PHD THESIS

Novel Mass Spectrometry-Based Approaches in the Screening and Diagnostics of Inborn Errors of Metabolism

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1. Introduction

Inborn errors of metabolism (IEMs) are a large group of rare genetic diseases that result from a defect in an enzyme or transport protein which results in a block of a metabolic pathway. The Hungarian newborn screening program dates back to the seventies and currently includes 26 IEMs. The common features of these disorders are that the early diagnosis and therapy, mainly in the asymptomatic phase, can be helpful in improving the outlook for many of these conditions. In the course of newborn screening, as the first step in the diagnostics of the definite IEMs, metabolites are determined in dried blood spots (DBS) by mass spectrometry (MS)-based method. The aim of the examination is to highlight all the positives and therefore, to keep the number of false negative cases at minimum, preferably zero. One of the disadvantages of this method arises from this fact, namely that the number of the false-positive cases are higher. Current screening does not result in a final diagnosis. Even though these diseases are due to the defect of specific enzymes, still these processes are not isolated, but interchained with several other enzymes. The product of an enzyme appears as the substrate of another reaction, therefore, if an enzyme does not work properly, many processes are at risk and can cause damage. This means that the increased level of a given metabolite can characterize several diseases, so a confirmatory examination is obligatory. The confirmatory examinations can be second-tier or secondary tests that offer precise diagnosis and can help to keep the number of false-positive cases at minimum. These methods are specific, unlike the ones used for screening, though require the application of further instruments and staff. Furthermore, the request of a new sample leads to the delay of the diagnosis [1-4]. The majority of the currently used confirmatory examinations consist of metabolite determinations of serum and urine samples and are carried out by gas or liquid chromatography-mass spectrometry-based methods. The requirement of new sampling often causes stress to the affected family due to the sampling's time requirement [5, 6]. Determining the activity of the given enzyme is also a confirmatory examination that leads to a final diagnosis. The disadvantages of the currently used enzyme activity measurements are that they have different sample requirements (leukocyte, serum, fibroblast) from the newborn sampling (dried blood spots, DBS), sample preparation is labour-intensive and time-consuming, and the low selectivity and sensitivity of the analytical methods (colorimetry, fluorimetry) result in a high false-positive rate.

2. Objectives

My PhD work was focused on the development of novel mass spectrometry-based methods that can eliminate the disadvantages of the currently used and diagnostically necessary confirmatory examinations. We also focused on avoiding the disadvantages of traditional enzyme activity measurements and aimed the novel methods to be applied to the sample type used for newborn screening.

During the research our aim was to develop a novel, high throughput, single stage high resolution mass spectrometry-based (HR-MS) method that uses nanospray ionization (nS) for the determination of the metabolite profile of the DBS samples. The basis for the sample preparation was the method used in newborn screening. For the validation of the new method, results were compared to the ones obtained from the traditional electrospray ionization tandem mass spectrometry (ESI-MS/MS) method. For thorough examination, phenylketonuria (PKU) and medium-chain acyl-coenzyme A dehydrogenase deficiency (MCADD) were chosen. Firstly, these are the most widely screened diseases across the world and secondly, due to their incidence there was a sufficient number and amount of samples available to study the applicability of the new method. The research focused on the HR-MS based identification and quantification of all the analytes affected by PKU and MCADD diseases, which can expectedly increase the selectivity of the screening and furthermore, the setup of diagnosis could be achieved in one step.

In the second part of my PhD work the aim was to improve the traditional, currently used enzyme activity measurements, which are crucial for the diagnosis of the IEMs. Our purpose was to develop an HPLC-MS-based enzyme activity determination which uses a new analytical approach where the enzyme reaction occurs in situ in the biological fluid freshly dropped on the filter paper. With this new method we intended to eliminate the disadvantages of the traditional methods, as well as to reduce the number of false-positive results deriving from the instability of enzymes and substrates in the DBS samples. Due to the suspected stability of the product, the in situ enzyme reaction expectedly results in a more robust and more easily reproducible method than the traditional enzyme assays. For the examination of the method's applicability biotinidase enzyme was chosen, which plays a role in the diagnosis of biotinidase deficiency (BD). In this case, sufficient number of samples and patients were available because the determination of biotinidase activity is included in the newborn screening program and confirmatory examinations in serum are also performed in our laboratory using LC-MS method. The aim of the research was to validate the developed

method, to study the stability of the applied sample and to compare the results obtained with the new method to both the colorimetric method used for newborn screening and the LC-MS method used for confirmatory test. Moreover, our goal was to examine the possible integration of this new method into the tandem mass spectrometric newborn screening protocol.

3. Methods

3.1. Metabolomics of newborn screening dried blood spot cards by nanospray ionization high resolution mass spectrometry

The high resolution mass spectrometry-based detection and identification of metabolites and the determination of cut-off levels were carried out with the analysis of 500 healthy samples. For the examination of the applicability of the novel method in diagnostics, 42 samples - 21 from genetically verified PKU patients and 21 from genetically verified MCADD patients - were analysed. DBS samples from 12 false-positive cases (babies screened out with Phe levels higher than 99 percentile but having normal PAH activity) were analysed using nS-HR-MS method. The examination of all 554 samples were performed in parallel with the traditional newborn screening ESI-MS/MS method.

Sample preparation:

- DBS samples (d=4.5 mm, ~10 µl whole blood) were placed in 96-well hydrophilic PTFE filter plates,
- extraction was carried out with 100 µl of methanolic solution, containing known concentration of stable isotope labeled standards (30 min, RT),
- methanolic extracts were subsequently filtered into 96-well polypropylene plates,
- and diluted with water in 1:1 ratio before nS-HR-MS analysis.

The calculation of concentration levels was based on the intensity rate of the analyte and the adherent internal standard, the latter in known concentration. Validation against traditional ESI-MS/MS method was carried out with the determination of Pearson correlation coefficient.

High resolution mass spectrometry-based detection and identification of analytes were performed using LTQ Orbitrap Discovery and Exactive instruments. The high-throughput screening method was developed using the Exactive instrument equipped with TriVersa NanoMate ion source.

3.2. ‘In-paper’ enzyme assay method for the determination of biotinidase enzyme activity

The method was based on the LC-MS determination of the product (p-aminobenzoic acid) resulting from the hydrolysis of the artificial substrate (N-biotinyl-p-aminobenzoic acid) catalysed by biotinidase enzyme. The fundamental idea of the novel assay comprises the addition of substrate to the sample carrier (filter paper) prior to its distribution to the sampling sites. As a result the enzymatic reaction took place consequently after the fresh blood sample was taken. Enzyme activity was expressed in PABA formation (µM/spot).

‘In-paper’ enzyme assay conditions according to the optimized steps:

- Sampling card was pretreated by the addition of 100 µl of aqueous solution of 10 mM B-PABA and 10 mM DL-dithiothreitol (DTT),
- DBS (d=6 mm) from calibration points, blank and samples were punched into a 96-well polypropylene plate,
- extraction was carried out with 160 µl of the extraction solution containing 150 µM 4-nitroaniline as internal standard and 5 mM DTT in water (30 min, RT),
- after extraction, 100 µl of the content of each well transferred into a 96-well 0.45 µm hydrophilic PTFE filter plates,
- and 200 µl 3% TCA solution was added for irreversible protein precipitation,
- 20 µl of the supernatant was analysed by LC-MS after filtration into 96-well polypropylene plates (figure 1).

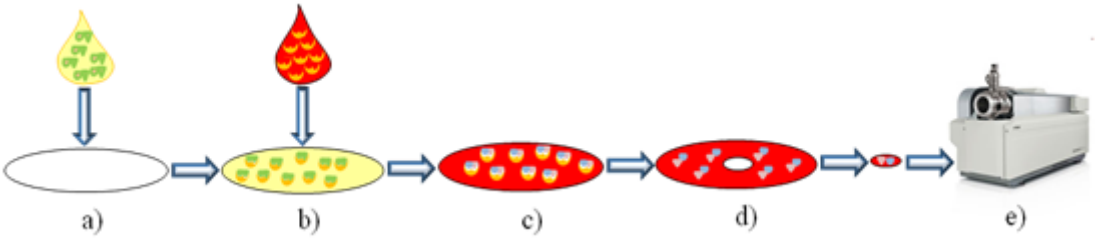

Figure 1. Schematic representation of ‘in-paper’ enzyme activity determination. Pretreatment of filter paper with B-PABA solution (a). Blood sampling on the pretreated collection card (b). ‘In-paper’ enzymatic reaction (c). Sample preparation (d). Analysis of resulted product by LC- MS technique (e).

For the comparison of the novel method with the traditional HPLC-MS based serum biotinidase assay, 46 pretreated DBS samples and sera were collected separately for activity measurements. Out of 46 samples 13 were healthy with lower enzyme activities (30-50%), 25 were patients with partial biotinidase deficiency (BD) (10-30%) and 8 were patients with

profound BD with residual activity below 10%. Furthermore, for the determination of the average enzyme activity in serum and in pretreated DBS, we examined 45 samples from healthy subjects (>50%). The enzyme activity besides PABA production rate was also expressed in percentage of average activity of negative controls to allow the comparison of the methods.

The quantitative determination of PABA was performed with the same LC-MS method in both assays. The HPLC system consisted of a PerkinElmer Series 200 LC pump and autosampler. The detection was carried out using an API 4000 Q-TRAP mass spectrometer equipped with a TurboIonSpray source operated in positive ion mode. Analytes were separated using reversed-phase liquid chromatography on a Merck Purospher Star C18 column (3 μm, 2.1 mm x 50 mm; Merck) using isocratic elution. The mobile phase of 10 mM ammonium acetate/0.1 % acetic acid in 1:3 acetonitrile/water mixture was used at a flow rate of 200 μl/min. Analytes were detected in multiple reaction monitoring mode using the 138 – 120 and 138 – 92 fragmentation channel for p-aminobenzoic acid and the 139 – 65 and 139 – 92 fragmentation channel for 4-nitroaniline.

We studied the stability of the novel assay since the main disadvantage of the traditional enzyme assays is originated from the enzyme instability [7]. We studied the substrate stability in the pretreated filter paper after storage at 25°C, 4°C and -20°C for 3, 5, 7, 10 and 18 days. We also examined the product stability in the pretreated DBS samples after storage at 25°C and 4°C for 3, 5, 7, 10 and 18 days and at -20°C for 3, 5, 7, 10, 18, 30 and 120 days.

4. Results and discussion

4.1. Metabolomics of newborn screening dried blood spot cards by nanospray ionization high resolution mass spectrometry

- Nanospray high resolution mass spectrometry analysis of the 554 DBS samples in both positive and negative ion mode (mass range of 70-1000 m/z) resulted in spectra featuring ~1150 different metabolic constituents (excluding isotopes and adduct peaks). The identification of ion signals was performed using accurate masses of ions determined at 50 000-100 000 fwhm (depending on actual m/z of the ion) nominal resolution with mass accuracy window of 1.0 ppm. In order to clarify the isomeric composition of ion signals corresponding to multiple known metabolites tandem mass spectrometry experiments were also carried out.

In positive ion mode, from the registered ~450 individual ion signals, proper identification was achieved for 102 species. In negative ion mode, from the detected ~300

ion signals, 185 was identified. The observed metabolites cover practically all amino acids and acylcarnitines relevant to newborn screening in addition to metabolites utilized for confirmatory diagnostics of IEMs, including organic acids, carbohydrates, carbohydrate phosphates, bile acids, fatty acids, eicosanoids, phospholipids and sphingolipids.

- Limit of detection (LOD) and linear range for a number of key metabolites were determined by extracting parallel samples with methanol containing various concentrations of isotope labeled internal standards.

The examination shows that the LOD values were always lower than the physiological (or pathological if disease results in lower than physiological concentration) levels of the metabolites. Calibration curves exceeding beyond the linear range always show saturation phenomenon at higher concentrations (i.e., no breakdown phenomenon was observed). This guarantees that all pathological values will be detected as positive, even if the determined level falls outside of the linear range.

- Both the traditional ESI-MS/MS method and the developed nS-HR-MS method were able to quantify a set of 20 amino acid and 20 acylcarnitine species. These analytes were used as a benchmark to validate the novel method against the traditional one.

Correlation plots generally reveal a linear relationship with correlation coefficient (Pearson product-moment correlation coefficient, r) in the range of 0.4 – 0.92. Correlation coefficients in case of phenylalanine and Phe/Tyr ratio (PKU screening) were 0.87 and 0.95, and in case of acetylcarnitine (MCADD screening) was 0.93. However, even though the correlation plots show systematic shifts between the values obtained by the two methods, this does not compromise the diagnostic value. While the healthy and diseased range may be different between the nS-HR-MS and the ESI-MS/MS approach, the separation of the ranges is similar or better for metabolites determined by the novel method.

- In case of phenylketonuria besides the traditionally screened phenylalanine concentration as the primary parameter and Phe/Tyr ratio as the secondary parameter, further analytes were detected in negative ion mode with the ns-HR-MS method. These are also accumulated in PKU due to the alternative metabolic pathways. From these additionally detected metabolites phenyllactic acid and phenylpyruvic acid have diagnostic value and the latter gives an even better separation between the healthy and the diseased population than the

traditionally screened phenylalanine. 12 false-positive samples were also analysed beside the 500 healthy and 21 PKU samples by the novel technique. All of the 12 false-positive samples analysed by nS-HR-MS revealed normal phenylpyruvic acid levels. In 5 of these cases, the elevated Phe level was associated with amino acid infusion, while in 3 cases the gestational age of babies was <36 weeks.

Although our results do not fully validate phenylpyruvic acid as a selective marker for PKU in cases of premature babies and babies receiving amino acid infusion, the observations strongly support its testing at a population level as an additional/confirmatory marker in the diagnostics of PKU.

- In case of MCADD besides the traditionally screened acylcarnitines, the nS-HR-MS method was able to detect further metabolites, such as dicarboxylic acids, acylglycines and fatty acids. Traditionally, these additionally detected metabolites are used as confirmatory parameters in the diagnostics of MCADD and are determined in urine by GC-MS method. During the research 500 healthy and 21 MCADD samples were analysed by the novel technique. Furthermore, 21 false-positive samples were also examined, in which at least 3 parameters were elevated from the detected– C8, C6, C10:1, C10:1/C2 – in the traditional screening. All these samples proved to be normal with confirmatory tests. The nS-HR-MS analysis of these samples yielded confirmatory metabolite concentrations lower than the 1st percentile of diseased population, between 3rd and 44th percentile of healthy population for octenedioic acid, between 65th and 89th percentiles for hexanoylglycine, and between 40th and 51th percentile for suberic acid. Accepting a case positive if all of the parameters exceed the estimated 1 percentile value of diseased population using these markers combined with C8 level 100% selectivity and sensitivity was achieved for the detection of MCADD in the set test.

With the developed nS-HR-MS method we were able to detect highly specific biomarkers which can be applicable in the diagnostics of MCADD. Introduction of the novel method into the population screening program could expectedly decrease the number of samples requested for secondary tests.

4.2. ‘In-paper’ enzyme assay method for the determination of biotinidase enzyme activity

- In our laboratory, in case of positive results highlighted in the newborn screening, currently we use an HPLC-MS-based serum biotinidase assay for confirmation of biotinidase

deficiency. All enzyme activity values measured by the ’in-paper’ assay were compared to values obtained from serum analysis.

Relative biotinidase activity values using the newly developed method were in good agreement with values obtained from serum. Pearson product-moment correlation coefficient was 0.93.

- For determination of the diagnostic applicability of the novel method, values were also compared to the ones obtained by the semi-quantitative colorimetric method in the newborn screening.

The enzyme activity values obtained by the ’in-paper’ assay showed definite separation between healthy and diseased populations. Moreover, samples from patients with the different forms (partial, profound) of biotinidase deficiency separated clearly, as well. According to these results the newly developed technique is suitable for the selective and sensitive diagnosis of the disorder.

- In stability tests the overall relative standard deviation of enzyme activity values were 7.8% at 25°C, 5.8 % at 4°C and 7.5% at -20°C during the examined interval. Accordingly, RSD values remained below 15%, which is acceptable in case of biological samples. These results show that PABA concentration in DBS was stable during storage at -20°C, 4°C and 25°C for at least 2 weeks, indicating that the substrate was also stable under the studied conditions. The long-term stability tests (30 and 120 days) also showed no decrease in PABA concentration, therefore neither in enzyme activity (RSD=8.9%).

Due to the immediate enzymatic reaction and the stability of the product, no special sample storage conditions are required in the newly developed DBS-based method, resulting in a more simple and reliable assay.

- For the examination of the possible integration of this new method into the tandem mass spectrometric newborn screening protocol we studied the influence of the coexisting PABA in the DBS card on amino acid and acylcarnitine profile. During this study concentration of 6 aminos acids and 17 acylcarnitines were determined in 20 pretreated samples from patients with partial BD.

RSD of the measured values were within 15% and concentrations stayed below the cut-off values in every case. Since the presence of PABA has no influence on amino acid and acylcarnitine profiles, theoretically the described assay might be introduced into the current newborn screening protocol.

5. Conclusions

In my PhD work we have developed a high resolution mass spectrometry method using nanospray ionization that could be a promising alternative to the traditional ESI-MS/MS method in the population level newborn screening of inborn errors of metabolism. The chip-based nanospray system used in this method has several advantages compared to the traditional ESI sample introduction, most importantly the elimination of cross contamination. This way the number of false results could be decreased and also the analysis time could be shortened. Opposite to the traditional screening method the presented technique enables the semi-quantitative determination of not only amino acids and acylcarnitines but also the metabolites specific in the diagnostics of IEMs. The routine use of this method would expectedly decrease the number of samples required for both second-tier and confirmatory examinations. On one hand this would disencumber the laboratory, on the other hand the stress on the affected families could also be avoided. The number of analysed samples in the pilot test does not properly represent the statistical difference among the healthy groups or the incidence frequency of the tested diseases, but based on the performed examinations a clear separation can be made between the healthy and the abnormal groups.

During the PhD research a novel method was developed with a brand new analytical approach that can determine the activity of the biotinidase enzyme. In the new method blood sampling occurs on a filter card which was pretreated with the substrate. The presence of the substrate enables the in situ enzymatic reaction. The HPLC-MS determination of the product generated by the in situ enzymatic hydrolysis shows enzyme activity values that correlate very well with the colorimetric results currently used for the newborn screening, as well as with the values measured with the HPLC-MS-based serum enzyme assay method. The method presented in this dissertation is faster and more robust than the traditional enzyme assays due to the instantaneous enzymatic reaction. The product generated in the enzymatic reaction proved to be stable in DBS in long-term, too, as opposed to the enzyme which can already be degraded in a few days during transportation or storage. Due to the mass spectrometry detection, the selectivity and sensitivity of the method significantly increases compared to the colorimetric method currently used for screening. The developed method generally results in lower false-positive and negative rates. Sample preparation, despite the necessity of the pretreatment of the filter card with a substrate, is a lot faster and easier to implement as only consists of a simple extraction step, where there is no need to create laborious enzyme assay conditions. Since the presence of the product PABA influences neither the amino acid nor the

acylcarnitine profiles of the DBS, the described assay can also be introduced into the tandem mass spectrometric newborn screening protocol, which could eliminate the separate biotinidase assay and improve the quantitative performance of the method. The developed method has further potential as the affected enzyme activity determination plays a crucial role in the diagnostics of IEMs to which this method is probably easily applicable.

Both methods developed in my PhD work could be applied to a population level screening with the examination of a bigger and statistically more relevant number of samples.

6. Publications related to the subject of dissertation

Szabó E, Balogh L, Szabó A, Szatmári I. *Ritka örökletes anyagcsere-betegségek diagnosztikája: laboratóriumi vizsgálati megközelítések.* **Orv Hetil.** 2017; 158(48): 1903–1907. (Impact Factor: 0.349)

Szabó E, Szatmári I, Szőnyi L, Takáts Z. *Quantitative Analytical Method for the Determination of Biotinidase Activity in Dried Blood Spot Samples.* **Anal Chem.** 2015 Oct 20;87(20):10573-8. doi: 10.1021/acs.analchem.5b02996. Epub 2015 Oct 9. (Impact Factor: 5.64)

Dénes J, Szabó E, Robinette SL, Szatmári I, Szőnyi L, Kreuder JG, Rauterberg EW, Takáts Z. *Metabonomics of newborn screening dried blood spot samples: a novel approach in the screening and diagnostics of inborn errors of metabolism* **Anal Chem.** 2012 Nov 20;84(22):10113-20. (Impact Factor: 5.64)

7. References

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