

A Miniaturized On-Line SPE-LC Solution for Direct Analysis of Drugs in Small-Volume Plasma Samples

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This presentation shows a highly-productive automated total analysis system (TAS), based on an UltiMate™ 3000 x2 Dual-Ternary Micro LC System, for therapeutic drug monitoring (TDM). The integrated sample preparation step involves two silica-based columns; a unique 20 × 1 mm i.d. restricted access material (RAM) solid phase extraction (SPE) column and a 50 × 2.1 mm i.d. C8 analytical column. Compared to a conventional-size on-line RAM SPE column setup, the miniaturized approach significantly speeds the analysis cycle time, reduces solvent consumption as well as required injection volume, and provides ideal flow rates for hyphenation with ESI-MS. This representative application demonstrates the fully automated analysis of antimycotic drugs in human plasma.

INTRODUCTION

Integrated Sample Clean-Up Versus Conventional Sample Clean-Up

Common off-line sample preparation methods (precipitation, liquid-liquid extraction, off-line SPE, etc.) for the therapeutic monitoring of drugs in body fluids with HPLC are usually time-consuming, error-prone, and costly. This is mainly because they typically involve manual pretreatment steps to eliminate the complex sample matrix.

Optimization of the clean-up of complex biofluids, such as plasma and urine, enables higher sample throughput and total automation while reducing costs and improving overall analytical quality. This is achieved by integrating the extractive sample clean-up process into the TAS, as shown in Figure 1. The instrumentation and method allow the direct injection and LC system-integrated SPE of native human plasma followed by the separation and quantitation of the target analytes.

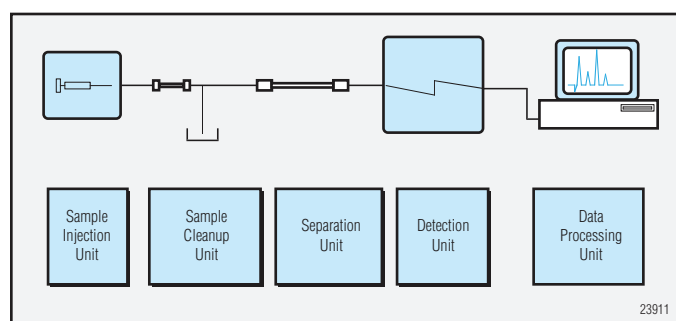


Figure 1. Total Analysis System (TAS).

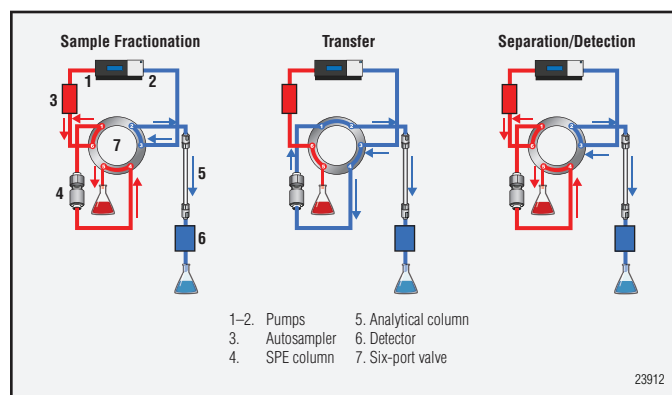


Figure 2. Typical instrumental setup of an on-line SPE-LC system with a six-port switching valve. In the sample fractionation step, the analytes are trapped on an SPE column while the matrix is flushed into waste. In the second step the analytes are transferred onto an analytical column. The UltiMate 3000 x2 dual-ternary system integrates both pumps in one enclosure and has a preinstalled switching-valve.

Principle of On-Line SPE-LC

A small SPE column packed with a special stationary phase selectively retains the target analytes from the sample matrix. The trapped and enriched analytes are transferred in a back-flush mode to a conventional analytical HPLC column for separation and detection afterwards. Both columns are controlled with a multi-port switching valve. This technique is commonly referred to as on-line SPE-LC (Figure 2). Its instrumental setup represents a TAS.

The Benefits of a Miniaturized and Highly Optimized Column Setup

A reduction of the SPE column bed volume minimizes the size of transferred volumes to the analytical column and thus optimizes the enrichment factors at a distinct sample load. A decrease in the diameter of the connected analytical column translates into lower flow rates and increased peak heights when a defined amount of analyte is transferred from the SPE column. At the same time, the liquid processing speed can be increased by applying higher linear velocities in the narrow bore columns without any need to exceed available flow rates of HPLC instruments. Cutting down the HPLC column length accompanied with adaptation of the gradient profile can further reduce the analysis cycle time.

The overall benefits of such a volume-miniaturized system are:

- Markedly lower sample consumption
- Significant solvent savings
- Reduced cycle times and thus higher analysis throughput
- Improved compatibility with electrospray mass spectrometry

UltiMate 3000 x2 Dual-Ternary Micro LC System for Miniaturized On-Line SPE-LC

Setup and operation of a coupled-column system traditionally required special technical and advanced chromatography expertise. Using micro-bore columns multiplies these challenges. The UltiMate 3000 x2 Dual-Ternary Micro LC System (Figure 3) overcomes such drawbacks by:

- Integrating two micro LC gradient pumps needed for on-line SPE-LC in one enclosure
- Providing the switching valve as a preinstalled part of the thermostated column compartment and minimizing all extracolumn volumes
- Combining these instrument features with single point Chromeleon® software control to provide easy method creation and automated operation with full traceability



Figure 3. UltiMate 3000 x2 Dual-Ternary Micro LC System.

Restricted Access Material (RAM)

In on-line SPE-LC the SPE column is an integral part of the TAS. As opposed to disposable cartridges used in off-line SPE, the column has to repetitively deplete the matrix of complex samples while providing a continuous high recovery level for the analytes of interest. For this purpose, special SPE-column packings known as RAM have been developed.^{1,2}

These stationary phases are based on porous silica or crosslinked copolymers. RAMs have a defined exclusion barrier for macromolecular matrix components (e.g., proteins, nucleic acids, polysaccharides) and a non-adsorptive outer particle surface. Low-molecular weight molecules, like the target analytes, can reach the adsorption sites located exclusively at the inner pore surface. This combination of two-dimensional chromatography, size exclusion chromatography (SEC) and reversed-phase chromatography (RPC), allows the extraction of drugs directly and selectively from the native biofluid. The extracted analytes are then transferred to the analytical column for separation and detection.

Figure 4 illustrates the surface topochemistry of a representative RAM (LiChrospher® ADS, Merck KGaA, Germany) applied in on-line SPE-LC of biofluids.

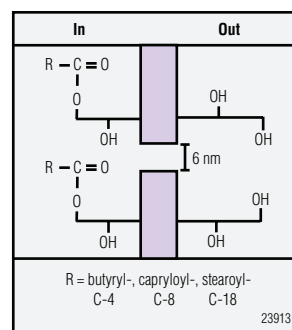


Figure 4: Topochemistry of the restricted access material LiChrospher® ADS.

INSTRUMENTS AND EXPERIMENTAL CONDITIONS

General Instrumentation

- UltiMate x2 Dual-Gradient Micro LC System with a PDA-3000 photodiode array detector controlled with Chromeleon 6.80 Chromatography Management Software (Dionex Corp.)
- Analytical detector flow cell (13 μ L, 10 mm) for conventional method, semimicro flow cell (3.1 μ L, 9 mm) for miniaturized method
- In-line filter: FK7400 (Recipe GmbH, Munich, Germany)
- All connection tubing optimized in length and at 125 μ m internal diameter

Column Setup and Chromatographic Conditions for Conventional On-Line SPE-LC

RAM SPE Column:	LiChrospher ADS RP-4, 25 μ m, 20 \times 4 mm, (Merck KGaA, Germany; VWR Intl.)
Guard Cartridge:	Acclaim [®] 120 C8, 5 μ m, 10 \times 4.3 mm (Dionex Corp.)
Analytical Column:	Acclaim 120 C8, 3 μ m, 150 \times 4.6 mm (Dionex Corp.)
Injection Volume:	50 μ L plasma sample
Fractionation Step:	2 minute loading with pump 1 at 2.0 mL/min (water/acetonitrile 98/2, v/v)
Transfer and Separation:	0.01 M ammonium acetate to acetonitrile gradient from 45 to 85% within 7.0 min at 1.2 mL/min with pump 2
Column Compartment Temperature:	40 $^{\circ}$ C (RAM SPE column operated at room temperature)
Detection:	UV 260 nm

Column Setup and Chromatographic Conditions for Miniaturized On-Line SPE-LC

RAM SPE Column:	LiChrospher ADS RP-8, 25 μ m, 20 \times 1 mm, (custom-made prototype)
Guard Cartridge:	Acclaim 120 C8, 5 μ m, 10 \times 2.0 mm (Dionex Corp.)
Analytical Column:	Acclaim 120 C8, 3 μ m, 50 \times 2.1 mm (Dionex Corp.)
Injection Volume:	20 μ L plasma sample
Fractionation Step:	1.75 minute loading with pump 1 at 0.4 mL/min (water/acetonitrile 95/5, v/v)
Transfer and Separation:	0.01 M ammonium acetate to acetonitrile gradient from 50 to 75% within 2.25 min at 0.4 mL/min with pump 2
Column Compartment Temperature:	58 $^{\circ}$ C (RAM SPE column operated at room temperature)
Detection:	UV 255 nm

Sample Preparation

Human plasma samples were stored at -20° C and centrifuged for 10 min at 15,000 g prior to injection.

RESULTS AND DISCUSSION

Steps of the Miniaturized On-Line SPE-LC Method

The fully automated SPE-LC analysis cycle using the Dionex UltiMate 3000 \times 2 Dual-Ternary Micro LC System consists of four distinct steps, each of them highly optimized to minimize the cycle time.

1. Matrix Depletion and Analyte Extraction

The sample, human plasma, is injected onto the RAM SPE column. While the sample matrix is flushed to waste with the mobile phase delivered by pump 1, the analytes (antimycotics) are retained on the stationary phase of the RAM SPE column.

2. Analyte Transfer from the RAM SPE Column to the Analytical Column

After switching the multi-port valve, the RAM SPE column and the analytical HPLC column are connected in series. The mobile phase delivered by pump 2 desorbs the retained analytes from the RAM SPE column in a back-flush mode and transfers them onto the analytical column.

3. Analyte Separation

The analytes of interest (e.g., voriconazole, ketoconazole, itraconazole, and the metabolite 1-OH-itraconazole) are separated using a linear gradient.

4. Reconditioning of the RAM SPE column and Re-equilibration of the Analytical Column

In order to prevent carry-over and retain full capacity, the RAM SPE column is first washed with 90% organic content and then reequilibrated with solvent (pump 1).

Figure 5 illustrates the 5.5-min scheme of the method.

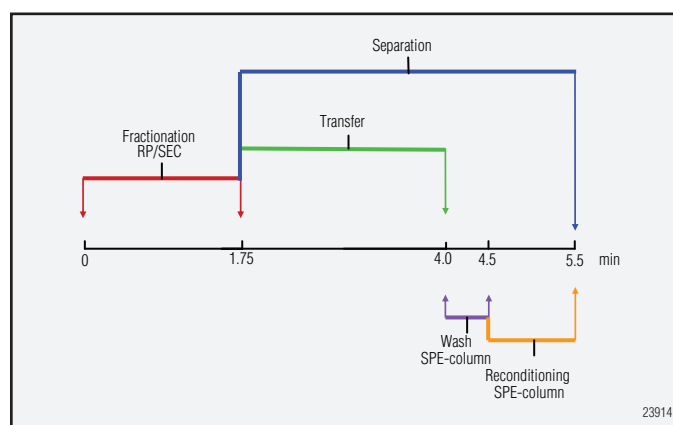


Figure 5. Timing scheme for miniaturized on-line SPE-LC-UV analysis of antimycotics.

Antimycotic Drugs as Application Example

Antimycotics such as ketoconazole, voriconazole and itraconazole represent broad spectrum antifungal agents against a variety of yeasts (e.g., candida albicans) and filamentous fungi (e.g., aspergillus). These compounds act as inhibitors of cytochrome P450 dependent lanosterol 14- α -demethylase, a key enzyme of the sterol biosynthesis pathway. They are used for the therapeutic treatment of serious systemic infections, e.g., in immunosuppressed patients suffering from AIDS.

Method Optimization Steps Overview

The miniaturized on-line SPE-LC method was based on a previous conventional scale LC method. Impressive benefits of the miniaturized method become obvious in direct comparison to a conventional scale method as will be shown in a later section. During the miniaturization process, the following alterations have been made:

- Miniaturizing the RAM SPE column from 20×4 mm to 20×1 mm in order to achieve the smallest possible transfer volume to the connected micro bore LC column and to generate a high linear velocity for a short cycle time even at low flow rate.
- Changing the RAM stationary phase from RP-4 to RP-8 in order to increase the adsorption capacity for the analytes of interest.
- Miniaturizing the LC column from 150×4.6 mm to a 50×2.1 mm format, to increase the absolute amount sensitivity, speeds up the analysis cycle, and decrease solvent consumption.
- Adapting the gradient conditions (window and slope) and column temperature optimizes the overall process with regard to the altered column dimensions and SPE materials.

Effect of the RAM SPE Column Miniaturization on Matrix Depletion Speed

The RAM SPE column internal diameter was down-scaled stepwise from 4 mm to 2 mm and eventually to 1 mm. For the 1 mm i.d. column, the stationary phase was adapted from RP-4 to RP-8. The alteration of the flow rate included a stepwise increase of the linear velocity with down-scaling of the i.d. It could be verified independently that the plasma fractionation process on the ADS RAM columns for small molecule target analytes is not affected in performance, even at linear velocities of up to 10 mm/s. Table 1 lists the flow rates applied and linear velocities achieved with the different column i.d.s. The elution profiles for raw human plasma injections are shown in Figure 6, which demonstrates the increased fractionation speed and decreased clean-up time due to higher linear velocity.

SPE Column i.d.	Flow Rate	Linear Velocity
4 mm	2 mL/min	2.6 mm/s
2 mm	1 mL/min	5.3 mm/s
1 mm	0.4 mL/min	8.5 mm/s

Table 1. RAM SPE column internal diameters, applied flow rates, and resultant linear velocities. Column length was 20 mm for each i.d.

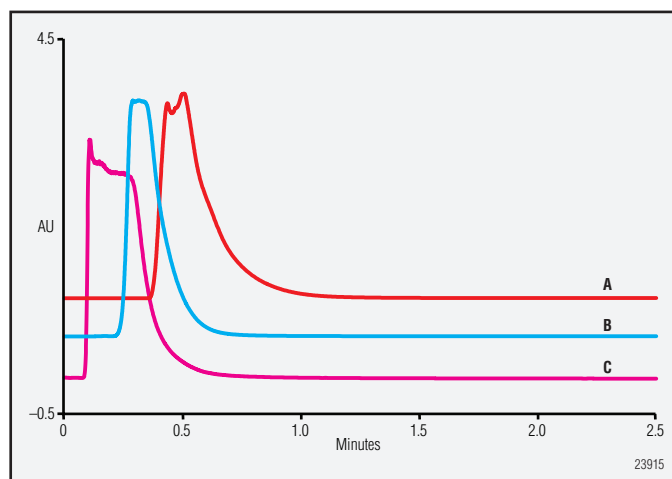


Figure 6. Elution profiles of the macromolecular matrix of a raw human plasma sample obtained using LiChrospher ADS SPE columns of different sizes. (A) RP-4 SPE column (20×4 mm i.d.), sample volume $50 \mu\text{L}$, flow rate 2 mL/min. (B) RP-4 SPE column (20×2 mm i.d.), sample volume $15 \mu\text{L}$, flow rate 1 mL/min. (C) RP-8 SPE column (20×1 mm i.d.), sample volume $20 \mu\text{L}$, flow rate 0.4 mL/min. The SPE columns were connected directly to a UV detector set at 280 nm.

Overall Performance Comparison between Miniaturized and Conventional Scale On-Line SPE-LC

To fully assess the benefits of the miniaturized on-line SPE-LC TAS, the results of its application to antimycotics analysis in plasma were directly compared to those obtained with a conventional on-line SPE-LC method for the same application. Therefore, a human plasma sample spiked with defined amounts of antimycotics was analyzed using both methods. Figure 7 and Table 2 show the results of an injection of a $50 \mu\text{L}$ plasma sample into the conventional system. Figure 8 and Table 3 show the results of an injection of a $20 \mu\text{L}$ sample into the miniaturized system. As can be deduced from these data, the overall analysis precision and recovery was not adversely affected by the miniaturization.

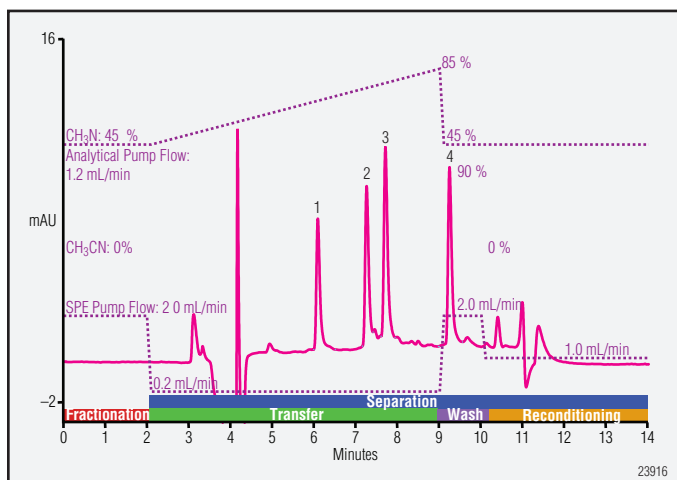


Figure 7. Conventional on-line SPE-LC analysis of 50 μ L plasma (spiked with (1) Voriconazole: 1.026 μ g/mL; (2) Ketoconazole: 1.524 μ g/mL; (3) 1-OH-Itraconazole: 0.7360 μ g/mL; (4) Itraconazole: 0.7560 μ g/mL) on a LiChrospher ADS RP-4 SPE column (20 \times 4 mm i.d.) coupled to a C8 modified analytical column (150 \times 4.6 mm i.d.). Refer to experimental section for method details.

Antimycotic Drug	Mean Peak Area	RSD	Calculated Recovery
Voriconazole	0.68 mAU min	\pm 1.00%	84%
Ketoconazole	0.84 mAU min	\pm 1.18%	80%
1-OH-Itraconazole	1.00 mAU min	\pm 0.94%	90%
Itraconazole	0.87 mAU min	\pm 0.88%	93%

Table 2. Conventional on-line SPE-LC method: analytical precision (10 injections) and analyte recovery (calculated versus direct injections of corresponding standard solutions on analytical column). For further details refer to Figure 7 and experimental section.

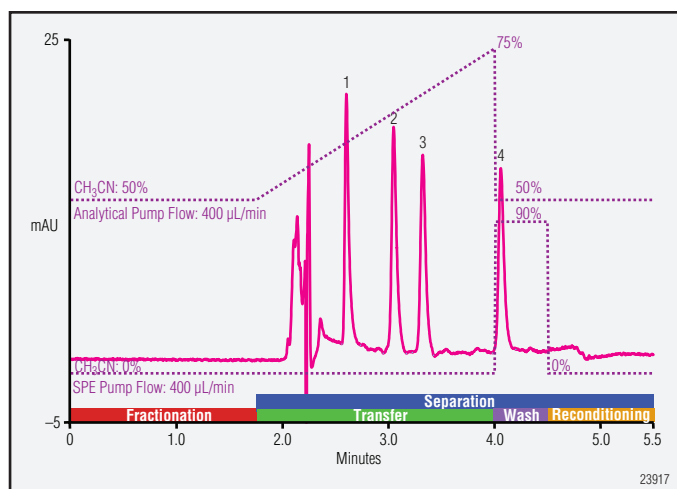


Figure 8. Miniaturized on-line SPE-LC analysis of 20 μ L plasma spiked with antimycotics (refer to Figure 7 for peak identification and analyte concentrations) on a LiChrospher ADS RP-8 SPE column (20 \times 1 mm i.d.) coupled to a C8 modified analytical column (50 \times 2.1 mm i.d.). Refer to experimental section for method details

Antimycotic Drug	Mean Peak Area	RSD	Calculated Recovery
Voriconazole	0.87 mAU min	\pm 0.73%	96%
Ketoconazole	0.99 mAU min	\pm 1.73%	91%
1-OH-Itraconazole	0.87 mAU min	\pm 1.25%	72%
Itraconazole	0.81 mAU min	\pm 1.40%	80%

Table 3. Miniaturized on-line SPE-LC method: analytical precision (10 injections) and analyte recovery (calculated versus matrix free direct injections of corresponding standard solutions on analytical column). For further details refer to Figure 8 and experimental section.

Figure 9 directly compares the chromatograms from a 20 μ L plasma sample analyzed using the two different systems. The comparison clearly displays the advantages of the miniaturization with respect to sample size, analytical sensitivity, and cycle time. The overall benefits of the miniaturization are listed in Table 4. The theoretical increase in sensitivity calculated from the i.d.s of the analytical columns would be 4.7. The observed increase was somewhat lower. This can be explained by the shorter detection path length of the semimicro flow cell and stronger influence of extra column effects. In addition to the increased sensitivity in UV detection in spite of the lower sample requirement, the table shows how analysis throughput could be improved and solvent could be saved. The use of the miniaturized method with MS or MS/MS detection would translate into further benefits as the 3 times lower flow rate would certainly increase the relative ionization yield in the electrospray source.

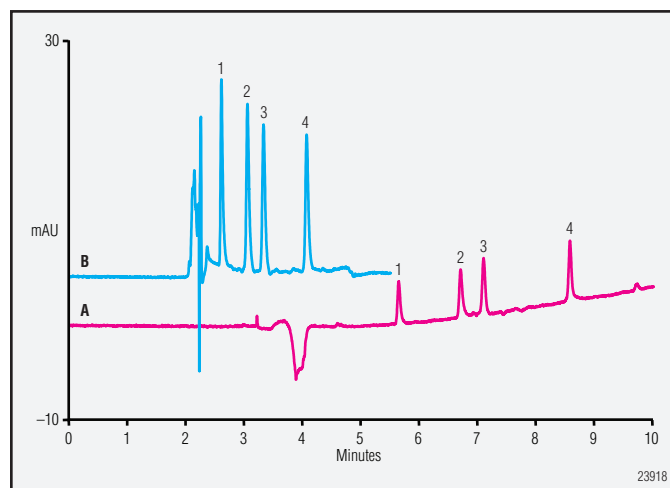


Figure 9. Direct comparison of an on-line separation of 20 μ L plasma spiked with antimycotics (refer to Figure 7 for concentrations) on (A) the conventional method (refer to Figure 7 for method details) and (B) the miniaturized method (refer to Figure 8 for method details). Detection is at 255 nm.

	Conventional On-line SPE-LC	Miniaturized On-line SPE-LC	Improvement Factor
Injected sample volume	50 µL	20 µL	2.5
Cycle time	13 min	5.5 min	2.4
Eluent consumption	26 mL	4.4 mL	5.9
Response factor (Itraconazol)	1 ng = 0.23 mAU	1 ng = 0.93 mAU	4.0
ESI-MS compatibility	Limited (1.2 mL/min flow rate)	Ideal (0.4 mL/min flow rate)	—

Table 4. Main benefits of the miniaturized on-line SPE-LC method over the conventional method for direct analysis of antimycotics in human plasma (data calculated from results shown in Figure 7 and Figure 8).

CONCLUSION

This poster shows that UltiMate 3000 ×2 Dual-ternary Micro LC systems with single point Chromeleon control provide a turn-key solution for fully integrated and automated sample clean-up at a micro bore column scale. In combination with a size-adjusted RAM SPE column, the system is ideally suited for the direct injection, extractive enrichment and subsequent separation and detection of drugs present in complex biofluids, especially when only a limited sample volume is available.

Compared to a conventional scale on-line SPE-LC system, users experience the following benefits:

- 2.5-fold lower sample volume required
- Fourfold lower limits of detection with UV
- Sixfold lower solvent consumption
- More than twice the analysis throughput
- Better compatibility with electrospray MS due to decreased flow rate

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2. Cassian, N. M.; Lima, V. V.; Oliveira, R. V.; de Pietro, A. C.; Cass, Q. B. *Anal. Biol. Chem.* **2006**, *384*, 1462-1469.

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