Small Particle Columns for Faster High Performance Liquid Chromatography

Dorothy J. Phillips, Mark Caparella, Zoubair El Fallah, and Uwe D. Neue

Abstract
The customer need for fast and efficient separations on complex samples such as pharmaceuticals and biologicals led Waters to develop fast, efficient, and reproducible Symmetry® 3.5 µm columns. Separations using 3.5 µm columns are 30 to 50% faster at equal plate-count compared to 5 µm columns. For many analytical methods, switching to Symmetry® 3.5 µm columns can save time and reduce costs.

Separation methodologies using Symmetry® 5 µm columns are easily modified to accommodate Symmetry® 3.5 µm columns because efficiency, resolution and sensitivity remain the same. It is shown that Symmetry® 3.5 µm columns have column lifetimes comparable to Symmetry® 5 µm columns and are more stable than other 3 and 3.5 µm columns.

Introduction
High-performance liquid chromatography (HPLC) columns packed with 3 µm packing materials were first commercialised in the early 1980s (1, 2, 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 required a shorter column to compensate for increased backpressure. 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.

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Waters
The extra column effects were a direct result of the HPLC system’s incompatibility with small column volumes. Columns made with 3 µm packing materials also had shorter lifetimes due to plugging of the frits. 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.

This paper compares the separation efficiency of the Symmetry® 3.5 µm column (4.6 x 100 mm) with the Symmetry® 5 µm column (3.9 x 150 mm and 4.6 x 150 mm). Chromatographic data on several pharmaceutical compounds show the speed advantage of the Symmetry® 3.5 µm column for fast chromatography. Column lifetimes for Symmetry® columns are compared with Merck Superspher® 100 RP-18 and Zorbax® SB C18 column.

Experimental
Sulphanilamide, sulphadiazine, sulphathiazole, sulphamerazine, sulphamethazine, succinylsulphathiazole, beclomethasone, paclitaxel (taxol), and tamoxifen were from Sigma Chemical Corporation (St. Louis, MO). Testosterone propionate, testosterone benzoate and testosterone acetate were from U.S. Pharmacopeia Reference Standards (Rockville, MD). Butalbital and butabarbital were from Alltech (Deerfield, IL). Rabbit serum was from Equitech-Bio (Ingram, TX). Buffers and mobile phases were made with reagent grade chemicals and HPLC grade solvents. Mobile phases were filtered and degassed.

Symmetry® C18 and C8 columns (3.9 x 150 mm — 5µm; 4.6 x 150 mm — 5µm; 4.6 x 100 mm — 3.5 µm) and Symmetry Sentry® guard columns were from Waters (Milford, MA). Merck Superspher 100 RP-18 (44 mm x 125 mm) was purchased from EM Separations (Gibbstown, NJ). Zorbax SB C18 (4.6 x 75 mm, 3.5 µm) was obtained from MacMod (Chadds Ford, PA).

HPLC Systems
The HPLC system used in this study consisted of either a Waters 625 or a 616 LC System, 717plus Autosampler and the 996 Photodiode Array Detector. Stability studies with biological samples were done on the Waters 600E Multisolvent Delivery System, 715 Ultra WISP Sample Processor and 490 Programmable Multimwavelength detector. Control and results management were provided by the Millennium® 2010 Chromatography Manager.

The components of the HPLC system for column lifetime studies with the standard sulpha 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.

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

The column stability study using 10 µL of the sulpha drug mixture (10 to 39 mg/mL) was injected on a Symmetry® C8 Sentry guard column (3.9 x 20 mm, 5 µm) in line with a Symmetry® C8 column (4.6 x 100 mm, 3.5 µm). The mobile phase was water/methanol/glacial acetic acid 79:20:1 and was recycled during the course of the experiment. The flow rate was 1.5 mL/min 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 minutes and spiked with 5 µg/mL of butalbital and 5 µg/mL of butabarbital. The mobile phase was 0.1 M potassium phosphate, pH 6.9/ acetonitrile/water, 20:30:50 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 (1.25 mL/min for the Superspher column). The detection wavelength was 214 nm.

Results and Discussion
Efficiency Theory
The objective of the Symmetry® 3.5 µm column is to offer fast separations without sacrificing chromatographic resolution. Resolution is a function of: the selectivity, $\alpha$, 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, $N$, expressed in theoretical plates. Equation 1 shows the dependence of resolution on $\alpha$, $k$ and $N$.

$$ R_s = \frac{1}{4} N^{1/2} (\alpha^{-1} \frac{k}{k+1}) $$

(Equation 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. Figure 1 shows a plot of the theoretical plate count, $N$, vs. flow rate for 5 µm and 3.5 µm Symmetry® columns and illustrates the following points:

- The 3.5 µm, 150 mm 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 backpressure.
- The short 75 mm 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, for example, to the same reduced plate height ($h$). Figure 2 shows the plot of reduced plate height versus linear velocity for the Symmetry® C$_{18}$ 5 µm column and the Symmetry® C$_{8}$ 3.5 µm 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 for 50:50 acetonitrile / water, whereas for 50:50 methanol / water, a more viscous mobile phase, the minimum occurs at 0.9 mL/min.

Efficiency and Speed

Figure 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 Symmetry® C$_{18}$ columns: a. 3.9 x 150 mm, 5 µm and b. 4.6 x 100 mm, 3.5 µm are shown. Both columns have a resolution of 18 which meets the U.S. Pharmacopeia assay (4) specification ($R_s \geq 5$). Both columns have similar plate counts of approximately 8000 and similar backpressures under the chromatographic conditions chosen. The analysis 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 µm to 3.5 µm.
Performance equivalence between 3.5 µm and 5 µm packing is further illustrated in Figures 4 and 5 where impurity profiles of pharmaceutical compounds are shown. Figure 4 and Figure 5 show the assays of tamoxifen and taxol, respectively, on a Symmetry® C18 (3.9 x 150 mm, 3.5 µm) column and a Symmetry® C18 (4.6 x 100, 5 µm) column. In Figure 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 (Figure 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. Figure 6 shows the separation of testosterone at 1.4 mL/min and 2.8 mL/min. The separation efficiency at 2.8 mL/min is still 6800 plates (compared to 7500 plates at the lower flow rate) and the run time is reduced by half. This is possible 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 backpressure. 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.
Column Lifetime

For column lifetime studies, a Symmetry® C8 column (4.6 x 100 mm, 3.5 µm) was used with Sentry guard columns containing 5 µm C8 packing material. The guard column was replaced every time the backpressure increased or resolution decreased. The sample was a mixture of sulphur drug standards. The results are shown in Figures 7 and 8. In Figure 7 the plate count of one of the analytes is plotted against the number of injections. In Figure 8 the system backpressure is plotted. As one can see, neither a significant decline in efficiency nor a significant increase in backpressure was observed over 10,000 injections. This demonstrates that excellent column life can be achieved with Symmetry® 3.5 µm columns, when they are protected by Sentry guard columns. This result parallels the results of a previous study using Symmetry® 5 µm packing.

In another lifetime study, deproteinated rabbit serum spiked with butalbital and buta was injected 1000 times on a Symmetry® C18 3.5 µm column, a 3 µm Merck Superspher 100 RP-18 column and a 3.5 µm Zorbax SB C18 column. Figure 9 is a comparison between theoretical plates versus number of injections for the Symmetry® and the Superspher column. The Superspher column had a 25 % loss in efficiency compared to a ≤ 5 % decrease for the Symmetry® column. As shown in Figure 10, the Zorbax column did not reach 1000 injections due to a sharp increase in backpressure ( < 3500 psi).

For more Information on

Symmetry® 3.5 µm columns, please check box 4 on the Business Reply Card and return today.
Symmetry® 5 µm columns, please check box 5 on the Business Reply Card and return today.
SymmetryPrep™ 7 µm columns, please check box 6 on the Business Reply Card and return today.
Compared to other 3 µm columns, Symmetry® has a larger mean particle size (3.5 µm). Also, the particle size distribution about the mean is very narrow (90% / 10% volume ratio of 1.5). The increase in particle size and the narrow distribution is the likely cause of the increase in the lifetime of the Symmetry® 3.5 µm columns.

**Conclusions**

The Symmetry® 3.5 µm columns demonstrate 30 to 50% faster analysis times over Symmetry® 5 µm columns. Separation methodologies using Symmetry® 5 µm columns can be easily translated into methods using Symmetry® 3.5 µm columns since the efficiency, resolution and sensitivity remain the same. Also, unlike other 3 µm columns, Waters Symmetry® 3.5 µm columns are as stable as their 5 µm counterparts.

**References:**

### Ordering Information

**Symmetry® Analytical Steel Columns**

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**Symmetry 3.5 µm Columns**

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**Symmetry® Steel Cartridge Columns** *(All cartridge columns require reusable endfittings.)*

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**Symmetry® Validation Kit** *(three steel columns or steel cartridges from 3 different batches of packing materials)*

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### Part No.

End Connector Kit *(includes 2 sets of reusable endfittings, 2 C-Clips and Kalrez o-rings)*

- Part No.: WAT037525

**Sentry™ Guard Columns* and Guard Holders**

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* For Symmetry® columns, please refer to box numbers 4, 5, 6 on the Business Reply Card. To receive information simply check the appropriate box number.

For more Information on *Sentry guard columns can be used with either the steel cartridge columns but require the appropriate guard holder.** For Waters 3.9 mm x 50 mm cartridge column, you must use the Universal Guard Holder.
Sample Dilution Increases Sensitivity and Resolution

Uwe D. Neue and Ed Serowik

Introduction and Overview
Marginal or insufficient sensitivity is not uncommon in chromatographic analysis. It may be encountered in the analysis of the metabolic fate of a drug or in the investigation of environmental pollutants. In this paper we look at a specific set of circumstances where low sensitivity can be improved through a simple operation.

The principle that we employed is the use of the analytical column as an enrichment column. Sample enrichment permits a significant increase in the injection volume which results in a proportional increase in sensitivity. A very specific example is used to demonstrate the principle. But the general principle is useful in many other circumstances as well.

In this example, the analytical method is an isocratic reversed-phase method. The sample is subjected to a solid-phase extraction step prior to chromatographic analysis for sample cleanup and enrichment. A typical instance is a metabolite assay from a biological matrix like serum or urine. In this case, the solid-phase extraction step frequently is a reversed-phase method as well, using a Waters Sep-Pak® cartridge with the same or similar chemistry as the analytical column. In a commonly followed procedure, the reversed-phase Sep-Pak cartridge is first conditioned and the sample is then loaded onto the cartridge. Then the adsorbed sample is washed with a polar eluent, typically water or buffer containing a small amount of organic solvent. Subsequently, the sample is eluted from the solid-phase extraction cartridge with an organic solvent such as methanol. The methanol extract is then evaporated to dryness, and the sample is redissolved in mobile phase for HPLC analysis. This sequence of steps usually results in an enrichment of the sample as well as a partial sample clean-up.

A significant disadvantage of this procedure is the fact that a fairly broad spectrum of hydrophobicity lies between the sample washing step and the elution step. Sample constituents with a larger hydrophobicity than the metabolites are eluted from the Sep-Pak cartridge together with the analytes. They are retained on the analytical column more strongly than the analytes of interest. Consequently, they will appear either late in the analytical chromatogram, resulting in excessively long analysis times, or they may even interfere with subsequent analyses. The latter case would necessitate a washing step after each chromatogram, which together with the accompanying reequilibration of the column significantly increases the run time for an assay and reduces productivity.

A better approach is an elution procedure, in which a solvent is used that is just strong enough to elute the metabolites efficiently, but leaves compounds of higher hydrophobicity on the Sep-Pak cartridge. If the bonded-phase used for sample preparation is the same as or similar to the bonded phase in the analytical column, a good elution solvent would be the chromatographic mobile phase with the amount of organic modifier increased by about 20%. Therefore, if the mobile phase for the analytical column is 30% methanol / 70% buffer, the elution solvent for the Sep-Pak cartridge should be 50% methanol / 50% buffer.

However, using mixtures of organic solvent and water or buffer as the elution solvent has one major disadvantage: it is very difficult and time-consuming to evaporate aqueous mixtures to dryness for reconstitution of the sample. If on the other hand, the sample is injected without this step, there is a limitation amount that can be injected onto the column without loss of resolution. To solve this dilemma, the method of simply diluting the sample with water or buffer, and injecting a larger sample volume was employed. To use a dilution step to increase sensitivity is counterintuitive, but the increased sample volume that can be injected overcompensates for the dilution.

Experimental
We used ethyl- and propylparaben at a concentration of 0.2 µg/mL as our model system. The column used was a Symmetry® C18 5 µm 3.9 mm x 150 mm column. The mobile phase consisted of 35% acetonitrile and 65% water, resulting in an elution time of about 5 minutes for ethylparaben and about 10 minutes for propylparaben. The sample was dissolved in 55% acetonitrile, 45% water to mimic the elution conditions from a C18 Sep-Pak cartridge needed for a quick and complete elution of the analytes. For the enrichment experiments, the sample was diluted with water in ratios of 1:1, 2:1, 4:1, 5:1, 6:1, 7:1, 11:1, and 15:1. The amount injected was varied in proportion to the dilution such that a constant mass of sample was injected.

Discussion
In order to maximise sensitivity under fixed mobile phase conditions, we would like to inject the largest mass of sample onto the column that is possible. Ideally, the entire sample should be injected (although there may be cases where one chooses to retain a portion of the sample for repeat analysis). The factor that limits how much can be injected is the loss of resolution due to volume overload. Sometimes, we have enough sample available that this limit is encountered using a column with a standard diameter. However, in most cases the sample amount is limited, and a column with a smaller diameter should be considered.

An increase in injection volume leads to a broadening of the peak and to a loss in resolution. Let us first consider the
case in which the sample is dissolved in mobile phase. Table 1 shows the injection volume as a function of the retention factor \( k \) for different column dimensions that result in either a 5 % or 10 % loss in resolution.

If we are willing to accept about 10 % loss in resolution between peaks, we can inject about 50 % of the peak-volume on the column. If we are willing to accept only a 5 % loss in resolution, we are still able to inject about 33 % of the peak-volume on the column. Therefore, typical tolerable injection volumes for a 3.9 mm x 150 mm, 5 µm column would be between 40 and 160 µL, depending on the retention factor of the sample and the resolution requirement.

The situation actually gets worse in the case when the sample is dissolved in a solvent that has a higher elution strength than the mobile phase. In Figure 1 we plot the % resolution versus injection volume for the paraben sample dissolved in 55 % acetonitrile with a mobile phase of 35 % acetonitrile for a 3.9 mm x 150 mm Symmetry\textsuperscript{\textregistered} C\textsubscript{18} column. Already at an injection volume of only 25 µL, the resolution has dropped by about 15 %. For a 50 µL injection, the resolution is decreased by 35 %.

However, the situation changes a great deal if the sample is injected in a solvent that has a higher water content than the mobile phase. We diluted the sample 5:1 with water, resulting in a water content of 90 %. At this dilution, we were able to inject 1200 µL of the sample without a loss in resolution (Figure 2). The mass of sample injected was the same as in the 200 µL of undiluted sample, which had resulted in a fourfold loss of resolution. Also, due to the fact that the peak-volume was smaller than in the former case, the height of the signal was larger by nearly a factor of 4. As can be seen in Figure 2, the peaks obtained at the larger injection volume are delayed compared to the peaks at the small injection volume. This is due to the injection volume itself. The 1.2 mL of sample is loaded onto the top of the column first, and peak migration will not start until the loading step is complete.

This method is based on the interplay between the dilution of the sample by water and the enrichment of the sample on the surface of the packing due to the increased water content of the sample solvent. The enrichment factor increases exponentially with the water content of the sample. For small molecules the enrichment increases by a factor of roughly 2.5 to 3 per 10% increase in water content. Therefore, as long as a 2:1 dilution results in a change in water content that exceeds 10 %, a gain in the amount injectable onto the column for a tolerable loss in resolution is realised. Since this is only a rule of thumb, experimentation is required to determine the actual optimal dilution.

<table>
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<th>Column I. D. [mm]</th>
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<th>10 %</th>
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<td></td>
</tr>
<tr>
<td>2</td>
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<td>10</td>
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</table>

Table 1: Maximum Injection Volume [µL] for a 5 % or 10 % Loss in Resolution for 5 µm, 150 mm Columns

Figure 1: Relative Resolution as a Function of the Injection Volume

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Figure 3 shows the results of some dilution experiments. Resolution is plotted against the amount of sample injected. In order to inject a constant amount of sample, the injection volume was increased in proportion to the dilution. For example, while a 40 ng injection corresponded to 200 µL of undiluted sample, it corresponded to 1600 µL of 1:7 diluted sample. One can see that for the 40 ng injection, a dilution of about 1:5 to 1:6 gave optimal results, while for the 20 ng sample any dilution between 1:2 and 1:7 gave equivalent results.

There is an optimal dilution factor that maximises the amount of sample that can be injected without peak distortion. It can be modeled mathematically, if the relationship of the retention factor with solvent composition is known. However, one can assume some typical values and use this model as a guide. In our model, we assumed that the sample has been eluted from the Sep-Pak cartridge using an eluent that contains 20% more organic solvent than the mobile phase. We then calculated the optimal dilution factor as a function of the mobile phase composition. The enrichment factor is the ratio of the sample volume that can be injected after dilution compared to the sample volume that can be injected if the sample is dissolved in mobile phase. Table 2 shows the results of the calculation for different mobile phase compositions. The lower values are more typical when methanol is used as organic modifier. The higher values are more typical for acetonitrile. It is clear that the dilution method works best for mobile phases containing large amounts of organic solvents.
Table 2: Optimal Dilution Factor and Resulting Enrichment Factors as a Function of the Mobile Phase Composition

<table>
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<th>Dilution Factor d</th>
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<td>20</td>
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<td>1 - 3.5</td>
</tr>
<tr>
<td>30</td>
<td>3 - 4.5</td>
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<td>80</td>
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As an example: the sample of interest has a $k'$ of 5 on a 3.9 mm x 150 mm column in 40% methanol / 60% buffer. It was eluted from the Sep-Pak cartridge with 60% methanol / 40% buffer and diluted by a factor of 4 based on the data in Table 2. The sample volume that would result in a 10 % loss in resolution in Table 1 is 122 µL, if the sample were dissolved in mobile phase. The enrichment factor from the last table is at least 10, so we can inject at least about 1200 µL on a 3.9 mm x 150 mm column. If the elution volume from the Sep-Pak cartridge was 500 µL and we diluted the sample to 2500 µL, then this procedure means that we can inject about 50 % of the total amount of sample available onto the column. That is quite reasonable.

In the experiment shown in Figure 3, the optimal dilution factor was found to be about 5, and the enrichment factor about 40, in good agreement with the estimation in Table 2. This means the method is quite robust.

**Conclusion**

A method for sample enrichment has been outlined that is simple in practice but complex in concept. We have provided some guidance on how the technique should be used. Also we have demonstrated in a model system that the technique works in practice.

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A Resolution Equation for EKC Based on Electrophoretic Mobilities

Jeffrey R. Mazzeo, Michael E. Swartz and Edward R. Grover

Abstract

A resolution equation for EKC was developed starting from the resolution equation for electrophoresis. The equation was used to predict the influence of the migration window and partitioning on resolution in EKC. It is theoretically shown that the migration window can have a dramatic effect on resolution in EKC. Using a novel chiral surfactant, the influence of the migration window and partitioning on the separation of benzoin enantiomers was experimentally determined. The results obtained agreed with predictions based on the equation. The ability to obtain very high resolution values by migration window manipulation was demonstrated for the separation of N-methylpseudoephedrine enantiomers ($\alpha = 1.3$). Specifically, the resolution was 2.4 under conditions of robust EOF, but increased to 11 using conditions of low EOF.

Introduction

Electrokinetic chromatography (EKC), invented by Terabe, is a subset of capillary electrophoresis (CE) (1). In EKC, analytes partition between the bulk aqueous CE phase and an additive. The most powerful technique of EKC is the use of micelles (MEKC). Resolution of two analytes is achieved in EKC by one or both of the following mechanisms:

1) differences in their mobilities in the bulk aqueous phase (capillary zone electrophoresis), and/or
2) differences in their partitioning between the bulk aqueous phase and the additive, with the further requirement that the mobility of the analyte-additive complex is different from the mobility of the analyte in the bulk aqueous phase.

The second mechanism results in a migration window in EKC. For instance, MEKC is usually performed with sodium dodecyl sulphate (SDS) micelles. SDS micelles are anionic and have an electrophoretic mobility towards the anode. Uncoated fused silica capillaries are typically used in MEKC, and a bulk electroosmotic flow toward the cathode is produced at pH > 2.0. Above pH 6.0, the electroosmotic velocity is usually faster than the electrophoretic velocity of the SDS micelles, causing the micelles to have a net movement toward the cathode. This situation leads to a migration window, which for neutral analytes, is defined by the electroosmotic flow marker (no partitioning) and the micelle marker (complete partitioning). All neutral analytes must migrate between these two boundaries.

The existence of a migration window leads to an additional term in the resolution equation for MEKC compared to the standard resolution equation for chromatography. As developed by Terabe (1), the resolution equation for neutral analytes in MEKC is:

$$R_s = \frac{N}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k_2^2}{k_1^2 + k_2^2} \right) \left( \frac{1 - t_0/t_m}{1 + t_0/t_m} \right)$$

where $N$ is the theoretical plate count, $\alpha$ is the selectivity term, $k_1$ and $k_2$ are the capacity factors for the two analytes, $t_0$ is the electroosmotic flow time, and $t_m$ is the micelle marker time.

Capacity factors of neutral compounds are calculated using the following equation (1):

$$k = \frac{t - t_0}{t_0} \left( \frac{1 - t_0}{t_m} \right)$$

where $t$ is the observed migration time of the analyte.

The resolution and capacity factor equations for MEKC were derived for neutral analytes under conditions where the micelles and the bulk aqueous phase move toward the same electrode (1). Charged analytes which do not interact with the micelles will not migrate at the electroosmotic flow time. Several researchers have proposed alternative equations for calculating capacity factors and resolution of charged analytes (2-3). We proposed that $t_{aq}$ be substituted for $t_0$ in the resolution and capacity factor equations, $t_{aq}$ being defined as the time in the aqueous phase (3).
The $t_{eq}$ value of a charged analyte will be a function of the electroosmotic mobility, its own electrophoretic mobility, and its interaction, if any, with non-micellised surfactant. The value of $t_{eq}$ can be determined by measuring the analyte’s electrophoretic mobility in the MEKC buffer without micelles, adding it to the electroosmotic mobility in the MEKC buffer with micelles, and converting the resulting mobility to a migration time. Analyte interaction with free surfactant molecules is assumed to be negligible.

It is possible for the micelles and the aqueous phase to have net mobilities toward opposite electrodes. This situation occurs when the electrophoretic velocity of the anionic micelles toward the anode is greater than the electroosmotic velocity toward the cathode (4). In this case, an unpartitioned neutral analyte will migrate toward the cathode, while a completely partitioned analyte will migrate toward the anode (4). Depending on its partitioning value, a neutral analyte can migrate toward the cathode with a migration time from $t_{eq}$ to infinity, or toward the anode with a migration time from $t_{mc}$ to infinity. The resolution and capacity factor equations are written with migration times, so the practice has been to use negative values for the migration times when migration is toward the anode (4). Consequently, negative resolution values can also be obtained. A negative resolution value indicates that the more highly retained analyte migrates faster (5).

The migration window or elution range is defined as $t_0/t_{mc}$, so the case where the net micelle movement is toward the anode has been referred to as a negative migration window.

We are interested in determining the influence of the migration window and partitioning on resolution in EKC, especially with negative migration windows. Therefore, we have derived a resolution equation which is applicable to all forms of EKC. This paper shows the derivation of this equation. Using the equation, the influence of the migration window and partitioning on resolution were predicted. The predictions were then verified experimentally.

### Theory

The resolution equation for two analytes in electrophoresis is defined as (6):

$$R_s = \frac{(N^2)}{4} \left( \frac{\mu_{app,1} - \mu_{app,2}}{(\mu_{app,1} + \mu_{app,2})} \right)$$

where $N$ is the average theoretical plate count, and $\mu_{app}$ is the apparent mobility. Assuming that diffusion is the only cause of band broadening, the average plate count for two analytes in CE is given by (7):

$$N = \frac{\left(\mu_{app,1} + \mu_{app,2}\right) V}{2DL}$$

where $V$ is the applied voltage, $L$ is the capillary length from injection to detection, $D$ is the diffusion coefficient, and $L$ is the total capillary length.

For two analytes partitioning between the aqueous phase and some additive (micelles, cyclodextrins, etc.), apparent mobilities can be calculated:

$$\mu_{app,1} = x_1 \mu_{additive,1} + (1-x_1) \mu_{fs,1}$$

$$\mu_{app,2} = x_2 \mu_{additive,2} + (1-x_2) \mu_{fs,2}$$

where $x$ is the fraction of time the analyte associates with the additive, $\mu_{additive}$ is the mobility of the analyte-additive complex, and $\mu_{fs}$ is the mobility of the analyte in the aqueous phase. The mobilities of the analyte-additive complexes for the two analytes are assumed to be the same in order to simplify the resulting equations.

The apparent mobilities are determined from the electrophoretic and electroosmotic mobilities:

$$\mu_{additive} = \mu_{additive,ep} + \mu_{os}$$

$$\mu_{fs} = \mu_{fs,ep} + \mu_{os}$$
Substituting equations 4-8 into equation 3 and canceling terms leads to the following equation:

The main assumptions of equation 9 are:

\[ R_s = \left\{ \frac{V}{16DL} \right\}^\frac{1}{2} \frac{\left[x_1 \left( \mu_{\text{additive,ap}} - \mu_{h,1,\text{ap}} \right) - x_2 \left( \mu_{\text{additive,ap}} - \mu_{h,2,\text{ap}} \right) + \mu_{h,1,\text{ap}} - \mu_{h,2,\text{ap}} \right]}{\left[\left(x_1 + x_2\right) \left( \mu_{\text{additive,ap}} - \mu_{h,\text{ap}} \right) + 2 \mu_a + 2 \mu_{h,\text{ap}}\right]^\frac{1}{2}} \]

1) diffusion is the sole cause of band broadening,
2) the analytes have the same diffusion coefficient, and
3) the mobilities of the analytes when interacting with the additive are the same.

The influence of EOF (and therefore the migration window) on resolution can be calculated using equation 9 assuming constant values for \( x_1 \), electrophoretic mobility of the additive, and free solution electrophoretic mobility of the two analytes. As an example, assume neutral analytes \( \mu_{\text{hs}} = 0 \), negatively charged micelles with \( \mu_{\text{additive}} = -4.4 \times 10^{-4} \text{ cm}^2/\text{Vs} \), \( x_1 = 0.50 \) and \( x_2 = 0.51 \) (\( \alpha = 1.04 \)). \( V = 15,000 \) volts, \( l = 60 \) cm, \( L = 52.5 \) cm and \( D = 8 \times 10^{-6} \text{ cm}^2/\text{Vs} \) (typical value for an electrolyte with MW < 500).

Since the analytes have the same electrophoretic mobility, equation 10 can be used. Resolution of the two analytes was plotted as a function of electrophoretic mobility, equation 10 can be used. Resolution of the two analytes was plotted as a function of electrophoretic mobility, Figure 1. A symmetrical curve was generated, centered about a point at which the resolution is infinity. This point occurs at an electrophoretic mobility of +2.222 \times 10^{-4} \text{ cm}^2/\text{Vs} for these assumed values.

Using equations 5, 6, 7, and 8, the apparent mobilities of each analyte can be calculated as a function of electrophoretic mobility. Using equation 4, the average apparent mobility decreases. Since the apparent mobility is directly proportional to the average apparent mobility, the plates also decrease.

For the given \( x \) values (0.50 and 0.51), at electrophoretic mobilities less than +2.222 \times 10^{-4} \text{ cm}^2/\text{Vs}, the average apparent mobility decreases. Since the plate count is directly proportional to the average apparent mobility, the plates also decrease.

Furthermore, below electrophoretic mobilities of +2.222 \times 10^{-4} \text{ cm}^2/\text{Vs}, the more highly retained analyte \( x_h \) has the higher mobility, while above +2.222 \times 10^{-4} \text{ cm}^2/\text{Vs}, the more highly retained analyte has the lower mobility. Therefore, an inversion of migration order is expected. Electrophoretic mobilities near +2.222 \times 10^{-4} \text{ cm}^2/\text{Vs} lead to very low analyte mobilities and long analysis times. Clearly, a tradeoff exists; higher resolution can be obtained at the expense of longer analysis time.
The influence of x on resolution for several migration windows can also be calculated using equation 12 in conjunction with equation 10. Note that when varying x, both the average apparent mobility and the difference in apparent mobility change (equation 10). Assume neutral analytes (µfs = 0), negatively charged micelles with µmic,app = -4.4 x 10^-4 cm^2/Vs, α=1.04, V = 15,000 volts, L = 60 cm, l = 52.5 cm and D = 8 x 10^-6 cm^2/Vs. The influence of x on resolution for several migration windows is shown in Figure 3.

With a finite migration window (i.e., the micelles move in the same direction as the electroosmotic flow but at a slower rate), an optimum value for x is seen and the resolution goes to zero when x = 1 (Figure 3a, µos = 6.0 x 10^-4 cm^2/Vs, µmc = 1.6 x 10^-4 cm^2/Vs). With an infinite migration window (the micelles do not move), resolution is higher at all x values and the optimum x value is higher (Figure 3b, µos = 4.4 x 10^-4 cm^2/Vs, µmc,app = 0 x 10^-4 cm^2/Vs). This shift in the optimum x value is in agreement with the Foley equation, which shows that the optimum capacity factor in MEKC depends on the migration window (10).

With a negative migration window where the micelles move slowly toward the anode, the optimum x value increases compared to an infinite migration window, and infinite resolution can be obtained (Figure 3c, µos = 4.4 x 10^-4 cm^2/Vs, µmc = -0.4 x 10^-4 cm^2/Vs). This shift in the optimum x value is in agreement with the Foley equation, which shows that the optimum capacity factor in MEKC depends on the migration window (10). With a symmetrical, negative migration window (i.e., the micelles move toward the anode but the bulk aqueous phase does not move, the optimum x value shifts lower compared to a symmetrical, negative migration window (Figure 3e, µos = 0 x 10^-4 cm^2/Vs, µmc = -4.4 x 10^-4 cm^2/Vs). Also note that the curve is the same shape as with an infinite migration window where the micelle does not move (3b), but is reversed.

To determine if equation 10 correctly predicts the influence of electroosmotic flow and x on resolution, the plots in Figure 1 and Figure 3d will be verified experimentally.

**Experimental**

Separations were performed on a Waters Quanta® 4000E system (Milford, MA). AccuSep™ uncoated fused silica capillaries (50 µm x 60 cm, 52.5 cm injection to detection) were employed. Its electrophoretic mobility (µphase,ep = -4.4 x 10^-4 cm^2/Vs) was constant over the pH range studied.

Finally, with an infinite migration window where the micelle moves toward the anode but the bulk aqueous phase does not move, the optimum x value changes lower compared to a symmetrical, negative migration window (Figure 3e, µos = 0 x 10^-4 cm^2/Vs, µmc = -4.4 x 10^-4 cm^2/Vs). Also note that the curve is the same shape as with an infinite migration window where the micelle does not move (3b), but is reversed.

**Results**

To demonstrate the influence of the migration window on resolution, the dependence of EOF on pH was exploited [4]. A phosphate-acetate buffer (25 mM each, sodium salts) was employed over the pH range 3-6, with the electroosmotic mobility ranging from 5.3 x 10^-4 cm^2/Vs (pH 6) to 0.5 x 10^-4 cm^2/Vs (pH 3). The enantiomers of the neutral (µfs = 0) analyte, benzoin, were separated (3:1 ratio of (+):(-)). A novel chiral surfactant, (S)-2-[[1-oxododecyl]amino]-3S-methyl-1-sulphooxypentane will be described in a future publication. The synthesis of (S)-N-dodecoxy-carbonylvaleine has been described [3]. Structures of the two surfactants are shown in Figure 4.

**Figure 4. Surfactant Structures**

![Surfactant Structures](image-url)
It was initially assumed that the $x$ values of the benzoin enantiomers would not change over the pH range of 3-6. The observed differences in $x$ over the pH range are attributed to joule heating as no attempt was made to perform the experiments at constant ionic strength.

Table 1 lists the values of $T_{m(+)}$, $T_{m(-)}$, $T_{m(c)}$, resolution, plate counts, $x$, and alpha at the different pH-values. From the apparent mobilities of the analyte, micellar phase, and electroosmotic flow, the $x$ value of the analyte was determined using the following equation:

$$x = \frac{\mu_{app} - \mu_c}{\mu_{phase} - \mu_e}$$  \[13\]

This equation is simply a rearrangement of equation 5. Alphas values were calculated using equation 12.

At pH 4.5, injection was performed at the cathode, while at pH 5.2, injection was performed at the anode. Some of the separations are shown in Figure 5. The separations at pH 5.2 (Figure 5b) and pH 4.5 (Figure 5c) show that the migration order of the enantiomers was reversed. Experiments were also performed at pH 4.8 and 5.0. However, injecting from both the anode and cathode led to no peaks up to 90 minutes. These pH-values led to electroosmotic mobilities close to the point where resolution approaches infinity; unfortunately analysis time approaches infinity as well.

Figure 6 is a graph of experimental resolution versus electroosmotic mobility. The experimental results show the same symmetrical profile as seen in Figure 1. Since the $x$ values and alpha values remained relatively constant with electroosmotic mobility, the change in resolution was due to changes in the migration window.

The ability of equation 10 to quantitatively predict changes in resolution with electroosmotic flow was tested for this data set. The diffusion coefficient of the enantiomers was calculated with equation 10 using the experimental

![Figure 5: Separation of Benzoin Enantiomers at pHs 5.5 (a), 5.2 (b), 4.5 (c) and 4.0 (d).](image)
resolution at pH 6.0 as well as the x values, electroosmotic mobility, additive electrophoretic mobility, capillary lengths and voltage. The diffusion coefficient was calculated to be \(6.45 \times 10^{-6} \text{ cm}^2/\text{s}\). Then, using this value for the diffusion coefficient and assuming constant x values, resolution was calculated as a function of electroosmotic mobility at each pH. Table 2 gives the calculated and experimental resolution at each pH, as well as the % difference. Generally, the predicted value using equation 10 is within 10% of the actual resolution value. The one exception is at pH 4.5, where the difference was 21%. This large difference is attributed to the fact that the x values, which were assumed to be the same as at pH 6.0, were significantly different at pH 4.5 (see Table 1). If the x values measured at pH 4.5 are used, the predicted resolution is 4.19, only a 3.7% difference from the experimental resolution. Another possible source of error could be improperly measured values for the electroosmotic flow and micelle marker time.

Trends in resolution similar to those in Figure 6 can also be generated with a negative, symmetrical migration window by changing the analytes’ x values (Figure 3d). In an equilibrium distribution process, such as MEKC, an analyte’s x value, or partitioning, can be altered thermodynamically (by changing the nature of one of the phases) or through phase ratio (by changing the concentration of one of the phases in relation to the other). The advantage of changing the partitioning through phase ratio is that selectivity (\(\alpha\)) does not change. To demonstrate this concept, the separation of benzoin enantiomers was performed at pH 5.0 with surfactant concentrations of 60, 30, 15 and 10 mM. At pH 5.0, \(\mu_{os} = +2.1 \times 10^{-4} \text{ cm}^2/\text{Vs}\) and \(\mu_{mc} = -2.3 \times 10^{-4} \text{ cm}^2/\text{Vs}\), which is essentially a symmetrical, infinite migration window. The separations at the four surfactant concentrations are shown in Figure 7. Note the similarity to those shown in Figure 5 and the fact that a reversal of migration order was obtained. The general trend in resolution with x (surfactant concentration) is similar to the theoretical curve (Figure 3d). Quantitative predictions of resolution as a function of x values using equation 10 are problematic since the electroosmotic flow changes with the change in surfactant concentration.

The alpha value for benzoin using (S)-2-[(1-oxododecyl)amino]-(3S)-methyl-1-sulphooxypentane is 1.04, which was sufficient for baseline resolution under all the conditions investigated. In many cases, lower selectivities are found. By optimising the migration window and x values for a given system using equation 9 or 10, simple pairs with alpha values less than 1.04 can be baseline resolved. Very high resolution values can be realised by optimising the migration window and/or x values when the alpha value is higher. To demonstrate this point, the separation of the enantiomers of N-methylpseudoeudrphedrine was performed with (S)-N-dodecoxycarbonylvaline (Figure 4b, \(\mu_{mc, ep} = -4.5 \times 10^{-4} \text{ cm}^2/\text{Vs}\)) under high and low EOF conditions. The alpha value for this pair of enantiomers is 1.3 with (S)-N-dodecoxycarbonylvaline (3). Separations of the enantiomers were performed in 50 mM Na2HPO4 adjusted to pH 8.0, where N-methylpseudoeudrphedrine is positively charged (\(\mu_{fs, ep} = +2.1 \times 10^{-4} \text{ cm}^2/\text{Vs}\)). Separations were performed in an uncoated capillary with robust EOF (\(\mu_{os} = +5.5 \times 10^{-4} \text{ cm}^2/\text{Vs}\), \(\mu_{mc} = +1.0 \times 10^{-4} \text{ cm}^2/\text{Vs}\)) and in a coated capillary with low EOF (\(\mu_{os} = +0.5 \times 10^{-4} \text{ cm}^2/\text{Vs}\), \(\mu_{mc} = -4.0 \times 10^{-4} \text{ cm}^2/\text{Vs}\)). In the uncoated capillary, a finite migration window was obtained, while with the coated capillary a negative migration window was obtained.

![Figure 6: Plot of Resolution versus Electroosmotic Mobility (Data in Table 1)](image)

Table 1: Migration Times, Resolution, Plate Counts, Partitioning (x), and Alpha at Each pH

<table>
<thead>
<tr>
<th>pH</th>
<th>(T_{m(+)})</th>
<th>(T_{m(-)})</th>
<th>(T_{os})</th>
<th>(T_{mc})</th>
<th>(R_s)</th>
<th>(N_{avg})</th>
<th>(x_1)</th>
<th>(x_2)</th>
<th>(\alpha)</th>
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<tr>
<td>6.0</td>
<td>13.193</td>
<td>13.397</td>
<td>6.590</td>
<td>38.890</td>
<td>2.15</td>
<td>263,000</td>
<td>0.60</td>
<td>0.61</td>
<td>1.04</td>
</tr>
<tr>
<td>5.5</td>
<td>16.187</td>
<td>16.540</td>
<td>7.393</td>
<td>116.990</td>
<td>2.64</td>
<td>230,000</td>
<td>0.58</td>
<td>0.59</td>
<td>1.04</td>
</tr>
<tr>
<td>5.2</td>
<td>35.037</td>
<td>36.827</td>
<td>9.890</td>
<td>(40.698)</td>
<td>4.17</td>
<td>107,000</td>
<td>0.59</td>
<td>0.60</td>
<td>1.04</td>
</tr>
<tr>
<td>4.5</td>
<td>(44.820)</td>
<td>(42.323)</td>
<td>21.875</td>
<td>(9.210)</td>
<td>4.35</td>
<td>82,000</td>
<td>0.52</td>
<td>0.53</td>
<td>1.04</td>
</tr>
<tr>
<td>4.0</td>
<td>(24.450)</td>
<td>(23.630)</td>
<td>43.750</td>
<td>(9.722)</td>
<td>2.80</td>
<td>155,000</td>
<td>0.55</td>
<td>0.56</td>
<td>1.04</td>
</tr>
<tr>
<td>3.0</td>
<td>(19.183)</td>
<td>(18.707)</td>
<td>69.750</td>
<td>(9.000)</td>
<td>2.32</td>
<td>210,000</td>
<td>0.55</td>
<td>0.56</td>
<td>1.04</td>
</tr>
</tbody>
</table>

All migration times are in minutes; values in parentheses indicate migration toward the anode.

Conditions: Used For Data Generated in Table 1:
Buffer: 25mM (S)-2-[(1-oxododecyl)amino]-(3S)-methyl-1-sulphooxypentane
25mM sodium acetate/25 mM monosodium phosphate
50 µm x 60 cm uncoated capillary; + or - 15 kV; 2 second hydrostatic injection
Sample: 3:1 ratio of (+):(-) benzoin, 0.4 mg/mL in buffer
Figure 7 shows the two separations, with the resolution increasing from 2.4 (uncoated capillary) to 11.0 (coated capillary). The same buffer was used for both separations, the only difference being the capillary employed (and hence the magnitude of electroosmotic flow).

**Conclusions**

A resolution equation for EKC based on electrophoretic mobilities is a useful tool in predicting the influence of the migration window and partitioning on resolution. Several points can be made about the simulated resolution vs. migration window and partitioning curves prepared using equation 10. First, the migration window is a powerful parameter to optimise resolution in EKC. Infinite resolution is possible. Second, for compounds which spend more time with the additive than in bulk solution, i.e., x values > 0.50, an infinite migration window where the additive does not move is preferable to an infinite migration window where the additive does move. For compounds with x values < 0.50, the latter case is preferred. This situation is due to the fact that the lower the average apparent mobility of the analytes, the better the resolution. When the additive does not move, analytes which spend more time with the additive will have lower average apparent mobility. When the bulk solution does not move, analytes which spend more time in it (and therefore less time with the additive) will have the lower average apparent mobility. Finally, through a combination of EOF and/or x value manipulation, reversals in migration order can be obtained in EKC.

The system employed here to verify the predictions of equation 10 is an ideal one. The analytes are enantiomers and neutral over a wide pH range; the surfactant is fully ionised over a wide pH range. However, the separation of enantiomers is an important practical problem, and the thought process demonstrated in this paper makes the optimisation of such a separation by chiral MEKC both rational and efficient.

### Table 2: Comparison of Experimental Resolution and Resolution Calculated using Equation 10

<table>
<thead>
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<th>pH</th>
<th>Experimental Rs</th>
<th>Calculated Rs</th>
<th>%Difference</th>
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<tr>
<td>5.5</td>
<td>2.64</td>
<td>2.43</td>
<td>8.0</td>
</tr>
<tr>
<td>5.2</td>
<td>4.17</td>
<td>3.81</td>
<td>8.6</td>
</tr>
<tr>
<td>4.5</td>
<td>4.35</td>
<td>3.42</td>
<td>21.4</td>
</tr>
<tr>
<td>4.0</td>
<td>2.80</td>
<td>2.58</td>
<td>8.0</td>
</tr>
<tr>
<td>3.0</td>
<td>2.32</td>
<td>2.39</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Figure 8: Separation of N-Methylpseudoephedrine Enantiomers using Uncoated (top) and Coated Capillary (bottom)

Conditions: 10 mM (S)-N-dodecyloxy carbonylvaline
50 mM Na2HPO4
pH 8.0
214 nm detection
10 second injection
2:1 ratio of (+):(-)-N-methylpseudoephedrine in buffer, 0.4 mg/mL

Uncoated Capillary:
50µm X 45 cm, 35 cm effective
+8 kV

Coated Capillary:
50µm X 45 cm, 35 cm effective
-8 kV

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References

Ordering Information

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**Literature Corner:**

**Pharmaceutical Applications:**

“Analysis of Cisapride in Neonatal Plasma Using High-Performance Liquid Chromatography with a Base-Stable Column and Fluorescence Detection”

The authors describe a method for measuring the level of staurosporine which is a protein-kinase inhibitor in blood. The assay required that the acetone extract be diluted 1:1 in order for the staurosporine to bind to the µBondapak C₁₈ Radial-Pak cartridge (8 mm x 100 mm). Direct injection of the plasma sample was not possible because the protein and drug would co-elute using a linear elution gradient of acetonitrile with 0.2% TFA. The UV detection allowed a range from 0.1 to 2 µg staurosporine. This method was also used to determine the pharmacokinetics of the drug in vivo.

Dorothy J. Phillips

“Development of a High Performance Liquid Chromatographic Method for the Analysis of Staurosporine”

The U. S. Pharmacopeia method for cephalixin (an antibiotic) requires the use of both an ion-pairing reagent and a silanol blocking agent. Also the method uses an uncommon internal standard. The British Pharmacopeia method for the analysis of cephalixin oralpreparation is an iodometric titration. The US Code of Federal Regulations describe both an iodometric titration and a microbiological method. The authors of this paper have developed a method which has a simple mobile phase (methanol/1.25% glacial acetic acid) with the readily available pairing reagent and a silanol blocking agent. Also the method uses an uncommon internal standard. The U.S. Pharmacopeia method for cephalixin (an antibiotic) requires the use of both an ion-pairing reagent and a silanol blocking agent. Also the method uses an uncommon internal standard. The British Pharmacopeia method for the analysis of cephalixin oralpreparation is an iodometric titration. The US Code of Federal Regulations describe both an iodometric titration and a microbiological method. The authors of this paper have developed a method which has a simple mobile phase (methanol/1.25% glacial acetic acid) with the readily available drug acetaminophen as the internal standard. The µBondapak C₁₈ column (3.9 mm x 300 mm) for the basic drug cisapride. The plasma extracts were analysed on a Waters Symmetry® C₈ column with a simple mobile phase of acetonitrile and phosphate buffer. No mobile phase additives were required for sharp, symmetrical peaks. The linear range for cisapride was 5 to 250 ng/mL. The specificity of the assay was confirmed by showing that cisapride did not co-elute with its metabolite nor with twelve other drugs used in neonatal pharmacotherapeutics. The short analysis time of 10 minutes also made the assay suitable for clinical assays. This is one of the first reported clinical applications in neonatal medicine of the Symmetry® column, which is a recently developed column from Waters that is ideally suited for this type of analysis.

Dorothy J. Phillips

“High-Performance Liquid Chromatographic Method for Potency Determination of Cephalexin in Commercial Preparations and for Stability Studies”

The authors developed a paired-ion HPLC method for the simultaneous determination of sweeteners (dulcin, saccharin-NA and ascesulphame-K), preservatives (acetic, sorbic, salicylic, benzoic, succinic, methyl-p-hydroxybenzoic, ethyl-p-hydroxybenzoic, n-propyl-p-hydroxybenzoic and i-propyl-p-hydroxybenzoic acids) and antioxidants (3-t-butyl-4-hydroxy-anisole and t-butyl-hydroquinone). Detailed sample preparation procedures are given for soy sauce, dried roast beef and sugared fruit using solid-phase extraction with Waters Sep-Pak C₁₈ cartridges.

Uwe D. Neue

“Stereospecific High-Performance Liquid Chromatographic Assay of Iomefloxacin in Human Plasma”

Several methods have been reported in the literature for the quantitation of iomefloxacin, a chiral quinolone carboxylic acid antibiotic. The literature methods are not able to discern the quantities of the individual enantiomers of this drug in biological fluids. The method reported in this paper describes an HPLC assay for quantitation of the enantiomers of iomefloxacin. The drug is extracted from plasma at pH 7 in the presence of the internal standard, racemic acetobutol. A two-step precolumn derivatization with unichiral (S)-(+-)-naphthylethyl isocyanate and achiral ethyl chloroformate is then carried out. The enantiomers are separated as diastereomers on Waters Nova-Pak® silica packing in the 8 mm x 100 mm Radial-Pak™ column segment.

Dorothy J. Phillips

Food Applications:

“Simultaneous Determination of Preservatives, Sweeteners and Antioxidants in Food by Paired-Ion Liquid Chromatography”

Simple, rapid and robust methods are most suitable for drug analyses. For the analysis of drugs in plasma a high degree of sensitivity and selectivity are also required. A simple, selective, sensitive and precise high-performance liquid chromatography method was developed for the basic drug cisapride. The plasma extracts were analysed on a Waters Symmetry® C₈ column (3.9 mm x 150 mm) with a simple mobile phase of acetonitrile and phosphate buffer. No mobile phase additives were required for sharp, symmetrical peaks. The linear range for cisapride was 5 to 250 ng/mL. The specificity of the assay was confirmed by showing that cisapride did not co-elute with its metabolite nor with twelve other drugs used in neonatal pharmacotherapeutics. The short analysis time of 10 minutes also made the assay suitable for clinical assays. This is one of the first reported clinical applications in neonatal medicine of the Symmetry® column, which is a recently developed column from Waters that is ideally suited for this type of analysis.

Dorothy J. Phillips
**Food Applications:**

“Structure of Hydroxycinnamic Acid Derivatives Established by High-Performance Liquid Chromatography with Photodiode-Array Detection”

L. Bengoechea, T. Hernández, C. Quesada, B. Bartolomé, I. Estrella and C. Gce Liquid Chromatography with Photodiode-Array

Hydroxycinnamic acid derivatives are widely distributed throughout the plant kingdom and play an important role in the secondary metabolism in plants. In this paper, hydroxycinnamic acid derivatives are separated by reversed-phase HPLC using a Nova-Pak C18 column and categorised using a Waters Model 991 Photodiode Array Detector. p-Coumaric acid derivatives are distinguishable from caffeic and ferulic acid derivatives, but it is not possible to distinguish between the latter two groups due to overlap of the ranges of spectral characteristics. Uwe D. Neue

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**The Choice is Yours**

**Waters™ Columns now available from Phase Separations**

We credit the tremendous success of Symmetry® columns to the fact that their development is a direct result of a dialogue with our customers. So in our search for a better way to sell and deliver Waters™ HPLC columns, we initiated another dialogue with our customers; both scientists and purchasing professionals.

What we’ve found is that you are happy with our products and support, but would like to purchase all of your chromatography supplies from a convenient, single source. While you would like to be able to purchase Waters™ columns from a chromatography “superstore”, you strongly cautioned us against taking any action that would compromise the high level of technical support you receive from Waters today.

Our search for a better way to sell and deliver Waters™ columns led us to Phase Separations, a well established United Kingdom based chromatography supplies company. With Phase Sep, you have one source for the broadest selection of chromatography columns and supplies in the world. They have skilled technical support to help you with your applications and they never offer any “substitute” brands.

With their new international presence, Phase Sep can help Waters give you what you want because we have given them exclusive third party distribution rights to Waters™ columns. Phase Sep can provide you with Waters™ columns as well as other column brands you may use. They can also support Waters™ columns the way you would like them to be supported. Of course, all of our products can still be purchased directly from Waters with the same guarantee of excellence in technical support.

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**Spherisorb® Columns now available from Waters**

Spherisorb®️, one of the world’s widely used HPLC columns was first developed by Phase Separations in the late 1970s. It has been Phase Sep’s historical practice to sell Spherisorb®️ packing material broadly to secondary manufacturing companies who then pack this material into a steel column and re-sell it under the Spherisorb®️ brand name. While this practice gained broad distribution for Spherisorb®️ products, given the critical nature of the assays performed by HPLC, Phase Sep and Waters are taking steps to bring a higher level of quality and consistency to the finished Spherisorb®️ column wherever and from whomever you purchase it.

Effective immediately, Phase Separations has granted exclusive manufacturing and distribution rights for Spherisorb®️ products to Waters. Spherisorb®️ columns will still be available from Phase Separations as well as from your current source, but you now have the option of purchasing them direct from Waters, and at a very attractive price.

For price and delivery information on Waters Spherisorb®️ columns, call your local sales office listed on page 22.
What’s new

New catalog available in May
Spherisorb® Columns now available from Waters (previous page)

The Waters Chromatography Columns and Supplies Catalog 1996-1997 will be available in early May. For your free copy, please check box 11 on the Business Reply Card and return today.
**EPA Method 604: Phenols**

**Details:**
Phenols are important industrial wastewater contaminants which must be monitored for compliance with discharge regulations. Phenols, particularly nitrophenols, often display inconsistent response when analyzed by GC, even when time consuming derivatization steps are employed.

EPA Method 604 phenols are separated in 20 minutes using a Symmetry® C8 5 µm column.

**System:**
The HPLC system consisted of the Waters 626 LC System, 717 plus Autosampler, and the 996 Photodiode Array Detector. Control and results management were provided by a Millennium® 2010 Chromatography Manager.
Millennium® 2010 Chromatography Manager

Advanced computer technology simplifies accessing, interpreting, and reporting your results.

The productivity of today’s chromatography is centered around the ability to find, generate, summarise, and report results in a way that meets your needs and complies with any regulatory requirements. The Millennium relational database lets you create your own view filters to customise the way you sort your information, such as “lotCode”.

- Using the database, you can track a method history giving you audit trail capability that ensures total security of your raw data and results. In addition, you can specify “Access Type” which allows you to “lock” your methods for even greater security.
- The database keeps track of your instruments, triggering an alarm when scheduled maintenance is required. The Millennium relational database lets you view the injection information on any calibration point at any particular point in time on a processing sequence. This gives you a detailed account of the processing method for that injection, ensuring an accurate audit trail for regulatory compliance.
- The Millennium Chromatography Manager can provide acquisition and control for as many as four chromatographic systems, including three HP5890 GC.
- The Millennium Report Publisher has the capability to give you exactly what you want.

Waters 996 Photodiode Array Detector

Complete Spectral Data–Complete Confidence in Peak Purity and Identity.

With Waters 996 Photodiode Array Detector, you can be confident that if there is an impurity in your sample, you will find it; that your compound is really what you report it to be and not something else. Every spectral detail of your sample is rapidly, easily, and accurately detected. Every peak is analysed with mathematical precision so you can be sure to assess the purity of peaks and confirm their identity. And you will never have to trade off resolution against sensitivity.

- The 996 PDA Detector lets you look at your results in a variety of ways - even while a separation is under way.
- Absorbency measurement over the entire 190-800 nm wavelength range.
- The MaxPlot feature of the Millennium PDA Software automatically detects UV/Vis absorbing compounds anywhere within the 190-800 nm range. It measures their absorbence maxima, and plots a peak for each compound at its absorbence maximum, creating a composite chromatogram of all the compounds it has detected.

Waters 717plus Autosampler

Waters 717plus Autosampler is a fully programmable autosampler for High-Pressure Liquid Chromatography.

Waters 626 LC System

Innovative LC technology.

The Waters 626 LC System combines advanced polymeric technology and low dispersion system volume into a liquid chromatograph that provides the highest level of performance available in a non-metallic system. The 626 LC System is optimised for ion exchange, gel filtration, affinity, hydrophobic interaction and reversed-phase chemistries used in microbore, analytical or micro preparative methods relevant to the chemist. The 626 LC System has maximum system operating pressure of up to 4000 psi.

For more Information on

For more information on these products please check box 17-21 on the Business Reply Card.
Rapid Analysis on Symmetry® 3.5 µm Column

Objective:
To demonstrate that 3.5 µm packing allows shorter analysis times without sacrificing efficiency or sensitivity.

Details:
Efficiency is directly related to column length. Therefore, reducing column length results in a decrease in efficiency. Decreasing the particle size from 5 µm to 3.5 µm gives an increase in efficiency which compensates for this loss when using shorter columns. Hence, faster analysis can be obtained with the shorter 3.5 µm column without a decrease in efficiency. Maintaining the efficiency on the similar volume columns also results in no change in sensitivity.

System:
The HPLC system consisted of the Waters 626 LC System, 717plus Autosampler, and the 996 Photodiode Array Detector. Control and results management were provided by a Millennium® 2010 Chromatography Manager.
Millennium® 2010 Chromatography Manager

Advanced computer technology simplifies accessing, interpreting, and reporting your results.

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• The database keeps track of your instruments, triggering an alarm when scheduled maintenance is required. The Millennium relational database lets you view the injection information on any calibration point at any particular point in time on a processing sequence. This gives you a detailed account of the processing method for that injection, ensuring an accurate audit trail for regulatory compliance.

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