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Clinica Chimica Acta 364 (2006) 180 – 187

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Quantitative determination of guanidinoacetate and creatine in dried blood spot by flow injection analysis-electrospray tandem mass spectrometry

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Received 23 February 2005; received in revised form 16 June 2005; accepted 17 June 2005
Available online 28 September 2005

Abstract

Background: Guanidinoacetate (GAA) and creatine (Cr) are reliable biochemical markers of primary creatine disorders. The aim of this study was to develop and validate a method for the determination of GAA and Cr in dried blood spot through the use of stable isotope dilution and flow injection analysis (FIA)-ESI-MS/MS.
Methods: Dried blood spots were extracted using methanol–water solution containing D3-Cr. After evaporation and formation of butyl esters, samples were analyzed using multiple reaction monitoring mode (*m*/*z* 174.2→101.1 for GAA, 188.3→90.1 for Cr and 191.3→93.1 for D3-Cr).
Results: The analysis was very fast (1 min). The detection limits were 0.34 μmol/l of blood and 0.30 μmol/l of blood for Cr and GAA, respectively, and the response was linear over the range 0.25–12.5 μmol/l of blood for GAA and 3.57–624.7 μmol/l of blood for Cr. Recovery range was 93–101% for Cr and 94–105% for GAA and between-run CVs were 5.3% for GAA and 4.5% for Cr. Ion suppression effect was also studied. The method was applied to spots obtained from two patients affected by GAMT deficiency, four patients affected by AGAT deficiency (including a newborn) as well as 282 healthy subjects.
Conclusions: The detection of GAA in dried blood spot by FIA-ESI-MS/MS is a highly reliable and high throughput method for the diagnosis of GAMT and AGAT deficiencies and a possible tool for newborn screening of both these tractable disorders.

Keywords: Guanidinoacetate; Creatine; Tandem mass spectrometry; Dried blood spot; GAMT; AGAT

1. Introduction

Primary creatine (Cr) disorders are caused by a defect of the enzymes guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2, OMIM 601240) and arginine:glycine methyltransferase (AGAT, EC 2.1.4.1, OMIM 602360), or by a defect at the Cr transporter level (CT1, SLC6A8, OMIM 300036). AGAT catalyzes the transfer of an amidino group from arginine to glycine forming ornitine and guanidinoacetate (GAA), while GAMT catalyzes the transfer of a methyl group from *S*-adenosylmethionine to GAA forming Cr. Brain Cr depletion is the common result of all these disorders, whose clinical features includes: early onset severe epilepsy (GAMT deficiency), progressive psychomotor delay (GAMT deficiency), movement disorders (GAMT deficiency, CT1 deficiency), pallidal lesions on brain MRI (GAMT deficiency), autism (GAMT and AGAT deficiencies), and severe mental retardation (GAMT, AGAT, and CT1 deficiencies). The first encouraging results of the

Abbreviations: Cr, creatine; GAMT, guanidinoacetate methyltransferase; AGAT, arginine:glycine methyltransferase; GAA, guanidinoacetate; ESI-MS/MS, electrospray tandem mass spectrometry; FIA, flow injection analysis.

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0009-8981/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2005.06.016

treatment of AGAT and GAMT deficiencies [1], support the hypothesis that an earlier diagnosis and treatment can improve the prognosis of these disorders.

The concentrations of GAA and Cr in physiological fluids are reliable diagnostic markers for both GAMT and AGAT deficiencies [2]: their assessment should be the first choice tool for the diagnosis of these diseases, while molecular analysis, brain ^{1}H-MRS assessment, as well as determination of enzyme activity should be confirmation tests.

Several analytical methods have been described for the determination of GAA and Cr in plasma and urine based on: liquid chromatography and post-column derivatization with ninhydrin [3], high performance liquid chromatography (HPLC) [2], gas chromatography-mass spectrometry (GC-MS) [4–7], liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) [8], and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [9,10]. Two methods have also been tested for the determination of GAA in dried blood spot on filter paper, one based on HPLC with fluorometric detection [11] and the other an extension of a LC-MS/MS method requiring previous chromatographic separation [10].

Electrospray tandem mass spectrometry (ESI-MS/MS) is a powerful as well as highly selective and sensitive analytical technique. In recent years, many applications in ESI-MS/MS have been developed for the study of inborn errors of metabolism and the number of new conditions detectable by ESI-MS/MS validated methods is increasing significantly [12].

The aim of this study was to develop and validate a method for the determination of GAA and Cr in dried blood spot by FIA (flow injection analysis)-ESI-MS/MS, as a diagnostic tool for the early diagnosis of GAMT and AGAT deficiencies.

2. Materials and methods

2.1. Chemicals and reagents

Creatine monohydrate and GAA were purchased from Sigma-Aldrich (Steinheim, Germany). *N*-methyl-D_3-creatine (D3-Cr) was from CDN Isotopes (Quebec, Canada). HPLC grade acetonitrile, methanol and glacial acetic acid were from MERK (Darmstadt, Germany). 3N HCl in *n*-butanol solution was purchased from REGIS Technologies Inc. (Morton Grove, USA). All solutions were prepared using highly purified water produced by a Millipore Milli-RO/Milli-Q system (Millipore, Bedford, Ma, USA) and used without any further purification.

Ten millimoles per liter Cr and ten millimoles per liter GAA stock solutions were prepared in water and stored frozen at −20 °C. Hundred micromoles per liter D_3-Cr stock solution was prepared in methanol/H_2O (90:10) and stored at 5 °C. In order to obtain a working solution of 0.1 μmol/l D3-Cr, daily dilutions (1:1000) were made using methanol/water (90:10).

2.2. Sample preparation

Whole blood from newborns and children was collected on Schleicher&Schuell 903 grade filter paper (Dassel, Germany), dried overnight under ambient conditions and stored at 5 °C in plastic bags containing desiccant. A 3-mm diameter dot was punched from DBS into a single well of 96-well micro plate. The volume of whole blood in 3-mm diameter spot corresponds to 2.8 μl [13]. The dried blood spot was eluted in 100 μl of working extraction solution containing D_3-Cr (0.1 μmol/l) in methanol/H_2O (90:10). The sample was shaken on the Incubator/Shaker (Labsystems, USA) for 30 min at 30 °C. Then 65 μl of supernatant was dried under a nitrogen flow at 45 °C using an EvapArray Sample Concentrator (Porvair Advanced Materials, UK). The extracted Cr and GAA were derivatized to butyl esters using 3 mol/l hydrochloric acid in *n*-butanol solution at 60 °C for 30 min. After derivatization, the sample was dried under a nitrogen flow at 45 °C and then recovered by 50 μl of methanol/water (80:20) containing 0.1% acetic acid. Twenty microliters of the diluted sample was injected in flow injection analysis (FIA) mode for the MS/MS experiments. Mobile phase was methanol/water (80:20) at a flow rate of 80 μl/min.

For the evaluation of the matrix effect, human blood containing heparin and intact cells was dispensed in 100 μl portions onto filter paper from which 3-mm spots were punched. The spots were extracted using 7 extraction solutions containing 0.1 μmol/l D3-Cr and containing different amounts of GAA: from 0.06 to 0.35 μmol/l (corresponding to the range 2.1–12.5 μmol/l of blood), and Cr from 3 to 17.5 μmol/l (corresponding to the range 107.1–624.7 μmol/l of blood). Ion suppression was assessed by comparing the signal of analytes (including the signal ratio to internal standard) in standard solutions with that in spiked blood extract.

In order to assess the recovery of the method, four stock solutions of 30, 60, 90 and 120 μmol/l GAA and 0.4, 1, 1.5 and 2 mmol/l Cr were prepared in phosphate saline buffer (PBS). Blood from a healthy volunteer containing heparin and intact cells (hematocrit adjusted at 55%) was divided into five aliquots of 1 ml. Each blood aliquot, after removing 100 μl of plasma, was spiked with 100 μl of PBS or 100 μl of GAA and Cr stock solutions yielding a final concentration of 0, 3, 6, 9 and 12 μmol/l of blood for GAA and 0, 40, 100, 150 and 200 μmol/l of blood for Cr. The blood was mixed gently, then dispensed into 100 μl portions onto filter paper, dried overnight and stored at −20 °C.

2.3. Instrumentation and operating parameters

A triple quadruple mass spectrometer Sciex API 2000 (PerkinElmer Sciex, Toronto, Canada) equipped with a TurboIonSpray source was used. A Series 200 micro pump (PerkinElmer, Norwalk, CT, USA) and a Series 200 auto sampler (PerkinElmer) were used for solvent delivery and automated sample introduction. Nitrogen was used as