High-performance capillary zone electrophoretic assay for markers of diabetic nephropathy in plasma and urine

Yu Kong[a], Ning Zheng[b], Zhichao Zhang[a], Ruyu Gao[a],*

[a]State Key Laboratory of Elemento-Organic Chemistry, Institute of Elemento-Organic Chemistry, Nankai University,
Tianjin 300071, China
[b]Tianjin Metabolic Disease Hospital, Tianjin 300071, China

Abstract

A new high-performance capillary zone electrophoretic assay for creatine (Cr), creatinine (Cn), urea (U) and uric acid (Ua), markers of human diabetic nephropathy, both in plasma and urine has been developed with UV detection at 200 nm. The plasma sample was deproteinized with trichloroacetic acid and centrifuged at 10 000 rpm for 10 min. The urine sample was diluted 20-fold with buffer before analysis. The optimum separation conditions for the markers was investigated with respect to the concentration of the buffer, the pH, the voltage and the capillary temperature. Baseline separation was achieved in 25 mmol/L phosphate buffer (pH 3.45) using a 21 cm×75 μm I.D. fused-silica capillary at 40 °C with an electric field of 1190 V/cm. The calibration curves showed good linearity in the range 3.5–1000, 0.18–700, 500–5000 and 2–800 μM ($r^2_{min} > 0.998$) for Cr, Cn, U and Ua, respectively. The proposed method also has a high reproducibility (peak area $RSD_{max} < 3\%$) and has been successfully applied to the determination of clinical samples.
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1. Introduction

Diabetic nephropathy (DN) is a serious complication of diabetes mellitus. It is estimated that death due to renal disease is 17 times more common in diabetics than in nondiabetics. It is now well established that the urinary excretion of albumin is predictive of morbidity and mortality due to renal complications and cardiovascular disease. However, when DN was diagnosed by this classical method, little could be done to prevent the progressive downhill course to renal failure. It would be more significant, therefore, if DN could be detected at an even earlier stage, before the appearance of microalbuminuria, so that intervention could reverse the process. Nonprotein nitrogen compounds, including creatinine (Cn), creatine (Cr), urea (U), uric acid (Ua), etc., could serve as markers for renal function. Cr, U and Ua are good indicators of the glomerular filtration rate (GFR) of the kidneys. Cn is the major breakdown product of phosphocreatine and Cr, which is one of the most widely used markers of renal function. Ua, which serves as a marker for tubular reabsorption of the nephrons in addition to the GFR, is the major product of the catabolism of adenine and guanine.

Numerous methods for the assay of these markers have been reported, such as enzymatic assays [1,2], spectrochemical methods [3], high-performance liquid chromatography (HPLC) [4–7] and capillary electrophoresis (CE) [8–10]. The enzymatic assays

and spectrochemical methods are limited due to the slow speed of analysis, the inability to test for multiple markers at one time, non-specificity for the analyte of interest and the consumption of large amounts of reagents and samples. The major drawbacks with HPLC methods are the relatively long analysis times, and large consumption of solvents, as well as the inability to effectively maintain the quality of the column in some cases. CE, in many cases, has advantages over HPLC due to its higher resolution, greater efficiency and smaller sample volumes.

The present CE methods, developed for the analysis of Cn, Cr, or Ua, have mostly been performed in the pH range from 5 to 10. However, under such conditions, U cannot be completely separated from Cn, Cr or Ua. In order to avoid interference from U, detection wavelengths above 214 nm have been selected [9] or sodium dodecyl sulfate has been added [10]. Few articles have reported the quantitative analysis of U and Cn, Cr and Ua simultaneously in both plasma and urine samples.

In this work, a new capillary zone electrophoresis (CZE) method for the analysis of the nonprotein nitrogen (Cn, Cr, U and Ua) in plasma and urine was developed. A systematic investigation of the effect of buffer concentration, pH, voltage and capillary temperature on the migration behavior and separation of Cn, Cr, U and Ua is presented. Baseline separation was achieved in a low-pH phosphate buffer (pH 3.45) within 15 min. Good reproducibility, a wide linearity range, and a relatively low detection limit were also obtained.

2. Experimental

2.1. Materials

Cr, Cn and Ua were obtained from Sigma (St. Louis, MO, USA). U, potassium phosphate, potassium dihydrogenphosphate, sodium monohydrogen-phosphate, phosphoric acid, potassium carbonate, sodium hydroxide and hydrochloric acid were purchased from Tianjin Chemical Reagent Co. (analytical-grade agent, Tianjin, China). Acetonitrile and methanol, HPLC-grade reagents, were purchased from Tianjin Juneng Chemical Co. (Tianjin, China).

Trichloroacetic acid (TCA) was obtained form Yuanhang Chemical Co. (analytical-grade agent, Shanghai, China). All solutions and samples were prepared in redistilled water and filtered through a 0.22 μm filter before use.

2.2. Apparatus

All CE experiments were performed using a Beckman P/ACE MDQ system (Beckman, Fullerton, CA, USA) equipped with a UV–Vis detector, an autosampler and a temperature controller (15–60±0.1 °C). Instrument control and data analysis were carried out by Beckman P/ACE system software (Ver. 1.5) on a personal computer. For pH measurements, a pH meter (pHS-25, Weiye, China) calibrated with a precision of 0.01 pH units was employed. Centrifugation was performed on a Biofuge status (Heraeus, Germany).

2.3. Sample preparation

Standard solutions (about 10 mmol/L) of Cr, Cn and U were prepared quantitatively using redistilled water. Ua (about 10 mmol/L) standard was dissolved in 30 mmol/L potassium carbonate buffer at 60 °C. All standard solutions were stored at 4 °C and diluted with buffer before use.

Plasma and urine samples were obtained from a DN patient. The plasma samples were deproteinized with acetonitrile, methanol or TCA. Acetonitrile or methanol was added directly to the plasmas at a ratio of 1:1 (v/v), and TCA was used to deproteinize plasma at a final concentration of 100 mg/mL. The proteins were separated by centrifugation for 10 min at 10 000 rpm and the supernatant was injected directly onto the CE system. The urine sample was diluted 20-fold with separation buffer prior to analysis.

2.4. Electrophoresis procedure

All separations were performed on a fused-silica capillary of 31 cm (effective length 21 cm)×75 μm I.D. (Yongnian Photoconductive Fibre Factory, Hebei, China) at 40 °C with a constant separation voltage of +25 kV. The separation buffer consisted

of 25 m*M* phosphate buffer (pH 3.45). The samples were injected in the pressure mode at 0.3 p.s.i. for 4 s (1 p.s.i.=6894.76 Pa). The detection wavelength was set at 200 nm. Between injections, the capillary was rinsed with 0.1 *M* NaOH for 3 min, 0.1 *M* HCl for 1 min, water for 1 min and buffer for 3 min to obtain a stable electroosmotic flow (EOF).

3. Results and discussion

3.1. Optimization of separation conditions

3.1.1. Influence of pH

The buffer pH plays an important role in separation in the CZE mode. In this work, the effect of buffer pH on migration times was investigated using 25 mmol/L phosphate buffer over the pH range 2–10. The results indicated that, when the pH is >4.4 or <2.45, the four markers can hardly be completely separated. Fig. 1 shows the effect of buffer pH on migration times of the four markers in the range 2.45–4.4. A satisfactory separation was obtained in this range. However, when the pH is >3.45, Cr was coeluted with the background impurity peak, and when the pH was reduced, the separation time increased as the EOF decreased. There-

fore, it was decided to carry out the separations at pH 3.45 considering the peak resolution and the separation time.

3.1.2. Effect of buffer concentration

The buffer concentration affected the thickness of the electric double layer on the capillary inner wall, thereby affecting the separation. Normally, separation was improved with increasing concentration of buffer. On the other hand, the migration time was delayed and the electric current was elevated, and, as a result, the Joule heat increased and the peak broadened. As a high electric current should be avoided in order to suppress Joule heat generation, the lowest concentration which provides a good separation is preferred. Fig. 2 shows the influence of buffer concentration on the migration times. The concentration was adjusted between 5 and 40 mmol/L. It can be seen that, when the concentration was <10 mmol/L, Cr and U could not be separated completely. From the electropherograms it can be observed that Cr could not be separated from the background impurity peak until the buffer concentration was 25 mmol/L. Therefore, a buffer concentration of 25 mmol/L was selected for further studies.
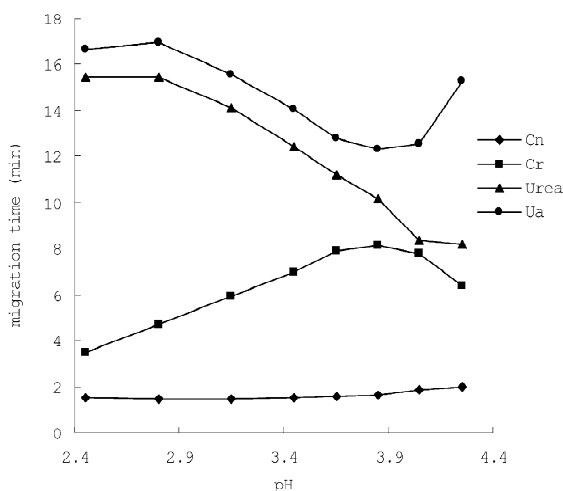

Fig. 1. Effect of buffer pH on migration times Conditions: fused-silica capillary (21 cm×75 μm), 25 mmol/L phosphate buffer, 25 kV, 20 °C, 200 nm. Between each run, the capillary was flushed as described in Section 3.2.
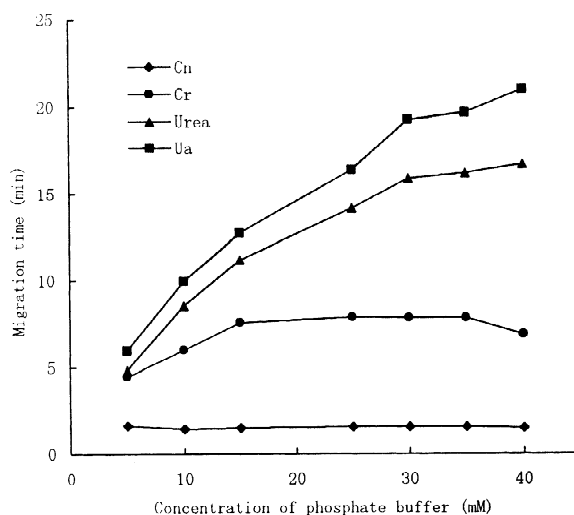

Fig. 2. Effect of buffer concentration on separation Conditions: fused-silica capillary (21 cm×75 μm), pH 3.45 phosphate buffer, 25 kV, 20 °C, 200 nm. Between each run, the capillary was flushed as described in Section 3.2.

3.1.3. Effect of voltage

In theory, increasing the applied voltage would improve the efficiency of the separation and shorten the migration time. However, with an increase of the applied voltage, the resolution (R_s) decreased and the increasing Joule heating affected the peak shape and method reproducibility. Fig. 3 shows the influence of voltage on the migration time. In the selected range, the markers could be baseline separated. Finally, a voltage of 25 kV, which produced a rapid (about 15 min) and complete separation, was selected as the best voltage.

3.1.4. Selection of capillary temperature

The capillary temperature is also important in CZE. In this study, the capillary temperature was increased from 20 to 50 °C. The effect of capillary temperature on migration time is shown in Fig. 4 and on electric current in Fig. 5. The results indicated that the markers could be separated completely in the selected temperature range. It was also found that the migration times of the four markers decreased and the current increased with increasing temperature, which was due to the decreasing viscosity of the buffer and increasing EOF. Cn, However, began to
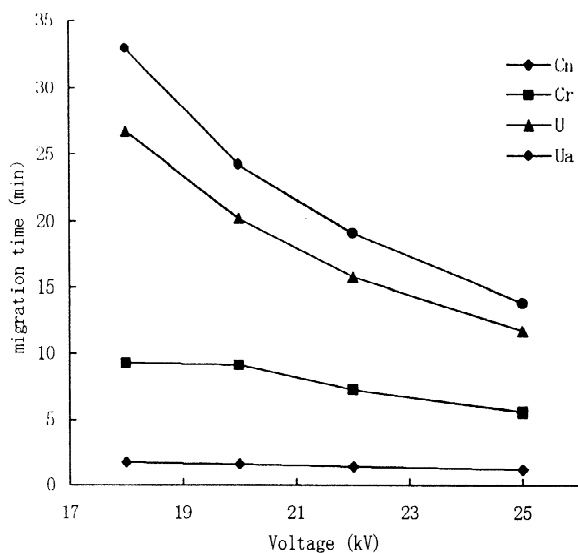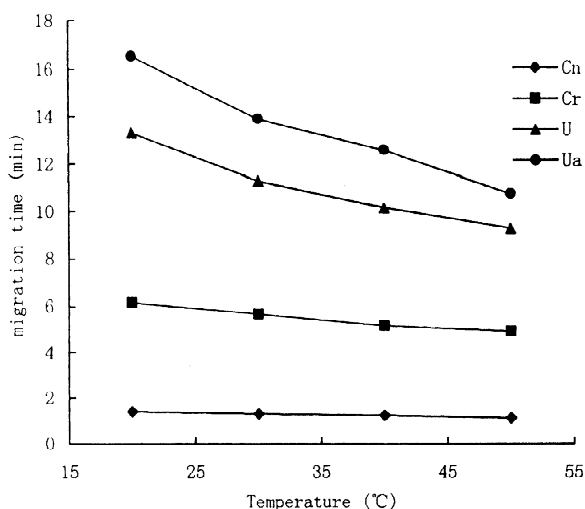

Fig. 4. Effect of temperature on migration time. Conditions: fused-silica capillary (21 cm×75 μm), 30 mmol/L pH 3.45 phosphate buffer, 25 kV, 200 nm. Between each run, the capillary was flushed as described in Section 3.2.

split into two peaks when the temperature was below 30 °C, and as the temperature increased above 40 °C, the baseline clearly shifted with migration time. Therefore, the capillary temperature was selected as 40 °C for the best peak shape of Cn and the shorter assay time.
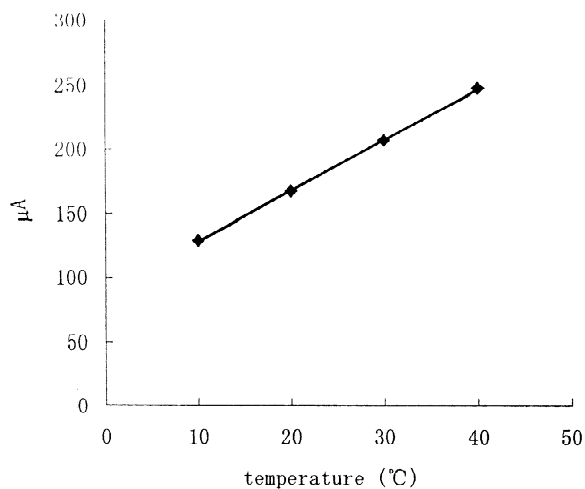

Fig. 3. Effect of voltage on separation. Conditions: fused-silica capillary (21 cm×75 μm), 25 mmol/L pH 3.45 phosphate buffer, 20 °C, 200 nm. Between each run, the capillary was flushed as described in Section 3.2.

Fig. 5. Effect of capillary temperature on electric current. Conditions: fused-silica capillary (21 cm×75 μm), 30 mmol/L pH 3.45 phosphate buffer, 25 kV. Between each run, the capillary was flushed as described in Section 3.2.

3.1.5. Separation results

Under optimum conditions (using a fused-silica capillary and 25 mmol/L pH 3.45 phosphate buffer with a separation voltage of $+25$ kV, at 40 °C, 200 nm), Cn, Cr, U and Ua were baseline separated. Electropherograms of the four standards are shown in Figs. 6b and 7b.

3.2. Reproducibility

The condition of the capillary inner-wall silanol greatly affects the reproducibility of the method. Normally, the capillary was rinsed with NaOH to refresh the silanol, and then (water and) separation buffer was used to equilibrate the capillary for the next separation. This section reports the application of a particular flushing step between each run to
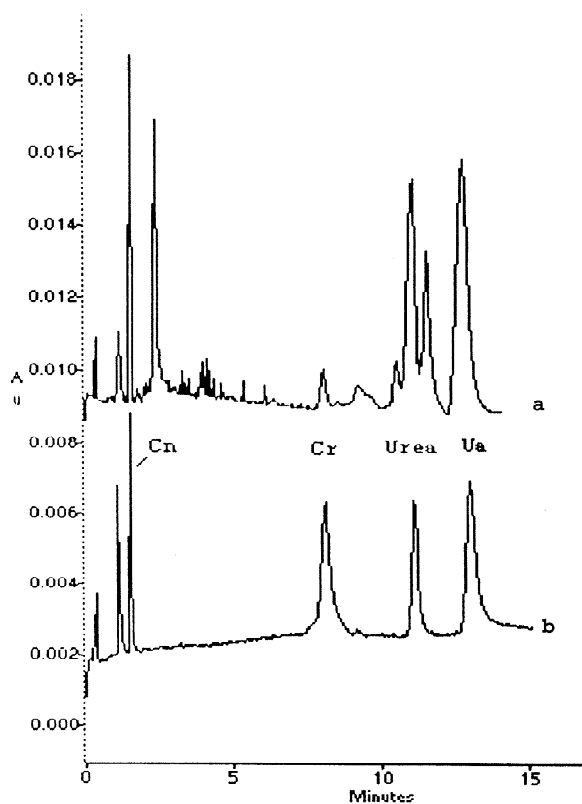

Fig. 6. Electropherograms of a DN plasma sample (b) and a standard sample (a). Conditions: fused-silica capillary (21 cm× 75 μm), 25 mmol/L pH 3.45 phosphate buffer, 25 kV, 40 °C, 200 nm. Between each run, the capillary was flushed as described in Section 3.2.

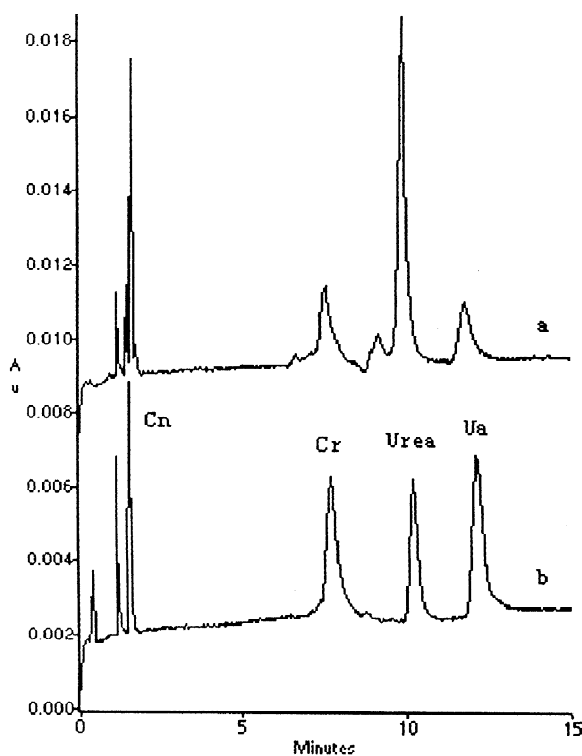Fig. 7. Electropherograms of a DN urine sample (a) and standard markers (b). Conditions: fused-silica capillary (21 cm×75 μm), 25 mmol/L pH 3.45 phosphate buffer, 25 kV, 40 °C, 200 nm. Between each run, the capillary was flushed as described in Section 3.2.

obtain better reproducibility. It was found that, by adding an HCl (0.1 mol/L) rinsing step between the NaOH and water rinsing step, better reproducibility of the migration time, peak height and peak area was obtained. The results are shown in Table 1.

Table 1
RSDs of several parameters

| | RSD (%) | | | | | | | |
| | Creatinine (n=5) | | Creatine (n=5) | | Urea (n=5) | | Uric acid (n=5) | |
	R	N	R	N	R	N	R	N
Time	0.5	1	0.8	3.4	1.8	5.8	1.9	7.1
Height	3	3.6	1.5	7.9	1	7	7	10
Area	1.6	4	0.6	8.1	2.7	12	2.2	8

Conditions: fused-silica capillary (21 cm×75 μm), using 25 mmol/L pH 3.45 phosphate buffer with a separation voltage of $+25$ kV, at 40 °C, 200 nm. For R and N, see text.

R represents the method that included the HCl-rinsing step and N represents the method that did not include the HCl-rinsing step. It was also found that, when the rinsing times of NaOH, HCl, water and buffer were longer than 3, 1, 1 and 3 min, respectively, the RSD_{max} values of the migration times were <2%. For the assay time, the shortest rinsing times, stated above, were chosen.

3.3. Calibration and limit of detection

Standard mixtures of Cn, Cr, U and Ua were tested at several different concentrations. The linear range was found, using the peak area as a function of the concentration, to be 0.18–700 μM for Cn, 3.5–1000 μM for Cr, 500–5000 μM for urea and 2–800 μM for Ua. The limits of detection for Cn, Cr, U and Ua were 0.18, 3.5, 500 and 2 μM, respectively ($S/N = 5$).

3.4. Separation of clinical samples

3.4.1. Choice of deproteinizing agent for the plasma sample

Three common deproteinizing agents (methanol, trichloroacetic acid and acetonitrile) were investigated. Three aliquots of plasma were treated with methanol, trichloroacetic acid and acetonitrile, respectively, as described in Section 2.3. The separation results indicated that the plasma sample deproteinized using TCA had less protein remaining than the plasma samples deproteinized using methanol or acetonitrile. TCA also seems not to interfere in the recoveries of these markers. Therefore, TCA was selected for preparation of the plasma samples.

3.4.2. Application of the method to clinical samples

The clinical samples were prepared as described in Section 2.3. Fig. 6 (Fig. 7) shows the electropherogram of the DN plasma (urine) sample and a mixture of the standard markers under optimum conditions. The results show that the samples were well assayed under the proposed conditions. The analysis results for the plasma and urine samples are shown in Table 2. The value in parentheses is the percent of U of total nonprotein nitrogen. The results for the DN plasma sample indicate that the total concentration of nonprotein nitrogen was much higher than the normal level (200–300 mg/L) and the percent of U of the total nonprotein nitrogen was 72%, which is also higher than the normal value (50%). On the contrary, the assay results for the DN urine sample show that the total concentration of nonprotein nitrogen in urine was a little lower than the normal level (1.5–5). This indicates that the renal function of the patient was slightly damaged.

4. Conclusion

A new CZE method for the separation of nonprotein nitrogen in plasma and urine was optimized in terms of buffer pH, buffer concentration, separation voltage, capillary temperature and rinsing steps. Good reproducibility and linearity were obtained. It is hoped that the method presented here will be suitable for routine urine and plasma assays in clinical studies and helpful for the diagnosis of DN.

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Table 2
Assay results for plasma and urine samples

	Cn	Cr	U	Ua	Total
Plasma (mg/L)	23.3	17.8	292.5 (72%)	69.9	403.5
Urine (mg/mL)	0.6	0.078	0.654	0.064	1.39

Conditions: fused-silica capillary (21 cm×75 μm), using 25 mmol/L pH 3.45 phosphate buffer with a separation voltage of +25 kV, at 40 °C, 200 nm. Between each run, the capillary was flushed as described in Section 3.2.

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