ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 939–945

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

A simple LC method with UV detection for the analysis of creatine and creatinine and its application to several creatine formulations

Alekha K. Dash *, Angeli Sawhney

Department of Pharmacy Sciences, School of Pharmacy and Allied Health Professions, Creighton University, 2500 California Plaza, Omaha, NE 68178, USA

Received 2 November 2001; received in revised form 28 March 2002; accepted 1 April 2002

Abstract

The objective of this study was to develop a simple and sensitive LC method for the determination of creatine and creatinine in various creatine supplement formulations. The chromatographic system comprised of a LC-600 pump, SCL-6B system controller, and SPD-6AV detector (Shimadzu, Japan). The mobile phase consisted of 0.045 M ammonium sulfate in water. The chromatographic separation was achieved at ambient temperature on a Betabasic C-18 column (250 × 4.6 mm, Keystone Sci.). The flow rate was maintained at 0.75 ml/min and effluents are monitored at 205 nm. 4-(2-Aminoethyl)benzene sulfonamide was used as an internal standard (IS). This method required less than 7 min of chromatographic time. The standard curves were linear over the concentration range of 1–100 μg/ml for creatine and 2–100 μg/ml for creatinine, respectively. The relative standard deviations (RSD) for the within-day and day-to-day precision for creatine were within 1.0–4.6 and 2.2–4.7%, respectively. The RSD for the accuracy of creatine assay was in the range of 2.4–4.7%. The RSD values for the within-day precision, day-to-day precision and accuracy for creatinine validation were 1.7–4.4, 2.3–5.4 and 2.4–4.8%, respectively. This method was used to determine: (i) the creatine concentration in various marketed products; (ii) saturated solubility of various creatine salts; and (iii) stability of creatine in aqueous solution. In conclusion, a simple and sensitive LC method with UV detection was developed for the simultaneous determination of creatine and creatinine in formulations. Di-creatine citrate salt showed a higher aqueous solubility (at 25 °C) as compared to creatine and creatine monohydrate. Some of the over-the-counter (OTC) products tested contained a very low level of creatine in contrast to their label claim. Substantial conversion of creatine into creatinine was noticed in liquid formulation. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Creatine; Creatinine; Stability; UV detection; Creatine formulation; Creatine to creatinine conversion

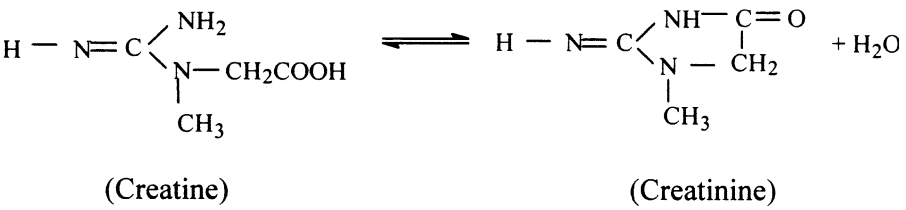
1. Introduction

Creatine is a naturally occurring guanidino compound mostly found in skeletal muscles [1].

* Corresponding author. Tel.: +1-402-280-3188; fax: +1-402-280-1883.
E-mail address: adash@creighton.edu (A.K. Dash).

0731-7085/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(02)00167-X

Creatine plays an important role in the metabolism of proteins. Therefore, it appears to increase lean body mass, high-intensity power output, and strength in humans. Recently, there has been a great interest among consumers and researchers concerning the therapeutic application of creatine and benefits of using creatine as a dietary supplement. Studies have also reported



Scheme 1. Creatine and creatinine equilibrium in aqueous solution.

Table 1
Within-day and day-to-day analytical precision for creatine

Within-day			Day-to-day	
Concentration (μg/ml)	Mean PAR[a]	RSD (%)	Mean PAR[b]	RSD (%)
0	0	0	0	0
1	0.02	4.7	0.02	2.7
20	0.31	3.6	0.31	1.7
40	0.59	3.1	0.61	4.7
50	0.74	3.5	0.78	3.3
70	1.04	2.2	1.06	1.4
100	1.45	2.7	1.49	1.0
Slope	0.015 ± 0.0001	0.94	0.0147 ± 0.0004	2.7

[a] $n = 4$.
[b] $n = 6$, over a period of 28 days.

Table 2
Within-day and day-to-day analytical precision for creatinine

Within-day			Day-to-day	
Concentration (μg/ml)	Mean PAR[a]	RSD (%)	Mean PAR[b]	RSD (%)
0	0	0	0	0
2	0.06	4.4	0.06	2.9
10	0.28	4.4	0.28	5.4
40	1.11	2.9	1.09	3.6
50	1.37	2.5	1.38	2.3
70	1.91	3.8	1.90	3.1
100	2.69	1.7	2.69	3.8
Slope	0.027 ± 0.0006	2.07	0.0269 ± 0.0008	3.2

[a] $n = 4$.
[b] $n = 6$, over a period of 28 days.

Table 3
Accuracy in the analysis of creatine in QCs

Actual concentration (μg/ml)	Measured concentration[a] (μg/ml)	Accuracy[b]
4	3.99 ± 0.11	99.9 ± 2.7
30	29.8 ± 0.75	99.2 ± 2.5
80	82.1 ± 2.2	102.6 ± 2.4

[a] Mean ± S.D.; $n = 7$.
[b] Accuracy = (measured concentration/actual concentration) × 100.

Table 4
Accuracy in the analysis of creatinine in QCs

Actual concentration (μg/ml)	Measured concentration[a] (μg/ml)	Accuracy[b]
4	4.11 ± 4.7	102.8 ± 4.8
30	31.1 ± 2.7	103.6 ± 3.7
90	90.5 ± 2.2	100.6 ± 2.4

[a] Mean ± S.D.; $n = 7$.
[b] Accuracy = (measured concentration/actual concentration) × 100.

that the use of creatine supplementation can reduce fatigue, accelerate both energy recovery and muscle growth, and increase muscle strength [2,3]. It also promotes muscle size without affecting body fat and regenerates ATP-energy to increase the amount of muscle working time.

Creatine is a very weak base and shown to be converted to creatinine at acidic conditions as shown in Scheme 1 [4]. Various HPLC methods have been reported for the analysis of creatine [5–10]. In most instances, reversed-phase ion pairing HPLC with UV-photometric detection was utilized. Secondly, all of these reported methods have validated the assay procedure only for creatine. In this investigation, a simple and sensitive HPLC method without the use of ion-pairing agent and an organic modifier in the mobile phase was developed and validated for both creatine and creatinine in aqueous samples. Therefore, this method could be reliable in determining creatine and creatinine simultaneously in marketed formulations. The application of this method includes determination of creatine and creatinine concentration in some marketed formulations, to study their solubility, and stability in aqueous solution.

2. Experimental methods

2.1. Materials

Creatine (Lot: 08912PS), creatinine (Lot: A011608003), and 4-(2-aminoethyl)benzene sulfonamide (Lot: 02816TP) (Sigma, St. Louis, MO, USA); water (HPLC grade, Lot: 007403), and ammonium sulfate (Lot: 50K0246) (Fisher Scientific, NJ, USA) were used as received. Three creatine formulations (two effervescent and one liquid) were obtained from commercial sources. Creatine effervescent powders (Creatine Edge and Creatine Clear) were obtained from FSI Nutrition, Omaha, USA. Creatine liquid formulation (Creatine Serum, Muscular Marketing, CA, USA) was obtained from a General Nutrition Center. The 0.45 μm syringe filters used in this study were obtained from Millipore (Millipore Inc., Bedford, MA, Lot: N6SMB345X).

2.2. Chromatography

The HPLC system consisted of a pump (Model LC-600) programmed by a system controller (Model SCL-6B), an UV–Visible spectrophotometric detector (model SPD-6AV), all from Shimadzu (Tokyo, Japan). The mobile phase consisted of 0.045 M ammonium sulfate in water and the flow rate was maintained at 0.75 ml/min and monitored at 205 nm. Chromatographic separation was achieved at room temperature on a Betabasic C-18 column (250 × 4.6 mm, Keystone Sci., IL, USA). 4-(2-Aminoethyl)benzene sulfonamide was used as an internal standard (IS).

2.3. Standard solutions and sample preparation

The stock standard solution was prepared by dissolving 0.1 g of creatine or creatinine in 100 ml mobile phase. Various standard solutions (1–100 μg/ml) were then prepared by diluting the resulting stock solution with mobile phase to yield

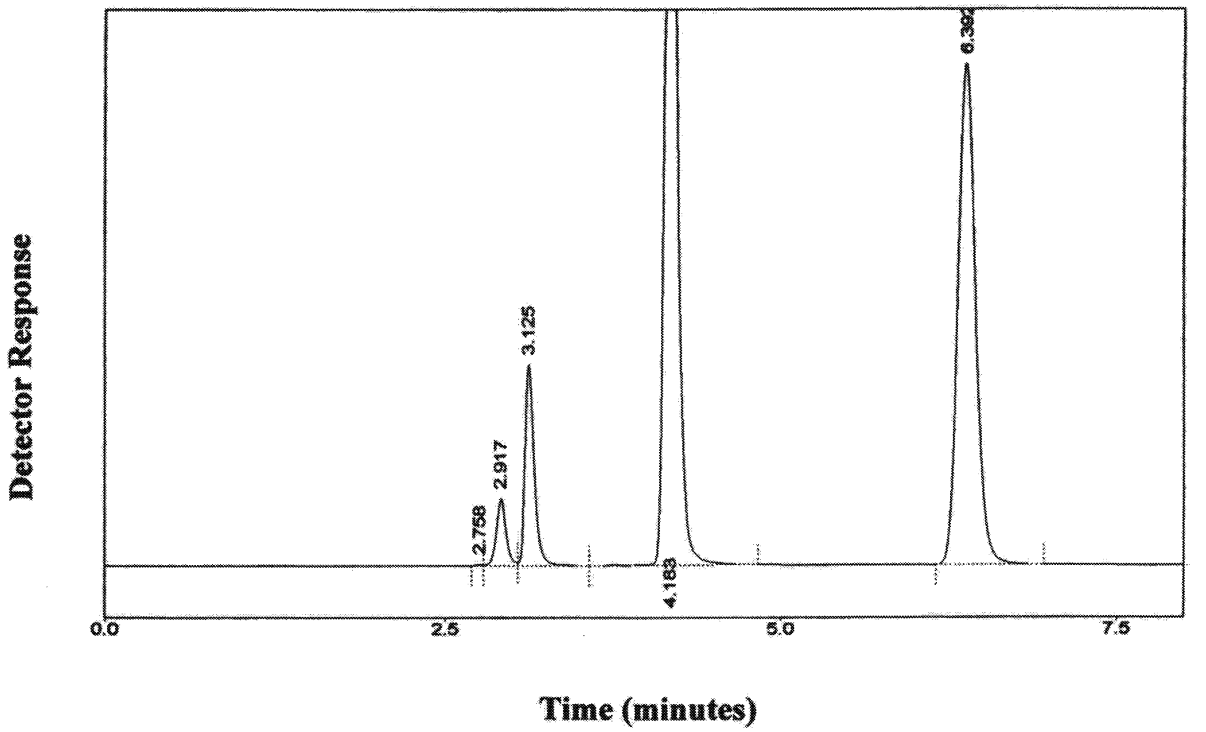


Fig. 1. A representative chromatogram obtained following injection of an aqueous sample containing creatine (13.1 μg/ml, retention time 3.1 min), creatinine (50.0 μg/ml, retention time 4.2 min) and the IS (90.0 μg/ml, retention time 6.4 min).

nominal concentrations over a range 1–100 μg/ml for creatine and 2–100 μg/ml for creatinine.

4-(2-Aminoethyl)benzene sulfonamide solution (300 μg/ml) was prepared in methanol. This solution was used as IS. This compound was used as an IS because of its complete base line separation from both the analytes of interest under the described chromatographic conditions.

The IS solution (60 μl) was added to borosilicate culture tube and evaporated to dryness at 40 °C in an oven. The standard solution or the sample to be analyzed (200 μl) was spiked to the test tube and vortexed for 10 s. An aliquot (20 μl) was analyzed by LC.

Mobile phase was prepared by mixing 5.9 g of ammonium sulfate in HPLC water and volume was adjusted to 1000 ml. This solution was filtered through a 0.45-μm MAGNA Nylon, 47-mm filter (MSI, MA, USA).

2.4. Determination of creatine and creatinine concentration in marketed formulations

Three marketed formulations (two effervescent powders and one liquid formulation) were used in this study. Known amount of the formulation was weighed into volumetric flask and volume was adjusted with mobile phase. Samples were filtered

Table 5
Solubility of various creatine salts and creatinine in water at 25 °C

Creatine salt	Solubility (mg/ml)[a]
Creatine	18.8 ± 2.8
Creatine monohydrate	16.6 ± 0.3
Dicreatine citrate	27.2 ± 1.9
Creatinine	178.6 ± 7.2

[a] Mean $\pm$ S.D.; $n = 3$.

Table 6
Quantitation of creatine in marketed formulations

Creatine sample (type)	pH	Level claim for creatine	Experimentally determined creatine level[a]
Creatine edge (effervescent powder)	4.3	100% (w/w)	99.2 ± 0.04
Creatine clear (effervescent powder)	4.3	100% (w/w)	99.6 ± 0.03
Creatine serum (liquid drop)	4.1	100% (w/v)	1.71 ± 0.18

[a] Mean ± S.D.; $n = 3$.

through a syringe filter (Millipore Inc., Bedford, MA, Lot: N6SMB345X) and 20 μl of the filtered sample was analyzed by LC.

2.5. Determination of saturated solubility of creatine and creatinine in water at 25 °C

The saturated solubility of creatine, creatine monohydrate, creatinine and di-creatine citrate was determined at 25 °C. Excess amount of the samples were added to 5 ml of water in borosilicate screw-capped glass bottles and shaken continuously in an Orbit Environ-Shaker (Lab-line Instruments, IL, USA). After 48 h, the samples were centrifuged at 2000 rpm for 5 min and supernatant was collected and 20 μl of the sample was injected on to the HPLC. In case of di-creatine citrate and creatinine, additional dilution of samples were needed prior to injection on to the LC system.

2.6. Stability of creatine in water at 25 °C

Stability of creatine in water at 25 °C was carried out over a period of 10 days. Creatine solutions in water (80 μg/ml) in triplicate were shaken in a water bath at 25 °C in screw-capped bottles. At definite time intervals, 1 ml sample was collected and analyzed for both creatine and creatinine.

3. Results and discussion

3.1. Assay validation

3.1.1. Linearity

Standard curves were constructed by plotting peak area ratio (PAR) (peak area of drug/peak area of the IS) versus concentration of the drug. Standard curves for creatine were linear over the concentration range of 1–100 μg/ml. The equation of the standard curve relating the PAR to creatine concentration (C in μg/ml) in this range was $\mathrm{PAR} = 0.0149C + 0.0122$, $R^2 > 0.999$. For creatinine, the standard curve was linear over 2–100 μg/ml and the equation of the line was $\mathrm{PAR} = 0.027C + 0.0142$, $R^2 > 0.999$.

3.1.2. Precision

Within-day precision of the assay was determined by analysis of replicate ($n = 4$) samples of six different concentration on the same day. To determine day-to-day precision, the same solutions on six different days were analyzed during a period of 28 days. The variability in the PAR at each concentration are presented in Tables 1 and 2. Within-day and day-to-day relative standard

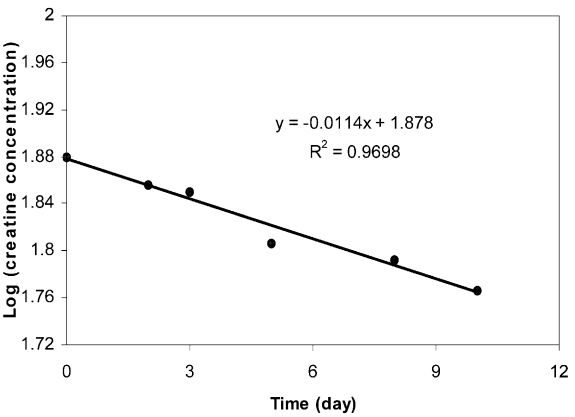


Fig. 2. A plot of log (creatine concentration) versus time to show the first-order degradation kinetics of creatine in aqueous solution. The data represents the mean of triplicate samples. The initial concentration of creatine was 80 μg/ml.

deviation (RSD) values for the creatine assay ranged from 1.0 to 4.7 and 2.2 to 4.7%, respectively. During this period, the stock solution and standard solutions were stored under refrigeration (4 °C). The within-day and day-to-day precision RSD values for creatinine were 1.7–4.4 and 2.3–5.4%, respectively.

3.1.3. Accuracy

Three quality control samples (QCs) for creatine and creatinine were refrigerated at 4 °C over a period of 28 days. These samples were analyzed seven times during this time and the accuracy of the assay was determined by comparing the measured concentration to its nominal value. The results of this study are depicted in Tables 3 and 4. The RSDs for creatine ranged from 2.2 to 4.7%. The RSDs for creatinine accuracy determination was from 2.4 to 4.7%.

3.1.4. Sensitivity

The lowest limit of reliable assay measurement criteria described by Oppenheimer et al. [11] was used to determine the sensitivity parameters. Seven different standard curves were used in this calculation. The critical level was defined as the assay response above which an observed response is reliably recognized as detectable. This value is also considered as the threshold value, defining detection. If the measured value exceed this value then the presence of analyte is detected, otherwise it is not. This was 0.09 ± 0.03 μg/ml (mean ± S.D.). The detection level is the actual net response, which may a priori be expected to lead to detection. This is the least value of the true concentration that is 'nearly sure' to produce a measured value that results in detection. This was 0.17 ± 0.06 μg/ml (mean ± S.D.). The determination level is the concentration at which the measurement precision will be satisfactory for quantitative determination was 0.46 ± 0.17 μg/ml (mean ± S.D.) for a level of precision of 10% RSD. For creatinine assay critical level was 0.06 ± 0.01 μg/ml (mean ± S.D.), the detection level was 0.12 ± 0.03 μg/ml (mean ± S.D.) and determination level was 0.33 ± 0.08 μg/ml (mean ± S.D.) for a level of precision of 10% RSD.

3.2. Applications of the HPLC method

The LC method was used in the assay of creatine and creatinine in aqueous solutions. Fig. 1 represents a typical chromatogram for creatine, creatinine and IS in aqueous solution. Both creatine and creatinine peaks had a good baseline separation with retention times of 3.1 and 4.2 min, respectively. Under similar chromatographic condition, the IS had a retention time of 6.4 min.

3.2.1. Saturated solubility of creatine and creatinine

This LC method was used to evaluate the saturated solubility of various creatine salts, and creatinine at 25 °C. The results of this study are depicted in Table 5. The solubility of dicreatine citrate was found to be higher than creatine and creatine monohydrate. However, the solubility of creatinine in water at 25 °C was found to be the highest.

3.2.2. Determination of creatine and creatinine concentrations in marketed formulations

Creatine and creatinine concentration in three marketed formulations was analyzed using this method and results were depicted in Table 6. Interestingly, the creatine concentration in the liquid formulation was less than 2% of the label claimed. Most of the active constituent was converted to creatinine. However, no significant conversion of creatine to creatinine was noticed in the two solid effervescence formulations. No interference from the inactive materials were also detected in the chromatograms of the three formulation studied.

3.2.3. Stability of creatine in water at 25 °C

Stability of creatine in water at 25 °C was carried out over a period of 10 days. A log (creatine concentration) versus time plot was linear indicating the degradation of creatine in solution followed first-order kinetics (Fig. 2). The first-order degradation rate constant for creatine calculated from the slope of the line and was 0.0263 per day at 25 °C.

4. Conclusions

A simple and sensitive method was developed and validated for quantitation of creatine and creatinine in pharmaceutical formulations. Some marketed formulations were found to contain less than 2% of the label-claimed creatine and the major component was detected to be creatinine. This method is useful in determining the saturated solubility of various creatine salts. Di-creatine citrate has the highest solubility as compared to creatine and creatine monohydrate. The degradation of creatine in water at 25 °C follows a first-order degradation kinetics with a rate constant of 0.0263 per day. This study further suggests that creatine liquid dosage forms should have an expiration date which can be calculated based on the degradation rate constant for that particular formulation.

Acknowledgements

This work was partly presented at the AAPS Annual Meeting and Exposition, Denver, CO, October 20–25, 2001 [12] and supported by FSI nutrition, Omaha, NE.

References

[1] A.L. Green, E.J. Simpson, J.J. Littlewood, I.A. Macdonald, P.L. Grrehaff, Acta Physiol. Scand. 158 (1996) 195.
[2] P.L. Greenhaff, A. Casey, A.H. Short, R. Harris, K. Soderlund, E. Hultman, Clin. Sci. 84 (1993) 565.
[3] C.P. Earnest, P.G. Snell, R. Rodriguez, A.L. Almada, T.L. Mitchell, Acta Physiol. Scand. 153 (1995) 207.
[4] G. Edgar, H.E. Shiver, J. Am. Chem. Soc. 47 (1925) 1170.
[5] E. Juenging, H. Kammermeier, L.O. Nordesjo, Scand. J. Clin. Lab. Invest. 35 (1974) 87.
[6] E. Harmsen, P.P. De Tombe, J.W. De Jong, J. Chromatogr. 230 (1982) 131.
[7] M. Dunnett, R.C. Harris, C.E. Orme, Scand. J. Clin. Lab. Invest. 51 (1991) 137.
[8] Y. Yokoyama, S. Horikoshi, T. Takahashi, H. Sato, J. Chromatogr. A 886 (2000) 297.
[9] Y.D. Yang, Biomed. Chromatogr. 12 (1998) 47.
[10] S. Takaaki, S. Susumu, O. Munenaka, S. Takeshi, H. Toshiyuki, Fish Sci. 63 (1997) 769.
[11] L. Oppenheimer, T.P. Capizzi, R.M. Weppelman, M. Mehta, Anal. Chem. 55 (1983) 638.
[12] A. Dash, AAPS Pharm. Sci. Suppl. 3 (3) (2001) M2249.