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Analysis of creatine and creatinine in urine by capillary electrophoresis

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Abstract

Creatine is found in the urine of subjects ingesting creatine monohydrate as an ergogenic aid. Creatinine, the catabolic breakdown product of creatine, is a major constituent of normal urine. It is of interest to follow the excretion of creatine and creatinine in urine as a function of time after creatine ingestion. In this study, creatine and creatinine were analyzed in urine by capillary electrophoresis. The optimization of the method was discussed, with the best results being obtained using a 30 m*M* phosphate–150 m*M* sodium dodecyl sulfate buffer at pH 6, with the detector set at 214 nm and an applied voltage of 15 kV across a 45 cm capillary. Verification of the method was provided by HPLC analysis and spiking. The application of the method was demonstrated by analysis of creatine and creatinine in urine samples collected in a 24-h period following creatine ingestion.

Keywords: Creatine; Creatinine

1. Introduction

Ingestion of a creatine (CR) supplement has been shown to increase the level of CR phosphate present in muscles [1], and to increase muscular power and endurance [2], with a concomitant increase in muscle size and body mass [3]. It has also been implicated as a possible means of reducing total plasma cholesterol (primarily very low density lipoproteins) and triacylglycerols [4]. CR is present in muscle tissue and blood, and although not present in large amounts in normal urine from adults [5], it is abundantly present in the urine of adults who have recently ingested CR supplements [6,7]. Creatinine (CN) is the end product of CR catabolism. It is found in muscle tissue, blood and urine, and is an important clinical marker of renal function [5]. High-performance liquid chromatography (HPLC) has been used to simultaneously analyze CR and CN in serum, plasma [8–10], tissues [11,12] and urine [13,14]. Capillary electrophoresis (CE) has been used to measure CN in tissue extracts [15] and there are a variety of CE methods available for the analysis of CN in urine and serum. The methods vary depending on the nature of the other compounds that the authors wish to include in their analysis.

One reported method [migration time (t_m) 4 min, 50-fold sample dilution in running buffer containing

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2% EDTA] of analyzing CN by CE involves use of a potassium phosphate buffer at pH 6.4, with a detector wavelength of 235 nm [16]. However, at pH values between 4.8 and 9.2, CN is zwitterionic and coelutes with neutral compounds, although these are usually present in significantly smaller amounts and do not necessarily absorb at the wavelength used for CN. At low pH values, CN has been determined along with calcium and other cations [17]. On the other hand, it has been shown that raising the pH to 10 with a borate buffer does not allow separation of the CN from neutrals of interest, such as nicotinamide and caffeine. Rather, these compounds have been analyzed in deproteinized blood sera using micellar electrokinetic chromatography (MEKC) with a 20 m*M* borate buffer (pH 9.0) containing 80 m*M* sodium dodecyl sulfate (SDS) [18]. To avoid peak tailing when CN was analyzed in undiluted urine and plasma, Miyake et al. included isopropanol in their phosphate–SDS buffer system (pH 9 for plasma, pH 6 for urine, t_m values~10 min) [19]. The separation of CN from vanilmandelic acid, homovanillic acid and uric acid in urine by MEKC was examined as a function of pH and buffer concentration and the best resolution was obtained using a 30 m*M* phosphate buffer (pH 7.0) containing 150 m*M* SDS with detection at 245 nm [20]. CN was the first substance to elute in these studies with a t_m of around 7 min. Very recently, the separation of urea, uric acid, CR and CN standards was reported. Yan et al. used a phosphate buffer containing sodium cholate to achieve their separation, but did not analyze any real samples nor give any indication of the concentrations of the standards used [21].

In this paper, we describe the optimization of the simultaneous analysis of CN and CR in urine by MEKC. We chose to use MEKC with a phosphate–SDS buffer and optimized for speed, resolution and reproducibility. The method was then used to follow CR and CN excretion in urine as a function of time after CR ingestion.

2. Experimental

2.1. Conditions

CE separations were carried out on a Waters Quanta 4000 CE system using a hydrostatic injection mode (10 cm elevation of sample) for 20 s, a positive power supply, and a detector wavelength of 214 nm. The capillary was either 60 cm×75 μm or 45 cm×75 μm (Polymicro Technologies, Phoenix, AZ, USA) with a 1.5 mm detector window 7.5 cm from one end. The data was collected using Lab Calc software from Galactic (Salem, NH, USA) with 1 V equal to 1 AU. Rinse times were 30 s 0.1 *M* sodium hydroxide, 30 s water, and 1 min buffer, using a vacuum pressure of 15 in.Hg (1 in.Hg=3386.38 Pa). Urine was diluted 100-fold with water before analysis.

HPLC analyses were carried out on a Gilson HPLC with 712 HPLC System Controller software. The column was a 250 mm×4.6 mm Econosil C_{18} 10 μm reversed-phase column (Cat. No.: 60147) made by Alltech Associates (Deerfield, IL, USA). The buffer was potassium dihydrogenphosphate (14.7 m*M*, 2.0 g l^{-1}) and tetrabutyl ammonium hydrogen sulfate (2.3 m*M*, 0.8 g l^{-1}) adjusted to pH 5.0 with aqueous potassium hydroxide [11]. A flow-rate of 1 ml min^{-1} and a detection wavelength of 210 nm were used. CN was analyzed directly from its peak height by reference to a standard curve. The concentration of CR was determined by the difference in peak height of the designated peak before and after the addition of creatinase, with adjustments being made as appropriate for dilution factors. To carry out the reaction with creatinase, 1 mg enzyme was dissolved in 0.5 ml 0.1 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.0, and 0.05 ml of this solution was added to the 0.5 ml urine sample which had been previously diluted 100-fold in the same Tris buffer. Samples were left to sit overnight to allow for complete digestion of the CR.

Standard deviations (SDs) were calculated using results from eight or more runs. The limits of detection (LODs) were calculated as the concentration that gave a signal equal to the blank plus three-times the SD of the blank.

2.2. Urine collection

CR monohydrate was prepared for ingestion as a solution in warm grape juice (heated to ensure that all the CR dissolved). Urine samples (24 h) were collected in 3-l urine collection containers (Fisher Scientific, Nepean, Canada) from subjects (young male athletes) who had been taking daily CR mono-

hydrate supplements of 0.1 g kg^{-1} lean body mass. The volume was recorded and a 20-ml sample was taken after mixing and stored at 4°C pending analysis, which was done as soon as possible after collection.

2.3. Reagents

CN standard solutions, creatinase and SDS were purchased from Sigma (Oakville, Canada). CR monohydrate was donated by a Canadian supplement company, Nu-Life Nutrition/Natraceuticals. Disodium hydrogenphosphate, sodium dihydrogenphosphate and potassium dihydrogenphosphate were from Fisher Scientific. Tetrabutyl ammonium hydrogen sulfate and Tris were from Aldrich (Mississauga, Canada). Sodium hydroxide was from J.T. Baker (Phillipsburg, NJ, USA). All water used was 10 MΩ cm resistivity (Millipore 4000S purification system).

3. Results and discussion

Our particular interest was in the analysis of both CR and CN in urine, as a means by which we could follow how much ingested CR was ''spilling over'', and find whether CN excretion was changed by ingestion of CR. We had previously confirmed that CR was present in much larger amounts than normal in 24 h urine samples from subjects on a CR regimen [7], and were next interested in seeing how fast the CR was excreted after initial ingestion. Because of the structural similarities of these two compounds, conditions for their separation must be chosen carefully, and both peaks must be away from major interferences. Although HPLC methods are available, as described above, the use of HPLC requires that all impurities must be eluted before injection of the next sample. Thus, although the elution times for CN and CR might be less than 5 min, all slower-moving impurities must be eluted before starting another sample analysis, and for urine analysis we have found that this requires at least an extra 12 min. This drawback can be overcome if CE rather than HPLC is used. In analyses carried out by CE, impurities migrating slower than the peaks of interest can be removed by purging once the analytes of interest have passed the detector, thus considerably increasing sample throughput. In addition, CE column replacement, even if necessary, is not expensive. This cannot be said for HPLC columns.

We began by using a 30 m*M* phosphate–150 m*M* SDS buffer at pH 7.0 [20] with a 60 cm (end-to-end) capillary. We chose to use 214 nm as our detection wavelength. CN and CR absorb strongly at this wavelength, while urea has negligible absorbance. (Injection of a 500 μg ml^{-1} solution of urea did not result in a peak). Injected water did, however, produce a peak. We attributed this to the difference in ionic strength produced as the water plug travels through with the electroosmotic flow. With a run voltage of 20 kV, CR eluted very close to CN. If the run voltage was lowered to 8 kV, baseline separation could be obtained, and maintained through subsequent runs. However, the time required for elution of both peaks was 16 min, which timewise gave no improvement over the HPLC method. We therefore examined the effect of the buffer pH on the separation, and the results are summarized in Fig. 1. Using 20 kV and changing to pH 6.0 gave much better resolution of the water peak, CN and CR. Decreasing the voltage to 17 kV gave even better resolution of the three peaks. Use of pH 5 was unsatisfactory, as the t_m for CN was greatly increased, in agreement with the findings of Shirao et al. [20].

The current produced by the buffer at pH 6.0 and 17 kV was ~160 μA. We investigated the use of less concentrated versions of the buffer, to try and prevent problems with Joule heating. Shirao et al. reported that decreasing either the phosphate or the SDS concentration had little effect on the t_m values of CN [20]. However, we found that decreasing the SDS concentration by a factor of 2 decreased the separation between the peaks for water and CR to the extent that they were no longer resolved. Thus the use of the higher concentration of SDS is justified. Decreasing the phosphate concentration also decreased the separation between the water and CR peaks. The addition of methanol (5%, 10%) as organic modifier to the buffer resulted in narrower peaks but significantly longer retention times (t_m values for CN of 11.6, 12.2 min, respectively), and the resolution between the water/methanol and CR peaks was not improved.

At this point we decided to try and optimise the analysis time and we changed to a 45 cm capillary. The smaller capillary length meant that the peaks

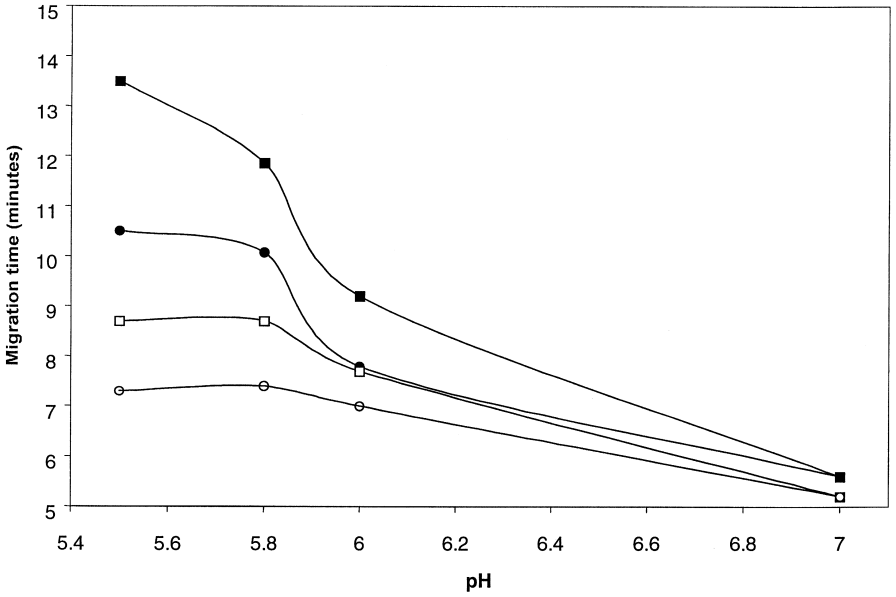

Fig. 1. Migration times of CN (□) and CR (○) in a 60 cm capillary with a phosphate–SDS (30–150 m*M*) buffer, pH 6.0, with an applied voltage of 17 kV (filled symbols) or 20 kV (open symbols) and detection at 214 nm.

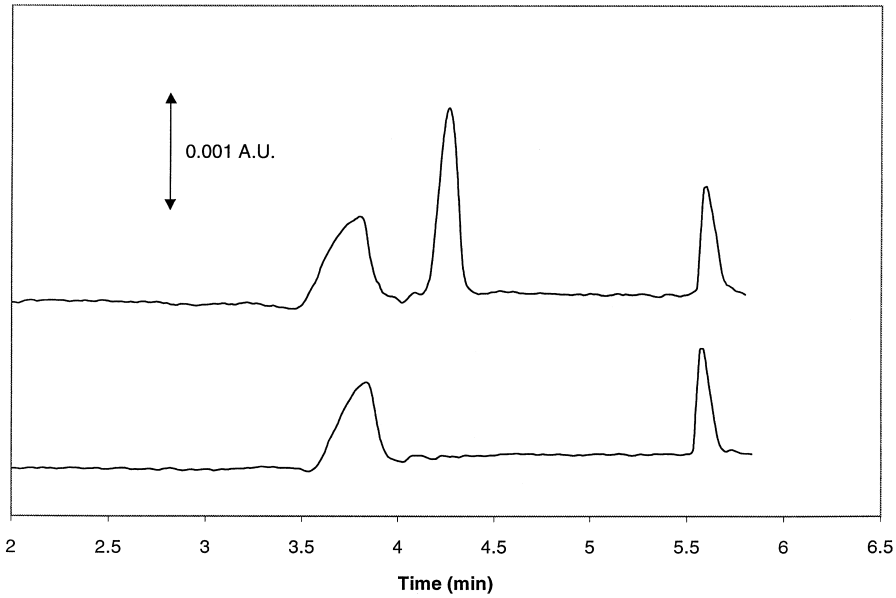

Fig. 2. Electropherograms of 100-fold dilutions of urine run in phosphate–SDS (30–150 m*M*) buffer, pH 6.0, with an applied voltage of 17 kV and detection at 214 nm. The lower trace is the urine itself; the upper trace is the urine spiked with CR to give a nominal concentration of 100 μg ml^{-1}. The first peak in each is due to water, the next to CR, and the last to CN. Other substances may have been seen at later times if the run times had been extended.

came out faster but closer together. By reducing the voltage to 15 kV, good separation was obtained in a time of 5.7 min.

Calibration curves were obtained by running known mixes of CR and CN and plotting peak area divided by t_m (min) versus the concentration (μg ml^{-1}). The curves were linear at least up to 500 μg ml^{-1} (eight-point curve) but the calibration curves were generally only prepared to 300 μg ml^{-1} (six-point curve) as the concentrations found in diluted urine were well below this. Typical equations were $y=9.21\cdot10^{-4}[CN]+5.78\cdot10^{-4}$ ($R^2=0.9997$, $SD_{slope}=1.0\cdot10^{-5}$, $SD_{intercept}=1.2\cdot10^{-3}$) and $y=4.15\cdot10^{-4}[CR]-3.88\cdot10^{-5}$ ($R^2=0.9986$, $SD_{slope}=6.0\cdot10^{-6}$, $SD_{intercept}=1.6\cdot10^{-3}$). Although some variance in t_m was found from day-to-day, on a particular day the relative standard deviation in both t_m and peak area was reduced to 2% by use of identical rinse cycles between runs. The total conditioning time between runs was only 2 min and each rinse was exactly timed. Increasing the rinse times beyond 2 min did not increase the reproducibility, but haphazard timing of the rinse cycles could lower reproducibility. Because of the acceptability of these results, it was not deemed necessary to use an internal standard. The LOD was 2 μg ml^{-1} for CR and 1 μg ml^{-1} for CN.

Use of a 100-fold dilution of urine brought CR and CN levels to a range appropriate to the above standard curves. Electropherograms of a ''normal'' urine sample, diluted as described, showed no peaks at the t_m for CR and subsequent spiking to 100 μg ml^{-1} produced peaks of the expected size (Fig. 2). Recoveries for CR obtained by spiking up to 100 μg ml^{-1} were 102±3%.

A HPLC method was set up to provide a comparison method. It involved using the buffer system and conditions described by Dunnett et al. [11] on a similar reversed-phase column. Green et al. also used this method for analysis of CR in urine [22]. Creatinase was used in our laboratory to positively identify the CR, as it is possible that another component of the urine might coelute with it. Thus creatinase was used to remove CR from urine samples diluted in buffer, and the difference was taken between the peak heights in the chromatograms of samples run before and after treatment with creatinase, with due allowance for dilution. A comparison of the results obtained by CE and HPLC is shown graphically in Figs. 3 and 4. The fact that the

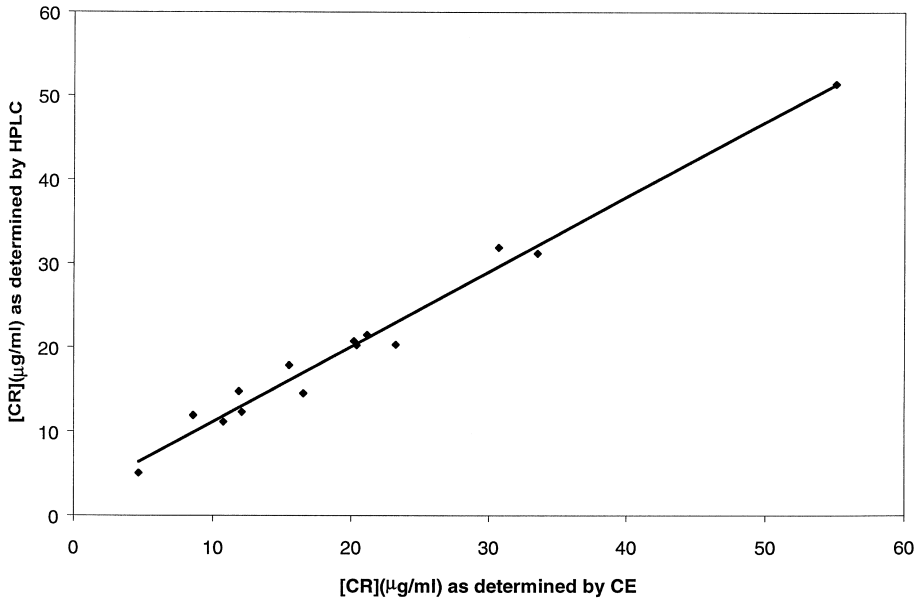

Fig. 3. Concentrations of CR in urine samples as determined by HPLC are plotted versus the concentrations found in the same samples by CE.

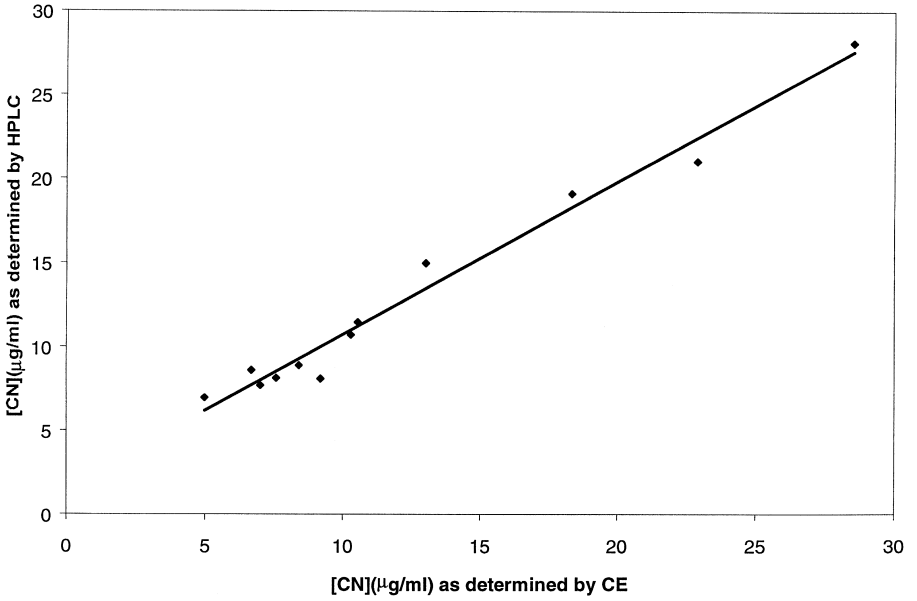

Fig. 4. Concentrations of CN in urine samples as determined by HPLC are plotted versus the concentrations found in the same samples by CE.

correlation between the CE and the corrected HPLC results was good validated the CE method. The results also invited further experimentation on excretion of creatine in urine during creatine supplementation, as the creatine content of these urine samples supports previous reports that a significant amount of ingested creatine is not absorbed by the body [6,7]. The HPLC method required 20 min per run to completely elute all the sample components, and required a blank to be run for correction purposes, thus emphasizing the convenience of the CE method, which required 8 min per sample, including the rinse times.

The CE method was then used to follow the excretion of CR and CN in urine as a function of time after CR ingestion by several young male athletes. Typical results for two such athletes are given in Tables 1 and 2. Athlete I (56 kg lean body mass, 70 kg total body mass) took 5.6 g CR as CR monohydrate. Athlete II (66 kg lean body mass, 80 kg total body mass) took 16.5 g CR as CR monohydrate. The output of CR in the 2.0 l of urine collected from athlete I in the 24 h period after CR ingestion was 1.6 g (29% of dose), with 1.3 g of this being excreted in the first 4.3 h after ingestion. For athlete II, the 2.9 l of urine collected contained 6.0 g CR (36% of dose). Of this amount, 5.3 g was excreted in the first 5.7 h.

These results indicate that a significant amount of creatine is excreted very soon after ingestion. However, no parallel jump in creatinine output could be discerned. It can also be seen that with a bigger dose, a larger absolute amount of CR is apparently retained, even though the fraction of the dose excreted is larger.

Table 1
Amounts of CR and CN excreted by athlete I at the specified times after ingestion of CR as determined using CE with a phosphate–SDS buffer at pH 6, applied voltage of 17 kV, and detection at 214 nm

Time (h)	CR (g)	CN (g)
0.9	0.17	0.26
2.3	0.79	0.08
4.3	0.37	0.14
7.7	0.12	0.24
10.2	0.04	0.15
13.6	0.02	0.19
21.3	0.05	0.43

Table 2
Amounts of CR and CN excreted by athlete II at the specified times after ingestion of CR as determined using CE with a phosphate–SDS buffer at pH 6, applied voltage of 17 kV, and detection at 214 nm

Time (h)	CR (g)	CN (g)
1.3	1.43	0.11
2.7	2.22	0.14
5.7	1.65	0.12
8.3	0.30	0.04
10.8	0.32	0.06
12.2	0.01	0.20
13.8	0.01	0.18
16.4	0.02	0.15
23.6	0.07	0.68

4. Conclusions

Simultaneous analysis of CR and CN in urine can be carried out using CE with a phosphate–SDS buffer at pH 6 and photometric detection at 214 nm. Results correlate well with those obtained by HPLC, but the CE method is considerably faster and more convenient.

The application of the described method to the analysis of CR and CN in urine samples as a function of time after CR ingestion showed fast elimination of a significant fraction of CR and suggest that further studies are in order to fully investigate this phenomenon. The design and execution of such studies are currently underway in our laboratories.

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