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Characteristics and applications of a new high-performance liquid chromatography guard column

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Abstract

A new HPLC guard column has been developed that significantly increases chromatographic efficiencies when placed in series with an analytical column. When compared with other commercially available guard columns, only the Sentry guard column was found to result in increased plates. All other guard columns evaluated resulted in a 10–60% loss of chromatographic efficiency.

By stressing the Sentry guard columns with injections of crude mouse serum it was shown that analytical column lifetimes could be extended. The Sentry guard columns have also been shown to have higher capacity for contaminants than most competitive guard cartridges. Over 8000 injections of a standard mixture have been made on the Symmetry C_8 analytical columns protected by Sentry guard columns with no significant change in the analytical columns' performances.

1. Introduction

Guard cartridge columns are widely used as a cost-effective means of prolonging analytical HPLC column lifetime [1–4]. They protect the analytical column from particles, sub-particles and molecular contaminants. These contaminants originate from the sample matrix, the mobile phase and/or the HPLC instrumentation (e.g., seals). The ideal guard column is designed to protect the analytical column from any or all three failure modes which are (1) a sample irreversibly adsorbing onto the stationary phase, (2) particulate in the sample plugging the inlet frit and (3) particulate in the sample passing through the frit and plugging the packed bed.

The first protection a guard column provides is a highly porous frit made of either stainless steel or an inert material that filters particles larger than its pore size. Particulate debris builds up on the guard column, resulting in an increase in backpressure. This problem is corrected on an unprotected analytical column by cleaning or changing the inlet filter, providing that the column design permits this operation. The analytical column can also be cleaned by reversing the flow direction in order to force the particulate out of the column [1].

The sub-particulate debris can pass through the column frit. The HPLC column can act as a depth filter for this sub-particulate debris, trapping these particles at the head of the column. This can result in a change in hydrodynamic flow at the column entrance which may have unfavorable effects on column performance (peak tailing and/or splitting). One common example of sub-

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particulates that can affect column performance are proteins. Injection of an aqueous protein sample on to a reversed-phase column may result in precipitation of the protein on the top of the column bed because of both filtration and adsorption. In an unprotected column this type of contamination would lead to an irreparable failure. The guard column protects the analytical column from this sub-particulate debris; this type of contamination leads to an increase in backpressure for the guard column.

Adsorption of significant amounts of molecular contaminants may mask the chromatographic surface and result in deviations in absorption behavior. Such molecular adsorption can cause changes in retention (reduction in k'), distorted peak shapes and a decrease in column performance. In this case the unprotected column would have to be washed with a strong solvent to recover performance.

The designs of most guard cartridges allow particulate and chemical contaminants to be removed; however, typical guard columns are usually poorly packed to keep costs low. This results in a significant reduction in the performance of the analytical column. Some guard cartridges cause as much as a 60% decrease in efficiency.

This report discusses a new guard column that not only has high capacity for mechanical and chemical filtration but also increases the efficiency of the analytical column. The efficiency and capacity of the Sentry guard column were compared to those of other commercial guard columns. Lifetime studies were done with Nova-Pak and Symmetry reversed-phase columns to show how the Sentry guard column extends column lifetime.

2. Experimental

2.1. Materials and reagents

Sulfanilamide, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine and succinylsulfathiazole were from Sigma (St. Louis, MO, USA). Carbamazepine, carbamazepine-10,11-epoxide, 2-ethyl-2-phenylmalonamide, primidone, phenytoin and phenobarbital were from Alltech (Deerfield, IL, USA). Mouse serum came from BioProducts for Science (Indianapolis, IN, USA). Acetonitrile, acetone, methanol, trifluoroacetic acid, acenaphthene and potassium phosphate, dibasic were from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid and potassium phosphate, monobasic were from EM Science (Cherry Hill, NJ, USA).

Sentry guard columns, Nova-Pak C_{18} and Symmetry C_8 columns were from Waters (Milford, MA, USA). Direct-Connect guard columns with Econosil (10 μm C_{18}) and with Adsorbosphere (5 μm C_{18}) (10 mm × 4.6 mm) were from Alltech. Upchurch ODS cartridge (10 mm × 4.3 mm) was from Upchurch Scientific (Oak Harbor, WA, USA). Zorbax Reliance Rx-C_8 (12.5 mm × 4.0 mm) was from MAC-MOD Analytical (Chadds Ford, PA, USA). Brownlee MPLC NewGuard cartridge (RP-18) and holder came from Applied Biosystems (Foster City, CA, USA).

The 0.45-μm Millex HV filter unit was from Millipore. The Eppendorf micro centrifuge Model 5415C was supplied by Brinkmann Instruments (Westbury, NY, USA). The Mistral column thermostat was from Euramark (Euro-American Marketing Groups, Mount Prospect, IL, USA). The Rheodyne injector is manufactured by Rheodyne (Cotati, CA, USA).

2.2. HPLC systems

The efficiency measurements on the guard columns alone and with the analytical column were performed using a low-dispersion or microbore HPLC system. The microbore system included the Waters 625 LC System, the Waters 484 tunable absorbance detector fitted with a microbore cell, and a Model 7520 Rheodyne injector equipped with a 0.5-μl polyether ether ketone (PEEK) rotor. This system had a bandspread of 30 μl at 0.2 ml/min flow-rate.

The lifetime studies with crude and spiked mouse serum were done on a Waters 625 LC System, a Waters 484 tunable absorbance detector or the Model 490 multiwavelength detector

and either a Waters Model 715 autoinjector or the WISP Model 712. The lifetime study with sulfa drugs used the Waters 590 programmable solvent-delivery module, Waters Model 440 absorbance detector and the WISP Model 712.

All data were acquired and analyzed using the Waters 845 Station with System Suitability Software Package and ExpertEase version 3.0.

2.3. Efficiency measurements

The plate count sample was prepared by dissolving 200 mg acenaphthene in 50 ml acetonitrile and then adding two 10-ml portions of water, shaking after each addition. As a void volume marker, 2.4 ml of acetone were added to the plate count sample. The injection volume was 0.5 ml.

The flow-rates for the efficiency measurements were 0.5 and 0.7 ml/min, respectively, for the 3.9 mm and 4.6 mm I.D. columns. The mobile phase was acetonitrile–water (50:50, v/v).

The efficiencies of the guard columns were measured on the microbore HPLC system, as well as the efficiencies of the analytical columns alone and in series with the guard columns. The guard columns and analytical columns that were connected in series were matched by the packing chemistry and the column inner diameter.

2.4. Extra-column band broadening

When a guard column is placed in series with an analytical column, the efficiency expected for the column alone ($N_{c,calc}$) can be calculated. The guard column contribution to peak broadening is subtracted out.

$$N_{c,calc} = \frac{25(t_{c+g} - t_g)^2}{w_{c+g4.4}^2 - w_{g4.4}^2}$$

where t_{c+g} is the retention time of the analyte with the guard column in line, t_g is the retention time of the analyte for the guard column alone, $w_{c+g4.4}$ is the width of the peak at 4.4% of peak height with the guard column in line and $w_{g4.4}$ is the width of the peak at 4.4% of peak height for the guard column alone.

2.5. Lifetime studies

Crude mouse serum

The mouse serum was filtered using a 0.45-μm Millex HV filter unit. The plate count of the Nova-Pak C_{18} column (150 mm × 3.9 mm) in series with a Sentry guard column was measured. Afterward five injections of filtered mouse serum were made. These series of injections (plate count analysis followed by five mouse serum injections) were repeated until the efficiency decreased 50%.

A gradient analysis was done for the mouse serum chromatography. The mobile phases were 0.1% aqueous trifluoroacetic acid (TFA) as solvent A and acetonitrile with 0.1% TFA as solvent B. The gradient was 0 to 100% solvent B in 10 min at 1 ml/min. The wavelength used to monitor absorbance was 254 nm.

Spiked mouse serum

The proteins were removed from the mouse serum by adding two parts acetonitrile to one part serum. After mixing, the sample was centrifuged at 1500 *g* for 5 min and the supernatant decanted and collected. The deproteinated mouse serum was then spiked with the antiepileptic drugs and their metabolites. The final serum sample contained 25 μg/ml of each drug which included carbamazepine, carbamazepine-10,11-epoxide, 2-ethyl-2-phenylmalonamide, primidone, phenytoin and phenobarbital.

The Nova-Pak C_{18} column was tested with and without the Sentry guard column. The mobile phase was 10 m*M* potassium phosphate, pH 7.0–acetonitrile–methanol (110:50:30, v/v/v). The flow-rate was 0.5 ml/min with temperature controlled at 40°C using the Mistral column thermostat. The absorbance was monitored at 214 nm.

The injections of spiked mouse serum were continued until there was a significant change in backpressure, efficiency, retention time or relative retention.

Sulfa drugs

The lifetime studies with the sulfa drugs were on Sentry guard columns in series with the

Symmetry C_8 column (150 mm × 3.9 mm). The mobile phase was water–methanol–glacial acetic acid (79:20:1, v/v/v). The mobile phase was recycled and changed approximately every 1000th injection. The chromatography was monitored at 254 nm and used a flow-rate of 1 ml/min and a run time of 11 min. The temperature was controlled at 25°C using a the Mistral column thermostat. The sample contained 10 μg/ml of sulfanilamide, 25 μg/ml of sulfadiazine and sulfathiazole, 29 μg/ml of sulfamerazine, and 39 μg/ml of sulfamethazine and succinylsulfathiazole. The injection volume of this sample was 10 μl.

Guard columns were changed either when the backpressure increased (30%) or exceeded 3000 p.s.i. (1 p.s.i. = 6894.76 Pa), or when the performance changed (30% decrease in efficiency or relative retention). The retention time and retention factor as well as peak asymmetry were also monitored.

3. Results and discussion

3.1. Guard column efficiency

Table 1 summarizes the results for the plate count tests for the Waters Sentry guard column and other commercial guard cartridges. The efficiency of the Sentry guard column approached analytical values (1000 plates/cm). The Alltech Direct-Connect Econosil and the Upchurch ODS cartridges have the next highest efficiencies, 480 and 450 plates/cm, respectively. The results for the Brownlee NewGuard, Zorbax Reliance and Direct-Connect Adsorbosphere guard cartridges are not close to analytical column values.

The geometry of the hardware and the packing process have an impact on the efficiency of the guard cartridge. In order to achieve efficiencies similar to those of analytical columns the guard columns should be filled by a high-performance packing technique. Dry filling or low-pressure packing probably contributed to the lower efficiencies observed for the guard cartridges. A high-pressure packing process using a slurry was developed for the Sentry guard columns to give both high efficiency and a stable bed.

3.2. Effect of guard column on HPLC column efficiency

A typical 5-μm Symmetry C_8 column (150 mm × 3.9 mm) had 12 400 plates. The 3.9 mm and 4.6 mm Nova-Pak C_{18} columns (150 mm length) averaged 13 200 and 14 000 plates, respectively. The 4-μm Nova-Pak columns were used with all commercial cartridges except the Zorbax Reliance cartridge. The Zorbax Rx-C_8 (150 mm × 4.6 mm) which had 11 500 plates was used with the Zorbax Reliance guard cartridge.

The diameters of the analytical columns and those of the guard columns were matched as closely as possible. The 4.6 mm Nova-Pak C_{18}

Table 1
Efficiencies of commercial guard columns

Guard Column	Dimensions	No. of plates
Waters Sentry Nova-Pak C_{18} (4 μm)	20 mm × 3.9 mm	2000
Alltech Direct-Connect	10 mm × 4.6 mm	
Econosil C_{18} (10 μm)		480
Adsorbosphere C_{18} (5 μm)		280
Brownlee MPLC NewGuard RP-18 (7 μm)	15 mm × 3.2 mm	90
Upchurch ODS Cartridge (5 μm)	10 mm × 4.6 mm	450
Zorbax Reliance Rx-C_8 (5 μm)	12.5 mm × 4.0 mm	60

The guard columns were analyzed on a microbore HPLC system. The mobile phase was acetonitrile–water (50:50, v/v). The sample contained acenaphthene (2.9 mg/ml) and acetone (34 μl/ml). The flow-rate was adjusted on the basis of the guard column inner diameter in order for all analysies to be at the same linear velocity (0.5 ml/min for 3.9 mm I.D. column). All analyses were at 254 nm.

column was used with the Alltech and Upchurch guard cartridges (4.6 mm I.D.) whereas the 3.9 mm column was used with the Brownlee cartridge (3.2 mm). Fig. 1 shows the effects of the guard cartridges on the analytical columns' efficiencies. The plate counts for the Nova-Pak C_{18} and Symmetry C_8 columns (150 mm × 3.9 mm) were enhanced when they were in series with the corresponding Sentry guard columns. The Direct-Connect Adsorbosphere cartridge caused only a slight decrease (2% lower) in the efficiency of the Nova-Pak C_{18} column. Greater decreases in the efficiency of the analytical columns were noted when placed in series with the other cartridges. A 60% decrease in plates was observed for the Zorbax Rx-C_8 column when the Zorbax Reliance Rx-C_8 cartridge was put in line.

A decrease in efficiency is expected when the diameter of the guard column is greater than that of the analytical column or when there is a large difference in particle size [2,5]. The deterioration in performance due to the guard column having the larger diameter has been well documented by users of the Waters Guard-Pak cartridges (5 mm I.D.) with the 3.9 mm I.D. HPLC columns. We observed a 30% decrease in plate count of the Nova-Pak column when in series with a Guard-Pak cartridge. No effects from inner diameter should have been apparent in this study since the guard and analytical columns were matched. In addition, particle size did not vary significantly in most cases. The only exception was the Direct-Connect Econosil cartridge that contains 10-μm particles and was used with the 4-μm Nova-Pak column. Therefore, the fact that the Direct-Connect Econosil cartridge caused the greatest decrease in efficiency for the Nova-Pak column

Table 2
Extra-column effects from guard column: plate counts for Nova-Pak columns

	No. of plates	
	Column length 50 mm	Column length 150 mm
Measured (column alone)	3900	13 200
Calculated (minus Sentry guard column contribution)	3500	12 800
Change (%)	−10	−3

Plate counts were measured for the Nova-Pak C_{18} columns (150 mm and 50 mm × 3.9 mm I.D.) alone and in series with the Sentry guard column. The sample contained acenaphthene (2.9 mg/ml) and acetone (34 μl/ml). The data are for the acenaphthene peak at 254 nm. Mobile phase was acetonitrile–water (50:50, v/v) at a flow-rate of 0.5 ml/min. See Experimental section for equation for calculations.

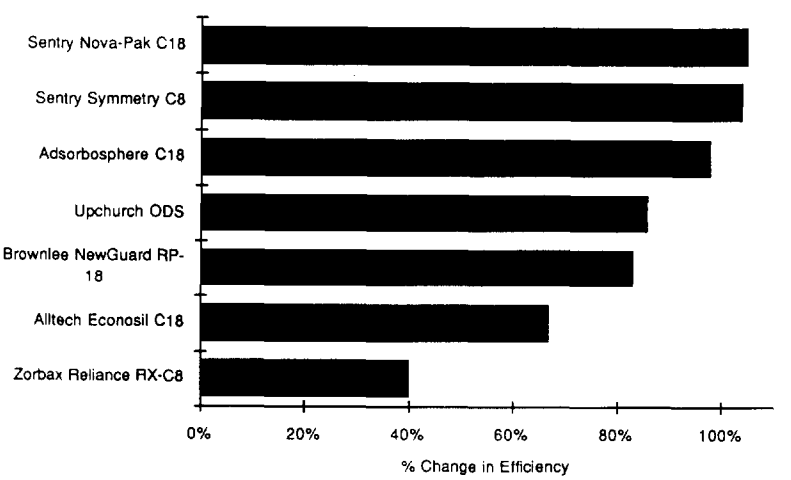


Fig. 1. Effect of guard columns on HPLC columns' efficiencies. Analytical column, Nova-Pak C_{18} (150 mm × 3.9 mm or 4.6 mm) except Zorbax Rx-C_8 (150 mm × 4.6 mm) with Zorbax Reliance cartridge. See Table 1 for dimensions of guard columns. Mobile phase, acetonitrile–water (50:50, v/v); flow-rate 0.5 ml/min for 3.9 mm I.D. and 0.7 ml/min for 4.6 mm I.D column; detection, 254 nm. The sample was 0.5 μl injection of acenaphthene (2.9 mg/ml) and acetone (34 μl/ml) in acetonitrile–water (70:30, v/v).

could be partially related to differences in particle size.

Extra-column volume was minimized by using low-dead-volume compression fittings and a minimal length of narrow-bore tubing (≤ 0.01 in. I.D., 1 in. = 2.54 cm)) to connect the guard cartridge to the analytical column [2,4]. The depression in analytical column efficiency is probably related to the efficiency of the guard cartridge itself. Most of the guard columns were not packed efficiently as noted in Table 1.

Since the Sentry guard column enhanced the analytical column efficiency, it was assumed that this guard column did not contribute to band broadening. When a guard column is placed in series with an analytical column the efficiency expected for the column alone can be calculated [4]. The guard column contribution to peak broadening is subtracted out. Using the formula given in the Experimental section, the plate count for the analytical column alone was calculated ($N_{c,calc}$) and compared to the measured value. The data are in Table 2. The % change in Table 2 is a qualitative measure of the contribution to peak broadening not merely due to volume increase by the guard column. The Sentry guard column showed little additional peak broadening, therefore, acting as an extension of the HPLC column length. These results support the enhancement of efficiency by this guard column.

In summary, the Sentry guard column causes an increase in efficiency when added to an analytical column. The design of the Sentry

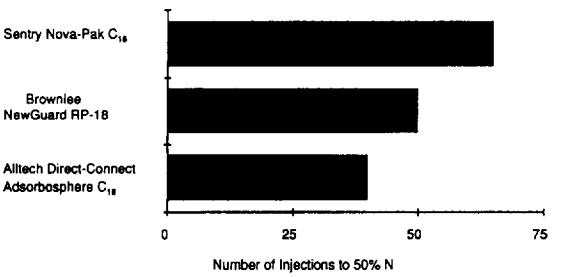


Fig. 3. Number of injections of crude mouse serum before a 50% decrease in plate count. Plate count measured following each set of five mouse serum injections. Figs. 1 and 2 give methods for plate count measurement and mouse serum injections, respectively.

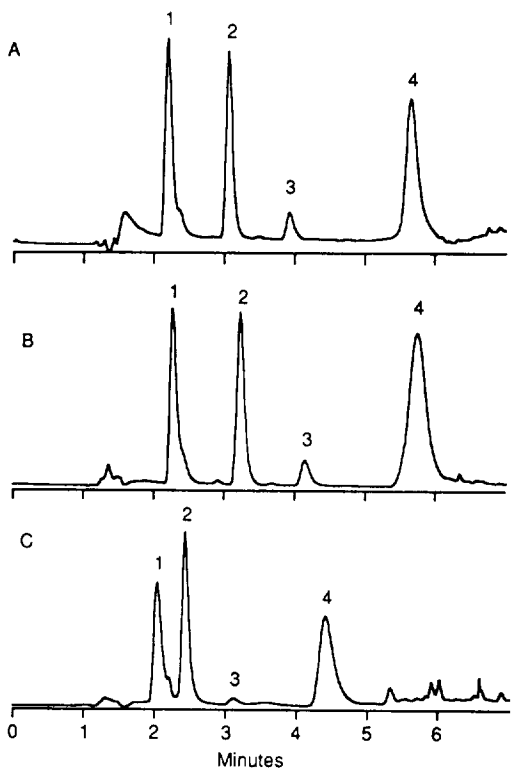


Fig. 2. Crude mouse serum capacity test with and without Sentry guard column. Analytical column, Nova-Pak C_{18} (150 mm × 3.9 mm) with and without Nova-Pak C_{18} Sentry guard column. Mobile phase A, 0.1% aqueous trifluoroacetic acid (TFA); mobile phase B acetonitrile with 0.1% TFA; gradient, 0 to 100% B in 10 min; flow-rate, 1 ml/min; detection, 254 nm. The sample was 10 μl filtered mouse serum. (A), (B) First and 55th injections, respectively, of mouse serum on column with Sentry guard column; (C) 55th injection on unprotected column. (The peaks were not identified; they are general serum proteins.)

Table 3
Change from 1st to 200th injection of spiked mouse serum

	Change (%)			
	Column alone		Column with Sentry	
	Peak 4	Peak 6	Peak 4	Peak 6
Retention time	−3.2	−4.5	2.6	2.8
Plates	−17	−10	2.3	−3.7

Experimental conditions same as in Fig. 4. Peaks 4 and 6 (from Fig. 4) = carbamazepine-10,11-epoxide and carbamazepine, respectively.

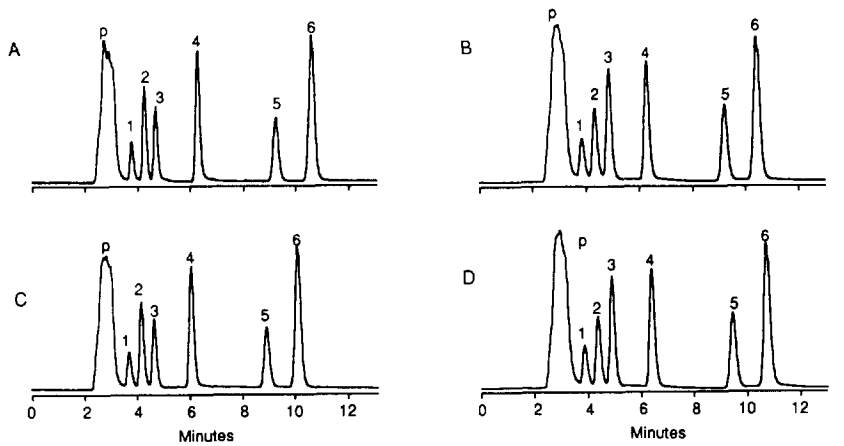
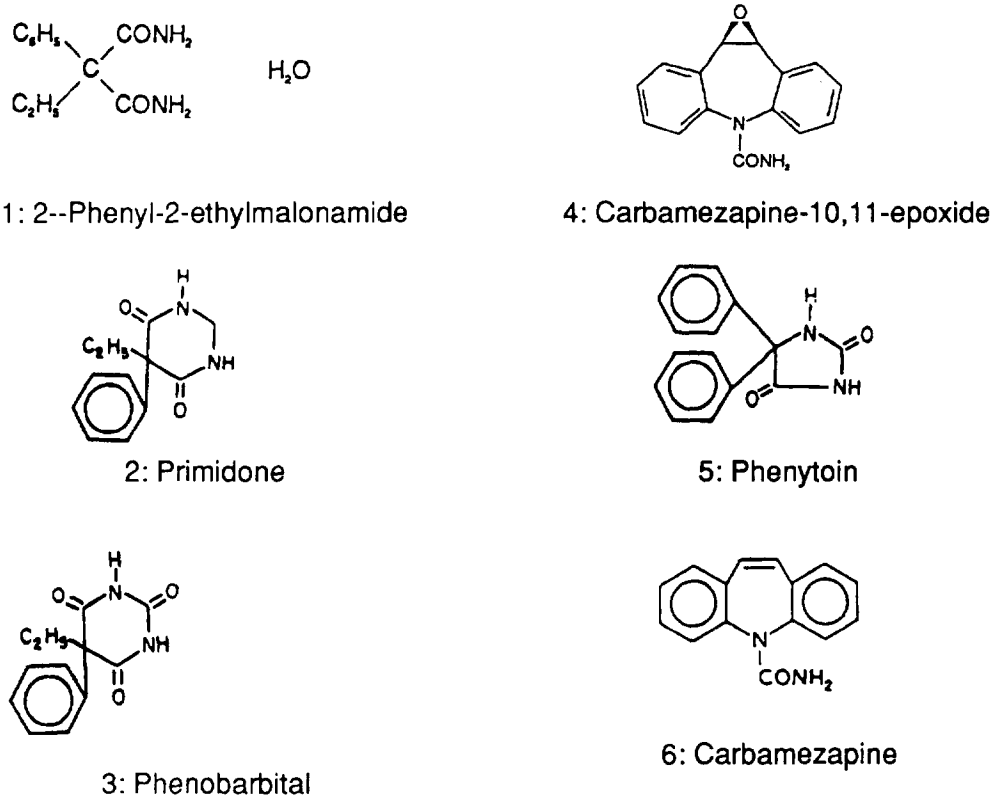


Fig. 4. Effect of guard column on column lifetime with spiked mouse serum sample. Analytical column, Nova-Pak C_{18} (150 mm × 4.6 mm); mobile phase, 10 m*M* potassium phosphate, pH 7.0–acetonitrile–methanol (110:50:30, v/v/v); flow-rate, 0.5 ml/min; temperature, 40°C; detection, 214 nm; injection volume, 5 μl of sample with 25 μg/ml of each component in mouse serum. Peaks: p = serum matrix; 1 = phenylethyl malonamide; 2 = primidone; 3 = phenobarbital; 4 = carbamazepine-10,11-epoxide; 5 = phenytoin; 6 = carbamazepine (see Fig. 5 for chemical structures). (A), (B) First injection on analytical column without and with matching Sentry guard column, respectively; (C), (D) 200th injection on analytical column without and with matching Sentry guard column, respectively.



guard column and the high quality of the packed bed are the reasons for this increase in efficiency. The Sentry guard column length (2 cm) increases the total packed bed length which should theoretically increase plate count. The Sentry guard column does not cause extra-column band-spreading because its diameter is the same or less than that of the analytical columns. Therefore, the expected increase in efficiency due to length is observed. The combination of the Sentry guard column with the analytical column has a higher plate count than the analytical column alone.

3.3. Crude mouse serum capacity test

An important benefit of the guard column is to protect the analytical column from debris. The guard column should be used anytime a sample contains anything that can be irreversibly bound to the analytical column. Mouse serum contains proteins that are not completely removed with each gradient run. Therefore, an accumulation of particulate debris and molecular contaminants on the guard columns was expected with this sample. The chromatography for the mouse serum injections on Nova-Pak C_{18} columns (150 mm × 3.9 mm) with and without the Sentry guard column are given in Fig. 2. The chromatography in Fig. 2C shows a major change after 55 injections on an unguarded Nova-Pak column; molecular contaminants resulted in a change in chromatographic performance. This column went high pressure after 59 injections probably due to the inlet frit being plugged. The addition of the Sentry guard column extended the lifetime of the column to more than 70 injections with no apparent changes in performance.

The data in Fig. 3 show the number of injections of mouse serum before the original plate count decreased 50%. The numbers are the average values for three different guard columns each in series with a Nova-Pak C_{18} column. The Sentry guard column withstood over 70 injections whereas the Brownlee NewGuard RP-18 cartridge and the Alltech Direct-Connect Adsorbosphere cartridge failed after less than 50 injections. The greater volume of the Sentry guard column (0.24 ml) than the Brownlee (0.12 ml) or the Upchurch (0.17 ml) cartridges probably contributed to its increased capacity for mouse serum. The efficiency of the guard column may also play a role in its ability to distribute the debris over the bed prior to failure. A poorly packed bed is more likely to result in poorer hydrodynamics that are most affected by subparticulate (such as protein) accumulation.

3.4. Spiked mouse serum capacity test

The pre-clinical and clinical studies for the approval of a new drug require analysis of the parent drug and its metabolites. The desire is to have HPLC columns that are used for this application be able to withstand several hundred

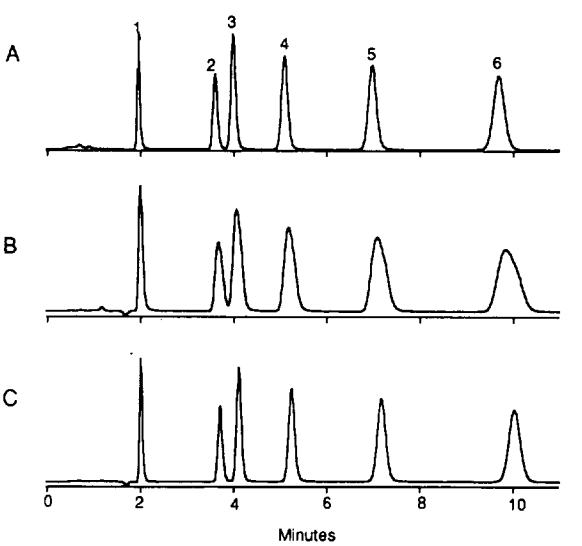
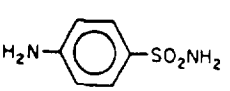


Fig. 6. Extension of column lifetime with guard column using a mixture of sulfa drugs as the sample. Analytical column, Symmetry C_8 (150 mm × 3.9 mm) with matching Sentry guard column; mobile phase, water–methanol–glacial acetic acid (79:20:1, v/v/v); flow-rate, 1 ml/min; temperature, 25°C; detection, 254 nm; injection volume, 10 μl of sulfa drug mixture (10 to 39 μg/ml of each drug). Peaks: 1 = sulfanilamide; 2 = sulfadiazine; 3 = sulfathiazole; 4 = sulfamerazine; 5 = sulfamethazine; 6 = succinylsulfathiazole (see Fig. 7 for chemical structures). (A) Initial injection on Symmetry C_8 Sentry guard column, (B) after 550 injections on same Sentry guard column, (C) new Sentry guard column for injection 551 on analytical column.

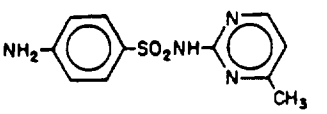
injections before chemical or mechanical failure. The guard column is a valuable tool for preserving column lifetime for these analyses. The Sentry guard column was effective in extending the lifetime of the Nova-Pak C_{18} column that was subjected to repeated injections of spiked mouse serum. The initial chromatography with and without the guard column in Figs. 4A and 4B for the antiepileptic drugs (primidone, carbamazepine and phenytoin) and their metabolites were very similar. After 200 injections a noticeable change was observed in the chromatography for the column alone in Fig. 4C. Table 3 quantitates the difference in the protected and unprotected column after 200 injections. On the unprotected Nova-Pak column the peaks for carbamazepine and its metabolite, carbamazepine-10,11-epoxide, had shifted in retention times as much as 4.5% and decreased in efficiency up to 17%. In contrast, the Nova-Pak column in series with the Sentry guard column continued to perform well with an improvement in the plate count for carbamazepine-10,11-epoxide. The unprotected column showed a change in retention time and peak shape probably from molecular contaminants (small molecules in deproteinated serum).

3.5. Lifetime study with standards

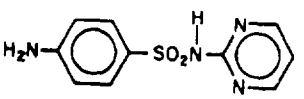
The Sentry guard columns with the Symmetry C_8 packing has prolonged the life of the Symmetry C_8 columns (150 mm × 3.9 mm). The Sentry guard column has been changed every time the pressure increased approximately 500 p.s.i. which was the predominant failure mode during the study. The guard column was also changed when performance deteriorated as noted by decreased resolution and plate count. Fig. 6 gives an example of the ability of the guard column to extend column lifetime. Fig. 6A shows the chromatography for the first sulfa drug injection on a new guard column; all peaks are sharp and there is baseline resolution between peaks 2 and 3. After 550 injections on this Sentry guard column the peaks became very broad leading to a loss of the baseline resolution between peaks 2 and 3 as shown in Fig. 6B.



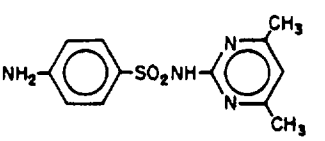
1: Sulfanilamide



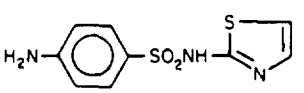
4: Sulfamerazine



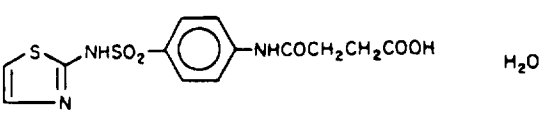
2: Sulfadiazine



5: Sulfamethazine



3: Sulfathiazole



6: Succinylsulfathiazole

Fig. 7. Structures of sulfa drugs in Fig. 6.

When a new guard column was put in line the chromatography returned to that observed originally as noted in Fig. 6C. The analytical column was not damaged and its use was continued for many more injections; over 8000 injections were obtained on these Symmetry C_8 columns. The average number of injections per guard column was approximately 800. By changing the guard column at regular intervals the life of the analytical column can be increased. The guard column is considered the disposable part of the HPLC system and used to maintain the analytical column [3].

4. Conclusions

The Sentry guard column connected in series with an analytical column enhances the performance of the HPLC column. Guard cartridges that are less efficiently packed result in decreased chromatographic performance.

All guard columns tested extended the lifetime of the analytical column. The Sentry guard column was the most effective of the commercial guard cartridges tested in prolonging column lifetime. The lifetime studies stress the importance of changing the guard column regularly for maximum protection of the analytical column.

In summary, the Sentry guard column has been designed to protect the HPLC analytical column from deleterious contaminants without compromising chromatographic performance.

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