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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ANTIBIOTICS ON MICROBORE COLUMNS

E. RODERICK WHITE* and DIANE N. LAUFER
Analytical, Physical and Structural Chemistry Department, Smith, Kline and French Laboratories, 1500 Spring Garden Street, Philadelphia, PA 19101 (U.S.A.)

SUMMARY

Isocratic and gradient microbore high-performance liquid chromatographic systems are described and are constructed from commercially available components. The microbore system has been found to be sixteen times more sensitive than a conventional system. Conditions are given for the separation and quantitation of a number of cephalosporin antibiotics, using a μBondapak C_{18} microbore column. It has been demonstrated that cephalosporin antibiotics can be determined with a relative standard deviation of 0.75%. Cephalosporin C levels in fermentation broths can be determined on the microbore system simply by injecting a diluted broth sample. It has been demonstrated that reversed-phase gradient elution can be carried out more easily and reliably on a microbore system than on a conventional system, and that microbore gradient elution is a viable technique for the determination of impurities in antibiotics.

INTRODUCTION

Reversed-phase high performance liquid chromatography (HPLC) has been shown to be well suited for the analysis of antibiotics[1–3]. One of the recent advances in HPLC which is applicable to the analysis of antibiotics is the development of microbore columns. The first columns of this type were described by Ishii and co-workers[4–10], who used short PTFE tubes, 0.25–0.50 mm I.D., packed with silica and reversed-phase materials. Scott and co-workers[11–17] pioneered the use of steel, 10–100 cm × 1 mm I.D., columns. Steel columns are now commercially available from a number of sources as well as various microbore HPLC instruments.

Microbore columns have several important advantages. Less column packing is used because of the small internal diameter (1 mm), and with typical flow-rates of the order of 10–20 μl/min, solvent consumption is reduced by as much as 95% over that of a conventional HPLC column. Higher mass sensitivities are also obtained with microbore columns. This allows trace analyses to be carried out on limited amounts of sample. A particularly attractive feature of microbore columns is their ability to be connected in series to provide ultra-high efficiencies, proportional to total column length. The low flow-rates and smaller sample sizes used enable micro-

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bore columns to be compatible with many sensitive and selective small-volume detectors. Examples are electrochemical, laser-induced fluorescence, laser-induced photo-acoustic and flame-based gas chromatographic detectors such as flame photometric and thermionic specific detectors. Microbore columns are especially well suited for qualitative identification by direct interface mass spectrometry (LC–MS) and Fourier transform infrared spectroscopy (LC–FT-IR).

This paper describes applications of reversed-phase microbore HPLC in isocratic and gradient modes, for the analysis of antibiotics. Unexpectedly, it was found that gradient analyses were much easier to carry out on a microbore column than on an analytical column. This makes microbore gradient HPLC especially useful for the determination of impurities in antibiotics and other pharmaceutical compounds.

EXPERIMENTAL

Isocratic instrument

A DuPont 870 pump (DuPont Instruments, Wilmington, DE, U.S.A.) was used for solvent delivery. This pump can provide flow-rates as low as 25 μl/min without modification. A Rheodyne 7413 sample valve (Rheodyne, Cotati, CA, U.S.A.) with internal loops of 0.5, 1.0 and 5.0 μl was used to inject samples. Column effluents were monitored with a Kratos Spectroflow 773 (Kratos, Ramsey, NJ, U.S.A.) variable-wavelength UV detector from which the heat exchanger had been removed. Micro flow cells of 0.5 μl (1-mm light path), 2.5 μl (3-mm light path) and 8.0 μl (10-mm light path) were used with this detector. A Waters 441 detector (Waters Assoc., Milford, MA, U.S.A.) with 1.9 and 12 μl (10-mm light path) flow cells was used for some experiments.

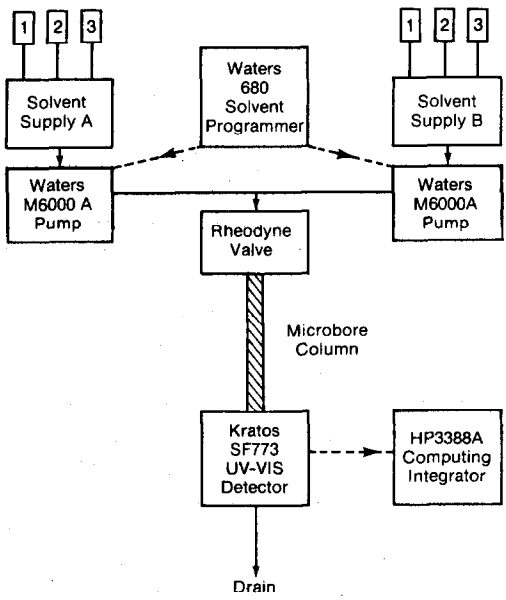

Fig. 1. Schematic for microbore gradient chromatograph.

Gradient instrument

The gradient instrument was identical to the isocratic instrument except for the pumping system, which consisted of two Waters 6000A pumps and a Model 680 gradient controller. The two pumps were connected together in the conventional manner, but the pulse dampeners were bypassed. A block diagram of the gradient system is shown in Fig. 1.

Reagents

All solvents used were HPLC grade, distilled in glass, and were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Phosphate salts were HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Materials

The antibiotics used in this study were obtained from the Investigational Products Laboratory of Smith, Kline and French Labs. The fermentation broths were provided by Charles Pan.

Column packings

μBondapak C_{18} packing was obtained from Waters. LiChrosorb RP-18, 5 μm, was obtained from EM Science (Gibbstown, NJ, U.S.A.).

Columns

The tubing used for the microbore columns was 1/8 in. O.D. × 1 mm I.D., glass-lined stainless steel (Alltech Assoc., Deerfield, IL, U.S.A.). The 2-μm porous frits were also obtained from Alltech Assoc. Parker-Hannifin end-fittings, Part No. 2-1ZHBZ7-SS, were obtained from Ecco Process and Control Equipment (Broomall, PA, U.S.A.). The columns were packed by a modified balanced density slurry procedure[18].

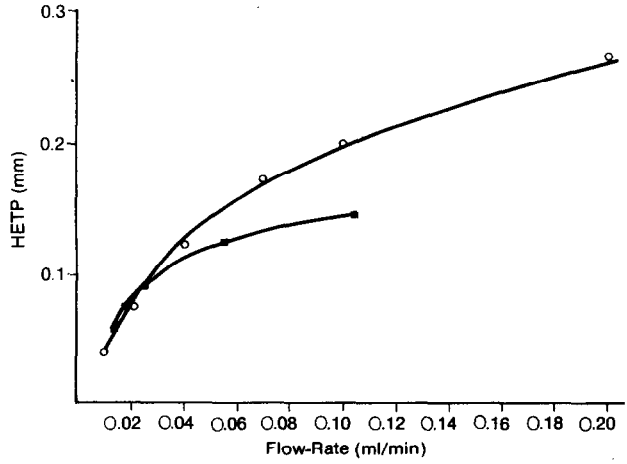

Fig. 2. HETP curves for cephradine. ■, 5-μm RP-18; O, 10-μm μBondapak C_{18}.

Mobile phase

The mobile phases used in this work consisted of methanol and water in various proportions with phosphate buffers at a concentration of 0.01 *M*, unless otherwise specified.

RESULTS AND DISCUSSION

Cephradine, a cephalosporin antibiotic, was chromatographed on a 15 cm × 1 mm I.D. RP-18, 5 μm column and on a 25 cm × 1 mm I.D. μBondapak C_{18}, 10 μm column. Height equivalent to theoretical plate (HETP) values were calculated as a function of flow-rate, over the range 10–200 μl/min. Fig. 2 shows the curves obtained for the two columns. These curves agree in general shape with those obtained by Scott[16]. At 50 μl/min the μBondapak C_{18} column has a plate number, *N*, of *ca.* 1800 while the corresponding number for the RP-18, 5 μm column is *ca.* 1300. These values, as well as the figure, show that there is no significant benefit to using the 5-μm column at lower flow-rates, so all subsequent work was done on the 10-μm μBondapak C_{18} column.

A comparison of the peak detection sensitivity between a microbore and a conventional column was made. The two columns had approximately the same efficiencies (*ca.* 2000 plates), capacity factors (*k'*) (*ca.* 2.3) for the test substance, cephradine, and the light paths of the flow cell thus making a direct comparison possible. Theoretically, the relative mass sensitivity of the microbore system is equal to the square of the ratio of the internal diameters of the columns, or $(4.6/1)^2 \approx 21$. The actual relative mass sensitivity obtained was 16. The difference between the actual and theoretical values represents the unavoidable extra-column effects that are common to all HPLC systems.

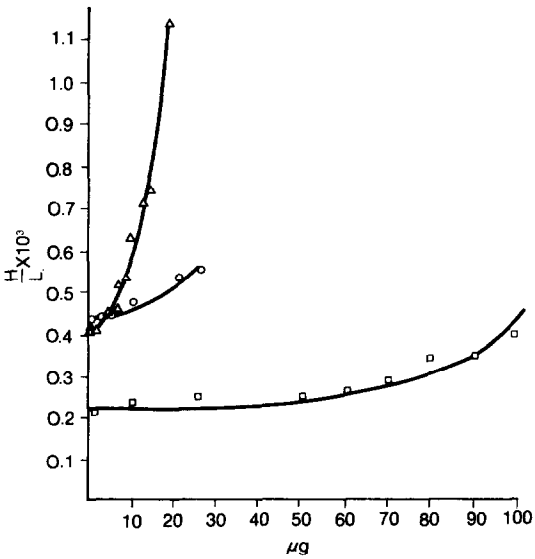

Fig. 3. HETP as a function of sample weight. □, Toluene; ○, ampicillin; △, cephradine.

The linearity of the microbore system was established by examining the response of ampicillin as a function of concentration, and a linear response was obtained through the 10-μg level.

Because of the smaller diameters and resulting reduced cross-sectional areas, microbore columns have reduced loading capacities. This is illustrated in Fig. 3. The quantity H/L (where H = HETP and L = column length) is plotted as a function of weight of sample injected on a 10-μm μBondapak C_{18} microbore column. Three compounds were used: toluene, ampicillin and cephradine. The toluene curve is fairly flat out to *ca.* 50 μg, indicating that this amount can be injected without overload. With ampicillin, overloading begins to occur at a much lower level (*ca.* 10 μg). Severe overloading occurs with cephradine at levels above *ca.* 2 μg. These results illustrate that the phenomenon of column overload is very dependent on sample type and should be investigated before any type of quantitative determinations are made. We routinely carry out assay determinations at levels of 1 μg or less in order to be certain that overload does not occur.

The precision of the microbore system was established by repetitively injecting a cephalosporin antibiotic solution onto a μBondapak C_{18} microbore column. The

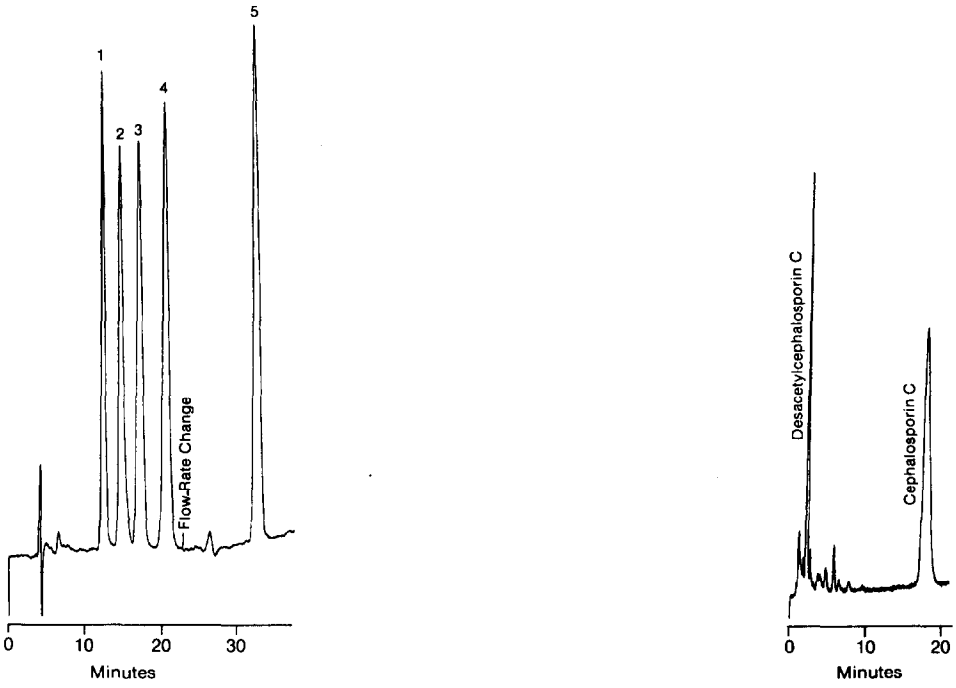

Fig. 4. Separation of a mixture of cephalosporin antibiotics on a microbore column. Column, 25 cm × 1.0 mm I.D. μBondapak C_{18}, 10 μm; mobile phase, 0.01 *M* sodium dihydrogen phosphate–methanol (75:25); flow-rate, 50 μl/min (initially), 150 μl/min (after 23 min); sample volume, 5 μl. Peaks: 1 = Cephalexin, 0.05 μg; 2 = Cefoxitin, 0.05 μg; 3 = Cephradine, 0.07 μg; 4 = Cephaloglycin, 0.10 μg; 5 = Cephalothin, 0.23 μg.

Fig. 5. Cephalosporin C fermentation broth on a microbore column. Column, 25 cm × 1 mm I.D. μBondapak C_{18}, 10 μm; mobile phase, 0.03% potassium dihydrogen phosphate; flow-rate, 150 μl/min; sample volume, 5 μl.

TABLE I

COLUMN EFFICIENCES AND SENSITIVITIES *VERSUS* FLOW CELL VOLUME

Cell (μl)	*Path length (mm)*	*Apparent efficiency*	*Relative absorbance*
0.5	1	2227	1
2.5	3	1276	2.2
8.0	10	1219	7.1
1.9	10	1742	

precision is excellent with the relative standard deviation of eight injections equal to 0.75%. It should be emphasized that this excellent precision was obtained without the need for an internal standard.

The effect of flow-cell volume on apparent column efficiency and detector sensitivity was determined with cephradine as the test substance. The results are shown in Table I. With the smallest volume flow cell, 0.5 μl (1-mm path length), an apparent column efficiency of 2227 plates was obtained. With a 2.5-μl (3-mm path length) flow cell the efficiency decreases to 1276 plates, which is an indication of diffusion within the cell. Three times more absorbance would be expected from the path length ratio, but a factor of only 2.2 relative absorbance was obtained. The difference, of course, is due to the extra diffusion within the 2.5-μl cell. Similarly, use of an 8.0-μl (10-mm path length) flow cell results in a further small decrease in efficiency and a relative absorbance of 7.1 instead of the theoretical maximum of 10. Use of a 1.9-μl (10-mm path length) flow cell in conjunction with the Waters Model 441 detector results in only a small loss of efficiency and represents a good compromise detector for obtaining high sensitivity. With the Kratos detector the 0.5-μl flow cell is used when

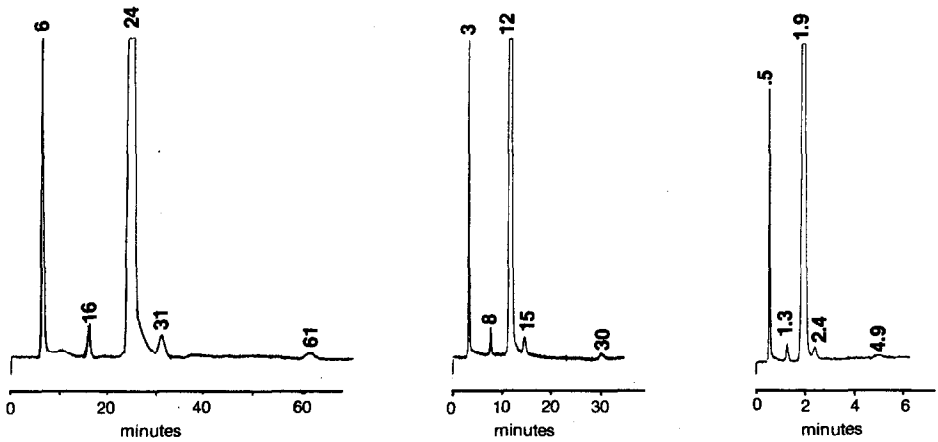

Fig. 6. Fast flow-rate separation of a cephalosporin antibiotic on a microbore column. Column, 25 cm × 1.0 mm I.D. μBondapak C_{18}, 10 μm; mobile phase, water–methanol–0.1 *M* ammonium dihydrogen phosphate (75:15:10); sample volume, 1 μl. Chromatogram A: flow-rate, 25 μl/min; pressure, 250 p.s.i. Chromatogram B: flow-rate, 50 μl/min; pressure, 500 p.s.i. Chromatogram C: flow-rate, 300 μl/min; pressure, 3000 p.s.i.

maximum column efficiency is desired. The 2.5- and 8.0-μl cells can be used to enhance sensitivity if k' values are greater than *ca.* 5.

The separation of a mixture of cephalosporin antibiotics on a μBondapak C_{18} microbore column is shown in Fig. 4. Baseline resolution of all five compounds is obtained with a flow-rate increase needed to elute cephalothin. Naturally, not all of these compounds would be present in a real mixture. However, as an example, the determination of cephalexin impurity in cephradine would be quite straightforward due to the large separation between these two compounds. The high mass sensitivity of microbore columns allow sub-microgram levels of each antibiotic to be determined easily with nearly full-scale response. An important point in connection with this separation is the fact that, depending on the compound being determined, one of the other cephalosporins could be used as a suitable internal standard.

Fermentation broths can be analyzed conveniently for cephalosporin C by HPLC on C_{18} columns[3,19]. When an analytical column is used it is necessary to centrifuge the broth in order to get rid of particulate matter which might lead to blockage of the column. With a microbore column, however, only a very dilute solution is needed for the highly sensitive system. Sample preparation consists of diluting the broth by a factor of 10 with mobile phase, allowing this solution to stand for *ca.* 20 min for settling of most of the particulate matter, then diluting again by a factor of 50 with mobile phase. Fig. 5 shows a chromatogram obtained on the final dilution which allows the quantitation of both desacetylcephalosporin C and cephalosporin C at μg/ml levels.

A significant advantage of microbore columns is the ability to run at low

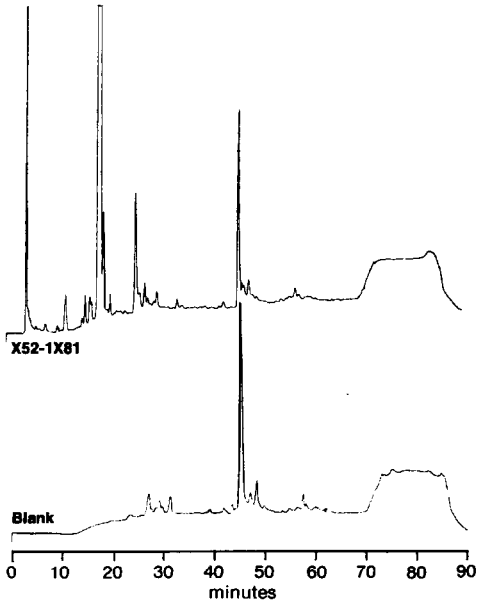

Fig. 7. Gradient separation of a cephalosporin antibiotic on an analytical column. Column, 30 cm × 4.6 mm I.D. μBondapak C_{18}, 10 μm; mobile phase: A, 0.01 *M* ammonium dihydrogen phosphate; B, 0.1 *M* ammonium dihydrogen phosphate–methanol (10:90); gradient, linear, 0–100% B in 1 h, 100% methanol after 1 h; flow-rate, 1.0 ml/min; sample volume, 20 μl.

flow-rates for high efficiency and at high flow-rates for speed, while keeping reasonable pressure drops across the column. An example of this is the separation of impurities in a cephalosporin antibiotic, shown in Fig. 6. At 25 μl/min and 250 p.s.i., the total separation requires *ca.* 1 h, while at 50 μl/min and 500 p.s.i. it requires only *ca.* 30 min. However, the analysis can be shortened even further to *ca.* 5 min by increasing the flow-rate to 300 μl/min and 3000 p.s.i., which is twelve times faster than the 25 μl/min run. Even these fast conditions do not present a problem for the pumping system. In contrast, to carry out an equivalent fast analysis on an analytical column requires a flow-rate of 6 ml/min and a high operating pressure of almost 5000 p.s.i. Both conditions place extra demands on the pumping system.

The analysis of pharmaceutical compounds for impurities can be carried out by gradient elution analysis. However, in many cases this approach is not feasible due to the lack of appropriate equipment. An alternative is to perform a step gradient analysis using an isocratic pumping system. A DuPont Model 870 pump was used and the gradient was generated by manually increasing the methanol concentration of the mobile phase in 10% increments at 15–20 min intervals. The amount of baseline upset, due to refractive index effects that accompany solvent changes, is minimal because of the short path length (1 mm) flow cell used with the microbore system. This leads to a relatively smooth, flat baseline and allows the detection of a number of significant impurities. A run of this type would be impossible to carry out on an analytical column because of the relatively large refractive index effects that occur in a 10-mm path length flow cell when solvent changes are made.

Reversed-phase gradient HPLC is very difficult to carry out on an analytical column because of artifactual peaks which interfere with the detection of impurities. The column was exhaustively cleaned with methanol and butyl chloride before use, and the highest quality mobile phase components were used. In spite of these preventive measures, artifactual peaks are still obtained. This is illustrated in Fig. 7,

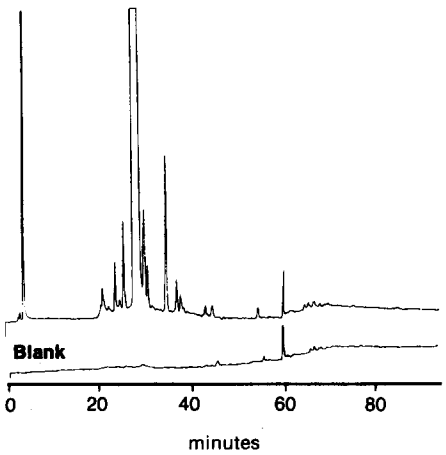

Fig. 8. Gradient separation of a cephalosporin antibiotic on a microbore column. Column, 25 cm × 1.0 mm I.D. μBondapak C_{18}, 10 μm; mobile phase: A, 0.01 *M* ammonium dihydrogen phosphate; B, 0.1 *M* ammonium dihydrogen phosphate, methanol (10:90); gradient, linear, 0–100% B in 1 h, 100% methanol after 1 h; flow-rate, 50 μl/min; sample volume, 5 μl.

TABLE II

PRECISION MEASUREMENTS BY MICROBORE GRADIENT ELUTION

Peak	*Retention time (min)*		*Area (μV · min)*		*Peak height (μV)*	
Impurity 1	3.77 ± 0.02	±0.5%	8704 ± 168	±1.9%	663 ± 9.4	±1.4%
Major component	26.85 ± 0.19	±0.7%	120,694 ± 6813	±5.6%	2257 ± 117	±5.2%
Impurity 2	29.16 ± 0.19	±0.7%	2601 ± 217	±8.3%	83.2 ± 3.8	±4.5%
Impurity 3	33.74 ± 0.18	±0.5%	2729 ± 168	±6.2%	140 ± 4.97	±3.5%
Impurity 4	35.73 ± 0.10	±0.3%	628 ± 60	±9.6%	27.5 ± 1.16	±4.2%
Range	0.3–0.7%		1.9–9.6%		1.4–5.2%	

which shows a blank gradient run and a cephalosporin antibiotic run made on an analytical μBondapak C_{18} column. Clearly, the artifactual peaks make it very difficult to determine the real impurities in the sample.

With a microbore column, however, the gradient run can be carried out easily with very little interference from artifactual peaks. This is illustrated in Fig. 8, which is the same sample run on a microbore μBondapak C_{18} column. There are no significant artifactual peaks which interfere in the analysis, except for the peak at 60 min which is easily discernible. The decreased artifactual behavior with microbore columns is, presumably, related to the small mobile phase volumes and the small amount of column packing used. When these variables are decreased, possible contaminants from the mobile phase and the column packing also decreased.

From the foregoing example it should be obvious that gradient elution on a microbore column represents a viable approach to the quantitative determination of impurities in pharmaceutical compounds such as antibiotics. This has been demonstrated by studying the reproducibility of the microbore gradient elution system. The run represented in Fig. 8 was repeated five times and the precision of the gradient elution experiment was determined. The data are summarized in Table II, where the mean values, standard deviations and relative standard deviations of retention time, peak area and peak height are tabulated. The retention time reproducibility is good and ranges between 0.3 and 0.7%. This range is in good agreement with the work of Scott and Kucera[14], who reported a range of 0.3–1.0% using similar equipment. The peak area reproducibility ranges between 1.9 and 9.6%, which is not particularly good. However, it should be noted that the largest deviations are for impurities 2, 3 and 4 which are all present at levels less than 1%. The range of 1.9–9.6% is in reasonable agreement with the work of Scott and Kucera[14]. We also found, as they did, that peak height reproducibility was better than peak area reproducibility and ranged from 1.4 to 5.2%. We agree that the preferred method for analysis should be peak heights when using gradient elution with microbore columns. It should be pointed out that the data given in Table II were generated from a linear gradient that ran from 0 to 90% methanol in water over a period of 1 h. These conditions represent a much more severe test of a gradient system than the one employed by Scott and Kucera[14], which only ran from 60 to 90% methanol in water.

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