Fast and Easy Solid-Phase Extraction with a New Reversed-Phase Sorbent

Dorothy J. Phillips, Laura L. Bean, Edouard S.P. Bouvier, Mark Capparella, Yung-Fong Cheng, Pamela C. Iraneta and Uwe D. Neue

The Challenge

Over the past five years, pharmaceutical companies have made a fundamental change in the way they conduct their drug discovery programs. Their preferred discipline now is combinatorial chemistry. Combinatorial chemistry integrates such tools as novel synthesis, reaction equipment and advanced information systems to accelerate the generation and optimisation of pharmaceutical candidates (1). The ultimate vision for a combinatorial or high throughput chemistry laboratory is the synthesis of millions of individual, pure compounds each day (1). The implementation of combinatorial chemistry in the discovery programs has resulted in an increase in the number of drug candidates in the preclinical phase. Drug metabolism groups now have many more candidates to screen and need to work even faster to shorten the drug discovery cycle. Hence, there is a critical need for more efficient methods to extract and analyze samples from biological fluids. The analytical technique that is stepping up to the challenge is LC/MS/MS which can analyze samples at a rate of one sample every 2–5 minutes. Sample preparation, required for the analysis of most drugs in biological fluids, is now the bottleneck and needs to be automated. But liquid-liquid extraction and protein precipitation methods do not lend themselves to automation. The method of choice is solid-phase extraction (SPE). Using robotics and miniaturisation has enabled GlaxoWellcome to process 50,000 samples a day (2). Many other pharmaceutical companies are struggling to increase sample throughput with vacuum manifolds and robotic sample processors.

Waters Oasis™ HLB extraction cartridge — the most important advance in solid-phase extraction in 20 years.
Traditional Problems

Development and implementation of solid-phase extraction methods can be tedious. It is a challenge to devise methods that deliver high recoveries reproducibly. For at least three reasons, traditional SPE products, whether used with manual vacuum manifolds or robotic systems, often do not achieve this goal.

First, the silica-based reversed-phase sorbents in these traditional SPE products weakly retain the more polar drugs and metabolites. Such weak retention causes sample breakthrough yielding unpredictable low recoveries.

Second, optimizing a method for reproducible recoveries can be time-consuming because traditional sorbents contain a heterogeneous population of surface silanol groups which have a strong and variable retentive effect on basic analytes.

Finally, perhaps the major contributor to irreproducibly low recoveries is the inability of the traditional silica- and polymer-based reversed-phase sorbents to be wetted with water. If these sorbents are allowed to run dry at any time before the sample loading step in an SPE protocol, only a fraction of the sorbent's available surface will be wetted by an aqueous sample. Adsorption capacity for drugs and metabolites will thereby be reduced, resulting in low, variable recoveries (3,4).

A Unique Solution

Waters new Oasis™ HLB extraction cartridges overcome these limitations of traditional silica- and polymer-based reversed-phase SPE products (3,4). Oasis™ HLB extraction cartridges contain a novel polymeric sorbent (patent pending) that makes it fast and easy to develop reproducible extraction methods yielding high recoveries. The simple reversed-phase extraction mechanism of this unique new sorbent offers stronger, more predictable retention of polar drugs and metabolites, including basic compounds.

But most important, Oasis™ HLB extraction cartridges eliminate the major cause of variability in solid-phase extraction. Unlike traditional hydrophobic reversed-phase sorbents, Oasis™ HLB sorbent is fully wettable with water. This means there is no longer a need to monitor vacuum levels, program robotic adjustments, or twist stopcocks to keep sorbent beds from drying out during the critical cartridge conditioning, equilibration, and sample loading steps.

Oasis™ HLB sorbent is a macroporous copolymer (poly(divinylbenzene-co-N-vinylpyrrolidone) composed of both lipophilic and hydrophilic monomer units. This hydrophilic-lipophilic balance gives Oasis™ HLB sorbent its unique reversed-phase extraction performance advantages (and its name):

- High and reproducible recoveries for acidic, basic and neutral drugs with a wide range of polarities.
- Simple and rapid methods development for drugs and metabolites.
- Easily automated methods for high sample throughput.
- Streamlined manual or automated analyses since the sorbent can run dry during the process without affecting the reproducibility of results.

Bouvier et al. discussed the theory and advantages of the Oasis™ HLB sorbent’s water wettability in earlier papers (3, 4). In this paper, we report the extraction of drugs from serum using Oasis™ HLB extraction cartridges. A simple SPE method gave reproducibly high recoveries for acidic and basic drugs of varying polarity.

Experimental

Reagents and Materials

Salicylic acid, benzoic acid, propranolol, dipropyl phthalate and butylparaben were purchased from Aldrich Chemical Company (Milwaukee, WI). Procainamide, ranitidine, acetaminophen, sultanilamide, propranolol, dexamethasone, phenacetin, phenylbutazone, tetracycline and demeclocycline were obtained from Sigma Chemical Company (St. Louis, MO). Acetonitrile, methanol, potassium phosphate, sodium phosphate, sodium chloride, glacial acetic acid and phosphoric acid were obtained from J.T. Baker (Phillipsburg, NJ). Porcine serum was obtained from Equitech-Bio (Ingram, TX). Oasis™ HLB extraction cartridges, 1 cc/30 mg were obtained from Waters Corporation (Milford, MA). Bond Elut® C18, 1 cc/100 mg solid-phase extraction (SPE) cartridges were purchased from Varian Sample Preparation Products (Harbor City, CA).

Solid-Phase Extraction of Drugs in Serum

Sample pretreatment for the tetracyclines, salicylic acid and benzoic acid involved adding 20 µL of phosphoric acid to 1 mL of spiked porcine serum before sample loading. Figure 1 outlines the general solid-phase extraction procedure developed on the Oasis™ HLB extraction cartridges (3). The cartridges were placed on a 20-position Waters Extraction Manifold attached to a Waters vacuum pump. The cartridges were conditioned with 1 mL methanol and equilibrated with 1 mL water; the specific flow rate was not important for these steps. Then a 1 mL serum sample containing the drug(s) was added at a flow rate of < 2 mL/min (vacuum level ~ 3-4” Hg). The cartridges were then washed with 5% methanol in water to remove polar interferences. The analyte was eluted at a flow rate of < 2 mL/min with methanol.

Figure 1: General SPE method developed for analysis of drugs in biofluids using Oasis™ HLB extraction cartridges.
Finally, the methanol eluate was evaporated to dryness at 40 °C under a nitrogen stream, and the sample was reconstituted in mobile phase at the desired concentration. Flow through the Oasis™ HLB extraction cartridges did not have to be monitored because the cartridges can run dry without loss of recovery or reproducibility.

In each study, a pair of porcine serum samples were prepared, each containing a different drug level, and then each sample was extracted in replicates of six, using an appropriate internal standard as indicated. For the tetracyclines study, the first serum sample contained tetracycline and minocycline each at 2.5 µg/mL; in the second sample, the concentrations of each were 0.5 µg/mL. Demeclocycline was added to both serum samples as an internal standard at 5.0 µg/mL. Similarly, for the acids experiments, salicylic and benzoic acids were added to a pair of porcine serum samples at respective drug levels of 1 µg/mL and 5 µg/mL. In this case, the internal standard, malonic acid at an effective level of 5 µg/mL, was added to the analyte-containing fraction from each extracted sample after its evaporation and reconstitution in mobile phase.

The same protocol above was repeated using the Bond Elut® C18 cartridges, with only one critical difference: with these silica-based C18 cartridges, the entire extraction of the tetracyclines is achieved with the simple general SPE method that was developed for the Oasis™ HLB extraction cartridges is outlined in Figure 1 (3). Due to the simplicity of the interaction of analytes with the Oasis™ HLB sorbent, it was possible to develop a general SPE method for extraction of many kinds of drugs and their metabolites from serum and plasma. This SPE method is applicable to a wide range of analytes; it can be modified or fine tuned to remove specific interferents or to extract compounds at the extremes of polarity (very hydrophilic or hydrophobic drugs).

Tetracyclines
One of the causes for low and variable recovery on the traditional silica-based reversed-phase cartridges is the strong retention of basic compounds by ionic interaction with deprotonated silanols. In addition, metals present in the silica packings can also retain drugs, such as tetracyclines, which are metal chelators. Higher and more consistent recoveries of such compounds would be expected from the Oasis™ HLB extraction cartridges which do not contain either silanols or metals. Indeed, when the amphoteric tetracyclines were extracted from serum using Oasis™ HLB extraction cartridges and the simple general SPE method, recovery and reproducibility were found to be significantly better than the results obtained when C18-silica SPE cartridges were used (see Table 1) (5).

The structures of the tetracyclines used in this study are shown in Figure 2. Under typical SPE conditions, tetracyclines are amphoteric and can chelate with metals. The results in Table 2 show that optimum extraction of the tetracyclines is achieved if the spiked serum is pretreated with acid before loading on the cartridges.
The tetracyclines are known to bind to serum proteins; the literature reports up to 75% retention of tetracyclines by serum proteins (6, 7). The three acids, 2% phosphoric acid, 1% TFA and 63 mM citric acid, were equally effective and resulted in essentially 100% recovery of minocycline and tetracycline. All three acids were able to disrupt the drug-protein interaction; phosphoric acid was selected for reasons of safety and convenience.

The chromatograms in Figure 3 of the blank serum (A) and the extracted drug-spiked sample (B) show that no endogenous peaks interfere with the HPLC analysis of the drugs or the internal standard. Choosing an absorption maximum wavelength of 270 or 350 nm allows detection of the tetracyclines where few endogenous compounds absorb. The excellent peak shapes on the SymmetryShield™ RP8 column also facilitated quantitation with high accuracy and precision. Extractions using the Oasis™ HLB extraction cartridges with our general method gave less than 1.5% RSDs and greater than 95% recoveries for two levels of minocycline and tetracycline; the data for both the 2.5 and 0.5 µg/mL levels are in Table 1.

With the method outlined in Figure 1, results obtained using Oasis™ HLB extraction cartridges were compared to those using the silica-based Bond Elut® C18 cartridges. Table 1 lists 40.7% and 67.4% recoveries from the Bond Elut® C18 cartridges versus 94.8% and 104% recoveries from the Oasis™ HLB extraction cartridges for minocycline and tetracycline, respectively. The reason(s) for the inferior recoveries obtained by SPE with the C18 cartridges are not clearly understood. Interactions of tetracyclines with the surface of silica-based reversed-phase sorbents are typically manifested in chromatograms by tailing and distorted peaks. In the literature, this behavior has been attributed to silanol interaction and/or complexing with metal contaminants. These complications clearly do not occur with the unique polymer sorbent in Oasis™ HLB extraction cartridges.

Table 1: Comparison of SPE results for tetracyclines.

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<td>Recovery (%)</td>
<td>RSD (%)</td>
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<td>Concentration (µg/mL)</td>
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<td>RSD (%)</td>
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<tr>
<td>0.5</td>
<td>97.8</td>
<td>1.4</td>
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Table 2: Recovery of tetracyclines as a function of the type of acid used for sample pretreatment. The SPE method using Oasis™ HLB extraction cartridges is described in the text.

<table>
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<tr>
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<th>2% Phosphoric Acid</th>
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<td>Tetracycline</td>
<td>97.2%</td>
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Figure 2: Structures of drugs and internal standards.
Polar Acidic Drugs

The Oasis™ HLB extraction cartridges retain drugs and their metabolites by the same reversed-phase mechanism as do the traditional silica-based reversed-phase cartridges. Acidic and neutral drugs do not interact secondarily with the silanols on the silica-based sorbents. Therefore, these compounds should interact with the Bond Elut® C18 and the Oasis™ HLB sorbents in a similar manner. Both cartridge types should show the same high recoveries. The only proviso is the polarity of the drug since the more polar drugs may be poorly retained on the C18 cartridges.

Salicylic and benzoic acids are polar compounds with pK_a's close to 3.0; the structures are shown in Figure 2. They were extracted by Oasis™ HLB extraction cartridges with the same SPE method employed for the amphoteric tetracyclines. Table 3 illustrates the importance of extracting the polar acids in protonated form. When salicylic acid was extracted from spiked saline at pH 7.0 only 62.5% recovery was obtained. In contrast, extraction of these acidic compounds in the protonated form at pH < 2 gave 100% recovery. A protonated acid is less polar than its corresponding ionic form and is therefore better retained on a reversed-phase SPE sorbent.

Excellent peak shapes were obtained for both salicylic and benzoic acid on the Symmetry-Shield™ RP8 column which enabled accurate quantitation of these compounds in serum or saline after solid-phase extraction. The chromatography is shown in Figure 4 for both the extracted acids and the serum blank. Table 4 shows the recoveries obtained when salicylic acid and benzoic acid at both 5 µg and 1 µg per mL levels were extracted from serum using Oasis™ HLB extraction cartridges. Recoveries greater than 85% percent and RSDs less than 5% were obtained.
Specifications and sorbent batch test results are reported on a Certificate of Analysis which is included in each box of product. As a result of this stringent quality control, excellent batch-to-batch reproducibility has been observed for the Oasis™ HLB sorbent. Figure 5 shows the high recovery obtained for seven compounds, ranging from the very polar neutral acetaminophen to the more lipophilic dipropyl phthalate and betamethasone valerate and including basic drugs such as procainamide, propranolol, doxepin and ranitidine.

Regardless of the nature of the drug, greater than 90% recoveries were observed. More important is the fact that the recoveries for all drugs were highly consistent from batch-to-batch. RSDs ≤ 3.67% were obtained across three different batches of Oasis™ HLB sorbent. These data along with the low % RSDs for replicates of six cartridges per analysis (Tables 2 and 4) illustrate the high reproducibility from batch-to-batch and cartridge-to-cartridge, respectively, for the Oasis™ HLB extraction cartridges.

Table 4: Recoveries of salicylic and benzoic acids extracted from serum using Oasis™ HLB extraction cartridges. The SPE method is described in the text.

<table>
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<tr>
<th>Compound</th>
<th>Concentration (µg/mL)</th>
<th>(%) Recovery</th>
<th>(%) RSD (n=6)</th>
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<td>Salicylic acid</td>
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<td>1.0</td>
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<td>Benzoic acid</td>
<td>5.0</td>
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<td>1.0</td>
<td>85</td>
<td>3.8</td>
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Figure 5: Batch-to-Batch Reproducibility of Oasis™ HLB Sorbent in 1 cc/30 mg Cartridges

Reproducibility
A high level of reproducibility is built into the synthetic design and the manufacturing process for the Oasis™ HLB sorbent. Each sorbent batch is quality controlled by eleven different tests. Physical property specifications include pore size, particle size, surface area, total pore volume and fines content (% of particle volume with particles less than 10 micron in size). Recoveries and relative standard deviation (n=6) are reported for the solid-phase extraction of three polar drugs—procainamide, ranitidine and acetaminophen. A sorbent selectivity test ensures that each batch has identical retention characteristics. This selectivity test involves packing the sorbent in an HPLC column to measure relative retentions and a capacity factor for a mixture of acidic (salicylic acid), basic (ranitidine) and neutral (caffeine, p-toluamide) compounds.

When the acids were extracted from acidified serum, using Bond Elut® C_{18} cartridges and the procedure detailed in Figure 1, being careful not to let the cartridges run dry, the results were similar to those obtained with the Oasis™ HLB extraction cartridges. Greater than 90% recoveries were obtained for salicylic acid at 1 µg/mL on both types of cartridges. Because acidic compounds interact with the SPE sorbents used here principally by a reversed-phase (hydrophobic interaction) mechanism, no differences were expected or observed between the results obtained using traditional bonded-silica C_{18} cartridges and those using Oasis™ HLB extraction cartridges. Many methods that have been developed on silica-based reversed-phase cartridges can be transferred directly to Oasis™ HLB extraction cartridges. The key advantage in doing this is that flow through Oasis™ HLB extraction cartridges does not have to be monitored. Cartridge drying as described above will not lead to low and/or variable recoveries.

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Conclusions

Oasis™ HLB extraction cartridges are a universal replacement for, and provide a solution to the problems associated with, traditional silica-based reversed-phase SPE cartridges. The absence of silanol interaction and the excellent retention of polar drugs on the Oasis™ HLB extraction cartridges make it possible to develop ideal sample preparation methods without difficulty. Compounds as different as tetracyclines, basic drugs, and polar acids were all extracted with high recoveries using the same simple, rugged and highly reproducible method. High and consistent recoveries were obtained across cartridges and batches of Oasis™ HLB sorbent regardless of an analyte’s polarity or chemical functionality. These new reversed-phase SPE cartridges make sample preparation fast and easy for the analytical chemists who are quantitating drugs and metabolites in biological fluids. Oasis™ HLB extraction cartridges should replace C18 cartridges in existing methods and should be the cartridge of choice for developing all new SPE protocols.

References


For more information on Oasis™ HLB Extraction Cartridges and Extraction Plate, please check box 4 on the business reply card.

Ordering Information

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To achieve the maximum advantage of the Oasis™ HLB extraction cartridge, we recommend the new Waters Extraction manifold (see page 18).

NEW Oasis™ HLB Extraction Plate

The Waters Oasis™ HLB Extraction Plate (96-well) is available with 30 mg of sorbent in each well. Each well contains a unique (patent pending) sorbent, a copolymer designed to have a Hydrophilic-Lipophilic Balance (HLB), that gives high and reproducible recoveries for acidic, basic, and neutral compounds—even if the sorbent in the wells runs dry.

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For your free copy of the Oasis™ HLB Extraction Cartridges Applications Notebook, please check box 3 on the business reply card.
Improving Method Development Using a New Reversed-Phase High-Performance Liquid Chromatography (HPLC) Column with Unique Selectivity

Uwe D. Neue, Dorothy J. Phillips, Michael S. Young, Thomas H. Walter, John E. O’Gara, Mark Capparella and Bonnie A. Alden

Introduction
The process of developing HPLC methods is a critical step in the work of an analytical chemist. Frequently, the investigator encounters a situation where the selectivity obtainable on a particular column fails to meet the separation requirements, even after systematic optimisation of the mobile phase (1,2). When this happens the chromatographer often resorts to use of unusual mobile phase compositions or additional mobile phase additives. In this paper, we demonstrate an alternative approach: when a separation does not respond to simple variations in mobile phase composition, it is often advantageous to change to a column that has significantly different selectivity characteristics.

The SymmetryShield™ RP₈ Packing Material
Waters has recently developed a unique patented reversed-phase packing material (SymmetryShield™ RP₈) possessing selectivity characteristics significantly different from traditional C₈ and C₁₈ materials (3). The modified selectivity arises from the insertion of a carbamate group into the alkylsilane bonded phase (Figure 1). The incorporation of polar groups into alkylsilane bonded phases has been shown to significantly modify their selectivity characteristics, due to perturbation of both analyte/stationary phase and mobile phase/stationary phase interactions (3-7). These polar functional groups compete with polar functional groups in the analyte molecule for the interaction with the surface silanols. Therefore, one particularly notable feature of all reversed-phases containing embedded polar group is the reduction in both retention and peak tailing of amines.

This reduced retention can be attributed to a shielding of the silanols by the embedded polar group.

Discussion
In the process of methods development, the investigator often encounters a situation where the selectivity obtainable on a particular packing does not entirely match the requirements of the application. In such a situation, the chromatographer usually struggles for extended periods of time with unusual mobile phase compositions or additional mobile phase additives. An alternative approach that has proven very valuable in our laboratory is the use of a column chemistry that provides similar retention characteristics but different selectivity compared to a classical reversed-phase column. The SymmetryShield™ RP₈ column provides this alternative. The addition of the carbamate group in the bonded phase significantly changes the interaction between polar functional groups in the analyte molecule and the silanol groups present on all bonded-phase packings. As a consequence, switching from a standard C₈ or C₁₈ column to a SymmetryShield™ RP₈ column can drastically affect the peak spacing and the elution order of analytes without the need to change mobile phase composition. This allows for a rapid comparison between a standard C₈ column and the SymmetryShield™ RP₈ column.

The orientation of the carbamate group should be emphasised. It is oriented such that after a hydrolytic loss of the ligand at the carbamate function, an interactively neutral alcohol function is formed. Although careful hydrolysis studies have not revealed any stability issues, we purposely designed the phase to avoid any inadvertent secondary interactions. This is not the case for other packing materials available on the market, which also rely on polar functional groups to prevent the interaction between analytes and surface silanols (7).

A selectivity study of SymmetryShield™ RP₈ was made using simple substituted benzenes as samples. It was found that, generally, the methylene group selectivity was lower on the SymmetryShield™ RP₈ column than on a standard Symmetry™ C₈ column. At the same time, the selectivity for polar functional groups was significantly different for both phases. There was a reduced retention for very polar functional groups on the SymmetryShield™ RP₈ column, especially for amino-, alcohol- and amide- functions, and an increased retention for phenols and acids at acidic pH. This indicates that the influence of acidic silanols on the retention of analytes is decreased using the SymmetryShield™ RP₈ column. An example of the selectivity differences is shown in Figure 2.
On the SymmetryShield™ RP8 column, we find a significant decrease in retention for benzyl alcohol and benzylamine, and a slight increase in retention for phenol and benzoic acid. It is believed that the reason for these selectivity differences is due to the fact that water strongly binds to the polar carbamate group. This tightly bound layer of water prevents interactions between the analytes and the underlying tether function as well as with the surface silanols of the underlying silica. As a consequence, surface silanols do not play as much of a role in the retention of analytes on the SymmetryShield™ RP8 column as they do on a standard C8 column. Also, good peak shapes are obtained for polar analytes, in addition to the differences in selectivity discussed above. Thus, the name of the column is quite appropriate: the surface silanols are “shielded” from interacting with the polar functional groups of analytes. Such differences in interaction can result in significant shifts in the selectivity of a separation.

As mentioned above, the SymmetryShield™ RP8 column often yields a separation when mobile phase modification on a C8 packing has failed to do so. An example is shown in Figure 3. On the left, the separation of furazolidone impurities is shown on a Symmetry® C8 column. It appears that there is only a single impurity present. However, as shown in the chromatogram on the right, the SymmetryShield™ RP8 column cleanly separates the impurity into two well resolved peaks. In this case, the selectivity difference between a SymmetryShield™ RP8 column and a Symmetry® C8 column is quite striking.

Another example of selectivity differences is shown in Figure 4 for the Alberta peptide standards. A significant reduction in retention is observed for these peptides on the SymmetryShield™ RP8 column, as compared to the standard Symmetry® C8 column.

**Figure 2: Selectivity differences between SymmetryShield™ RP8 and Symmetry® C8 packings**

| Columns: | 3.9 mm x 150 mm SymmetryShield™ RP8, 5 µm and Symmetry® C8, 5 µm |
| Mobile phase: | 80% 50 mM KH2PO4, pH 3.0, 20% acetonitrile |
| Flow rate: | 1 mL/min |
| Detection: | Absorbance at 254 nm |

**Figure 3: Selectivity comparison for furazolidone impurities**

| Column: | a. Symmetry® C8, 5 µm  
| b. SymmetryShield™ RP8, 5 µm 3.9 mm x 150 mm |
| Mobile Phase: | Gradient at 1%/min acetonitrile starting at 10% acetonitrile/ 90% 0.1% acetic acid, pH 3.3 |
| Flow Rate: | 0.7 mL/min |
| Detection: | Absorbance at 370 nm |
| Injection: | 10 µL |
| Sample: | Furazolidone and its impurities |
This reduction in retention is due to a decrease in the interactions of these basic peptides with the SymmetryShield™ RP8 column compared to the Symmetry™ C8 column. This reduced interaction is completely in line with the expectations outlined above. It appears that the SymmetryShield™ RP8 column provides additional separation capabilities for peptide samples where the options for mobile phase manipulation are more limited.

A further example in Figure 5 shows the separation of phenoxyacid herbicides and phenols on a SymmetryShield™ RP8 column. The separation of all 16 EPA 555 analytes is accomplished without difficulties using this column. The same separation is not possible using standard reversed-phase columns. Once again, the advantage of the different selectivity achievable with the SymmetryShield™ RP8 column is well demonstrated for this application.

For separations of compounds with differences in the pK_a significant variations in elution order have been observed between a standard C8 column and the SymmetryShield™ RP8 column. An example is the separation of the weak acid, acetaminophen; the strong base, codeine and caffeine at pH 3 (Figure 6). On the Symmetry® C8 column, codeine elutes after acetaminophen. Using the identical mobile phase conditions on a SymmetryShield™ RP8 column, significantly different retention times were observed. Most importantly, the elution order of acetaminophen and codeine changed. The basic compound codeine exhibits less retention than acetaminophen on the SymmetryShield™ RP8 column, while it elutes after acetaminophen on the Symmetry® C8 column. This is completely in line with the expectations outlined above.

In addition to the selectivity differences demonstrated, SymmetryShield™ RP8 columns also perform better than standard reversed-phase columns in highly aqueous mobile phases.
Figure 6: Gradient separation of acetaminophen, codeine and caffeine

Conclusion

The SymmetryShield™ RP8 column represents a powerful new tool for reversed-phase separations. The selectivity of this column for compounds with polar functional groups can be significantly different from the selectivity of standard reversed-phase columns. This property can be used advantageously in methods development. This is especially true in circumstances where a good separation with standard reversed-phase columns appears to be difficult to achieve. The difference in the selectivity characteristics observed between the SymmetryShield™ RP8 column and standard reversed-phase columns is primarily due to the shielding of the surface silanols by the carbamate group incorporated in the column. Another advantage of this column is its good wettability in highly aqueous mobile phases. The SymmetryShield™ RP8 column significantly enhances the tools available to the methods development chemist and opens a new dimension in reversed-phase selectivity.

References

1. M.Z. El Fallah, Waters Column, Vol. 6 (1) 1995, 1
### Ordering Information

#### SymmetryShield™ RP<sub>8</sub> 5 µm Columns

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#### SymmetryShield™ RP<sub>8</sub> 5 µm Cartridge Columns*

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#### SymmetryShield™ RP<sub>8</sub> 5 µm Method Validation Kits (three columns from 3 different batch materials)

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#### SymmetryShield™ RP<sub>8</sub> 3.5 µm Method Validation Kits (three cartridge columns* from 3 different batch materials)

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#### SymmetryShield™ RP<sub>8</sub> 3.5 µm Method Validation Kits (three columns from 3 different batch materials)

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#### Description

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<td>Integrated Guard Holder (for Waters steel cartridge columns only)</td>
<td>WAT037525</td>
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<tr>
<td>Universal Guard Holder (for any HPLC column)</td>
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* Requires reusable endfittings

** Guard columns require the appropriate Sentry Guard Holder.

[3.9 x 50 mm cartridge columns must use the Universal Guard Holder]

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For complete Symmetry® ordering information, please check box 5 on the business reply card.

For your free copy of the Symmetry® Applications Notebook, please check box 6 on the business reply card.
Evaluation of Imitation \textit{µBondapak™} C\textsubscript{18} Columns

Waters \textit{µBondapak™} C\textsubscript{18} column was introduced in 1973 as the first 10 micron particle size bonded reversed-phase column. Twenty-four years later, it remains one of the most widely used HPLC columns, with applications in many different scientific fields. The selectivity of the \textit{µBondapak™} C\textsubscript{18} column is unique, the result of a carefully controlled combination of hydrophobicity and silanol activity. \textit{µBondapak™} C\textsubscript{18} columns are manufactured only at Waters’ ISO 9002-certified Material Synthesis Facility in Taunton, Massachusetts, using proprietary procedures.

The popularity of Waters \textit{µBondapak™} C\textsubscript{18} columns has inspired many attempts at imitation. To determine how successful these attempts have been, we recently characterised three imitation columns using a sensitive chromatographic selectivity test. The results are described in this article.

\textbf{Experimental}

The columns evaluated were all 3.9 x 300 mm steel columns, except for the 4.6 x 216 mm Imitation C column. The reference column was an authentic Waters \textit{µBondapak™} C\textsubscript{18} 3.9 x 300 mm column. The chromatography was carried out using a Waters 625 LC System, a 717plus Autosampler, a 490 Programmable Multiwavelength Detector, and a Millennium® Workstation (all from Waters Corporation). The columns were thermostatted at 23.4 (± 0.5) °C using a Mistral® column thermostat.

\textbf{Results and Discussion}

The columns were first tested for efficiency using acenaphthene as the marker, and a mobile phase of 60/40, v/v, acetonitrile/water flowing at 2.0 mL/min. The results are summarised in Table 1.

The authentic Waters \textit{µBondapak™} C\textsubscript{18} column was found to be the most efficient of the four columns, and also gave the best peak symmetry. The imitation columns were found to have efficiencies that were 25—30% lower, and all showed significant peak fronting (USP Tailing < 1). This is an indication that the imitation columns are not as well packed as the authentic Waters \textit{µBondapak™} C\textsubscript{18} column.

To evaluate the selectivity of the columns we used a test mixture containing acidic, basic, and non-ionisable compounds (see Figure 1). The mobile phase contained methanol (65% v/v) and a pH 7.20 mM potassium phosphate buffer. Under these conditions, the strong base, propranolol (\(pK_a \approx 9.6\)) is protonated, and interacts with ionised silanol groups through an ion-exchange mechanism. This causes the propranolol peak to tail noticeably.

The chromatograms obtained on the four columns are shown in Figure 2. The retention factors and several relative retentions are summarised in Table 2. The imitation columns clearly do not match the separation obtained using the authentic Waters \textit{µBondapak™} C\textsubscript{18} column. The largest differences are observed for the strong base, propranolol (marked with arrow). On the authentic Waters \textit{µBondapak™} C\textsubscript{18} column, propranolol elutes midway between toluene and acenaphthene. On the Imitation A C\textsubscript{18} columns, however, propranolol partially coelutes with acenaphthene.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Column & Efficiency\textsuperscript{1} & USP Tailing \\
\hline
\textit{µBondapak™} C\textsubscript{18} & 30,200 & 1.01 \\
Imitation A C\textsubscript{18} & 20,800 & 0.92 \\
Imitation B C\textsubscript{18} & 22,100 & 0.93 \\
Imitation C\textsubscript{18} & 21,900 & 0.98 \\
\hline
\end{tabular}
\caption{Efficiencies and Tailing Factors for Authentic Waters \textit{µBondapak™} C\textsubscript{18} and Imitation C\textsubscript{18} Columns}
\label{table1}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
 & \textit{µBondapak™} & Imitation A & Imitation B & Imitation C \\
\hline
Retention Factors & & & & \\
Propyl paraben & 1.47 & 1.29 & 1.17 & 0.98 \\
Toluene & 2.43 & 2.40 & 1.90 & 1.72 \\
Propranolol & 4.52 & 6.35 & 1.61 & 13.1 \\
Aacenaphthene & 7.60 & 7.69 & 6.23 & 5.09 \\
Dibutyl phthalate & 12.3 & 11.4 & 9.61 & 7.85 \\
\hline
Relative Retention versus Aacenaphthene & & & & \\
Propyl paraben & 0.193 & 0.167 & 0.188 & 0.193 \\
Toluene & 0.320 & 0.312 & 0.305 & 0.339 \\
Propranolol & 0.594 & 0.825 & 0.258 & 2.57 \\
Dibutyl phthalate & 1.62 & 1.48 & 1.54 & 1.54 \\
\hline
\end{tabular}
\caption{Retention Factors and Relative Retentions for Authentic Waters \textit{µBondapak™} and Imitation C\textsubscript{18} Columns}
\label{table2}
\end{table}
This indicates that the silanol activity of the alphaBond™ column is higher than that of an authentic Waters µBondapak™ C_{18} column. On the Imitation C_{18} column, the silanol activity is so much higher that propranolol elutes last, as a barely discernable peak. In contrast, on the Imitation B C_{18} column propranolol elutes before toluene, indicating reduced silanol activity. Clearly, none of the imitation columns matches the selectivity of an authentic Waters µBondapak™ C_{18} column.

**Conclusions**

These results show that there are significant differences between authentic Waters µBondapak™ C_{18} columns and the imitation columns. The imitation columns were found to have lower efficiencies, and produced fronting peaks. In addition, we found large differences in selectivity, particularly evident in the retention of basic compounds relative to neutral compounds. This is not surprising, because controlling the retention of basic compounds has been one of the most difficult problems in the synthesis of C_{18}-silica packings. Research has shown that base retention is affected not only by the bonding processes, but also by many properties of the silica support. In the final analysis, no imitation column matches the performance of an authentic Waters µBondapak™ C_{18} column.

For your copy of the Waters Column Selection Guide for USP Specifications, please check box 7 on the business reply card.
### Ordering Information

<table>
<thead>
<tr>
<th>Column</th>
<th>Particle Size</th>
<th>Porc Size</th>
<th>Dimensions</th>
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<td>4.6 mm x 250 mm</td>
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  - Fax: 80 8969 7157

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  - Fax: 787 747 8448

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  - Fax: 3 704 8599

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  - Fax: 3 704 8599

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  - Fax: 02 706 9704

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  - Fax: 01923 219012

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  - Address: 34 Maple Street Milford, MA 01757 USA
  - Tel: 508 476 2000
  - Toll-free: 1 800 252 4752
  - Fax: 508 872 1990

**http://www.waters.com/**
Papers to be presented at HPLC ‘97

Simple and Rugged Solid-Phase Extraction (SPE) Method for the Determination of Tetracyclines in Serum by HPLC Using a Volatile Mobile Phase

Yung-Fong Cheng, Dorothy J. Phillips and Uwe D. Neue
Waters Corporation, 34 Maple Street, Milford, MA 01757 USA

Due to its high sensitivity, high selectivity and high sample throughput, LC/MS/MS has become the choice for the analysis of drugs and their metabolites in biological fluids such as serum and urine. This paper reports a simple and reproducible SPE method for the determination of tetracycline (TC), minocycline (MC) and demeclocycline (DCC) in porcine serum using a volatile HPLC mobile phase.

High pH Stability of Silica-Based Reversed-Phase HPLC Packings

Tom Walter, Bonnie Alden and Ray Fisk
Waters Corporation, 34 Maple Street, Milford, MA 01757 USA

Recently there has been considerable interest in the stability of silica-based reversed-phase packings when exposed to high pH mobile phases. The most common failure mode is loss of efficiency and peak symmetry due to voiding, as a result of dissolution of the silica particles. In contrast to the results reported by other researchers, we find no correlation between stability and the method of preparation of the silica particles.

Applications of a Polymeric Extraction Sorbent for Isolating Highly Polar Drugs From Biological Fluids

Dorothy J. Phillips, Edouard S. P. Bouvier, Mark Capparella, Yung-Fong Cheng, Pamela Iraneta, Uwe D. Neue and Laura L. Bean
Waters Corporation, 34 Maple Street, Milford, MA 01757 USA

Method development experts in the pharmaceutical industry are striving to streamline sample preparation. Considerable time and effort are consumed in choosing the appropriate solid-phase extraction (SPE) sorbent and extraction protocol. The new Waters Oasis™ HLB extraction cartridges address the problem of low recovery of polar analytes. This paper includes the extraction procedures, HPLC methods and the results for several highly polar drugs.

Development of Methods for Extraction of Neutral Steroids From Biological Matrices With a Polymeric Solid-Phase Sorbent

Dorothy J. Phillips, Mark Capparella, Yung-Fong Cheng, Pamela Iraneta, Uwe D. Neue
Waters Corporation, 34 Maple Street, Milford, MA 01757 USA

Steroids have many biological functions, making their analysis important in biomedical applications. The ratio of the amount of a steroid to that of extraneous material is very low in biological fluids. Therefore, extraction of the steroid from the biological matrix is necessary prior to HPLC analysis. The solid-phase extraction method development process, giving details at each step from sample preparation to elution, will be presented.

Simple and Fast Method Development For HPLC and Sample Preparation

Yung-Fong Cheng, Dorothy J. Phillips, Uwe D. Neue and Laura Bean
Waters Corporation, 34 Maple Street, Milford, MA 01757 USA

The development of assays for drugs and their metabolites from serum is time-consuming since optimisation of the HPLC method and the sample preparation is difficult and tedious. It would be desirable to have a well thought out strategy for HPLC method development which can provide good peak shapes and good resolution for a wide range of compounds. In this paper, a carefully planned HPLC method development protocol using Waters Symmetry™ columns for the separation of chlordiazepoxide and its metabolites (ranging from basic to weakly acidic in nature), will be presented.
Sample preparation is a necessary step in the analysis of drugs from biological fluids including serum and urine. This paper reports a simple, fast and rugged solid-phase extraction (SPE) method for determination of two basic tricyclic antidepressants and their metabolites using Waters Oasis™ HLB extraction cartridges. Extraction of other basic compounds using the same SPE method will also be presented.

Solid-phase extraction (SPE) is a routinely used chromatographic technique for sample enrichment and sample cleanup. Processing aqueous samples by SPE is predominantly performed using cartridges containing C18-bonded silica. Waters has introduced a porous polymeric sorbent for reversed-phase solid-phase extraction. This sorbent possesses greater water-wetting properties than traditional C18-silica sorbents. We have measured wetting properties for several hydro-organic mixtures on both C18-silica and the polymeric sorbent and have correlated the results to performance of the sorbents in solid-phase extraction.

The behavior of reversed-phase HPLC packings is strongly dependent on the interaction of the mobile phase with the stationary phase. We have developed chromatographic protocols to observe the wetting of reversed-phase packings, and determined the extent of wetting using water/methanol and water/acetonitrile mobile phases of varying water content. Results will be presented for a range of C18- and C8-silica packings showing the effects of different pore size distributions and bonded-phase coverages.

Recently, a new cartridge has been introduced for reversed-phase solid-phase extraction (SPE). The Oasis™ HLB cartridge contains a hydrophilic/lipophilic balanced polymeric resin which has been demonstrated effective for the extraction of polar pharmaceuticals and metabolites. In this research, the cartridge is shown to be highly effective for the demanding analysis of water samples for polar pesticides and related compounds. Among the applications discussed in this presentation are sulfonylurea herbicides, triazine herbicides and metabolites, phenolic compounds, and water soluble pesticides such as acephate. For most analytes, using a 75 mL water sample and a 1 mL elution volume, detection limits at or below 100 ng/L are routinely achieved.
**What’s New**

**ElectroChromatography**

Capillary Zone Electrophoresis (CZE) has been demonstrated to be a highly efficient separation technique for charged analytes. However, in its native form, it is incapable of separating neutral molecules. On the other hand, high-performance liquid chromatography (HPLC) is an established technique with a vast number of stationary phases available, but it is restricted in the use of small particles due to pressure constraints. Capillary ElectroChromatography (CEC) uses an electric field rather than hydraulic pressure to drive the mobile phase through the packed bed. Since the resulting flow is very uniform, very high efficiencies are achieved.

In our next issue, we will explore the fundamental principles of CEC highlighting several exciting applications. The author of the article is Norman Smith of Innovatech, one of the leading innovators in the field of CEC. CEC is a highly promising technique that couples the very high efficiencies associated with CZE with reversed-phase chromatography. The theory predicts a two to three-fold increase in efficiency going from a pressure driven system (HPLC) to an electrically driven system (CEC) at equal column length. Due to the differences in the driving mechanism, however, gains of over a factor of 10 can be achieved by going from an HPLC separation to a CEC separation.

Waters is now offering a line of Capillary ElectroChromatography products manufactured by Innovatech Ltd. for Waters.

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<thead>
<tr>
<th>Description</th>
<th>Dimension</th>
<th>Part No.</th>
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Copies of this article can be obtained now by checking box 12 on the business reply card.

For information on the Waters Edge Frequent Buyer Program, please check box 9 on the business reply card.

For the new Waters Spherisorb® columns brochure, please check box 10 on the business reply card.

For information on the Waters Extraction Manifold, please check box 11 on the business reply card.

**The Waters EDGE Frequent Buyer Program**

With the Waters Edge Frequent Buyer Program, you can earn points with every purchase of Gelman Sciences filtration products from Waters. You can then redeem these points for products, services and merchandise found in the Waters Edge Frequent Buyer brochure. The points are automatically shipped from Waters with the Gelman Sciences products.

**Waters Spherisorb® Columns**

Waters Spherisorb® columns are superior in quality and competitively priced. They are available everywhere through the Waters worldwide distribution network. Buying your Spherisorb® column from Waters assures you the best packed Spherisorb® column you can buy.

**The New Waters Extraction Manifold**

The new extraction manifold has enhanced features designed for use with conventional silica-based solid-phase extraction cartridges as well as modifications that allow you to take full advantage of the unique performance characteristics of our new Oasis™ HLB extraction cartridges.
Androgens: Testosterone Esters

Solid-Phase Extraction Procedure

Oasis™ HLB 1 cc Extraction Cartridge

System:
A Waters 717plus Autosampler and a Waters 625 LC System. Detection was by UV using a Waters 486 Tunable UV/VIS Absorbance 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>Testosterone</td>
<td>1.00</td>
<td>98%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.200</td>
<td>94%</td>
<td>3.7%</td>
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<tr>
<td>Testosterone</td>
<td>1.00</td>
<td>100%</td>
<td>1.9%</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.200</td>
<td>94%</td>
<td>4.3%</td>
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<tr>
<td>Testosterone</td>
<td>1.00</td>
<td>94%</td>
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<tr>
<td>Benzoate</td>
<td>0.200</td>
<td>87%</td>
<td>3.2%</td>
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</table>
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.

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.

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 “IotCode”:

- 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.

The quality management system of Waters manufacturing facility, Milford, Massachusetts, complies with the International Standard ISO 9001 Quality Management and Quality Assurance Standards. Waters quality management system is periodically audited by the registering body to ensure compliance.

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.
Morphine and Its Metabolites

Solid-Phase Extraction Procedure
Oasis™ HLB 1 cc Extraction Cartridge

- Condition Cartridge: 1 mL methanol/1 mL water
- Equilibrate Cartridge: 1 mL water
- Load Sample: 1 mL sample spiked porcine serum
- Wash Cartridge: 1 mL water
- Elute Analytes: 0.5 mL 3% triethylamine
- Evaporate and Reconstitute Analytes: No evaporation—sample injected directly

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

Column: SymmetryShield™ RP8, 3.9 mm x 150 mm
Sample: 100 µL of porcine serum extract
Mobile phase: 20 mM potassium phosphate, pH 6.4
Flow rate: 1.0 mL/min
Detection: Fluorescence: excitation at 280 nm, emission at 355 nm

Peaks:
1. Morphine-3 glucuronide
2. Morphine-6 glucuronide
3. Morphine

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<th>% Recovery</th>
<th>% RSD (n=6)</th>
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<tbody>
<tr>
<td>M3G</td>
<td>0.48</td>
<td>90.7</td>
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<td>M6G</td>
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<td>Morphine</td>
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<tr>
<td></td>
<td>0.73</td>
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<td>3.2</td>
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</tbody>
</table>
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**Waters Fluorescence Detector**

Fluorescence detection offers both greater sensitivity and selectivity for compounds that fluoresce naturally, or can react reproducibly with reagents to form stable fluorescent compounds. Waters Scanning Fluorescence Detector enables you to easily apply this technology with unbeatable performance and versatility. A dual monochromator design provides independent programming of both excitation and emission wavelengths to improve individual analyte detectability. The Waters Fluorescence Detector offers the sensitivity you need for applications where very low concentrations of targeted compounds are analysed.

**Waters 616 LC System**

Waters has incorporated the latest technological advances into the new Waters 616 LC system. The 616 is the culmination of leading innovative technology—fluidic, electronic, and software—designed to deliver superior performance in a premier pumping system. The 616 offers pulse-free flow, low delay volume, efficient gradient mixing, precision, accuracy, and reliability.

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