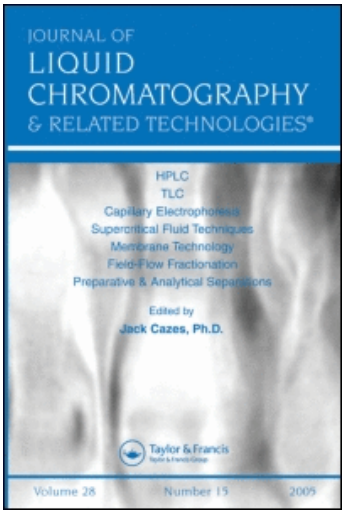

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Original Article

CAPILLARY ELECTROPHORESIS AS A DIAGNOSTIC TOOL: DETERMINATION OF BIOLOGICAL CONSTITUENTS PRESENT IN URINE OF NORMAL AND PATHOLOGICAL INDIVIDUALS

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ABSTRACT

A quantitative ultraviolet detection method for the determination of urinary metabolites is described using capillary zone electrophoresis. The determination of these metabolites is simple, fast, reproducible and utilizes very small amounts of sample. This method is linear between 1.0 x 10^{-4} and 1.0 x 10^{-2} M for creatinine and between 1.0 x 10^{-1} and 1.0 M for urea. The ultraviolet method shows detection limits for creatinine in the picogram (femtomol) range, and for urea in the nanogram (picomol) range.

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INTRODUCTION

Capillary electrophoresis is a separation nanotechnique which resolves chemical species according to their differential rate of migration through solution in the presence of an electric field. Open-tube capillary electrophoresis separation is carried out within a narrow tube filled with a buffer solution. The capillary tube dissipates heat efficiently and prevents disruption of separations by thermally driven convection currents, as observed when large electric fields are used. This separation technique has been successfully applied to the analysis of a variety of compounds ranging from ions to macromolecules (1-7).

Determination of urinary metabolite levels have been used as one of the convenient clinical indices to measure a variety of physiological and pathophysiological conditions in human and in different animal species. For example, the determination of urea and creatinine levels in urine have been used primarily to assess kidney glomerular function. Currently, several methods are available to measure both metabolites in urine, including colorimetric, enzymatic, chromatographic and electrophoretic techniques (8-12). Although some of the advantages of these methods are their simplicity and wide clinical acceptance over many years of use, several limitations still need to be overcome. Thus, we have evaluated the use of capillary electrophoresis as an analytical tool to quantitatively and qualitatively identify urea and creatinine in urine collected from normal and pathological individuals.

EXPERIMENTAL

Reagents and samples

High purity grade sodium tetraborate, Trizma base, sodium acetate, urea, creatine, creatinine, sodium hydroxide, potassium hydroxide, and hydrochloric acid were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Millex disposable filter units (0.22

μm) were obtained from Millipore Corporation (Bedford, MA, U.S.A.). Fused-silica capillary columns were purchased from Scientific Glass Engineering (Austin, TX, U.S.A.), and from Polymicro Technologies (Phoenix, AZ, U.S.A.).

Triply distilled and deionized water was used for the preparation of buffer solutions. Both buffers and samples were routinely degassed with helium after filtration (with disposable Millipore filter units).

Urine specimens collected during a 24 hr period were obtained from four individuals: two normal persons, one dehydrated patient, and one patient undergoing strenuous exercise. All urine donors were adult males of approximately 70-90 kg body weight.

Instrumentation

The capillary electrophoresis apparatus was similar to that previously described (5,6,13,14). The system is a full-featured instrument (Princeton Biochemicals, Inc., Princeton, NJ, U.S.A.) that includes an autosampler or autoloader; a modified on-column UV, variable-wavelength, detection system; a direct current power supply (30-kV, 1000 μA) used in the positive voltage mode, modified to obtain a reverse field polarity capability; a fraction collector; an on-line degassing system (using a small flow of helium, through a narrow capillary tubing, into every sample and buffer); and an automated cleaning device to clean the capillary column. Data collection for quantitation of electropherographic peaks was carried out with an integrator model D-2500 Chromato-Integrator (Hitachi Instruments, Inc., Danbury, CT, U.S.A.). Electropherograms were generated with a strip chart recorder model L-6512 (Linseis Inc., Princeton Junction, NJ, U.S.A.) at 20 cm/hr and 1 mV output. For specific technical details of the capillary electrophoresis instrument see references 5,6,13, and 14.

Procedure

For each individual, urine samples were collected for 24 hours. During the collection period, urine specimens were refrigerated be-

tween voidings without the use of preservatives. At the end of the collection period, samples were mixed gently but throroughly, their total volume measured, and an aliquot was immediately used for analysis.

Each sample was filtered, degassed, and then injected by electrokinetic migration, into a buffer-filled, surface untreated fused-silica capillary column. Each injection was carried out for 10 sec at 5 kV (or approximately 9 nl of sample injection). The capillary column (75 μm x 150 cm) was primed with 0.05 M sodium tetraborate buffer, pH 8.3. The components in the urine sample were separated at 15 kV (100 V/cm) for 100 min, maintaining the capillary column at constant temperature (25°C). Separated components were monitored at 210 nm. The rate of electroosmotic flow was calculated as previously described (5). In order to ensure optimal performance of the column, the capillary was cleansed (after every sixth sample injection) by a minor modification of the potassium hydroxide-water-buffer cycle described elsewhere (15). Briefly, this procedure was carried out automatically for 10 min using a cleaning device attached to a fluid trap and a miniaturized pump to aspirate solutions (14,16). Two minute aspirations were performed with 2% (v/v) phosphoric acid, followed by two minutes each of water, 1 N potassium hydroxide, water, and finally equilibrated for two minutes with the appropriate buffer.

Calibration curves for both urea and creatinine standards were performed under identical conditions as for the urine samples. The criteria to qualitatively identify these urine metabolites in each sample was based on: a) the migration time of the standards comparatively to the urine specimen constituents; and b) by spiking the urine sample (before injection) with the known standards, and comparing their migration times and peak areas to the electropherogram of the untreated urine.

RESULTS

As shown in Figure 1, several components were observed in the electropherograms (generated from urine specimens of normal

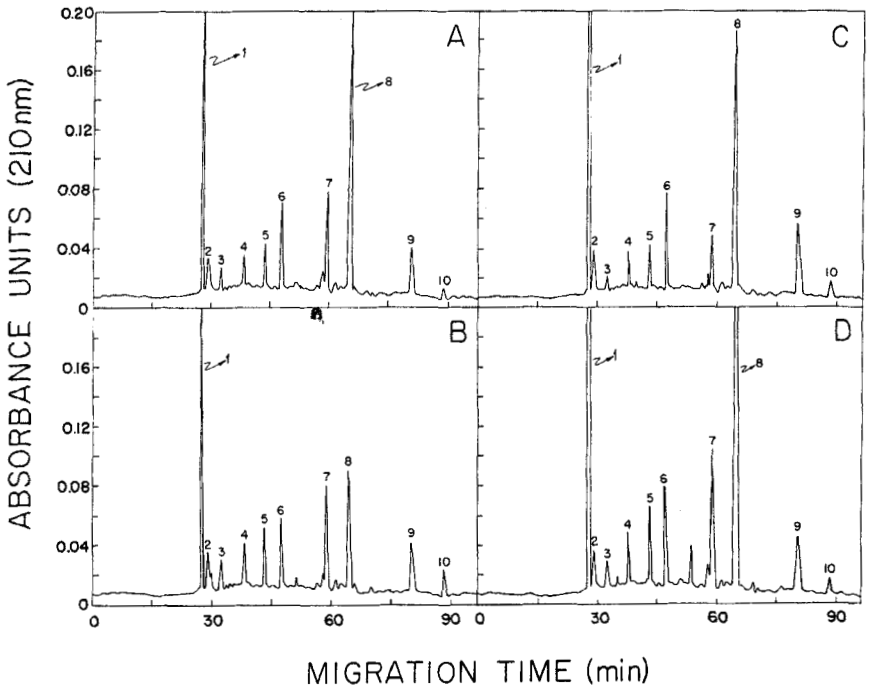


FIGURE 1. Typical electropherograms of human urine using ultraviolet detection methods. Approximately 10 major components were observed in the capillary electrophoresis profiles of urine specimens. **A.** urine profile from normal person-1; **B.** urine profile from normal person-2; **C.** urine profile from dehydrated patient; and **D.** urine profile from patient undergoing strenuous exercise. Peak 1 in all four electropherograms shows a migration time corresponding to that of urea and creatinine.

and abnormal individuals) when an ultraviolet detection method was used to monitor and quantitate urine metabolites. In preliminary experiments, several buffers of different ionic strenghts, ranging from pH 3.0 to 9.0, were used to optimize the separation conditions of the urinary metabolites (data not shown). Apparently, the best conditions were obtained with 0.05 M sodium tetraborate buffer, pH

8.3 (Figure 1). In addition, several attempts were made to identify the major components resolved to completion by capillary electrophoresis. Logically, we started by identifying those metabolites present in large concentration in the urine, such as creatine, creatinine, and urea. Only urea and creatinine were identified under the conditions described above (Peak 1 in Figure 1-A,B,C,D), but their separation was not resolved to completion because the two urinary metabolites co-migrated as a single peak in the electropherogram (Figure 2). Since urea and creatinine differ significantly in structural properties, i.e., in the number of double bonds (Figure 3), the extinction coefficient of creatinine at 210 nm is several-fold greater than urea at this wavelength (Figure 2).

Although quantitation of urea and creatinine in urine was only possible as combined values of both metabolites (under our experimental conditions), differences in values of total urinary urea-creatinine from normal and pathological individuals were observed. For example, higher levels of these metabolites (when compared to normal urines) were observed in urines derived from the dehydrated patient (concentrated urine), and from the patient under strenuous exercise (due to increased degradation of creatine from muscle tissue) (see below). As shown in Table 1, quantitative values were obtained for both urea and creatinine standard solutions. These calibration parameters were used to obtain quantitative results of both metabolites in the urine samples (Table 2). Due to the symmetry and sharpness of the peaks, no significant differences were observed between quantitative values calculated by the maximum peak absorbance method or when calculated by using the total peak area method (data not shown). As shown in Table 2, the two urines from the normal persons (sample-1 and sample-2) contained 17 and 22 mg/kilo/24 hr of creatinine-urea, respectively; and the urine from the dehydrated patient contained a higher value but not significantly elevated (26 mg/kilo/24 hr). However, the urine from the patient undergoing strenuous exercise had a significantly higher value (34 mg/kilo/24 hr) of creatinine-urea. Also, a slight elevation of other metabolites and the appearance of new metabolites was observed.

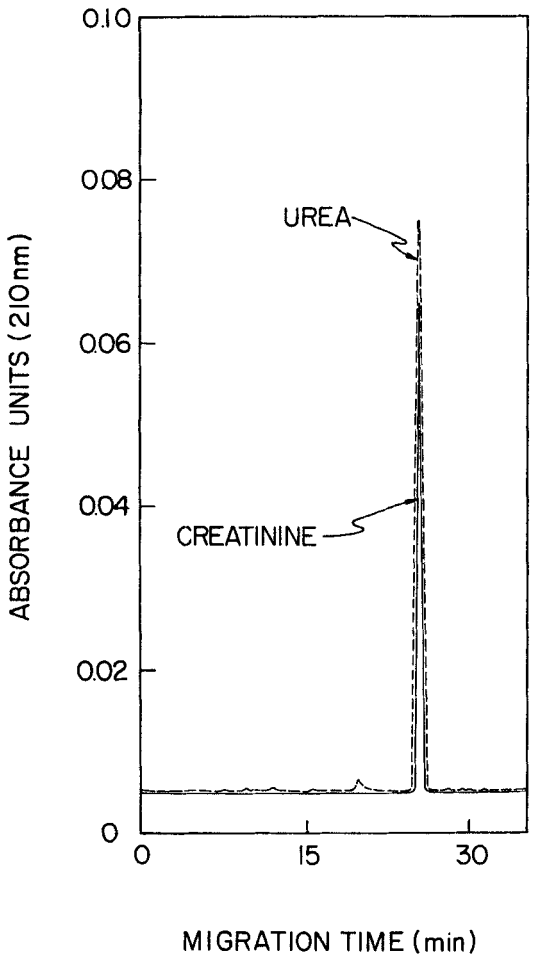
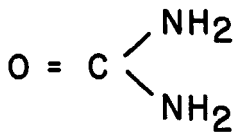
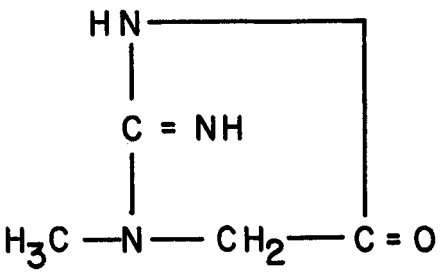


FIGURE 2. Co-migration of urea and creatinine peaks under the conditions described in text. Both metabolites were injected onto the capillary column under identical experimental conditions except that the concentration of metabolites were different (1 M urea, 1 x 10^{-4} M creatinine).



UREA



CREATININE

FIGURE 3. Molecular structures of urea and creatinine. Creatinine has more double bonds, which is probably one of the main features of its strong absorbance at 210 nm.

The calibration curves for both urine metabolites (Table 1) demonstrated that the method was linear between 1 x 10^{-4} and 1.0 x 10^{-2} M for creatinine, and between 1.0 x 10^{-1} and 1.0 M for urea. The ultraviolet method showed detection limits for creatinine in the picogram (femtomol) range, and for urea in the nanogram (picomol) range. Thus, quantitative values for physiological urea levels in urine were negligible under the experimental conditions described

TABLE 1. LINEARITY STUDIES FOR STANDARDS OF UREA AND CREATININE BASED ON ARBITRARY ABSORBANCE UNITS VERSUS CONCENTRATION

STANDARD	CONCENTRATION (M)	ARBITRARY ABSORBANCE UNITS AT 210 nm
UREA	1.0	0.0676
	5.0×10^{-1}	0.0354
	1.0×10^{-1}	0.0180
	5.0×10^{-2}	0.0035
	1.0×10^{-2}	0.0002
CREATININE	1×10^{-2}	0.3560
	5×10^{-3}	0.2051
	1×10^{-3}	0.1153
	5×10^{-4}	0.0502
	1×10^{-4}	0.0104
	5×10^{-5}	0.0041
	1×10^{-5}	0.0019

TABLE 2. QUANTITATIVE ANALYSIS OF URINARY METABOLITES

URINE SPECIMEN	CREATININE-UREA* (mg/kilo/24 hr)
Sample-1	17
Sample-2	22
Sample-3	26
Sample-4	34

* Values are based on an aliquot of approximately 9 nl of urine specimen injected onto the capillary column (see text). Values are expressed as mg of urinary metabolite present in the volume collected during a 24 hr period, corrected for one kilogram of body weight. Sample-1, control patient-1; sample-2, control patient-2; sample-3, dehydrated patient; sample-4, patient undergoing strenuous exercise.

here. Therefore, Peak 1 in Figures 1-A,B,C,D represent quantitative values for creatinine in urine specimens.

Since the performance of any analytical technique is characterized in terms of accuracy, precision, reproducibility and dynamic range, we have determined the precision of the capillary electrophoresis method in terms of creatinine peak areas and migration times. As shown in Table 3, the precision of the method was carried

TABLE 3. PRECISION OF CREATININE PEAKS AREAS AND MIGRATION TIMES OF SAMPLE-1

Run Number	Peak Area (arbitrary units)	Migration Time (seconds)
1	10143	1654
2	9846	1614
3	9964	1763
4	10012	1592
5	10218	1584
6	9904	1603
Mean	10014.50	1635.00
Standard deviation	142.19	67.32
Coefficient of variation (%)	1.42	0.30

out in a run-to-run operation for 6 sample injections. The reproducibility of the parameters obtained here were excellent, and this has been also demonstrated by other investigators (using various capillary electrophoresis instruments, different analytes, and different buffer conditions) (1-7,17).

DISCUSSION

The differentiation and analysis of urinary metabolites has substantially contributed to our knowledge of physiological and pathophysiological processes associated with glomerular filtration and tubular catabolism of plasma constituents. By use of a high resolution biochemical separation technique, such as capillary electrophoresis, urinary metabolites can be identified in a simple manner in a short period of time without any proccessing of collected urine, other than a filtration step to remove cells and particulated matter.

Urea and creatinine have been used as indices in many aspects of physiological and pathophysiological conditions. Historically, urea clearance was the first procedure useful as an index to measure overall renal function (8-10). Later, the urea clearance assay was replaced by the creatinine test which is currently one of the most popular test for measuring the actual capacity of the kidney (8-10). In addition, urea and creatinine have been used as markers in pathophysiological conditions other than the kidney (8,9). For example, the major determinant of the size of the creatinine pool in an individual is the total muscle mass. Therefore, pathophysiologic states associated with muscle wasting and reduced muscle mass (for example, muscular dystrophy, muscular paralysis and dermatomyositis) feature a reduced creatinine generation. Urinary creatinine levels has been used also as an index compound to compare excretion rates of other metabolites (11).

Although analyses of metabolic products by capillary electrophoresis have already been performed both in human (5,18,19) and in mouse urine (20), no attempts were made to identify and quantitate the separated urinary constituents. In this communication we report the identification and quantitation of two more components present in human urine (using capillary electrophoresis). Previously, using this high-resolution technique, we were able to identify and quantitate urinary phosphate (21). In addition, it was shown that storage of urine samples under low temperature

conditions changed the quantitative profile exclusively of phosphates (when compared to the capillary electrophoresis profile derived from freshly analyzed urine specimens). Attempts to identify other urinary constituents by capillary electrophoresis have been presently unsuccessful in our laboratory. Therefore, our intention is to further identify the unknown components found in urine by exploring: (a) new methods to optimize the separation conditions of the components present in urine using capillary electrophoresis (i.e., modification of the chemical groups at the surface of the capillary, and/or modification of the buffer composition); (b) the **in tandem** connection of capillary electrophoresis to mass spectrometry; and (c) new detection methods, such as electrochemistry, fluorescence and laser-induced fluorescence microscopy (22,23). With these detection methods we hope to improve the sensitivity of the analysis of substances present in urine and other biological fluids, in order to identify other analytes that cannot be detected by ultraviolet detection methods. For example, using conductivity detection, Zare and co-workers (24,25) determined the presence of ions in human serum, thus opening the possibility of using capillary electrophoresis as a possible diagnostic tool for analyzing sodium and potassium in biological fluids. In addition, capillary electrophoresis has been useful clinically for monitoring therapy for patients suffering from mental diseases, such as acute mania, when lithium was the therapeutic agent (24,25). Similarly, Roach ***et al.*** (26) used capillary electrophoresis in the analysis of the anticancer drug, methotrexate, and its major metabolite, 7-hydroxymethotrexate, in human serum samples. High-dosage methotrexate therapy, used in the treatment of several cancers, requires careful monitoring of the drug in serum to ensure minimal toxic effects. More recently, various laboratories have demonstrated that capillary electrophoresis is the most promising analytical technique for routine clinical laboratory work and, in turn, a valuable tool for the diagnosis of human metabolic diseases when constituents present in biological fluids were analyzed (27-30).

The work described herein opens new avenues for capillary electrophoresis, i.e., the monitoring of metabolites and drugs secreted

in urine, or present in kidney dialyzates, or in other biological fluids. Furthermore, capillary electrophoresis could have a tremendous potential as diagnostic tool, particularly as a confirmatory technique when false positive or negative results are obtained using other technologies. Although in many instances this technology might not be practical for routine sample analysis (i.e., mass screening of urine specimens), its unprecedented resolution and accuracy recommends it as a reference method and for validating other methods. In addition, it will probably complement or compete with the **in vitro** enzymatic gene amplification technique, polymerase chain reaction, used in diagnosis and forensic medicine (31-34), because of the high resolution, sensitivity, and simplicity.

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