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Semipreparative Capillary Electrochromatography

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Capillaries with inner diameters of 550 μm have successfully been packed with 1.5-μm octadecyl silica particles using frits made of macroporous polymers by the UV photopolymerization of a solution of glycidyl methacrylate and trimethylolpropane trimethacrylate. This type of frit is found superior to one made of low-melting point poly(styrene-*co*-divinylbenzene) beads. Bubble formation is not observed to occur within these capillary columns under our experimental conditions. Separations can be achieved with sample injection volumes as high as 1 μL. To demonstrate its semipreparative use, a mixture of 500 nL of taxol (20 mM) and its precursor, baccatin III (30 mM), is separated using such a column with a Tris buffer.

Capillary electrochromatography (CEC) is a powerful separation technique that combines high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). It has attracted much interest in the past few years because it has (1) high separation efficiency, (2) high selectivity, (3) low solvent consumption, and (4) low operational costs. Recently, several research groups have presented many impressive applications of CEC. A broad variety of different species, such as corticosteroids,[1] amino acids,[2−4] proteins and peptides,[5−8] carbohydrates,[9] environmental contaminants,[10,11] natural products,[12,13] and pharmaceuticals,[14−16] have successfully been separated using CEC. Several analyses indicate that the superiority of CEC over HPLC is maintained for complex biological species and pharmaceuticals. To our knowledge, analyses of such complex molecules using CEC in a semipreparative scale have never been reported**,** with the motivation being not only to separate but also to isolate the purified components of the mixture. Some researchers have been devoting their efforts toward the development of semipreparative techniques based upon CE.[17−20] The use of wide-bore (up to 180 μm) capillaries is one of the approaches being investigated[18] in an attempt to increase sample loading without increasing the injection plug length.

Our approach is to use wide-bore capillaries packed with octadecyl silica (ODS) particles so that both charged and neutral compounds can be separated on a semipreparative scale. To date, most of the CEC columns used have an inner diameter (i.d.) of 50−100 μm and an effective length of 15−20 cm**.** In this work, we have successfully employed capillaries with much larger inner diameters (≥550 μm), each with a packed bed of about 16−17 cm in length.

Among CEC studies, over 70% of the work utilizes packed CEC columns. In comparison to open tubular CEC, packed CEC columns have the advantages of higher capacity and larger retention capabilities. Various types of HPLC stationary phases can also be packed in capillaries, making packed CEC versatile. However, frit fabrication is one of the major challenges in using packed columns. Several different approaches toward solving this problem have been reported to date. The preparation of monolithic columns by a polymerization process within the confines of the capillary has been one of the dominating alternatives.[21−24] Tapered capillaries without frits have been studied as well.[25] Other

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approaches include the use of surface-functionalized open-tubular columns, [26−28] sintering of silica particles within a column,[3] entrapment of silica particles using sol−gel techniques,[29−31] and silicate polymerization for preparation of the frit.[32] Despite the simplicity of their preparation, monolithic columns remain in the shade of packed columns since a large number of practitioners of CEC arrived at this area from HPLC where the column packings were perfected to a very high degree.

Several frit fabrication methods have been applied to large-bore capillaries. We discovered that it is very difficult to fabricate frits using the conventional preparation methods. In these methods, heat is required to fuse together a section of packing material, but heat does not easily penetrate through the silica particles. Therefore, the center of the packing may not fuse as well as the region near the capillary wall. Entrapment of silica particles using sol−gel techniques[29−31] that have been performed in our group earlier requires extremely well-controlled conditions since the sol−gel process leads to a dramatic shrinkage. This might lead to the creation of excessively large cavities within the large-bore capillary column.

Two approaches to frit preparation in large-bore capillaries have succeeded in our laboratory. One approach is to use low-melting point poly(styrene-*co*-divinylbenzene) beads with a diameter of 6 μm. We have also recently invented a way of preparing frits by photopolymerization.[33] The later procedure has been adapted to large-bore capillaries and possesses several advantages over the former method: (1) simplicity of the preparation process, (2) significantly shortened preparation time, and (3) reduced bubble formation. In this paper, we describe and compare the frit preparation procedures for large-bore capillaries using the two different frit fabrication methods. The resulting preparative CEC columns have been evaluated under various running conditions and employed to separate taxol, which is used in the treatment of ovarian and breast tumors, from its precursor, baccatin III.

EXPERIMENTAL SECTION

Materials. The monomers 2,3-epoxypropyl methacrylate (glycidyl methacrylate, GMA) and trimethylolpropane trimethacrylate (TRIM) were of the highest purity (Aldrich, Milwaukee, WI). Toluene and 2,2,4-trimethylpentane (isooctane) were used as porogenic solvents (Sigma, St. Louis, MO). The fused capillaries were purchased from Polymicro Technologies (Phoenix, AZ). The 1.5-μm spherical ODS particles were provided by Micra Scientific Inc. (Lafayette, IN). Poly(styrene-*co*-divinylbenzene) (latex beads, 6-μm diameter), α-methoxy-α-phenylacetophenone (benzoin methyl ether, 99%), thiourea, 2-methylnaphthalene, sodium phosphate, tris(hydroxymethyl)aminomethane (Tris), and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, MO). Water was purified with an Ultrapure water system from Millipore (Milford, MA).

Frit Fabrication Using Latex Beads and Column Packing. The detection window of a 34-cm-long capillary column with a 550-μm i.d. was made by using a thermal wire stripper (Teledyne Electronic Technologies, Los Angeles, CA) to remove a 1-mm-long section of polyimide coating at the middle of the column. An inlet frit, which was 17 cm away from the detection window, was made by heating a small section of silica particles (10−40 μm in diameter) that were tapped into the tip of the capillary column. Glass beads (90 μm in diameter) were then filled into the capillary by gravity until they reached the detection window. This filling process took 3−4 min. About 100 μL of an aqueous latex bead suspension was introduced into the capillary, and the beads accumulated near the detection window. The plug length of the accumulated particles was ∼2 mm. Water in the column was removed by using a syringe pump. This plug of beads was heated with a thermal wire stripper for ∼30 s. The beads fused together and adhered to the inner wall of the capillary, becoming the outlet frit. The inlet frit was cut off, and glass beads were washed from the column with a syringe pump. Using a slurry packing method,[34] 1.5-μm ODS particles were packed into the capillary column having the outlet frit formed from polymer beads. The inlet frit was made by a method similar to that of the outlet frit. The column was then rinsed with the running buffer for over 6 h before use.

Frit Fabrication Using Photopolymerization and Column Packing. Polyimide coating at two sections (1−2 mm long each, 16 cm apart) of the 550-μm capillary was removed using a thermal wire stripper. A GMA/TRIM monomer mixture[35] was filled into the capillary with a syringe. The inlet section of the capillary column was covered with aluminum foil, and the capillary was exposed to 365-nm UV light provided by a Spectrolinker XL 1500A (Spectronics Corp., Westbury, NY) for ∼1 h. The unreacted solution and porogenic solvents were washed from the monolithic frit in the capillary with a syringe pump at 200 psi. The column was then packed with 1.5-μm ODS particles using a slurry packing method.[34] The length of the segment packed after 1 h was ∼17 cm, which is sufficiently long to cover the window cut at the inlet end of the bed. This end of the capillary was filled with the polymerization mixture containing GMA and TRIM using a syringe pump. The inlet frit of the column was then formed by UV-initiated polymerization of the exposed part of the capillary while the rest was masked with aluminum foil. Once the polymerization was completed, the excess of capillary was cut off, thus exposing the frit at the end of the capillary column. Because the entire packing process and monolithic frit fabrication proceeds at room temperature, the column did not dry out. Running buffer was then used to rinse the column for ∼3 h before use.

Capillary Electrochromatography. The CEC experiments were performed with a homemade CE instrument equipped with an UV absorbance detector (254 nm). No pressure or cooling was applied to the CEC experiments. Samples were injected electrokinetically at the anodic end of the capillary column. The mobile phases used in the CEC separations were mixtures of 5 mM phosphate (pH 7.0) or 10 mM Tris (pH 7.6) buffer solutions with acetonitrile. The percentage of acetonitrile varied from 50 to 80%. The applied voltage ranged from 2 to 7 kV across the capillary

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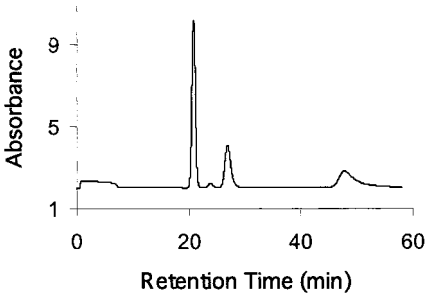

Figure 1. Electrochromatogram of four neutral compounds, which eluted in the order thiourea, benzyl alcohol, benzaldehyde, and 2-methylnaphthalene. Conditions: capillary column, 550-μm i.d. packed with 1.5-μm ODS particles having poly(styrene−divinylbenzene) beads as frits; mobile phase, 5 mM phosphate (pH 7.6) with 50% acetonitrile, UV detection at 254 nm; applied voltage, 2 kV; sample concentration, 2 mM of each compound; injection, 2 kV for 5 s.

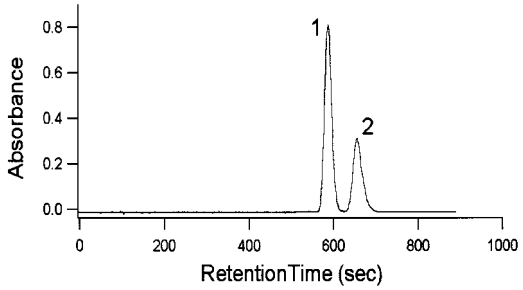

Figure 2. Electrochromatogram of thiourea and 2-methylnaphthalene obtained with a 550-μm-i.d. column packed with 1.5-μm ODS particles having photopolymers as frits. Peak 1, thiourea; peak 2, 2-methylnaphthalene. Conditions: mobile phase, 5 mM phosphate (pH 7.6) with 80% acetonitrile; applied voltage over the 17-cm effective length, 5 kV; injection volume, 60 nL.

Table 1. Electrical Conductivity (μS) of 5 mM Phosphate and 5 mM Tris with Different Percentages of Acetonitrile

buffer	0% ACN	40% ACN	50% ACN	80% ACN
phosphate	400	na[a]	190	150
tris	270	100	86.3	18

[a] Not available.

columns with a packed length of 16−17 cm. Separations were performed at a temperature of 20 °C.

RESULTS AND DISCUSSION

Frit Stability. *Fused Beads.* Because the polymer beads can be fused at a much lower temperature than that required to sinter silica beads, the polyimide coating does not need to be stripped from the surface of the capillary to fabricate the frits. Therefore, the detection window remains the only weak point in the column. The outlet frit easily withstands a short exposure to the high pressure of over 4000 psi used during the packing procedure. This behavior indicates that the poly (styrene-*co*-divinylbenzene) frit strongly adheres to the inner wall of the capillary. The openings between the fused polymer particles are sufficiently small to avoid the passing of silica beads through the frit during both the packing procedure and the CEC operation. The separation of four neutral compounds, shown in Figure 1, was performed in a 550-μm-i.d. capillary provided with frits prepared from polymer beads using an applied voltage of less than 120 V/cm. All four compounds are well resolved. At higher voltages, bubble formation was observed, which may be due to the considerable difference between the surface chemistries of the silica particles and the polymer beads. Despite the success in separating neutral compounds using capillary columns with poly(styrene-*co*-divinylbenzene) frits, this approach mimicking the "traditional" method of frit fabrication also has several weaknesses. For example, the porosity of the frits, which largely depends on the temperature and time used to fuse the polymer beads, is difficult to control. Therefore, we sought other means of fabrication.

Monolithic Frits. Our recent discovery that photopolymers can be used as frits in narrow-bore capillaries[33] represents an alternative solution to the problem of frit fabrication in large-diameter capillaries. This process is simple and robust. A detailed study[35] demonstrated that no radial gradient in pore size distribution existed in 4-mm-diameter photopolymer structures. Therefore, this method appears to be particularly suitable for fabricating frits in large-bore capillaries. Because the frit preparation process does not require elevated temperature, both the frits and the packing material of the column remain wetted. Therefore, the conditioning time for the column prior to its use is shortened significantly. During this study, more than 10 550-μm-i.d. capillary columns were successfully prepared using the photopolymer frits and tested for the separation of two neutral compounds, thiourea and 2-methylnaphthalene. A representative separation is shown in Figure 2. In contrast to fused frits, the columns with photopolymer frits can be used at electric field strengths as high as 420 V/cm. This improved performance may be caused by the differences in both surface chemistry and porous properties between these two types of frit structures. The major advantage of the monolithic frits is that the pore size of the material can easily be controlled. The average pore size of the frit is ~2.7 μm, as determined using a mercury intrusion porosimeter. In our previous study, a pressure of 180 psi and the addition of sodium dodecyl sulfate (SDS) were applied to avoid bubble formation. Although we did not use these conditions while running the CEC separation in large-bore capillary columns, no bubbles were observed even at currents over 20 μA. It was found that, by hydrolyzing the epoxide moiety of glycidyl methacrylate, the hydrophobicity of the frit could be modified.[36] Being able to tune the surface chemistry of frits allows us to match the properties of the frits with the properties of different stationary phases including reversed-phase chromatographic materials. Thus, the discrepancy in EOF between the packed bed and the frits can be avoided.

Fabrication of monolithic frits using photopolymerization has many advantages over fused polymer frits, such as the short and simple preparation process, fine control of porous properties as well as the location of the frits, and the elimination of bubble formation. Therefore, we focused our efforts on the performance of preparative columns with photopolymer frits.

CEC Separations. *Effect of Buffer.* Table 1 shows the comparison of the electrical conductivities of phosphate and Tris buffer solutions that were used as running buffers. Control of conductiv-

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Table 2. Comparison of Column Efficiency, Plate Height, and Resolution between Two Different Running Buffers (5 mM Phosphate with 80% Acetonitrile and 10 mM Tris with 80% Acetonitrile) Using a 550-μm-i.d. × 32-cm Column (16 cm Packed with 1.5-μm ODS Particles)

buffer	N (m)	H (μm)	R (s)
phosphate	30 000	33	1.1
tris	40 000	25	1.5

ity is an important issue in CEC because Joule heating results in band broadening. This heating also currently represents the major problems preventing larger diameter CE columns from being used. Tris buffer has a much lower electrical conductivity than phosphate buffer. Consequently, less Joule heating is produced using Tris buffer under the same running conditions. Therefore, Tris is a more suitable buffer for our desired preparative CEC separations. Table 2, which lists the efficiency, N, the plate height, H, and the resolution, R, of a packed column with 16-cm effective length, shows an additional benefit of using Tris buffer. The separation characteristics obtained with Tris are better than those with phosphate buffer. For example, the column efficiency increases ~33% for Tris.

Composition of Mobile Phase. The percentage of acetonitrile (ACN) in the running buffer is varied from 50 to 80%. A significant improvement in resolution is achieved on lowering the percentage of ACN. As shown in Figure 3, the resolution of thiourea and 2-methylnaphthalene is 4 times higher using the mobile phase containing 50% acetonitrile than that with 80% ACN. The decrease in percentage of ACN affords a mobile phase with higher viscosity, which decreases the velocity of the electroosmotic flow (EOF) as demonstrated by the increasing elution times of thiourea. Because thiourea is an unretained neutral molecule, it moves along the column at the speed of the electroosmotic flow. In addition, the bandwidth for thiourea changes insignificantly with the variation of percentage of ACN in the mobile phase. In contrast, there is a significant increase in retention time and band broadening for 2-methylnaphthalene. This behavior can be mainly ascribed to the stronger interaction between the hydrophobic stationary phase and this retained compound in higher aqueous content mobile phases. It is worth noting that a resolution as high as 4.8 was obtained for the separation of thiourea and 2-methylnaphthalene.

Voltage. Figure 4 shows the effect of increasing voltage on electroosmotic velocity. The electroosmotic flow becomes unstable for phosphate buffer at electric field strengths exceeding 380 V/cm. In addition, bubble formation also starts to occur at this electric field strength. In contrast, Tris buffer allows electric field strengths as high as 440 V/cm without affecting the stability of EOF. This behavior may again be ascribed to the lower electrical conductivity of Tris buffer compared to phosphate buffer.

Column Efficiency. The flow velocity in CEC is controlled by the applied voltage across the column length. As always in chromatography, column efficiency depends on the flow velocity of the mobile phase in the column. Figure 5 shows a van Deemter plot of theoretical plate height versus flow velocity obtained with thiourea as an unretained analyte. This curve has a typical shape with a minimum at a plate height of 21 μm. Although this plate

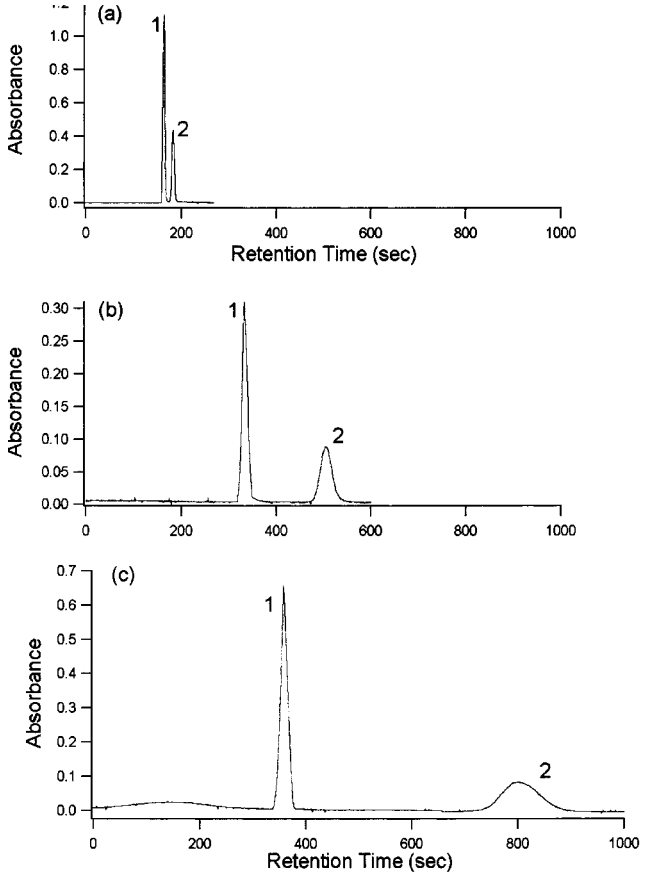

Figure 3. Electrochromatograms showing how the performance varies with changes in the percentage of acetonitrile in Tris buffer: (a) 80, (b) 60u, and (c) 50% ACN. Conditions: capillary column used is similar to the one used for Figure 2 except its effective length is 16 cm; mobile phase, 10 mM Tris (pH 7.6) with various amount of acetonitrile. For other conditions, see Figure 2.

height is higher than that observed for packed 75−100-μm-i.d. capillaries with similar size beads, it still represents a column efficiency of 45 000 plates/m, larger than that of many preparative HPLC columns. Because the column performs best at a flow velocity of 0.45 mm/s, which corresponds to an electric field strength of 300 V/cm, these conditions were also used to study sample injection.

Effect of Sample Volume. One of the major factors influencing the column band broadening is the length of injected sample plug. The plug length L_{inj} is given by[37]

$$L_{inj} = u_{eo} t_{inj} \quad (1)$$

where u_{eo} is the electroosmotic migration velocity and t_{inj} is the injection time. Theoretically, the effect of the injected plug length on the total peak variance can be expressed by[37]

$$\sigma_T = [(L_{inj})^2/12 + L^2/N]^{1/2} \quad (2)$$

where σ_T is the total variance of the column, L is the effective column length, and N is the plate number. On the basis of eq 2,

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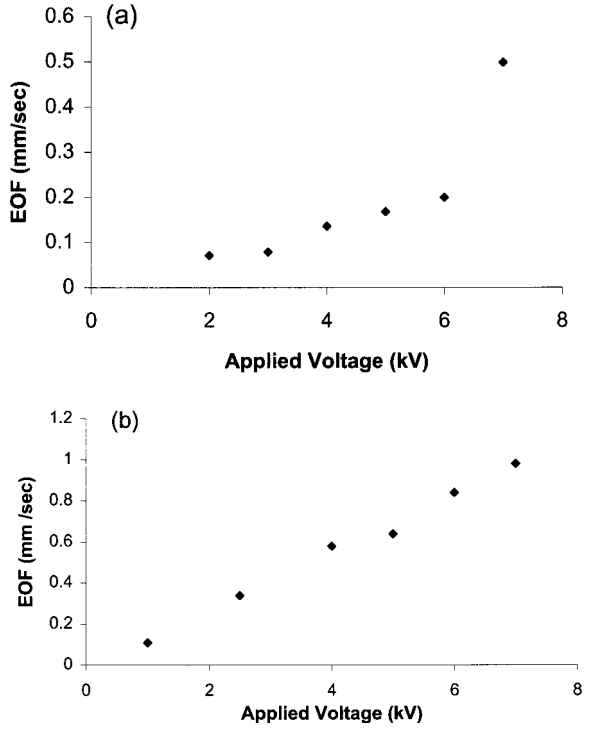

Figure 4. Comparison of EOF dependence on applied voltage using two different running buffers: (a) 5 mM phosphate (pH 7.6) with 80% acetonitrile and (b) 10 mM Tris (pH 7.6) with 80% acetonitrile. The column is the same as in Figure 3; injection, ~60 nL of thiourea (2.0 mM) was injected in each run.

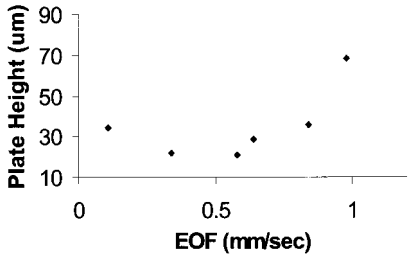

Figure 5. van Deemter plot of plate height vs electroosmotic flow rate for thiourea. Conditions: capillary column is the same as in Figure 3; mobile phase, 10 mM Tris (pH 7.6) with 60% acetonitrile. For other conditions, see Figure 2.

the shorter the injection plug, the less it contributes to band broadening. In contrast, the column efficiency decreases rapidly for injected amounts exceeding a specific threshold volume. Figure 6 shows the column efficiency as a function of the sample plug length for a column having an effective length of 16 cm and an efficiency of 40 000 plates/m. The threshold value of L_{inj} for this column is ~1.5 mm. The injected sample volume corresponding to this plug length is ~150 nL, which is more than 50 times higher than the volumes typically injected in capillary electrophoresis.

CEC Separations of Taxol and Baccatin III. Semipreparative CEC can be potentially useful for organic synthesists. Collected products along the synthetic pathways can be analyzed using semipreparative CEC to identify the possible components that have been synthesized. Each separated component can be further analyzed by using mass spectrometer or NMR. The most common method currently used is HPLC, where 1 mg of sample is required for such test. A smaller quantity of sample is required using semipreparative CEC and higher resolution can be achieved.

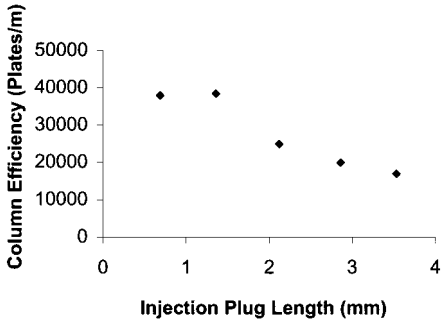

Figure 6. Plot of column efficiency vs injection plug length. The column is the same as in Figure 3, and the running conditions are the same as in Figure 5. The injection volumes of thiourea (2.0 mM) vary from 50 to 300 nL.

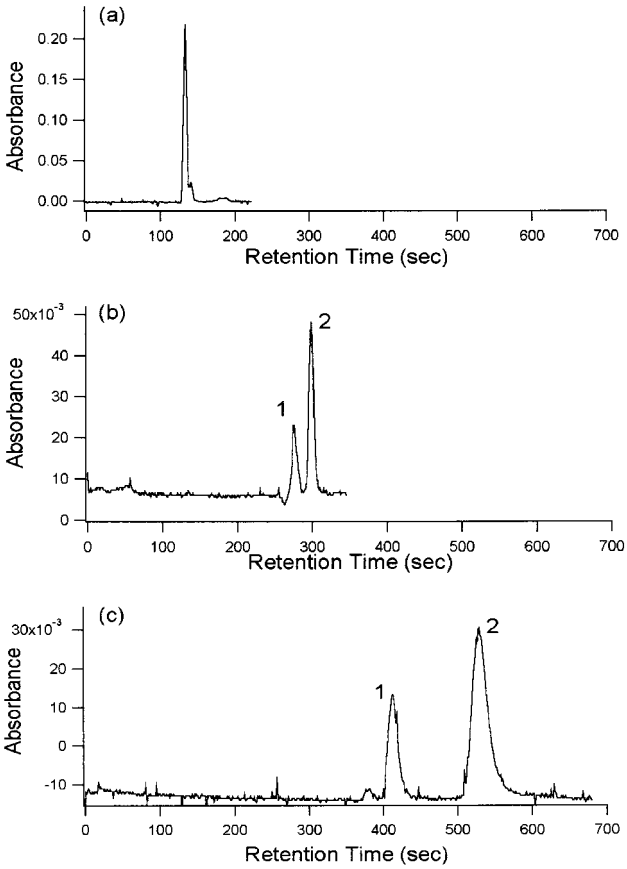

Figure 7. Electrochromatograms showing how the resolution of baccatin III (peak 1) and taxol (peak 2) varies with the percentage of acetonitrile in 10 mM Tris (pH 7.6) buffer: (a) 80, (b) 60, and (c) 50% ACN. Conditions: capillary column use is the same as the one used in Figure 3; applied voltage, 4 kV; injection, 4 kV for 2 s.

Separations of a popular anticancer drug taxol and its precursor, baccatin III, are studied to demonstrate this application.

Composition of Mobile Phase. The percentage of ACN in the running buffer for taxol and baccatin III is varied from 50 to 80% as it is for neutral test molecules. The resolution is significantly improved when the percentage of ACN is lowered. Figure 7 shows the separation of taxol and baccatin III at different percentages of acetonitrile. Taxol is a larger and more hydrophobic molecule than baccatin III and therefore more highly retained on the stationary phase. The resolution improves to 2.0 when the percentage of acetonitrile drops to 50%. The retention time

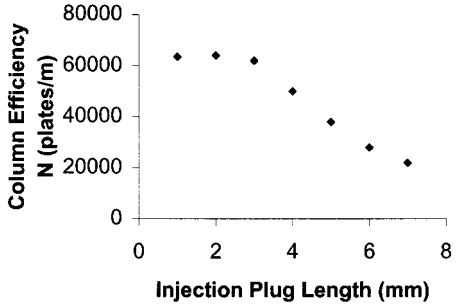

Figure 8. Column efficiency vs taxol injection plug length. Conditions: capillary column used is the same as the one used in Figure 3.; mobile phase, 10 mM Tris (pH 7.6) with 50% acetonitrile; applied voltage, 4 kV. The taxol (20 mM) injection volumes vary from 50 to 500 nL.

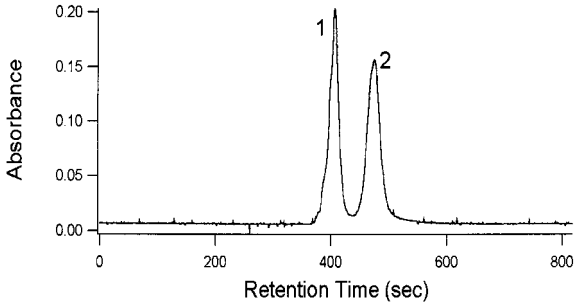

Figure 9. Electrochromatogram of baccatin III (peak 1) and taxol (peak 2). Conditions: capillary column used is the same as the one used for Figures 7 and 8; injection, 8.5 μg of taxol (20 mM, 500 nL) and 9 μg of baccatin III (30 mM, 500 nL); applied voltage, 4 kV. For other conditions, see Figure 7.

increases linearly with the decrease in percentage of acetonitrile. This behavior indicates that the partitioning of analyte between the reverse phase and the mobile phase is the dominating mechanism in this semipreparative CEC system.

Effect of Sample Volume. As discussed previously, the shorter the sample injection plug, the less it contributes to the band broadening. The column efficiency deteriorates rapidly for injected amounts exceeding a specific threshold volume. Figure 8 shows how the column efficiency is affected by the sample plug length for a column having an effective length of 16 cm and an efficiency of 60 000 plates/m. The threshold value of L_{inj} for this column is ~2.5 mm. The injected sample volume corresponding to this plug length is ~250 nL.

Maximum Sample Injection Amount. Experiments were carried out to determine the maximum amount of taxol and baccatin III that could be injected onto the column and still achieve a satisfactory separation. Figure 9 shows the separation of 8.5 μg of taxol and 9 μg of baccatin III. The resolution falls to 1.0, but a 500-nL mixture of the two compounds can still be resolved. Two hundred times higher sample injection volume is achieved using large-bore capillary columns compared with that typical of the common CEC columns. The present study has concerned only the separation process, not the collection of the separated components of the mixture.

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