

Volumetric absorptive microsampling combined with impact-assisted extraction for hematocrit effect free assays

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Aim: Volumetric absorptive microsampling (VAMS) is a recent technology available for sampling and analyzing low blood volume. The present work describes the utilization of VAMS for the quantitation of naproxen and ritonavir in human blood using a novel bead-based impact-assisted extraction (IAE) procedure. **Results:** Sampling volume accuracy of the VAMS device was independent of the blood hematocrit (HCT) level, however analyte recovery decreased with increasing HCT when extracted using ultrasonication. In contrast, IAE was unaffected by HCT, resulting in quantitative recovery for all levels evaluated. Precision and accuracy batches, as well as matrix effect evaluation, met acceptance criteria. **Conclusion:** The IAE procedure coupled with VAMS is immune to HCT biases affecting sampling volume and recovery.

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In the past several years, the use of reduced sample volume has increased in importance as the collection of small blood volume is extremely beneficial for reducing the number of sacrificed animals, while making sample collection less invasive. Despite the numerous benefits of low blood volume sampling [1], there are several drawbacks depending upon the microsampling technique used. In the case of fixed-diameter sub-punch approaches for dried blood spot (DBS), only a portion of the sample is extracted. Further, blood hematocrit levels affect viscosity, leading to variability in the actual blood volume collected [2,3]. In contrast, full blood spot punching, wherein the entire blood spot is extracted, has been shown to eliminate HCT-related sampling bias [4,5]. The technique of precut DBS [5] also utilizes the full blood spot for extraction. This latter approach eliminates the need of punching at the point of analysis by utilizing precut discs of substrate. However, full blood spot approaches require generating spots of an accurate volume at the clinic, making them impractical for large scale studies [1]. Capillary microsampling (CMS) techniques have also been shown to circumvent the HCT effect [6], but their collection and processing are tedious, and drugs exhibiting nonspecific binding or a lack of stability are problematic.

A recent alternative to DBS/CMS is volumetric absorptive microsampling (VAMS) marketed by Neoteryx under the trade name MitraTM. In this microsampling approach, an accurate volume of blood is absorbed onto a hydrophilic polymeric tip by capillary action, overcoming the HCT effects related to blood sampling [4,7] while simplifying the processing difficulties associated with CMS and full-sample punch DBS. As reported with DBS, the impact of HCT level on extraction efficiency is still a major concern with VAMS. As HCT increases, a higher proportion of red blood cells entrapped in the VAMS sorbent can obstruct the analyte desorption process [8,9]. Factors affecting analyte recovery from the VAMS sorbent as a function of hematocrit level include the choice of extraction solvent [10], the type and strength of mixing, as well as the duration of agitation. Previously reported mixing processes include vortexing [9,10], lateral displacement [11,12] and combinations of sonication and vortexing [13,14]. Numerous examples demonstrate that extraction-related hematocrit effects for VAMS assays can be both challenging and a limitation to the success of the technique. For example, a study conducted by Parker *et al.* [11] for fosfomycin, in

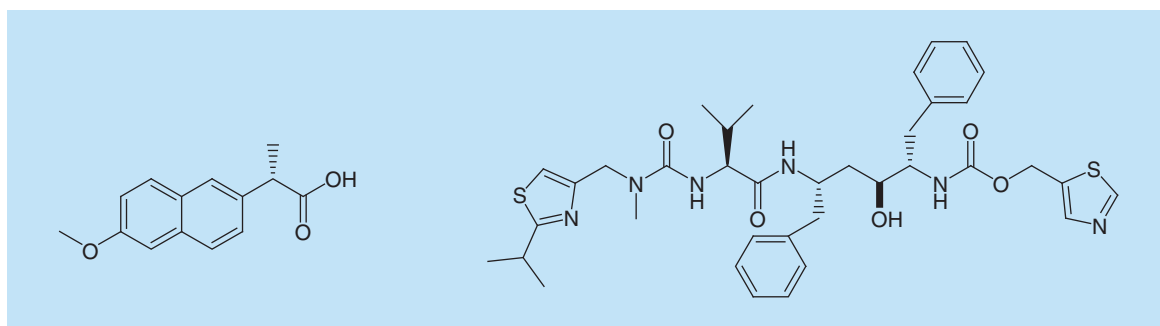


Figure 1. Molecular structures of naproxen (left) and ritonavir (right).

which 30 min of lateral shaking was used for extraction, indicated a trend of decreasing recovery with increasing HCT (from ~62% recovery at 27% HCT to ~47% at 52% HCT). Denniff *et al.* [12] also report the use of lateral shaking (60 min) to extract paracetamol from rat blood with an identical relationship of reduced recovery with increased HCT, although concentration was found to be within 11% of nominal value. A recent article from Ye and Gao [10], in which Mitrasamples were vortexed for 1 h in the presence of various extraction solvents demonstrated lower overall recovery as the HCT increased from 20 to 70% for the five analytes evaluated (metoprolol, midazolam, atorvastatin, tamoxifen and amiodarone). The disparity in recovery across hematocrit levels was minimized through the use of methanol:acetonitrile 50:50% v/v as the extraction solvent, rationalized from the dried blood solvation ability of methanol and the elution strength of acetonitrile. Using this solvent allowed the authors to successfully demonstrate accuracy (curve with 44% HCT) for all the quality control samples fortified in blood at various HCT levels. In a VAMS assay for miltefosine, Kip *et al.* [8] used 15 min of vortex mixing at 1250 r.p.m. and demonstrated that the impact of HCT on assay accuracy was reduced when compared with conventional DBS sampling; however, results still indicated a decline in recovery with increased hematocrit. A publication from Mano *et al.* [14], in which 15 min of sonication followed by 10 s of vortexing was used, reported between 14 and 20% less recovery at 66% HCT versus 20% HCT for E6005 and its *O*-desmethylated metabolite. Overall, reduced recovery with increased blood HCT is observed to various degrees in most VAMS-based assays, and thus remains a major challenge for the adoption of the technique.

The mechanical disruption process via bead-based impact (hereafter referred to as impact-assisted extraction [IAE]) is commonly implemented for tissue homogenization. Devices such as the 2000/2010 Geno/Grinder from SPEX CertiPrep, Inc. (NJ, USA) or FastPrep-96™ from MP Biomedicals, LLC (CA, USA) can be used for high-throughput bead impact homogenization in the 96-well plate format. The applicability of IAE has been previously demonstrated on a precut DBS substrate [15] and successfully applied to the extraction of naproxen from full sample punch DBS, resulting in quantitative recovery regardless of blood HCT.

The current study determines the extent to which IAE can facilitate analyte recovery from VAMS sorbent, independent of HCT, such that a simplified and universal sample preparation strategy might be adopted. Both naproxen (calculated logP = 2.99) and ritonavir (calculated logP = 5.22) (Figure 1) were selected as diagnostic compounds to characterize the applicability of IAE as a viable extraction technique for VAMS.

Experimental

Chemicals, reagents & materials

Naproxen and ritonavir were purchased from USP (MD, USA). Internal standards (IS) were stable isotope-labeled naproxen-D3 and ritonavir-D8, which were purchased from Toronto Research Chemicals, Inc. (ON, Canada) and Synthèse AptoChem, Inc. (QC, Canada), respectively. Acetonitrile (ACN) and methanol (MeOH) were supplied by EMD Millipore Corporation (ON, Canada). Ammonium hydroxide, formic acid (HCOOH) and propionic acid were supplied by Fisher Scientific Limited (ON, Canada). Human plasma and human whole blood (K₂EDTA) were obtained from Biological Specialty Corp. (PA, USA) and Milli-Q water (18.2 MW·cm resistivity) was generated in-house by a Millipore water distribution system. Desiccants were provided by Dessicare, Inc. (MS, USA). VAMS devices marketed as Mitra™ 10 µl by Neoteryx LLC (CA, USA) were used in all cases. The polymeric mat (WebSeal mat, 96 rd flat, 7 mm) used to seal the plate during bead impact was supplied by Thermo Scientific (ON, Canada). Stainless steel grinding balls (5/32") were purchased from VWR International LLC (ON, Canada).

Solution preparation

Naproxen stock and intermediate solutions were prepared in MeOH at concentrations of 10.0, 1.00, 0.10 and 0.01 mg/ml. Naproxen-D3 stock solution was prepared at a concentration of 100 µg/ml in MeOH and used to prepare a 300 ng/ml IS working solution (ISWS) in MeOH. Stock solutions of ritonavir (500 µg/ml) and ritonavir-D8 (100 µg/ml) were prepared in ACN:H₂O 50:50% v/v. Ritonavir-D8 ISWS was prepared at 15 ng/ml in ACN:H₂O 75:25% v/v. Spiking solutions of ritonavir in ACN:H₂O 50:50% v/v were prepared from the stock and used to spike calibrants and quality control samples (QCs) in order to reach the desired concentration in blood. All solutions were stored at 4°C.

Calibration standards & QCs

Human whole blood, collected using K₂EDTA as anticoagulant, was fortified at a temperature of 22°C by adding naproxen stock and intermediate solutions or ritonavir spiking solutions. Analytical ranges in human blood were 0.500–100.0 µg/ml for naproxen and 10.0–5000.0 ng/ml for ritonavir. Sampling onto the Mitra™ device was performed on the same day as blood calibrant and QC preparation. As recommended by the manufacturer, human blood calibrants and QCs were absorbed onto the Mitra device positioned at an angle of 45° relative to the sample. Only the lower part of the tip was dipped into the sample. Upon the Mitra™ tip turning fully red, an additional 2 s of sampling time was added. After each sampling, the Mitra™ was air-dried for 15 min, then sealed in an air tight bag containing desiccant. A minimum drying period of 24 h was used prior to sample extraction.

Sample extraction

The Mitra™ sampler tips were transferred to a 1 ml polypropylene 96-well plate (Thermo Scientific 60180-P201) by angular displacement and a 5/32" stainless steel grinding ball placed in each well. Naproxen-D3 (400 µl) or ritonavir-D8 (300 µl) ISWS were then added and the plate was sealed with a polymeric mat. Tips were left presoaking for 10 min at room temperature followed by 5 min of mixing using a 2000 Geno/Grinder (SPEX CertiPrep, Inc.) at a speed of 1900 vertical strokes/min. After this initial stage of IAE, Mitra™ samples were left soaking for an additional 5 min. The cycle of mixing and soaking was then repeated prior to centrifugation at 4612 × *g* for 5 min. For naproxen samples, 100 µl of supernatant was diluted with 100 µl of MeOH:H₂O 20:80% v/v while for ritonavir, 100 µl of supernatant was diluted with 100 µl of ACN:H₂O 10:90% v/v. Samples were then vortexed (1 min at 2000 r.p.m.) and centrifuged (4612 × *g*, 2 min). Ultrasonication experiments were conducted with a VWR 550 T ultrasonic bath which was operated at room temperature for a period of 1 h followed by centrifugation and dilution as detailed above for IAE.

LC-MS/MS instrument & conditions

All analyses were performed using an Agilent Technologies Series 1100 LC system equipped with a solvent degasser, binary pump, column heater and autosampler (Agilent Technologies, CA, USA). Acquisition and data processing were controlled by SCIEX Analyst software.

All chromatographic separations were performed at 35°C from an autosampler temperature of 4°C.

Naproxen extracts were chromatographed at 0.60 ml/min under isocratic conditions with MeOH:propionic acid, 0.1% (60:40% v/v) using a Waters XBridge C₁₈ column (30 × 2.1 mm, 3.5 µm) for a run time of 1.25 min. Naproxen was detected in multiple reaction monitoring mode (MRM) using an API 3000 triple quadrupole mass spectrometer (Sciex, ON, Canada) equipped with a TurboIonSpray source operated at 550°C in negative ionization mode at an electrospray voltage potential of -2.0 kV. Declustering potential was -20 V, focusing potential -100 V, entrance potential -4 V, collision energy -23 V and collision cell exit potential -10 V. MRM *m/z* transition pairs were 229.1 → 170.1 and 232.1 → 173.1 for naproxen and naproxen-D3, respectively.

Ritonavir extracts were chromatographed using a Waters XBridge C₁₈ column (30 × 2.1 mm, 5 µm) for a run time of 1.30 min at 1.00 ml/min under gradient conditions with 0.5% NH₄OH (*aq*) and ACN as mobile phase (MP) components A and B, respectively. Ritonavir was eluted with MP-B held constant at 44% for 0.40 min; MP-B was then increased to 85% and the column washed for 0.40 min, after which 0.50 min was allowed for re-equilibration. Ritonavir was detected in MRM using an API 5000 triple quadrupole mass spectrometer (Sciex, ON, Canada) equipped with a TurboIonSpray source operated at 700°C in positive ionization mode with an electrospray voltage potential of 3.0 kV. Other potentials were 70 V (declustering potential), 100 V (focusing potential), 15 V (entrance potential), 40 V (collision energy) and 15 V (collision cell exit potential). MRM *m/z* transition pairs were 721.33 → 268.10 and 729.38 → 276.10 for ritonavir and ritonavir-D8, respectively.

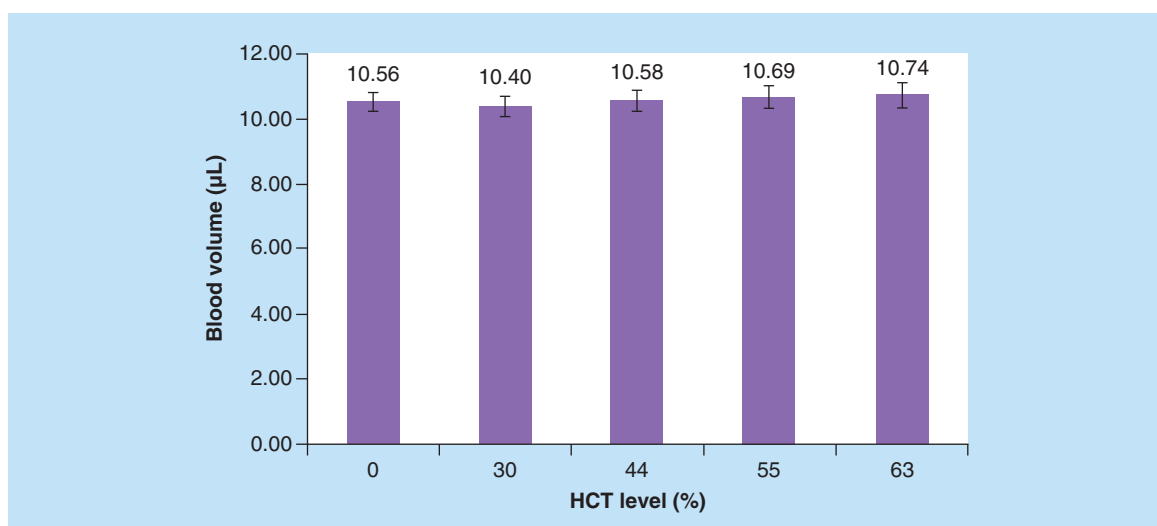


Figure 2. Volume of blood absorbed by volumetric absorptive microsampling device at various HCT levels (n = 12).

Preparation of blood at different HCT levels

Blood lots at various hematocrit levels were prepared in-house by centrifuging human whole blood ($1500 \times g$, 10 min, 4°C) and mixing the collected plasma in various proportions with the bottom fraction containing red blood cells. Precise hematocrit levels were determined by Dynacare[®] (QC, Canada).

Results & discussion

Two essential conditions must be fulfilled in order to achieve a hematocrit effect free assay:

- Blood sampling volume should be accurate and independent of the HCT level; and
- Recovery must be consistent and ideally quantitative. Investigations were performed to determine if both of these conditions could be met when using VAMS in combination with IAE.

Sampling volume determination

The HCT level impacts blood viscosity, and in classic DBS, it has been well-established that this can translate to variability in blood spot area [2,3], thereby biasing sampling volume when a sub-punch is taken. Further, depending upon extraction conditions and the physicochemical properties of the analyte, blood HCT can also deleteriously affect recovery. This combined bias in concentration arising from variability in sampling volume and recovery can compromise an assays' suitability in supporting critical studies.

Therefore, our first evaluation of the VAMS device consisted of verifying if the HCT level had any impact on the volume of blood absorbed onto the polymeric tip of the 10 µl MitraTM. Typical human HCT levels in the normal healthy population range from 41 to 50% for men and 36 to 44% for women, however ranges from 28 to 67% have been reported in certain populations [2]. The current research covers an HCT range of 0% (plasma) to 63%. For each HCT level evaluated, gravimetric analysis coupled with blood density measurements enabled the accurate determination of sampling volume and indicated that the 10 µl MitraTM device absorbed an average volume of 10.6 µl, without significant differences in volume between HCT levels (Figure 2). The average MitraTM sampling volume of 10.6 µl was factored into calculations used to evaluate naproxen and ritonavir recovery and is consistent with results reported elsewhere [7]. It is prudent to note that the procedure described by the manufacturer for collecting blood on the MitraTM tip was carefully followed.

Development of the IAE technique

Previous precut DBS work with IEA [15] involved the testing of different bead sizes with 5/32" stainless steel grinding balls furnishing optimal results. The same beads were chosen for the current research. By placing a bead on top of the MitraTM tip, it was observed that this effectively protects the bottom of the 96-well plate, allowing

Table 1. Recovery and matrix factor for naproxen and ritonavir extracted from Mitra™ (blood HCT 39%) using various solutions combined with Impact-Assisted Extraction (n = 3).

Extraction solution	Naproxen		Ritonavir	
	Recovery (%)	Matrix factor	Recovery (%)	Matrix factor
ACN:HCOOH 99.9:0.1% v/v	87.9	0.95	–	–
ACN	15.0	1.01	–	–
ACN:H ₂ O 90:10% v/v	86.8	0.99	76.3	0.83
ACN:H ₂ O 75:25% v/v	92.4	0.79	91.5	0.80
ACN:H ₂ O 50:50% v/v	96.0	0.81	89.3	0.84
MeOH:HCOOH 99.9:0.1% v/v	95.8	0.85	–	–
MeOH	94.1	0.92	–	–
MeOH:H ₂ O 90:10% v/v	94.5	0.85	90.3	0.82
MeOH:H ₂ O 75:25% v/v	92.9	0.87	83.9	0.85
MeOH:H ₂ O 50:50% v/v	93.4	0.86	81.0	1.04

the utilization of a higher number of strokes/minute compared with that previously used for DBS samples as the Mitra™ polymer absorbs much of the impact; the Mitra™ tip itself remains intact during the IAE process.

Extraction solution optimization

Optimization of the VAMS extraction solution included an examination of MeOH and ACN mixed in various aqueous proportions (Table 1). Preliminary testing with ritonavir suggested that adding water in the extraction solution improved recovery (data not shown); therefore, pure organic solvents were not further evaluated for this analyte. Since naproxen is deprotonated at physiological pH, acidified MeOH and ACN solvents were also assessed.

Recovery of analyte from Mitra™ for each extraction solvent was determined using high QC samples compared against matrix blanks fortified with drug post-extraction. For matrix factor (MF), the same matrix blank fortified with drug post-extraction was compared in terms of peak area response against pure solution containing analyte. An ideal MF of 1.00 indicates a lack of suppression or enhancement, while MF <1.00 suggests analyte response is suppressed. High recovery, low%CV between the extracted replicates, and minimal ion suppression were the primary factors governing the final selection of extraction solution. Multiple extraction solutions met these criteria (Table 1), among which MeOH and ACN:H₂O 75:25% v/v were further chosen for evaluation of naproxen and ritonavir, respectively.

The low recovery using ACN showed poor recovery and is in agreement with VAMS results reported elsewhere [10,14]. Acidifying ACN with 0.1% v/v formic acid markedly improved naproxen recovery versus its nonacidified counterpart. This might be explained by an improved solubilization of dried blood under these conditions and/or the neutralization of the acidic moiety of the drug. Acidified MeOH had little impact on recovery, and in fact, extracts were a reddish/brown hue, suggesting partial resuspension of potentially undesirable components from blood; a similar phenomenon was noted for extraction solutions of higher aqueous content.

IAE recovery versus hematocrit level

Under the optimal extraction conditions established for each analyte, recovery was >93% for all blood HCT levels investigated (Figure 3). The oft reported decrease in recovery as HCT level increases was not observed for either naproxen or ritonavir using IAE, but was noted for ultrasonication [12,14]. The novel bead impact conditions used here successfully circumvented the issue.

Precision, accuracy & matrix effect

A precision and accuracy batch including matrix effect evaluation from four control donors was extracted for naproxen and ritonavir using the optimized VAMS-IAE workflow (Tables 2 & 3). Matrix effect samples demonstrated excellent accuracy, while precision and accuracy (linear weighted $[1/x^2]$ regression for both analytes) data met acceptance criteria for a validatable method.

IAE versus sonication

VAMS coupled with IAE was compared against extraction by ultrasonication (for 1 h in a water bath at 22°C) using QC samples in blood with 39% HCT. For this comparison, conditions for ultrasonication were chosen based upon

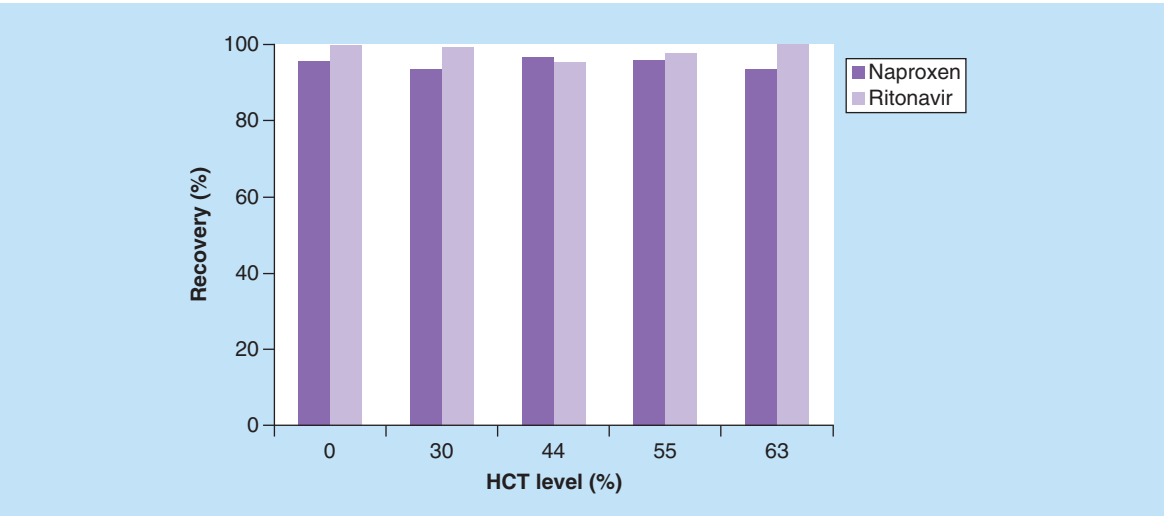


Figure 3. Recovery of naproxen and ritonavir from Mitra™ at various HCT levels using impact-assisted extraction (n = 6).

Table 2. Precision, accuracy and matrix effect for naproxen.			
Evaluation	QC concentration (µg/ml)	Accuracy	%CV
Precision and accuracy (n = 6)	0.5	102.8	7.8
	1.5	96.5	3.8
	20.0	105.7	6.4
	75.0	99.5	3.8
Matrix effect (n = 3 for each lot) (4 lots)	1.5	93.1	4.0
		92.9	8.9
		94.1	7.5
		95.2	4.3
	75.0	96.3	1.6
		94.3	0.1
		101.6	3.1
		96.7	2.2

Table 3. Precision, accuracy and matrix effect for ritonavir.			
Evaluation	QC concentration (ng/ml)	Accuracy	%CV
Precision and accuracy (n = 6)	10.0	105.4	5.5
	30.0	95.3	8.4
	600.0	96.3	10.5
	3750.0	95.3	9.6
Matrix effect (n = 3 for each lot) (4 lots)	30.0	95.5	6.1
		97.2	0.8
		98.1	11
		94.4	2.7
	3750.0	95.6	7.2
		102.9	6.3
		97.6	8.8
		95.0	12.6

literature precedent, which typically include prolonged agitation approaches involving vortexing, lateral shaking or sonication [8,10,12,14]. Using the optimal extraction solution determined for IAE, ultrasonication showed lower recoveries for both naproxen and ritonavir (Figures 4 & 5). The recovery comparison of IAE against the more commonly reported ultrasonication technique confirms that IAE is the more efficient physical-disruptive process, providing near quantitative results desired to eliminate possible HCT-related bias.

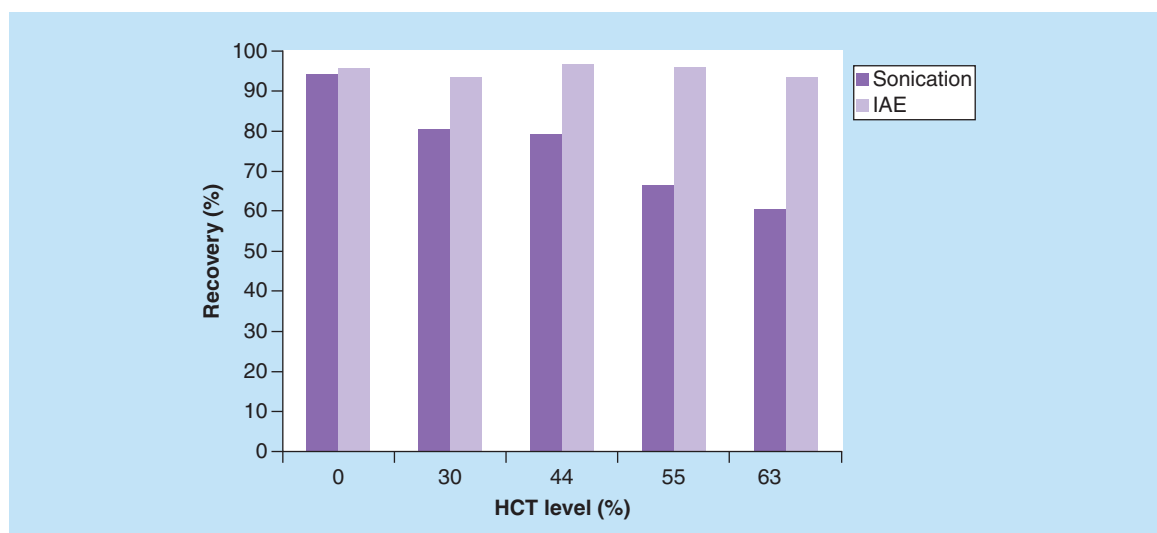


Figure 4. Recovery comparison for naproxen extracted by impact-assisted extraction versus sonication (39% blood HCT, $n = 3$).

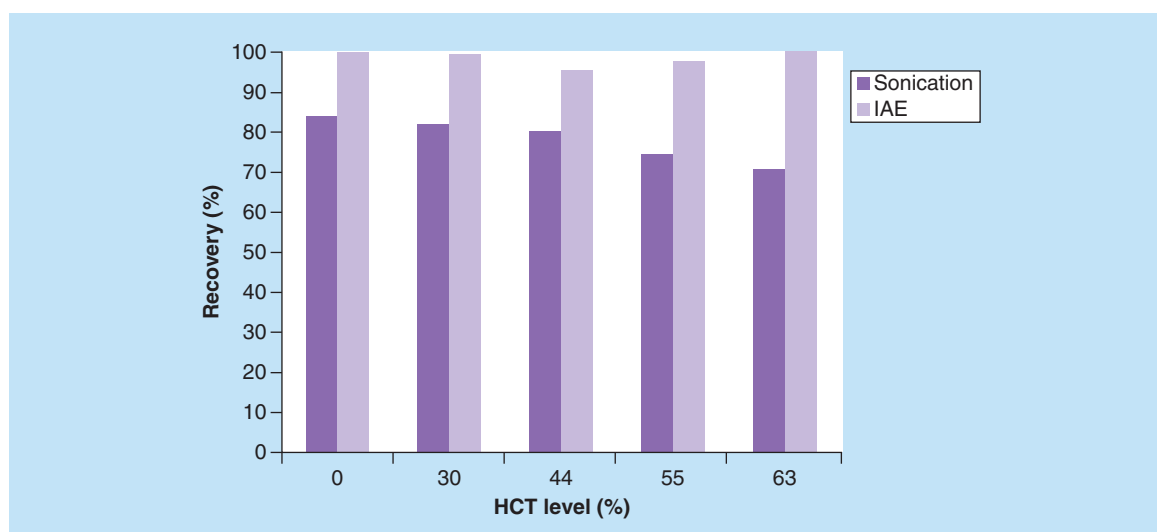


Figure 5. Recovery comparison for ritonavir extracted by impact-assisted extraction versus sonication (39% blood HCT, $n = 3$).

Conclusion

Gravimetric analysis coupled with blood density measurements enabled the accurate determination of Mitra™ sampling volume, indicating that the 10 μ l nominal VAMS device absorbed an average volume of 10.6 μ l, independent of the HCT level (0–63% HCT). The novel IAE approach yielded higher recovery for both ritonavir and naproxen than the more commonly implemented ultrasonication procedure. Following IAE solution optimization, recoveries for ritonavir and naproxen were $\geq 93\%$, demonstrated without HCT bias. The IAE procedure is believed to be responsible for the absence of HCT-related bias from extraction. A precision and accuracy batch with matrix effect (four donors) was extracted for each compound and met acceptance criteria for a validatable method.

In summary, the VAMS method coupled with IAE represents a simple collection and extraction procedure independent of the HCT biases affecting sampling volume and recovery. It is also a procedure compatible for high-throughput analysis of blood samples in the context of a regulated environment, and is scalable for large studies. In addition, bead impact apparatus, such as the 2000 or 2010 Geno/Grinder, is readily available in many

bioanalysis laboratories. Future work will include evaluating the impact of presoaking duration and extraction solution temperature on a range of compounds with varying logP and stability.

Future perspective

At this stage of the VAMS evolution, we believe widespread adoption will be dependent upon a complete absence of hematocrit effect, once demonstrated for an expanded number of compound types. Therefore, the novel IAE workflow described in this research is anticipated to contribute to this adoption through the elimination of the HCT-related recovery bias previously associated with VAMS. Future research endeavors include the evaluation of several small molecules representing a broader range of logP to determine if IAE continues to eliminate the hematocrit recovery bias independent of compound polarity. Additionally, the application of VAMS and IAE will be explored for both peptides and large molecules, the latter using the bottom-up approach for quantitation.

Executive summary

Aim

- Volumetric absorptive microsampling (VAMS) is a recent option available for sampling and analyzing low blood volumes. The present work describes the utilization of VAMS for the quantitation of naproxen and ritonavir in human blood using a novel Impact-Assisted Extraction (IAE) procedure.

Experimental

- Blood lots of various hematocrit levels were prepared in-house and their exact hematocrit level was subsequently determined.
- The VAMS sampler tips were transferred to a 96-well plate and a 5/32" stainless steel grinding ball was added. Internal standard working solutions were then added and the plate was sealed with a polymeric mat and the tips were left to soak and were treated by IAE. Samples were diluted and analyzed by LC-MS/MS.

Results & discussion

- 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 μ L, independent of the HCT level (0–63% range).
- Following extract solution optimization, MeOH and ACN:H₂O 75:25% v/v were chosen, respectively, for naproxen and ritonavir, as these solvents offered a combination of high recovery, minimal variability between replicates and a lack of matrix effect.
- Using IAE, the often reported decrease in recovery with increasing HCT was not observed for either naproxen or ritonavir; recoveries of >93% were achieved at all HCT levels. The aggressive mixing process of IAE is believed to be responsible for the absence of extraction-related HCT bias.
- A precision and accuracy batch with matrix effect (four donors) was extracted for each analyte and met all acceptance criteria for a validatable method.
- The novel IAE procedure produced superior recoveries for naproxen and ritonavir when compared with ultrasonication.

Conclusion

- The simple IAE technique described for naproxen and ritonavir in blood sampled by VAMS is independent of HCT biases affecting sampling volume and recovery.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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