Internal standard addition for dried blood spot analysis based on flow through desorption solid-phase extraction and mass spectrometry

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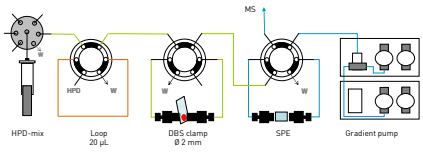
Introduction

Dried Blood Spot (DBS) analysis has many advantages with respect to sampling, shipment and storage. Multiple studies have been performed (e.g. by GSK) to demonstrate the benefits of DBS processing. A major issue in DBS analysis is the addition of the internal standard, because adding the internal standard to the sample before applying it on the filter paper is hardly possible. In this paper an on-line DBS extraction/analysis concept is presented that utilizes flow through desorption of the bloodspot, coupled directly to mass spectrometric analysis via online solid-phase extraction. Moreover, various ways of adding the internal standard are investigated and discussed.

Experimental conditions

Online DBS-SPE (Spark Holland)



DBS

Filter card:	Whatman FTA™ DMPK-C Card (Cat No Wb129243)
Sample:	15 µL Human blood (K_3-EDTA)
Desorption solvent:	1 mL water 0.2% FA at 2 mL/min (= sample transfer)
Clamp flush:	1 mL 80/20 acetonitrile/water 0.2% FA at 5 mL/min
	1 mL water 0.2% FA at 5 mL/min

SPE

Cartridge:	HySphere C18HD 10x2 mm, 7 µm
Conditioning:	1 mL acetonitrile at 5 mL/min
Equilibration:	1 mL water 0.2% FA at 5 mL/min
Sample transfer:	1 mL water 0.2% FA at 2 mL/min
Cartridge wash:	1 mL 5/95 acetonitrile/water 0.2% FA at 5 mL/min
Elution:	Gradient A) water 0.2% FA; B) acetonitrile 0.2% FA

Gradient:

time (m:s)	flow (mL/min)	A %	B %
00:01	1.0	95	5
00:05	1.0	95	5
01:45	1.0	75	25
02:50	1.0	75	25
02:51	1.0	95	5
03:10	1.0	95	5

Clamp flush: 1 mL 80/20 acetonitrile/water 0.2% FA at 5 mL/min

ESI-MS (Acquity TQD, Waters)

ESI-MS/MS conditions (positive mode)

MS-settings

Compound	Parent (m/z)	Daughter (m/z)	General settings	
Used as internal standard in loop / on card			Capillary voltage	2.00 kV
Propranolol	260.1	116.1	Cone voltage	40 V
Imipramine	281.3	85.9	Cone gas	50 L/hr
Verapamil	455.3	165.2	Source temp	150 °C
			Desolvation temp	350 °C
Used as internal standard in blood			Desolvation gas	500 L/hr
Haloperidol	376.1	165.1	Collision gas	0.20 mL/min
Amitriptyline	278.1	90.8	Dwell time	0.05 s

Results and Discussion

Internal standard addition via loop

A 20 µL loop is filled with IS-solution (2 ng/ml propranolol in 5/95 ACN/water).

The IS is flushed over a card, spotted with blank blood, towards the SPE cartridge by means of a high pressure dispenser (HPD).

A gradient is run over the C18HD cartridge to facilitate some chromatographic separation before ESI-MS/MS detection.

A relative standard deviation of 2.5% was obtained (n=8) indicating a good precision for this new IS-addition approach.

The test was repeated with multiple compounds in the IS-solution (2 ng/ml propranolol, imipramine and verapamil in 5/95 ACN/water). Furthermore, blood spiked with IS (200 ng/mL haloperidol and amitriptyline) is spotted on the filter card.

The following results were obtained for the complete DBS-SPE-MS/MS approach.

IS addition via loop (n = 8)

	Propranolol	Imipramine	Verapamil	Haloperidol	Amitriptyline
Average peak area	419.9	1993.8	1923.6	11152.6	5076.6
Repeatability (% RSD)	4.7	4.1	5.2	3.9	4.5

- Approach is comparable to the punch-out and desorb methodology in which IS is added to the desorption liquid.
- Good repeatability of this automated IS addition methodology is obtained.
- RSD is comparable with ideal situation in which IS is added to blood sample (haloperidol, amitriptyline). The latter being difficult to achieve in practice.
- High flexibility is obtained as various internal standards can be used per / between DBS-SPE-MS/MS runs.
- IS addition approach does not compensate for variations in spot desorption.

Internal standard addition on blank Card

15 µL of IS-solution (200 ng/ml propranolol, imipramine and verapamil in MeOH) is pipetted onto a filter card and dried for 1 hr.

The IS is desorbed from the card, towards the SPE cartridge utilizing the flow-through desorption methodology.

A gradient is run over the C18HD cartridge to facilitate some chromatographic separation before ESI-MS/MS detection.

The following results were obtained for the complete DBS-SPE-MS/MS approach.

IS addition on card (n = 8)

	Propranolol	Imipramine	Verapamil
Average peak area	496.8	2750.0	1575.5
Repeatability (% RSD)	5.0	3.7	4.9

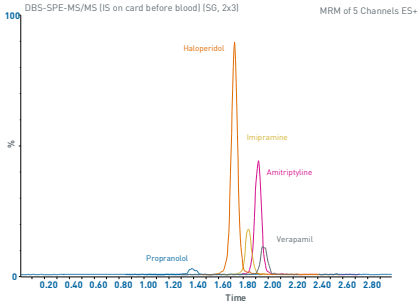
- Internal standard can be spotted on the filter paper with good precision.
- This IS addition approach is less flexible as filter cards should be pretreated with a specific compound before a blood sample can be spotted.
- Preliminary tests with a sprayer to apply the IS onto a card showed bad repeatability. RSD values of 10-25% have been found, and many factors still need to be optimized to reach the same (or better) performance as achieved by pipetting.

Internal standard addition on Card before blood spotting

15 µL of IS-solution (200 ng/ml propranolol, imipramine and verapamil in MeOH) is pipetted onto a filter card and dried for 1 hr.

Subsequently 15 µL of spiked blood (200 ng/ml haloperidol and amitriptyline) is spotted onto the filter card and dried for at least 2 hr.

Automated DBS-SPE-MS/MS is performed to determine the applicability of the IS-addition approach.

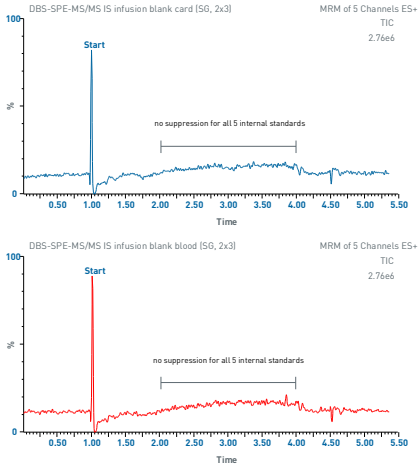


IS addition on card before blood spotting (n = 8)

	Propranolol	Imipramine	Verapamil	Haloperidol	Amitriptyline
Average peak area	283.0	1568.7	1260.0	10789.7	4677.6
Repeatability (% RSD)	8.9	4.2	3.7	3.8	3.2

- The repeatability of IS addition is within the acceptance criteria, but the average peak area of the initial IS on card is reduced (that of IS in blood remains the same).

Ionization suppression cause of reduced IS peak area?



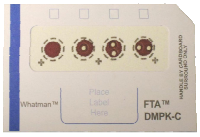
Drying time of IS on card cause of reduced IS peak area?

Effect IS drying time (min) on measured IS peak area (n = 3)

Drying time	Propranolol	Imipramine	Verapamil
30	237.4	1467.1	1211.4
60	258.0	1563.0	1190.6
90	288.3	1541.6	1040.9
160	312.5	1732.3	1239.7

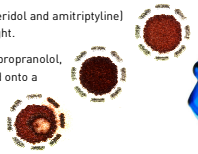
- The IS drying time may affect the measured IS level as shown for propranolol.

Spot desorption position cause of reduced IS peak area?



- No differences in the measured IS levels were found across the spot.
- Supplementary tests indicate that the IS migrates on the paper during blood spotting.

Internal standard addition on Card after blood spotting



15 µL of blood spiked with IS (200 ng/ml haloperidol and amitriptyline) is spotted onto the filter card and dried overnight.

Subsequently 7.5 µL of IS-solution (200 ng/ml propranolol, imipramine and verapamil in MeOH) is pipetted onto a filter card and dried for 1 hr.

The use of highly aqueous IS solutions is not recommended as it affects the blood spot.

Automated DBS-SPE-MS/MS is performed to determine the applicability of the IS-addition approach.

IS addition on card after blood spotting (n = 8)

	Propranolol	Imipramine	Verapamil	Haloperidol	Amitriptyline
Average peak area	1100.5	5510.1	4123.3	9385.5	4180.2
Repeatability (% RSD)	7.3	2.3	4.3	3.7	2.7

- The repeatability of IS addition is within the acceptance criteria, but the average peak area of IS on card is increased compared to that on a blank card (that of IS in blood remains the same).

Volume of IS applied on paper the cause of increased IS peak area?

Effect IS volume (µL) on measured IS peak area (n = 3)

IS volume	Propranolol	Imipramine	Verapamil	Haloperidol	Amitrityline
7.5	1122.2	5828.8	3929.9	9275.9	4309.6
15	1146.9	5609.8	3724.6	9436.6	4249.1

Spot size of IS on paper the cause of increased IS peak area?

IS spot dimensions (7.5 µL)

IS spot	Diameter (mm)	Area (mm²)	Remark
Blank card	8.85	61.5	area ratio is 1.9, thus
Card with DBS	6.54	32.7	IS is more concentrated

- The volume of IS solution applied on a blood spot does not affect the measured IS level, even when it "overflows" the blood spot.
- It was found that the IS solution spreads out over a smaller area when applied on a dried blood spot.

Conclusion

- The concept of dried blood spot desorption by means of a new flow through concept proved to be easy to use and allows for various ways of IS addition.
- Blood samples were directly flushed from the paper towards the SPE cartridge and sufficient clean-up was obtained to measure the IS model compounds Propranolol, Imipramine, Verapamil, Haloperidol and Amitriptyline by means of MS/MS.
- Internal standard addition to the blood before spotting is most ideal as it can compensate for extraction variations as well, though it requires labeled compounds and conflicts with some of the benefits of dried blood spot sampling.
- Internal standard addition via a loop during desorption of a blood spot provides the best overall precision and accuracy. It is flexible but might not compensate for some dedicated assay aspects such as desorption recovery, however.
- Internal standard addition on card can be done with good precision when applied with a pipette. Spraying techniques investigated so far did not meet the bioanalysis guidelines, but are still under investigation.
- Internal standard addition on card before blood spotting is possible but the IS can be flushed from the paper during blood spotting. On the other hand it might compensate for variations in spot sizes during sampling (more research needed).
- Internal standard addition on card after blood spotting is flexible and can be used to compensate for desorption recovery differences. Nevertheless, this approach might affect the original spot depending on the solvent used to apply the IS.
- Various ways of IS addition have been tested and can be used in practice. The question "where should it compensate for" will determine our best choice.

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