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Determination of oxalate in parenteral nutrition solutions by capillary electrophoresis

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Abstract

A rapid, sensitive and reliable capillary electrophoretic (CE) method based on indirect UV absorbance detection has been developed, optimized and applied to the separation and determination of low (mg/l) levels of oxalate in commercial parenteral nutrition preparations. The determination of oxalate requires no extraction, derivatization or other complex pre-analysis steps. The method used chromate as the UV-absorbing background electrolyte and oxalate was detected indirectly at 254 nm. The analysis time for oxalate is just under 7 min per sample with oxalate migrating at approximately 4 min. The method calibration curves were shown to be linear over a minimum of 1.5 orders of magnitude with a limit of detection for oxalate of approximately 240 μg/l. The methods linear dynamic range for oxalate was shown to extend over more than 2.5 orders of magnitude. The final optimized method was used to separate and quantify oxalate in parenteral nutrition solutions that had been submitted to 24-h neonatal phototherapy procedures.

Keywords: Parenteral nutrition; Food analysis; Oxalic acid; Organic acids

1. Introduction

Oxalic acid ($H_2C_2O_4$) or oxalate ($C_2O_4^{2-}$) is an end product of metabolism in man and it has no clear nutritional value or physiological importance [1]. Renal metabolic disorders and renal diseases, such as primary hyperoxaluria, chronic glomerulonephritis, chronic pyelonephritis, renal tubular acidosis, acute tubular necrosis and nephrocalcinosis are directly related to the presence of excess oxalate [2]. Most of this excess oxalate is excreted unchanged in the urine, but an unknown proportion of the oxalate contributes to the formation of nephrocalcinosis and renal calculi. Deposition of calcium oxalate, CaC_2O_4, contributes to the incidence of nephrocalcinosis in premature infants with attendant morbidity [3–6]. Calcium oxalate is very insoluble in aqueous environments (0.00067 g/100 ml water) [7] and small increases in the physiological oxalate concentration can have profound effects on the calcium oxalate saturation in the body [8,9]. It has

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been postulated that the infusion of parenteral nutrition (PN) solutions can act as a direct source of excess oxalate introduction into premature infants [4,9–13]. PN solutions contain, among many other biologically active components, the major oxalate precursor, ascorbic acid (ascorbate). Ascorbate in parenteral nutrition solutions is known to oxidize to oxalate via a non enzymatic pathway in vitro [14,15], thus acting as a "direct" source of oxalate introduction into premature infants via parenteral infusions. Increased levels of oxalate have also been reported to cause hyperoxaluria, elevated serum oxalate levels and systemic oxalosis in adults [14,16].

Many analytical techniques for the determination of oxalate in biological matrices have been reported [17,18] and the results are used to help understand and diagnose renal related diseases and disorders. Most of these techniques are concerned with the determination of oxalate in either blood plasma, blood serum or urine. The measurement of oxalate in biological matrices has traditionally proven difficult due to the much higher concentrations of other anions such as chloride, sulfate and phosphate in the samples and/or because of specific interferences from proteinaceous materials and metal cations. Early analytical techniques for the determination of oxalate in biological matrices were based on colorimetric reactions, enzymatic reactions, fluorimetric reactions, isotopic dilutions, precipitate formations, solvent extractions and titrimetric analyses [17]. These techniques were labor intensive, insensitive, expensive and frequently inaccurate, giving poor recovery due to extensive sample handling [17]. Later analytical techniques are based mostly on the direct physical determination of oxalate [18]; these include improved enzymatic reactions [19], flow injection analyses [20], gas chromatographic separations [21], ion chromatographic separations [22] and liquid chromatographic separations [23]. These physical/instrumental techniques often show greater precision and tend to give more accurate results than the chemical techniques in the determination of oxalate in biological matrices. However, because of the high concentration of other biological components (ions, salts, proteins) in the samples, many of the later techniques remain laborious; often requiring extraction of oxalate from the sample, oxalate derivatization schemes or other complicated sample clean-up procedures.

Capillary electrophoresis (CE) is a highly efficient and rapid technique for the separation and quantitative analysis of a wide range of charged, and to a lesser extent, uncharged species [24–26]. The technique has been extensively used for the separation of inorganic and organic anions (including oxalate) in pure aqueous samples [27–37]. Most recently, CE has been successfully applied to the specific determination of oxalate in the rich biological matrix of urine [38–40]. CE has also been used to monitor metal ions in parenteral solutions [41].

In the present work, a simple and reliable coelectroosmotic CE method based on indirect UV absorbance detection was developed and applied to the determination of low (mg/l) levels of oxalate in commercial preparations of PN solutions. Initial efforts were focused on the optimization of the analytical conditions, i.e., the type and concentration of the electrolyte, the pH of the electrolyte and the type of charge-reversal reagent on the overall determination of oxalate. The effect of including a chelating agent, such as EDTA, within the sample matrix and within the separation electrolyte, was examined in terms of oxalate signal response. The method allows baseline separation between oxalate and other low and high concentration anions present in the parenteral solutions, and additionally, the determination of oxalate requires no extraction, derivatization or complicated pre-CE sample clean-up steps. The developed method was used to determine oxalate in five specific PN preparations that had been submitted to 24-h neonatal phototherapy.

2. Experimental

2.1. Instrumentation and equipment

Initial method development was conducted with a GTI/SpectroVision (Groton Technology, Concord, MA, USA) modular CE system with the electrodes in the reversed configuration (sample injection at negative electrode, detection at positive electrode). The system consisted of an AD-200 variable-wavelength absorbance detector, a DA-30 high-voltage power supply and a DS-5 sample injection unit. Between

injections, the capillary was rinsed using a Beckman solvent delivery module 112 HPLC pump (Beckman Instruments, Palo Alto, CA, USA). All later experiments, method optimizations and final data collection were performed on a Beckman P/ACE 2100 CE system (Beckman Instruments, Fullerton, CA, USA) with the electrodes also in the reversed configuration. The system was equipped with an automatic sample changer, a multi-wavelength UV detector and temperature-controlled capillary cartridges. The detector time constant was set at 0.1 s and the data collection rate was 10 points/s. A Hewlett-Packard 386 microcomputer (Hewlett-Packard, Palo Alto, CA, USA) provided system control. For both CE systems, indirect UV absorbance detection was accomplished with a deuterium lamp and a 254-nm optical filter. Electropherographic data was collected and integrated with a Spectra-Physics 4270 integrator (Spectra-Physics, San Jose, CA, USA). A Corning 125 pH meter (Corning, Medfield, MA, USA) was used to check and adjust the pH of samples, standards and electrolytes.

Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75-μm I.D.×375-μm O.D. were used. The effective length (length to the detector, L_d) of all capillaries was 60 cm. Polypropylene (Nalgene) volumetric flasks (100 ml) (Nalge, Rochester, NY, USA) were used to make all oxalate calibration standards. Polyethylene sample vials (0.5 and 1.5 ml) with screw caps (Bio-Rad, Richmond, CA, USA) were used with the GTI/SpectroVision CE system while 4.0-ml borosilicate glass sample vials with 0.5-ml polyethylene sample inserts (Beckman Instruments, Fullerton, CA, USA) were used with the Beckman CE system. Disposable polyethylene transfer pipettes (Bio-Rad Labs., Hercules, CA, USA) were used to fill all sample vials. Nylon membrane filters (0.45 μm) (Nalge) were used to filter all electrolytes.

2.2. Reagents

All chemicals were of analytical-reagent grade unless otherwise noted. Cetyltrimethyl-ammonium bromide (CTAB), tetradecyltrimethlyammonium bromide (TTAB) and sodium chromate were purchased from Aldrich (Milwaukee, WI, USA). Potassium hydrogenphthalate, potassium phosphate, sodium acetate, sodium bisulfite, sodium chloride, sodium hydroxide, sodium metabisulfite, sodium sulfate, L-ascorbic acid, disodium ethylenediaminetetracetate (EDTA) dihydrate and oxalic acid dihydrate were purchased from Sigma (St. Louis, MO, USA). Mesityl oxide, methanol (HPLC grade) and sulfuric acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Purified water (18 MΩ), prepared using a Millipore Milli-Q purification system, (Milipore, Bedford, MA, USA) was used to prepare all solutions.

2.3. Preparation of capillaries

The capillaries for both the GTI/SpectroVision and the Beckman CE systems were cut to standard lengths. For the former, the total length (L_t) of the capillary was 100 cm and the length to the detector (L_d) was 60 cm. For the latter system, the capillaries were cut to the dimensions of L_t=67 cm and L_d=60 cm and inserted into Beckman capillary cartridges. The detection window of all capillaries was prepared by burning off a 1-cm section of the polyimide outer coating of the capillary. Methanol was used to wipe the window section clean.

For the GTI/SpectroVision CE work, capillaries were rinsed using the Beckman HPLC pump. All new capillaries were initially conditioned by rinsing 0.1 *M* NaOH (collected 500 μl) through the capillary, followed by distilled water (collected 500 μl), and finally, the appropriate running electrolyte (collected 1000 μl). Between each injection, the capillaries were rinsed with fresh electrolyte solution (collected 100 μl). The collection of 100 μl of electrolyte was sufficient to guarantee that the contents of the previous injection were eliminated and that the capillary was filled with fresh electrolyte. When changing to different electrolyte solutions, the capillaries were rinsed with 0.1 *M* NaOH (collected 100 μl), followed by distilled water (collected 100 μl), and finally, the new electrolyte solution (collected 200 μl).

For the Beckman CE work, all new capillaries were initially conditioned by rinsing 0.1 *M* NaOH (5 min) through the capillary, followed by distilled water (10 min) and finally the appropriate running electrolyte (10 min). Between each injection, the capillaries were rinsed with fresh electrolyte solution

(2 min). Rinsing for 2 min was sufficient to guarantee that the contents of the previous injection were eliminated and that the capillary was filled with fresh electrolyte. When changing to different electrolyte solutions, the capillaries were rinsed with 0.1 *M* NaOH (2 min), followed by distilled water (10 min), and finally, the new electrolyte solution (10 min).

2.4. Preparation of electrolytes

2.4.1. Stock solutions

Stock solutions of 100 m*M* sodium chromate (Na_2CrO_4), 100 m*M* potassium hydrogenphthalate ($KC_8H_5O_4$), 20 m*M* TTAB, 20 m*M* CTAB and 100 m*M* disodium EDTA were prepared using Milli-Q water and vacuum filtered through 0.45-μm nylon membrane filters.

2.4.2. Chromate electrolyte

To a 500-ml glass volumetric flask, 250 ml of Milli-Q water was added, followed by 50 ml of the 100 m*M* sodium chromate stock solution. Next, 12.5 ml of either 20 m*M* CTAB or 20 m*M* TTAB was added to the flask, followed by 500 μl of 100 m*M* EDTA. The contents were mixed well and then the flask was filled to within approximately 5 ml of the mark with Milli-Q water. The pH of the electrolyte solution was checked and adjusted as necessary to pH 8.0 with dilute solutions of sodium hydroxide or sulfuric acid, as needed. The volumetric flask was then filled completely to the mark with Milli-Q water. The final makeup of the chromate electrolyte solution consisted of 10 m*M* Na_2CrO_4, 0.5 m*M* cationic surfactant (CTAB or TTAB) and 0.1 m*M* EDTA. Fresh chromate electrolyte was prepared weekly or as needed.

2.4.3. Phthalate electrolyte

To a 500-ml glass volumetric flask, 250 ml of Milli-Q water was added, followed by 50 ml of the 100 m*M* potassium hydrogen phthalate stock solution and 12.5 ml of either 20 m*M* CTAB or 20 m*M* TTAB. The contents of the flask were mixed well and then the flask was filled to within approximately 5 ml of the mark with Milli-Q water. The pH of the electrolyte solution was checked and adjusted as necessary to pH 6.0 with dilute solutions of sodium hydroxide or sulfuric acid. The volumetric flask was then filled completely to the mark with Milli-Q water. The final makeup of the phthalate electrolyte solution consisted of 10 m*M* $KC_8H_5O_4$ and 0.5 m*M* cationic surfactant (CTAB or TTAB). Fresh phthalate electrolyte was prepared weekly or as needed.

2.5. Preparation of oxalate calibration standards

Oxalate standard solutions were prepared individually in 100-ml Nalgene volumetric flasks by diluting the appropriate amount of 100 m*M* EDTA and either a 1000 or a 10 000 mg/l oxalate stock solution. All calibration standards were prepared in Milli-Q water immediately prior to CE analysis using Eppendorf pipettes and disposable pipette tips. Calibration standards were analyzed in triplicate.

2.6. Preparation of recovery samples

One in-house and three commercially available stock PN solutions (Individual Additives, MVI Pediatric MultiVitamin Infusion, Trace Elements Injection and TrophAmine) were compounded to formulate the five PN samples used in the study. The complete combination of all four stock PN solutions is the total parenteral nutrition (TPN) solution. Table 1 shows how the five samples in the study were prepared from the individual stock PN solutions. Solution 1 (Individual Additives) was composed of various inorganic salts and dextrose. Solution 2

Table 1
Parenteral nutrition solution samples submitted to 24-h phototherapy

Sample	PN Solution	Solution identities
1	2	MVI
2	2+3	MVI+Trace Elements
3	2+4	MVI+TrophAmine
4	2+3+4	MVI+Trace Elements+TrophAmine
5	1+2+3+4 (TPN)	Additives+MVI+Trace Elements+TrophAmine

(MVI Pediatric MultiVitamin Infusion) was composed of oil and water soluble vitamins. Solution 3 (Trace Elements Injection) consisted of the chloride salts of trace metals. Solution 4 (TrophAmine) was composed of amino acids. The most important nutrition solution for the study was solution 2 (MVI Pediatric), since it contained the direct oxalate precursor, ascorbic acid. MVI is the name commonly given to any one of several commercially available PN solutions which acts as a daily multivitamin supplement when diluted to the proper dosage level. Specifically, MVI consists of 13 vitamins, 1 complex sugar, 2 emulsifiers and 2 antioxidants. All research was carried out in reference to solution 2 and specific combinations of solution 2 with the other three PN solutions.

In order to determine oxalate recovery values, samples of the MVI Pediatric solution and the TrophAmine solution were spiked with known amounts of oxalate using concentrated oxalate stock solutions. Additionally, samples of the TPN solution were spiked with oxalate. Recovered amounts of oxalate (in terms of total percent recovery) were calculated based on external standard calibration peak areas.

2.7. Preparation of phototherapy samples

A major proportion of premature infants undergo 24-h phototherapy treatment. Most of these infants obtain their nourishment through a predetermined regimen of PN infusions. Phototherapy has been shown to be an effective means of reducing serum bilirubin levels to safe levels in normal and premature infants [42]. Premature infants are much more susceptible to hyperbilirubinemia and its associated problems [42] than normal infants. The main problem that arises from hyperbilirubinemia is the development of kernicterus (brain damage) [42] due to excess, unconjugated bilirubin entering the brain. By the action of phototherapy at 460 nm, unconjugated serum bilirubin can be isomerized [43] to a more hydrophilic molecule. This hydrophilic "photobilirubin" molecule [42] can then be directly excreted in the bile. The overall effect is to cause the serum bilirubin level to fall to safe levels. In order to simulate the hospital conditions for the in vitro formation of oxalate from the in vitro oxidation of ascorbate, the PN and TPN solutions were submitted to 24-h neonatal phototherapy conditions. The manner in which this was done was designed to simulate the actual intravenous infusion of PN and TPN solutions into premature infants.

The phototherapy samples used in this study were prepared at the Baystate Medical Center (Springfield, MA, USA). All samples were mixed using a Micromix Compounder (Baxter Healthcare, Deerfield, IL, USA). The general procedure was to prepare the experimental PN solution in a 250-ml Clintec All-in-One polyvinylchloride bag (Baxter Healthcare). The bag was allowed to hang on a stand directly beside an Isolette Infant Incubator (Healthdyne, Hatboro, PA, USA). The temperature inside the incubator was held constant at 30.2°C. A straight length (335 cm× 12 mm I.D.) of intravenous tubing (Baxter Healthcare) ran from the bag into the infant incubator. A part of the tubing (76 cm) was allowed to lay inside the incubator, where it was exposed to the phototherapy lights. The free end of the tubing exited the incubator through a sealed opening in the incubator siding. The open end of the tubing was allowed to drip into a closed plastic container throughout the duration of the phototherapy procedure. The phototherapy lights (2 blue and 2 white) were inside of the incubator. The blue lights were Philips Westinghouse lamps F20T 12BB Special Blue 20 W lights. The white lights were Vitalite Test 20 W lights. The phototherapy system was an AirShields Fluoro-Lite Phototherapy Unit (Narco Scientific, Hatboro, PA, USA). The wavelength of the emitted radiation was approximately 460 nm. The radiant flux of the lights was 3 $\mu W\ cm^{-2}\ nm^{-1}$ measured at a distance 18 in. (1 in.=2.54 cm) with an AirShields PR phototherapy radiometer (Narco Scientific). The PN solution inside the bag was pumped (5 ml h^{-1}) through the length of tubing by the action of a peristaltic pump (Travenol FLO-GARD). Samples were collected from the free end of the tubing at seven set time points during the 24-h procedure. However, the initial sample (0 h sample) was collected directly from the bag before the phototherapy procedure began. The samples (1 ml) were collected directly into 1.5-ml polyethylene sample vials. Each sample was immediately sealed and frozen at −20°C. Prior to CE analysis, all PN and TPN sample sets were prepared in an identical manner. Initially, the ex-

perimental sample set was removed from the freezer and allowed to thaw in a laboratory refrigerator at 4°C. All preparations were carried out with Eppendorf pipettes. From each 1.5-ml sample vial, a 495-μl aliquot of sample was removed and transferred to a new 1.5-ml sample vial. Exactly 5 μl of 100 m*M* EDTA was added to the vial and the vial was sealed and shaken (the concentration of EDTA in each sample was 0.1 m*M*. Each sample vial was then loaded onto the Beckman autosampler, with oxalate calibration standards spaced between each sample. All samples were injected 3 times for quantitative purposes.

2.8. Final optimized CE method

An uncoated fused-silica capillary with L_t=67 cm, L_d=60 cm, I.D.=75 μm was used for the PN phototherapy sample analyses. Indirect UV absorbance detection of oxalate was accomplished at 254 nm using an electrolyte that contained 10 m*M* chromate–0.5 m*M* TTAB–0.1 m*M* EDTA, pH 8.0. Initially, samples were injected electrokinetically, but in the final optimized method, all samples were injected by pressure for 10 s and electrophoresis was carried out at −15 kV for 7 min.

3. Results and discussion

3.1. Indirect UV absorbance detection

Prior to the application of CE to the determination of oxalate in the parenteral solutions, the parameters affecting the indirect UV absorbance detection and separation of oxalate in simple aqueous samples were examined in order to obtain optimum oxalate response. This type of CE is often referred to as capillary ion electrophoresis (CIE) or (Waters' trade name Capillary ion analysis CIA) [32]. Indirect UV detection is based on the use of a highly absorbing background electrolyte. Some common background electrolytes are benzoate, chromate, phthalate, pyromellitate, salicylate, sorbate, trimellitate, *o*-benzylbenzoate, *p*-hydroxybenzoate, 2-sulfobenzoate and 2,6-naphthalenedicarboxylate [31,33,34,44,45]. Analytes which have negligible or very low UV absorbance, such as inorganic and organic anions, displace molecules of the highly absorbing background electrolyte. When the zones pass through the detection window, there is a vacancy in the otherwise constant background absorbance of the electrolyte which is recorded as an analytical signal. For the indirect detection of anions in CE, several important criteria must be met initially [26]. First, the molar absorptivity (ε) of the background electrolyte needs to be substantial in order to provide a strong and constant background absorbance. Secondly, the dynamic reserve (D_r) of the background electrolyte, expressed mathematically as the ratio of the background signal to the noise level in the signal needs to be large in order to allow for a detectable signal. Thirdly, the concentration of the background electrolyte has to be optimized in order to guarantee the sensitive detection of both low level and high level analytes. The electrolyte concentration has to be high enough to allow for a sufficiently large dynamic range, but it should not be so high that it surpasses the linear range of the detector. Fourthly, the displacement ratio (R) of the analytes to the electrolyte should approximate unity. The displacement ratio is the charge balance for the spatial displacement of one molecule of electrolyte with one molecule of analyte of identical charge. For optimum electrophoretic stability, the displacement ratio should be close to 1:1. Finally, depending upon the particular analytes that are being determined, the electrophoretic mobility of the electrolyte has to match or be very close to the electrophoretic mobility of the analytes to preclude peak tailing or peak fronting.

3.2. Optimization of separation electrolyte

In the indirect determination of non-absorbing analytes like oxalate, a background electrolyte was needed that had a relatively large molar absorptivity to ensure the sensitive detection of the oxalate anion, and at the same time, the electrolyte needed to be similar in electrophoretic mobility to the oxalate anion to provide adequate peak symmetry. Both a potassium hydrogenphthalate ($KHC_8H_4O_4$) based electrolyte and a sodium chromate (Na_2CrO_4) based electrolyte were tested since both of these compounds have relatively high molar absorptivities at

254 nm (phthalate=1357 L mol^{-1} cm^{-1} and chromate=3180 L mol^{-1} cm^{-1}) [33]. Aqueous standards of 10 mg/l oxalate were injected electrokinetically (−5 kV for 10 s) and analyzed (−15 kV for 10 min) using either 10 m*M* phthalate–0.5 m*M* CTAB, pH 6.0 or 10 m*M* chromate–0.5 m*M* CTAB, pH 8.5. Detection was at 254 nm using an uncoated fused-silica capillary with L_t=100 cm, L_d=60 cm and I.D.=75 μm. The average migration time for oxalate in each electrolyte was under 10 min. In the phthalate electrolyte, the oxalate peak was very broad with severe fronting, while in the chromate electrolyte, the oxalate peak was sharp and did not exhibit any fronting or tailing. All subsequent work was done with the chromate electrolyte.

Next, the concentration of the chromate electrolyte was optimized to give the lowest detection limits for the oxalate anion without sacrificing the linear response of the UV detector. The minimum limit of detection of an analyte using indirect UV detection in CE can be calculated by Eq. (1) [26]:

$$C_{lim} = \frac{C_{BGE}}{RD_r} \quad (1)$$

where C_{lim} is the minimum limit of detection in terms of concentration for any particular analyte, C_{BGE} is the concentration of the background electrolyte, R is the displacement ratio for the analyte and the background electrolyte and D_r is the dynamic reserve of the electrolyte. As C_{lim} is directly proportionally to C_{BGE}, it was important that the concentration of chromate used for the detection of oxalate was at a high enough level to permit low detection limits to be attained. Two different concentrations of chromate were tested using standard samples of oxalate covering the range between 100 and 1000 μg/l oxalate. Each standard was injected three times electrokinetically and the average peak area response of oxalate was calculated. Oxalate gave a higher and a more reproducible response in 10 m*M* chromate electrolyte than in 15 m*M* chromate electrolyte as measured by peak area counts (Fig. 1). This suggested that the 1:1 theoretical displacement ratio for the chromate ion–oxalate ion did not hold in the 15 m*M* electrolyte. The 10 m*M* chromate electrolyte was chosen as the optimum electrophoresis electrolyte.

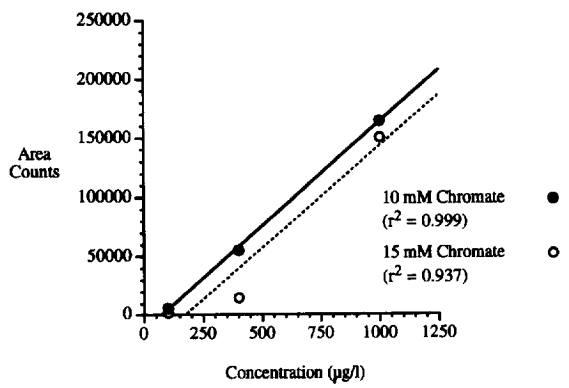

Fig. 1. Comparison of oxalate response in 10 m*M* chromate vs. 15 m*M* chromate electrolyte. Experimental conditions: CE instrument=GTI/SpectroVision; uncoated fused-silica capillary, L_t=100 cm, L_d=60 cm, I.D.=75 μm; electrolyte=10 m*M* or 15 m*M* chromate–0.5 m*M* CTAB, pH 8.5; detection=indirect UV at 254 nm; injection=−5 kV for 30 s, electrophoresis=−15 kV for 10 min.

3.3. Optimization of charge reversal reagent

The fused-silica wall of an uncoated capillary nominally possesses a negative charge due to ionized silanol groups. In this state, the electroosmotic flow (μ_{eof}) in the capillary is in the direction of the cathode (toward the detector). The migration order of the analytes passing in front of the detector is thus cations, followed by neutral molecules (unresolved) and finally anions. However, if a charge reversal reagent, such as a cationic surfactant, is added to the electrolyte below the critical micelle concentration (CMC), it can act to neutralize the negative charge on the capillary wall and, at the same time, dynamically reverse the direction of the electroosmotic flow in the capillary. The electroosmotic flow is now in the direction towards the anode (away from the detector) and the order of ionic migration is also reversed. To carry out electrophoresis, the polarity of the power supply must be reversed to allow analytes to pass in front of the detector. The new order of analyte migration is anions, followed by neutral molecules (unresolved) and finally cations. The reversal of the electroosmotic flow provides for a faster determination of anionic species.

The typical cationic surfactants used to reverse the electroosmotic flow are long-chain alkylammonium salts. Two different alkylammonium salts were test-

ed; CTAB ($C_{19}H_{42}BrN$) and TTAB ($C_{17}H_{38}BrN$). A standard solution of 10 mg/l oxalate was analyzed in 10 m*M* chromate electrolyte with either 0.5 m*M* CTAB or 0.5 m*M* TTAB added to the electrolyte as the charge reversal reagent. The CE experimental conditions were the same as in Fig. 1. The results indicated that there were no basic differences between the migration behavior of the oxalate anion in the presence of either cationic surfactant. In both TTAB and CTAB, the oxalate migration time was approximately 7 min, but it was noted that the electrolyte solutions prepared with CTAB did tend to become cloudy over time. To avoid unpredictable behavior with the subsequent determination of oxalate in the PN solutions, it was decided that TTAB would be a more stable charge reversal reagent to use with the chromate electrolyte.

3.4. Optimization of the electrolyte pH

It was imperative that oxalic acid not exist in its un-ionized state in the separation electrolyte. In aqueous solutions in which the pH is higher than or equal to the second pK_a of oxalic acid (pH≥4.19) [46], oxalic acid should be completely ionized and exist mostly as the di-anion ($C_2O_4^{2-}$) in solution. These oxalate anions would migrate through the capillary at a velocity that would be proportional to their charge to mass ratios. Further, as doubly charged anions, the oxalate anions would be able to displace equivalent amounts of doubly charged chromate anions in the electrolyte solution on a 1:1 basis, fulfilling a requirement for optimum indirect UV detection. This would lead to Gaussian peak shapes and to better detection sensitivity. It was necessary to optimize the pH of the chromate electrolyte for the most sensitive determination of oxalate, adjusting the predominance of chromate ion (CrO_4^{2-}) or dichromate ion ($Cr_2O_7^{2-}$) form in solution. In actuality, both ion forms exist in solution in a pH-dependent equilibrium. Eq. (2) illustrates this equilibrium.

$$2CrO_4^{2-} + 2H^+ \leftrightarrow Cr_2O_7^{2-} + H_2O \quad (2)$$

The pK_a values of chromic acid (H_2CrO_4), pK_1 = 0.74 and pK_2 = 6.49 at 25°C [46] indicate that the stable chromate di-anion (CrO_4^{2-}) will predominate in solution when the pH is above 6.49. Since this pH value is higher than the pH value required for the oxalate ion to exist in its di-anionic form (pH 4.19) in solution, the chromate electrolyte system should be ideal for the determination of oxalate in aqueous samples.

Stock solutions of 10 m*M* chromate electrolyte were titrated to three different basic pH values (pH 7.5, pH 8.0 and pH 8.5) using dilute solutions of sodium hydroxide or sulfuric acid as needed. Aqueous standards of 10 mg/l oxalate were injected electrokinetically and measured in each of the three chromate electrolytes. The CE experimental conditions were the same as previously described, except that the electrolyte contained TTAB instead of CTAB. The results indicated that the pH 8.0 chromate electrolyte was optimum. The average migration time (min) and the average signal response (area counts) for oxalate in each electrolyte are shown in Table 2. As the pH of the chromate electrolyte was increased, the migration time of the oxalate peak decreased, i.e., the migration velocity of the oxalate ion increased due to the increase in the electroosmotic flow in the capillary. Both the pH 8.0 and the pH 8.5 electrolytes gave acceptable migra-

Table 2
Results from the pH optimization of the chromate electrolyte[a]

Parameter	Chromate (pH 7.5)	Chromate (pH 8.0)	Chromate (pH 8.5)
Migration time[b] (average±%R.S.D.)	10.01±5.9	8.25±1.5	7.39±1.8
Area Counts[c] (average±%R.S.D.)	61 718±5.2	71 547±8.5	53 827±10.7

[a] Electrolyte was 10 m*M* chromate–0.5 m*M* TTAB, variable pH. Injection = −5 kV for 10 s; electrophoresis = −15 kV for 10 min; detection = indirect at 254 nm. Capillary = uncoated fused-silica, L_t = 100 cm, L_d = 60 cm, I.D. = 75 μm.
[b] Migration time in min represents the average of three injections.
[c] Area counts represent the average of three injections. Work was conducted on the GTI/SpectroVision CE.

tion times and Gaussian peak shapes for oxalate, but the pH 7.5 electrolyte gave a long migration time and a tailing peak for oxalate.

The pH 8.0 electrolyte gave the best overall signal response for oxalate in respect to peak area counts. The high electroosmotic flow velocity of the pH 8.5 electrolyte dramatically reduced the residence time of the oxalate anion zone within the UV detector window, thereby producing the lowest oxalate peak area counts of the three electrolytes tested. Because the pH 8.0 electrolyte performed equally well in terms of the migration time, the peak shape and the overall area count response for oxalate in the aqueous standards, this pH was adopted as the standard electrolyte pH.

3.5. Mode of injection

PN solutions contain many negatively charged species of differing concentrations, such as chloride, acetate, phosphate, sulfate and metabisulfite ions. When the electrokinetic mode of injection is used with coelectroosmotic CE, high mobility ions such as chloride are preferentially injected into the capillary, to the partial or total exclusion of the lower mobility ions [35,47,48]. Experiments showed that the oxalate response (area counts) in the presence of chloride was significantly less than the response for an equal concentration of oxalate injected from pure standards. This was due to a combination of various factors, the most important being the higher electrophoretic mobility (μ_e) of the chloride ion and the larger contribution of the chloride ion to the overall conductivity of the sample. The electrophoretic mobility of a given ion is directly proportional to its ionic equivalent conductance. The ionic equivalent conductances for chloride and oxalate are $7.63 \cdot 10^{-3}$ and $7.41 \cdot 10^{-3}$ S m^2 mol^{-1}, respectively, at 25°C [30]. The chloride ionic equivalent conductance is larger, and subsequently, the chloride ion has a higher electrophoretic mobility during injection, i.e., chloride migrates more rapidly than oxalate into the capillary.

In order to prevent analyte injection bias, the mode of sample injection was changed from electrokinetic injections to pressure injections. Even though extremely low limits of detection (low μg/l) for ionic analytes can be attained by electrokinetic injection, biasing makes it difficult to quantitate samples accurately. With pressure injection, a completely representative portion of the sample is introduced into the capillary. The inability to attain extremely low limits of detection for ionic analytes using pressure injections is balanced by the capability of achieving unbiased sample introduction. The injection time period was optimized to 10 s on the Beckman CE system. In order to provide quicker analysis, the total length of the capillary (L_t) was decreased from 100 cm to 67 cm. This reduced the average migration time of oxalate from 7.0 min to 4.0 min.

3.6. Effect of EDTA

It was found that the addition of 1 m*M* EDTA to aqueous oxalate standards significantly increased the relative precision of the measured oxalate response. Experiments were conducted with 10 mg/l standards of oxalate. Standards were prepared with and without added EDTA and analyzed for oxalate using the optimized pressure injection procedure. The results showed, for replicate injections, that the oxalate standards prepared with 1 m*M* EDTA exhibit almost a two-fold improvement in response precision (3.3% R.S.D. vs. 6.3% R.S.D.) over oxalate standards prepared without 1 m*M* EDTA (see Table 3).

Table 3
Comparison of signal response precision of oxalate standards with and without added EDTA ($n=15$)

Standard	Area counts±95% CI[a]	Area counts (%R.S.D.)
10 ppm Oxalate	51 524±1797	6.3
10 ppm Oxalate–1 m*M* EDTA	49 288±908	3.3

Electrolyte was 10 m*M* chromate–0.5 m*M* TTAB, pH 8.0. Injection = pressure for 10 s; electrophoresis = −15 kV for 10 min; detection = indirect at 254 nm. Capillary = uncoated fused-silica, $L_t = 67$ cm, $L_d = 60$ cm, I.D. = 75 μm. Work was conducted on the Beckman P/ACE 2100 CE.

[a] 95% CI = confidence interval of the mean response at the 95% confidence level.

Confidence intervals (CI) of the mean responses calculated at the 95% confidence level indicate that both standards give the same overall response in terms of peak area counts. Hence, the addition of EDTA to the standards does not create a positive or a negative bias towards the absolute oxalate response, but the addition does enhance the oxalate measurement precision. A suggested reason for the increased response precision for the oxalate–EDTA standards may be related to the better trace metal chelating ability of EDTA versus oxalate in aqueous solutions. It is probable that the sodium chromate (Na_2CrO_4) electrolyte (98% pure analytical reagent) contains trace metal (cationic) impurities. Organic acids can form weak chelates with metal impurities in solution which tends to decrease the acids' overall charge and charge density [49]. A reduction in charge density also reduces the relative electrophoretic mobilities of the organic acids. There exists a rapid, yet variable, ionic equilibrium between the complexed form of the acid and the free form of the acid which is dependent upon the concentration of the metal impurities in the solution. This variable equilibrium between the complexed acid and the free acid forms can affect the measured signal response precision because the quantity of electrolyte ions displaced by the analyte ions (during indirect UV detection) is proportional to the concentration and to the total charge density of the analyte ions migrating within the electrophoretic zone. If the total charge density of the ion zone is slightly variable, then the amount of electrolyte ions displaced will be slightly variable which will tend to reduce the signal response precision. Another factor contributing towards signal response imprecision may involve the differences in the relative electrophoretic mobilities of the complexed and the free acid forms. However, when oxalate and EDTA are introduced into the capillary together during the pressure injection procedure, both the oxalate ions and the EDTA ions can interact with and form complexes with the metal impurities in the electrolyte. The probability of stronger and more stable complex formations between EDTA and the metal impurities is higher than the probability of stable complex formations between oxalate and the metal impurities because EDTA is a much stronger chelator than oxalate, especially at the alkaline pH value (pH 8.0) of the chromate electrolyte. Thus, the addition of EDTA to the standard–sample solution acts to prevent the formation of equilibrium organic acid forms with variable charge densities and electrophoretic mobilities. The precision of the measured oxalate response is increased because the displacement of the chromate electrolyte ions is dominated by the uncomplexed oxalate ions migrating as an ion zone of uniform charge density and constant electrophoretic mobility, and not as a mixture of uncomplexed and complexed acid ions. To try to keep the analytical parameters identical between the CE analyses of the oxalate standards and the CE analyses of the PN solutions, a 1 m*M* spike of EDTA was also added to each of the PN phototherapy samples prior to analysis.

The incorporation of EDTA within the chromate electrolyte itself would further serve to prevent trace metal impurities from complexing with oxalate or from catalyzing the arbitrary oxidation of ascorbate in the PN solution samples. These metals could originate from a number of possible sources including, but not limited to, metals that are natural components of the PN samples, metal leachates from the walls of sample containers or metals that are present as impurities in the water supply. EDTA acts as a chelating agent to trap the trace metals which prevents them from oxidizing ascorbate to oxalate.

Experiments were performed to determine the effect of adding EDTA directly to the chromate electrolyte. Controlled concentrations of EDTA (0, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 m*M*) were incorporated into the chromate electrolyte. Experiments were carried out using 1, 2 and 5 mg/l oxalate–1 m*M* EDTA standards under the CE conditions described in Table 3. The addition of EDTA concentrations higher than 0.1 m*M* to the chromate electrolyte had a number of detrimental effects; the baseline became extremely unstable and noisy, the migration time of oxalate steadily increased (4.3 min to 5.1 min), the peak area response for oxalate became more irreproducible and the separation current steadily increased (29.5 μA to 39.2 μA). Accordingly, 0.1 m*M* EDTA was incorporated as a regular component of the chromate electrolyte.

3.7. Calibration and dynamic range

The calibration linearity and dynamic range were

studied. It was determined, based upon spiking and recovery experiments, that external standard calibration graphs could be prepared and used for the quantitation of oxalate in the various phototherapy samples. Individual calibration curves were prepared from a set of seven oxalate–EDTA standards: 0, 1, 2, 4, 10, 20 and 40 mg/l oxalate–1 m*M* EDTA. The response for oxalate showed good linearity over the range 1–40 mg/l oxalate with a corresponding regression coefficient (r^2) equal to 0.999 ($n=3$). The linearity of the oxalate response extended over 1.5 orders of magnitude which was sufficient for the determination of oxalate in the phototherapy samples.

The linear dynamic range was also determined. The linear dynamic range for oxalate extends over the range 1–400 mg/l oxalate with a corresponding regression coefficient (r^2) equal to 1.000 ($n=3$) The dynamic range of the oxalate response shows linearity over more than 2.5 orders of magnitude. The average migration time of oxalate under the conditions of the method was 4.10±0.02 min (0.02 equals the standard deviation).

3.8. Limit of detection

The method limit of detection (LOD) for oxalate was determined. Based upon the response for a sample blank (1 m*M* EDTA), the LOD for oxalate was determined to be 240 μg/l ($S/N=3$).

3.9. Analytical precision

The within-run and between-run signal response (area counts) precision and migration time (min) precision for the determination of oxalate were evaluated. The data given in Table 4 represents the results from replicate analyses of a 10 mg/l oxalate–EDTA standard injected on three different occasions. High precision within and between the three different sample sets is evident for both the oxalate response and the oxalate migration time.

3.10. Relative recovery

Standard calibration curves were used to calculate the relative recovery of oxalate from the PN solutions. Oxalate recovery experiments were performed on select PN and TPN solution standards to determine the feasibility of using external standard calibration graphs to measure oxalate in the actual PN and TPN phototherapy samples. It was concluded that oxalate could be quantitated in each of the five phototherapy samples using external standard calibration graphs based upon the quantitative area count recovery results from spiked PN and TPN solution standards (see Table 5). Each study was conducted with a new uncoated capillary using the CE conditions given in Table 5.

3.11. Determination of oxalate in phototherapy samples

The developed method was applied to the determination of oxalate in the PN and TPN solution samples (Table 1) submitted to 24-h neonatal phototherapy. The phototherapy samples were analyzed for oxalate using the CE conditions used for the analysis of the recovery samples. The mobility of the electroosmotic flow in a typical capillary was determined by measuring the migration time for 0.1% mesityl oxide, a neutral compound which migrates at

Table 4
Precision and reproducibility data ($n=5$)

Set	Area counts (counts±95% CI)[a]	Area counts (%R.S.D.)	Migration time (min±95% CI)[a]	Migration time (%R.S.D.)
1	49 491±2365	3.8	4.11±0.02	0.30
2	49 158±2526	4.1	4.09±0.02	0.41
3	49 216±1557	2.5	4.11±0.02	0.32

Electrolyte was 10 m*M* chromate–0.5 m*M* TTAB–0.1 m*M* EDTA, pH 8.0. Injection=pressure for 10 s; electrophoresis=−15 kV for 7 min; detection=indirect at 254 nm. Capillary=uncoated fused-silica, $L_t=67$ cm, $L_d=60$ cm, I.D.=75 μm. Work was conducted on the Beckman P/ACE 2100 CE.

[a] 95% CI=confidence interval of the mean response or migration time at the 95% confidence level.

Table 5
Relative recovery values for oxalate in PN and TPN solutions

PN solution	Solution identities	Recovery range (mg/l)	Recovery (%) (average±S.D.)[a]
2	MVI	2–20	98±6
4	TrophAmine	2–20	100±4
2+4	MVI+TrophAmine	2–20	98±2
1+2+3+4	TPN[b]	5–40	97±4[c]

Electrolyte was 10 m*M* chromate–0.5 m*M* TTAB–0.1 m*M* EDTA, pH 8.0. Injection = pressure for 10 s; electrophoresis = −15 kV for 7 min; detection = indirect at 254 nm. Capillary = uncoated fused-silica, L_t = 67 cm, L_d = 60 cm, I.D. = 75 μm.
[a] n = 4; S.D. = Standard deviation.
[b] The TPN recovery solution was formulated from an Invidual Additives PN solution that did not contain supplementary chloride or sulfate.
[c] Injection pressure for the TPN samples was reduced to 5 s, all other experimental conditions remained the same.
Work was conducted on the Beckman P/ACE 2100 CE.

the same velocity as the electroosmotic flow. The experimental mobility of the electroosmotic flow in the capillary was calculated to be $-2.19 \cdot 10^{-4} \pm 1.36 \cdot 10^{-6}$ cm^2 V^{-1} s^{-1} (average±S.D.). The R.S.D. of the determinations was 0.62% (n = 3).

A representative electropherogram of a typical MVI/Trace Elements/TrophAmine phototherapy sample is shown in Fig. 2. Peak identities were confirmed by co-injection of standards. The oxalate ion (peak 3) migrates at 4.0 min and is well resolved from the sulfate ion (peak 2) and the 1 m*M* EDTA spike (peak 4). Over the 24-h phototherapy sampling period, it was noted that the sulfate peak size increased as the metabisulfite (not shown in this electropherogram) in the sample was oxidized to sulfate. Even though the amount of sulfate increased, it did not affect the quantitation of oxalate in the samples. Fig. 3 shows a representative electropherogram of oxalate determination in a TPN phototherapy sample. The quantitation of the oxalate ion is not compromised by the presence of excessively high levels of phosphate (peak 5) and acetate (peak 6) ions in the sample. Both the phosphate and the acetate ions have lower electrophoretic mobilities than the oxalate ions (peak 4), and thus, the phosphate and acetate ions migrate after the oxalate ions. Even though the EDTA spike is present in this sample, the EDTA peak is hidden within the phosphate peak.

A summary of the results from the determination of oxalate in the five PN preparations submitted to 24-h neonatal phototherapy is given in Table 6 which indicates that the direct infusion of oxalate into premature infants on a PN or TPN solution regimen can be quite substantial. The values in the table are based on an infusion of 120 ml of complete solution over a 24-h period. These values are relatively high,

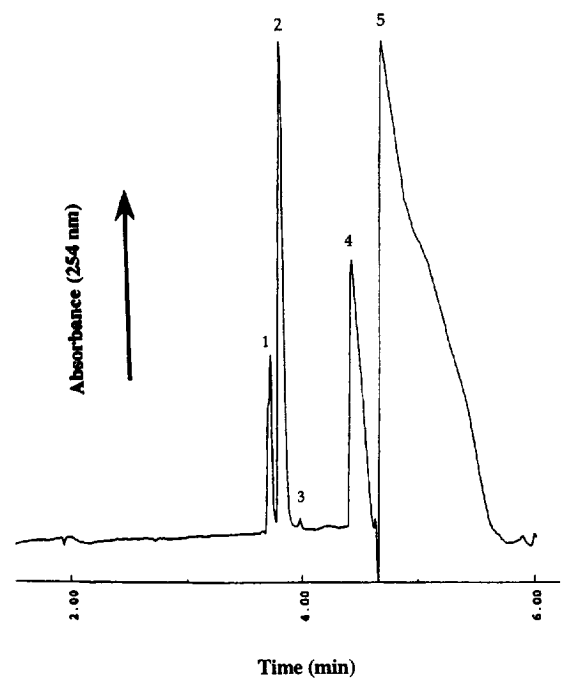

Fig. 2. Representative electropherogram of a typical MVI/Trace Elements/TrophAmine phototherapy sample. Experimental conditions: CE instrument = Beckman P/ACE 2100; uncoated fused-silica capillary, L_t = 67 cm, L_d = 60 cm, I.D. = 75 μm; temperature = 25°C; electrolyte = 10 m*M* chromate–0.5 m*M* TTAB–0.1 m*M* EDTA, pH 8.0; detection = indirect UV at 254 nm; injection = pressure for 10 s, electrophoresis = −15 kV for 7 min. Peak identities: 1 = unknown component and chloride co-migrating; 2 = sulfate; 3 = oxalate (1.6 mg/l); 4 = EDTA (1 m*M*); 5 = acetate (~3739 mg/l).

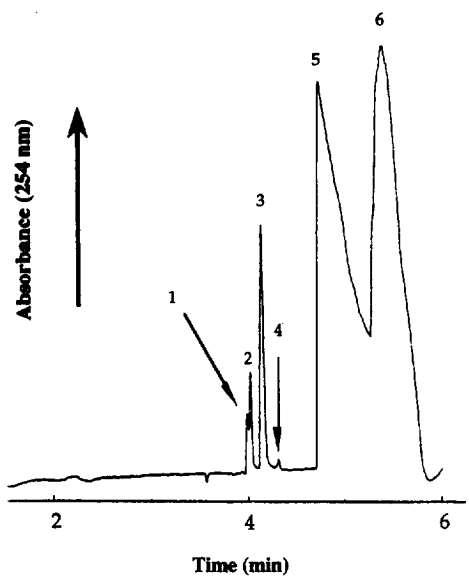

Fig. 3. Representative electropherogram of a typical TPN phototherapy sample. Experimental conditions: CE instrument = Beckman P/ACE 2100; uncoated fused-silica capillary, $L_t = 67$ cm, $L_d = 60$ cm, I.D. = 75 μm; temperature = 25°C; electrolyte = 10 m*M* chromate–0.5 m*M* TTAB–0.1 m*M* EDTA, pH 8.0; detection = indirect UV at 254 nm; injection = pressure for 5 s, electrophoresis = −15 kV for 7 min. Peak identities: 1 = unknown component; 2 = chloride; 3 = sulfate; 4 = oxalate (4.6 mg/l); 5 = phosphate (~1984 mg/l); 6 = acetate (~3739 mg/l).

considering that any amount of oxalate introduced into a premature infant is an unnecessary and extra metabolic burden. A normal infant excretes 24±3 mg/l (average mg/l±standard error of the mean) oxalate per day [9]. This value was used to calculate the percentages shown in the last column of Table 6. It is evident that a significant percentage of the urinary oxalate excreted by the infants can be attributed to the direct introduction of oxalate via parenteral nutrition solutions. An infant on total parenteral nutrition (TPN) has the largest burden, having as much as 29% of its urinary oxalate coming directly from parenteral nutrition. Hence, it is probable that renal oxalate saturation in premature infants occurs more rapidly in infants on parenteral nutrition regimens. Renal oxalate saturation can lead to secondary hyperoxaluria, and in turn, to the clinical condition of nephrocalcinosis.

4. Conclusions

A simple, rapid and reproducible CE method for the quantitative determination of oxalate in parenteral nutrition preparations has been developed. The analytical oxalate response has been optimized in respect to the type and concentration of the back-

Table 6
Summary of the results from the 24-h parenteral nutrition phototherapy study

Solution identities	Oxalate determined[a] (mg/l h±95% CI)	Oxolate infused[b] (mg/day±95% CI)	% of Urinary oxalate excretion[c]
MVI	3.0±0.2	8.6±0.6	12.5±2.4
MVI + Trace Elements	3.6±0.2	10.4±0.6	15.0±2.7
MVI + TrophAmine	2.0±0.1	5.8±0.3	8.3±1.5
MVI + Trace Elements + TrophAmine	1.6±0.1	4.6±0.3	6.7±1.3
TPN[d]	7.0±0.6	20.2±1.7	29.2±6.1

Electrolyte was 10 m*M* chromate–0.5 m*M* TTAB–0.1 m*M* EDTA, pH 8.0. Injection = pressure for 10 s; electrophoresis = −15 kV for 7 min; detection = indirect at 254 nm. Capillary = uncoated fused-silica, $L_t = 67$ cm, $L_d = 60$ cm, I.D. = 75 μm.

[a] The average result is reported based on the analysis of seven independent samples collected from 0–24 h. For each independent sample, $n = 3$. All values calculated via oxalate external standard calibration graphs. Values reported are average mg/l h oxalate±95% confidence limit.

[b] Values reported are mg/day oxalate±95% confidence interval, based on an infusion of 120 ml of solution over a 24-h period.

[c] Values reported are % oxalate±95% confidence interval. Calculated values based on the average amount of oxalate (24 mg/day) excreted from a normal infant over a 24-h period (see Ref. [9]).

[d] The TPN phototherapy sample was formulated from an Invidual Additives PN solution that did not contain supplementary chloride or sulfate. Injection pressure for the TPN samples was reduced to 5 s, all other experimental conditions remained the same.

Work was conducted on the Beckman P/ACE 2100 CE.

ground electrolyte, the pH of the background electrolyte and the type of charge-reversal reagent used for the analysis. This study illustrates the utility of CE as a powerful bioanalytical technique for the separation and measurement of organic acid analytes in biological matrices. The results give support to the establishment of a possible link between the infusion of parenteral nutrition solutions into premature infants and the onset or the advancement of nephrocalcinosis in those infants.

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