Anal. Chem. **1998,** *70,* 2439−2445

Hydrophilic Interaction Chromatography−Electrospray Mass Spectrometry Analysis of Polar Compounds for Natural Product Drug Discovery

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For the drug discovery efforts currently taking place within the pharmaceutical industry, natural product extracts have been found to provide a valuable source of molecular diversity which is complementary to that provided by traditional synthetic organic methods or combinatorial chemistry. However, there exists a need for analytical tools that can facilitate the separation and characterization of components from these sources in a rapid manner. Specifically, the evaluation of highly polar compounds (i.e., compounds that cannot be retained on traditional reversed-phase stationary phases) has been challenging, and a hydrophilic interaction chromatography−electrospray ionization mass spectrometry (HILIC−ESI-MS) method was developed to meet this need. In this investigation, amide-, Polyhydroxyethyl Aspartamide-, and cyclodextrin-based packings provided superior performance for the analysis of a set of polar natural product compounds. The properties of the mobile-phase buffers also greatly impacted the separations, and relative to other volatile buffering agents, ammonium acetate at a concentration of approximately 6.5 mM was determined to facilitate optimal HILIC retention, reproducibility, and durability. An optimized HILIC−ESI-MS system was successfully applied for the analysis of complex natural product mixtures. The techniques described in this report should also prove useful for the analysis of polar compounds from synthetic sources of molecular diversity such as combinatorial chemistry.

Within the pharmaceutical industry, the identification of novel active compounds through the use of high-throughput screening (HTS) can be considered to be one of the most important functions for success. The chemical diversity of the collection of samples that undergo HTS is crucial, and vast libraries consisting of thousands of compounds generated by biological (e.g., natural products) or synthetic methods (e.g., combinatorial chemistry) are being created to provide this diversity. Samples isolated for these libraries often are initially generated as complex mixtures consisting of multiple components, and powerful analysis tools are required for both the separation and characterization of the compounds in these mixtures in a rapid manner, either prior to or following HTS. Specifically, the intrinsic complexity of natural product extracts represents both a source of extremely high compound diversity and a significant separation/deconvolution challenge.

Traditionally, reversed-phase liquid chromatography (RPLC) has served as an effective technique for the separation of many of the components in complex mixtures that are under evaluation for drug discovery. Reversed-phase column packings have been successfully utilized in conjunction with volatile mobile phases to provide rapid high-throughput characterization of samples via liquid chromatography−electrospray ionization mass spectrometry (LC−ESI-MS). However, a significant portion of chemical diversity lacks the hydrophobic character necessary for suitable retention on an octadecyl (C18) reversed-phase packing and, therefore, requires alternative methods for analysis. Because of the difficulties associated with their high-throughput processing, including the fact that most natural product fermentation media components behave similarly, these "polar compounds" are often not pursued as viable drug candidates at the HTS stage. If a durable, rapid, and simple separation and analysis system could be designed for the high-throughput evaluation of polar compounds from natural product, combinatorial chemistry, or other sources, the post-HTS characterization (including the determination of novel structures and the identification of known compounds) of samples of this nature could be effectively integrated within drug discovery programs.

Normal-phase liquid chromatography (NPLC) has historically been employed for separations using nonaqueous mobile phases such as hexane or chloroform together with silica or alumina stationary phases. However, the application of NPLC to the separation of samples of biological origin has been extremely limited because of the problems associated with dissolving hydrophilic materials in nonaqueous mobile phases. Another difficulty associated with the use of NPLC for complex mixture characterization is the fact that several commonly used organic solvents such as benzene and toluene are not compatible with the electrospray process now prevalently utilized for molecular mass determinations.[1] NPLC using mobile phases containing a low percentage of an aqueous component has been employed, but the chromatography obtained in these systems has been of notoriously poor reproducibility.[2]

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S0003-2700(98)00227-3 CCC: $15.00
Published on Web 05/30/1998

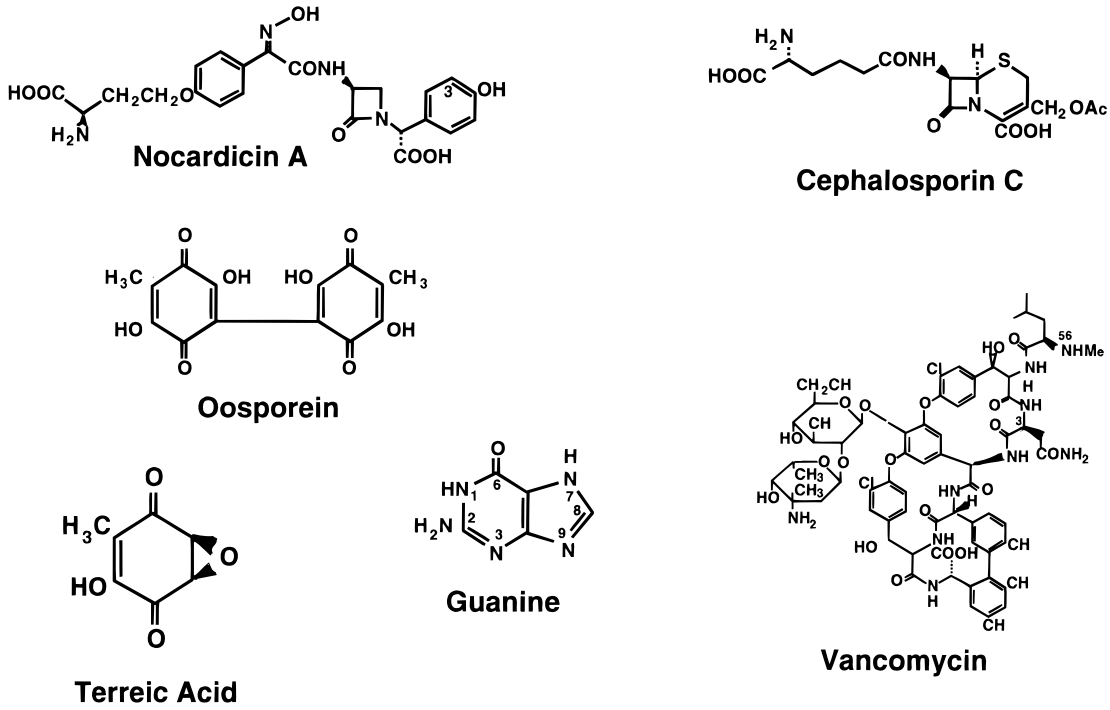

Figure 1. Structures of the natural product test standards.

An alternative to NPLC for the chromatography of natural product extracts is hydrophilic interaction chromatography (HILIC). HILIC is similar to NPLC in that elution is promoted by the use of polar mobile phases, but is unique in that the presence of water in the mobile phase is crucial for the establishment of a stagnant enriched aqueous layer on the surface of the stationary phase into which analytes may selectively partition, as described by Alpert.[3] The HILIC separations of carbohydrates[4−6] and peptides[7−9] have been reported, together with some examples of preliminary evaluations of the LC−MS technique.[5,7] However, the employment of HILIC−MS as a tool for the evaluation of polar compound mixtures for drug discovery has not yet been investigated or exploited. In this study, the influence of parameters upon the retention and resolution of polar natural product compounds was evaluated, with the goal of optimizing the utility of HILIC−ESI-MS for use in high-throughput drug discovery processes.

EXPERIMENTAL SECTION

Apparatus. The HPLC system employed for this investigation consisted of an Alliance model 2690 separations module equipped with a Model 996 photodiode array (PDA) detector (both from Waters Corp., Milford, MA). ESI spectra were collected on a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray source.

Reagents. All of the natural product standards and fermentation broth were provided by Eli Lilly and Co. (Indianapolis, IN). Keystone Carbohydrate (4.6 mm i.d. × 30 cm, 5 μm), BetaBasic CN (4.6 mm i.d. × 25 cm, 5 μm, 150 Å), and Shodex Asahipak NH2P-50 (4.6 mm i.d. × 25 cm, 5 μm) packings were obtained from Keystone Scientific (Bellefonte, PA). Metasil CN (4.6 mm i.d. × 25 cm, 5 μm, 80 Å) was purchased from MetaChem (Torrance, CA). TSK-Gel Amide-80 (4.6 mm i.d. × 25 cm, 5 μm, 80 Å) Polyhydroxyethyl Aspartamide (4.6 mm i.d. × 20 cm, 5 μm, 300 Å), and Cyclobond III (4.6 mm i.d. × 25 cm, 5 μm) were obtained from TosoHaas (Montgomeryville, PA), PolyLC (Columbia, MD), and Advanced Separations Technologies (Whippany, NJ), respectively. Ammonium acetate and ammonium formate buffers were adjusted to pH 5.5 and pH 4.4 through the addition of either concentrated acetic or formic acid. Phosphate buffers were prepared by dilution of concentrated phosphoric acid (pH 2.5 buffer) or through the use of monobasic ammonium phosphate, pH adjusted with concentrated phosphoric acid.

Methods. Gradient separations were performed using mobile phases consisting of acetonitrile (ACN) and purified water, with or without buffer as noted. All buffered mobile phases were prepared by the addition of a 10% (w/v) buffer stock solution and the required volume of water to a volumetric flask, followed by the addition of ACN to a level several milliliters below the mark. After mixing and degassing, ACN was added to the mark. For the HILIC development experiments, the natural product test compounds were dissolved in 75% ACN/25% water at a concentration of 0.5 mg/mL. Sample (20 μL) were injected to provide a 10-μg on-column load for each separation which resulted in chromatographic peaks ranging in UV absorbance intensity from 0.5 to 1.5 AU, facilitating simple and effective identification of the test compounds via the unique UV−visible absorbance spectrum of each compound. The flow rate was 1.0 mL/min, all analyses were performed at ambient temperature, and elution was

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monitored by PDA UV−visible detection scanning from 200 to 600 nm.

For HILIC−ESI-MS, a 100 μL/min flow was diverted from the HPLC column effluent into the electrospray source, and the mass spectra were acquired at 1 scan/s over a *m*/*z* scan range of 100−2000. A capillary temperature of 200 °C was used, along with a spray voltage of 4.41 kV, and nitrogen was employed as both the sheath and auxiliary gas. Data collection and processing were performed on a PC using the Navigator 1.1 software (Finnigan MAT, San Jose, CA). A solution of caffeine with a molecular weight of 194.2 in 50% methanol with 0.05% ammonium acetate was used for tuning and calibration of the mass scale.

RESULTS AND DISCUSSION

Effects of Stationary Phase. The chromatography of a set of natural product standards was evaluated by performing HILIC using a variety of hydrophilic stationary phases. The test standards utilized for this study included terreic acid, oosporein, nocardicin A, and guanine (see Figure 1). Because these compounds were not retained by traditional RPLC using a C18-derivatized packing and ammonium acetate pH 5.5-buffered mobile phase containing 2% ACN (data not shown), it was presumed that they would effectively represent the behavior of highly polar natural product structures. Cephalosporin C and vancomycin, which are weakly and moderately retained by RPLC, respectively, were also included in this study as controls. These six compounds were also chosen for this investigation because as a set they represented both acidic and basic characters and each individual analyte possessed unique UV−visible absorption characteristics which facilitated the identification of its retention time within an elution profile.

Because unbuffered ACN/aqueous mobile-phase systems have historically been used with great success for the HILIC chromatography of carbohydrates,[4] an initial assessment of the use of HILIC for polar natural product compounds was performed under these conditions. The HILIC retention of the set of standards obtained through the use of seven different stationary phases and a 40-min 10−25% aqueous gradient, followed by a 40-min hold at 25% aqueous phase, is presented in Figure 2. These conditions were employed because it had been reported that the HILIC retention mechanism is significant in the presence of >70% ACN for peptide analyses.[3] Data points at 80-min retention represent compounds that did not elute during the 80-min chromatographic window. In the cyano, Amide-80, Polyhydroxyethyl Aspartamide, and Cyclobond III packings, the compounds that had remained on-column in the presence of the 25% aqueous mobile phase were successfully eluted by 40% aqueous phase, an environment not supportive of "pure" HILIC retention. This result suggested that, under these conditions, a significant normal-phase-type adsorption (i.e., direct analyte−stationary-phase interaction) had occurred.

The results demonstrated that, in the absence of buffer, none of the compounds were eluted from either of the two amino stationary phases, Keystone Carbohydrate and Asahipak NH2P. A column wash with 40% aqueous phase also did not remove the solutes from these packings. This was not surprising, since the amino groups on the surface of these packings may serve as weak anion exchangers which electrostatically associate with the acidic functionalities of five of the test compounds. Guanine, which is highly basic, may interact directly with accessible negative charges inherent in the backbone of these stationary phases. The cyano

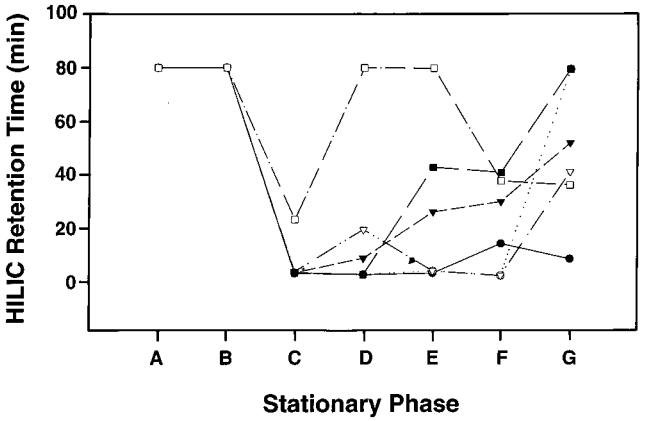

Figure 2. HILIC retention time plotted vs stationary phase for the standards (●) terreic acid, (○) oosporein, (▼) guanine, (▽) nocardicin A, (■) cephalosporin C, and (□) vancomycin in the stationary phase packings (A) Keystone Carbohydrate, (B) Asahipak NH2P, (C) BetaBasic Cyano, (D) Metasil Cyano, (E) TSKGel Amide-80, (F) Cyclobond III, and (G) Polyhydroxyethyl Aspartamide obtained in a nonbuffered system using a 40-min 10−25% aqueous gradient, followed by a 40-min hold at 25% aqueous phase. Data points at 80 min represent compounds that did not elute under these conditions.

columns, BetaBasic CN and Metasil CN, represented packings that display relatively weak polar character.[10] With the exception of vancomycin, none of the test compounds displayed retention times of >20 min in the Metasil column, and none were retained by the BetaBasic packing (the analytes were also poorly retained (i.e., <20 min under equivalent gradient conditions) by the cyano packings in the presence of 6.5 mM ammonium acetate buffer (data not shown)). The remaining three stationary phases, Amide-80, Polyhydroxyethyl Aspartamide, and Cyclobond III, which consist of highly polar, yet intrinsically noncharged, functional groups (carbamoyl, polymeric carbamoyl, and α-cyclodextrin, respectively), provided superior retention within the chromatographic window and were, therefore, employed for further optimization studies.

Effects of Buffer Salt Concentration. Typically, the use of buffered mobile phases is crucial for the achievement of acceptable reproducibility for chromatographic separations of charged species, since electrostatic interactions between the solute and stationary phase impacting retention are influenced and controlled by the buffer. However, although volatile buffering agents are compatible with ESI-MS, it is known that electrospray ionization is adversely affected by buffer salts due to ionization suppression.[11] It has also been determined that HILIC retention is inversely proportional to mobile-phase ionic concentration, since increases in salt concentration result in increases in solution hydrophilicity, as had been observed in a study of the HILIC separation of dipeptides comparing retention in the presence of 10 and 40 mM triethylamine phosphate.[3] Therefore, an investigation of the effects of volatile buffer salt concentration at the low levels required for optimal retention and ESI-MS sensitivity was performed.

A study of the effects of mobile-phase ammonium acetate pH 5.5 concentration at five levels (0, 3.3, 6.5, 13.0, and 26.0 mM) upon the retention of the test compounds by the Amide-80,

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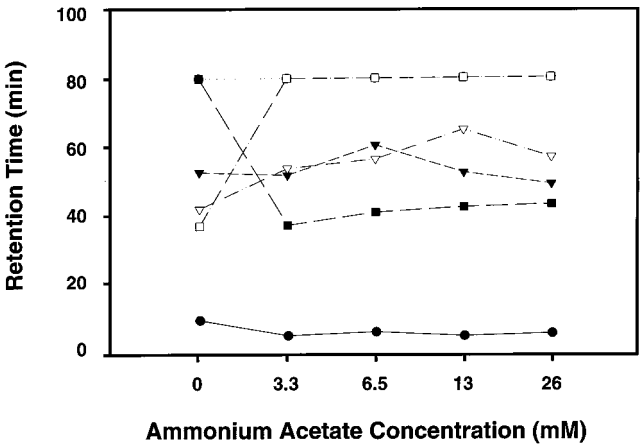

Figure 3. HILIC retention time plotted vs ammonium acetate concentration for the standards (●) terreic acid, (○) oosporein, (▼) guanine, (▽) nocardicin A, (■) cephalosporin C, and (□) vancomycin obtained in a Polyhydroxyethyl Aspartamide packing using a 40-min 10−25% aqueous gradient, followed by a 40-min hold at 25% aqueous phase. Data points at 80 min represent compounds that did not elute under these conditions.

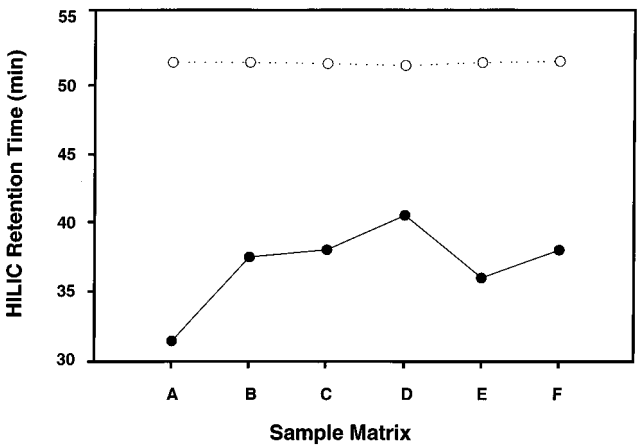

Figure 4. HILIC retention time of nocardicin A plotted vs the sample matrixes (A) 75% ACN; (B) 75% ACN, 25 mM sodium phosphate pH 7.0; (C) 75% ACN, 25 mM sodium phosphate pH 7.0, 100 mM sodium chloride; (D) 75% ACN, 25 mM sodium phosphate pH 7.0, 500 mM sodium chloride; (E) 75% ACN, 25 mM sodium phosphate pH 4.5; and (F) 75% ACN, 25 mM sodium phosphate pH 8.5 obtained in a TSKGel Amide 80 packing and (●) a nonbuffered system and (○) 6.5 mM ammonium acetate pH 5.5, using a 90-min 10−40% aqueous gradient.

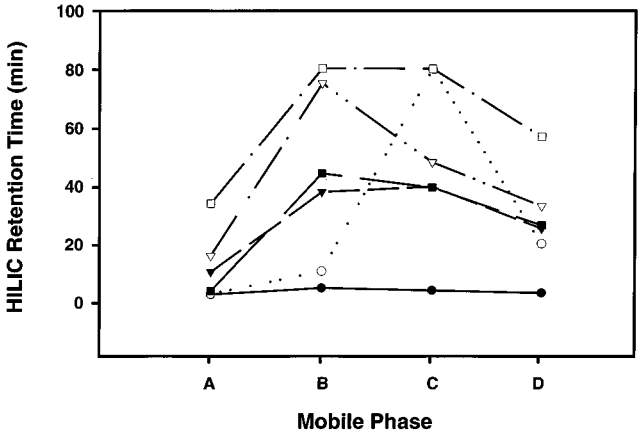

Figure 5. HILIC retention time plotted vs the mobile phase buffers (A) 6.5 mM trifluoroacetic acid; (B) 7.9 mM ammonium formate pH 4.4; (C) 6.5 mM ammonium acetate pH 5.5; and (D) 6.3 mM ammonium bicarbonate pH 7.9 for the standards (●) terreic acid, (○) oosporein, (▼) guanine, (▽) nocardicin A, (■) cephalosporin C, and (□) vancomycin obtained in a TSKGel Amide 80 packing using a 40-min 10−25% aqueous gradient, followed by a 40-min hold at 25% aqueous phase. Data points at 80 min represent compounds that did not elute under these conditions.

Polyhydroxyethyl Aspartamide, and Cyclobond III packings was performed. Relatively marked changes in test compound retention and elution order were observed at buffer salt concentrations between 0 and 3.3 mM in all three packings (see the poly(hydroxyethyl aspartamide) data in Figure 3 as an example). The presence of only 3.3 mM buffer salt was also observed to significantly improve analyte peak shape and efficiency. The factors influencing sample HILIC retention in extremely low buffer salt environments are complex and may include effects such as ion exclusion and/or ion exchange (the carbamoyl functionality of both the Amide-80 and Polyhydroxyethyl Aspartamide packings has been reported to exhibit a weak positive charge with a pK_a at pH 4.4[6]), in addition to the impact of the salt upon mobile-phase hydrophilicity. Ion exclusion effects in the absence of buffer salt appeared to have an influence upon the retention of nocardicin A and vancomycin, as evidenced by an increase in retention time in the presence of 3.3 mM salt. Retention of the test compounds appeared to be relatively stable in all three packings at ammonium acetate concentrations of 6.5 mM or greater. These results suggest that an ammonium acetate concentration of at least 6.5 mM is necessary to provide acceptable HILIC−MS reproducibility, while still minimizing the impact of ESI signal ion suppression that will occur with the employment of higher buffer concentrations.

Since the chromatographic analysis of natural product extracts often involves the injection of sample matrixes exhibiting a range of pH and ionic strength (i.e., fermentation broth, plant and marine organism material, etc.), it was important to verify the durability of the HILIC separations in response to changes in sample matrix. Samples of nocardicin were prepared in solutions of 75% ACN and 25 mM sodium phosphate over a range of pH, with or without added sodium chloride. These samples were then injected into both a buffered (6.5 mM ammonium acetate pH 5.5) and a nonbuffered HILIC system in triplicate to verify data reproducibility, and chromatography was performed using an Amide-80 packing and a 90-min 10−40% aqueous gradient. The influence of the sample matrix upon mean retention is demonstrated in Figure 4, where it is evident that, in the absence of mobile-phase buffer, nocardicin A retention increases in response to an increase in both sample solution pH and ionic strength. These effects were minimized through the use of the ammonium acetate buffer.

Effects of Buffer Character and pH. To determine an optimal volatile buffering system to use for the HILIC analysis of natural product extracts, the chromatography of the set of standards was evaluated using an Amide-80 packing and mobile phases buffered by 6.5 mM trifluoroacetic acid (TFA) pH 1.5, 7.9 mM ammonium formate pH 4.4, 6.5 mM ammonium acetate pH 5.5, and 6.3 mM ammonium bicarbonate pH 7.9 together with a 40-min 10−25% aqueous gradient, followed by a hold at 25%

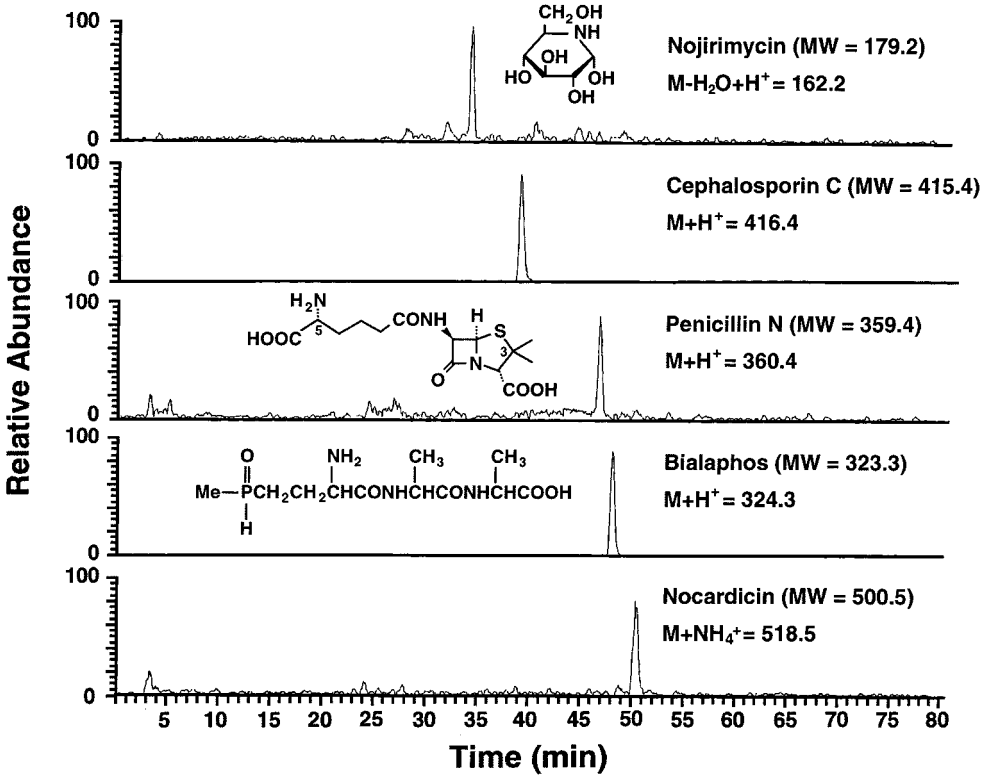

Figure 6. HILIC−ESI-MS separation of a mixture of five polar natural product standards, represented as the selected ion mass chromatogram of each structure in positive ion ESI. The molecular weight and the identity of the major ion observed for each of the standards are indicated, along with the structures of nojirimycin, penicillin N, and bialaphos. The chromatography was obtained using a TSKGel Amide 80 packing, 7.9 mM ammonium formate pH 4.4-buffered mobile phases, and a 90-min 10−40% aqueous gradient.

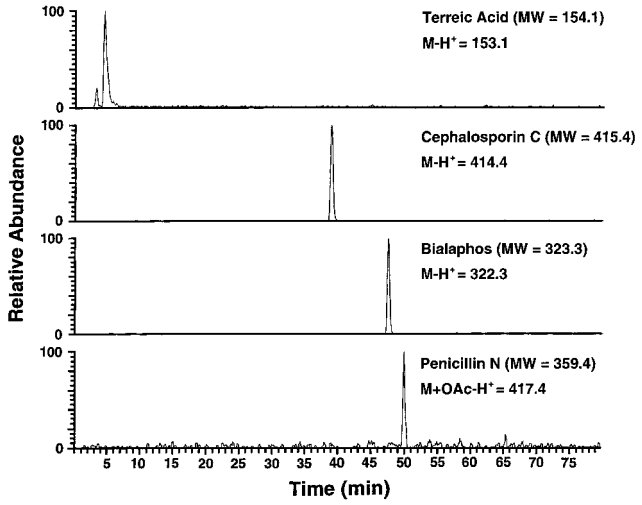

Figure 7. HILIC−ESI-MS separation of a mixture of four polar natural product standards, represented as the selected ion mass chromatogram of each structure in negative ion ESI. The molecular weight and the identity of the major ion observed for each of the standards are indicated. The chromatography was obtained using a TSKGel Amide 80 packing, 6.5 mM ammonium acetate pH 5.5-buffered mobile phases, and a 90-min 10−40% aqueous gradient.

aqueous phase. The results from this experiment are displayed in Figure 5. Mobile phases containing TFA have been employed successfully for the normal phase HILIC chromatography of polar peptides,[12] but under the conditions of this study, TFA provided unacceptably weak retention of the natural product compounds, probably in large part due to ion-pairing effects which serve to decrease the hydrophilicity of the analytes. In general, in the remaining buffered mobile phases, retention of the test compounds appeared to follow the pattern acetate ≈ formate > bicarbonate, which corresponded to a relationship in the differences of anion polarity. In terms of structural dipole moment, acetate (followed closely by formate) will represent the most unsymmetrical anion of this group and, therefore, following the association of the anion with the analyte, would be expected to theoretically have the greatest effect upon the inducement of analyte hydrophilicity. Similar results have been reported for the HILIC separation of peptides, where retention in the presence of TFA, formic acid, and acetic acid was compared and the differences were attributed to the effectiveness of these acids in minimizing ion-exchange interactions.[12] It is important to note that analyses performed using mobile phases buffered with 0.05% ammonium bicarbonate pH 7.9 were not found to have had any adverse effects upon the stationary phase, such as silica dissolution, since the separation of the standards obtained using ammonium acetate buffer could successfully be reestablished following the 12-h period during which the packings were exposed to pH 7.9.

Since both the acidic and basic test compounds behaved in a relatively similar fashion from one buffer system to another, the results suggested that the effects of the pH upon solute retention were minimal in comparison to the changes that had occurred within the stationary-phase-directed adsorption/desorption processes, as influenced by the character of the buffer anion. To

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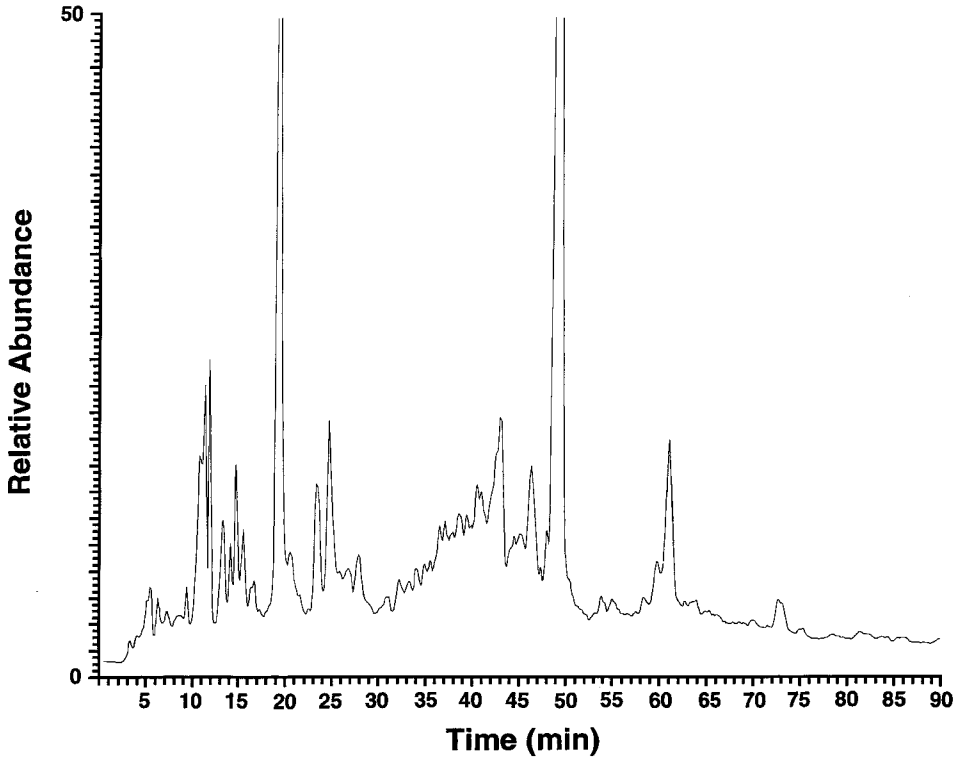

Figure 8. HILIC−ESI-MS separation of the polar components (unretained by reversed-phase solid-phase extraction pretreatment) of a fermentation extract, represented as a total ion chromatogram in positive ion ESI. The chromatography was obtained using a TSKGel Amide 80 packing, 6.5 mM ammonium acetate pH 5.5-buffered mobile phases, and a 90-min 10−40% aqueous gradient.

serve as a control to verify these conclusions, separations of the test compounds were obtained in a set of 10 mM ammonium phosphate-buffered systems at pH 2.5, pH 3.8, and pH 5.5. In this experiment, the retention of all six test compounds remained relatively consistent, varying by no more than 10% over the pH range, providing further evidence for the marked impact of the character of the buffer anion in volatile-buffered systems for HILIC−ESI-MS.

HILIC−ESI-MS. To investigate the feasibility of the analysis of polar natural product compounds by HILIC−ESI-MS, mixtures of natural products were prepared and analyzed by both positive and negative ion LC−MS. Figure 6 displays the selected-ion chromatograms obtained following the single 20-μL injection of a mixture of the five compounds nojirimycin, cephalosporin C, penicillin N, bialaphos, and nocardicin A (each at a concentration of 50 μg/mL) into a system consisting of an Amide-80 stationary phase, 7.9 mM ammonium formate pH 4.4-buffered mobile phases and a 90-min 10−40% aqueous gradient. The structures of compounds not previously displayed in Figure 1 are indicated, along with the identity of the major ion observed in the positive ion mode. All five compounds possessed amino functionalities which carried the positive charge responsible for the ESI signal obtained under these conditions.

A mixture of terreic acid, cephalosporin C, bialaphos, and penicillin N was evaluated by negative ion HILIC−ESI-MS under the same conditions as described above with the exception of the substitution of 6.5 mM ammonium acetate pH 5.5 as the mobile-phase buffer (see Figure 7). The acidic hydroxyl group in terreic acid and the carboxylic acid functionalities in the other three compounds supported a negatively charged site for ESI signal generation in this environment. As predicted by the results of Figure 5, cephalosporin C demonstrated equivalent retention times within the two different buffer systems employed for the positive and negative ion HILIC−ESI-MS analyses, and bialaphos also behaved similarly.

To demonstrate the application of this method to a complex mixture, a fermentation broth sample was prepared by reversed-phase solid-phase extraction using an Oasis (Waters Corp.) extraction cartridge, and the nonretained, highly polar material was then dried and resolublized to the original volume in 75% ACN; a 200 μL injection was separated by HILIC using the Amide-80 packing and a 10−40% aqueous gradient in 6.5 mM ammonium acetate pH 5.5 buffered mobile phases. The HILIC−ESI-MS positive ion total ion chromatogram is displayed in Figure 8, where >70 components appear to be resolved by the chromatography. For the analysis and identification of biologically active components present in complex samples such as that represented by the fermentation broth extract, the ability to perform an additional dimension of "deconvolution" via the generation of selected ion chromatograms (as was performed for the mixtures of natural product standards in Figures 6 and 7) is crucial.

The techniques described in this report should also prove useful for the analysis of polar compounds present as complex mixtures from synthetic sources of molecular diversity, such as combinatorial chemistry. Regardless of the origin of the samples, the most rapid and effective approach for generating HILIC−ESI-MS data will be the concurrent generation of positive and negative ion mass spectra during the separation, a function that was not feasible in the ion trap mass spectrometer used for this study but that is practical in other commercially available instruments.

Together with on-line ESI-MS and PDA detection, the analytical system may be interfaced with fraction collection into a 96-well plate for subsequent biological activity evaluation, and following the identification of biologically active fractions, the corresponding chromatographic retention times may be analyzed for the presence of both ESI and UV−visible absorbance spectra which indicate the identity or novelty of potential drug molecules.[13,14] The integration of a mass-sensitive detection system such as evaporative light scattering detection (ELSD) with the HILIC−ESI-MS system can also facilitate the on-line determination of component concentrations, information that can be extremely valuable for the estimation of the potency of potential drug candidates. It is important to note that other separation techniques such as capillary electrophoresis and capillary electrochromatography may also provide effective resolution of polar molecules, but at this time appear to be less attractive than HILIC for drug discovery applications because of the limitations of these capillary-based methods for providing microgram-scale fraction collection in addition to concurrent ESI-MS, PDA, and ELSD detection. One potential limitation that may be presented by the use of HILIC−ESI-MS for the analysis of mixtures of unknown polar compounds may be limited analyte solubility in the solutions of relatively high organic (>60% ACN) which are required by the chromatography. This issue may be at least partially alleviated through the use of stationary phases possessing greater hydrophilic character than amide, Polyhydroxyethyl Aspartamide, or cyclodextrin to facilitate HILIC under conditions of a higher aqueous environment.

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ACKNOWLEDGMENT

The author acknowledges Mr. Steve Lawrence and Mr. Larry Sachs for their technical assistance and Dr. Andrew Alpert for insightful discussions.

Received for review February 27, 1998. Accepted May 6, 1998.

AC9802271