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A simple HPLC method with pulsed EC detection for the analysis of creatine

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Abstract

The objective of this study was to develop a simple and sensitive LC method for the determination of creatine in aqueous solutions as well as in rat plasma using electrochemical detection. The chromatographic system consisted of a GP50 gradient pump, an ED40 pulsed electrochemical detector, and an AI-450 chromatography automation system (Dionex). The mobile phase consisted of a mixture of water, acetonitrile, 0.01 M sodium acetate, and 1.0 M sodium hydroxide (2.5:2.5:90:5, V/V/V/V) at a flow rate of 1.0 ml/min. The chromatographic separation was achieved at 45 °C on a column with a polyhydroxylated glucose and sulfonated stationary phase. The retention times of creatine and creatinine was 3.50 and 4.73 min, respectively, with creatine fully resolved from its major degradation product, creatinine. The standard curves were linear over the concentration range of 0–20 μg/ml. Within-day and day-to-day relative standard deviations (R.S.D.) were less than 10%. This method was used to study dissolution characteristics of various creatine salts in water.
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Keywords: Creatine; Creatinine; Pulsed electrochemical detection; HPLC

1. Introduction

Creatine is a naturally occurring guanidino compound mostly found in skeletal muscles [1]. Creatine plays an important role in the metabolism of proteins. Since it appears to increase lean body mass along with high-intensity power output and strength in humans, there has been great interest among consumers and researchers concerning the therapeutic application of creatine and benefits of using creatine as a dietary supplement [1]. Studies have shown that the use of creatine supplementation reduces fatigue, accelerates muscular energy recovery, and increases 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 [4].

Chemically, creatine is a weak base. In aqueous solution, and under acidic condition it converts to

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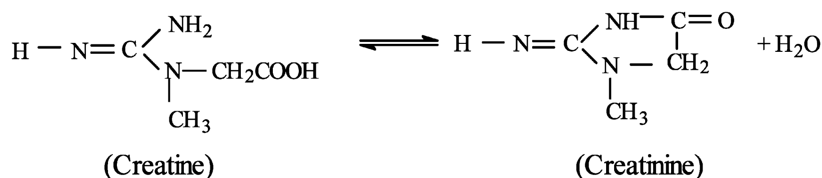

Scheme 1. Equilibrium between creatine and creatinine in aqueous solution.

creatinine. The creatine–creatinine equilibrium in aqueous solution is shown in Scheme 1 [5]. According to this study, the ratio of creatinine to creatine is around 32 at pH 2. However, at pH 7 this ratio is around 1. It was also evident the rate of conversion is more prominent in the acidic range (pH 1–2.5) as compared with the basic pH [5]. Various HPLC methods have been reported for the analysis of creatine [6–10]. In most instances, reversed-phase ion-pairing HPLC with UV–photometric detection was utilized. Sodium dodecyl sulfate (2 mM) has been utilized as the ion-pairing agent in the mobile phase. Low-cost cation exchange chromatography using an ODS column dynamically coated with hexadecylsulfonate with ethylenediammonium as eluting ion has been used for the quantitation of creatine and creatinine [8]. This procedure provides fixed-site ion exchangers that eliminates the addition of ion-pairing agent in the mobile phase. The other advantages of this method include greater chromatographic efficiency, lower cost, increased selectivity for specific ions, and flexibility in tuning ion-exchange capacity. However, not a single study has utilized electrochemical detection for the determination of creatine in aqueous and biological samples. Electrochemical detection not only offers superior sensitivity and limits of detection (LOD) for non-aromatic compounds when compared with UV detection, but potentially can be done with a minimal of sample preparation. Electrochemical detection also offers the following potential advantages for future work in situations such as: (i) when direct analysis of samples with minimal sample volume is needed, and (ii) when online detection is essential to determine the tissue distribution of this analyte (e.g. microdialysis sampling techniques). The work presented here was undertaken to demonstrate the use of LC–PED in the determination of creatine in the presence of creatinine. The goal was to develop a sensitive and specific HPLC method for the determination of the creatine in both aqueous and plasma samples using a simple dilution procedure. This method was further utilized to study the in vitro dissolution characteristics of various creatine salts.

2. Experimental

2.1. Materials

Creatine, creatinine (Sigma, St. Louis, MO, USA), water (HPLC grade), acetonitrile, sodium acetate, sodium hydroxide (Fisher Scientific, St Louis, MO, USA), rat plasma (Taconic, NY, USA) were used as received.

2.2. Chromatography

The HPLC system consisted of a GP50 gradient pump, LC25 column oven, and ED40 pulsed electrochemical detector interfaced to a thin-layer amperometric cell with a 1-mm diameter gold working electrode. The reference electrode for the electrochemical cell was a pH-Ag/AgCl combination electrode. The counter electrode was provided by the upper half of the detection cell, made of titanium. The system was interfaced to a PC (Gateway Inc.) through an AI-250 chromatography automation system using PEAKNET® software (Dionex Inc., Sunnyvale, CA). Sample injection was provided by an electronically-activated injector equipped with a 25 μl sample loop. The mobile phase for the initial work with aqueous samples consisted of a mixture of water, acetonitrile, 0.01 M sodium acetate, and 1.0 M sodium

hydroxide (2.5:2.5:90:5, V/V/V/V). In contrast, the plasma systems used a mixture of 1.0 M sodium hydroxide and water (5:95, V/V). Chromatographic separation was achieved at 45 °C on a polyhydroxylated glucose and sulfonated column (Jordi Glucose-DVB, 250 × 4.6 mm, Alltech, Deerfield, IL) using a flow rate of 1.0 ml/min.

2.3. Standard solutions

2.3.1. Aqueous samples

The stock standard solution was prepared by dissolving 16.97 mg of creatine in 500 ml water (33.94 μg/ml). Various standard solutions were then prepared by diluting the resulting stock solution with water to yield nominal concentrations over a range 0–20 μg/ml.

2.3.2. Biological samples

Here, 'blank plasma' is defined as unspiked plasma with endogenous creatine. This plasma was obtained from a commercial source. Rat-plasma standard solutions were prepared daily by adding 5–500 μl of the stock solutions and appropriate volume (495–995 μl) of water to 5 μl of blank plasma to yield the same total volume (1000 μl). Samples were vortexed for 20 s and injected directly without further sample preparation. For creatine in the plasma system, the standard curve was linear over the concentration range of 0–20 μg/ml.

2.4. Calculations

A standard addition procedure was used for the quantitation of creatine. Peak height (PH) was plotted versus creatine concentration (C) to obtain a working calibration curve for both aqueous and plasma samples. When working with plasma samples, the peak height for creatine in the blank plasma was subtracted from each spiked plasma sample to account for the endogenous creatine. Once the calibration curve was generated for creatine in plasma, it was used to determine the amount of endogenous creatine present in the blank plasma prior to it being spiked.

2.5. Application of the HPLC method

Application of this HPLC method includes the in vitro dissolution profiles of various creatine salts and formulation.

2.5.1. In vitro dissolution studies

The in vitro dissolution characteristics of various creatine salts and a commercially available creatine supplement formulation in water were studied using a USP dissolution apparatus (Hanson Research Corporation, Chatsworth, CA) at 37 °C. At predetermined time intervals, 1.0 ml of the sample was collected and replaced with 1.0 ml of water. The creatine concentration in the dissolution medium was determined as discussed above.

3. Results and discussion

The determination of highly polar aliphatic amine compounds is most often done using liquid chromatographic separation with in-line spectrophotometric detection of the compounds after they have been derivatized with a chromophoric adduct [11,12]. Direct photometric detection of creatine and creatinine is possible yet does not result in very low LOD. The LOD that are reported for creatine using direct photometric detectors was 0.09 μg/ml [10] and 0.69 μg/ml [9] as opposed to 0.01 μg/ml reported in this study. When considering electrochemical detection, constant (DC) detection is not possible because anodic current for aliphatic compounds at many working electrodes quickly decays below detectable levels when using a constant applied potential. This loss of electrode sensitivity is attributed to either (1) passivation of the electrode surface by the oxidative products of the reaction providing analytical current; or (2) the transient nature of the catalytic conditions (e.g. surface oxides) that are short-lived when a constant potential is applied at the working electrode [13].

Pulsed electrochemical detection at noble metal electrodes was introduced for aliphatic compounds separated by HPLC [14,15]. The utility of PED is largely realized because of its multi-step potential

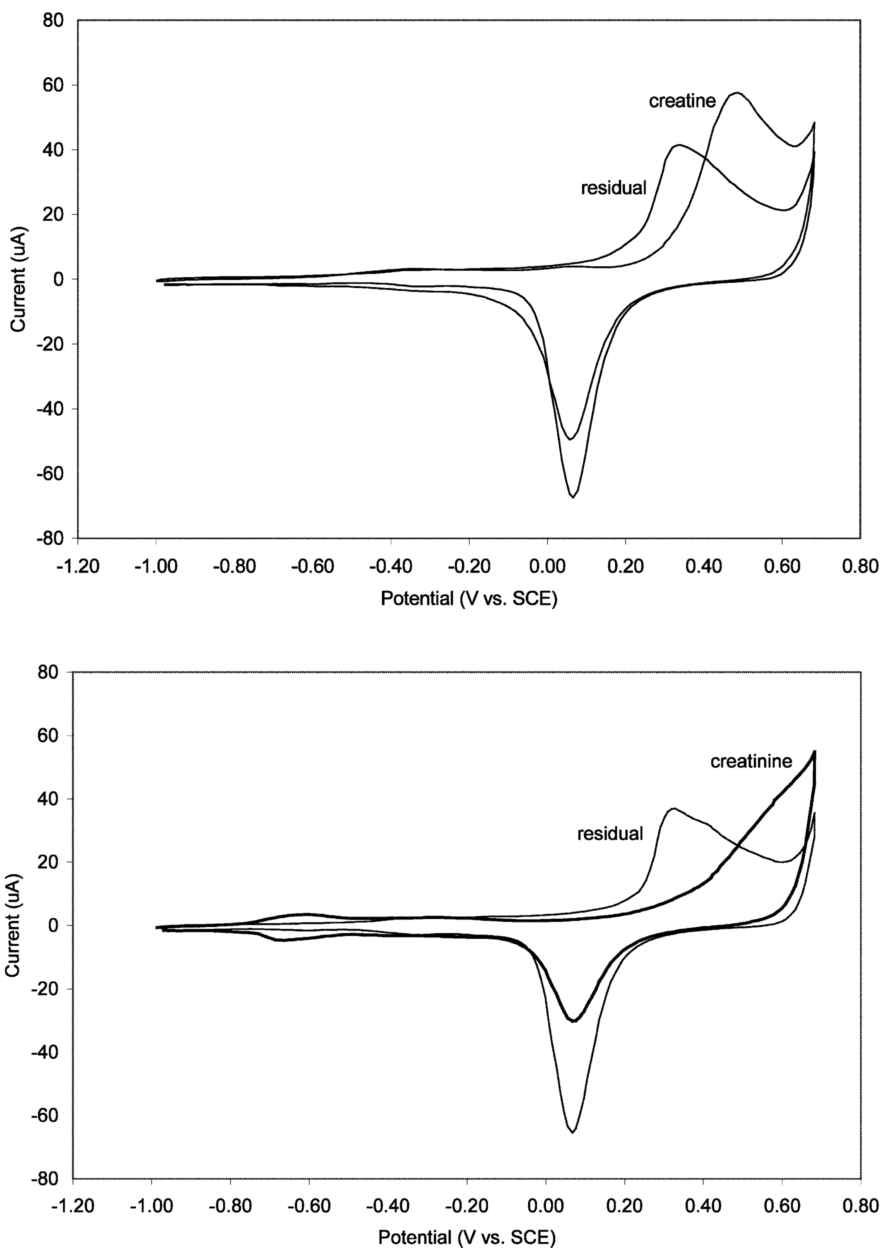

Fig. 1. (a) Voltametric response of 13.1 μg/ml creatine in 0.1 M NaOH; (b) voltametric response of 11.3 μg/ml creatinine in 0.1 M NaOH at a gold rotating disk electrode at 400 rpm. Scan rate = 100 mV/s.

waveform, which continuously regenerates the conditions responsible for providing analytical signal at the working electrode. The application of PED to amines and amino acids has been extensively reviewed elsewhere [16,17]. In the work presented here, the separation of creatine and creatinine was achieved with alkaline ($pH > 13$) mobile phases on a polymeric column, thus

Table 1
PED waveform parameters

Time (s)	Potential (V)	Integration period
0.00	0.30	
0.20	0.60	Begin
0.50	0.80	
0.60	0.35	
0.70	0.30	End
0.71	1.00	
0.90	1.00	
0.91	−0.40	
1.00	−0.40	

obviating the need for post-column addition of hydroxide as was required in previous PED methods for amines [18,19].

The PED detection strategy was optimized based upon data generated by cyclic voltammetry (CV) and pulsed voltammetry at a rotated gold disk electrode. For this, the electrode was rotated at 400 rpm and the potential cycled between −1.0 and 0.7 V versus a standard calomel electrode (SCE). The supporting electrolyte was 0.10 M NaOH. Representative results are shown in Fig. 1. The residual response represents the current obtained at the gold electrode in the absence of analyte. The most obvious features of this residual are the anodic wave due to oxide formation at the gold surface during the positive scan from 0.20 to 0.70 V, and the large cathodic peak corresponding to the reduction of this same gold oxide at 0.00 V during the negative scan. The response at the gold electrode with 13.1 μg/ml creatine and 11.3 μg/ml creatinine is shown in Fig. 1(a and b), respectively. During the positive scan, no current for the oxidation of creatine and creatinine is seen until the potential of the electrode is made greater than 0.30 V, corresponding to the potential at which oxides are formed at the gold electrode. This anodic current for the oxidation of creatine continues to increase as the electrode potential is scanned even more positive, in this case to the limit of 0.70 V. If the potential is held constant at any value, the current for both creatine and creatinine quickly approaches zero. This result and other data (not shown) verified that generation of catalytic gold oxides is absolutely necessary for oxidation of creatine and creatinine. Thus, the HPLC–PED waveform chosen for this work uses a detection step in which the potential is first stepped well into the region of oxide formation and subsequently scanned to more positive values to generate as much catalytic oxide as possible (see Table 1). By doing so, anodic current (signal) for creatine and creatinine was also maximized.

3.1. Assay validation

The validation of creatine assay in the rat plasma included the following parameters.

3.1.1. Specificity

Representative chromatograms for creatine and creatinine in aqueous solution is shown in Fig. 2. The representative chromatogram for both creatine and creatinine in biological sample (rat plasma) is shown in Fig. 3. No interfering peaks were detected in the chromatograms of aqueous samples as well as that of the rat plasma. However, endogenous creatine was detected in the untreated rat plasma. Therefore, the response for endogenous creatine in unspiked samples was subtracted from that of the spiked samples in preparing and statistically analyzing standard curves. Complete separation of creatine and creatinine required less than 6 min of the chromatographic time, with the peaks for creatine and creatinine having retention times of 3.37 and 4.73 min, respectively.

3.1.2. Linearity

The standard curves of creatine in rat plasma were linear over the concentration range of 0–20 μg/ml. The equation for the standard curve relating the peak height (PH) to creatine concentration (C in μg/ml) in this range was: $PH = 89052C - 11012$, $R > 0.98$ ($n = 5$).

3.1.3. Precision

Within-day precision of the assay was determined by analysis of replicate ($n = 4$) samples of five different concentration on the same day. To determine day-to-day precision, the same aqueous standard solutions were analyzed five different times over a period of 28 days. The variability in the peak height at each concentration was used to

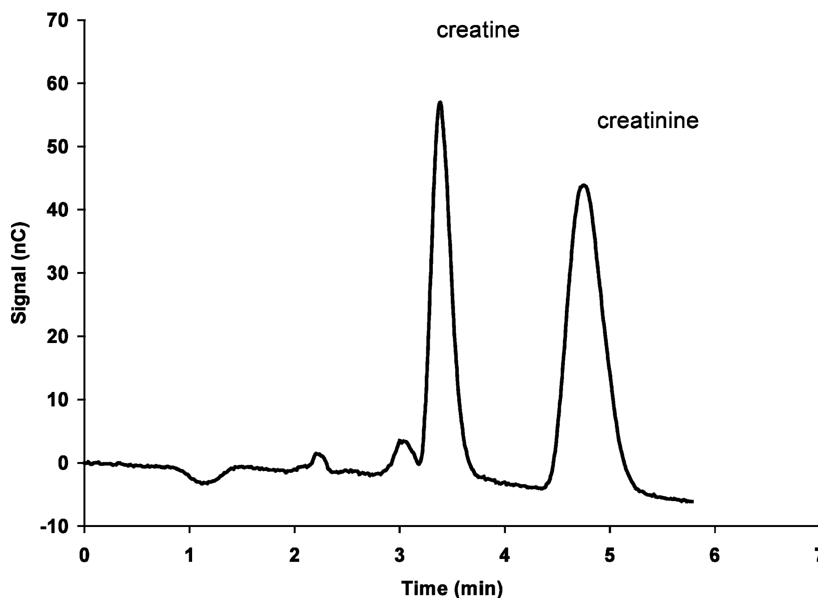

Fig. 2. Representative chromatogram of 13.1 μg/ml creatine and 11.3 μg/ml creatinine in aqueous samples.

determine the precision of the assay procedure and depicted in Table 2. Within-day and day-to-day relative standard deviation (R.S.D.) values ranged from 0.4 to 5.4 and 2.3 to 10.4%, respectively. During this period, the stock and standard solutions were stored under refrigeration (4 °C).

3.1.4. *Accuracy and sensitivity*

Three quality control samples (QCs) and standard solutions were refrigerated at 4 °C over a period of 28-days. These samples were analyzed six times during this period and the accuracy of the assay was determined by comparing the measured

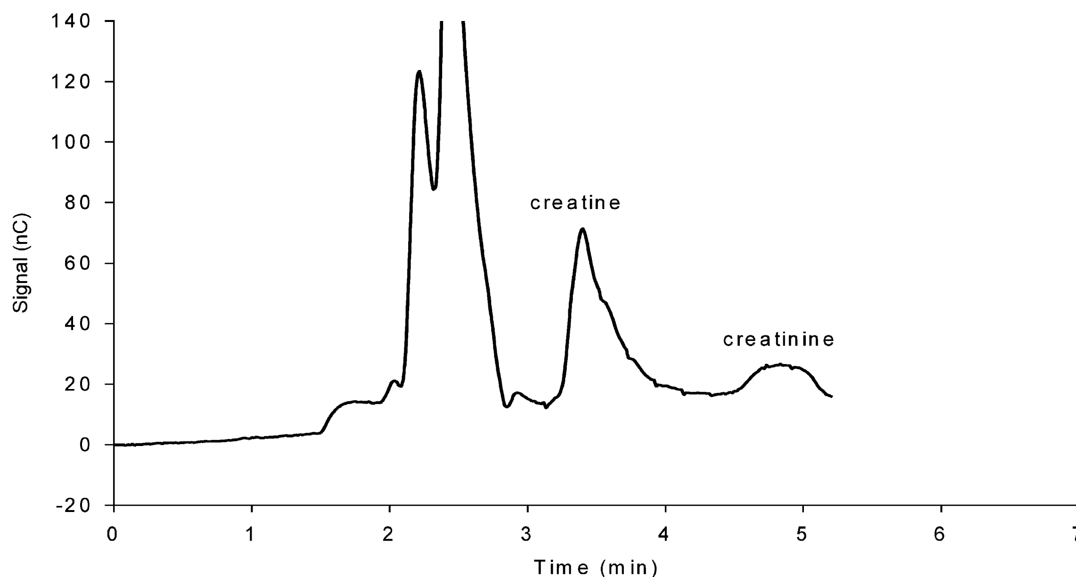

Fig. 3. Representative chromatogram of creatine and creatinine in rat plasma.

Table 2
Within-day and day-to-day analytical precision of creatine in plasma samples

Within-day[a]			Day-to-day[b]	
Concentration (μg/ml)	Mean peak-height[c]	R.S.D. (%)	Mean peak-height[d]	R.S.D. (%)
0	0		0	
5.9	46 5369	5.4	43 0025	10.4
9.8	93 1850	1.7	98 2243	5.2
11.8	1 05 7485	0.4	1 04 7322	3.7
13.8	1 11 0542	1.4	1 17 0250	6.8
19.7	1 73 3013	2.8	1 73 4462	2.3
Slope	87 529 ± 2426	2.8	89 052 ± 3197	3.6

[a] Analyzed on the same day.
[b] Analyzed on five different days within a period of 28-days.
[c] Mean; $n = 4$.
[d] Mean; $n = 5$.

concentration to its nominal value and results are shown in Table 3. The R.S.D. ranged from 5.6 to 7.8%. The LOD ($S/N > 3$) were better than 0.0134 μg/ml for creatine and 0.0113 μg/ml for creatinine, respectively.

3.1.5. Applications of the HPLC method

This HPLC method was used to determine the in vitro dissolution profiles of various salt forms of creatine in water using this LC method. The dissolution profiles of various forms of creatine are shown in Fig. 4. Results from this study revealed that effervescent creatine supplement formulation had better dissolution characteristics as compared with the other three salts tested. Creatine monohydrate, the most used creatine salt in creatine supplement formulation had the poor dissolution in water as compared with the all other samples tested. The low aqueous solubility of creatine monohydrate as compared with the other salts used in this investigation is responsible for its poor dissolution properties.

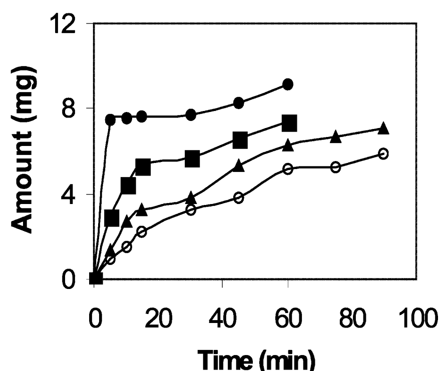

Fig. 4. In vitro dissolution profiles of, (○) creatine monohydrate; (▲) creatine; (■) dicreatine citrate; and (●) an effervescent creatine suplement formulation.

Table 3
Accuracy in the analysis of creatine in QC samples

Actual concentration (μg/ml)	Measured Concentration (μg/ml)[a]	Accuracy[b]	R.S.D. (%)
3.9	4.3 ± 0.34	110.1 ± 8.62	7.8
7.9	7.9 ± 0.46	100.2 ± 5.81	5.8
15.7	15.5 ± 0.87	98.68 ± 5.57	5.6

[a] Mean ± S.D.; $n = 6$.
[b] Accuracy = (measured concentration/actual concentration) × 100.

4. Conclusions

A simple and sensitive LC method with pulsed electrochemical detection was developed and validated for the quantitation of creatine in aqueous solution as well as in rat plasma. The application of this method further includes the evaluation of the in vitro dissolution characteristics of various creatine salts and formulations.

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