Tranexamic acid quantification in human whole blood using liquid samples or volumetric absorptive microsampling devices

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Background: Recent clinical trials demonstrate the benefits of the antifibrinolytic drug tranexamic acid but its pharmacokinetics remain to be investigated more in depth. Although pharmacokinetics studies are usually performed with plasma, volumetric absorptive microsampling devices allow us to analyze dried whole blood samples with several advantages. Materials & methods: High-sensitivity LC–MS/MS methods for the quantification of tranexamic acid in human whole blood using liquid samples or dry samples on volumetric absorptive microsampling devices were developed and validated based on International Association from Therapeutic Drug Monitoring and Clinical Toxicology, European Medicines Agency and US FDA guidance. Conclusion: The method performances were excellent across the range of clinically relevant concentrations. The stability of tranexamic acid in blood samples stored up to 1 month at +50°C was demonstrated. The methods’ suitability was confirmed with clinical samples.

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Tranexamic acid (TXA) is an antifibrinolytic drug that reduces surgical bleeding and bleeding-related deaths in trauma and postpartum hemorrhage [1–6]. Early initiation of TXA treatment is essential. The intravenous route is the preferred route in acute life-threatening bleeding in part because the pharmacokinetics with alternative routes, for example, oral solution or intramuscular injection, is as yet poorly described. Although TXA is not a new drug, there is indeed no agreed dosing regimen for each indication and pharmacokinetic studies are needed to determine the optimal dosing and administration route in each context [7–12].

Although plasma or serum are the classical samples used for therapeutic drug monitoring or pharmacokinetic studies, other samples may have considerable interests. Pharmacokinetic studies involve administering the drug to patients and taking blood samples (sometimes up to ten) within hours of administration. Venous blood samples must be taken by qualified personnel and sent under suitable storage conditions to a laboratory for processing. This includes a centrifugation step to obtain plasma or serum, which will then be aliquoted, frozen and sent under appropriate conditions to the laboratory for analysis. New volumetric absorptive microsampling (VAMS) devices have recently been introduced for the collection of capillary blood and therapeutic drug monitoring [13–17]. In contrast with dry blood spot sampling, volumetric absorptive microsampling devices allow us to collect a precise volume of blood, ensuring accuracy of drug quantification. Other advantages are the ease of collection and the savings in personnel costs (no need to hire professional staff to collect blood samples, as these devices can be used at anytime, anywhere and by anyone), whole blood work that avoids laboratory processing of the sample (no centrifugation or aliquoting of the plasma), and the storage and dispatch of samples at room temperature [18].

Although several LC–MS/MS methods have been described for the quantification of tranexamic acid in human samples, all used plasma or serum [19–23]. We aimed to develop methods for the quantification of tranexamic
acid in human whole blood using either liquid samples or dried samples collected with volumetric absorptive microsampling devices and to validate these methods based on the guidance from the International Association from Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) for the development and validation of dried-blood spot-based methods [24], and from the European Medicines Agency (EMA) and the US FDA for the validation of bioanalytical methods. Since microsampling devices are also intended to facilitate pharmacokinetic studies in environments away from established laboratories, an extensive stability study was also conducted. The methods were finally applied to both liquid and dried whole blood samples from five healthy volunteers participating to a pharmacokinetic study.

**Materials & methods**

**Chemicals, reagents & materials**

Tranexamic acid [4-(aminomethyl)cyclohexanecarboxylic acid] (C8H15NO2; monoisotopic mass: 157.110 g/mol; logP: -2), the internal standard (IS) 4-aminocyclohexanecarboxylic acid (C7H13NO2; monoisotopic mass: 143.095 g/mol; logP: -2.2) and LC–MS-grade formic acid were supplied by Sigma Aldrich (Saint Quentin Fallavier, France). LC–MS-grade ammonium formate, methanol, acetonitrile and water were obtained from Fisher Scientific (Illkirch, France). Blank human whole blood was obtained from Etablissement Français du Sang (Rungis, France). Volumetric absorptive microsampling devices (Mitra®) were supplied by Neoteryx (CA, USA).

**Preparation of solutions, calibration standards & quality control samples**

Stock solutions of tranexamic acid (10 g/l) and IS (1 g/l) were prepared in water/methanol (50/50, v/v). Working solutions of tranexamic acid (1000.0, 100.0 and 10.0 mg/l) and IS (200.0 mg/l) were prepared by dilution of the stock solutions in methanol and stored at -20°C. Calibration standards (CS) were prepared at eight concentrations (0.1, 1.0, 10.0, 50.0, 100.0, 250.0, 500.0 and 1000.0 mg/l) by dilution of the stock or working solutions in human whole blood. Quality control (QC) samples were prepared separately at 0.3, 25.0, 400.0 and 750.0 mg/l by dilution of the appropriate solution in human whole blood and stored in aliquots at -20°C.

**Sample preparation**

**Liquid whole blood samples**

Whole blood (10 μl) were put into a conic 1.5 ml tube. Five microliters of IS solution (4-aminocyclohexanecarboxylic acid at 200.0 mg/l in methanol) and 150 μl of methanol were added. Samples were vortexed, shaken for 20 min and centrifuged at 17,000 × g for 10 min. Fifty microliters of the supernatant were taken to injection vials for LC–MS/MS analysis.

**Whole blood sampled with the volumetric absorptive microsampling devices**

Volumetric absorptive microsampling devices (10 μl devices) were immersed for 2 s in the calibration standard, quality control or patient sample (capillary blood obtained with finger stick) and dried for 24 h at room temperature, according to manufacturer's instructions. Then, the extremity of the devices was put into conic 1.5 ml tubes. Five microliters of IS solution (4-aminocyclohexanecarboxylic acid at 200.0 mg/l in methanol) and 150 μl of water/methanol (20/80, v/v) were added. Samples were vortexed, put in ultrasonic bath for 30 min, shaken on an orbital shaker for 6 h at 300 r.p.m. at 37°C and centrifuged at 17,000 × g for 10 min. Fifty microliters of the supernatant were taken to injection vials for LC–MS/MS analysis.

**LC–MS/MS system & analysis procedure**

The chromatography and mass spectrometry detection were performed as previously described [19]. The multiple reaction monitoring transitions were as follows (precursor ion (m/z) → product ions (m/z) (collision energy (%)): 158.1 → 67.2 (25), 95.1 (16), 123.2 (10) for tranexamic acid and 144.1 → 81.1 (22), 109.4 (15), 126.1 (11) for the internal standard; the first transition being used for quantification. Data acquisition and processing were performed using Xcalibur v4.2.28.14 (Thermo Fisher Scientific).

**Method validation procedure**

The two methods for liquid whole blood and dried whole blood on volumetric absorptive microsampling devices were validated based on the EMA guideline [25] and FDA guideline [26] for selectivity, carryover, lower limit of quantification, calibration curve, accuracy, precision, matrix effect and stability.
Selectivity & carryover

Human whole blood samples from six different donors were used. First, six samples without tranexamic acid and internal standard added (blank samples) were processed and compared with samples from the same donors spiked with tranexamic acid at the LLOQ and internal standard. A blank sample was analyzed immediately after the highest CS in each run to evaluate the carry over. Assay selectivity was defined by the ratio between the area of tranexamic acid or IS in samples spiked at the LLOQ and their area in blank samples, whose mean had to be greater than 5 for tranexamic acid and greater than 20 for IS.

Lower limit of quantification

The LLOQ is defined as the lowest measurable concentration for which the accuracy is between 80 and 120% and the CV of ± 20% or less. Signal has to be superior or equal to five-times the signal of a blank sample.

Calibration curve

Calibration curves ranged from 0.1 mg/l (LLOQ) to 1000.0 mg/l (ULOQ) and a blank sample, a zero sample and eight CS. For quantification, the peak area ratios of tranexamic acid to the IS were plotted versus theoretical concentrations and analyzed with by least-square regression. For at least six CS, the back-calculated concentrations have to be in the range 85–115% of the nominal concentrations. For the LLOQ, the accepted range is 80–120%.

Accuracy & precision

Accuracy (measured concentration/nominal concentration) and precision (CV) were calculated for the QC and the LLOQ. Each QC level was processed six-times the same day for the intraday assay and on three different days for the interday assay. The data were analyzed using variance analysis (ANOVA). Accuracy had to be within 85–115% of the nominal concentrations and precision of ± 15%. For the LLOQ, an accuracy between 80–120% and a precision of ± 20% were accepted.

Extraction recovery, matrix effect & overall recovery

Human whole blood samples from six different donors and matrix-free samples were used to investigate extraction recovery, matrix effect and overall recovery. Three procedures (A, B and C) were performed at two QC concentrations (0.3 and 750.0 mg/l) for liquid samples (LS) or VAMS devices to evaluate these parameters: (A) 10 microliters of each spiked whole blood sample (LS) or dried VAMS device extremities loaded with spiked whole blood were put into conic 1.5 ml tubes. Then, IS was added and the complete sample preparation procedure was carried through; (B) blank whole blood samples (LS) or dried VAMS device extremities loaded with blank whole blood were prepared according to the complete sample preparation procedure, supernatant was transferred into a vial at the end of the precipitation step, then tranexamic acid and the IS were spiked and the preparation procedure finished; (C) tranexamic acid and the IS were spiked in methanol (LS) or water/methanol (20/80, v/v; VAMS) and directly injected. The peak areas obtained using the three procedures for the two lowest and highest QC levels were analyzed for both tranexamic acid and IS. Extraction recovery was defined by the ratio between the peak area obtained in procedure A and the peak area obtained in procedure B. The matrix factor, defined as the ratio between the peak area obtained in procedure A and the peak area obtained in procedure B, was calculated to assess matrix effect. The IS-normalized matrix factor is defined as the matrix factor of tranexamic acid divided by the matrix factor of the IS and its CV has to be ≤15%. Overall recovery corresponds to the ratio between the peak area of procedure A to the peak area of procedure C. For IS, overall method recovery had to be ± 15% of that of tranexamic acid.

Stability

The stability of tranexamic acid was previously shown in solutions stored at -20°C for 5 years, in whole blood samples after storage for 6 h at +4°C, in serum samples for 6 years at -20°C or after three freeze and thaw cycles and in processed samples up to 7 days in the autosampler [19,20]. In the present study, due to the analysis of new matrices with specific sample preparation procedures and to storage conditions which may be encountered in real conditions, the stability of whole blood sampled on volumetric absorptive microsampling devices was studied after storage at + 50°C for 1 month. The stability of processed samples was also studied after storage in the autosampler (+ 8°C) for 1 week.
Analysis of study samples
To confirm the suitability of the method for the analysis of clinical samples, it was applied to measure tranexamic acid concentration in whole blood samples from five volunteers participating in a clinical trial (PharmacoTXA, NCT03777488). Patients gave written informed consent for their participation to the study. Those patients received either 1 g TXA through the intravenous or intramuscular route or 2 g through the oral route and one liquid whole blood sample and one capillary blood sample on a VAMS device were collected 3–6 h after drug administration. The liquid blood sample was obtained by venipuncture and stored frozen at -80°C while the fingerstick method was used for VAMS capillary blood sampling and the devices stored at ambient temperature.

Results & discussion
Optimization of sample preparation for volumetric absorptive microsampling devices
Tranexamic acid is a hydrophilic compound (logP: -2.2), therefore several polar solvents were tested for the extraction of the drug from whole blood dried on volumetric absorptive microsampling devices. Acetonitrile, methanol and water/methanol (20/80, v/v) were compared with devices spiked with whole human blood at four concentrations (0.1, 1.0, 10.0 and 100.0 mg/l). For this step, samples were vortexed for 30 s, shaken for 1 h and centrifuged at 17,000 × g for 10 min. The use of acetonitrile resulted in obtaining chromatographic peaks with very poor quality, whereas good peak shape was obtained with methanol and water/methanol (20/80, v/v), with similar peak area between the two solvents. When compared with liquid whole blood samples prepared with the same method, the average ratio of the peak area between VAMS devices and liquid samples was 0.6, suggesting that the extraction recovery was not optimal. The sample preparation method was therefore modified according to previous reports [27,28]: samples were vortexed for 30 s, then put in ultrasonic bath for 30 min, shaken for 20 min and centrifuged at 17,000 × g for 10 min. With adding this step of ultrasonic bath, the peak area ratio between VAMS and liquid samples was improved to about 0.8. In a last step, shaking was extended to 6 h at 37°C, which resulted in extraction recoveries of 93–101% when water/methanol (20/80, v/v) was used as an extraction solvent. Consequently, these conditions were retained as the final sample preparation procedure.

In order to have a comprehensive overview of method performances, the influence of hematocrit was studied. Three hematocrit levels (30, 50 and 70%) were generated with three blood samples which were then spiked at 20 mg/l tranexamic acid and loaded on VAMS devices. For the three hematocrit levels, the measured concentrations were 22.1, 20.0 and 23.2 mg/l, respectively (min: 18.1; max: 25.0 mg/l), suggesting that hematocrit does not affect tranexamic acid measurements with VAMS devices.

Method validation
Selectivity & carryover
The mean of the ratio between the tranexamic acid peak area in samples spiked at the LLOQ and blank samples were 40 and 36 for human liquid whole blood and human whole blood dried on VAMS devices, respectively; for the internal standard, the mean ratio was greater than 100,000, demonstrating the selectivity for both molecules. The peak area of tranexamic acid and internal standards in blank samples injected after the highest CS was lower than 5% of the peak area at the LLOQ (n = 6), therefore no carry-over occurs after the analysis of samples with tranexamic acid concentrations up to the ULOQ.

Lower limit of quantification
The LLOQ was set at 0.1 mg/l, providing a signal superior to five-times the signal of a blank sample and with intra- and interday CVs of 2.9 and 10.9% for human liquid whole blood samples and 6.3 and 12.6% for human whole blood samples on VAMS devices. Intra- and interday accuracies were between 91.8–100.2 and 95.6% for liquid samples and 90.7–99.2% and 93.6% for VAMS devices (Tables 1 & 2). Representative chromatograms are shown in Figures 1 and 2 for liquid whole blood samples and VAMS devices, respectively.

Calibration curve
The calibration range has been determined with the eight calibration standards in the range 0.1–1000.0 mg/l. The data were fitted with an ordinary least square model, as guided by the visual inspection of the graphs plotting the peak area ratios of tranexamic acid to the IS versus concentration. Data heteroscedasticity were suggested by heterogeneity in the pattern of residuals plotted as a function of the concentration. Therefore, a weighted least square regression model was applied with a 1/x weighing factor [29]. Means fitted equation obtained from seven...
Table 1. Accuracy and precision data for tranexamic acid in liquid whole blood.

| QC concentration (mg/l) | Concentration found each day (mean ± SEM) | Intraday (n = 6) | Interday (n = 6) |
|-------------------------|------------------------------------------|-----------------|-----------------|
|                         |                                          | CV (%)          | Accuracy (%)    | CV (%)          | Accuracy (%)    |
| 0.3                     | D1: 0.26 ± 0.01                          | 6.5             | 85.9–90.6       | 6.6             | 88.0            |
|                         | D2: 0.27 ± 0.01                          |                 |                 |                 |                 |
|                         | D3: 0.26 ± 0.002                         |                 |                 |                 |                 |
| 25.0                    | D1: 26.1 ± 0.3                           | 1.8             | 104.3–110.8     | 8.2             | 108.5           |
|                         | D2: 27.7 ± 0.1                           |                 |                 |                 |                 |
|                         | D3: 27.6 ± 0.2                           |                 |                 |                 |                 |
| 400.0                   | D1: 405.3 ± 2.3                          | 1.7             | 101.3–105.5     | 5.1             | 103.7           |
|                         | D2: 417.4 ± 2.9                          |                 |                 |                 |                 |
|                         | D3: 422.0 ± 3.4                          |                 |                 |                 |                 |
| 750.0                   | D1: 730.2 ± 6.0                          | 1.9             | 95.5–101.1      | 7.0             | 98.0            |
|                         | D2: 716.6 ± 6.4                          |                 |                 |                 |                 |
|                         | D3: 757.9 ± 4.4                          |                 |                 |                 |                 |

QC: Quality control; SEM: Standard error of the mean.

Table 2. Accuracy and precision data for tranexamic acid in dried whole blood sampled on volumetric absorptive microsampling devices.

| QC concentration (mg/l) | Concentration found each day (mean ± SEM) | Intraday (n = 6) | Interday (n = 6) |
|-------------------------|------------------------------------------|-----------------|-----------------|
|                         |                                          | CV (%)          | Accuracy (%)    | CV (%)          | Accuracy (%)    |
| 0.3                     | D1: 0.27 ± 0.002                         | 3.0             | 85.2–93.2       | 11.3            | 89.8            |
|                         | D2: 0.28 ± 0.003                         |                 |                 |                 |                 |
|                         | D3: 0.26 ± 0.004                         |                 |                 |                 |                 |
| 25.0                    | D1: 28.2 ± 0.1                           | 1.4             | 111.5–112.8     | 1.4             | 112.2           |
|                         | D2: 28.0 ± 0.2                           |                 |                 |                 |                 |
|                         | D3: 27.9 ± 0.2                           |                 |                 |                 |                 |
| 400.0                   | D1: 436.3 ± 4.9                          | 2.4             | 109.1–110.5     | 1.7             | 109.9           |
|                         | D2: 442.0 ± 4.5                          |                 |                 |                 |                 |
|                         | D3: 441.2 ± 3.7                          |                 |                 |                 |                 |
| 750.0                   | D1: 769.6 ± 13.2                         | 4.1             | 102.6–103.7     | 1.5             | 103.3           |
|                         | D2: 777.9 ± 14.5                         |                 |                 |                 |                 |
|                         | D3: 777.3 ± 11.3                         |                 |                 |                 |                 |

QC: Quality control; SEM: Standard error of the mean.

curves for human liquid whole blood and whole blood dried on VAMS devices were $y = 0.00454 \ (\pm 0.00058) x - 0.00080 \ (\pm 0.00074; \text{mean} \pm \text{SD})$ and $y = 0.00572 \ (\pm 0.00094) x - 0.00084 \ (\pm 0.00054)$, respectively, with corresponding mean least square linear regression correlation coefficient ($r^2$) of 0.9997 ($\pm 0.0002$) and 0.9994 ($\pm 0.0005$).

The mean bias of the back-calculated concentrations of the calibration standards (for the two methods) ranged from -7.2 to 4.1% and the interday CVs ranged from 0.7 to 9.5%.

**Accuracy & precision**

Intraday and interday precision and accuracy of the QC samples are presented in Table 1 for liquid whole blood and in Table 2 for dried whole blood sampled on volumetric absorptive microsampling devices. Intraday precision was in the range 1.4–6.5% with an accuracy between 85.2 and 112.8%. Interday precision was between 1.4 and 12.6% with an accuracy from 88.0 to 112.2%. The developed methods are therefore precise and accurate in a wide range of concentrations.

**Extraction recovery, matrix effect & overall recovery**

Table 3 summarizes the results for both liquid and dried whole blood samples. The extraction recovery of tranexamic acid was in the range 90.9–100.7%. The matrix effect was in the range 19.2–38.7% for both tranexamic acid and internal standard, at the two concentration levels. However, the IS-normalized matrix effect for the two methods was in the range 89.1–104.5%, demonstrating the appropriateness of the internal standard. The CVs of the IS-normalized matrix effect were lower than 10% for the two QC levels, meeting all validation requirements.
**Figure 1.** Representative LC–MS/MS chromatograms obtained for tranexamic acid assay in liquid whole blood. Representative LC–MS/MS chromatograms of tranexamic acid (two upper graphs in each panel) and internal standard (two lower graphs in each panel) obtained after the analysis of a blank sample (A), a calibration standard at the LLOQ (B), at the ULOQ (C) and of a patient sample (D).

**Table 3.** Extraction recovery and matrix effect of tranexamic acid and internal standard in human whole blood and human whole blood sampled on volumetric absorptive microsampling devices (mean ± standard error of the mean% (coefficient of variation%)).

| Quantity of tranexamic acid | Extraction recovery | Matrix factor | IS-normalized matrix factor |
|-----------------------------|---------------------|---------------|-----------------------------|
| Liquid whole blood          |                     |               |                             |
| 0.3 mg/l (n = 6)            | 90.9 ± 6.7 (18.1)   | 19.2 ± 2.2 (28.0) | 89.7 ± 5.4 (14.6) |
| 750.0 mg/l (n = 6)          | 98.4 ± 5.5 (13.7)   | 32.8 ± 3.3 (24.7) | 109.8 ± 5.2 (11.6) |
| Internal standard           | 116.5 ± 7.0 (22.5)  | 24.2 ± 2.7 (31.2) | NA                         |
| Whole blood dried on VAMS devices |                   |               |                             |
| 0.3 mg/l (n = 6)            | 93.1 ± 4.0 (10.5)   | 26.5 ± 1.6 (14.4) | 89.5 ± 3.1 (8.6) |
| 750.0 mg/l (n = 6)          | 100.7 ± 10.9 (26.5) | 38.7 ± 2.2 (13.8) | 104.5 ± 3.5 (8.2) |
| Internal standard           | 87.8 ± 3.1 (12.3)   | 33.5 ± 1.8 (17.4) | NA                         |

IS: Internal standard; VAMS: Volumetric absorptive microsampling.

**Stability**

The stability of processed samples (for the two methods) was studied after storage in the autosampler (+8°C) for 1 week and all the differences between the measured and nominal concentrations were in the range -6.5–13.6% (n = 6). The stability of whole blood sampled on VAMS devices was studied after storage at +50°C for 1 month and
Quantification of TXA in human whole blood  
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Figure 2. Representative LC–MS/MS chromatograms obtained for tranexamic acid assay in dry whole blood on volumetric absorptive microsampling devices. Representative LC–MS/MS chromatograms of tranexamic acid (two upper graphs in each panel) and internal standard (two lower graphs in each panel) obtained after the analysis of a blank sample (A), a calibration standard at the LLOQ (B), at the ULOQ (C) and of a patient sample (D).

the mean difference between the measured and nominal concentrations was 1.5% (-13.5; 14.8%; min–max; n = 5 for each level), demonstrating the stability of tranexamic acid sampled on VAMS devices for 1 month at +50°C.

Analysis of study samples

Representative chromatograms for patient analysis are shown in Figures 1 (liquid whole blood) and 2 (dry whole blood on VAMS devices) and quantitative values in the samples from the five patients are depicted in Figure 3. Tranexamic acid was quantified in all samples in the range 4.0–7.7 mg/l for liquid whole blood obtained by venepuncture and in the range 2.6–10.0 for dry capillary whole blood on VAMS devices obtained with fingerstick. For the five volunteers, the ratio of the dried to the liquid whole blood concentration was 0.95, 0.96, 0.65, 1.35 and 0.86. The excellent agreement between concentrations measured in both sample types demonstrates the feasibility of measuring TXA in those whole blood samples and a clinical validation study is ongoing.

Conclusion

This study reports the first method for the quantification of tranexamic acid in human whole blood using either liquid samples or dried samples on volumetric absorptive microsampling devices. These methods use very small, 10 μl sample size; have a wide range of the calibration curve, relevant to concentrations reported in clinical studies and were validated based on the EMA and FDA guidelines. The suitability was demonstrated using clinical samples, with an excellent agreement between TXA concentrations measured in liquid blood samples obtained by venepuncture and in dried capillary blood samples obtained by fingerstick on VAMS devices. These methods can
be used in subsequent studies for a better understanding of the pharmacokinetics and pharmacology of tranexamic acid.

**Future perspective**
Tranexamic acid is on the WHO list of essential medicines but its pharmacokinetics (PK) remains poorly known in a range of patient populations. The setting up of pharmacokinetic studies may be laborious, involve qualified paramedical and laboratory personal. The suitability of a method to quantify tranexamic acid in dried whole blood sampled on volumetric absorptive microsampling devices, stable for several weeks at +50°C, will allow to perform further PK study to improve dosage regimen in all patient populations.

**Summary points**

| Background |
| --- |
| To investigate PK a LC–MS/MS method to quantify tranexamic acid in human liquid or dry whole blood was developed. |

| Experimental |
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| Volumetric absorptive microsampling (VAMS) devices were used for sample collection and compared with liquid whole blood samples. |
| This method was validated based on the European Medicines Agency and US FDA guidelines. |

| Results & discussion |
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| The PK of tranexamic acid can be investigated with whole blood sampled on VAMS devices. |
| VAMS devices are easy to sample and can be stored up to 1 month at +50°C. |

| Conclusion |
| --- |
| The assay is considered suitable to quantify tranexamic acid in human liquid or dry whole blood samples. |

**Financial & competing interests disclosure**
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**Ethical conduct of research**
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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