Published in final edited form as:

Anal Biochem. 2009 December 1; 395(1): 91–99. doi:10.1016/j.ab.2009.07.037.

Simultaneous Assay of Isotopic Enrichment and Concentration of Guanidinoacetate and Creatine by Gas Chromatography-Mass Spectrometry

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Abstract

A gas chromatographic-mass spectrometric (GC-MS) method for the simultaneous measurement of isotopic enrichment and concentration of guanidinoacetic acid and creatine in plasma sample for kinetic studies is reported. The method, based on preparation of the bis(trifluoromethyl)-pyrimidine methyl ester derivatives of guanidinoacetic acid and creatine, is robust and sensitive. The lowest measurable m_1 and m_3 enrichment for guanidinoacetic acid and creatine, respectively, was 0.3%. The calibration curves for measurements of concentration were linear over a range of 0.5–250 μM guanidinoacetic acid and 2–500 μM for creatine. The method was reliable for inter-assay and intra-assay precision, accuracy and linearity. The technique was applied in a healthy adult to determine in vivo fractional synthesis rate of creatine using primed- constant rate infusion of $[1-^{13}\text{C}]$ glycine. It was found that isotopic enrichment of guanidinoacetic acid reached plateau by 30 min of infusion of $[1-^{13}\text{C}]$ glycine, indicating either a small pool size or a rapid turnover rate or both, of guanidinoacetic acid. In contrast, 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 rate of synthesis of creatine in-vivo in human and animal studies.

Keywords

creatine; guanidinoacetate; guanidinoacetate N-methyltransferase; muscle wasting; creatine kinetics

Introduction

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

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Mass isotopomers are designated as m_n , where n is the number of atomic mass units above molecular weight of the unlabeled isotopomer m.

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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 ~1.5 % of the total body creatine pool per day (corresponding to 0.9-1.7 g per day for 70 kg man) [1]. In subjects with omnivorous diet approximately half of the required creatine is obtained from the diet and remaining synthesized in the body. In vegetarians, all the daily requirements of creatine are met by its endogenous synthesis [3].

Creatine synthesis and utilization is an inter-organ process involving kidney, liver and muscle. The first step in creatine synthesis is catalyzed by the reversible arginine: glycine amidinotransferase (AGAT, EC 2.1.4.1) which transfers amidino group from arginine to glycine yielding ornithine and guanidinoacetic acid (GAA) (Fig. 1). Irreversible methylation of GAA with guanidinoacetate N-methyltransferase (GAMT, EC 3.5.3.2) utilizes S-adenosylmethionine (SAM) as a methyl donor and results in the production of creatine and S-adenosylhomocysteine (SAH) [1]. In rats, AGAT is mainly expressed in the kidney, while 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 [9]. There is only a minor outflow of muscle creatine into the extracellular compartment and muscle creatine is lost predominantly as creatinine [10].

Creatine plays an important role in the maintenance of skeletal muscle mass and satellite cell proliferation and differentiation [11]. Several pathological conditions associated with the 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 human is difficult because of the large pool size, multicompartamental distribution and the slow rate of turnover of creatine. Creatine kinetics in human have been estimated by isotopic dilution of [^{14}C]- and [^{15}N]creatine [13]. After intravenous infusion of either radioactive or stable isotopic labeled creatine to human, the half life ($t_{1/2} = 50$ days) and the fractional rate of disappearance of creatine ($k_t = \sim 1.5\%/ \text{day}$) were determined from the disappearance curve of the labeled creatine. The long duration of the study (8 to 11 days) makes these techniques less favorable for routine purpose. In addition, application of radioactive [^{14}C]creatine imposes health concerns, while [^{15}N]creatine technique requires labor intensive isolation and multi-step 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 [14-18], 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 GC-MS technique for simultaneous measurement of isotopic enrichment and concentration of GAA and creatine in biological samples. We have applied this technique for the estimation of the rate of creatine synthesis in human. 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.

Material and Methods

Sterile, pyrogen-free [$1\text{-}^{13}\text{C}$]glycine for infusion studies, [^{15}N]glycine and [$^{13}\text{C}_2$, ^{15}N]glycine for the synthesis of [^{15}N]- and [$^{13}\text{C}_2$, ^{15}N]guanidinoacetate were obtained from Cambridge Isotope laboratory (Andover, MA). N-[$^2\text{H}_3$]methylcreatine was purchased from C/D/N ISOTOPES (Quebec, CA). Hexafluoroacetone was obtained from Sigma (St. Luis, MO). Creatine and guanidinoacetate were purchased from Aldrich (Milwaukee, MO). All other chemicals were obtained from Fluka.

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

[^{15}N]GAA and [$^{13}\text{C}_2$, ^{15}N]GAA were prepared from [^{15}N]glycine and [$^{13}\text{C}_2$, ^{15}N]glycine and cyanamide according to described method [19]. Briefly, the [^{15}N]- or [$^{13}\text{C}_2$, ^{15}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]- or [$^{13}\text{C}_2$, ^{15}N]GAA was filtered and washed subsequently with ice cold water (3 $\times$ 5 ml) and with acetone (3 $\times$ 10 ml). The total yield of products were 78% and 69% for [^{15}N]GAA and [$^{13}\text{C}_2$, ^{15}N]GAA, respectively. The chemical purity of [^{15}N]GAA and [$^{13}\text{C}_2$, ^{15}N]GAA were verified by GC-MS after derivatization with hexafluoroacetylacetone and acidic methanol, based on absence of interfering peaks. The isotopic enrichment of [^{15}N]GAA and [$^{13}\text{C}_2$, ^{15}N]GAA were 99 and 98 atom %, respectively. [^{15}N]GAA and [$^{13}\text{C}_2$, ^{15}N]GAA were used for constructing the calibration curves for m_1 enrichment and for measurement of concentration 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 [20]. An N-propyl-n-acetyl ester derivative of glycine was prepared according to the method of Adams [21,22]. Positive chemical ionization and selected ion monitoring were used to monitor mass-to-charge ratio (m/z) for ions 160 and 161 representing unlabeled (m_0) and ^{13}C labeled (m_1) glycine.

Creatine and GA assay

Sample preparation: The derivatization of creatine and GAA utilizes two step procedure involving the reaction of guanidino group with hexafluoroacetylacetone to form a bis(trifluoromethyl)pyrimidine ring structure (adapted from [23]) followed by derivatization of the carboxyl group with acidic methanol (Fig. 2). Briefly, saturated aqueous sodium bicarbonate (50 μl), toluene (1 ml) and hexafluoroacetylacetone (35 μl) were added to 200 μl plasma. After incubating at 80 $^\circ\text{C}$ for 2 hours 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 used for the second step derivatization. The carboxylic group was derivatized with 200 μl of methanol/acetyl chloride (10:1, v/v) mixture at 80 $^\circ\text{C}$ for 1 hour. After drying under nitrogen gas, 0.5 ml 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 μl of ethyl acetate and 1 μl of this solution was injected into GC-MS.

GC-MS conditions: Creatine and GAA derivatives were separated on a GC-MS system (Agilent Technologies, Santa Clara, CA) using a Supelco Wax-10 fused silica capillary column (30m $\times$ 0.25mm $\times$ 0.25 μm). The injector port temperature and auxiliary temperatures were 250 $^\circ\text{C}$. The oven temperature ramp was set as follows: the initial oven temperature was 80 $^\circ\text{C}$ and was increased to 180 $^\circ\text{C}$ at 10 $^\circ\text{C}$ per minute, and then to 250 $^\circ\text{C}$ at 50 $^\circ\text{C}$ per minute and kept at 250 $^\circ\text{C}$ for 7 min. Creatine and GAA derivatives were eluted at

7.5 min 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 and selected ion monitoring were used to monitor mass-to-charge ratio (m/z) for ions 303, 304, 305 and 317, 318, 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 concentrations, 200 μl plasma was spiked with $[^2\text{H}_3]\text{creatine}$ (10 nmol) and $[^{13}\text{C}_2, ^{15}\text{N}]\text{GAA}$ (0.5 nmol) and analyzed as above. The m/z 317 (m^-) and 320 ($(m+3)^-$) for creatine and 303 (m^-) and 306 ($(m+3)^-$) ion peak areas were integrated for calculation of GAA concentrations. Analysis of pure $[^2\text{H}_3]\text{creatine}$ and $[^{13}\text{C}_2, ^{15}\text{N}]\text{GAA}$ show that these labeled compounds have undetectable quantities of unlabeled 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 μM) and GAA (0.5 -250 μM) and constant amount of $[^2\text{H}_3]\text{creatine}$ and $[^{13}\text{C}_2, ^{15}\text{N}]\text{GAA}$ internal standards. The linear regression equations that 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 concentration. The first set of the samples included 200 μl of solution of creatine and GAA in water at three different concentrations (9, 37 and 185 $\mu\text{mol/L}$ creatine and 3, 12.5 and 25 $\mu\text{mol/L}$ GAA). The second set of samples consisted of duplicates of 200 μl 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 μl plasma. All eight samples were spiked with $[^2\text{H}_3]\text{creatine}$ (10 nmol) and $[^{13}\text{C}_2, ^{15}\text{N}]\text{GAA}$ (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 non spiked plasma and pure standards.

The precision, i.e., intra-assay and inter-assay reproducibility of the method for creatine and GAA concentration was determined by multiple analyses of plasma sample from pooled plasma. Intra-assay variability was determined by analyzing one sample 5 times. Inter-assay 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 previously described GC-MS technique [17]. Three different plasma samples in duplicate were aliquoted and spiked with $[^2\text{H}_3]\text{creatine}$ (10 nmol) and $[^{13}\text{C}_2, ^{15}\text{N}]\text{GAA}$ (1.2 nmol) internal standards. One set of samples were converted to hexafluoroacetylacetone-methyl derivative and analyzed as described above. The second set of samples were converted to hexafluoroacetylacetone-pentafluorobenzyl derivative and analyzed as previously described [17].

Human study: We investigated GAA and creatine kinetics in a healthy adult who participated in a study on the effect of an intravenous infusion of intralipid with heparin on $[1-^{13}\text{C}]\text{glycine}$ metabolism in human. The study examined the kinetics of glycine in the basal state and in response to infusion of intralipid (manuscript submitted for publication, 2009). The study protocol was approved by the Institutional Review Board (IRB) of the Cleveland Clinic. The study subject was placed on a weight maintenance diet containing at least 70 gm of protein/day for seven days prior to the tracer study. Following a 12h

overnight fast, two indwelling cannulae were placed in the dorsal vein of each hand, one for tracer infusion and the other to obtain blood samples. In order to obtain an arterialized blood, the sampling site was kept warm by placing the hand in a thermostat controlled warm blanket. After a priming dose of $[1-^{13}\text{C}]$ glycine ($16 \mu\text{moles}\cdot\text{kg}^{-1}$) a sterile solution of $[1-^{13}\text{C}]$ glycine in 0.45% saline was infused at a constant rate ($16 \mu\text{moles}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). Following the basal study (4 h), triglyceride solution (20%) (Intralipid®) with heparin ($0.2 \text{ units}\cdot\text{kg}^{-1}$) was infused at $40 \text{ ml}\cdot\text{h}^{-1}$. Blood samples (1 ml), in EDTA coated tubes, were obtained at time zero, prior to tracer infusion, and at 20-30 minute 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 percent enrichments of glycine, GAA and creatine were determined as molar fraction of m_1 isotopomer over m_0 , m_1 , and m_2 species. The measured mass isotopomer distributions were corrected for natural enrichments.

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

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

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

Data were presented as mean $\pm$ SD.

Results

Creatine and GAA were derivatized using a two step procedure involving reaction of hexafluoroacetylacetone with guanidino group followed with esterification of carboxyl group with acidic methanol (Fig. 2). The GC-MS chromatograms of creatine and GAA are presented in Fig. 3 (top panel). Despite the lower polarity of tertiary amino group in creatine compared to secondary amino group in GAA, the higher molecular weight creatine derivative eluted ahead of GAA derivative on GC column. As shown (Fig. 3, bottom panel), in negative chemical ionization (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 $[^{15}\text{N}]\text{GAA}$, $[^{13}\text{C}_2, ^{15}\text{N}]\text{GAA}$ and $[^2\text{H}_3]\text{creatine}$ yield ions with m/z 304, 306 and 320, respectively. As shown in Fig 4A, because of isotopic fractionation of $[^2\text{H}]$ -labeled compounds, $[^2\text{H}_3]\text{creatine}$ derivative (m/z 320) eluted ahead of m_0 creatine (m/z 317) on gas chromatography column. In contrast, $[^{13}\text{C}_2, ^{15}\text{N}]$ - and unlabeled GAA (m/z 306 and 303, respectively) did not separate on gas-chromatography (Fig 4B).

Assay validation

Fig 5 shows the standard curves of [$^2\text{H}_3$]creatinine (Panel A) and [^{15}N]GAA (Panel B) molar enrichments. The standard mixtures were prepared by adding increasing amounts of [$^2\text{H}_3$]creatinine and [^{15}N]GAA to a constant (10 nmol) amount of unlabeled creatine and GAA. Both calibration curves were linear in the 0.3-9% 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 6 shows the calibration curve for creatine (Panel A) and GAA (Panel B) concentrations 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}N]GAA (5 nmol). The curves were linear over a range of 2-500 μM and 0.5-250 μM for creatine and GAA, respectively. The lower limit of quantification (LOQ) with a signal/noise ratio greater than 10:1 was 0.25 μM and 1 μM for GAA and creatine, respectively. These ranges cover the GAA and creatine concentrations found in human plasma and urine.

In order to test the effect of plasma matrix on creatine and GAA assays we analyzed 100 μl , 200 μl and 300 μl 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 coefficient of variation less than 5%.

Fig 7 presents the results of the accuracy study for the assay of creatine (Panel 7A) and GAA (Panel 7B) concentrations. Plotting of nmols of creatine and GAA determined in plasma against creatine and GAA added to plasma resulted in lines that were linear with slopes and regression coefficient 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 recoveries of creatine and GAA from plasma were 92-96% and 89-109%, respectively. Application of stable isotope labeled internal standards compensates for any loss of analytes during derivatization and extraction steps and permits to consider the recovery results as the total recovery during the assay conditions. Intra-assay and inter-assay coefficient 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 [$1\text{-}^{13}\text{C}$]glycine. A near steady state tracer enrichment of GAA was reached by 30 min. The enrichment of GAA was ~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 this subject ($1.7 \pm 0.12 \mu\text{M}$) was similar to values reported using other techniques [16; 17]. The plasma concentration of creatine ($25.7 \pm 2.1 \mu\text{M}$) of this subject was less than that reported in healthy adults in the literature ($46 \pm 2 \mu\text{M}$) [17]. However, the plasma concentrations of creatine ($59.4 \pm 2.2 \mu\text{M}$ and $63.4 \pm 0.81 \mu\text{M}$) of two other healthy adults determined by our technique was similar to values reported in literature. In addition, our verification study demonstrated that two independent assays of creatine and GAA concentrations using this technique and previously established method yield similar results.

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 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 report we present a new technique for simultaneous measurement of creatine and GAA enrichment and concentration in human plasma. Utilization 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. The simultaneous measurement of GAA, creatine enrichment, and concentration in this study can be used to measure the rate of creatine synthesis in human.

The low micromolar concentrations of creatine and GAA in plasma combined with slow turnover rate of creatine in humans dictate a sensitive method for measurement of the concentration of creatine and GAA and their enrichments is needed. Several liquid chromatography-mass spectrometric (HPLC-MS) [16; 24; 25] and gas chromatography-mass spectrometric (GC-MS) methods [26; 27] for the analysis of creatine and GAA concentrations in biological fluids, including plasma have been described. Although HPLC-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 and therefore their GC-MS analysis requires converting them to nonpolar groups in order to improve their volatility. Derivatization procedures that have been used include reaction of these compounds with hexafluoroacetylacetone followed by either pentafluorobenzylbromide [17; 28] or alkyl silicone compounds. Utilization of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (TMS) [18; 27] and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (TBDMS) [14] for derivatization of carboxyl group increase the baseline natural abundance and therefore can not 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 [17; 28]. However, analysis of natural enrichment of creatine and GAA standards revealed that m_1 enrichment was higher than theoretical, 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 m_1 ion could be related to the ionization condition of 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 [26]

In this study we elected to prepare bis(trifluoromethyl)pyrimidine methyl ester derivative for the following reasons: (i) methyl group adds only one carbon and therefore has negligible effect on baseline natural enrichment of the native compound and (ii) fluorinated derivatives increase analytes electron affinity and improve their detection in sensitive NCI mode.

Derivatization of guanidino group with hexafluoroacetylacetone was adapted from previous studies [17; 23]. By examining various parameters (pH, t° , reaction time, ratio of aqueous to organic volume) it was determined that derivatization is complete in two hours at 80 °C, pH 9 with the aqueous/organic ratio below 0.4 and 50 μl of hexafluoroacetylacetone. We found

that if the plasma was buffered with freshly prepared saturated NaHCO_3 to pH 9, there is minimal hydrolysis of hexafluoroacetylacetone and therefore 35 μl of this reagent is sufficient for derivatization. The second step methylation of the carboxyl group with 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 and therefore extra care needs to be taken to avoid such a problem. We have not determined the yield of the derivatization reactions. However, since the endogenous metabolites (creatine and GAA) were derivatized simultaneously with the stable isotope labeled internal standards ($[\text{}^2\text{H}_3]\text{creatine}$ and $[\text{}^{13}\text{C}_2, \text{}^{15}\text{N}]\text{GAA}$), the yield of derivatization reactions did not affect the calculated concentrations of GAA and creatine. The validation of this technique for the measurement of creatine and GAA concentrations demonstrates that it is linear, accurate, precise and free of any appreciable plasma matrix effect. Although the sensitivity (limit of quantification) of this method is lower than those previously described GC-MS [17] and LC-MS [24; 25] methods, this technique allows the simultaneous measurement of creatine and GAA concentrations found in human plasma with good accuracy and precision. This technique was verified by the measurement of creatine and GAA concentrations using previously described GC-MS technique [17].

This technique was used for investigation of creatine metabolism in human. In human infused with $[1\text{-}^{13}\text{C}]\text{glycine}$ m_1 enrichment of glycine plateaued at about 9.6%, while m_1 GAA was about 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 their isotopic labeling will become similar.

Using GAA as immediate precursor pool, we calculated that FSR of plasma creatine to be 37%/day (Table 3). This is the first study estimating the rate of creatine synthesis in human using a steady state glycine tracer infusion. Previously the creatine turnover in human was determined based on creatinine excretion rate. The rate of creatine turnover ($\sim 1.5\%$ /day or ~ 1.5 g/day) in these studies reflects the turnover rate of total body creatine pool, predominantly in the muscle ($\sim 95\%$ of total creatine pool). Creatine in plasma and in most tissues has much higher turnover rate than that creatine in skeletal muscle [29]. Creatine in plasma rapidly equilibrates with nonmuscle creatine pool; it does not appear to equilibrate rapidly with the large pool of creatine in skeletal muscle [30;31]. This is in agreement with our estimate of creatine kinetics calculated based on the miscible extra-skeletal muscle pool (estimated to be $\sim 5\text{g}$ in 70 kg adult or 5% of total creatine pool). Based upon the extra-skeletal muscle creatine pool, the rate of creatine synthesis estimated in this study is 1.85 g/day (37% of 5.0 g), which is similar to values reported in literature ($\sim 1.5\text{g/day}$) [29]. Therefore we conclude that the calculated FSR of plasma creatine in this study represents the kinetics of creatine pool in nonmuscle tissues and in plasma.

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

Acknowledgments

We thank the GCRC staff for their help with the studies, and Mrs. Joyce Nolan for secretarial assistance. The study was supported by CCF institutional support (to SSK).

Supported by: This work was supported in part by National Institutes of Health, National Center for Research Resources, CTSA 1UL1RR024989, Cleveland, Ohio, and by Cleveland Clinic Foundation.

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Abbreviations used

GC-MS	gas chromatography-mass spectrometry
MPE	molar percent enrichment
TMS	trimethylsilyl
NCI	negative chemical ionization
GAA	guanidinoacetic acid

Fig.1.

Creatine biosynthesis. The first step in creatine synthesis involves the reversible transfer of 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.

Fig. 2.

Preparation of bis(trifluoromethyl)pyrimidine methyl derivatives of guanidinoacetate (X = H) and creatine (X = CH₃). Guanidino group of creatine and guanidinoacetate was derivatized with hexafluoroacetylacetone and carboxyl groups were esterified with acidic methanol.

Fig.3. Gas chromatography-mass spectrometry chromatograms of creatine and guanidinoacetate standards (top panel). Creatine and guanidinoacetate mass spectra and structures of corresponding derivatives are also shown (bottom panel).

Fig. 4.

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

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

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

Fig. 7.

Accuracy study for the creatine and guanidinoacetate concentration analysis. Known amounts of creatine (0, 2.7, 11 and 37 nmol) and guanidinoacetate (0, 0.9, 3.75 and 7.5 nmol) were added to pooled plasma samples (200 μ l each) and the amounts of creatine and guanidinoacetate were determined. Panel A is a plot of nmols creatine (endogenous + added creatine) versus nmols creatine added. Panel B is a plot of nmols guanidinoacetate (endogenous + added guanidinoacetate) versus nmols guanidinoacetate added. Chromatographic conditions and quantification procedures are described in the text.

Fig. 8. M1 molar percent enrichment (MPE) of glycine and guanidinoacetate in plasma of a human subject. $[1-^{13}\text{C}]$ glycine in 0.45 % saline was administered as prime-constant infusion for 8 hours. Steady state enrichment was apparent in both glycine and guanidinoacetate pools within 30 min

Fig. 9.
M1 molar percent enrichment (MPE) of creatine from same experiment as Fig.8

Table 1
Intra- and inter-assay variability for creatine and guanidinoacetate (GAA) analysis

	Creatine	CV	Guanidinoacetate	CV
	($\mu\text{mol/L}$)	(%)	($\mu\text{mol/L}$)	(%)
Intra-assay (n=5)	21.05 $\pm$ 0.24	1.2	2.63 $\pm$ 0.07	2.7
Inter-assay (n=6)	19.84 $\pm$ 0.78	3.9	2.50 $\pm$ 0.11.	4.4

Table 2

Verification of creatine and GAA concentration analysis. Three different plasma samples were analyzed by GC-MS using hexafluoroacetylacetone-methyl (current study) and hexafluoroacetylacetone-pentafluorobenzyl (previously described [17]) derivatives. Data are shown as the average of 4 replicate injections.

Sample #	Hexafluoroacetylacetone- Methyl		Hexafluoroacetylacetone- Pentafluorobenzyl	
	[Creatine] ($\mu\text{mol/L}$)	[Guanidinoacetate] ($\mu\text{mol/L}$)	[Creatine] ($\mu\text{mol/L}$)	[Guanidinoacetate] ($\mu\text{mol/L}$)
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

Table 3
Concentrations and stable isotope enrichments of glycine, GA and creatine and creatine FSR in a healthy adult after overnight fasting

[1- ¹³ C]glycine infusion rate (μmol.kg ⁻¹ .h ⁻¹)	15.48
[1- ¹³ C]glycine plateau MPE (%)	9.6 ± 0.51
[1- ¹³ C]guanidinoacetic acid plateau MPE	3.7 ± 0.3
Slope of creatine labeling	0.00097
Glycine (μmol.L ⁻¹)	385
Guanidinoacetic (μmol.L ⁻¹)	1.7
Creatine (μmol.L ⁻¹)	25
Creatine FSR (%/day)	37