Bioanalysis of Emixustat (ACU-4429) in Whole Blood Collected with Volumetric Absorptive Microsampling by LC–MS/MS

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Introduction

The analysis of dried-blood spots (DBS) for assessment of PK exposure shows some advantages over traditional plasma-based methods, including simplified collection, processing, storage and shipment. However, bioanalysis of DBS does have challenges related to method validation in accordance with regulatory guidance. Considerations include potential cross contamination during automated excision, sample dilution procedures, application of the internal standard, stability assessments and the effect of hematocrit on spot size and homogeneity.

Volumetric absorptive microsampling (VAMS) (Figure 1) is a novel technology designed to allow for precise microsampling of biological fluids. The Mitra™ Volumetric Absorptive Microsampling (VAMS) device (trademark application of Neoteryx, LLC, CA, USA) provides a simple and efficient workflow that allows direct sampling of blood from a subject (i.e. fingerstick collection), followed by drying and extraction in a fashion similar to current practices with DBS. The Mitra™ VAMS device was designed to absorb a fixed volume (10 µL in this case) (Figure 2) of blood regardless of hematocrit level which is then entirely extracted. This approach to obtain a dried blood sample for quantitative bioanalysis was intended to overcome the area bias, hematocrit and homogeneity issues associated with conventional DBS where a subpunch is extracted (Figure 3).

Emixustat (CAS number 1141777-14-1, see Figure 4) is a novel, orally administered small-molecule visual cycle modulator and is being investigated in clinical trials for the potential treatment of geographic atrophy (GA) associated with dry age-related macular degeneration (AMD). In the clinical setting, AMD is evaluated by specialized imaging techniques. The ophthalmology offices are not equipped to collect and process venous (wet) blood for plasma samples and store and ship these samples under frozen conditions. In addition, AMD is a disease of the geriatric population who present their own challenges for sample collection due to phlebotomy difficulties. The blood-to-plasma partitioning and plasma protein binding properties of emixustat are consistent across a broad concentration range. Based on these characteristics, dried blood microsampling may be a suitable approach for PK sample collection during clinical development of Emixustat.

Methods

Based on its *in vitro* properties in blood and plasma, Emixustat (ACU-4429) was chosen as the test compound to evaluate the merits of the Mitra™ VAMS device for DBS bioanalysis. The whole blood was applied to the Mitra tips (10.0 µL), per the manufacturer's instructions. After drying, the analyte was extracted from the Mitra tip by vortexing for 15 minutes at low speed (~1100 RPM) in the presence of 1% ammonium hydroxide in methanol. Then the Mitra tips were removed and the extract was dried down. The samples were reconstituted in 0.1% formic acid in MeOH/H_2O (30:70, v:v) and analyzed via LC-MS/MS utilizing an AB SCIEX® 5500 mass spectrometer, Shimadzu Prominence 20 series pumps (Figure 5).

Results

Precision and Accuracy

Accuracy and precision were investigated at four concentration levels in the curve range 0.0500 ng/mL (LLOQ) to 10.0 ng/mL (ULOQ) (Figure 6a and 6b). The inter-run overall precision (RSD) was 13.1, 9.5, 6.5 and 5.2% and the accuracy was 109, 99.3, 109 and 106% at 0.0500, 0.150, 1.00 and 7.50 ng/mL, respectively. The intra-run precision (repeatability) was 17% or below for all levels, and the accuracy was within 96-115% (Table 1).

Linearity

Linearity was determined from three calibration graphs during accuracy and precision runs with coefficients of correlation (R2) of 0.9947, 0.9958 and 0.9956, respectively. Accuracy of the back-calculated concentrations of the calibration samples at all eight levels was within 83.4-112% during all 11 runs (Table 2). Mean accuracy at all calibration levels ranged from 94.9 to 104% and inter-run precision (%RSD) was <6.6%.

Recovery

The overall mean recovery was 65.6% and 80.2% for emixustat and IS, respectively, and it is consistent across the concentration levels.

Matrix Factor

The overall normalized matrix factor at the LQC concentration was 0.968 and the HQC was 0.980, with an RSD of 4.0% and 1.6%, respectively.

Carryover

For each accuracy and precision run, extracted blank blood samples were analyzed after the high calibration (10.0 ng/mL) and second highest (8.00 ng/mL) samples. No significant carryover was observed, and was well below 20% of the LLOQ (0.0500 ng/mL) (Figure 6c).

Selectivity

Selectivity was investigated by analyzing spiked blood from six individuals at LQC (0.150 ng/mL) in three replicates. The overall accuracy at this concentration close to the LQC was 102% and the overall precision was 8.5%. All six individuals were also tested without drug or IS, no interference peaks were observed at the retention time of the drug or IS.

Hematocrit Effect

Whole blood collected with NaF/KOx anticoagulant was tested at seven hematocrit levels: 15, 20, 30, 40, 45, 50 and 55%. Accuracy and precision was acceptable at hematocrit ranges from 30 to 55% and ranged from -10.0 to 3.5% and 2.5 to 11.8%, respectively. Accuracy of the samples prepared in blood with hematocrit of 15 and 20% showed a high bias at HQC concentrations, with accuracies of 123 and 125% of nominal. Table 3 summarizes the hematocrit effect.

Stability

Reinjection Reproducibility

The reinjection reproducibility was investigated by reinjecting sample extracts of calibration standards and QCs after storage at refrigerated temperature for 122 h. Accuracy was within 96–108% and imprecision was below 7.4%.

Room Temperature, Refrigerated and Frozen Stability of Emixustat on VAMS Device in Blood Collected with and without Anticoagulant

To investigate the viability of using VAMS devices not pretreated with anticoagulant, whole blood samples without anticoagulant were evaluated alongside whole blood collected with NaF/KOx anticoagulant. Although blood collected without anticoagulant readily clots at room temperature, freshly drawn blood from a single donor was collected without anticoagulant and immediately spiked with the appropriate working solution of Emixustat and gently mixed and agitated while kept on wet ice until multiple VAMS devices had the blood applied over approximately a 15 min period. Pooled whole blood collected with NaF/KOx as the anticoagulant was spiked with emixustat and applied to the VAMS devices in a similar fashion and allowed to dry for at least 2 h at room temperature. This anticoagulant has been demonstrated to stabilize collected blood and plasma during previous bioanalytical projects. Samples appear stable at all concentrations (LQC and HQC) when stored at ambient conditions up to 1 week (Table 4a), refrigerated up to 1 month (Table 4b) and frozen up to 3 months (Table 4c) both with and without anticoagulant.

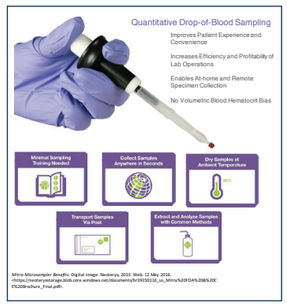

Figure 1. The general illustrations of the advantages for Mitra™ microsampling tips.

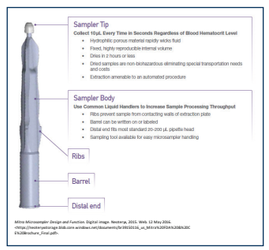

Figure 2. a) The detailed structure for the Mitra™ microsampler

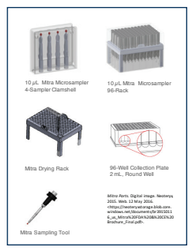

Figure 2. b) The related products for Mitra™ tips during the sample preparation.

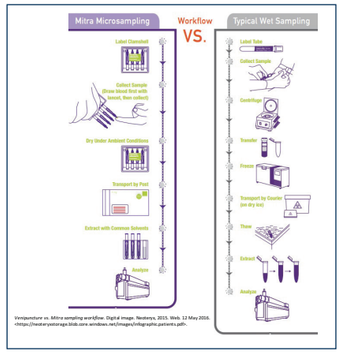

Figure 3. The comparison of the workflows between the regular wet plasma method vs the microsampling using Mitra™ tips showing the advantages of Mitra™ tips.

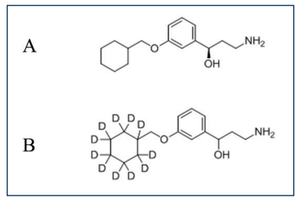

Figure 4. Structures of (A) Emixustat (ACU-4429) and (B) its IS, ACU-4946.

a) **After the blood adsorbed Mitra tips were dry at RT for 2~4h**

Extraction Procedure

1) Add 400 µL 1% NH_4OH in MeOH
2) Add 20.0 µL internal standard
3) Insert the dried Mitra Tips into the extraction plate wells
4) **Sonicate** the plate 15 mins and then Vortex mix the plate for 15 mins at low speed (**~1100RPM**)
5) Remove Mitra tips from the extraction plate with caution and discard them
6) Dry down under N_2 and reconstitute with 150 µL of 0.1% FA in MeOH/H_2O (30:70, v:v)

b) **MS**: API 5500
LC: Shimadzu Prominence 20 Series
Column: Phenomenex Synergi MAX-RP, 50 x 2mm, 4 µm particle size

Time Program

Time	Module	Events	Parameter
0.10	Pumps	Pump B	Conc. 30
0.80	Pumps	Pump B	Conc. 30
0.81	Pumps	Pump B	Conc. 95
1.40	Pumps	Pump B	Conc. 95
1.41	Pumps	Pump B	Conc. 30
2.20	System Controller		Stop

Figure 5. a) The optimized extraction procedures, and b) the chromatography conditions in this study.

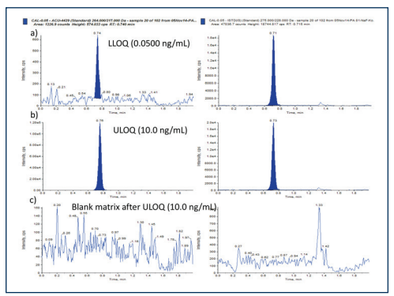

Figure 6. Representative chromatogram a) LLOQ at 0.0500 ng/mL in whole blood, b) ULOQ at 10.0 ng/mL in whole blood, c) Blank matrix injected directly after the ULOQ injection for the carryover evaluation.

Table 1. Accuracy and Precision of Emixustat Spiked in Human Blood Collected in NaF/KOx and Extracted from Volumetric Absorptive Microsampling Device

QC Level	Spiked Emixustat Conc. (ng/mL)	Run # (n=6)	Intra-run Mean Bias (%)	Intra-run Precision (%RSD)	Inter-run Mean Bias (%)	Inter-run Precision (%RSD)
LLOQ	0.050	1	15.0	16.3	9.4	13.1
		2	11.4	9.7		
		3	1.8	10.7		
LQC	0.150	1	-2.0	13.6	-0.7	9.5
		2	4.0	6.7		
		3	-3.3	6.1		
MQC	1.00	1	10.0	7.6	9.0	6.5
		2	12.0	6.1		
		3	5.0	4.1		
HQC	7.50	1	2.4	5.7	6.3	5.2
		2	8.3	4.8		
		3	8.1	3.8		
DQC	50.0	1	-7.2	6.7		

Table 2. Accuracy and Precision of Emixustat Calibration Standards (ng/mL)

Run Number	CAL 1 0.0500 ng/mL	CAL 2 0.100 ng/mL	CAL 3 0.500 ng/mL	CAL 4 1.00 ng/mL	CAL 5 2.00 ng/mL	CAL 6 5.00 ng/mL	CAL 7 8.00 ng/mL	CAL 8 10.0 ng/mL
1	0.0500	0.102	0.483	0.912	1.91	5.40	7.71	11.1
2	0.0523	0.0926	0.449	1.01	2.01	5.07	8.37	10.6
3	0.0524	0.0892	0.538	0.957	2.00	4.83	8.34	10.2
4	0.0506	0.0971	0.496	1.06	2.06	5.07	7.95	9.30
5	0.0507	0.0985	0.459	1.06	1.90	5.52	7.76	9.97
6	0.0507	0.0984	0.466	0.969	2.19	5.35	7.49	10.0
7	0.0505	0.0985	0.503	0.982	1.83	5.29	8.03	10.4
8	0.0501	*0.0834	0.514	0.946	1.84	5.16	8.37	10.2
9	0.0495	0.102	0.515	*0.838	1.76	5.27	7.93	10.3
10	0.0523	0.0917	0.478	0.964	2.03	5.23	8.02	10.5
11	0.0524	0.0906	0.498	1.00	1.85	5.04	8.95	9.89
Mean	0.0510	0.0949	0.491	0.973	1.94	5.20	8.08	10.2
%Bias	102%	94.9%	98.2%	97.3%	97.0%	104%	101%	102%
%RSD	2.14%	6.17%	5.46%	6.53%	6.48%	3.73%	4.99%	4.49%

Table 3. Hematocrit Effect for Emixustat in Human Whole Blood

Hematocrit Level (%)	QC Level	Spiked Emixustat Conc. (ng/mL)	Mean Bias (%) n=6	Precision (%RSD)
15	LQC	0.150	13.3	4.1
	HQC	7.50	22.5	3.4
20	LQC	0.150	14.0	4.2
	HQC	7.50	24.7	2.7
30	LQC	0.150	-4.7	3.6
	HQC	7.50	0.9	3.5
40	LQC	0.150	-7.3	3.1
	HQC	7.50	-0.3	5.1
45	LQC	0.150	-1.3	11.8
	HQC	7.50	3.5	5.8
50	LQC	0.150	-10.0	11.8
	HQC	7.50	-2.5	4.8
55	LQC	0.150	-7.3	2.5

Table 4. Storage Stability of Emixustat

a)

Storage Time (days)	Anticoagulant	QC Level	Spiked Emixustat Conc. (ng/mL)	Mean Bias (%) n=6	Precision (%RSD)
0	NaF/KOx	LQC	0.150	4.0	6.7
		HQC	7.50	8.3	4.8
	None	LQC	0.150	14.7	4.9
		HQC	7.50	9.2	4.2
1	NaF/KOx	LQC	0.150	5.3	5.5
		HQC	7.50	1.5	2.6
	None	LQC	0.150	12.7	4.3
		HQC	7.50	0.80	2.5
2	NaF/KOx	LQC	0.150	-2.0	4.0
		HQC	7.50	-4.3	3.6
	None	LQC	0.150	9.3	9.5
		HQC	7.50	0.3	7.2
3	NaF/KOx	LQC	0.150	2.0	14.0
		HQC	7.50	1.6	6.4
	None	LQC	0.150	-1.3	6.8
		HQC	7.50	5.1	5.2
4	NaF/KOx	LQC	0.150	-7.3	12.0
		HQC	7.50	-0.7	5.8
	None	LQC	0.150	4.0	4.5
		HQC	7.50	4.3	3.3
7	NaF/KOx	LQC	0.150	-12.0	8.0
		HQC	7.50	-6.0	5.7
	None	LQC	0.150	-3.3	11.9
		HQC	7.50	4.0	4.5
92	NaF/KOx	LQC	0.150	-30.0	5.9
		HQC	7.50	-34.8	4.5
	None	LQC	0.150	-31.3	8.5
		HQC	7.50	-29.2	6.3

b)

Storage Time (weeks)	Anticoagulant	QC Level	Spiked Emixustat Conc. (ng/mL)	Mean Bias (%) n=6	Precision (%RSD)
1	NaF/KOx	LQC	0.150	0.7	6.5
		HQC	7.50	2.1	5.4
	None	LQC	0.150	12.7	4.1
		HQC	7.50	5.2	3.3
4	NaF/KOx	LQC	0.150	-2.7	7.0
		HQC	7.50	12.4	5.6
	None	LQC	0.150	6.7	6.4
		HQC	7.50	9.3	5.3
12	NaF/KOx	LQC	0.150	-10.0	3.2
		HQC	7.50	-19.5	5.6
	None	LQC	0.150	-8.7	5.4
		HQC	7.50	-19.1	3.7

c)

Storage Time (weeks)	Anticoagulant	QC Level	Spiked Emixustat Conc. (ng/mL)	Mean Bias (%) n=6	Precision (%RSD)
1	NaF/KOx	LQC	0.150	9.3	5.5
		HQC	7.50	0.1	6.1
	None	LQC	0.150	14.0	6.3
		HQC	7.50	3.5	7.0
4	NaF/KOx	LQC	0.150	5.3	6.1
		HQC	7.50	3.6	2.9
	None	LQC	0.150	17.3	8.5
		HQC	7.50	15.7	5.1
12	NaF/KOx	LQC	0.150	4.7	5.9
		HQC	7.50	-9.2	3.6
	None	LQC	0.150	1.3	10.7
		HQC	7.50	-5.1	2.1

a) Room temperature storage stability of Emixustat on volumetric absorptive microsampling device after application of spiked human blood collected with and without anticoagulant
b) Refrigerated stability of Emixustat on volumetric absorptive microsampling device after application of spiked human blood collected with and without anticoagulant
c) Frozen storage stability of Emixustat on volumetric absorptive microsampling device after application of spiked human blood collected with and without anticoagulant.

Conclusions

- The method described has been validated and is suitable for the bioanalysis of ACU-4429 in human whole blood collected by Mitra™ volumetric absorptive microsampling devices.
- The validated assay allows for the comparison of data from both fingerstick and venous blood samples absorbed onto VAMS devices to simultaneously collected traditional plasma samples.
- Assuming strategic criteria are acceptable in future clinical bridging studies, this method could be used for PK sample collection during late-stage clinical trials.

Acknowledgements

The author would like to thank the following people for their contributions to the this work: D. Fast, T. Steege, R. Kuen and N. Shefte (Covance); V. Kuksa and R. Eyre (Acucela); and B. Milan and B. Virasingh (Phenomenex).

Presented at ASMS 2016

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