Published in final edited form as:

Electrophoresis. 2005 February ; 26(3): 633–639. doi:10.1002/elps.200410061.

pH-mediated acid stacking with reverse pressure for the analysis of cationic pharmaceuticals in capillary electrophoresis

Julie A. Gillogly and Craig E. Lunte

Department of Chemistry, The University of Kansas, Lawrence, KS, USA

Abstract

When using capillary electrophoresis (CE) for the analysis of biological samples, it is often necessary to employ techniques to overcome peak-broadening that results from having a high-conductivity sample matrix. To improve the concentration detection limits and separation efficiency of cationic pharmaceuticals in CE, pH-mediated acid stacking was performed to electrofocus the sample, improving separation sensitivity for the analyzed cations by 60-fold. However, this method introduces a large titrated acid plug into the capillary. To overcome the limitations this low-conductivity plug poses to stacking, the plug was removed prior to the separation step by applying reverse pressure to force it out of the anode of the capillary. Employing this technique allows for roughly twice the volume of sample to be injected. A maximum sample injection time of 240 s was attainable with baseline peak resolution compared to a maximum sample injection time of 120 s without reverse pressure, leading to a twofold decrease in the limits of detection of the analytes used. Separation efficiency overall is also improved when utilizing the reverse pressure step. For example, a 60 s sample injection time results in 94 000 theoretical plates as compared to 60 500 theoretical plates without reverse pressure. This reverse-pressure method was used for detection and quantitation of several cationic pharmaceuticals that were prepared in Ringer's solution to simulate microdialysis sampling conditions.

Keywords

On-column concentration; pH-mediated stacking; Stacking

1 Introduction

The high separation efficiencies associated with capillary electrophoresis (CE) make it an attractive alternative separation technique to conventional liquid chromatography for the analysis of complex biological samples. However, CE is generally not compatible with high-ionic-strength sample matrices due to band-broadening, or destacking, that occurs because of the differences in field strengths between the sample zone and the separation background electrolyte (BGE), and due to the diluting effects of the formed discontinuous electrolyte system controlled by the Kohlrausch regulation function [1]. This problem usually results in the necessity for a sample extraction step prior to analysis of most biological samples due to their high ionic strength. Several stacking methods have been developed to overcome the destacking effect apparent in the analysis of biological samples [2].

The most common stacking method is field-amplified stacking [3,4]. In this method, the sample is diluted in water before injection so that the sample matrix is low-conductive relative to the

BGE. The analyte ions will then stack into a narrow sample plug, overcoming the destacking effect. The apparent limitation of this method is the need to dilute the sample matrix, which requires pretreatment prior to analysis and limits the mass of analyte that is able to be loaded onto the column. Another stacking method prevalent in the literature is sweeping [5–7]. This technique utilizes partitioning of hydrophobic analytes into micelles to enhance stacking; however, sweeping also requires a low-conductivity sample matrix.

In order to increase the volume capacity of CE, large-volume sample stacking has been developed by Chien and Burgi [8–12]. In this approach, a sample volume approximately half of the capillary capacity is hydrodynamically injected and reverse polarity is applied so that anionic analytes move toward the detection end while the sample buffer is backed out of the capillary. Once the electrophoretic current has returned approximately to its original value, indicating sufficient sample buffer has exited the capillary, the polarity is switched to normal mode and the separation occurs in the normal manner. As in the previous stacking methods, this technique requires the sample matrix to be low-conductive, necessitating a pretreatment step for biological samples. Isotachopheresis (ITP) is another separation method that is applicable to a variety of analytes [13–21]. ITP does not require a low-conductivity sample matrix and is therefore particularly suited for biological samples. This method relies on a discontinuous buffer system to trap the analyte between differing electrolyte zones, creating an electric potential gradient. Several other methods take a similar approach to ITP using the chemical differences in discontinuous buffer zones to enhance peak efficiencies [22–27]. A counterflow transient ITP method was developed by Touissant and coworkers [28] that utilizes a counterpressure step to move the sample zone near the inlet of the capillary once ITP-focused so a larger amount of capillary is available for separation.

Our group has developed a method termed pH-mediated stacking to overcome the difficulties associated with high-ionic-strength samples without needing sample pretreatment [29–33]. For pH-mediated acid stacking, the salt of a weak acid, such as acetate, is used as the BGE. During the electrokinetic injection of a sample in high-ionic-strength matrix, such as Ringer's solution, the acetate ions of the BGE displace the sample anions. Following the sample injection, a plug of strong acid is electrokinetically injected. This acid plug titrates the acetate in the sample zone, creating a low-conductivity neutralized acetic acid region across which the cationic sample ions stack. This method has also been shown to be applicable to anionic analytes using an ammonium salt buffer with reversed separation polarity and reversed electroosmotic flow, and using a strong base plug as opposed to the strong acid plug.

The disadvantage of utilizing this pH-mediated acid stacking method for large injection volumes is that a significant amount of the capillary has been used in the stacking step, leaving a shorter length of capillary available for the separation step. In addition, the low-conductivity acetic acid region which remains in the capillary will have a large field strength drop across it. To overcome these limitations, it would be desirable to remove this neutralized plug before separation. We have previously described using a double-capillary system for this purpose, where pH-mediated stacking is performed in one capillary and the other is used for separation [30]. A zero dead volume "T" union was used to connect the two capillaries. However, this "T" union system is very fragile and difficult to construct. This report describes an alternative to the double-capillary design where the use of pH-mediated acid stacking in conjunction with a reverse pressure step to remove the undesired neutralized plug is used to analyze a variety of pharmaceutical compounds in high-ionic-strength sample matrices. This method increases the possible sample volume without compromising the peak efficiency of the analytes.

2 Materials and methods

2.1 Chemicals

Eletriptan, dofetilide, and doxazosin (Fig. 1) were provided by Pfizer UK (Tadworth, Surrey, UK) and used as received. Stock solutions were made by dissolving sample standards in 100% HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ, USA). Sample solutions were further diluted into Ringer's solution to the appropriate concentration. Ringer's solution consisted of 155 mM NaCl, 2.3 mM CaCl₂, and 5.5 mM KCl (all Fisher Scientific). The BGEs used were 100 mM sodium acetate (Fisher Scientific) and 100 mM lithium acetate (Fisher Scientific) titrated to pH 4.75 with concentrated acetic acid. BGEs and Ringer's solutions were prepared in nanopure water from a Labconco Water Pro Plus water system (Fisher Scientific). All solutions were stored at 4°C when not in use.

2.2 CE

All experiments were performed using a home-built CE unit with an ISCO capillary electrophoresis UV-Vis detector (ISCO, Lincoln, NE, USA). Detection was set at 220 nm. 100 cm length of a 75 µm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used as the separation capillary. A 0.5 cm detection window was burned 50 cm from the injection end through the polyimide coating using a microtorch. Samples were introduced by electrokinetic injection at 5 kV. Separations were performed at 20 kV. Data was collected with a CB-50LP connector block connected to a computer containing a PCI-6025E data acquisition board, and displayed using LabView 5.1 data acquisition program (all National Instruments, Austin, TX, USA). Data was analyzed using Microcal Origin 6.0 (OriginLab, Northampton, MA, USA).

2.3 Pressure system

A pressure system was built to apply reverse pressure to the outlet end of the capillary at a pressure of 14 kPa using helium gas and an automatic timer box (built in-house) and small 3-way direct solenoid switching valve (Model #225B-111CAAA; MAC Valves, Wixom, MI, USA). Pressure was applied to the outlet buffer vial using a gas-tight plastic union tee with septa (Fischer Scientific) and epoxy glue (Pacer Technology, Rancho Cucamonga, CA, USA). Routine checks for leakage were performed by immersing the outlet buffer vial and tee system into water and checking for bubble formation.

3 Results and discussion

3.1 Mechanism of pH-mediated acid stacking with reverse pressure

In order to counteract destacking effects that result from having a high-ionic-strength sample matrix, pH-mediated acid stacking with reverse pressure was utilized. Figure 2 shows a schematic of the steps performed in the analysis and the field strength profile across the capillary at each step. When a high-ionic-strength sample plug is electrokinetically injected into the capillary filled with an acetate BGE (Fig. 2A), the field strength drop is primarily across the BGE region in the capillary due to its higher resistivity in comparison with the low resistivity of the sample plug. The anions in the sample plug, such as chloride, will be displaced by the acetate ions. Figures 2B–D show the electrokinetic injection of a strong acid (HCl) immediately after the sample injection. The protons from the acid plug move quickly through the sample plug neutralizing the acetate ions to create an acetic acid region of high resistivity, shown in gray. This neutralized zone allows the sample cations to accelerate to the boundary with the BGE, where they stack into narrow bands. However, now the majority of the field strength drop in the capillary is across the titrated acetic acid zone. Figure 2E shows the application of pressure from the outlet to force the titrated zone out the inlet of the capillary

once stacking has occurred. This not only allows the largest field strength drop to be across the sample plug, but also backs up the analyte bands to the inlet of the capillary so that there is a longer length of the capillary available for separation. Once the titrated zone is sufficiently removed from the capillary, the separation proceeds in the normal fashion, as shown in Figure 2F.

3.2 Optimization of the reverse-pressure step

In order to determine the length of time needed to apply reverse pressure to the outlet of the capillary to sufficiently remove the titrated zone, three different approaches were considered. The first approach involved calculating the volume of acid injected electrokinetically and equating that result to the volume of BGE injected hydrodynamically from the outlet. The second approach was to observe the current profile upon applying reverse pressure when the electrophoretic current of the capillary had returned to a normal value for when the capillary has only BGE present. However, this approach required a small potential to be applied to the capillary during the hydrodynamic reverse pressure in order to obtain a current profile, which was undesirable. The third approach involved several trial-and-error runs to determine experimentally the necessary reverse-pressure time by comparing peak areas of the electroosmotic flow and the analytes. Reverse pressure was applied for varying amounts of time in 2 s increments until the peak areas of the analytes began to decrease. The time point just before any loss of peak area was used as the amount of time to apply reverse pressure. This approach was taken at each of the sample injection times used, although a trend developed to give an approximate guess to use as a starting point for each optimization.

3.3 Comparison of separations without acid stacking, with acid stacking, and of acid stacking with reverse pressure

Figure 3 shows the separation of three cationic pharmaceuticals in a Ringer's solution sample matrix used to demonstrate the increased peak efficiencies gained with acid stacking. Figure 3A demonstrates the compounds without acid stacking. It is apparent that band-broadening has occurred, and peak separation is problematic. Figure 3B shows the injection of the strong acid plug after sample injection, which greatly increases peak efficiencies and baseline resolution is easily achieved. The amount of strong acid to inject has previously been optimized as 1.6 times the sample injection time [29,31]. Figure 3C illustrates the acid stacking with reverse pressure at the same injection time. Peak migration is slightly shifted due to two conflicting forces. The analytes have a farther distance to travel in order to reach the detector, which would result in a longer migration time, but also there is a larger field strength across the capillary as a result of removing the low-conductivity titrated region with reverse pressure, resulting in faster migration. The summation of these two factors results in the analytes only having a slightly longer migration time than without the reverse-pressure step.

3.4 Comparison of the analyte peak efficiencies of acid stacking and of acid stacking with reverse pressure

Table 1 summarizes the results of one representative compound, dofetilide, with and without acid stacking, and with acid stacking and reverse pressure. Peak efficiency (N) was calculated by using the equation $N = 5.54 (t_m/w_{1/2})^2$ where t_m is the peak migration time and $w_{1/2}$ is the peak width at half peak height (PH). All trials were repeated in triplicate and standard deviations for each are presented.

The effect of sample injection time on separation efficiency for the three injection modes is shown in Fig. 4. Separation efficiency degrades rapidly with injection time when stacking is not employed. Injection times are only possible up to 40 s with severe loss in efficiency. Acid stacking results in much higher efficiency for the same injection time. Employing reverse pressure gives similar peak efficiency as compared to without reverse pressure up to 40 s

injection times. For longer injection times, acid stacking efficiency starts to decrease, and peak resolution is not sufficient after 120 s. However, acid stacking with reverse pressure still has adequate peak efficiency for 240 s sample injection times with sufficient peak resolution, using approximately 80% of the effective capillary length for sample introduction. Clearly employing the additional reverse-pressure step to acid stacking allows for approximately twice the sample injection volume as acid stacking alone.

3.5 Comparison of the analyte peak width at half height and peak height of acid stacking and of acid stacking with reverse pressure

Figure 5 shows the relationship between sample injection time and peak width at half height and peak height for dofetilide. As similar to the peak efficiency trend, at 60 s the two methods start to differentiate, and the samples without reverse pressure become wider and shorter than the acid stacked with reverse-pressure at the same sample injection time. It is of interest to note that the parabolic flow of the hydrodynamic reverse pressure step does not appear to degrade the narrow peak band to any sufficient degree.

3.6 Advantages and disadvantages of acid stacking with reverse pressure

It is clear from the data obtained that reverse pressure does achieve the desired goal of increasing sample volume possible for pH-mediated acid stacking. The method does require the additional reverse-pressure step before the separation can proceed. While this method was optimized manually for reverse pressure, automation of the injection process should ease the difficulty of analysis so that the additional reverse-pressure step is not a throughput barrier to analysis using pH-mediated acid stacking. Another concern is the introduction of a hydrodynamic parabolic flow in the reverse-pressure step. Although the deleterious effects of a hydrodynamic flow step were not apparent in decreasing peak efficiencies in the results presented here, this is certainly another possible drawback to the method proposed.

4 Concluding remarks

From the results obtained, we have concluded that employing reverse pressure in conjunction with pH-mediated acid stacking can increase the sample volume that can be used while still providing sufficient peak efficiencies and adequate resolution. Since this technique does require an additional injection step, one would have to decide if the gain of doubling the sample volume is worth the additional step. Automation of this method in a commercially available instrument would greatly simplify the process so that the additional step would not be deterrent to the analyst. This method is applicable to high ionic strength biological samples without the need for a pretreatment step. Compared to the previously mentioned double-capillary design, which allowed for a fivefold increase in sensitivity, the incorporation of reverse pressure is not as much of an improvement over traditional pH-mediated stacking; however, the application of the reverse pressure is much simpler than the construction of the zero dead volume “T” junction used in the double-capillary method, and the ability to use a commercial instrument is possible with this reverse-pressure method.

Acknowledgements

This work was supported in part by the National Institutes of Health grant R01EB00247. The author wishes to thank Ken Saunders of the Department of Drug Metabolism, Pfizer Central Research, Sandwich, Kent, UK for supplying eletriptan, dofetilide, and doxazosin for use in this project.

References

1. Křivánková L, Pantùèková P, Boček P. J Chromatogr A 1999;838:55–70.
2. Chien RL. Electrophoresis 2003;24:486–497. [PubMed: 12569539]

3. Chien RL, Burgi DS. *J Chromatogr* 1991;559:141–152.
4. Chien RL, Burgi DS. *Anal Chem* 1992;64:489A–496A.
5. Quirino JP, Terabe S. *Anal Chem* 1999;71:1638–1644.
6. Quirino JP, Terabe S. *Anal Chem* 2000;72:1023–1030. [PubMed: 10739207]
7. Quirino JP, Terabe S, Otsuka K, Vincent JB, et al. *J Chromatogr A* 1999;838:3–10.
8. Chien RL, Burgi DS. *Anal Chem* 1992;64:1046–1050.
9. McGrath G, Smyth WF. *J Chromatogr B* 1996;681:125–131.
10. Harland GB, McGrath G, McClean S, Smyth WF. *Anal Commun* 1997;34:9–11.
11. Martinez D, Borrull F, Calull M. *J Chromatogr A* 1997;788:185–193.
12. Quirino JP, Terabe S. *Electrophoresis* 2000;21:355–359. [PubMed: 10675015]
13. Mazereeuw M, Tjaden UR, van der Greef J. *J Chromatogr A* 1994;677:151–157.
14. Kašička V, Prusik Z. *J Chromatogr* 1991;569:123–174. [PubMed: 1939486]
15. Udseth HR, Loo JA, Smith RD. *Anal Chem* 1989;61:228–232.
16. Bergmann J, Jaehde U, Mazereeuw M, Tjaden UR, et al. *J Chromatogr A* 1996;734:381–389. [PubMed: 8673244]
17. Křivánková L, Boček P. *J Chromatogr B* 1997;689:13–34.
18. Mazereeuw M, Spikmans V, Tjaden UR, van der Greef J. *J Chromatogr A* 2000;879:219–233. [PubMed: 10893038]
19. Shihabi ZK. *Electrophoresis* 2002;23:1612–1617. [PubMed: 12179979]
20. Kong Y, Zheng N, Zhang Z, Gao R. *J Chromatogr B* 2003;795:9–15.
21. Li M, He YZ, Gan WE, Lin XQ, et al. *Sepu* 2001;19:176–178.
22. Britz-McKibbin P, Chen DDY. *Anal Chem* 2000;72:1242–1252. [PubMed: 10740866]
23. Britz-McKibbin P, Terabe S. *Chem Record* 2002;2:397–404. [PubMed: 12469351]
24. Cao CX, Zhou SL, Qian YT, He YZ, et al. *J Chromatogr A* 2001;922:283–292. [PubMed: 11486874]
25. Cao CX, Zhou SL, He YZ, Qian YT, et al. *J Chromatogr A* 2001;907:347–352. [PubMed: 11217043]
26. Cao CX, Zhou SL, He YZ, Zheng XY, et al. *J Chromatogr A* 2000;891:337–347. [PubMed: 11043794]
27. Britz-McKibbin P, Bebault GM, Chen DDY. *Anal Chem* 2000;72:1729–1735. [PubMed: 10784134]
28. Toussaint B, Hubert P, Tjaden UR, van der Greef J, et al. *J Chromatogr A* 2000;871:173–180. [PubMed: 10735297]
29. Park S, Lunte CE. *J Microcol Sep* 1998;10:511–517.
30. Zhao Y, Lunte CE. *Anal Chem* 1999;71:3985–3991. [PubMed: 10500485]
31. Weiss DJ, Saunders K, Lunte CE. *Electrophoresis* 2001;22:59–65. [PubMed: 11197180]
32. Arnett SD, Lunte CE. *Electrophoresis* 2003;24:1745–1752. [PubMed: 12783451]
33. Zhao Y, McLaughlin K, Lunte CE. *Anal Chem* 1998;70:4578–4585. [PubMed: 9823717]

Figure 1.
Chemical structures of the compounds analyzed: (A) eletriptan, (B) dofetilide, (C) doxazosin.

Figure 2.

Schematic of the pH-mediated acid stacking mechanism showing reverse pressure. (A) A sample in a high-ionic-strength matrix is electrokinetically injected. The field strength drop is primarily across the BGE region of the capillary due to the low resistivity of the sample matrix. (B) A plug of strong acid is next injected electrokinetically. (C) The strong acid titrates the sample region to neutral, creating a zone of high resistivity. (D) As the acid titrates, the analytes are stacked into narrow bands at the boundary of the titrated region and the BGE. The primary field strength drop is now across the titrated zone. (E) Pressure is applied from the outlet of the capillary, pushing the titrated zone out of the capillary, and pushing the analytes near the inlet. The field strength drop is now across the sample zone, and the entire capillary length is

available for analyte separation. (F) The separation voltage is applied and the separation proceeds.

Figure 3.

Separation of three cationic pharmaceuticals eletriptan, dofetilide, and doxazosin in Ringer's solution (A) without acid, (B) acid-stacked, and (C) acid-stacked with reverse pressure.

Analytes were each 50 μ M. Injection was performed electrokinetically at 5 kV, and separation was performed at 20 kV. The BGE was 100 mM lithium acetate buffer, pH 4.75.

Figure 4. Plot showing the effect of sample injection time on separation efficiency of dofetilide in Ringer's solution (Δ) without acid, ($\bullet$) acid-stacked, and ($\square$) acid-stacked with reverse-pressure.

Figure 5. Effect of sample injection time (A) on peak width at half height and (B) peak height for dofetilide in Ringer's solution acid-stacked ($\blacktriangle$) and acid-stacked with reverse pressure ($\square$).

Table 1

Effect of sample injection time (T_{inj}) on migration time (T_{mig}), width at half height ($W_{1/2}$), efficiency (N), peak height, and peak area for dofenilide in Ringer's solution without acid stacking, with acid stacking, and with acid stacking and reverse pressure

T_{inj} (s) sample/acid	T_{mig} (s)	$W_{1/2}$ (s)	$N/1000$	Peak height (mAU)	Peak area/1000
Ringer's solution without acid stacking					
5	320.2 ± 4.8	2.8 ± 0.1	72.1 ± 3.3	2.3 ± 0.2	6.8 ± 0.6
10	322.5 ± 6.2	2.6 ± 0.2	85.5 ± 7.4	3.8 ± 0.3	14.1 ± 1.1
20	321.9 ± 3.4	3.1 ± 0.4	59.2 ± 9.2	5.9 ± 0.4	23.8 ± 1.9
30	322.0 ± 4.9	4.3 ± 0.6	31.7 ± 3.8	6.5 ± 0.5	34.4 ± 2.9
40	324.5 ± 7.1	6.8 ± 0.6	13.4 ± 1.4	6.7 ± 0.6	45.3 ± 4.4
With acid stacking					
5/8	286.1 ± 2.4	1.4 ± 0.1	231.2 ± 5.9	3.2 ± 0.1	6.6 ± 0.4
10/16	288.3 ± 1.8	1.6 ± 0.1	179.6 ± 8.2	5.9 ± 0.2	12.2 ± 0.8
20/32	291.7 ± 2.2	1.7 ± 0.2	162.4 ± 7.7	14.4 ± 1.0	25.1 ± 1.1
30/48	285.0 ± 2.1	1.6 ± 0.1	176.7 ± 4.9	19.6 ± 1.4	34.9 ± 1.4
40/64	297.7 ± 2.9	2.0 ± 0.1	121.9 ± 2.5	21.9 ± 3.2	46.8 ± 1.6
50/80	299.3 ± 4.0	2.5 ± 0.2	79.0 ± 4.7	24.3 ± 6.1	61.2 ± 2.1
60/96	313.6 ± 3.6	3.0 ± 0.1	60.5 ± 2.8	22.7 ± 3.5	72.0 ± 3.2
70/112	315.2 ± 5.1	3.6 ± 0.2	42.7 ± 2.5	22.9 ± 4.7	83.7 ± 3.9
90/144	312.2 ± 5.4	4.8 ± 0.3	23.1 ± 2.2	21.6 ± 4.6	108.5 ± 7.3
120/192	314.8 ± 7.8	5.9 ± 0.5	16.3 ± 1.8	23.9 ± 5.4	141.8 ± 11.8
With acid stacking and reverse pressure					
5/8	316 ± 2.1	1.5 ± 0.1	246.2 ± 9.1	3.4 ± 0.2	6.2 ± 0.4
10/16	315 ± 2.3	1.4 ± 0.2	280.8 ± 9.4	6.7 ± 0.3	11.9 ± 0.9
20/32	321 ± 1.9	1.7 ± 0.1	198.3 ± 7.7	12.1 ± 0.4	23.8 ± 1.4
30/48	321 ± 2.4	1.9 ± 0.1	158.1 ± 5.8	17.3 ± 1.7	35.9 ± 1.8
40/64	322 ± 2.2	2.2 ± 0.2	119.0 ± 3.6	18.7 ± 3.0	48.4 ± 2.0
50/80	324 ± 3.4	2.4 ± 0.1	101.6 ± 4.9	22.4 ± 4.6	61.1 ± 2.7
60/96	326 ± 3.2	2.5 ± 0.1	94.0 ± 2.8	25.5 ± 5.2	71.7 ± 3.5
70/112	325 ± 5.1	2.3 ± 0.1	111.7 ± 3.3	31.9 ± 5.4	84.3 ± 4.3
90/144	327 ± 8.6	2.8 ± 0.2	76.4 ± 2.3	33.6 ± 6.1	108.5 ± 7.0
120/192	329 ± 9.6	3.2 ± 0.1	59.2 ± 3.1	38.2 ± 5.7	144.0 ± 7.5
150/240	330 ± 8.2	3.6 ± 0.1	47.3 ± 3.4	43.7 ± 6.3	177.9 ± 12.2
180/288	334 ± 9.3	4.8 ± 0.3	27.9 ± 3.6	37.6 ± 4.9	216.4 ± 15.3
210/336	338 ± 9.0	5.6 ± 0.3	20.2 ± 3.2	39.2 ± 5.8	253.8 ± 19.6
240/384	342 ± 9.1	6.1 ± 0.5	17.4 ± 2.4	41.5 ± 5.2	285.8 ± 23.1

Values are presented as mean ± standard deviation from experiments run in triplicate.