

Distance Learning Module II: Using Capillary Electrophoresis for Qualitative and Quantitative Analysis

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Objective:

To determine the effect of varying injection parameters for capillary electrophoresis, to establish the best conditions for sample introduction and to use this information to perform qualitative and quantitative analysis of an unknown sample.

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Learning Outcomes

Upon successful completion of Learning Module II, researchers will be able to:

- (1) formulate protocol for sample introduction in capillary electrophoresis;
- (2) perform quantitative and qualitative analysis of an unknown using capillary electrophoresis

Introduction

This learning module is written for undergraduate chemistry researchers who are already familiar with fundamentals and basic aspects of capillary electrophoresis system such as those outlined in Learning Module I. The experiment outlined in this Module may be accomplished using a commercial or custom-built capillary electrophoresis instrument. The first 8 steps of this learning module explore sample introduction for capillary electrophoresis. Steps 9 and 10 require the user to formulate protocol for quantitative and qualitative analysis by capillary electrophoresis. The last step (11) requires an assistant or colleague to prepare a sample of unknown concentration that will be used for a practical laboratory experiment. This exercise will allow the user to assess her or his laboratory skill and knowledge of the operation of a capillary electrophoresis instrument. The experiment outlined here is performed with recommended chemicals (atenolol, mesityl oxide, naproxen). The experiment may be completed with other compounds, and alternative chemicals are outlined in the instructor's manual accompanying this material. Successful completion of Learning Module II requires you perform runs under various injection conditions and draw conclusions regarding the optimal injection protocol; (2) document standard protocol for quantitative and qualitative analysis; and (3) assess your proficiency at qualitative and quantitative analyses.

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Background

Fundamentals. In Learning Module 1, we have discussed the mechanics of transport in capillary electrophoresis. Another important consideration is zone broadening, or separation efficiency. In the simplest sense, this is a measure of how skinny a peak is in a separation. A method with high separation efficiency will have smaller band spreading, or peak width. Since separation peaks are ideally Gaussian, peak width is defined by σ , or variance of the Gaussian function used to fit the separation peak. The quantitative descriptor of efficiency is theoretical plates, N , which is related to analyte migration time, and the variance in migration time, σ^2 , of the Gaussian fit (equation 2.1). You will find other variations of this equation that include width at half height or width of base rather than variance. If you are using Igor Pro, software, the program will report migration time and variance in seconds, and you should use equation 2.1 to calculate theoretical plate count. In an ideal case, the only source of zone broadening is the longitudinal diffusion. In reality, Joule heating may introduce convective flow, and analyte may adsorb to the capillary surface. The detection cell may be a source of variance, for example if the capillary is connected to the detection cell in such a way to allow the analyte band to spread. A key factor in capillary electrophoresis is the band spreading introduced in the sample introduction. For example, if a sample plug is particularly large (for example, 10% of the capillary length), upon separation, the detected analyte peak will generally be larger than the injected plug.

$$N = \frac{t^2}{\sigma^2} \quad (\text{equation 2.1})$$

Sample Introduction. Sample introduction is generally achieved with pressure, voltage, or siphoning. Pressure-assisted injections involve the application of reproducible pressures in the range of 0.5-5 p.s.i. Gravity-based injections involve raising the injection end of the capillary, immersed in a sample vial, higher than the detection end to promote siphoning. Voltage based injections introduce sample differentially depending of sample mobility. Thus, cations will preferentially load onto a capillary as compared to neutral compounds or anions. A successful injection introduces a small analyte plug in a reproducible manner. Reproducibility is achieved with electronic timing.

Basic Protocol. Sample introduction in capillary electrophoresis system requires knowledge of the performance, standard operation procedures and a working knowledge of the figures of merit to quantitatively assess whether the injection technique is adequate. Most users learn what is important through experience. To streamline this process,

we have outlined a systematic evaluation of different conditions for pressure-assisted injections. The researcher will record migration time and peak width for triplicate runs obtained at various pressure and injection durations. It is important that all other parameters remain the same (capillary dimensions, analyte, buffer, separation voltage...). In doing this, the researcher can effectively gauge the effect of changing either injection pressure or duration. Ultimately, this exercise will assist new researchers in documenting strategies for sample introduction.

Application. Capillary electrophoresis is frequently employed for quantitative analysis. Reproducible and efficient injections are important for quantitation. The researcher will devise a protocol for quantitative analysis, for example, using a 3-point standard calibration curve. The final exercise of this learning module is a lab practical. A colleague or mentor will provide a sample containing analyte above the limit of quantification. The user will then implement the strategies she/he has devised and determine the analyte concentration as a means of assessing her/his lab skills.

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Materials

In order to complete Learning Module II you will need the materials listed below.

- (1) A capillary electrophoresis system that includes the five components (injection, capillary, high voltage, detection, analog-to-digital converter). We recommend you use a bare fused silica capillary with an inner diameter of ~25 microns.
- (2) Chemicals: atenolol, deionized water, mesityl oxide, naproxen, sodium hydroxide, tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES).
- (3) Standard laboratory equipment: electronic balance, pH meter, volumetric pipets, sonicator (for degassing running buffer).

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Safety Precautions

Consult the safety guidelines and Chemical Hygiene Plan provided by your Institution before beginning any experiment. The safety guidelines of your home Institution supercede any recommendations outlined here.

Consult the MSDS and the label prior to using a chemical and adjust your laboratory procedures accordingly.

Personal protective equipment, such as goggles, safety glasses, laboratory coat or apron, gloves, or a respirator, should be used as appropriate for the hazards involved and as recommended on the label and in the MSDS.

Use chemical fume hoods as advised in the MSDS

Store and handle all chemicals appropriately.

Do not consume anything in the laboratory.

Do not smoke, chew gum, or use smokeless tobacco in the laboratory.

Remove your gloves and thoroughly wash your hands before leaving the laboratory.

Practical advice regarding use of the high voltage power supply:

There is potential for electrical shock from the high voltage power supply. Typical currents employed in capillary electrophoresis are less than 100 microamps. According to the OSHA tutorial cited below, AC currents of 1mA result in a tingling sensation. However, the degree of danger of such exposure depends upon: (1) if the skin is wet or dry, (2) if the shock may potentially throw the victim away from the electrical connection (for example into an acid bath behind the researcher), or (3) if the exposed person undergoes muscle contraction that does not allow them to let go of the electrical circuit. See the following website for an OSHA tutorial of the risks of electrical shock: http://www.osha.gov/SLTC/etools/construction/electrical_incidents/eleccurrent.html

We recommend the following precautions to prevent electrical shock or minimize the effects in the event of accidental exposure.

- (1) Implement the interlock safety switch outlined in the assembly protocol to facilitate “guarding by location”.
- (2) Turn on the voltage only after closing the interlock box with the integrated interlock switch. Turn off the voltage before you intend to open the Plexiglas box with the integrated interlock switch. In doing this, the circuit will never have the potential to be live when you open the Plexiglas box. Should you ever unsafely open the box with the power supply turned on, the interlock switch is the back-up that will prevent electrical exposure. If you press the interlock switch down with the lid to the Plexiglas open, you are no longer protected from accidental exposure to the high voltage. You may further ensure the safety of the systems by wiring an audible alarm to sound when the interlock switch is closed, completing the electrical circuit. This will supplement the visual indicator created with implementation of the interlock switch (power on green button on the front of the high voltage power supply lights up when the circuit is live).
- (3) Check that the interlock switch is fully functional, using a voltmeter to measure resistance, every day prior to using the instrument.
- (4) Set the current limiting knob so that the power supply can provide a maximum current of 100 microamperes. Use the voltage limiting knob to adjust the applied voltage as necessary.
- (5) Be sure your skin is dry, when you are using the instrument. If you, or the device, are sweating, do not operate the instrument.

Consult the safety guidelines provided by your Institution before beginning any experiment. The safety guidelines of your home Institution supercede any recommendations outlined here.

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Procedures: The Module

1. Use the run parameters outlined below to obtain triplicate runs of a standard containing both a neutral marker (for example, dimethylformamide or mesityl oxide) and atenolol. Report the migration times, peak height, and calculated theoretical plates for the neutral marker and atenolol for each run. Calculate the mean, standard deviation (SD) of the mean, and relative standard deviation (RSD) of the mean for each parameter. You may use the table below to report your data.

Preparation of capillary: Assuming this is the first run of the day, the capillary should be flushed as follows: 30-min, 20 psi flush with 0.1 N NaOH, followed by 15-min, 20 psi flush with deionized water, followed by a 30-min, 20-psi flush with running buffer. This flushing protocol is necessary only at the beginning of the day. If exercises 1-6 are completed within a single lab period, repeating this initial capillary flush should not be necessary.

Sample: 0.65 millimolar (0.005 % v/v) dimethylformamide, 75 micromolar atenolol in 25 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffered at pH 7.0.

Injection parameters: 1 second injection at 2 psi pressure

Run conditions: running buffer 25 mM TES pH 7.0, 25 micron i.d. fused silica capillary, 42.8 cm total length, 32.9 cm to detection window, separation voltage: 15 kV, range 0.001, rise time 0.3 seconds, 220 nm

Injection parameters: 1 second injection at 2 psi pressure				
Atenolol	Trial 1	Trial 2	Trial 3	Mean
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				
Neutral Marker				
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				

2. Use the run parameters outlined below to obtain triplicate runs of a standard containing both a neutral marker and atenolol. Report the migration times, peak height, and calculated theoretical plates for the neutral marker and atenolol for each run. Calculate the mean, SD and RSD for each parameter. You may use the table below to report your data.

Sample: 0.65 millimolar (0.005 % v/v) dimethylformamide, 75 micromolar atenolol in 25 mM TES pH 7.0.

Injection parameters: 1 second injection at 4 psi pressure

Run conditions: running buffer 25 mM TES pH 7.0, 25 micron i.d. fused silica capillary, 42.8 cm total length, 32.9 cm to detection window, separation voltage: 15 kV, range 0.001, rise time 0.3 seconds, 220 nm.

Injection parameters: 1 second injection at 4 psi pressure				
Atenolol	Trial 1	Trial 2	Trial 3	Mean
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				
Neutral Marker				
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				

3. Use the run parameters outlined below to obtain triplicate runs of a standard containing both a neutral marker and atenolol. Report the migration times, peak height, and calculated theoretical plates for the neutral marker and atenolol for each run. Calculate the mean, SD and RSD for each parameter. You may use the table below to report your data.

Sample: 0.65 millimolar dimethylformamide, 75 micromolar atenolol in 25 mM TES pH 7.0.

Injection parameters: 1 second injection at 1.5 psi pressure

Run conditions: running buffer 25 mM TES pH 7.0, 25 micron i.d. fused silica capillary, 42.8 cm total length, 32.9 cm to detection window, separation voltage: 15 kV, range 0.001, rise time 0.3 seconds, 220 nm

Injection parameters: 1 second injection at 1.5 psi pressure				
Atenolol	Trial 1	Trial 2	Trial 3	Mean
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				
Neutral Marker				
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				

4. Use the run parameters outlined below to obtain triplicate runs of a standard containing both a neutral marker and atenolol. Report the migration times, peak height, and calculated theoretical plates for the neutral marker and atenolol for each run. Calculate the mean, SD and RSD for each parameter. You may use the table below to report your data.

Sample: 0.65 millimolar dimethylformamide, 75 micromolar atenolol in 25 mM TES pH 7.0.

Injection parameters: 2 second injection at 2 psi pressure

Run conditions: running buffer 25 mM TES pH 7.0, 25 micron i.d. fused silica capillary, 42.8 cm total length, 32.9 cm to detection window, separation voltage: 15 kV, range 0.001, rise time 0.3 seconds, 220 nm

Injection parameters: 2 second injection at 2 psi pressure				
Atenolol	Trial 1	Trial 2	Trial 3	Mean
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				
Neutral Marker				
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				

5. Use the run parameters outlined below to obtain triplicate runs of a standard containing both a neutral marker and atenolol. Report the migration times, peak height, and calculated theoretical plates for the neutral marker and atenolol for each run. Calculate the mean, SD and RSD for each parameter. You may use the table below to report your data.

Sample: 0.65 millimolar dimethylformamide, 75 micromolar atenolol in 25 mM TES pH 7.0.

Injection parameters: 4 second injection at 2 psi pressure

Run conditions: running buffer 25 mM TES pH 7.0, 25 micron i.d. fused silica capillary, 42.8 cm total length, 32.9 cm to detection window, separation voltage: 15 kV, range 0.002, rise time 0.3 seconds, 220 nm

Injection parameters: 4 second injection at 2 psi pressure				
Atenolol	Trial 1	Trial 2	Trial 3	Mean
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				
Neutral Marker				
Peak Height				
Migration Time (sec)				
σ (sec)				
Plates ($\times 10^3$)				

6. Use the run parameters outlined below to obtain a single run of a standard containing both a neutral marker and atenolol. Report the migration times, peak height, and calculated theoretical plates for the neutral marker and atenolol for each run.

Sample: 0.65 millimolar dimethylformamide, 75 micromolar atenolol in 25 mM TES pH 7.0.

Injection parameters: 2 second injection at 4 kV applied voltage (normal polarity, no pressure!)

Run conditions: running buffer 25 mM TES pH 7.0, separation voltage: 15 kV, range 0.001, rise time 0.3 seconds, 220 nm

2 s, 4 kV	
Atenolol	Trial 1
Peak Height	
Migration Time (sec)	
σ (sec)	
Plates ($\times 10^3$)	
Neutral Marker	
Peak Height	
Migration Time (sec)	
σ (sec)	
Plates ($\times 10^3$)	

7. Use your findings from the exercises outlined in steps 1-5 to complete the table below. What conclusions can you draw from these studies? What injection protocol is best suited for analyses of 75 micromolar atenolol?

Injection Conditions	4s, 2psi	2s, 2psi	1s, 4psi	1s, 2psi	1s, 1.5psi
Atenolol					
Height, Mean					
Height, %RSD					
Time (s), Mean					
Time, %RSD					
Plates					
Neutral Marker					
Height					
Height, %RSD					
Time (sec)					
Time, %RSD					
Plates					

8. Independent Excursion: As you can see from this exercise, the injection protocol can have a marked effect on various aspects of the separation. Many other injection protocol, concerns and criteria are important to other applications of capillary electrophoresis. For instance, in addition to hydrodynamic injection (forcing sample on using pressure), injections may also be facilitated by voltage and siphoning. The result produced in step 6 of this lab exercise, might lead you to devise another set of experiment designed to find optimal injection parameters using electrophoretic injection. Difference in the ionic strength of the sample and running buffer can also effect the separation. Below you will find two proposed experiments that might provide insight in these two areas. After you have had time to ponder the experiment performed in steps 1-6, you may attempt the independent experiments below, or design your own experiment.

Injection facilitated by siphoning:

Elevate the anodic buffer reservoir and the separation capillary such that the liquid line (top of vial) and capillary end in are 8 cm higher in the anodic reservoir relative to the cathodic reservoir. Determine the mean value for migration time (n=2) for both a neutral marker and tolmetin. Repeat this exercise with the liquid line (top of vial) and capillary end are at identical heights in both the anodic and cathodic reservoirs.

Determination of analyte in matrix of ionic strength different from running buffer*

Use identical pressure-based injection and run conditions for the samples outlined below. Determine the mean values for migration time, height and theoretical plate count for triplicate runs of each of the solutions listed below. Be sure to report the uncertainty in your answers.

- (A) neutral marker and 100 micromolar mesityl oxide, and 300 micromolar tolmetin dissolved in deionized water
- (B) neutral marker and 300 micromolar tolmetin dissolved in background electrolyte (25 mM TES pH 7.0)
- (C) neutral marker and 300 micromolar tolmetin dissolved in background electrolyte (25 mM TES pH 7.0) that also contains 500 mM sodium chloride

*Note: this exercise may be attempted using voltage-based injections (electrophoretic) or siphoning.

9. Outline the experimental strategy to determine the concentration of atenolol in an aqueous solution.

10. Lab Practical

Through a faculty mentor or collaborator, you should arrange to receive an unknown solution containing either naproxen or atenolol diluted in background electrolyte at a concentration above the limit of quantification for your capillary electrophoresis system. Once you receive the unknown solution, you are to determine the analyte composition and concentration in the solution. Your final report should include pertinent data and a clear explanation of your results. When you report your final value, be sure to include uncertainty. You should have access to standard solutions as necessary.

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Follow-up Activities

Now that you have completed Learning Module II, you have documented procedures for sample introduction and quantitative analysis with capillary electrophoresis. Following the separation you completed in the lab practical, consider whether you would revise any of the protocol you developed in this Learning Module. As you expand your use and knowledge of capillary electrophoresis, you will undoubtedly expand and modify the documented operating procedures. Take a look at the answer key we have provided for Learning Module II. If you are in contact with other researchers who have completed this Learning Module, you should consider sharing your responses with others. You may find subtle differences or explanations that you find useful.

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Conclusions and Future Direction

If you have mastered the learning outcomes for Learning Module II, congratulations! Capillary electrophoresis is a flexible separation technique that requires some experience for developing methods that are providing the efficiency and/or selectivity requisite for a particular application. Learning Module III is a self-guided exercise designed to teach you the basics of a different mode of electrophoresis based on secondary separation equilibria: micellar electrokinetic capillary chromatography. This will assist you in devising separation strategies for future capillary electrophoresis analyses. The procedures you have mastered in Learning Module II will be required to further refine your laboratory skills in the third Module.