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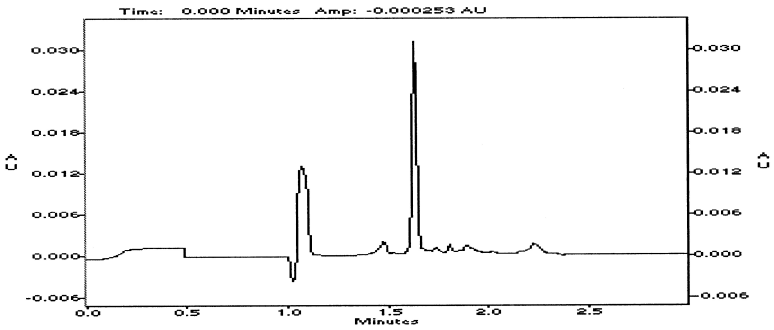

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Analysis of Combinatorial Chemistry Samples by Micellar Electrokinetic Chromatography

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A micellar electrokinetic chromatography (MEKC) method has been developed that can evaluate the purity of samples generated in combinatorial chemistry libraries. This method uses an open tube capillary (27 cm × 50 μm) along with a run buffer composed of sodium dodecyl sulfate (SDS), hydroxypropyl-β-cyclodextrin, and sodium tetraborate coupled with UV detection. Neutral compounds and compounds that were insoluble in aqueous buffers could be analyzed under these conditions in approximately 3 min. The concentration of SDS and the concentration of hydroxypropyl-β-cyclodextrin effected the separation. The affect on selectivity resulting from the addition of an organic modifier to the run buffer was examined. The low background absorbency of the run buffer made for easy detection of compounds that absorbed at low UV wavelengths. The quick analysis time made this suitable for analysis of combinatorial chemistry samples.

Introduction

The advent of combinatorial chemistry has had a significant effect on early stage drug discovery in that it allows a medicinal chemist to synthesize and identify a large number of potential drug candidates in a short period of time. The use of combinatorial chemistry has posed a new type of challenge for the analytical chemist; it requires the analysis of a large number of compounds with the data reported to the medicinal chemist rapidly and effectively. Traditional methods like high-performance liquid chromatography (HPLC) can be used to assess the purity of a combinatorial library, but these assays need to be rapid and to provide good resolution of all the components to ensure confidence in the data.

Capillary electrophoresis (CE) has been used for a variety of applications;[1−3] however, most of the compounds analyzed by this method are water-soluble and usually larger biomolecules. CE cannot analyze neutral compounds or poorly dissolved compounds under these conditions. The advantage to CE is that it only requires a small amount of buffer and sample to perform several analyses. Micellar electrokinetic chromatography (MEKC) has gained in popularity as an alternative method to HPLC because of the ability to get more efficient separations using a small amount of material.[4−6] It has been used recently to estimate the *n*-octanol−water partitioning coefficients of compounds.[7−9] Hyphenated techniques are being developed with MEKC such as CE−MS.[10−14] Most systems use a negatively charge micelle, and the most popular choice is sodium dodecyl sulfate (SDS). Because of the negative charge, the electrophoretic mobility will cause the micelle to migrate toward the cathode when a voltage is applied across the capillary; however, the electroosmotic flow (EOF) of the system is stronger than the electrophoretic mobility of the micelle toward the cathode. The result is that the micelle eventually migrates toward the anode but at a much slower time than the EOF. Analytes will elute on the basis of the distribution coefficient between the micelle and the aqueous portion of the run buffer. Analytes that are extremely hydrophilic will elute close to or with the EOF. Very hydrophobic analytes will be completely incorporated into the micelle and elute with it, giving very little resolution. This partitioning allows samples that are neutral or not soluble in the aqueous run buffer to be analyzed by capillary electrophoresis conditions. Resolution of chiral compounds can be achieved by modifying the micelle with compounds such as cyclodextrin.[15−19] The small amounts of solvent and sample used and the high resolving power of MEKC make it an attractive tool for combinatorial analysis. Through the application of this technique to combinatorial chemistry analysis, one can analyze a large number of compounds with good resolution in a short period of time without consuming large amounts of reagents. This allows accurate estimates of the purity using very small amounts of sample. The low UV absorbance of the run buffer and the absence of background noise from HPLC pumps help increase the sensitivity at low wavelengths, which gives good detection of compounds that have poor UV absorbance. A previously developed assay using MEKC that focused on known pharmaceuticals and excipients was examined but did not provide the resolution needed to assess the purity on a large number of combinatorial chemistry samples.[20]

We now report on a MEKC method for analyzing combinatorial chemistry libraries using a sodium dodecyl sulfate, hydroxypropyl-β-cyclodextrin run buffer. Separation

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of chiral compounds and detection of poor-absorbing compounds can be achieved by this method with good resolution and with an analysis time of 3 min or less.

Experimental Section

Reagents. Sodium tetraborate ($Na_2B_4O_7$), sodium dodecyl sulfate (SDS), hydroxypropyl-β-cyclodextrin, hydrocortisone, hydrocortisone 21-hemisuccinate, hydrocortisone 21-cypionate, and 0.1 N sodium hydroxide (NaOH) were purchased from Sigma Chemical Co., St Louis, MO. The high-purity water, acetonitrile, and the trifluoroacetic acid (TFA) were purchased from Fisher Scientific Co., Inc., Pittsburgh, PA. The fused silica uncoated capillary tubing and the 0.2 μm filters (40 and 90 mm diameter) were purchased from Alltech Associates, Inc., Deerfield IL. The C18 Monochrom column (50 mm $\times$ 4.6 mm) was purchased from Ansys, Inc, Torrence, CA. All CE analyses were performed on a Beckman P/ACE 5000 capillary electrophoresis instrument, which was equipped with a variable-wavelength UV detector. The data were collected using the Beckman System Gold software. Reversed-phase HPLC was carried out using a Dionex Summit system equipped with a P580 high-pressure pump and UVD170S variable-wavelength UV detector. Samples were injected using an ACI-100 autosampler, and the data were collected using Chromeleon chromatography software. LC−MS analysis was performed using a Finnigan LCQ MS−MS system equipped with a Waters 2690 Alliance HPLC system. The UV data were collected on a 2487 variable-wavelength detector. The data were collected using the Xcalibur software system.

Preparation of Run Buffers. $Na_2B_4O_7$ (0.3814 g) and SDS (2.884 g) were placed in a 100 mL volumetric flask. The flask was diluted to the mark with high-purity water, giving a final concentration of 10 mM $Na_2B_4O_7$ and 100 mM SDS. Ten milliliters of this solution was added to 0.730 g of hydroxypropyl-β-cyclodextrin, resulting in a final concentration of 50 mM. The run buffer was filtered through a 0.2 μm nylon filter (40 mm) prior to use.

Reversed-Phase HPLC Conditions. The mobile phase was prepared by taking 1000 mL of acetonitrile and adding 1 mL of TFA, resulting in a final solution of acetonitrile (0.1% TFA). The 0.1% TFA solution in water was prepared by taking 1000 mL of water and adding 1 mL of TFA. Both solvents were filtered through a 0.2 μm nylon filter (90 mm).

The gradient conditions used were as follows: 0 to 0.5 min 10:90 acetonitrile/water/0.1%TFA to 40:60 acetonitrile/water/0.1%TFA hold for 1 min at 1.5 min ramp to 90:10 acetonitrile/water/0.1% TFA in 3 min and hold at 90:10 acetonitrile/water/0.1% TFA for 1 min at 5.6 min. The gradient was adjusted back to initial conditions, and the column was equilibrated for 3 min. The flow rate used for the analysis was 3.5 mL/min.

Capillary Electrophoresis Conditions. The capillary used was a 27 cm $\times$ 50 μm open tube fused silica capillary. It was packed according to the Beckman P/ACE manual. The capillary was cleaned daily with 0.1 N NaOH for 5 min, followed by a high-purity water rinse for 5 min. Prior to each analysis the capillary was rinsed with run buffer for 30 s. The samples were injected for 1 s using a pressure injection

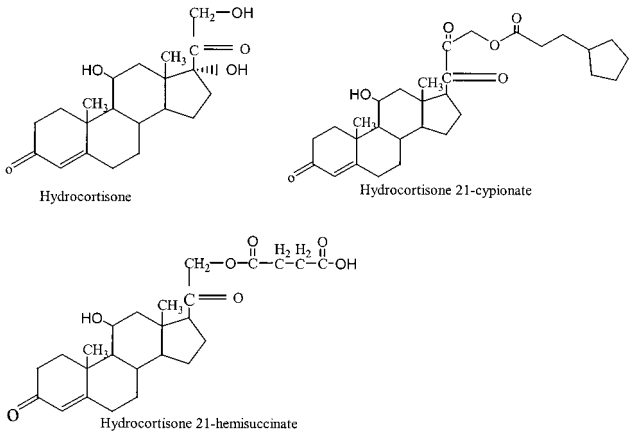

Figure 1. Structures of hydrocortisone, hydrocortisone 21-hemisuccinate, and hydrocortisone 21-cypionate.

(20 psi). A total of 20 kV was applied across the capillary. The capillary was kept at a constant temperature of 20 °C for adequate heat dissipation. The compounds were detected at 254 or 200 nm using a variable-wavelength detector.

LC−MS Conditions. The column used for LC−MS analysis was a Supelco Discovery C18 column (50 mm $\times$ 2.1 mm). The mobile phase used was prepared in the same manner as the mobile phase used for HPLC analysis, except 0.1% formic acid was used in place of 0.1% TFA. The gradient used was identical to the gradient used for the HPLC analysis; however, the flow rate was 0.75 mL/min. The MS−MS detector was equipped with an atmospheric pressure chemical ionization (APCI) probe. The probe was held at a constant temperature of 450 °C and used a sheath gas flow of 80 units, a discharge current of 5 μA, a discharge voltage of 6.0 kV, a capillary temperature of 200 °C, and a capillary voltage of 10 V for analysis. UV data were collected at 254 nm.

Results and Discussion

It was not practical to develop this assay using combinatorial chemistry samples because combinatorial libraries are diverse and large. The assay was developed using various standard compounds, and the conditions were then applied to four 400-compound combinatorial libraries. Efforts were focused on developing a method that could separate a large number of compounds that differed in polarity and structure, while using only one run buffer. Additional characteristics of the system included (1) a stable run buffer, (2) good resolution for many samples, (3) a short analysis time, and (4) the ability to use a variety of wavelengths. SDS was selected as the micelle because of its popularity with this technique and its ease of use. Hydrocortisone, hydrocortisone 21-hemisuccinate, and hydrocortisone 21-cypionate were used as the standard mixture (Figure 1). These compounds were selected because they were similar in structure but differed in polarity. The hydrocortisone 21-cypionate contained an impurity that gave roughly the same peak intensity. This impurity was not fully characterized but had a molecular weight that was 2 amu higher than that of hydrocortisone 21-cypionate. This was believed to be a hydro form of hydrocortisone 21-cypionate. Since HPLC is typically used to monitor compound purity in combinatorial libraries, the

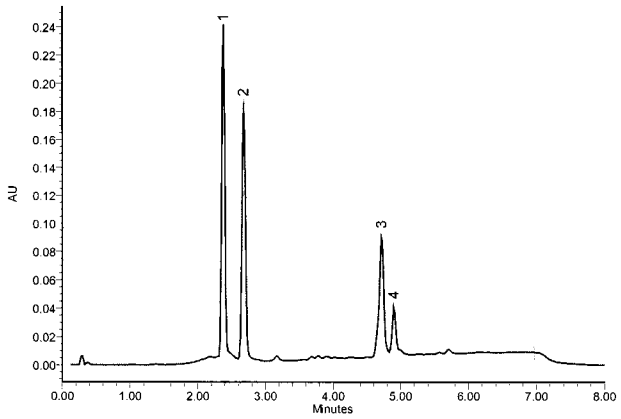

Figure 2. Reversed-phase HPLC chromatogram: (1) hydrocortisone 21-hemisuccinate, (2) hydrocortisone, (3) hydrocortisone 21-cypionate, and (4) impurity in hydrocortisone 21-cypionate. The chromatogram was generated using a 50 mm × 4.6 mm C18 column along with an acetonitrile/water/trifluoroacetic acid gradient at a flow rate of 3.5 mL/min. See Experimental Section for the gradient conditions. The compounds were detected at 254 nm for HPLC.

mixture of the hydrocortisone standards was analyzed by HPLC. This served as a prototype of resolution we would need to ensure good separation of combinatorial samples by MEKC. Figure 2 shows the chromatogram of the hydrocortisone standards analyzed on a C18 column using an acetonitrile/water/0.1% trifluoroacetic acid gradient mobile phase. Hydrocortisone, hydrocortisone 21-hemisuccinate, hydrocortisone 21-cypionate, and the impurity were well resolved under these conditions.

There are many choices of micelles that can be used for MEKC; however, it was desirable to use a micelle that had roughly the same number of carbons as the stationary phase used for C18 columns. Therefore, SDS was chosen as the micelle for this system because of the C16 side chain and its popularity. When the hydrocortisone standards were analyzed with run buffers at SDS concentrations of 50, 75, and 100 mM, no resolution was obtained between hydrocortisone 21-cypionate and its impurity. This is consistent with what has been reported in the literature for MEKC analysis of steroids using SDS micelles, which shows that the best separations were obtained using sodium cholate as the micelle.[21] Using sodium cholate in place of SDS was considered; however, because of its hydrophobic nature, it was decided that this micelle might not be adequately resolved or might retain fewer hydrophobic compounds. Two approaches were taken to improve the resolution: (1) addition of an organic modifier to the micelle and (2) addition of a neutral micelle to the run buffer. After acetonitrile (10%) was added to the run buffer, some improvement in resolution was achieved; however, peak shapes for hydrocortisone and hydrocortisone 21-cypionate were much broader. In addition, the analysis time had to be increased to approximately 5 min (data not shown).

Hydroxypropyl-β-cyclodextrin was added to the run buffer as a neutral micelle. This compound was selected because it has a greater solubility in aqueous solutions than β-cyclodextrin. In MEKC, cyclodextrins are electrically neutral and have no electrophoretic mobility. They are not assumed to be incorporated into the micelle because of the hydrophilic nature of the outside surface of the molecule. Cyclodextrin cavities are hydrophobic and can include hydrophobic molecules. Therefore, when cyclodextrin is added to the run buffer the separation is based on the equilibrium distribution between the analyte in the aqueous phase, the cyclodextrin cavity, and the SDS micelle. The analyte molecule included by the cyclodextrin migrates at the same velocity as the EOF because electrophoretically cyclodextrin behaves as the bulk aqueous phase. Therefore, addition of cyclodextrin reduces the apparent distribution coefficient and enables the separation of highly hydrophobic analytes, which otherwise would be totally incorporated into the micelle in the absence of cyclodextrin.[16] This results in shorter analysis times and improved resolution when compared to run buffers that do not contain cyclodextrin. Figure 3 shows electropherograms of the hydrocortisones generated using 0 to 50 mM hydroxypropyl-β-cyclodextrin. The sample run with just SDS did not give resolution of hydrocortisone 21-cypionate and its impurity. Addition of 10 mM hydroxypropyl-β-cyclodextrin did not resolve the compounds, but it did decrease the analysis time. Resolution was not obtained until 50 mM hydroxypropyl-β-cyclodextrin was added to the run buffer. The order of elution for these compounds using the MEKC method and reversed-phase HPLC was similar. It was important to note that when hydroxypropyl-β-cyclodextrin was added to the run buffer, the compounds eluted in less than 2.5 min, which allowed the run time to be decreased to 3 min.

An Ohm's law plot was made to determine the maximum voltage that could be applied to this system at a constant temperature of 20 °C. This temperature was selected because it allowed us to apply the highest current and to maintain the rapid analysis time. When the current at each applied voltage was recorded, an ideal Ohm's law plot should yield a straight line, indicating that the heat being generated inside the capillary was adequately dissipated. Deviation from a straight line is an indication of inadequate joule heat dissipation, which leads to irreproducible results. The Ohm's law plot from 5 to 25 kV was linear with a r^2 value greater than 0.99. At 30 kV the line deviated from linearity, which yielded a maximum applied voltage of 25 kV. However, 20 kV was selected as the voltage to be used in the analysis because of the improved resolution obtained due to the slower EOF.

The run buffer was examined for reproducibility by making several injections with the same run buffer and plotting migration time vs injection number. The data showed that over 60 successful injections were made from the same run buffer vial with almost no change in migration time. Figure 4 shows an electropherogram of the initial injection and the 60th injection of the hydrocortisones. There was little change in migration time or resolution from the first injection to last injection.

After the running conditions were optimized, the system was used to evaluate combinatorial chemistry samples that were generated in four 400-compound libraries (structures not provided). The libraries were selected at random and were analyzed by HPLC and MEKC. Figure 5 shows samples obtained from these libraries that were analyzed by MEKC.

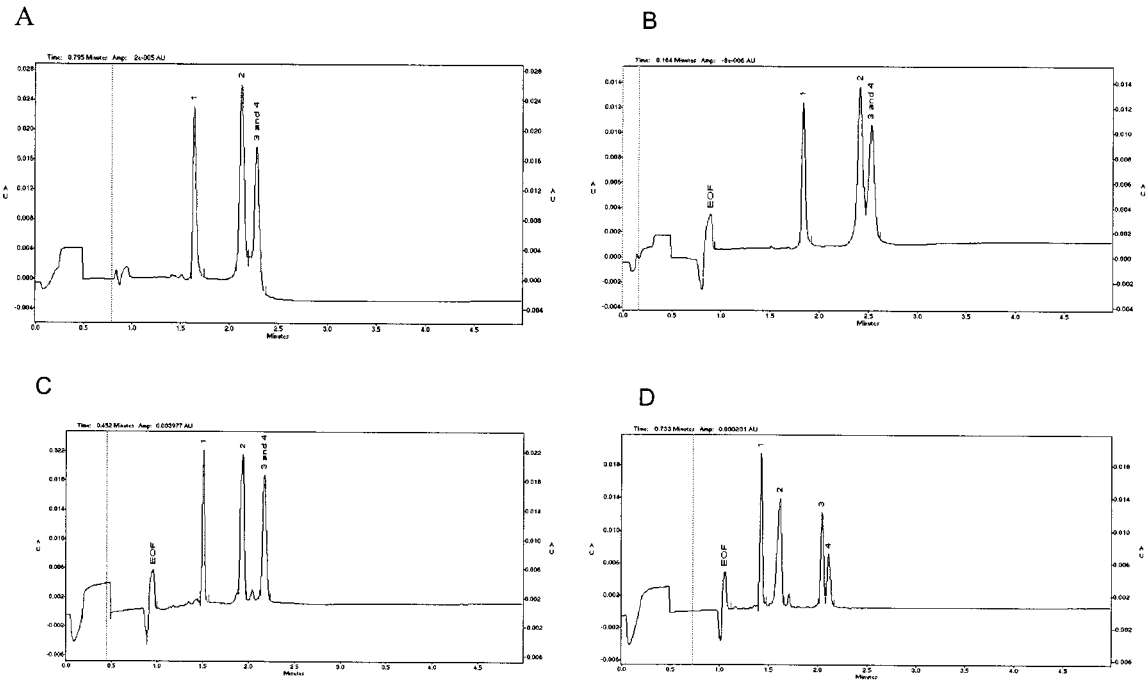

Figure 3. Electropherograms generated using (A) 0 mM hydroxypropyl-β-cyclodextrin, (B) 10 mM hydroxypropyl-β-cyclodextrin, (C) 25 mM hydroxypropyl-β-cyclodextrin, and (D) 50 mM hydroxypropyl-β-cyclodextrin added to the 100 mM SDS, 25 mM sodium tetraborate run buffer. All the samples were detected using UV at 254 nm. See Figure 2 for peak identification.

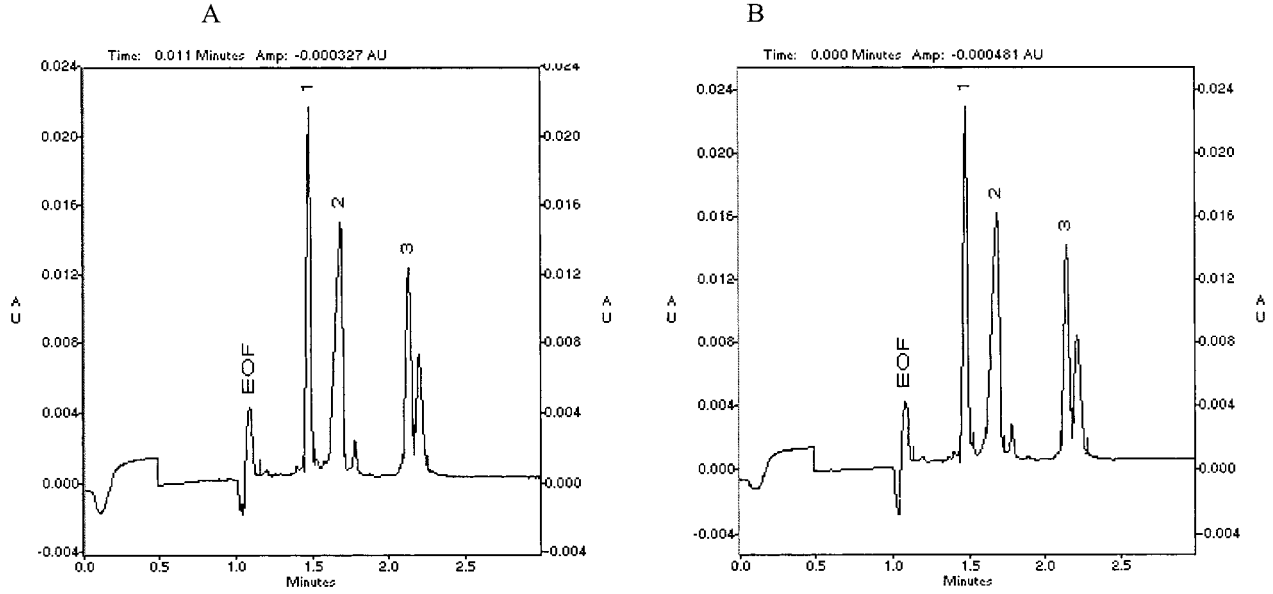

Figure 4. Electropherograms of (A) first injection and (B) 60th injection of the (1) hydrocortisone 21-hemisuccinate, (2) hydrocortisone, (3) hydrocortisone 21-cypionate, and (4) impurity using a 27 cm $\times$ 50 μm open tube capillary with 10 mM sodium tetraborate, 100 mM SDS, and 50 mM hydroxypropyl-β-cyclodextrin

The component of interest was identified using LC−MS. Since the order of elution was the same for both MEKC and HPLC, the order of elution was used to determine which peak was the compound of interest in the electropherogram. Table 1 compared the purity data obtained by HPLC to the data obtained by MEKC. In all cases the purity data were similar. Figure 6 compared data generated by HPLC and MEKC where the samples eluted in the void volume in HPLC. Under these conditions it is difficult to assess the purity of these samples because multiple compounds could be eluting in the void. When the samples were analyzed by MEKC, all the peaks were retained and a better assessment of the purity could be made. In MEKC the solvent contains no organic modifier, whereas the HPLC mobile phase contains 10% acetonitrile. The lack of organic modifier caused inclusion to occur in the micelle, allowing for some retention of compounds that would not be retained by HPLC. In addition, better separation was obtained on a few samples that showed additional impurities. The area for each sample was taken at 254 nm. The areas might vary at different wavelengths. Decreasing the UV wavelength from 254 to 200 nm produced a stable baseline, showing that there is little interference from the run buffer. There was very little change in baseline going from 254 to 200 nm. Samples could

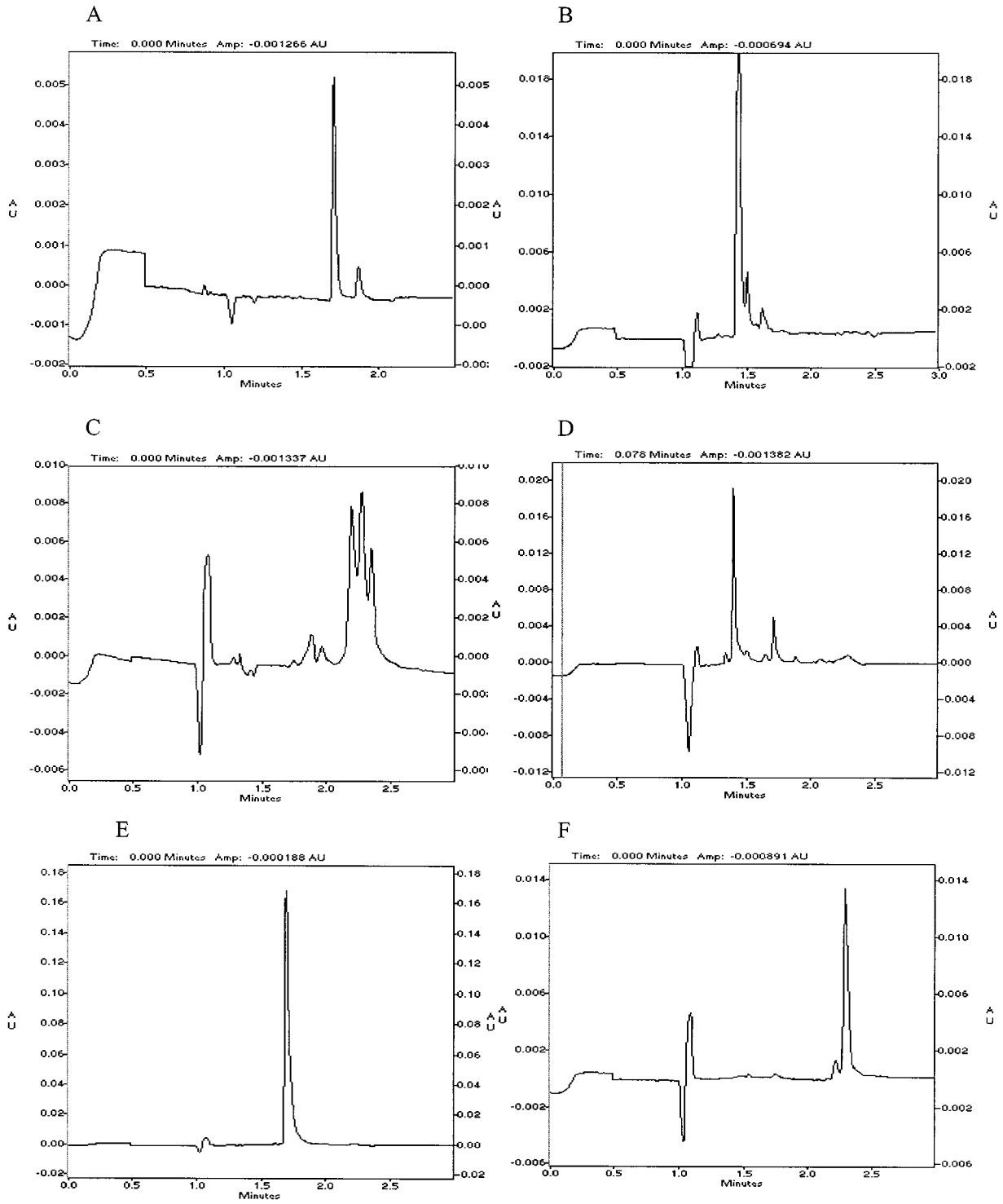

Figure 5. Electropherograms of compounds generated from four 400-compound libraries using a 27 cm × 50 μm open tube capillary with a 100 mM SDS, 50 mM hydroxypropyl-β-cyclodextrin, 10 mM sodium tetraborate run buffer. UV detection was at 254 nm. The electropherograms show the different levels of purity of each sample and resolution that can be obtained in a short period of time.

be injected and dissolved in a variety of solvents without affecting the analysis. There was no precipitation of sample or loss of current during analysis when samples were dissolved in solvents such as chloroform, acetone, or DMSO.

Conclusion

The MEKC method using a run buffer of 100 mM SDS and 50 mM hydroxypropyl-β- cyclodextrin and 10 mM sodium tetraborate was useful for analyzing combinatorial chemistry samples. Samples with low solubility in aqueous

Table 1

figure letter	percent purity MEKC	percent purity HPLC
A	89	87
B	82	87
C	35	31
D	62	62
E	100	100
F	93	91

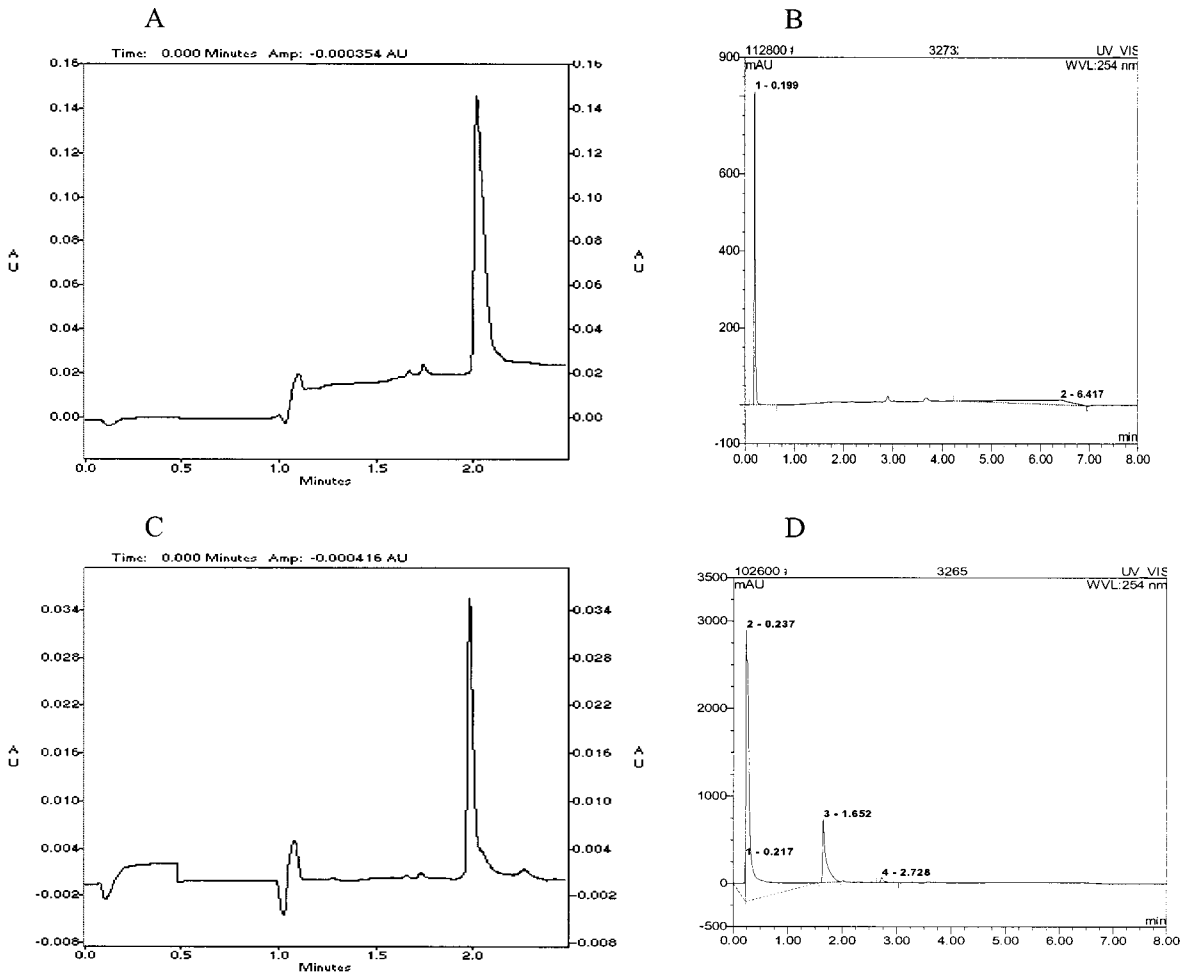

Figure 6. Compareison of (A, C) electropherograms and (B, D) chromatograms of combinatorial chemistry samples generated from the synthesis of four 400-compound libraries. The samples elute close to the void in HPLC and were retained by MEKC. The MEKC samples were analyzed using a 27 cm × 50 μm open tube capillary with a 100 mM SDS, 50 mM hydroxypropyl-β-cyclodextrin, 10 mM sodium tetraborate run buffer. The chromatograms were generated using a 50 mm × 4.6 mm C18 column along with an acetonitrile/water/trifluoroacetic acid gradient at a flow rate of 3.5 mL/min. See Experimental Section for the gradient conditions. The compounds were detected at 254 nm with both methods.

solutions could be analyzed under these conditions. The system allowed for rapid analysis of samples with unknown purity and provided good resolution for those samples when compared to HPLC. When the compound purity levels from four combinatorial libraries generated by HPLC and MEKC were compared, the levels were close to one another. The analysis could be completed within 3 min, and at least 60 injections could be made using the same run buffer vial. In addition, samples that eluted in the void volume using HPLC were retained by MEKC. MEKC also resolved additional impurities from certain compounds, giving a better assessment of the purity. Samples analyzed by MEKC in this study were detected using UV. The commonly used detectors for CE and MEKC analysis are UV and laser-induced fluorescence detectors. Currently, there are no reported literature references where MEKC analysis uses evaporative light or nitrogen chemiluminescent detectors. The low-flow characteristics of CE make it difficult to interface these detectors. CE−MS methods using MEKC systems have been developed, but these systems have not been applied to combinatorial chemistry samples.[10−14] Sample and reagent consumption was greatly reduced using this method, which reduced the use of valuable sample and decreased the accumulation of hazardous waste. The inject-to-inject time is approximately 3.5 min, and this would allow for 400 samples to be analyzed on one instrument within a given 24 h period. We feel that this method is a nice alternative to HPLC when the need to reduce the use of solvents and sample is a concern. In addition, it is an extra tool for assessing the purity of combinatorial chemistry libraries.

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