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In-capillary determination of creatinine with electrophoretically mediated microanalysis: characterization of the effects of reagent zone and buffer conditions

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Abstract

Previous work has demonstrated proof-of-concept for carrying out the clinically useful Jaffe reaction between creatinine and picrate within a capillary tube using electrophoretically mediated microanalysis (EMMA). Here, it is shown that careful control of reagent plug length as well as concentration and pH of the background electrolyte (BGE) can result in a marked improvement in the sensitivity of this assay. Increasing the length of the picrate reagent zone is shown to give rise to as much as a 3–4 fold enhancement, and increasing the concentration and/or pH of the borate buffer also results in an additional, albeit modest, improvement in sensitivity. Interestingly, borate BGE concentrations approaching 100 mM give rise to an unexplained drop in reaction efficiency, an effect which can be avoided by utilizing lower borate concentration with higher pH. The improvements appear to primarily minimize electrodispersion of the picrate reagent, allowing higher picrate concentration in the reaction zone. The same conditions also appear to minimize the electrodispersion of the in-line product as well. With optimized EMMA parameters, the sensitivity of the inline Jaffe chemistry can be enhanced to an extent that there is no need for the two capillary “high sensitivity” detection system required in previous work. Using optimized conditions, three different human serum samples spanning the expected clinical range of creatinine concentrations were successfully analyzed. Overall, this work illustrates the importance of systematically characterizing the conditions under which EMMA analyses are carried out.

Keywords

EMMA; creatinine; picrate; in-line reaction; borate buffer; stacking

1. INTRODUCTION

The Jaffe method is an extensively used clinical procedure, performed in batch mode, for quantitatively determining creatinine in urine and blood serum samples [1–3]. The chemical reaction between creatinine and picrate, under basic conditions, is reasonably specific, and leads to the formation of the Janovski complex, which absorbs strongly in the 485–500 nm range. Creatinine levels in blood serum range from less than 1 mg/dL in healthy adults to over

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10 mg/dL for patients with renal failure. A few analytical assays for creatinine that use capillary electrophoresis have recently been reported [4–7]. Prior work in our lab has shown that the chemistry of Jaffe chemistry can be accurately accomplished within a capillary with electrophoretically mediated microanalysis (EMMA), but this approach suffers from relatively poor concentration sensitivity, requiring the use of a cumbersome and leak-prone high sensitivity cell for samples in the normal range of healthy adults [6]

EMMA, first reported by Boa and Regnier [8,9], involves electrophoretic mixing of nanoliter volumes of chemical reactants within the confines of a narrow bore capillary tube. EMMA is an attractive option for chemical analysis, as minimal sample volumes are required, negligible waste is generated, and rapid analyses are possible. Avila and Whitesides introduced the plug-plug EMMA methodology, which made use of discrete plugs of two reagents, an enzyme and its substrate, and mixed them based on differences in electrophoretic mobilities [10]. Indeed, most of the reported work with EMMA involves the use of enzymatic reaction schemes in which enzyme turnover can affect relatively large changes in substrate and product concentrations. However, the plug-plug EMMA methodology has also been applied to reactions involving only small molecules [6,11–15], and some of these experiments have been accomplished using commercially available UV detection systems, albeit with relatively poor concentration sensitivity [6,11–13].

One of the challenging aspects of EMMA is electrodispersion of ionic reagent zones which can adversely affect an in-line EMMA reaction, resulting in lower local concentrations of the reagent, and poor reagent zone overlap giving rise to poor reaction yield. The factors that govern electrodispersion are the same factors that govern widely studied stacking phenomena, including local field strength differences between reagent zones and the background electrolyte (BGE) as well as ionic mobility mismatch(es) between sample and BGE buffering ions [16–20]. Deliberate control of the ionic conditions to minimize electrodispersion of the ionic reagents used with EMMA will result in improved in-line reaction yield. Moreover, minimizing electrodispersion of an ionic product as it is formed in the capillary will have a direct effect on the method sensitivity.

Field amplified sample stacking (FASS), the simplest mode of stacking an ionic analyte [19], involves concentrating an ionic analyte within a sample plug by focusing the analyte at an ionic boundary between the sample plug and an adjacent zone which has a higher conductivity. As an analyte ion migrates across the ionic boundary, into the zone of higher conductivity, the ion experiences a drop in electric field strength, and a corresponding decrease in migration velocity, resulting in a narrowing of the analyte plug width. Electrodispersion is observed when the ion migrates into a less conductive zone. With EMMA, significant electrodispersion of a charged reagent is not uncommon, since one often employs a high concentration of reagents to drive the reaction, and at the same time is limited, by the onset of Joule heating, to only moderately conductive background electrolytes. Consequently, some care should be given to minimizing electrodispersion of the reactant zones in EMMA. Dovichi's group has successfully used this approach to enhance in-line labeling reaction yields for the analysis of bacterial proteomes [21]. Here we extend this concept to the small-molecule chemistry of the Jaffe reaction.

In this work, we systematically study the EMMA reaction and separation conditions to minimize electrodispersion of the ionic reagents used in the Jaffe reaction and characterize the system in terms of the increase in sensitivity that is obtained. The largest improvements in sensitivity are achieved with conditions that maximize the local concentration of the anionic reagent picrate by minimizing electrodispersion. Injecting a wider zone of the picrate solution is shown to give rise to a 3–4 fold increase in the local concentration of the non-dispersive edge of picrate. In addition, higher background electrolyte (BGE) concentration and/or pH results in sharper picrate zones, and correspondingly higher in-line reaction yield, further

boosting sensitivity. The conditions that minimize the electrodispersion of the reagent picrate also minimize the dispersion of the anionic product. These approaches, illustrated here with this clinically relevant small molecule system, should be generally applicable to any EMMA approach employing at least one charged reagent.

2. EXPERIMENTAL SECTION

2.1. Chemicals

Creatinine was purchased from Sigma Chemical Company (St. Louis, MO, USA). Picric acid (1.2% w/v aqueous solution) was purchased from VWR Scientific (West Chester, PA, USA). Boric acid was obtained from J.T. Baker Chemical Company (Phillipsburg, NJ, USA) and sodium hydroxide was purchased from Fisher Scientific (Fairlawn, NJ, USA). All chemicals were reagent grade, and were used as received.

2.2. Solutions

All solutions were prepared using 18 M Ω -cm water. Borate buffer solutions were prepared by dissolving boric acid in water and adjusting to the desired pH with 1.0 M NaOH. Aqueous standard solutions of creatinine were prepared at a concentration of 20.0 mM. The picrate reagent solution (47 mM, pH ca. 12.3) was prepared by mixing 1 volume of 1.0 M NaOH with 9 volumes of the commercial 1.2% w/v picric acid solution. All solutions were filtered through 0.45 μ m PTFE membrane syringe filters (Whatman Clifton, NJ, USA) and transferred into 1 mL polypropylene vials (Agilent Technologies, Palo Alto, CA, USA) immediately prior to use.

2.3. Serum Preparation

Human blood serum was obtained from Sigma Aldrich (St. Louis, MO) and a pooled sample of uremic serum from patients with renal failure was obtained from Geisinger Medical Center in Danville, PA. Three samples for analysis were prepared by mixing commercial and local serum samples to obtain creatinine concentrations in the low, normal and elevated levels. Each serum sample for analysis was deproteinized as follows: 180 μ L of serum and 20 μ L of either water or a 2.0 mM aqueous standard of creatinine were vortexed for 30 sec in an Eppendorf microcentrifuge tube. 200 μ L of acetonitrile (Aldrich Chemical Company, St. Louis, MO) was added followed by an additional 30 sec of vortex mixing. The precipitated protein was then spun down in a Galaxy mini benchtop centrifuge (VWR Scientific, West Chester, PA). The supernatant was loaded directly into a 250 μ L polypropylene CE sample vial (Agilent Technologies, Palo Alto, CA).

2.4. Instrumentation and CE conditions

An Agilent 3D capillary electrophoresis instrument (Palo Alto, CA, USA) with photoiode array UV/Vis detection and HP Chemstations software was used for all analyses. A fused silica capillary (Polymicro, Phoenix, AZ, USA) with inner and outer diameters of 50 μ m and 360 μ m, respectively, was kept at 25 $^{\circ}$ C during experimentation. The total capillary length was 50.0 cm, and the detection point was 41.5 cm from the inlet. New capillaries were conditioned by flushing with 1.0 M sodium hydroxide and ultrapure water, each for 3 min. Prior to each injection, capillaries were flushed with the BGE solution for 3 min. Hydrodynamic injections (50.0 mbar) of picrate (3–18 sec.), creatinine standard or serum sample (3 sec.), and the BGE borate buffer (3 sec.) were performed. Application of an overlap potential was then applied to allow the reactant plugs to interpenetrate and overlap, as previously described [6,8]. Briefly, the duration of time that a particular overlap potential was applied was determined based on the difference in electrophoretic mobilities, as determined by CZE experiments in the BGE, and the length of the injected reagent plugs. The overlap conditions differed for each BGE buffer and capillary, but were in the range of 10 to 12 kV for a duration of less than 3 sec. After

a six minute incubation period (no applied potential), the reaction components were separated by CZE and detected spectrophotometrically at 210 and 485 nm (8 nm bandwidth). All experiments were carried out in triplicate.

3. RESULTS AND DISCUSSION

The Jaffe method for creatinine determination involves the reaction of anionic picrate with neutral creatinine under alkaline conditions, and this chemistry has previously been carried out within a capillary with voltage-driven mixing of the reagents [6], albeit with the aid of a “high sensitivity” detection cell. Typical electropherograms obtained for the in-line Jaffe assay are shown in Figure 1. For selectivity, the product can be best observed at 485 nm or higher. However, when the data are viewed at 210 nm, it is clear that a significant amount of unreacted creatinine remains, leaving room for improvements in sensitivity through greater reaction yield. The characteristic sawtooth peak shape of the picrate zone indicates that this reagent is significantly broadened by electrodispersion, which likely leads to lower than anticipated picrate concentration in the reaction zone and correspondingly slower reaction kinetics.

The picrate peak symmetry and width can be improved by increasing the concentration of the BGE (*vide infra*), but doing so can eventually lead to Joule heating. Increasing the initial concentration of picrate used in the assay would be the most direct way to increase the in-column reaction rate. However, picric acid is an explosive compound in solid form, and is commercially available only as a 1.2% w/v solution. We have found that increasing the total amount of picrate by increasing the picrate plug zone length can minimize the deleterious effect of electrodispersion, allowing increased formation of product (Figure 2). Indeed, a 2–3 fold increase in peak area, and a 50–70% increase in peak height is observed simply by injecting longer picrate reagent zones. When the length of the injected picrate plug is increased, with all other conditions held constant, both product peak area and peak height are increased, indicating an enhanced reaction yield. It is noteworthy that the injection of wider zones of picrate does not markedly increase the width of the product peak as the product peak width remains limited by the width of the creatinine zone. The local concentration of picrate within the portion of the picrate zone that is overlapped with creatinine during the zero-potential dwell time is apparently higher when electrodispersion is minimized, and this leads to increased product formation. The tail end of the picrate zone is more highly concentrated (as evidenced by higher peak height) when a wider picrate zone is injected, indicating that electrodispersion on the front end of the zone has less of an effect on the back edge of the zone as the zone becomes wider. Since the back edge of the picrate zone is where the reagents are expected to overlap, it follows that increased product formation results when wider picrate zones are used.

It should also be possible to manipulate the local concentration of the picrate reagent by changing the concentration and/or pH of the BGE. In general, higher buffer conductivity is expected to minimize the sample electrodispersion. As seen in Figure 3, a higher concentration of borate BGE indeed results in an increase in picrate peak height and a concurrent narrowing of picrate peak. This allows for increased rates of reaction, and amplified product formation, as illustrated in the insets in Figure 3. As shown, a small shoulder begins to appear on the product peak at higher borate concentration; the origin of this shoulder is unknown but spectral data obtained across the main product peak and the shoulder region are consistent, suggesting this shape is the result of a nonlinear electromigration effect rather than the presence of multiple products. Thus, the total peak area including the shoulder has been used for quantification.

The observed Jaffe product peak area, as a function of picrate injection length for several borate concentrations are shown in Figure 4. In general, increasing the volume of picrate solution injected (x-axis) and/or increasing the concentration of the borate BGE (traces A → D) gives rise to increased product formation. The unexpected crossover seen with 60 and 80 mM borate

and short (3–6 sec) injections of picrate may indicate that the slower EOF rate with higher BGE concentration leads to incomplete overlap conditions; this complication is overcome when higher picrate injection volumes are used. Interestingly, when 100 mM borate buffer is employed, an adverse effect on the assay is immediately apparent. This reproducible phenomenon, observed only at high borate concentration, is under further investigation in our lab. Since the measured current, which ranged from 6 to 24 μA , was linear with borate concentration, the effect was likely not simply a result of excessive Joule heating. Regardless, a four-fold increase in product formation can be achieved as borate concentrations are increased up to 80 mM.

An alternative way to increase the conductivity of the borate BGE, and thereby decrease the electrodispersion of picrate, is by increasing the pH of the BGE, causing increasing levels of borate deprotonation. Figure 5 shows Jaffe product results obtained with 40.0 mM borate under conditions of varied pH between 8.6 and 10.3. Each buffer solution was prepared by dissolving the same amount of boric acid into 18 M Ω -cm water and adjusting the pH via the dropwise addition of ca. 0.1 M NaOH. The ionic strength of each BGE is a function of the sodium ion concentration which is nominally equal to the concentration of the $\text{B}(\text{OH})_4^-$ ion concentration. As expected, elevated BGE pH increases the ionic strength of the BGE, lessening the severity of picrate electrodispersion, giving rise to an increased product peak area. For example, increasing the pH of 40 mM borate from 8.6 to 10.3 led to a 50–70% increase in product peak area and a concurrent 25–30% increase in product peak height. Importantly, the deleterious effects that were observed at borate BGE concentrations above 80 mM in Figure 4 can be avoided by pH alteration. That is, manipulating the pH, rather than the concentration of borate, to increase the BGE conductivity appears to be effective for minimizing electrodispersion without a drop-off in product formation (*vide infra*, Figure 7B). Increased BGE pH results in more anionic $\text{B}(\text{OH})_4^-$ ions, and the concentration of that ion turns out to be a predictor of the picrate peak height, as seen in Figure 6. The chemistry of borate systems is more complicated than this simple treatment implies, but at the concentrations and pH employed here, this treatment is within reason and certainly provides significant insight. Indeed, data obtained with various borate BGEs containing 29.3 mM of each Na^+ and $\text{B}(\text{OH})_4^-$, prepared with boric acid and adjusted with NaOH (40.0 mM at pH 9.68; 60.0 mM at pH 9.22; and 80.0 mM at pH 9.00) gave rise to essentially the same amount of product (Figure 7A) and a current of 16–18 μA passed across the capillary. Further, unlike increasing the borate concentration at pH 9.00 (Figure 4), increasing the ionic strength of the BGE by increasing the pH of borate did not lead to any dramatic drop in product peak area. Finally, Figure 7B illustrates how the low peak area observed with 100 mM borate at pH 9.00 (◆ in Figure 7B) can be “restored” to the expected value of ca. 1400 mAu·s by using lower concentration but higher pH borate BGE systems of equal ionic strength (36.7 mM Na^+ and $\text{B}(\text{OH})_4^-$). Thus, for this in line reaction, elevating the pH appears to be the preferred method for increasing the conductivity of the BGE.

Finally, three samples of pooled human serum were successfully analyzed via EMMA using the optimized conditions without the need for the high sensitivity detection cell. The creatinine concentrations in the three serum samples were determined to be 4.5 ± 0.7 , 1.4 ± 0.2 , and 0.3 ± 0.1 mg/dL, spanning the range from elevated to normal to slightly low, respectively. The method of standard addition was employed, followed by deproteinization of the serum samples prior to analysis by EMMA. The optimized EMMA conditions, which included a pH 10.29 borate BGE at 40.0 mM and a 600 mbar·s injection of 47 mM picrate, gave rise to narrower product peaks, and peak areas that were more than 5 times higher than the previously published conditions (20.0 mM borate at pH 9.00 and 150 mbar·s picrate injection plug) without the aid of the high sensitivity cell. Indeed, with the previous conditions, a product peak could only be detected for one of the three samples, the serum sample with 4.5 mg/dL creatinine. However, a product peak was detected for all samples using the optimized conditions, with the S/N ratio of the most dilute sample of about 8:1.

4. CONCLUDING REMARKS

Electrodispersion of the ionic reagent can be a limiting factor in EMMA. This effect has been illustrated using the Jaffe reaction between anionic picrate and creatinine as a model system. Through judicious selection of reagent plug length as well as BGE concentration and pH, the local concentration of an ionic reagent was maximized allowing significant improvements in the sensitivity of EMMA based methods.

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Figure 1.

Typical electropherograms obtained at 210 nm (A) and 485 nm (B) for the inline Jaffe reaction accomplished via EMMA. Indicated time includes 6.0 minute zero-potential reaction time.

Peak identification: 1 = unreacted creatinine, 2 = unreacted picrate, 3 = product. Conditions: 50.0 μ m i.d. capillary, L_t = 50.0 cm, L_d = 41.5 cm; pressure injections of (i) 150 mbar·s of 47 mM alkaline picrate, pH 12.3 (ii) 150 mbar·s of 20.0 mM aqueous creatinine standard and (iii) 150 mbar·s of borate BGE (20.0 mM at pH 9.0); 16.7 kV overlap potential for 2.2 s; 6.0 min zero-potential incubation, 15.0 kV separation voltage.

Figure 2.

Plot of the Jaffe product peak area as a function of picrate plug length, in relation to length of creatinine plug. Inset: raw data at 485nm showing product peaks corresponding to picrate plug volumes 1, 3, and 5 times that of the creatinine plug. Error bars correlate to the standard deviation (n=3). Other conditions as in Figure 1.

Figure 3.

Effect of borate buffer concentration on electrophoretic data at 210 nm and on the product peak at 485 nm (inset). Peak identity: creatinine (1), picrate (2), and Janovski product (3).

Conditions: pH 9.00 borate background electrolytes at 20.0 (A), 40.0 (B), and 60.0 (C) mM. Picrate injection 600 mbar*s (4 times the creatinine injection zone); other conditions as in Figure 1.

Figure 4.

Effect of changing the borate BGE concentration on the trend of increasing product areas with increasing the picrate zone volume. Borate BGE at pH 9.00 and concentrations of 20.0 mM (A, •), 40.0 mM (B, ▼), 60.0 mM (C, ■), 80.0 mM (D, ◆), and 100.0 mM (E, ▲). Error bars represent standard deviation (n=3).

Figure 5. Effect of the pH of the borate BGE on the trend of increasing product areas with increases in the picrate zone volume. Borate BGE at a concentration of 40.0 mM and pH of 8.59, (A, •), 9.00 (B, ▼), 9.33 (C, ■), 9.68 (D, ◆), and 10.29 (E, ▲). Error bars represent standard deviation (n=3).

Figure 6.

A comparison of the relationship between the calculated concentration of $B(OH)_4^-$ borate species in pH 9.00 borate BGE (A) and the observed picrate peak at 210 nm (B). Calculations considered only the first pK_a of boric acid and assumed no borate self-complexation. 450 mbar-s injections of 47 mM picrate.

Figure 7.

Janovski product peak areas obtained by EMMA analyses in borate BGE systems with differing pH but the same ionic strengths of (A) 29.3 mM and (B) 36.7 mM. Key: Panel A: 40.0 mM borate at pH 9.68 (•), 60.0 mM borate at pH 9.22 (▼), and 80.0 mM at pH 9.00 (■). Panel B: 40.0 mM borate at pH 10.29 (•), 60.0 mM borate at pH 9.43 (▼), 80.0 mM borate at pH 9.17 (■), and 100.0 mM borate at pH 9.00 (◆). Error bars are standard error of the mean (n=3). All data collected at 485 nm with a bandwidth of 8 nm.