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Hydrophilic interaction chromatography using amino and silica columns for the determination of polar pharmaceuticals and impurities

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Abstract

Hydrophilic interaction chromatography (HILIC) is described as a useful alternative to reversed-phase chromatography for applications involving polar compounds. In the HILIC mode, an aqueous–organic mobile phase is used with a polar stationary phase to provide normal-phase retention behavior. Silica and amino columns with aqueous–acetonitrile mobile phases offer potential for use in the HILIC mode. An examination of the retention and separation of several pyrimidines, purines, and amides on silica and amino columns from three manufacturers revealed that mobile phases should contain a buffer or acid for pH control to achieve similar and reproducible results among columns from different sources. Amino columns may also be used in an anion-exchange mode, which provides an advantage for some applications. In some cases, silica can provide different selectivity and better separation than an amino column. Example applications include: low-molecular-mass organic acids and amides as impurities in non-polar drug substances, 5-fluorouracil in 5-fluorocytosine, guanine in acyclovir, and different selectivity for polar basic compounds compared to an ion-pairing system. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Hydrophilic interaction chromatography; Pharmaceutical analysis; Mixed-mode chromatography; Stationary phases, LC; Pyrimidines; Purines; Amides

1. Introduction

The determination of polar compounds is needed in many fields and often arises during pharmaceutical development. Polar analytes may include raw materials and intermediates used to synthesize drug substances or the drug substance itself. Polar reagents, raw materials, intermediates and reaction by-products may also be present as impurities in a drug substance or synthetic intermediate. Suitable methods are needed to determine polar drugs and metabolites in various biological sample matrices.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is widely used in pharmaceutical analysis because of its selectivity and sensitivity for a large range of compounds. For many polar compounds however, mobile phases employing little or no organic modifier must be used to achieve retention and separation. These highly or totally aqueous mobile phases can lead to the collapse of the octyl or octadecyl alkyl chains commonly used for RP-HPLC [1]. Stationary phase collapse can lead to poor retention and selectivity for polar analytes as well as poor reproducibility. Modified stationary phases employing an alkyl chain with an embedded polar group (often an amide) have been developed to help address this problem [2,3]. The increased

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polarity of the stationary phase close to the silica surface imparts more hydrophilic character to the stationary phase, which keeps the alkyl chains from collapsing under aqueous conditions. However, some polar compounds are still difficult to retain under these conditions. An example is the use of four octadecyl columns in series to separate uracil and 5-fluorouracil [4]. A relatively non-retentive column was used in this analysis but even an effective column length of 1.2 m provided only slight separation from the void volume. In many analyses, a polar impurity is present in a relatively non-polar sample, e.g., a polar reagent impurity in a drug substance. The predominant component may not be eluted using the low-organic-content mobile phase required to retain the polar analyte and thus would build up on the column. A related aspect is that a non-polar sample may not be soluble in a highly aqueous solvent. This can limit the concentration of the sample solution and the sensitivity of the method. With RP-HPLC, a sample solvent containing higher concentrations of organic solvent could be used but with the risk of poor peak shapes and interference with the analyte response, especially for early-eluting solutes.

There is an alternative mode of chromatography wherein a polar stationary phase, such as propylamine bonded to silica, is used with aqueous–organic mobile phases. This mode is similar to normal-phase chromatography in that polar compounds are retained longer than non-polar compounds and the polar mobile phase component, water, is the strong solvent. This method has been used for many years for polar compounds, particularly sugars [5]. Alpert used the term ''hydrophilic interaction chromatography'' (HILIC) to describe the use of a polar stationary phase with aqueous–organic mobile phases [6]. He developed a poly(hydroxyethyl)aspartamide column for the separation of proteins, peptides and other polar compounds. Other reports using the HILIC mode with various polar columns have appeared for carbohydrates [7,8], peptides [9,10], histones [11]and natural product extracts [12]. HILIC has been used in a mixed-mode fashion with cation-exchange chromatography for peptide separations [13–15]. Mechanistic studies and various applications using bare silica as a polar stationary phase with aqueous–organic mobile phases have also been reported [16–21].

The current report describes the use of propylamine-bonded and bare silica stationary phases for the determination of polar compounds in the HILIC or mixed HILIC–ion-exchange mode. Advantages, disadvantages and method development considerations are examined using several compounds representing polar analytes of pharmaceutical interest. Several applications are also presented to demonstrate the utility of this approach to real situations.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Model L-6200A pump (Hitachi, Naperville, IL, USA), a Model 728 autoinjector (Alcott, Norcross, GA, USA) with a fixed-loop injection valve (Valco, Houston, TX, USA), and a Model 757 variable-wavelength UV detector (Applied Biosystems, Ramsey, NJ, USA). Chromatograms were recorded using a laboratory-developed data acquisition system. The following columns were used: Zorbax NH_2 and SIL, 70 Å pore diameter (MacMod, Chadds Ford, PA, USA), Nucleosil NH_2 and silica, 100 Å pore diameter (Phenomenex, Torrance, CA, USA), YMC silica and YMC-Pack NH_2, 120 Å pore diameter (Waters, Milford, MA, USA), polyhydroxyethyl aspartamide, 200 mm (PolyLC, Columbia, MD, USA), TSK-Gel Amide-80 (Tosohaas, Montgomeryville, PA, USA). All columns were 250 mm×4.6 mm I.D. with 5 μm particles unless indicated otherwise. Void volumes were approximately 2.7 ml for amino columns and 2.0 ml for silica columns.

2.2. Reagents

HPLC-grade acetonitrile, methanol and *n*-propanol were from EM Science (Gibbstown, NJ, USA). Phosphoric acid, *N*,*N*-dimethylformamide and potassium phosphate buffer salts were also from EM Science. Uracil, 5-fluorouracil, 5-fluorocytosine, oxalic acid, oxamic acid, oxamide and acetamide were from Aldrich (Milwaukee, WI, USA). Guanine and acyclovir (acycloguanosine) were from Sigma (St. Louis, MO, USA). Formamide was from Fisher

(Fairlawn, NJ, USA). Other chemicals and samples were from Eli Lilly.

2.3. Chromatographic conditions

Phosphate buffers were prepared using the desired concentration of phosphoric acid or potassium phosphate salt with 5 *M* potassium hydroxide used to adjust the pH to the desired value. The mobile phase consisting of 25 m*M* potassium phosphate, pH 6.5–acetonitrile (20:80, v/v) was close to the solubility limit of the buffer, depending on the temperature of the solution (note that unless otherwise stated, buffer concentrations and pH values refer to the aqueous portion alone). If haziness was observed in the mobile phase, addition of about 0.5% water was sufficient to dissolve the buffer. The flow-rate was 1.0 ml/min and the injection volume was 20 μl for all experiments.

3. Results and discussion

3.1. Compounds and columns for general studies

Several types of polar compounds (Fig. 1) were used to investigate and compare the separation properties of various stationary phases in the HILIC mode. Pyrimidines and purines are polar compounds of significant biological and pharmaceutical interest. Included in this class is uracil, which is often employed as a void volume marker in RP-HPLC since it is not retained. Cytosine, 5-fluorouracil and 5-fluorocytosine were other pyrimidines investigated. The separation of purines guanine and acyclovir was also evaluated. Oxamide, oxamic acid and oxalic acid were used to examine the potential of a mixed-mode HILIC–anion-exchange retention mechanism on an amino column. A mixture of low-molecular-mass amides including oxamide, acetamide and formamide was used to investigate closely-related neutral compounds that are difficult to retain and separate by RP-HPLC.

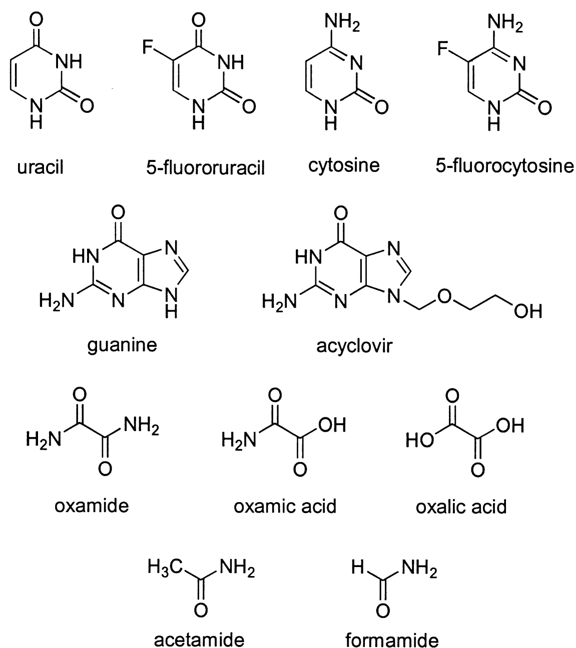

Fig. 1. Compounds studied.

Propylamine (amino) and silica columns from three manufacturers (Zorbax, Nucleosil, YMC) were evaluated. Observations made among different column suppliers were thought to be more informative than comparisons made with multiple columns from the same supplier. Other columns (polyhydroxyethyl aspartamide, TSK-Gel Amide-80) that have been used for polar compounds in the HILIC mode were also briefly examined.

3.2. Pyrimidines and purines

Initial work with a Zorbax amino column showed normal-phase behavior where increases in the aqueous content of the mobile phase produced decreased retention times. This is shown in Fig. 2 for the separation of uracil and cytosine over a range of water content from 10 to 30%.

The pyrimidines and purines shown in Fig. 1 were used for further evaluation of amino columns. Chromatograms given in Fig. 3 show comparable retention and separation on the three columns tested. The aqueous portion of the mobile phase for the data in Fig. 3 was 25 m*M* potassium phosphate at pH 6.5. Initial work with unbuffered aqueous acetonitrile mobile phases showed significant differences among the columns and a dependence of retention on column history. For example, 5-fluorocytosine was strongly retained with poor peak shape on a new YMC column using an unbuffered mobile phase. When 5 m*M* phosphoric acid was added to a mobile phase of acetonitrile–water (60:40), the retention of

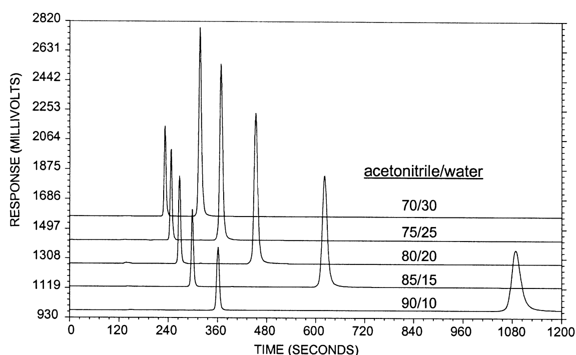

Fig. 2. Separation of uracil and cytosine as a function of mobile phase composition. Conditions: Zorbax NH_2 column; mobile phase acetonitrile–water as indicated; 205 nm detection; uracil (first peak, 0.04 mg/ml), cytosine (0.06 mg/ml).

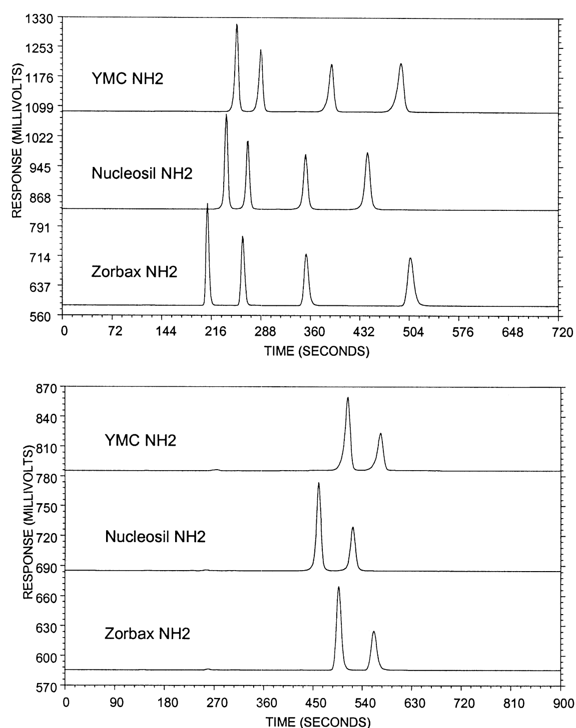

Fig. 3. Pyrimidines (upper) and purines (lower) on amino columns. Conditions: mobile phase acetonitrile–25 m*M* potassium phosphate, pH 6.5 (80:20); 275 nm detection; pyrimidines, 0.03 mg/ml (in order of elution): uracil, 5-fluorouracil, cytosine, 5-fluorocytosine; purines (in order of elution): acyclovir (0.02 mg/ml), guanine (0.009 mg/ml).

5-fluorocytosine was reduced to 3.4 min and the peak shape was greatly improved. When the mobile phase was switched back to unbuffered acetonitrile–water, the initially strong retention of 5-fluorocytosine could not be reproduced. A similar phenomenon may explain the observation by Strege that guanine was not eluted from amino columns using aqueous acetonitrile mobile phases [12]. As shown in Fig. 3, guanine was easily eluted from the amino columns using a buffered mobile phase. Therefore, although some separations could be carried out using aqueous acetonitrile with no buffering, most subsequent work utilized buffered systems to improve reproducibility among different brands of columns and avoid effects due to individual column history.

The buffer type, concentration, and pH were not optimized for the separations shown in Fig. 3. At pH 6.5 the amine groups of the stationary phase were protonated, which improved the reproducibility of the separations as mentioned above. Lower pH values and alternative buffer salts could be employed but were not investigated in depth. Phosphate at a relatively high pH was chosen as an aggressive challenge to test the ruggedness of the amino stationary phase as described below. Phosphate buffers also have the advantage of good transparency for low-wavelength UV detection. Volatile buffers would clearly be advantageous for mass spectrometric (MS) or evaporative light scattering detection.

Substituting alcohols in place of water as the strong mobile phase solvent was investigated. Methanol and *n*-propanol were examined to determine their eluting strength compared to water and for potential selectivity advantages. Hydrophilic interaction behavior was still observed, but greater proportions of methanol (approximately double) were required to obtain roughly equivalent retention compared to water. The peaks also exhibited greater tailing with methanol as the strong solvent. Uracil and cytosine were not eluted with 100% *n*-propanol as the mobile phase, apparently due to the decrease in hydrophilic character caused by the longer alkyl chain. Addition of 10% water to *n*-propanol eluted cytosine in about 7.2 min compared to 18.1 min for 10% water in acetonitrile, suggesting that *n*-propanol is a stronger eluting solvent in HILIC than acetonitrile. Since no advantage in selectivity was apparent with methanol as the strong solvent and greater peak

tailing was observed, additional work was focussed on acetonitrile–aqueous mobile phases.

Separations of pyrimidines and purines on silica columns were conducted using dilute phosphoric acid as the aqueous component of the mobile phase (Fig. 4). As with the different amino columns, silica columns from different suppliers gave comparable retention and separation. The Zorbax silica column gave longer retention and greater tailing for 5-fluorocytosine and cytosine than the other silica columns. The same retention order was obtained on silica columns compared to amino columns, but band spacing was sometimes different. This suggests that the choice of stationary phase may offer some opportunity to gain resolution but that dramatic changes in selectivity are not likely.

The separation of pyrimidines was also investigated using silica-based stationary phases bonded with poly(hydroxyethyl)aspartamide or carbamoyl groups (TSK-Gel Amide-80). The poly(hydroxyethyl)aspartamide column provided HILIC retention behavior for uracil and cytosine but the peaks tailed significantly relative to the amino columns. The TSK-Gel Amide-80 column provided separations comparable to those obtained on the amino columns although the peak efficiency was about 70% and tailing was higher (1.45 vs. 1.13) compared to the Zorbax amino column. Although they might be suitable alternatives to amino and silica columns, the polyhydroxyethyl aspartamide and TSKgel Amide-80 columns were not pursued further for this study.

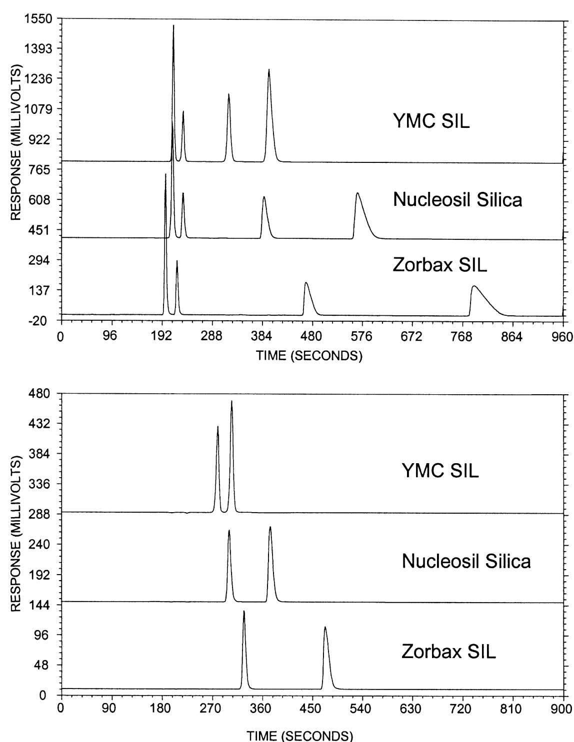

Fig. 4. Pyrimidines (upper) and purines (lower) on silica columns. Conditions: mobile phase 5 m*M* phosphoric acid in acetonitrile–water (75:25) for pyrimidines, (70:30) for purines; 275 nm detection; pyrimidines, ~0.05 mg/ml (in order of elution): 5-fluorouracil, uracil, 5-fluorocytosine, cytosine; purines, ~0.02 mg/ml (in order of elution): acyclovir, guanine.

3.3. Low-molecular-mass amides

Low-molecular-mass amides were investigated as examples of neutral polar compounds. The separation of oxamide, acetamide and formamide on amino and silica columns is shown in Fig. 5. Retention was achieved on both columns for these compounds that are poorly retained on reversed-phase columns. The amino columns afforded incomplete separation, while the amides were baseline resolved on the silica columns. Some selectivity difference was observed for formamide, which was eluted last on the amino column but between the other two peaks using silica. Note that the simple mobile phases used with either column allow low wavelength detection that is necessary for these relatively non-UV-absorbing compounds.

3.4. Hydrophilic interaction–ion-exchange mixed-mode separations using amino columns

Propylamine bonded stationary phases will be protonated at neutral and acidic mobile phase pH values. For compounds that are anions in the mobile phase, this offers the possibility of an anion-exchange mechanism in addition to hydrophilic interactions. The following analytes were used to investigate this possibility: oxamide (neutral), oxamic acid (monocarboxylic acid), oxalic acid (dicarboxylic acid). The pH of the aqueous portion of the mobile phase was held at 7.0 where both acids were in ionic form. The ion-exchange nature of the separation was demonstrated as shown in Fig. 6, where the aqueous–acetonitrile ratio of the mobile phase was

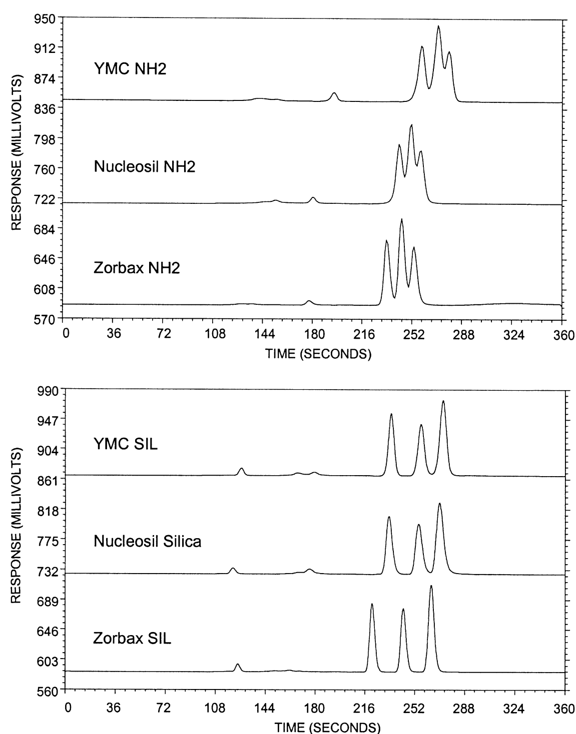

Fig. 5. Separation of amides on amino and silica columns. Conditions: mobile phase for amino columns acetonitrile–25 m*M* potassium phosphate, pH 6.5 (80:20), silica columns acetonitrile–water (80:20); 205 nm detection; oxamide (0.004 mg/ml), acetamide (0.12 mg/ml), formamide (0.06 mg/ml). Elution order on amino columns: oxamide, acetamide, formamide. Elution order on silica columns: oxamide, formamide, acetamide.

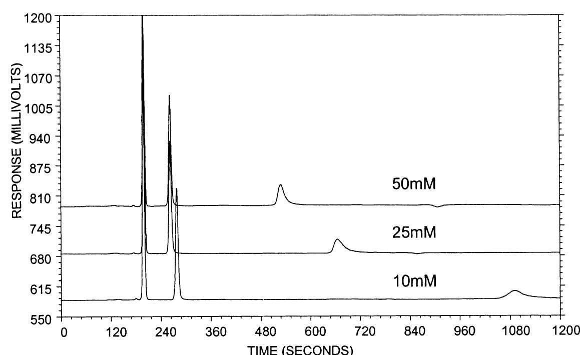

Fig. 6. Effect of mobile phase buffer concentration on separation of of oxamide, oxamic acid, and oxalic acid. Conditions: Zorbax NH_2 column; mobile phase acetonitrile–potassium phosphate buffer (concentration before mixing with acetonitrile, as given in figure), pH 7.0 (60:40); 205 nm detection; elution order: oxamide (0.08 mg/ml), oxamic acid (0.08 mg/ml), oxalic acid (0.06 mg/ml).

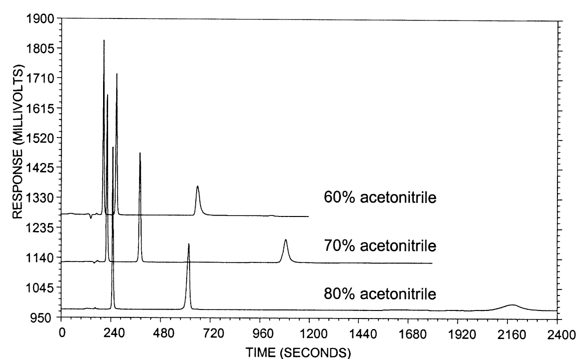

Fig. 7. Effect of aqueous–acetonitrile ratio at constant buffer strength on the separation of oxamide, oxamic acid, and oxalic acid. Conditions: Zorbax NH_2 column; mobile phase percentage acetonitrile as indicated with aqueous phosphate buffer, pH 7.0 to give overall mobile phase buffer concentration of 10 m*M*; 205 nm detection; elution order: oxamide (0.03 mg/ml), oxamic acid (0.06 mg/ml), oxalic acid (0.15 mg/ml).

constant but the buffer concentration was varied. As expected for ion-exchange, higher buffer concentrations resulted in less retention of the anionic components but did not affect retention of the neutral oxamide. In Fig. 7 the overall buffer concentration of the mobile phase was constant but the aqueous–acetonitrile ratio was varied. In this case, the hydrophilic interaction mechanism produced lower retention for all three components as the mobile phase aqueous content increased. Operation in a mixed-mode with amino columns, sometimes viewed as undesirable, can offer additional options for optimization of separations involving anions.

4. Applications

4.1. 5-Fluorouracil in 5-fluorocytosine

The United States Pharmacopeia monograph for 5-fluorocytosine includes a thin-layer chromatographic (TLC) test for 5-fluorouracil with a specification limit of 0.1% [22]. Another TLC method employing fourth derivative UV spectrophotometry had a detection limit of 0.01% [23]. A method involving derivatization followed by gas chromatography (GC)–MS analysis has also been described [24]. This analysis is easily accomplished in the HILIC mode using either an amino or silica column. Fig. 8 illustrates the detection of 5-fluorouracil at

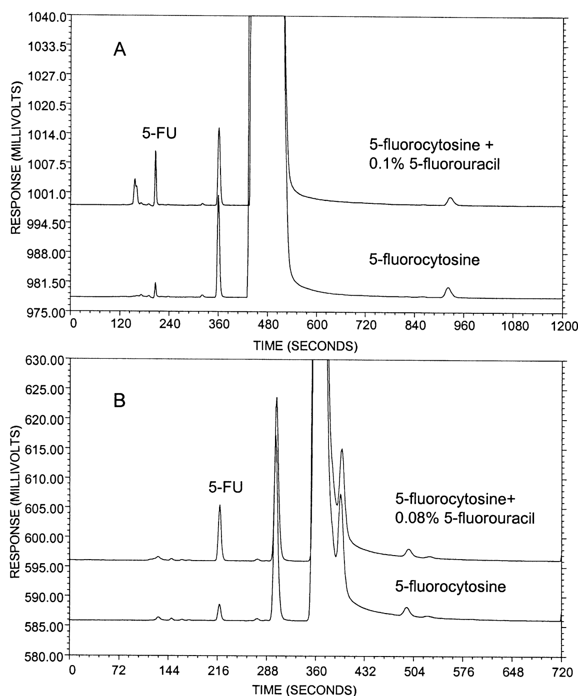

Fig. 8. Determination of 5-fluorouracil in 5-fluorocytosine. (A) Zorbax SIL column; mobile phase acetonitrile–water (75:25), 5 m*M* phosphoric acid; 275 nm detection; 5-fluorocytosine (1.0 mg/ml). (B) Zorbax NH_2 column; mobile phase acetonitrile–25 m*M* potassium phosphate, pH 6.5 (80:20); 275 nm detection; 5-fluorocytosine (1.0 mg/ml).

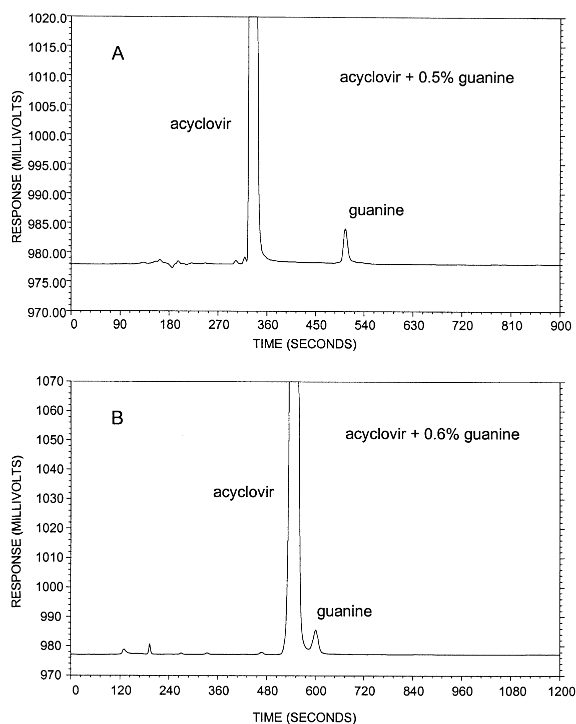

Fig. 9. Determination of guanine in acyclovir. (A) Zorbax SIL column; mobile phase acetonitrile–water (70:30), 5 m*M* phosphoric acid; 275 nm detection; acyclovir (0.22 mg/ml). (B) Zorbax NH_2 column; mobile phase acetonitrile–25 m*M* potassium phosphate, pH 6.0 (80:20); 205 nm detection; 5-fluorocytosine (0.20 mg/ml).

levels below 0.1%. Note that several unknown impurities were also detected in the sample of 5-fluorocytosine. Either of these conditions offers a good possibility for quantifying 5-fluorocytosine at or below 0.1% in contrast to the USP method which is given as a limit test.

4.2. Guanine in acyclovir

A totally aqueous mobile phase is used with RP-HPLC in a pharmacopeial method to quantify guanine in acyclovir at a limit of 0.7% [25]. Other RP-HPLC methods with mobile phases containing 5–10% methanol have also been reported [26,27]. Fig. 9 shows HILIC mode chromatograms for this application on amino and silica columns. Detection of guanine in acyclovir at the specification limit of 0.7% is easily accomplished with either column. In this case, the silica column provides greater resolution of the guanine impurity from the main peak compared to the amino column.

4.3. Acetamide in a synthetic intermediate

A method was needed to determine acetamide at a level of 0.05% in a pharmaceutical synthetic intermediate. The relatively low volatility and detectability of acetamide limit the usefulness of gas chromatography, while poor retention is a problem with RP-HPLC. In this application, the sample compound was only soluble at appreciable concentrations in tetrahydrofuran, which would function as a strong solvent in RP-HPLC and cause an injected solvent effect for poorly-retained analytes. Fig. 10 shows chromatograms of spiked and unspiked sample solutions using an amino column with an aqueous–acetonitrile (20:80) mobile phase. The non-polar main sample component was eluted early in the

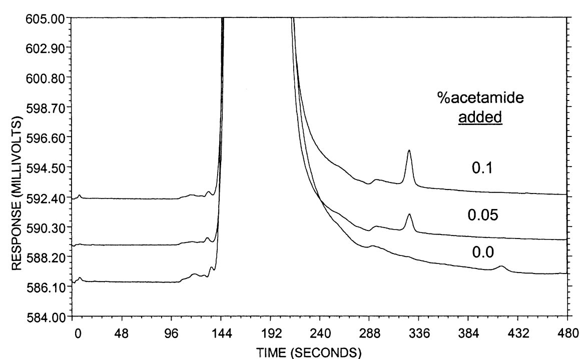

Fig. 10. Determination of acetamide in a pharmaceutical intermediate. Conditions: Zorbax NH_2 column; mobile phase acetonitrile–water (90:10); 205 nm detection; sample concentration: 4 mg/ml in tetrahydrofuran.

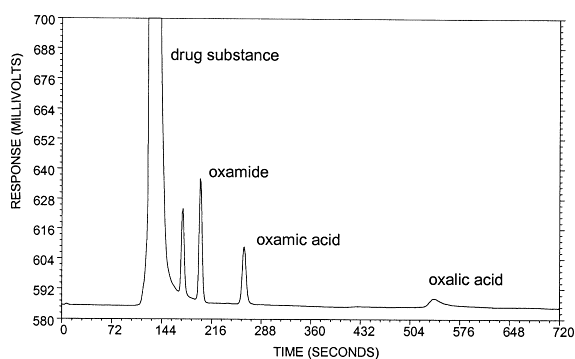

Fig. 11. Determination of oxamide, oxamic acid and oxalic acid in a drug substance. Conditions: Zorbax NH_2 column; mobile phase acetonitrile–50 m*M* potassium phosphate, pH 7.0 (60:40); 205 nm detection; sample concentration: 4.9 mg/ml; standard concentrations: oxamide (0.01 mg/ml), oxamic acid (0.01 mg/ml), oxalic acid (0.03 mg/ml).

chromatogram and acetamide was retained long enough to be detected at the required level. The simple mobile phase allowed detection at the low wavelength needed for acetamide. The analysis was also attempted on a silica column using a mobile phase of 20% water in acetonitrile. Although acetamide was retained under these conditions, the sample matrix peak was broad enough to obscure the acetamide peak.

4.4. Ion-exchange applications

Oxamide, oxamic acid and oxalic acid were all potential impurities in a drug substance. This determination was previously accomplished using ion-exclusion chromatography [28] but was also possible in a HILIC–ion-exchange mode. As shown in Fig. 11, the polar analytes were all detectable in the drug substance, which was not retained. This prevented sample build-up on the column, alleviating a concern with the ion-exclusion method.

4.5. Alternate selectivity

The normal-phase retention behavior afforded by HILIC offers the potential for dramatic changes in selectivity compared to RP-HPLC. An example of this is shown in Fig. 12. The RP-HPLC system utilized an ion-pairing mobile phase to achieve retention of the polar compounds in the sample. Even then, compound 1 (dihydroxy substitution) suffered interference from the baseline disturbance often observed with injections in ion-pairing systems. In the HILIC mode, the retention order for the three most polar compounds was reversed, with compound 1 being retained the longest and all three being retained longer than compounds 4–6. Interestingly, compounds 4–6, while all eluting earlier than 1–3, kept the same relative retention order compared to each other in the RP-HPLC method.

The HILIC mode can provide increased retention, separation, and freedom from baseline artifacts for compounds difficult to retain by RP-HPLC. Another potential application is that non-polar compounds requiring gradient elution by RP-HPLC might be eluted early in an isocratic HILIC system, thereby simplifying the overall analysis.

4.6. Column lifetime

Column lifetime is very application dependent, as are judgments about acceptable column lifetimes. A number of cautions can be made concerning the use of amino and silica columns. Irreversible adsorption of sample components is probably a bigger concern with these columns than with reversed-phase columns. Also, amino and silica columns offer a more reactive stationary phase compared to alkyl phases. Lack of shielding or protection of the stationary

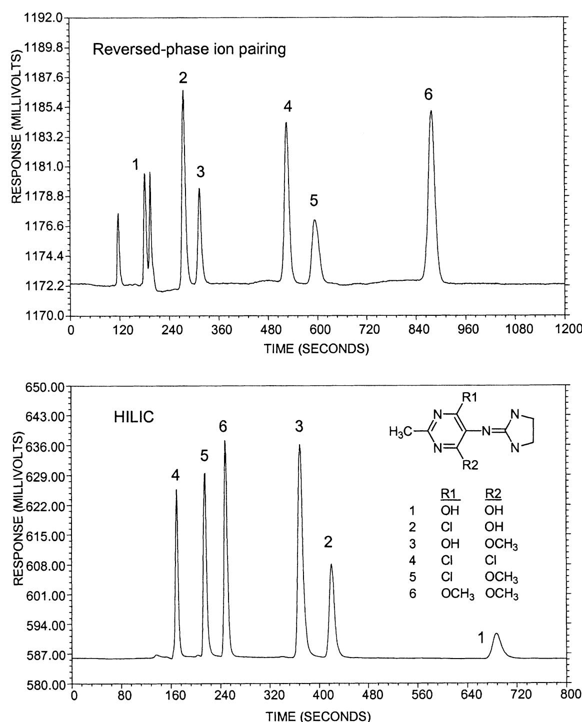

Fig. 12. Comparison of selectivity provided by reversed-phase ion pairing and HILIC separation modes. Reversed-phase conditions: LiChrospher RP-Select B, 250 mm×4.6 mm I.D., 5 μm column; mobile phase acetonitrile–20 m*M* sodium pentanesulfonate (14:86), pH 3.5 with sulfuric acid; 230 nm detection; 1.2 ml/min; column temperature 40°C. HILIC conditions: Zorbax NH_2 column; mobile phase acetonitrile–5 m*M* potassium phosphate, pH 6.5 (65:35); 230 nm detection. Compound numbering is the same for both chromatograms.

phase surface from mobile phase could lead to dissolution of the packing, particularly at higher pH values. The separation of pyrimidines as in Fig. 3 was monitored on a Zorbax amino column over a period of several weeks. Eleven separate runs were made and over 1800 column volumes of mobile phase (25 m*M* potassium phosphate, pH 6.5–acetonitrile, 20:80) was passed through the column. Phosphate buffer at a pH higher than necessary was chosen as a fairly aggressive challenge to the column. The number of theoretical plates obtained for each peak was greater than 94% of the initial value and peak tailing did not increase. Retention and selectivity also indicated no sign of column deterioration.

5. Conclusions

HILIC using amino or silica columns offers an attractive, if not superior alternative to RP-HPLC for the determination of polar analytes in pharmaceutical matrices. Simple acetonitrile–aqueous buffer mobile phases with high acetonitrile content can be employed to retain polar compounds while eluting non-polar compounds relatively quickly. This mode avoids problems associated with highly aqueous mobile phases used for polar compounds with reversed-phase systems and also provides different separation selectivity. Methanol can be used as a strong solvent in the HILIC mode, but appears to offer no advantages over aqueous solvents. Simple mobile phases allow detection at low wavelengths for analytes with poor UV chromophores. Comparable separations were obtained using different brands of amino and silica columns, suggesting that these separations should be robust toward changes in column characteristics. An anion-exchange mechanism can also be exploited using amino columns. Using appropriate precautions, amino columns appear to be rugged enough for routine use applications.

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