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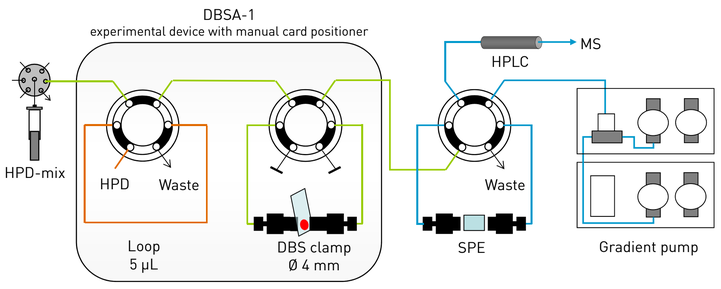
Method Development and Validation for Dried Blood Spot Analysis based on Flow Through Desorption, Solid-Phase Extraction and Mass Spectrometry

Introduction

Reduction of blood volume required for bioanalysis is of great importance for pharmacological data from pre-clinical studies and for animal welfare. Ideally, blood volumes taken should allow single-animal pharmacokinetic (PK) studies. For small animals such as rats and in particular mice, this is challenging, since only a few µL of blood can be withdrawn at every time point. In an earlier study we showed that small blood sample volumes could be collected on specially designed "sorbent sampling" cartridges and analyzed using a Spark Holland Symbiosis system.[1] The technique was successfully applied to record full PK data from single mouse. Recently, a new technology for online desorption of dried blood spots cards has replaced the sorbent sampling format.[2] In this work the results of a first evaluation of this new technology for very small blood volumes are shown.

Experimental conditions

Online DBS-SPE-LC (Spark Holland)



DBS

Filter card:	Ahlstrom DMPK-300
Sample:	1 µL mouse blood
Desorption:	0.75 mL 5/95 ACN/water 0.2% FA at 0.75 mL/min
Clamp flush:	1 mL 80/20 ACN/water 0.2% FA at 5 mL/min
	1 mL 5/95 ACN/water 0.2% FA at 5 mL/min

SPE

Cartridge:	HySphere C18HD 10x2 mm, 7 µm
Conditioning:	1 mL ACN at 5 mL/min
Equilibration:	1 mL 5/95 ACN/water 0.2% FA 5 mL/min
Sample transfer:	see Desorption solvent
Cartridge wash:	1 mL 10/90 ACN/water 0.2% FA at 2mL/min
Elution:	LC gradient

LC

Mobile phase: Gradient A) water 0.2% FA; B) ACN 0.2% FA

Gradient:

Time (mm:ss)	Flow (mL/min)	A %	B %
00:01	0.7	95	5
00:20	0.7	95	5
03:10	0.7	0	100
03:40	0.7	0	100
03:45	0.7	95	5
04:30	0.7	95	5

Column: Waters XBridge C8 (3.5 µm, 3 x 50 mm)

Pre-column: Phenomenex Guard Cartridge C8 endcapped (4 µm, 4 x 2.0 mm)

Column oven: 40 °C

APCI-MS (4000 QTrap, AB Sciex)

APCI-MS/MS conditions (positive mode)

MS settings

Compound	Parent (m/z)	Daughter (m/z)	General settings	
IS 1 (Steroid)	292	97		
IS 2	277	202		
IS 3	568	536	Curtain gas	25 psi
			Temperature	450 °C
Compounds			Gas 1	25 psi
A: $C_{17}H_{16}ClNO$	286	44	Dwell time	0.05 s
B: $C_{17}H_{16}N_2O$	265	233	CAD	8 psi
C: $C_{28}H_{30}O_4$	431	135	Needle current	5 µA
D: $C_{27}H_{33}ClN_4O_3$	497	126	Entrance potential	10 V
E: $C_{23}H_{28}N_6O_3S_2$	501	428		
F: $C_{12}H_3N_3O_2H_{10}$	232	147		

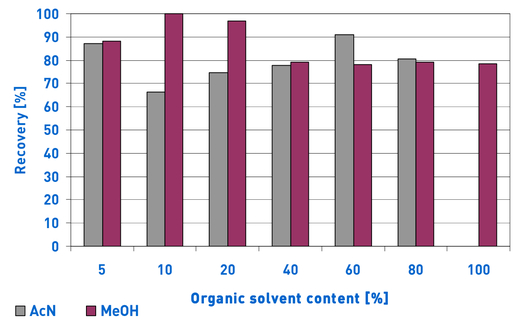
Results and Discussion

Desorption of whole DBS from card

1 µL blood is pipetted on the sample paper using a gel-saver tip. The entire blood spot is clamped within the 4 mm internal clamp diameter by careful manual positioning. No sample is wasted. The amount of blood is limited to 1 µL due to the punch size of 4 mm. Bigger punches would allow the use of larger blood spots. Herewith, a better precision of sample application and a better sensitivity can be expected.

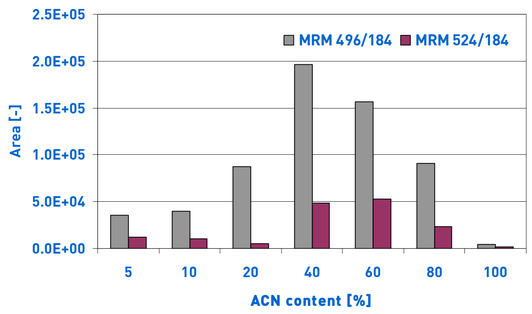
Effect of desorption solvent composition

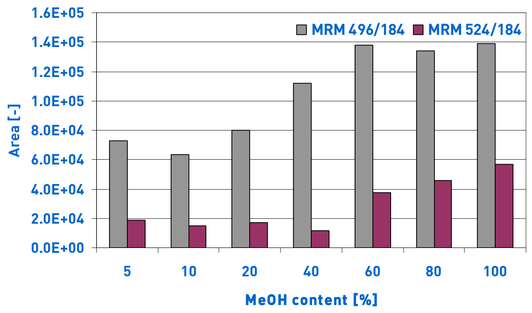
1 µL-blood spots containing 1 µmol/L internal standard 3 are desorbed with 750 µL of desorption solvents of different organic liquid contents. The flow rate is 750 µL/min. Fractions of the samples are collected in vials at the outlet of the DBS clamp and injected into the LC-MS/MS system.



- No response is observed with 100% ACN as desorption solvent!
- No major differences in IS 3 recovery by different desorption solvent compositions are observed.

The presence of phospholipids (PLs) is also investigated in the collected fractions. Therefore, LC-ESI/MS/MS measurements in positive ion mode are carried out. The following two literature-known MRM transitions are monitored as representative for a number of frequently occurring PLs:
496 → 184 and 524 → 184.

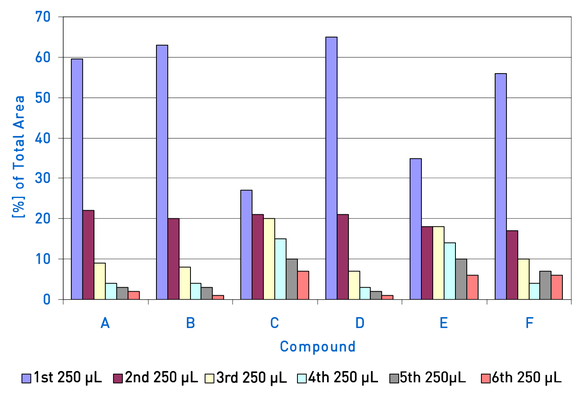




- PLs desorption is dependent on the organic content of the desorption solvent.
- Almost no PLs response is observed with 100% ACN as desorption solvent!
- Low organic contents are advantageous as low PL concentration and high analyte recoveries are obtained.

Dependence of analyte recovery on desorption volume

A 1 µL DBS containing compounds A-F is desorbed in 6 steps à 250 µL of 5%ACN 0.2%FA in water at 750 µL/min (total desorption volume = 1500 µL). For every step the desorbed DBS sample is trapped on a new SPE cartridge and after clean-up eluted to MS/MS.



- A total desorption volume of 1000 µL is necessary to obtain a recovery ≥ 80% for all compounds (typical drugs covering a wide range of physicochemical parameters → log D, pKa).

Validation

The DBS method is validated with respect to precision, selectivity, carry-over and sensitivity. The results are compared with those obtained by a LC-MS/MS method based on protein precipitation (PP). For sample preparation, 50 µL blood is mixed with 50 µL IS 3 (in DMSO) and 250 µL ACN (which contains IS 1 and IS 2). The supernatant (5 µL) is directly injected into the LC/MS system.

Precision of internal standard

The precision of the IS determination is measured. The average peak areas of the IS added to 44 different standards and quality controls are calculated.
In case of DBS analysis IS1 and IS2 are added via a loop. Therefore, the loop is filled with the ISs by means of a High Pressure Dispenser (HPD). The IS solution is afterwards flushed over the DBS card towards the SPE together with the desorption solvent. For PP IS 1 and IS 2 are added together with the precipitation solvent ACN. Blood is spiked with IS 3 in all experiments.

IS Precision

Compound		Average Area [-] (n=44)	CV [%]
IS 1	DBS	8.2E+05	14.1
	PP	3.8E+05	5.4
IS 2	DBS	4.9E+05	6.9
	PP	1.3E+05	5.2
IS 3	DBS	1.9E+05	7.3
	PP	1.7E+05	6.2

- The precision of the IS measurements is comparable for DBS and PP. In case of IS 1 precision the CV for DBS analysis is higher but still acceptable.

Accuracy and Precision of the method

The accuracy and precision of the measurements of 100 and 4000 ng/mL QCs is determined. The DBS results shown are based on IS 2 (added via a loop).

Analyte Accuracy and Precision

Compound		100 ng/mL Average Accuracy [%] (n = 4)	100 ng/mL CV [%]	4000 ng/mL Average Accuracy [%] (n = 4)	4000 ng/mL CV [%]
A	DBS	90	11.1	96	4.0
	PP	104	5.3	93	4.0
B	DBS	107	8.0	97	4.6
	PP	95	6.4	106	4.4
C	DBS	106	6.4	109	10.6
	PP	99	2.7	105	5.4
D	DBS	112	8.5	112	9.9
	PP	102	6.6	100	5.2
E	DBS	105	13.3	114	16.7
	PP	102	6.3	101	11.5
F	DBS	103	6.1	100	4.5
	PP	99	5.1	105	2.6

- A good overall accuracy is obtained for all DBS and PP measurements.

Carry-over

The carry-over is investigated by measuring a blank DBS (reference), a high standard and a blank DBS in turn. For the high standard, mouse blood spiked with 5000 ng/mL of compounds A-F is used.

For DBS analysis an additional clamp flush using a blank filter card is carried out between the measurements.

Carry-over Compound E (10000 ng/mL)

	No Clamp Flush Peak Area	No Clamp Flush Carry-over [%]	Clamp Flush Peak Area [-]	Clamp Flush Carry-over [%]
Sample	2.07E+06		1.89E+06	
Blank	8.43E+03	0.41	7.77E+02	0.04

- No peak was detected at all in the reference blank measurements.
- Carry-over is for all measurements within the acceptance range (≤0.05%).
- For most of the compounds less carry-over is observed for DBS analysis than for PP (data not shown).

Sensitivity

The sensitivities are determined by measuring blood spiked with compounds A-F in the concentration range 1 – 5000 ng/mL. The LLOQs mentioned are based on calculations with internal standard 3.

Sensitivity

	LLOQ [ng/mL] A	B	C	D	E	F
DBS	100	5	5	1	1	20
PP	5	2	1	1	1	1

- LLOQs are slightly higher for DBS. Improvement could possibly be achieved by using bigger punches.

Conclusion

- A new SPE-LC-MS/MS method for analysis of very small blood volumes from DBS cards was developed and validated.
- 1 µL-blood spots are desorbed in a flow through manner directly onto a SPE-LC-MS/MS system cartridge for online clean-up, followed by LC/MS/MS analysis.
- Low organic liquid content in the desorption solvent can avoid co-elution of large amounts of phospholipids.
- Using 100% ACN as desorption solvent no DBS compounds are detected. This observation is possibly based on wetting properties of aprotic ACN. Further investigations with different ananlytes have to be carried out.
- Validation of the method is done in comparison to a method based on offline protein precipitation. The two methods yield comparable results.
- Carry-over is negligible for DBS analysis executing a clamp flush step between the measurements.
- Precision for DBS using IS loop injection is satisfying and comparable to the traditional PP method.
- For some compounds the LLOQ is higher for DBS analysis in comparison to LC. The improvement of the DBS sensitivity can be expected by using bigger punch sizes.
- Online DBS desorption combined with online SPE-LC-MS/MS provides a highly automated analytical concept for analysis of very small blood samples in support of pre-clinical bioanalysis.
- As the DBS technique allows the application of very small blood volumes, full PK data can be expected from individual mice. Former investigations already indicated that PK curves obtained from individual animals are a better representation of the kinetics than those of multiple animals.[2]

Literature: **[1]** BA Ingelse, G Vogel, M Botterblom, D Nanninga, B Ooms. Rapid Commun Mass Spectrom 2008;22(6):834-40; **[2]** JA Ooms, L Knegt, EHM Koster. Bioanalysis 2011;3(20):2311-20