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SHORT COMMUNICATION

A simple screening method using ion chromatography for the diagnosis of cerebral creatine deficiency syndromes

Takahito Wada · Hiroko Shimbo · Hitoshi Osaka

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Abstract Cerebral creatine deficiency syndromes (CCDS) are caused by genetic defects in L-arginine:glycine amidinotransferase, guanidinoacetate methyltransferase or creatine transporter 1. CCDS are characterized by abnormal concentrations of urinary creatine (CR), guanidinoacetic acid (GA), or creatinine (CN). In this study, we describe a simple HPLC method to determine the concentrations of CR, GA, and CN using a weak-acid ion chromatography column with a UV detector without any derivatization. CR, GA, and CN were separated clearly with the retention times (mean ± SD, $n = 3$) of 5.54 ± 0.0035 min for CR, 6.41 ± 0.0079 min for GA, and 13.53 ± 0.046 min for CN. This new method should provide a simple screening test for the diagnosis of CCDS.

Keywords Cerebral creatine deficiency syndromes · HPLC · Creatine · Guanidinoacetic acid · Creatinine

Abbreviations

CCDS	Cerebral creatine deficiency syndromes
AGAT	Arginine:glycine amidinotransferase
GAMT	Guanidinoacetate methyltransferase
CR	Creatine
GA	Guanidinoacetic acid
CN	Creatinine

T. Wada · H. Shimbo · H. Osaka (✉)
Division of Neurology, Kanagawa Children's Medical Center, 2-138-4 Mutsukawa, Minami-ku, Yokohama, Kanagawa 232-8555, Japan
e-mail: hosaka@kcmc.jp

Introduction

Creatine (CR) plays an important role in the storage and transmission of ATP-derived energy (Walker 1979; Wyss and Kaddurah-Daouk 2000). Normal levels of cellular CR are maintained through both diet and biosynthesis in the kidney and liver. Recently, it has been shown that brain must synthesize an important part of its CR, due to the very restricted permeability of blood–brain barrier for CR (Braissant et al. 2011). This synthesis requires the action of two enzymes, arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) and guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2) (Fig. 1). AGAT transfers an amidino group from arginine to glycine, yielding ornithine and guanidinoacetate (GA), while GAMT transfers a methyl group from S-adenosylmethionine to GA, forming CR. However, tissues are incapable of CR biosynthesis such as the brain, the levels of CR are dependent on a creatine transporter 1 encoded by the *SLC6A8* to transport CR against the concentration gradient (Fig. 1).

The cerebral CR deficiency syndromes (CCDS) are a group of disorders that include two recessive conditions that impair the synthesis of CR, namely, AGAT deficiency (OMIM 612718) (Item et al. 2001) and GAMT deficiency (OMIM 612736) (Stockler et al. 1996), as well as one X-linked condition, namely, SLC6A8 deficiency (OMIM 300036) (Salomons et al. 2001).The common clinical features of all CCDS are mental retardation, speech delay, autistic behavior, and seizures.

In GAMT deficiency, the urinary GA/CN concentration ratio increases 2- to 30-fold (Mercimek-Mahmutoglu et al. 2006). AGAT deficiency shows decreased ratios of both CR/CN and GA/CN concentrations in urine (Carducci et al. 2002). The X-linked-SLC6A8 deficiency is characterized by high urinary CR/CN concentration ratios. Therefore,

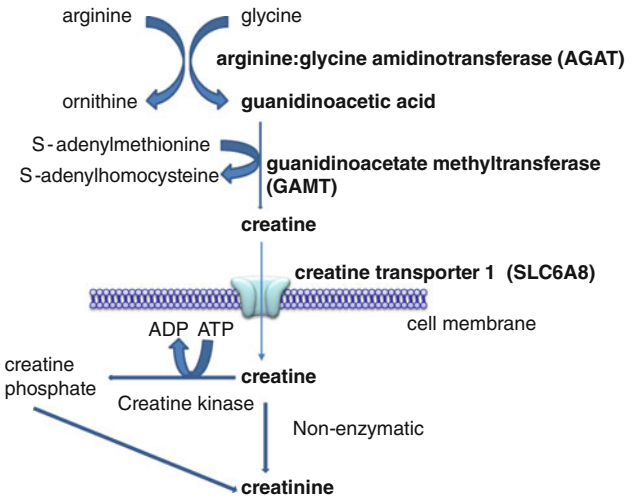

Fig. 1 Synthesis and transport of creatine. L-arginine:glycine amidinotransferase, (AGAT) synthesizes guanidinoacetate from arginine and glycine. Guanidinoacetate methyltransferase (GAMT) transfers a methyl group from S-adenosylmethionine to guanidinoacetate, thus, generating creatine. Creatine enters cells in the brain through the CT1 creatine transporter encoded by the SLC6A8 gene. Creatine is phosphorylated by creatine kinase to phosphocreatine, which is a reversible reaction and contribute to the storage and swift supply of ATP. Both creatine and phosphocreatine, lead non-enzymatically to the formation of creatinine that is excreted in urine

Table 1 Urine creatine and guanidinoacetic acid levels in patients with defects of creatine synthesis and transport

Disease	GA	CR/CN
AGAT deficiency	Low	Low
GAMT deficiency	High	Normal
CR transporter deficiency	Normal	High (4017.5 ± 286.4)

(), values from one patient with CR transporter deficiency (μmol/mmol): HPLC method; 4017.5 ± 286.4; mean ± SD, $n = 2$

Normal values with this study from 15 samples are 798.6 ± 574.8 (CR/CN (μmol/mmol)) (HPLC method; mean ± SD)

AGAT arginine:glycine amidinotransferase, *GAMT* guanidinoacetate methyltransferase, *CR* creatine, *GA* guanidinoacetic acid; *CN* creatinine

evaluation of CR/CN and GA/CN concentrations in urine allows the diagnosis of three types of CCDS (Table 1). So far, 4 cases from two families of AGAT deficiency and ~40 cases with GAMT-related CCDS have been reported (Longo et al. 2011). The SLC6A8 deficiency is the most frequent cause of CCDS. The SLC6A8 deficiency is reported to constitute 0.8–5.4% of the cases of X-linked intellectual disability, and represents the second frequent cause of non-syndromic X-linked intellectual disability following a fragile-X syndrome (Rosenberg et al. 2004; Newmeyer et al. 2005; Clark et al. 2006; Lion-Francois et al. 2006; Arias et al. 2007; Betsalel et al. 2008; Puusepp et al. 2009; Ardon et al. 2010). However, the majority of these patients may remain unrecognized. In this study, we sought to establish an easy and inexpensive high performance liquid chromatography (HPLC) method with UV detection technique to quantify CR, GA, and CN in urine. We chose a weak acidic cation-exchange column of hydrophilic polymer beads with carboxyl groups on their surface and phosphoric acid (H_3PO_4) as the mobile phase. Our results show a complete separation and detection of the three compounds by UV at 210 nm without derivatization.

Materials and methods

All reagents were of analytical grade or better. Acetonitrile, phosphoric acid (85%), creatine monohydrate (98%), guanidinoacetic acid (98%), and creatinine (99%) were obtained commercially (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Ultrapure water was prepared by a Milli-Q system (Millipore, Tokyo, Japan). Standard solutions of CR, GA, and CN were prepared, respectively, by dissolving weighed amounts of the reagents in 2 mM phosphoric acid.

Urine samples collected from healthy individuals or patients were stored at −20°C and thawed just before analyses. Urine (500 μl) of urine was treated with acetonitrile (500 μl) to precipitate proteins, and centrifuged (13,000 rpm, 10 min) after 10 min. The supernatant (50–100 μl) was diluted with 2 mM phosphoric acid (400–950 μl), and a diluted sample (25 μl) was analyzed under the standard condition.

The HPLC set-up comprised a pump, LC-6A, (Shimadzu Ltd., Kyoto, Japan), a Rheodyne injector fitted with a 100 μl loop, an UV–vis spectrophotometric detector, SPD-6A (Shimadzu Ltd.) and a Chromatopack integrator, CR-6A (Shimadzu Ltd.). The separation was performed on a weak acidic cation-exchange column, IC YS-50 (Shodex Ltd., Kawasaki, Japan. 4.6 mm × 125 mm i.d.) using aqueous phosphoric acid as the mobile phase with flow rate of 1.0 ml/min. The analytics were monitored with UV detection at 210 nm.

Standard solutions (25 μl each) of creatine monohydrate (2 mg/l: 134 μmol/l), guanidinoacetic acid (6.7 mg/l: 572 μmol/l), and creatinine (2 mg/l: 177 μmol/l) were loaded into the 100 μl loop, and injected. Analyses were carried out under the following concentrations of mobile phase: 1, 2, 5, and 10 mM H_3PO_4. All analyses were performed at room temperature (32 ± 2°C).

Our new HPLC method was compared to a conventional enzymatic method. Briefly, creatinine amidohydrolase catalyzes CN to CR. Creatine amidinohydrolase and sarcosine oxidase generate sequentially CR to hydrogen peroxide, which is measured at 510 nm in a reaction catalyzed by horseradish peroxidase (Fossati et al. 1983). Fifteen

urine samples were used to determine urinary CR, CN, and the CR/CN ratio. Moreover, one urine sample from a patient with X-linked-SLC6A8 deficiency was analyzed. The results were analyzed by Pearson's correlation coefficient using the PRISM software (La Jolla, CA).

All parents of individuals participating in this study gave a written informed consent after full explanation of the study. The design of this study was approved by the ethical committee of Kanagawa Children's Medical Center.

Results and discussion

CR and GA, not separated with 5 and 10 mM H_3PO_4, were clearly separated with 1 and 2 mM H_3PO_4. However, 2 mM H_3PO_4 produced a complete separation of CR, GA and CN, with retention times (mean ± SD, $n = 3$) of 5.54 min (±0.0035) for CR, 6.41 min (±0.0079) for GA, and 13.53 min (±0.046) for CN using 2 mM H_3PO_4 at a flow rate of 1.0 ml/min (Fig. 2). Since 1 mM H_3PO_4 required a longer retention time (>20 min.), we used 2 mM of H_3PO_4 for subsequent analyses.

Standard solutions (25 µl) of CR (10–1,000 µmol/l), GA (50–4,000 µmol/l), and CN (10–1,000 µmol/l) were analyzed under the above conditions. We obtained a linear correlation between peak areas and concentrations. The linear regression equations for peak area (y; in arbitrary units) and concentration (µmol/l) of the injected calibrator (x) were: $y = 59.821x$ ($R^2 = 0.9971$) for CR; $y = 12.746x$ ($R^2 = 0.9982$) for GA; and $y = 61.604x$ ($R^2 = 0.9968$) for CN. The calibration curves (0–1,000 µmol/l) covered the range of CR, GA, and CN concentrations typically found in urine (when diluted 10- to 40-fold).

Next, we analyzed 15 normal urine samples and compared the values obtained from our new method with those obtained using a conventional enzymatic method. Good correlations were obtained between our new method and the enzymatic method [CR; $r = 0.9276$ ($p < 0.001$, $R^2 = 0.8605$), CN; $r = 0.9370$ ($p < 0.001$, $R^2 = 0.8780$)]. In addition, the CR/CN ratio showed a good correlation between the two methods ($r = 0.984$, $p < 0.001$, $R^2 = 0.9682$). We also obtained a good correlation in a patient with SLC6A8 deficiency of CR/CN (µmol/mmol): Enzymatic method; 4439.5 ± 375.5, HPLC method; 4017.5 ± 286.4; mean ± SD, $n = 2$) The Jaffe reaction and enzymatic methods have been used to determine CN and CR levels (Husdan and Rapoport 1968; Fossati et al. 1983). In addition, Shirokane et al. (1991) described an accurate and simple enzymatic determination for urinary GA (Shirokane et al. 1991). However, this method is not easily accessible because it utilizes guanidinoacetate kinase

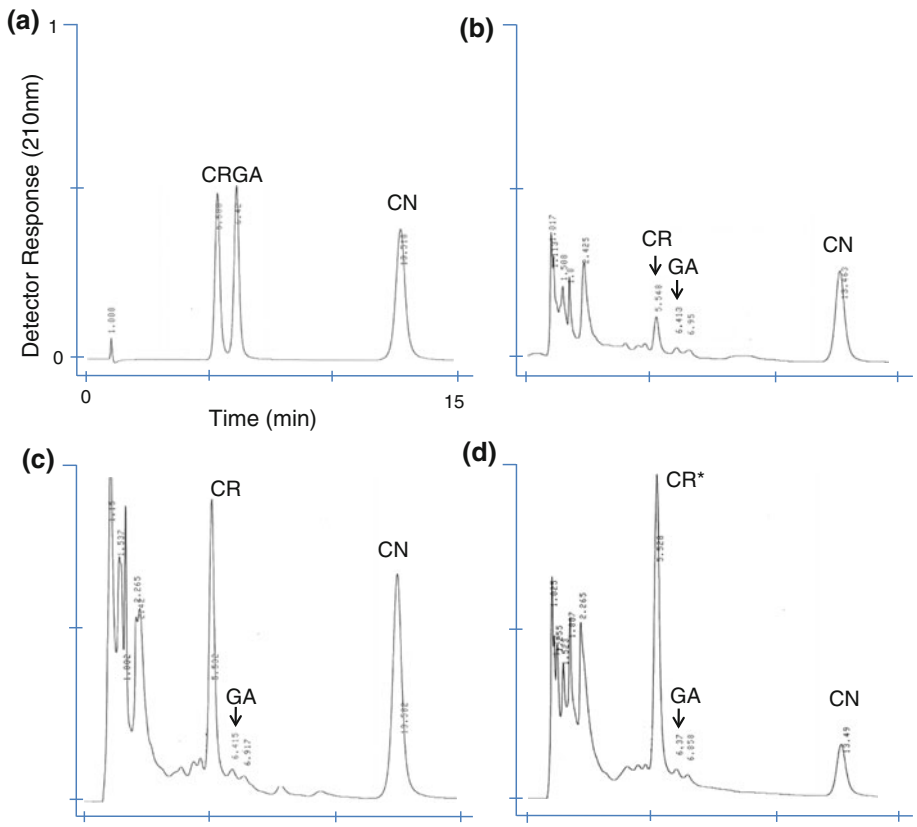

Fig. 2 Examples of chromatograms. Chromatograms of **a** standard samples, and urine sample from **b** a healthy individual-1, **c** a healthy individual-2, and **d** a male patient with SLC6A8 deficiency. Compared to healthy individuals, the patient with SLC6A8 deficiency shows a marked increase in creatine (CR) (*asterisk*)

purified from a polychaete, *Perineresis sp*., which is not commonly available.

So far, simultaneous HPLC determination of CR, GA, and CN in urine and blood has been reported using the reversed-phase and strong acidic cation-exchange modes, which require derivatization for fluorescence detection (Natelson 1984; Carducci et al. 2001).

Analytical methods using mass spectrometry (MS) have been described recently, including gas chromatography–mass spectrometry (GC-MS) (Hunneman and Hanefeld 1997), liquid chromatography–mass spectrometry (LC-MS) (Yasuda et al. 1997), liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Carling et al. 2008; Cognat et al. 2004), and flow injection analysis-electrospray ionization-tandem mass spectrometry (FIA-ESI-MS/MS) (Carducci et al. 2006). Techniques using MS are highly reliable and should contribute to the diagnosis CCDS.

Here, we report a simple HPLC method using weak-acidic ion chromatography column with UV detection that does not require derivatization. Three components, CR, GA, and CN, needed for CCDS diagnosis eluted within 15 min with complete separation. Our new method allows the quantitation of these three compounds without the use of MS. This new method should contribute to patient screening, and allow early interventions for patients with CCDS.

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Conflict of interest The authors declare that they have no conflict of interest.

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