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Quantification of metabolites in dried blood spots by direct infusion high resolution mass spectrometry

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HIGHLIGHTS

- Separate analyses of metabolite groups are often needed for optimally assessing the patients metabolic status.
- Quantification of these different markers has a long turnaround time.
- Chip-based nanoESI HRMS is a good alternative for current methods.
- CChip-based nanoESI HRMS is extremely fast (with analytical run times of 4.5 min per sample).
- Chip-based nanoESI HRMS is very efficient (many different markers in one run).

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ABSTRACT

Diagnosis and treatment of inborn errors of metabolism (IEM) require the analysis of a variety of metabolites. These compounds are usually quantified by targeted platforms. High resolution mass spectrometry (HRMS) has the potential to detect hundreds to thousands of metabolites simultaneously. A chip-based nanoelectrospray source (chip-based nanoESI) enables the direct infusion of biological samples. Major advantages of this system include high sample throughput, no sample carryover, and low sample consumption. The combination, chip-based nanoESI-HRMS enables untargeted metabolomics of biological samples but its potential for quantification of metabolites has not been reported.

We investigated whether chip-based nanoESI-HRMS is suitable for quantification of metabolites in dried blood spots (DBS). After addition of internal standards, metabolites were extracted with methanol. Aliquots of each extract were analysed by chip-based nanoESI-HRMS operating in both positive and negative mode with an *m/z* window of 70–600 and a resolution of 140,000. Total run time was 4.5 min per sample and a full report could be generated within 40 min. Concentrations of all 21 investigated diagnostic metabolites in DBS as quantified by chip-based nanoESI-HRMS correlated well with those obtained by targeted liquid chromatography-tandem mass spectrometry. We conclude that chip-based nanoESI-HRMS is suitable for quantification.

1. Introduction

Targeted analysis of small molecules in biological samples (e.g. plasma, urine, cerebrospinal fluid and dried blood spots (DBS)) by (UP)LC-MS/MS platforms is routinely used in diagnostic settings. For diagnosing inborn errors of metabolism (IEM) or follow up of therapy, different platforms are usually needed (e.g. glycine, free carnitine, propionylcarnitine and methylcitrate (MCA) are

Abbreviations: IEM, inborn errors of metabolism; HRMS, high resolution mass spectrometry; nanoESI- HRMS, nanoelectrospray ionization coupled to high resolution mass spectrometry; DBS, dried blood spots; SRM, selective reaction monitoring; PKU, phenylketonuria; MCA, methylcitrate; SA, succinylacetone; HPA, hyperphenylalaninemia; MCAD, medium-chain acyl-CoA dehydrogenase; VLCAD, very long-chain acyl-CoA dehydrogenase; MAT1A, methionine adenosyltransferase I.

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requested in cases of follow up of propionic academia patients). This approach is laborious and leads to long turnaround times (sample preparation, analysis time and processing of results) which is especially detrimental when there is need for rapid availability of analytical results.

High-resolution mass spectrometry (HRMS) [1] is often applied for untargeted metabolomics. Due to its high mass-resolving power, HRMS enables the determination of masses of thousands of metabolites simultaneously, thereby providing a broad overview of the content of the sample (extract). In addition, HR mass spectrometers have an extended dynamic range, a high mass extraction window and a high mass accuracy. Thus, HRMS provides good discrimination between compounds and interferents (for review see Refs. [2,3]). The performance of HRMS enables direct infusion rather than sample introduction after chromatographic separation. In direct infusion, the sample is continuously flowed into the MS ion source and the sum of the mass signals is used for data analysis. Chip-based direct infusion nanoelectrospray ionization system was reported some years ago [4] and has been applied successfully [5–7]. Application of this chip-based nanoESI is analogous to flow-injection analysis, but has the advantage of directly introducing the sample without a mobile phase (thus avoiding any dilution caused by the mobile phase). The system offers all the advantages of conventional nanoESI plus automated, high-throughput analyses without analyte carryover. Elimination of chromatographic separation results in shorter analysis time, improved reproducibility and simplified data analysis.

The present study explores the potential of this chip-based ESI-HRMS to quantify a diversity of clinically relevant metabolites in a single run. For this purpose, we selected metabolites that are quantified to diagnose metabolic dysfunction commonly associated with, but not limited to IEM. As a matrix, DBS were used since sampling is a patient-friendly alternative to venapuncture and DBS can easily be transported and stored [8].

2. Material and methods

2.1. Chemicals and materials

Phenylalanine, tyrosine, citrulline, arginine, methionine, lysine, serine, proline, ammonium formate, C0-carnitine, SA and guanidino acetate were purchased by Sigma-Aldrich (Steinheim, Germany). Glycine, hydrochloric acid and creatine monohydrate were obtained from Merck (Darmstadt, Germany). Methylcitric acid, 2-methyl-$^{2}H_3$-citric acid, $^{2}H_3$-creatine monohydrate, NSK-A-amino acids reference standards (containing ^{15}N; 2-^{13}C-glycine, $^{2}H_4$-alanine, $^{2}H_3$-leucine, $^{2}H_3$-methionine, $^{13}C_6$-phenylalanine, $^{13}C_6$-tyrosine, $^{2}H_3$-aspartate, $^{2}H_3$-glutamate, $^{2}H_2$-ornithine, $^{2}H_2$-citrulline and $^{2}H_4$;^{13}C-arginine), NSK-B- C0-carnitine and acylcarnitine reference standards (containing $^{2}H_9$-carnitine, $^{2}H_3$-acetylcarnitine (C2), $^{2}H_3$-propionylcarnitine (C3), $^{2}H_3$-butyrylcarnitine (C4), $^{2}H_9$-isovalerylcarnitine (C5), $^{2}H_3$-octanoylcarnitine (C8), $^{2}H_9$-myristoylcarnitine (C14) and $^{2}H_3$-palmitoylcarnitne (C18)) and NSK-T-succinylacetone reference standard (containing $^{12}C_5$-SA) were purchased from Cambridge Isotope Laboratories, via Buchem bv (Apeldoorn, The Netherlands). $^{13}C_2$-guanidino acetate, C3-, C6-, C8- and C14-carnitine were prepared by Organic Synthesis Laboratory, VU Medical Centre (Amsterdam, The Netherlands). Methanol (ULC/MS grade) and formic acid were purchased from Biosolve BV (Valkenswaard, The Netherlands). All solutions were prepared using highly purified water produced by a MilliQ system (Millipore, Bedford, Ma, USA) unless otherwise described. MassCheck® Amino Acids/Acylcarnitines Dried Blood Spot Controls at two levels, were purchased from Chromsystems (Lochhem, Germany).

2.2. Stock solutions, calibration curves and internal standard solutions

Separate stock solutions of analytes were prepared in MilliQ; C0-carnitine (6.79 mM), SA (10.1 mM), methionine (28.3 mM), methylcitric acid (10.6 mM), C3-carnitine (1.65 mM), C6-carnitine (1.28 mM), C8-carnitine (1.42 mM), C14-carnitine (1.12 mM), guanidino acetate (10.0 mM) and creatine (10.0 mM). Remaining stock solutions were prepared in 0.1 M HCl; phenylalanine (52.4 mM), tyrosine (11.0 mM), lysine (154 mM), glycine (107 mM), citrulline (51.8 mM), arginine (51.9 mM), serine (26.2 mM) and proline (78.5 mM). From these stock solutions, a twelve points mixed calibration curve was prepared in MilliQ with final concentrations of C0-carnitine (67.9 μM), SA (101 μM), methionine (565 μM), methylcitric acid (106 μM), C3-carnitine (49.4 μM), C6-carnitine (5.11 μM), C8-carnitine (45.41 μM) and C14-carnitine (8.93 μM), guanidino acetate (3.98 μM), creatine (153 μM), phenylalanine (1048 μM), tyrosine (220 μM), lysine (3080 μM), glycine (2142 μM), citrulline (1036 μM), arginine (1039 μM), serine (525 μM) and proline (1570 μM) respectively. To test the linearity of the calibration curve a twelve points mixed high concentration calibration curve was prepared containing C0-carnitine (1220 μM), SA (1000 μM), methionine (5650 μM), methylcitric acid (1050 μM), C3-carnitine (90 μM), C6-carnitine (50 μM), C8-carnitine (450 μM), C14-carnitine (90 μM), guanidino acetate (500 μM), creatine (5000 μM), phenylalanine (21300 μM), tyrosine (4440 μM), lysine (22580 μM), glycine (16000 μM), citrulline (7760 μM), arginine (7790 μM), serine (3675 μM) and proline (10470 μM).

Internal standard stock solutions were prepared for 2-methyl-$^{2}H_3$-citric acid (1 mM), $^{13}C_2$-guanidino acetate (2.5 mM) and $^{2}H_3$-creatine (1.8 mM). A mixed internal standard working solution was prepared in methanol achieving concentrations of 0.72 μM 2-methyl-$^{2}H_3$-citric acid, 4.7 μM $^{13}C_2$-guanidino acetate, 184 μM $^{2}H_3$-creatine, 25 μM ^{15}N; 2-^{13}C-glycine, 5 μM $^{2}H_4$-alanine, $^{2}H_3$-leucine, $^{2}H_3$-methionine, $^{13}C_6$-phenylalanine, $^{13}C_6$-tyrosine, $^{2}H_3$-aspartate, $^{2}H_3$-glutamate, $^{2}H_2$-ornithine, $^{2}H_2$-citrulline and $^{2}H_4$;^{13}C-arginine, 1.52 μM $^{2}H_9$-carnitine, 0.38 μM $^{2}H_3$-acetylcarnitine (C2), 0.076 μM $^{2}H_3$-propionylcarnitine (C3), $^{2}H_3$-butyrylcarnitine (C4), $^{2}H_9$-isovalerylcarnitine (C5), $^{2}H_3$-octanoylcarnitine (C8), $^{2}H_9$-myristoylcarnitine (C14), 0.152 μM $^{2}H_3$-palmitoylcarnitne (C18)) and 10 μM $^{12}C_5$-succinyl acetone.

2.3. Sample collection and storage

Blood samples were obtained from patients who were referred to our Department for diagnostics (and follow-up). Blood samples were collected by venous puncture in heparin containing tubes. Aliquots of whole heparinised blood were aspirated and spotted onto Guthrie card filter papers (Whatman no. 903 Protein saver TM cards, formerly Schleicher & Schuell, Keene, USA). DBS were inspected visually to make sure that the blood spots circle was completely filled. All Guthrie card filter papers with spotted blood were left to dry for at least 4 h at room temperature and were stored at −80 °C (for a maximum of 3 years) in a foil bag with desiccant package pending further analysis. Collection of DBS was approved by the Medical Ethics Review Committee of the University Medical Centre (UMC) Utrecht.

Concentrations of phenylalanine, tyrosine and different acylcarnitines in DBS quantified by chip-based nanoESI-HRMS were compared to values obtained in the same DBS measured by UPLC–MS/MS. For this purpose we used clinical samples from (un) treated (newborns and pediatric) patients with confirmed (enzymatic or molecular) IEM.

C0-, C2-, C6-, C8-, and C10-carnitine were measured in DBS obtained from patients with medium-chain acyl-CoA