Evaluation of an automated SPE method for samples derived from capillary microsampling

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Introduction

Alternatives to animal testing and the development of methods that help reducing the number of animals in preclinical studies are much sought after. In capillary micro sampling, a low volume of blood (for example 30 µL) is collected in a capillary, centrifuged and an exact volume of plasma (for example 10 µL) transferred into another capillary. For analysis, the plasma is washed or blown out into a sample tube and diluted to a volume, that can be handled reliably and reproducibly. In contrast to DBS techniques, plasma is processed and analyzed, thus avoiding investigation of critical parameters like effect of hematrocit and offering standard procedures for addition of stabilizers or internal standards during analysis. Even though necessary, dilution should be minimized as it directly affects the achievable limits of quantification. In this poster we describe an automated SPE procedure using small, single use cartridges in a modified CTC liquid handling auto sampler. Results from this approach are compared to results obtained from identical samples processed manually by protein precipitation.

Experimental

General: Samples were processed by dilution of spiked plasma with water containing the internal standard followed by online sample-preparation described below.

Working-solutions and sample preparation: The analyte (MW 581) was dissolved in DMSO and diluted in ACN/DMSO (50:50, v/v) to obtain working-solutions at concentrations twenty times higher than those of the final CAL and QC samples. Aliquots of the working-solutions were spiked into blank rat plasma and diluted with water containing the internal standard in a ratio of 1:10 (v/v). The samples were vortexed, centrifuged and transferred to the ITSP sample-preparation plate.

Online-sample-preparation: ITSP (ITSP Solutions Inc.) cartridges were conditioned with 80µL MeOH and 80µL 0.2% aqueous formic acid before loading 10µL sample. After drying the cartridge with 80µL air, the ITSP cartridges were washed twice with 80µl 0.2% aqueous formic acid and dried again. The compounds were eluted using 80µL EtOH and 10µL of the eluate was injected onto the analytical column. Sample-preparation of each plasma sample was performed directly before the injection (see Figure 1 for the workflow and the results of the optimization process).

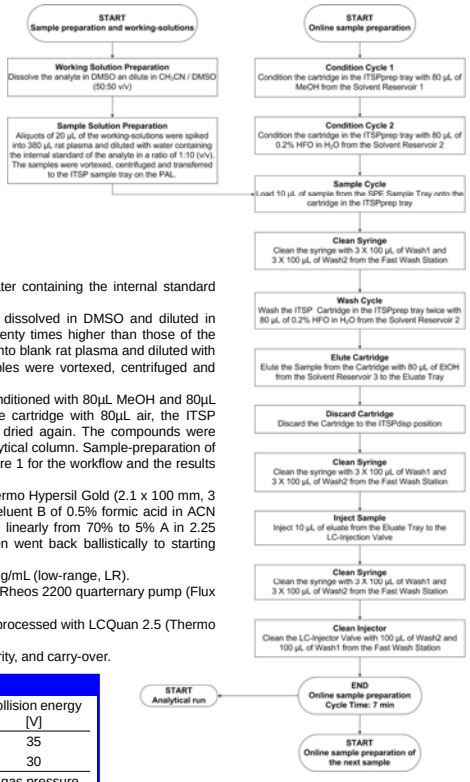
Chromatography: 10 µL of the extracted samples were injected onto a Thermo Hypersil Gold (2.1 x 100 mm, 3 µm) analytical column. Eluent A consisted of 0.5% aqueous formic acid, eluent B of 0.5% formic acid in ACN and LC flow rate was 0.5 mL/min. The eluent composition was changed linearly from 70% to 5% A in 2.25 minutes. Eluent composition was kept at 5% B for one minute and then went back ballistically to starting conditions.

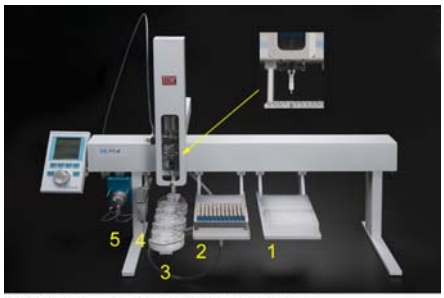
Calibration range: 50.0 - 25'000 ng/mL (high-range, HR) and 5.00 - 2'500 ng/mL (low-range, LR).

Instrumentation and MS-method: TSQ Quantum Ultra (Thermo Scientific), Rheos 2200 quarternary pump (Flux Instruments), HTX PAL autosampler (CTC Analytics).

Data-Processing: Raw data were acquired with Xcalibur 2.0 software and processed with LCQuan 2.5 (Thermo Scientific).

Validation parameters: Intra- and inter-assay accuracy and precision, linearity, and carry-over.





1: TrayHolder 1	Front Position: SPE Sample Tray [MT1-Frnt] Rear Position: Filtration Sample Tray [MT2-Rear / ITSPsmpl]
2: TrayHolder 2	Front Position: ITSP Cartridge Tray [ITSPprep] Rear Position: Eluate & Filtrate Tray [MT2-Rear / ITSPelut]
3: Solvent Reservoir	Position1: MeOH Position2: 0.2% aq. HFo Position3: EtOH
4: WashStation	Wash1: H2O/EtOH (50:50 v/v) Wash2: ACN/MeOH/i-Prop (1/1/1 v/v/v) Waste
5: Injection Valve	

Results

Blank rat plasma was spiked with the analyte and diluted with water containing the internal standard in a ratio of 1:10 (v/v). CALs and QCs were prepared to cover concentrations of 50.0 - 25'000 ng/mL in the high-calibration range and 5.00 - 2'500 ng/mL in low calibration range. The diluted plasma samples were transferred to the ITSP sample-preparation plate and automatically extracted directly before analysis. Sample preparation and LC-MS/MS measurements were performed in parallel using the "Prep Ahead Function" of the CTC autosampler (Figure 4). Chromatograms of the lowest calibration standard Cal1 are presented in Figure 2 and compared with the chromatograms of the same calibration standard prepared by protein-precipitation.

Three independent precision/accuracy runs were conducted for both calibration ranges and the intra- and inter-run variabilities are presented in Table 2. An example of a typical calibration curve for each range is presented in Figure 3. Both assays gave very similar results and the intra- and inter-run performance is equivalent to the data obtained using a standard protein-precipitation method.

The carry-over in the analytical assay does not meet the acceptance criteria of the EMA guideline but can be significantly reduced by using 0.3% Tween as an additive in the aqueous wash-solution (see Table 3).

The influence of the amount of (diluted) plasma loaded onto the cartridge and the volume of the extraction solvent is presented in Figures 6 and 7. Elution from the ITSP cartridge is complete with as little as 20 µL of extraction solvent (Figure 7) resulting in a factor 4 higher concentration of the analyte in the eluate compared to the standard-elution method. The capacity of the cartridges has been tested between 2 to 40 µL diluted plasma (Figure 6). The area ratio of analyte to internal standard is constant across this range and demonstrate excellent performance of the method across a wide range of sample volumes.

The sample-preparation time and the overall run-time of the automated and manual method are compared in Figure 5. Since only minimal manual intervention is required for the automated approach, this workflow offers significant advantages by high sample-workload and longer LC-MS/MS run-times.

Table 1. MS-parameter (SRM-assay).

Analyte	Parent ion mass [Da]	Product ion mass [Da]	Collision energy [V]
Analyte	582.3	255.1	35
Internal Standard	588.3	487.2	30

ESI positive, spray voltage 3.5 kV, capillary temperature 350 °C, collision gas pressure (Ar) 1.5 mTorr, scan-time 100 msec

Figure 1. Workflow and results of the optimization process.

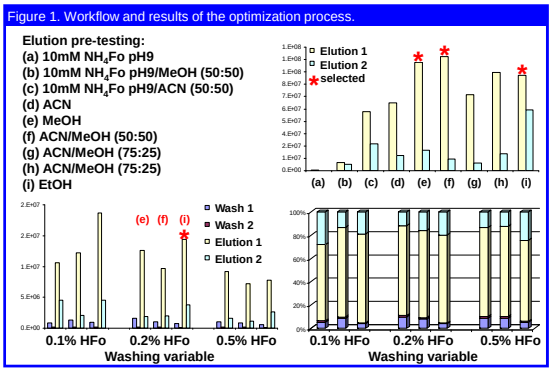


Table 2. Intra- and inter-run precision and accuracy (3 runs, [%]).

	at LLOQ (LR)	at LLOQ (HR)	> LLOQ (LR)	> LLOQ (HR)
$Prec_{intra}$	9.7-10.5%	5.0-6.0%	1.5-10.5%	1.4-8.3%
Acc_{intra}	93.2-107.4%	95.7-113.0%	101.0-105.2%	95.5-113.5%
$Prec_{inter}$	11.1%	8.9%	4.4-9.5%	2.5-8.1%
Acc_{inter}	100.6%	105.1%	101.0-105.2%	104.8-108.6%

Figure 2. Comparison of protein-precipitation (PP) and ITSP at LLOQ.

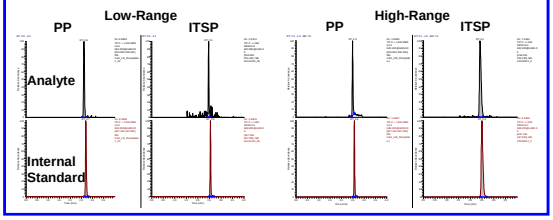


Figure 3. Calibration-curves (low-range assay left, high-range assay right).

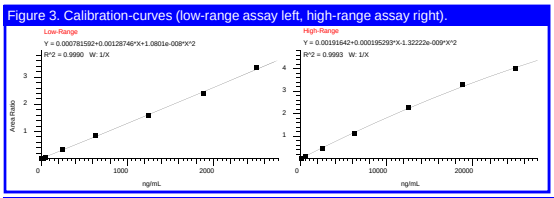


Figure 4. Prep Ahead Function of the CTC autosampler.

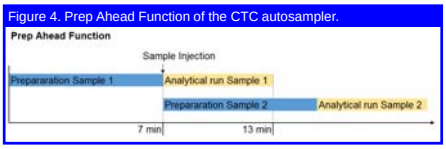


Figure 5. Comparison of protein-precipitation (PP) and ITSP (# Samples: variable; gradient-time: variable; ITSP: sample-preparation 7min in parallel; PP: pre-injection-time 1.0min).

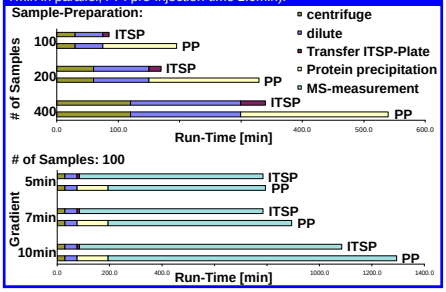


Figure 6. Linearity of the loading-volume and capacity of cartridges.

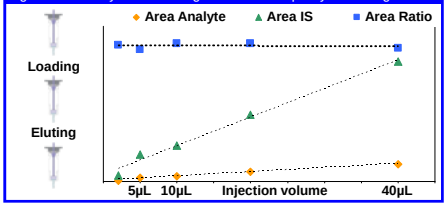


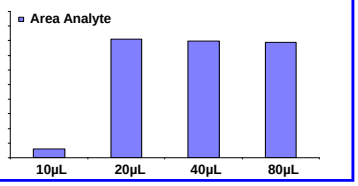
Table 3. Carry-over.

Calibration-Range	Carry-over* [% of CAL8]	Carry-over* [% of Cal1]
Low-Range [1st Blank after Cal8]	0.6 **	229.7 **
Low-Range [2nd Blank after Cal8]	0.2 **	80.3 **
High-Range [1st Blank after Cal8]	0.7	278.6
High-Range [2nd Blank after Cal8]	0.3	108.5

* Standard-Wash-solution; Wash 1: H_2O/EtOH (50:50, v/v); Wash 2 ACN/MeOH/i-Prop (1:1:1, v/v/v)

** Carry-Over can be significantly reduced using 0.3% Tween in Wash-solution 1 (below 30% of Cal1 for the 2nd Blank after Cal8)

Figure 7. Volume of the elution solvent EtOH (area of analyte normalized to concentration).



Conclusions

•The automated workflow described above gave equivalent results (LLOQ, precision, accuracy) compared to samples processed manually by protein precipitation. The overall time/sample is slightly higher for the automated protocol. However, since only minimal intervention is required, process safety is superior and human resources are freed up for other tasks.

•The automated protocol may be further optimized in several aspects. As shown in Figure 7, a volume of 20 µL is sufficient for complete elution of the analyte from the cartridge. Figure 6 indicates that an loading volume of 2-5 µL diluted plasma is sufficient allowing the use of low-volume samples. Both aspects are important for further optimizing this protocol for analysis of samples originating from studies using microsampling techniques.

•Methods for the automated optimization of the SPE conditions exist and will be applied in future studies.

Reference: Nilsson LB, Ahnoff M, Jonsson O; Bioanalysis (2013) 5(6), 731–738