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Short communication

Quantification of total and unbound tranexamic acid in human plasma by ultrafiltration liquid chromatography/tandem mass spectrometry: Application to pharmacokinetic analysis

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ABSTRACT

The present work describes the development and validation of rapid, sensitive and accurate liquid chromatography method, coupled with tandem mass spectrometry detection, for quantification of tranexamic acid in human plasma using isotopically labeled internal standard (IS). A one-step plasma protein precipitation was performed with acetonitrile. UPLC BEH amide column was used for chromatographic separation. Tranexamic acid and IS were detected in multiple reaction monitoring in electrospray positive ionization. The method was linear over the concentration range of 0.8–200 mg/L. The intra- and inter-day precision values were below 11.5% and accuracy was better than 9.6%. Total analysis time was reduced to 6 min including sample preparation. The present method was successfully applied to pharmacokinetic pilot study in patients undergoing orthopedic surgery. Ultrafiltration allowed confirming the weak binding to plasma proteins and confirming that total plasma TXA concentration is measured by this assay.

1. Introduction

Tranexamic acid (trans-4-(aminomethyl)cyclohexanecarboxylic acid) (TXA) is a synthetic antifibrinolytic agent used to prevent or reduce bleeding during and after surgery [1–4]. TXA is a lysine analog that blocks lysine receptors on plasminogen, plasmin, and tissue activator of plasminogen [5]. This mechanism explains how TXA inhibits plasmin generation, limiting this way fibrin degradation, and can delay natural fibrinolysis and then clot degradation [5]. TXA pharmacokinetic shows a distribution volume from 9 to 12 L, a weak hepatic metabolism and major renal elimination with terminal half-life of 2 h [6].

Since aprotinin withdrawal in 2007, interest is growing on formally overwhelmed TXA. However, sparse and divergent pharmacokinetic data for this old drug, developed in 1960s, are available. For this reason, the optimum dose and administrations schedules of TXA are still debated in the literature.

Several assays using chromatographic method have been published. Most of these methods use GC or HPLC-UV for TXA quantification [7–10]. Among them, four used mass spectrometry detection [11,14]. One of them rely on complex sample preparation [11], others present reverse phase (C18) chromatographic separation [12–14] which does not seem to be suitable regarding the polarity of the molecule.

The purpose of this work was to develop and validate a method for quantification of TXA in human plasma. The affinity for lysine receptor suggests that TXA can be fixed on other proteins containing this receptor. It might therefore be interesting to evaluate pharmacokinetics of total and unbound forms for a better understanding of TXA protein binding during analytical process. The method was applied to pharmacokinetic study of TXA during and after orthopedic surgery.

2. Materials and methods

2.1. Chemicals and reagents

Tranexamic acid (98% purity) and the internal standard (IS) $^{13}C_2$,^{15}N,*cis*-tranexamic acid (98% isotopic purity) were supplied by Toronto Research Chemicals (Toronto, Canada). LC MS grade acetonitrile was purchased from Biosolve (Dieuze, France). Distilled water was obtained from Aguettant (Lyon, France). Perchloric acid was obtained from Sigma Aldrich (Paris, France). Human plasma

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and serum were collected by "Etablissement Français du Sang" (Saint-Etienne, France).

2.2. Calibration standards and quality control samples

Stock solutions of TXA at 50 g/L and IS at 1 g/L were prepared in distilled water. Two stock solutions of tranexamic acid were prepared: one for calibration standards preparation and another for quality control (QC) preparation. Tranexamic acid working solutions of were prepared by dilution of appropriate stock solution in distilled water. The IS solution was prepared at a concentration of 0.5 mg/L by diluting stock solution in acetonitrile.

Calibration standards at 200, 100, 50, 12.5, 3.125, and 0.8 mg/L were obtained by serial dilution in human plasma. QC samples at concentrations of 1.6, 10 and 62.5 mg/L were also prepared by dilution of the appropriate working solutions in blank plasma.

2.3. Sample preparation

Blood samples were centrifuged 10 min at 3500 × *g* to obtain plasma or serum. Several protein precipitation reagents were tried: perchloric acid, trichloroacetic acid, methanol and acetonitrile. For the organic acids, 100 μL of 0.1 M acids was added to 100 μL of plasma or serum, vortexed and then diluted with 500 μL of distilled water containing IS (0.5 mg/L). For the organic solvents, 100 μL of samples was deproteinizated by adding 500 μL of solvent containing IS (0.5 mg/L). Then the mixture was vortexed during 30 s and centrifuged at 13 000 rpm for 4 min. Finally, 10 μL of clear supernatant was injected into the system.

2.4. Liquid chromatography

Analyses were performed on an Acquity ultra-performance LC system coupled to a Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) probe (Waters®, Saint-Quentin, France).

Different analytic columns were tested for tranexamic acid: Luna C18 Mercury 20 mm × 4 mm × 3 μm (Phenomenex, Le Pecq, France), BEH Amide 50 mm × 2.1 mm × 1.7 μm (Waters) and Kinetex HILIC 30 mm × 3 mm × 2.6 μm (Phenomenex). Retention time, peak shape and Height Equivalent to a Theoretical Plate (HETP) were compared for each columns. Assay temperature was set to 40 °C. The mobile phase was a mixture of A: distilled water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. A 0.2 μm polyvinylidene fluoride filter provided by Interchim (Montluçon, France) was used to filtered mobile phases.

2.5. Tandem mass spectrometry

The ESI was used in positive mode. Acquisitions were performed in multiple-reaction monitoring (MRM) mode. Nitrogen was used for desolvatation and nebulization and argon for collision gas. Mass spectrometer parameters were set as follows: source temperature 140 °C, capillary voltage 3 kV, desolvatation temperature 400 °C, desolvatation gas flow 400 L/h, cone gas flow 50 L/h. Direct introduction of TXA and IS solutions (1 mg/L in distilled water) in the mass spectrometer were performed to optimize cone voltage, collision energy and determine parent and daughter ions masses. Data acquisition and quantification were performed using Masslynx V 4.1 software (Waters).

2.6. Method validation

The method was validated for sensitivity, linearity, precision, accuracy, stability, selectivity and matrix effect according to "Guidance for Industry-Bioanalytical Method Validation" recommended by the US Food and Drug Administration [15]. For these tests, a calibration curve (0, 0.8, 3.125, 12.5, 50, 100, and 200 mg/L) and QC (1.6, 10, and 62.5 mg/L) were used.

2.6.1. Sensitivity

Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ) were determined with decreasing amounts of TXA in plasma samples. LLOQ was defined as the concentration giving a signal-to-noise ratio of 10. LOD was the concentration giving a signal-to-noise equal to 3.

2.6.2. Linearity

Calibration range covered the expected therapeutic concentrations. Linear regression analysis was carried out on the standard curve generated by plotting peak areas ratio of TXA and IS versus concentration of TXA. Seven standard curves were constructed by least-squares linear regression analysis using a weighting factor of 1/concentration.

2.6.3. Accuracy and precision

Seven sets of 3 QC levels were used to determine accuracy and precision. Seven replicates of each QC levels were also analyzed the same day to determine intra-day assay. For inter-day assay, seven replicates of each QC level were processed on seven consecutive days. Precision was estimated by computing the coefficient of variation for each level across sets. The mean accuracy was determined by comparing the measured concentrations against the theoretical concentration ((1 − (theoretical concentration − mean concentrations)/theoretical concentration) × 100) for the 3 level QC samples.

2.6.4. Stability

Stability of TXA was evaluated by: (1) short term stability after storage of whole blood samples and QC samples ($n = 3$) during 4 and 24 h at room temperature and for one week at +4 °C; (2) long term stability, after storage at −18 °C during one, two weeks and one month for the three QC levels ($n = 3$); (3) for freeze–thaw stability, the three QC levels ($n = 3$) were analyzed after three freeze (−18 °C) and thaw cycles (complete thawing at room temperature). To evaluate tranexamic acid stability, the obtained concentrations were compared to freshly prepared samples. Tranexamic acid was considered stable when the differences did not exceed 15%.

2.6.5. Selectivity

To investigate whether other endogenous components in plasma interfered with the TXA assay, samples from 10 different TXA free patients were analyzed.

2.6.6. Matrix effect

The matrix effects were investigated by direct infusion of TXA and IS into the mass spectrometer during the chromatographic analysis of blank plasma extracts. The standard solutions of TXA and IS at 1 mg/L were infused at a flow rate of 30 μL/min during the chromatographic analysis. The chromatographic signals in each MRM transition were analyzed to check for any signal disturbance at the analytes' retention times.

2.7. Pharmacokinetic study

2.7.1. Influence of blood collection tubes

To evaluate whether the presence of anticoagulants influences the determination of TXA, serum and plasma were collected. Serum was obtained in tubes without polymer gel or anticoagulants. Plasmas were drawn on lithium heparin or buffered sodium citrate solution.