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Research Article

Determination of Guanidinoacetic Acid in Urine by High-Performance Liquid Chromatography-Fluorescence Detection Method

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ABSTRACT: Creatine deficiency syndromes are newly recognized inherited metabolic disorders. Creatine deficiency is classified into three groups according to the synthesis steps: guanidinoacetate N-methyl transferase (GAMT) deficiency, arginine-glycine amidinotransferase (AGAT) deficiency, and creatine transporter disorder. The common biochemical finding in all three diseases is cerebral creatine deficiency. Low urinary guanidinoacetate (GAA) levels are observed in AGAT deficiency and high urinary GAA levels are observed in GAMT deficiency. In this study, we developed a new, simple, inexpensive, and rapid chromatographic analysis method for the quantitative determination of guanidinoacetate in urine samples. Guanidinoacetate was chromatographically separated with a buffer containing 50 mM formic acid and methanol, 1.0 mL/min flow, C18 (150 mm 4.6 mm, 5.0 µm) analytical column and fluorescence detector at λex: 390 nm, λem: 470 nm.

Keywords: *Guanidinoacetic Acid, Creatine deficiency, High-performance liquid chromatography-fluorescence detection (HPLC-FLD)*

1 INTRODUCTION

Creatine (α-N-methyl-guanidino-acetic acid) is a nitrogen-containing organic acid and is involved in the storage, utilization, and transport of energy, especially in organs with high energy requirements such as the brain and muscles. In humans, dietary creatine provides half of the daily requirement, and the remaining 50% is synthesized endogenously. In mammals, creatine is synthesized in a two-reaction pathway. In the first step, guanidino-acetate (GAA) and ornithine are formed from arginine and glycine by the enzyme arginine-glycine amidino transferase (AGAT). In the second step, GAA is converted to creatine by a methylation reaction mediated by the enzyme guanidinoacetate methyl transferase (GAMT). In this conversion, S-adenosyl methionine (SAM) is used as the methyl donor [1,5].

1.1 Arginine-Glycine Amidino Transferase Deficiency (AGAT)

The enzyme arginine-glycine amidino transferase catalyzes the first step in creatine biosynthesis. In enzyme deficiency, the production of GAA, the precursor molecule in

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creatine synthesis, is impaired. Arginine-glycine amidino transferase deficiency (OMIM#612718) is an autosomal recessive disorder caused by mutations in GAMT [6-8]. AGAT deficiency should be considered in all patients with muscle weakness/myopathy in addition to developmental delay, behavioral problems, and cognitive or mental retardation, but these findings are not specific and can be seen in many neurometabolic disorders. Low GAA levels in urine samples are important in the diagnosis [7,8].

1.2 Guanidino-Acetate Methyl Transferase Deficiency

The enzyme guanidino-acetate methyl transferase constitutes the second step in creatine synthesis and enables the formation of creatine from guanidino-acetate with the help of SAM. GAMT deficiency (OMIM #612736) is an autosomal recessive creatine synthesis defect [9,10]. The disease develops due to mutations in the GAMT gene. The most important biochemical features of GAMT deficiency are cerebral creatine deficiency and elevated GAA. Cerebral creatine deficiency can also be seen in AGAT deficiency and creatine transporter defects, but elevated GAA is specific for GAMT deficiency. Elevated GAA can be detected in urine, blood, and blood-cerebrospinal fluid (CSF) [9,10]. Elevated GAA levels in body fluids are not associated with disease severity. Urine GAA screening is known to be an effective screening method in individuals with intellectual disability [10]. In cases of GAMT and AGAT deficiency, early diagnosis and treatment with creatine monohydrate leads to clinical improvement.

Simultaneous measurement of GAA and creatinine in biological samples is difficult and often requires advanced instrumental analysis techniques. The analytical methods used for analysis were reported as high-pressure liquid chromatography (HPLC) [11], liquid chromatography sequential mass spectrometry (LC/MS/MS) [12], gas chromatography-mass spectrometry (GC/MS) [13], capillary zone electrophoresis ultraviolet detector (CZE-UV) [14]. This study aimed to develop a new, simple, inexpensive, and rapid chromatographic analysis method for the quantitative determination of GAA in urine samples. GAA and creatinine were chromatographically separated simultaneously with 50 mM formic acid and methanol buffer, 1.0 mL/min flow, C18 (150 mm 4.6 mm, 5.0 µm) analytical column, and fluorescence detector at λex: 390 nm, λem: 470 nm.

2 MATERIAL AND METHOD

2.1 Chemicals and Synthetic Urine

GAA and creatinine were obtained from Sigma/Aldrich (St. Louis, MO, USA). Potassium hydroxide, trichloroacetic acid, ascorbic acid, Tris-HCI, phosphoric acid, and formic acid (98-100% extra pure) were purchased from Merck (Darmstadt, Germany).

Methanol and ninhydrin (puriss. p.a.) were purchased from Riedel-de Han (Seelze, Germany). The synthetic urine used in the study was purchased from Artificial Urine, Pickering Laboratories (Mountain View, California, USA). Synthetic urine is a ready-to-use and stable solution with a pH of 6.5, similar in composition to real human urine, and used in standard laboratory studies. The solutions used in this study were prepared using ultrapure water obtained from the Elga Purelab brand Option-7-15 model pure water system.

2.2 HPLC

HPLC Nexera-i LC-2040C 3D Plus high-performance liquid chromatography (Shimadzu, Kyoto, Japan) was used for GAA and creatinine analysis. A photo diode array PDA) detector was used in the system. LabSolutions software was used for instrument control, data acquisition, and data analysis. Chromatographic studies and analytical separation were performed on a Vertical GES C18 HPLC column (Tailand) (150 mm 4.6 mm, 5.0 µm) at 25°C.

2.3 Running Buffer and Optimum Conditions

The optimized mobile phase for simultaneous analysis of GAA and creatinine was formed from a gradient of 50 mM formic acid and methanol from 5% to 90% methanol over 19 min. The flow rate was set to 1.0 mL/min. [1]. The injection volume was 10 µL and the fluorescence detector was measured at λex: 390 nm, λem: 470 nm. The chromatogram obtained from this working buffer and optimized conditions is shown in Figure 1.

2.4 Sample Preparation

To precipitate the protein in urine samples, 100 µL of 30% trichloroacetic acid (TCA) was added to 20-fold diluted urine, centrifuged at 10000 rpm for 5 minutes, and filtered with a 25 mm 0.2 µm nylon filter. For GAA and creatinine analysis, 400 µL of urine sample was taken from the filtered sample, 300 µL of 1.3 M KOH, and 150 µL of 0.9%

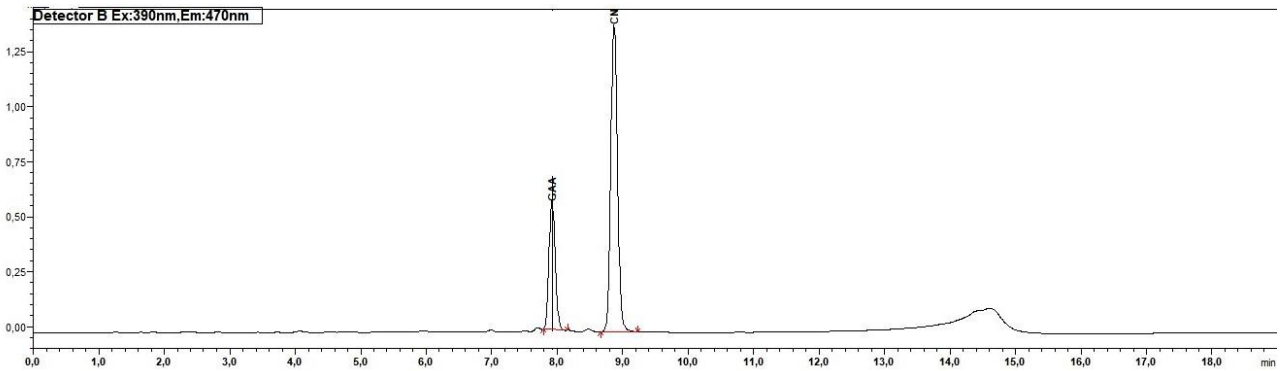

Figure 1. Chromatogram of peaks of 10 nmol/mL standard GAA and 10000 nmol/mL creatinine in optimized buffer solution medium.

ninhydrin was added to derivatize, the solution was mixed thoroughly and incubated for 15 minutes at room temperature [11]. Then 100 µL of 5% ascorbic acid and 100 µL of 5 M phosphoric acid were added and the solution was thoroughly mixed and incubated at 90 ◦C for 30 minutes. After cooling to room temperature, 10 µL of the sample was injected into the HPLC device.

3 RESULT

3.1 Validation

Validation of the proposed method was performed according to Eurachem guidelines [15].

3.1.1 Linear Range, the minimum limit of detection (LOD), and the minimum limit of quantification (LOQ)

The calibration line was plotted against the corrected peak areas (A/t) over the concentration range of 1, 5, 10, 25, 50, and 100 nmol/mL guanidinoacetate for GAA in pure water and 20-fold diluted urine. The regression parameters obtained for the calibration equation at these limits are given in Table 1. Three times the signal value of the noise detected from three different regions in the chromatogram was considered as LOD and this value was found to be 1.0 nmol/mL. Quantitatively, LOQ value was found to be 3.51 nmol/mL, 10 times the noise signal (Table 1).

3.1.2 Matrix Effect

The matrix effect was analyzed by calculating the ratio of the slopes of the matrix-matched calibration curve and the pure solvent calibration curve. We used 20-fold diluted urine for matrix-matched calibration and ultrapure water for pure solvent calibration. The matrix effect was found to be 8%. Since this value was not between -10% and 10%, the matrix effect was not significant (Table 1).

Table 1. Regression parameters, limit of observability, and limit of detection.

Regression parameters	In urine	In Aqueous Solution
Regression coefficient (R^2)	0,996	0,997
Linear range (nmol/mL)	1,0-100	1,0-100
Regression equation	y=165,5x + 138636	
Number of observations	6	6
Limit of dedection (nmol/mL)	1,0	
Limit of quantification (nmol/mL)	3,51	
Recovery (%)	91-101	

3.1.3 Repeatability

The precision of the method was checked by 7 successive injections of 5, 25, 50, and 100 nmol/mL GAA standard solution. The precision values for migration times and corrected peak areas (A/t) are given in Table 2 as intra-day and inter-day precision values as % RSD.

Table 2. Repeatability and recovery values obtained from synthetic urine samples under optimized conditions.

Added amount nmol/mL	Recovery %	Time (min)	Adjusted Area
5	91	7,92	5,58
25	98	7,93	5,67
50	101	7,91	5,84
100	97	7,92	5,72

3.1.4 Accuracy of the method

The accuracy of the method was determined by adding three different concentrations of 5, 50, and 100 nmol/mL GAA standards to the urine sample and taking 7 different measurements for each of them. Recovery values were between 91 and 101% (Table 2).

4 DISCUSSION

In conclusion, the determination of GAA levels in urine is of vital importance in creatine synthesis disorders. In this study, it was observed that it could be easily determined in 7.92 minutes in 50 mM formic acid-methanol buffer using a C18 (150 mm 4.6 mm, 5.0 µm) analytical column in a HPLC system with fluorescence detector. It was found to be a highly sensitive method in terms of the results obtained. It has been demonstrated that GAA can be used in routine analysis and clinical research.

5 AUTHOR CONTRIBUTIONS

Hypotesis: M.S.C., E.I.; Design: M.S.C., E.I.; Literature review: M.S.C., E.I.; Data Collection: E.I.; Analysis: E.I.; Manuscript writing: M.S.C., E.I.

6 CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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