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Determination of creatinine and other uremic toxins in human blood sera with micellar electrokinetic capillary electrophoresis[1]

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Abstract

We have been interested in the clinical use of capillary electrophoresis (CE) to monitor low-molecular-mass uremic toxins in body fluids. Creatinine, an important clinical marker for renal failure, is zwitterionic over a fairly wide pH range (pH 5–9) and can not be resolved from neutral components using free solution CE under these conditions. We report here a micellar electrokinetic capillary chromatography method using an sodium dodecyl sulfate–borate buffer system at pH 9.0 to determine creatinine levels in human serum. This method, performed on deproteinized sera, is also suitable for determining multiple ionic components. Moreover, this method compares favorably with an enzymatic method for creatinine performed in a clinical laboratory and thus appears to be a promising method in terms of potential clinical use.

Keywords: Creatinine; Toxins

1. Introduction

Because of the speed of analysis, capability for single-run multi-component determinations and minimal waste generation, the use of capillary electrophoresis (CE) for the analysis of biological fluids [1–10] in the clinical medical laboratory is an attractive alternative to many current analysis schemes. We have become particularly interested in exploring the use of CE for the analysis of blood serum for a number of low-molecular-mass components that approach toxic levels in patients afflicted with chronic renal failure. We have recently reported a free solution CE method employing borate buffer at pH 9.0 to determine several key metabolites, including hippuric acid, uric acid, hypoxanthine and pseudouridine, in ultracentrifuged sera [10]. Uniformly higher levels of these four components were found in uremic sera when compared to "normal" sera. Unfortunately, creatinine could not be determined with this free solution CE method. Creatinine, the most widely used clinical marker for renal dysfunction [11–13], is zwitterionic at pH 9.0 and therefore coelutes with neutral serum components when free solution CE is performed at this pH. Creatinine has pK_a values at 4.8 and at 9.2 [14]. In principle, creatinine can be separated from other serum components using free solution CE at a pH below 4.8 (where it is cationic) or at a pH above 9.2 (where it is anionic). The former has been reported [8] and is useful for creatinine determinations, but results in the coelution of many acidic metabolites.

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The latter has proven ineffective in our laboratory for separating creatinine from other neutral components, including nicotinamide and caffeine, at least with borate buffer systems with pH values as high as 10.00. Alternatively, zwitterionic creatinine can be separated from neutrals by the addition of a micellar pseudo-stationary phase into the CE buffer. This approach has been used by Schmutz and Thormann [9] and by Miyake et al. [4] to qualitatively and quantitatively determine creatinine in serum, respectively. Both of these reports employ direct serum injection. We report here a micellar electrokinetic capillary electrophoresis (MEKC) method performed on deproteinized sera that offers improved separation efficiency and reproducibility to that reported with the direct serum injection methods.

In recent years, several groups have reported CE analysis schemes for determining creatinine in biofluids [4,8,9]. The use of a low pH buffering system that included 18-crown-6 as a chelating agent and pyridine as the chromophore for indirect detection of creatinine in the cationic form, as well as of several inorganic cations in urine has been reported [8]. Miyake et al. [4] have successfully used MEKC to determine creatinine and uric acid in both blood sera and urine using antipyrene as an internal standard and a phosphate–sodium dodecyl sulfate (SDS)–isopropanol buffer system at pH values of 9 and 6, respectively. In this work, the serum samples were directly analyzed without any attempt to remove proteins from the sera prior to injection. More recently, Schmutz and Thormann [9] have explored various CE operating conditions for determining several low-molecular-mass drugs and metabolites, including creatinine, using direct serum injection with a phosphate–borate–SDS buffer system. In the above applications, the low-molecular-mass components were found to elute in a "window" before the higher molecular mass proteins in the sera. Interactions between the proteins and the inner walls of the capillary were minimized by using SDS or urea to help solubilize the proteins, or by employing buffers with high ionic strength. Despite the presence of serum proteins, these methods were reasonably effective; however, a flushing routine that involves rinsing the capillary with NaOH was required prior to each analysis in order to ensure reproducible results. In addition, separation efficiency can be compromised by the presence of the proteins.

We report here an alternative CE method employing a simple SDS–borate buffer system to separate and quantify creatinine levels in deproteinized sera. The deproteinization, which is accomplished via ultracentrifugation, removes problematic proteins from the serum sample and allows the use of an operational buffer with moderate ionic strength. By removing the proteins prior to the separation, the difficulties associated with erratic flow and coelution of late-eluting small molecule components with the early eluting proteins are minimized. Moreover, the need for frequent caustic rinsing of the capillary is eliminated. The analysis scheme described is used to determine creatinine levels in blood sera obtained from Geisinger Medical Center (Danville, PA, USA) and our findings are compared to those obtained in the clinical laboratory at Geisinger with an accepted enzymatic method. In addition to creatinine determination, this method is also capable of profiling the other low-molecular-mass components, including hippuric acid and uric acid. Only creatinine is quantitatively determined in this work.

2. Experimental

2.1. Chemicals

Indican, 2-hydroxyhippuric acid, indole-3-acetic acid, kynurenic acid, pseudouridine, theobromine and theophylline were purchased from Sigma (St. Louis, MO, USA). 1-Methylnicotinamide iodide was purchased from Lancaster Laboratories (Winham, NH, USA). Allopurinol, hypoxanthine, 4-hydroxyphenylacetic acid, xanthine, caffeine, nicotinamide and creatinine were purchased from Aldrich (Milwaukee, WI, USA). Ferulic acid and nicotinic acid were purchased from ICN Biochemicals (Cleveland, OH, USA) and sodium dodecyl sulfate and nicotinamide-1-oxide were purchased from ICN Biomedicals (Aurora, OH, USA). Boric acid and hippuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Uracil, L-(−)-tyrosine and L-(−)-tryptophan were purchased from Eastman Kodak (Rochester, NY, USA). Uric acid was purchased from Mallinckrodt (St. Louis, MO, USA). All reagents were used as purchased without further purification.

2.2. Solutions

The CE borate buffer solutions were prepared by dissolving boric acid in HPLC-grade water, adjusted to pH 9.0 with 1.0 *M* sodium hydroxide and vacuum filtered through a Supelco Nylon 66 membrane (0.2 μm, Supelco, Bellefonte, PA, USA). MEKC running buffer solutions were prepared by dissolving SDS in the pH-adjusted borate solution. The buffer solutions were filtered with a Whatman syringe filter (0.45 μm, Whatman, Clifton, NJ, USA) prior to use.

Standard solutions composed of 21 "target" compounds were prepared by dissolving the appropriate amounts in 20 m*M* borate buffer at pH 9.0. The standard solutions served to determine the characteristic elution parameters and UV spectra for each compound that was used for identification of peaks in serum separations.

A series of standard calibration solutions for creatinine ($1.0 \cdot 10^{-4}$ to $1.00 \cdot 10^{-2}$ *M*), each of which also contained $1.00 \cdot 10^{-4}$ *M* theobromine as an internal standard (IS), were prepared in 18 MΩ water.

2.3. Serum

Blood serum samples from healthy and uremic individuals were obtained from Dr. Joe Bisordi and Dr. Jay Jones (Geisinger Medical Center). Serum samples were deproteinized prior to analysis by CE. An internal standard (100 μl of 1.00 m*M* theobromine) was combined with 900 μl of serum in a Centrifree Micropartition filter (M_r cut-off = 30 000; Amicon, Beverly, MA, USA) and this mixture was centrifuged at 1580 *g* for 1 h. The ultrafiltrate was used directly in the CE analysis.

2.4. Capillary electrophoresis

A Hewlett-Packard ^{3D}CE capillary electrophoresis system (Hewlett-Packard, Wilmington, DE, USA) equipped with a diode-array UV detector was used to perform all the separations. Electropherograms were collected at 210 and 254 nm, and spectra were obtained for all peaks from 190 to 300 nm with the aid of an HP Vectra 486/66U computer and Hewlett-Packard ^{3D}CE Chemstation software.

Hewlett-Packard extended light path capillaries (50 μm I.D.) were used for all separations. For the MEKC separations, the running and flushing buffer for all analyses was 80 m*M* SDS–20 m*M* borate, pH 9.0. After each serum analysis, the capillary was flushed with the buffer for 5 min. Injection was performed by applying 5 kPa (50.0 mbar) of pressure to the inlet vial for a duration of 5.0 s. All serum separations were carried out at 25°C using a 72-cm effective length (80.5 cm total length) capillary and a separation voltage of 22 kV, generating a maximum of ca. 22 μA of current across the capillary.

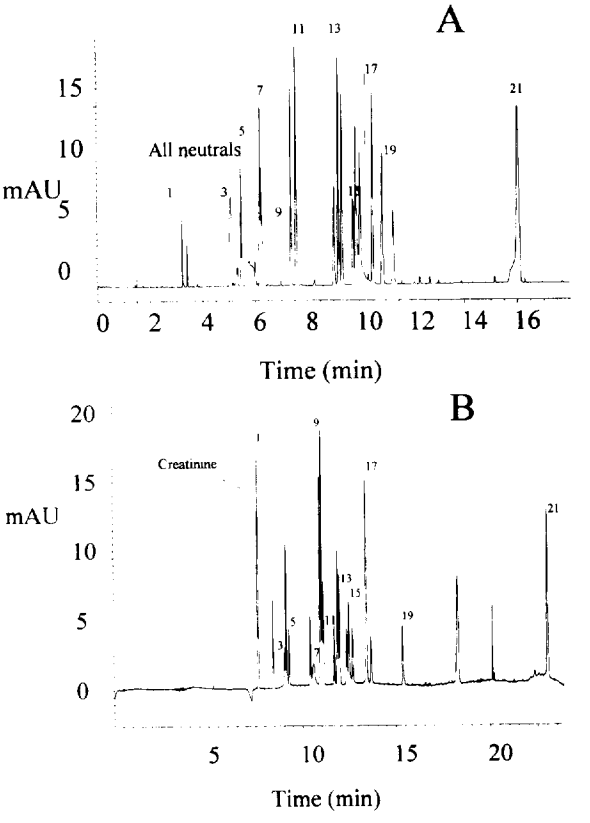

Fig. 1. CZE (A) and MEKC (B) separations of a standard mixture of target compounds illustrating the ability of MEKC to separate creatinine from other neutral analytes. (A) CZE separation showing coelution of creatinine with other neutrals. CZE separation conditions: 150 m*M* borate, pH 9.0; 80.5 cm (72.0 cm injector to detector), 50 μm I.D. capillary; 24 kV (36 μA); 7 s, 5 kPa (50 mbar) injection; UV detection at 210 nm; analytes ca. $5 \cdot 10^{-4}$ *M*. (B) MEKC separation showing the resolution of creatinine from other species. MEKC separation conditions: 20 m*M* borate–80 m*M* SDS, pH 9.0; 72 cm effective length (80.5 cm total), 50 μm I.D. capillary; 22 kV (22 μA); 5 s, 5 kPa (50 mbar) injection; UV detection at 210 nm (shown) and 254 nm with UV spectra collection within each peak; analytes ca. $1 \cdot 10^{-4}$ *M*, with the exception of creatinine ($5 \cdot 10^{-4}$ *M*).

2.5. Data analysis

Elution time, relative elution time (ratio of elution time to time of flow marker), retention factor and effective retention factor were used for primary peak identification in the MEKC separations. In addition, UV spectra were used to aid in the assignment of unknown peaks in serum sample separations. Quantification of creatinine was accomplished by using the peak-area ratio of creatinine to theobromine and by comparing this ratio with the calibration curve obtained with authentic standards. Calibration curves were constructed by plotting the peak-area ratio of creatinine to theobromine vs. creatinine concentration for a series of external calibration standards.

3. Results and discussion

A comparison of the elution profiles for capillary zone electrophoresis (CZE) and MEKC separations of several compounds expected to be present in biofluids is given in Fig. 1. A list of the compounds in this mixture of authentic standards and the corresponding peak numbers in each separation is given in Table 1. Fig. 1A clearly illustrates the use of CZE with a borate buffer system to separate many of these important metabolic waste products; however, creatinine coelutes with all other neutral species in peak 3. A similar set of metabolites is separated using an SDS–borate buffer system in Fig. 1B. With the MEKC system, creatinine is well resolved from the other analytes. Creatinine, a zwitterionic compound at pH 9.0, does not interact appreciably with the hydrophobic interior of the micelles and therefore elutes early in the separation.

Typical MEKC electropherograms obtained upon injection of the deproteinized sera obtained from healthy individuals (normal) and from patients diagnosed with chronic renal failure (uremic) are given in Fig. 2. Qualitative assignment of peak identity can be accomplished by using elution time and/or retention

Table 1
List of target compounds considered in this study, along with corresponding peak numbers for the separations shown in Fig. 1

Compound	CZE peak number Fig. 1A	MEKC peak number Fig. 1B
Creatinine	3[a]	1
Nicotinamide	3[a]	2
Uracil	7	N/A
Tyrosine	8	3
Theobromine	N/A	4
Allopurinol	6	5
Hypoxanthine	9	6
Pseudouridine	N/A	7
Tryptophan	5	8
Caffeine	3[a]	9
Theophylline	10	10
Hippuric acid	11	11
Kynurenic acid	12	12
Indole-3-acetic acid	13	13
4-Hydroxyphenylacetic acid	14	14
Uric acid	15	15
Xanthine	16	16
Ferulic acid	17	N/A
Indican	18	17
Nicotinic acid	19	18
1-Methyl nicotinamide	1	19
2-Hydroxyhippuric acid	20	20
Phenyltriethylammonium ion	2	N/A
Quinine (micelle marker)	N/A	21

[a] Denotes metabolites that coelute.

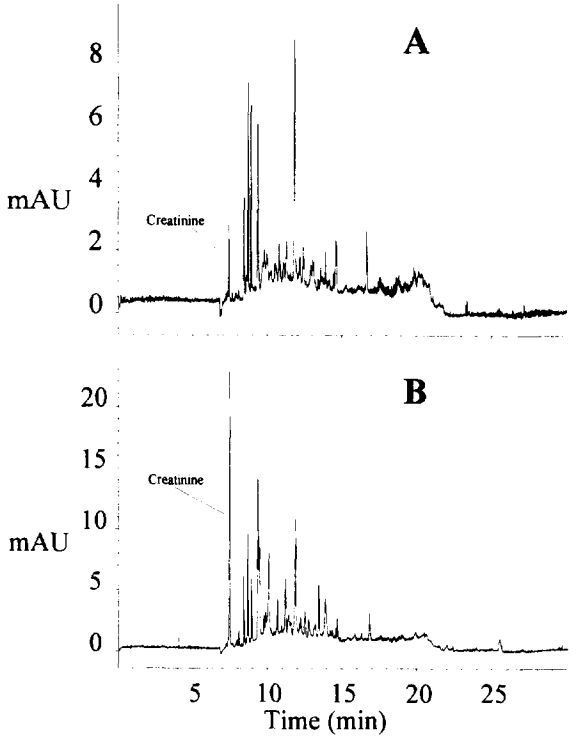

Fig. 2. MEKC separations of (A) a "normal" serum sample and (B) a serum sample from a patient with chronic renal failure. Serum samples have been deproteinized by ultrafiltration prior to CE analysis. Separation conditions: 20 m*M* borate–80 m*M* SDS, pH 9.0; 80.5 cm (72.0 cm injector to detector), 50 μm I.D. capillary; 22 kV (21 μA); 5 s, 5 kPa (50 mbar) injection; UV detection at 210 nm (shown) and 254 nm with UV spectra collection within each peak. Creatinine concentrations in these samples were determined to be 0.14 and 1.60 m*M*, respectively.

factor. The use of various successful methods of peak assignment are summarized in Table 2. With CE separations, the sole use of elution time is often inadequate for peak identification, due to matrix effects and unpredictable fluctuations in flow-rates. The relative elution time can be calculated by normalizing the elution time of the analyte to the time that the flow marker passes the detector (t_o indicated by the "dip" at about 7 min in Fig. 2). The most rigorous treatment of separation parameters that can be used for peak identification, however, is the retention factor. Retention factors have been calculated for each of the target species by first obtaining their electrophoretic mobilities by performing a separation in the absence of micelles. Then, using the determined electrophoretic mobility in conjunction with the MEKC data, the true value of the retention factor describing the analyte equilibrium between micelle and free solution can be calculated [15]. This method, although thorough, requires two analyses; a CZE and a MEKC separation. A more practical approach, given in the final column in Table 3, involves the calculation of an effective retention factor. The effective retention factor is calculated by incorporating the effects of the electrophoretic mobility of the free ion and that of the ion when it is bound to the micelle into a single parameter. This treatment is mathematically identical to assuming that all species have zero electrophoretic mobility. This treatment is not rigorous, but is a practical way (as long as both t_o and t_{mc} are known) to qualitatively identify unknown peaks without the need to perform a separation in the absence of micelles. With each of the methods given in Table 2, the creatinine peak in a serum sample can be easily identified when coupled with the power of the UV spectral data. As shown in Table 3, the reproducibility of elution time and relative elution time are sufficient for qualitative identification of creatinine in serum. It should be noted that the relative elution time for creatinine in standard solutions and of that in serum are slightly different, presumably due to sample matrix effects. For later-eluting species, this difference is minimized. Nonetheless, the peak can be easily identified using this method. Also listed in Table 3 is the efficiency of separation observed as measured using the creatinine peak. This value compares favorably with values obtained using direct serum injection [4].

In addition to the elution time and retention parameters, UV spectral characterization of the creatinine peak aids in the conclusive identification of this peak. A spectrum obtained from the creatinine peak in Fig. 2B (uremic sera) is overlaid with the spectrum obtained upon injection of an authentic creatinine standard in Fig. 3. The match is nearly perfect, supporting our assignment of the origin of this peak to creatinine.

The differing levels of creatinine in the two samples analyzed in Fig. 2 are easily discerned with the MEKC method. Quantitation of creatinine was accomplished using theobromine as an internal standard at a constant concentration of 0.100 m*M* in all samples. A calibration plot for creatinine (eight data points ranging in concentration from 0.100 to 10.0

Table 2
Elution parameters for MEKC separation of standard solutions of the target analytes considered in this study for separation with a 20 m*M* borate–80 m*M* SDS buffer system, as seen in Fig. 2B

Compound	Elution time (min)	Relative elution time[a] (min)	Retention factor[b]	Effective retention factor[c]
Creatinine	7.67	1.04	0.074	0.074
Nicotinamide	8.47	1.16	0.252	0.252
Tyrosine	9.07	1.24	0.879	0.398
Theobromine	9.18	1.26	0.333	0.426
Allopurinol	9.34	1.28	0.157	0.467
Hypoxanthine	10.46	1.43	0.162	0.788
Pseudouridine	10.62	1.45	0.124	0.840
Tryptophan	10.98	1.50	0.684	0.961
Caffeine	11.08	1.52	0.992	0.992
Theophylline	11.16	1.53	0.285	1.021
Hippuric acid	11.74	1.61	0.217	1.233
Kynurenic acid	11.93	1.63	0.238	1.308
Indole-3-acetic acid	12.02	1.64	0.242	1.344
4-Hydroxyphenylacetic acid	12.40	1.70	0.262	1.505
Uric acid	12.51	1.71	0.266	1.554
Xanthine	12.77	1.75	0.286	1.671
Indican	13.45	1.84	0.384	2.015
Nicotinic acid	13.71	1.87	0.393	2.156
1-Methyl nicotinamide	15.42	2.11	13.65	3.339
2-Hydroxyhippuric acid	18.34	2.51	1.259	7.344
Quinine	23.09	3.16	–	–

[a] Relative elution time calculated by dividing the elution time by the time of the flow-rate indicator (negative dip in the baseline).
[b] Retention factors were calculated as described by Nelson and Foley [15] and are reproducible using standards with R.S.D. (%) values of less than 1. The μ_{ep} for each compound was determined via CZE with 20 m*M* borate, pH 9.00, as the operational buffer.
[c] Effective retention factors were calculated assuming all species are neutral (i.e. $\mu_{ep}=0$) and are reproducible using standards with R.S.D. (%) values of less than 1.

m*M*) was constructed by using the peak-area ratio of creatinine to theobromine (at 254 nm) vs. the concentration of creatinine. The relationship was linear with a slope of 4090 and an intercept of 0.40 (r^2= 0.997, standard error=0.949). Using this calibration plot, the creatinine level of nine different uremic serum samples was determined. R.S.D. values obtained for three parallel creatinine analyses of each serum sample using the ultrafiltration–MEKC method reported here were typically between 5 and 13% (even for the lower "normal" concentrations of creatinine). A comparison of our findings with that obtained for the sera samples in the clinical laboratory at Geisinger is given in Table 4. Our values were within 30% of the values obtained with the enzymatic method in the clinical laboratory [16]. A creatinine concentration at, or above, 2 mg/dl (0.18 m*M*) is considered abnormally high and may be

Table 3
Reproducibility of several separation parameters for the MEKC determination of creatinine in serum samples

	Average	S.D.	R.S.D.(%)
Retention time (min)	7.40	0.16	2.16
Relative retention time (unitless)	1.020	0.006	0.64
Separation efficiency, *N* (plates)	$1.89 \cdot 10^5$	$0.16 \cdot 10^5$	8.22

For all values, $n=27$ (nine serum samples analyzed three times each).

Table 4
MEKC quantification and comparison of creatinine concentration using the MEKC method and the clinical method

Sample	Creatinine concentration (m*M*)	R.S.D.[a] (%)	Percentage difference from clinical laboratory results
1	0.68±0.02	2.9	10
2	0.143±0.002	1.4	25
3	0.83±0.10	12	−6
4	0.84±0.12	14	17
5	0.43±0.01	2.3	−31
6	0.82±0.03	3.6	12
7	1.17±0.05	4.3	8
8	0.86±0.04	4.6	−7
9	0.94±0.06	6.4	13

Errors represent standard deviation ($n=3$).
[a] Relative standard deviation of three independent analyses of each serum sample via the ultrafiltration–MEKC analysis scheme.

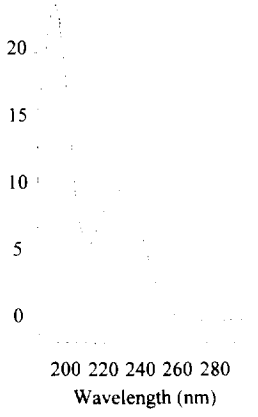

Fig. 3. Identification of creatinine by UV spectral match. Solid line represents a spectrum obtained upon injection of a creatinine standard; dotted line represents spectrum obtained from the identified creatinine peak in Fig. 2B.

indicative of renal failure. Clearly, our method is capable of determining "elevated" levels of creatinine.

4. Conclusions

The reported MEKC method is capable of accurately determining creatinine concentrations in human blood sera. This method may find clinical use due to the multi-component determination capability of this separation-based analysis scheme. Using this method, one could determine several uremic toxins, including creatinine, in a single 20 to 30 min MEKC analysis.

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