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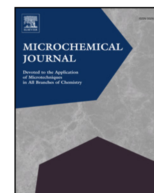

Determination of tranexamic acid in human plasma by UHPLC coupled with tandem mass spectrometry targeting sub-microgram per milliliter levels

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ABSTRACT

Tranexamic acid (TXA) is an antifibrinolytic drug, with the ability to inhibit lysine binding at plasminogen receptors, used in adult trauma patients with on-going or at risk of significant haemorrhage. To understand the pharmacokinetics and pharmacodynamics of this drug in variable age groups undergoing surgeries with high blood loss, effective methods for determination of TXA in biological samples at sub-μg mL^{-1} are still required. We describe herein the development and validation of a method based on ultra-high performance liquid chromatography coupled to triple quadrupole-tandem mass spectrometry to quantify TXA in human plasma. An inexpensive, simple and efficient sample clean-up was implemented, not requiring matrix-matching calibration. Sample preparation consisted in protein precipitation using acetonitrile containing 0.5% (v/v) formic acid, followed by hydrophilic interaction based chromatographic separation, with elution in isocratic mode using a combination of acetonitrile and water (75:25, v/v), with quantification of TXA based on selected reaction monitoring. Good linearity was achieved ($r^2 > 0.997$) for TXA concentrations ranging from 30 to 600 ng mL^{-1}, with LOD of 18 ng mL^{-1} in plasma. The developed method proved to be selective, sensitive, accurate (96.4–105.7% of nominal values) and precise (RSD ≤ 4.5%). TXA was found to be stable in plasma extracts standing 24 h at room temperature (20 °C) or in the autosampler, and after three freeze-thawing cycles. Mean recovery values of TXA spiked plasma samples were ≥91.9%. No significant matrix effects were observed. The proposed methodology was successfully applied to the clinical study of plasma samples recovered during scoliosis surgery of pediatric patients pretreatment with TXA.

1. Introduction

Tranexamic acid [*trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid] (TXA, Fig. 1) has been recently included in the World Health Organization (WHO) core list of essential medicines for use in adult trauma patients with on-going significant haemorrhage, or at risk of significant haemorrhage within 8 h of injury [1]. Despite its recognition as an important antifibrinolytic drug [2–5], there is a lack of pharmacokinetic and pharmacodynamic data for TXA concerning variable age groups undergoing surgeries with high blood loss. Clinical trials performed so far suggest a wide variability in response to TXA and, therefore, the optimum dose and administration schedules of TXA are still subject of research, aiming at a safe inhibition of fibrinolysis in the perioperative period. Hence, effective methods for determination of TXA in biological samples, including detection at low levels, e.g. ng per milliliter levels, are of utmost importance.

Several methods have been proposed for quantification of TXA in biological fluids, including human plasma and serum [6]. Due to the complexity of biological matrices, most of the techniques used to quantify TXA require a careful sample pre-treatment to remove potential interferences, namely proteins and branched-chain amino

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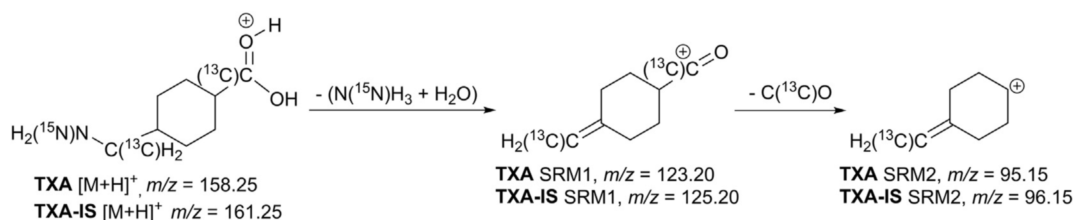

Fig. 1. Structure of *pseudo*-molecular ion of tranexamic acid (TXA) and corresponding internal standard, the isotopically labeled TXA ($^{13}C_2$,^{15}N-TXA).

acids. As TXA has a structural resemblance to amino acids, the concomitant presence of these molecules in biological samples can interfere during preparative separation procedures. In most cases, a deproteinization step with acetonitrile is selected, after addition of an appropriate internal standard (IS) whenever required.

The most commonly used methods for quantification of TXA associate reversed-phase HPLC to fluorescence [7–10] or UV [11–13] detection. Since TXA does not possess in its chemical structure a chromophore or fluorophore, exhibiting therefore a poor absorption at 220 nm, a derivatization step is required. MS detection, however, enables straightforward analyte identification and a tendency to use this technique has been observed in more recent reports [14–22]. Detection limits ranging from 0.01 to 0.5 μg mL^{-1} in blood plasma were so far attained using LC-MS/MS [14,16,18–20,22]. Although LC-MS/MS allows increased sensitivity and the ability to measure down to the ng mL^{-1} range in complex matrices such as plasma or serum, it still requires attention and development due to issues concerning sample treatment, matrix effects on ion suppression/enhancement, and studies on analyte ionization efficiency.

In this context, the aim of this work was to develop and validate a UHPLC-MS/MS method for the quantification of TXA in human plasma. Minimal sample treatment was pursued for high-throughput applications, aiming also to reduce the manipulation of biological materials. The proposed methodology was successfully applied to the clinical study of samples recovered during scoliosis surgery of pediatric patients dosed with TXA.

2. Material and methods

2.1. Chemicals

Tranexamic acid and $^{13}C_2$,^{15}N,*trans*-tranexamic acid (Fig. 1), used as internal standard (TXA-IS), were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada), through LGC standards (Barcelona, Spain). Acetonitrile (LiChrosolv LC-MS grade) and formic acid were acquired from Merck (Darmstadt, Germany). Ammonium bicarbonate (LC-MS grade) was acquired from Fluka (Buchs, Switzerland). Water from arium water purification system (resistivity > 18 MΩ cm, Sartorius, Göttingen, Germany) was used for the preparation of all solutions. Two different mixtures of acetonitrile and water containing 10 mM NH_4HCO_3, pH 7.4 (A and B) were used for studying mobile phase composition. Component A consisted of acetonitrile-water-aqueous ammonium bicarbonate (pH 7.4; 100 mM) (30:60:10, v/v/v) whereas component B consisted of acetonitrile-water-aqueous ammonium bicarbonate (pH 7.4; 100 mM) (80:10:10, v/v/v). Prior to use, both mobile phase components were filtered through 0.45 μm Millipore (Billerica, MA) HVHP filters and degassed in an ultrasonic bath for 15 min.

2.2. Preparation of standard solutions

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 20000 and 1500 ng mL^{-1} in mobile phase, *i.e.* acetonitrile-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v), and further diluted in the same solvent to achieve final concentrations of 30, 60, 90, 150, 300, 450 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}.

2.3. Sample preparation and quality control samples (QC)

Human plasma samples containing TXA (100 μL) were mixed with acetonitrile (500 μL), containing 0.5% (v/v) of formic acid to precipitate proteins. The samples were then mixed vigorously by vortexing for 30 s and centrifuged at 18000 ×*g* for 6 min at 4 °C. Supernatants were collected, TXA-IS was added at 300 ng mL^{-1} and, lastly, 0.2 μL of the extract were injected into the UHPLC-MS/MS for analysis. Whenever required, the supernatant was diluted 20× in acetonitrile-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v) before analysis. Quality control (QC) samples at three different levels (low, medium and high) were prepared in mobile phase and in plasma extract at concentrations of 90, 300 and 600 ng mL^{-1}.

2.4. UHPLC-MS/MS analysis

Chromatographic analysis was performed in a Nexera X2 UHPLC system comprising two LC-30AD pumps, a DGU-20A5R degassing unit, a 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). The UHPLC-MS/MS system was also equipped with an additional LC-20AD pump and a diverter valve FCV-20AH2 (Shimadzu Corporation) that permit to divert to waste non-relevant portions of chromatographic runs, thus minimizing fouling of the ESI probe and sample cone.

A BEH Amide column (50 × 2.1 mm, 1.7 μm; Waters, Milford, MA, USA), maintained at 40 °C, was used as stationary phase. The chromatographic separation was performed in isocratic mode using as mobile phase a mixture of acetonitrile-aqueous ammonium bicarbonate (pH 7.4; 10 mM) (75:25, v/v), at a flow rate of 0.1 mL min^{-1}. The total run time was 8.0 min, with a retention time of 6.4 min for TXA. During each chromatographic run, the column eluate was diverted to waste from 0 to 5.5 min, directed to MS interface between 5.5 and 7.6 min and again diverted to waste until 8.0 min.

The MS was operated in positive ionization mode (ESI+) and data was 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. $^{13}C_2$,^{15}N,*trans*-TXA was employed as internal standard and monitored at *m/z* transitions 161.25 > 96.15 and 161.25 > 125.20 with similar purposes. The following parameters were used for analysis: nebulizing gas (N_2) flow rate at 1.5 L min^{-1}, drying gas (N_2) flow rate at 18.0 L min^{-1}, desolvation line temperature at 280 °C, heat block temperature at 400 °C, detector voltage at 1.88 kV, 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).

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