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Extractability-mediated stability bias and hematocrit impact: High extraction recovery is critical to feasibility of volumetric adsorptive microsampling (VAMS) in regulated bioanalysis

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ABSTRACT

Volumetric absorptive microsampling (VAMS), a new microsampling technique, was evaluated for its potential in supporting regulated bioanalysis. Our initial assessment with MK-0518 (raltegravir) using a direct extraction method resulted in 45–52% extraction recovery, significant hematocrit (Ht) related bias, and more importantly, unacceptable stability (>15% bias from nominal concentration) after 7-day storage. Our investigation suggested that the observed biases were not due to VAMS absorption, sampling techniques, lot-to-lot variability, matrix effect, and/or chemical stability of the compound, but rather the low extraction recovery. An effort to improve assay recovery led to a modified liquid–liquid extraction (LLE) method that demonstrated more consistent performance, minimal Ht impact (Ht ranged from 20 to 65%), and acceptable sample stability. The same strategy was successfully applied to another more hydrophilic model compound, MK-0431 (sitagliptin). These results suggest that the previously observed Ht effect and "instability" were in fact due to inconsistent extractability, and optimizing the extraction recovery to greater than 80% was critical to ensure VAMS performance. We recommend adding Ht-independent recovery as part of feasibility assessment to de-risk the long-term extractability-mediated stability bias before implementing VAMS in regulated bioanalysis.

1. Introduction

Volumetric absorptive microsampling (VAMS), marketed as Mitra™, was introduced as a novel microsampling device in 2014 [1], and has been the focus of increasing attention in bioanalysis since then [2]. Compared to conventional dried blood spot (DBS), VAMS delivers all the benefits of microsampling, including small sample volume, and convenient sample handling in terms of collection, storage, and shipment. Most importantly, its potential to overcome hematocrit (Ht) effects [3,4], a well-known potential limitation associated with DBS card-based microsampling [5,6], becomes an attractive feature for VAMS implementation.

For any new technology, an in-depth evaluation is warranted to assess its performance and explore any potential issues, before implementation in the regulated bioanalytical environment. Over the past three years, there have been numerous reports on using VAMS for quantification of various drugs/drug candidates [7–20]. Among these publications, only one case reported acceptable stability for up to one month [9], while most of the other compounds were stable on VAMS for either up to 7–10 days or shorter at room temperature storage. For the latter set of compounds, some were not evaluated for longer storage times [16,17,19], others, such as fosfomycin, hydroxyurea, and piperacillin, etc., could be explained by compound degradation due to chemical instability [11,14,20]. However, some incidences could not be explained by chemical instability. For example, tacrolimus was reported stable on DBS card for at least 30 days under ambient condition [21], while its VAMS QC sample showed <85% accuracy within 3 days of room temperature (RT) storage [7]. Similarly, itraconazole was stable in human whole-blood samples for up to 48 h at room tempera-

Abbreviations: VAMS, volumetric absorptive microsampling; Ht, hematocrit; LLE, liquid–liquid extraction; GLP, good laboratory practice; LC–MS/MS, liquid chromatography coupled with triple quadrupole mass spectrometry; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; QC, quality control; IS, internal standard; %CV, correlation variation; RH, relative humidity; FDA, US Food and Drug Administration; EMA, European Medicines Agency.

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ture [22], but not on VAMS – significant analyte loss was observed after 48 h RT storage [10]. Nevertheless, a 7- or 10-day stability is insufficient for most regulated bioanalysis, especially for late stage clinical sample handling, which generally involves inventory at central laboratories and shipment from worldwide clinical sites to analytical laboratories for analysis. Some mitigating actions were implemented by storing the VAMS samples at −20 °C or −80 °C [7,11,15,18,20] or extracting the analyte from VAMS within 24 h of sample collection and then keeping the extract at −80 °C for long-term storage [8,10]. However, these alternative storage/shipping conditions would diminish the advantage of handling dried blood matrix, and may not be feasible for clinical PK studies, especially for home collection.

Another concern related to VAMS is the impact of hematocrit on assay performance. Although it has been demonstrated by gravimetric determination that the VAMS tip can accurately absorb a fixed volume of blood (10 μL) regardless of hematocrit values [3], an inverse correlation between accuracy of analyte and hematocrit was reported in some instances – a positive bias was observed at low Ht values, while a negative bias was found for blood samples with a high Ht [9,14]. In certain cases, the Ht impact could be even more profound than that observed in DBS; but it could be corrected by changing extraction solvents [23,24]. These observations led to the conclusion that Ht-independent recovery is key for bioanalysis using VAMS devices, which can be achieved by careful selection of extraction methods [23,24]. However, no publication thus far has linked the Ht effect with the observed stability bias.

For regulated bioanalysis, stability is an essential requirement to ensure sample integrity and data consistency over long term studies. Understanding the root-cause of the apparent "instability", and subsequently improving the sample stability on VAMS are critical assessments to allow implementation of VAMS to support GLP and clinical studies in the future. In this report, we share our results from VAMS evaluation using model compounds, MK-0518 (raltegravir) and MK-0431 (sitagliptin), and focus on the two major concerns/challenges discussed above. Based on our data, we propose that extractability could be a critical reason for the apparent instability and the observed hematocrit impact. Improving extraction recovery to achieve age-independent and Ht-independent recovery is the key for VAMS performance. To ensure appropriate usage of VAMS technology, we recommend including recovery optimization and evaluation as part of bioanalytical feasibility assessments in our strategic guidance on microsampling implementation [25,26]. To our best knowledge, this is the first report to explicitly elaborate the relationship among the three factors, i.e. Ht effect, stability and recovery, for VAMS. We believe this information will be helpful to the analysts during method development, and make VAMS suitable/applicable to more programs in regulated bioanalysis in the future.

2. Materials and methods

2.1. Chemicals, reagents and instruments

Reference standards for raltegravir (MK-0518) and sitagliptin (MK-0431) and their respective isotope-labeled internal standards, [$^{13}C_6$]-MK-0518 (IS-1) and [$^{2}H_4$]-MK-0431 (IS-2) (Fig. 1), were obtained in house (Merck & Co., Inc., Kenilworth, NJ, USA). DMSO was purchased form Sigma Aldrich (Missouri, USA). Optima LC/MS grade acetonitrile (ACN), methanol (MeOH), 0.1% formic acid (FA) in acetonitrile, 0.1% formic acid in water, methyl-*t*-butyl either (MtBE), and ammonium acetate (NH_4Ac) were obtained from Thermo Fisher Scientific (MA, USA). Water was purified by a Milli-Q ultra-pure water system from Millipore (MA, USA).

VAMS units (10 μL tips in 96-well format) were obtained from Neoteryx (CA, USA). Human control blood with K_2EDTA as anticoagulant was purchased from Biological Specialty Corp. (Colmer, PA) with certificate of analysis (COA), and used within 10 h of blood draw. MC5 microbalance was from Sartorious (Goettingen, Germany). Sonication bath was purchased from Thermo Fisher Scientific. Humidity chamber with a temperature range of 20–75 °C and up to 100% relative humidity (RH) was purchased from Thermo Fisher Scientific.

2.2. LC–MS/MS conditions

Both MK-0518 and MK-0431 were analyzed using a Waters Acquity™ UPLC system coupled with a Sciex API4000 or API5000 triple quadrupole mass spectrometer under positive electrospray mode and multiple reaction monitoring (MRM). Unit resolution was used for both compounds and their internal standards. Peak area ratios were calculated using Analyst Software 1.6. Calibration curves were obtained by weighted ($1/x^2$) linear regression of the peak area ratio of analyte to IS vs. nominal concentration (x) of the analyte.

For MK-0518, samples were analyzed on a Waters BEH Shield RP column (2.1 × 50 mm, 1.7 μm). The mobile phase consisted of (A) 0.1% FA in water and (B) 0.1% FA in acetonitrile. A gradient elution with a flow rate of 0.6 mL/min was employed with linear increase of %B from 30 to 90% in 1 min, held at 90%B for 0.2 min, repeating the step gradient from 30% to 90% for two cycles in 1.7 min to minimize carryover, and then equilibrating the column to 30%B for 0.6 min. The total analytical run time was 3.5 min. The column was maintained at 40 °C, and the autosampler was kept at 10 °C. The MRMs monitored were m/z 445 → 361 for MK-0518 and m/z 451 → 367 for IS-1. The instrument setting was adjusted to maximize the response for the analyte and IS, respectively. The ion source gas 1 (GS1), gas 2 (GS2), collision gas (CAD) and curtain gas (CUR) were set at 50, 60, 5 and 30 L/min, respectively, with a source temperature at 500 °C. The ion-spray voltage, declustering potential (DP), collision energy (CE) were set at 4200 V, 51 V, and 25 V, respectively. The dwell time was 100 milliseconds for MK-0518 and IS-1, respectively.

For MK-0431, samples were analyzed on a Supelco Ascentis Express HILIC column (50 × 2.1 mm 2.7 μm) with a mobile phase of 10 mM NH_4Ac (pH 4.5) in 80%ACN at a flow rate of 0.3 mL/min. The analytical run time was 2 min. The column was maintained at 40 °C and the autosampler was kept at 10 °C. The MRMs monitored were m/z 408 → 235 and m/z 412 → 239 for MK-0431 and IS-2, respectively. GS1, GS2, CAD and CUR were set at 40, 70, 6 and 30 L/min, respectively, with a source temperature of 550 °C. The settings for ion spray voltage, DP, and CE were 5500, 70, 28 V, respectively. The dwell time was 100 milliseconds for MK-0431 and IS-2, respectively.

2.3. Preparation of standards and quality control (QC) samples on VAMS

MK-0518 primary stock was prepared in DMSO at 1 mg/mL. Working standard solutions were prepared by serial dilution with 50% acetonitrile to obtain a concentration range of 500–500,000 ng/mL for MK-0518. The whole blood standards and quality control samples were prepared by spiking 20 μL of the working solution into 980 μL EDTA human control blood to yield the corresponding MK-0518 standards with concentrations ranging from 10 to 10,000 ng/mL in blood, and MK-0518 QC samples at four levels: LLOQ (10 ng/mL), low QC (LQC; 30 ng/mL), middle QC (MQC; 500 ng/mL) and high QC (HQC; 7500 ng/mL).

MK-0431 primary stock and working standard solutions were prepared in 50% acetonitrile. Working standard solutions were prepared from stock solutions (1 mg/mL) with concentrations ranging