Amino acid profiling in urine by capillary zone electrophoresis – mass spectrometry

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Abstract

Analysis of amino acid profiles in urine and plasma is an essential part of modern clinical diagnostic routine. Here we present an approach for the analysis of amino acids in urine by capillary electrophoresis/time-of-flight (TOF) mass spectrometry. At first a method combining improved separation, high dynamic range, and high sensitivity is presented. Detection limits in the mid nM-range are achieved through the use of pH-mediated stacking injection in combination with modern TOF detection technology. The method can be easily applied to detect differences in the amino acid profile in urine in a clinical context. Moreover, beside amino acids low molecular weight amines, peptides and related metabolites can be profiled. As a proof of concept, urine samples from patients suffering from osteoarthritis have been analyzed. Finally, the introduction of multivariate data analysis in the workflow was evaluated on spiked urine samples and real clinical material.

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Analysis of the amino acid composition of body fluids (cerebrospinal fluid, serum and urine) is an important part of clinical routine. Profiling of amino acids and their derivates helps to access several important clinical categories: such as for example gastrointestinal function; it may reveal dietary protein deficiency and/or maldigestion [1]. The cellular energy metabolism is another function assessed by amino acids profiling, here amino acids patterns have been used for in depth analysis of chronic fatigue syndrome [2]. Proline, hydroxyproline and hydroxylysine levels are important indicators of connective tissue status [3,4]. In metabolic disorders, the knowledge of the concentration of one amino acid or related group of amino acids is essential for correct diagnosis.

There are several analytical approaches for amino acid detection currently used in routine clinical practice. Individual species are often measured with the help of specialized assays like the nitrosonaphthol test for tyrosine or the cyanide-nitroprusside test for cysteine and homocystine. Such tests are practical, robust, cheap but extremely limited and their analytical foundation is, sometimes, a bit outdated [5]. Much more advanced is amino acid profiling with dedicated HPLC-based analyzers. Commercial amino acid analyzers offer almost “plug-and-play” solutions for amino acid profiling, but derivatization (pre-column, post-column or on-column) is an essential step and the variety of compounds subjected for analysis is still limited.

Capillary electrophoresis (CE) is not an entirely new method for the clinical laboratory and it has already proven itself as a valuable addition to the clinical chemists arsenal especially in the field of molecular diagnostics [6]. Amino acids were one of the first compound classes investigated by CE because they are charged in solution [7]. The combination of capillary electrophoresis and mass spectrometry (MS) opens even more analytical possibilities due to the increased sensitivity, specificity and the potential identification of unknown amino acids or related compounds [8–14]. The analytical qualities of CE combined with mass spectrometry provide an opportunity to...
analyze the amino acid content of body fluids without complicated sample pretreatment, derivatization, and make use of such dedicated instruments as amino acid analyzers rather unpractical. The feasibility of CE/MS for the amino acids analysis as well as some applications has already been demonstrated by Soga and co-workers [9,10] and by others [8,12,14,15]. These investigators addressed a number of important technical issues such as an influence of background electrolyte composition on resolution and peak shape, they showed the limits of detection for different amino acids and suggested some basic rules for sheath liquid selection. All together these results provide a very solid analytical background for development of a tailored clinical application. On the other side we would like to stress several points which are not covered by previous studies. First of all, the mass analyzers selected by Soga and coauthors (quadrupole and triple-quadrupole) demand compound fragmentation for identification. Moreover to obtain the maximum sensitivity of quadrupole with selected ion monitoring (SIM) and triple-quadrupole instrument with Multiple Reaction Monitoring (MRM) one has to generate a list of compounds, which will certainly bias further analysis. One has to pay attention to a number of selected compounds, because the more compounds need to be detected in a given time interval the less sensitive the detection in quadrupole-based MS instruments is. Here, we propose to use an orthogonal time-of-flight (TOF) instrument, which provides a mass accuracy sufficient to identify compounds only on basis of accurate mass measurement and migration time respectively. All amino acids and related compounds (amines, small peptides, etc.) can be detected simultaneously without restriction to expected or known compounds. This, in turn, allows experiments where amino acids are measured in their metabolic context. This context or “background” contains important information about the metabolical state of the organism, which can be used for diagnostic as well as for analytical purposes.

To demonstrate the feasibility of the approach we selected urine samples of osteoarthritis patients. Osteoarthritis, the most common form of arthritis, is a chronic joint disease characterized by progressive destruction of articular cartilage, resulting in impaired movement, pain and, ultimately, disability [16,17]. Diagnosis of osteoarthritis is based on a combination of clinical symptoms and laboratory examinations with imaging techniques (such as radiology or MRI imaging) in the first place. Imaging techniques provide a kind of gold standard for diagnosis, but degenerative changes become visible only in late stages of disease progression and at this time cartilage degradation is irreversible. Numerous efforts have been directed at development of noninvasive, early diagnostic of osteoarthritis which has resulted in a number of clinically approved markers [18]; however, early diagnostics is still an important issue. So far, osteoarthritis has never been associated with any amino acids aberration in urine, but there are reports on the difference in metabolite composition between healthy donors and osteoarthritis patients [19]. In the current study we used an improved CE separation method in combination with TOF mass analyzer and subsequent multivariate data analysis of entire data set to reveal differences in urinary amino acids patters between healthy controls and patients with different stages of osteoarthritis.

1. Material and methods

HPLC-grade acetonitril, methanol and water were supplied by J.T. Backer. Amino acids standards were purchased from Sigma.

CE was performed on Hewlett Packard (Agilent) or Beckman Coulter PA 800. Uncoated fused silica capillaries (100 cm) with 50 μm i.d. were used for separation. 20% methanol with 2 M formic acid was used as background electrolyte. For injection 50 μl of sample was equilibrated with 1 μl of background electrolyte. Sample injection was performed hydrodynamically with pH-mediated stacking: a small plug (50 mbar, 9 s) of 12.5% NH₄OH was injected before the sample plug (50 mbar, 45 s).

MS was performed using the microTOF (Bruker Daltonik, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer. Transfer parameters were optimized by direct infusion of a tune mix (Agilent) or sodium formate clusters. Spectra were collected with time resolution 1 s. Post-run internal mass scale calibration were performed using the sodium formate cluster ions Na(NaCOOH)₁₋₉ ranging from 90.9766 to 634.8760 m/z, which are formed by the fast migrating Na ions and the BGE.

CE/MS coupling was realized by a co-axial sheath liquid interface (Agilent Technologies, Waldbronn, Germany) with 50% isopropanol and 0.1% formic acid as a sheath liquid. The following spray conditions were used: sheath liquid flow −4 μl/min, dry gas temperature − 180°C, nitrogen flow − 5 l/min, nebulizer − 0.4 bar. Electrospray in positive ionization mode was achieved by the application of −4.5 kV on the inlet of the mass spectrometer. Holding the electrospray potential at ground results in no influence of CE separation voltage on the electrospray, and, thus, easy method optimization and the use of high currents in the CZE.

2. Results and discussion

2.1. Separation

Besides the early work by Lu et al. [12] who used 10% acetic acid all other studies have been performed using formic acid at high concentrations of 0.3–1 M as background electrolyte (BGE) [8,9,14,15]. Good separation (and ionization efficiency in ESI positive mode) is achieved; however, the critical pair of leucine isoleucine is not baseline separated. We optimized the CE conditions in respect of separation efficiency and sensitivity. Higher concentration of formic acid resulted in a better separation but as well in higher current which interfere again the separation. Therefore, we tested various organic modifiers (isopropanol, acetonitril, and methanol) and achieved the best separation using a BGE consisting of 2 M formic acid and 20% methanol. A separation of a standard solution of 16 amino acids is shown in Fig. 1. A good separation was obtained for the amino acids (i.e. among basic, neutral and acidic amino acids,
2.2. Sensitivity

The most important drawback of CE is its low “loadability”, which leads to low concentration sensitivity in comparison to HPLC methods. Usually less than one percent of the capillary can be filled with sample (<20–40 nl) but the concentration sensitivity can be improved through the increase of injection volume. Through the injection of a basic plug in front of the acidic sample an injection of more than 10% of the capillary can be used for the analysis of peptides [20]. This pH-mediated stacking has been applied here for the analysis of amino acids (transient isotachophoresis can explain the preconcentration step as well: in fact both principles apply here and act in the same direction, namely to sharpen the injection zone before the actual zone electrophoretic separation starts). The injection of up to 10% of the capillary was applied not only for standards but also for urine samples. In order to obtain a good focusing of the sample zone the urine had to be acidified prior to injection. To this end, 1 μl of BGE has been added to 50 μl of sample. In this way detection limits of well below 50 nM could be achieved for most amino acids. This is about 1–2 orders of magnitude better concentra-

2.3. Evaluation of dynamic range

Every analytical method has its own limits and optimal performance range. With the introduction of a new detection technique in the ESI-TOF, i.e. the use of an analogue-to-digital converter, the dynamic range is not limited anymore by the mass analyzer rather than by the ionization process itself. To evaluate a dynamic range of our set-up we ran amino acids standards in dilution series ranging from 50 nM to 250 μM. Fig. 2 represents data on glutamic acid: the response is linear for concentrations between 50 nM and 250 μM. A similar range of linearity was observed for basic and neutral amino acids. Thus, the method covers concentrations over range of more than three orders of magnitude.

2.4. Mass accuracy and compound identification

One of the advantages of using mass spectrometry in combination with separation techniques is the possibility to reveal
the chemical identity of the measured species on the basis of its mass-to-charge ratio. In previous reports on CE/MS methods for amino acids analysis two types of mass analyzers have been used: Agilent 1100 MSD and API 3000 triple-quadrupole [9]. The last one was applied for on line compound identification using MRM. In contrast to these instruments, TOF analyzer offer the possibility of compound identification on the basis of accurate mass measurement with accuracy better than ±3 mDa and make MS/MS not necessary, at least in routine cases. Fig. 3 shows the mass-deviation of methylhistidine measured in the same urine sample over 20 consecutive runs. There is no visible trend in mass fluctuations and the consistency in combination with the mass accuracy is the basis for compound identification. For any given \( m/z \) value a range of possible molecular formulas can be calculated, which represents a set of possible elemental compositions, which fall into the specified error tolerance of the measured mass. Furthermore, narrow mass traces down to about ±3 mDa can be generated, since the applied TOF MS does not show any mass drift with intensity. This is exemplarily shown in Fig. 4 for the identification of creatinine in urine. This nicely shows the gain in selectivity by reducing the mass range of the extracted ion electropherogram from ±30 to ±3 mDa.

2.5. Application of principal component analysis (PCA) to the data processing

The presented method does not only reveal target compounds but is able to detect “all” molecules with similar electrophoretic properties (e.g., amines, small peptides and related metabolites) in the limited mass range (as defined by transfer settings of the TOF MS). Thus, unsupervised statistics on all these compounds can be performed, even they are not identified at first. Application of multivariate statistics to CE/MS data has one principal complication: the relatively strong migration time shifts in samples containing a real biological matrix (urine, CSF, plasma). Algorithms for migration time correction are available, but their implementation for routine analysis of multiple data sets is still challenging for ordinary biological lab. Therefore we tested first a tolerance of our PCA algorithm for migration time shift and amino acids concentration fluctuations. For that purpose we created an experiment with twelve urine samples, where four samples were left without modifications, and other eight were spiked with two different sets of amino acids (Set I – Alanine, Lysine, Phenylalanine, Tyrosine and Set II – Proline, Valine, Tryptophane, Arginine). The concentration was different for each amino acid in a way that the spiked concentration was about a factor of 2–10 higher than the initial concentration in the sample. However, since the amino acid concentration varies within the sample group in similar orders of magnitude the actual spiking ratio varies as well. Samples were run under standard conditions (see Section 2) and analyzed using the software package Profile Analysis (Bruker Daltonics). As Fig. 5 shows the PCA algorithm had no difficulties to resolve all three groups even without any correction for retention time. This result demonstrates clearly that the combination of CE/MS experiments with PCA algorithms from Profile Analysis is robust enough for processing data without correction for migration time.

2.6. Analysis of clinical samples

The next logical step was to apply our method to real clinical samples. As a trial model we selected a set of osteoarthritis samples including 10 patients with severe cartilage breakdown, 10 patients with mild cartilage breakdown and 10 healthy controls. To avoid instrumental bias we ran all samples independently in two laboratories. At the start of sample sequence three concentration steps of amino acids standards (5, 10, and 50 μM)
were injected and an additional standard was injected after every fifth sample in order to control MS detector and capillary performance. All samples were injected into the capillary with practically no sample pretreatment, apart from the mentioned pH adjustment with BGE. All traces were examined visually before data processing to avoid data sets with anomalous migration time shifts. On the basis of this preliminary examination we had to exclude three data sets from further analysis. PCA analysis showed that control samples fall in a distinct group, whereas all samples from diseased patients grouped into another (Fig. 6). At the same time there was no difference between severe and mild cartilage breakdown, and these samples fall more or less in the same cluster. Examination of score and loadings plot showed that control samples fall in a distinct group, whereas all samples from diseased patients grouped into another (Fig. 6). At the same time there was no difference between severe and mild cartilage breakdown, and these samples fall more or less in the same cluster. Interpretation of the biological significance of this finding is outside the scope of the current manuscript. The number of samples used in our work is just sufficient for the test of analytical workflow. We have larger numbers of samples available to test the clinical relevance of this method for monitoring the presence, severity and progression of OA. However, we have to mention that abnormalities of histidine metabolism in context of osteoarthritis were demonstrated before [21]. For now, it is important to emphasize the fact that the combination of CE with TOF-MS is directly applicable for analysis of clinical samples and provides a solid foundation for more detailed clinical study.

3. Conclusions

An improved method for CE/MS analysis of amino acids in body fluids was presented and its efficiency was sufficient for baseline separation of the isobaric pair leucine and isoleucine. The sensitivity of the method was improved by increasing the injection volume with the help of pH mediated stacking. In this way detection limits of well below 50 nM could be achieved for most amino acids. This is about 1–2 orders of magnitude better concentration sensitivity compared to that reported in previous studies [8,9,14] This combination of CE with TOF mass spectrometry tested for sensitivity and dynamic range. The linearity of the method was demonstrated for concentrations between 50 nM and 250 μM. It was shown that the mass accuracy and stability of TOF instrument are providing a good basis for compound identification (i.e. derivation of the elemental composition) using the accurate mass and migration time only. Finally, the introduction of multivariate data analysis in the work flow was evaluated on spiked urine samples and real clinical material. The presented work demonstrates that CE-TOF MS is a powerful tool for the characterization of body fluids in the clinical context based on profiling of amino acids, amines, small peptides, and related metabolites.

References