Method Development for the Separation of Sulfonamides by Supercritical Fluid Chromatography

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Abstract

Supercritical fluid chromatographic separation of eight regulated sulfonamides is achieved within 20 min by coupling a packed-silica column (250 × 4.6 mm) to an aminopropyl-packed column (250 × 4.6 mm) employing ultraviolet detection. The chromatographic conditions are as follows: 10% methanol is used for 5 min, then increased 2.5% per minute to 30% methanol. The column oven temperature is 65°C, the outlet CO_2 pressure is 300 bar, and the liquid flow rate is 2.5 mL/min. Other stationary phases investigated are Amino 2, Deltabond CN, and Alltima CN (all columns are 250 × 4.6 mm, 5-µm dp). Column deactivation is found to decrease the resolution of the polar sulfonamides by decreasing analyte interaction with active sites. The retention time of sulfathiazole is found to decrease by nearly 50% using an Amino 2 deactivated column, compared with a Spherisorb amino column. Retention behavior of some of the analytes is greatly affected by a subtle change in temperature.

Introduction

Supercritical fluid chromatography (SFC) has received considerable attention as a viable separation technique. SFC is complementary to both gas chromatography (GC) and high-performance liquid chromatography (HPLC) and is not restricted by volatility or thermal lability of the analyte, as is GC. Packed-column SFC is also more efficient per unit of time than HPLC. Packed-column SFC permits larger sample loadings than open-tubular SFC, but the addition of a polar modifier is often required to elute moderately polar to polar analytes from packed columns. Modifiers are seldom used with open-tubular columns.

A large portion of the literature on SFC concerns the analysis of relatively nonpolar materials (1–3). However, applications of polar analytes are becoming much more prevalent with modified fluids. For example, the separation of nitrogen-containing pharmaceutical compounds, including caffeine, tetrazepam, diphenhydramine, phenobarbital, and tetrandin, has been reported by Shi et al. (H. Shi, L.T. Taylor, and E.M. Fujinari. Chemiluminescent nitrogen detection for packed column supercritical fluid chromatography with methanol-modified CO_2. *J. Chromatogr.,* in press.) using chemilumi-

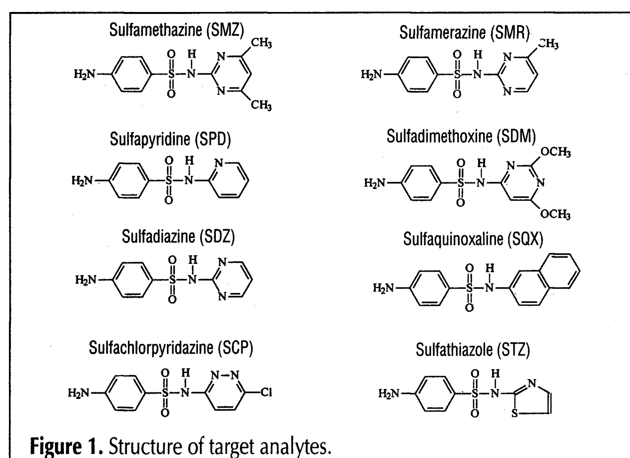

Figure 1. Structure of target analytes.

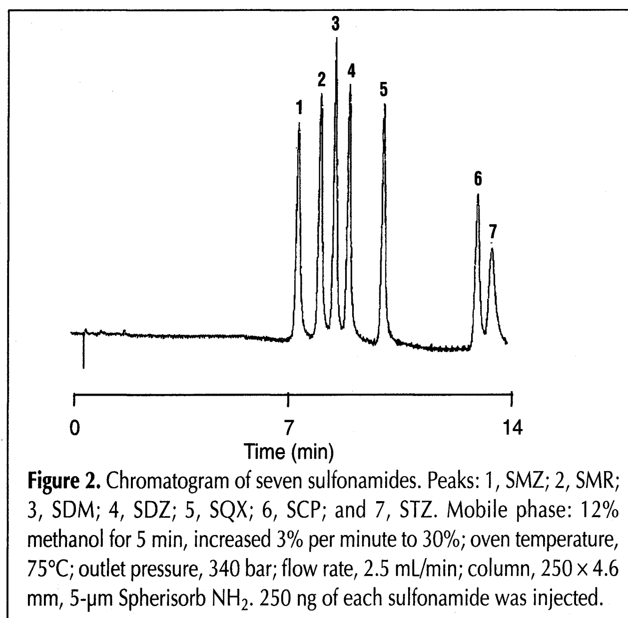

Figure 2. Chromatogram of seven sulfonamides. Peaks: 1, SMZ; 2, SMR; 3, SDM; 4, SDZ; 5, SQX; 6, SCP; and 7, STZ. Mobile phase: 12% methanol for 5 min, increased 3% per minute to 30%; oven temperature, 75°C; outlet pressure, 340 bar; flow rate, 2.5 mL/min; column, 250 × 4.6 mm, 5-µm Spherisorb NH_2. 250 ng of each sulfonamide was injected.

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nescence nitrogen detection (CLND) (150- × 4.6-mm column, 5-µm Valuepak amino; 8% methanol-modified CO_2 held 2 min, increased at 1.5% per minute to 15%; 250–300 atm CO_2 at 10 atm/min at 50°C with a flow rate of 2 mL/min). SFC of phenylurea herbicides using electron-capture detection (ECD) was published by Strode et al. (4). The separation conditions also required a pressure program and a mobile phase gradient program on a Hypersil silica column. SFC separation of lanthanide β-diketonates has also been illustrated (5) with a packed column and ethanol-modified CO_2.

The SFC analysis of sulfonamides has been relatively limited. Games et al. (6) reported analysis of drug residues by combined SF extraction (SFE)–SFC–mass spectrometry (MS)–MS (100- × 4.6-mm column, 5-µm Spherisorb NH_2; 100% CO_2 for 8 min to 20% methanol-modified CO_2 in 0.5 min, held for 10 min; column pressure was maintained at 302 bar at 75°C with a flow rate of 4 mL/min, employing a moving belt MS interface) from freeze-dried pig kidney. Berry et al. (7) and Perkins et al. (8) have further investigated the utility of SFC and SFC–MS for the analysis of sulfonamides. These workers discovered that separation of the eight sulfonamides we were interested in could not be achieved due to peak coelution on both an amino column (100- × 4.6-mm column, 5-µm Spherisorb NH_2; 15% methanol-modified CO_2 for 4 min, increased to 25% with a column pressure of 361 bar at 90°C and a flow rate of 4 mL/min) and a silica column (100- × 4.6-mm column, 5-µm Spherisorb; 12% methanol-modified CO_2 for 5 min, increased to 20% with a column pressure of 263 bar at 75°C and a flow rate of 4 mL/min) (8). Commenting on the density of methanol–carbon dioxide mixtures, Berger (9) found that sulfamethoxypyridizine and sulfimidazole exhibited distinctly different elution behavior with changing temperature. Specifically, chromatographic peak reversal occurred with increasing temperature (150- × 2-mm column, 3-µm Zorbax CN; 5% methanol-modified CO_2 with an outlet pressure of 182 bar and flow rate of 1 mL/min).

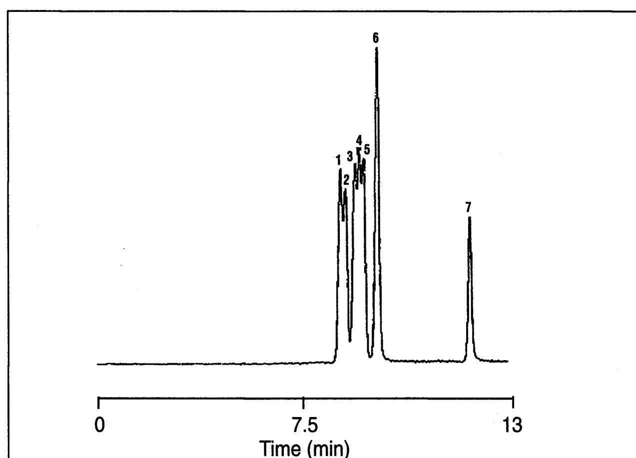

Figure 3. Chromatogram of seven sulfonamides. Tentative peak assignment: 1, SMZ; 2, SMR; 3, SDM; 4, SDZ; 5, SQX; 6, SCP; and 7, STZ. Mobile phase: 2% methanol for 5 min, increased 2% per minute to 15%; oven temperature, 50°C; outlet pressure, 300 bar; flow rate, 2.5 mL/min; column, 250 × 4.6 mm, 5-µm Alltima CN.

The objective of this report was to successfully separate eight regulated sulfonamides (Figure 1) by packed-column SFC with ultraviolet (UV) detection and to present a systematic approach to methods development in SFC. A method development outline for SFC by Berger (10), in brief, consists of: (*a*) determining the solubility of the solutes in a desirable mobile phase; (*b*) matching the column and mobile phase polarity with the polarity of the analyte (a polar analyte requires a polar stationary phase, often with the addition of a polar modifier); (*c*) choosing an appropriate detection system for the needs of the analysis, and (*d*) a step-by-step speed resolution optimization procedure that includes methods for reducing peak tailing and asymmetric peaks, selectivity adjustments, and resolution enhancement, all of which are described in considerable detail in reference 10.

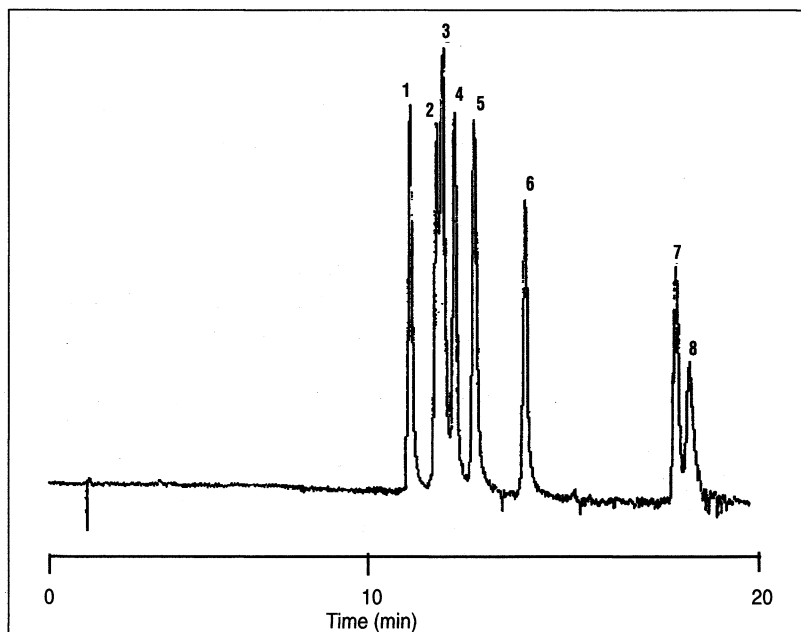

Figure 4. Chromatogram of eight sulfonamides. Tentative peak assignment: 1, SMZ; 2, SMR; 3, SPD; 4, SDM; 5, SDZ; 6, SQX; 7, SCP; and 8, STZ. Mobile phase: 10% methanol for 5 min, increased 2% per minute to 30%; oven temperature, 75°C; outlet pressure, 320 bar; flow rate, 2.5 mL/min; column, 250 × 4.6 mm, 5-µm Alltima CN in series with 250 × 4.6 mm, 5-µm Spherisorb NH_2.

Experimental

A Hewlett-Packard (HP) (Little Falls, DE) model G1205 SF chromatograph was used for all the separations in this study. The system pressure was maintained electronically by a computer-controlled back-pressure regulator, which allowed the flow rate and pressure to be controlled independently. The mobile phase flow rate was measured as liquid at the pump. Organic modifier was added via an auxiliary high-pressure pump. An internal 5-µL loop was used. A standard HP model 1050 multiwavelength detector (MWD) that employed a

13-μL high-pressure flow cell was exclusively used. The sample wavelength was fixed at 270 nm with a 4-nm bandwidth. The reference wavelength was 450 nm with an 80-nm bandwidth. The columns used were: 250 × 4.6 mm, 5-μm Spherisorb NH_2 (Alltech, Deerfield, IL); 250 × 4.6 mm, 5-μm Amino 2 (Keystone Scientific, Bellefonte, PA); 250 × 4.6 mm, 5-μm LiChrosorb SI-100 (Alltech); 250 × 4.6 mm, 5-μm Deltabond CN (Keystone Scientific); and 250 × 4.6 mm, 5-μm Alltima CN (Alltech). All chromatographic conditions are noted in the figure captions.

SFE–SFC-grade carbon dioxide without helium headspace was obtained from Air Products and Chemicals (Allentown, PA). HPLC-grade methanol was purchased from EM Science (Gibbstown, NJ). All sulfonamides (sulfamethazine [SMZ], sulfamerazine [SMR], sulfapyridine [SPD], sulfadimethoxine [SDM], sulfadiazine [SDZ], sulfaquinoxaline [SQX], sulfachlorpyridazine [SCP], and sulfathiazole [STZ]) in this study were obtained from the United States Department of Agriculture Agricultural Research Service (Philadelphia, PA), courtesy of Robert Maxwell. Sulfonamide standard solutions were prepared at 50 ng/μL in methanol unless otherwise noted.

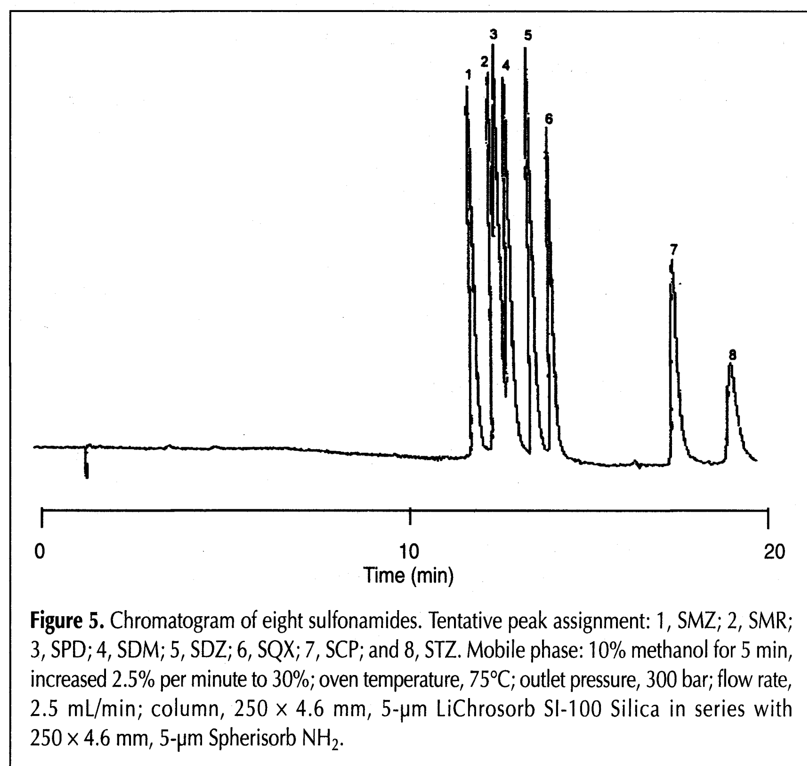

Figure 5. Chromatogram of eight sulfonamides. Tentative peak assignment: 1, SMZ; 2, SMR; 3, SPD; 4, SDM; 5, SDZ; 6, SQX; 7, SCP; and 8, STZ. Mobile phase: 10% methanol for 5 min, increased 2.5% per minute to 30%; oven temperature, 75°C; outlet pressure, 300 bar; flow rate, 2.5 mL/min; column, 250 × 4.6 mm, 5-μm LiChrosorb SI-100 Silica in series with 250 × 4.6 mm, 5-μm Spherisorb NH_2.

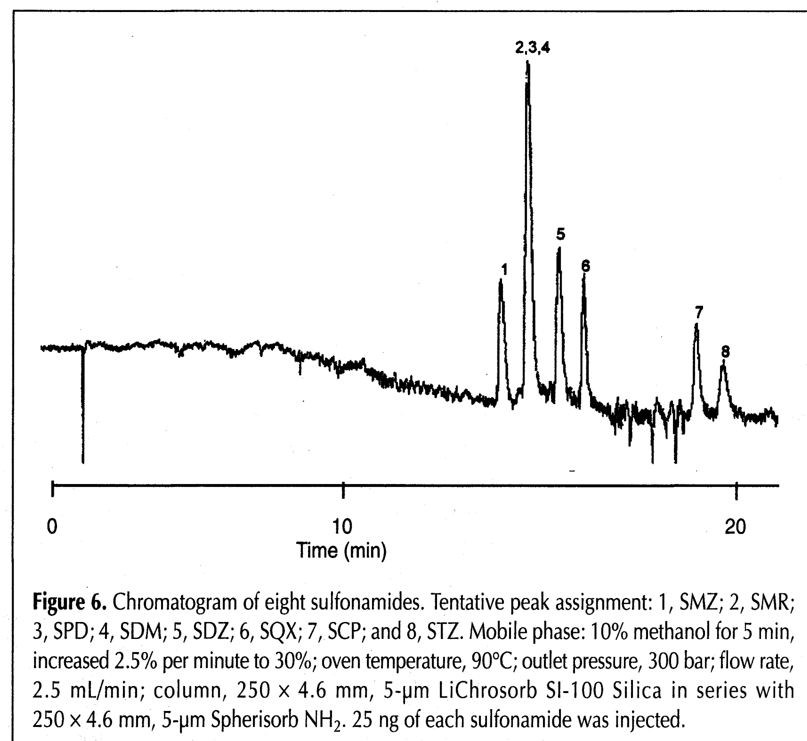

Figure 6. Chromatogram of eight sulfonamides. Tentative peak assignment: 1, SMZ; 2, SMR; 3, SPD; 4, SDM; 5, SDZ; 6, SQX; 7, SCP; and 8, STZ. Mobile phase: 10% methanol for 5 min, increased 2.5% per minute to 30%; oven temperature, 90°C; outlet pressure, 300 bar; flow rate, 2.5 mL/min; column, 250 × 4.6 mm, 5-μm LiChrosorb SI-100 Silica in series with 250 × 4.6 mm, 5-μm Spherisorb NH_2. 25 ng of each sulfonamide was injected.

Results and Discussion

The objective of this study was to develop a separation of eight regulated veterinary drugs, (e.g., sulfonamides). The choice of stationary phase and mobile phase and the effect of temperature on selectivity were investigated in an attempt to provide sufficient and timely resolution of all compounds without the use of density programming.

Based on previous work by Perkins et al. (8), we felt that a CO_2 pressure gradient would not be successful. A combination of multiple stationary phases, modifier gradient, and eventually a subtle temperature change was required to obtain the desired separation.

The first column investigated was a 5-μm Spherisorb NH_2 designed for HPLC. Figure 2 shows the separation obtained with seven sulfonamides. Sufficient resolution can be obtained for all compounds (resolution [*R*] was greater than 3.7 for peaks 2, 3, and 4). However, the addition of SPD caused coelution with SMR. Perkins et al. (8) also reported coelution of SPD and SMR with the use of an aminopropyl silica stationary phase under similar chromatographic conditions. Due to the coelution, an alternate amino column was investigated. The trace obtained using an Amino 2 column, which was said to be a highly deactivated column, decreased the retention time of STZ nearly 50%, for example. This decreased retention also caused a loss in resolution of the early eluting peaks, which also precluded the use of the Amino 2 column.

Since the aminopropyl silica did not provide an adequate separation, a Deltabond cyanopropyl phase was investigated. Deltabond CN consists of a polymeric coated silica particle that results in a highly deactivated phase. As with the deactivated amino phase, a poor separation was obtained for the seven investigated

sulfonamides. Therefore, another cyano stationary phase was attempted. An Alltima CN column was used. This column is deactivated by double endcapping. The chromatogram obtained is shown in Figure 3. Seven compounds can be observed, although baseline resolution was not obtained (R = 1.10 for peaks 3 and 4), and the SPD was not added to the mixture.

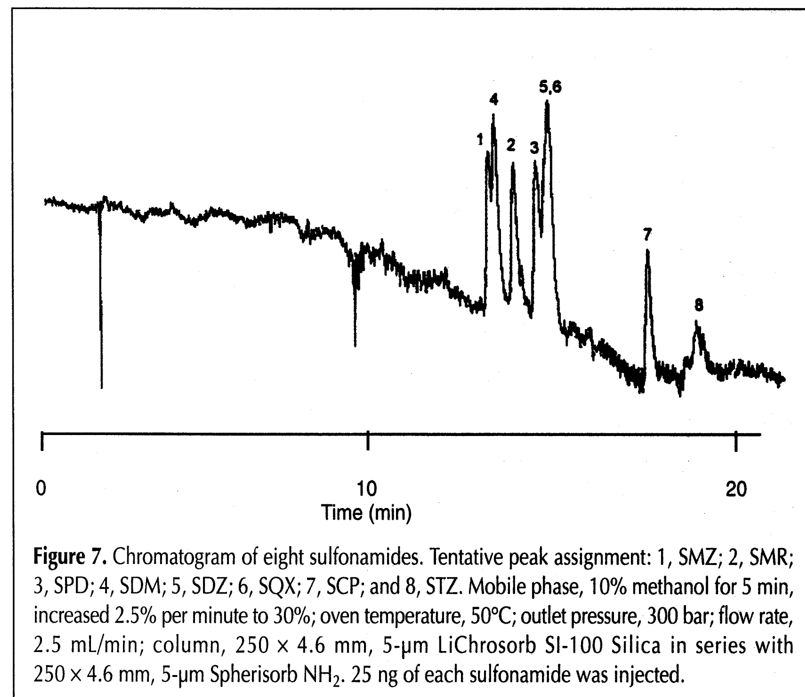

Figure 7. Chromatogram of eight sulfonamides. Tentative peak assignment: 1, SMZ; 2, SMR; 3, SPD; 4, SDM; 5, SDZ; 6, SQX; 7, SCP; and 8, STZ. Mobile phase, 10% methanol for 5 min, increased 2.5% per minute to 30%; oven temperature, 50°C; outlet pressure, 300 bar; flow rate, 2.5 mL/min; column, 250 × 4.6 mm, 5-µm LiChrosorb SI-100 Silica in series with 250 × 4.6 mm, 5-µm Spherisorb NH_2. 25 ng of each sulfonamide was injected.

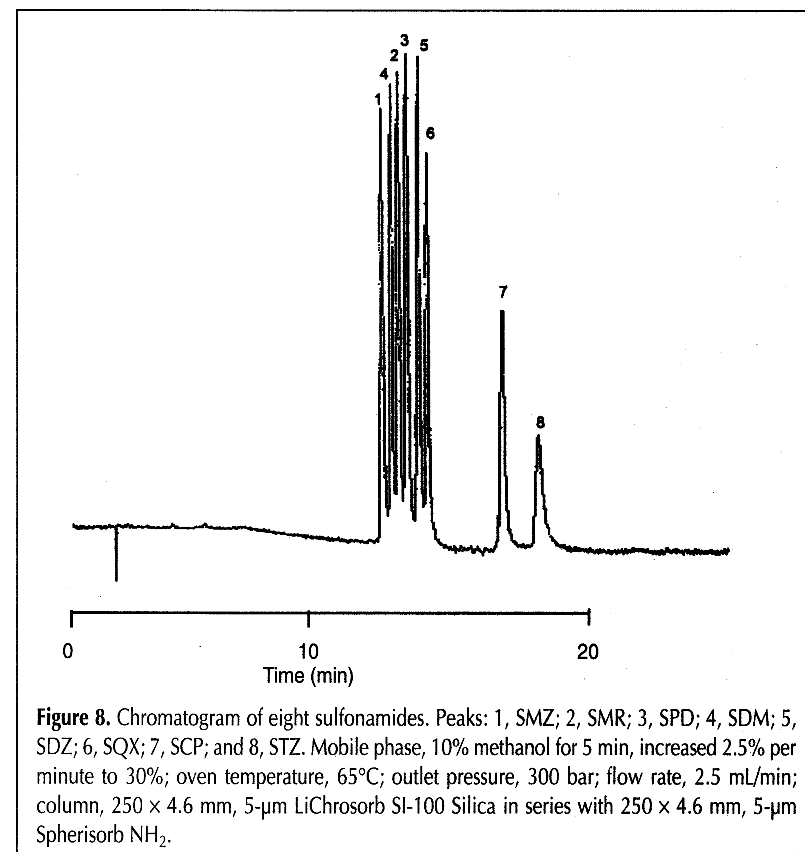

Figure 8. Chromatogram of eight sulfonamides. Peaks: 1, SMZ; 2, SMR; 3, SPD; 4, SDM; 5, SDZ; 6, SQX; 7, SCP; and 8, STZ. Mobile phase, 10% methanol for 5 min, increased 2.5% per minute to 30%; oven temperature, 65°C; outlet pressure, 300 bar; flow rate, 2.5 mL/min; column, 250 × 4.6 mm, 5-µm LiChrosorb SI-100 Silica in series with 250 × 4.6 mm, 5-µm Spherisorb NH_2.

Because coelution with the addition of SPD on the Spherisorb amino phase was observed, two columns of different selectivities were run in series (Figure 4) (e.g., the Alltima CN column, followed by the Spherisorb NH_2). With this configuration, we were able to separate the eight compounds of interest with moderate resolution (R = 1.24 for peaks 2 and 3, and R = 2.67 for peaks 3 and 4). Two different stationary phases were used in an attempt to separate SPD and SMR on the cyano column, followed by elution on the amino column. However, additional attempts to improve the separation were unsuccessful.

Perkins et al. (8) achieved adequate separation of seven of the sulfonamides that we were interested in on either an amino or silica stationary phase. However, when SPD was added to the mixture, coelution occurred with SMR on the amino column and with SDZ on the silica column. In light of these events, we used a silica column followed by an amino column in an attempt to separate one pair of coeluting peaks on the silica column and maintain separation of the other components on the amino column (Figure 5). Separation of the eight drugs was achieved under these conditions, but without baseline resolution (R = 1.31 for peaks 2 and 3, and R = 2.02 for peaks 3 and 4). Injection of a smaller quantity of material under the same chromatographic conditions had little effect on the separation.

We then looked at altering the temperature from 75°C to improve separation. Peaks 2–4 coeluted when the temperature was increased to 90°C, as shown in Figure 6. The chromatogram was more noisy because only 10% of the original amount was injected. We next reasoned that if an increase in temperature could harm the separation, a decrease in temperature might improve the existing resolution. At a temperature of 50°C, however, more retention differences were observed (Figure 7). Peaks 5 and 6 then coeluted, whereas baseline separation of these peaks was obtained at both 75 and 90°C. A more important observation was that peaks 2–4 were resolved at 50°C, although peaks 1 and 4 coeluted, and peak 3 coeluted with peaks 5 and 6. An intermediate temperature was then attempted in order to obtain the positive effects of lowering the temperature while maintaining separation of the later eluting peaks. Figure 8 shows the chromatogram obtained at 65°C. By using a temperature between those previously investigated, a complete separation was achieved for all eight compounds of interest within 20 min (R > 2.3 for all peaks). The separation was reproducible; relative standard deviations (RSDs) of retention times were less than 1.15% (two replicates), and the RSD of peak area was less than 3.6% (two replicates).

In retrospect, to obtain the desired results, a

systematic strategy of method development was performed. First, the suitability of the mobile phase was known, based on previous studies. Stationary phase polarity was matched with the analyte polarity. However, complete separation could not be obtained with a single stationary phase (either silica, cyano, or amino); two dissimilar phases were required. Variation of the CO_2 pressure and mobile phase composition was required to improve the separation. A modifier gradient was also necessary to achieve separation of early eluting peaks and to speed the elution of late eluting peaks. Changing the temperature of the separation altered the analyte selectivity and was required in our study to obtain the desired separation.

Conclusion

The objective of this study was to obtain a separation of eight regulated sulfonamides. Several different columns were tested, including aminopropyl, deactivated aminopropyl, deactivated cyanopropyl, and silica. A satisfactory separation was achieved by coupling a silica column to an aminopropyl column. Column deactivation was found to decrease the resolution of the polar sulfonamides by decreasing interaction with active sites. Temperature was found to play a vital role in the separation.

Acknowledgments

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