



Analysis of Antimycotic Drugs in Biofluids by On-Line SPE-LC

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INTRODUCTION

This poster gives an application example for the direct analysis of untreated, native human plasma. The method consists of fully automated extraction, analytical separation and quantitation of drugs relevant for therapeutic monitoring. We describe the on-line SPE-LC-UV analysis of antimycotics using an UltiMate™ 3000 ×2 Dual-Gradient HPLC System.

Conventional Approach

Common HPLC methods for the therapeutic monitoring of drugs in body fluids are often time-consuming, error-prone and costly. This is because they typically involve manual pretreatment steps to eliminate the complex sample matrix. This conventional pretreatment process comprises:

- Precipitation/centrifugation
- Liquid-liquid extraction/evaporation/reconstitution
- Membrane filtration
- Solid Phase Extraction (SPE) in off-line mode

Integrated Sample Cleanup

To achieve higher sample throughput and total automation with a simultaneous cost reduction and improvement of the overall analytical quality, the cleanup of complex biofluids such as plasma and urine needs to be optimized. A major improvement can be achieved by integrating the extractive sample cleanup process into a Total Analysis System (TAS), as shown in Figure 1. Instrumentation and methods allow the direct injection and LC-system integrated SPE of native human plasma followed by the separation and quantitation of the target analytes.

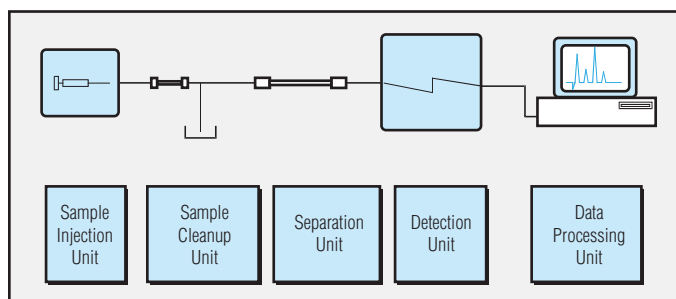


Figure 1. Total Analysis System (TAS).

On-Line SPE-LC Principle

One of the possible setups for a TAS includes a small, dedicated SPE column to selectively extract the target analytes from the matrix (integrated sample cleanup). The trapped analytes are transferred in backflush mode to a conventional analytical HPLC column for analyte separation and detection. During this step the SPE column can be reconditioned. The SPE column and the analytical column are connected via a 2-position 6-port switching valve. This technique is commonly referred to as on-line SPE-LC (Figure 2).

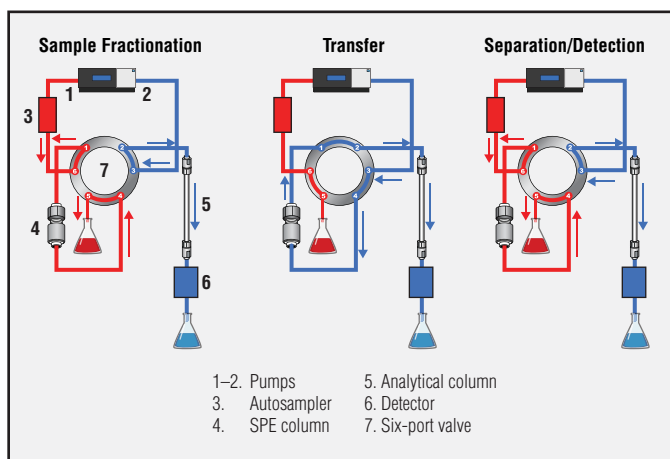


Figure 2. Typical setup of an on-line SPE-LC system with a 6-port switching valve. In the sample fractionation step, the analytes are trapped on an SPE column while the matrix is flushed to waste. In the second step the analytes are transferred to the analytical column. An UltiMate 3000 ×2 Dual-Gradient system integrates two pumps in one enclosure and provides a preinstalled thermostated switching valve.

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UltiMate 3000 x2 Dual-Gradient System for On-Line SPE-LC

Until now, setup and operation of a coupled-column system has required special technical and advanced chromatography knowledge. The UltiMate 3000 x2 Dual-Gradient System (Figure 3) overcomes this drawback by:

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



Figure 3. UltiMate 3000 x2 Dual-Gradient 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 in off-line SPE, it has to repetitively deplete the matrix of complex samples while providing continuous high recovery levels for the analytes of interest.

For this purpose, special SPE-column packings have been developed called restricted access materials (RAM). They have been reviewed in Reference 1. These stationary phases are based on porous silica or cross-linked copolymers. These materials have a defined exclusion barrier for macromolecular matrix components (e.g., proteins, nucleic acids, polysaccharides) and a nonadsorptive outer particle surface. Low molecular weight molecules, like the target analytes, can advance to the adsorption sites located exclusively at the inner pore surface. This combination of two-dimensional chromatography (e.g., 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.

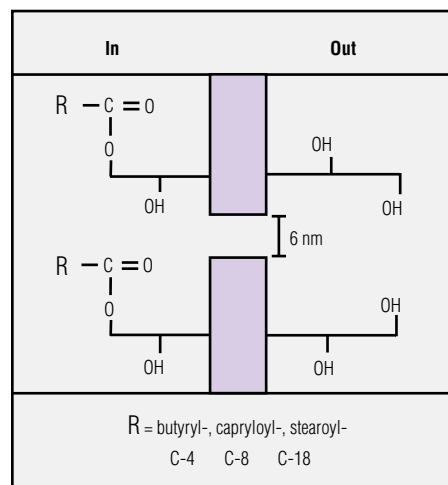


Figure 4. Topochemistry of the restricted access material LiChrospher ADS.

INSTRUMENTS AND EXPERIMENTAL CONDITIONS

Instrumentation

- Dionex ×2 Dual-Gradient HPLC System with Chromeleon 6.60 Chromatography Management System
- In-line filter: FK7400 (Recipe GmbH, Munich, Germany)
- RAM SPE column: LiChrospher ADS RP-4, 25 µm, 25 × 4 mm
 - Molecular weight cutoff: 15 kDa
 - (Merck KGaA, Germany; VWR Intl.)
- Guard cartridge: Dionex Acclaim® 120 C8, 5 µm, 10 × 4.3 mm
- Analytical column: Dionex Acclaim 120 C8, 3 µm, 150 × 4.6 mm

Chromatographic Conditions

- Injection volume: 100 µL
- Fractionation step (Pump 1): mobile phase (P1) water/MeCN (98/2, v/v) at 2.0 mL/min (1 mL/min during transfer and separation phase)
- Transfer and separation (Pump 2): mobile phase (P2) 0.01 M ammonium acetate and MeCN at 1.2 mL/min
- RAM SPE column wash step: mobile phase water/MeCN (10/90, v/v) at 1 mL/min
- Column compartment temperature: 40 °C
- Transfer and separation gradient: as displayed in Figure 7
- Detection: UV 260 nm

Sample Preparation

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

METHOD, RESULTS, AND DISCUSSION

Method

A fully automated SPE-LC analysis cycle using Dionex UltiMate 3000 ×2 Dual-Gradient HPLC Systems consists of four distinct steps (Figures 2 and 5):

1. Matrix depletion and analyte extraction

The sample (standard solution or 100 µL of untreated human plasma) is injected onto the RAM SPE column. While the sample matrix is flushed to waste with mobile phase P1 in 2 min, the analytes (antimycotics) are retained on the RP-4 stationary phase of the RAM SPE column.

2. Analyte transfer from the RAM SPE column to the analytical column

After switching the 2-position 6-port valve, the RAM SPE column and the analytical HPLC column are connected in line. The mobile phase delivered by pump 2 desorbs the retained analytes from the RAM SPE column in backflush mode and transfers them to the analytical column.

3. Analyte separation

The antimycotics voriconazole, ketoconazole, and itraconazole—as well as its metabolite 1-OH-itraconazole—are separated using a linear gradient RAM SPE column.

4. Reconditioning of the RAM SPE column and reequilibration of the analytical column

In order to prevent carryover and retain full capacity, the RAM SPE column is washed for 1 min and then reequilibrated with solvent P1. Following this procedure, the RAM SPE column can be used for >300 injections of 100 µL each of raw human plasma. The analytical column is reequilibrated in 3 min.

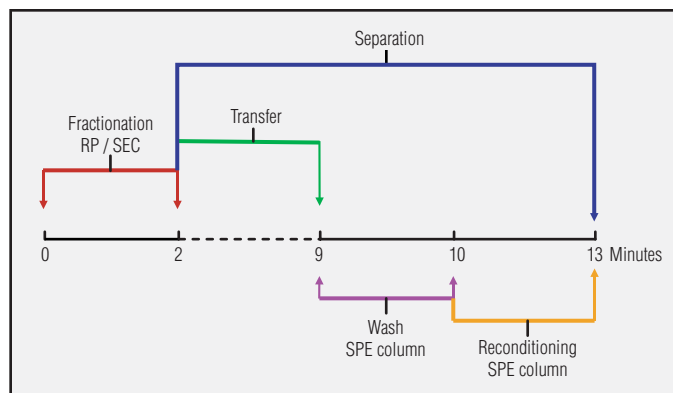


Figure 5. Timing scheme for on-line SPE-LC-UV analysis of antimycotics.

Antimycotic Drugs

Antimycotics such as ketoconazole, voriconazole, and itraconazole (Figure 6) 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., for immunosuppressed patients suffering from AIDS).

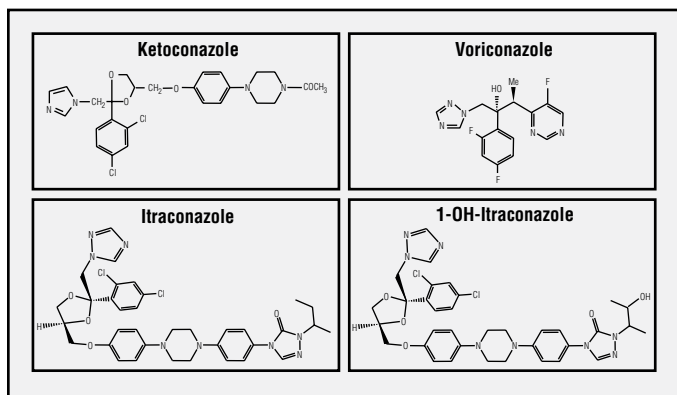


Figure 6. Chemical structures of the investigated antimycotics.

RESULTS

Figure 7 shows the chromatogram of a human plasma sample spiked with the four antimycotics investigated. All analytes are baseline resolved within 13 min. PumpLeft and PumpRight are the names of the two pumps incorporated in the UltiMate 3000 Dual-Gradient pump. In this case PumpLeft is used as the analytical gradient pump. PumpRight serves for sample fractionation, analyte transfer, and reconditioning of the RAM SPE column.

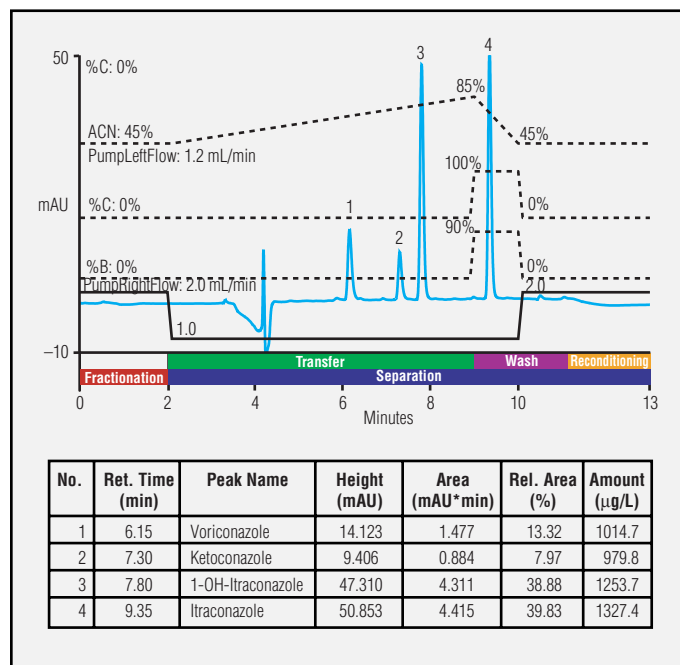


Figure 7. On-line SPE-LC-UV of antimycotics standards in human plasma.

Figure 8 shows the chromatogram of a plasma sample taken from a patient treated with itraconazole (Peak 4). As expected the metabolite 1-OH-itraconazole (Peak 3) is also present.

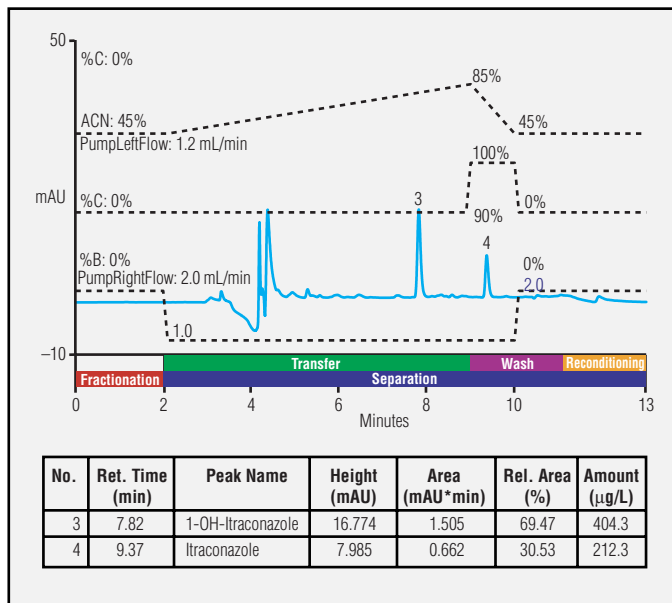


Figure 8. On-line SPE-LC-UV of plasma from a patient treated with itraconazole.

Figure 9 shows the chromatogram of a plasma sample of a patient initially treated with itraconazole and changed to voriconazole.

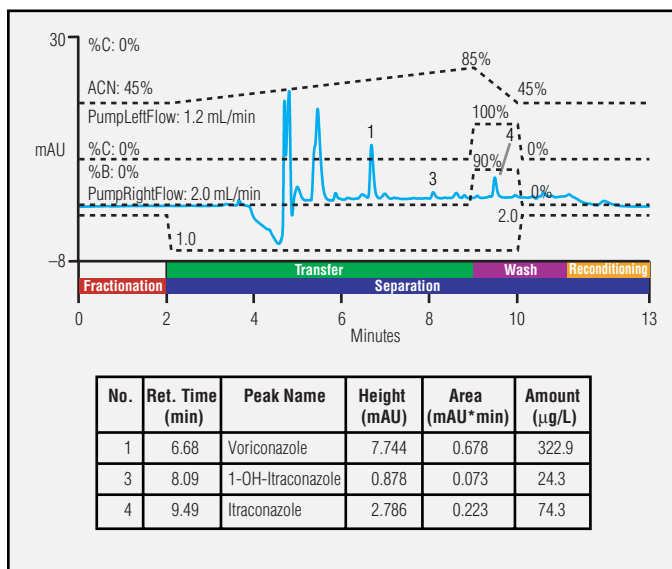


Figure 9. On-line SPE-LC-UV of plasma from a patient undergoing a change of treatment from itraconazole to voriconazole.

CONCLUSION

This poster shows that UltiMate 3000 ×2 Dual-Gradient HPLC systems with single-point Chromeleon control provide a solution for the fully integrated and automated sample cleanup. In combination with a tailored RAM SPE column this system is ideally suited for the direct injection, extractive enrichment and subsequent separation and UV detection of drugs present in complex biofluids (on-line SPE-LC-UV).

With this setup users benefit from the following advantages:

- Elimination of time-consuming labor and cost-intensive off-line operations
- A closed system that provides enhanced operator safety
- Sophisticated automation possibilities enabling unattended operation overnight or over the weekend
- Full instrument control, advanced diagnostics, automated validation functions
- Complete documentation and data traceability of all sample cleanup and analytical separation steps
- Improved quality of the overall analytical process
- Repetitive use of tailored SPE RAM columns for integrated and automated solid phase extraction for at least 300 plasma injections, 100 µL each

REFERENCES

1. Boos, K.-S.; Grimm, C. H. *Trends Anal. Chem.* **1999**, *18*, 175.

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