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Short communication

Simultaneous determination of creatine and guanidinoacetate in plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Sara Boenzi[a,*], Cristiano Rizzo[b], Vincenzo Maria Di Ciommo[c], Diego Martinelli[a], Bianca Maria Goffredo[b], Giancarlo la Marca[d], Carlo Dionisi-Vici[a]

[a] Division of Metabolism, Bambino Gesù Pediatric Hospital, Rome, Italy
[b] Laboratory of Metabolic Biochemistry, Bambino Gesù Pediatric Hospital, Rome, Italy
[c] Unit of Epidemiology and Biostatistics, Bambino Gesù Pediatric Hospital, Rome, Italy
[d] Department of Pharmacology, University of Florence, Florence, Italy

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ABSTRACT

Background: Guanidinoacetate (GAA) and creatine are reliable biochemical markers for primary and secondary creatine defects. We describe a method by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for simultaneous determination of plasma GAA and creatine. We analyzed 283 healthy subjects from 0 to 63 years old to obtain age-related control values.
Methods: Plasma samples were extracted with acetonitrile containing 13C2-GAA and d3-creatine. Samples were analyzed by LC–MS/MS in positive ionisation mode, after derivatization to butyl-esters. Optimal chromatographic separation was achieved using a column Supelcosil^{TM} LC-4.6 mm with isocratic elution in 5 min.
Results: Run time was 5 min. Standard curves were linear from 0.05 to 200 μmol/L for creatine and from 0.02 to 40 μmol/L for GAA. Limit of detection (LOD) and limit of quantitation (LOQ) were respectively 0.005 and 0.05 μmol/L for creatine; LOD and LOQ were 0.002 and 0.02 μmol/L respectively for GAA. Intra and inter-assay CVs for creatine and GAA were <8%. Recovery experiments adding 50 and 100 μmol/L creatine and 10 and 20 μmol/L GAA were 102.1% and 101.2%, for creatine; 102.95% and 96.45% for GAA. The method was applied to 283 plasma controls from healthy subjects to obtain control values in three specific age ranges: 0–12, 13–20, >20 years old.
Conclusion: A rapid and high sensitive LC–MS/MS method was developed and validated for determination of creatine and GAA in plasma and it could also be applied to other biological materials, such as CFS and urines. This method is useful for diagnoses of primary and also for secondary creatine defects that may occur in inherited metabolic diseases in which precursors of creatine biosynthesis are involved.
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1. Introduction

Creatine and phosphocreatine play an essential role in energy storage and transmission in several tissues. Creatine is synthesized mainly in the liver and pancreas by two reaction involving two enzymes: the first arginine:glicine amidinotransferase (AGAT; EC 2.1.4.1) transfers the amidino-group from arginine to glycine, producing ornithine and guanidinoacetate (GAA); the second enzyme is S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2), which transfers a methyl group to GAA producing creatine. Creatine is transported to tissues by the creatine transporter (CrT) and is non-enzymatically converted to creatinine [1]. Three inherited defects in the biosynthesis and transport of creatine have been described: AGAT [2] and GAMT [3] deficiencies and the functional defects in the creatine transporter (SLC6A8) [4]. A common feature in all these disorders, called Creatine Deficiency Syndromes (CDS), is the complete lack of Cr/PCr in the brain measured by in vivo magnetic resonance spectroscopy (MRS). In AGAT and GAMT deficiencies the creatine pool can be partially reverted by creatine supplementation, whereas male patients with the X-linked creatine transporter defect are unresponsive to this supplementation [5]. Biochemical detection of CDS relies on the determination of two main metabolites in biological fluids: creatine and GAA [6,7]. The accumulation of GAA in biological fluids in GAMT deficiency was demonstrated to be reliable diagnostic marker of this disease. In contrast, for diagnostic investigation for AGAT deficiency is recommended to analyse creatine and GAA in both urine and plasma in which the levels of these two metabolites are decreased. X-linked creatine transporter deficiency patients are characterized by increased urinary creatine/creatinine ratio and

* Corresponding author at: Division of Metabolism, Bambino Gesù Children's Hospital, Piazza S. Onofrio 4, 00165 Rome, Italy. Tel.: +39 06 68592225; fax: +39 06 68592791.
E-mail address: sara.boenzi@opbg.net (S. Boenzi).

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plasma creatine concentration usually within the reference range or slightly increased [8].

There are also a few secondary creatine defects, in particular, two inherited conditions causing hyperornithinemia have been associated to secondary creatine deficiency: deficiency of ornithine delta-aminotransferase activity (OAT) [9], and hyperornithinemia–hyperammonemia–homocitrullinuria syndrome (HHH) [10,11]. In these cases, treatment with creatine can improve some clinical aspects of the disease.

Creatine and GAA concentrations in physiological fluids are reliable diagnostic markers for primary creatine deficiency syndromes (AGAT, GAMT), but also to recognize secondary creatine deficiencies that may possibly occur in other metabolic diseases in which precursors of creatine biosynthesis are involved. Therefore, it is important to develop highly selective and sensitive analytical techniques for the determination of creatine and its precursor GAA. We developed and validated a reliable and sensitive method by liquid chromatography coupled with tandem mass spectrometry for quantitative determination of creatine and GAA as buthyl-esters in plasma. We analyzed a large number of control samples (283) by this method for the identification of age-related reference ranges, as a diagnostic tool for early diagnosis of primary and secondary creatine deficiencies. The importance to detect with reasonable precision any creatine and GAA alterations is because, in addition to the classic primary creatine deficiencies, increasing number of inherited metabolic diseases occur with slight changes of plasma creatine. Moreover, in those cases in which the concentrations of GAA and creatine are very low, as for AGAT deficiency, is necessary a high sensitive method, enough to safely detect values below the normal range. Previous methods [12–16] are useful for great increasing but not for a decrease of two metabolites. Bodamer et al. [14] using the same metabolites derivatization procedures did not report LLOQ for creatine neither for GAA but only high limits of detection, especially for GAA, 1.2 μmol/L. Our data demonstrate that this value is a typical concentration detectable in a normal plasma sample.

2. Materials and methods

2.1. Reagents

The labeled internal standard d_3-creatine was synthesized by Dr. H. Ten Brink (VU University Medical Center, Amsterdam, The Netherlands) and $^{13}C_2$-GAA were obtained from CDN isotopes (Quebec, Canada). Creatine and GAA were obtained from Sigma–Aldrich (Steinheim, Germany). Ammonium acetate was purchased from Merk KGaA (Damstadt, Germany). HPLC grade acetonitrile and water were purchased from Romil Ltd. (The Source Convent Drive Waterbeach Cambridge, United Kingdom). Formic acid 98/100% (FOA) was obtained from BDH (VWR Ltd., Poole, England). Butanolic-HCl was from Regis Technologies (Morton Grove, IL, USA).

2.2. Preparation of standard solutions

To investigate the molecular fragmentation a to obtain calibration curves for creatine and GAA, 10 mmol/L d_3-creatine and $^{13}C_2$-GAA stock solutions (Stock-1) were prepared in water and stored frozen at −20 °C. 5 mmol/L d_3-Cr and 1 mmol/L of $^{13}C_2$-GAA were prepared by dilution in acetonitrile–water (50:50, v/v) in the same solution (Stock-2) and stored frozen at −20 °C. “Daily standard solution” containing 10 μmol/L $^{13}C_2$-GAA and 50 μmol/L d_3-Cr was prepared by dilution of Stock-2 solution 1:100 (v/v) with acetonitrile.

2.3. Sample treatment procedure

Plasma sample was obtained from blood samples collected in EDTA K2 tubes that were immediately centrifuged, for 5 min at 3000 × g, plasma was separated from red cells and immediately stored frozen at −20 °C.

Fifty microliters of plasma sample was mixed with 25 μL of “Daily standard solution”. Four hundred microliters of acetonitrile was added to precipitate proteins and extract the metabolites. Each vial was mixed by vortex for 2 min, then centrifuged for 5 min at 10,000 rpm and the supernatant fluid was transferred to a clean vial and dried at 40 °C under nitrogen steam. Eighty microliters of butanolic-HCl was added for derivatization of metabolites as buthyl-esters and heated at 65 °C for 15 min. Samples were dried again at 40 °C under nitrogen steam and finally they were reconstituted with 200 μL of acetonitrile–water 50:50 (v/v) containing 0.05% FOA to obtain pH 4, the appropriate pH for metabolites ionization.

2.4. Liquid chromatography

Chromatography was performed on a Agilent series 1200 pump and autosampler (Agilent Technologies Inc., Wilmington, DE, USA). The column for chromatographic separation was a Supelcosil™ LC-8, 3 μm, 7.5 cm × 4.6 mm (Supelco, Bellefonte, PA, USA). The mobile phase was solution A (0.005 mol/L ammonium acetate containing 0.03% FOA) and solution B (acetonitrile containing 0.03% FOA). Chromatography separation of metabolites was obtained with isocratic elution solvent A-solvent B (30–70, v/v) at the flow rate of 1.0 mL/min. 5 μL of sample was injected into the column. The total run was 5 min.

2.5. Mass spectrometry

Tandem mass spectrometry experiments were carried out on an API3200 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Toronto, Canada), equipped with a Turbo Ion Spray Source operating in positive ion mode with a needle potential of 5500 V; the source temperature was 550 °C. Declustering Potential (DP), Collision Cell Exit Potential (CXP) and Collision Energy (CE) were optimized introducing 5.0 μmol/L aqueous standard solution of 50% acetonitrile containing 0.05% FOA of each metabolite into spectrometer infusion at flow rate of 10 μL/min. The resulting DP was +41 for creatine and +36 V for GAA. Optimal CE and CXP were found at 27 and 5 V for creatine, and 21 and 3 V for GAA, respectively. The following transitions were monitored: m/z 174.1 > 101.0 for GAA, 176.1 > 103.0 for $^{13}C_2$-GAA, 188.1 > 90.0 for creatine and 191.1 > 93.0 for d_3-creatine. The dwell times was 60 ms and the pause between the mass transitions was 5 ms.

2.6. Standard curves for quantification

Aqueous standard solutions of 40, 20, 10, 5, 2, 1, 0.1, 0.02 μmol/L for GAA and 200, 100, 50, 25, 10, 5, 0.5, 0.05 μmol/L for creatine were analyzed with the same procedure of plasma samples. The acquired data were processed using Analyst version 1.4.2 software (Applied Biosystems-Sciex), including option for chromatographic and spectral interpretation and for quantitative information generation. Calibration curves were constructed with the Analyst Quantitation program using a linear least-square regression non-weighted.

The limit of detection (LOD) was determined by progressive dilutions of calibrator solutions of each analyte and was considered as the lowest concentration for which the signal-to-noise ratio (S/N) was indicated by the Analyst software to be at least 3. The limit of quantification (LLOQ) was determined by preparing cali-

Table 1
Precision data for the LC–MS/MS assay.

	Mean ± SD (μmol/L)	CV (%)
Intra-day (n = 10)		
Creatine	53.42 ± 1.81	3.39
GAA	2.66 ± 0.16	6.01
Inter-assay (n = 18)		
Creatine	54.71 ± 2.73	4.99
GAA	2.87 ± 0.22	7.66

Table 2
Accuracy data for Creatine and GAA.

	Mean ± SD (μmol/L)	CV (%)	Recovery (%)
Creatine added (n = 5)			
0 μmol/L	54.14 ± 2.62	4.84	n.c.
50 μmol/L	106.31 ± 3.40	3.20	102.08
100 μmol/L	155.92 ± 3.91	2.51	101.15
GAA added (n = 5)			
0 μmol/L	2.54 ± 0.19	7.48	n.c.
10 μmol/L	12.91 ± 0.46	3.56	102.95
20 μmol/L	21.74 ± 0.97	4.46	96.45

brator solutions with decreasing concentration of each analyte and was considered as the lowest concentration for which the signal-to-noise ratio (S/N) was indicated by the Analyst software to be at least 10.

2.7. Sample collection of patients and controls

Children's plasma, for reference values, was obtained from hospitalized neurologically healthy subjects and adults' plasma was obtained from anonymous blood donor. Plasma from two patients previously diagnosed with GAMT deficiency was studied. All samples were treated as described in Section 2.3.

2.8. Statistical analysis

A preliminary test (Kolmogorov–Smirnov) was performed to assess normality of distributions of creatine, age, and GAA. Differences among the three age groups were tested using ANOVA for creatine and Kruskal–Wallis test for GAA.

3. Results

3.1. Chromatography and mass spectra

Fig. 1 shows an extract ion chromatogram from a 5 μmol/L GAA and 20 μmol/L creatine standard solution. Multiple Reaction Monitoring (MRM) was used for the detection of the specific transitions in positive ion mode for the monitoring of each molecule. Under the conditions described above, the system was efficient for the separation of GAA and creatine that were eluted with their respective labeled internal standard at the retention times of 3.96 and 4.18 min, respectively. Moreover, in plasma samples were separated by these conditions two additional interfering peaks presenting to the same GAA MRM transition, m/z 174.1 > 101.0, at different retention times, 1.20 and 4.76 min, that could interfere with the GAA quantification (Fig. 4).

3.2. Linearity

Calibration curves were linear over concentrations of 0.05–200 μmol/L for creatine and 0.02–40 μmol/L for GAA which covers and exceed the concentration ranges found in plasma for both metabolites. The coefficient of linear correlation (r^2) was 0.9997 for creatine and 0.9998 for GAA.

3.3. Limit of detection, precision and recovery

The minimal detectable concentrations, LOD of the analytes were 0.005 μmol/L for creatine (S/N ratio 7.5) and 0.002 μmol/L for GAA (S/N ratio 4.6). LLOQ were 0.05 μmol/L for creatine (S/N ratio 12.3) and 0.02 μmol/L GAA (S/N ratio 12.9).

All validation experiments were performed with pooled plasma from healthy controls and the validation data of the presented method are listed in Tables 1 and 2. The intra-assay variation was assessed from 10 replicates within one day (n = 10) and inter-assay from three times on six different days (n = 18); CVs for creatine and GAA in plasma were in all cases <8%. Recovery experiments were performed at two different concentrations: pooled plasma was spiked with creatine (50 and 100 μmol/L) and with GAA (10 and 20 μmol/L). The creatine recovery was 102.1% and 101.2%, respectively, of the expected amount; GAA recovery was 102.95% and 96.45%, respectively.

3.4. Biological variation of creatine and GAA in plasma

We measured creatine and GAA concentrations in plasma sample of 283 healthy subjects from 0 to 63 years old. Our results are summarized in Table 3 were we arbitrarily individuated three age group ranges: 0–12 years, 13–20 years and >20 years. The differences among the three age groups are highly significant for creatine (F = 40.920; p < 0.001) and for plasma GAA control values (p < 0.001) (Fig. 2).

Plasma GAA and creatine were significantly correlated with age: creatine shows an inverse correlation with age (rho = −0.450; p < 0.001) and GAA a direct correlation with age (rho = 0.719; p < 0.001) (Fig. 3). For this reason, it is not preferable to use one reference age range to detect any abnormal creatine and GAA concentration.

Given the broad distribution of values especially for the younger age cohorts, we estimate reference values for both creatine and GAA by use of control plasma sample with percentile distribution (2.5th and 97.5th percentiles).

3.5. Patients samples analysis

The LC–MS/MS method was used to analyse two samples from two diagnosed GAMT patients to testify the ability of this method to differentiate them from controls. Plasma sample from a 6 years old patient showed an increase of GAA concentration 12.4 μmol/L (range 0–12 years: 2.5th–97.5th percentile 0.8–1.6 μmol/L) and reduction of creatine concentration 7.4 μmol/L (range 0–12 years: 2.5th–97.5th percentile 16.2–97.0 μmol/L). For the second patient 11 years old plasma GAA was 11.6 μmol/L and creatine 20.1 μmol/L relatively to the same 0–12 years control values. These levels of creatine and GAA are readily distinguishable from controls and

Table 3
Creatine and GAA control values in μmol/L (n = 283).

Age range	0–12 (n = 93)	13–20 (n = 96)	>20 (n = 94)
Creatine			
2.5th percentile	16.2	14.1	13.2
Median	60.0	47.9	31.4
97.5th percentile	97.0	92.0	56.8
GAA			
2.5th percentile	0.3	0.6	0.7
Median	0.8	1.1	2.2
97.5th percentile	1.6	2.1	3.3

Fig. 1. Extract ion chromatogram (upper panel) with multiple reaction monitoring experiments (lower panels) for plasma sample. GAA and $^{13}C_2$-GAA (RT: 3.96 min); creatine and d_3-creatine (RT: 4.18 min).

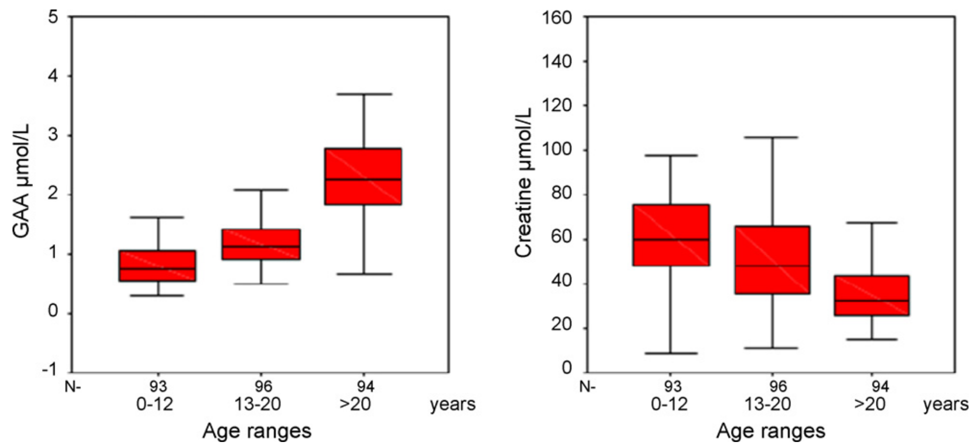

Fig. 2. Box plots for creatine and GAA concentrations (µmol/L) in the three different age ranges: 0–12, 13–20 and >20.

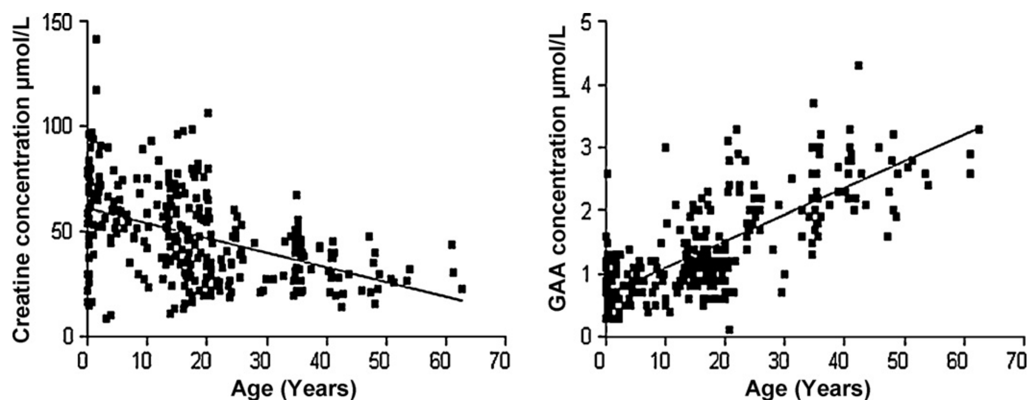

Fig. 3. Scatter plot of creatine and GAA concentration (μmol/L) vs age expressed in years. Plasma creatine concentration shows an inverse correlation with age, while GAA concentration shows a direct correlation.

are consistent with characteristic biochemical findings of GAMT deficiency [3].

4. Discussion

Patients with plasma creatine reduction could be easily treated with creatine or its precursors supplementation, causing, in some cases, a rapid improvements of clinical conditions. Early diagnosis of primary and secondary deficiencies can improve the prognosis of these disorders. We have developed a sensitive and specific method for determination of creatine and GAA concentrations in plasma. In recent years, introduction in the laboratory of liquid chromatography coupled with mass spectrometry, have greatly increased the diagnosis of creatine primary defects that were not previously easily diagnosed with traditional analytical techniques. Several methods for creatine and GAA determination have been described, including stable isotope dilution gas chromatography–mass spec-

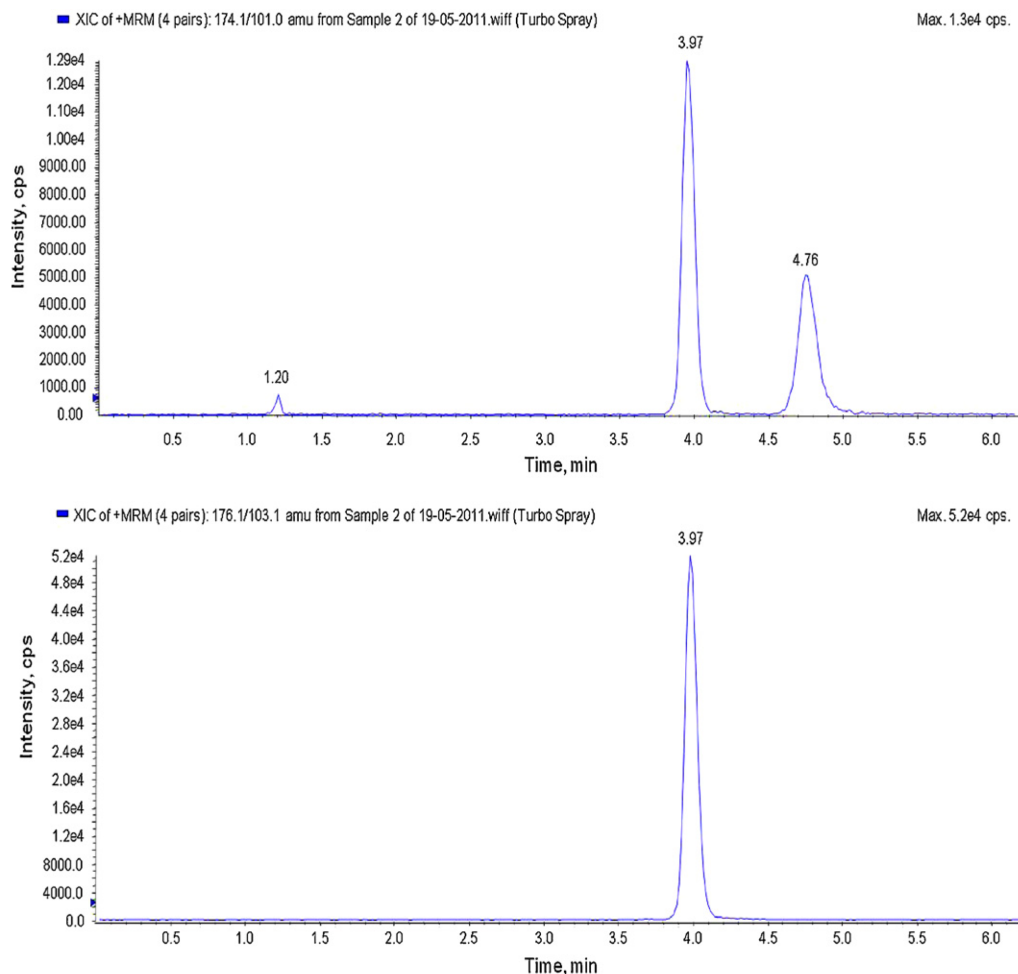

Fig. 4. Plasma sample chromatogram. Upper panel: extract ion chromatogram with multiple reaction monitoring (MRM) experiment *m/z* 174.1 > 101.0 of GAA (RT: 3.97 min) and other two unknown peaks (RT: 1.20, 4.76 min); Lower panel: extract ion chromatogram with MRM experiment *m/z* 176.1 > 103.0 of $^{13}C_2$-GAA (RT: 3.97 min).

trometry (GC–MS) [12,13], and HPLC [6] and LC–MS/MS [14–16] but these methods reach less sensitivity and specificity. All these GC–MS methods require a long time for several steps of extraction and derivatization. New method described in this paper has a satisfactory intra-day precision 3.39% for creatine and 6.01% for GAA and inter-day precision 4.99% and 7.66% respectively, similar to those described by R.S. Carling et al. [15] by un-derivatized LC–MS/MS, that were 2.7% and 6.4% for intra-day precision but new data are slight better for inter-day assay precision that were 6.2% and 9.5% respectively for creatine and GAA; LOD and LLOQ were 0.1 μmol/L and 0.4 μmol/L for both creatine and GAA, higher compared with the new method: LOD 0.005 μmol/L for creatine, 0.002 μmol/L for GAA, LLOQ 0.05 μmol/L for creatine and 0.02 μmol/L GAA. Probably, new better data are due to the chromatographic separation of two additional interfering peaks presenting to the same GAA MRM transition at different retention times that could interfere with the GAA (Fig. 4).

Cognat et al. [16] published in 2004 a method by LC–MS/MS, reaching LOD of 0.01 μmol/L for creatine and 0.025 μmol/L for GAA, and LLOQ 0.05 μmol/L and 0.1 μmol/L respectively for creatine and GAA, slight higher than our new data. The intraday imprecision were 9% for GAA and 2% for creatine in plasma while the interday imprecision were 13% and 9% respectively, higher especially for GAA compared with our intra-day and inter assay CVs. Probably, different data obtained mainly for GAA, are due to the different evaluation of interferences that occurs in plasma samples analysis.

Similarly, Bodamer et al. [14] give only few information about chromatography and no chromatogram with the peaks or their retention times has shown, so it is unknown if the two metabolites were well separated or not, or if background noise or such interference were present or not. Intra-day and inter-day assay are similar to those obtained by new method, but limits of detection were very high: 7.4 and 1.2 μmol/L respectively for creatine and GAA. New method is 2 min longer than the others LC–MS/MS methods but it allow to separate the unknown peaks that correspond to unknown two substances with the same molecular weight and that produce the same fragment *m*/*z* 101 of GAA butyl-derivative. Theoretically they could interfere with the GAA quantification, if not well separated. Therefore, it is difficult to explain the reason for the increased sensitivity of our method respect the others by LC–MS/MS. Probably, it is due to both the instrumentation and the chromatographic separation by the column we used, that differs from previously study published. The column we used perform a good separation of creatine, GAA and some interference that could occur, as showed in the reported figure of chromatogram (Figs. 1 and 4) with two distinct retention times allowing high sensitivity for the quantitative determination of both metabolites, confirmed by very low LOD and LLOQ values. It could be suitable for detecting lower GAA and creatine concentrations, such as for AGAT deficiency, for several secondary creatine deficiencies, and possibly also to expand the analysis to CFS in which preliminary studies demonstrate that GAA concentration is almost 10 times lower than in plasma, however much lower than detectable concentrations with all methods previous published.

Moreover, Carling et al. [15] mentioned that the validity of existing reference ranges quoted in literature is questionable by the relatively small numbers of subjects used to determine them and not always specified modality storage. Specification of storage modality of samples analyzed is very important since it is known that creatine, over time and changing pH of medium, varies its concentration transforming itself into creatinine [15,17]. Our plasma creatine and GAA reference values are determined by large group of control subject allowing us to obtain three age-related ranges, clearly indicating storage modality of samples. Our data confirm those already published about the correlation of plasma creatine with the age of subjects [18] but in our study, the number of control values are very large respect previous publications in which there was analyzed up to 30 samples per age range, without any description of sample storage [14–16]. Therefore using our control values, it is possible to estimate even slight changes of creatine and GAA concentration in a defined age range. Furthermore, the applicability of the method is illustrated by the determination of GAA and creatine in plasma of two patients affected with GAMT, in which GAA was highly elevated and creatine was decreased respect the corresponding age related control values. In summary, it is known that it is possible to detect large alterations of plasma creatine and GAA, even with low sensitive methods. The LC–MS/MS method described in this paper is a rapid and useful tool to diagnose primary creatine deficiencies and also to recognize treatable secondary creatine defects that may occur in inherited metabolic diseases in which precursors of creatine biosynthesis are involved. The development of this method is just to detect small changes compared to controls and to detect great decreases of two metabolites, such as for AGAT deficiency, in which it is known that creatine and GAA concentrations are very low. Therefore, it will be possible to assess any variation of creatine and GAA in inherited metabolic diseases and will be possible to explore the relationship between circulating creatine levels and others metabolic pathways.

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