Short communication

Critical sample pretreatment in monitoring dried blood spot citrulline

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Abstract

Background: Plasma Citrulline concentration has been correlated to functional enterocyte mass. Dried blood spot (DBS) analysis using LC-MSMS reduces sample amount needed. We optimized DBS elution to increase precision and accuracy of DBS LC-MSMS analysis.

Method: DBS control samples were eluted in varying pH (2.2–7.0) and for varying times (15–75 min) and Cit, Arg and Orn were analyzed using LC-MSMS, with and without derivatization. In 20 volunteers, the DBS LC-MSMS assay was correlated with a plasma ion exchange HPLC method.

Results: For Citrulline an extraction optimum was obtained at pH 2.6, whereas lower Arginine concentrations were found using low extraction pH. Increasing elution times lead to increased concentrations. Within-run CV was higher with, compared to without derivatization. No close association could be found between plasma HPLC and DBS LC-MSMS concentrations.

Conclusion: Analysis of amino acids on DBS using LC-MSMS should be optimized regarding the purpose of the assay. In our study, most optimal results were obtained without derivatization and elution in pH 2.6 for 45 min. Cellular amino acids in DBS might influence the correlation of Cit with severity of enteral disorders. Therefore, further evaluation of DBS Cit as a marker for enteral disorders is warranted.

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1. Introduction

Citrulline (Cit) is a nonessential amino acid that is not incorporated into proteins [1]. Serum Cit levels arise almost exclusively from enterocyte metabolism, where glutamate is converted by carbamoyl phosphate synthase to carbamoyl phosphate which condenses with ornithine to form Cit. The key enzyme of this glutamate-to-ornithine pathway pyrroline-5-carboxylate synthase is expressed mainly in the intestinal mucosa [2,3]. Cit is largely metabolized into arginine in the kidney through a partial urea cycle involving argininosuccinate synthetase and argininosuccinate lyase [1,4]. Plasma Cit concentration has been reported to be a reliable marker of remnant functional enterocyte mass and subsequently intestinal failure in short bowel syndrome [5] and in celiac and nonceliac villous atrophy [6]. Lower plasma Cit levels were also seen in patients with intestinal damage following radiation [7] and chemotherapy [8]. Delayed normalization of Cit levels following intestinal transplantation is correlated with allograft rejection [9,10].

Preterm infants are prone to numerous complications including hyperammonemia, respiratory distress syndrome, intraventricular hemorrhage, necrotizing enterocolitis and sepsis [11]. In the Very Low Birth Weight (VLBW) infants necrotizing enterocolitis or enteral dysfunction are common causes of malnutrition leading to poor growth and development delay. Rhoads et al [12] observed that serum Cit correlated better with enteral tolerance, an indirect measure of calorie absorption, than with bowel length in children with short bowel syndrome. Furthermore, in premature infants, they observed that Cit concentrations were normal at the onset of necrotizing enterocolitis but dropped below normal subsequently [12]. Plasma Cit levels therefore are a useful marker for enteral dysfunction in preterm neonates. Although, amino acid analysis

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based on plasma ion-exchange HPLC is the reference method, LC-MSMS on dried blood spot (DBS) on filter paper has the advantage of allowing large-scale studies and easy sample transport. DBS LC-MSMS has shown to be a valuable tool for neonatal screening for inherited metabolic disorders, drug monitoring, infection surveillance and epidemiological studies [13]. Neonatal metabolic screening using LC-MSMS often includes Cit measurement in DBS [14]. Furthermore, DBS Cit analysis has been reported to be a marker for monitoring graft function following intestinal transplantation [15]. However, the sample preparation conditions were not mentioned in this publication. Solubility in the internal standard solution and instability in the presence of the acidified butanol derivative are important influencing factors in the assay performance. To minimize inaccuracy and imprecision of the measurement of Cit and its metabolites Arg and Orn in DBS, we evaluated an LC-MSMS method without derivatization and optimized the DBS elution.

2. Materials and methods

2.1. Materials

The stable isotope $^2$H$_4$-Lys and calibrators Cit, Arg and Orn were purchased from Sigma-Aldrich (Bornem, Belgium). Methanol, acetonitrile and water were HPLC-grade (Sigma-Aldrich, Bornem, Belgium). Sodium citrate, disodium phosphate, butanol, and acetylclorete were purchased from VWR (Leuven, Belgium).

2.2. Samples

A DBS control sample with normal amino acid concentration was prepared, spotting whole blood of a healthy volunteer on filter paper Schleicher & Schuell No. 903. The same whole blood sample was spiked with 70 μmol/L Cit, 84 μmol/L Arg and 92 μmol/L Orn and spotted on filter paper. These DBS samples were used as high amino acid concentration control sample.

To study the correlation between the current DBS LC-MSMS assay and a plasma HPLC method, a DBS and plasma sample were taken in 20 patients from our pediatric consultation who had to undergo blood sampling for amino acid analysis and gave an informed consent. Of these patients, 3 had proven argininosuccinic aciduria. None of the other patients had an amino acidopathy. The group consisted of 12 men, 8 females and aged between 4 days and 32 years. DBS samples were obtained according to the National Committee on Clinical Laboratory Standards (NCCLS) criteria [13].

2.3. Sample preparation

Internal standard solution was prepared dissolving 6.0 μmol/L $^2$H$_4$-Lys in a 30% citrate phosphate buffer 0.1 mol/L in methanol. For the evaluation of the influence of the pH of the internal standard solution on the extraction capacity, the internal standard solution was prepared using citrate phosphate buffers 0.1 mol/L with pH 2.2, 2.6, 3.0, 4.0, 5.0, 6.0 and 7.0. For all the other analyses, the internal standard solution with citrate phosphate buffer 0.1 mol/L pH 2.6 was used.

A disc of 3.2 mm diameter, containing approximately 3.5 μL blood, was punched in a microtiter plate, 100 μL internal standard solution was added and the plate was covered. The influence of the elution time was evaluated using varying elution times of 15, 30, 45, 60, and 75 min at room temperature under gentle shaking of the microtiter plate. For all the other analyses, an extraction time of 45 min was used.

For all analyses except the evaluation of derivatization, the solvent after elution was transferred into a new microtiter plate and analyzed immediately. For the derivatization study, the solvent after elution was transferred into a new microtiter plate and dried under a stream of nitrogen. Derivatization was performed with 50 μL of butanol acidified with acetylclorete (10:1, v/v) at 56°C for 15 min. Immediately after derivatization, the samples were dried under a stream of nitrogen and resolved in 100 μL acetonitrile 80% in water.

2.4. LC-MSMS analysis

LC-MSMS analysis was performed using an Alliance 2795 (Waters, Milford, MA, USA) HPLC and a QuattroMicro triple quadrupole tandem mass spectrometer (Micromass UK Ltd, Cheshire, UK). The LC-MSMS analysis was performed according to Zytkovicz et al. [16]. Without derivatization, the compounds were monitored using multiple reaction monitoring [17]. With derivatization, the compounds were monitored using neutral loss [16].

2.5. Amino acid HPLC analysis

Amino acid analysis in plasma was performed using ion-exchange chromatography on a Biochrom-20 amino acid analyser with ninhydrin detection using 200 μL plasma for deproteinization, according to Jones et al. [18]. Within-run and between-run precision in plasma were 3.4 and 3.8% for Cit at concentrations 18.6 and 60.9 μmol/L, 3.0 and 4.2% for Orn at concentrations 33.4 and 54.5 μmol/L, and 2.3 and 3.2% for Arg at concentrations 11.3 and 65.0 μmol/L.

2.6. Statistics

The influence of the pH of the internal standard solution and the elution time on the amino acid concentration was tested using Kruskal–Wallis. Correlation between plasma HPLC and DBS LC-MSMS concentrations were evaluated using the Pearson test and Bland and Altman plots were drawn.

3. Results

3.1. Influence of the pH of the internal standard solution on the extraction capacity

The control DBS samples with normal and high amino acid concentration were analyzed in 5-fold using the internal

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Fig. 1. Influence of the pH of the elution buffer on Cit, Arg and Orn concentration in a normal (dotted line) and high (full line) dried blood spot control sample. The figure shows the median with the 5th and 95th percentile (n = 5).
standard solutions with varying pH: 2.2, 2.6, 3.0, 4.0, 5.0, 6.0 and 7.0. The pH had a significant effect on Cit (p<0.002) and Arg (p<0.001). For Cit, an extraction optimum was obtained at pH 2.6, whereas lower Arg concentrations were found using low extraction pH (Fig. 1). There was no significant influence of the extraction pH on the concentrations of Orn (p>0.05).

3.2. Influence of the extraction time

Using the internal standard solution containing the citrate phosphate buffer pH 2.6 the control samples were analyzed using varying extraction time of 15, 30, 45, 60, and 75 min. In the high control, the amino acid concentrations increased significantly (p<0.001 for Orn and Arg and p<0.02 for Cit). In the low control, the elution time had only a significant effect in Cit and Arg (p<0.02) (Fig. 2).

3.3. Influence of derivatization

The normal and high control samples were analyzed in tenfold with and without derivatization using the internal standard solution containing the citrate phosphate buffer pH 2.6 (Table 1). Within-run CV were higher with than without derivatization. Amino acid concentrations were significantly higher without derivatization compared to with derivatization for Arg and Orn. Without derivatization, between-run precision was 21, 18 and 20% for Cit, Orn and Arg, respectively. The recovery of the added amino acids in the high DBS control versus the normal DBS control was 0.58; 0.18; 0.61 with derivatization and 0.59; 0.72; 0.94 without derivatization, for Cit, Arg, Orn respectively.

3.4. Correlation with amino acid HPLC

LC-MSMS analysis was performed in 20 DBS samples using an elution buffer of pH 2.6 and elution time of 45 min and without derivatization. From the same volunteers, plasma samples were obtained and analyzed using ion-exchange HPLC chromatography. DBS analysis correlated with plasma ion-exchange chromatographic analysis in Cit (R=0.97) and Orn (R=0.73). For Arg the correlation coefficient was low (R=0.58). Bland and Altman plots of Cit, Arg and Orn DBS LC-MSMS versus plasma HPLC are shown in Fig. 3.

4. Discussion

Although, amino acid analysis based on plasma ion-exchange HPLC is the reference method, LC-MSMS on dried blood spot (DBS) on filter paper has the advantage of allowing large scale studies with extremely easy sample transport. LC-MSMS could be a valuable alternative for measurement of Cit concentrations in large-scale studies evaluating enteral dysfunction in VLBW infants. The LC-MSMS analysis of amino acids in neonatal screening programs is usually performed with derivatization of the extracted DBS samples and an internal standard in 100% methanol [14,16,19–20]. Zytkovicz et al. [16] reported imprecisions of 9% for Leu, Orn, Met, Phe, Cit and Tyr, and 22% for Arg. When performing the DBS LC-MSMS assay according to Zytkovicz et al. [16] within-run precision varied between 3.1 and 14.2% and between-run precision between 6.2 and 21.3% for Leu, Met, Phe, and Tyr, which is comparable to the literature. For Cit and Orn, however, intolerable high imprecision was found (within-run 21.2 and 26.9%; between-run 82.6 and 46.7%, respectively). No explanation could be found why in the method with derivatization the imprecision was so high. The precision clearly improved to 6–10% within-run and 18–21% between-run when analysis was performed without derivatization.

The use of DBS not only affects precision but also the concentration, since the blood volume in a blood spot punch

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Table 1: Comparison of mean amino acid concentration (μmol/L), SD (μmol/L) and CV (%) (n = 10) between the LC-MSMS analysis with and without derivatization in a normal and high dried blood spot control.

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<tr>
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<th>With derivatization</th>
<th>Without derivatization</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Normal DBS control</td>
<td>Cit 16.1 6.63 41.2</td>
<td>25.9 2.59 10.0</td>
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<tr>
<td></td>
<td>Arg 7.0 1.12 15.9</td>
<td>39.9 3.32 8.3</td>
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<tr>
<td></td>
<td>Orn 54.3 27.5 50.6</td>
<td>104.5 6.77 6.5</td>
</tr>
<tr>
<td>High DBS control</td>
<td>Cit 56.6 15.6 27.6</td>
<td>67.0 3.99 6.0</td>
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<tr>
<td></td>
<td>Arg 21.8 2.87 13.1</td>
<td>100.7 6.42 6.4</td>
</tr>
<tr>
<td></td>
<td>Orn 110.5 27.2 24.6</td>
<td>191.3 12.7 6.6</td>
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depends on hematocrit and position of the disk [21]. Canepa et al. [22] demonstrated only few significant correlations between amino acid concentrations in plasma, red blood cells (RBC) and polymorphonuclear leukocytes (PMN). This lack of correlation indicates that there is no close association in the same subject between individual free amino acid concentrations in various types of cells, presumably because they differ with respect to metabolism and function [22]. Our correlation study between DBS LC-MSMS and plasma HPLC showed that Cit, Orn and Arg concentrations in DBS clearly differed to those in plasma as was expected in view of the finding of a lack of correlation by Canepa et al. [22]. Furthermore, a clear difference in Cit correlation could be seen in the arginosuccinic aciduria patients. This suggests that indeed the association between Cit concentration in plasma and cells differs with respect to metabolism and function as stated by Canepa et al. [22]. Since the Cit levels in cells might have an influence on the correlation with enteral dysfunction, the association of plasma Cit cannot be extrapolated to DBS. Therefore, the utilization of DBS Cit levels as marker for severity in various enteral disorders should be evaluated.

Recovery for Arg and Orn was poor when analyzed with derivatization, yet increased to 72 and 94%, respectively. In contrast, the recovery was equally low for Cit with and without derivatization. Our measured recoveries are in agreement with those reported by Sanders et al. [23], demonstrating recoveries for Cit in DBS of 65.1% at concentration 114 μmol/L and 55.9% at 285 μmol/L in a method using derivatization.

Extraction of Cit out of DBS improved using a more acidic pH. Because of its polar lateral chain, Cit is relatively soluble in water but barely in methanol and ethanol, resulting in a poor elution in the internal standard based on methanol, as commonly used in neonatal screening programs. Cit is a diacid with pKa’s around 2.4 and around 9.4 [4]. The molecule is a zwitterion under physiological conditions and protonated under acidic conditions (pH 2 to 3). The elution buffer of pH 2.6 in the internal standard will therefore protonate Cit and increase its solubility. Our results show that increasing the extraction time gave slightly higher concentrations of all measured amino acids. To avoid lengthy sample preparation times, we chose to extract during 45 min.

In conclusion, the analysis of Cit and its metabolites on DBS using LC-MSMS has potential in large-scale studies. When using DBS, the elution method should be optimized regarding the main purpose of the assay. Varying characteristics of the amino acids require different sample preparation for optimal elution and precise analysis. The presence of cellular amino acids in DBS might have an influence in the correlation of Cit with severity of enteral disorders. Therefore, the use of DBS Cit as a marker for enteral disorders should be further evaluated.

References


