Minimizing hematocrit effects in Dried Blood Spot analysis using online DBS-SPE-MS/MS with maximum recovery

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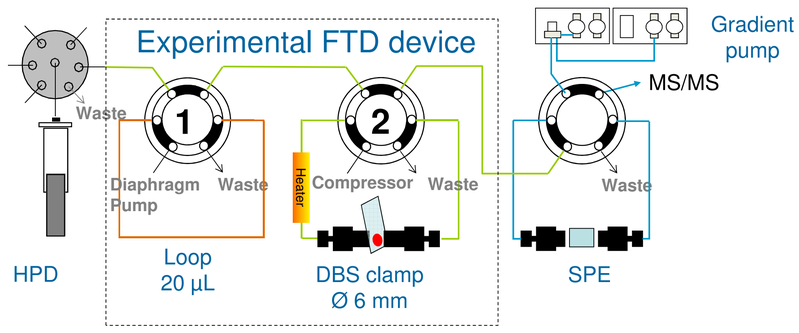
Introduction

Increasing interest in dried blood spot (DBS) analysis is based on benefits as less-invasive sample collection, small sample volumes and easy sample shipping and storage. Recent developments regarding online bloodspot desorption and automated sample preparation procedures improve DBS applications with respect to throughput and error prevention. However, variation in the blood hematocrit (Ht) level still is a serious issue in DBS analysis as it influences spot size and consequently causes deviations in the sample aliquot when only a portion of the spot is used for analysis [1]. The most obvious approach to solve this problem is using the entire spot for analysis (full-spot analysis). This would eliminate the issue of variable spot size, but it requires accurate application of a known volume of blood on the DBS card. Successful implementation of full-spot methods for clinical studies therefore requires robust and easy to use pipetting devices. Not only spot size but also recovery of analytes is influenced by Ht fluctuations [2]. A possible strategy toward a solution here would be to apply more rigorous desorption conditions, aiming at re-dissolution of the entire blood sample. Obviously, because all matrix components are re-dissolved as well, efficient sample clean-up is mandatory. Flow-through desorption of DBS coupled online to SPE and (LC)MS/MS combines total workflow automation with efficient front-end sample clean-up and seems perfectly positioned to follow this strategy successfully [3]. We have explored this approach using full-spot analysis of 5 µl DBS samples. A prototype micropipette (Drummond) was evaluated for ease of use and volumetric precision. An experimental device for flow-through desorption was used in full-spot desorption mode and coupled directly to a system for online SPE-MS/MS. Desorption conditions including pH, organic content and temperature were optimized to achieve maximum dissolution of dried blood. A novel method for measuring DBS recovery and matrix ionization suppression was developed. Recovery was measured for a mixture of test compounds in blood for Ht values ranging from 0.3 - 0.7.

Online DBS-SPE-MS/MS



The experimental Flow-Through Desorption (FTD) device for DBS cards comprised a card clamp, a sliding holder for positioning the card in the clamp and two switching valves. Valve 2 is used for switching the clamp online or offline with the solvent flow to the SPE cartridge and valve 1 for loop injection of a standard solution. High Pressure Dispenser 1 (HPD, Spark Holland) delivers conditioning and equilibration solvent to the SPE cartridge (DBS clamp offline) and desorption solvent via the DBS card (DBS clamp online). A heater placed upstream of the clamp allows heating of the desorption solvent up to 80 °C. The same HPD delivers wash solvent to the cartridge to remove matrix components from the SPE cartridge after trapping (DBS clamp offline). The diaphragm pump is used to fill the loop with standard solution. The loop is switched into the desorption flow stream simultaneously with the start of the desorption. Before opening the clamp, air provided by a mini-compressor flushes solvent from the clamp to waste to avoid spillage. A standard SPE cartridge exchange system (ACE, Spark Holland) is used to provide a new SPE cartridge for every analysis and to switch the SPE cartridge between trapping and elution position. A gradient pump system (Symbiosis LC pump, Spark Holland) elutes the trapped components from the SPE cartridge to the mass spectrometer. The entire system is controlled using SparkLink instrument control software (Spark Holland).

Experimental conditions

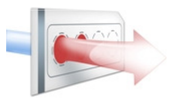
Preparation of spiked blood

50 µL of a standard mix in 40% acetonitrile was added to 950 µL human blood (K_3-EDTA) to obtain blood spiked with Chlortalidone (3.0 µg/ml), Hydrochlorothiazide (0.5 µg/ml), Acebutolol (0.1 µg/ml), Haloperidol (0.1 µg/ml), Verapamil (0.1 µg/ml) and Propranolol (0.2 µg/ml). The spiked sample is equilibrated for at least 1 hour at room temperature and gently mixed several times. For the (approximate) determination of the Ht value, blood was centrifuged for 15 minutes at 3000 rpm. Subsequently, the Ht value was calculated by dividing the volume of blood cells by the total blood volume. Blood with different Ht values was prepared by the addition of plasma (low Ht) or red blood cells (high Ht) to blood.

Standard mixture for recovery measurement using loop injection

The volume of the "20 µL" loop was accurately determined to be 21.87 µL.
For loop injection of equivalent amounts of compounds as present in 5 uL blood spots the following standard mixture of compounds was prepared in 10% acetonitrile with 0.2% FA: Chlortalidone (0.68 µg/ml), Hydrochlorothiazide (0.11 µg/ml), Acebutolol (0.023 µg/ml), Haloperidol (0.023 µg/ml), Verapamil (0.023 µg/ml), Propranolol (0.46 µg/ml).

DBS



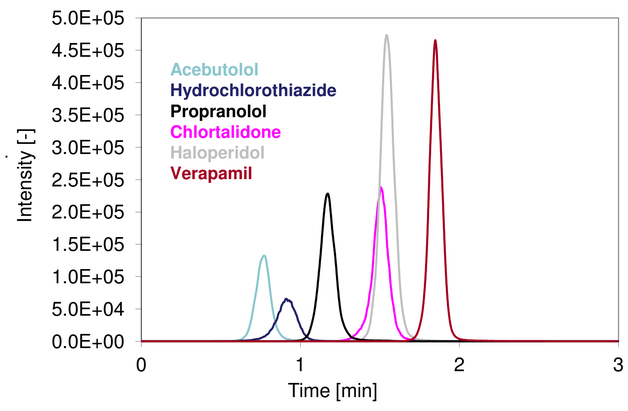
Filter card:	Whatman FTA™ DMPK-C
Sample:	5 µL blood was spotted on the card using a prototype capillary dispenser (Drummond Scientific, Broomall, PA)
Desorption:	1 mL water 0.2% FA at 2mL/min at 80°C + 1 mL 5% methanol 0.2% FA at 5 mL/min at 80°C
DBS Clamp diameter:	6 mm
DBS Clamp flush:	1 mL 80/20 acetonitrile/water 0.2% FA at 5 mL/min
	1 mL water 0.2% FA at 5 mL/min

Combined SPE/LC

SPE Cartridge:	HySphere C18HD (7 µm) 10x2 mm for Acebutolol, Haloperidol, Verapamil and Propranolol HySphere Resin GP (5-15 µm) 10x2 mm for Chlortalidone and Hydrochlorthiazide
Conditioning:	1 mL acetonitrile at 5 mL/min
Equilibration:	1 mL water 0.2% FA at 5 mL/min
Cartridge wash:	1 mL 5/95 acetonitrile/water 0.2% FA at 2 mL/min
SPE 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
Elution:	Solvent: A) water 0.2% FA; B) acetonitrile 0.2% FA Post-cartridge flow-split to 200 µL/min

Elution 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
03:10	1.0	95	5
03:20	1.0	95	5



Example of an online DBS-SPE-MS/MS chromatogram of spiked blood (superposition of the positive mode and negative mode chromatograms)

ESI-MS/MS (ABSciex API 4000)

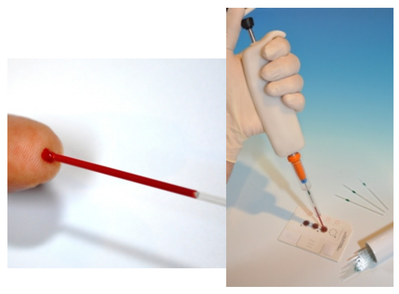
Compound	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP (V)	General settings	
Positive mode						IS (V)	+ 3000 / - 4200
Acebutolol	337.2	116.1	26.0	33.0	10.0	TEM (°C)	550
Haloperidol	376.3	165.2	66.0	35.0	30.0	CAD	4
Verapamil	455.4	165.1	76.0	39.0	30.0	CUR (psi)	15
Propranolol	260.1	116.1	61.0	27.0	22.0	GS1 (psi)	55
Negative mode						GS2 (psi)	70
Chlortalidone	336.8	190.0	- 90.0	-24.0	-11.0	EP (V)	+ 10 / - 10
Hydrochlorthiazide	296.0	268.8	-95.0	-26.0	-21.0	Dwell time (msec)	100

Measuring recovery and matrix effects on ionization

Recovery of the analytes from the blood spot and matrix effects on ionization are measured using a loop on valve 1 with a precisely known volume. The procedure involves 3 subsequent experiments :

1. First a "normal" analysis of a blood spot was performed using spiked blood as the sample
2. Then the loop was filled with the standard mix of test compounds and a blood spot analysis was performed using blank blood as the sample. By switching the loop into the desorption solvent stream at the moment of starting the desorption, blank blood and analyte mix are flushed over the SPE cartridge simultaneously. By comparing 1 and 2, any loss of analyte due to incomplete recovery from the blood spot can be determined independent of SPE recovery and ionization suppression
3. Finally, step 2 was repeated, but now with a totally blank card (no blood spots) in the clamp. By comparing 3 and 2, signal loss or enhancement due to matrix effects on ionization can be determined.

Exact sample application using the Drummond DBS Multi-dispenser



The Drummond DBS Multi-dispenser has been especially designed for aspiration of finger-prick blood and volumetric spotting on a DBS card. A calibrated disposable capillary is mounted into the dispenser and blood is aspirated by capillary suction into the capillary. To mimic a finger-prick sample, a drop of blood (spiked or blank) was placed on a flat inert surface. When the capillary is filled up to a mark on the capillary, the blood can be dispensed in 5 sequential volumes of 5 µl, (strokes are pre-set on the dispenser) to make 5 different spots on the DBS card. Touching the card with the capillary is no problem, in fact, spot size appeared to be smaller when deliberately touching the card. Spot size of sample volumes up to 10 µL can thus be kept small enough to allow full spot analysis using the 6 mm clamp.

Results and Discussion

Precision of the full-spot online DBS-SPE-MS/MS method using the Drummond DBS Multi-dispenser

Precision of 5 µL spotting was as measured by full-spot online DBS-SPE-LC-MS/MS analysis of spiked bloodspots taking verapamil for calculating the precision. 5 capillaries were used to spot 5x5 µL blood on DBS cards (i.e. 5 spots from one capillary). As shown in table 1, high %RSD was found initially, when using the results of all (5) spots from one capillary. However, it appeared that only the first 5 µL showed a high variance (*). When discarding the first 5 µL, the dispenser works fine, with spot volume precision ranging from 1.4 – 5.2 % RSD for individual capillaries and 3.4 % RSD for a series of 20 spots from 5 capillaries.

Table 1 – precision of online DBS-SPE-MS/MS using the Drummond DBS Multi-dispenser to apply 5 µL blood volumes

Capillary	Precision [%RSD Verapamil] 5 spots per capillary	Precision [%RSD Verapamil] 4 spots per capillary (first spot not used)	Overall Precision [% RSD Verapamil] 20 spots, 4 spots per capillary, first spot not used
1	31.6	3.3	3.4
2	5.9	2.5	
3	1.5	1.4	
4	21.0	5.2	
5	8.0	3.7	

(*) Drummond is working on an improved version to solve this issue.

Recovery

Using the procedure for recovery measurement as described under experimental conditions (exp 1 and 2) , the recoveries were measured for all compounds spiked to blood of different Ht levels. The blank blood spot used for reference had Ht 0.45 for all recovery measurements. The results (Table 2) show recoveries ranging from 88.9 to 107.9% for all compounds over the entire Ht range. No correlation is observed between Ht value and recovery. **Clearly, the "harsh" desorption conditions efficiently remove blood from DBS card to SPE cartridge, independent of Ht.**

Table 2 – recovery (%) for all test compounds at different Ht levels (n = 3)

Compound	Ht 0.3	Ht 0.45	Ht 0.7
Chlortalidone	96.8	96.0	97.2
Hydrochlorothiazide	95.6	103.6	92.6
Acebutolol	98.5	95.8	94.5
Haloperidol	101.1	96.9	88.9
Verapamil	106.7	101.3	99.7
Propranolol	91.0	104.3	107.9

Matrix ionization effects

Using the procedure as described under experimental conditions, matrix effects on ionization can be assessed in one simple extra experiment (exp. 3). As shown in table 3, only Chlortalidone shows an effect that is clearly more than the usual variation in recovery measurement. This effect is considered small enough to allow reliable analysis.

Table 3 – effect of blood matrix (Ht 0.45) on ionization for all test compounds (n = 3)

Compound	Matrix Effect [% deviation between exp. 2 and exp. 3, taking exp. 3 as 100%]
Chlortalidone	- 16.7
Hydrochlorothiazide	+ 2.7
Acebutolol	+ 9.9
Haloperidol	+ 5.4
Verapamil	- 11.6
Propranolol	- 9.6

Conclusion

- The prototype Drummond DBS Multi-dispenser is an easy to use device for sampling capillary blood from a finger prick. Blood volumes as small as 5 µL can be applied on DBS cards with good precision. Touching the DBS card with the tip of the capillary, helps to keep the spot small. It also simplifies blood application and showed no negative effect on precision.
- "Harsh" desorption conditions resulted in almost 100% desorption of analytes from the blood spot independent of Ht value.
- Online SPE removed sufficient matrix to reduce ionization effects to an acceptable level.
- Full-spot online DBS-SPE-MS/MS with harsh desorption conditions, overcomes the Ht issues in DBS analysis. Full-spot DBS analysis is therefore a more robust micro-sample analysis concept than partial-spot DBS analysis in an environment where some training of personnel (or patients) at the sampling site is acceptable and feasible.

Acknowledgement

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References

[1] Denniff P, Spooner N: The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis*, 2 (8), 2010, pp 1385-1395
[2] Knegt L, Hempen C and Ooms JA; Instrumental approach to eliminate the hematocrit issue using online DBS analysis, poster presented at EBF symposium Brussels June 2012
[3] Ooms JA, Knegt L, Koster EH: Exploration of a new concept for automated dried blood spot analysis using flow-through desorption and online SPE-MS/MS**.** *Bioanalysis,* 3(20), 2011, pp 2311-2320

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