Hematocrit effect: Does it influence the analysis of plasma samples or is it just a DBS concern?

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OVERVIEW

Purpose

- Evaluation of hematocrit effect on plasma preparation. Drug quantifications are performed using LDTD-MS/MS

Method

- Liquid-Liquid extraction of plasma sample
- DBS card extract of blood sample
- DBS In tips extract of blood sample
- LazWell plate spotting, evaporate to dryness and LDTD-MS/MS analysis

Quantification

- Linearity: $r^2 > 0.995$ over the calibration range
- **Samples analyzed with a run time of 9 seconds using LDTD-MS/MS system**

INTRODUCTION

The "hematocrit effect" is a major concern when dry blood spots (DBS) are analyzed. Usually drugs are analyzed in plasma samples and no mention is made about the hematocrit effect in this matrix. Does the level (high or low) of hematocrit have an effect on the reported concentration of the analyzed drugs?

In this study, blood samples with low, standard and high hematocrit levels are prepared. The hematocrit effect on blood samples was investigated using two different devices (DBS cards and DBS In tipss) and the plasma obtained from blood fraction. Measured concentration of the drugs is evaluated as a function of blood hematocrit level. The extracted samples are analyzed with LDTD-MS/MS.

LUXON Ionization Source:

The LUXON Ion Source (**Figure 1**) is the second generation sample introduction and ionization source based on the LDTD® technology for mass spectrometry. LUXON ion source uses Fiber-Coupled Laser Diode (**Figure 2**) to obtain unmatchable thermal uniformity giving more precision, accuracy and speed. The process begins with dry samples which are rapidly evaporated using indirect heat. The thermally desorbed neutral molecules are carried into a corona discharge region. High efficiency protonation and strong resistance to ionic suppression characterize this type of ionization, and is the result of the absence of solvent and mobile phase. This thermal desorption process yields high intensity molecular ion signal in less than 1 second sample to sample and allows working with very small volumes.

Figure 1 LUXON Ion Source

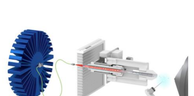

Figure 2 Schematic of the LUXON ionization source

METHOD

Blood sample preparation:

- 20 mL of Human EDTA-K2 Blood are pooled.
- 4 mL are transferred in three tubes and sit on ice for 1 hour to get plasma separation by gravity.
- 1 mL of plasma from Tube 3 is removed and added in Tube 1. All tubes are mixed.
  - Tube 1: Low Hematocrit (Level: 38.5%)
  - Tube 2: Medium or Standard Hematocrit (46.7%)
  - Tube 3: High Hematocrit (57.1%)

Blood spiking and sample preparation:

- A calibration curve and QC of Methadone are spiked in Human EDTA-K2 Blood
- Hematocrit test samples are spiked at medium QC Level in:
  - Low Hematocrit Blood (LH)
  - Medium Hematocrit Blood (MH)
  - High Hematocrit Blood (HH)
- DBS in Card: 15 µL of calibration curve, QCs and Hematocrit test samples (HL, HM and HH) are added on Whatman 903 card and dried for 12 hours.
- DBS In tips: 2 µL of internal standard solution followed by 2 µL of calibration curve, QCs and Hematocrit test samples (HL, HM and HH) are added on TBD in tip. Dried for 12 hours.
- 1 mL of calibration curve, QCs and Hematocrit test samples (HL, HM and HH) are centrifuged and upper layer plasma is collected for analysis.

Plasma sample extraction:

- 5 µL plasma sample, 5 µL internal standard and 60 µL Carbonate buffer (500 mM, pH10) are added in tube and mixed.
- 400 µL Hexane:Ethyl Acetate (1:1) is added.
- After mixture and centrifugation, 4 µL of upper layer are deposited on LazWell plate and evaporated to dryness.

DBS card sample extraction:

- 3 mm punch of DBS card is added in tube.
- Add 60 µL NaOH (0.05N in water)
- Add 5 µL internal standard solution
- Add 200 µL Hexane:Ethyl Acetate (1:1)
- After mixture and centrifugation, 4 µL of upper layer are deposited on LazWell plate and evaporated to dryness.

DBS In tips sample extraction:

- 24 µL of Methanol is aspirated in the DBS In tips (**Figure 3**) followed by 16 µL air gap using RTC system (**Figure 4**).
- Extracts are then expelled directly on a LazWell plate and evaporated to dryness.

Figure 3 DBS In tips with blood sample (left) and blank tip (right).

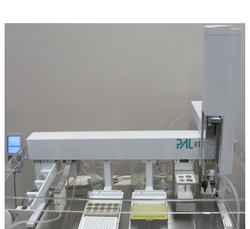

Figure 4 RTC system using Pipet tool for DBS In tips extraction.

INSTRUMENTAL CONDITIONS

Instrumentation

- LDTD model: LUXON
- MS: Sciex 5500 QTrap®

LDTD Parameters

- Laser power pattern :
  - ➢Increase laser power to 45 % in 3 sec
  - ➢Decrease laser power to 0 %
- Carrier gas flow : 3 L/min (Air)

MS Parameters

- APCI (+)
- DP: 100 V
- Dwell: 50 msec
- MRM mode **(Table 1)**

Table 1 MRM transitions

Compound	Q1	Q3	CE
Methadone	310	265	20
Methadone-d9	319	268	20

RESULTS

Linearity results:

A standard calibration curve ranging from 10 to 500 ng/mL was prepared in blank blood matrices and analyzed. In Table 2, linearity coefficients for each experiment are reported.

Table 2 Linearity results

Test	R^2
Plasma	0.9977
DBS card	0.9970
DBS In tips	0.9984

Intra-run

Low, medium and High QCs were spiked in blood sample at 30, 150 and 350 ng/mL respectively. QCs were extracted and their concentrations were evaluated against the calibration curve. The intra-run precision and accuracy are reported in **Table 3** for each experiment.

Hematocrit effect evaluation

Methadone was spiked in blood matrix having Low (HL), Medium (MH) and High (HH) hematocrit levels at a concentration of 150 ng/mL. Samples were than extracted with their respective methods and analyzed using LDTD-MS/MS method. The precision and accuracy are reported in **Table 4** for each experiment. DBS Card shows a clear deviation of the QC levels as a function of hematocrit concentration. Plasma recovered from high hematocrit levels also show overestimation in a high QC. Using plasma instead of whole blood induces a bias in the measured concentration of the drug based on the hematocrit level. DBS In tips use a volumetric deposit of blood which is not affected by the hematocrit.

Table 3 Precision and accuracy intra-run results

	Test	QC-Low	QC-Med	QC-High
Plasma	Precision (%CV)	7.0	8.8	2.8
	Accuracy (%Nom)	101.3	111.9	108.4
DBS card	Precision (%CV)	1.9	4.3	3.1
	Accuracy (%Nom)	103.0	92.2	95.5
DBS In tips	Precision (%CV)	3.4	5.0	5.7
	Accuracy (%Nom)	94.5	93.4	101.1

Table 4 Precision and accuracy hematocrit effect experiment

	Test	HL	HM	HH
Plasma	Precision (%CV)	2.8	3.7	1.5
	Accuracy (%Nom)	97,8	99.8	**118.4**
DBS card	Precision (%CV)	2.5	4.9	7.3
	Accuracy (%Nom)	**86.7**	99.8	**116.4**
DBS In tips	Precision (%CV)	6.2	2.6	7.4
	Accuracy (%Nom)	104.4	97.6	97.4

CONCLUSION

- **When DSB card is used, Hematocrit effect is confirmed**
- **When Plasma is prepared with blood having high hematocrit levels, it results in an over estimation of the drug's concentration**
- **When DBS In tips is used, No Hematocrit effect is obtained**
- **Fast quantification of Methadone in the 3 experiments' extractions was performed in 9 seconds per sample using LDTD-MS/MS**