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Review

Separation methods applicable to urinary creatine and creatinine

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Abstract

Urinary creatinine has been analyzed for many years as an indicator of glomerular filtration rate. More recently, interest in studying the uptake of creatine as a result of creatine supplementation, a practice increasingly common among bodybuilders and athletes, has lead to a need to measure urinary creatine concentrations. Creatine levels are of the same order of magnitude as creatinine levels when subjects have recently ingested creatine, while somewhat elevated urinary creatine concentrations in non-supplementing subjects can be an indication of a degenerative disease of the muscle. Urinary creatine and creatinine can be analyzed by HPLC using a variety of columns. Detection methods include absorption, fluorescence after post-column derivatization, and mass spectrometry, and some methods have been automated. Capillary zone electrophoresis and micellar electrokinetic capillary chromatography have also been used to analyze urinary creatine and creatinine. Creatine and creatinine have also been analyzed in serum and tissue using HPLC and CE, and many of these separations could also be applicable to urinary analysis.

Keywords: Reviews; Creatine; Creatinine

Contents

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1. Introduction

Creatine has recently achieved great popularity as an ergogenic aid [1]. This popularity is based on the fact that creatine is converted to phosphocreatine in muscle in a reversible reaction with adenosine triphosphate (ATP), facilitated by creatine kinase [2]. When muscle contractions deplete the immediate supply of ATP, the phosphocreatine can rephosphorylate ADP to replenish the supply of ATP. Creatine supplementation has been demonstrated to be effective in increasing peak force and peak power during short-duration, high-intensity activity [3,4], but to have no effect on endurance [5].

Over 90% of bodily creatine is present in muscle, with the remainder being in the plasma (usually <5 mg/l) and brain [6]. Creatine is synthesized in the body using glycine, arginine (kidney) and methionine (liver) [7]. The main dietary source of creatine is red meat, which suggests why vegetarians have been shown to have lower levels of creatinine in urine and lower levels of creatine and creatinine in serum [8]. No cleavage or rearrangement of creatine occurs in the body other than the non-enzymic cyclization to creatinine [7].

Creatinine is the means by which creatine and phosphocreatine are excreted from the body and this occurs at fractional rates of 0.016 and 0.03 per day for creatine and phosphocreatine, respectively. The amount of creatinine in the urine is proportional to the amount of creatine and creatine phosphate present in the body [9] and hence also to the muscle mass. The 24-h creatinine content of urine remains roughly constant for an individual [7], and is often used as a normalization factor when measuring other urinary components. Changes in urinary creatinine content can indicate renal problems. It is an indicator of the glomerular filtration rate, and as such it is widely used in clinical diagnoses.

Patients with muscle wasting diseases such as muscular dystrophy exhibit reduced concentrations of muscle creatine, high levels of creatinurea, and decreased excretion of creatinine [10,11].

A typical 24-h sample of urine contains 1–3.3 g creatinine, 0.07–1 g uric acid, 20–35 g urea [12], about 1–43 mg creatine [13], as well as a variety of other components that are in lower concentrations and tend not to interfere. Creatine concentrations rise dramatically upon supplementation. In a 24-h urine sample, typical volumes range from 600 to 1600 ml or more, depending on water intake. The pH varies from 4.6 to 8 but is normally around 6.5. Actual concentrations fluctuate with the time of day and the intake of water, and thus it is usual to analyze 24-h totals rather than measuring actual concentrations at any time. During oral supplementation of creatine, where the dose can vary from a few grams per day to as much as 20 g/day, up to 12 g creatine may be found in a 24-h sample [14], although lower values are more common [15].

The structures of creatine and creatinine are shown in Fig. 1. The pK_a values for creatinine are 4.8 and 9.2, while those for creatine are 2.6 and 14.3. The absorbance of creatine is insignificant above 225 nm, but rises as the wavelength is decreased. It is generally monitored at 210 nm. Creatinine also has a strong absorbance at this wavelength, but in addition has an absorbance peak at 234 nm of similar intensity. Uric acid also absorbs at these wavelengths [16].

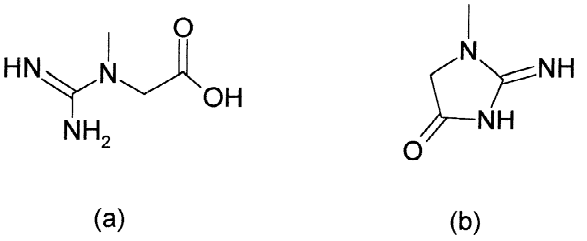

Fig. 1. Structures of creatine (a) and creatinine (b).

1.1. Sample preparation

As it is often difficult to immediately analyze all samples that are collected, samples are routinely refrigerated or frozen prior to analysis to avoid contamination and compositional changes. Above about pH 6, the equilibrium between creatine and creatinine is at approximately equimolar concentrations, but at 0 °C conversion will be very slow [17]. Cooling can lead to precipitation of protein, not all of which necessarily redissolves on warming to room temperature. Subsequent dilution can help, and samples are often diluted before creatine and creatinine analysis because of the high concentrations typically present. Precipitated proteins can be removed by centrifugation, but there is always a concern that some creatine and/or creatinine may have been lost by adsorption on the precipitate. Urine may also be deproteinized by centrifugation after the addition of acetonitrile.

Some analyses are reported to give superior results when the sample is first divided into acidic, basic and neutral fractions. The basic fraction is held up on a cation-exchange column while the acidic and neutral species are eluted in water. Subsequent elution with 0.1 *M* NH_3 releases the basic fraction [18]. Freeze-drying of this fraction, or acidification with phosphoric acid, removes ammonia. Creatine and creatinine have also been separated and isolated from urine using chromatography on a strong cation-exchange resin followed by chromatography on a weak cation-exchange resin [19].

2. HPLC

2.1. Analysis of urinary creatinine

Reversed-phase HPLC has been used to analyze urinary creatinine alone [20] and in conjunction with a variety of other species, including iothalmic acid [21], estriol [22] and amino acids and organic acids [23]. Enzymatic detection methods have been shown to be an alternative to UV detection, where co-eluting compounds can be a problem [24]. Cation-exchange columns have also been used [18,25,26]. Determinations of just creatinine [27], and simultaneous determinations of serum creatinine and uric acid [28], pseudouridine [29], organic acids [30], or amino acids, bioactive amines and nucleic acid bases [31] have also been carried out using ion-pair chromatography with sodium dodecyl sulfate as the ion-pairing reagent. Some analyses have involved the use of several columns [32].

2.2. Analysis of creatine and other substances

HPLC has been used to analyze creatine and phosphocreatine simultaneously with adenine nucleotides in muscle fibres [33] and plasma and heart tissue [34]. The eluents were phosphate buffers with quaternary ammonium ion-pairing reagents. Creatine has also been analyzed in deproteinized serum using 43 m*M* Na octyl sulfate in 50 m*M* ClH_2COOH, pH 3, with fluorescent detection after post-column derivatization with ninhydrin [35].

2.3. Analysis of creatine and creatinine in non-urine matrices

Simultaneous HPLC analyses of creatine and creatinine, and a variety of other species, have been carried out on serum, plasma and muscle tissue. Some methods were included in a recent review on the determination of uremic toxins in biofluids [36], and in Table 1 a summary of analyses of creatine and creatinine in non-urine matrices is given. The conditions for these separation methods are partly dictated by the need to separate and detect the other specified compounds, and partly by the fact that creatine and creatinine are very similar and are thus difficult to separate from each other and from other species in the sample matrix which tend to elute early and cause interference. In some chromatograms a largely unidentified peak elutes just before creatine and is referred to as ‘‘unretained species’’ [37]. Numerous buffer variations have been tried, particularly ones containing ion-pairing reagents [37–40].

2.4. Analysis of creatine and creatinine in urine

As supplementation with creatine gained popularity in the 1990s, much research was carried out on the efficiency of creatine uptake and the factors that influence it. Such research is still ongoing. The

Table 1
Table of conditions used for the HPLC separation of creatine and creatinine in a variety of matrices

Matrix	Buffer	pH	Column	Detection	Ref.
Serum	30 m*M* SDS in 100 m*M* Na H_2PO_4–MeCN (3:1)	2.1	CLC-ODS 5 μ, 150 mm	210 nm	[38]
Serum	30 m*M* K_2HPO_4–MeOH (99.5:0.5), MeOH gradient	7.5	Partisil ODS 3 5 μm, 250 mm	210 nm, 234 nm	[16]
Serum	10 m*M* Na citrate, 5 m*M* Na octanesulfonate, CH_3CN gradient	4.5	Porous graphite 3.5 μ, 50 mm	Fluorescence with ninhydrin	[39]
Fish tissue	2 m*M* SDS–10 m*M* KH_2PO_4/ 25% CH_3N	3.5	Wakosil-II 5C18 HG 5 μ, 150 mm	210 nm	[40]
Equine muscle	14.7 m*M* KH_2PO_4, 2.3 m*M* TBAHS	5.0	Spherisorb ODS 2 5 μ, 150 mm	210 nm	[37]

difference between the urinary creatine content and the ingested dose gives a good indication of the amount of creatine taken into the muscles [14]. Thus the analysis of creatine and creatinine in urine is a common requirement in many physiology labs.

While plasma and tissue samples contain significant amounts of protein and other interfering substances, serum is effectively plasma with the proteins removed, and presents a simpler matrix. Urine has a fairly small protein content, but the concentrations of creatinine, urea and uric acid are about 70, 60 and 20 times greater than the respective values in plasma or serum. Thus urine samples are usually diluted before analysis, and often do not require any sample preparation. The composition of urine reflects the composition of serum, and methods developed for serum analysis should be equally applicable to urine analysis. This is not as true for muscle, although Dunnett et al.'s [37] method (Tables 1 and 2 and Fig. 2) for the analysis of creatine and creatinine in equine muscle has subsequently been used for the analysis of creatine in plasma and for the analysis of creatine and creatinine in urine [41]. Inamoto et al.'s [39] method (Table 1) should also be applicable to urine, as the fluorescent detection was specific for guanidino compounds. These were shown to be well separated (Fig. 3) under the specified conditions (Table 2). The LOD for creatine was 8 ng/ml using fluorescent detection, but for creatinine was 1 μg/ml. The advantage of this method is its specificity. However, the high LOD for creatinine could necessitate performing analyses at different dilutions to get optimal determinations of both creatine and creatinine.

Werner et al. [16] found that use of a pre-column packed with NH_2-packing material facilitated separation of impurities that otherwise co-eluted with serum. This could be of similar use for urine. They also demonstrated the usefulness of a diode array detector, using wavelengths of 210 nm for creatine and 234 nm for creatinine (Fig. 4). Details are shown in Tables 1 and 2.

Table 2
Summary of chromatographic data for HPLC analyses applicable to the analysis of urinary creatine and creatinine

Other peaks	Retention times (min)			Reference
	Creatine	Uric acid	Creatinine	
Unretained species	2.3	10.8	2.8	Dunnet et al. [37]
Other guanidino species	5.4	N/A	23.1	Inamoto et al. [39]
None	3.9	5.2	6.4	Werner et al. [16]
Unretained species	0.7	0.95	1.3	Yang [13]
Xanthine	2.8	5.4	3.7	Samanidou et al. [43]
Histidines	3.5	N/A	4.5	Yokoyama et al. [44]
Less-retained species	3.5	N/A	7.5	Gu and Lim [45]

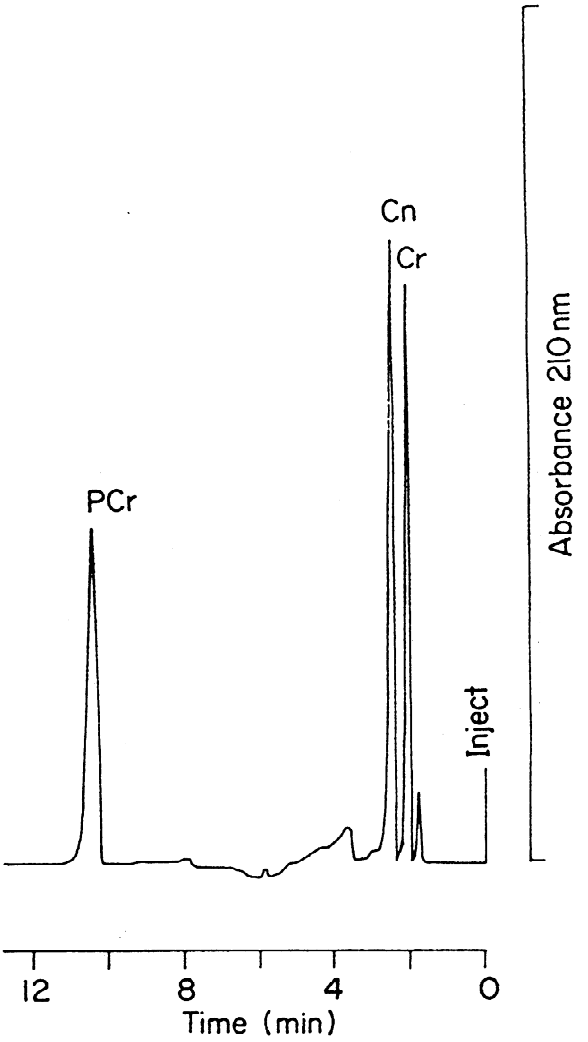

Fig. 2. Chromatogram of a mixture of phosphocreatine (PCr), creatine (Cr) and creatinine (Cn) standards obtained using a 5-μm, 4.6×250-mm Spherisorb ODS 2 column, a potassium phosphate (14.7 m*M*)–tetrabutylammonium hydrogen sulfate (2.3 m*M*) mobile phase, pH 5.0, and detection at 210 nm. From ''Reverse-phase ion-pairing high-performance liquid chromatography of phosphocreatine, creatine and creatinine in equine muscle'' [37] with permission from Taylor and Francis AS: www.tandf.no/sjcli

2.4.1. Reversed-phase columns

Rossiter et al. [42] diluted urine 10-fold with distilled water, and eluted with 15 m*M* potassium phosphate buffer, pH 6.4, from a 3-μm Spherisorb™ ODS II, 4.6×150-mm column. No retention times were given. Yang [13] reported a separation of urinary creatine, creatinine, uric acid and hippuric acid (Fig. 5). A 3.9×150-mm Nova-Pak C_{18} column was used with a 20 m*M* potassium phosphate buffer, pH 6.5, and detection at 220 nm. Samples were diluted 10-fold with mobile phase before injection. The retention times of the creatine and creatinine (Table 2) were found to be independent of pH from pH 3 to 8, but uric acid was found to elute slightly faster at higher pH. The run must continue until the hippuric acid has eluted, but this occurs within 6 min of injection. Samanidou et al. [43] determined urinary creatine, creatinine, uric acid and xanthine on a 5-μm, 4×250-mm Kromasil C_8 column with 10 m*M* KH_2PO_4 as the mobile phase and detection at 200 nm (Table 2 and Fig. 6). Urine was analyzed after a 400–500-fold dilution, with a detection limit of 0.2 ng/ml for both creatine and creatinine. The precision and accuracy of this method were well documented. A run time of just under 8 min was necessary.

These three methods all allow fast, facile analysis of creatine and creatinine with little sample preparation and isocratic elution on a common configuration of an HPLC. The dilution of the sample ensures that there are no components present in sufficient concentration to interfere. Samanidou et al. report a very low LOD, but slightly better resolution of creatine from the less retained species was obtained by Yang [13].

2.4.2. Ion-pair chromatography

Urinary creatine and creatinine were analyzed by Yokoyama et al. [44] using a 5-μm 4.6×150-mm Capcell PAK ODS UG80 reversed-phase column coated with sodium hexadecylsulfonate, and an eluent containing 5 m*M* ethylenediamine and 15 m*M* phosphate (pH 5), with detection at 210 nm (Table 2 and Fig. 7). However, significant sample preparation was carried out before sample analysis. The sample was passed through a cation-exchange column in the hydrogen form, and eluted with water. Creatine and creatinine were eluted in 1 or 2 min using 0.1 *M* NH_4OH, then freeze-dried to remove ammonia. The residue was dissolved in water before analysis to give an effective 10-fold dilution from the original sample. A number of reversed-phase columns were compared and it was found that coated ODS columns were easily damaged when pH changes were made to an organic-free eluent. The advantage of using this

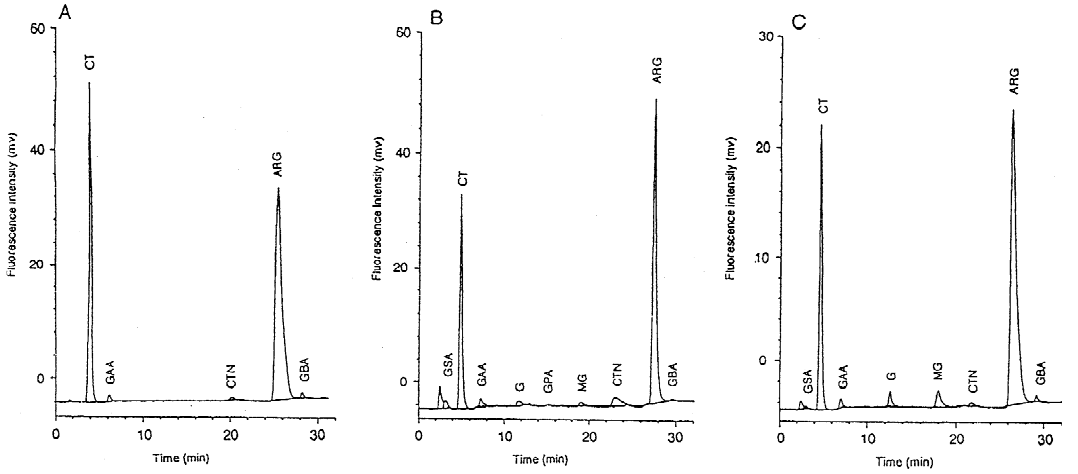

Fig. 3. Chromatograms of guanidino compounds in sera from a control patient (A), from a patient undergoing continuous ambulatory peritoneal dialysis after renal transplantation (B) and from a patient with membrane proliferative glomerulonephritis (C). Ion-pair LC with a 4.6×50-mm I.D. column packed with 3.5-μm porous graphitic carbon was used. The eluent was changed in four steps from 10 m*M* citrate bufffer (pH 4.5) containing 5 m*M* sodium octanesulfonate to 10 m*M* citrate buffer containing 20% (v/v) acetonitrile. Detection was by fluorescence with excitation at 392 nm and emission at 500 nm after post-column reaction with 0.6% ninhydrin and 1 *M* NaOH solutions flowing at 0.2 ml/min. From ''Liquid chromatography of guanidino compounds using a porous graphite carbon column and application to their analysis in serum'' [39] with permission from Elsevier Science.

method is that a wide range of amino acids can be determined along with creatine and creatinine. The LODs were 65 and 23 ng/ml for creatine and creatinine, respectively.

2.4.3. Porous graphitic columns

Porous graphitic columns have also been used for the analysis of urinary creatine and creatinine. Gu and Lim [45] used a 4.6×100-mm Hypercarb column with a mobile phase of 3% acetonitrile in 0.1% trifluoroacetic acid (ion-pairing reagent). Sample preparation involved removal of hydrophobic contaminants in a C_8 extraction cartridge after treatment with trifluoroacetic acid. Porous graphitic columns are extremely hydrophobic and the retention times are longer than on octadecyl reversed-phase columns, as shown in Table 2. The longer preparation and analysis time can be weighed against the very good separation of creatine from the ''unretained species'' and creatinine (Fig. 8).

2.4.4. Automatic analyzers

An automatic HPLC analyzer was developed using a cation-exchange column, gradient elution, and fluorescence detection after post-column derivatization with benzoin and KOH [46].

An amino acid analyzer with a cation-exchange column, sodium citrate buffer and fluorescence (ninhydrin) detection was used to analyze creatine and creatinine in serum [47]. The method would be equally applicable to urine, and would greatly speed up analysis time. Creatine has also been detected using post-column fluorescent derivatization with glucose [48].

3. High-performance thin-layer chromatography (HPTLC)

Urinary creatine and creatinine, along with uric acid and carbohydrates, were analyzed on an amino-modified HP–TLC plate without sample preparation [49]. The LODs were reported to be below physiological range. Creatine and creatinine have also been separated on 300MN cellulose in a chilled propanol–acetone–ammonia–water (5:2:4:1) buffer [50]. Visualization involved treatment with picric acid or α-naphthol.

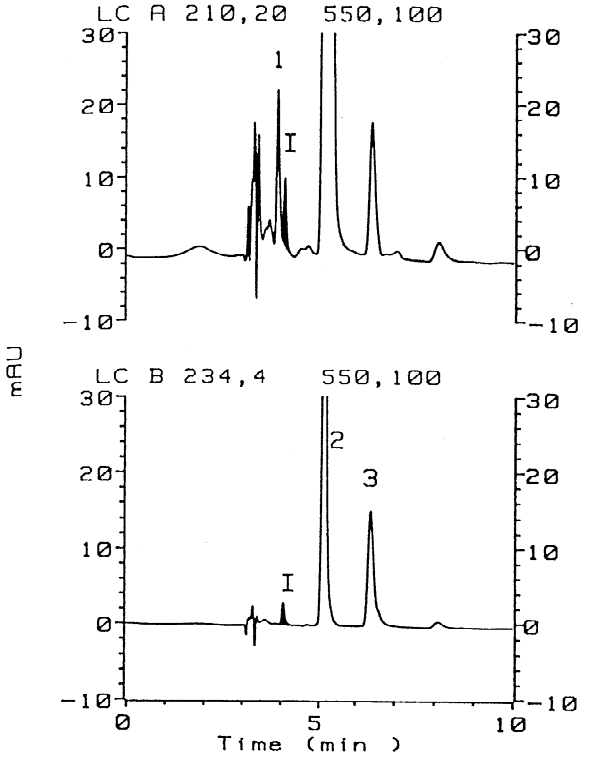

Fig. 4. Chromatograms at 210 and 234 nm for a serum sample run through a NH_2 pre-column (4.6×40 mm) before HPLC analysis on an ODS column (4.6×250 mm, 5 μ) with a 0.03 *M* potassium phosphate (pH 7.5)–methanol eluent (99.5:0.05, v/v) for 8 min followed by a 2-min gradient to 20% methanol. Peaks: (1) creatine (162 μmol/l); (2) uric acid (5.2 mg/dl); (3) creatinine (1.55 mg/dl); (I) unknown interference. From ''Simultaneous determination of creatine, uric acid and creatinine by high-performance liquid chromatography with direst serum injection and multi-wavelength detection'' [16] with permission from Elsevier Science.

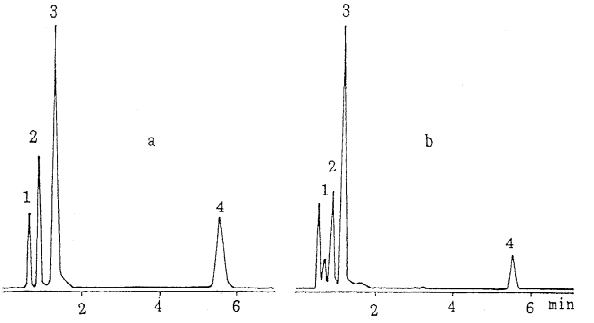

Fig. 5. Chromatograms of a standard mixture (a) and normal urine diluted 1:10 (b) run using a Nova-Pak C_{18} column (150×3.9 mm) with 0.02 *M* potassium phosphate (pH 6.5) as eluent (1.6 ml/min) and detection at 220 nm. Peaks: (1) creatine; (2) uric acid; (3) creatinine; (4) hippuric acid. In the standard, the concentrations of creatine, uric acid and hippuric acid were 100 mg/l and that of creatinine was 200 mg/l. From ''Simultaneous determination of creatine, uric acid, creatine and hippuric acid in urine by high performance liquid chromatography'' [13] with permission from John Wiley & Sons Limited.

4. Capillary electrophoresis (CE)

4.1. Analysis of creatinine by CE

In 1990, Guzman et al. [51] reported that urea and creatinine comigrated in a borate buffer, as both were neutral under the specified conditions. Jia et al. [52] reported that at pH values below 7.0, other UV-absorbing species comigrate with creatinine, while Petucci et al. [53] reported that this was true over the pH range 6–9. The detection sensitivity at 210 or 214 nm is 10^4 better for creatinine than for urea on a molar basis and therefore creatinine could easily be determined by these methods without significant interference from urea. Other species in urine are present in significantly smaller amounts than creatinine and thus should not cause a problem in its analysis. Shi et al. [54] analyzed creatinine in urine in 4 min, using a phosphate buffer with a pH of 6.4 and detection at 235 nm. They reported that urea was not able to be detected under the analysis conditions unless 1.5 *M* urea was used, and then it appeared as a dip at 2.5 min. As part of their sample preparation, they added 2% EDTA to complex metal ions to prevent them complexing with creatinine. Creatinine has also been analyzed simultaneously with a variety of cations, including calcium, using pyridine, tartaric acid and 18-crown-6 in a buffer at pH 4.05, and indirect photometric detection [55]. CE has been used to analyze creatine in tissue samples simultaneously with the analysis of other metabolites, including adenine nucleotides [56,57].

Separation of creatine and creatinine has been accomplished with actual electrophoresis [58], but the use of CE is much more convenient. Clark et al. [59] compared three run buffers for the separation of creatine, creatinine, uric acid. In 10 m*M* sodium borate buffer (pH 9.5) creatine and creatinine were

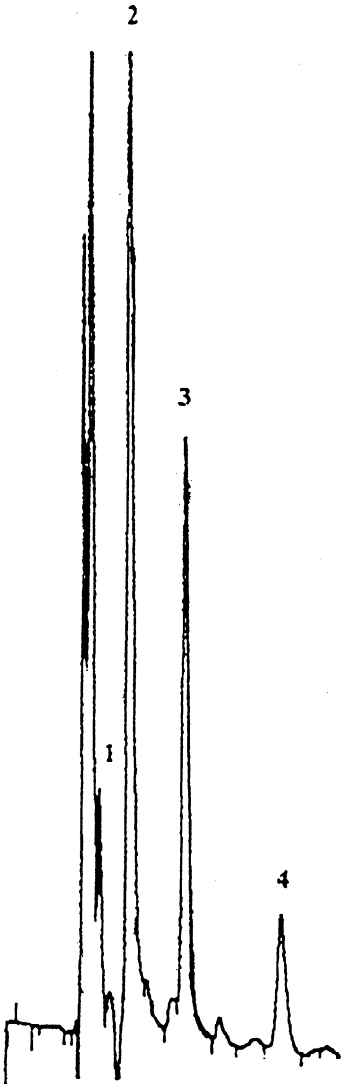

Fig. 6. Chromatogram of urine sample after 400-fold dilution, as obtained with a 4×250-mm Kromasil column, a 10 m*M* KH_2PO_4 mobile phase and detection at 200 nm. Peaks: (1) creatine; (2) creatinine; (3) uric acid; (4) xanthine (retention time 7.5 min). From ''Direct simultaneous determination of uremic toxins:creatine, creatinine, uric acid, and xanthine in human biofluids by HPLC'' [43] with permission from Marcel Dekker, Inc.

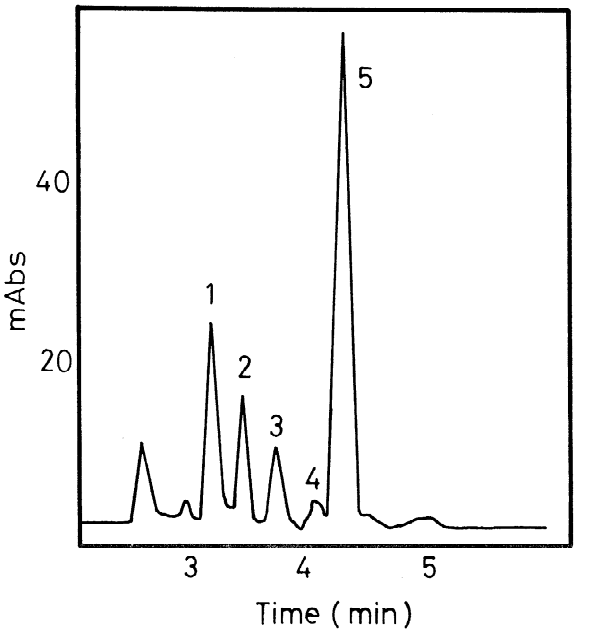

Fig. 7. Portion of chromatogram of the basic fraction from a urine sample from a patient with phenylketonurea, corresponding to 10-fold dilution of intact urine, obtained using a 4.6×150-mm Capcell PAK ODS UG80 column coated with sodium hexadecylsulfonate, 5 m*M* ethylenediamine–15 m*M* phosphate (pH 5) as eluent and detection at 210 nm. Peaks: (1) 1-methylhistidine; (2) creatine; (3) 3-methylhistidine; (4) histidine; (5) creatinine. Phenylalanine eluted after 47 min. From ''Low-capacity cation-exchange chromatography of ultraviolet-absorbing urinary basic metabolites using a reversed-phase column coated with hexadecylsulfonate'' [44] with permission from Elsevier Science.

not baseline resolved, but were well separated from uric acid, which migrated much faster. In 30 m*M* phosphate buffer (pH 7.5) migration times were longer and resolution was still not complete. Use of 50 m*M* phosphate (pH 5.5) allowed baseline resolution of creatine, creatinine and uric acid (Fig. 9). At this lower pH, creatine and creatinine eluted before uric acid. The LOD was 0.65 μg/ml for creatine and 0.09 μg/ml for creatinine, with a linear range to 65 μg/ml. Prior to analysis, urine samples were diluted 50-fold in 50 m*M* phosphate, pH 5.5, containing 2% EDTA. The EDTA caused a dip to occur just before the creatinine peak (Fig. 9A). A better-looking electropherogram, with longer migration times, and of course a relatively smaller EDTA dip, was obtained with a 1:1 dilution (Fig. 9 B). It may be that an intermediate dilution factor would be

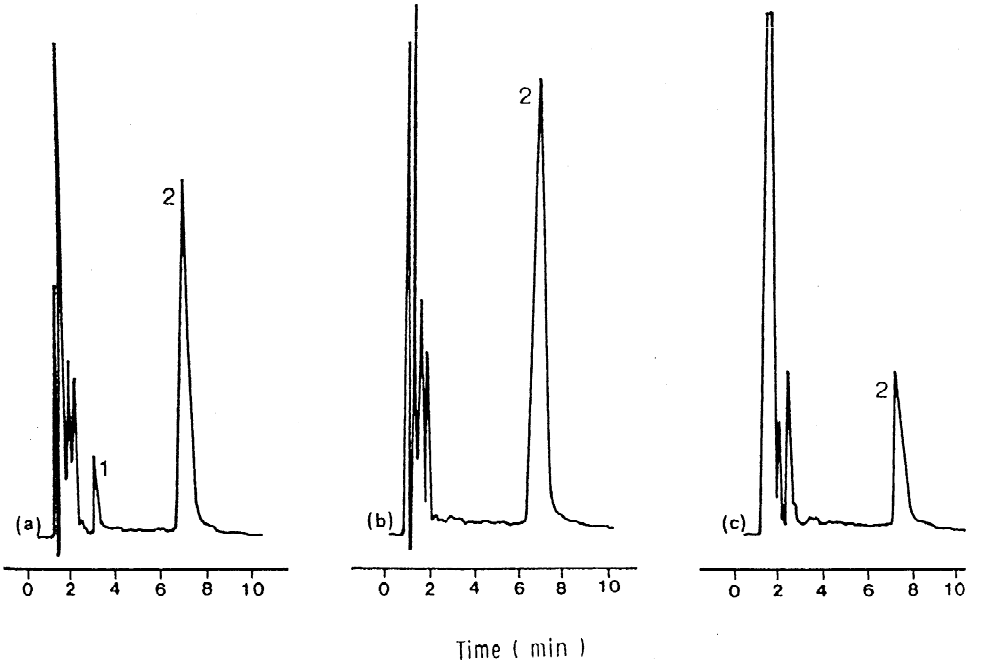

Fig. 8. Chromatograms of baby urine (a), adult urine (b) and adult serum (c) after treatment with trifluoroacetic acid and passage through a C_8 extraction cartridge. A Hypercarb column (4.6×100 mm, 7 μm) was used on the HPLC, with an eluent of 3% acetonitrile in 0.1% TFA (1 ml/min) and detection at 210 nm. Peaks: (1) creatine; (2) creatinine. From ''Separation of anionic and cationic compounds of biomedical interest by high-performance liquid chromatography on porous graphitic carbon'' [45] with permission from Elsevier Science.

most successful. A separation method is most robust if slight shifts in migration rate or pH will not destroy the separation.

4.2. Micellar electrokinetic capillary chromatography

The addition of a micellar phase allows for separation of neutral species that would otherwise migrate together. Separation of other species can also be enhanced. Serum creatinine has been analyzed using an 80 m*M* SDS–20 m*M* borate buffer, pH 9 [60], and urinary creatinine was analyzed in 150 m*M* SDS–30 m*M* phosphate, pH 7 [61]. Creatinine and uric acid in urine were analyzed simultaneously using 50 m*M* SDS as the micellar phase in a pH 9.0 phosphate buffer containing 5% isopropanol [62].

Yan et al. [63] reported that all the major constituents of urine—creatine, creatinine, urea and uric acid—could be determined using 0.75 *M* sodium cholate in 0.77 *M* phosphate buffer at pH 7.4 with detection at 200 nm. However, the method was not actually used to analyze any clinical samples, and neither the concentrations of standards used to obtain the electropherograms shown, nor LODs, were reported. Although the four specified compounds were able to be separated, the method would not be considered particularly robust as the urea, creatinine and creatine were just resolved under the optimum conditions shown (Fig. 10). The complete separation took upwards of 23 min depending on the particular conditions chosen, but if uric acid was not of interest the analysis could be completed in half this time.

In our lab, 150 m*M* SDS in a 30 m*M* phosphate buffer, pH 6, was used to analyze urinary creatine and creatinine, after 100-fold dilution of the sample with water [64]. Under these conditions, urea was

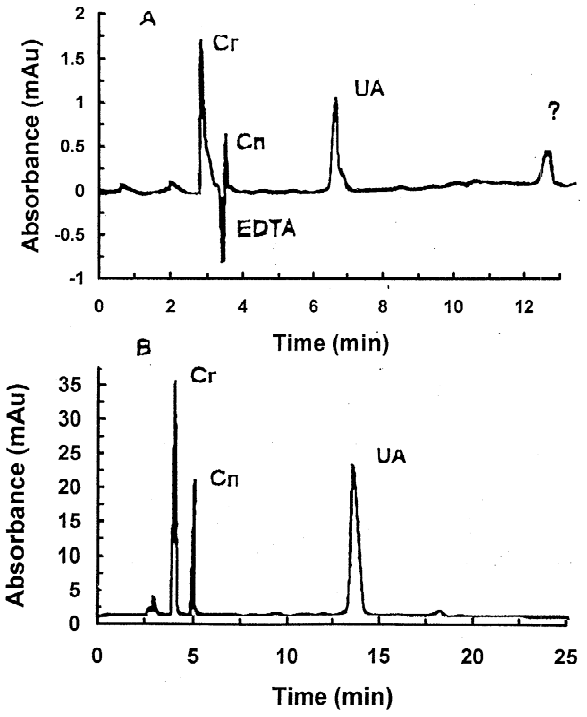

Fig. 9. Electropherograms of a urine sample diluted 1:50 (A) and 1:1 (B) in buffer containing 2% EDTA. The separations were carried out at 25 kV with injection for 5 s at 0.5 p.s.i., the buffer was 50 m*M* phosphate (pH 5.5) and detection was by absorbance at 214 nm. Peaks: (Cr) creatine; (Cn) creatinine; (UA) uric acid; (EDTA) ethylenediaminetetraacetic acid. From ''High-throughput multi-analyte screening for renal disease using capillary electrophoresis'' [59] with permission from Elsevier Science.

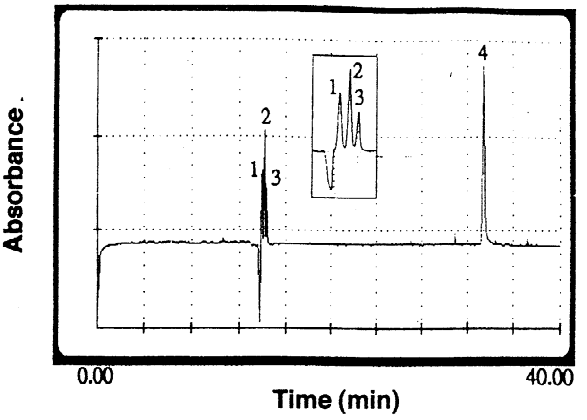

Fig. 10. Electropherogram of standard solution. The separation was carried out at 25 kV with injection for 5 s, the buffer was 0.077 *M* phosphate with 0.75 *M* sodium cholate, and detection was by absorbance at 200 nm. Peaks: (1) urea; (2) creatinine; (3) creatine; (4) uric acid. From ''Separation of urea, uric acid, creatine, and creatinine by micellar electrokinetic capillary chromatography with sodium cholate'' [63] with permission from Preston publications, a Division of Preston Industries, Inc.

not able to be detected when run alone in concentrations typical of those in urine. In urine samples, peaks were seen only at 3.7 min (system peak), 4.3 min (creatine) and 5.6 min (creatinine) in a 60-cm capillary with an applied voltage of 17 kV (Fig. 11). As the pH of the buffer was increased to 7, the resolution dramatically decreased. Lowering the pH led to unnecessarily long elution times for the creatinine. The LOD was 2 μg/ml for creatine and 1 μg/ml for creatinine, and the standard curves were linear to 500 μg/ml. The advantage of a CE method is that once the species of interest have passed the detector, the rest of the sample can be sucked through. The disadvantage of the method is that rinsing procedures between samples are essential and must be carefully timed. However, the instruments are easily programmed to do this automatically. High buffer concentrations cause significant Joule heating, and instruments with good temperature control are

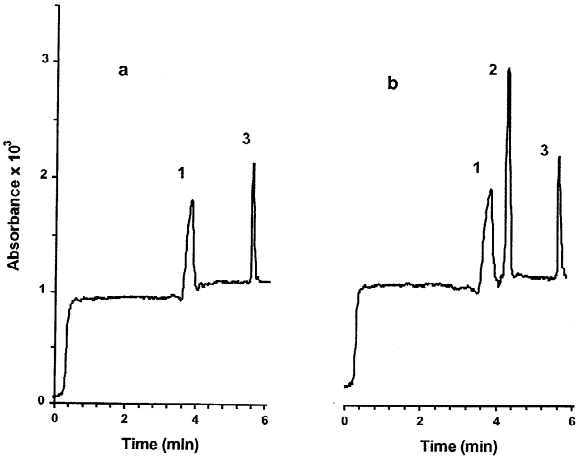

Fig. 11. Chromatograms of normal urine diluted 1:100 (a) and diluted urine spiked with creatine (b). The separation was carried out at 17 kV with hydrostatic injection for 20 s, the buffer was 30 m*M* phosphate–150 m*M* sodium dodecyl sulfate (pH 6.0) and detection was by absorbance at 214 nm. Peaks: (1) system peak; (2) creatine; (3) creatinine.

required to ensure that migration times remain constant.

5. Hyphenation procedures

5.1. HPLC–mass spectrometry

An isotope dilution method based on analysis by discharge-assisted thermospray liquid chromatography–mass spectrometry has been reported for creatinine [65]. A d_3-labeled isomer was used, the eluent was 10 m*M* ammonium acetate, and the $[MH]^+$ ion was monitored.

Isotope dilution electrospray tandem MS, using d_3-labeled creatine as the standard, has been used for the simultaneous assay of plasma for creatine and guanidinoacetate after their derivatization as butyl esters [66]. No sample clean-up was necessary before derivatization. This procedure would be equally useful for urine. The only disadvantage is that this procedure does not allow for determination of creatinine.

Urinary creatine, creatinine and guanidinoacetic acid have been determined using HPLC–MS [67]. The HPLC eluent was 2% acetonitrile in 100 m*M* ammonium acetate, chosen because of its volatility. The column was 5-μm Inertsil ODS-2 (250×4.6 mm), and an atmospheric pressure chemical ionization (APCI) interface was used. The determination was carried out by scanning the $[MH]^+$ ions for each compound. Representative mass spectra are shown in Fig. 12. As would be expected, use of higher percentages of acetonitrile in the eluent decreased the separation of the creatine and creatinine peaks. The sample preparation was relatively complicated as it involved passage of the samples through two ion-exchange columns. Urine or serum was mixed with 2% sulfosalicylic acid, passed through a cation-exchange column, eluted with ammonium hydroxide, evaporated to dryness, and eluted through an anion-exchange column with acetic acid before a final evaporation to dryness. Recoveries were ~94%. As creatine and creatinine can be separated easily by HPLC, it seems that their analysis by HPLC–MS should be possible with much less sample preparation, particularly if they are the only components of interest. We are investigating this possibility.

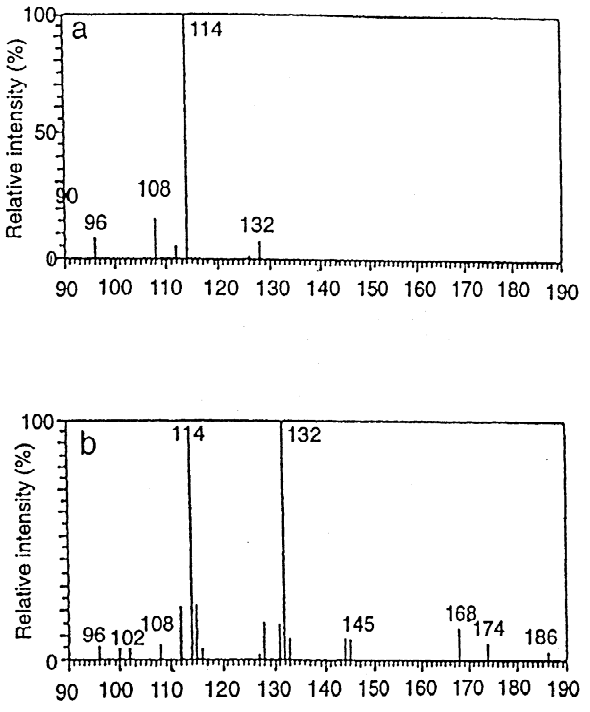

Fig. 12. Atmospheric pressure chemical ionization mass spectra of creatinine (a) and creatine (b) as determined after HPLC separation on an ODS-2 column using a 100 m*M* ammonium acetate buffer containing 2% acetonitrile. From ''Simultaneous determination of creatinine, creatine, and guanidinoacetic acid in human serum and urine using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry'' [67] with permission from Academic Press, FL.

5.2. GC–mass spectrometry

Several methods involving isotope dilution GC–MS have been suggested as reference methods for the determination of creatinine in serum or urine. Creatine and creatinine must be derivatized to facilitate their movement through a GC column, and as both commonly form the same derivatives, the creatinine is first separated from creatine by passage through a weakly acidic cation-exchange column in the hydrogen-form (wash with water to remove creatine, elute creatinine with 1–2 *M* ammonia) [68,69], or by HPLC [70]. This initial isolation step, and the specificity of MS, means that a method developed for serum should be equally applicable to urine analysis. If the creatine-containing fraction was

kept after the initial separation process, this too could be derivatized and analyzed. Such a method is currently under development in our lab.

Derivatives which have been used include *O*-trifluoroacetylcreatinine [71], the di-trifluoroacetate of the (2-hydroxy, 2-methyl)ethyl derivative, made by reaction with 1,2-epoxypropane then trifluoroacetic acid (ions at m/e 294 and m/e 296) [70], the ethyl ester of *N*-(4,6-dimethyl-2-pyrimidinyl)-*N*-methylglycine made by reaction with 2,4-pentanedione [69], and the trimethylsilyl derivative made by reaction with *N*-methyl-*N*-trimethylsilyl-trifluoroacetic amide (MSTFA) [68]. Of these, the reaction with 2,4-pentanedione is the most facile. However, although analysis of the $^{13}C_2$-creatinine pentanedione derivative was reproducible, allowing it to be used as a standard, analysis of the d_3-labelled species was not [69]. We have also experienced reproducibility problems for GC–MS of the pentanedione derivative of d_3-creatine. It seems that some separation of the two derivatives occurs. This is unfortunate, as the d_3-species is cheaper than the other isotopically labelled species. $^{15}N_2$-labeled creatinine [70], and $^{13}C_2$,$^{15}N_2$-creatinine [68] have successfully been used as standards for isotope dilution, and therefore would also be suitable as metabolic tracers

6. Final comments and conclusions

The method to be chosen for an analysis of creatine and creatinine would first of all depend on whether any compounds other than creatine and creatinine were required to be analyzed at the same time. For example, Yokoyama et al.'s [44] simultaneous analysis of creatine and creatinine with 1- and 3-methylhistidines (Fig. 7) would be very useful for following muscle breakdown at the same time as analyzing creatine uptake during supplementation.

If the concentrations of creatine are large and similar to that of creatinine, larger dilution factors can be used to help peak resolution. Creatine concentrations are particularly elevated when subjects are supplementing with creatine.

The HPLC and CE methods without precolumn treatments are the simplest options for analysis of urinary creatine and creatinine. The method of choice would be isocratic HPLC with a phosphate buffer [13,42,43]. Second choice would be CE separation using a phosphate–SDS buffer [64]. Although this method requires more attention to detail to ensure reproduciblity than does the HPLC analysis, solvent consumption is reduced.

Better HPLC separations can be achieved using more complicated sample pretreatments, and should be used when very exact quantitation of smaller amounts of creatine is required, or when a variety of other substances need to be analyzed. When following the uptake of creatine during supplementation, interfering peaks are small compared to the enhanced creatine levels and would not significantly alter results if the resolution was slightly less than desired.

The choice of column is critical, even among columns that are purportedly the same but from different manufacturers. Dunnett et al. [37] reported that a Spherisorb column gave better separation than Lichrosphere RP18, Apex ODS and Hypersil ODS. There are also new reversed-phase columns on the market that are designed for use in aqueous eluents.

The main checks of quantitation reliability have been carried out for HPLC using the Jaffe method [43] or GC–MS [16]. The correlation was excellent. Analysis by MS can be used just as an alternative, or may be required if positive identification of a species is needed, or if isotopic ratios are to be found. No derivatization is required with HPLC–MS [67] and d_3-labelled creatinine has been successfully used. On the other hand, the reported procedure included an extensive sample workup.

For all the methods discussed, the LOD is well below the typical concentrations for creatine and creatinine, and in fact sample dilution is usually necessary. This often means that potentially interfering substances are well below the LOD.

Overall, there are a variety of methods which can enable creatine and creatinine to be determined and the choice will depend on the instrumentation available and the nature of the information that is being sought.

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