A New Water-Wettable Solid Phase Extraction Sorbent for the Analysis of Drugs in Biological Fluids

Edouard Bouvier, Donna Martin, Pamela Iraneta, Mark Capparella, Dorothy Phillips, Yung-Fong Cheng and Laura Bean

Introduction

Waters is working closely with methods development experts in the pharmaceutical industry to streamline sample preparation. We have learned that considerable time and effort is consumed in choosing an appropriate Solid Phase Extraction (SPE) sorbent and extraction protocol. The limitations of today's sorbents require the analyst to watch carefully and control closely the extraction procedure. Even then it is difficult and time-consuming to achieve high, reproducible recoveries for analysis of important polar drugs and metabolites. A new polymeric SPE sorbent has now been developed that allows more samples to be processed in one batch, resulting in a significant increase in throughput. This is especially important in the pharmaceutical industry, where quantitation of drugs and their metabolites is performed using LC/MS/MS, with typical analysis times of less than 5 minutes. The bottleneck at the sample preparation step can now be significantly reduced or eliminated.

Traditional Methods for Solid Phase Extraction

Solid phase extraction is typically performed by loading a sample onto a pre-conditioned extraction cartridge containing a reversed-phase chromatographic sorbent. The most commonly used sorbents are porous silica particles surface-bonded with C_{18} or other hydrophobic alkyl groups.

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Prior to use, the sorbent must first be conditioned with a water-miscible organic solvent and then equilibrated with water or buffer solution. Because these sorbents are not hydrophilic, or water wettable, care must be taken to ensure that the sorbent stays wet before loading the aqueous sample. When reversed-phase silica-based cartridges run dry, they lose activity because water cannot wet the sorbent. This is a major cause of low analyte recoveries and poor assay-to-assay reproducibility (1-7).

Now, Waters has developed Oasis™ HLB extraction cartridges to overcome these limitations of reversed-phase SPE and to streamline the sample preparation process. The key to this advancement has been the development of a novel (patent pending) polymeric reversed-phase sorbent. This macroporous copolymer [poly(divinylbenzene-co-N-vinylpyrrolidone)] exhibits both hydrophilic and lipophilic surface characteristics. HLB is an acronym for Hydrophilic-Lipophilic Balance which describes two major features of this sorbent: the unique abilities to (1) remain wetted with water, and (2) retain a wide spectrum of both polar and nonpolar compounds. Oasis™ HLB extraction cartridges deliver higher, more reproducible recoveries for a wide range of analytes using rapid, straightforward extraction protocols. These results can be achieved without having to worry about watching your samples as they are processed, because the cartridges can be allowed to dry out during the extraction procedure.

**Experimental**

**Chemistry and Equipment**

Acetaminophen, butyl paraben, doxepin, procainamide, propranolol, and sulfanilamide were obtained from Aldrich Chemical Company (Milwaukee, WI). Sodium hexanesulfonate, ibuprofen, naltrexone, oxycodeone, salbutamol and ranitidine were purchased from Sigma Chemical Company (St. Louis, MO). Naproxen was obtained from Alltech (Deerfield, IL). Acetonitrile, methanol, ammonium acetate, glacial acetic acid, potassium phosphate and phosphoric acid were obtained from J.T. Baker (Phillipsburg, NJ). Porcine serum was obtained from Equitech-Bio (Ingram, TX). One cc/30 mg Oasis™ HLB extraction cartridges were obtained from Waters Corporation (Milford, MA). One cc/100 mg reversed-phase C₁₈ SPE cartridges were obtained from Varian Sample Preparation Products (Harbor City, CA).

The HPLC system used in this study consisted of a Waters 717+ plus Autosampler and a 600E Multi Solvent Delivery System or 625 IC System. Detection was by UV using a Waters 486 Tunable Absorbance Detector or a 996 Photodiode Array Detector, or by fluorescence using a Waters 470 Scanning Fluorescence Detector. Data acquisition was performed using either a Waters 860 Chromatography Data System or a Millennium® 2010 Chromatography Manager. A 20 position vacuum manifold with a vacuum pump (Waters Corporation) was used to process solid phase extraction cartridges.

**Effect of Cartridge Drying on Recovery**

Freshly thawed porcine serum was spiked either with procainamide, acetaminophen and ranitidine (Sample 1) at a final concentration of 10 µg/mL each, or with doxepin and propranolol (Sample 2), at 5 µg/mL and 20 µg/mL, respectively. Three replicate extractions were performed for each sample. One cc/100 mg reversed-phase C₁₈ and 1 cc/30 mg Oasis™ HLB extraction cartridges were conditioned in the vacuum manifold with 1 mL of methanol. When the methanol reached the top frit of the cartridges, the vacuum was maintained for 0, 10, 30, 60, 120, 240, or 480 seconds to vary the cartridge drying time. 1 mL of water was then loaded onto the cartridge followed by 1 mL of porcine serum spiked with Sample 1 or Sample 2. Cartridges were then washed with either 1 mL of deionised water, for the reversed-phase C₁₈ cartridges, or 1 mL of 5% methanol in water for the Oasis™ HLB extraction cartridges. The analyte was eluted from the sorbent with 1 mL of methanol. Ten µL of 1000 µg/mL internal standard (either sulfanilamide or butyl paraben) was added to this eluate. Sample 1 eluates were diluted 1:1 with water. Samples were vortexed and then analysed by HPLC.

**Solid Phase Extraction and HPLC Analysis of Drugs in Serum**

Freshly thawed porcine serum was spiked, at the levels indicated in Figure 4, with one or more of the following: ibuprofen, naproxen, acetaminophen, procainamide, ranitidine, oxycodone, propranolol, naltrexone, salbutamol, doxepin. Samples spiked with ibuprofen and naproxen were also acidified with 20 µL phosphoric acid per mL of serum to minimise protein binding. A schematic of the solid phase extraction procedure is shown in Figure 1. The SPE procedure was performed on six replicate samples for each drug analysed.

**Chromatographic Conditions**

For the analysis of procainamide, ranitidine and acetaminophen, a Waters SymmetryShield™ RP₈ column (3.9 x 150 mm) coupled with a Sentry™ SymmetryShield™ RP₈ guard column was used for the HPLC assay. The column temperature was controlled to 30 °C. The mobile phase was 20 mM potassium phosphate, 30 µL of 200 mM sodium acetate, glacial acetic acid, potassium phosphate and phosphoric acid were obtained from J.T. Baker (Phillipsburg, NJ). Porcine serum was obtained from Equitech-Bio (Ingram, TX). One cc/30 mg Oasis™ HLB extraction cartridges were obtained from Waters Corporation (Milford, MA). One cc/100 mg reversed-phase C₁₈ SPE cartridges were obtained from Varian Sample Preparation Products (Harbor City, CA).
pH 3/methanol 97:3 (v/v). The flow rate was set to 1.0 mL/min. The detector wavelength was 220 nm.

For the analysis of ibuprofen and naproxen, a Waters SymmetryShield™ RP₈ column (3.0 x 150 mm) coupled with a Sentry™ SymmetryShield™ RP₈ guard column was used at a temperature of 25 °C. The mobile phase consisted of 1.2% glacial acetic acid in water/acetonitrile 50:50 (v/v). Flow rate was 0.6 mL/min; detection was at 254 nm.

Naltrexone and oxycodone were analysed on a Waters SymmetryShield™ RP₈ column (3.0 x 150 mm) coupled with a Sentry™ SymmetryShield™ RP₈ guard column at 25 °C using a mobile phase of 20 mM ammonium acetate, pH 5/acetonitrile 90:10 (v/v). Flow rate was 0.6 mL/min; detection was at 215 nm.

Salbutamol was analysed on a Symmetry® C₁₈ column (3.9 x 150 mm) coupled with a Sentry™ Symmetry® C₁₈ guard column. The mobile phase was 5 mM sodium hexanesulfonate/methanol 75:25 (v/v), with 1% glacial acetic acid added. Flow rate was 1.0 mL/min. Detection was by fluorescence, with an excitation wavelength of 275 nm and an emission wavelength of 310 nm.

For doxepin and propranolol, a Waters Symmetry® C₁₈ column (3.9 x 150 mm) coupled with a Sentry™ Symmetry® C₁₈ guard column was used. The mobile phase was 20 mM potassium phosphate buffer, pH 7/methanol 35:65 (v/v). The flow rate was 1 mL/min; detection was at 254 nm.

**Results and Discussion**

### Effect of Cartridge Drying

It is important to condition reversed-phase silica-based SPE cartridges properly before loading the sample. The conditioning solvent (typically methanol or acetonitrile) acts as a wetting agent and plays a key role by fully solvating the hydrocarbon chains on the sorbent surface. Once the surface is wetted, water can displace this solvent, resulting in a sorbent surface that is in intimate contact with the water phase. Without the conditioning step, the aqueous sample cannot penetrate a reversed-phase silica-based sorbent’s pores, where most of the analyte retention occurs. Improperly conditioned cartridges which have been allowed to dry out do not provide the same retention as cartridges that remain wetted. This leads to reduced recoveries with higher analytical variability.

Figure 2A shows what happens when reversed-phase silica-based cartridges dry out after the conditioning step. Reversed-phase C₁₈ cartridges conditioned with methanol were allowed to dry under vacuum-induced flow for varying amounts of time prior to the equilibration and loading of spiked porcine serum.

The results show that recovery decreases markedly for all analytes even after only one minute of cartridge drying. The loss is most pronounced for more polar compounds, such as acetaminophen. Thus, if care is not taken to condition the C₁₈ cartridges properly and keep them wetted, significant variability can be observed in the recovery data. From a practical standpoint, the requirement to ensure that the solvent level in an SPE cartridge remains above the top of the sorbent bed reduces the number of samples that an analyst can process at one time on a vacuum manifold.

When Waters Oasis™ HLB extraction cartridges are dried out after conditioning, recovery is unaffected (Figure 2B). This means that stringent monitoring of liquid levels is no longer necessary. More consistent recoveries and more rugged SPE methods can result. Sample processing thus becomes faster, easier and more efficient. Samples can be processed under continuous vacuum without using stopcocks at all, eliminating not only tedium, but also a potential source of contamination and/or sample loss.

### A More Universal Sorbent

Reversed-phase silica-based sorbents have become the most widely used materials for HPLC and SPE. This is due to their broad utility and ruggedness. However, silica-based sorbents have at least three drawbacks.

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Figure 2. Effect on recovery of cartridge drying after methanol conditioning for pharmaceutical drugs in serum. Results with (A) 1 cc/100 mg reversed-phase C₁₈ cartridges, and (B) 1 cc/30 mg Oasis™ HLB extraction cartridges.
One major issue is the complication caused by surface silanol groups. These silanols provide a site for strong ion-exchange interaction with basic compounds. In HPLC, silanols can cause significant peak tailing of bases. Similarly, in SPE, these silanol interactions must be minimised when isolating basic compounds. Otherwise low recoveries or excessively large elution volumes may result. The degree of silanol interactions will vary depending on the pH and ionic strength of the sample matrix. It may take more time to explore the adjustments to the sample matrix and/or the sorbent to ensure good reproducibility, to minimise silanol interactions, and to optimise a particular protocol. Each developed application may therefore require a somewhat different procedure, which can increase the possibility of operator error or confusion.

Limited pH stability represents a second serious drawback of reversed-phase silica-based sorbents. One key tool in developing an HPLC or SPE method is to vary the pH of the sample either to promote or suppress ionisation of the analyte and/or sorbent surface. However, the high solubility of silica at a pH greater than 8 and the potential for hydrolysis of the bonded phase at a pH less than 2 limits the useable pH range.

A third problem is that highly polar analytes may not be sufficiently retained by hydrophobic reversed-phase sorbents. This can result in reduced recoveries due to analyte breakthrough during the sample loading step. With the increased importance of more polar drugs (and their metabolites), C_{18} and C_{8} SPE sorbents may not provide satisfactory recoveries.

The polymeric Oasis™ HLB sorbent overcomes all of the problems associated with reversed-phase silica-based SPE sorbents. As a result, Oasis™ HLB extraction cartridges allow use of a simple, more universal SPE protocol. Because there are no troublesome ion-exchange interactions, the mechanisms for affecting drug and metabolite extraction are simpler. When elution sorbents at pH extremes deliver optimal results, Oasis™ HLB extraction cartridges are stable over a much broader pH range (from pH 1-13) than silica-based sorbents.

To demonstrate the universal nature of Oasis™ HLB extraction cartridges, a similar protocol was used to extract a wide range of compounds (Figure 3) from serum. A schematic of this protocol is outlined in Figure 1. The only required modification to the protocol, in some instances, was the acidification of serum samples, necessary to minimise drug-protein binding. Percent recoveries, shown in Figure 4, were determined for all analytes by HPLC with UV or fluorescence detection. Recovery for each analyte tested using this protocol was greater than 90%. RSDs, based on six replicate extractions, were less than 5% in all cases for a broad range of acidic, basic and neutral compounds. Even ionised analytes are quantitated with consistently high recoveries, due to the much stronger retention on the HLB sorbent as compared to C_{18}. A typical chromatogram of a serum extract after SPE is shown in Figure 5.

Figure 3. Structures of pharmaceutical compounds extracted from porcine serum using Oasis™ HLB extraction cartridges.
Conclusions
Waters Oasis™ HLB extraction cartridges provide major new benefits and eliminate the problems associated with using reversed-phase silica-based SPE cartridges. Unlike conventional reversed-phase silica-based sorbents, the Oasis™ HLB sorbent does not lose activity when cartridges dry out during sample processing. This eliminates the need for the tedious, time-consuming manipulation of stopcocks when processing samples on vacuum manifolds.

Oasis™ HLB extraction cartridges simplify and speed up methods development. There is no need to worry about silanol interactions. Simple SPE protocols can be employed for a wider range of compounds, including analytes previously not amenable to isolation by SPE using traditional sorbents. The greater range of pH stability with Oasis™ HLB extraction cartridges increases your flexibility for solving tough problems. Since analytes are more strongly retained on Oasis™ HLB extraction cartridges than on traditional C18 Cartridges, higher, more reproducible recoveries, especially for polar compounds, may result. Sample preparation is thus easier and faster with higher recoveries and more reproducible results.

References

Figure 5. Chromatograms of (A) serum blank and (B) serum spiked with acetaminophen, using sulfanilamide as internal standard. Chromatographic conditions are described in the text.
A Solid Phase Extraction Procedure for Determination of Priority Pollutant Phenols in Aqueous Samples

Michael S. Young

Introduction

Phenolic compounds are important industrial chemicals of environmental concern. Phenols are intermediates in the production of polymers, pharmaceuticals, agricultural chemicals, and dyes. They are used as disinfectants and pesticides. Pentachlorophenol is a common wood preservative. Halogenated phenols may be produced as byproducts from chlorination of natural organic matter or as occurs in wood pulp bleaching or from water disinfection. Phenols are toxic and impart undesirable characteristics of taste, odor and color to water. Because of their potential for environmental harm, the EPA currently requires the analysis of municipal and industrial effluents for 11 phenols (1). The procedure described in this report is suitable for confirmatory analysis for samples analysed by EPA method 604.

Phenols are organic acids with the degree of acidity increasing with increasing substitution of halogen or nitro groups. Thus phenol is a weak acid (pKa~10), mononitrophenols are somewhat stronger (pKa~8) and dinitrophenols stronger still (pKa~5). The acidity and reactivity of phenols can complicate analytical procedures such as extraction and chromatography. Phenols are typically analysed using gas chromatography or liquid chromatography and each procedure has its advantages and limitations. Although improvements in gas chromatographic procedures allow for analysis of free phenols, derivatisation is often utilised for GC analysis of phenols to generate less reactive compounds which give better and more consistent response and show better peak symmetry. Among the derivatisation reactions commonly utilised for GC are acylation (acetic anhydride), silylation (BSTFA), and formation of alkyl ethers (diazomethane, pentafluorobenzyl bromide). For LC applications, there is less need for derivatisation to generate less active compounds because free phenols give consistent symmetrical peaks on modern reversed-phase LC columns such as Waters Symmetry® columns. The best sensitivity for HPLC analysis of free phenols is obtained with the electrochemical detector, but the availability of this detector is still limited for many analysts. Therefore, derivatives are often prepared to enhance UV or fluorescence sensitivity by addition of an appropriate chromophore to the phenolic molecule. Among the derivatisations employed for HPLC analysis of phenols are acylation (fluorescamine, imparts enhanced fluorescence) (2) and esterification with aromatic acids (for example, 2-fluorenesulfonic acid, enhances UV or fluorescence) (3).

The recent development of the photodiode array detector (PDA) allows for the sensitive, reproducible determination of free phenols by HPLC without derivatisation. Although the response of a PDA detector, at any given wavelength, is similar to a more traditional variable wavelength detector, the PDA provides more usable sensitivity and much more information. First, the PDA allows the analyst to independently measure each constituent at its maximum wavelength to provide optimum sensitivity. Furthermore, the ability of the PDA to measure peak purity and to compare unknown spectra to library spectra provides the analyst with tools to verify the integrity of the analysis. Therefore, the PDA allows for the sensitive, reproducible determination of free phenols by HPLC without derivatisation (4).

Phenols may be extracted from water using a variety of techniques, most commonly liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Although the less cumbersome SPE procedure is generally preferable to LLE, there may be problems with low recovery of some phenols on traditional C18 silica cartridges or disks. For this study, the samples were analysed by SPE using Porapak® Rx Sep-Pak® cartridges. The Porapak® Rx resin employed in these cartridges shows enhanced reten-

Experimental Section

Chemical Standards. Standard phenols were obtained from AccuStandard Inc. (New Haven, CT).

Sources of Water. Detection limit studies were conducted on high purity reagent water obtained from a Milli-Q® Water System (Millipore, Bedford, MA). Finished drinking water was obtained at Waters Corporation, Milford, MA.

HPLC Conditions. All analyses were performed on a Waters’ HPLC system comprised of a Model 600 Solvent Delivery System, a Model 712 WISP™ Autosampler, a Model 616 pump, and a Model 996 Photodiode Array.
The results of this experiment indicated that the detection limit for these compounds is lower than 1 µg/L.

**Best Solvent for Sample Elution.** Initial experiments were performed using methanol to elute the analytes from the Sep-Pak® cartridges. However, inconsistent recovery of pentachlorophenol led to an investigation of other solvents to give consistent and high recovery of all compounds. Good recovery of all analytes was obtained using tetrahydrofuran.

**Recovery from Reagent Water.** Seven replicate 500 mL samples were prepared using Milli-Q water. Each sample was spiked to yield a sample concentration of 10 µg/L of each analyte. The results are presented in Table 1.

Detector (PDA). The Millennium® Chromatography Manager was used for system control, data acquisition, and data analysis. The analytical column was a Waters Symmetry® C8, 3.9 mm x 150 mm. The aqueous portion of the mobile phase (Solvent A) was 1% acetic acid in Milli-Q water. The organic portion of the mobile phase (Solvent B) was 1% acetic acid in acetonitrile. All solvents were obtained from J.T. Baker (Philipsburg N.J.) and were HPLC grade; all reagents were also from J.T. Baker and were ACS grade.

The gradient chosen for this analysis was 30% B initial, with a linear gradient to 100% B in 20 minutes. The flow rate was 1.2 mL/min. and the injection volume was 40 µL.

**Solid Phase Extraction with Porapak® Rtx Cartridges.** The conditions chosen for the extraction of phenols were based on the procedure developed for analysis of nitroaromatic and nitramine explosives (6). All cartridge conditioning and sample enrichment was performed at 10 mL per minute. A Waters SPE manifold was used for processing up to 12 cartridges at a time. The sample size was 500 mL.

The Porapak® Rtx Sep-Pak® cartridges were preconditioned with 15 mL of methanol followed by 30 mL of Milli-Q water. The aqueous samples were adjusted to pH 1.5-2 by addition of 2 mL of 1 N HCl, and were then processed through the Sep-Pak cartridges at approximately 10 mL per minute. Following sample enrichment, the cartridges were air dried for 10 minutes before elution with 5 mL of methanol or tetrahydrofuran. The sample extracts were then diluted 1:1 with Milli-Q water before analysis by HPLC.

**Results and Discussion**

**Detection Limit.** According to a procedure recommended by the EPA (7), the detection limit for an analyte may be estimated from the magnitude of the standard deviation in the results obtained from seven or more replicate spiked samples. In this experiment, seven identical 500 mL samples were spiked at a level of 10 µg/L of all components and were analysed by the new Porapak® Rtx Sep-Pak® method.

Recovery is presented for data collected at 280 nm except for pentachlorophenol which was monitored at 294.3 nm.

**Recovery from a Municipal Drinking Water.** Five replicate 500 mL samples were prepared at a spike level of 20 µg/mL using Milford, MA tap water. Before spiking, each sample was treated with 30 mg of sodium sulfite to remove any residual chlorinating agent. The results are presented in Table 2. All results were obtained at 280 nm except for pentachlorophenol (294.3 nm). Figure 1 shows a typical HPLC chromatogram obtained from this experiment (extracted wavelength chromatogram at 280 nm).

### Table 1. Recovery of Phenols from Reagent Water (spike level 10 µg/mL, 7 replicates)

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<th>% Recovery</th>
<th>% RSD</th>
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<td>99.5</td>
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<tr>
<td>4-nitrophenol</td>
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<td>2-chlorophenol</td>
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<td>pentachlorophenol</td>
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### Table 2. Recovery of Phenols from Drinking Water (spike level 20 µg/mL, 5 replicates)

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The results show excellent recovery for all the phenols tested. Of particular importance is the excellent recovery shown for phenol, which is among the most volatile and water soluble of the priority pollutant phenols. Traditional solid phase extraction on C18 silica has shown acceptable recovery for many of these analytes but gives poor recovery for phenol. The Porapak® RDX Sep-Pak® cartridge method described here is fast, accurate and reproducible for the determination of phenol and other priority pollutant phenols in aqueous samples. Initially, the results of all analytes were obtained at 280 nm, a general phenolic absorbance wavelength commonly utilised for analysis of phenols. Using the PDA, it was determined that an interfering compound was coeluting with pentachlorophenol. The response of this interference was from 240 to 285 nm while the PDA indicated that the maximum absorbance for pentachlorophenol was at 294.3 nm. Therefore, when the pentachlorophenol response was measured at 294.3, the interference was eliminated.

Conclusion

The Porapak® RDX Sep-Pak® cartridge has been shown to be a highly effective solid phase extraction device for the determination of phenols in aqueous samples. Recovery was greater than 95% for all analytes. The high recovery of phenol is of particular note, recovery of this analyte using traditional C18 solid phase extraction cartridges is not generally acceptable.

The Symmetry® C8 column employed for this study proved to be rugged and reliable. Although some performance loss was noted at the end of the study, after some 300 injections in various matrices, the column performance was still acceptable for sub ppb analysis.

References


For more information on Symmetry® columns, please check box 5 on the Business Reply Card.
Ordering Information

Porapak® RDX Cartridges and Accessories

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For more information on Porapak® RDX Sep-Pak® Cartridges, please check box 6 on the Business Reply Card.

 Corrections to an article in Issue 1, 1996 of the Waters Column

Many of our Waters Column readers notified us of some errors in an article titled “A New Solid Phase Extraction Method for Determination of Triazine Herbicides and Metabolites in Aqueous Samples” that appeared in our Volume VI, Issue 1, 1996.

On page 16, the reference to drinking water contaminants monitored under the Safe Drinking Water Act, the limits for atrazine in the United States should read 3 µg/L, the European limit is 0.1 µg/L for atrazine or any individual regulated pesticide, and 0.5 µg/L for the sum of all pesticides.

On page 17, Experimental Section: HPLC Conditions, in the last paragraph, the injection volume should read 50 µL. In the Results and Discussion: Detection Limit, in the last line in column two, seven samples were spiked at a level of 0.400 µg/L, in the third column, first paragraph, last sentence; The results of this experiment indicated that the detection limit for these compounds is lower than 0.1 µg/L. In Table 1, the header should read “Recovery of Triazine Herbicides and Metabolites from a Pond Water Source (spike level 2 µg/L, 5 replicates)”.

On page 18, Recovery from a Municipal Drinking Water: samples were prepared at a spike level of 0.4 µg/L. In Table 2, the header should read “Recovery of Triazine Herbicides and Metabolites from a Municipal Drinking Water Source (spike level 0.4 µg/L, 5 replicates)”.

Our apologies to Michael S. Young and to our readers.
Simple Strategies for the Isolation of Drug Impurities by Preparative Chromatography

Yung-Fong Cheng, M. Zoubair El Fallah and Uwe D. Neue

Introduction

The current guidelines of the Food and Drug Administration (FDA) and the International Conference for Harmonisation (ICH) scrutinise the analytical methods used in the pharmaceutical industry. The guidelines require the identification and qualification of all impurities and degradation products present at concentrations down to 0.1% of the parent drug. A certain amount of these impurities and degradants is needed for their initial characterisation and identification.

This paper reports on a simple strategy for the isolation of impurities from the main component. First, the separation is developed on an analytical column. The method is then directly transferred to a preparative column without sacrificing resolution and separation efficiency. We also demonstrate a simple fraction collection method. The importance of the properties of the column for achieving good loading capacity, peak shape and resolution is stressed.

Theory

The adsorption of an analyte is often described by a Langmuir isotherm as shown in the following equation:

\[
\frac{q}{q_s} = \frac{b \cdot c}{1 + b \cdot c}
\]  
(Eq. 1)

where \( c \) is the solute concentration in the mobile phase;
\( q \) is the solute concentration in the stationary phase;
\( q_s \) is the maximum concentration of the analyte in the stationary phase at saturation;
\( b \) is a constant.

With the derivative of the above isotherm, equation 1, and rearrangement of the equation, one can obtain the retention factor \( k \) as a function of the concentration in the mobile phase as follows:

\[
\frac{k}{k_0} = \frac{1}{(1 + b \cdot c)}
\]  
(Eq. 2)

where \( k_0 \) is the retention factor obtained when a very low mass of analyte is injected onto the column. From this equation, we can see how the retention factor decreases with an increase of the concentration \( c \) of the sample in the mobile phase. What we would like to obtain is the relationship between the concentration at the peak maximum \( c_{\text{max}} \) and the amount of sample injected, \( m \). This can be simply achieved by integrating over the peak. The result is as follows:

\[
m = V_s \cdot q_s \cdot \frac{b^2 \cdot c_{\text{max}}^2}{1 + b \cdot c_{\text{max}}} \]  
(Eq. 3)

A convenient parameter of preparative chromatography is the loading factor. It is defined as the amount of analyte injected onto the column, \( m \), divided by the mass that could be loaded onto the column to saturate the stationary surface, \( m_s \). With the combination of equation 3 and the definition of the loading factor, the loading factor can be expressed as follows:

\[
L_i = \frac{m}{m_s} = \frac{V_s \cdot q_s}{V_s \cdot q_s} \cdot \frac{b^2 \cdot c_{\text{max}}^2}{1 + b \cdot c_{\text{max}}} \]  
(Eq. 4)

where \( V_s \) is the volume of stationary phase in the column.

Substituting equation 3 into equation 4, the loading factor can be expressed as a function of the retention factor at the peak maximum, \( k_{\text{max}} \), and the end of the peak, \( k_0 \), at low sample mass:

\[
L_i = \left( 1 - \frac{k_{\text{max}}}{k_0} \right)^2
\]  
(Eq. 5)

If a given mass injected onto a column results in a large loading factor, the column is more overloaded than a column that gives a small loading factor. A large loading factor at a given load means low loadability, while a small loading factor means high loadability. High sample loadability generates better peak shape and higher resolution than low loadability. Thus, one can judge the capacity of a packing for the analyte unambiguously from a single injection under overload conditions.

Experimental Section

Equipment

The HPLC system for both analytical and preparative chromatography consisted of the Waters® 616 LC System, a 717plus Autosampler and a 996 Photodiode Array Detector. The Millennium® 2010 Chromatography Manager was used for system control and data acquisition. For preparative chromatography, a special sample loop (2000 µL) was installed into the autosampler and the standard 250 µL syringe was replaced with a 2500 µL syringe. A Foxys® Fraction Collector from ISCO was used for fraction collection.

Comparison of Different Packings

Aliquots of 14 µL of a 5 mg/mL tamoxifen solution were injected onto two different columns with the same column dimension of 4.6 mm x 150 mm: a SymmetryPrep™ C18, 7 µm and a Kromasil® C18, 7 µm. The mobile phase was 44% acetonitrile/56% 50 mM phosphate buffer (pH 3.0) and 40% acetonitrile/60% 50 mM phosphate buffer (pH 3.0) for the Kromasil® column and for the SymmetryPrep™ column, respectively. The effluent was monitored at 254 nm.

Study of the Loading Capacity

Samples of buspirone were prepared at two different concentrations: 1.2 mg/mL and 12 mg/mL. Mass loads of 0.3, 0.6, 1.2 and 2.4 mg
were achieved by injecting the 1.2 mg/mL sample at injection volumes of 0.25, 0.5, 1.0 and 2.0 mL, respectively. Mass loads of 4.8, 9.6 and 19.2 mg were generated by injecting 0.4, 0.8, and 1.6 mL of the 12 mg/mL sample. Separations were performed using a SymmetryPrep™ C18, 7 µm column (3.9 mm x 150 mm). The mobile phase was 28% acetonitrile/72% 0.18% triethylenetetramine (TETA) acidic acid buffer (pH 7.0) at a flow rate of 1.0 mL/min. The effluent was monitored at 360 nm.

Scale-up Study
In the scale-up study, a Symmetry® C18, 5 µm column (3.9 mm x 150 mm) and two preparative SymmetryPrep™ C18, 7 µm columns were used. A 0.5 mg mass load of diltiazem was injected onto the scale-up column at a flow rate of 0.7 mL/min. Mass loads of 2.0 mg and 11.9 mg were injected onto the 7.8 mm x 150 mm and the 19 mm x 150 mm columns, respectively. The flow rates were 2.8 mL/min and 16.6 mL/min for both columns, respectively. The mobile phase was 30% acetonitrile/70% 0.1% aqueous TFA solution.

Fraction Collection
Valerophenone concentrations of 7.7 mg/mL and 14.7 mg/mL were prepared in 50% acetonitrile/50% water and in 60% acetonitrile/40% water, respectively. A 3.9 mm x 150 mm Symmetry C18, 5 µm column was used for the scaling study, while a SymmetryPrep™ C18, 7 µm column with a dimension of 7.8 mm x 300 mm was used for the preparative separation. Detection wavelength was 340 nm.

For the scaling study, volumes of 50, 100 and 250 µL were injected onto a Symmetry C18, 5 µm column (3.9 mm x 150 mm). The mobile phase was 60% acetonitrile/40% water at a flow rate of 0.7 mL/min.

For the preparative separations, 2000 µL of 14.7 mg/mL of valerophenone was injected onto a SymmetryPrep™ C18, 7 µm column with the same mobile phase at a flow rate of 3.6 mL/min. Fractions of the column effluent were collected every 0.2 min starting 6.2 min after the injection. The fractions were subsequently analysed using the analytical column. The injection volume was 100 µL.

Results and Discussion

Effect of the Loading Capacity on the Separation of Impurities
For the separation of impurities and degradation products, a column with a high separation efficiency is required. In addition, for the isolation of these products, it is necessary to maintain this high separation efficiency at high sample load.

To determine the loadability of a column for a particular sample, the loading factor can be measured at a given amount of sample injected. Large loading factors correspond to a large column overload and accordingly a small loading capacity of the column. To compare the loadability of SymmetryPrep™ and Kromasil® C18 packings, 14 µL of 5 mg/mL solution of tamoxifen was injected onto 4.6 mm x 150 mm columns prepared from each packing. The results are shown in Figure 1. The loading factor was calculated to be 0.013 for the Kromasil column, and 0.008 for the SymmetryPrep™ column. Therefore, the loadability of SymmetryPrep™ is approximately 60% higher for this sample and method. The Kromasil packing exhibits a higher silanol activity than the SymmetryPrep™ packing, which can be seen from the need to add 4 % more organic modifier for the Kromasil packing to obtain the same retention on both columns. The higher silanol interaction on the Kromasil column is most likely the reason for its lower loadability.

Effect of Increasing Load on the Separation of Impurities
How much of an impurity can be isolated in a single preparative run depends on the relative retention between the compound of interest and the parent compound, its position in the chromatogram and the concentration ratio. We wanted to study the effect of sample load on the separation of impurities in the presence of a large excess of the parent compound.

Figure 1. Comparison of Loading Capacities on SymmetryPrep™ and Kromasil® Columns.

<table>
<thead>
<tr>
<th>Column</th>
<th>Mobile Phase</th>
<th>Flow Rate</th>
<th>Sample</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>a: Kromasil® C18 7 µm (4.6 x 150 mm)</td>
<td>a: 44% acetonitrile / 56% 50mM potassium phosphate buffer, pH 3.0</td>
<td>1.0 mL/min</td>
<td>14 µL of 5 mg/mL tamoxifen solution</td>
<td>UV at 254 nm</td>
</tr>
<tr>
<td>b: SymmetryPrep™ C18 7 µm (4.6 x 150 mm)</td>
<td>b: 40% acetonitrile / 60% 50mM potassium phosphate buffer, pH 3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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For this purpose, an increasing amount of buspirone was injected onto a SymmetryPrep™ C18 column under the experimental conditions described above. The results are shown in Figure 2. The three major buspirone impurity peaks are marked with arrows and the numbers 1, 2 and 3. As the amount of buspirone injected was increased to 2.4 mg, the buspirone peak became increasingly broader and its retention time decreased steadily (Figure 2a). However, the increased load did not impact the shape or the position of the impurity peaks.

As the load was further increased (Figure 2b) to 4.8 mg, the first impurity peak was still well resolved, but the second impurity peak became broader and coeluted with the main peak. At a load above 9.6 mg, the parent peak merged with the impurity peaks eluting early in the chromatogram, and a separation was no longer visible. On the other hand, the peak shape of the third impurity, which eluted after the main compound, barely changed, even at a load of 19.2 mg of the parent compound. This demonstrated that the effect of increasing load on the separation of impurities depends strongly on the position of the peak in the chromatogram.

For preparative chromatography, it is most desirable that the minor components of the sample elute after the main component. For the preparation of impurities eluting after the parent peak, 10 to 100-fold higher loads can be applied compared to the situation when the impurities elute before the parent compound.

The highest load that we could apply and still maintain analytical-grade resolution was 2.4 mg or approximately 1 mg per mL of column volume. This is typical for a high-quality preparative column.

For more information
SymmetryPrep 3.9 x 150 mm and 4.6 x 150 mm columns are not available as standard product. For ordering information, please call 1-800-252-4752.
Scaling up from a Small Column to a Preparative Column

The best path from an analytical separation to a preparative separation contains three steps:

1. Optimisation of the analytical separation
2. Optimisation of sample load using the analytical column
3. Scaling the separation to a preparative column

The optimisation of sample load has been discussed in the previous section. The scaling to a preparative column is straightforward, provided that a preparative column with the same high-resolution packing as used in the scaling column is available. This is the case for laboratory-scale preparative chromatography with SymmetryPrep™ columns.

The scaling of all parameters of the separation should always be done in proportion to the column volume. Conversely, the most appropriate column for the preparative separation is selected based on the amount of sample to be purified and the loadability information obtained from the loading studies. Once we have decided on a suitable preparative column, the column load or injection volume and the flow rate are all scaled from the small column to the preparative column in proportion to the column volume. If the separation is a gradient separation, gradient volumes are also scaled in proportion to the volume ratio of the preparative column to the analytical column. If we do this, identical separations are obtained.

Figure 3 shows the scaling of the isolation of diltiazem impurities from an analytical Symmetry™ C_{18} 5 µm column to a preparative SymmetryPrep™ C_{18} 7 µm column. Figure 3a shows the injection of 0.5 mg of diltiazem at 0.7 mL/min on a 3.9 x 150 mm analytical column. The sample load and flow rate was scaled fourfold to 2.0 mg of analyte at 2.8 mL/min on a 7.8 x 150 mm SymmetryPrep™ column (Figure 3b). A 25-fold scaleup was achieved using a SymmetryPrep™ 7 µm 19 x 150 mm column (Figure 3c). 11.9 mg of diltiazem was injected at a flow rate of 16.6 mL/min.

The peak shape, retention times and resolution obtained on the scale-up column and on the preparative columns were indistinguishable.

Simple Strategy for Increasing the Throughput in Preparative Chromatography

As we have seen in the loading studies, an increase in sample load results in the apparent loss of resolution between the major compound and the impurities eluting before the major compound. However, the high load of the major compound results in a displacement of the early eluting impurities: they migrate as sharp bands in front of the major peak. This effect can be used with advantage in an attempt to purify the early eluting compounds.

The strategy uses two separate preparative runs. In the first preparative separation, a significant overload of the major compound is applied to the point that it completely covers the early part of the chromatogram. An example is the last separation shown in Figure 2b.

Fractions of this separation are collected and analysed using analytical chromatography. Some of the fractions will contain pure or highly purified impurity compounds. Even the last fraction, containing the major compound and the impurity peaks eluting closest to the major compound, contains the impurity with a significant enrichment compared to the original sample. The enrichment can be up to 2 orders of magnitude.
A further purification of this sample can then be obtained in a second preparative separation, where the load of the parent compound is significantly reduced.

We used the separation of valerophenone and its impurities to demonstrate the displacement effect. The separation was first optimised on an analytical scale. Then the load was optimised and the method was transferred to a lab-scale preparative column.

Two different sample concentrations were prepared: a concentration of 7.7 mg/mL in 50% acetonitrile/50% water and a concentration of 14.7 mg/mL in 60% acetonitrile/40% water. Figures 4a to 4c show the chromatograms obtained with increasing load using the first sample, and Figures d to f using the second sample. The results are generally similar to the results obtained with buspirone. A further increase in sample load was prohibited by the limited solubility of the sample in the mobile phase.

The separation was then scaled directly to a Symmetry® C18, 7 µm, 7.8 mm x 300 mm column (Figure 5). As expected, the chromatograms obtained with the analytical column (Figure 4f) and with the preparative column (Figure 5) were indistinguishable.

The fractions of the preparative separation were collected. A total of 11 fractions were obtained in intervals of 0.2 minutes starting from approximately 6.2 minutes after injection. These fractions were then analysed with the analytical column, and the relevant chromatograms are shown in Figure 5. The third fraction showed mainly the impurity eluting just before the main component. The fourth fraction contained the same impurity peak together with the main peak. As expected, the peak area ratio of the impurity peak to the parent peak was much larger in this fraction than in the original sample. The next fractions (6 to 11) contained the pure parent peak.

Figure 4. Loading study for valerophenone on a Symmetry® 5 µm column.

Column: Symmetry® C18, 5 µm
Mobile Phase: 60% ACN / 40% water
Flow Rate: 0.7 mL/min
Sample: 7.7 mg/mL valerophenone in 50% ACN / 50% water
Detection: 340 nm
**Conclusion**

In summary, the SymmetryPrep™ column has an excellent loading capacity allowing good peak shape and resolution to be maintained even at high sample loads. This property of the packing makes it ideally suitable for the isolation of impurities and degradants, which are present in significantly lower concentration than the main compound. Moreover, due to the fact that the chemistry of the SymmetryPrep™ column is identical to the chemistry of the analytical Symmetry® column, and because of the high reproducibility of this packing, scaling of the separation from analytical to the lab-scale is straightforward. In addition, a simple fractionation method can drastically increase the throughput of the isolation of minor impurities eluting before the main compound at high sample load.

For your free copy of the Symmetry® Applications Notebook, please check box 8 on the Business Reply Card.
### Ordering Information

#### Symmetry® 3.5 µm Columns

<table>
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<tr>
<th>Dimension</th>
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<th>Part No.</th>
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#### Symmetry® 3.5 µm Cartridge Columns*

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#### SymmetryShield™ RP® 5 µm Columns

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#### SymmetryShield™ RP® 5 µm Cartridge Columns*

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#### SymmetryPrep™ 7µm Columns

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<td>WAT066225</td>
<td>WAT066228</td>
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<td>19 mm x 150 mm</td>
<td>WAT066245</td>
<td>WAT066240</td>
<td>WAT066230</td>
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### Description

- **Endfittings**: WAT037525
- **Coupling**: WAT044390
- **Integrated Guard Holder** (for Waters steel cartridge columns only): WAT046905
- **Universal Guard Holder** (for any HPLC column): WAT046910

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** Require use of coupling with endfittings

*** Guard Columns require the appropriate Sentry™ Guard Holder

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Antiarrhythmic: Propranolol

Solid Phase Extraction Procedure
Oasis™ HLB 1 cc Extraction Cartridge

- Condition: 1 mL methanol/1 mL water
- Load: 1 mL spiked porcine serum
- Wash: 1 mL 5% methanol in water
- Elute: 1 mL methanol with 5 µg butyl paraben
- Evaporate and Reconstitute: 40 °C under nitrogen stream 200 µL methanol

Chromatogram of Serum Extracts: A) Blank B) Spiked Sample

System:
A Waters 717plus Autosampler and a 600E Multi Solvent Delivery System. Detection was by UV using a Waters 486 Tunable Absorbance Detector. Data Acquisition was performed using a Waters 860 Chromatography Data System. A 20 position vacuum manifold with a vacuum pump was used to process solid phase extraction cartridges.

SPE Spiked Serum Results

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration µg/mL</th>
<th>% Recovery</th>
<th>% RSD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>0.800</td>
<td>85.9%</td>
<td>2.1%</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>96.4%</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

For more information on Oasis™ HLB extraction cartridges, please check box 11 on the Business Reply Card.
**Waters 717plus Autosampler**

The Waters 717plus Autosampler combines reliability, versatility and low cost of ownership into a fully automated, all-electric, easy-to-use instrument that will meet and exceed your requirements. The 717plus Autosampler incorporates a highly reliable and extremely precise fluid path design that is versatile enough to use for all your methods and applications. Advanced automation capabilities make it easy to develop methods using automated derivatisation techniques or to automatically add reference standards or diluents. The optional Heating/Cooling module allows you to work with a wide range of samples, from heat labile biological samples to viscous polymer samples, with exceptional precision and reliability.

**The Waters 600E Multisolvent Delivery System**

A no-compromise approach to single pump gradient systems

Waters 600E Multisolvent Delivery System offers no-compromise high-performance in a true single pump design. By combining two Waters patented technologies, time-proven solvent delivery and innovative Random Phase Synchronization (RPS™) software, we can offer you unmatched performance compared with any other single pump gradient system.

- An exceptionally wide total flow range from 0.01 to 20.00 mL/min (with 45.0 mL/min optional) gives you the versatility to perform analytical and semi-preparative separations.
- You can centralise operations through single-keyboard system programming with either the new Waters 600E controller or with an integral, on line PC.
- For comprehensive programming of all pump, autosampler, and detector parameters directly from the controller keypad, the PowerLine™ software embedded in the 600 controller allows you to store up to 15 completed instrument methods for easy method retrieval.
- By linking your 600E to the Millennium® 2010 Chromatography Manager, you can take advantage of complete system control and information management.

**The Waters 860 Networking Computer System**

The Waters 860 is designed for large, multi-user, multisystem data network. Information processed and stored on the 860 can be shared among every chromatographer in your lab, while resources such as printers, plotters and storage facilities, can be easily shared and added. And the 860 offers unlimited expansion capabilities with the ease of single keyboard system control.

**The Waters 486 Tunable UV/VIS Absorbance Detector**

The performance you expect with the flexibility you need.

The Waters 486 Tunable UV/VIS Absorbance Detector is the highest performing UV/VIS detector with the flexibility to accommodate virtually all your HPLC applications. Our exclusive Taper-Cell® flow cell design eliminates refractive index effects, providing exceptional baseline stability.

- The 486 detector is tunable from 190 to 600 nm wavelength range with reproducibility and precision.
- The unique modular cell assemblies for microbore, analytical, preparative, high-pressure mass spectrometry, and nonmetallic flow paths give your application flexibility.
Keratolytic: Salicylic Acid and Related Compounds

Solid Phase Extraction Procedure

Oasis™ HLB 1 cc Extraction Cartridge

1. **Condition**
   - 1 mL methanol/1 mL water

2. **Load**
   - 1 mL spiked porcine serum, acidified with 20 µL phosphoric acid

3. **Wash**
   - 1 mL 5% methanol in water

4. **Elute**
   - 1 mL methanol

5. **Evaporate and Reconstitute**
   - 40°C under nitrogen stream

   - 1 mL 5 µg/mL nalidixic acid in mobile phase

**Chromatogram of Serum Extracts: A) Blank B) Spiked Sample**

**Peaks:**
1. Benzoic acid
2. Nalidixic acid (I.S.)
3. Salicylic acid

**System:**
A Waters 717 plus Autosampler and a 625 LC System. Detection was by UV using a Waters 996 Photodiode Array Detector. Data Acquisition was performed using a Waters Millennium® 2010 Chromatography Manager. A 20 position vacuum manifold with a vacuum pump was used to process solid phase extraction.

**SPE Spiked Serum Results**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration µg/mL</th>
<th>% Recovery</th>
<th>% RSD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoic Acid</td>
<td>0.100</td>
<td>84.8%</td>
<td>3.8%</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>87.1%</td>
<td>3.0%</td>
</tr>
<tr>
<td>Salicylic Acid</td>
<td>0.100</td>
<td>92.3%</td>
<td>5.0%</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>91.3%</td>
<td>3.3%</td>
</tr>
</tbody>
</table>

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**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 systems.
- 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.
- Absorbancy 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 absorbance maxima, and plots a peak for each compound at its absorbance maximum, creating a composite chromatogram of all of the compounds it has detected.

**Waters 625 LC System**

The Waters 625 LC System combines advanced polymeric technology and low dispersion system volume into a single liquid chromatograph providing the highest performance available in a non-metallic system. This instrument is designed to handle a wide variety of life science applications such as high-resolution protein purification, microbore peptide mapping, nucleic acid isolations, and purification and analysis of oligosaccharides. These applications require an array of different column chemistries. The Waters 625 LC System is optimised for ion-exchange, gel filtration, affinity, hydrophobic interaction and reversed-phase column chemistries used in microbore, analytical or micropreparative methods relevant to the biochemist. The Waters 625 LC System is designed to be easy to operate, and to maintain, for reliable daily operation.