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Electrophoretically mediated microanalysis of calcium

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Abstract

Electrophetically mediated microanalysis (EMMA) was used for the determination of calcium. The faster analyte zone containing the calcium was injected spatially behind a slower zone of *o*-cresolphthalein complexone in a capillary electrophoresis based system. Upon application of the electric field the calcium zone was electrophoretically mixed with the reagent and product was formed. The bulk electroosmotic flow carried the product to the detector where the absorbance of the resulting complex at 575 nm was measured.

Quantitation using an internal standard yielded a linear response with an R.S.D. of 8.1%. An inter-method comparison was performed with the standard bulk method and yielded results that did not significantly differ. The advantages of EMMA with respect to traditional methods were addressed.

1. Introduction

Traditionally capillary electrophoresis has been applied to the separation of species on the basis of differences in electrophoretic mobilities as dictated by the chosen electrophoretic medium. However, as we recently described [1], electrophoretically mediated microanalysis (EMMA) utilizes this phenomenon to bring spatially distinct zones of chemical reagents of different mobilities into physical contact in order to perform a reaction-based chemical analysis. Capillary electrophoretic systems, as employed in EMMA, are capable of performing each of the tasks required in reaction based chemical analysis: (1) the metering of the analyte(s) and analytical reagent(s); (2) the mixing of analyte(s) and analytical reagent(s); (3) a time period in which the quantitative analytical reaction is allowed to occur and (4) the detection of one or more species whose production or depletion is indicative of the quantity or concentration of analyte(s) present.

Electrophoretic mixing offers numerous advantages over the mixing of bulk solutions employed in traditional chemical analysis. As species electrophorese essentially independently of the bulk solution, electrophoretic mixing interpenetrates zones with differential mobilities under the application of an electric field without an additive change in volume and, therefore, without mixing-induced dilution of the zones. Additionally, electrophoretic mixing allows for zones to be merged without the need for turbulent flow and the concurrent loss of efficiency experienced in traditional methods of mixing. Further advantages of the EMMA method include the separative power of capillary electrophoretic systems. This capability allows for the development of assays which do not require the

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production or depletion of a species with unique detection properties. EMMA's separative capacity also permits multiple analytes to be determined if the analytes or respective analytical reagents or products possess unique electrophoretic properties in the chosen electrophoretic medium [2]. The small dimensions of capillary electrophoretic systems are a major benefit of the EMMA method as they allow for the microanalysis of nanoliter scale volumes of analyte utilizing sub-microliter scale volumes of analytical reagents.

We have recently reported EMMA methods for the determination of enzymes [1–4] and substrates [1,5]. Liu and Dasgupta [6] demonstrated the applicability of this phenomenon to complexation reactions using a flow-injection analysis (FIA) format. This paper describes an EMMA method for the determination of calcium by reaction with *o*-cresolphthalein complexone. Calcium is one of the many analytes routinely determined in the clinical laboratory. The clinical significance of calcium is vast and has been well documented [7]. Calcium ions decrease neuromuscular excitability, are an active agent in blood coagulation and neurotransmitter release, activate many enzymes, and play an important role in inorganic ion transport across cell membranes [8]. It is, therefore, significant to monitor the calcium levels in serum and urine to detect conditions, such as hypercalcemia and hypocalcemia, which could have serious detrimental effects, such as tetany. Many different methods have been used to determine the calcium concentrations in biological fluids, such as redox titrations [9,10], precipitation reactions [11,12], EDTA titrations [13,14], emission flame photometry [15], atomic absorption spectrophotometry [7], and reaction with *o*-cresolphthalein complexone [16,17]. Of these methods the latter has become the choice for most clinical laboratories due to its simplicity and ease of automation. The reaction of *o*-cresolphthalein and calcium forms a purple complex whose absorbance at 575 nm is indicative of the amount of calcium present. However, the monitoring of calcium levels in interstitial fluids has frequently proven to be difficult using traditional methods due to the micro-sample volumes. Due to its minimal sample requirements, EMMA offers potential for the detection of calcium in such sampling environments.

2. Experimental

2.1. Instrumentation

The assays were carried out using an in-house design. Polyimide-coated, fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D., 360 μm O.D., and 35 cm in total length were uses as the columns. The separation length (distance from anodic injection end to detection window) was 20 cm. Detection was achieved with an ISCO (Lincoln, NE, USA) CV^4 capillary electrophoresis absorbance detector at 575 nm. A Spellman (Plainview, NY, USA) Model FHR 30P 60/EI power supply was utilized to apply the electric field across the capillary. The spectrophotometry experiments were performed with a Spectronic 20D (Milton Roy Company, Niagara Falls, NY, USA) operated at 575 nm.

2.2. Chemicals

The *o*-cresolphthalein complexone reagent and buffer solutions were obtained from Sigma (St. Louis, MO, USA). The analytical reagent solution was prepared by mixing the *o*-cresolphthalein complexone reagent and buffer solutions in a 1:1 ratio. The resulting 250 m*M* 2-amino-2-methyl-1,3-propanediol working buffer at a pH of 9.0 contained the *o*-cresolphthalein complexone at a concentration of 0.189 m*M* and 8-hydroxyquinoline at a concentration of 8.61 m*M* and was allowed to sit for no more than 4 h before being replaced. 8-Hydroxyquinoline is utilized to eliminate interference from magnesium. Calcium chloride standards were prepared by serial dilutions form a 20 m*M* standard purchased from Sigma. The bromophenol blue used as an internal standard was obtained from

Aldrich (Milwaukee, WI, USA). All sample solutions were prepared from in-house double-distilled-deionized water and were degassed to remove interferences from air bubbles.

2.3. Electrophoresis procedures

The capillaries were treated with 1 *M* NaOH for 10 min and then rinsed with buffer solution for 10 min prior to use. The assays were effected by filling the capillary and the buffer reservoirs with the analytical reagent solution and injecting a plug of calcium analyte solution or a plug of pre-reacted complex. In the latter case, the reaction was allowed to go to completion by sitting for three minutes prior to the injection. Hydrodynamic injections were made into the anodic end by siphoning for a fixed time (1–2 s) at a fixed height (10–15 cm). Following injection, a constant potential was applied, and the absorbance at 575 nm was monitored. In order to limit the operating current to 40–60 μA, electric field strengths of 85 to 114 V/cm were applied. All assays were performed without temperature control at ambient temperature.

2.4. Spectrophotometry procedures

The spectrophotometric determination of calcium in samples was performed as described in the manual accompanying the Sigma Diagnostic kit [18].

3. Results and discussion

3.1. EMMA methodology

The *o*-cresolphthalein complexone reaction with calcium is a simple reaction that can be utilized to demonstrate EMMA's ability to perform sensitive chemical analysis with a rapid sample throughput while utilizing small amounts of both analyte and analytical reagent. Electrophoretic mixing requires that the reagents involved in an EMMA reaction, calcium and *o*-cresolphthalein in this assay, differ in electrophoretic mobility so that spatially distinct zones of the reagents will migrate at different rates and thereby merge under the influence of an electric field. In the chosen electrophoretic medium, calcium and *o*-cresolphthalein complexone have a differential mobility of $8.2 \cdot 10^{-4}$ $cm^2/(Vs)$ as indicated by the electrophoretic and mobilities listed in Table 1. This differential mobility allows the two species to be rapidly electrophoretically mixed under the influence of an applied potential.

Since the magnitude of the electrophoretic mobility of the negatively charged *o*-cresolphthalein complexone is less than the magnitude of the electroosmotic flow, the *o*-cresolphthalein complexone has a net apparent mobility in the direction of the cathode. Therefore, both the *o*-cresolphthalein complexone and the positively charged calcium will migrate from anode to cathode. However, since the apparent mobility of the calcium is greater than that of the *o*-

Table 1
Electrophoretic mobilities of chemical species at pH 9

Chemical species	Electrophoretic mobility [$cm^2/(Vs)$]	Apparent electrophoretic mobility [$cm^2/(Vs)$][a]
Calcium	$4.3 \cdot 10^{-4}$	$9.7 \cdot 10^{-4}$
o-Cresolphthalein complexone	$-3.9 \cdot 10^{-4}$	$1.5 \cdot 10^{-4}$
Calcium complex	$-9.1 \cdot 10^{-5}$	$4.5 \cdot 10^{-4}$
Bromophenol blue	$-2.5 \cdot 10^{-4}$	$2.9 \cdot 10^{-4}$

[a] Apparent electrophoretic mobilities were calculated as the sum of the electrophoretic mobility and the electroosmotic flow [$5.4 \cdot 10^{-4}$ $cm^2/(Vs)$].

cresolphthalein complexone, the *o*-cresolphthalein complexone must be introduced into the capillary prior to the injection of the calcium at the anode so that the faster migrating zone of calcium can overtake the slower migrating zone of *o*-cresolphthalein complexone.

The EMMA mode chosen was to initially fill the capillary and the buffer reservoirs with a solution containing the *o*-cresolphthalein complexone buffered at pH 9.0. A plug of solution containing the calcium analyte was then hydrodynamically injected at the anodic end of the capillary, and a potential was applied across the capillary. Under the influence of the electric field, the calcium ions quickly exited their injection environment and penetrated the adjacent zone containing the *o*-cresolphthalein complexone. The time required to completely electrophoretically mix an analyte zone in a broader analytical reagent zone t_{mix} can be estimated as

$$t_{mix} = \frac{w_A}{\Delta\mu_{ep,A-R}E} \quad (1)$$

where w_A is the width of the analyte zone, $\Delta\mu_{ep,A-R}$ is the difference in electrophoretic mobilities between the analyte and analytical reagent zones, and E is the electric field strength. Based upon their differential mobility and a typical experimental electric field strength of 100 V/cm, a 0.5-mm calcium plug was fully merged within the adjacent *o*-cresolphthalein complexone region in approximately 600 ms following the application of the potential.

As the reagent zones were electrophoretically mixed, the calcium and *o*-cresolphthalein complexone reacted to form the product, which was transported toward the cathode. Since the product differed in electrophoretic mobility from the analyte [$\Delta\mu_{ep} = 5.2 \cdot 10^{-4}$ cm^2/(Vs)], as the plug of unreacted calcium traversed the zone of *o*-cresolphthalein complexone, the product formed was electrophoresed away from the vicinity of the unreacted analyte at a rate V_{diff} equal to the product of this differential mobility $\Delta\mu_{ep,P-A}$ and the electric field strength:

$$v_{diff} = \Delta\mu_{ep,P-A}E \quad (2)$$

As a result, if the reaction was not immediate, the product peak would have been skewed with the asymmetry attributable to the kinetics of the reaction. Since the analyte had a greater apparent mobility than the detected product, the first product formed was the last to reach the detector position as it must traverse the greatest portion of the separation distance of the capillary with a lower mobility, that of the product. The last product formed migrates with a higher apparent mobility, that of the analyte, for the greatest period of time and was thus the first product to react the detection position.

At the concentrations of calcium and *o*-cresolphthalein complexone utilized in these experiments, the reaction approached completion very rapidly as indicated by the sharp product peaks obtained. To confirm that the mixing of reagents and the reaction are rapid in the EMMA assay, calcium samples were assayed both by the EMMA method and by reacting the calcium and *o*-cresolphthalein complexone prior to injection. A calcium sample was first injected at the anodic end of the capillary. After application of the potential for approximately 2.9 min a previously reacted calcium *o*-cresolphthalein complexone complex was injected. As illustrated in Fig. 1, indistinguishable product peaks for the two in-

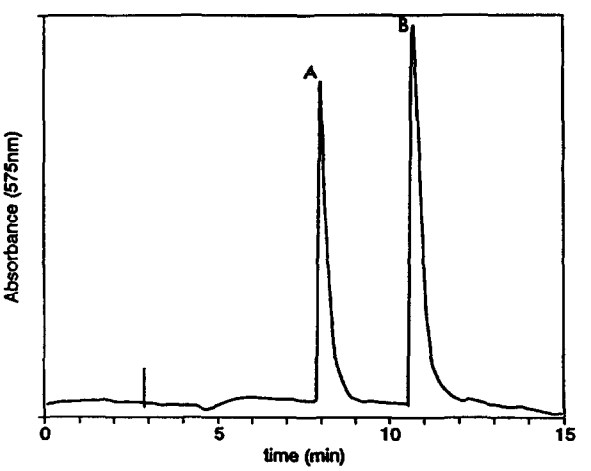

Fig. 1. A determination of a 1 m*M* calcium sample injected at the anode by the EMMA method and a pre-reacted 1 m*M* calcium *o*-cresolphthalein complexone complex injected after the application of potential for 2.9 min; (A) product peak from the EMMA determination and (B) pre-reacted product peak. The applied field strength was 93 V/cm.

jections were obtained in the resulting electropherogram. Identical calculated electrophoretic mobilities indicated that the mixing and reaction processes occurred nearly instantaneously. If the calcium had existed in its more mobile, uncomplexed form for a significant time during the EMMA assay, the migration time of the product would have differed for that formed in the EMMA analysis and that reacted prior to injection. Furthermore, the similarity in peak shapes signifies that any kinetic effects manifested as peak skewness were insignificant relative to the effects of diffusional broadening, thus again indicating that the mixing and reaction processes occurred rapidly. While the immediate nature of the reaction would seemingly allow the use of a narrower plug of analytical reagent, it was decided to use a broad zone of *o*-cresolphthalein complexone so that the analyte and product would remain merged within the analytical reagent zone throughout their migration to the detection position thereby inhibiting dissociation of the product complex.

Since product peak area determines the quantity, rather than concentration, of calcium injected, the poor reproducibility of hydrodynamic injection volumes for capillary electrophoresis required the use of an internal standard as a measure of injection volume for the determination of analyte concentrations by EMMA. The dye bromophenol blue was selected due to its large absorbance at 575 nm as well as its differential mobility [$\Delta\mu_{ep} = 1.59 \cdot 10^{-4}$ $cm^2/(V\ s)$] from the product thereby preventing comigration of the internal standard and product peaks. A typical electropherogram of an EMMA determination of calcium using the internal standard is shown in Fig. 2.

3.2. Linearity and reproducibility of EMMA method

To determine the linearity of the EMMA calcium determination, a calibration curve (Fig. 3) was constructed. The concentration of the internal standard was held constant at 0.75 m*M* while the concentration of the calcium was varied between 0.010 and 1.0 m*M*. Six trials

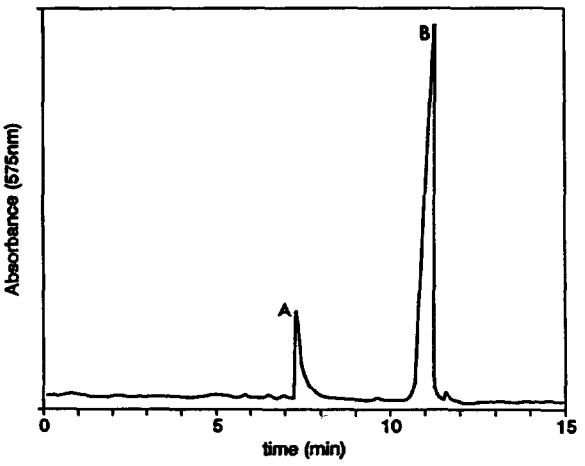

Fig. 2. A typical electropherogram obtained by the EMMA determination of a 0.75 m*M* calcium standard; (A) calcium *o*-cresolphthalein complexone complex (analytical reaction product) and (B) bromophenol blue (internal standard) peaks. The applied field strength was 103 V/cm.

were performed for each of the eight samples. Quantitation was based upon the relative peak areas observed for the complex and the internal standard. The EMMA method proved to be linear over this entire calcium concentration range with a correlation coefficient of 0.9936. The lower limit of detection, based upon a signal-to-noise ratio of 3, was found to be ap-

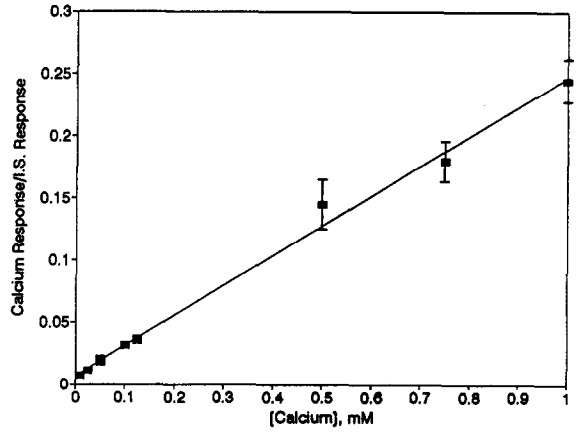

Fig. 3. A calibration curve for the EMMA determination of calcium. Points are the mean of six replicates, and the brackets represent the 95% confidence intervals. The line represents the line regression of 0.01 to 1 m*M* data ($R^2 = 0.9936$). Conditions stated in text. I.S. = internal standard.

proximately $5 \cdot 10^{-3}$ m*M*. Assuming an injection volume of about 2 nl, this detection limit corresponded to the determination of $1 \cdot 10^{-14}$ mol of calcium. This lower limit of detection does not approach that observed in the previously published EMMA determination of enzymes [2–5] due to the non-amplifying nature of the complexatory reaction.

Reproducibility ranged from 6.5 to 10.2% relative standard deviations (R.S.D.) with an average value of 8.1% R.S.D. This R.S.D. is larger than that obtained for the spectrophotometric determination of the samples (average of 3.1%) presumably due to error in the determination of peak areas by the cut and weigh methodology.

3.3. Comparison of EMMA to spectrophotometric determination

To assess the validity of the EMMA method of calcium determination, an inter-method correlation with the Sigma Diagnostic spectrophotometric method was performed. Four calcium samples varying between 0.058 and 0.22 m*M* were assayed by both techniques. Four determinations were made of each sample by each method. Fig. 4 compares the results of the two assays. Least-squares regression yielded y (Sigma) = $0.989x$ (EMMA) − 0.00140 m*M* with a correlation coefficient of 0.9992. A paired Student's t calculation showed that the two methods did not yield significantly different values at a 95% confidence level. The two methods agreed well indicating that the EMMA technique is a viable procedure for the determination of calcium.

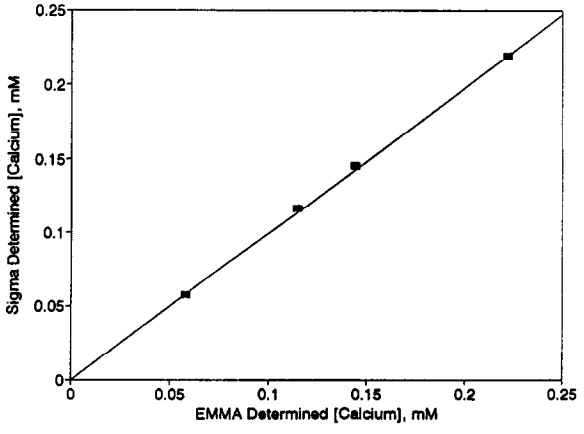

Fig. 4. An inter-method correlation of four calcium determinations by EMMA and Sigma spectrophotometric methods. The line represents the linear regression results (R^2 = 0.9992). Conditions stated in text.

3.4. Advantages of EMMA determination

Although the reproducibility of the Sigma method was superior to the EMMA assay, the EMMA technique holds many advantages over the bulk methodology including its ability to allow the analyte to encounter many times its volume in analytical reagent without concurrent dilution due to electrophoretic mixing. As previously described, any unreacted calcium will continue to traverse the *o*-cresolphthalein complexone region thereby continually encountering unreacted analytical reagent. In the mode described, the volume of analytical reagent encountered relative to the initial volume of analyte, R_{vol} can be estimated as

$$R_{vol} = \frac{\Delta\mu_{ep,A-R} l}{(\mu_{ep,A} + \mu_{eo}) w} \quad (3)$$

where l is the distance from injection to detector, $\mu_{ep,A}$ is the electrophoretic mobility of the analyte, and μ_{eo} is the electroosmotic flow. Based upon the differential mobility of the calcium and *o*-cresolphthalein complexone, as a 0.5 mm wide plug of calcium passed from injection point to the detection window through a typical separation distance of 20 cm, the calcium zone effectively encountered approximately 340 times its own initial volume in *o*-cresolphthalein complexone. This advantage allows for the effective linear range to be extended without the detrimental dilution exhibited by the bulk mixing of increased volumes of solutions.

One major advantage EMMA possesses over standard spectrophotometric methods is its small sample and analytical reagent requirements. As demonstrated by this study, EMMA was capable of analyzing nanoliter scale sample volumes, such as that encountered in the determination of

analytes in interstitial tissues. Furthermore, the volumetric consumption of the reagent/buffer solution was minimal. Based upon the bulk electroosmotic flow-rate, at an experimental electric field strength of 100 V/cm with an inner capillary diameter of 75 μm, the volumetric consumption of the *o*-cresolphthalein complexone/buffer solution V_f was approximately 15 μl/h as calculated by

$$V_f = \pi r^2 \mu_{eo} E \quad (4)$$

where r is the radius of the capillary. Our experimental determinations involved runs of approximately 12 min from injection of analyte to detection of product peak due to the 20 cm separation length utilized. This limitation was imposed by the spatial restrictions of our instrumental design. However, the immediate nature of the mixing and reaction processes exhibited by the EMMA determinations would allow an experimental apparatus with capillaries on the order of a centimeter in separation length to be utilized. With such a design, the analysis could be completed in less than 1 min. Increased sample throughput can also be obtained by running many samples sequentially in the capillary. For the stated experimental apparatus and parameters, an injection would be made every 2.7 min without overlapping of product or internal standard peaks thereby allowing a sample throughput of 22 assays/h. Furthermore, the availability of commercial capillary electrophoresis instruments with autoinjectors would allow the EMMA method to be readily automated.

4. Acknowledgements

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