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Validation of a simple liquid chromatography assay for creatine suitable for pharmacokinetic applications, determination of plasma protein binding and verification of percent labeled claim of various creatine products

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Abstract

Creatine has been quantified in various tissues by a range of methodologies. This paper reports on the development and validation of a simplified HPLC assay to determine plasma creatine, plasma protein binding of creatine, creatine in microdialysate and creatine in over-the-counter products. An isocratic, reversed-phase (C_{18}) HPLC assay, using potassium phosphate monobasic (pH 4) as a mobile phase, was validated in human plasma and microdialysis perfusion fluid (normal saline). The lower limit of quantification for the assay was 1 mg l^{-1} in saline and 5 mg l^{-1} in plasma. The RSD was below 6% and accuracy was below 12% in both matrices. Protein binding in human plasma was found to be negligible (<10%). Over-the-counter creatine monohydrate products tested contained 100% creatine monohydrate. This assay was found to be suitable for pharmacokinetic studies and the assessment of plasma creatine and skeletal muscle microdialysate.

Keywords: Protein binding; Creatine

1. Introduction

Research on creatine (Cr) has become popular because of the positive effects on muscle strength and muscle performance in both healthy populations [1–3] and patient populations [4–6]. Although pharmacological effects of Cr have been investigated, the pharmacokinetics of Cr has yet to be elucidated in blood or skeletal muscle. There are several important points that need to be considered to reliably determine Cr pharmacokinetics.

The first issue in determining the clinical pharmacokinetics of Cr is the development of a reliable assay that has the capability of quantifying baseline levels of endogenous Cr in blood (7–13 mg l^{-1}). Previously, various methods have been used to quantify Cr in urine [7–12], skeletal muscle via

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biopsy [13–23] and in blood [7,11,24–27]. The ultimate goal of Cr supplementation is to increase muscle levels of Cr; microdialysis is a novel method to sample interstitial muscle levels and may be useful in understanding Cr disposition. To date, Cr has never been measured in microdialysate samples and therefore an assay is needed to quantify Cr in microdialysate (i.e., interstitial muscle Cr concentrations). Microdialysis has the advantage over biopsy technique in that it is less invasive, gives information about a definitive compartment (i.e., interstitial space), and allows one to frequently monitor concentrations over time.

The most important consideration in analyzing Cr in any biological fluid is the separation from its dehydration degradation product, creatinine (Crn). Previous assays used to quantify Cr in biological samples include both separation and non-separation methods. Non-separation methods include enzymatic assays [7,12,28–31] and a fluorometric assay [9]. The problem with previously published non-separation assays is the possibility of cumbersome analytical methodology. Separation methods have included capillary electrophoresis [32,33] and high-performance liquid chromatography (HPLC) [10,11,13,14,16,19,22,23,34,35]. However, the majority of previous HPLC methods included ion-pairing agents [16,18–21,36], ion-exchange columns [23], or gradient elution/organic modifiers because of interest in simultaneously detecting Cr, phosphocreatine and/or other nucleotides [19,37]. The addition of these factors can potentially complicate HPLC methodology.

The second point in the clinical determination of Cr disposition is that of product purity. Dietary supplements like Cr are not regulated with respect to purity or labeled claim of content. Accuracy of the dose administered is important for pharmacokinetic studies. It is also important to the consumer that products contain the reported labeled amounts. For these reasons, various over-the-counter products will be tested. A recent study also has addressed the issue of product purity and other physiochemical properties of Cr [38,39].

Lastly, plasma protein binding data is also essential to pharmacokinetics. Protein binding influences volume of distribution, metabolism and elimination but also plays an essential part in the interpretation of microdialysis data. To date there are no reported determinations of plasma protein binding of Cr in humans or animals. Our goal was to assess plasma protein binding in this study.

The overall goal of this work was to establish a simple, reliable and relatively easy to use HPLC analysis for the quantification of Cr in plasma and microdialysis perfusion fluid. Ultimately, this methodology will be used to determine Cr concentrations in blood and muscle microdialysis samples for pharmacokinetic analysis. Therefore, this paper describes a simple, isocratic HPLC assay in regards to its validation in plasma and microdialysis fluid, normal saline. This validated assay will then be applied in testing percent labeled claim of various over-the-counter creatine monohydrate (CrM) products that will be used for future human studies. Additionally, the plasma protein binding properties of Cr are unknown and could play an important role in Cr pharmacokinetics; therefore Cr protein binding will also be determined in human plasma.

2. Methods

2.1. Chemicals

Standard reagents creatine monohydrate (minimum 99% pure), creatinine, perchloric acid, potassium phosphate monobasic, phosphoric acid and acetonitrilie were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human plasma was obtained from Civitan Blood Bank (Gainesville, FL, USA). Normal saline (USP) and lactated Ringers (USP) were obtained from Shand's Hospital (Gainesville, FL, USA).

2.2. Preparation of samples

Plasma samples were acidified in a 2:1 ratio of plasma to 6% perchloric acid. Samples were centrifuged at 20 000 *g* for 5 min at room temperature. The supernatant was removed and directly injected for analysis.

2.3. Apparatus and chromatographic conditions

The mobile phase was KH_2PO_4 (50 m*M*, 6.8 g l^{-1}) adjusted to pH 4.0 with H_3PO_4. The mobile phase was degassed prior to use and the flow-rate for the assay was 1 ml min^{-1}. Chromatography was performed using a Waters C_{18} ODS(2) 250×4.6 mm, 5 μm analytical column (Waters, Milford, MA, USA) with a 10 mm guard column packed with Pellicular ODS (Whatman, Maidstone, UK). A Perkin-Elmer 200 Series liquid chromatograph system equipped with a UV–Vis detector (Shimadzu SPD-10A) monitoring at a wavelength of 210 nm was used. The injection volume was 25 μl for normal saline with a run time of 6 min, and 50 μl for plasma with a run time of 27 min. Peak height was used to quantify Cr using TurboChrom software package (Perkin-Elmer, Norwalk, CT, USA) because this parameter was less sensitive to disturbances in overlapping peaks. At the end of each validation run (or after 60 injected samples during sample analysis), the system was flushed with 100% distilled water for 10 min followed by acetonitrile:water (65:35) for 20 min and stored in this final solution. The guard column was also replaced after each run.

2.4. HPLC validation

Standard stock solutions were prepared by dissolving commercial preparations of CrM (Sigma) in normal saline. Calibration curves were generated in both normal saline and human plasma. Standard curves and quality controls were prepared independently. The range for the standard curve for saline was 0, 1, 5, 10, 25, 50, 75, 100 mg l^{-1} and for plasma was 0, 1, 5, 10, 25, 50, 75, 100 mg l^{-1}. All samples were frozen in individual aliquots at −20 °C for no longer than 1 month. Analyzing quality control samples at the beginning and end of each validation run was used to assess bench top stability in plasma. Freeze–thaw stability was assessed by comparing the slope of a freshly prepared standard curve in normal saline against samples frozen for 3 and 8 days. Recovery was assessed by comparing peak height of acidified plasma samples against normal saline. Lower limit of quantification was defined as the lowest tested concentration at which the relative standard deviation (RSD) was <15%.

2.5. Application to microdialysis

In vitro: Three standard CrM solutions (10, 25, and 75 mg l^{-1}) were prepared in lactated Ringers to mimic expected in vivo concentrations after oral dosing of CrM. The microdialysis probe (CMA 60, CMA Microdialysis, Sweden) was placed into lactated Ringers (15 ml) containing 0 mg l^{-1} Cr at 37 °C. The ''no net flux'' method of probe calibration was performed by perfusing the probe at a rate of 2 μl min^{-1} with the three standard solutions. The dialysate and perfusion media was then analyzed by HPLC. The procedure was repeated to quantify a known concentration of Cr solution (25 mg l^{-1}). During this experiment an additional perfusate concentration containing 0 μg ml^{-1} of Cr was included in the ''no net flux'' procedure. Lactated Ringer's was used in these experiment because it was the perfusion fluid used in previous human microdialysis experiments in our department; however, lactated Ringer's had an interfering peak with Crn and was therefore not used for the human Cr microdialysis or the HPLC validation procedure.

In vivo: Three microdialysis samples (~30 μl per sample) were obtained from healthy human volunteers during the baseline portion of retrodialysis (2 μl min^{-1}, lactated Ringers) from a previous study performed by the Department of Pharmaceutics at the University of Florida [40]. These samples were examined for peaks co-eluting with Cr. Due to a small sample volume, only one injection was possible and therefore we were unable to spike microdialysis samples with Cr. However, Cr was spiked into lactated Ringers for comparison and these samples were compared to samples from the in vitro ''no net flux'' experiments.

As part of a human study previously published by our laboratory [41], unbound concentrations of Cr in the interstitial space in thigh muscle (vastus lateralis) were measured by microdialysis. One dialysis probe (CMA 60, CMA Microdialysis) was inserted into a thigh muscle and calibrated as previously described [41]. The disappearance rate through the membrane was employed as the in vivo recovery by plotting the

concentration into the probe or perfusate (C_{in}) against the difference between the concentration out of the probe or dialysate and the perfusate ($C_{out}-C_{in}$). The in vivo probe recovery value was the slope of regression; the point of ''no net flux'', or when $C_{out}-C_{in}=0$, was utilized as the endogenous Cr levels.

2.6. Protein binding

Protein binding was determined by ultrafiltration [42]. Human plasma was spiked with 50 mg l^{-1} Cr and loaded into an ultrafiltration tube (Centrifree, Millipore, Bedford, MA, USA). Samples were kept at room temperature for 30 min and centrifuged for 5 min at 1000 *g* (Dynac II Centrifure, Clay Adams, Franklin Lake, NJ, USA). Ultrafiltrate and unfiltered plasma were acidified with plasma–6% perchloric acid (2:1) and centrifuged for 5 min at 20 000 *g*. Binding was calculated as [1−(filtrate/unfiltered plasma)].

2.7. Product quality

Various over-the-counter (OTC) CrM products (CreaTeam, Nutrasense, Shawnee Mission, KS, USA; Pure Creatine Monohydrate, Weider Nutrition, Salt Lake City, UT, USA; Micronized Creatine, MET-Rx Substrate Tech., Irvine, CA, USA; Creatine Fuel Capsules, Twin Labs., Ronkonkoma, NY, USA; Pure Creatine Monohydrate, General Nutrition, Pittsburgh, PA, USA) were analyzed for percent labeled claim. All products were labeled as 100% creatine monohydrate powder except Creatine Fuel Capsules which were soft gelatin capsules. In the latter case, capsules were opened and powder removed and it was not necessary to separate active ingredient from excipients. For each product a 50 mg l^{-1} solution was prepared in normal saline. Products were randomized and blinded prior to analyzing for purity.

3. Results

3.1. Normal saline

Validation was performed in normal saline because it is the perfusion media to be used during microdialysis experiments in our laboratory as well as the solvent used to assess percent labeled claim in OTC products. Validation of the assay in normal saline was performed on three different occasions (Table 1). Elution times for Cr and Crn in normal saline were 3.2 and 4.0 min, respectively (Fig. 1A). Linearity was tested from 1 to 100 mg l^{-1}, however, Cr in normal saline was only linear in the range of 1–75 mg l^{-1} with an *R*-value of 0.999. The lower limit of quantification was 1 mg l^{-1}. The resulting regression after injecting 25 μl was $y=6937(x)+3191$.

To examine the effects of freezing on Cr stability, freshly prepared standards in saline were compared to samples frozen for 3 and 8 days at −20 °C. There was no significant difference between peak height for freshly prepared samples and those frozen for 3 and 8 days. The variability of these samples was within the upper limit of the RSD for the assay (6%). It was not necessary to test bench stability of Cr in normal saline because it was previously reported that Cr is most stable at neutral to high pH ($k_{DEGRAD}=0.0171$

Table 1
Validation of HPLC assay using normal saline as a matrix

	Day 1			Day 2			Day 3		
	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3
Nominal value	5	25	75	5	25	75	5	25	75
Mean	4.8	25.3	74.9	4.4	25.2	75.2	4.8	25.6	75.6
SD	0.02	0.07	0.82	0.05	0.02	1.43	0.28	0.36	1.53
n	3	3	3	3	3	3	3	3	3
RSD (%)	0.42	0.26	1.1	1.1	0.072	1.9	5.9	1.41	2.0
Accuracy (%)	−3.1	1.2	−0.12	−12	0.83	0.26	4.8	2.4	0.75

QC=Quality control, RSD=relative standard deviation (SD/mean), SD=standard deviation, *n*=number of replicates, accuracy=[(nominal−mean)/mean].

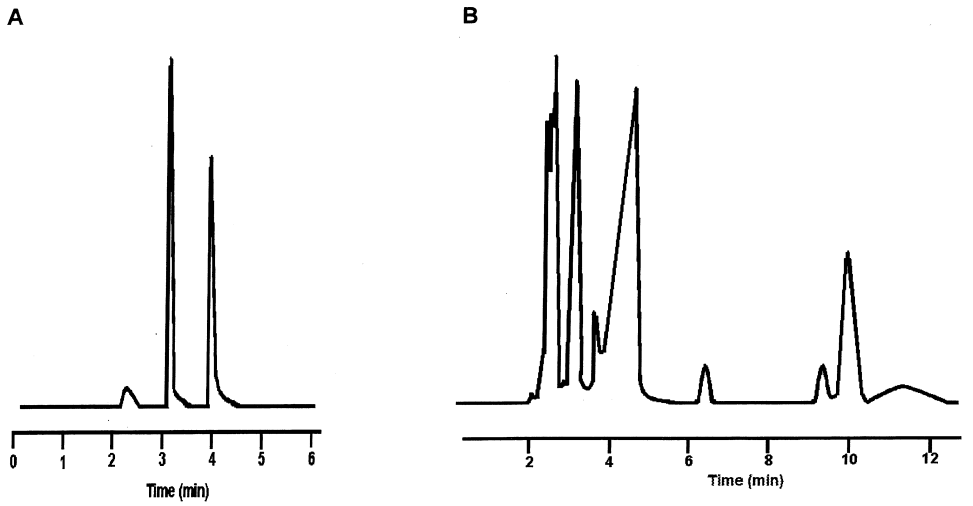

Fig. 1. (A) Chromatogram after injection of 25 μl of normal saline spiked with a final creatine concentration was 50 mg l^{-1}. Creatine=3.2 min, creatinine=4.0 min. (B) Chromatogram after injection of 50 μl of human plasma spiked with creatine monohydrate for a final concentration of 25 mg l^{-1}. Creatine 3.2 min and creatinine=3.8 min.

day^{-1} at pH 6.9 and 0.0093 day^{-1} at pH 8.5) [43]. Cr in normal saline produces a basic solution (pH >7).

3.2. Plasma samples

Elution times for Cr and Crn after spiking human plasma were 3.2 and 3.8 min, respectively (Fig. 1B). A co-eluting peak with Crn was detected at 4 min but was only present in certain individuals. Calibration was linear in the range of 5 through 50 mg l^{-1} with an *R*-value of 0.998. The RSD values for within and between days can be found in Table 2. The lower limit of quantification was 5 mg l^{-1}. The resulting regression after injecting 50 μl of sample was $y=16\,666(x)+29\,828$. The higher slope of the regression data compared to the saline regression is most likely a function of the larger injection volume (50 μl for plasma versus 25 μl for saline). To examine bench stability, acidified samples were injected at the beginning and end of a validation run. There was <1.5% decrease in peak height of acidified plasma samples over a validation run period (time=5.5 h). This confirms the predicted rates of Cr degradation in solution in that the rate constants for the conversion of Cr to Crn in solution at pH 1.4 and 3.7 are 0.0127 and 0.0895 day^{-1}, respectively [43]. Based on these rate constants, in 6 h <0.5% of Cr will be lost at pH 1.4 and <2.5% at pH 3. Recovery from plasma was estimated at 89%.

Table 2
Validation of the HPLC assay using human plasma as a matrix

	Day 1				Day 2				Day 3			
	QC 1	QC 2	QC3	QC 4	QC 1	QC 2	QC3	QC 4	QC 1	QC 2	QC3	QC 4
Nominal value	5	10	20	40	5	10	20	40	5	10	20	40
Mean	4.5	10.5	21.3	39.3	4.5	9.8	20.2	37.8	4.5	9.7	19.3	37.6
SD	0.11	0.22	0.43	2.26	0.23	0.15	0.77	0.44	0.17	0.07	0.17	0.75
n	6	6	6	6	6	6	6	6	6	6	6	6
RSD (%)	2.6	2.1	2.0	5.7	5.0	1.6	3.8	1.2	3.7	0.7	0.9	2.0
Accuracy (%)	−10.8	5.0	6.4	−1.7	−9.4	−2.3	0.9	−5.4	−10.2	−2.6	3.3	−6.0

QC=Quality control, RSD=relative standard deviation (SD/mean), SD=standard deviation, *n*=number of replicates, accuracy=[(nominal−mean)/mean].

3.3. Microdialysis

From the in vitro experiments, the ‘‘no net flux’’ method of calibration of unspiked lactated Ringer’s is shown in Fig. 2A. The graph shows good linearity and estimates the point of no net flux at 0.65 mg l^{-1}; recovery from the probe was 93%. Fig. 2B illustrates the results of ‘‘no net flux’’ when the solution was spiked with 25 mg l^{-1} of Cr. The measured concentration of the solution by the HPLC method was 28.3 mg l^{-1}; the ‘‘no net flux’’ method estimated the concentration of Cr at 31.3 mg l^{-1}.

Microdialysis samples from healthy human volunteers from a previous study were screened for interfering peaks eluting with Cr. No interfering peaks were detected in these samples (data not shown). This is not unexpected as interstitial levels of Cr should be lower than plasma concentrations due to active transport processes that remove Cr from the interstitial space. Fig. 2C is a representative calibration curve from ‘‘no net flux’’ obtained from a single human volunteer from a previously published study involving the microdialysis of Cr [41]. Recovery in this subject was 47% and baseline interstitial concentration, taken as the point of no net flux, was calculated as 0.23 mg l^{-1}, baseline plasma Cr in this subject was 14.8 mg l^{-1} thus supporting the hypothesis of lower interstitial levels due to active

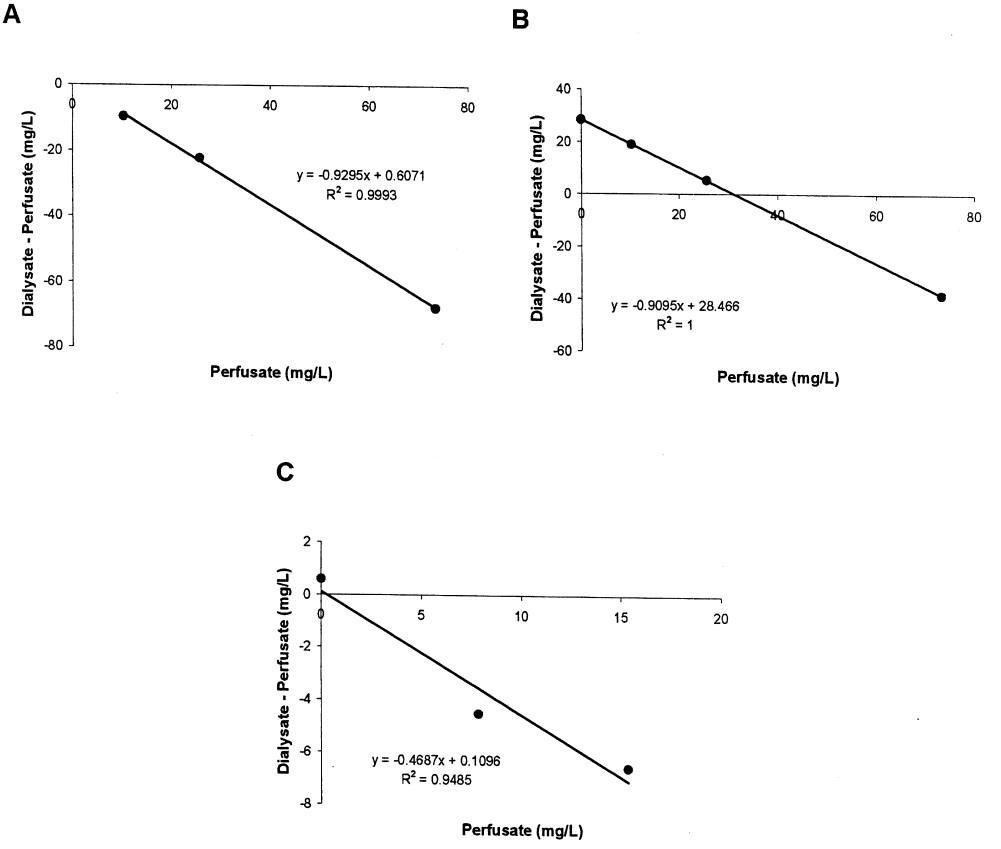

Fig. 2. Representative ‘‘no net flux’’ calibration curves from (A) lactated Ringers solution with 0 mg l^{-1} Cr, (B) lactated Ringers solution with 25 mg l^{-1} Cr, and (C) from one human volunteer collected in normal saline. Recovery is determined from the slope of the regression line.

transport into muscle. Full interstitial Cr versus time profiles can be found elsewhere [41].

3.4. Protein binding

Plasma spiked with 50 mg l^{-1} Cr was found to have a plasma protein binding of 3.8±4.6% (mean±standard deviation, $n=4$) ($f_u=0.96$). Unspiked plasma had a protein binding value of −10.1±5.6% ($n=4$) ($f_u=1.10$); this value is mostly likely a result of being close to the lower limit of quantification and is not reflective of true protein binding. Normal saline spiked with 50 mg l^{-1} Cr resulted in a 5.4±0.33% ($n=3$) loss of Cr.

3.5. Product purity

Five OTC Cr products (Met-Rx, General Nutrition, Twin Labs., Nutrasense, Weider) were randomly chosen to assess percent Cr from powder dissolved in normal saline. Fig. 3 shows the percent labeled claim for these products. One product, Twin Labs., was an encapsulated powder that contained excipients (i.e., magnesium stearate) but the other four products' ingredient label claim only creatine monohydate. There were no detectable peaks other than Cr in any product chromatograms (data not shown).

4. Conclusion

This HPLC assay was developed and validated for use in a clinical pharmacokinetic study but was not fully validated according to recent US Food and Drug Administration (FDA) guidelines. Previous methods using HPLC to assay Cr in muscle, urine, and blood, used ion-pair agents [16,36], ion-exchange columns [23,34] or gradient elution [44]. Previous assays also used lower concentrations of potassium phosphate buffer [16] and some others have used lower [34] or higher pH [16] buffers than the current study. The higher concentration of buffer and lower pH used in the current study enhanced the separation of Cr from Crn and improved peak shape in this particular assay. Previous methods have shown similar ranges of linearity [10,11,35], similar RSDs [16,23,34,44] and similar retention times for Cr and Crn [16,34,36] as this current assay; however, this assay did not use ion-pairing agents or gradient elutions. Accuracy and RSD of this current method are within the specified FDA guidelines and are comparable with other published assays without the concerns of ion-pair equilibrium, organic solvents, or specialty columns.

Resting plasma levels of creatine are between 7 and 13 mg l^{-1} but after an oral 5 g dose of CrM (a

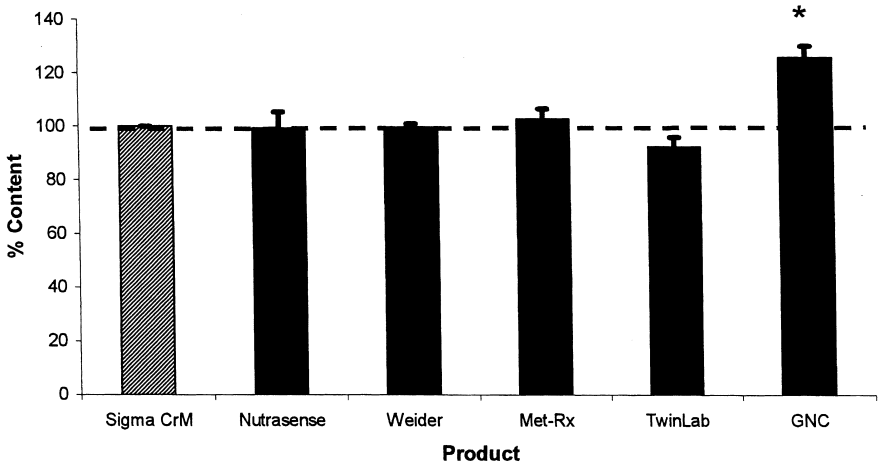

Fig. 3. Percent labeled content of selected OTC creatine products ($n=3$). Dotted line indicates 100%. Sigma CrM (hatched column) was used as the reference compound. * $P<0.05$ for one-way ANOVA with Tukey's HSD post-hoc analysis.

typical CrM dose), peak levels can reach >150 mg l^{-1}. This assay is sensitive enough to quantify basal levels in plasma but dilution would be necessary for samples around the maximum plasma concentrations (C_{MAX}). This is a possible limitation of most HPLC assays but enzymatic assay may be better suited to quantify these higher levels but reliability and accuracy of these assays are suspect at lower concentrations.

Normal saline was used in the validation process since it is the perfusion media for microdialysis studies. Interstitial concentrations should reflect unbound levels of Cr in the plasma. In this case of Cr, almost all Cr should be unbound to plasma protein as protein binding was found to be low (<10%). Additionally, recovery from microdialysis probes is an issue that needed to be addressed since recovery is generally <100% [45]. The results from this study found that in vitro recovery was nearly 100%, however in vivo recovery was <50%. The assay in normal saline showed greater sensitivity than plasma and will be able to detect interstitial concentrations for microdialysis even with lower recovery (<10%). We screened human microdialysis samples from several healthy volunteers and found no Cr or co-eluting peaks in these samples and results from a previous Cr study confirm these findings [41].

In conclusion, a simple and precise HPLC assay for plasma and microdialysis samples was developed and validated. This assay was used to determine protein binding of Cr to plasma proteins and quantify amount of creatine monohydrate in some over-the-counter creatine products. This assay does have limitations of a long run time for plasma samples (27 min), possible instability of a C_{18} column due to a pure aqueous mobile phase and the need for dilution of plasma values around the maximal concentrations after an oral dose. The advantages of this assay: (1) little need of organic solvents, (2) no need for ion-pairing agents and (3) no need for ion-exchange columns and therefore offer an alternative to previously published assays.

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