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Analysis of anion constituents of urine by inorganic capillary electrophoresis

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ABSTRACT

Inorganic capillary electrophoresis (ICE) is a new separations technology which melds the technique of classical electrophoresis with the separations approach of ion chromatography. Matrices which have been difficult to deal with using ion chromatography have proven amenable to analysis by ICE. The simultaneous analysis of weak acid anions, oxalate and citrate and inorganic anions, chloride, sulfate, nitrate, phosphate and carbonate in diluted urine was achieved using ICE. The determination of the oxyanions of arsenic (*i.e.* arsenite and arsenate) in urine was also performed.

INTRODUCTION

The determination of the urinary levels of oxalate has long been recognized as an important factor in the study of renal stone formation. Additionally, citrate in urine can form soluble complexes with calcium oxalate monohydrate for example, which tends to reduce the rate of mineralization of this stone-forming material [1]. There have been a number of papers describing the use of ion chromatography (IC) to analyze oxalate [2–6], citrate [1,7] or nitrate [8] in urine individually.

IC is employed most commonly to analyze strong acid anions such as chloride and sulfate by ion-exchange chromatography. Weak acid anions, such as short-chained organic acids (*e.g.* formate and acetate), are usually separated by ion-exclusion chromatography. Typically, the simultaneous analysis of both weak and strong acid anions must be performed by gradient ion chromatography [9] or coupled techniques [10]. Inorganic capillary electrophoresis (ICE) was recently introduced as an alternative that allows the simultaneous analysis of both groups in a simple and rapid manner [11]. This preliminary investigation was undertaken in an effort to analyze oxalate, citrate and nitrate simultaneously using ICE. This analysis is possible due to the very high efficiency of the separation. Additionally, the separation of chloride, sulfate, phosphate and carbonate occurs within the same analytical run as oxalate, citrate and phosphate. The advantage of ICE with respect to ion chromatography (IC) is that the former results in different selectivities that are predictable based on the charge-to-size ratio of the analyte.

A second aim of this investigation was to examine the possibility of the analysis of the oxyanions arsenite and arsenate in urine. Speciation of these anions is important in the determination of the toxicological effects of each.

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EXPERIMENTAL

Instrumentation

A Waters Chromatography Division of Millipore Corporation Quanta 4000 Capillary Electrophoresis instrument was used with a Model 820 data station. Since the analysis times of the technique are extremely short and very narrow peak widths are obtained, it was necessary to collect the data at 20 points per second. The separations were performed on an Waters AccuSep polyimide-coated, 60 cm × 50 μm I.D. fused-silica capillary. The injection mode used was hydrostatic in which the sample was elevated to constant height (10 cm) for 60 seconds. The electrolyte was prepared daily, filtered and degassed using a Waters Solvent Clarification Kit and Millipore (Bedford, MA, U.S.A.) HATF Membrane filter (0.45 μm). Indirect photometry at 254 nm was used as the detection mode. Operating conditions are provided as captions to the figures.

Reagents

All solutions were prepared using 18 MΩ water purified using a Millipore Milli-Q Water Purification System. ACS grade sodium chromate tetrahydrate, ascorbic acid and boric acid were obtained from Aldrich (Milwaukee, WI, U.S.A.). A 0.5% (w/v) solution of sodium arsenite was obtained from Ricca Chemical (Arlington, TX, U.S.A.). Sodium arsenate was obtained from Mallinckrodt (Paris, KY, U.S.A.). All other anion standards used were prepared from the analytical grade sodium salts purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). NICE-Pak OFM Anion-BT, the electroosmotic flow modifier, is a proprietary chemical obtained from Waters Chromatography Division of Millipore Corporation.

RESULTS AND DISCUSSION

Instrumental configuration for ICE

In conventional capillary electrophoresis, sample introduction is carried out at the anodic electrode and detection is carried out at the cathodic electrode. There is a bulk fluid flow, referred to as the electroosmotic flow (EOF), toward the cathode at most pH values, that is dependant on the charge on the capillary wall [11]. In ICE, a proprietary compound, NICE-Pak OFM Anion-BT, is used to reverse the electroosmotic flow. Since the EOF, with the osmotic flow modifier present, is toward the anode, injection is carried out at the cathodic electrode and detection at the anodic electrode in ICE.

Migration in capillary electrophoresis is dependant on the charge-to-size ratio of each of the analytes, and the inorganic and organic anions analyzed by ICE are typically low-molecular-weight species, so this ratio is relatively large. Therefore, the anionic species migrate toward the anode preceding the EOF. This results in a very efficient and short analysis. The typical value for theoretical plates is in excess of 250 000 and analysis times are within 6 min.

There are two commonly employed injection modes in capillary electrophoresis. The first, used exclusively in this work, is termed hydrostatic (or gravity) injection. This mode introduces a representative sample of a homogeneous mixture into the capillary. The capillary, immersed in the sample, is raised to a constant height of 10 cm

for a user-specified time. The capillary is then lowered into electrolyte and the voltage is applied. The second mode, electrostatic injection, selects a sample which is biased toward faster-migrating species. The capillary is immersed in sample and a pre-selected sampling voltage is applied which causes anions in the sample to migrate into the capillary. Analysis then occurs as described above. Detection occurs by the process of indirect photometry at 254 nm as the electrolytes chosen have significant absorbance at this wavelength.

Determination of oxalate and citrate in urine

The determination of inorganic anions and short-chained organic acids in urine is a challenging analytical problem. Undiluted urine contains approximately 6000 ppm chloride, 1200 ppm phosphate, 180 ppm sulfate, 100–500 ppm citrate and 10–50 ppm oxalate. These very high levels of inorganic ions can cause severe interferences and quantitation difficulties when analyzing for citrate or oxalate by IC. In all the cited cases, a dilution of the urine was performed before ion chromatographic analysis. A difficult situation exists in that the urine must be diluted sufficiently to prevent overloading of the analytical column but yet not so much as to dilute the oxalate and citrate beyond the detection limits for these species. These IC approaches have yielded analyses for either oxalate or citrate but not both together, nor with the inclusion of the other inorganic ionic species which can also be important in urinary studies.

Fig. 1 shows an electropherogram, henceforth referred to as a pherogram, of eight commonly analyzed anions using ICE with a 50 μm I.D. capillary and a high mobility chromate electrolyte. The very short analysis times allow rapid methods development. It is important to note that a different selectivity is obtained when using this technique as compared to conventional anion-exchange chromatography. The normal elution order using IC is fluoride carbonate, chloride, nitrite, bromide, nitrate, phosphate and sulfate, column efficiencies are on the order of a few thousand plates, and analysis times commonly range from 10 to 15 min. The migration order of these ions using ICE is bromide, chloride, sulfate, nitrite, nitrate, fluoride, phosphate and carbonate, the efficiency of the separation ranges upwards of 250 000 plates, and the analysis time is under 6 min. The combination of selectivity differences, extremely high efficiencies, and fast analysis time make ICE ideal for investigating the analysis of anionic species in urine.

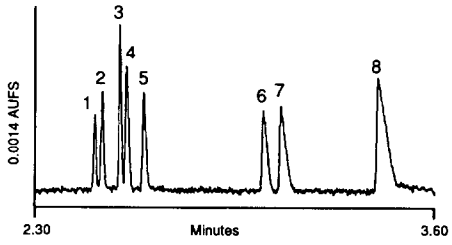

Fig. 1. Pherogram of standard anions. Conditions; Capillary: 60 cm × 50 μm I.D. fused silica. Electrolyte: chromate at pH 8.0 with NICE-Pak OFM Anion-BT (patent applied for); injection: hydrostatic for 60 s; detection: indirect UV at 254 nm; potential: 20 kV. Solutes: 1 = bromide (4 ppm); 2 = chloride (2 ppm); 3 = sulfate (4 ppm); 4 = nitrite (4 ppm); 5 = nitrate (4 ppm); 6 = fluoride (1 ppm); 7 = phosphate (4 ppm); 8 = carbonate (4 ppm).

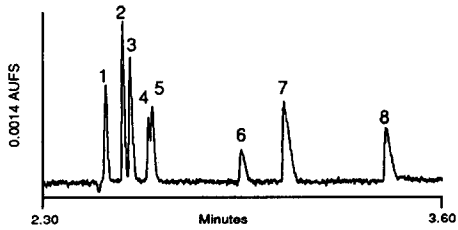

Fig. 2. Pherogram of standard anions expected in urine. Conditions as for Fig. 1 except; solutes: 1 = chloride (2 ppm); 2 = sulfate (4 ppm); 3 = nitrite (4 ppm); 4 = nitrite (2 ppm); 5 = oxalate (2 ppm); 6 = citrate (2 ppm); 7 = phosphate (4 ppm); 8 = carbonate (4 ppm).

A standard was prepared to contain citrate and oxalate (2 ppm each) in addition to other anions expected in diluted urine (*i.e.* chloride, sulfate, nitrite, nitrate, phosphate and carbonate) and then analyzed. The resultant pherogram is shown in Fig. 2. Citrate is well resolved from phosphate but nitrate and oxalate are only partially resolved. Upon closer examination of this pherogram, (Fig. 3) it can be seen, however, that there is sufficient resolution to allow accurate quantitation. The important feature of this separation to note is apparent when contrasted with IC. Oxalate is a strongly retained species using IC and commonly elutes after sulfate. Citrate is so strongly retained that, in most cases, a very strong eluent must be used. Use of such eluents generally sacrifices the ability to analyze for the early-eluting species such as chloride and nitrate. More complex approaches, such as the use of either gradient ion chromatography [9] or a coupled system [10], allow for the analysis of citrate without the loss of resolution of the early eluting anionic species. However, analysis times are significantly lengthened. Figs. 2 and 3 show the advantage of the different selectivity of ICE without the disadvantage of increasing analysis time to incorporate both weak and strong acid anions in one chromatographic run.

A pherogram of a fresh midstream void sample (author), diluted 1:50 in 18 MΩ water, is shown in full-scale view in Fig. 4. Due to the very high efficiency of ICE, the adjacent peaks, chloride and sulfate are well resolved even though present in a 14:1 ratio. A magnification of this pherogram (Fig. 5) shows that in addition to the five peaks seen in Fig. 4, a small peak (0.5 ppm) for nitrate is apparent. For the complexity of this matrix, the pherogram obtained is relatively simple, In IC, cations and water in the sample appear early in the chromatogram at the void volume using anion-exchange

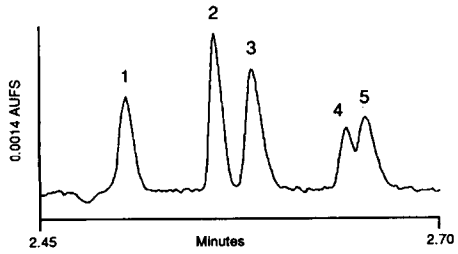

Fig. 3. Expanded view of peaks 1–5 of Fig. 2. Conditions as for Fig. 2.

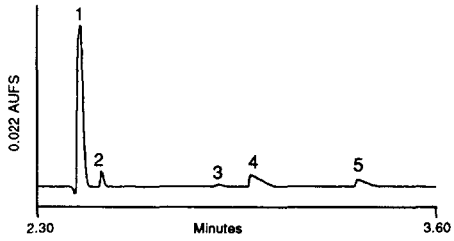

Fig. 4. Pherogram of diluted urine. Conditions as for Fig. 1 except; sample preparation 50 × dilution in deionized water; solutes: 1 = chloride (109 ppm); 2 = sulfate (7.9 ppm); 3 = citrate (3.2 ppm); 4 = phosphate (16.2 ppm); 5 = carbonate (12.2 ppm).

separations. This large void peak can interfere in the quantitation of early-eluting anions. An advantage of ICE is that cations in the sample do not appear in the pherogram as they migrate in the opposite direction, toward the cathode hence, do not interfere with the analyte anions. In this particular sample, no oxalate was found so the diluted urine sample was then spiked with 2 ppm oxalate and 2 ppm citrate and reanalyzed (Fig. 6). Nitrate appears as a shoulder on the leading edge of the oxalate peak but it is quantitatively recovered. The recoveries of oxalate (95%) and citrate (94%) are acceptable.

Speciation of arsenite and arsenate in urine

There is some interest in the speciation of arsenic oxyanions in urine to determine the independent toxicological effect of each upon ingestion of contaminated fish. A second aim of this work was to investigate the feasibility of using ICE to analyze for each of these species. Fig. 7 is a pherogram of chloride, sulfate, nitrite, nitrate, phosphate, arsenate, carbonate and arsenite using chromate electrolyte at pH 10. It was necessary to operate the electrolyte at pH 10 to ensure the presence of the anionic form of arsenite as the pK_a of arsenite is 9.22. Good resolution of arsenate, phosphate and carbonate is seen. At pH 10, fluoride and phosphate co-migrate (compare Fig. 1) but this is inconsequential as little or no fluoride is expected in urine. It is important to use plastic sample vials when using electrolytes at elevated pH values rather than the commonly used borosilicate glass vials. When the glass vials were used, a peak identified as the borate anion was found due to a leaching of this anion from the vial wall.

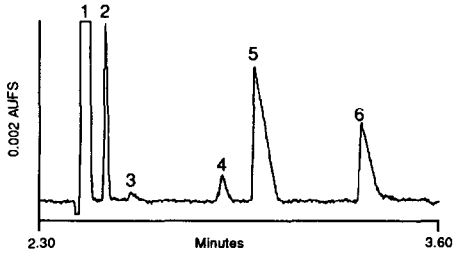

Fig. 5. Expanded view of Fig. 4. Condition as for Fig. 4 except; solutes: 1 = chloride (109 ppm); 2 = sulfate (7.9 ppm); 3 = nitrate (0.5 ppm); 4 = citrate (3.2 ppm); 5 = phosphate (16.2 ppm); 6 = carbonate (12.2 ppm).

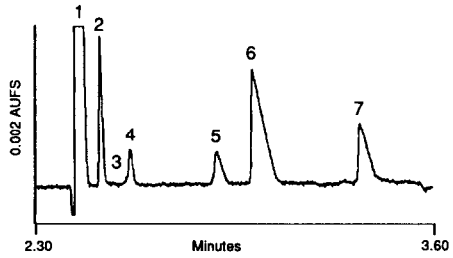

Fig. 6. Pherogram of diluted urine spiked with oxalate and citrate. Conditions as for Fig. 4 except; solutes: 1 = chloride (109 ppm); 2 = sulfate (7.9 ppm); 3 = nitrate (0.5 ppm); 4 = oxalate (1.9 ppm); 5 = citrate (4.9 ppm); 5 = phosphate (16.3 ppm); 6 = carbonate (9.3 ppm).

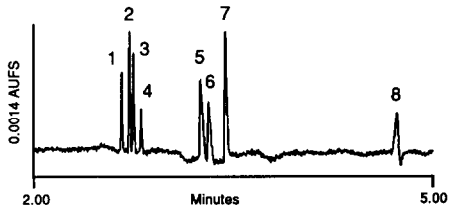

Fig. 7. Pherogram of standard anions and arsenite and arsenate. Conditions as for Fig. 1 except; electrolyte: chromate at pH 10.0 with NICE-Pak OFM Anion-BT; solutes: 1 = chloride (2 ppm); 2 = sulfate (4 ppm); 3 = nitrite (4 ppm); 4 = nitrate (2 ppm); 5 = phosphate (4 ppm); 6 = arsenate (5 ppm); 7 = carbonate (4 ppm); 8 = arsenite (5 ppm).

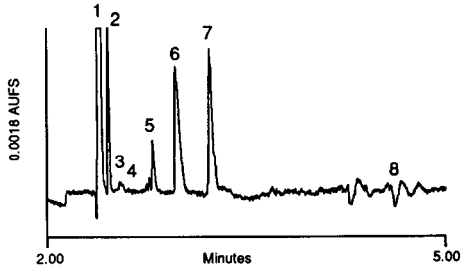

Fig. 8. Pherogram of diluted urine. Conditions as for Fig. 7 except; sample preparation 50 × dilution in deionized water, solutes: 1 = chloride (67.7 ppm); 2 = sulfate (9.6 ppm); 3 = nitrate (1.0 ppm); 4 = oxalate (0.6 ppm); 5 = citrate (4.6 ppm); 6 = phosphate (11.5 ppm); 7 = carbonate (7.4 ppm); 8 = ascorbate (0.7 ppm).

A freshly voided midstream sample of urine was collected and diluted 1:50 in 18 MΩ water and analyzed using the conditions above (Fig. 8). Nitrate and oxalate are seen as partially resolved peaks 3 and 4. It was apparent that this sample also contains a small amount of ascorbate (peak 8), which appears as a negative peak (increasing absorbance) as this compound exhibits appreciable absorbance at 254 nm. There are also unidentified peaks appearing before the citrate peak (peak 5). As expected neither arsenite nor arsenate were found. The same urine sample was spiked with 10 ppm each of arsenite and arsenate and reanalyzed. The pherogram (Fig. 9) indicates good resolution between both the arsenic oxyanions and the surrounding peaks.

Future investigations

As this work was undertaken as a feasibility study, non-preserved urines were used. In clinical practice, urine is preserved with acid to approximately pH 2 to prevent precipitation of calcium oxalate species and also to prevent the conversion of urinary ascorbate to oxalate. Continuation of the present work will proceed with several main aims. The first two, improved resolution of nitrate and oxalate and the ability to analyze low-pH-preserved samples may be concurrently solved. At pH 8.0 both oxalate and citrate are totally ionized. There exist a number of alternate electrolytes to chromate which can be used at lower pH values. The use of these electrolytes at pH 3 for example, would allow the analysis of preserved samples and also change the selectivity of both oxalate and citrate since they would each exist as the monovalent species instead of the di- and trivalent species as occurs at pH 8. Since their sizes remain relatively constant, the charge-to-size ratios decrease and both will exhibit increased migration times. Nitrate, being a strong acid remains unaffected so the nitrate–oxalate pair become separated. The third aim is to analyze urine using less dilution to increase the peak size of nitrate and oxalate. Given that oxalate and citrate migrate later using an electrolyte of lower pH, a less-diluted sample may be analyzed without concern for increased resolution problems. A disadvantage however, is the possible loss of the ability to quantitate chloride and sulfate. The fourth aim is to investigate the analysis of two other important urinary anions glyoxalate and glycolate.

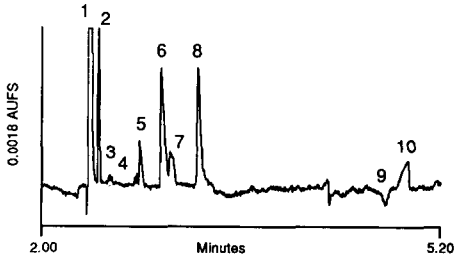

Fig. 9. Pherogram of diluted urine spiked with arsenate and arsenite. Conditions as for Fig. 8 except; solutes: 1 = chloride (67.7 ppm); 2 = sulfate (9.6 ppm); 3 = nitrite (1.0 ppm); 4 = oxalate (0.6 ppm); 5 = citrate (4.6 ppm); 6 = phosphate (11.5 ppm); 7 = arsenate (10.0 ppm); 8 = carbonate (7.4 ppm); 9 = ascorbate (0.7 ppm); 10 = arsenite (10.0 ppm).

CONCLUSIONS

The feasibility of the simultaneous analysis of chloride, sulfate, nitrate, oxalate, citrate, phosphate and carbonate in diluted urine by inorganic capillary electrophoresis has been demonstrated. This technique provides rapid and highly efficient separation of both weak and strong acid anions. The extension of the technique to properly preserved samples with increased resolution between nitrate and oxalate remains the subject of future investigation.

The speciation of the oxyanions of arsenic in diluted urine has also been demonstrated.

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REFERENCES

1 R. P. Singh and G. H. Nancollas, *Kidney Int.*, 28 (1985) 985.
2 C. J. Mahle and M. Menon, *J. Urol.*, 127 (1982) 159.
3 R. P. Singh and G. H. Nancollas, *Anal. Letters*, 19 (1986) 1487.
4 R. L. Orwell, D. S. Scurr, A. Smith and A. G. Robertson, *Fortschr. Urol. Nephrol.*, 20 (1982) 263.
5 M. Menon and C. J. Mahle, *Clin. Chem.*, 29 (1983) 369.
6 W. G. Robertson, D. S. Scurr, A. Smith and R. L. Orwell, *Clin. Chem. Acta*, 126 (1982) 91.
7 Y. Ogawa, M. Morozumi, T. Tanaka and K. Yamaguchi, *J. Urol.*, 135 (1986) 178.
8 J. P. Witter, S. J. Gatley and E. Balish, *J. Chromatogr.*, 229 (1982) 450.
9 R. D. Rocklin, C. A. Pohl and J. A. Schibler, *J. Chromatogr.*, 411 (1987) 107.
10 W. R. Jones, P. Jandik and M. Swartz, *J. Chromatogr.*, 473 (1989) 171.
11 W. R. Jones and P. Jandik, *Am. Lab.*, June (1990) 51.