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Separation of Saturated and Unsaturated Free Fatty Acids Using Capillary Electrophoresis with Indirect Photometric Detection

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Abstract

The capillary zone electrophoresis separation of various mixtures of saturated and unsaturated free fatty acids (FFAs) is performed using adenosine monophosphate (AMP) as an indirect photometric detection reagent. The presence of Brij in the electrolyte and a temperature of 40°C in the running buffer are required to improve solubility of the very long chain FFAs. The separation of C₁₂–C₃₁ differing by only one carbon is achieved using N-methylformamide (NMF)–dioxane (3:2), 40mM Tris, 2.5mM AMP, and 0.5% (w/v) Brij in approximately 40 min. However, baseline resolution of unsaturated isomers of FFAs (C₁₄–C₂₂) requires the presence of water, because water enhances the solubility of unsaturated isomers, resulting in improved resolution. A mixture of 13 saturated and unsaturated isomers of C₁₄–C₂₂ FFAs is baseline resolved in 37 min using a mixture of NMF–dioxane–water (5:4:1).

Introduction

Free fatty acids (FFAs) are an important class of naturally occurring compounds that can be found in living cells. These compounds differ in their chain length, branches, degree of unsaturation, position, and configuration of double bonds (1). The separation of FFAs is important in studying the biological activity of cells. In addition, the analysis of food for FFAs is required for quality control (2).

Numerous methods employing gas chromatography (GC) (3–5) and high-performance liquid chromatography (HPLC) (6–9) have been described for the separation of FFAs. However, both GC and HPLC often require precolumn derivatizations to enhance the volatility and detectability, respectively.

FFAs contain an acidic hydrogen because of their carboxylic acid functional groups. Therefore, these compounds predominantly exist as anions in basic solutions. Considering the differences in charge-to-mass ratios, both saturated (10–12) and unsaturated (13) FFAs can be separated by capillary zone electrophoresis (CZE). Saturated FFAs exhibit weak absorption in the region of 200 nm. Therefore, capillary electrophoresis (CE) with direct ultraviolet (UV) detection is problematic and results in limited sensitivity. In addition, the use of low wavelengths not only impairs the utility of many organic solvents and buffer systems, it also results in increased interference from the biological matrix. The key element for indirect photometric detection (IPD) is to maintain a large, continuous background absorbance signal at the UV detector by employing a detectable ionic (chromophoric) species in the running electrolyte. If the concentration of the light-absorbing ions remains constant in the electrical double layer in CE columns, a steady background absorbance translated as a stable baseline is displayed on the electropherogram. When a non-UV detectable ionic species passes the detection window, the original high level of the
absorbance signal is decreased because of the dilution of the chromophoric compound by the transparent analyte molecules. This technique provides a simple, easy, and time-efficient approach for the detection of FFAs. Micellar electrokinetic chromatography (MEKC) with direct detection (14) or IPD (15) has also been developed to separate long chain FFAs (C₈–C₂₀).

The CZE separation of very long chain (C₂₁–C₃₁) saturated and unsaturated FFAs is difficult using either aqueous or partially aqueous electrolyte. First, C₂₁–C₃₁ FFAs have poor solubility, and they tend to form micelles. Although the use of a higher fraction of organic solvents disrupts the micelles, it also results in longer analysis time. Second, the difference in electrophoretic mobility between two consecutive homologues of FFAs rapidly decreases with increased alkyl chain length for those possessing greater than 20 carbon atoms. For these reasons, the use of a non-aqueous electrolyte for the CZE separation of very long chain FFAs has been recently developed by Drange and Lundanes (10). The authors showed that separation of C₁₄–C₂₆ FFAs could be conveniently accomplished in 15 min using anthraquinone-2-carboxylic acid (ANT) in N-methylformamide (NMF) and dioxane. However, their method was not optimized for the resolution of unsaturated FFAs. In addition, the separation of only even chain numbers of C₁₄–C₂₆ was reported. The separation of long chain (C₁₂–C₂₀) and very long chain (C₂₁–C₃₁) FFAs differing by only one carbon atom requires the investigation of a new IPD reagent.

Adenosine monophosphate (AMP) has been shown to be a useful chromophore for IPD in aqueous and partially aqueous solutions (16–19). In this report, the potential of AMP as an IPD electrolyte for the separation of saturated FFAs (C₁₂–C₃₁) differing only by one carbon atom is investigated. In addition, a partially aqueous CZE system is optimized for the separation of a complicated mixture of unsaturated (C₁₄–C₂₂) FFA isomers.

**Experimental**

**Instrumentation**

The CE instrument was a BioFocus 3000 CE (Bio-Rad, Hercules, CA) equipped with a UV detector. Untreated fused-silica capillary (50-μm i.d., 320-μm o.d., 45-cm effective length) was purchased from Polymicro Technologies (Phoenix, AZ).

**Reagents and chemicals**

NMF and dioxane were purchased from Fluka (Bellefonte, PA). The monosodium salt of AMP (99%), ANT, and Trizma base (Tris) were obtained from Sigma (St. Louis, MO). Saturated FFAs such as lauric acid (C₁₂₁₀), tridecanoic acid (C₁₃₁₀), myristic acid (C₁₄₁₀), pentadecanoic acid (C₁₅₁₀), palmitic acid (C₁₆₁₀), heptadecanoic acid (C₁₇₁₀), stearic acid (C₁₈₁₀), oleic acid (C₁₈₁₀), erucic acid (C₂₂₁₀), and linoleic acid (C₁₈₂₀) were all obtained from Sigma (Bellefonte, PA).
**Preparation of solutions**

A running electrolyte solution of AMP and 40mM Tris buffer was prepared in different concentrations (v/v) of NMF–dioxane. The final buffer was filtered through a 0.45-µm Nalgene (Rochester, NY) Nylon filter and used without any pH adjustment. All FFA standards were dissolved in NMF–dioxane (4:1, v/v), sonicated, and filtered prior to use.

**Procedure**

A new capillary was subjected to a standard wash cycle for 30 min using 1M NaOH and 10 min using triple-deionized water at room temperature. As a daily routine, a capillary was flushed with 1M NaOH for 10 min and water for 5 min. Between injections, the capillary was flushed for 2 min with each of the following solutions: 0.1M NaOH, triple-distilled water, and the running electrolyte. Samples were pressure injected for 1 s. The capillary temperature was controlled with an aqueous coolant. Separations were performed at +20 kV. IPD was performed at 259 nm and 264 nm using AMP (18) and ANT (10), respectively.

**Results and Discussion**

Several parameters were studied to optimize the separation of saturated (C12–C31) FFAs and unsaturated (C14–C22) isomers under nonaqueous and partially aqueous CZE conditions. These parameters included the concentration and choice of the IPD reagent, volume fraction of organic modifier, and temperature.

**Type and concentration of IPD reagent**

To perform the separation of FFAs differing by one carbon with a reasonable peak capacity and efficiency, an IPD reagent with electrophoretic mobility similar to the analyte ions should be used. Mobility matching between the analyte ions and the IPD reagent reduces peak dispersion, thus two analyte ions with small difference in mass-to-charge ratio can be resolved (20).

The effect of AMP concentration on the sensitivity of FFA signals was studied using 1, 2.5, and 5mM solutions. With respect to electrophoretic separation and detection, a 2.5mM AMP solution was found to be a good compromise. In the literature, ANT has also been introduced as an IPD reagent for nonaqueous CZE (10). The effect of ANT concentration on the migration behavior of saturated FFAs was studied (data not shown). At 7mM concentration, ANT electrolyte completely absorbed the UV light. In addition, ANT provided poor sensitivity for very long chain FFAs (Cn, n > 26). Moreover, AMP provides more rapid separation of FFAs (electropherogram not shown). Therefore, it was concluded that AMP is a better IPD reagent for the separation and detection of long chain FFAs.

**Effect of organic modifier on separation of FFAs**

To achieve the best separation efficiency for both saturated and unsaturated FFAs, optimization of solvent composition was necessary. The propensity of long chain FFAs to form micelles and their poor solubility in aqueous electrolytes causes serious problems in the separation of this group of compounds. CZE separations of FFAs (Cn, n < 18) have been reported using 60% methanol (11). In addition, the separation of FFAs containing up to 20 carbons has been achieved using acetonitrile and nonionic surfactants such as Brij (15). The use of Brij facilitates the solubility of long chain FFAs. As discussed earlier, Drange and Lundanes separated only even chain number FFAs (C14–C20) in nonaqueous media (10). In order to separate FFAs differing by only one carbon, a better understanding of organic solvent composition is required. Figure 1 shows the variation of the relative migration time (tR/t0) of C12–C24 FFAs versus the percentage
(v/v) of dioxane in NMF. As shown, at high NMF content, the variation of \( t_{\text{R}}/t_{0} \) values of long chain FFAs (e.g., C24 and C33) are not as pronounced as with shorter chains (e.g., C12 and C13) due to the fast electroosmotic flow (EOF). Decreasing the percentage of NMF (increasing the percentage of dioxane) decreases the EOF. Additional solvent studies indicated that dioxane improves the solubility of the very long chain FFAs to a certain degree. For example, baseline resolution of C28 and C29 was obtained at 40% (v/v) dioxane. However, dioxane did not affect the resolution of C30 and C31. At concentrations above 50% (v/v) dioxane, no significant improvement in the resolution of C28–C31 was observed.

To optimize the CZE conditions for unsaturated FFAs, C18 isomers were chosen. The \( t_{\text{R}}/t_{0} \) of C18 isomers versus the percentage (v/v) of NMF is shown in Figure 2. At 100% NMF, all five isomers coeluted, and at 60% NMF–40% dioxane (v/v), maximum difference in \( t_{\text{R}}/t_{0} \) values of the five isomers was obtained. Figure 3A shows the CZE separation of the C18 isomers using optimized non-aqueous (60% NMF–40% dioxane) conditions. All isomers were baseline resolved except for C18:2 and C18:3. The partial resolution between C18:2 and C18:3 is possibly caused by the aggregation of these two isomers. To achieve baseline resolution and to overcome the aggregation, 10% (v/v) water was added to the running buffer. Under such conditions, a near-baseline separation of all five C18 isomers was obtained (Figure 3B). However, a water content of less than 10% resulted in decreased resolution.

**Effect of temperature**

Dioxane and NMF have relatively high boiling points (102°C and 200°C, respectively); therefore, the effects of temperature on the separation of C12–C24 FFAs were studied. Different temperatures (20–30°C and 40°C) under optimum conditions (NMF–dioxane, 3:2) were compared, and the results showed that at 40°C, more sensitive signals for short and long chains can be obtained in comparison with lower temperatures. However, temperature does not influence the resolution of the five unsaturated C18 isomers.

Reproducibility for saturated FFA migration times between sequential runs was investigated at several temperatures. The average relative standard deviations for the migration times of five peaks (C12–C10) from 10 different runs was found to be 0.8%. The results showed that temperature does not have a significant effect on reproducibility. In addition, a stable baseline at 40°C was obtained without any significant increase in current.

**Separation of saturated FFAs**

Separation of saturated FFAs was performed using 2.5mM AMP and 40mM Tris in NMF–dioxane (3:2) at 40°C (Figure 4). Solubility of the very long chain (C26–C31) FFAs in NMF dioxane was poor. To improve the solubility of C26–C31 FFAs and to achieve a resolution between C30 and C31, Brij was added to the running buffer. Nonionic surfactants such as Brij have been shown to improve the solubility of long chain FFAs (15); however, Brij slows the EOF and increases the viscosity. In this work, the effect of Brij concentration on the migration time of long chain FFAs was investigated. Below the critical micelle concentration of Brij, small surfactant aggregates improved the solubility of the very long chain FFAs. At concentrations below 0.5%, C30 and C31 coelute; at concentrations greater than 0.5%, the elution time of FFAs increases drastically. In addition, the presence of Brij results in peak broadening for FFAs with an alkyl chain length of less than 10 carbon atoms. The optimum concentration of Brij in the running buffer was found to be approximately 0.5% (Figure 4B).

As expected in a mixture of C12–C31 FFAs, the longest chain FFA (C31) eluted first and the shortest chain (C12) FFA eluted last. This is because the longer chain FFAs are less mobile and are rapidly swept toward the negative electrode (detection end) by the EOF. It should be reiterated that the difference in electrophoretic mobility of FFAs decreases with an increase in alkyl chain length. Therefore, the resolution among C26–C31 FFAs is less than that among C12–C16 FFAs. Further studies showed that elimination of Brij from the electrolyte under optimum conditions resulted in the separation of C5–C31 FFAs using a single CE run. However, long-chain FFAs exhibited poor sensitivity and short-chain FFA peaks were broadened. It should be noted that short chain FFAs (C1–C10) can be conveniently separated in aqueous solution using 2.5mM AMP and 40mM Tris.

**Separation of mixtures of saturated and unsaturated free fatty acid isomers**

As discussed earlier, the use of a nonaqueous electrolyte results in the partial resolution of C18, and the presence of water improves the resolution of unsaturated isomers. Therefore, a combination of NMF, dioxane, and water in the ratio of 5:4:1 was required for the separation of complicated mixtures of unsaturated FFAs (C14–C22 with 0, 1, and 2 double bonds). Again, lowering the NMF content in the running electrolyte down to 50% (v/v) results in a slow separation and the loss of peak capacity. In contrast, at 0% NMF (dioxane–water, 50:50, v/v), the separation time

![Figure 6. Electropherogram of unsaturated FFA (C14–C22). Conditions were the same as in Figure 5.](https://academic.oup.com/chromsci/article-lookup/doi/10.1093/jcrom/37.4.103)
increased to 90 min. Figure 5 shows the separation of C_{14}–C_{22} saturated and unsaturated isomers. Isomers with three degrees of unsaturation for even chain length (e.g., C_{22:3}) coeluted with the saturated forms of the next even chain length homologues (e.g., C_{20:0}). Similarly, C_{20:3} and C_{18:3} coeluted with C_{18:0} and C_{16:0}, respectively. However, under the same optimum conditions, single-, double-, and triple-unsaturated isomers of C_{18}–C_{20} FFAs (and nearly C_{22} isomers) can be baseline separated in a single CE run (Figure 6). In addition, the baseline separations of six different isomers of C_{20} (0, 1, 2, 3, 4, and 5 double bonds) and five isomers of C_{22} (0, 1, 2, 3, 4, and 6 double bonds) can be achieved in 25 and 30 min, respectively (electropherogram not shown).

Conclusion

The advantage of using AMP as an IPD reagent is that AMP is soluble in aqueous, partially aqueous, and nonaqueous media. Therefore, the separation of different saturated and unsaturated FFA homologues is feasible using AMP. In addition, standard mixtures of very long chain saturated (C_{21}–C_{31}) FFAs can be separated in a single run with long chain (C_{12}–C_{21}) FFAs in the presence of 0.5% (v/v) Brij in nonaqueous electrolyte.

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