

Recent Developments in On-line SPE for HPLC and LC-MS in Bioanalysis

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Introduction

On-line SPE-LC-MS is becoming increasingly popular for automation of bioanalytical assays, especially in the pharmaceutical industry (1). Total automation, high precision and high sensitivity are among the most favoured features. For high-speed bioanalysis based on SPE-LC-MS, the challenge for on-line SPE is to keep pace with the very short LC-MS cycle times. Ideally, on-line SPE should be faster than, and run in parallel with, LC-MS analysis to avoid any limitation of assay cycle time. Another major challenge in drug analysis that should be faced is the reduction of assay development time. A generic method for SPE and LC is urgently needed to eliminate method development for preclinical assays or as a universal starting point for the development of high-quality, high-throughput clinical assays.

In this technical article, we present new instrumentation for on-line SPE that has been designed to achieve SPE cycle times of less than one minute. With dual SPE cartridge clamps, SPE runs entirely in parallel with the LC-MS run. Results of high-speed SPE and fast generic gradient SPE-LC-MS-MS show that on-line SPE is no longer a speed-limiting element in high-speed SPE-LC-MS assays. In addition, dedicated system configurations and strategies for automated and smart method development are presented that can be used to rapidly select optimum SPE conditions including temperature.

Table 1: Results of generic SPE-LC-MS-MS analysis of spiked porcine serum.

Compound	SRM (m/z)	Recovery (%)	Linearity (ng/mL)
Sulfadiazine	251.4 → 156.2	99	1-1000
Sulfamerazine	264.8 → 156.2	103	1-1000
Taxol	854.5 → 285.9	97	5-500
Propranolol	259.8 → 183.0	100	2-1000
Carbamazepine	237.6 → 193.8	99	2-1000
Procainamide	236.4 → 163.4	98	2-1000
Caffeine	195.2 → 138.2	99	1-10000
Ranitidine	315.2 → 176.0	98	2-5000
Theophylline	181.2 → 124.0	99	2-10000
Acetaminophen	152.2 → 110.2	100	5-1000
Theobromine		101	-

System Set-up

Parallel SPE and elution was performed using a Prospekt•2 (Spark Holland, Emmen, The Netherlands) with dual cartridge clamps (Figure 1). SPE solvents for solvation, equilibration, sample application and clean-up are provided by the high-pressure dispenser (HPD) at programmable flow-rates, independent of SPE cartridge back pressure. Raw sample is introduced by the autosampler. After SPE on the left cartridge, the cartridge is transferred to the right clamp for elution into the LC-MS system. During LC-MS-MS analysis, SPE of the next sample is performed on a new cartridge in the left clamp. The SPE cartridge exchange is programmable per analysis.

Experimental Conditions

Generic SPE: SPE cartridge = HySphere Resin GP (10 × 2 mm and 10 × 3 mm); solvation = 1.5 mL MeOH (5.0 mL/min); equilibration = 1.5 mL H₂O (5.0 mL/min); sample application and clean-up = 2.0 mL H₂O (2.0 mL/min).

Generic fast gradient LC: Column = C18, 30 × 4 mm i.d., 3 μm particles; flow-rate = 2.0 mL/min; solvent A = 5% ACN, 0.1% HCOOH, 10 mM NH₄Ac; solvent B = 95% ACN, 0.1% HCOOH, 10 mM NH₄Ac; gradient = 100% A for 0.1 min, to 100% B in 1.3 min, hold at 100% B for a further 0.1 min, then to 100% A in 0.5 min (total gradient run time = 2 min).

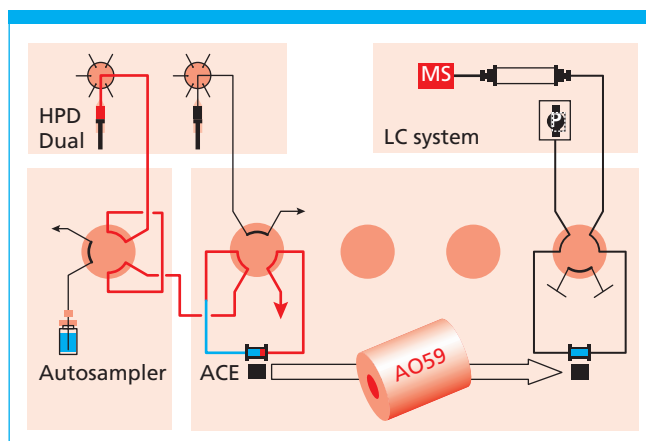


Figure 1: Parallel SPE and elution with Prospekt•2™ with dual cartridge clamps.

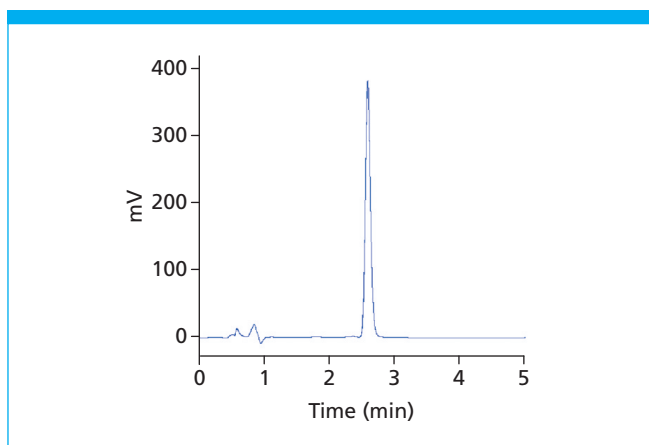


Figure 2: On-line SPE-LC-UV of carbamazepine in serum as performance test for the 35 s SPE method, column: C18, 4.6×75 mm, $3.5 \mu\text{m}$; detection: 254 nm mobile phase: water/ACN 60/40; flow-rate: 1 mL/min.

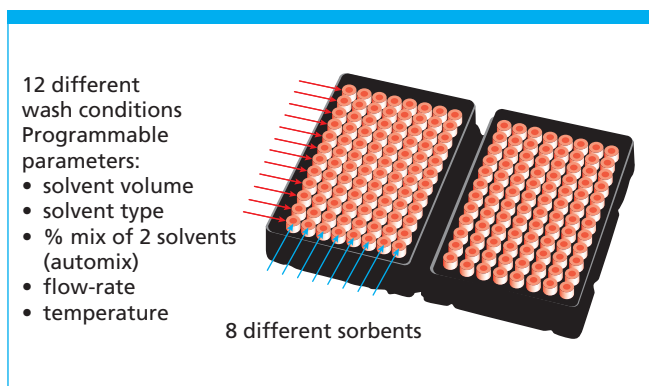


Figure 3: Method development trays.

Mass spectrometry: Instrument = API 3000 with LINAC collision cell (Applied Biosystems, Foster City, California, USA); flow = split to $400 \mu\text{L}/\text{min}$; ionization mode = turbo ion spray, positive ion mode; scan mode = selective reaction monitoring.

Results

Table 1 shows the results of generic SPE-LC-MS-MS analysis of $100 \mu\text{L}$ porcine serum, spiked with 11 different compounds. Compounds with identical colours were determined in a single run. $20 \mu\text{L}$ phosphoric acid per mL sample was added to release the strongly protein bound sulfa's and a 3 mm SPE cartridge was used for the polar acetaminophen and theobromine. MS detection of theobromine failed and recovery was determined using diode array detection. With the dual cartridge clamps allowing one sample to be extracted as the previous one is analysed, the overall assay cycle time equals the longest of the two parallel processes, namely the 2 min gradient LC-MS-MS.

How Fast Can On-line SPE Be?

For the generic SPE-LC-MS method shown, on-line SPE is not required to be faster than 2 min because of the time required for LC-MS. However, some bioanalytical LC-MS-MS methods run even faster than 1 min. Can on-line SPE keep up with these methods? In order to answer this question we tested the extraction performance of a 35 s SPE method in an on-line SPE-LC-UV determination of carbamazepine in serum.

Protocol:

- take SPE cartridge (HySphere C18 HD, 10×2 mm, $7 \mu\text{m}$)
- solvation: ACN, 0.5 mL, 10 mL/min
- equilibration: H_2O , 0.5 mL, 10 mL/min
- sample application ($25 \mu\text{L}$ serum): H_2O , 0.5 mL, 10 mL/min
- wash: 0.5 mL $\text{H}_2\text{O}/\text{ACN}$ (10/90), 0.5 mL, 10 mL/min
- transfer cartridge to other clamp for elution
- start next SPE cycle

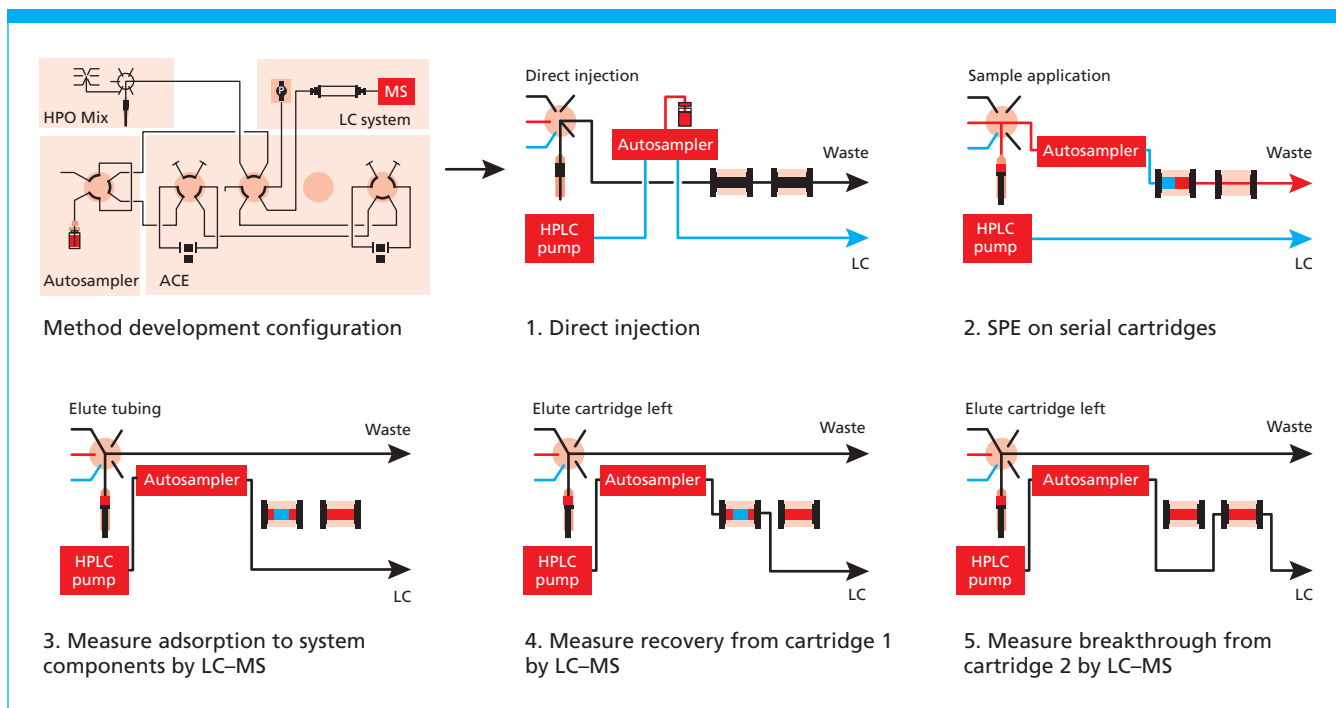


Figure 4: Intelligent method development.

Figure 2 shows the results of this analysis. Recovery was greater than 98%, linearity (r) was greater than 0.999 and precision was less than 2% RSD ($n = 20$). Thus, high flow-rates for all SPE steps do not appear to compromise SPE performance and permit on-line LC-MS-MS with total assay cycle times of less than one minute.

Automated Method Development

This procedure involves using data from the generic method as a starting point. Subsequent screening of SPE performance for different sorbents as a function of all clean-up solvent variables is then effected. Figure 3 shows an example method development tray. The configuration and procedure outlined in Figure 4 are adopted for intelligent method development in order to measure % recovery, % breakthrough and % adsorption to system components for every combination of sorbent and clean-up parameters. As an example, sorbent capacity was tested for on-line SPE on xanthenes (Figure 5). SPE-LC-UV traces from cartridge 1 and cartridge 2 reveal that HySphere GP has a 100% recovery for all xanthenes (no breakthrough) and that C18 can be used for selective determination of caffeine.

Temperature-Assisted On-line SPE

The optimization of wash temperature for on-line SPE-LC, as an alternative to modifier addition, was then performed. Figure 6 shows the chromatograms of xanthenes in human plasma after on-line SPE-LC incorporating a 0.5 mL wash with water at different temperatures. As can be seen, elevating wash temperature enables the removal of early eluting matrix compounds. However, temperatures greater than 47 °C leads to breakthrough of polar xanthenes.

Conclusions

The combination of high flow-rates for all SPE steps (10 mL/min) and parallel SPE allows on-line coupling of SPE to fast LC-MS-MS bioanalysis, for assay cycle times shorter than one minute. A generic SPE-LC-MS protocol leads to new assays for preclinical bioanalysis with (almost) no method development. For assays under more stringent regulations (i.e., clinical trials), the application of the intelligent SPE method development system enables fast automated testing of all SPE variables. The system

provides information on recovery, breakthrough and adsorption to system components. For MS detection, this information is very helpful to discriminate between analyte loss and ionization suppression in case of reduced analyte signal.

Acknowledgement

We wish to thank Anniek Schellen and TNO Pharma, Zeist, The Netherlands for assisting in the generic SPE-LC-MS experiments.

Reference

(1) M.S. Lee and E.H. Kerns, *Mass Spectrometry Reviews*, **18**, 187-279, (1999).

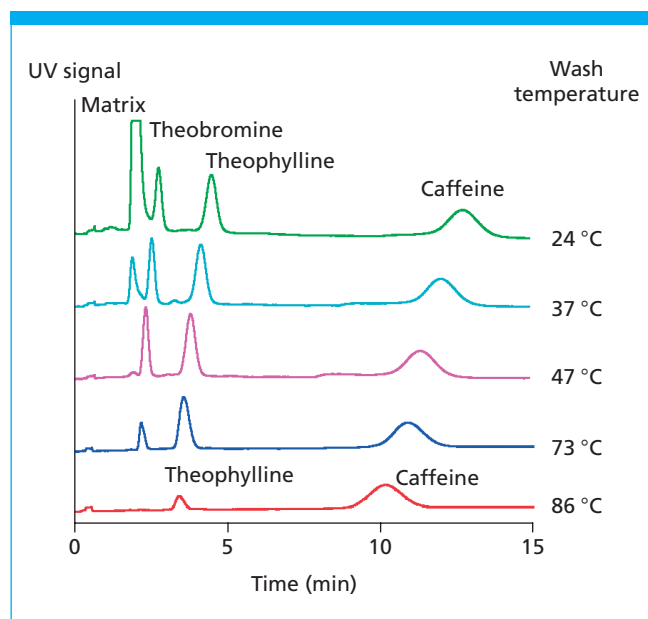


Figure 6: Analysis of xanthenes in human plasma after on-line SPE-LC using a HySphere resin GP cartridge and a 0.5 mL wash with water at different temperatures.

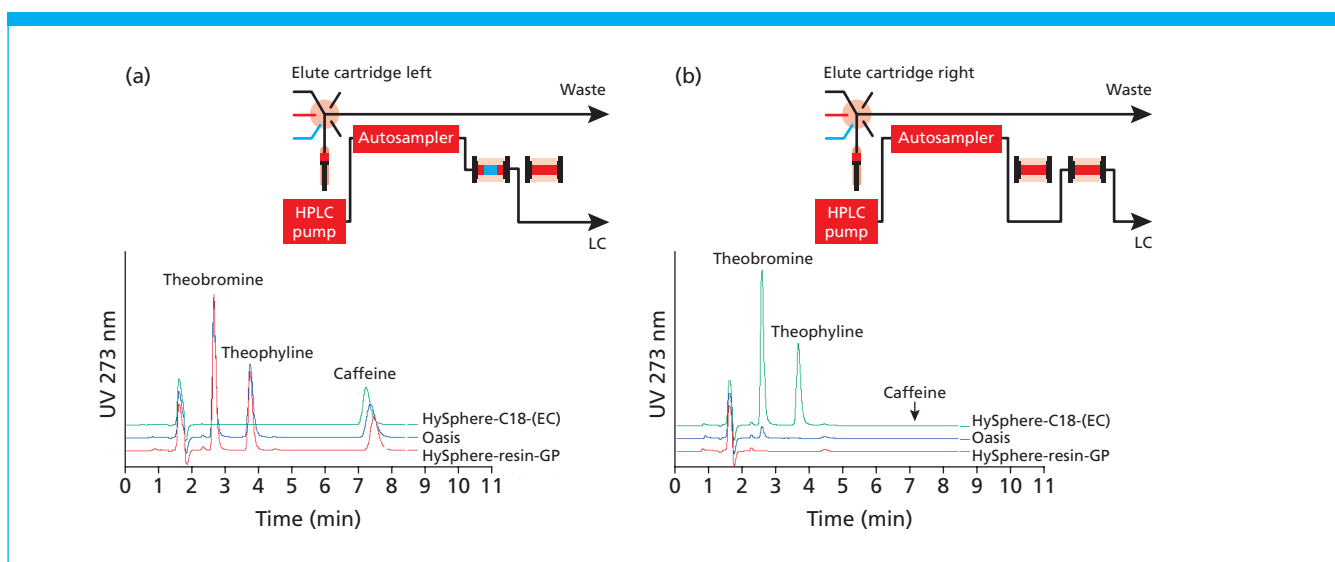


Figure 5: Testing of sorbent capacity for on-line SPE of xanthenes. (a) SPE-LC-UV elution profiles from cartridge 1 to measure recovery. (b) SPE-LC-UV elution profiles from cartridge 2 (strong hydrophobic sorbent) to measure breakthrough from cartridge 1.