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DETERMINATION OF TRANEXAMIC ACID IN HUMAN SERUM BY LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETER - A REVIEW

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ABSTRACT

Tranexamic acid is synthetic lysine used to reduce bleeding disorders. TXA inhibits plasmin(ogen) binding to fibrin and reduces fibrinolysis. TXA antifibrinolytic activity is typically measured by clot lysis; however, effects on plasmin generation (PG) are unclear due to a lack of tools to measure PG in plasma.

In this study, we developed and validated a method for the determination of TA in human serum by liquid chromatography with mass spectrometer. Serum sample (100 µL) was dehydronation 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

INTRODUCTION:

Tranexamic acid (TXA) is a medication used to treat or prevent excessive blood loss from major trauma, postpartum bleeding, surgery, tooth removal, nosebleeds, and heavy menstruation. Tranexamic acid (TA), *trans*-4-(aminomethyl) cyclohexane carboxylic 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 **(Figure 1)**.

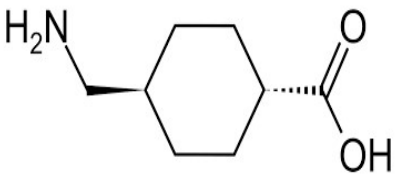


Figure 1: Structure of Tranexamic acid

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]**. 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.

MATERIALS AND METHODS:

Chemicals and Reagents:

Tranexamic acid (*trans*-4-(aminomethyl) cyclohexanecarboxylic acid, **Figure 2A**), *cis*-4 aminocyclohexanecarboxylic acid, **Figure 2B** (used as the internal standard (IS)), 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.

A.Tran-4-aminomethyl cyclohexanecarboxylic acid B. Cis-4-aminocyclohexanecarboxylic acid
Figure 2: Chemical structure of tranexamic acid and Cis-4-aminocyclohexanecarboxylic acid

Liquid Chromatographic System:

HPLC analysis was carried out on a Shimudzu Model 20A liquid chromatography. The chromatographic separation was performed using a 5 µm Hypurity C18 Thermohypersil column (150x2.1 mm i.d.) maintained at 25^0C. The column was protected with a precolumn (Hypurity C18 Thermohypersil, 5µm, 10x2.1 mm i.d.). The mobile phase consisted of 2 ml 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.

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-sprayn 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 275^0C. 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 3A and B)**. The data was processed using the instrument software (AB Sciex, Analyst version 1.4.2).

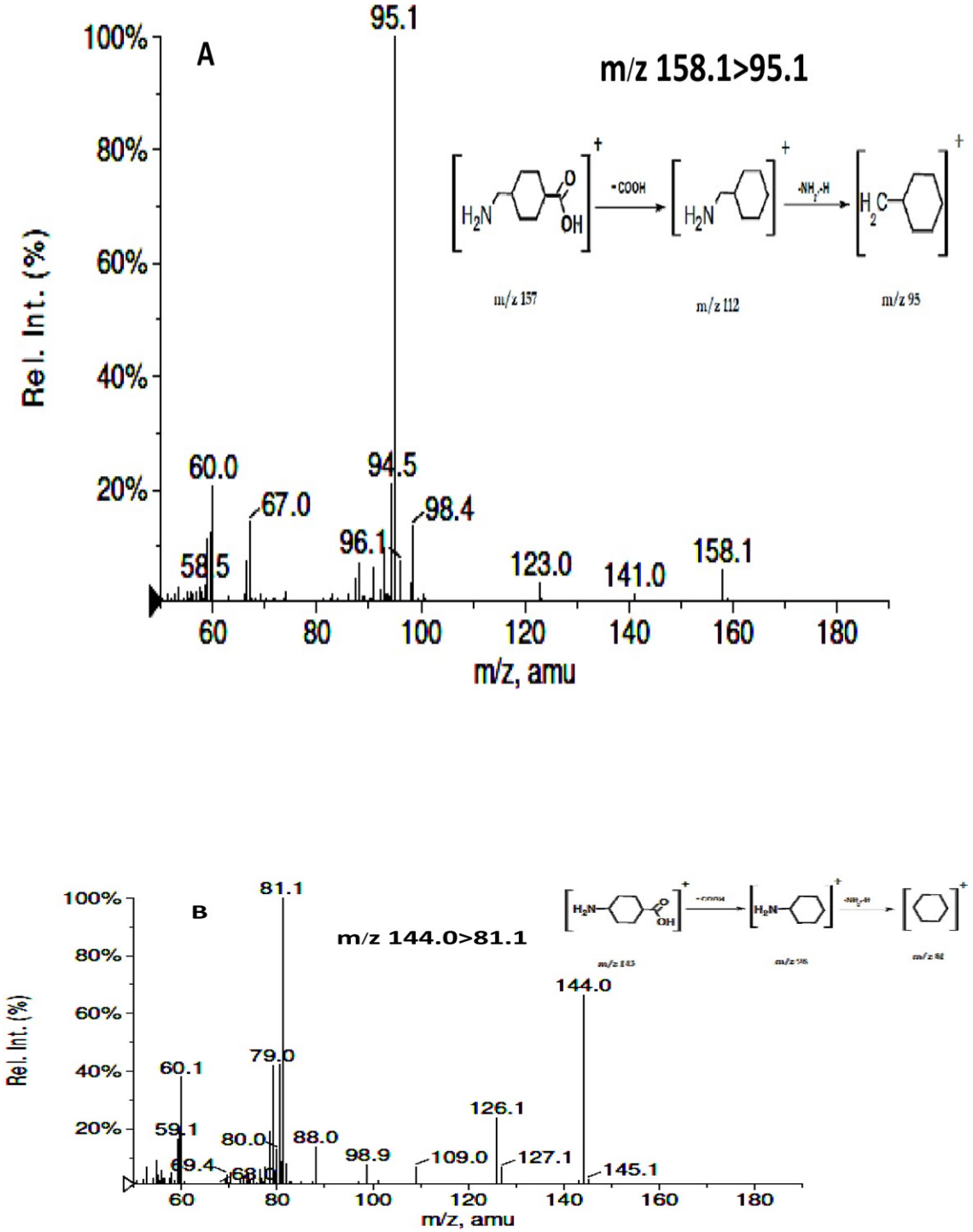


Figure 3: Quantitation was performed using the multiple reaction mode (MRM) with the ion transitions[M+H]+ m/z 158.1 to m/z 95.1 [M+H]+ m/z 144.0 to m/z 81.1 for TA and IS respectively

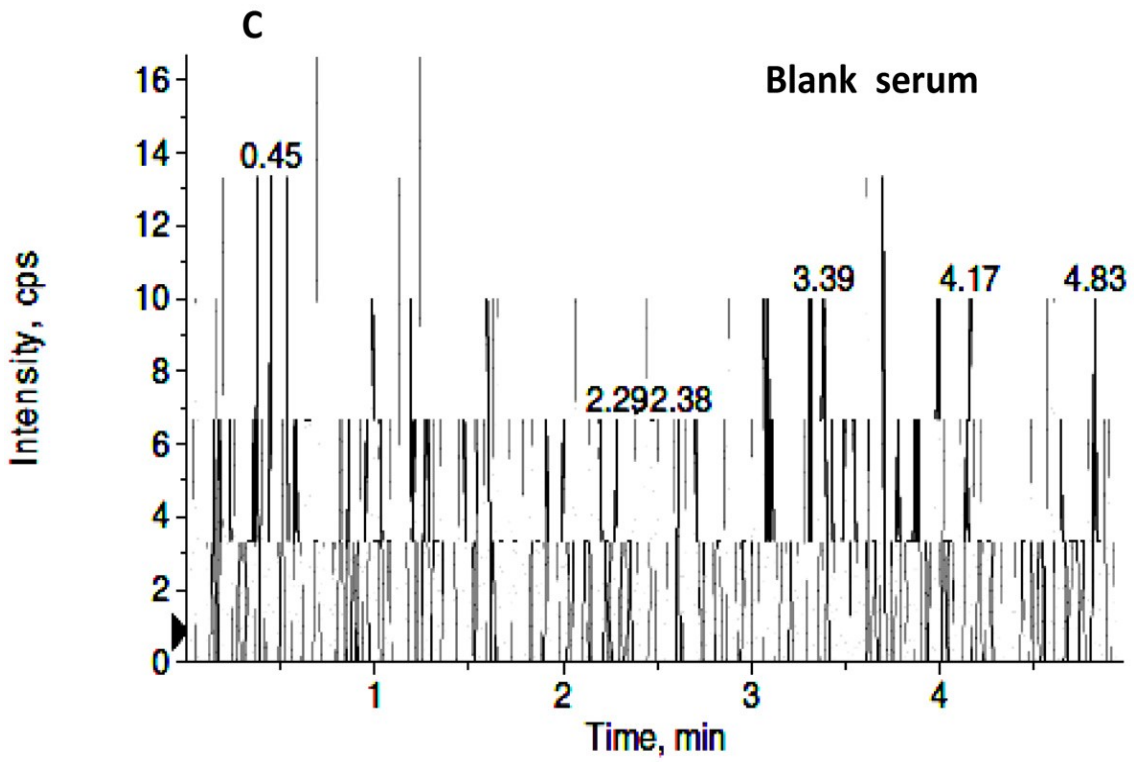
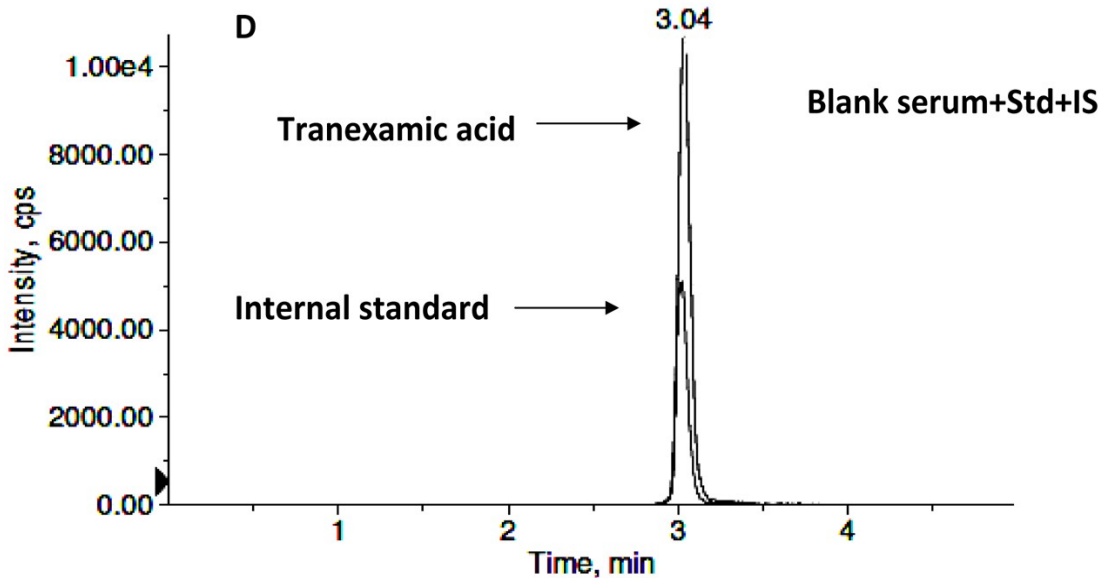


Figure 4: The effect of matrix on determination of TA in serum



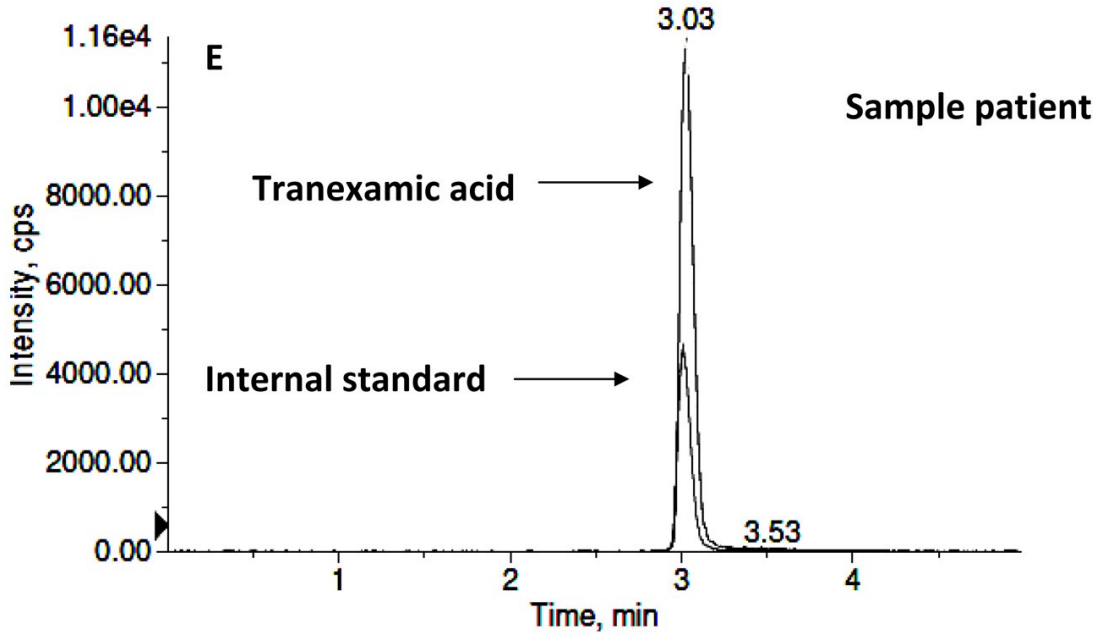


Figure 4: Product ions mass spectrum of TA(precursor ion [M+H]+m/z 158.1 (A) and IS (precursor ion [M+H]+ m/z 144.0 (B). Chromatogram of blank serum (C), blank serum spiked with standard TA at 10 µg/mL and IS at 50 µg/mL (D), and patient serum with IS at 50 µg/mL (E)

Calibration Standards:

The stock solutions of TA (1 mg/mL) and IS (1mg/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^{0}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.

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, vortexly 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.

VALIDATION OF THE METHOD

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.

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.

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.

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 ((CT-CS)×100)/C Std, where CT is the total concentration (n=5), CS is the sample concentration and C Std is the spiked concentration.

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.

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 haemoglobin concentration. A hemoglobin concentration of 13.6 g/dL was used as indicator of 100% hemolysis.

RESULTS

Standard Curve and Matrix Effect

Figure 5 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 (r2) = 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. 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.

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.

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 dertermination of TA; hemolysis up to 20% increased TA levels only 0.8%.

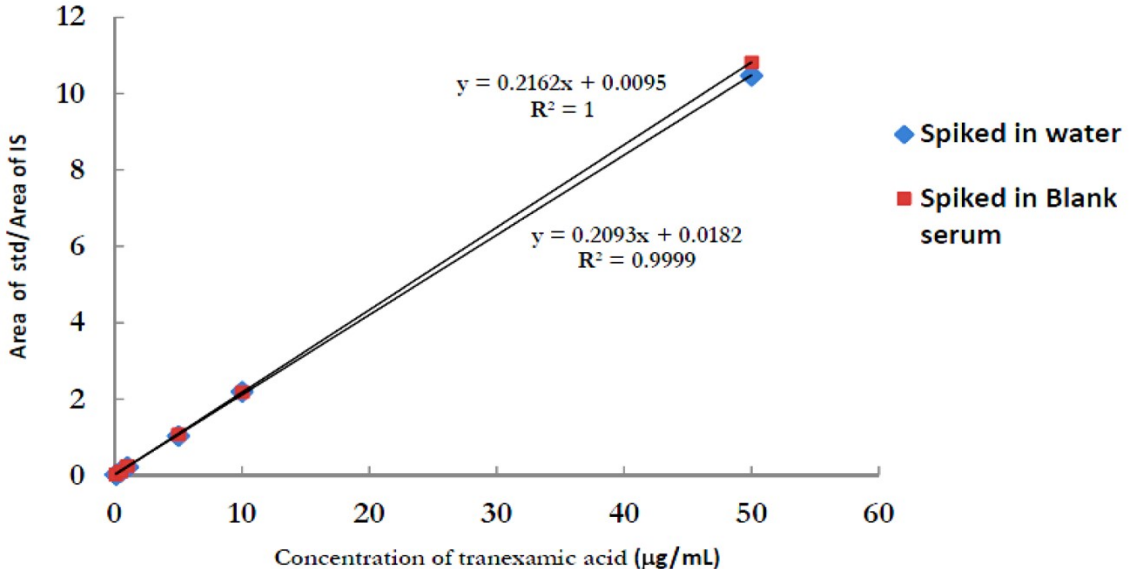


Figure 5: Calibration curves for TA spiked in water and blank serum

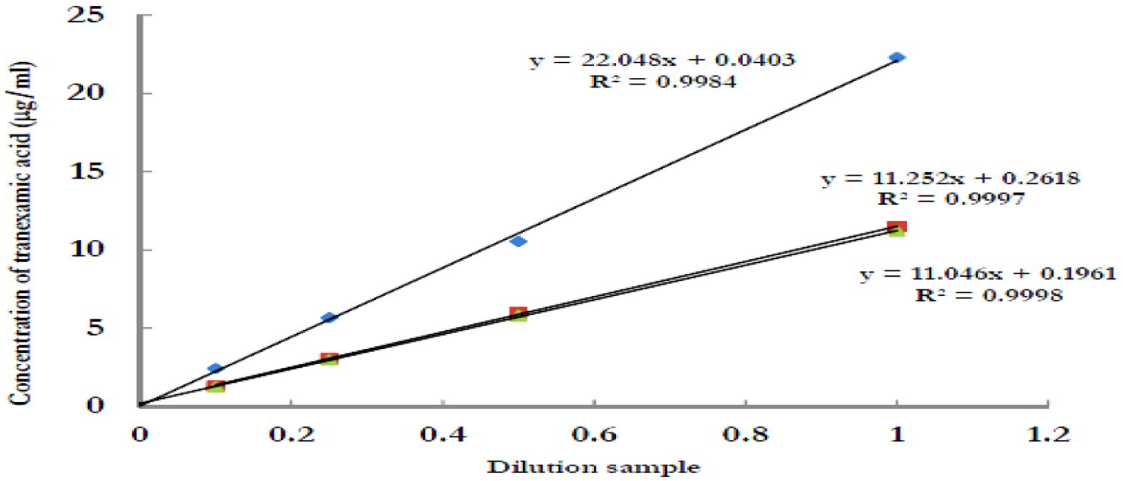


Figure 6: Values of tranexamic acid plotted against serial dilution of serum for 3 samples

Table 1: The recovery of TA from serum was 92.5 to 99.3% (Intra and inter-day imprecision of the method (mean±SD, n=5)

DAY	Sample 1		Sample 2	
	TA (µg/mL)	22.1±0.4	TA (µg/mL)	9.6±0.2
Intra-day	22.1±0.4	1.8	9.6±0.2	1.2
Inter-day	22.6±0.5	2.1	9.6±0.2	2.0

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)

Table 2: Recovery of the method (mean±SD, n=5)

Sample	Conc. of TA (µg/mL)	Std. TA added (µg/mL)	Conc. of TA After adding std. (µg/mL)	%recovery
Blank serum	0	0.5	0.5±0.0	98.6±3.9
	0	10	9.8±0.3	98.0±3.3
	0	50	46.2±0.7	92.5±1.4
Serum Pt 1a	22.1	10	31.5±0.4	94.7±4.2
Serum Pt 2a	9.6	10	19.6±0.4	99.3±3.5

a=Serum samples obtained from TKA patient 2 hours after intra-articular injection of TA

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

Condition	Storage time					Difference (%) (range)
	Day 0	Day 1	Day 4	Day 7	Day 30	
Room temp.	10.2±0.3	10.1±0.1	9.9±0.3	-	-	-0.9 to -2.4
4°c	10.2±0.3	10.1±0.2	10.2±0.2	9.5±0.1	9.7±0.3	0 to -6.8
-20°c	10.2±0.3	10.0±0.2	9.9±0.2	9.4±0.2	10.1±0.2	-1.8 to -7.3
-80°c	10.2±0.3	9.9±0.2	10.2±0.2	9.6±0.0	9.9±0.4	-0.9 to -6.4

Table 4: Hemolysis interference in serum tranexamic acid measurement (mean±SD, n =4)

Hemolysis (%)	Serum TA (µg/mL)	Difference (%)
0	9.6±0.3	
2.5	9.7±0.4	1.7
5	9.6±0.4	0.7
10	9.5±0.1	-0.5
20	9.6±0.2	0.8

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 iontrap. 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 r2 = 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.

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%.

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.

ACKNOLEDGEMENT

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