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High-throughput multi-analyte screening for renal disease using capillary electrophoresis

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Abstract

End-state renal disease (ESRD) affects 300000 people in the United States each year. A large percentage of these individuals ($\sim 20\%$) die within the first year after diagnosis. Current methods of determining renal function rely on the measurement of a single marker using slow and frequently non-specific colorimetric methods. In this report, capillary zone electrophoresis was used to perform a multi-analyte assay for markers of renal function in urine. This method tested for creatinine (Cr), creatine (Cn), uric acid (UA), and *p*-aminohippuric acid (PAH) levels. The limits of detection ($S/N = 3$) were found to be 5 μM for Cr, 0.75 μM for Cn, and 1.5 μM for UA and PAH. Linear ranges were determined to be 5–500 μM for Cr, 0.75–500 μM for Cn, and 1.5–250 μM for UA and PAH. These ranges included the expected concentrations of the markers in human urine after 50-fold dilution. This screening method proved to be a simple and fast way to perform a high throughput analysis for multiple renal function indicators.

Keywords: Capillary electrophoresis; Creatinine; Creatine; Uric acid; PAH; High throughput screening

1. Introduction

The kidneys play a vital role in maintaining the body's stable internal chemical environment. They are responsible for filtering impurities from the blood, draining the body of its waste products, and maintaining the necessary balance of chemicals in the liquid form [1]. However, renal failure affects more than 300000 people annually in the United States (about 0.1% of the population) [2]. Because of the body's inability to keep a clean and stable internal environment, renal failure can be a fatal disorder. The death rate of patients in their first year of treatment for end-stage renal disease (ESRD) is above 20% [2]. Kidney disease affects people of all races, sexes, and genders and is frequently associated with other medical disorders. It is very prevalent in diabetic patients, accounting for about one third of all ESRD. This prevalence in diabetics is due to the elevated plasma glucose level, which puts too much strain on the proximal tubules of the nephrons in the

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kidneys. In addition to diabetes, renal failure is also closely associated with hypertension. About one fourth of all ESRD patients suffer from hypertension [2,3].

Several compounds in urine can serve as markers for renal function. Urine levels of creatinine (Cr), urea, and uric acid (UA) are good indicators of the glomerular filtration rate (GFR) of the kidneys, the amount of fluid filtered per unit time. Creatinine is the major breakdown product of both phosphocreatine and creatine (Cn), which are major compounds used in muscular activity. It is one of the most widely used markers of renal function. Uric acid is the major product of catabolism of adenine and guanine. It serves as a marker for tubular reabsorption of the nephrons in addition to the GFR. An imbalance in UA level can lead to gout, the formation of urate crystals in the joints. *p*-Aminohippuric acid (PAH) levels in the urine are an indicator of the tubular secretion of the nephrons [4,5]. A determination of PAH clearance is used to measure effective renal plasma flow, ERPF, the amount of plasma flow to the kidneys per unit time [6].

The current methods of testing for these renal markers in urine are limited due to slow analysis times, the inability to test for multiple markers at a time, and the consumption of large amounts of reagent and sample. In many cases, the tests are also non-specific for the analyte of interest. For example, determination of Cr is largely carried out by the Jaffé reaction. In this test, a picrate ion is added which reacts with Cr to form a red-orange adduct. However, several other substances in the urine (both endogenous and exogenous) will also form a colored adduct with the picrate ion, preventing an accurate measure of Cr level [7]. The presence of proteins, glucose, ascorbic acid, guanidine, acetone, or α-keto acids will interfere with the measurement of Cr [4]. Most analysis of urea content in urine is carried out by the Fearon reaction. Diacetyl condenses with urea to form diazine, which absorbs at 540 nm. However, this method has many complications. The diacetyl must be formed in the reaction mixture because of its instability. Also, thiosemicarbazide and Fe^{3+} must be added to the reaction mixture to stabilize the color [4]. The two predominant analysis methods used for the determination of UA are the phosphotungstic acid (PTA) method and the uricase method. In the PTA method, urate reduces PTA to form a blue product. The concentration is determined by spectrophotometry. The presence of proteins can interfere with light absorbance by unpredictable quenching. In addition to this interference, there are many exogenous and endogenous compounds, such as glucose, acetaminophen, and caffeine that can also reduce PTA to yield a blue product. In the uricase analysis method, urate is oxidized by uricase oxidoreductase. This method has less interference than the PTA method, but the presence of certain compounds, such as guanine and xanthine, can lead to inaccurate results [4]. PAH levels in the urine are largely analyzed by colorimetric tests. The presence of glucose or certain drugs can cause the tests to become difficult and inaccurate by interfering with the colorimetric reactions [6].

Numerous methods have been developed for the quantification of Cr and Cn. Madaras and coworkers have pioneered work in enzyme-based electrochemical sensors for Cr [8,9]. In these sensors, an enzyme cascade generates hydrogen peroxide, which is electrochemically active, from Cr. The presence of Cn however, is problematic to accurate quantification as the first step in the enzyme cascade is the conversion of Cr to Cn. Other electrochemical methods have been developed for the measurement of UA [10–12]. Uric acid has a well-known oxidation potential and is easily detected at physiological concentrations. Direct detection of UA from urine is limited because of the similar oxidation potentials of common analytes such as ascorbic acid.

The use of a separation method allows for the accurate quantification of renal function markers. Capillary zone electrophoresis (CZE) is a technique that allows for simultaneous separation and quantitation of multiple analytes from urine [7,13,14]. This powerful separation method is able to overcome many of the shortcomings of current analyses for indicators of renal function. The advantages of capillary electrophoresis include fast analysis times, high resolution, and very low sample volumes [13]. Also, diluted urine samples can be directly injected into the capillary without complicated sample preparation procedures.

The goal of this project was to use CZE to develop a simple separation and quantify of multiple renal function markers in human urine. By quantitating multiple markers at a single time, a better diagnostic value can be obtained. Separation conditions were optimized using a 500 μM solution of the markers. A successful separation of Cr, Cn, UA, and PAH was accomplished. The limit of detection for Cr was 5 μM, with a linear range of 5–500 μM. The limit of detection for Cn was 0.75 μM, with a linear range of 0.75–500 μM. Uric acid and PAH had a limit of detection of 1.5 μM and a linear range of 1.5–250 μM. Urine samples were analyzed for each of the markers. Creatinine, Cn, and UA were found in each sample. PAH was not detected in any of the urine samples although the expected concentration of PAH in urine lies well within the detection limits of the developed method. Peak identity was confirmed by spiking, and the renal function markers in the urine samples were quantified by comparing peak height to the linear calibrations. To demonstrate the high throughput analysis capabilities of this method, 46 urine samples were analyzed for Cr, Cn, and UA.

2. Experimental

2.1. Chemicals

Urea, potassium phosphate (monobasic anhydrous), and PAH were obtained from Sigma (St. Louis, MO). Creatinine monohydrate, Cn, and UA were purchased from Acros Organics (New Jersey). Etylenediamine-tetraacetic acid (EDTA), disodium salt dihydrate was obtained from Aldrich (Milwaukee, WI). Sodium hydroxide and sodium borate were purchased from Fisher (Fair Land, NJ). All chemicals were used as received.

2.2. Apparatus and analytical conditions

All CE experiments were performed on a Beckman P/ACE MDQ fitted with a UV absorbance detector. Absorbance was monitored at 214 nm for all experiments. Data collection and analysis were carried out by Beckman P/ACE System MDQ software on a personal computer. Fused silica capillary (Polymicro Technologies, Phoenix, AZ) with 360 μm outer diameter and 50 μm inner diameter was used for all experiments. The total length of the capillary was 49 cm with an effective length of 38 cm. New capillaries were treated with a 60 min pressure wash of 1 M NaOH and a 10 min pressure wash of deionized water at 20 psi. Each morning, the capillary was rinsed for 10 min with 1 M NaOH, 5 min with deionized water, and 5 min with run buffer at 20 psi. Before each run, the capillary was rinsed for 4 min with 1 M NaOH, 1 min with deionized water, and 3 min with run buffer at 20 psi. The capillary was dried at the end of each day. Once the separation parameters were optimized, the same capillary was used during all calibration and urine analysis experiments. The working temperature of the capillary was held at 25.0°C. Samples were pressure injected for 5 s at 0.5 psi. Separations were carried out at 25 kV, giving a field strength of 510 V/cm. The current ran between 55 and 58 μA for the pH 5.5 separation protocol.

2.3. Sample and solution preparation

Three run buffers were prepared. A pH 9.5 borate buffer was made by dissolving 0.95 g of sodium borate ($Na_2B_4O_7$) in 200 ml of deionized water, titrating to pH 9.5 with 1.0 M NaOH, and diluting to 250 ml with deionized water to make a 10 mM sodium borate buffer. A pH 7.5 buffer was prepared by dissolving 1.0 g of KH_2PO_4 in 200 ml of deionized water, titrating to pH 7.5 with 1.0 M NaOH, and diluting to 250 ml with deionized water to make a 30 mM phosphate buffer. A pH 5.5 buffer was made by dissolving 1.7 g of KH_2PO_4 in 200 ml of deionized water, titrating to pH 5.5 with 1.0 M NaOH, and diluting to 250 ml with deionized water. This made a 50 mM phosphate buffer at pH 5.5. Fresh buffer was made each week. A 2% (w/v) EDTA solution was used as the urine dilution buffer and was prepared by dissolving 0.6 g of EDTA in 30 ml of the pH 5.5, 50 mM run buffer. The 2% EDTA in the urine dilution buffer served to complex the metal ions in the urine in order to prevent the ions from complexing with any of the renal markers and altering their migration times [14].

Stock solutions of Cr, Cn, PAH, and UA were prepared weekly by dissolving the compounds in deionized water to a concentration of approximately 10 mM and stored at 4°C until use. Solutions of these compounds were made daily by diluting the stocks to the appropriate concentration with run buffer. Urine samples were collected in sterile sample containers and frozen until use. On the day of analysis, a sample was thawed and diluted 50 fold with the urine dilution buffer. All solutions (diluted stock compounds, diluted urine samples, and run buffers) were passed through syringe filters with 0.2 μm pore size (Whatman) and degassed for 2 min in a sonicator (Fisher Scientific, FS 20) prior to use.

3. Results and discussion

3.1. Selection of buffer pH

The first step was the optimization of separation parameters. The optimum resolution for CZE separations frequently occurs at an intermediate pH relative to the pK_a of the analytes. However, this can result in long analysis times for acidic species as the electroosmotic flow is greatly reduced. The separation of the four renal markers (Cr, Cn, PAH, and UA) was attempted at pH 9.5 and 500 μM concentration [13] (Fig. 1a). At this pH, UA appeared first at 2.9 min. Creatinine and Cn appeared at 6.3 and 7.2 min, respectively, but were not baseline resolved. PAH appeared at 7.9 min. The unusual peak shape for Cr and Cn were consistent from run-to-run, however no attempt was made to determine the cause of this behavior. The pH was lowered to 7.5 in an attempt to improve resolution between Cr and Cn. At this pH, Cr and Cn were still not baseline resolved and the migration times had slowed in comparison to the pH 9.5 buffer system. Creatinine and Cn eluted first with migration times of 11.5 and 12.9 min, respectively. No other peaks appeared within the first 30 min of separation. Analysis at this pH was abandoned because of long analysis times and incomplete resolution. By lowering the buffer pH from 9.5 to 7.5 the EOF slowed without sufficiently increasing the charge of the markers. An analysis was performed at pH 5.5 to further increase the positive charge on the markers [7,14]. At this pH, Cr and Cn are positively charged while UA and PAH are negatively charged. A baseline separation of Cr, Cn, UA, and PAH was obtained, as shown in Fig. 1b. Respective migration times for Cr, Cn, UA, and PAH were 3.1, 3.8, 6.3, and 10.6 min.

3.2. Limits of detection and linearity determination

Standard mixtures of Cr, Cn, UA, and PAH were tested at ten different concentrations between 0.75 and 500 μM (Table 1). Experimentally determined limits of detection ($S/N = 3$) were found to be 5 μM for Cr, 0.75 μM for Cn, and 1.5 μM for UA and PAH. Relative standard deviation (R.S.D.) values of peak height were lower than those of peak area for all of the four renal markers (Table 2). Therefore, linear regression

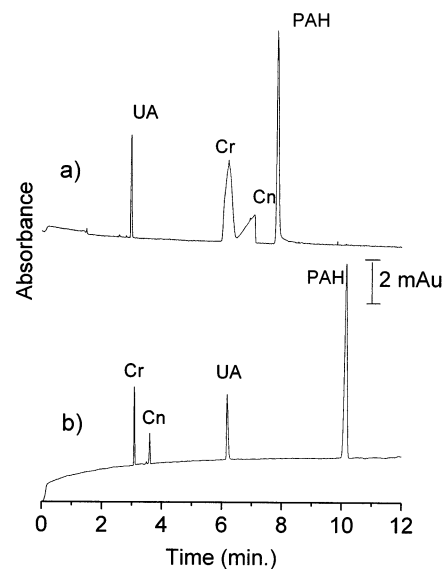

Fig. 1. Electropherograms of 500 μM standard mixture of Cr, Cn, UA, PAH, and urea. Separations were carried out at 25 kV. (a) Separation in pH 9.5 buffer. Uric acid eluted first. Creatinine and Cn eluted next with incomplete baseline resolution, followed by PAH. A urea peak was not observed. (b) Separation in pH 5.5 buffer. A baseline separation was accomplished for four of the markers (Cr, Cn, UA, and PAH, respectively).

Table 1
Linear calibration data for Cr, Cn, UA, and PAH[a]

	Cr	Cn	UA	PAH
Limit of detection (μM)	5	0.75	1.5	1.5
Linear range (μM)	5–500	0.75–500	1.5–250	1.5–250
Slope ((μA/μM)	9.43 (±0.29)	3.49 (±0.06)	13.36 (±0.37)	25.87 (±0.98)
Intercept (μAu)	50.9 (±62.2)	65.0 (±10.5)	51.8 (±35.6)	70.7 (±95.2)
R^2	0.998	0.999	0.998	0.996

[a] Data was collected using the experimental conditions listed for Fig. 1b.

data to be used for marker analysis in urine samples was calculated from peak heights. The linear ranges of response as determined from peak area and height were 5–500 μM for Cr, 0.75–500 μM for Cn, and 1.5–250 μM for UA and PAH. These linear ranges were adequate to cover all renal marker concentrations of human urine after a 50-fold dilution. Expected concentrations in the urine of an average 66 kg male are 6 mM for Cr, 1 mM for Cn, 2 mM for PAH, and 3 mM for UA [15]. Therefore, a 50-fold dilution of the urine will put all of the concentrations in the linear range of detection (120 μM for Cr, 20 μM for Cn, 40 μM for PAH, and 60 μM for UA).

3.3. Identification of peaks in urine samples

Two urine samples were collected and analyzed for the presence of the four markers. Both diluted urine samples showed four peaks in the first 14 min of separation (Fig. 2A). A negative peak due to the EDTA in the dilution buffer appeared at 3.7 min. EDTA was confirmed as the cause of the negative peak by the appearance of the peak when a separation was performed using only the urine dilution buffer. Spiked urine samples were run with an additional 1.0 mM concentration of the marker to be tested (Fig. 3a–d). This allowed for positive identification of Cr (3.1 min), Cn (3.8 min), and UA (6.3 min) peaks. However, the sample spiked with PAH revealed that the fourth peak in the urine samples, appearing at 11.8 min, was not due to PAH. In the spiked sample, the PAH had a migration time of 10.6 min, but the unknown peak appeared at 11.8 min. A peak for PAH in the urine samples was not detected, even though the expected concentration of PAH in the diluted samples (approximately 40 μM) was well above the limit of detection. This lack of PAH in the samples may be the result of either levels of PAH below the limit of detection after dilution or an indication of PAH breakdown before analysis occurred. A more concentrated urine sample (2-fold dilution) was tested in an attempt to detect the PAH, but no PAH was seen (Fig. 2B). The peak height for Cr, Cn, and UA increased as expected in the more concentrated sample. The migration times of the markers at this dilution were slower than at a 50-fold dilution. This is the result of the increased ionic strength of the sample, as the rate of migration is known to decrease with increasing ionic strength. PAH could also be chemically unstable in the storage conditions used, causing it to breakdown before the analysis could proceed. Experiments were run using spiked PAH in urine where the samples were spiked and the peak height monitored as a function of time to determine if breakdown was occurring. No decrease in peak height was seen with time.

Table 2
Comparison of area vs. height R.S.D. values for the four different analytes[a]

	Average peak area R.S.D. (%)	Average peak height R.S.D. (%)
Cr	21.3	10.9
Cn	11.0	8.0
UA	16.8	9.4
PAH	13.9	11.2

[a] Peak height quantification was noticeably more reproducible.

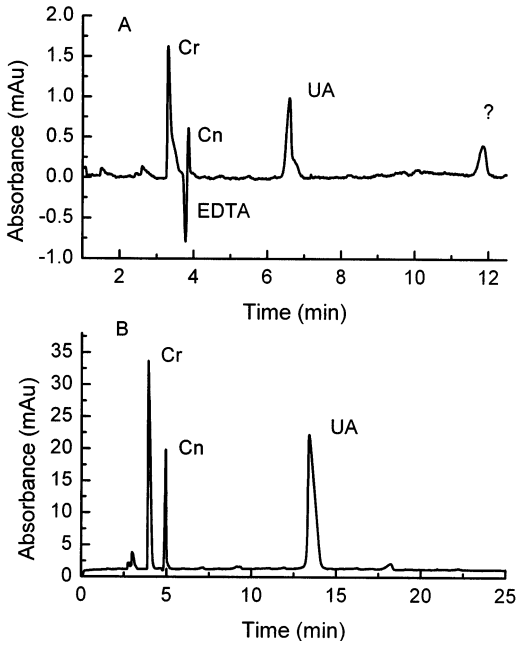

Fig. 2. (A) Electropherogram of a diluted (50-fold dilution) urine sample. (B) Electropherogram of a concentrated (1:1 dilution with urine dilution buffer) urine sample. Separations were carried out at 25 kV with injection for 5 s at 0.5 psi. Absorbance detection at 214 nm. Urine dilution buffer consisted of 50 mM pH 5.5 phosphate with 2% EDTA.

3.4. High throughput analysis

After the successful analysis of two urine samples, a high throughput screen was performed on samples from 46 subjects in 1 day (Fig. 4). The migration times for the renal function indicators were slower in the 46 samples ran than in the analysis of the initial two samples. However, the negative peak due to EDTA served as a reference point in the electropherograms. Migration times of renal function markers were compared with the EDTA peak time to identify the compounds of interest. All 46 of the electropherograms contained Cr and Cn peaks. Only 44 samples were run for an adequate amount of time for UA elution. Uric acid peaks were detected in 37 of these 44 samples. In the other samples, the UA concentration was below the limit of detection. Concentrations of the desired compounds were determined from the peak height linear regression data and shown in Table 3. All of the measured

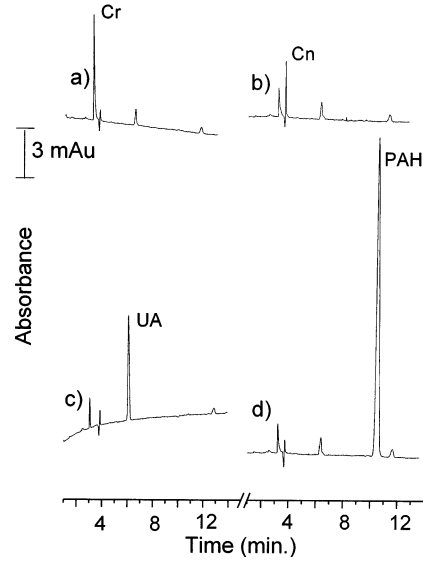

Fig. 3. (a–d) Urine samples diluted 50 fold with urine dilution buffer and spiked with 1 mM concentration of (a) Cr, (b) Cn, (c) UA, and (d) PAH in 50 mM pH 5.5 phosphate buffer. Same conditions as in Fig. 2 (dilute samples).

values fell within the limits of normal output for a single urine sample. This demonstrates the method's ability to rapidly screen large numbers of samples.

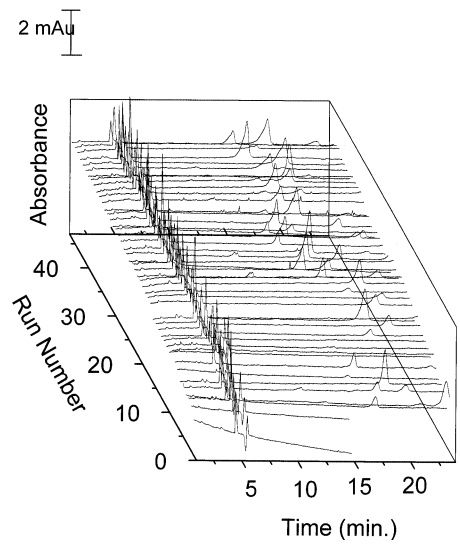

Fig. 4. Electropherograms of 46 urine samples demonstrating the high throughput analysis capabilities of the method. Same conditions as in Fig. 2 (dilute samples).

Table 3
Summary of the high-throughput screening run shown in Fig. 4[a]

	High	Low	Average
Cr (mM)	35.2	0.87	13.6
Cn (mM)	42.1	1.72	20.9
UA (mM)	11.0	0.01	4.76
[Cr]/[Cn]	1.23	0.3	0.66
[Cr]/[UA]	14.7	1.69	9.2

[a] Quantification based on the linear calibration data shown in Table 1.

4. Conclusion

A high throughput multi-analyte screening method for markers of renal function has been described. The method requires only dilution and filtration of the urine sample. The capillary electrophoresis analysis of renal markers offers many advantages to current testing methods, such as a fast analysis time, high specificity, minimal sample volume, reduced reagent consumption, and the ability to simultaneously analyze multiple markers.

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