



Simultaneous assay of isotopic enrichment and concentration of guanidinoacetate and creatine by gas chromatography–mass spectrometry

Takhar Kasumov, Lourdes L. Gruca, Srinivasan Dasarathy, Satish C. Kalhan *

Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA

Department of Hepatology and Gastroenterology, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH 44195, USA

ARTICLE INFO

Article history:

Received 30 June 2009

Available online 29 July 2009

Keywords:

Creatine

Guanidinoacetate

Guanidinoacetate *N*-methyltransferase

Muscle wasting

Creatine kinetics

ABSTRACT

A gas chromatography–mass spectrometry (GC–MS) method for the simultaneous measurement of isotopic enrichment and concentration of guanidinoacetate (GAA) and creatine in plasma sample for kinetic studies is reported. The method, based on preparation of the bis(trifluoromethyl)pyrimidine methyl ester derivatives of GAA and creatine, is robust and sensitive. The lowest measurable m_1 and m_3 enrichment for GAA and creatine, respectively, was 0.3%. The calibration curves for measurements of concentration were linear over ranges of 0.5 to 250 μM GAA and 2 to 500 μM for creatine. The method was reliable for inter- and intraassay precision, accuracy, and linearity. The technique was applied in a healthy adult to determine the in vivo fractional synthesis rate of creatine using primed-constant rate infusion of [1- ^{13}C]glycine. It was found that isotopic enrichment of GAA reached a plateau by 30 min of infusion of [1- ^{13}C]glycine, indicating either a small pool size or a rapid turnover rate (or both) of GAA. In contrast, the tracer appearance in creatine was slow (slope = 0.00097), suggesting a large pool size and a slow rate of synthesis of creatine. This method can be used to estimate the rate of synthesis of creatine in vivo in human and animal studies.

© 2009 Elsevier Inc. All rights reserved.

Creatine plays an indispensable role in buffering and in translocation of energy in vertebrates [1]. The creatine phosphate, produced by phosphorylation of creatine in the mitochondria, shuttles energy to cytosol, where ATP is regenerated when there is an acute demand, for example, muscle contraction or neuronal action [2]. Creatine and creatine phosphate are nonenzymatically transformed to creatinine, which is excreted in urine. The dietary intake and endogenous synthesis of creatine is matched by creatinine excretion, estimated to be approximately 1.5% of the total body creatine pool per day (corresponding to 0.9–1.7 g/day for a 70-kg man) [1]. In subjects with an omnivorous diet, approximately half of the required creatine is obtained from the diet and the remainder is synthesized in the body. In vegetarians, all of the daily requirements of creatine are met by its endogenous synthesis [3].

Creatine synthesis and utilization is an interorgan process involving kidney, liver, and muscle. The first step in creatine synthesis is catalyzed by the reversible arginine:glycine amidinotransferase (AGAT,¹

EC 2.1.4.1), which transfers the amidino group from arginine to glycine, yielding ornithine and guanidinoacetate (GAA) (Fig. 1). Irreversible methylation of GAA with guanidinoacetate *N*-methyltransferase (GAMT, EC 3.5.3.2) uses *S*-adenosylmethionine (SAM) as a methyl donor and results in the production of creatine and *S*-adenosylhomocysteine (SAH) [1]. In rats, AGAT is expressed mainly in the kidney, whereas GAMT is localized in the liver [4,5]. Recently, it has been postulated that human liver may have a complete pathway of creatine synthesis [3,6,7]. Creatine produced in the liver is released into circulation and taken up by the muscle by a specific sodium- and chloride-dependent creatine transporter against a concentration gradient [8]. There is only a minor outflow of muscle creatine into the extracellular compartment, and muscle creatine is lost predominantly as creatinine [9].

Creatine plays an important role in the maintenance of skeletal muscle mass and satellite cell proliferation and differentiation [10]. Several pathological conditions associated with muscular dystrophy and neurological muscular atrophy have been related to defects in creatine synthesis and transport [1]. The mechanisms responsible for altered creatine homeostasis in human subjects with these disorders are not fully understood. The variations in muscle creatine levels could be the result of modified retention of creatine by muscle, creatine synthesis, uptake, and/or loss. Understanding regulation of creatine synthesis is important for designing effective strategies aimed to maintain creatine homeostasis.

The measurement of creatine kinetics in humans is difficult because of the large pool size, multicompartmental distribution, and

* Corresponding author. Address: Department of Pathobiology, Lerner Research Institute, NE 40, 9500 Euclid Avenue, Cleveland Clinic, Cleveland, OH 44195, USA. Fax: +1 216 636 1493.

E-mail address: sck@case.edu (S.C. Kalhan).

¹ Abbreviations used: AGAT, arginine:glycine amidinotransferase; GAA, guanidinoacetate; GAMT, guanidinoacetate *N*-methyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; GC–MS, gas chromatography–mass spectrometry; NCI, negative chemical ionization; EDTA, ethylenediaminetetraacetic acid; MPE, molar percentage enrichment; FSR, fractional synthesis rate; LOQ, lower limit of quantification; LC, liquid chromatography; TMS, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; TBDMS, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide.

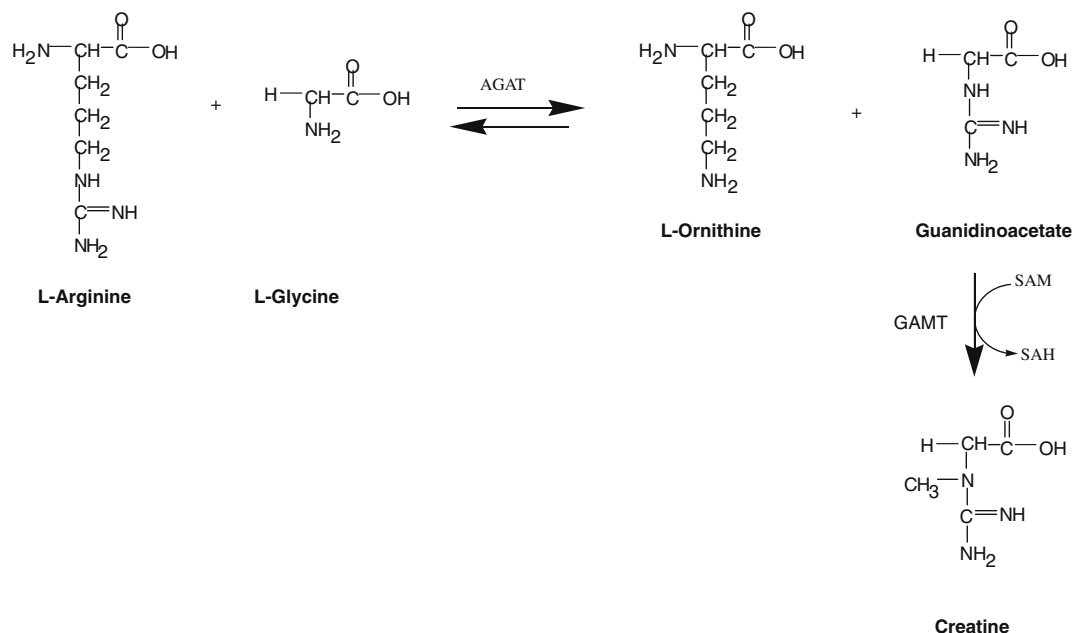


Fig. 1. Creatine biosynthesis. The first step in creatine synthesis involves the reversible transfer of the amidino group of L-arginine to L-glycine with the production of L-ornithine and guanidinoacetate via L-arginine:glycine amidinotransferase (AGAT). In the second step, guanidinoacetate is irreversibly methylated with S-adenosyl-L-methionine by S-adenosyl-L-methionine:guanidinoacetate N-methyltransferase (GAMT) with the production of S-adenosyl-L-homocysteine and creatine.

slow rate of turnover of creatine. Creatine kinetics in humans has been estimated by isotopic dilution of [^{14}C]creatine and [^{15}N]creatine [11]. After intravenous infusion of either radioactive or stable isotope-labeled creatine to humans, the half life ($t_{1/2} = 50$ days) and the fractional rate of disappearance of creatine ($kt \sim 1.5\%/day$) were determined from the disappearance curve of the labeled creatine. The long duration of the study (8–11 days) makes these techniques less favorable for routine purposes. In addition, the application of radioactive [^{14}C]creatine imposes health concerns, whereas the [^{15}N]creatine technique requires labor-intensive isolation and multistep transformation of creatine nitrogen for the isotope ratio mass spectrometric analysis. Although several methods for the measurement of concentration of creatine and its precursor GAA in biological fluids have been described [12–16], currently there is no rapid method for measurement of isotopic enrichment of GAA and creatine in biological samples.

In this study, we have developed a gas chromatography–mass spectrometry (GC–MS) technique for simultaneous measurement of isotopic enrichment and concentration of GAA and creatine in biological samples. We applied this technique for the estimation of the rate of creatine synthesis in humans. The fractional rate of creatine synthesis was determined in a healthy human subject using primed-constant rate infusion of [$1\text{-}^{13}\text{C}$]glycine and a single-pool model.

Materials and methods

Sterile, pyrogen-free [$1\text{-}^{13}\text{C}$]glycine for infusion studies and [^{15}N]glycine and [$^{13}\text{C}_2,^{15}\text{N}$]glycine for the synthesis of [^{15}N]guanidinoacetate and [$^{13}\text{C}_2,^{15}\text{N}$]guanidinoacetate were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). $N\text{-}[^2\text{H}_3]$ Methylcreatine was purchased from C/D/N Isotopes (Quebec, Canada). Hexafluoroacetylacetone was obtained from Sigma (St. Louis, MO, USA). Creatine and guanidinoacetate were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Fluka (Bucks, Switzerland).

Synthesis of [^{15}N]GAA and [$^{13}\text{C}_2,^{15}\text{N}$]GAA

[^{15}N]GAA and [$^{13}\text{C}_2,^{15}\text{N}$]GAA were prepared from [^{15}N]glycine and [$^{13}\text{C}_2,^{15}\text{N}$]glycine and cyanamide according to the method described previously [17]. Briefly, the [^{15}N]glycine or [$^{13}\text{C}_2,^{15}\text{N}$]glycine (0.1 g, 1.32 mmol) and cyanamide (1.78 mmol) were dissolved in water (2.5 ml). After adding concentrated ammonium hydroxide (65 μl), the mixture was stirred at room temperature for 3 days. The precipitated [^{15}N]GAA or [$^{13}\text{C}_2,^{15}\text{N}$]GAA was filtered and washed subsequently with ice-cold water (3×5 ml) and with acetone (3×10 ml). The total yields of products were 78 and 69% for [^{15}N]GAA and [$^{13}\text{C}_2,^{15}\text{N}$]GAA, respectively. The chemical purities of [^{15}N]GAA and [$^{13}\text{C}_2,^{15}\text{N}$]GAA were verified by GC–MS after derivatization with hexafluoroacetylacetone and acidic methanol based on the absence of interfering peaks. The isotopic enrichments of [^{15}N]GAA and [$^{13}\text{C}_2,^{15}\text{N}$]GAA were 99 and 98 atom%, respectively. [^{15}N]GAA and [$^{13}\text{C}_2,^{15}\text{N}$]GAA were used for constructing the calibration curves for m_1 enrichment and measuring the concentrations of GAA, respectively.

Analytical methods

Glycine assay

Glycine, along with other amino acids in the plasma, was separated using mixed-bed ion exchange chromatography as described previously [18]. An N -propyl- n -acetyl ester derivative of glycine was prepared according to the method of Adams [19,20]. Positive chemical ionization and selected ion monitoring were used to monitor the mass-to-charge ratio (m/z) for ions 160 and 161 representing unlabeled (m_0) glycine and ^{13}C -labeled (m_1) glycine.

Creatine and GAA assay

Sample preparation. The derivatization of creatine and GAA uses a two-step procedure involving the reaction of the guanidino group with hexafluoroacetylacetone to form a bis(trifluoromethyl)pyrimidine ring structure (adapted from Ref. [21]) followed by derivatization of the carboxyl group with acidic methanol (Fig. 2). Briefly,

saturated aqueous sodium bicarbonate (50 μ l), toluene (1 ml), and hexafluoroacetylacetone (35 μ l) were added to 200 μ l of plasma. After incubating at 80 $^{\circ}$ C for 2 h with continuous stirring, the mixture was allowed to cool down to room temperature. The upper phase was transferred to another test tube and dried completely under nitrogen gas and was used for the second step of derivatization. The carboxylic group was derivatized with 200 μ l of a methanol/acetyl chloride (10:1, v/v) mixture at 80 $^{\circ}$ C for 1 h. After drying under nitrogen gas, 0.5 ml of water was added and creatine–hexafluoroacetylacetone–methyl and GAA–hexafluoroacetylacetone–methyl derivatives were extracted with 4 ml of ethyl acetate and dried under nitrogen. The residue was dissolved in 70 μ l of ethyl acetate, and 1 μ l of this solution was injected into a GC–MS system.

GC–MS conditions. Creatine and GAA derivatives were separated on a GC–MS system (Agilent Technologies, Santa Clara, CA, USA) using a Supelco Wax-10 fused silica capillary column (30 m \times 0.25 mm \times 0.25 μ m). The injector port temperature and auxiliary temperature were 250 $^{\circ}$ C. The oven temperature ramp was set as follows: the initial oven temperature was 80 $^{\circ}$ C and was increased to 180 $^{\circ}$ C at 10 $^{\circ}$ C/min and then to 250 $^{\circ}$ C at 50 $^{\circ}$ C/min and kept at 250 $^{\circ}$ C for 7 min. Creatine and GAA derivatives were eluted at 7.5 and 11.1 min, respectively (Fig. 3, top panel). Methane was used as the carrier and the ionization gas at a flow rate of 40 ml/min. Negative chemical ionization (NCI) and selected ion monitoring were used to monitor m/z for ions 303 to 305 and ions 317 to 319 representing unlabeled (m_0) and 13 C-labeled (m_1 and m_2) GAA and creatine, respectively. For simultaneous measurement of creatine and GAA enrichment and concentration, 200 μ l of plasma was spiked with [2 H $_3$]creatine (10 nmol) and [13 C $_2$, 15 N]GAA (0.5 nmol) and analyzed as above. The m/z 317 (m^-) and 320 [$(m+3)^-$] for creatine and m/z 303 (m^-) and 306 [$(m+3)^-$] ion peak areas were integrated for calculation of GAA concentrations. Analysis of pure [2 H $_3$]creatine and [13 C $_2$, 15 N]GAA shows that these labeled compounds have undetectable quantities of unlabeled species and less than 1% residual ($m+1$) labeled species.

Validation studies

Linearity. Calibration curves for measurement of creatine and GAA concentrations were constructed with different concentrations of creatine (2–500 μ M) and GAA (0.5–250 μ M) and a constant amount of [2 H $_3$]creatine and [13 C $_2$, 15 N]GAA internal standards. The linear regression equations derived from calibration curves

were used for the calculation of creatine and GAA concentrations in plasma samples.

Accuracy and recovery. Three sets of samples were prepared for accuracy and recovery study for creatine and GAA concentrations. The first set of the samples included 200 μ l of solution of creatine and GAA in water at three different concentrations: 9, 37, and 185 μ mol/L creatine and 3, 12.5, and 25 μ mol/L GAA. The second set of samples consisted of duplicates of 200 μ l plasma samples. For the third set, samples with the three different concentrations (same as in set 1) of creatine and GAA were each spiked with 200 μ l of plasma. All eight samples were spiked with [2 H $_3$]creatine (10 nmol) and [13 C $_2$, 15 N]GAA (1.2 nmol) internal standards and analyzed. All plasma samples were aliquoted from the same pooled plasma. After quantification of creatine and GAA in all samples, the recovery of the assay was calculated as the ratio of analyte (creatine or GAA) concentrations in spiked plasma to the sum of nonspiked plasma and pure standards.

The precision (i.e., intra- and interassay reproducibility) of the method for creatine and GAA concentrations was determined by multiple analyses of plasma samples from pooled plasma. Intraassay variability was determined by analyzing one sample five times. Interassay variability was established by processing the same sample in six different preparations on different days over 2 weeks.

Methods for the analysis of creatine and GAA concentrations were compared with a previously described GC–MS technique [15]. Three different plasma samples in duplicate were aliquoted and spiked with [2 H $_3$]creatine (10 nmol) and [13 C $_2$, 15 N]GAA (1.2 nmol) internal standards. One set of samples was converted to hexafluoroacetylacetone–methyl derivative and analyzed as described above. The second set of samples was converted to hexafluoroacetylacetone–pentafluorobenzyl derivative and analyzed as described previously [15].

Human study

We investigated GAA and creatine kinetics in a healthy adult who participated in a study on the effects of an intravenous infusion of Intralipid with heparin on [1 - 13 C]glycine metabolism in humans. The study examined the kinetics of glycine in the basal state and in response to infusion of Intralipid (manuscript submitted for publication). The study protocol was approved by the institutional review board of the Cleveland Clinic (Cleveland, OH, USA). The study subject was placed on a weight maintenance diet containing

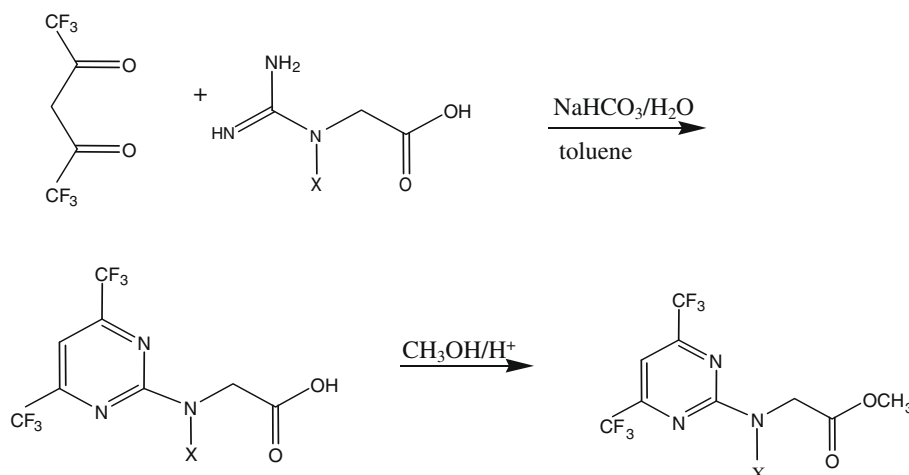


Fig. 2. Preparation of bis(trifluoromethyl)pyrimidine methyl derivatives of guanidinoacetate (X = H) and creatine (X = CH₃). The guanidino group of creatine and GAA was derivatized with hexafluoroacetylacetone, and the carboxyl groups were esterified with acidic methanol.

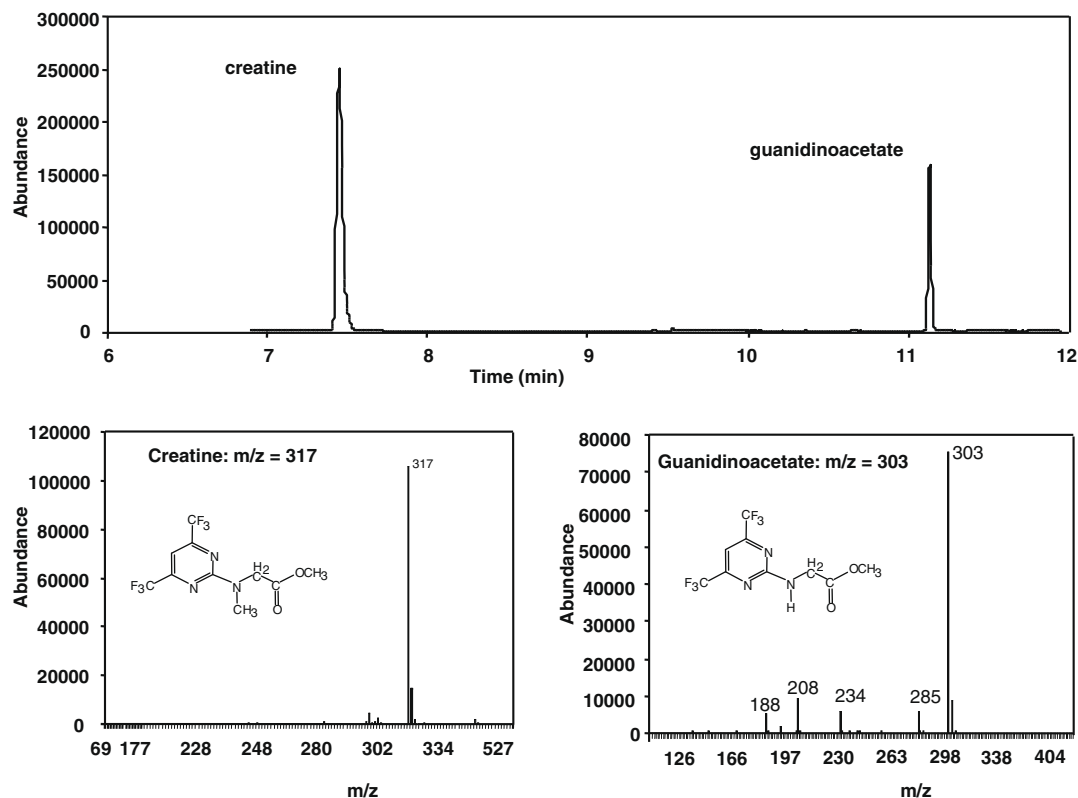


Fig. 3. Top panel: GC–MS chromatograms of creatine and GAA standards. Bottom panels: Creatine and GAA mass spectra and structures of corresponding derivatives.

at least 70 g protein/day for 7 days prior to the tracer study. Following a 12-h overnight fast, two indwelling cannulas were placed in the dorsal vein of each hand: one for tracer infusion and the other to obtain blood samples. To obtain arterialized blood, the sampling site was kept warm by placing the hand in a thermostat-controlled warm blanket. After a priming dose of [1- ^{13}C]glycine (16 $\mu\text{mol kg}^{-1}$), a sterile solution of [1- ^{13}C]glycine in 0.45% saline was infused at a constant rate (16 $\mu\text{mol kg}^{-1} \text{h}^{-1}$). Following the basal study (4 h), triglyceride solution (20%, Intralipid) with heparin (0.2 U kg^{-1}) was infused at 40 ml h^{-1} . Blood samples (1 ml) in ethylenediaminetetraacetic acid (EDTA)-coated tubes were obtained at time zero, prior to tracer infusion, and at 20- to 30-min intervals during the tracer infusion.

Calculations

GAA and creatine concentrations were determined from the peak area ratios 303/306 and 317/320 for GAA and creatine, respectively. Molar percentage enrichments (MPEs) of glycine, GAA, and creatine were determined as molar fraction of m_1 isotope over m_0 , m_1 , and m_2 species. The measured mass isotopomer distributions were corrected for natural enrichments.

Because GAA is the immediate precursor of creatine, we used GAA enrichment at steady-state for the calculation of the fractional synthesis rate (FSR) of creatine synthesis. The fractional rate of creatine synthesis was calculated as follows, assuming that creatine synthesis has the zero-order kinetics:

$$\text{FSR} (\text{day}^{-1}) = (\text{slope of creatine labeling})/E_{\text{GAA}} * 60 * 24,$$

where the slope is the rate of increase in the ^{13}C enrichment (m_1) of plasma creatine during [1- ^{13}C]glycine infusion, E_{GAA} is the steady-state ^{13}C enrichment of GAA, and the factors 60 and 24 convert FSR (min^{-1}) to FSR (day^{-1}).

Data were presented as means \pm standard deviations.

Results

Creatine and GAA were derivatized using a two-step procedure involving reaction of hexafluoroacetylacetone with the guanidino group followed by esterification of the carboxyl group with acidic methanol (Fig. 2). The GC–MS chromatograms of creatine and GAA are presented in Fig. 3 (top panel). Because of the lower polarity of the tertiary amino group in creatine compared with the secondary amino group in GAA, the higher molecular weight creatine derivative eluted ahead of the GAA derivative on the GC column. As shown (Fig. 3, bottom panel), in NCI mode the mass spectrum of bis(trifluoromethyl)pyrimidine methyl ester derivatives of GAA and creatine yield only a few fragment ions with low intensity. GAA and creatine are characterized by molecular ions m/z 303 and 317, respectively. These ions are derived from intact GAA and creatine derivatives after gaining one electron in NCI mode. GAA and creatine peaks were free of interfering peaks on chromatograms. The measured mass isotopomer distribution pattern of GAA ($m_0 = 88.97\%$, $m_1 = 10.05\%$, and $m_2 = 0.93\%$) and of creatine derivatives ($m_0 = 88.09\%$, $m_1 = 12.06\%$, and $m_2 = 1.03\%$) were in agreement with the theoretical values. The corresponding labeled analogs [^{15}N]GAA, [$^{13}\text{C}_2,^{15}\text{N}$]GAA, and [$^2\text{H}_3$]creatine yield ions with m/z 304, 306, and 320, respectively. As shown in Fig. 4A, because of isotopic fractionation of ^2H -labeled compounds, [$^2\text{H}_3$]creatine derivative (m/z 320) eluted ahead of m_0 creatine (m/z 317) on the GC column. In contrast, [$^{13}\text{C}_2,^{15}\text{N}$]GAA and unlabeled GAA (m/z 306 and 303, respectively) did not separate on the GC column (Fig. 4B).

Assay validation

Fig. 5A and B show the standard curves of [$^2\text{H}_3$]creatine and [^{15}N]GAA molar enrichments, respectively. The standard mixtures were prepared by adding increasing amounts of [$^2\text{H}_3$]creatine

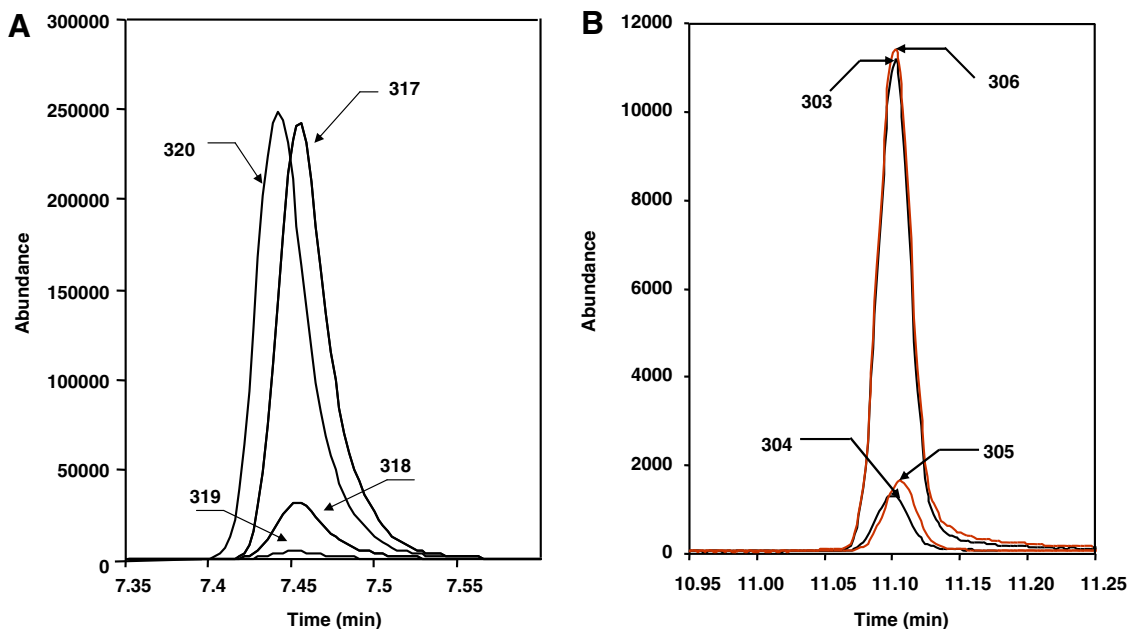


Fig. 4. Ion chromatograms of creatine (A) and GAA (B) assayed as bis(trifluoromethyl)pyrimidine methyl derivatives. The analyses were performed on human plasma (200 μ l) spiked with [$^2\text{H}_3$]creatinine (10 nmol) and [$^{13}\text{C}_2, ^{15}\text{N}$]GAA (0.5 nmol). The deuterated standard of [$^2\text{H}_3$]creatinine eluted ahead of the unlabeled and ^{13}C -labeled isotopomers, whereas $^{13}\text{C}_2, ^{15}\text{N}$ -labeled GAA molecules elute together with the unlabeled and ^{13}C -labeled species.

and [^{15}N]GAA to a constant (10 nmol) amount of unlabeled creatine and GAA. Both calibration curves were linear in the 0.3 to 9% enrichment range. We limited the calibration curve to the maximum of anticipated tracer enrichment in the in vivo study. The correlation coefficients of isotopic enrichments were close to unity (0.99 and 1.04 for creatine and GAA, respectively).

Fig. 6A and B show the calibration curves for creatine and GAA concentrations, respectively, made by adding increasing amounts of unlabeled creatine and GAA to constant amounts of [$^2\text{H}_3$]creatinine (10 nmol) and [$^{13}\text{C}_2, ^{15}\text{N}$]GAA (5 nmol). The curves were linear over ranges of 2 to 500 μM and 0.5 to 250 μM for creatine and GAA, respectively. The lower limits of quantification (LOQs) with a signal/noise ratio greater than 10:1 were 0.25 and 1 μM for GAA and creatine, respectively. These ranges cover the GAA and creatine concentrations found in human plasma and urine.

To test the effect of plasma matrix on creatine and GAA assay, we analyzed 100, 200, and 300 μ l of pooled plasma samples spiked with the same quantity of [^{15}N]GAA (5 nmol) and [$^2\text{H}_3$]creatinine (10 nmol) internal standards and calculated the concentrations of GAA and creatine in each sample. These three assays with different volumes of plasma yielded similar results with coefficients of variation less than 5%.

Fig. 7A and B present the results of the accuracy study for the assays of creatine and GAA concentrations, respectively. Plotting of nanomoles of creatine and GAA determined in plasma against creatine and GAA added to plasma resulted in lines that were linear with slopes and regression coefficients close to 1. The y intercepts correspond to plasma concentrations of creatine (8.0 nmol/0.2 ml plasma or 40.0 μM) and GAA (0.76 nmol/0.2 ml plasma or 3.8 μM). Based on these analyses, the calculated percentage recov-

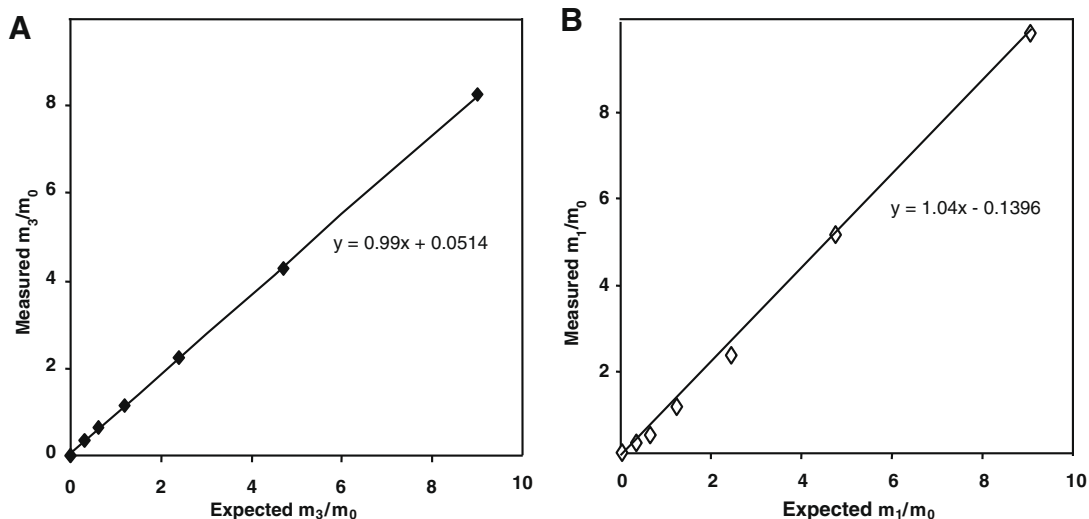


Fig. 5. Calibration curves of m_3 enrichment of creatine (A) and m_1 enrichment of GAA (B). The constant amounts of m_0 creatine and GAA were spiked with increasing quantities of [$^2\text{H}_3$]creatinine and [^{15}N]GAA, respectively.

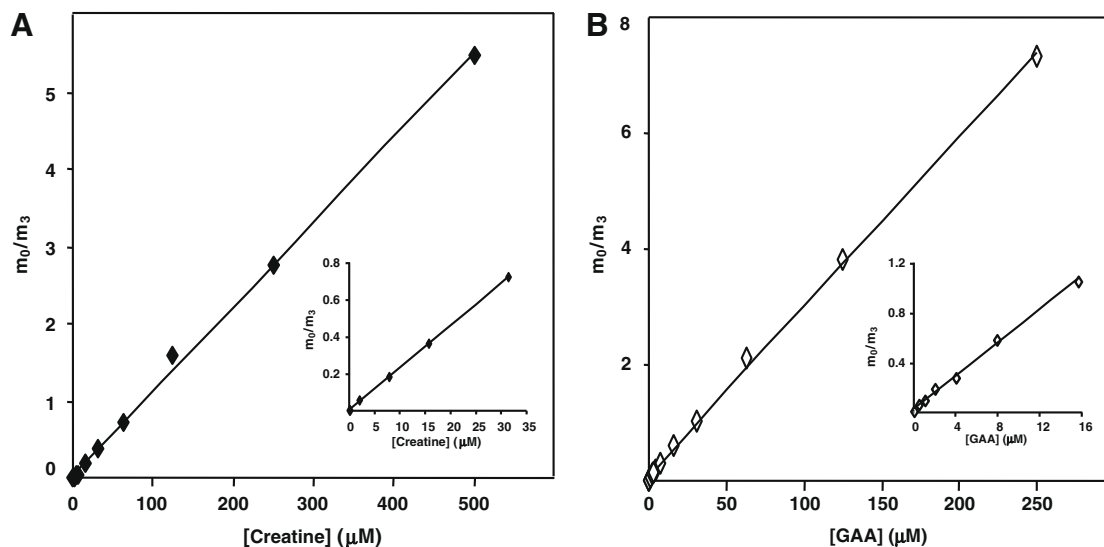


Fig. 6. Calibration curves of creatine (A) and GAA (B) concentrations. The increasing quantities of unlabeled creatine and GAA were spiked with the constant amounts of $[^2\text{H}_3]$ creatine and $[^{13}\text{C}_2, ^{15}\text{N}]$ GAA, respectively. The insets magnify the lower range parts of the calibration curves.

eries of creatine and GAA from plasma were 92 to 96% and 89 to 109%, respectively. Application of stable isotope-labeled internal standards compensates for any loss of analytes during derivatization and extraction steps and permits considering the recovery results as the total recovery during the assay conditions. Intra- and interassay coefficients of variation for the assay were 1.2 and 3.9% for creatine and 2.7 and 4.4% for GAA, respectively (Table 1).

The verification study of the assay of concentrations of creatine and GAA is presented in Table 2. The analysis of plasma samples from three different sources reveals that two independent assays of creatine and GAA concentrations are similar.

Fig. 8 shows the time profile of m_1 enrichment of glycine and GAA in a healthy adult who was infused with $[1-^{13}\text{C}]$ glycine. A near steady-state tracer enrichment of GAA was reached by 30 min. The enrichment of GAA was approximately 30% of glycine. Infusion of Intralipid did not have a significant impact on glycine and GAA enrichment.

There was no change in GAA and creatine concentrations during the glycine infusion study. The concentration of GAA in plasma of

Table 1

Intra- and interassay variability for creatine and GAA analysis.

	Creatine ($\mu\text{mol/L}$)	CV (%)	GAA ($\mu\text{mol/L}$)	CV (%)
Intraassay ($n = 5$)	21.05 ± 0.24	1.2	2.63 ± 0.07	2.7
Interassay ($n = 6$)	19.84 ± 0.78	3.9	2.50 ± 0.11	4.4

Note. CV, coefficient of variation.

this subject ($1.7 \pm 0.12 \mu\text{M}$) was similar to values reported using other techniques [14,15]. The plasma concentration of creatine of this subject ($25.7 \pm 2.1 \mu\text{M}$) was less than that reported in healthy adults in the literature ($46 \pm 2 \mu\text{M}$) [15]. However, the plasma concentrations of creatine of two other healthy adults (59.4 ± 2.2 and $63.4 \pm 0.81 \mu\text{M}$) determined by our technique were similar to values reported in the literature. In addition, our verification study demonstrated that two independent assays of creatine and GAA concentrations using this technique and previously established methods yielded similar results.

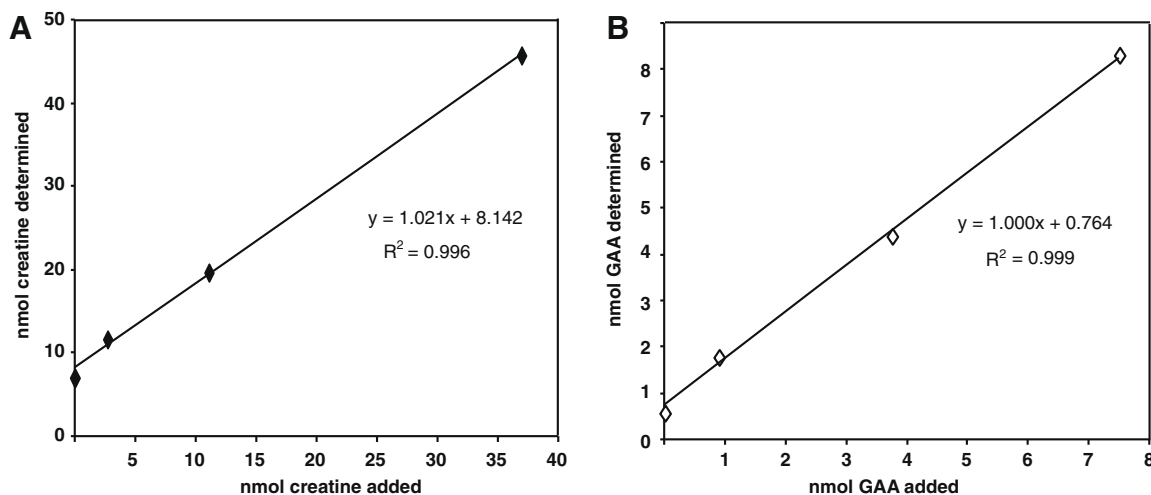


Fig. 7. Accuracy study for the creatine and GAA concentration analysis. Known amounts of creatine (0, 2.7, 11, and 37 nmol) and GAA (0, 0.9, 3.75, and 7.5 nmol) were added to pooled plasma samples (200 μl each), and the amounts of creatine and GAA were determined. (A) Plot of nanomoles of creatine (endogenous + added creatine) versus nanomoles of creatine added. (B) Plot of nanomoles of GAA (endogenous + added GAA) versus nanomoles of GAA added. Chromatographic conditions and quantification procedures are described in the text.

Table 2
Verification of creatine and GAA concentration analysis.

Sample	Hexafluoroacetylacetone-methyl		Hexafluoroacetylacetone-pentafluorobenzyl	
	[Creatine] ($\mu\text{mol/L}$)	[GAA] ($\mu\text{mol/L}$)	[Creatine] ($\mu\text{mol/L}$)	[GAA] ($\mu\text{mol/L}$)
1	36.1	4.5	33.5	3.8
2	22.2	2.2	20.9	2.7
3	25.3	2.3	23.3	2.4

Note. Three different plasma samples were analyzed by GC–MS using hexafluoroacetylacetone-methyl (current study) and hexafluoroacetylacetone-pentafluorobenzyl [15] derivatives. Data are shown as the averages of four replicate injections.

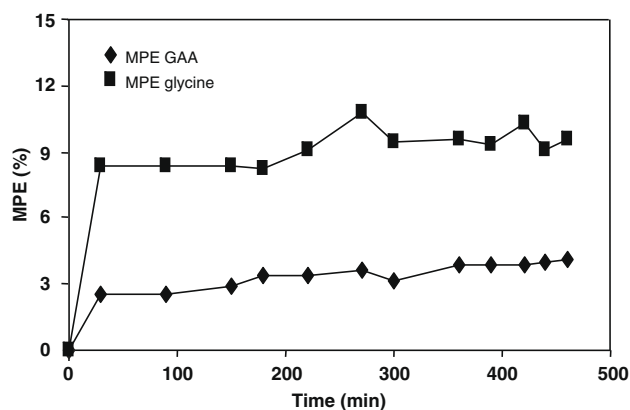


Fig. 8. m_1 MPE of glycine and GAA in plasma of a human subject. $[1-^{13}\text{C}]$ glycine in 0.45% saline was administered as a prime-constant infusion for 8 h. Steady-state enrichment was apparent in both glycine and GAA pools within 30 min.

Fig. 9 shows the m_1 enrichment of creatine in the subject infused with $[1-^{13}\text{C}]$ glycine. The incorporation of $[^{13}\text{C}]$ glycine into creatine increased linearly throughout the tracer infusion. The slope of creatine labeling (0.00097) was used for calculation of the fractional rate of synthesis of creatine. Using the precursor–product relationship and using GAA as the immediate precursor, the FSR of plasma creatine was estimated to be 37%/day (Table 3).

Discussion

In this article, we have presented a new technique for simultaneous measurement of creatine and GAA enrichment and concentration in human plasma. The use of labeled ($m+3$) internal standards, $[^2\text{H}_3]$ creatine, and $[^{13}\text{C}_2,^{15}\text{N}]$ GAA allowed accurate quantification of concentrations of creatine and GAA in the plasma.

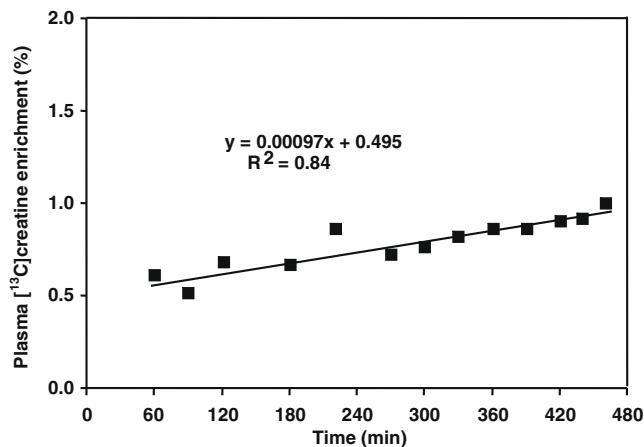


Fig. 9. m_1 MPE of creatine from the same experiment as in Fig. 8.

The simultaneous measurement of GAA, creatine enrichment, and concentrations in this study can be used to measure the rate of creatine synthesis in humans.

The low-micromolar concentrations of creatine and GAA in plasma combined with the slow turnover rate of creatine in humans dictate that a sensitive method for measurement of the concentration of creatine and GAA and their enrichments is needed. Several liquid chromatography (LC)–MS [14,22,23] and GC–MS [24,25] methods for the analysis of creatine and GAA concentrations in biological fluids, including plasma, have been described. Although LC–MS assays are more sensitive and do not require derivatization, a GC–MS instrument is easily available and widely used for the stable isotopic tracer enrichments. Creatine and GAA have the polar guanidino and carboxyl groups; therefore, their GC–MS analysis requires converting them to nonpolar groups to improve their volatility. Derivatization procedures that have been used include reaction of these compounds with hexafluoroacetylacetone followed by either pentafluorobenzylbromide [15,26] or alkyl silicone compounds. The use of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (TMS) [16,25] and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (TBDMS) [12] for derivatization of the carboxyl group increases the baseline natural abundance and, therefore, cannot be used for measurement of low isotopic enrichment of creatine. Originally, we used bis(trifluoromethyl)pyrimidine pentafluorobenzyl derivative for the assay of both guanidine compounds in NCI mode. It has been shown that this derivative allows measuring low concentrations of creatine and GAA in plasma [15,26]. However, the analysis of natural enrichment of creatine and GAA standards revealed that m_1 enrichment was higher than the theoretical values, indicating the interference of unknown ions with the analyte ions. A similar effect was observed when $[^2\text{H}_3]$ creatine and $[^{15}\text{N}]$ GAA were analyzed. Because of the similar observations with $[^2\text{H}_3]$ creatine and $[^{15}\text{N}]$ GAA analysis, we assumed that contamination of the m_1 ion could be related to the ionization condition of the mass spectrometer source. Manipulation of source temperature and ionization potential did not resolve the problem. Creatine also has been derivatized with trifluoroacetic anhydride; however, this derivative does not differentiate creatine from creatinine and, therefore, requires prior separation of these two related compounds [24].

In this study, we elected to prepare bis(trifluoromethyl)pyrimidine methyl ester derivative for the following reasons: (i) the methyl group adds only one carbon and, therefore, has a negligible

Table 3

Concentrations and stable isotope enrichments of glycine, GAA, and creatine and creatine FSR in a healthy adult after overnight fasting.

$[1-^{13}\text{C}]$ glycine infusion rate ($\mu\text{mol kg}^{-1} \text{h}^{-1}$)	15.48
$[1-^{13}\text{C}]$ glycine plateau MPE (%)	9.6 ± 0.51
$[1-^{13}\text{C}]$ GAA plateau MPE (%)	3.7 ± 0.3
Slope of creatine labeling	0.00097
Glycine ($\mu\text{mol L}^{-1}$)	385
GAA ($\mu\text{mol L}^{-1}$)	1.7
Creatine ($\mu\text{mol L}^{-1}$)	25
Creatine FSR (%/day)	37

effect on baseline natural enrichment of the native compound; (ii) fluorinated derivatives increase analytes' electron affinity and improve their detection in sensitive NCI mode.

Derivatization of the guanidino group with hexafluoroacetylacetone was adapted from previous studies [15,21]. By examining various parameters (pH, t° , reaction time, and ratio of aqueous volume to organic volume), it was determined that derivatization is complete in 2 h at 80 °C at pH 9.0 with the aqueous/organic ratio below 0.4 and 50 μ l of hexafluoroacetylacetone. We found that if the plasma was buffered with freshly prepared saturated NaHCO₃ to pH 9.0, there is minimal hydrolysis of hexafluoroacetylacetone and, therefore, 35 μ l of this reagent is sufficient for derivatization. The second step, methylation of the carboxyl group with a methanol/acetyl chloride (10:1, v/v) mixture, is a standard procedure for the derivatization of organic acids. In both derivatization steps, the major concern is evaporation of organic solvents; therefore, extra care needs to be taken to avoid such a problem. We did not determine the yield of the derivatization reactions. However, because the endogenous metabolites (creatinine and GAA) were derivatized simultaneously with the stable isotope-labeled internal standards (²H₃creatinine and [¹³C₂,¹⁵N]GAA), the yield of derivatization reactions did not affect the calculated concentrations of GAA and creatinine. The validation of this technique for the measurement of creatinine and GAA concentrations demonstrates that it is linear, accurate, precise, and free of any appreciable plasma matrix effect. Although the sensitivity (LOQ) of this method is lower than the values described previously for GC–MS [15] and LC–MS [22,23] methods, this technique allows the simultaneous measurement of creatinine and GAA concentrations found in human plasma with good accuracy and precision. This technique was verified by the measurement of creatinine and GAA concentrations using the previously described GC–MS technique [15].

This technique was used for investigation of creatinine metabolism in humans. In humans infused with [1-¹³C]glycine, m_1 enrichment of glycine plateaued at approximately 9.6%, whereas m_1 GAA was approximately 3.7%. The synthesis of GAA is catalyzed by reversible AGAT reaction with an apparent equilibrium constant $K' = 1$ at pH 7.5 and 37 °C [1]. This suggests that in physiological conditions the intracellular glycine pool equilibrates rapidly with GAA so that the isotopic labeling will become similar.

Using GAA as the immediate precursor pool, we calculated the FSR of plasma creatinine to be 37%/day (Table 3). This is the first study to estimate the rate of creatinine synthesis in humans using a steady-state glycine tracer infusion. Previously, the creatinine turnover in humans was determined based on the creatinine excretion rate. The rate of creatinine turnover in these studies (~1.5%/day or ~1.5 g/day) reflects the turnover rate of the total body creatinine pool, predominantly in the muscle (~95% of total creatinine pool). Creatinine in plasma and in most tissues has a much higher turnover rate than creatinine in skeletal muscle [27]. Creatinine in plasma rapidly equilibrates with the nonmuscle creatinine pool; it does not appear to equilibrate rapidly with the large pool of creatinine in skeletal muscle [28,29]. This is in agreement with our estimate of creatinine kinetics calculated based on the miscible extracellular muscle pool (~5 g in a 70-kg adult or 5% of total creatinine pool). Based on the extracellular muscle creatinine pool, the rate of creatinine synthesis estimated in this study is 1.85 g/day (37% of 5 g), similar to values reported in the literature (~1.5 g/day) [27]. Therefore, we conclude that the calculated FSR of plasma creatinine in the current study represents the kinetics of the creatinine pool in non-muscle tissues and in plasma.

In conclusion, we have developed and validated an isotopic dilution assay for simultaneous measurement of isotopic enrichment and concentration of GAA and creatinine in plasma. This technique could be used to study creatinine synthesis and its relation to diseases associated with altered metabolism of creatinine.

Acknowledgments

This work was supported in part by the National Institutes of Health, the National Center for Research Resources, Clinical and Translational Science Awards (CTSA, 1UL1RR024989, Cleveland, OH), and the Cleveland Clinic Foundation. We thank the General Clinical Research Center (GCRC) staff for their help with the studies and Joyce Nolan for secretarial assistance. The study was supported by Cleveland Clinic Foundation (CCF) institutional support (to S.C.K.).

References

- [1] M. Wyss, R. Kaddurah-Daouk, Creatine and creatinine metabolism, *Physiol. Rev.* 80 (2000) 1107–1213.
- [2] M. Wyss, O. Braissant, I. Pischel, G.S. Salomons, A. Schulze, S. Stockler, T. Wallimann, Creatine and creatine kinase in health and disease: a bright future ahead?, *Subcell Biochem.* 46 (2007) 309–334.
- [3] J.T. Brosnan, M.E. Brosnan, Creatine: endogenous metabolite, dietary, and therapeutic supplement, *Annu. Rev. Nutr.* 27 (2007) 241–261.
- [4] J.F. Van Pilsom, G.C. Stephens, D. Taylor, Distribution of creatine, guanidinoacetate, and enzymes for their biosynthesis in the animal kingdom: implications for phylogeny, *Biochem. J.* 126 (1972) 325–345.
- [5] R.P. da Silva, I. Nissim, M.E. Brosnan, J.T. Brosnan, Creatine synthesis: hepatic metabolism of guanidinoacetate and creatine in the rat in vitro and in vivo, *Am. J. Physiol. Endocrinol. Metab.* 296 (2009) E256–E261.
- [6] E.E. Edison, M.E. Brosnan, C. Meyer, J.T. Brosnan, Creatine synthesis: production of guanidinoacetate by the rat and human kidney in vivo, *Am. J. Physiol. Renal Physiol.* 293 (2007) F1799–F1804.
- [7] A.A. Sandberg, H.H. Hecht, F.H. Tyler, Studies in disorders of muscle: X. The site of creatine synthesis in the human, *Metabolism* 2 (1953) 22–29.
- [8] R.J. Snow, R.M. Murphy, Creatine and the creatine transporter: a review, *Mol. Cell. Biochem.* 224 (2001) 169–181.
- [9] C.D. Fitch, R.P. Shields, Creatine metabolism in skeletal muscle: I. Creatine movement across muscle membranes, *J. Biol. Chem.* 241 (1966) 3611–3614.
- [10] S. Olsen, P. Aagaard, F. Kadi, G. Tufekovic, J. Verney, J.L. Olesen, C. Suetta, M. Kjaer, Creatine supplementation augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training, *J. Physiol.* 573 (2006) 525–534.
- [11] D. Rittenberg, The application of isotope technique to problems of biology and medicine, *J. Mt. Sinai Hosp. NY* 14 (1948) 891–907.
- [12] R. Fingerhut, Stable isotope dilution method for the determination of guanidinoacetic acid by gas chromatography/mass spectrometry, *Rapid Commun. Mass Spectrom.* 17 (2003) 717–722.
- [13] C. Carducci, M. Birarelli, V. Leuzzi, C. Carducci, R. Battini, G. Cioni, I. Antonozzi, Guanidinoacetate and creatine plus creatinine assessment in physiologic fluids: an effective diagnostic tool for the biochemical diagnosis of arginine:glycine amidinotransferase and guanidinoacetate methyltransferase deficiencies, *Clin. Chem.* 48 (2002) 1772–1778.
- [14] A. Arias, A. Ormazabal, J. Moreno, B. González, M.A. Vilaseca, J. Garcáa-Villoria, T. Pámpols, P. Briones, R. Artuch, A. Ribes, Methods for the diagnosis of creatine deficiency syndromes: a comparative study, *J. Neurosci. Methods* 156 (2006) 305–309.
- [15] L.S. Almeida, N.M. Verhoeven, B. Roos, C. Valongo, M.L. Cardoso, L. Vilarinho, G.S. Salomons, C. Jakobs, Creatine and guanidinoacetate: diagnostic markers for inborn errors in creatine biosynthesis and transport, *Mol. Genet. Metab.* 82 (2004) 214–219.
- [16] C. Valongo, M.L. Cardoso, P. Domingues, L. Almeida, N. Verhoeven, G. Salomons, C. Jakobs, L. Vilarinho, Age related reference values for urine creatine and guanidinoacetic acid concentration in children and adolescents by gas chromatography–mass spectrometry, *Clin. Chim. Acta* 348 (2004) 155–161.
- [17] M.M. Daly, Guanidinoacetate methyltransferase activity in tissues and cultured cells, *Arch. Biochem. Biophys.* 236 (1985) 576–584.
- [18] S.C. Kalhan, K.Q. Rossi, L.L. Gruca, D.M. Super, S.M. Savin, Relation between transamination of branched-chain amino acids and urea synthesis: evidence from human pregnancy, *Am. J. Physiol.* 275 (1998) E423–E431.
- [19] R.F. Adams, Determination of amino acid profiles in biological samples by gas chromatography, *J. Chromatogr.* 95 (1974) 189–212.
- [20] S.C. Kalhan, L.L. Gruca, P.S. Parimi, A. O'Brien, L. Dierker, E. Burkett, Serine metabolism in human pregnancy, *Am. J. Physiol. Endocrinol. Metab.* 284 (2003) E733–E740.
- [21] P. Erdtmansky, T.J. Goehl, Gas–liquid chromatographic electron capture determination of some monosubstituted guanido-containing drugs, *Anal. Chem.* 47 (1975) 750–752.
- [22] C. Carducci, S. Santagata, V. Leuzzi, C. Carducci, C. Artioli, T. Giovannello, R. Battini, I. Antonozzi, Quantitative determination of guanidinoacetate and creatine in dried blood spot by flow injection analysis – electrospray tandem mass spectrometry, *Clin. Chim. Acta* 364 (2006) 180–187.
- [23] O.A. Bodamer, S.M. Bloesch, A.R. Gregg, S. Stockler-Ipsiroglu, W.E. O'Brien, Analysis of guanidinoacetate and creatine by isotope dilution electrospray tandem mass spectrometry, *Clin. Chim. Acta* 308 (2001) 173–178.

- [24] I. Nissim, M. Yudkoff, T. Terwilliger, S. Segal, Rapid determination of [guanidine-¹⁵N]arginine in plasma with gas chromatography-mass spectrometry: application to human metabolic studies, *Anal. Biochem.* 131 (1983) 75–82.
- [25] D.H. Hunneman, F. Hanefeld, GC-MS determination of guanidinoacetate in urine and plasma, *J. Inherit. Metab. Dis.* 20 (1997) 450–452.
- [26] E.A. Struys, E.E. Jansen, H.J. ten Brink, N.M. Verhoeven, M.S. van der Knaap, C. Jakobs, An accurate stable isotope dilution gas chromatographic-mass spectrometric approach to the diagnosis of guanidinoacetate methyltransferase deficiency, *J. Pharm. Biomed. Anal.* 18 (1998) 659–665.
- [27] C.D. Fitch, D.W. Sinton, A study of creatine metabolism in diseases causing muscle wasting, *J. Clin. Invest.* 43 (1964) 444–452.
- [28] C.D. Fitch, D.D. Lucy, J.H. Bornhofen, G.V. Dalrymple, Creatine metabolism in skeletal muscle: II. Creatine kinetics in man, *Neurology* 18 (1968) 32–42.
- [29] C.D. Fitch, S. Maben, Intestinal loss of creatine by normal and vitamin E-deficient rabbits, *Am. J. Physiol.* 207 (1964) 627–630.