Separation of Urea, Uric Acid, Creatine, and Creatinine by Micellar Electrokinetic Capillary Chromatography with Sodium Cholate

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Abstract

The capillary electrophoretic separation of the four nonprotein nitrogenous compounds (NPNs; urea, uric acid, creatine, and creatinine) typically employed in clinical and medical settings for the monitoring of renal function is described. Successful resolution of these compounds is achieved with the use of a bile salt micelle system composed of sodium cholate at phosphate buffer pH 7.4. The elution patterns of four NPNs are obtained within 30 min with a voltage of 30 kV. The effect of varying the applied voltage, temperature, and the mole ratio of phosphate buffer with bile salt surfactant on the migration behavior is also examined.

Introduction

The nonprotein nitrogen (NPN) fraction in blood consists of about 15 compounds of clinical interest. Urea, uric acid, creatine, and creatinine are four major NPN components and are routinely determined in clinical settings. They are used to monitor renal function.

Urea constitutes nearly half of the NPN in the blood. The level of urea in the plasma is markedly affected by renal perfusion, the protein content of the diet, and the level of protein catabolism. A high-protein diet, fever, major illness, or stress may increase urea levels. A low-protein diet or higher rate of protein synthesis during the periods of late pregnancy and infancy may decrease the urea levels.

In humans, uric acid is the final breakdown product of purine metabolism. Monosodium urate, a salt of uric acid, is relatively insoluble in the physiological condition and is saturated at levels above 6.4 mg/dL (1). There are three major causes for elevated levels of uric acid: gout, renal disease, and a higher rate of nucleic acid breakdown. High levels of uric acid are also found secondary to a variety of diseases, such as glycogen storage disease and congenital enzyme deficiency. A congenital enzyme deficiency results in the production of metabolites that may compete for urate secretion. Hyperuracemia is common to toxemia of pregnancy and lactic acidosis.

Creatine serves as a high energy source. It spontaneously loses water to form its anhydride, creatinine. Afterwards, it is excreted into plasma. Creatinine is removed from circulation almost entirely and excreted into the urine. Plasma levels of creatinine can reflect endogenous production and the glomerular filtration rate. Therefore, it is an excellent indicator for the assessment of renal function. Furthermore, serum creatinine level in combination with blood urea nitrogen have been used to differentiate between prerenal and renal causes of azotemia (2). Plasma creatine levels are not elevated in renal disease. Both creatine and creatinine levels in urine are often elevated in muscle diseases, such as muscular dystrophy, poliomyelitis, and hyperthyroidism.

Currently, coupled enzymatic methods are used to measure NPNs (3–5). One of the major disadvantages of these methods is that the endogenous enzymes (e.g., lactic dehydrogenase) compete for NADH in the reaction mixture, thus interfering with the indication reaction (4). Several HPLC methods have been developed to analyze uric acid (6–8) and creatinine (8–11). Because creatine assays are not readily available in most clinical laboratories, the creatine kinase levels are measured instead (12,13).

Capillary electrophoresis (CE) was developed to separate the charged molecules in a buffer-filled capillary by the application of a very high voltage (14). Micellar electrokinetic capillary chromatography (MECC) allows the resolution of uncharged molecules by adding surfactants to modify the conditions to extend the application of CE (15,16). Based on the differential binding to the micellar phase, the partition between the slow moving micelle and the fast moving aqueous phase causes differential resolution of the solutes. Sodium dodecyl sulfate (SDS) has proven very useful for separating water-soluble analytes (e.g., ascorbic acid) (17) and nonionic molecules (18,19). Cole et al. (20) successfully adopted bile salts instead of SDS to optimize the resolution of binaphthyl enatiomer separation. Hsiao et al. (21) successfully separated steroids in phosphate buffer with sodium cholate.

CE methods for urine samples have been developed. Guzman

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et al. (22) and Jenkins et al. (23) demonstrated the co-elution of urea and creatinine in borax buffer. The determination of creatinine and uric acid in phosphate buffer (pH 9.0) was developed by Mikaye et al. (24). However, the simultaneous detection of these four analytes has not been reported. In this paper, the successful separation of these four analytes in phosphate buffer (pH 7.4) containing sodium cholate and the other effects of such NPN separation is reported.

Experimental

Instrumentation

A Beckman (Fullerton, CA) 5500 P/ACE electrophoresis system equipped with Gold software for data collection was employed for CE. The P/ACE system included a temperature-controlled cartridge enclosing a capillary column, an autosampler, a wavelength-selectable detector, and an electric interface. Spectra were collected with the use of the 168 diode-array detector using the "scan graphic" option. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) used for CE had 50-µm inner diameters and 350-µm outer diameters. The capillaries were 80 cm long between the injection end and the detection window. The length of the capillary between the inlet and the outlet was 87 cm. The P/ACE instrument was controlled automatically via an IBM-compatible personal computer with Gold system software. Data were collected with the P/ACE software system. This package provided automated instrument control and sophisticated data analysis capability.

Reagents

Urea, uric acid, creatine, creatinine, sodium hydroxide, sodium cholate, sodium monophosphate, and sodium diphosphate were obtained from Sigma (St. Louis, MO). All chemicals were of analytical grade or better and were used as received without further purification. They were dissolved in double-distilled water obtained from a Millipore (Bedford, MA) water purification system with at least 18.2 MW of water resistance.

Procedure

Stock solutions of the four NPNs were prepared independently by dissolving different analytes in double-distilled water. Sample solutions were obtained by diluting the stock solutions in running buffers to give an adequate peak height. Varied concentrations of running buffers were prepared by dissolving sodium monophosophate and sodium diphosphate to give a constant pH of 7.4. Sodium cholate was added to the phosphate buffer solutions for running CE. Initially, the new capillary was rinsed with 1N HCl for 5 min and then with regeneration solution (0.1N NaOH) for 10 min before it was rinsed with deionized water for 5 min. Samples were introduced to the column using pressure injection for 5 s, corresponding to a volume of 3.1 nL. After each run, the capillaries were rinsed with 0.1N NaOH solution for 10 min followed by deionized water for 5 min. After the buffer was changed, separation voltage was applied for 10 min to condition the column with the new buffer. All the surfactant solutions were filtered through 0.45-µm membrane filters. Analytes were detected with an ultraviolet detector at 200 nm.

Results and Discussion

CE

Figure 1 shows the electropherograms of the four NPNs. The resolution of these four NPNs was not achieved in the buffer range of 0.038M to 0.097M in the absence of sodium cholate. The creatine peak was better resolved as the buffer concentration increased (Figure 1B–D), but urea and creatinine still co-migrated at these chosen concentrations. The co-migration of urea and creatinine in borax buffer has been reported by Guzman et al. (22) and Jenkins et al. (23).

MECC with sodium cholate

In CE, the resolution, separation proficiency, selectivity, and elution time of the analytes can be optimized by the capacity factor, k' (e.g., assorted surfactants, surfactant concentrations, organic modifiers, temperature, voltages, etc.), defined as $(t_R - t_0)/t_0$, where t_R is the migration time of the solute and t_0 is the migration time of unretained solute (16). However, the separation of these NPNs by MECC with the addition of SDS or sodium cholate in the 0.038M phosphate or 0.10M borate buffer solution was not achieved in the pH range of 7–9. Another approach of adding organic modifiers (e.g., methanol) in such a system was also unable to separate these NPNs under the experimental conditions.

Figure 2 shows the resolution of MECC electropherograms of the four NPNs in the 0.077M phosphate buffer (pH 7.4) with 0.075M sodium cholate at 250°C and 25 kV. The variation of the concentration of phosphate buffer with sodium cholate to adust k' and separate NPNs was conducted. The phosphate buffer concentrations had different fixed sodium cholate concentrations, and the data are shown in Figure 3 and Table I.

Figure 3 shows the plot of k' with the concentration of phosphate buffer from 0.038M to 0.097M and sodium cholate from 0.05M to 0.10M. The k' values increased with an increase of phosphate buffer concentration. The difference in k' in Figure 3 could be attributed to the difference in micellar volume. Uric acid was separated. Creatinine co-migrated with either creatine or urea

Table I. Resolution Factors* of Urea–Creatinine and Creatinine–Creatine Running in 0.077M and 0.097M Phosphate Buffer with 0.075M and 0.10M Sodium Cholate Added

	R_s urea–creatinine	R_s creatinine–creatine
0.077M Phosphate–0.075M sodium cholate	1.18	1.12
0.077M Phosphate–0.10M sodium cholate	1.69	0.93
0.097M Phosphate–0.075M sodium cholate	0.99	1.21
0.097M Phosphate–0.10M sodium cholate	1.67	1.23

* R_s is defined as $2(X_2 - X_1)/(W_1 + W_2)$, where X_1 is the migration distance of the analyte 1, X_2 denotes the slower moving component, and W is the width of the peak at the baseline.

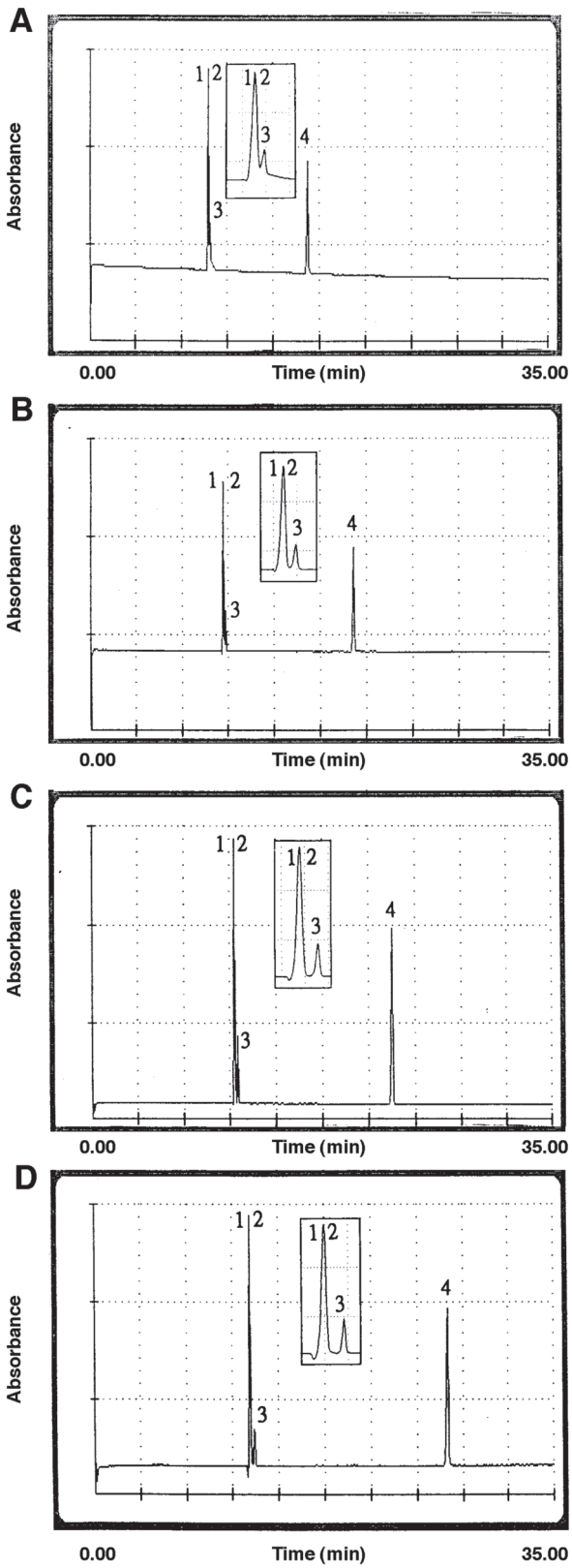

Figure 1. Electropherograms of four NPNs in capillary zone electrophoresis at phosphate buffer concentrations of 0.038M (A), 0.058M (B), 0.077M (C), and 0.097M (D). Conditions: pH, 7.4; temperature, 25°C; voltage, 25 kV. Peaks: 1, urea; 2, creatinine; 3, creatine; 4, uric acid.

when the phosphate buffer concentrations were 0.038M or 0.057M. The four NPNs were resolved when the phosphate buffer concentrations were 0.077M and 0.097M.

In Table I, as 0.075M sodium cholate was added into the phosphate buffer system, the phosphate buffer concentration varied from 0.077M to 0.097M, and the resolution factor R_s of creatinine–creatine increased from 1.12 to 1.21, but that of urea–creatinine decreased from 1.18 to 0.99. Conversely, as sodium cholate concentration increased from 0.075M to 0.10M with 0.077M phosphate buffer, the R_s of urea–creatinine

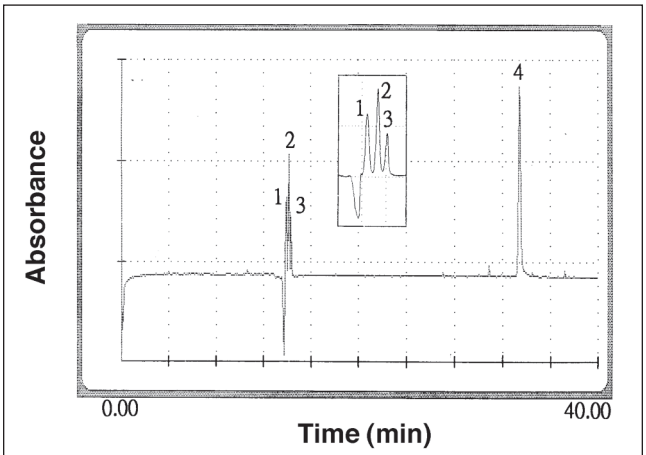

Figure 2. Electropherogram of NPNs in 0.077M phosphate buffer solution with 0.075M sodium cholate added. Conditions: pH, 7.4; temperature, 25°C; voltage, 25 kV. Peaks: 1, urea; 2, creatinine; 3, creatine; 4, uric acid.

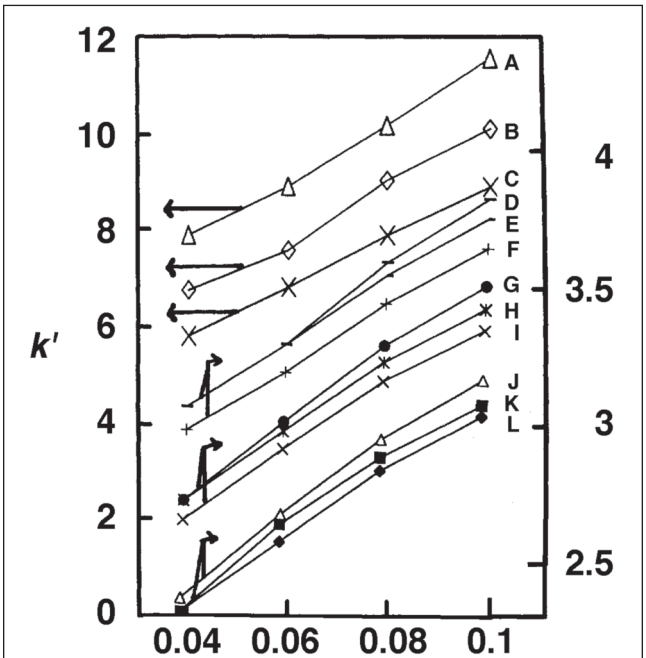

Figure 3. Plot of capacity factor k' versus the concentrations of phosphate buffer. Solutes are uric acid (sodium cholate 1.0M) (A), uric acid (sodium cholate 0.75M) (B), uric acid (sodium cholate 0.5M) (C), creatine (sodium cholate 0.10M) (D), creatinine (sodium cholate 0.10M) (E), urea (sodium cholate 0.10M) (F), creatine (sodium cholate 0.075M) (G), creatinine (sodium cholate 0.075M) (H), urea (sodium cholate 0.075M) (I), creatine (sodium cholate 0.05M) (J), creatinine (sodium cholate 0.05M) (K), urea (sodium cholate 0.05M) (L).

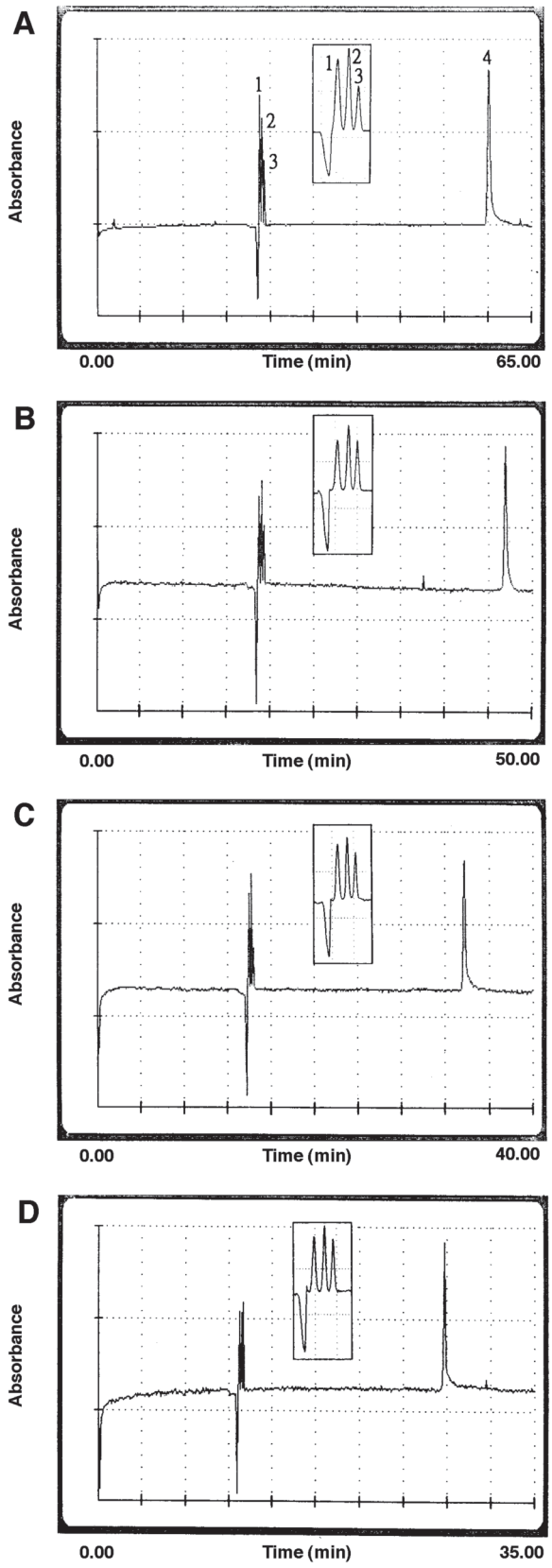

Figure 4. Electropherograms of NPNs in 0.077M phosphate buffer solution with 0.075M sodium cholate added at 15 kV (A), 20 kV (B), 25 kV (C), and 30 kV (D). Conditions: pH, 7.4; temperature, 25°C.

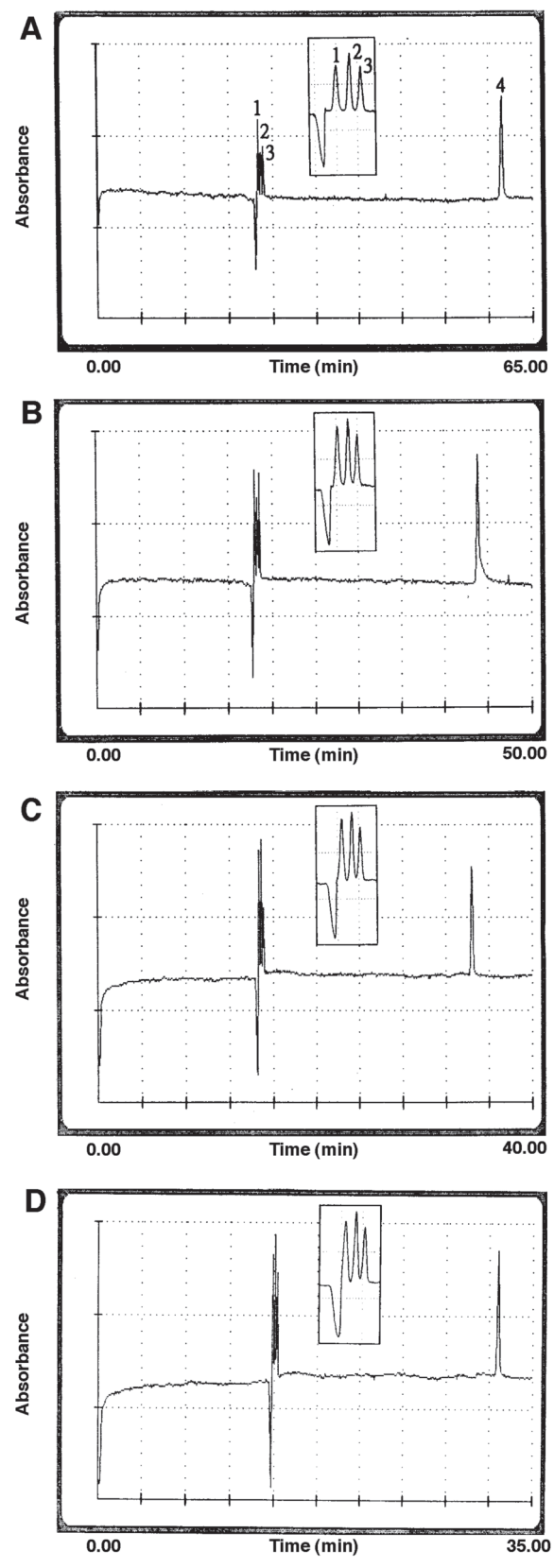

Figure 5. Electropherograms of NPNs in 0.077M phosphate buffer solution with 0.075M sodium cholate added at 20°C (A), 30°C (B), 35°C (C), and 40°C (D). Conditions: pH, 7.4; voltage, 25 kV.

increased from 1.18 to 1.69, but that of creatinine–creatine decreased from 1.12 to 0.93. As phosphate buffer increased from 0.077M to 0.097M with 0.10M sodium cholate, the R_s of creatinine–creatine increased from 0.93 to 1.23, but that of urea–creatinine did not change significantly. Furthermore, as sodium cholate concentration increased from 0.075M to 0.10M with 0.097M phosphate buffer, the R_s of urea–creatinine increased from 0.99 to 1.67, but that of creatinine–creatine did not change significantly.

It should be pointed out that the resolution of creatine and creatinine is affected by the phosphate buffer concentrations, and the resolution of urea and creatinine is affected by the sodium cholate concentrations. The results also indicate that an optimal ratio between phosphate buffer and sodium cholate concentrations for the simultaneous separation of these three analytes is necessary (Figure 2).

Variation of voltage

The variation of voltage can have effects on migration time, resolution, peak sharpness, EOF, and joule heating. The joule heating resulting from an increase in voltage may lead to changes in EOF, ion mobility, analyte diffusion, and band broadening. Figure 4 shows the electropherograms of the four NPNs running in 0.077M phosphate buffer with 0.075M sodium cholate added at different applied voltages. The migration time decreased when the applied voltage was increased (Table II), but the elution sequence did not change with different applied voltages. Joule heating effects (e.g., peak broadening) were not observed.

Temperature effect

Viscosity is a function of temperature. Therefore, as the temperature increases, the viscosity decreases and electrophoretic mobility and EOF increase. Some analytes may not be stable at higher temperatures, and the variation of temperature may lead to conformational change. Figure 5 shows the electropherograms of the four NPNs running in 0.077M phosphate buffer with 0.075M sodium cholate added at different applied temperatures. The migration sequence did not change with the application of different temperatures. An increase in EOF and electrophoretic mobility from increased temperature leads to a shorter analysis time (Table III).

Table II. Migration Time of NPNs Running in 0.077M Phosphate Buffer with 0.075M Sodium Cholate at Different Applied Voltages

	Migration time (min)			
	15 kV	20 kV	25kV	30 kV
Urea	24.339	18.641	13.903	11.291
Creatinine	24.721	18.950	14.118	11.473
Creatine	25.052	19.196	14.300	11.617
Uric acid	58.672	46.920	33.708	27.778

Table III. Migration Time of NPNs Running in 0.077M Phosphate Buffer with 0.075M Sodium Cholate at Different Applied Temperatures

	Migration time (min)			
	20°C	30°C	35°C	40°C
Urea	16.432	12.538	10.959	9.875
Creatinine	16.714	12.734	11.118	10.015
Creatine	16.942	12.897	11.244	10.130
Uric acid	41.892	30.701	25.802	23.000

Conclusion

In conclusion, the four NPNs are separated by MECC in 0.77M phosphate buffer and 0.75M sodium cholate at 25°C and 25 kV. This MECC approach can potentially be applied to clinical settings.

Acknowledgments

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