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Journal of Chromatography B, 827 (2005) 210–215
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Analysis of creatine, creatinine, creatine-d_3 and creatinine-d_3 in urine, plasma, and red blood cells by HPLC and GC–MS to follow the fate of ingested creatine-d_3

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Received 22 January 2005; accepted 4 September 2005
Available online 22 September 2005

Abstract

Creatine, which is increasingly being used as an oral supplement, is naturally present in the body. Studies on the fate of a particular dose of creatine require that the creatine be labeled, and for studies in humans the use of a stable isotopic label is desirable.

The concentrations of total creatine and total creatinine were determined using HPLC. Creatine and creatinine were then separated using cation exchange chromatography and each fraction was derivatized with trifluoroacetic anhydride and the ratio of the deuterated:undeuterated species determined using GC–MS. Ratios of creatine:creatine-d_3, and creatinine:creatinine-d_3, and the concentrations of each of these species, were able to be determined in urine, plasma and red blood cells. Thus, the uptake of labeled creatine into plasma and red blood cells and its excretion in urine could be followed for a subject who ingested creatine-d_3. Creatine-d_3 was found in the plasma and red blood cells 10 min after ingestion, while creatine-d_3 and creatinine-d_3 were found in the urine collected after the first hour.
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Keywords: Creatine; GC–MS; Deuterated creatine; Deuterated creatinine; Urine; Plasma; Red blood cells

1. Introduction

The oral intake of creatine, through the use of supplements or the ingestion of large amounts of meat or fish, can increase the body's overall creatine pool [1–3]. Oral ingestion of creatine can increase the creatine and phosphocreatine stores in skeletal muscles [4], and in combination with an exercise program can enhance exercise performance more than exercise alone [5]. Researchers are looking at creatine supplementation as a possible treatment for muscle wasting and neurodegenerative diseases [6,7].

Creatine is naturally present in the body and studies on the fate of a particular dose of creatine require that the creatine be labeled. Some older studies on the turnover of creatine in the body were carried out using C-14-labeled creatine [8] but such studies are now mostly confined to animals. Studies on humans are more appropriately done using creatine labeled with a stable isotope such as N-15 [3,9], C-13 [10,11], or H-2 (deuterium) [12].

GC–MS has been used as a reference method for creatinine analysis in biological samples [13,14] using isotope dilution analysis. Creatinine must be derivatized to allow passage through the GC, but in most cases it forms the same derivative as creatine does, necessitating removal of creatine before derivatization of the creatinine in a sample. Derivatives of creatinine that have been used include trimethylsilylated creatinine [13,15], the ethyl ester of *N*-(4,6-dimethyl-2-pyrimidinyl)-*N*-methylglycine [13] and *O*-trifluoroacetylcreatinine [16]. Before analysis, creatine has been removed from the sample using cation-exchange [17] chromatography. Creatine is zwitterionic in the pH range of 2–14 and is not held up on a cation exchange column. It was removed by rinsing the column with water before collecting the creatinine, which was eluted with ammonia.

In the study described in this paper, both the initial fraction containing creatine as well as the fraction eluted with ammo-

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doi:10.1016/j.jchromb.2005.09.011

nia, were collected. Separate derivatization of these fractions allowed us to analyze isotopic ratios for both labeled creatine and labeled creatinine. Quantitative analysis of the samples was carried out using HPLC so that actual concentrations of creatine, creatinine, creatine-d_3 and creatinine-d_3 could be calculated. The method was then applied to the analysis of blood and urine from a volunteer who had ingested a 5 g dose of creatine-d_3. We were able to follow the resulting increases in creatine-d_3, creatine, creatinine and creatinine-d_3 in plasma, red blood cells (RBC), and urine.

2. Experimental

2.1. Reagents

Amberlite weakly acidic cation exchange resin, creatine monohydrate, creatinine and creatininase (3.5.2.10) were supplied from Sigma Chemical (St. Louis, MO). Creatine-d_3 monohydrate (*N*-methyl-d_3) and creatinine-d_3 (*N*-methyl-d_3), each 99 at.% were supplied from CDN Isotopes (Pointe-Claire, PQ). Trifluoroacetic anhydride, tris(hydroxymethyl)aminomethane (Tris) and tetrabutylammonium hydrogen sulfate were supplied by Aldrich Chemical (Milwaukee, WI). Ammonium hydroxide was supplied by Fisher Scientific (Nepean, Ont.) and acetonitrile (HPLC grade) was supplied by Caledon Laboratories (Georgetown, Ont.). Sodium hydroxide and potassium hydroxide were purchased from BDH (Toronto, Ont.) and potassium dihydrogen phosphate was purchased from BDH (Poole, England).

2.2. Subject

Prior to the commencement of the study, the participant, a 32-year-old male, was made aware of all tests and procedures, and informed written consent was obtained. The subject weighed 86 kg, with a lean body mass of 67.6 kg and typically worked out four times a week. He had not supplemented with creatine in the previous 120 days. He was informed that he could withdraw from the study at any time without penalty. The study was granted approval by the Research Ethics Board at St. Francis Xavier University.

2.3. Supplementation

Creatine-d_3 monohydrate (5.06 g) was dissolved in 500 mL water and ingested at 8 a.m. of the day following baseline urine collection.

2.4. Urine collection

Samples were collected in 4 L urine collection containers (Fisher Scientific, Nepean, Ont.) and the total volume recorded. Three 10 mL aliquots of each sample were frozen at −80 °C until analysis. A 24 h baseline urine sample was collected the day before ingestion of labeled supplement. After ingestion, urine was collected at each micturation for the first 24 h, then as 24 h samples for the following 5 days. Three 10 mL aliquots of each sample were frozen at −80 °C until analysis. This resulted in seven individual collections in the first 24 h: at 10:15 a.m., 11:00 a.m., 12:05 p.m., 3:45 p.m., 9:40 p.m., 12:00 a.m. and 7:15 a.m.

2.5. Blood collection

Collection of a 10 mL blood sample from an antecubital vein of the participant was done immediately prior to the ingestion of the creatine-d_3 monohydrate and at time intervals of 10, 30 and 60 min after ingestion. Each sample was collected in a 10 mL heparinized Vacutainer tube using a 22 gauge Vacutainer brand blood collection needle (Becton Dickinson, Franklin Lakes, NJ) and immediately centrifuged (IEC Medispin, Needham Heights, MA) at 1228 g for 15 min to separate RBC and plasma. Samples of RBC and plasma were transferred into 1.5 mL microcentrifuge tubes (Fisher Scientific, Nepean, Ont.) and frozen at −80 °C until analysis. Freezing the RBC causes cell lysis to occur [18].

2.6. Sample preparation

2.6.1. Urine

Urine samples were thawed at room temperature and a 0.3 mL aliquot was removed and deproteinized with 1.0 mL of acetonitrile in a 1.5 mL microcentrifuge tube [19]. The tube was vortexed (Vortex-Genie™, Fisher Scientific, Bohemia, NY) for 15 s to mix solutions and centrifuged for 15 min. A 0.2 mL sample of the supernatant was transferred to a second 1.5 mL microcentrifuge tube, frozen in liquid nitrogen and placed in a vacuum until all acetonitrile had evaporated. The sample was dissolved in 1.5 mL of triply deionized water before analysis.

2.6.2. RBC and serum

Samples were thawed at room temperature and a 0.2 mL aliquot was removed and diluted with 0.2 mL of triply deionized water in a 1.5 mL microcentrifuge tube to reduce the viscosity. The tube was vortexed and the sample deproteinized using 1.0 mL of acetonitrile [19]. The tube was vortexed for another 15 s and centrifuged for 15 min. A sample of the supernatant was removed (0.28 mL for RBC, 0.6 mL for serum), frozen in liquid nitrogen and placed in a vacuum until all acetonitrile had evaporated. The sample was then dissolved in 0.2 mL of blood analysis buffer for analysis by HPLC.

2.7. HPLC

HPLC analysis was performed on a Shimadzu LC10AD VP Liquid Chromatograph using a Shimadzu SPD-10AD *VP* Variable wavelength Detector set at 210 nm and a 250 mm × 4.6 mm Synergi Hydro-RP 80 10 μm column preceded by a security guard column (KJO-4282), both supplied by Phenomenex (Torrence, CA). The data was collected using Peak Simple software (SRI Instruments, Torrance, CA).

The buffer used for the analysis of urine samples was potassium dihydrogen phosphate (20 mM, 2.72 g L^{-1}) adjusted to pH 5.0 with aqueous potassium hydroxide. A potassium dihydrogen phosphate (14.7 mM, 2.0 g L^{-1}) and tetrabutylammonium hydrogen sulfate (3.5 mM, 1.2 g L^{-1}) adjusted to pH 6.5 with

aqueous potassium hydroxide was used as the buffer for all red blood cell and plasma analysis.

Creatine and creatinine mixed standards of concentrations up to 100 μg mL^{-1} (made by serial dilution from 1000 μg mL^{-1} stock solutions) were used to determine standard curves and regression equations for creatine and creatinine analysis on the HPLC. Resulting peak heights were measured and regression equations calculated using Excel software (Microsoft). Equations with a R^2 value greater than 0.990 were considered acceptable. The LOD was 0.02 μg mL^{-1} ($k = 3$). Reproducibility in terms of repeat injections on the same day was ±1.4% for creatine (50 μg mL^{-1}, $n = 10$). Long-term reproducibility was ±6% for 5 and 10 μg mL^{-1} creatine and ±8% for 20 and 50 μg mL^{-1} ($n = 8$, over 2 months). Thus, standard curves were run daily.

Reproducibility of the actual urine analysis, including protein precipitation and freeze drying (aliquots of the same urine sample were individually deproteinated then analyzed) was ±1.0% for creatine (35 μg mL^{-1}, $n = 8$) and ±1.4% for creatinine (7 μg mL^{-1}, $n = 8$). Samples themselves were generally run in duplicate, but sometimes in triplicate. The buffer flow was reduced to 0.2 mL min^{-1} overnight. After the flow was increased to 1 mL min^{-1}, a standard was injected until the peak heights and retention times were seen to be stable. This usually required only three injections. Initially a 1 mL sample was freeze dried in a 1.5 mL centrifuge tube after protein precipitation with acetonitrile, but it was found that much more reproducibility was attained if only a 0.2 mL sample was used for the freeze drying.

2.8. Identification of creatinine by means of creatininase

Creatininase (0.35 mg) was dissolved in 5 mL 0.1 M Tris buffer (pH 7.0). A 400 μL aliquot of sample was placed in a 1.5 mL microcentrifuge tube and 30 μL enzyme solution was added with vortex mixing. The sample was immediately analyzed by HPLC and then again every 8 min for 24 min. A creatinine standard of approximately the same concentration was treated with enzyme and analyzed by HPLC in the same way to ensure that the enzyme was active.

2.9. Benchtop column separation of creatine and creatinine

Amberlite weakly acidic cation exchange resin was soaked in water overnight. Approximately 15 mL of this resin was poured into a 25 mL glass burette blocked with glass wool. The separation was initially optimized for a 1 mL standard containing 50 μg mL^{-1} of both creatine and creatinine. The eluate was collected in 5 mL fractions and constituents were confirmed using HPLC. The eluent for removal of creatine was 55 mL triply deionized water. The majority of the creatine eluted in the first 5–10 mL. It was absent from the last four fractions. It has previously been shown using creatinine-^{14}C and creatine-^{14}C that 99% of creatinine is retained on a weak acid resin during a 200 mL water wash, while greater than 99.9% of the creatine elutes in the water [14]. The eluent for removal of creatinine was 1.0 M NH_4OH, and these fractions were neutralized with 1.2 M HCl before analysis by HPLC. Creatinine eluted in the first five fractions, with the highest concentration in the fourth fraction. The column was then rinsed with 45 mL ammonia, regenerated with 1.2 M HCl until the eluate was acidic, then rinsed with water until the eluate was neutral. At this point, 1 mL of water put on the column and rinsed through with water then ammonia led to fractions that were free of both creatine and creatinine. When urine samples (1 mL) were passed through the column, the HPLC traces of the creatine-containing fractions eluted with water showed a peak that had a retention time close to that of creatinine. This peak was not identified but was shown not to be creatinine because the addition of creatininase to a sample did not remove the peak. Addition of creatininase to a sample containing creatinine standard caused peak reduction, indicating that the enzyme was active in the conditions used for the experiments.

2.10. TFAA derivatization

Creatine and creatinine were both derivatized using a modification of the method published by Nissim et al. [20]. Sample (0.1 mL 1000 μg mL^{-1} standard or ∼15 mL of column effluent) was placed in a suitable container and the solvent was allowed to evaporate. TFAA (1 mL) was added via pipette and the solution was sonicated for 2 min and then heated at 60 °C until all solvent had evaporated, which took approximately 5 min. Acetonitrile (1 mL) was added to the beaker and sonicated for 2 min. The solution was transferred to a screw top 2 mL GC vial for analysis.

2.11. GC–MS

GC–MS analysis was performed on a Varian CP3800 Gas Chromatograph using a Varian Saturn 2000 GC/MS/MS mass spectrometer. The carrier gas was UHP helium, the injector temperature was held at 220 °C, and electron impact was used for ionization. The CP-SIL 8CB-MS column (Varian) was 30 m × 0.25 mm with a 0.25 μm film thickness. The initial column temperature was held at 50 °C for 1 min, and then increased from 50 to 270 °C at a rate of 20 °C min^{-1}.

Standards containing creatine and creatine-d_3 or creatinine and creatinine-d_3 in ratios up to 100:1 were prepared using 10 μl of 1000 μg mL^{-1} creatine-d_3 solution and the appropriate amount of 1000 μg mL^{-1} creatine. Each delivery was weighed to increase the accuracy. The resulting solutions were evaporated to dryness and derivatized with trifluoroacetic anhydride as described above. The derivatives were then dissolved in acetonitrile and run in triplicate by GC–MS. The amount of acetonitrile was adjusted to give a signal in the MS of ∼10^5 to 10^6 ions. Resulting areas were measured and plotted versus the actual mass ratios. The regression equations were calculated using Excel software (Microsoft).

3. Results and discussion

A number of buffers have been previously used for the analysis of creatine and creatinine in plasma or urine by reverse phase

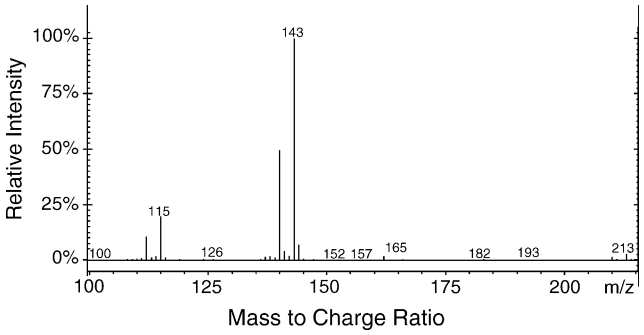

Fig. 1. Mass spectrum of a 1:2 mix of creatine and creatine-d_3. The spectrum was obtained using a CP3800 GC interfaced to a Saturn 2000 MS (Varian) with an ion trap detector. The CP-SIL 8CB-MS column (Varian) was 30 m × 0.25 mm with a 0.25 μm film thickness. The carrier gas was UHP helium and the injector temperature was 220 °C. The column was held at 50 °C for 1 min, and then the temperature was increased from 50 to 270 °C at a rate of 20 °C min^{-1}. The ionization (electron impact) time was 3781 μs with the filament emission current set at 10 μA. The ion count for the base peak (143) was 30318.

HPLC [21]. Those developed for serum or muscle analysis typically use a phosphate buffer containing tetrabutyl ammonium hydrogen sulfate (TBAHS), which has been shown to better separate impurities from the creatine peak. However, a simple phosphate buffer is preferable for urine analysis [22,23]. We found that different C_{18} reverse phase columns give different separations between creatine, creatinine and uric acid. If the protein was not precipitated from urine samples before HPLC analysis, in accordance with the method being followed for serum and RBC [19], the lifetime of the column was greatly decreased, the guard columns had to be changed too frequently, and the columns degraded resulting in broad split peaks in the chromatogram.

The mass spectra for the TFAA derivatives of creatine and creatinine were identical to those described previously for *O*-trifluoroacetylcreatinine [16]. A mass spectrum of a 1:2 mix of *O*-trifluoroacetylcreatinine and *O*-trifluoroacetylcreatinine-d_3 is shown in Fig. 1. We chose to use the areas of the peaks at 140 and 143 for determining the ratios of the derivatives of creatine/creatinine to creatine-d_3/creatinine-d_3. Experiments were carried out to validate this choice. When a 1000 μg mL^{-1} solution containing a 5.08:1 ratio of creatine and creatine-d_3 was derivatized, diluted 25-fold and injected into the GC–MS, the resulting area ratio was 5.31 ± 0.08 (n = 16). When the series of derivatized creatine standards (see Section 2) was run, the plot of the experimentally determined ratio versus the mass ratio had a slope of 0.933 ± 0.014 with an intercept of 0.65 ± 0.56. The R^2 was 0.994. In the analogous experiment using creatinine, the plot had a slope of 0.89 ± 0.03 with an intercept of 0.60 ± 0.64 and a R^2 of 0.98. A ratio of ∼100:1 was the limit for detecting creatine-d_3 in the presence of creatine. A sample of acetonitrile injected in the GC–MS gave no peaks above baseline in the 139–143 region.

The samples of plasma and red blood cells collected from the subject chosen to ingest creatine-d_3 for the case study were found to contain creatine in the amounts shown in Table 1. The baseline plasma creatine concentration was typical for a male [24], but the increase was less than reported for three other subjects who ingested 5 g [4]. Creatine-d_3 was found in the plasma after 10 min. In the 30 min sample, the increase in the total creatine can be attributed directly to the presence of creatine-d_3 absorbed from the ingested sample. In contrast, creatine-d_3 only accounted for 81 μM (40%) of the increase in plasma creatine concentration between the 30 and 60 min blood collections. The remaining ∼120 μM must have come from the creatine pool already present in the body, the majority being in the muscle. This indicates that there is fairly fast exchange of plasma creatine with the creatine pool in the body. As over 90% of this pool is in the muscle, our results suggest that although there is net uptake into the muscle, there is also exchange. The plasma concentrations gradually decreased after the first hour as the creatine was excreted or absorbed by the muscles.

The baseline creatine concentration in the red blood cells was higher, as expected, than that in the plasma, but did not increase after supplementation. Creatine-d_3 was found in the first sample of red blood cells post ingestion (10 min) and the concentration gradually increased, although the overall creatine concentration was not affected. We have previously found an increase in RBC

Table 1
Concentrations of total creatine and creatine-d_3 (and standard deviation) in the plasma and RBC of a 32-year-old male at various times after the ingestion of 5.06 g creatine-d_3 monohydrate

Time after ingestion (min)	Plasma		Red blood cells	
	Total creatine (μM)	Creatine-d_3 (μM)	Total creatine (μM)	Creatine-d_3 (μM)
0.0	34 ± 1	<0.03	142 ± 3	<1.8
10	52 ± 1	9 ± 3	141 ± 4	7 ± 2
30	282 ± 1	252 ± 9	167 ± 5	18 ± 2
60	493 ± 3	334 ± 12	157 ± 3	35 ± 4
90	478 ± 3	312 ± 12	155 ± 4	52 ± 5
120	274 ± 1	145 ± 8	130 ± 4	59 ± 5
150	188 ± 1	96 ± 5	147 ± 5	77 ± 7
180	132 ± 1	66 ± 5	124 ± 3	82 ± 5

Concentrations of total creatine were determined using HPLC (14.7 mM potassium dihydrogen phosphate and 3.5 mM tetrabutylammonium hydrogen sulfate adjusted to pH 6.5 with aqueous KOH). Creatine and creatinine were separated on a cation exchange column, then derivatized with trifluoroacetic anhydride, and the ratio of deuterated to non-deuterated derivative was determined using GC–MS (30 m × 0.25 mm CP-SIL 8CB-MS column, 0.25 μm film. The column was held at 50 °C for 1 min, and then increased to 270 °C at 20 °C min^{-1}).

Table 2
Concentrations of total creatine, creatine-d_3, total creatinine, and creatinine-d_3 (and standard deviation) in the urine of a 32-year-old male at various times after the ingestion of 5.06 g creatine-d_3 monohydrate

Time period for collection	Urine			
	Total creatine	Creatine-d_3	Total creatinine	Creatinine-d_3
24 h prior to ingestion	0.138 ± 0.001 g	<0.002 g	2.45 ± 0.01 g	<0.01 g
1 h after ingestion	0.27 ± 0.02 g	0.17 ± 0.02 g	0.43 ± 0.02 g	0.31 ± 0.04 g
2 h after ingestion	0.34 ± 0.03 g	0.34 ± 0.03 g	0.09 ± 0.02 g	0.06 ± 0.03 g
3 h after ingestion	0.05 ± 0.02 g	0.03 ± 0.02 g	<4 μg	<4 μg
7 h after ingestion	0.12 ± 0.02 g	<0.002 g	0.37 ± 0.02 g	<0.01 g

Concentrations of total creatine and creatinine were determined using HPLC (C_{18} column with a buffer of 20 mM potassium dihydrogen phosphate adjusted to pH 5.0 with aqueous KOH). Creatine and creatinine were separated on a cation exchange column, then derivatized with trifluoroacetic anhydride, and the ratio of deuterated to non-deuterated derivative in each sample was determined using GC–MS (30 m × 0.25 mm CP-SIL 8CB-MS column, 0.25 μm film. The column was held at 50 °C for 1 min, and then increased to 270 °C at 20 °C min^{-1}).

creatine after 5 days of supplementation [25], while no change was reported in the first few hours after ingestion of a 5 g dose [26,27]. The creatine-d_3 presumably entered because of slow exchange with creatine in the plasma. Such an exchange has been seen previously for blood cells in vitro [28].

A relatively large amount of creatine was found in the urine from the first micturation after ingestion of the creatine. This reflects the elevated [creatine] in the plasma. Both creatine-d_3 and creatinine-d_3 were also present. It is well established that only a few percent of creatine at the most is converted to creatinine at body temperature under neutral conditions in a 24 h period, while at the low temperatures at which urine samples are stored, conversion should be negligible [29–31]. However, some conversion of creatine to creatinine during ingestion should not be unexpected given the acidic conditions of the stomach. There is much less creatinine-d_3 in the second urine sample and none detected in the later samples. Overall ∼10% of the creatine was converted to creatinine. The fact that we collected the urine at each micturation rather than as a 24 h sample was instrumental in detecting this. Creatine-d_3 was also mostly excreted in the urine within the first 2 h. There was an uptake of 78% of the creatine ingested, considering the creatine-d_3 and creatinine-d_3 excreted. This gives a much better estimate of the uptake than by looking at the total creatine and total creatinine excreted in a 24 h period with no label to distinguish the origin of the excreted substances (Table 2).

4. Conclusions

Ratios of creatine:creatine-d_3, and creatinine:creatinine-d_3 in urine, plasma and red blood cells can be determined by GC–MS after separation of creatine from creatinine on a cation exchange column, then derivatization of each fraction using trifluoroaceticanhydride. This data can be combined with the HPLC determination of total creatine and creatinine to give concentrations of all four species.

The fate of an ingested bolus of creatine-d_3 can thus be followed. In our case study on a 32-year-old male subject, creatine-d_3 appeared in the plasma and red blood cells within 10 min of ingestion, while creatine-d_3 and creatinine-d_3 were excreted in the first 2 h after ingestion.

Use of the labeled species allowed a better estimate of uptake efficiency than is otherwise possible.

Acknowledgements

We thank Brian Haines for technical assistance on the GC–MS, and NSERC for funding.

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