Denaturing Solid-Phase Extraction for Reduced Protein Interference in Bioanalytical SPE–LC–MS

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Introduction

Pharmaceutical drug discovery and development is mainly based on data obtained from the quantitative determination of drugs (and related compounds) from biological samples.[1] Fast evaluation of possible drug candidates can be provided by assays that measure more than one analyte simultaneously. Automated SPE–LC–MS[2] has contributed to fast, sensitive and selective assays that provide quantitative data for many compounds much faster than ever possible in the past. Yet there are still some difficulties with reproducibility and accuracy when low concentrations of drugs have to be analysed in complex biological samples. Interferences apparent in LC with UV detection are not directly observed in LC–MS and affect the response in a different way. Especially in ESI-MS, interferences can reduce the extent of analyte ionization, which is often observed as a loss in signal. The effect of ionization suppression can be monitored by use of an infusion pump that delivers a constant amount of analyte into the effluent stream that enters the ion source of the MS.[3] Any interfering compound causes a variation in the ESI response of the infused analyte. To reduce suppression effects, LC run times are generally extended to separate matrix components from the target drug. The aim of this study is to obtain more insight in the origin and nature of serum matrix compounds that are responsible for ionization suppression effects in SPE-LC-ESI-MS and to develop strategies to remove these matrix compounds during SPE clean-up. Especially, the effects of denaturing wash conditions look promising.

Experimental Conditions *(On-line SPE–LC)*

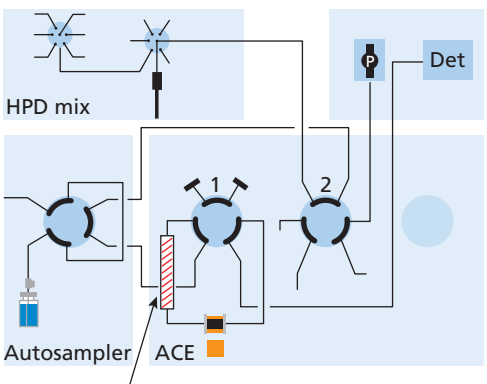
On-line SPE: PROSPEKT-2 (Spark Holland, Emmen, The Netherlands) comprising:

Table 1: MS settings for infused analytes.

Compound	Q1 mass	DP voltage	EP voltage
Acetaminophen	152.00	46.0	-7.0
Carbamazepine	237.20	61.0	-7.0
Procainamide	235.90	16.0	-5.0
Ranitidine	315.00	16.0	-6.0
Sulfadiazine	251.00	36.0	-8.0
Theophyline	181.00	56.0	-12.0

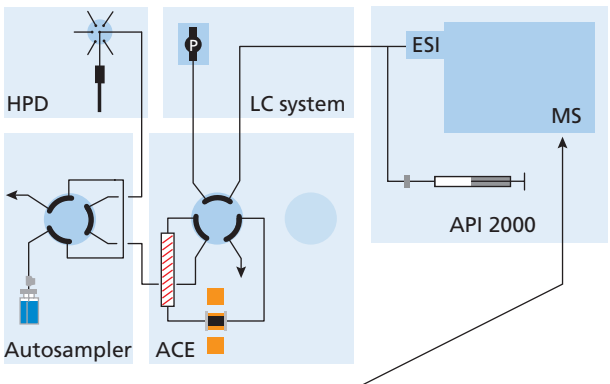
Compounds are dissolved in mobile phase (~2 µg/mL).

Figure 1: Experimental setup for UV monitoring of on-line SPE clean-up.



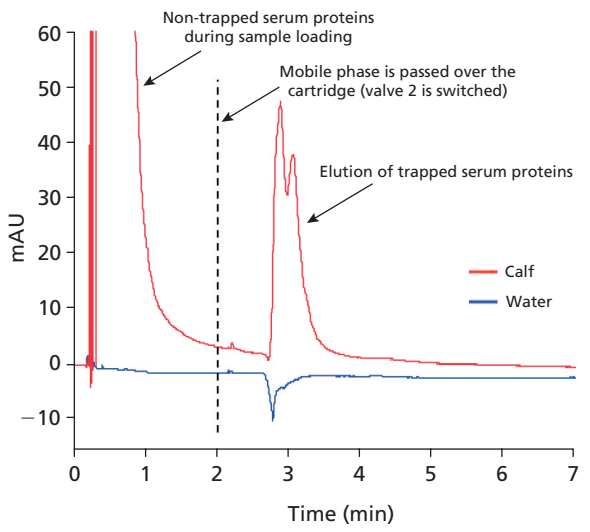
The temperature of the solvent entering the SPE cartridge is contolled from ambient up to 90 °C by the tubing heater (prototype).

Figure 2: Experimental setup for suppression monitoring.



Relatively low analyte concentrations should be infused as otherwise no differences in ionization suppression effects can be observed.

Figure 3: SPE–UV trace of calf serum compared with blank water.



Autosampler: Midas with serum needle option; injection volume = 25 µL partial loop fill or 100 µL full loop; needle wash = 500 µL methanol/water 40/60, 0.1% formic acid.
SPE: ACE with dual clamp and TASPE, HPD with solvent-mix option; cartridge = HySphere™ C18 HD 2 × 10 mm and 1 × 10 mm; solvation = 2 mL methanol, 5 mL/min; equilibration = 2 mL water (0.1% formic acid), 5 mL/min; sample load = 2 mL water (0.1% formic acid), 1 mL/min; wash = 1 mL wash solvent, 5 mL/min then 1.5 mL water, 5 mL/min; elution = isocratic for 5 min with LC mobile phase.
LC: System Gold® (Beckman Coulter, Fullerton, California, USA); pump = programmable solvent module 126; detector = diode array detector model 168 operated at 280 nm; mobile phase = methanol/water 40/60, 0.1% formic acid; flow-rate = 1 mL/min.

Experimental Conditions *(UV Monitoring of Clean-up)*

The experimental setup for UV monitoring of on-line SPE clean-up is shown in Figure 1.

Before analysis a cartridge is put into the clamp of the automatic cartridge exchanger (ACE). Subsequently the cartridge is conditioned and equilibrated with methanol and water, delivered by the high-pressure dispenser (HPD). After this, an aliquot of the serum sample

Figure 4: Infusion profiles of 3 different analytes after loading 25 µL of human serum or water with 2 mL water 0.1% formic acid.

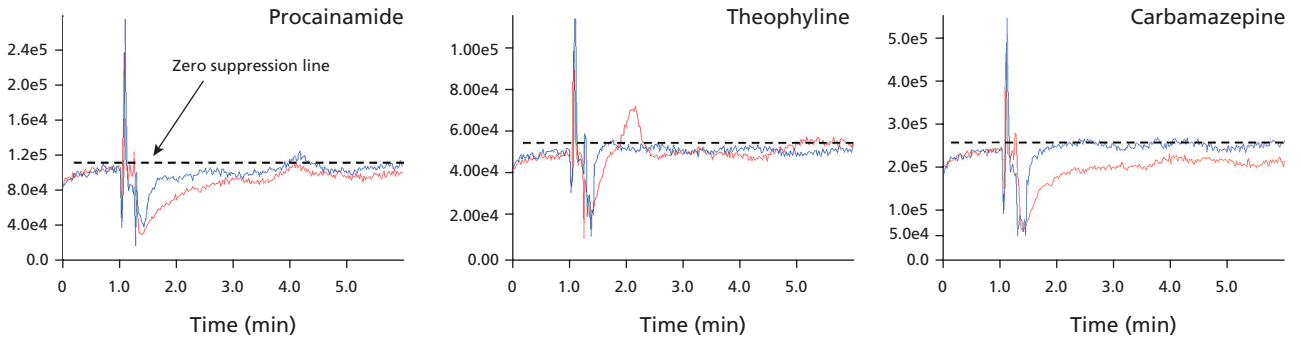
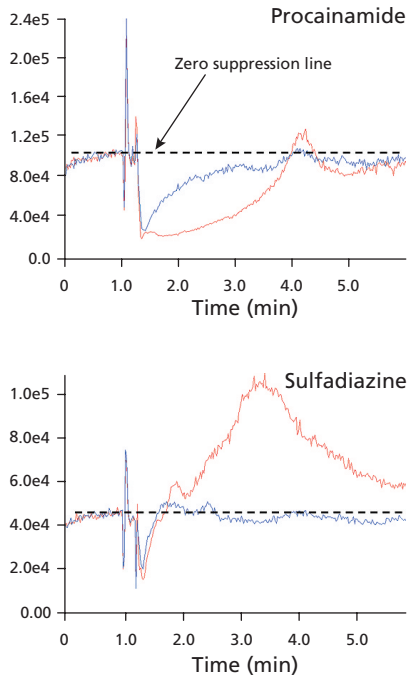


Table 2: Peak heights (mAU at 280 nm) of elution fractions after sample loading (water, 0.1% formic acid) and washing under various denaturing conditions.

Wash conditions	Calf serum (100 µL)	Porcine serum (25 µL)	Human serum (25 µL)
Sample loading at 1 mL/min (2 mL)	100	700	155
Sample loading at 10 mL/min (2 mL)	100	80	120
Sample loading at 1 mL/min (4 mL)	90	500	150
1 mL H_20*	110	70	120
1 mL trifluoroacetic acid (TFA 10%)*	55	15	45
1 mL sodium hydroxide (NaOH 0.1M)*	55	10	50
1 mL zinc sulfate ($ZnSO_4$ 50 mg/mL)*	85	35	35
2 mL H_20 at 90 °C	28	12	22
SPE after off-line denaturation with $ZnSO_4$	80	60	110
SPE after off-line denaturation with TCA	280	160	190

* After sample loading (2 mL, 1 mL/min) the wash step is performed at 5 mL/min, followed by a 1.5 mL water flush (5 mL/min) to remove the wash solvent from the cartridge before elution.

Figure 5: Infusion profiles of 2 different analytes after loading 25 µL of human serum with 2 mL water 0.1% formic acid and washing with water (blue trace) or $ZnSO_4$ (red trace).



can be pulled into the sample loop of the autosampler. After switching the injection valve, the sample is loaded onto the cartridge with acidified water delivered by the HPD. Non-retained matrix compounds are flushed to waste via the UV detector.

Subsequently the cartridge is washed with solvents selected by the HPD. Finally valve 2 is switched: mobile phase elutes the retained matrix compounds to the UV detector.

Experimental Conditions *(Suppression Monitoring)*

Experimental setup for suppression monitoring is shown in Figure 2. ESI-MS = API 2000 (Applied Biosystems, Foster City, California, USA); ion monitoring mode = positive; curtain gas (CUR) = 40 psi; ionspray voltage (IS) = 3000 V; temperature (TEM) = 350 °C; ion source gas 1 (GS1) = 40 psi; ion source gas 2 (GS2) = 85 psi; declustering potential (DP): see Table 1; focusing potential (FP) = 360 V; entrance potential (EP) = see Table 1; dwell time = 200 ms; Harvard syringe pump = 10 µL/min; LC flow-rate = 200 µL/min.

Results

UV monitoring of clean-up: Figure 3 shows typical SPE–UV traces that are used to estimate the amount of serum proteins that are washed to waste or elute (see Figure 1 for experimental setup).

95–99% of the serum proteins go directly to waste during sample loading with 2 mL water/0.1% formic acid. Less than 1% of the serum proteins elute with mobile phase, and less than 4% remain on the cartridge after elution.

The effect of various wash conditions on clean-up was investigated using UV monitoring (Table 2). High-speed sample

Figure 6: Infusion profiles of 3 different analytes after loading 25 µL of human serum with 2 mL water 0.1% formic acid and washing with water (blue trace) or TFA (red trace).

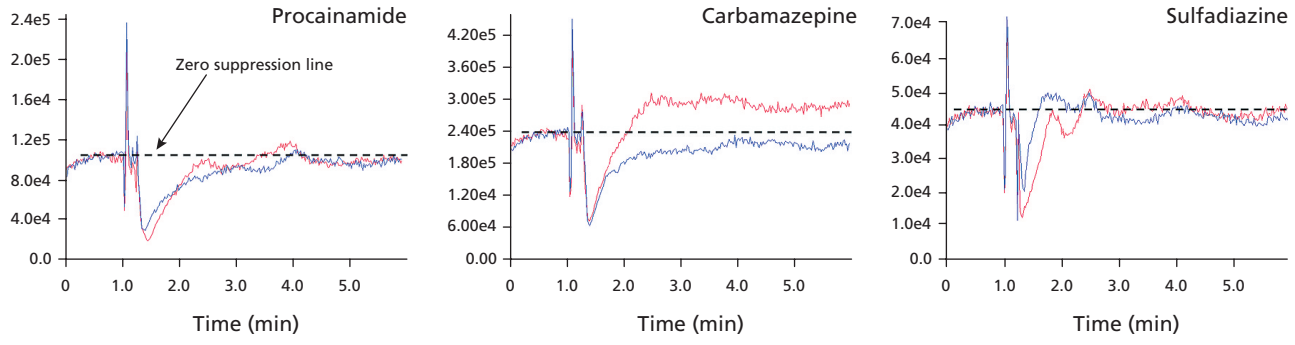


Table 3: Analyte recovery after clean-up of spiked calf serum (25 µL) under various load and wash conditions.

Wash conditions	Carbamazepine	Ibuprofen	Propranolol
Sample loading at 1 mL/min (2 mL)	>97%	>97%	>97%
Sample loading at 10 mL/min (2 mL)	>97%	<50%	>97%
1 mL H_20*	>97%	>97%	>97%
1 mL trifluoroacetic acid (TFA 10%)*	>97%	>97%	>97%
1 mL sodium hydroxide (NaOH 0.1M)*	>97%	>97%	>97%
1 mL zinc sulfate ($ZnSO_4$ 50 mg/mL)*	>97%	>97%	>97%
2 mL H_20 at 90 °C	>97%	>97%	<50%

* After sample loading (2 mL, 1 mL/min) the wash step is performed at 5 mL/min, followed by a 1.5 mL water flush (5 mL/min) to remove the wash solvent from the cartridge before elution.

loading (10 mL/min) gives cleaner extracts. On-line denaturing SPE improves clean-up significantly and also results in cleaner extracts than traditional off-line denaturation followed by SPE.

Analyte recovery: The recovery of analytes under varying load and wash conditions is shown in Table 3. High-speed sample loading (10 mL/min) gives a recovery loss for Ibuprofen. Washing at 5 mL/min after sample loading at 1 mL/min gives no recovery loss. With the exception of propranolol, a 90 °C water wash does not reduce analyte recovery.

Suppression monitoring: Using the system as described in Figure 2 we investigated the suppression effects of human serum, as compared with water, on the ionization of three different analytes (Figure 4).

The suppression effect observed for the water sample results from introduction of the water content of the cartridge after loading the sample. The effect of ionization suppression by matrix compounds is strongly dependent on the type of infused drug.

Analyte infusion profiles for ZnSO4 wash: Figure 5 shows the effect of a denaturing wash with ZnSO4 on the ionization suppression of two different analytes compared with a water wash. Washing with ZnSO4 does not result in less matrix effects on the ionization of procainamide and sulfadiazine. On the contrary, additional suppression is observed for procainamide and a strong enhancement effect for sulfadiazine. Similar effects were found for acetaminophen, carbamazepine, ranitidine and theophyline. Apparently, reduction of the elution fraction observed by UV monitoring when washing with ZnSO4 does not automatically translate into reduction of ionization suppression.

Analyte infusion profiles for TFA wash: Figure 6 shows the effect of a denaturing wash with TFA on the ionization suppression of three different analytes compared with a water wash. Washing with TFA has no favourable effect on the ionization of procainamide, carbamazepine and sulfadiazine. However, compared with ZnSO4 , washing with TFA gives less fluctuations in the ionization profiles. Similar effects were found for acetaminophen, ranitidine and theophyline. Apparently, reduction of the elution fraction observed by UV monitoring when washing with TFA does not automatically translate into reduction of ionization suppression.

Analyte infusion profiles for a 90 °C water wash: Figure 7 shows the effect of a denaturing wash with 90 °C water on the ionization suppression of three different analytes compared with a water wash at ambient temperature.

In contrast to ZnSO4 and TFA, washing with 90 °C water clearly reduces matrix ionization suppression of procainamide, acetaminophen and ranitidine. Similar effects were found for carbamazepine, sulfadiazine and theophyline.

Conclusions

High-speed loading and denaturing wash conditions for on-line SPE result in a significant reduction of matrix compounds eluting to the LC–MS as observed by UV monitoring. On-cartridge denaturing with ZnSO4 or TFA resulted in additional ionization suppression or enhancement. On-cartridge denaturing with hot water (90 °C) resulted in a reduction of ionization suppression. On-line SPE with on-cartridge denaturing provides better clean-up than off-line protein precipitation prior to on-line SPE and is completely automated.

References

1. J.D. Gilbert, T.V. Olah and D.A. McLoughlin, *Biochemical and Biotechnological Appl. of Electrospray Ionization Mass Spectrometry*, American Chemical Society (1995).
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3. R.Bonfiglio et al., *Rapid Commun. Mass Spectrom.*, **13** (1999) 1175–1185.

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Figure 7: Infusion profiles of 3 different analytes after loading 25 µL of human serum with 2 mL water 0.1% formic acid and washing with water at ambient temperature (blue trace) or water at 90 °C (red trace).