ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1389–1395

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

A new small particle packing for faster analysis with high resolution[1]

Dorothy J. Phillips [a,*], Mark Capparella [a], Uwe D. Neue [a], Zoubair El Fallah [b]

[a] *Waters Corporation, 34 Maple St., Milford, MA 01757, USA*
[b] *McGaw, Irvine, CA, USA*

Received 22 August 1996; accepted 15 October 1996

Abstract

The need for fast and efficient separations of complex samples such as pharmaceuticals and biologicals led to the development of fast, efficient, and reproducible 3.5 μm columns. Separations using 3.5 μm column are 30–50% faster at equal plate-count compared to 5 μm columns. The results show that the 3.5 μm columns (100 mm length) give the same efficiency and resolution of drug impurities as the 5 μm columns (150 mm length). For many analytical methods, switching to 3.5 μm columns saves time and reduces costs. Separation methodologies using 5 μm columns are easily modified to accommodate 3.5 μm columns of the same chemistry because efficiency, resolution and sensitivity remain the same. It is shown that 3.5 μm columns have lifetimes comparable to 5 μm columns of the same brand. © 1997 Elsevier Science B.V.

Keywords: Reversed-phase HPLC; Fast chromatography; Small particle HPLC packings; Column lifetime; Analysis of pharmaceuticals

1. Introduction

High performance liquid chromatography columns packed with 3 μm packing materials were first commercialized in the early 1980s [1–3]. Until recently, however, HPLC system compatibility and column lifetime issues have precluded the widespread use of the 3 μm columns. Relative to the traditional 5 μm analytical packing materials, the 3 μm size required a shorter column to compensate for increased back pressure. The smaller column volume of the shorter columns resulted in an increase of the influence of extra column effects, which negated the separation efficiency improvements expected from the smaller particle size. The extra column effects were a direct result of the HPLC system incompatibility with small column volumes. Columns made with 3 μm packing materials also had shorter lifetimes due to plugging of the frits or the packing. The smaller pore diameter frits are more prone to plugging by particulates that are either present in the sample or arise from abrasion of system seals.

* Corresponding author.

[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August 1996, Osaka, Japan.

PII S0731-7085(96)02049-3

This paper compares the separation efficiency of the 3.5 μm column (4.6 × 100 mm) with the 5 μm column (3.9 × 150 mm and 4.6 × 150 mm).

Chromatographic data on several pharmaceutical compounds show the speed advantage of the 3.5 μm column for fast chromatography. The results also show that column lifetimes for the 3.5 μm columns are comparable to 5 μm columns for standards and biological samples.

2. Experimental

2.1. Materials

Sulfanilamide, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, succinylsulfathiazole, beclomethasone, paclitaxel (taxol), and tamoxifen were from Sigma (St. Louis, MO). Testosterone propionate, testosterone benzoate and testosterone acetate were from US Pharmacopeia Reference Standards (Rockville, MD). Butalbital and butabarbital were from Alltech (Deerfield, IL). Rabbit serum was from Equitech-Bio (Ingram, TX0). Buffers and mobile phases were made with reagent grade chemicals and HPLC grade solvents. Mobile phases were filtered and degassed.

Symmetry® C_{18} and C_8 columns (3.9 × 150 mm and 4.6 × 150 mm) with 5 μm particles and 4.6 × 100 mm with 3.5 μm particles and Symmetry® Sentry Guard columns were from Waters (Milford, MA).

2.2. Instrumentation

The HPLC system used in this study consisted of either a Waters 625 or a 616 LC System, 717 plus Autosampler and the 996 Photodiode Array detector.

Stability studies with biological samples were done on the Waters 600 E Multisolvent Delivery System, 715 Ultra WISP Sample Processor and 490 Programmable Multiwavelength detector. Control and results management were provided by the Waters Millennium™ 2010 Chromatography Manager.

The components of the HPLC system for column lifetime studies with the standard sulfa drug mixture consisted of a Waters Model 712 WISP™, 590 Programmable Solvent Delivery Module, and 441 Fixed Wavelength UV/Visible detector. The Waters 845 Chromatography Data and Control Station with Expert-Ease v 3.0 was used for system control and data acquisition.

2.3. Stability studies

Stability studies with sulfa drugs and rabbit serum were done with pre-column filters between the pumps and the injectors. The temperature was controlled at 25°C on each HPLC system using a EuraMark™ Spark Holland Mistral® Column Thermostat.

The column stability study using 10 μl of the sulfa drug mixture (10–39 mg ml^{-1}) was injected on a Symmetry® C_8 Sentry Guard Column (3.9 × 20 mm, 5 μm) in line with a Symmetry® C_8 Column (4.6 × 100 mm, 3.5 μm). The mobile phase was water-methanol-glacial acetic acid (79:20:1 v/v/v) and was recycled during the course of the experiment. The flow rate was 1.5 ml min^{-1} and the detection wavelength was 254 nm.

Rabbit serum was deproteinated by mixing two parts acetonitrile to one part serum. The sample was then centrifuged at 1500 × *g* for 5 min and spiked with 5 μg ml^{-1} of butalbital and 5 μg ml^{-1} of butabarbital. The mobile phase was potassium phosphate (pH 6.9, 0.1 M)-acetonitrile-water (20:20:50 v/v/v). In this study, the mobile phase was not recycled. The injection volume was 15 μl and the mobile phase flow rate was 1.4 ml min^{-1} (1.25 ml min^{-1} for the Superspher column). The detection wavelength was 214 nm.

3. Results and discussion

3.1. Efficiency theory

The objective of the 3.5 μm column is to offer fast separations without sacrificing chromatographic resolution [4]. Resolution is a function of: the selectivity, α, which is a measure of the relative retention of two components in a mixture, the capacity factor, k, which is a measure of how well the analyte is retained and the efficiency, N, ex-

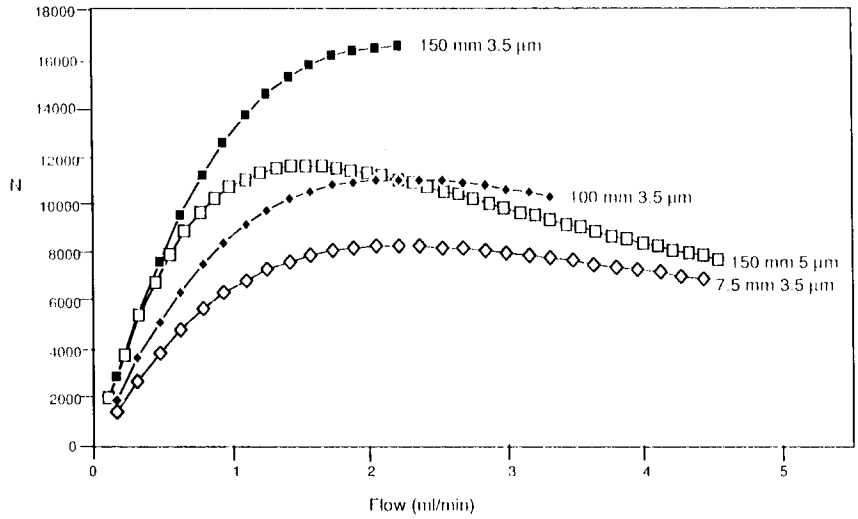

Fig. 1. Plate count N vs. flow rate for 5 and 3.5 µm Columns.

pressed in theoretical plates. Eq. (1) shows the dependence of resolution on α, k and N.

$$Rs = 1/4\, N^{1/2}(\alpha - 1)[k/(k+1)] \quad (1)$$

The same efficiency can be obtained in different ways: one can either use long columns with larger particles or short columns packed with smaller particles. The key to faster separations is to use shorter columns packed with smaller particles. In this manner, the increase in efficiency due to the smaller particle size compensates for the loss in plate count due to a shorter column length. Fig. 1 shows a plot of the theoretical plate count, N vs. flow rate for 5 and 3.5 µm columns and illustrates the following points:

- The 3.5 µm 15 cm column gives a higher efficiency than the 5 µm column of the same length, but is limited in flow rate due to the high column back pressure.
- The short 7.5 cm column packed with 3.5 µm particles yields very fast separations with still a good plate-count.
- The 100 mm 3.5 µm columns and the 150 mm 5 µm columns give similar efficiencies at a given flow rate, which translates into a faster analysis time due to the decreased column length.

In order for the theoretical results to hold true experimentally, the columns need to be packed to the same quality, i.e., to the same reduced plate height (h). Fig. 2 shows the plot of reduced plate height vs. linear velocity for the 5 µm C_{18} columns and the 3.5 µm C_8 column. The curves overlap, showing that the columns are packed equally well. Also, the reduced plate height of 2.4 obtained at the minimum is excellent. It should be noted that the minimum of the HETP occurs at different flow rates depending on the mobile phase viscosity. For example, the minimum is found at 1.8 ml min^{-1} for acetonitrile-water (50:50 v/v), whereas for methanol-water (50:50 v/v), a more viscous mobile phase, the minimum occurs at 0.9 ml min.

3.2. Efficiency and speed

Fig. 3 illustrates the similarity in efficiency between 5 µm 150 mm columns and 3.5 µm 100 mm columns. Chromatograms for beclomethasone and its internal standard testosterone propionate using two C_{18} columns: (a) 3.9 × 150 mm, 5 µm and (b) 4.6 × 100 mm, 3.5 µm are shown. Both columns have a resolution of 18 which meets the US Pharmacopeia assay [5] specification ($Rs \geq 5$). Both columns have similar plate counts of approximately 8000 and similar backpressures (1350 psi for 5 µm vs 1570 psi for 3.5 µm) under the chromatographic conditions chosen. The analysis

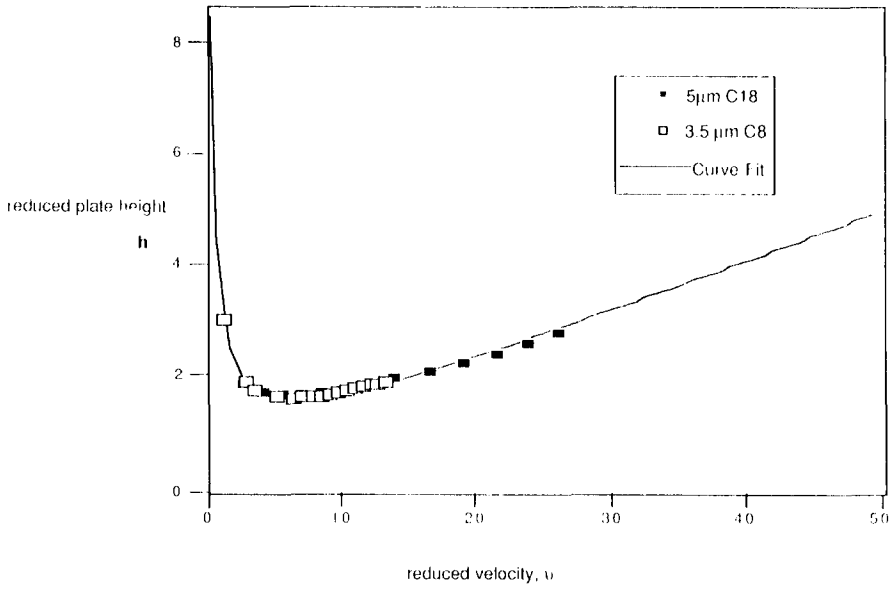

Fig. 2. Reduced plate height vs. linear velocity.

time for the 3.5 μm column (b) is about 50% lower compared to the 5 μm column (a). The reduction in analysis time clearly illustrates the point of fast chromatography without a sacrifice of separation efficiency. Many analytical methods could become more cost effective by merely changing particle size from 5 to 3.5 μm.

Performance equivalence between 3.5 and 5 μm packing is further illustrated in Figs. 4 and 5 where impurity profiles of pharmaceutical com-

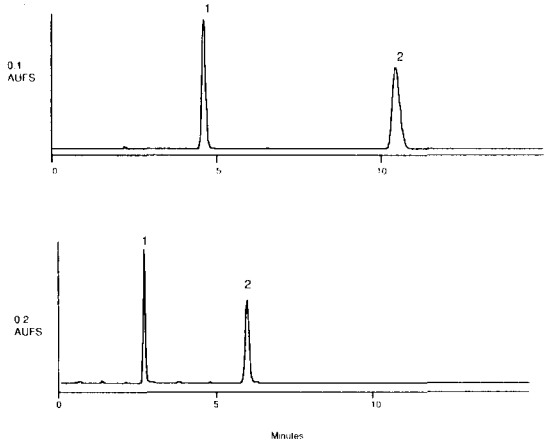

Fig. 3. Beclomethasone USP Method: comparison of 5 and 3.5 μm columns. Columns: Symmetry® C_{18} (a) 5 μm, 3.9 × 150 mm; (b) 2.3 μm, 4.6 × 100 mm. Mobile phase: water-acetonitrile (36:65, v/v). Flow: (a) 1.0 ml min^{-1}; (b) 1.4 ml min^{-1}. UV: 254 nm. Injection: 15 μl, 0.1 mg ml^{-1} in methanol of each drug. Peak 1 = beclomethasone, peak 2 = testosterone propionate (internal standard).

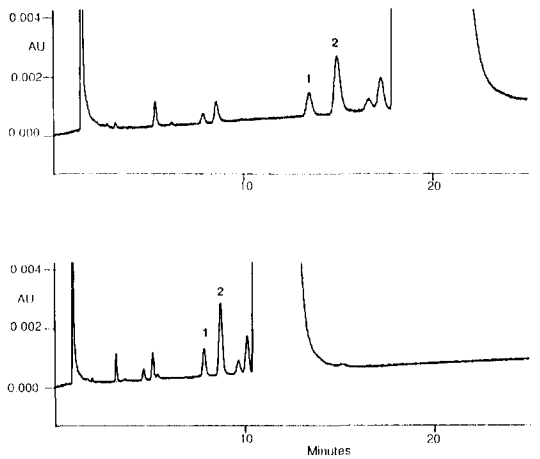

Fig. 4. Tamoxifen: comparison of 5 and 3.5 μm columns. Columns: Symmetry® C_{18} column (a) 5 μm, 3.9 × 150 mm; (b) 3.5 μm, 4.6 × 100 mm. Mobile phase: potassium phosphate (pH 3, 50 mM)-acetonitrile (60:40, v/v). Flow: (a) 0.7 ml min^{-1}; (b) 1.0 ml min^{-1}. UV: 240 nm. Injection: 4 μl, 5 mg ml^{-1}. Peaks 1 and 2 = impurities of tamoxifen.

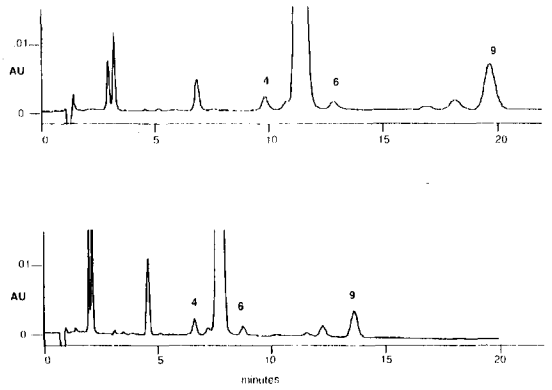

Fig. 5. Comparison of Taxol Impurities Profile on 5 μm and 3 μm Columns. Columns: Symmetry® C_8 (a) 5 μm 3.9 × 150 mm; (b) 3.5 μm 4.6 × 100 mm. Mobile phase: ammonium acetate (pH 5, 20 mM)-acetonitrile-methanol (50:40:10, v/v/v). Flow: (a) 1.0 ml min^{-1}; (b) 1.4 ml min^{-1}. UV: 230 nm. Injection: 25 μl, 100 μg ml^{-1}. Peaks 4, 6 and 9 = impurities of paclitaxel (taxol).

pounds are shown. Fig. 4 and Fig. 5 show the assays of tamoxifen and taxol, respectively, on a 3.9 × 150 mm, 5 μm column and a C_{18} 4.6 × 100, 3.5 μm column. In Fig. 4, both columns resolve the impurity peaks. However, the 3.5 μm column separation is markedly faster. Also, a calculation of the plate counts for the impurity peaks actually shows a slight improvement in efficiency for the shorter 3.5 μm column.

Essentially, the same observations were made with the taxol assay (Fig. 5). The shorter 3.5 μm column gave a much shorter analysis time combined with a slight improvement in plate count and resolution. A plate count of 8900 was measured for the taxol peak on the 100 mm 3.5 μm column, while a plate count of 7100 was obtained on the 150 mm 5 μm column. This is an added benefit, since this increased resolution provides an additional buffer against column deterioration.

The 3.5 μm columns can be operated at even faster flow rates to further reduce analysis times. Fig. 6 shows the separation of testosterone at 1.4 and 2.8 ml min^{-1}. The separation efficiency at 2.8 ml min^{-1} is still 6800 plates (compared to 7500 plates at the lower flow rate) and the run time is reduced by half. This is possibly due to the use of acetonitrile as the organic modifier in the mobile phase. Acetonitrile-water mixtures have a much lower viscosity than methanol-water mixtures, resulting in a much reduced back pressure. For still faster analysis or to accommodate highly aqueous mobile phases within normal pressure limits, the 3.5 μm packing is also available in 75 mm columns.

3.3. Column lifetime

For column lifetime studies, a C_8 column (4.6 × 100 mm, 3.5 μm) was used with guard columns containing 5 μm C_8 packing material. The guard column was replaced when back pressure increased or resolution decreased. The sample was a mixture of sulfa drug standards. The results are shown in Fig. 7 and Fig. 8. In Fig. 7 the plate count of one of the analytes is plotted against the number of injections. In Fig. 8 the system backpressure is plotted. As one can see, neither a significant decline in efficiency nor a significant increase in back pressure was observed over 10 000 injections. We interrupted the study after 10 000 injections. This demonstrates that excellent column life can be achieved with 3.5 μm columns, when they are protected by a guard columns. This result parallels the results of a previous study using a 5 μm packing.

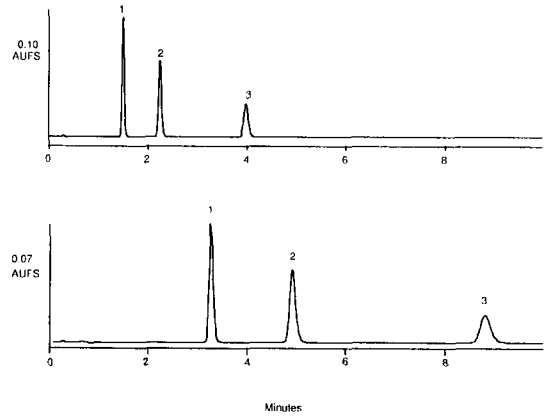

Fig. 6. Faster Testosterone Analysis on 3.5 μm Columns. Columns: Symmetry® C_{18} 3.5 μm, 4.6 × 100 mm. Flow: (a) 2.8 ml min^{-1}; (b) 1.4 ml min^{-1}. Mobile phase: water-acetonitrile (30:70, v/v). UV: 254 nm. Injection: 10 μl, 100 μg ml^{-1}. Peak 1 = testosterone acetate, 2 = testosterone propionate, 3 = testosterone benzoate.

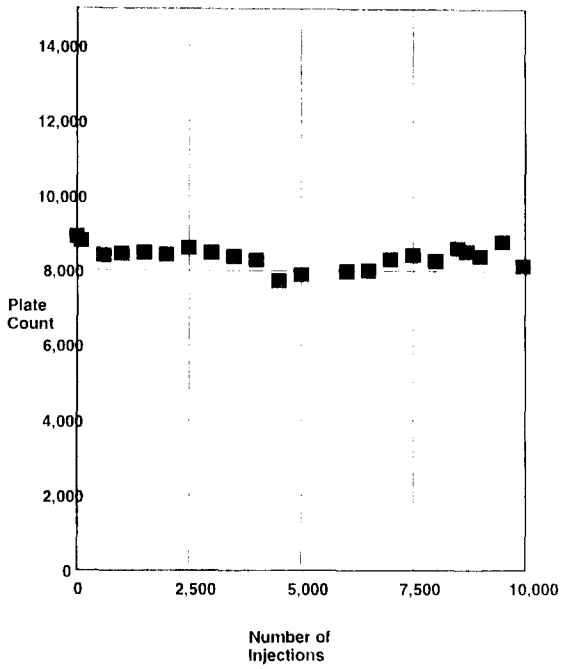

Fig. 7. Efficiency of Succinylsulfathiazole vs. Number of Injections. The plate count of peak 6 in the chromatography for the sulfa drug mixture was monitored during the lifetime study. The plate count was measured by tangent or the US Pharmacopeia method for the Symmetry® C_8 column 3.5 μm, 4.6 × 100 mm with Symmetry® Sentry™ Guard Column 5 μm, 3.9 × 20 mm.

In another lifetime study, deproteinated rat serum spiked with butalbital and butabarbital was injected 1000 times on a 3.5 μm C_{18} column. Fig. 9 is a plot of the theoretical plates vs number of injections for the 3.5 μm column. Only a $\leq 5\%$ decrease was noted. As shown in Fig. 10, the 3.5 μm column did not exhibit an increase in back pressure over the 1000 injection. Previously, a similar study was made with the 5 μm column which also showed no increase in backpressure and less than a 5% decrease in efficiency over the 1000 injections. The 3.5 μm column was as stable as the 5 μm column even for multiple injections of drugs in a biological matrix. The increase in particle size to 3.5 μm and the narrow particle size distribution (90%/10% volume ratio of 1.5) are the likely causes of the stability of the Symmetry 3.5 μm columns.

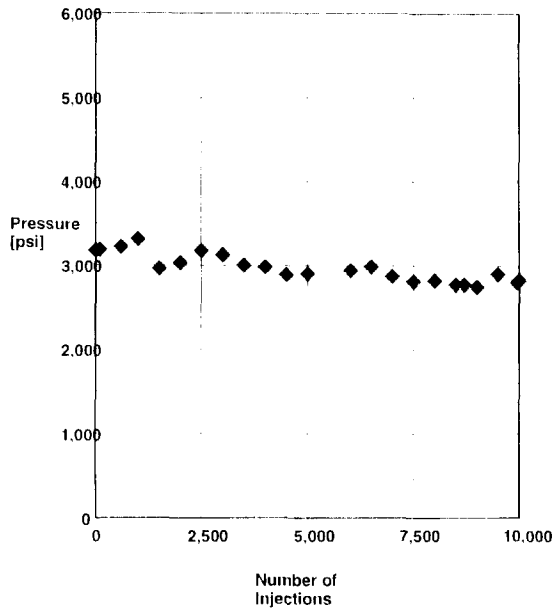

Fig. 8. Backpressure vs. Number of Injections: Stability Study with Sulfa Drugs on Symmetry® C_8 column, 3.5 μm, 4.6 × 100 mm, with Symmetry® Sentry™ Guard Column 5 μm, 3.9 × 20 mm.

4. Conclusions

The 3.5 μm columns demonstrate 30–50% faster analysis times over 5 μm columns. Separation methodologies using 5 μm columns can be easily translated into methods using 3.5 μm columns since the efficiency, resolution and sensi-

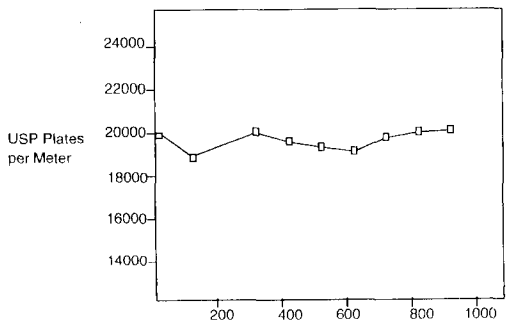

Fig. 9. Butalbital Efficiency vs. Number of Injections: spiked serum stability study for Symmetry® C_{18} column 3.5 μm, 4.6 × 75 mm. Efficiency of butalbital peak was measured by tangent or US Pharmacopeia method.

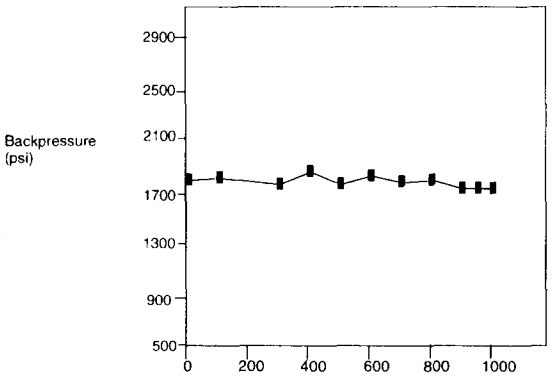

Fig. 10. Backpressure vs. Number of Injections: spiked serum stability study for Symmetry® C_{18} column 3.5 μm 4.6 × 75 mm.

tivity remain the same. Also for a given column chemistry the 3.5 μm Symmetry® columns are as stable as their 5 μm counterparts.

References

[1] L.R. Snyder, J.L. Glajch and J.J. Kirkland, in Practical HPLC Method Development, Wiley, 1988, pp. 46–40.
[2] R.W. Stout, J.J. DeStefano and L.R. Snyder, J. Chromatogr., 261 (1983) 189–212.
[3] L.R. Snyder and P.E. Antle, LC Mag., 3 (1985) 98–109.
[4] J.J. Kirkland, J. Chromatogr. Sci., 31 (1993) 193–197.
[5] US Pharmacopeia, Vol. 23, 1995, pp. 167.