A Sensitive Method for the Determination of Tranexamic Acid in Human Serum by Liquid Chromatography with Tandem Mass Spectrometer

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ABSTRACT
Tranexamic acid (TA) is a synthetic lysine analog used for the management of bleeding disorders. In this study, we developed and validated a method for the determination of TA in human serum by liquid chromatography with Q-trap mass spectrometer. Serum sample (100 μL) was deproteinated with perchloric acid, and after pH adjustment, chromatographic separation was performed on a C18 column and isocratically eluted using a mobile phase consisting of ammonium acetate buffer (pH 3.8) /acetonitrile (95:5, v/v) at a flow rate of 200 μL/min. The total run time was 5 minutes. Detection and quantitation were performed with the mass spectrometer using multiple reaction monitoring mode with the ion transition m/z 158.1 to m/z 95.1 for TA and m/z 144.0 to m/z 81.1 for the internal standard (cis-4-aminocyclohexanecarboxylic acid). The results were linear over the concentration range of 0.1-100 μg/mL of TA, with limit of quantitation of 0.03 μg/mL. The intra-day and inter-day assay coefficient of variations for serum were less than 1.8% and 2.1%, respectively, and the recovery of added standard TA was 92.5 to 99.3%. In conclusion; a simple and sensitive LC-MS/MS method has been developed for the determination of TA in human serum. The method showed excellent linearity, sensitivity, recovery and precision. This method is suitable for clinical pharmacokinetic studies.

Keywords: tranexamic acid, LC-MS/MS, human serum

1. INTRODUCTION
Tranexamic acid (TA), trans-4-(aminomethyl) cyclohexanecarboxylic acid, is a synthetic derivative of the amino acid lysine that exerts an antifibrinolytic effect through a reversible blockage of the lysine binding site on the plasminogen molecules [1]. The biological half life of TA is about three hours and total accumulative excretion after an intravenous dose is approximately 90% after 24 hours. Due to its potent
antifibrinolytic activity and lack of effect on blood clotting parameter, TA has been used to reduce postoperative blood loss and blood transfusion in a wide range of hemorrhagic conditions, such as acute upper gastrointestinal bleeding, oral surgery, gynaecologic bleeding, and in cardio surgery.

Several methods for the determination of TA in human plasma, serum and urine have been reported in the literature. These methods include HPLC-UV [2-4], HPLC-fluorescence [5], and gas chromatography-electron capture detector (GC-ECD) [6]. However, they require either pre-column or post-column derivatization which is complicated, and time consuming. Thus they are unsuitable for analysis of large numbers of samples. In addition, derivatization may introduce large assay variation. Newer methods that overcome such requirements have been developed based on LC-MS/MS [7,8] and UPLC-MS/MS [9].

One publication reported a LC-MS/MS method for the measurement of TA in plasma or serum; it required a relatively large volume of sample (200 μL) and was linear in a rather short range of concentrations (0.02 - 10 μg/mL) [7]. Another publication reported a higher limit of quantitation (1 μg/mL) [8]. The present method assayed TA levels in serum following the method of Delyle et al.[8] but using a Q-trap mass analyzer which improved the sensitivity of determination. This study developed and validated a more sensitive liquid chromatography-mass spectrometer method suitable for the determination of TA in human serum over a wide range of concentrations. The method has been tested with sera from patients undergoing total knee arthroplasty two hours after intra-articular injection of TA.

2. MATERIALS AND METHODS
2.1 Chemicals and Reagents
Tranexamic acid (trans-4-(aminomethyl) cyclohexanecarboxylic acid, Figure 1A), cis-4-aminocyclohexanecarboxylic acid, Figure 1B (used as the internal standard (IS)), and ammonium acetate were obtained from Sigma Aldrich (St.Louis, USA.), and ammonium acetate were obtained from Sigma Aldrich (St.Louis, USA.). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Labscan limited (Bangkok, Thailand). Formic acid was purchased from Fisher Scientific (Loughborough, UK). All other reagents were of analytical grade and were obtained from BDH Laboratory (England, UK) and Merck Chemical Co (Darmstadt, Germany). De-ionized water was used throughout the study.

Figure 1. Chemical structure of tranexamic acid (A) and Cis-4-aminocyclohexanecarboxylic acid (B).
2.2 Liquid Chromatographic System

HPLC analysis was carried out on a Shimadzu Model 20A liquid chromatography (Kyoto, Japan). The chromatographic separation was performed using a 5 μm Hypurity C₁₈ ThermoHypersil column (150×2.1 mm i.d.) maintained at 25°C. The column was protected with a precolumn (Hypurity C₁₈ ThermoHypersil, 5 μm, 10×2.1 mm i.d.). The mobile phase consisted of 2 mM ammonium acetate adjusted to pH 3.8 with formic acid and acetonitrile (95:5, v/v), with isocratic elution at a flow rate of 200 μL/min. The total run time was 5 min per sample.

2.3 Mass Spectrometry

An AB Sciex API 4000 mass spectrometer equipped with electro-spray ionization (ESI) source and quadrupole linear ion-trap mass spectrometer was used (Framingham, USA). The electro-spray ionization was performed in the positive mode with the main operating parameters set as follow: nebulizer gas (gas 1) 60 psi, auxiliary gas (gas 2) 60 psi, cone voltage 45 kV, collision energy 15 eV for fragmentation of TA and IS and desolvation temperature of 275°C. Quantitation was performed using the multiple reaction mode (MRM) with the ion transitions [M+H]+ m/z 158.1 to m/z 95.1 and [M+H]+ m/z 144.0 to m/z 81.1 for TA and IS respectively, (Figures 2A and B). The data was processed using the instrument software (AB Sciex, Analyst version 1.4.2).
Figure 2. Product ions mass spectrum of TA (precursor ion $[M+H]^+$ m/z 158.1) and IS (precursor ion $[M+H]^+$ m/z 144.0). Chromatogram of blank serum (C), blank serum spiked with standard TA at 10 μg/mL and IS at 50 μg/mL (D), and patient serum with IS at 50 μg/mL (E).
2.4 Calibration Standards

The stock solutions of TA (1 mg/mL) and IS (1 mg/mL) were prepared separately in methanol : aqueous containing 0.1% formic acid (1:1, v/v). The solutions were aliquoted into screw cap tubes and stored at -20°C. They were stable for at least 2 months. Working standard solutions of TA (1, 5, 10, 50, 100, 500, and 1000 μg/mL) and IS solution (500 μg/mL) were freshly prepared by dilution of the stock solutions with water.

Calibration curves were prepared by adding 10 μL of each working standard solution to blank serum (100 μL) to give concentrations of 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL, respectively.

2.5 Sample Preparation

Samples were prepared according to the method of Delyle et al [8] but with some modifications. To a 100 μL serum in a 1.5 mL plastic eppendorf tube, 10 μL water and 10 μL IS solution (500 μg/mL) were added. The sample was vortexed briefly. Then 100 μL of perchloric acid (2.5% w/w) was added, vortex mixed again for 1 min and centrifuged at 14,000 rpm for 10 min. The aqueous supernatant (100 μL) was transferred to another tube and 25 μL of sodium hydroxide (0.6 M) added, vortex mixed for 1 min and transferred to an injection vial for analysis (injection volume 5 μL).

Blank sera were obtained from normal subjects. Sera were obtained from patients 2 hours after intra-articular injection of 500 mg TA, and who had undergone total knee arthroplasty (TKA).

This study was approved by the human ethics committee of Ramathibodi Hospital, Mahidol University, and informed consents were obtained prior to collection blood.

3. VALIDATION OF THE METHOD

3.1 Linearity

Serum spiked with TA to give concentration of 0.1-100 μg/mL, were analyzed. Calibration curves were constructed by plotting the peak area ratio of tranexamic acid to the internal standard against corresponding concentration of tranexamic acid. Regression analysis was employed to evaluate the linearity.

3.2 Sensitivity

The limits of detection and quantitation were estimated from the SD of the mean value of 8 determinations of the same serum sample with a low TA concentration (0.03 μg/mL). The limits of detection and quantitation were defined as 3 times of SD and 10 times of SD, respectively.

3.3 Matrix Effect

Two procedures were performed to study the effect of sample matrix. (A) A direct comparison of slopes of calibration was made. Six concentrations of TA (0.1, 0.5, 1, 5, 10 and 50 μg/mL) and IS (50 μg/mL) were prepared in water and blank sera. The slopes for the two linear curves were compared. (B) Sera for three patients with known concentrations of TA were diluted with water in the ratio 1:2, 1:4 and 1:10, respectively and analyzed for TA. A linear line was then fitted for measured concentration of TA against dilution.

3.4 Precision and Recovery

Sera from two patients with known concentrations of TA (22.1 and 9.6 μg/mL) were analyzed in five replicates on the same day to determine the intra-day precision and after day 3 and day 10 to determine inter-day precision. Recovery was determined
by spiking three TA standard solutions 0.5, 10 and 50 μg/mL into blank serum and also spiking TA standard solution 10 μg/mL into serum from two patients. Percentage recovery was calculated using the formula \( (\frac{(C_T - C_S) \times 100}{C_{Std}}) \), where \( C_T \) is the total concentration \( (n=5) \), \( C_S \) is the sample concentration and \( C_{Std} \) is the spiked concentration.

### 3.5 Stability

The effects of temperature and storage on the stability of TA in serum were evaluated by spiking standard TA, 10 μg/mL, in fresh blank serum. The samples were aliquoted and stored at room temperature, 4°C, -20°C and -80°C respectively. A day 0, day 4, day 7 and day 30, the samples were analyzed for TA in quadruplicate. The differences between the values at day 0 and values at various storage temperatures and times were used to evaluate the stability.

### 3.6 Hemolysis

The effect of hemolysis on TA measurement was investigated by spiking standard TA, 10 μg/mL, into blank sera with 2.5%, 5%, 10% and 20% hemolysis. The values of TA in sample on various degree of hemolysis were compared to the value in sample without hemolysis (non-hemolysis). Percentage of hemolysis was calculated from the hemoglobin concentration. A hemoglobin concentration of 13.6 g/dL was used as indicator of 100% hemolysis.

### 4. RESULTS

#### 4.1 Standard Curve and Matrix Effect

Figure 2 shows mass spectra of standard TA and IS (A, B) and chromatogram of blank serum (C), serum spiked with TA standard, 10 μg/mL and IS 50 μg/mL (D) and patient serum with IS 50 μg/mL (E). Typical retention times were 3.04 min for TA and 3.03 min for IS, respectively.

The seven-point calibration curve was linear over the concentration range of 0.1-100 μg/mL with regression coefficient \( (r^2) = 0.9999 \). The effect of matrix on determination of TA in serum are shown in Figure 3. The slopes of the calibration lines for the two matrixes, water and blank serum, were similar 0.209±0.045, 0.216±0.043 respectively, (Figure 3). Matrix effect was further assessed by evaluating the curves of TA values plotted against 3 serial dilutions of sera of three patients. All the three curves were straight lines passing through the origin indicating that the matrix did not interfere in determination of TA as shown in Figure 4.

The detection limit of the method was determined by analyzing the lowest serum concentration of TA, 0.03 μg/mL in eight replicates. The mean±SD was 0.031±0.003 μg/mL. The limit of detection (LOD) was 0.01 μg/mL and the limit of quantitation (LOQ) was 0.03 μg/mL.
Figure 3. Calibration curves for TA spiked in water and blank serum.

Figure 4. Values of tranexamic acid plotted against serial dilution of serum for 3 samples.

**4.2 Precision and Recovery**

The intra-day and inter-day precision and recovery of the assay are summarized in Tables 1 and 2. The imprecision, present as CV (%), ranged from 1.2 to 1.8 and 2.0 to 2.1 for intra-day and inter-day, respectively (Table 1). The recovery of TA from serum was 92.5 to 99.3% (Table 2).

**Table 1. Intra and inter-day imprecision of the method (mean±SD, n = 5).**

<table>
<thead>
<tr>
<th>Day</th>
<th>Sample 1a</th>
<th>Sample 2a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA (μg/mL)</td>
<td>%CV</td>
</tr>
<tr>
<td>Intra-day</td>
<td>22.1±0.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Inter-day b</td>
<td>22.6±0.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

a= Serum samples from TKA patient after intra-articular injection of TA 500 mg at 2 hours  
b= analyzing over a period of 10 days (n = 10)
4.3 Stability and Hemolysis

Table 3 shows the data for the stability of TA in serum when stored at RT, 4°C, -20°C, and -80°C for 1 day, 4 days, 7 days, and 30 days respectively. Difference among TA values at various storage temperature and time ranged from 0 to -7.3%, indicating good stability. Hemolytes had no effect of determination of TA; hemolysis up to 20% increased TA levels only 0.8% (Table 4).

### Table 3. Stability of serum tranexamic acid measurement (μg/mL) (mean±SD, n=4) at various storage times and temperature.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sample</th>
<th>Conc. of TA (μg/mL)</th>
<th>Std. TA added (μg/mL)</th>
<th>Conc. of TA after adding std. (μg/mL)</th>
<th>%recovery</th>
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</thead>
<tbody>
<tr>
<td>Room temp.</td>
<td>Blank serum</td>
<td>0</td>
<td>0.5</td>
<td>0.5±0.0</td>
<td>98.6±3.9</td>
</tr>
<tr>
<td></td>
<td>Serum Pt 1a</td>
<td>0</td>
<td>10</td>
<td>9.8±0.3</td>
<td>98.0±3.3</td>
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<tr>
<td></td>
<td>Serum Pt 2a</td>
<td>0</td>
<td>50</td>
<td>46.2±0.7</td>
<td>92.5±1.4</td>
</tr>
<tr>
<td>4°C</td>
<td>22.1</td>
<td>10</td>
<td>31.5±0.4</td>
<td>94.7±4.2</td>
<td></td>
</tr>
<tr>
<td>-20°C</td>
<td>9.6</td>
<td>10</td>
<td>19.6±0.4</td>
<td>99.3±3.5</td>
<td></td>
</tr>
<tr>
<td>-80°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

Table 4. Hemolysis interference in serum tranexamic acid measurement (μg/mL) (mean±SD, n = 4).

<table>
<thead>
<tr>
<th>Hemolysis (%)</th>
<th>Serum TA (μg/mL)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.6±0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>2.5</td>
<td>9.7±0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>9.6±0.4</td>
<td>-0.5</td>
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<tr>
<td>10</td>
<td>9.5±0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>20</td>
<td>9.6±0.2</td>
<td>0.5</td>
</tr>
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5. DISCUSSION

Tranexamic acid has been used in a variety of clinical conditions in which antifibrinolytic therapy is beneficial. Use of this drug is attractive because of its ability to inhibit fibrinolysis while having no apparent effect on clotting parameters. Minimizing bleeding and transfusion are desirable [10]. However this agent may increase the risk of venous thrombosis. It is, therefore, necessary to study the safety and efficacy of this drug. In this regard monitoring of blood TA level is very useful.

Existing published methods for determination of TA (i.e. HPLC, GC) are inadequate and/or inconvenient for pharmacokinetic studies, due to tedious pre...
or post column derivatization procedures. In recent years, high performance liquid chromatography with tandem mass spectrometry has been shown to be a powerful technique for the quantitative determination of TA in plasma and serum. The present method employed a quadrupole to select the precursor ion and linear ion-trap for collision induced dissociation and analysis of the product ions. This technique had led to increase sensitivity over that of Delyle et al [8] who employed a simple ion-trap. The present report described the development and validation of a modified liquid chromatography-tandem mass spectrometer method for the determination of TA in human serum. It has been tested in serum patients who had undergone TKA.

The stock standard solutions of TA and IS can be kept at -20° C for at least two months. The calibration slopes for freshly prepared standard solutions, and solutions stored for 2 months were 0.213±0.024 and 0.197±0.041 respectively.

The present method was validated for linearity, precision and recovery. The seven-point calibration curves exhibited excellent linearity in the concentration range of 0.1-100 μg/mL with linear regression correlation coefficient $r^2 = 0.9999$. The serum matrix had no affect the determination of TA as shown in Figures 3 and 4. Limit of detection and quantitation were 0.01 and 0.03 μg/mL respectively. The present method improved detection limit 30 fold as compared with previous method of Delyle et al which has a LOQ of 1 μg/mL. [8]. The imprecision of determination of TA concentrations 22.1 μg/mL and 9.6 μg/mL expressed as %CV was less than 2.1%. The recovery of TA from serum was 92.5% to 99.3% respectively. These results indicated that the present method is precise, sensitive and reproducible for quantitation of TA in serum over a wide dynamic range. Also the present method is applicable for quantitation of TA in pharmaceutical products (data not shown).

The stability of TA in serum and hemolysis interference were examined as shown in Tables 3 and 4. The results showed that TA in serum was stable at RT for 4 days and over 30 days at 4°C, -20°C and -80°C. Hemolysis up to 20% did not interfere with TA determination, less than 0.8%.

6. CONCLUSIONS
A simple and sensitive LC-MS/MS method has been developed for the determination of TA in human serum. This method showed excellent linearity over a wide range of TA. It is sensitive and precise. This method is suitable for clinical pharmacokinetic studies.

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REFERENCES


