Automatic and renewable micro-solid-phase extraction based on bead injection lab-on-valve system for determination of tranexamic acid in urine by UHPLC coupled with tandem mass spectrometry

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Analytical and Bioanalytical Chemistry Editorial Office

Porto, July 23, 2021

Subject: Revision of manuscript **ABC-01045-2021**

Dear Editor,

Please find in attachment the revised version of the paper entitled "*Automatic and renewable micro-solid-phase extraction based on bead injection lab-on-valve system for determination of tranexamic acid in urine by UHPLC coupled with tandem mass spectrometry*", to be considered for publication in **ABC 20th Anniversary Issue**.

Tranexamic acid is an antifibrinolytic agent used in the prevention of bleeding in haemorrhagic scenarios, including surgical procedures with a high risk of significant blood loss. The significance of the work rests on the possibility of quantifying tranexamic acid at a large interval range (spanning from 300 ng mL^{-1} to 12 mg mL^{-1}), without matrix effects on mass spectrometry detection when low sample dilution is required. The novelty of the work, compared to existing methods, is the automation of sample treatment by a flow-based method, requiring no intervention from the operator and providing a fresh batch of sorbent for each sample (no carry over). Concerning the contribution to the field, analysis of a non-invasive sample (urine) is available to researchers working on pharmacokinetic studies concerning tranexamic acid, particularly for establishing/monitoring the therapeutic regimen of pediatric patients.

We have addressed all comments provided by the Editor and reviewers in order to improve the overall quality of the manuscript. We hope the work is now suitable for publication.

Yours sincerely,

Marcela Segundo

Reply to Referees' comments (ABC-01045-2021):

Editor Comments:

This manuscript requires major revision, as per the reviewers' comments, prior to consideration for publication in Analytical and Bioanalytical Chemistry.

We appreciate the comments and suggestions from the three referees, and we have revised the manuscript accordingly. All comments and recommendations are addressed below.

Referee A:

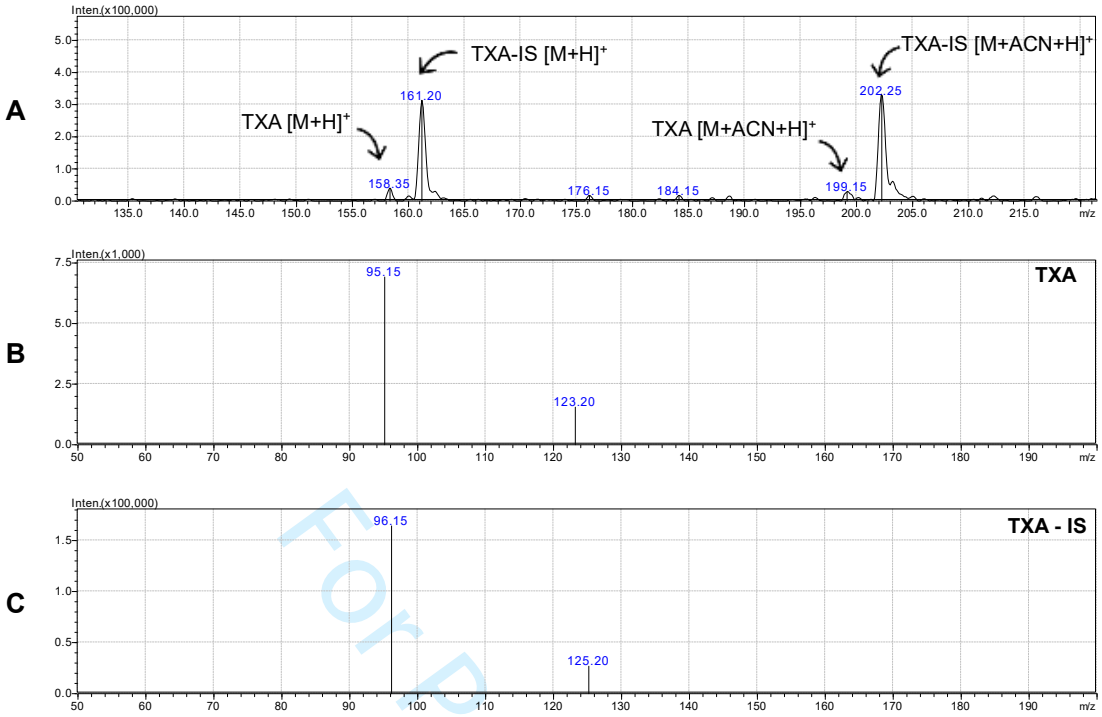
"In opinion of this reviewer, the manuscript deserves publication in Analytical and Bioanalytical Chemistry after minor revision, according to the following comments:

1. In tables 1, 2 and 4 the number of replicated measurements (n) should be given. The same for tables S6 and S7.

Reply: We appreciate this suggestion and we have introduced the number of replicated measurements (n) in tables 1, 2, 4, S6 and S7. The experiments reported in Tables 1 and 2 consisted on two independent experiments, for which extracts were analyzed in duplicate. The results present in the other tables were from one experiment with two replicate measurements.

"2. The MS of tranexamic acid in an analyzed sample (e.g. fig. 4B) should be shown."

Reply: We appreciate this suggestion and we have introduced a new figure (Figure 5) in the revised manuscript, showing the SCAN spectrum (*Q1*) and MS/MS product ion spectra for TXA and TXA-IS, obtained for the urine sample represented in Fig. 4B. The text was modified accordingly to frame the addition of this information as follows "*Furthermore, the SCAN spectrum (Q1) and MS/MS product ion spectra for TXA and TXA-IS, obtained for the urine sample show in Fig. 4B, are also presented (Fig. 5).*".



*"**Fig. 5.** SCAN spectrum (Q1) and MS/MS product ion spectra for precursor ion $[M+H]^+$ of: (B) TXA at m/z 158.35 and (C) TXA-IS at m/z 161.20, obtained for the urine sample represented in Fig.4B."*

"3. In table 3, CV(%) values for Recovery (TXA) at 1500 ng mL^{-1} is much higher than for 3000 ng mL^{-1}. Also, ion ratio (TXA) shows higher value. However, for IS both recovery and ion ratio show more uniform values. Have authors any explanation for it?"

Reply: We acknowledge this comment and a possible explanation for the higher CV (%) values obtained for Recovery (TXA) at 1500 ng mL^{-1} can be attributed to absolute signal intensity variability resulting from differences in ionization efficiency. However, as described by other authors (e.g. *A. Furey, M. Moriarty, V. Bane, B. Kinsella, M. Lehane, Ion suppression: A critical review on causes, evaluation, prevention and applications, Talanta 115 (2013) 104-122*), this effect can be normalized using internal standards. In fact, when the recovery values were evaluated for the area ratios TXA/TXA-IS, CV (%) values of 2.5, 1.8 and 0.3% were obtained for 450, 1500 and 3000 ng mL^{-1}, respectively. These values show that the differences in CV(%) were due to fluctuations in the area that were normalized by the area of the internal standard. This information was added as a footnote to Table 3 in the revised manuscript as follows: "*CV < 2.5% for all tested*

concentrations regarding the recovery values calculated from the area ratio TXA/TXA-IS."

"4. Please consider to include the word Mass Spectrometry in keywords as it is the determinative technique included in the title"

Reply: We appreciate this suggestion and we have introduced the word Mass Spectrometry in keywords.

"Minor comments:

- In abstract, the abbreviation TXA should be given the first time the analyte is mentioned (line 29)."

Reply: We acknowledge this suggestion and we have introduced the abbreviation of TXA in the first time the analyte is mentioned.

"- P. 12 line 271: first word is "charge" (not "change")."

Reply: We acknowledge this correction and we have changed the word in the revised manuscript.

Referee B:

"If the current strategy of ABC is not against application-type papers, the manuscript can be published after minor revision.

1. Lines 73 to 76 say that a drawback of dilute and shoot approaches is the "potential presence of particles and sediment". In my opinion it is good practice to filtrate analysis solution in all cases, not just in case of dilute-and-shoot. The sentence should be changed."

Reply: We acknowledge and agree that particles and sediment can be removed by filtration and/or centrifugation as a good practice for sample handling prior to LC analysis. We have meant that the dilution-and-shoot approach will leave traces of the sample matrix, which can contribute to sample instability after dilution (and further filtration/centrifugation) eventually precipitating when exposed to low temperatures in auto-samplers. Hence, we have changed the text, by deleting "*potential presence of*

particulates and sediment" and by adding "*potential sample instability from matrix traces*".

"2. Lines 83 and 86, lines 94 to 96: In the literature quite often the technical terms for a (column-based) miniaturized solid-phase extraction and the term solid-phase-microextraction (SPME) are mixed up, and this has also happened in the present manuscript. In lines 83 to 86 the authors say correctly that their approach is a micro-solid phase extraction approach (correct, because it is based-on a small column). However, in lines 94 to 96 it is stated that micro-solid phase extraction has been reported for quantitation of TXA in plasma and serum samples, and references 11 and 29-31 are given. However, these references deal with solid-phase microextraction (SPME), which is (contrary to SPE based on a small column) a technique where only one partitioning equilibrium of the analyte between solution and sorbent is established (contrary to any SPE where the column allows to establish repeated equilibria). Therefore, the techniques in references 11 and 29-31 should not be called micro-solid phase extraction."

Reply: We share the same opinion as Referee B. Hence, we have corrected the text in order to provide a correct information about the techniques used in the references 11 and 29-31 as follows: "*Solid phase microextraction (SPME) methodologies combined with LC-MS have been reported in the literature for the quantification of TXA in plasma and serum samples [11, 29-31], and only recently for urine samples [10], despite the potential of this type of sample for non-invasive analysis. However, the use of μSPE coupled with LC-MS for the quantification of this analyte in biological samples has not been reported so far.*"

"3. Lines 123 and 124 describe how the bead suspension was prepared. Lines 186 and 187 say that the bead suspension was placed in the bead reservoir. What volume of the bead suspension was used?"

Reply: We acknowledge this comment. Before starting the sample treatment procedure, approximately 200 µL of suspension were placed in the bead reservoir and refilling took place every two or three cycles. This information has also been added to the revised manuscript as follows: "*Next, 200 µL of bead suspension were placed in the bead reservoir connected to port 4.*".

"4. Lines 400 to 403 mention an SPME procedure published previously, and it is stated that despite high sample throughput "it still requires organic solvent for TXA desorption". This sounds as if the procedure reported in the present work would not require an organic solvent for elution (which is not correct). The sentence should be rephrased."

Reply: We acknowledge this comment and we would like to clarify what was meant. Concerning SPME procedures, the analyte(s) desorption from the coating can be performed by thermal (*M. Lashgari, V. Singh, J. Pawliszyn, A critical review on regulatory sample preparation methods: Validating solid-phase microextraction techniques, Trac-Trends Anal. Chem. 119 (2019) 11.*) or solvent desorption (*E. Boyaci, A. Rodriguez-Lafuente, K. Gorynski, F. Mirnaghi, E.A. Souza-Silva, D. Hein, J. Pawliszyn, Sample preparation with solid phase microextraction and exhaustive extraction approaches: Comparison for challenging cases, Anal. Chim. Acta 873 (2015) 14-30*). The first approach is clearly more environmental benign as it does not require organic solvent. However, the procedure offered by *Looby et al.* (2021) uses the second approach, requiring organic solvent, not profiting for green credentials of thermal desorption. In order to eliminate an unfair comparison, we have revised the text as follows: "*Despite the high sample-throughput (96 samples in parallel in 25 min), it also uses organic solvent for TXA desorption (1 mL of 90:10 water/methanol) and previous sample dilution (1:3) for buffering.*".

"5. Last, but not least, it would be fair to mention in the manuscript that the BI-LOV approach is still an off-line sample preparation procedure, but (miniaturized) SPE can also be done on-line. Instrumentation for on-line SPE coupled with HPLC is commercially available and provides advantages when it comes to full automation of the analysis."

Reply: We appreciate this comment and we have introduced two sentences in the *Conclusion* section about the potentialities of the proposed method as follows: "*The at-line hyphenation of the µSPE-BI-LOV method with UHPLC-MS/MS analysis is an important contribution for the quantification of TXA in biological samples comparatively to previous works since this methodology permits to perform a fully automatic and miniaturized SPE without human intervention. The proposed method has still the potential for a future on-line hyphenation with UHPLC-MS/MS, fostering a completely*

automated procedure with competitive features regarding commercially available alternatives."

Referee C:

"The authors propose a miniaturized, automated method for determination of tranexamic acid in urine samples. This is an interesting approach, and the method was properly characterized. In addition, the method was applied to urine samples collected from a cohort of patients. As methodologically the method is interesting, this could be accepted for publication in ABC. However, there are some limiting aspects that should be clarified before final acceptance. In fact, these aspects are critical to highlight the real novelty of the proposed research:

• The authors should explain if the method is fully automated, which means that a sequence of analyses can be programmed without human intervention or a unique analysis is automated, which means that the analyst should be involved in consecutive analyses."

Reply: We appreciate this comment. The present method was characterized as an automatic sample preparation method. Hence, it was implemented in order to perform the automatic sample preparation step without human intervention before analysis by LC-MS. The extracts were collected and loaded into the autosampler of LC-MS, where a sequence of analyses can be programmed, preferably overnight. In order to clarify this aspect, we have introduced the following text in the conclusion section: "*The at-line hyphenation of the μSPE-BI-LOV method with UHPLC-MS/MS analysis is an important contribution for the quantification of TXA in biological samples comparatively to previous works since this methodology permits to perform a fully automatic and miniaturized SPE without human intervention. The proposed method has still the potential for a future on-line hyphenation with UHPLC-MS/MS, fostering a completely automated procedure with competitive features regarding commercially available alternatives.*"

"• LODs and LOQs seem not to be particularly low for a compound that should be easily ionized. High ionization suppression is found in urine analysis. However, a comparison

with a method involving a direct analysis by UPLC-MS/MS is mandatory. Ultra-high-performance separation would minimize the ionization suppression effects. In fact, readers would appreciate the limitation associated to matrix effects with UPLC-MS/MS."

Reply: We acknowledge this comment. Nevertheless, compared to the other study where the quantification of this analyte was performed in urine samples, the LOQ value achieved using our method was about 300 times lower (25000 ng/mL vs 65 ng/mL). Furthermore, the evaluation of matrix effects was performed through the direct analysis of diluted urine samples by UPLC-MS/MS (dilute-and-shoot approach), showing clearly the presence of matrix effects (Fig. 3). The analysis of these samples revealed a presence of matrix effects. Moreover, when the same samples were processed using the proposed μSPE-BI-LOV method, these effects were avoided. The information obtained about this issue is present in the manuscript and in Fig. 3: "*Nevertheless, as shown in Figure 3, a reduction in signal intensity was observed (>15%) for the lowest dilution level (5x), indicating the existence of matrix effects that are circumvented by the implementation of the proposed μSPE-BI-LOV method.*"

"• Why were the sorbents compared using in batch SPE? Can the results be transferred despite scale differences?"

Reply: We appreciate this comment and we agree that results cannot be transferred directly between batch and flow SPE conditions. Batch SPE has been applied only in preliminary assays for evaluating the performance of the three sorbents towards the target analyte, namely chemical conditions of operation (presence of solvent modifiers, pH). The selection of the sorbent and operation conditions was accomplished after performing flow-based μSPE with the three sorbents (Table 1, section Selection of the sorbent and eluent). In fact, flow-based μSPE allows a more efficient control of the different variables that affect the extraction process, namely the time of contact between solutions and the solid phase. All these aspects were studied and tuned as described in section Study of the μSPE-BI-LOV conditions. No change was introduced in the manuscript regarding this comment.

"• Do the authors consider that batch SPE was properly optimized for a suited comparison?"

Reply: We appreciate this comment. Nevertheless, in this work, the optimization of batch SPE was not an objective. Batch SPE has been applied only in preliminary assays for

evaluating the performance of the three sorbents towards the target analyte, namely chemical conditions of operation (presence of solvent modifiers, pH). To clarify this aspect in the revised manuscript, we have changed the text as follows: "*Preliminary experiments were undertaken in batch SPE mode in order to evaluate the performance of the three sorbents (Table S2) regarding chemical aspects.*"

"• The main novel aspects of µSPE-BI-LOV for sample preparation of biological samples is well-known. Here, a method was proposed for determination of a unique analyte. Could the authors consider that this method deserves publication in ABC according to novel aspects from an analytical perspective?"

Reply: We acknowledge this comment. The scope of ABC is *"broad, and ranges from novel measurement platforms and their characterization to multidisciplinary approaches that effectively address important scientific problem"*. In this sense, the proposed automatic µSPE method resulted of a challenge from the clinical practice, regarding the need to process a large number of samples in short time span with minimal human intervention. This work was also an analytical challenge, with regard to the development of a method that would permit the determination of very low levels of the analyte (around 300 ng/mL to low µg/mL), thus allowing it to be applied to long duration pharmacokinetic studies (> 48 h). Furthermore, despite to the main aspects of µSPE-BI-LOV for sample preparation of biological samples is well-known, the use of this methodology to urine samples represents a significant advance due to the fact that the application to this type of sample is scarce (< 7 published reports).

In the case of TXA, the proposed method represents a significant advance in the quantification of TXA in urine samples, since only one recent work described its quantification in this type of biological sample. Furthermore, the application to urine samples allows the determination of TXA using a non-invasive sample and contributes to a better understanding of the drug elimination profile. Additionally, when this method is used, no previous derivatization is necessary, and it is possible to reduce matrix effects and no additional clean-up is required before injecting in the LC-MS/MS system. The association of µSPE-BI-LOV to LC-MS/MS is also innovative from an analytical point of view as only few papers have present it so far (< 6 reports), which shows that more work is required in this field.

Thus, taking into account all the aspects mentioned above and the scope of the journal, we believe that our work deserves to be published in ABC. In fact, we believe that this

method is innovative compared to other previously published, in particular the fact that it is applied to urine samples, and in addition, in the specific case of the TXA, it allows obtaining relevant information for the establishment of adequate therapeutic regimens namely in pediatric patients. No change was introduced in the manuscript regarding this comment.

Automatic and renewable micro-solid-phase extraction based on bead injection lab-on-valve system for determination of tranexamic acid in urine by UHPLC coupled with tandem mass spectrometry

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Abstract

An automatic micro-solid phase extraction (μSPE) method using on-line renewable sorbent beads followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was established for the determination of tranexamic acid (TXA) in urine. The μSPE method was based on the bead injection (BI) concept combined with the mesofluidic lab-on-valve (LOV) platform. All steps of the μSPE-BI-LOV were implemented by computer programming, rendering enhanced precision on time and flow events. Several parameters, including the type of sorbent, volume and composition of the conditioning solution, washing solution and eluent composition, were evaluated to improve the extraction efficiency. The best results were obtained with hydrophilic-lipophilic balanced mixed-mode sorbent, decorated with sulfonic acid groups (Oasis MCX), and 99% acetonitrile-water (50:50, v/v) - 1% ammonium hydroxide as eluent. Chromatographic separation was performed using BEH amide column coupled to MS/MS detection in positive ionization mode. Good linearity was achieved ($r^2 > 0.997$) for TXA concentrations in urine ranging from 300 to 3000 ng mL^{-1}, with LOD and LOQ of 30 and 65 ng mL^{-1}, respectively. Dilution integrity was observed for dilution factors up to 20,000 times, providing the extension of the upper limit of quantification to 12 mg mL^{-1}. The method was validated according to international guidelines and successfully applied to urine samples collected during scoliosis surgery of pediatric patients treated with TXA.

Keywords: Bead injection; Lab-on-valve; Automation; Mass Spectrometry; Tranexamic acid; Urine.

Introduction

Tranexamic acid (TXA) is an important antifibrinolytic agent used in the prevention of bleeding in several haemorrhagic scenarios [1, 2]. It is a synthetic analogue of the essential amino acid lysine and its haemostatic effects result from the competitive inhibition of plasminogen and, consequently, the inhibition of its conversion in plasmin, which is an enzyme responsible for degrading fibrin in blood clots [2, 1, 3, 4]. Regarding pharmacokinetics and pharmacodynamics of TXA, both from oral and intravenous administration, bioavailability was reported as 33-34% [1, 4, 2]. Furthermore, the elimination half-life of the intravenous form of TXA was estimated at about 2 h and, in the time span of 24 h, up to 90% of the given dose was excreted unchanged in the urine [2, 1, 4, 5].

Assuming that urinary excretion is the main route of elimination of TXA, the measurement of TXA concentration in urine assumes particular importance in the study of the clearance and in the establishment of the adequate doses and administration schedules of this drug [2, 6, 7]. Furthermore, there is a lack of information on the TXA concentration in urine, as only few works reported this information [8, 9, 7, 10]. Hence, the development of effective methodologies for the determination of this drug in urine as a non-invasive sample is of the utmost importance.

Several methods have been reported for the quantification of TXA in biological fluids, particularly in serum and plasma [6, 11]. Currently, the combination of liquid chromatography - mass spectrometry (LC-MS) is the most common strategy due to its robustness and the possibility of determining TXA without need of a previous derivatization [6]. Nevertheless, most of the methods employed for quantifying TXA in biological fluids require sample preparation procedures (such as deproteinization or solid phase extraction) to remove any potential interfering species or to concentrate the analyte

[6, 12-14]. Moreover, MS based methods for urine analysis often rely on dilute-and-shoot approaches [15, 16]. Nevertheless, this strategy comprehends several drawbacks, namely reduction of analyte detectability, potential sample instability from matrix traces, retention-time shift, ion suppression or ion enhancement, and occurrence of potential interfering peaks [17].

Solid phase extraction (SPE) is one of the most versatile and frequently used sample-processing methods [18-20, 12]. This technique has advantages over other sample preparation techniques – namely, low consumption of reagents and solvents, simplicity of use, low cost, short time of sample preparation, and the fact that analyte retention is achievable using any of a variety of commercially available sorbents with different polarities and chemical structures [18-20]. Furthermore, SPE can be easily miniaturized and automated using flow extraction approaches, including lab-on-valve (LOV) [21, 22]. The LOV technique is a suitable approach for implementing automatic micro-solid phase extraction (μSPE) methodologies using the bead injection (BI) concept [23-26] for on-line renewable/disposable μSPE. Due to the dimension of micro-channels suitable to the fluidic manipulation of solid phases in LOV, BI-SPE procedures present enhanced precision with exact control of time events [27, 28]. Furthermore, the automation of all steps allows the removal of sorbent at the end of each analytical cycle, rendering a fresh, new sorbent column for each sample. This feature is particularly important for biological samples, where fouling agents can deteriorate extraction performance for successive samples.

Solid phase microextraction (SPME) methodologies combined with LC-MS have been reported in the literature for the quantification of TXA in plasma and serum samples [11, 29-31], and only recently for urine samples [10], despite the potential of this type of sample for non-invasive analysis. However, the use of μSPE coupled with LC-MS for the

quantification of this analyte in biological samples has not been reported so far. Therefore, this research aimed at developing an automated μSPE methodology using BI in a mesofluidic LOV system for sample preparation prior to UHPLC - tandem mass spectrometry for the determination of TXA in urine. Parameters affecting the μSPE-BI-LOV sample pre-treatment method, namely the sorbent type, the eluent composition and variables related to fluid dynamics, are assessed. The application to human urine samples collected during scoliosis surgery is pursued.

Material and methods

Chemicals and solutions

All reagents used were of an analytical reagent grade with no further purification. Ultra-pure water (resistivity > 18 MΩ cm) from Arium water purification system (Sartorius, Göttingen, Germany) was used for the preparation of all aqueous solutions. Acetonitrile (ACN, LiChrosolv LC-MS grade) and formic acid were acquired from Merck KGaA (Darmstadt, Germany). Ammonium bicarbonate (LC-MS grade) was purchased from Fluka (Buchs, Switzerland). Ammonium hydroxide was acquired from Merck. Tranexamic acid (Fig. 1) and $^{13}C_2$, ^{15}N, *trans*-tranexamic acid (TXA-IS, internal standard) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada), through LGC standards (Barcelona, Spain).

For the BI-LOV system, the carrier solution consisted of ultra-pure water degassed in an ultrasonic bath for 15 min prior to use. The sorbent conditioning solution was a mixture of 99.9% ACN-H_2O (50:50, v/v) - 0.1% (v/v) formic acid and the eluent solution consisted in a mixture of 99% ACN-H_2O (50:50, v/v) - 1% (v/v) NH_4OH. The washing solution (for matrix removal) was 0.1% (v/v) formic acid. Three commercially available sorbents (particle diameter 60 μm) with different chemical properties were tested:

hydrophilic-lipophilic balance (Oasis HLB, Waters, Milford, MA), mixed-mode/cationic-exchange (Oasis MCX, Waters) and mixed mode/anionic exchange (Oasis MAX, Waters). Bead suspensions were prepared by mixing 200 mg of sorbent with 2 mL of sorbent conditioning solution.

Stock solutions of TXA and TXA-IS were prepared in water at 1 mg mL^{-1} and stored at – 20 °C. Intermediate solutions of TXA were prepared daily at 20 µg mL^{-1} and 1.5 µg mL^{-1} in mobile phase (acetonitrile-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v)) and also in water with 0.1% (v/v) formic acid for SPE extraction. They were subsequently diluted in the same solvents to achieve final concentrations of 60, 90, 150, 300, 450, 500 and 600 ng mL^{-1}. The internal standard (TXA-IS) was added to each TXA standard solution in order to obtain a final concentration of 300 ng mL^{-1}.

UHPLC-MS/MS analysis

The selection of chromatographic and mass spectrometry conditions used in this work are detailed elsewhere [32]. Chromatographic analysis was performed by a Nexera X2 UHPLC system comprising two LC-30AD pumps, a DGU-20A5R degassing unit, an SIL-30AC autosampler and a CTO-20AC oven (Shimadzu Corporation, Kyoto, Japan). The MS/MS system was a triple quadrupole LCMS-8040 mass spectrometer equipped with an electrospray ionization source (ESI) (Shimadzu Corporation).

Chromatographic separation was achieved using a BEH Amide column (50 x 2.1 mm, 1.7 µm; Waters) maintained at 40 °C. Elution was performed in isocratic mode using a mixture of acetonitrile-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v) as mobile phase at a flow rate of 0.1 mL min^{-1}.

The mass spectrometer was operated in positive ionization mode (ESI+) and data were acquired in selected reaction monitoring (SRM) mode. The product ions monitored for

TXA were *m/z* 158.25 > 95.15 for quantification, and 158.25 > 123.20 for identification. TXA-IS was monitored at *m/z* transitions 161.25 > 96.15 and 161.25 > 125.20 for quantification and identification, respectively.

The following parameters were used for analysis: nebulizing gas (N_2) at a flow rate of 1.5 L min^{-1}, drying gas (N_2) at a flow rate 18 L min^{-1}, desolvation line temperature at 280 ºC, heat block temperature at 400 ºC, detector voltage at 1.88 kV and collision gas (argon) at 230 kPa. The injection volume was 0.2 µL. Peak detection and quantification were performed using LabSolutions software version 5.60 SP2 (Shimadzu Corporation).

Lab-on-valve configuration

The flow system proposed for this study is depicted in Fig. 2. It comprised a multisyringe pump (Crison Instruments, Allela, Spain) from which one syringe was selected as propulsion unit (2500 µL, Hamilton, Bonaduz, Switzerland). A three-way commutation valve (NReseach, Caldwell, NJ, USA) placed at the head of the syringe was used for controlling the access to the solution reservoirs ('off' position) or to the LOV ancillary ports ('on' position). The multisyringe pump was connected to a customized poly(methylmethacrylate) lab-on-valve unit containing a central channel and eight peripheral ports (with microchannels of 1.5 mm i.d. each) incorporated atop an eight-port multi-position selection valve (MPV, Crison Instruments). The microchannels communicate to the central channel (CC), which connects to a holding coil (HC), allowing access to the eight peripheral ports, one at a time. All ports possess different functions, which are as follows: (1) SPE column; (2) sample; (3) air; (4) bead suspension; (5) eluent; (6) conditioning solution; (7) washing solution, and (8) waste. The bead suspension container consisted of a pipette tip (1 mL) attached to a polyetheretherketone (PEEK) nut, which was fixed onto port 4 of the LOV. In order to retain the beads in the

SPE column, a 1-mm thick polypropylene frit with a pore diameter of 35 µm (MoBiTec, Göttingen, Germany, ref #M523515) was placed between the outlet of port 1 and a PEEK nut. In order to connect the LOV ports to the solution reservoirs, polytetrafluorethylene tubing (Omnifit, Cambridge, UK) of 0.8 mm i.d was used throughout. As for the HC (with a 3.5 mL capacity) and the connections to the relevant solution flasks and syringes, 1.5 mm i.d. tubing of the same material was used.

Using BASIC programming (Quick Basic 4.5, Microsoft, Redmond, WA, USA), all steps of the analytical procedure were defined and implemented. The steps controlled by software were as follows: the direction and speed (flow rate) of piston movement on the multisyringe apparatus, the position of commutation valves, and the selection of the different ports in the selection valve and LOV.

Protocol Sequence

Prior to carrying on with the sample treatment procedure, the syringe pump and the HC were filled with ultra-pure water. The ports 2, 5, 6 and 7 were primed with the respective solutions. Next, 200 µL of bead suspension were placed in the bead reservoir connected to port 4. When the beads settled at the bottom of the channel connecting to port 4, the system was ready for operation.

The procedure used for µSPE under the BI-LOV format comprises four stages: (1) sorbent conditioning and in-line formation of the SPE column; (2) sample loading and matrix removal; (3) sample elution, and (4) in-line sorbent removal. A brief description of the automatic µSPE-BI-LOV method is given below and more detailed information can be found in Table S1:

(1) *Sorbent conditioning and in-line formation of the SPE column.* First, the beads in the reservoir were re-suspended with 125 µL of ACN-H_2O (50:50, v/v) - 0.1% (v/v) of formic

acid. Subsequently, a volume of 100 µL of bead suspension was collected and beads were loaded into channel 1 (SPE column), after which they were immediately perfused with ACN-H_2O (50:50, v/v) - 0.1% (v/v) of formic acid and water (carrier). Finally, the excess of beads that remained in the HC was discarded by sending 700 µL of carrier through port 8 (waste).

(2) *Sample loading and matrix removal.* To prevent dilution of the sample or contamination of the carrier (water), an air plug of 350 µL was aspirated from port 3 before sample aspiration. Afterwards, 1000 µL of sample was aspirated into the HC. A portion of the HC content (1200 µL) was dispensed through the SPE column (port 1) at 1.0 mL min^{-1}, the sample was loaded into the sorbent and the target analyte was retained followed by 200 µL air. Then, 500 µL of washing solution was dispensed through the column, washing off the sample matrix and the non-retained species.

(3) *Sample elution.* As occurred in the previous stage, an air plug (300 µL) was aspirated prior to aspiration of the eluent (1000 µL). Elution was then performed using a flow rate of 0.5 mL min^{-1} for the maximum contact between eluent and sorbent.

(4) *Sorbent removal.* The beads packed into channel 1 were first wetted with 400 µL of ACN-H_2O (50:50, v/v) - 0.1% (v/v) of formic acid (conditioning solution), previously stored in the HC. Next, the beads were aspirated back into the HC and subsequently disposed off to waste (port 8) using a high flow rate (5 mL min^{-1}). Finally, the LOV column port was cleaned with 200 µL of carrier solution (water). After this step, the system was ready for processing the next sample.

Application to urine samples collected during scoliosis surgery

The study followed internationally accepted rules of good clinical practices and was approved by Ethics Committee for Health at Centro Hospitalar do Porto (process no.

2015.083(077-DEFI/072-CES)). All participating subjects have given their written informed consent. Briefly, the patients had an initial bolus of 10 mg kg^{-1} of TXA during 15 min, 15 min before surgical incision, followed by continuous infusion of 1 mg kg^{-1} h^{-1} from surgical incision to closure of the surgical wound. Urine samples were collected at 30 min after starting the surgery, before blood transfusion, at the end of surgery, and 24 h later. The urine samples were stored at −20 ºC after collection until analysis.

The urine samples were diluted with 0.2% (v/v) of formic acid (5 to 20,000x) and filtered using a Corning ® syringe filter (constituted by a regenerated cellulose membrane, pore size of 0.2 µm), before the µSPE-BI-LOV procedure. The resulting eluate from the µSPE-BI-LOV method was acidified with formic acid in order to achieve a final content of 0.2% (v/v), and submitted to centrifugation at 18,000 × *g* for 10 min at 4 °C. Supernatants were collected, TXA-IS was added at 300 ng mL^{-1} and, lastly, the extract was loaded in the auto-sampler for UHPLC-MS/MS analysis (injection volume of 0.2 µL). Quality control (QC) samples at three levels (low, medium and high) were prepared in urine (diluted 5x with 0.2% (v/v) of formic acid) at concentrations of 450, 1500 and 3000 ng mL^{-1} TXA, and were subjected to the same treatment procedure described above for the urine samples.

Results and Discussion

Selection of the sorbent and eluent

Selection of the sorbent and eluent was performed by batch SPE (using 60 mg of sorbent) and by µSPE-BI-LOV (using 10 mg of sorbent). The sorbents tested in the present work were selected considering their physicochemical properties, regarding the presence of charged groups (sulfonic acid groups for Oasis MCX and carboxyl groups for Oasis MAX) and also their hydrophilic-lipophilic balanced sorptive capacity (conferred by two

monomers, hydrophilic N-vinylpyrrolidone, and lipophilic *m*-divinylbenzene). The physicochemical properties of TXA were also considered as it contains two ionizable groups in its structure and is therefore a highly polar compound that exists as a zwitterion at physiological pH (Fig. 1 and S1).

Preliminary experiments were undertaken in batch SPE mode in order to evaluate the performance of the three sorbents (Table S2) regarding chemical aspects. Briefly, standards containing 500 ng mL^{-1} of TXA and prepared in 0.1 %(v/v) formic acid (for OASIS HLB and OASIS MCX) or ammonium bicarbonate buffer (pH 7.4; 10 mM, for OASIS MAX) were used. For the steps of conditioning and washing, ACN, water and 0.1% (v/v) formic acid were applied. The retained analytes were eluted with 1 mL of the eluent, that is, mobile phase for OASIS HLB and OASIS MCX (ACN-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v)), and a mixture of ACN - H_2O (75:25, v/v) - 0.1% formic acid for OASIS MAX. The same conditions were applied in the µSPE-BI-LOV procedure. Also, the fractions corresponding to critical steps of the SPE procedure (sample loading, washing of sorbent, and elution) performed under batch and µSPE conditions were collected and analysed by UHPLC-MS/MS.

The results obtained with OASIS HLB and OASIS MAX revealed that a high percentage of TXA was not retained by the sorbent, since >85% of initial TXA was found in the fractions corresponding to sample loading and matrix removal for batch SPE (Table 1). This is most likely due to the net positive charge of TXA during loading through the reversed-phase Oasis HLB sorbent, and the zwitterion nature of the target analyte in the loading step for OASIS MAX. For in-line µSPE, analyte breakthrough occurred even earlier, with 63% and 84% of TXA present in the sample loading fraction for OASIS HLB and OASIS MAX, respectively (Table 1). On the other hand, for OASIS MCX under SPE batch format (conditions described in Table S2), the analyte (with a net positive

charge) was retained by the sorbent, but the elution of the compound was not observed, which indicated that the eluent used was not appropriate.

These results revealed the influence of acidity/alkalinity in the ionization of the analyte and the interaction of TXA with sorbent. As mentioned above, TXA contains two ionizable groups in its structure - a carboxyl and an amino group (pKa 4.3 and 10.6, respectively, see Fig. S1). Hence, the predominant form at pH 2.6 is the cationic species while, at pH 5.5, the zwitterionic form will predominate. Taking into account the properties of both TXA and the OASIS MCX sorbent, the interaction of TXA (present in 0.1% (v/v) of formic acid - pH 3.0) would be expected to occur between the sulfonic group of the sorbent and the positively charged amine group of TXA. This group would remain positively charged during the elution at pH 7.4, with ionization of the carboxylic group, and thus elution is not observed because of the predominance of the electrostatic interactions between the protonated amine and the sulfonic moieties of the sorbent.

Therefore, taking into account the properties of both sorbent and compound (see Fig. S1), eluents having a higher alkaline elution strength, namely ACN and mixtures of ACN-H_2O containing different amounts of ammonium hydroxide were tested (Table S3). Hence, the solutions of ACN-H_2O containing 2% (v/v) NH_4OH provided the best results (recoveries 93-102%). However, in order to reduce the amount of ammonium hydroxide used and, consequently, the amount of formic acid needed for acidification of samples before the mass spectrometric analysis, 99% ACN-H_2O (50:50, v/v) - 1% (v/v) NH_4OH was selected for testing under μSPE format, providing an acceptable recovery (88%, Table 1). In fact, the automation of the SPE process enhances the control of time events, namely, the control of the applied flow rate and the consequent contact time between all solutions and the sorbent, fostering quantitative recoveries.

Study of the μSPE-BI-LOV conditions

The main variables affecting the μSPE procedure were tested, namely, the composition of sorbent conditioning solution, the washing solution composition and volume, the volume of eluent, and the elution mode.

The first step in the μSPE procedure is usually the conditioning of the sorbent, performed in order to prepare the surface of the particles, rendering a suitable interface for sample application. Thus, mixtures of ACN-H_2O (50:50, v/v) and ACN-H_2O (50:50, v/v)-0.1% (v/v) of formic acid were tested as conditioning solution for Oasis HLB MCX. Results showed (Table 2) that better analyte recoveries were obtained when formic acid was added to the conditioning solution (with analyte recoveries of 88.0 ± 1.1% for ACN-H_2O (50:50, v/v)-0.1% (v/v) of formic acid and 85.9 ± 0.5% for ACN-H_2O (50:50, v/v). Hence, a mixture of ACN-H_2O (50:50, v/v) - 0.1% (v/v) of formic acid was chosen as conditioning solution.

Matrix removal is also an important step in the μSPE procedure. In this step, undesired contaminant species are selectively removed, while the target analyte remains retained on the sorbent. Thus, the composition of the matrix removal solution is an important parameter for the success of the sample preparation method and avoiding ion suppression effects in the MS detector. In this case, water (carrier solution) and a solution of 0.1% (v/v) of formic acid were tested. The results showed that the use of formic acid provided a slight increase in analyte recoveries (>1%) without appreciable changes in ion suppression in the ESI source with either washing solution. A solution of 0.1% (v/v) of formic acid was finally selected to wash the sorbent column after sample loading. The influence of its volume was inspected, using volumes of 1000 and 500 μL. The analyte recovery values obtained for both volumes were similar (Table 2) and, in order to

minimize the consumption of reagents and the volume of produced waste, 500 µL was selected.

In addition, the volume of eluent was evaluated, as well as the elution mode (continuous or with flow stop). Volumes between 200 and 1500 µL were evaluated. As shown in Table 2, higher values for analyte recovery were obtained when using larger volumes of eluent (> 88% for volumes equal or larger than 1000 µL). However, the use of larger volumes of eluent leads to a higher consumption of solvents and reagents and possible excessive dilution of the analyte. In this way, two different elution approaches (stopped-flow and continuous flow) were also tested (using 500 µL of eluent), in order to decrease the volume of eluent required for quantitative recovery. As shown in Table 2, the use of the stopped-flow approach enables analyte recovery values higher than those obtained when continuous-flow was used for the same eluent volume ($p > 0.05$). The stopped-flow strategy enables the best analyte recovery values but leads to an increase in analysis time (ca. 10 min). Thus, 1000 µL of elution volume and the continuous flow strategy were selected, as a compromise between shorter analysis time and acceptable consumption of solvents and reagents.

Analytical performance of µSPE-BI-LOV methodology

To evaluate the performance of the proposed µSPE-BI-LOV method, the figures of merit concerning linearity, precision and trueness (Table S4), limits of detection (LOD) and quantification (LOQ), matrix effect and recovery (Table 3) were established.

Calibration curves were established using six standards of TXA prepared in urine blank (300, 450, 750, 1500, 2250 and 3000 ng mL^{-1}, corresponding to 60 - 600 ng mL^{-1} after 1:5 dilution), processed by the µSPE-BI-LOV system and analyzed by UHPLC-MS/MS. Standards prepared in mobile phase (60 - 600 ng mL^{-1}) were also directly analyzed by

UHPLC-MS/MS. Data were fitted to least squares linear regression concerning peak area ratio (TXA/TXA-IS) versus TXA concentration, for an injection volume of 0.2 µL, providing a typical linear calibration curve for urine (diluted 5x) processed by µSPE-BI-LOV of $y = (0.00252 \pm 0.00005)x + (0.23 \pm 0.02)$, $R > 0.9977$ and for mobile phase standards of $y = (0.00238 \pm 0.00004)x + (0.20 \pm 0.01)$, $R > 0.9981$. Statistical analysis was performed in order to compare the slopes of the two calibration curves by *t-student* test and no statistical differences were observed ($p < 0.05$). Moreover, back-calculated concentrations were also obtained, including interpolating the urine standards into the mobile phase calibration curve. The back calculated concentrations presented deviations <15% from the nominal value, meeting the requirements of EMA guidelines [33]. Therefore, there is no need to use matrix-matching calibration, indicating the efficiency of the µSPE process for sample clean-up.

LOD and LOQ values for TXA in urine were determined by the signal-to-noise (S/N) ratio approach, defined as the concentrations that originated S = 3N and S = 10N, respectively. For urine extracts, LOD and LOQ values were 6 and 13 ng mL^{-1}, corresponding to 30 and 65 ng mL^{-1} in raw urine samples (dilution 5x), respectively.

Intra- and inter-day precision and trueness of the TXA assay were estimated at three concentration levels (450, 1500 and 3000 ng mL^{-1}) representative of the calibration range, by spiking TXA in urine collected from healthy volunteers. Intra- and inter-day precision and trueness exhibited values that met EMA requirements for bioanalytical assay validation, i.e., the precision, represented as CV, did not exceed 15% and the relative recovery range was between 98.1 and 110.9 %. The intra-day precision was ≤1.2% and the inter-day precision was ≤7.1% (Table S4).

The absolute recovery of TXA from urine samples was evaluated at three QC concentration levels (450, 1500 and 3000 ng mL^{-1}), by spiking urine collected from

healthy volunteers with TXA and TXA-IS before sample processing (dilution 5x). The absolute recovery was calculated by comparing the peak area of spiked samples processed by μSPE-BI-LOV with peak area of standard solutions prepared in eluent and not processed by μSPE-BI-LOV system (Table 3). Absolute recovery of TXA was repeatable (CV ≤ 4.6%) with mean recovery values of 102.7% whereas for the internal standard (TXA-IS) mean recovery values of 105.4% with CV ≤ 2.8% were attained, proving the method suitability for reliable bioanalysis. Dilution integrity was also assessed, providing satisfactory recoveries of 106.8, 96.7, 98.8 and 89.5% for samples diluted 200, 1000, 4000 and 20,000x before μSPE-BI-LOV processing (Table S5).

For confirmatory analysis of TXA in samples, a strategy based on four identification points (one precursor ion and two product ions) was applied. Therefore, the relative abundance of qualifier (*q*) and quantifier (*Q*) ions was determined, with the maximum permitted tolerance for relative ion abundance as ± 30% because the *q/Q* value belongs to the range 10-20% [34]. Mean ion ratio values of 13.5 ± 1.0% and 15.0 ± 1.3% were obtained for TXA and TXA-IS in mobile phase (Table S6). Mean ion ratio values of 11.0 ± 1.5% were obtained for TXA standards prepared in urine, and the ion ratio values ranged from 9.6 ± 0.5% and 12.6 ± 1.5% (Table 3). For TXA-IS, mean ion ratio values of 17.0 ± 0.9% were obtained (Table 3). Therefore, all values complied with the maximum permitted tolerance. Moreover, as shown in Table S7, the ion ratio values were within the acceptance range for all tested urine samples.

Previously reported methods for the determination of TXA in urine samples involved LC–UV [8] or paper-based separation [9] comprising also derivatization of the target analyte. Recently, a method using SPME followed by LC-MS/MS has been proposed [10]. The method proposed by Erickson *et al.* [9] is based on solid-phase separation by entrapment of TXA on paper and elution with methanol, followed by a direct

spectrophotometric determination after derivatization. The use of this methodology does not guarantee selectivity towards TXA. The method based on LC-UV analysis [8] presents figures of merit (linear range, LOD and LOQ) comparable to our proposed methodology, but cannot unequivocally identify TXA. In addition, a derivatization procedure, followed by heating and solvent switching before analysis, is required. The method developed by Looby *et al.* [10] use a hydrophilic-lipophilic balance (HLB)-coated SPME device for sample treatment before analysis by LC-MS/MS. Despite the high sample-throughput (96 samples in parallel in 25 min), it also uses organic solvent for TXA desorption (1 mL of 90:10 water/methanol) and previous sample dilution (1:3) for buffering. Furthermore, severe carryover effects were reported, which are clearly circumvented in our approach as a fresh portion of sorbent is used for each sample. Thus, the combination of automatic μSPE-BI-LOV with UPHLC-MS/MS analysis represents a significant advance in the quantification of TXA in urine samples.

Our methodology permitted to accomplish the determination of TXA without a previous derivatization procedure and assures the selectivity for TXA, which represents advantages compared to other proposed methodologies. Furthermore, the use of the μSPE-BI-LOV system for sample pre-treatment reduces the matrix effects without additional clean-up procedures before LC-MS/MS analysis. Additional advantages of our method are the low limits of detection and quantification compared to previous works, which allows it to be applied to the quantification of TXA in samples at low levels, particularly in studies that aim to follow pharmacological effects after 24-48 h of drug administration. This fact is important for pharmacokinetic studies and the establishment of the adequate doses and administration schedules of this drug.

As compared to traditional SPE procedures that normally use ≥ 60 mg sorbent for TXA determination in urine samples, our automatic sample treatment method has proved to be

rapid (15 min) and cost-effective, resulting in a simple and repeatable method with minimal intervention of the operator. As a result of downscaling of the SPE procedure, waste disposal, solvent and sorbent consumption were minimized (1260 µL of organic solvent and 10 mg of sorbent per assay).

Application to urine samples collected during scoliosis surgery

The applicability of the developed methodology was evaluated by analyzing four urine samples collected during scoliosis surgery. The analysis of the samples revealed a high range of concentration levels (from µg mL^{-1} up to mg mL^{-1}). Hence, larger sample dilution was required, making the dilute-and-shoot a possible strategy for sample treatment before analysis by LC-MS/MS. Nevertheless, as shown in Figure 3, a reduction in signal intensity was observed (>15%) for the lowest dilution level (5x), indicating the existence of matrix effects that are circumvented by the implementation of the proposed µSPE-BI-LOV method.

The concentrations of TXA in urine were compared with the concentrations of TXA found in plasma samples collected for the same patient and analyzed by UHPLC-MS/MS [32]. The values for TXA determination are presented in Table 4, along with chromatograms of urine sample extracts from a patient after TXA administration and from a healthy donor (Fig. 4). Furthermore, the SCAN spectrum (*Q1*) and MS/MS product ion spectra for TXA and TXA-IS, obtained for the urine sample show in Fig. 4B, are also presented (Fig. 5).

TXA concentration in the analyzed urine samples ranged from 35-7191 µg mL^{-1}. The maximum concentration was obtained for the sample collected before blood transfusion (sample 2) and the lowest concentration was obtained for the sample collected 30 min after starting the surgery (sample 1). As referred before, the elimination half-life of

intravenous TXA was estimated at about 2 h, according to the TXA elimination profile, in which approximately 90% of the given dose was excreted unchanged in the urine in the time span of 24 h [1, 2, 4, 5]. Therefore, sample 1 corresponded to a time for which TXA excretion occurred but was still limited. For the sample collected at the end of surgery (sample 3), a significant decrease in the concentration of TXA was observed. This decrease can be associated with a possible dilution effect after blood transfusion. Concerning the sample collected 24 h after surgery (sample 4), TXA plasma concentration was quite low (0.327 ± 0.007 µg mL^{-1}), and a higher amount was detected in urine (45.7 ± 0.8 µg mL^{-1}). Therefore, urine analysis might be used as a non-invasive indicator for the presence of TXA 24 h after administration and even after a longer time by using a lower dilution factor (eg. 5x).

Conclusion

A µSPE-BI-LOV method prior to UHPLC-MS/MS has been developed to carry out the determination of tranexamic acid in urine samples. The proposed methodology capitalizes on the combination of flexibility, miniaturization and simplification in sample preparation integrated within the LOV mesofluidic platform and the efficient chromatographic separation supported on the use of UHPLC-MS/MS.

The at-line hyphenation of the µSPE-BI-LOV method with UHPLC-MS/MS analysis is an important contribution for the quantification of TXA in biological samples comparatively to previous works since this methodology permits to perform a fully automatic and miniaturized SPE without human intervention. The proposed method has still the potential for a future on-line hyphenation with UHPLC-MS/MS, fostering a completely automated procedure with competitive features regarding commercially available alternatives.

The proposed method showed an adequate sensitivity and selectivity for the determination of TXA in urine samples, and it was successfully applied to the analysis of real samples collected during scoliosis surgery. Moreover, the novel method represents a significant advance in the quantification of TXA in biological samples given that it allows the determination using a non-invasive sample, and the information obtained might enable a better understanding of the drug elimination profile of TXA.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics declarations

The study protocol was approved by Ethics Committee for Heath at Centro Hospitalar do Porto (process no. 2015.083(077-DEFI/072-CES). Experiments were performed to

follow all internationally accepted rules of good clinical practices. All participants in the study have provided written informed consent prior to collection of samples.

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Figure captions

Fig. 1. Chemical structure of tranexamic acid.

Fig. 2. Scheme of the μSPE-BI-LOV system for tranexamic acid determination. S: syringe; V: three-way solenoid valve, HC: holding coil; Ca: carrier; E: eluent; SPE C: SPE column; Sa: sample; A: air; BS: bead suspension; CS: conditioning solution; CC: central channel; WS: washing solution; EL: eluate; W: waste.

Fig. 3. Chromatograms of urine spiked with TXA and diluted 5x (grey line) or 200x (black line), reaching a concentration of 300 ng mL^{-1} after dilution. (A) acquisition in SCAN mode (total ion count), (B) acquisition in SRM mode tuned for TXA detection (*m/z* 158.25 > 95.15).

Fig. 4. Representative chromatograms for (A) standards prepared in mobile phase with 60 ng mL^{-1} (black line) and 300 ng mL^{-1} (grey line) of TXA; (B) Urine sample extract from a patient after TXA administration (grey line, dilution 200x) and from a healthy donor (black line).

Fig. 5. (A) SCAN spectrum (*Q1*) and MS/MS product ion spectra for precursor ion $[M+H]^+$ of: (B) TXA at *m/z* 158.35 and (C) TXA-IS at *m/z* 161.20, obtained for the urine sample represented in Fig. 4B.

Table 1. Analyte recovery profile for different sorbents using batch SPE and μSPE-BI-LOV methodology.

Sorbent	Methodology	TXA recovery (%)[a]		
		Loading	Washing	Eluate
OASIS HLB	Batch SPE	58.9 ± 2.2	34.1 ± 0.6	<LOD
	μSPE	63.4 ± 6.2	<LOD	<LOD
OASIS MCX	Batch SPE	<LOD	<LOD	79.9 ± 0.6
	μSPE	<LOD	<LOD	88.0 ± 1.1
OASIS MAX	Batch SPE	73.8 ± 9.8	16.0 ± 4.9	<LOD
	μSPE	84.2 ± 4.9	<LOD	<LOD

[a] [TXA] = 500 ng mL^{-1}, % as w/w, mean ± SD (n = 4)

Table 2. Influence of different parameters on the μSPE-BI-LOV methodology.

Parameters	TXA recovery (%)[a]
Composition of sorbent conditioning solution	
ACN-H_2O (50:50, v/v)	85.9 ± 0.5
ACN-H_2O (50:50, v/v) - 0.1% (v/v) formic acid	88.8 ± 1.1
Volume of matrix removal solution	
1000	85.0 ± 2.3
500	85.9 ± 0.5
Volume of elution solution	
1500	93.1 ± 0.8
1000	88.0 ± 1.1
500	85.1 ± 0.4
200	66.1 ± 2.4
Elution mode	
Continuous-flow	85.1 ± 0.4
Stopped-flow	89.0 ± 0.3

[a] [TXA] = 500 ng mL^{-1}, % as w/w, mean ± SD (*n* = 4)

Table 3. Absolute recovery and ion ratio values obtained for TXA and TXA-IS determination in urine[a]

TXA concentration	Recovery (TXA)		Ion ratio (TXA)		Recovery (IS)[b]		Ion ratio (IS)	
(ng mL^{-1})	Mean (%)	CV (%)[c]	Mean (%)	CV (%)	Mean (%)	CV (%)	Mean (%)	CV (%)
450	106.5	0.8	9.6	5.7	108.7	1.8	17.8	1.8
1500	99.7	4.6	10.8	15.1	101.7	2.8	17.3	2.2
3000	101.9	0.2	12.6	12.2	105.7	0.1	16.1	3.5

TXA, Tranexamic Acid; IS, Internal Standard, $^{13}C_2$,^{15}N, *trans*-tranexamic acid

[a] TXA and TXA-IS were spiked in urine.

[b] TXA-IS at constant concentration of 300 ng mL^{-1}

[c] CV < 2.5% for all tested concentrations regarding the recovery values calculated from the area ratio TXA/TXA-IS.

Table 4. Quantification of TXA in urine and plasma samples collected during scoliosis surgery.

Samples [a]	TXA concentration (μg mL^{-1})[b, c]
Sample 1	35.1 ± 0.3
Sample 2	7191 ± 1
Sample 3	2810 ± 2
Sample 4	45.7 ± 0.8

[a] Samples were collected at 30 min after starting the surgery (Sample 1), before blood transfusion (Sample 2), at the end of surgery (Sample 3), and 24 h later (Sample 4).

[b] Each value corresponds to the mean ± standard deviation (n = 2). Before μSPE-BI-LOV, the urine samples were diluted in 0.2% (v/v) formic acid (sample 1 and 4: 200x; sample 2 and 3: 20000x).

[c] For each sample, TXA plasma level was also assessed, providing values of 30.9 ± 0.8, 11.5 ± 0.1, 9.5 ± 0.2, and 0.327 ± 0.007 μg mL^{-1} for samples 1-4, respectively.

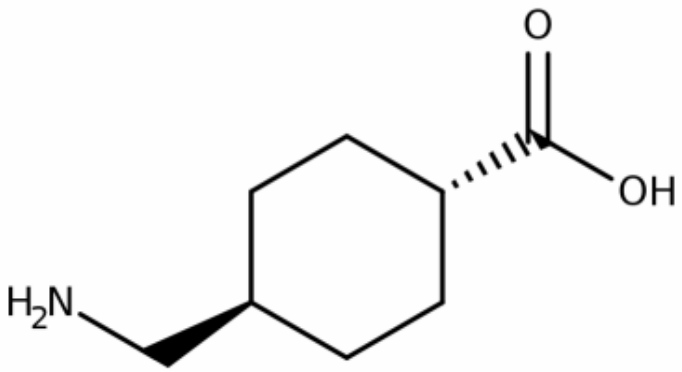


Figure 1

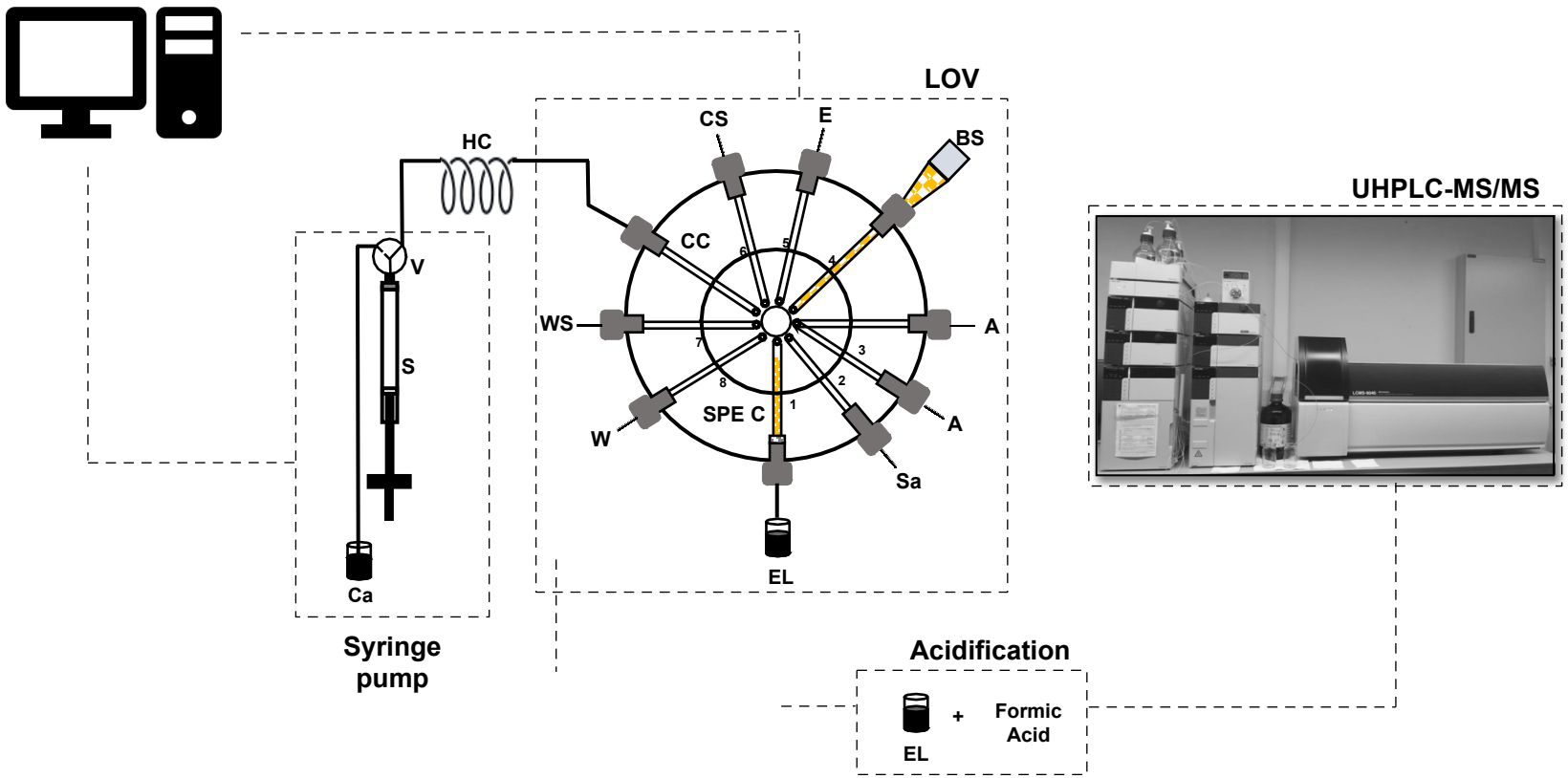
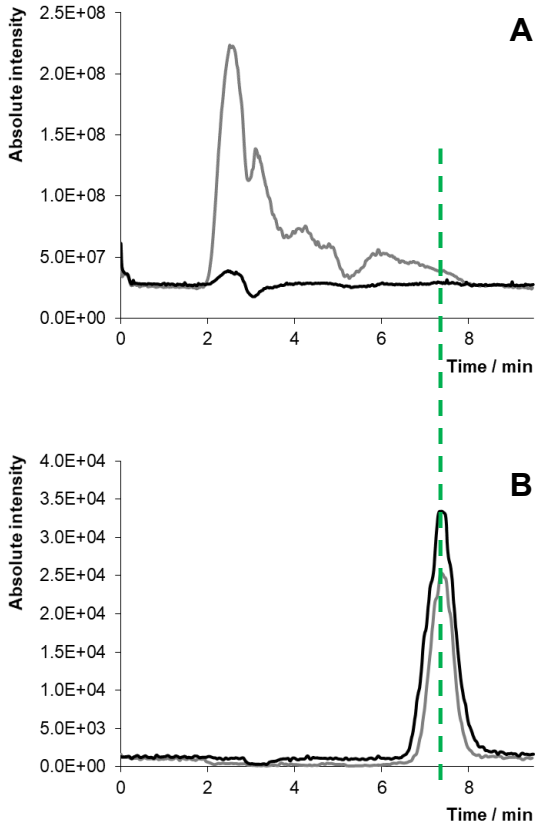

LOV
HC
CS
E
BS
CC
WS
A
W
A
Sa
SPE C
EL
V
S
Ca
Syringe pump
UHPLC-MS/MS
Acidification
EL
+
Formic Acid

Figure 2


A
Absolute intensity
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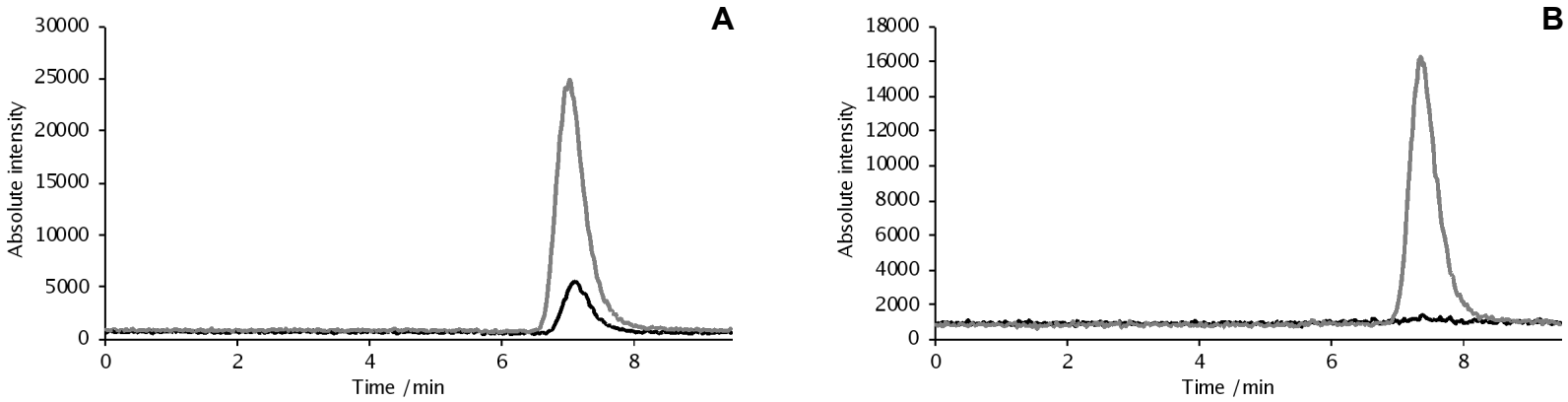
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
0
2
4
6
8
Time / min
B
Absolute intensity
4.0E+04
3.5E+04
3.0E+04
2.5E+04
2.0E+04
1.5E+04
1.0E+04
5.0E+03
0.0E+00
0
2
4
6
8
Time / min

Figure 3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25000
20000
15000
10000
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Absolute intensity
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2
4
6
8
Time /min
B
18000
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Absolute intensity
0
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8
Time /min

Figure 4

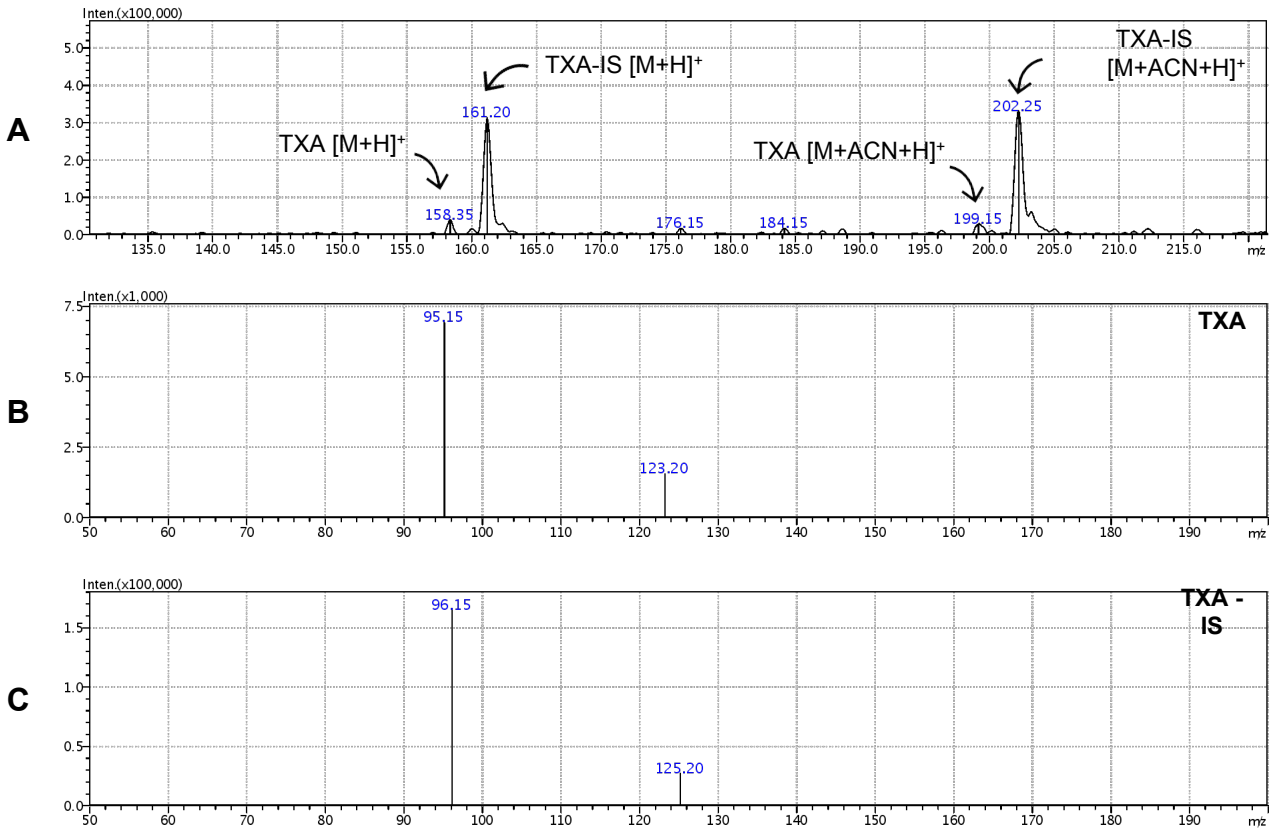
A
Inten.(x100,000)
TXA-IS [M+H]+
TXA-IS
[M+ACN+H]+
161.20
202.25
TXA [M+H]+
TXA [M+ACN+H]+
158.35
176.15
184.15
199.15
mz
B
Inten.(x1,000)
95.15
TXA
123.20
mz
C
Inten.(x100,000)
96.15
TXA -
IS
125.20
mz

Figure 5

Supplementary data

Automatic and renewable micro-solid-phase extraction based on bead injection lab-on-valve system for determination of tranexamic acid in urine by UHPLC coupled with tandem mass spectrometry

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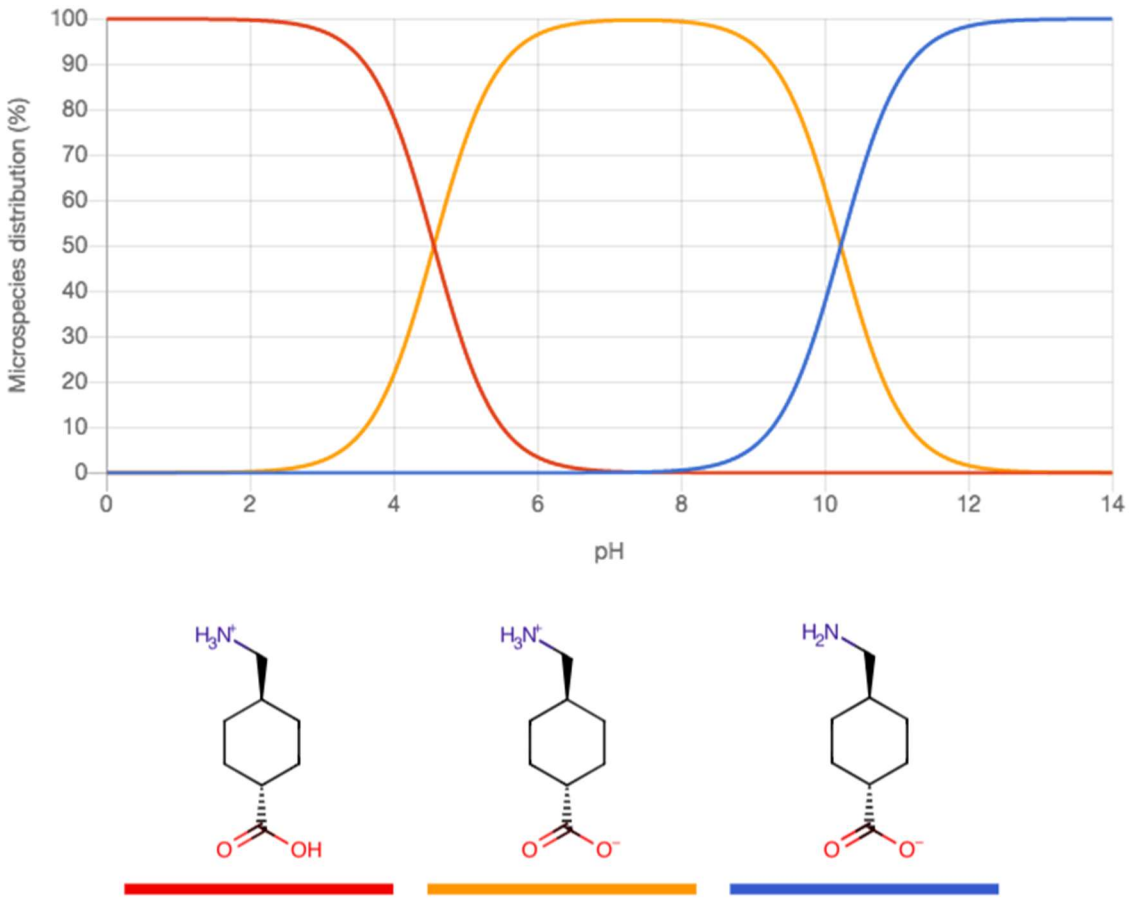

Figure S1. Tranexamic acid structure featuring group ionization at different pH values. Calculations were performed using Chemicalize software (https://chemicalize.com/).

Table S1. Analytical cycle performed during automatic BI-LOV microextraction of tranexamic acid from urine samples

1. Column formation and sorbent conditioning					
Event	Port	Volume (μL)	Flow rate (mL min^{-1})	Direction	Syringe valve
Aspiration of conditioning solution	6	550	2.5	a	1
Resuspension of beads	4	125	5	b	1
Carrier solution into syringe	4	1375	5	a	0
Aspiration of beads	4	100	0.5	a	1
Column formation and conditioning	1	1200	2	b	1
Rejection of exceeding beads	8	700	5	b	1

2. Sample loading and matrix removal					
Event	Port	Volume (μL)	Flow rate (mL min^{-1})	Direction	Syringe valve
Carrier solution into syringe	2	400	5	a	0
Sample prime	2	300	2	a	1
Washing of CC	8	700	5	b	1
Air aspiration	3	350	3	a	1
Sample aspiration	2	1000	2	a	1
“Dummy step”	2	150	5	b	0
Sample is loaded through SPE column	1	1200	1	b	1
Carrier solution into syringe	1	1500	5	a	0
Washing of central channel	8	1500	5	b	1
Aspiration of washing solution	7	500	5	a	1
Sample matrix removal	1	500	2	b	1
Air aspiration	3	300	5	a	1
Air is sent through SPE column	1	200	1	b	1
Piston adjustment	1	100	5	b	0

Table S1. Analytical cycle performed during automatic BI-LOV microextraction of tranexamic acid from urine samples (continuation)

3. Elution of compounds retained in the sorbent					
Event	Port	Volume (μL)	Flow rate (mL min^{-1})	Direction	Syringe valve
Carrier solution into syringe	1	1100	5	a	0
Air aspiration	3	300	2	a	1
Eluent aspiration	5	1000	2	a	1
"Dummy step"	5	250	5	b	0
Analyte elution	1	1200	0.5	b	1
Washing of central channel	8	950	5	b	1

4. Removal of sorbent particles					
Event	Port	Volume (μL)	Flow rate (mL min^{-1})	Direction	Syringe valve
Carrier solution into syringe	6	1000	5	a	0
Aspiration of conditioning solution	6	450	2.5	a	1
Conditioning solution is sent through SPE column	1	400	2	b	1
Sorbent slurry aspiration	1	500	5	a	1
Rejection of beads through waste	8	1350	5	b	1
Washing of column port	1	200	2	b	1

Table S2. Preliminary solutions used in the different steps of the batch SPE procedure.

Sorbent	Conditioning + loading	Washing	Eluate
OASIS HLB	*Conditioning:* 1 mL of ACN + 1 mL of H_2O *Standard solvent:* 0.1% (v/v) formic acid	1 mL of H_2O	1 mL of ACN-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v)
OASIS MCX	*Conditioning:* 1 mL of ACN + 1 mL of 0.1% (v/v) formic acid *Standard solvent:* 0.1% (v/v) formic acid	1 mL of 0.1% (v/v) formic acid	1 mL of ACN-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v)
OASIS MAX	*Conditioning:* 1 mL of ACN + 1 mL of H_2O *Standard solvent:* ammonium bicarbonate buffer (pH 7.4; 10 mM)	1 mL of H_2O	1 mL of ACN - H_2O (75:25, v/v) - 0.1% formic acid

Table S3. Evaluation of eluate composition, collected during batch SPE procedure using OASIS MCX.[a]

Eluent	TXA (%)[b]
ACN	<LOD
99.5% ACN - 0.5% (v/v) NH_4OH	<LOD
98% ACN - 2% (v/v) NH_4OH	<LOD
ACN-H_2O (50:50, v/v)	<LOD
99.5% ACN-H_2O (50:50, v/v) - 0.5% (v/v) NH_4OH	50.5
99% ACN-H_2O (50:50, v/v) - 1% (v/v) NH_4OH	79.9
98% ACN-H_2O (50:50, v/v) - 2% (v/v) NH_4OH	102.0
99% ACN-H_2O (75:25, v/v) - 1% (v/v) NH_4OH	62.3
98% ACN-H_2O (75:25, v/v) - 2% (v/v) NH_4OH	93.1

[a] The solutions used in the other steps of the batch SPE procedure are described in Table S2.

[b] [TXA] = 500 ng mL^{-1}, % as w/w.

Table S4. Trueness and precision for the analysis of TXA in urine.

Matrix	Nominal concentration (ng mL^{-1})	Intra-day			Inter-day		
		Measured concentration			Measured concentration		
		Mean (ng mL^{-1})	Trueness (relative recovery) (%)	CV (%)	Mean (ng mL^{-1})	Trueness (relative recovery) (%)	CV (%)
Urine	450	499	110.9	1.1	480	106.8	4.8
	1500	1575	105.0	1.2	1622	108.2	3.8
	3000	2943	98.1	0.7	3121	104.0	7.1

Table S5. Absolute recovery for TXA determination in urine diluted 5, 200, 1000, 4000 and 20000x.

Dilution factor [a]	[TXA] in urine [b] (ng mL^{-1})	Recovery (TXA)	
		Mean (%)	CV (%)
5x	3000	101.9	0.2
200x	12 000	106.8	8.2
1000x	600 000	96.7	6.8
4000x	2 400 000	98.8	1.3
20000x	12 000 000	89.5	1.5

[a] Dilution with 0.2% (v/v) formic acid

[b] TXA was spiked in urine before dilution.

Table S6. Ion ratio values obtained for TXA and TXA-IS analysis in standard solutions prepared using mobile phase.

TXA concentration (ng mL^{-1})	Ion ratio (TXA) (%)	Ion ratio (TXA-IS) (%)
60	12.5 ± 2.6	13.0 ± 0.3
90	14.5 ± 2.1	16.3 ± 0.2
150	12.6 ± 1.9	15.2 ± 1.3
300	12.8 ± 1.3	14.7 ± 1.5
450	14.3 ± 1.9	14.1 ± 1.1
600	14.5 ± 0.6	16.4 ± 1.6

TXA, Tranexamic Acid; IS, Internal Standard, $^{13}C_2$,^{15}N, *trans*-tranexamic acid

Each value corresponds to the mean ± standard deviation (n = 2).

Table S7. Ion ratio values obtained for TXA and TXA-IS analysis in human urine samples collected during scoliosis surgery.

Sample	Ion ratio (TXA) (%)	Ion ratio (TXA-IS) (%)
Sample 1	14.4 ± 1.4	14.8 ± 0.2
Sample 2	15.6 ± 0.8	15.7 ± 0.6
Sample 3	14.6 ± 0.5	15.0 ± 0.4
Sample 4	15.1 ± 0.4	14.9 ± 0.8

TXA, Tranexamic Acid; IS, Internal Standard, $^{13}C_2$,^{15}N, *trans*-tranexamic acid

[b] Each value corresponds to the mean ± standard deviation (*n* = 2).