Application Note: 414

Sample Clean-up Strategies for the LC/MS Analysis of Small Molecules in Serum

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Key Words

- Hypersil GOLD™ Column
- BioBasic™ SEC-60 Column
- HyperSep™ C18 Column
- Surveyor Plus™ HPLC
- LCQ Deca XP™ LC/MS"
- Solid Phase Extraction (SPE)
- Size Exclusion Chromatography (SEC)

Overview

The removal of matrix interferants from biological samples using off-line SPE adds a time-consuming step to analytical separations. On-line sample clean-up utilizing SEC minimizes the number of steps, minimizes the total time required for analysis, and has the added advantage that it can be automated.

Introduction

Drug discovery/detection commonly focuses on analytes which are present in biological matrices. Direct injection of such samples onto LC and LC/MS systems is problematic as analytes of interest can be 'lost' in the concentrated matrix peak (particularly if being polar they elute close to the solvent front) and instrument and column contamination readily occurs.

Traditionally, solid phase extraction (SPE) has been used for sample pre-treatment and clean-up, however, this can often be time consuming and adds an additional stage to the analytical method. The ability to automate biological assays, minimize the number of steps and overall analysis time, particularly in the pharmaceutical arena, is becoming increasingly popular and important. On-line sample clean-up utilizing size exclusion chromatography (SEC) fulfills this criteria.

Elution in SEC is dependent on the hydrodynamic volume (size) of analytes. SEC stationary phases possess pores with a variety of volumes. Small molecules can enter such pores whereas larger molecules are excluded, thus traveling through the column quickly and eluting rapidly. In SEC chromatographic resolution is dependent on the pore size of the stationary phase. SEC with a small pore size column can be utilized to separate small molecules from biological matrices–the analyte molecules are retained in the stationary phase pores whereas the serum/plasma constituents, being larger, are excluded and eluted quickly.

On-line SEC is a two-dimensional approach in which serum is injected directly onto a small pore size exclusion column and then the analytes, once separated from the matrix, are eluted onto an analytical column. On-line SEC reduces sample preparation time, minimizes potential analyte loss and results in increased productivity and higher sample throughput.

In this application note the use of off-line SPE and on-line SEC for the extraction and subsequent analysis of three anti-retroviral drugs (abacavir, lamivudine and zidovudine) is discussed.

Methods

Off-Line SPE

Phase: HyperSep C18 (Thermo Scientific, Bellefonte, PA)
Volume: 3 mL
Bed Weight: 200 mg
Conditioning: 2 mL MeOH, 2 mL H_2O
Sample Application: 10 mL serum
Washing: 1 mL H_2O, 2 × 1 mL Heptane
Elution: 4 × 1 mL MeOH (1st aliquot left on bed for 1 min before vacuum applied)
Vacuum: ~ 3 mm Hg
Drying: Sample dried under nitrogen gas (~ 5.5 bar)
Sample Reconstitution: 1 mL H_2O

Off-Line SPE Analytical Method

Column: Hypersil GOLD 1.9 µm, 50 × 2.1 mm (Thermo Scientific, Bellefonte, PA)
Instrumentation: Surveyor HPLC system–quaternary MS pump with degasser, autosampler and photodiode array UV detector (Thermo Scientific, San Jose, CA)
Mobile Phase: A = 10 mM Ammonium Formate, pH 3.0
B = MeOH
Gradient: 5–45% B in 4 min then 4 min re-equilibration
Flow Rate: 500 µL/min
Temperature: 45°C
Detection: UV at 265 nm
Injection Volume: 1 µL

On-Line SEC Analytical Method

Columns: BioBasic SEC-60 5 µm, 150 × 2.1 mm, Hypersil GOLD 3 µm, 50 × 2.1 mm (Thermo Scientific, Bellefonte, PA)
Instrumentation: Surveyor HPLC system–quaternary MS pump with degasser, autosampler and photodiode array UV detector, LCQ Deca XP Mass Spectrometer (Thermo Scientific, San Jose, CA)
Mobile Phase: A = 10 mM Ammonium Formate, pH 3.0
B = MeOH

Gradient:

Time (min)	% B
0	0*
0.75	0*
0.80	13
7.00	13**
7.10	100
10.10	100
10.20	13
13.20	13

*Eluent diverted to waste
**Before gradient begins BioBasic SEC-60 column is equilibrated in 100% buffer for 2 minutes, eluent diverted to waste

Flow Rate: 500 µL/min
Temperature: BioBasic SEC-60 at ambient temperature, Hypersil GOLD at 45°C
Detection: +ve ESI
Injection Volume: 2 µL

Results

Figure 1 details the chromatographic separation of the three anti-retroviral drugs following extraction from serum using off-line SPE. Resolution is achieved in under 4 minutes using a simple organic gradient. The drugs were present in serum at 'typical body concentrations' achieved after administration of anti-retroviral combination tablets such as Trizivir (abacavir 200 ng/mL, lamivudine 400 ng/mL, zidovudine 100 ng/mL). The percentage recoveries for abacavir, lamivudine and zidovudine from serum, at these levels, were 83, 77, and 89%, respectively.

Figure 2 provides a schematic diagram to illustrate the system/column configuration which was used for on-line SEC serum extraction and subsequent analytical separation.

In order to determine the duration for which the eluent should be directed to waste after injection, serum and serum dosed with individual analytes were injected onto the SEC column alone. Figure 3 details the resultant UV trace for blank serum. Lamivudine (the least retained of the analytes) was found to elute at ~ 0.8 minutes, therefore, the switch valve was set to direct flow to the analytical column at 0.75 minutes. As can be seen from the chromatogram, the major serum peak elutes at ~ 0.5 minutes and, therefore, is prevented from contaminating the analytical column.

The separation of the drugs from serum with on-line SEC teamed with isocratic analytical separation is depicted in Figure 4. Because of the isocratic conditions necessitated in order to achieve resolution of abacavir and zidovudine the analytical separation is almost double the length of the gradient method employed with off-line SPE. The percentage recoveries achieved using this method were > 100, 89, and 79% for abacavir, lamivudine and zidovudine respectively. The spectra for the analytes are displayed in Figure 5.

Table 1 details the variation in retention time observed for ten serum SEC extraction runs. The relative standard deviation observed was low (< 3.5 %) illustrating that the method is robust and reproducible. During the course of experimentation, over 110 runs have been performed with no significant deterioration in column performance observed.

Table 2 compares the solvent usage and total experimental time for the two methodologies. As can be seen, both in terms of solvent consumption and analytical time, the on-line SEC separation is superior. The greatest time limiting factor for the off-line SPE extraction is the removal of MeOH/drying of the sample under a constant flow of nitrogen.

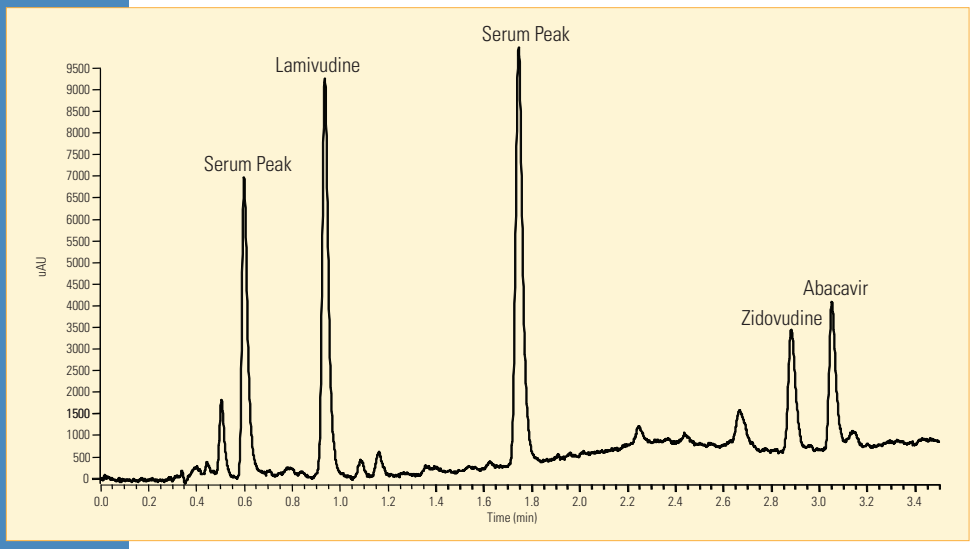

Figure 1: Anti-Retroviral Drugs Extracted from Serum at ng/mL Concentrations after Off-Line SPE

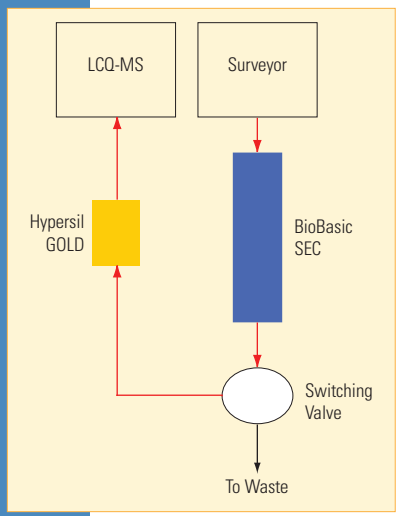

Figure 2: System Setup Diagram

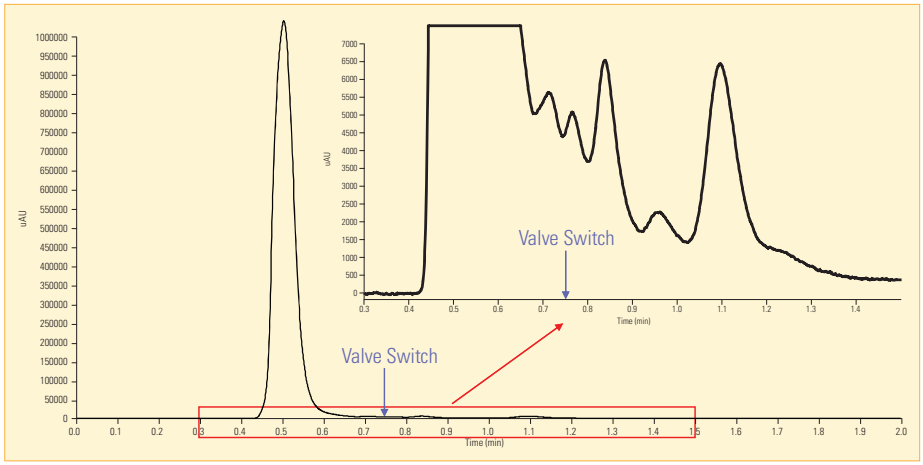

Figure 3: Blank Serum SEC Extraction

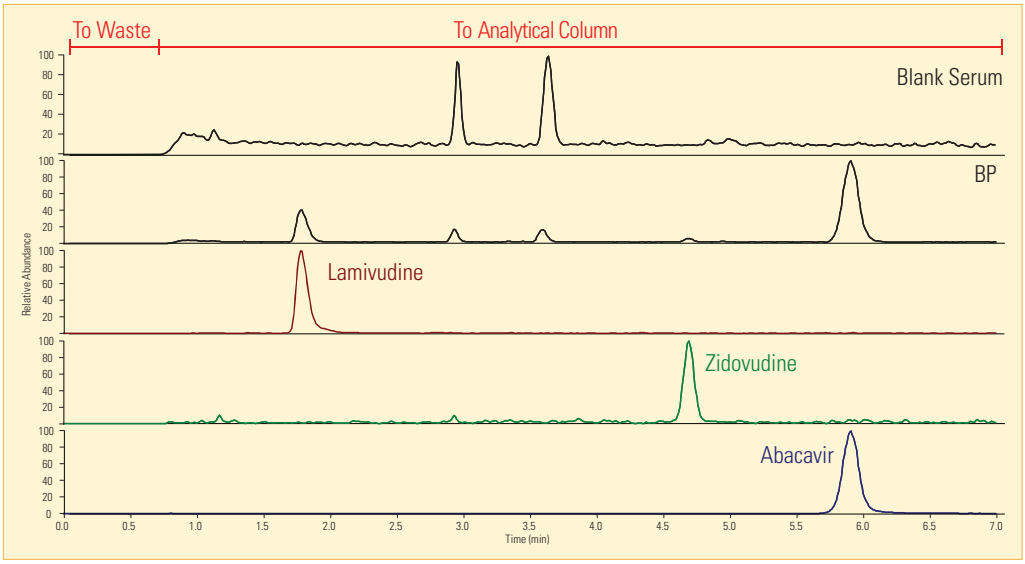

Figure 4: On-Line SEC Extraction of Anti-Retrovirals from Serum.

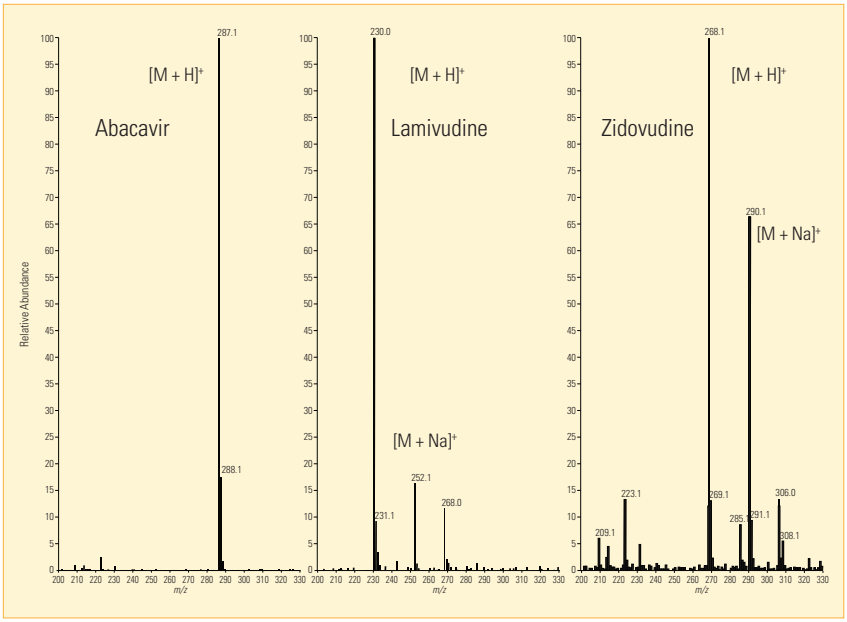

Figure 5: Analyte Spectra

Run	Retention Time (mins)		
	Lamivudine	Zidovudine	Abacavir
1	1.83	4.79	6.01
2	1.95	4.90	6.13
3	1.84	4.79	6.04
4	1.79	4.73	5.99
5	1.78	4.69	5.91
6	1.80	4.73	5.96
7	1.76	4.67	5.91
8	1.74	4.69	5.87
9	1.75	4.66	5.89
10	1.76	4.70	5.91
Average	1.80	4.74	5.96
RSD (%)	3.5	1.5	1.4

Table 1: On-Line SEC Data Repeatability

	Off-Line SPE	Off-Line SPE Analytical Separation	On-Line SEC Analytical Separation
Total Experimental Time (mins)	>120	8	15.2
Solvent Consumption (mL)	12	4	7.6

Table 2: On-Line and Off-Line Extraction Method Comparison

Conclusions

This application note successfully demonstrates the use of on-line SEC for the extraction of analytes from serum.

- On-line SEC analysis provides a suitable alternative to off-line SPE for the clean-up of samples in biological matrices.
- The methodology is robust and reproducible.
- Unlike off-line SPE, the process can be automated resulting in increased sample throughput, productivity and saving operator time.

Additional Information

For additional information, please browse our Chromatography Resource Center which can be accessed from: www.thermo.com/columns.

View additional Thermo Scientific LC/MS application notes at: **www.thermo.com/appnotes**

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