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Short communication

Plasma and cerebral spinal fluid tranexamic acid quantitation in cardiopulmonary bypass patients

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ABSTRACT

A method for the determination of tranexamic acid (TXA) in human plasma and cerebral spinal fluid (CSF) was developed. Analyses were performed by ultra performance liquid chromatography with tandem mass spectrometry detection (UPLC–MS/MS) using ε-aminocaproic acid (ACA) as an internal standard. TXA and ACA were extracted from a 50 μL sample of plasma or CSF using a methanol protein crash protocol, and chromatographic separation was performed on an ACQUITY™ TQD mass spectrometer using a UPLC C18 BEH 1.7 μm column with a water and methanol gradient containing 0.1% formic acid. The detection and quantitation was performed by positive ion electrospray ionization using the multiple reaction monitoring (MRM) mode. The method was linear over the concentration range of 0.1–10.0 μg/mL, with lower limit of quantitation of 0.1 μg/mL for TXA. The intra- and inter-assay precision was less than 12% and 13% respectively at the plasma and CSF TXA concentrations tested. The present method provides a relatively simple and sensitive assay with short turn-around-time. The method has been successfully applied to assess the plasma and CSF concentrations of tranexamic acid achieved with only one dosing regimen of tranexamic acid in patients undergoing cardiopulmonary bypass surgery (CPB).

1. Introduction

Tranexamic acid (trans-4-(aminomethyl)cyclohexanecarboxylic acid) is a synthetic derivative of the amino acid lysine that possesses antifibrinolytic properties. TXA exerts its antifibrinolytic effects by inhibiting the interaction of plasminogen and the heavy chain of plasmin with lysine residues on the surface of fibrin through the reversible blockage of lysine binding sites on plasminogen molecules. Although the plasmin forming ability is still present, it is unable to bind to and degrade fibrin [1]. Tranexamic acid is used to treat a variety of bleeding disorders, and to reduce postoperative blood loss in various types of surgery. Intravenously administered TXA decreases postoperative blood loss and blood transfusion requirements in patients undergoing CPB [2–4].

Several previous methods for the determination of TXA in plasma have been published using HPLC-UV [5,6], HPLC-fluorescence [7–11], GC-MS [12], and electrophoresis [13,14]. However, most of these methods include pre-column and post-column derivatization procedures or sample pretreatment to eliminate interfering branched-chain amino acids. Newer methods that overcome such requirements have been developed based on LC–MS/MS [15,16].

The previously reported LC–MS/MS methods for the measurement of TXA in plasma required a larger sample volume and was not linear over the whole measurement range [15], or has reported a high LLOQ [16]. The present method measures TXA levels in plasma and CSF and offers the advantage of low sample requirement of 50 μL. This is an important factor in in-patients or pediatric patients where larger blood volumes are not easily obtained. Importantly, this method has an LLOQ of 0.1 μg/mL, which is more adequate when measuring plasma TXA levels to determine clearance of the drug from the system following surgery in our patient population. A lower LLOQ was previously reported [15], which is expected since the method used a higher initial sample volume (200 μL).

This paper describes a rapid and sensitive ultra performance liquid chromatography positive electrospray ionization tandem mass spectrometry (UPLC–MS/MS) method to detect and quantitate TXA in human plasma and CSF. The method described uses a smaller sample volume than previously published and has been applied to assess TXA levels in plasma and CSF of adults undergoing CPB.

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2. Experimental

2.1. Chemicals and reagents

TXA (purity better than 98%) was obtained from Acros Organics (Geel, Belgium). ε-aminocaproic acid (6-aminohexanoic acid) internal standard (IS) was obtained from Hospira, Inc (Lake Forest, IL, USA) at 250 mg/mL. ammonium acetate was obtained from Sigma–Aldrich (St. Louis, MO, USA), methanol (LC–MS grade) from EMD Chemicals (Gibbstown, NJ, USA), and formic acid from Fisher Scientific (Pittsburg, PA, USA). De-ionized water was used throughout the study.

2.2. Preparation of calibration standards and stock solutions

The stock solutions of TXA (10 mg/mL) and the IS ACA (250 mg/mL) were prepared in methanol and were stored at 4 °C. The standard working solutions of TXA (1 and 100 μg/mL) and ACA (100 μg/mL) were prepared by dilution with water. Standard calibration samples were prepared by spiking blank human plasma or CSF with the appropriate working solution of TXA to yield concentrations of 0, 0.5, 1, 4, 7, and 10 μg/mL. Quality control samples were prepared at concentrations of 1, 4, and 8 μg/mL.

2.3. Sample preparation

Blood was collected in buffered sodium citrate tubes. Plasma was separated by centrifugation at 4000 × *g* for 10 min. CSF was collected in sterile tubes. A 50 μL aliquot was added to 500 μL of methanol containing the IS at 1 μg/mL. The sample was vortexed for 30 s and then centrifuged at 16,000 × *g* for 10 min. 500 μL of the aqueous supernatant was transferred to a glass tube (12 mm × 75 mm), dried under N_2 gas at 40 °C, and the residue was re-dissolved with 500 μL of mobile Phase A (2 mM ammonium acetate in water; 0.1% formic acid). After brief vortexing, 150 μL was transferred to an auto-sampler vial and 5 μL was subsequently injected into the UPLC–MS/MS system.

2.4. Calibration curve

The calibration standards were prepared and assayed as described above. Plasma and CSF standards were used to prepare standard curves when assaying plasma and CSF samples, respectively. The standard curves were constructed by plotting the peak area ratio of TXA/ACA versus TXA concentration. The calibration equation was obtained by weighted linear least squares regression analysis.

2.5. UPLC–MS/MS conditions

Chromatography was performed using a BEH C18 1.7 μm column (2.1 mm × 50 mm) (ACQUITY™ UPLC system, Waters Inc. Milford, MA, USA). The injected solutions were eluted with a gradient mobile phase of water (A) and methanol (B) both containing 2 mM ammonium acetate and 0.1% formic acid. The flow rate was 0.25 mL/min with a total run time of 5 min for each sample. A TQD (Waters, Inc. Milford, MA, USA) mass spectrometer was used in the positive ion electrospray ionization mode. Main working parameters were set as follows: cone 30.00 V, collision energy 15.0 keV for TXA and 10.0 keV for ACA, and desolvation temperature at 350 °C. Quantitation was performed using the MRM of the hydrogen adduct molecular ion to predominant product ion pair, m/z 158.2 > 95.2 for TXA and 132.1 > 114.0 for ACA. Data was processed using MassLynx software (version 4.1).

2.6. Validation of the method

2.6.1. Matrix effect and recovery

The matrix effect was evaluated by a method adapted from Matuszewski et al. [17] by comparing the peak area of TXA spiked in pre-extracted pooled plasma (3 different plasma pools each comprised of 15 individual healthy donor plasma) at a concentration of 5 μg/mL to that of TXA spiked in the aqueous mobile phase at the same concentration. The matrix effect of CSF was determined by comparing plasma and CSF spiked with 5 μg/mL. Percent ion suppression was calculated as: $(A_M - A_p)/A_M \times 100$, where A_M is the peak area of the TXA spiked in mobile phase *A* and A_p is the mean peak area of the TXA spiked in plasma. The absolute recovery was determined by assaying spiked plasma samples (2 and 8 μg/mL) and calculating percentage recovery by: $(C_R/C_S) \times 100$, where C_R is the mean recovered concentration ($n = 5$) and C_S is the spiked concentration.

2.6.2. Sensitivity and linearity

The lower limit of quantitation (LLOQ) was selected based on the criterion of repeated sampling ($n = 20$) of the lowest TXA analyte response resulting in a CV of less than ≤20%. The upper limit of quantitation (ULOQ) was selected when the method no longer displayed linearity within 20% of the calibration curve. The calibration curves of TXA were constructed by plotting the peak area ratio of TXA/ACA versus TXA concentration. Linearity was determined using a linear regression.

2.6.3. Precision and carryover

Two QC samples (4 and 8 μg/mL) were analyzed on the same day to determine the intra-assay precision ($n = 20$), and on separate days to determine the inter-assay precision ($n = 20$).

The zero and highest standard (10 μg/mL) were used to investigate carryover. The zero standard was assayed once before and three times after assaying the high level standard 3 consecutive times.

2.6.4. Interference

Hemolysis interference was investigated by spiking plasma samples containing various levels of hemoglobin with 5 μg/mL of TXA. Two samples of drug free plasma were obtained from a healthy donor. Hemolysis was induced by freezing one sample prior to centrifugation. The concentration of hemoglobin in plasma was determined on a Beckman Coulter LH750 analyzer. The various levels of hemoglobin in plasma samples were obtained by serial dilutions of the hemolyzed and the non-hemolyzed plasma.

2.6.5. Stability

Freeze-thaw stability testing was used to study the stability of TXA in plasma, by using QC samples that were prepared as previously described and were stored at −80 °C. The QC samples were then thawed prior to analysis, and then were stored again at −80 °C. The process was repeated 5 times.

2.7. Patient study

This method was applied to assess the TXA levels in plasma and CSF of patients undergoing CPB. A weight adjusted dosage regimen was followed at a dose of 15 mg/kg body weight with a 50 mg/mL infusion for 10 min and then an additional infusion, if needed, at the rate of 7.5 mg/kg/h for an hour. Blood and CSF samples were collected at specific time points; pre-dose (0 min), 15 min post-dose, and 90 min post-dose. The blood was separated and plasma and CSF samples were stored at −80 °C until analysis. This study was approved by the Emory University Institutional Review Board (00029932).

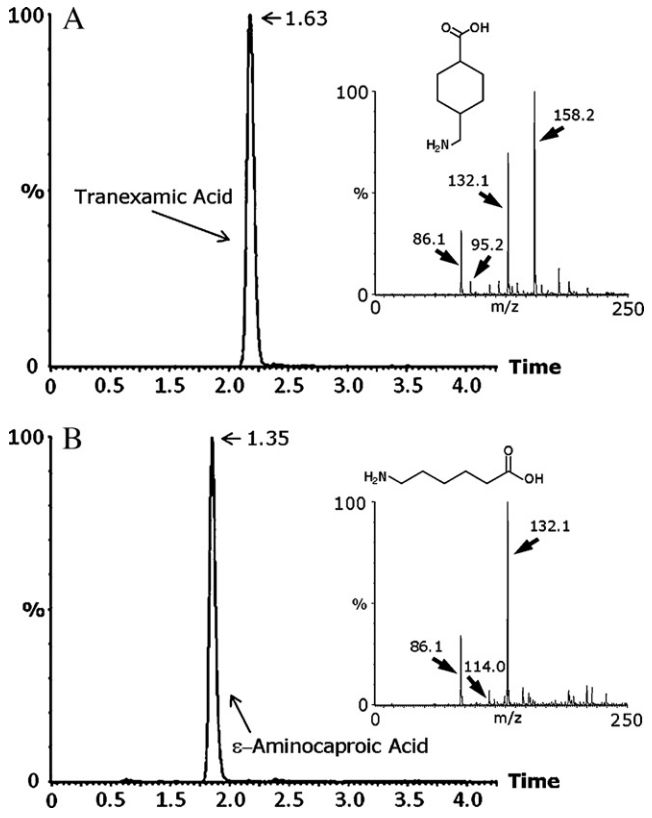

Fig. 1. UPLC–MS/MS chromatograms of plasma spiked with (A) tranexamic acid and (B) ε-aminocaproic acid. Full-scan mass spectra and chemical structures of tranexamic acid (A, inset) and ε-aminocaproic acid (B, inset).

3. Results and discussion

Fig. 1 shows a representative analysis of the chromatographic separation of TXA and ACA in plasma samples. The retention times were 1.63 min (Fig. 1A) and 1.35 min (Fig. 1B) for TXA and ACA respectively. Insets of Fig. 1 show the mass spectra of TXA and ACA. The precursor ion of TXA displays an m/z 158.2 (Fig. 1A, inset) and the precursor ion of ACA has an m/z 132.1 (Fig. 1B, inset).

Sample preparation was simplified when compared to a recent study that measured TXA in plasma. The method reported here employed no additional pH adjustment step [16]. The average ion suppression of pooled plasma was 12% ($n = 3$), when compared to aqueous mobile phase spiked with the same TXA concentration. The mean overall recovery of TXA from plasma was determined to be 108.4% ± 3.7% and 89.4% ± 1.5% at concentrations 2.0 and 8.0 μg/mL respectively ($n = 5$). The mean overall recovery of TXA in CSF compared to plasma was 108% at concentration of 5.0 μg/mL ($n = 5$).

The six-point calibration curve was linear over the concentration range of 0.1–10.0 μg/mL. A mean ($n = 5$) linear regression coefficient ($r^2 = 0.9903 \pm 0.0138$) was obtained. The mean linear regression equation obtained from 6 curves was: $y = 17.2$ (± 4.8)$x + 1565.2$ (± 776.3) for plasma and $y = 16.7$ (± 7.3)$x + 1124.7$ (± 333.8) for CSF. The inter-assay CV ranged from 2.5% to 4.9% for the 6 calibration standards. Using this method, the LLOQ was 0.1 μg/mL for plasma and CSF with a CV of 11.6% and 17.8% respectively. The ULOQ was 10.0 μg/mL for plasma and CSF with a CV of 3.3% and 1.3% respectively.

Intra-assay and inter-assay precision data of plasma and CSF QC samples are summarized in Table 1. Intra-assay precision ranged from 2.8 to 3.7% ($n = 20$) for plasma, and 7.9 to 12% ($n = 20$) for CSF. The inter-assay precision ranged from 4.4 to 6% for plasma, and 7.4 to 13% for CSF. Carryover studies were performed in order to determine any carryover effects that the high level standard might have on following samples. Even in the presence of 100 μg/mL concentrations of TXA, our method showed no carryover and due to the specificity of the MS/MS detection system (Fig. 1), no interfering peaks were observed from the blank plasma.

In addition, we have noticed that in our patient population plasma samples obtained from some surgical patients are commonly hemolyzed; therefore, we investigated the interference of

Table 1
Intra-assay and inter-assay precision and accuracy of the method for determination of tranexamic acid in human plasma and cerebral spinal fluid (CSF).

Concentration (μg/mL)	Intra-assay ($n = 20$)		Inter-assay ($n = 20$, four runs)	
	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)
1 (plasma)	2.8	83	6.0	108
4	3.5	80	5.4	93
8	3.7	107	4.4	104
1 (CSF)	12.0	96	13.0	91
4	11.1	96	7.4	96
8	7.9	109	9.6	107

Table 2
Hemoglobin interference in plasma tranexamic acid measurements.

Hgb concentration (g/dL)	TXA concentration (μg/mL)	Difference (%)
0	5.1	0
3.4	5.2	+2
6.8	5.2	+2
10.2	5.0	−1
13.6	3.9	−23

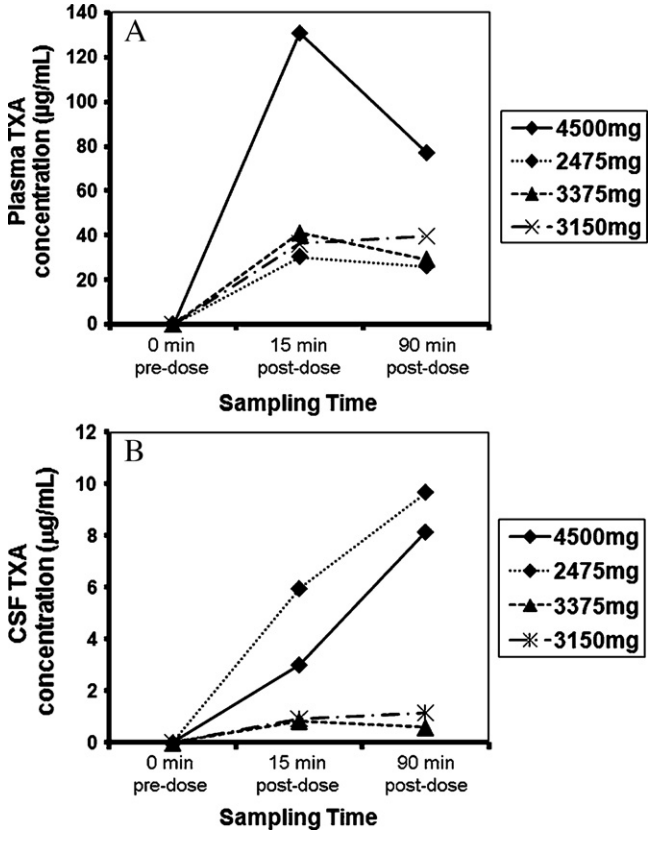

Fig. 2. Plasma concentration-time profile of tranexamic acid in (A) plasma and (B) cerebral spinal fluid from 4 patients following weight-adjusted dosage regimens.

Table 3
Plasma and cerebral spinal fluid tranexamic acid concentrations at 0 min, 15 min postdose, and 90 min post-dose.

Patient dose (mg)	Plasma (μg/dL)			Cerebral spinal fluid (μg/dL)		
	0 min	15 min	90 min	0 min	15 min	90 min
4500	0	130.8	77.1	0	3.0	8.1
2475	0	30.3	26.1	0	5.9	9.7
3375	0	41.0	29.3	0	0.8	0.6
3150	0	36.2	39.8	0	0.9	1.1

hemolysis on TXA measurements, which was not reported in previous LC–MS/MS method(s). Hemoglobin concentrations of up to 10.2 g/dL do not interfere with quantitation, and interference from hemolysis is only seen at a very high free hemoglobin concentration (13.6 g/dL), which is an excessively hemolyzed sample and not adequate for analytical measurements. We tested whether our CSF samples were contaminated with blood by testing hemoglobin concentrations.

Hemoglobin at levels of 0, 3.4, 6.8, and 10.2 g/dL did not interfere with plasma TXA measurements, as shown in Table 2. A hemoglobin level of 13.6 g/dL, corresponding to 100% hemolyzed plasma, showed a decrease of the TXA peak area by 23%. TXA was determined to be stable in plasma for 6 freeze thaw cycles. No significant degradation (>10%) of TXA in plasma was seen during that period. The CV ranged from 2.8 to 5.5% ($n = 5$).

This study was designed to assess the concentrations of plasma and CSF TXA achieved with a weight based dosing regimen of TXA of patients undergoing CPB. Despite the available literature describing the safety and effectiveness of TXA administration in patients undergoing CPB, some patients suffer from side effects such as seizures after receiving the drug. The plasma and CSF concentration time profiles are shown in Fig. 2. The patients received a weight-adjusted TXA doses at 4500 mg, 2475 mg, 3375 mg, and 3150 mg respectively. The plasma and CSF concentrations at times 0 min, 15 min post-dose, and 90 min post-dose, respectively, are listed in Table 3. These results support the finding that TXA crosses the blood brain barrier and demonstrate that the concentrations of plasma TXA may not reflect CSF concentrations in all patients receiving TXA. Our study is insufficiently powered to determine if increasing CSF TXA levels correlate with adverse side effects of the drug. However, the variability of TXA plasma and CSF concentrations observed, despite the use of a weight-based dosing regimen, suggests that other factors may affect the TXA concentrations in plasma and CSF. Additional studies are required to elucidate the potential adverse effects and untoward toxicity when high CSF TXA levels are observed in patients undergoing CPB.

4. Conclusion

A sensitive and reliable UPLC–MS/MS method was developed for the determination of TXA levels in plasma and CSF from CPB patients. This method showed acceptable sensitivity, linearity, and precision and uses a smaller sample volume than previously published methods [16]. Sample preparation was also simplified, when compared to previous studies [16], with no observed deleterious effect on method validation, and the level of hemolysis at which interference is observed using this method has been demonstrated. This method was successfully applied to assess the TXA levels of plasma and CSF TXA at specific time points during CPB.

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