate, 0,6% PEO (pH 9.3)</td>
<td>50 cm, 75 µm ID</td>
<td>15 kV</td>
<td>38</td>
</tr>
<tr>
<td>LIF</td>
<td>Human cerebrospinal fluid, saliva</td>
<td>SIFA</td>
<td>100 mM SDS, 100 mM boric acid (pH 9.6)</td>
<td>60.2 cm, 75 µm ID</td>
<td>15 kV</td>
<td>7</td>
</tr>
<tr>
<td>LIF</td>
<td>Human saliva</td>
<td>FITC</td>
<td>20 mM borate buffer (pH 9.5)</td>
<td>58 cm, 75 µm ID</td>
<td>20 kV</td>
<td>16</td>
</tr>
<tr>
<td>LIF</td>
<td>Human urine</td>
<td>FITC</td>
<td>80 mM borate buffer (pH 9.2) containing 45 mM α-cyclodextrin</td>
<td>60 cm, 50 µm ID</td>
<td>-15 kV</td>
<td>22</td>
</tr>
<tr>
<td>LIF</td>
<td>HUVEC, ECV304, R1 stem cells</td>
<td>5-IAF</td>
<td>20 mmol/l sodium phosphate, 16.5 mmol/l boric acid, 100 mmol/L N-methyl-D-glucamine (pH 11.2)</td>
<td>60 cm, 50 µm ID</td>
<td>30 kV</td>
<td>30</td>
</tr>
<tr>
<td>CCD</td>
<td>Human plasma</td>
<td>No</td>
<td>1.7 M acetic acid containing 0.1% hydroxyethylcellulose (pH 2.2)</td>
<td>80 cm, 75 µm ID</td>
<td>20 kV</td>
<td>23</td>
</tr>
<tr>
<td>CCD</td>
<td>Human urine and saliva, beer, yeast, herb extracts</td>
<td>No</td>
<td>2.3 M acetic acid and 0.1% hydroxyethylcellulose</td>
<td>80 cm, 50 µm ID</td>
<td>30 kV</td>
<td>21</td>
</tr>
<tr>
<td>CCD</td>
<td>Amniotic fluid</td>
<td>No</td>
<td>1.7 M acetic acid and 0.1% hydroxyethylcellulose (pH 2.15)</td>
<td>80 cm, 75 µl ID</td>
<td>20 kV</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1: Table summarizing some experimental conditions for determination of AAs using CE analysis.
<table>
<thead>
<tr>
<th>MS</th>
<th>Human dried blood spots</th>
<th>No</th>
<th>3 mM NH₄Ac (pH 10.7)</th>
<th>40 cm, 50 µm ID</th>
<th>25 kV</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Child plasma</td>
<td>No</td>
<td>50 mM aqueous formic acid (pH 2.5)</td>
<td>105 cm, 50 µm ID</td>
<td>30 kV</td>
<td>56</td>
</tr>
<tr>
<td>MS</td>
<td>Human urine</td>
<td>No</td>
<td>20% methanol, 2M formic acid</td>
<td>100 cm, 50 µm ID</td>
<td>Unknown</td>
<td>52</td>
</tr>
<tr>
<td>MS</td>
<td>Human urine</td>
<td>No</td>
<td>1 M formic acid (pH 1.8)</td>
<td>130 cm, 50 µm ID</td>
<td>30 kV</td>
<td>57</td>
</tr>
</tbody>
</table>

**Abbreviations:** 5-IAF 5-iodoacetamidofluorescein; NBD-F 4-fluoro-7-nitro-2,1,3-benzoxadiazole; SIFA N-hydroxysuccinimidyl fluorescein - O-acetate; NDA naphthalene dicarboxaldehyde; FITC fluorescein isothiocyanate; PEO poly(ethylene oxide)
3. Conclusion

The CE represents a powerful tool for determination of amino acids in complex body fluids due to the high resolving power, small reagents consumption and separation efficiency. There are a number of detection methods such as optical method, electrochemical method or CE in combination with MS. Problem occurs in photometric and fluorescence detection by analytes without the presence of chromophore or fluorophore. In this case derivatization procedures prior CE are used. Effective alternative without derivatization procedure is CE-CCD or CE-MS. CE-MS represents powerful analytical tool with highly sensitive for selective quantification and determination relative molecular weights and molecular structure of substances. However, the limiting factor in the use of this method in routine practice can be demanding requirements to use interface.

Whereas, CE-CCD permits the analysis of the whole profile of proteinogenic AAs in native form without derivatization and demanding requirements.

Financial support from CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged.

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE “uniform requirements” for biomedical papers.

References