

Distance Learning Module I: Getting to Know Your Capillary Electrophoresis System

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Objective:

To become familiar with the components of a capillary electrophoresis instrument and to translate fundamental capillary electrophoresis concepts into anticipated experimental observations.

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Learning Outcomes

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

- (1) predict analyte migration;
- (2) determine pertinent figures of merit related to capillary electrophoresis data;
- (3) establish standard protocol for future capillary electrophoresis experiments.

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Introduction

This learning module is written to help undergraduate researchers to understand practical aspects of the operation of a capillary electrophoresis system and combines information about fundamentals of the method and standard construction and operation of a capillary electrophoresis instrument. The experiment may be accomplished with a commercial or custom-built capillary electrophoresis system. The first 21 steps of this learning module are a dry-lab, and therefore may be completed without access to a capillary electrophoresis instrument. The last step requires a capillary electrophoresis separation and knits together the background information formulated in the dry lab to demonstrate the need for a practical understanding of the methodology. These experiments are outlined with recommended chemicals (tolmetin, atenolol, and mesityl oxide). 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 I requires you: (1) identify and name components of the system; (2) define and describe figures of merit that are frequently invoked in capillary electrophoresis; (3) document protocol for making standards and recording information in your notebook; and (4) formulate strategies for qualitative and quantitative analyses.

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Background

Fundamentals. There are two types of transport in free zone capillary electrophoresis. The first is electrophoretic transport, which is based on the charge attraction or repulsion of the analyte to a cathode (or anode). This is affected by frictional drag, so electrophoretic transport is related to the size-to-charge ratio of the analyte. Electrophoretic velocity, v_{eph} , is the product of electrophoretic mobility, μ_{eph} , applied voltage, V , and capillary length, L , (equation 1.1). The second type of transport in electrophoresis is a bulk flow, termed electroosmotic flow. The negative surface charge on the surface of the separation capillary leads to a double layer. When the high voltage is applied to the capillary, the electrolyte moves towards the cathode and induces bulk liquid flow with a plug profile. Electroosmotic velocity, v_{eof} , is the product of the electroosmotic mobility, μ_{eof} , applied voltage, V , and capillary length, L (equation 1.2).

$$v_{eph} = \frac{\mu_{eph}V}{L} \quad (\text{equation 1.1})$$

$$v_{eof} = \frac{\mu_{eof}V}{L} \quad (\text{equation 1.2})$$

The entire process occurs in the presence of the applied electric field and charged surface sites on the wall of the fused silica capillary. In a typical capillary electrophoresis configuration the anode is located at the injection side of the capillary and the cathode at the detection end of the capillary. In such a configuration, the electroosmotic flow moves from the injection to the detection end of the capillary (anode to cathode). These two transport mechanisms are combined for net flow. The electrophoretic mobility of cations drives them towards the detection end of the capillary. Small highly charged cations have a faster electrophoretic velocity than large highly charged cations, and all cations are transported by the sum of electroosmotic and electrophoretic transport. Neutral compounds have no electrophoretic mobility and are transported solely by electroosmotic flow. Thus, a neutral analyte is not separated from other neutral compounds. The electrophoretic mobility of anions drives them towards the injection end of the capillary (against the electroosmotic flow). Small highly charged anions have a faster electrophoretic velocity than large highly charged anions, and all anions are transported by the difference of electroosmotic and electrophoretic transport. The net effect of these transport mechanisms is visually represented in Figure 1.1.

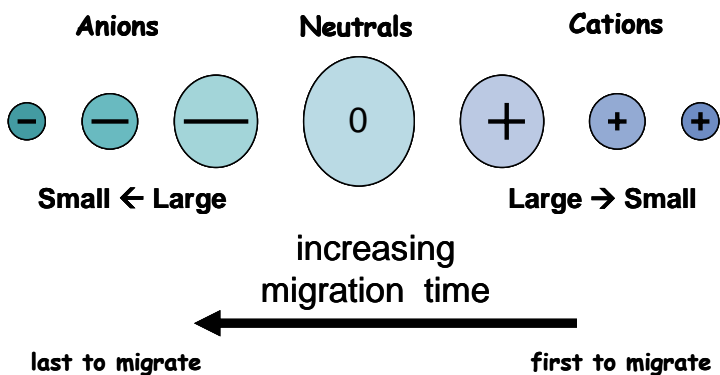


Figure 1.1 visually depicts analyte migration in a typical capillary electrophoresis system (anode at injection end, cathode at detection end). Cation migration is a result of the sum of electrophoretic and electroosmotic velocities. Neutral compounds migrate after cation. Anion migration is a result of the difference of electroosmotic flow toward the detector and electrophoretic transport away from the detector.

The Instrument. A capillary electrophoresis instrument can be described in terms of five components: the injector, the capillary, the voltage source, the detector, the digital-to-analog converter. The purpose of the injection mechanism is to introduce discrete plugs of sample into the separation capillary. This may be accomplished using siphoning, pressure or voltage. No matter which of these injection methods is used, to obtain reproducible data injection parameters, such as the capillary height, applied pressure, or applied voltage, must be well-defined. In addition, each mode requires the duration of the injection is well-defined. Thus, a timer or timing device is often necessary.

The second component of a capillary electrophoresis system is the separation capillary. These capillaries are typically constructed of fused silica, although they have also fabricated from other materials such as borosilicate glass or Teflon [1-6]. The surface charge of the capillary affects the electroosmotic flow. Furthermore, the surface may be covalently modified to display different functional groups, or additives to the running buffer itself may effect the surface charge [7-13]. The inner diameter generally ranges from 10 to 100 microns, although capillaries of < 1 micron inner diameter have been successfully used [14-16]. Fused silica capillary used in capillary electrophoresis is typically coated with polyimide. This polymer imparts flexibility to the capillary making it more practical for use. Both ends of the separation capillary are immersed in vials of ~2-5 mL volume and the vials and capillary are filled with a solution capable of conducting current. Usually, this is an aqueous solution buffered to a certain pH value using a good buffer producing separation currents less than 100 microamperes. Low separation current is desirable in a capillary electrophoresis separation because of resistive heating. If heat is not adequately dissipated from the separation capillary, convective flow will degrade the separation efficiency. At the extreme, solvent will boil, and current flow will cease due to bubble formation within the capillary. Smaller diameter capillaries have lower separation currents, and therefore generate lower resistive heating. In addition, smaller capillaries more efficiently dissipate heat generated in the separation. The drawback to small inner diameter capillaries is that they plug more frequently than larger diameter capillaries. Like inner diameter, the length of the separation capillary may also vary.

The third component of a capillary electrophoresis system is the high voltage power supply. This is used to apply voltage to either the anodic or cathodic reservoir, via a platinum electrode in contact with the background electrolyte in the buffer reservoir. Voltage (either positive or negative) is applied to one reservoir, while the other reservoir is grounded. Platinum electrodes are used as a means to connect the high voltage to the capillary electrophoresis running buffer because platinum is relatively inert. It is important to remember that electrochemical side reactions will undoubtedly occur. For this reason, the running buffer volume is at least 1 mL and buffer is used to control the pH of the solution of background electrolyte. For an in-depth investigation of how capillary electrophoresis running buffer can be modified by the application of separation voltage see [17-21].

The fourth component is the detector. Capillary electrophoresis has been coupled to a number of different detection devices and can be made compatible with different detection strategies. The most common modes of detection in capillary electrophoresis are UV-visible absorbance detection, laser induced fluorescence, mass

spectrometry, or electrochemical detection. Both UV-visible and fluorescence detection are usually performed on-column by removing a small portion of the polyimide coating to make an optically transparent window. Electrochemical and mass spectrometric detection are performed at the end of the capillary. The work outlined in this experiment is based on UV-visible absorbance detection coupled on-column with the electrophoresis capillary. Absorbance detection is a nearly universal technique, since most analyte absorbs radiation in the UV or visible region. The absorbance of incident radiation is linearly related to concentration (Beer's Law). UV-visible absorbance detection can provide quantitative and qualitative information using standards, or spectral analysis.

The fifth component of a capillary electrophoresis instrument is the equipment that enables conversion of the analog data output by the detector to digital format for software analysis. The custom-built instrument outlined in these materials incorporates a computer with a data card that performs analog-to-digital conversion. Any card must be addressed using code or software. The instructions we have provided for building a capillary electrophoresis system invoke commercially available software that drives the analog-to-digital conversion card and provides a convenient means to fit the resulting data and return quantitative information (peak moment, height, width). The Igor driver and software we have proposed for use process the information collected by the card. We have included some startup tips for using software from this vendor. A commercial instrument will also incorporate a means of reporting data in digital form as well as software to allow data analysis. If you are using a commercial instrument, refer to the instrument manual for information regarding data collection and analysis.

Basic Protocol. Successful application of a capillary electrophoresis instrument requires knowledge of the performance, standard operating procedures and a working knowledge of the figures of merit that will be necessary for analysis. Most users learn what is important through experience. To streamline this process, we have posed questions in a dry-lab format that will assist new researchers in documenting strategies for use and operation. As a result, this learning module requires the user to devise and document operating procedures for recording data in a laboratory notebook, preparing the separation capillary, and making chemical standards and running buffer. Each lab will have unique protocol based on practices and available equipment. Most standard operating procedures undergo multiple revisions as laboratory users become more experienced and focus on particular applications. Thus, we expect the protocol outlined for Learning Module I will undergo continual revision.

Application. Capillary electrophoresis is useful for rapid efficient analyses of a variety of compounds including ions, small molecules, drugs, amino acids, peptides, proteins, DNA, RNA, oligonucleotides, lipids, other polymers, carbohydrates, and other compounds. The method may be applied for qualitative or quantitative analysis. In either case, the user must have formulated the experimental procedures necessary to implement these analyses. To emphasize fundamental concepts, Learning Module I requires the user to anticipate migration order in free zone capillary electrophoresis for anionic, neutral and cationic compounds (tolmetin, mesityl oxide, atenolol). Following completion of the dry-lab, the user will implement the protocol she/he has devised by completing a single capillary electrophoresis separation of these compounds.

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Materials

In order to complete Learning Module I 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 50 microns.
- (2) Chemicals: atenolol, deionized water, mesityl oxide, sodium hydroxide, tolmetin, 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. Draw a schematic representation of a capillary electrophoresis system. Include instrumental components. Make the diagram as detailed as possible.
2. How are analytes separated by free zone capillary electrophoresis? Describe the fundamental principles that govern the method.
3. What type of detector will you use for this research project? What fundamental spectrophotometry principle(s) govern(s) analyte response in the detector?
4. Define the following terms: quantitative, qualitative, mean, median, standard deviation, relative standard deviation, significant figure, linear range, dynamic range, reproducibility, limit of detection, limit of quantification, analyte, sample, standard, and calibration curve.

Quantitative.

Qualitative

Mean

Median

Standard Deviation

Relative Standard Deviation

Significant Figures

Linear Range

Dynamic Range

Reproducibility

Limit of Detection

Limit of Quantification

Analyte

Sample

Standard

Calibration Curve

5. Write the standard operating procedure to make a stock solution of 1mM tolmetin dissolved in deionized water. Include a sample calculation.
6. Write the standard operating procedure to dilute a 1 mM stock solution of aqueous tolmetin to a final concentration of 100.µM. Note: the 100.µM tolmetin must be diluted in 50mM pH 7.0 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES). Include a sample calculation.

7. Mesityl oxide and tolmetin were separated using free zone electrophoresis under the following conditions: 50 micron i.d., 42 cm fused silica capillary, 50 mM pH 7.0 phosphate buffer, separation voltage 16kV, detection at 206 nm. Using the information provided below, report the mean migration time, mean peak height, mean peak area, mean plate count/meter for mesityl oxide as well as tolmetin. For each mean value determined, also report the standard deviation and relative standard deviation.

| Run # | Analyte | Migration Time | Peak Width at Base | Peak Height | Peak Area |
|-------|---------------|----------------|--------------------|-------------|-----------|
| 1 | mesityl oxide | 3.682 | 0.10 | 21932 | 59390 |
| | tolmetin | 7.975 | 0.19 | 11295 | 99031 |
| 2 | mesityl oxide | 3.758 | 0.11 | 22841 | 65364 |
| | tolmetin | 8.175 | 0.20 | 13255 | 110354 |
| 3 | mesityl oxide | 3.725 | 0.10 | 22780 | 63392 |
| | tolmetin | 8.329 | 0.20 | 12326 | 105155 |

Mesityl Oxide

Mean Migration Time = ____ (SD = ____ RSD = ____ %)

Mean Peak Height = ____ (SD = ____ RSD = ____ %)

Mean Peak Area = ____ (SD = ____ RSD = ____ %)

Mean Plate Count/Meter = ____ (SD = ____ RSD = ____ %)

Tolmetin

Mean Migration Time = ____ (SD = ____ RSD = ____ %)

Mean Peak Height = ____ (SD = ____ RSD = ____ %)

Mean Peak Area = ____ (SD = ____ RSD = ____ %)

Mean Plate Count/Meter = ____ (SD = ____ RSD = ____ %)

8. List the compounds of interest for the first phase of research.
9. By what mechanism(s) do the compounds comprising the answer to question 8 separate via capillary electrophoresis?
10. You are given three samples to analyze using a capillary electrophoresis-UV-visible absorbance system. Sample A contains ascorbic acid and mesityl oxide, sample B contains tolmetin and mesityl oxide, sample C contains mesityl oxide and either ascorbic acid or tolmetin. All analyte are at a concentration of 100 micromolar TES buffered at pH 7. Outline the procedure you would use to analyze these samples with your instrument and determine the identity of the analyte peaks in sample C. Assume you have access to standards if necessary. Provide rationale for your outlined procedures. Note any anticipated difficulties you might encounter.
11. Write a standard operating procedure for entering information into your laboratory notebook.

12. You are trying to separate a sample that contains both mesityl oxide and ascorbic acid. What is the order of migration of each analyte? (i.e. which analyte comes off the capillary first?) Explain/justify your answer.
13. How do you determine the identity of analyte peaks in an electropherogram?
14. How do you determine the concentration of analyte in a sample using CE-UV-visible absorbance detection (assume no sample matrix effects)?
15. Why is flushing/rinsing the capillary important? What flush/rinse procedures should you use?
16. What safety hazards must you consider before beginning this project?
17. Write the standard operating procedure to analyze the samples listed below using flow injection analysis with your CE-UV-visible absorbance instrument. Analytes: mesityl oxide, tolmetin, atenolol.
18. What is the molecular weight of each analyte listed below? Analytes: mesityl oxide, tolmetin, atenolol.
19. What is the net charge of each analyte in aqueous solution buffered at pH 7.0? Analytes: mesityl oxide, tolmetin, atenolol.
20. In a conventional free zone capillary electrophoresis system (i.e. anode at injection end, cathode at detection end), what is the order of migration of the following analytes: mesityl oxide, tolmetin, atenolol (assume background electrolyte is aqueous, buffered at pH 7.0)?
21. Write the standard operating procedure to analyze the sample listed below using free zone capillary electrophoresis with your CE-UV-visible absorbance instrument. Be sure to include protocol for injection, detector operation, and flushing/rinse. Sample contains the following analytes: mesityl oxide, tolmetin, atenolol.
22. Provide an electropherogram of a single sample containing the following three analytes: neutral marker (mesityl oxide or dimethylformamide), tolmetin, atenolol, each at a concentration of 100 micromolar. Note, this solution must be diluted with background electrolyte. Identify each peak in the electropherogram. Indicate how you verified the identity of each peak experimentally.

23. Using the electropherogram provided for question 22, complete the following information:

| | |
|------------------------------------|-----------------------------|
| sample: | run date and time: |
| run name: | computer filename: |
| notebook entries: | capillary dimensions: |
| background electrolyte: | |
| flushing protocol: | |
| injection duration: | injection pressure: |
| separation voltage: | separation current: |
| detection wavelength: | detection range: |
| detection rise time: | |
| tolmetin migration time: | tolmetin peak height: |
| tolmetin width (σ): | tolmetin plate count: |
| neutral marker migration time: | neutral marker peak height: |
| neutral marker width (σ): | neutral marker plate count: |
| atenolol migration time: | atenolol peak height: |
| atenolol width (σ): | atenolol plate count: |

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Follow-up Activities

Now that you have completed Learning Module I, you have documented procedures for operating a capillary electrophoresis system. Following the separation you completed in steps 22 and 23, consider whether you would revise any of the protocol you developed before your first use of the instrument. 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 I. 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 I, congratulations! Capillary electrophoresis is a flexible separation technique that requires some experience for developing methods that provide the selectivity and/or efficiency requisite for a particular application. Learning Module II is a self-guided exercise designed to teach you the basics of sample introduction and to introduce you to quantitative analysis. This will assist you in optimizing your run protocol for future capillary electrophoresis analyses. The procedures you have mastered in Learning Module I will be required to further refine your laboratory skills in the second Module.

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