

Elimination of Hematocrit Effect using Volumetric Absorptive Microsampling for the Determination of Ritonavir and Naproxen in Whole Blood by LC-MS/MS

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Overview

Purpose

- To introduce a novel Impact-Assisted Extraction procedure to negate the negative influence of blood hematocrit on recovery when using Volumetric Absorptive Microsampling (VAMS).

Method

- Blood donors of differing hematocrit level were fortified with ritonavir or naproxen and sampled using a VAMS Mitra™ device.
- Sampled Mitra™ tips were subjected to impact-assisted extraction with a stainless steel bead in a 96-well plate containing extraction solvent.

Results

- Gravimetric analysis coupled with blood density measurements enabled the accurate determination of sampling volume, and indicated that the VAMS device absorbed an average volume of 10.6 µL over the HCT range 0–63%; sampling volume was immune to blood HCT.
- Impact-assisted extraction was found to yield greater recoveries for both analytes when compared to vortex mixing or sonication-based extraction.
- Under optimal impact-assisted extraction conditions for each analyte, recovery was > 90% for both naproxen and ritonavir, regardless of blood HCT.
- Precision and accuracy data including matrix effect (4 lots) met acceptance criteria for a validatable method.

Introduction

Despite the numerous benefits of sampling low blood volume, challenges exist depending on the microsampling technique used. In the case of DBS, the hematocrit (HCT) of the blood affects its viscosity, giving rise to different-sized blood spots. While capillary microsampling (CMS) techniques circumvent the HCT effect, collection and processing is tedious, and drugs which exhibit non-specific binding to glass or require matrix stabilization are problematic. A recent alternative to DBS/CMS is Volumetric Absorptive Microsampling (VAMS), wherein an accurate volume of blood is absorbed onto a hydrophilic polymeric tip, overcoming the HCT effect and simplifying the processing difficulties associated with CMS. Theoretically, accurate sampling volume combined with near quantitative recovery should mitigate HCT effect and other related analytical challenges. The current research examines the characteristics of VAMS as applied to the determination of naproxen and ritonavir in human blood (Figure 1).

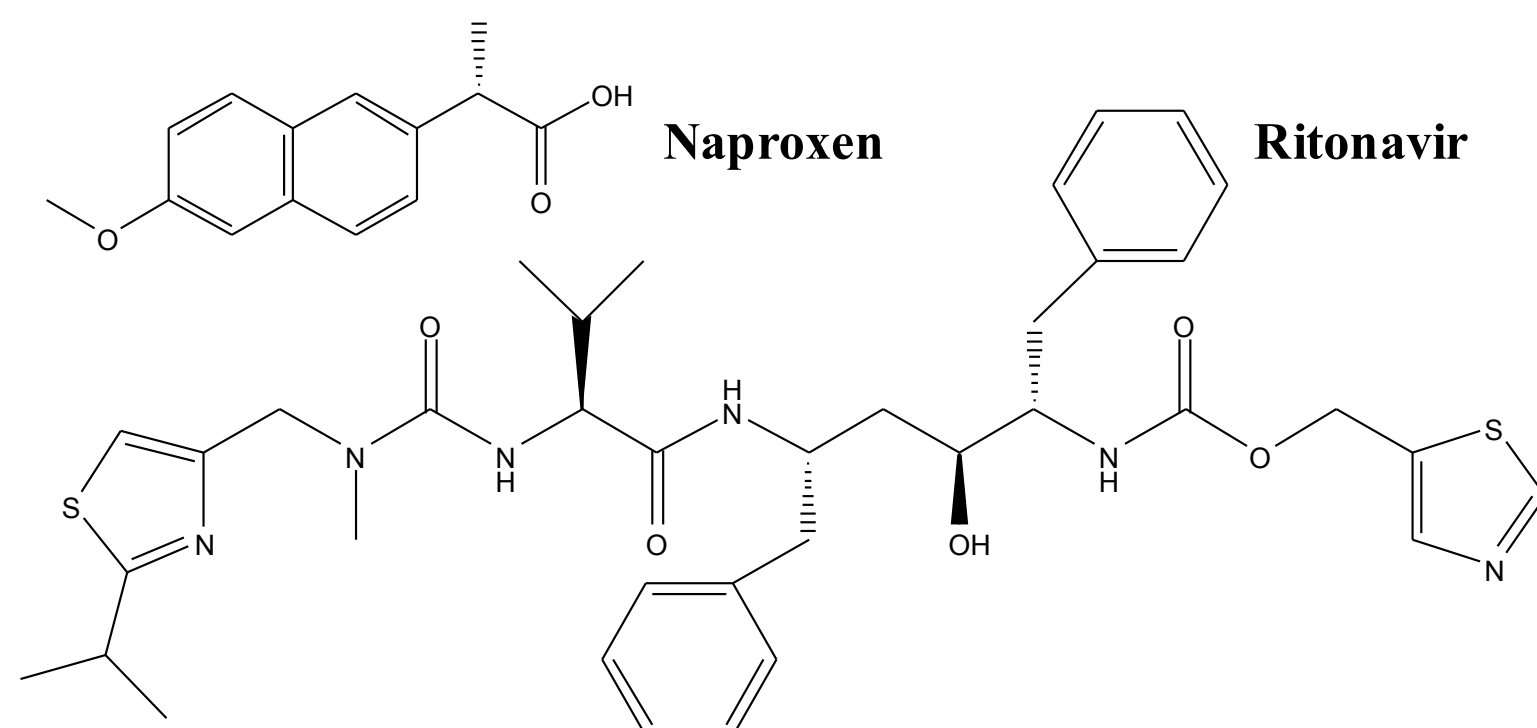


Figure 1. Structures of Naproxen and Ritonavir.

Methods

Sample Extraction

Human blood was absorbed onto a 10 µL Mitra™ microsampling device (Neoteryx) and dried a minimum of 24 hours at room temperature in the presence of desiccant. Dried tips were then loaded into a 96-well plate on which extraction solvent containing deuterated internal standard was added (400 µL naproxen-D3 in MeOH or 300 µL ritonavir-D8 in ACN:H₂O, 3:1). A single stainless steel bead was placed in each well, and sample desorbed from the Mitra™ tip by impact-assisted extraction at 1600 vertical strokes/min using a Geno/Grinder homogenizer. Following extraction, the plate was centrifuged, supernatant transferred, diluted, mixed, centrifuged again and analyzed by LC-MS/MS.

Chromatography and Detection

Analytes were separated isocratically on a C₁₈ column using mobile phase compositions of aqueous propionic acid/MeOH (naproxen), or aqueous ammonia/ACN (ritonavir), with detection by negative ESI/MRM (*m/z* 229 > *m/z* 170) or positive ESI/MRM (*m/z* 721 > *m/z* 268) on a SCIEX API 5000, respectively.

Results and Discussion

Sampling Volume Determination

Various blood HCT levels were evaluated in order to determine impact on Mitra™ sampling volume. Gravimetric analysis coupled with blood density measurements enabled this accurate determination and indicated that the nominally rated 10 µL sampling tip absorbed an average volume of 10.6 µL, without significant difference between HCT levels (Figure 2).

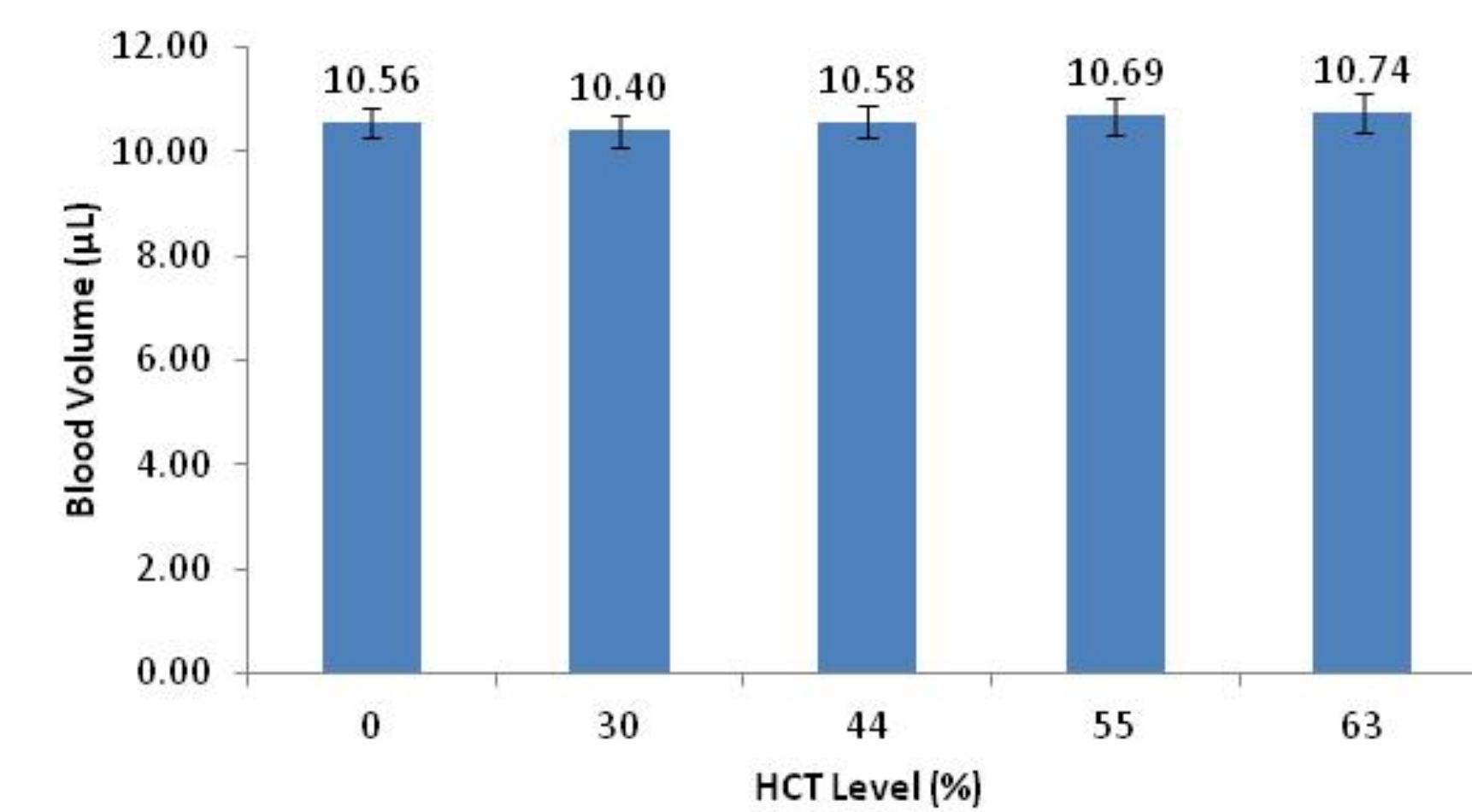


Figure 2. Blood sampling volume for the nominally rated 10 µL Mitra™ device as a function of HCT. Each data point represents the average of 12 determinations with error bars corresponding to SD.

Results and Discussion (Continued)

Extraction Solution Optimization

Optimization of the extraction solvent included an examination of MeOH and ACN mixed in various aqueous proportions. High recovery, low chemical noise, and the absence of ion suppression/enhancement from co-extracted endogenous components were the primary factors governing the final selection of solvent.

Recovery from each solvent was evaluated by extracting a high QC (75.0 µg/mL for naproxen and 3750.0 ng/mL for ritonavir) and comparing against a blank fortified with drug post-extraction; this same blank was compared against the response of drug in pure solution to determine matrix factor (Table 1). Amongst the extraction solvents evaluated, MeOH and ACN:H₂O (3:1) were chosen to conduct further assay evaluations for naproxen and ritonavir, respectively.

Table 1. Recovery (%) and matrix factor of naproxen and ritonavir from human blood (HCT=30%) for various extraction solutions using VAMS Impact-assisted extraction.

Extraction Solution	Naproxen			Ritonavir		
	Recovery (%)	CV (%)	Matrix Factor	Recovery (%)	CV (%)	Matrix Factor
	ACN:HCOOH 99.9:0.1% v/v	87.9	7.0	0.95	----	----
ACN	15.0	3.3	1.01	----	----	----
ACN:H ₂ O 90:10% v/v	86.8	9.4	0.99	76.3	5.2	0.83
ACN:H ₂ O 75:25% v/v	92.4	4.4	0.79	91.5	1.2	0.80
ACN:H ₂ O 50:50% v/v	96.0	3.9	0.81	89.3	1.7	0.84
MeOH:HCOOH 99.9:0.1% v/v	95.8	6.2	0.85	----	----	----
MeOH	94.1	0.5	0.92	----	----	----
MeOH:H ₂ O 90:10% v/v	94.5	4.3	0.85	90.3	7.3	0.82
MeOH:H ₂ O 75:25% v/v	92.9	4.7	0.87	83.9	1.2	0.85
MeOH:H ₂ O 50:50% v/v	93.4	5.4	0.86	81.0	2.0	1.04

The low recovery obtained in pure ACN for naproxen could be explained by its poor solvation characteristics for dried blood and the likelihood of protein precipitation occurring within the sampling tip. This observation is in agreement with results reported elsewhere in which low recoveries were obtained with pure ACN even for drugs soluble in this solvent. Acidification of ACN with 0.1% HCOOH markedly improved naproxen recovery vs. its non-acidified counterpart. This might be explained by improved solubilization of dried blood and/or release of drug from a protein-drug binding complex. In contrast, acidification of MeOH had little impact on recovery; further, extracts were reddish, suggesting partial re-suspension of blood components which might lead to matrix effect. A similar phenomenon was observed for extraction solutions containing higher aqueous content.

Results and Discussion (Continued)

Impact- vs. Ultrasonication-Assisted Extraction

Different extraction processes were interrogated in order to optimize the desorption of analyte from the Mitra™ tip into solvent. These included static desorption or physical disruption using impact-assisted or ultrasonication-assisted extraction. As outlined in Table 2, impact-assisted extraction furnished greater recovery than that observed by ultrasonication for both naproxen and ritonavir.

Table 2. Effect of impact- vs. ultrasonication-assisted extraction on recovery (%) of naproxen and ritonavir from human blood (HCT 39%, n ≥ 3) using MeOH and ACN:H₂O (3:1) as the extraction solvents, respectively.

Mixing Process	Naproxen		Ritonavir	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Sonication	84.6	5.8	67.5	3.4
Bead Impact	95.9	4.2	103.7	6.4

Impact-Assisted Extraction Recovery vs. Blood HCT

Under the optimal extraction conditions for each analyte, recovery was > 90% at each HCT level (Table 3). Thus, the oft reported decrease in recovery with increasing HCT when using VAMS was not observed for either analyte when using impact-assisted extraction. In contrast, the dependency of naproxen recovery with HCT level using pre-cut dry blood spot has previously been documented by our group.

Table 3. Recovery of naproxen and ritonavir from human blood at various HCT levels using MeOH and ACN:H₂O (3:1) as extraction solvents, respectively.

HCT Level	Naproxen		Ritonavir	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
0	95.6	3.4	101.0	1.3
30	93.6	6.1	99.4	1.8
44	96.7	0.6	95.5	1.4
55	96.0	2.7	97.8	3.9
63	93.6	3.5	102.8	3.2

Precision and Accuracy and Matrix Effect

Precision and accuracy data including matrix effect assessments using impact-assisted extraction of VAMS tips met all acceptance criteria for a validatable method (Tables 4 and 5). For matrix effect, low and high QCs from four blood lots were back-calculated against a calibration curve. Peak areas for the drugs and their respective stable-isotope internal standard were comparable in all cases, suggesting the absence of suppression/enhancement. Additionally, these QCs were all within the acceptable criteria.

Table 4. Precision, accuracy and matrix effect assessments (39% HCT) of naproxen using impact-assisted extraction in MeOH.

Curve Quality Control Samples (n=6 per level)			Matrix Effect Quality Control Samples (n=3 per lot)			
Nominal Concentration (µg/mL)	Accuracy (%)	CV (%)	Nominal Concentration (µg/mL)	Matrix Lot	Accuracy (%)	CV (%)
0.5	102.8	7.8	1.5	1	93.1	4.0
1.5	96.5	3.8	1.5	2	92.9	8.9
20.0	105.7	6.4	1.5	3	94.1	7.5
75.0	99.5	3.8	1.5	4	95.2	4.3
				Average	93.8	5.6
			75.0	1	96.3	1.6
			75.0	2	94.3	0.1
			75.0	3	101.6	3.1
			75.0	4	96.7	2.2
				Average	97.2	3.4

Table 5. Precision, accuracy and matrix effect (39% HCT) of ritonavir using impact-assisted extraction in ACN:H₂O 3:1.

Curve Quality Control Samples (n=6 per level)			Matrix Effect Quality Control Samples (n=4 per lot)			
Nominal Concentration (ng/mL)	Accuracy (%)	CV (%)	Nominal Concentration (ng/mL)	Matrix Lot	Accuracy (%)	CV (%)
10.0	105.4	5.5	30.0	1	95.5	6.1
30.0	95.3	8.4	30.0	2	97.2	0.8
600.0	96.3	10.5	30.0	3	98.1	11.0
3750.0	95.3	9.6	30.0	4	94.4	2.7
				Average	96.3	6.0
			3750.0	1	95.6	7.2
			3750.0	2	102.9	6.3
			3750.0	3	97.6	8.8
			3750.0	4	95.0	12.6
				Average	97.8	8.7

Conclusion

Gravimetric analysis coupled with blood density measurements enabled the accurate determination of sampling volume and indicated the Mitra™ device absorbed an average volume of 10.6 µL. Further, sampling volume was independent of blood HCT level. Unlike ultrasonication, the novel technique of impact-assisted extraction demonstrated quantitative recovery for naproxen and ritonavir. As well, recovery was not influenced by the blood HCT level and thus impact-assisted extraction represents an unbiased approach to quantitation using VAMS technology.