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Liquid chromatography–tandem mass spectrometry method for the determination of tranexamic acid in human plasma

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Abstract

A new method for the determination of tranexamic acid (TA) in human plasma using high performance liquid chromatography with tandem mass spectrometric detection was described. TA and the internal standard, methyldopa, was extracted from a 200 μl plasma sample by a one-step deproteination using perchloric acid. Chromatographic separation was performed on an Xtrra^{TM} MS C_{18} Column (2.1 mm × 100 mm, 3.5 μm) with the mobile phase consisting of 10% acetonitrile in 2 mM ammonium acetate buffer (pH 3.5) at a flow rate of 0.15 ml/min. The total run time was 5 min for each sample. Detection and quantitation was performed by the mass spectrometer using the multiple reaction monitoring of the precursor-product ion pair *m/z* 158 → 95 for TA and *m/z* 212 → 166 for methyldopa, respectively. The method was linear over the concentration range of 0.02–10.00 μg/ml with lower limit of quantification of 0.02 μg/ml for TA. The intra- and inter-day precision was less than 11% and accuracy ranged –10.88 to 11.35% at the TA concentrations tested. The present method provides a relatively simple and sensitive assay with short turn-around time. The method has been successfully applied to a clinical pharmacokinetic study of TA in 12 healthy subjects.

Keyword: Tranexamic acid

1. Introduction

Tranexamic acid (TA) is a synthetic derivative of amino acid lysine. It is chemically designated as trans-4-(aminomethyl) cyclohexanecarboxylic acid (Fig. 1). Due to its potent antifibrinolytic activity and lack of effect on blood clotting parameters, TA has been used in a wide range of haemorrhagic conditions [1,2].

For the determination of TA pharmacokinetics in humans, a simple and valid method for concentration determination in biological fluid such as plasma is needed. The published analytical methods in the literature include reversed-phase HPLC with ultraviolet [3,4] or fluorescent detection [5,6], and gas chromatography (GC) [7]. However, these methods involve either a pre-column [3–5] or post-column [7] derivatization procedure, which is very cumbersome and not suitable for large number of sample determination. In addition, the complicated derivatization procedure may introduce large assay variations. Furthermore, the stability of the derivate may be an additional concern.

This paper describes the development and validation of a new liquid chromatography–tandem mass spectrometry method for the determination of TA in human plasma. The method has been successfully applied to a clinical pharmacokinetic study of TA in healthy subjects.

2. Experimental

2.1. Chemicals and reagents

TA (purity better than 99%) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methyldopa was used as the internal standard (IS) and was purchased from USP (USP Science, Rockville, MD, USA). Acetonitrile (HPLC grade), methanol (HPLC grade) and dichloromethane (analytical grade) were obtained from Labscan (Asia) Co. Ltd. (Bangkok, Thailand). All other reagents were of analytical grade and were purchased from BDH Laboratory Supplies (Dorset, UK) or Merck Chemicals Co. (Darmstadt, Germany). De-ionized water was prepared using a Barnstead-nanopore® water purification system and used throughout the study.

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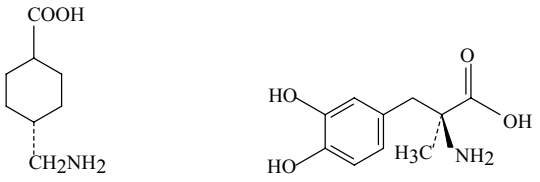

Tranexamic acid (TA) Methyldopa (IS)

Fig. 1. Chemical structures of tranexamic acid (TA) and methyldopa (IS).

2.2. Calibration standards and quality control samples

The stock solutions of TA (1 mg/ml) and IS (methyldopa, 0.5 mg/ml) were prepared separately in methanol–water (1:1, v/v) containing 0.1% formic acid. The solutions were stored at −20 °C and were found to be stable for at least 6 months. The standard working solutions of TA (0.4, 1, 2, 4, 10, 20, 40, 100, and 200 μg/ml) and IS solution (125 μg/ml) were prepared by serial dilution of their stock solutions with water.

Standard calibration samples were prepared by spiking the blank human plasma (200 μl) with 10 μl of the appropriate working solution of TA to yield concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/ml. Similarly, quality control samples at concentrations of 0.035, 0.35, 1.5, and 7.5 μg/ml were also prepared.

2.3. Sample preparation

To a 0.2 ml plasma in a 1.5 ml plastic Eppendorf tube, 20 μl of IS solution (125 μg/ml) was added. The sample was vortexed briefly, and then deproteinated by addition of 50 μl 10% (w/v) aqueous perchloric acid. After vortex-mixing for 30 s, the mixture was centrifuged at 16, 000 × *g* for 10 min. The aqueous supernatant was transferred into an auto-sampler vial, and 10 μl was subsequently injected into the LC–MS system.

2.4. Calibration curve

The calibration samples (0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/ml) were prepared in duplicate and assayed as described above. The calibration curve was constructed by plotting the peak area ratio of TA/IS versus TA nominal plasma concentration. In order to avoid undue bias in the low concentrations, the calibration curve was split into two concentration ranges, 0.02–0.5 and 0.5–10 μg/ml.

The calibration equation was obtained by linear least-squares regression analysis (without weighting) with the aid of Microsoft Excel (Microsoft Corporation):

$$y = ax + b$$

where, y is the peak area ratio, x the concentration, a the slope, and b the intercept of the regression line.

2.5. Liquid chromatography

A Perkin-Elmer LC system (Perkin-Elmer Norwalk, CT, USA) equipped with two series 200 micropumps and an autosampler was used. The chromatographic separation was performed using a Xtrra^{TM} MS C_{18} Column (2.1 mm × 100 mm, 3.5 μm, Waters, Milford, USA) eluted with a mobile phase of 10% acetonitrile in 2 mM ammonium acetate buffer (pH 3.5) at a flow rate of 0.15 ml/min. The total rum time was 5 min for each sample.

2.6. Mass spectrometry

A Perkin-Elmer Sciex API-2000 mass spectrometer equipped with electrospray ionization (ESI) source was used for the mass analysis and detection. The electrospray ionization was performed in the positive mode, with main working parameters set as follows: nebulizer gas (Gas1) 20 psi; auxiliary gas (Gas2) 40 psi; curtain gas 15 psi; orifice voltage 56 V; ring voltage 60 V and turboionspray temperature 400 °C. Quantitation was performed using the multiple reaction monitoring (MRM) of the protonated molecular ion to predominant product ion pair, *m/z* 158 → 95 for TA and 212 → 166 for IS (see mass spectrum in Fig. 2).

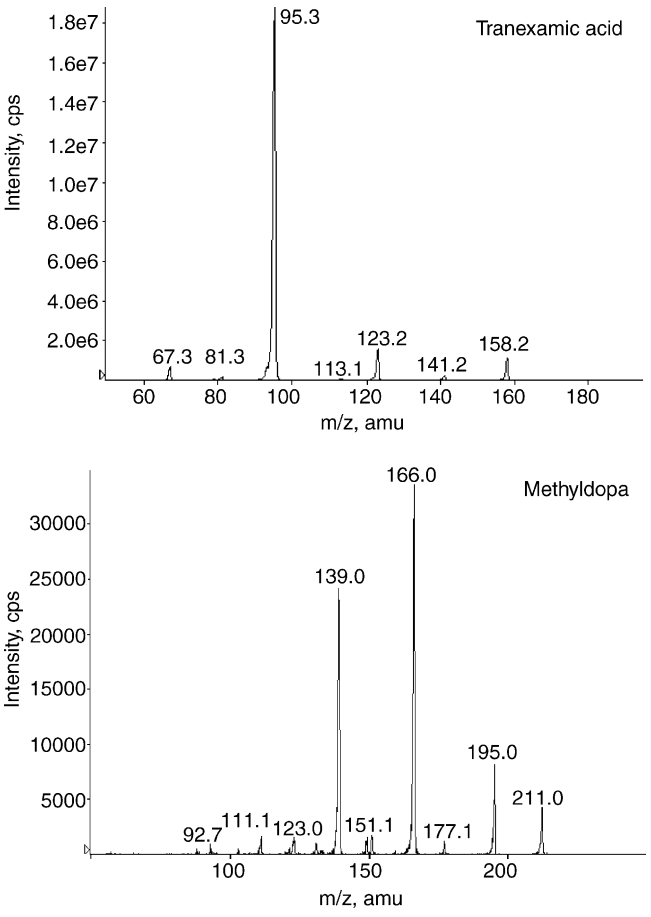

Fig. 2. Mass spectrum of tranexamic acid (LA) and methyldopa (IS).

The mass spectrometer was interfaced with a Macintosh computer, and data processed by the MassChrom software (version 1.1, Sciex).

2.7. Validation of the method

The matrix effect (i.e. potential ion suppression or enhancement effects of co-eluting and undetected matrix components in plasma) as well as lot-to-lot matrix variation were first investigated. The assay sensitivity, intra-day and inter-day accuracy and precision, extraction recovery, and stability of TA in plasma were also determined. Four quality control samples (0.035, 0.35, 1.5, and 7.5 μg/ml) were utilized for these tests using the assay procedures described in the previous section.

2.7.1. Matrix effect

The "absolute" matrix effect was evaluated by comparing the peak area of TA spiked in pre-extracted plasma samples (prepared in five replicates at each QC concentration using pooled blank plasma) to that of the aqueous standards at equivalent concentrations. Percent ion suppression was calculated as $100 \times (\mathrm{As} - \mathrm{Ap})/\mathrm{As}$, where, Ap was the mean peak area of TA from pre-extracted plasma samples (blank plasma extracted and spiked with TA after extraction) and As was the mean peak area of TA from the directly injected aqueous standards. The potential ion suppression effect of IS was evaluated by comparing the mean peak area of TA when the blank plasma sample was spiked with or without IS. To assess the lot-to-lot matrix variation, six different lots of blank plasma were used to prepare the QC samples (triplicates for each lot) at a concentration of 1.5 μg/ml. The relative standard deviation (R.S.D.) in peak area ratio among the six lots of plasma was calculated as an indicator of the inter-lot matrix variability.

2.7.2. Sensitivity

The lower limit of quantification (LLOQ) for TA was determined based on the criteria that (1) the analyte response at LLOQ is five times of the baseline noise; (2) the analyte response at LLOQ can be determined with precision ≤20% and accuracy of 80–120%. The limit of detection (LOD) was defined as the lowest concentration which gives a signal-to-noise ratio of 3.

2.7.3. Precision, accuracy, and recovery

QC samples (0.035, 0.35, 1.5, and 7.5 μg/ml) in five replicates were analyzed on the same day to determine the intra-day precision and accuracy, and on each of five separate days to determine inter-day precision and accuracy. The absolute recovery was determined in five replicates by comparing the peak areas of the extracted samples to those of the unextracted standards at equivalent concentration. The unextracted sample was prepared by mixing 0.2 ml of phosphate buffer solution (pH 7.4) with 10 μl of water, 20 μl of IS solution, and 50 μl of 10% perchloric acid.

2.7.4. Stability

The stability of TA was determined in three ways: (1) For storage stability, samples (four replicates at each QC concentration) were prepared and stored at −80 °C for 35 days. On day 36, all samples were thawed and analyzed along with the calibration standard samples which were freshly prepared. (2) For freeze-thaw stability testing, the samples (five replicates at each QC concentration) were determined after three freeze (−80 °C) and thaw (23 °C) cycles, and the concentrations were compared to their nominal concentrations. (3) To assess the injector stability of the processed samples, the plasma samples (five replicates at each QC concentration) were extracted and placed in the auto-sampler at 23 °C for 24 h, and then injected into the LC–MS system for analysis.

3. Results

3.1. Chromatography and matrix effect

Fig. 3 shows the representative LC–MS–MS chromatograms of a pooled blank plasma (A), a spiked plasma sample containing 0.02 μg/ml of TA and 12.5 μg/ml of methyldopa (B), and the plasma sample from a healthy subject at 5 h following an oral dose of 500 mg TA. The typical retention times were 2.65 min for TA and 2.60 min for IS, respectively. The results show that high and low TA concentrations can be clearly detected. Due to the high specificity of MS/MS detection, no interference peaks were observed from the blank plasma as well as the pre-dose plasma samples from the healthy subjects who participated in a clinical pharmacokinetic study of TA.

Neither matrix components in plasma nor IS caused significant changes in the MS/MS response of TA. The percent of ion suppression was <2.09% across the QC levels. No significant lot-to-lot matrix variation was observed. For the six lots of plasma spiked with 1.5 μg/ml of TA, the inter-lot variation in peak area ratio (R.S.D.%) was found to be 3.84%.

3.2. Linearity and sensitivity

The calibration curves of TA were linear over the concentration ranges of 0.02–0.5 and 0.5–10 μg/ml, respectively. The mean linear regression equations (from four runs) were:

0.02–0.5 μg/ml (low concentration range)	$y = 0.65088\ (\pm 0.03906)x + 0.00269\ (\pm 0.00226)$, $r = 0.9990 \pm 0.0006$
0.5–10 μg/ml (high concentration range)	$y = 0.57231\ (\pm 0.01587)x + 0.08033\ (\pm 0.04396)$, $r = 0.9997 \pm 0.0002$

Using the present method, the lower limit of quantification (LLOQ) was 0.02 μg/ml, and the LOD was 0.01 μg/ml.

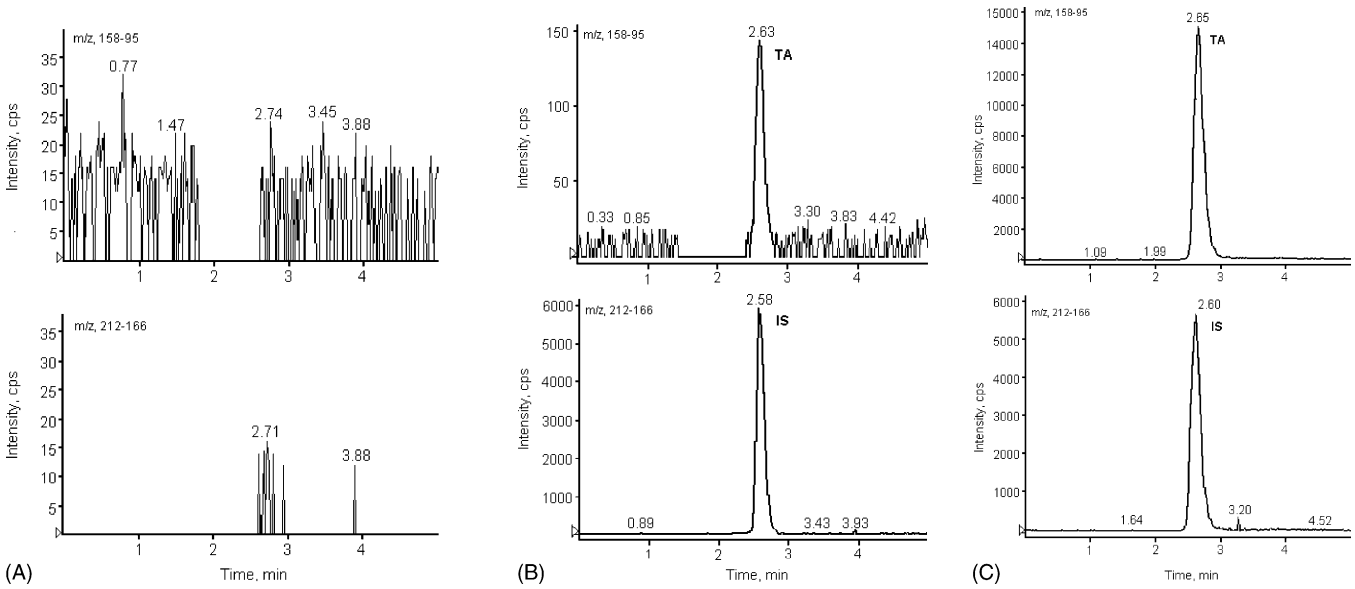

Fig. 3. Representative LC–MS–MS chromatograms of a pooled blank plasma (A), a spiked plasma sample containing 0.02 μg/ml tranexamic acid (TA) and 12.5 μg/ml methyldopa (IS) (B), and the plasma sample from a healthy subject at 5 h following an oral dose of 500 mg TA (TA plasma concentration was determined to be 4.79 μg/ml) (C).

3.3. Precision, accuracy, and recovery

The intra-day, inter-day precision and accuracy of the assay are summarized in Table 1. The precision, presented as percentage of relative standard deviation, ranged from 1.12 to 10.23% and 3.98 to 9.08% for intra-day and inter-day determination, respectively. The accuracy, presented as percentage of bias against the nominal concentration, ranged from −10.88 to 11.35%, and −1.89 to 6.21% for intra-day and inter-day assay, respectively. The absolute recovery of TA from plasma was determined to be 102.09 ± 6.64%, 99.49±10.50%, 99.07±5.29%, and 101.16±6.18% at concentrations of 0.035, 0.35, 1.5, and 7.5 μg/ml, respectively. The recovery of IS averaged 97.93 ± 4.90% ($n = 20$).

3.4. Stability

TA was found stable in plasma when stored at −80 °C for at least 35 days. No significant degradation of TA was observed for the plasma samples after three freeze-thaw cycles, or placed in the auto-sampler at 23 °C for up to 24 h (Table 2).

Table 1
Intra- and inter-day precision and accuracy of the method for determination of TA in human plasma

Nominal concentration (μg/ml)	Parameters	Intra-day					Inter-day
		Day 1 ($n = 5$)	Day 2 ($n = 5$)	Day 3 ($n = 5$)	Day 4 ($n = 5$)	Day 5 ($n = 5$)	5 Days ($n = 25$)
0.035	Mean	0.034	0.037	0.035	0.034	0.031	0.034
	S.D.	0.003	0.002	0.001	0.003	0.003	0.003
	R.S.D. (%)	9.98	5.92	3.34	7.90	10.23	9.08
	Bias (%)	−2.80	5.63	1.03	−2.54	−10.88	−1.89
0.35	Mean	0.39	0.37	0.37	0.33	0.37	0.37
	S.D.	0.02	0.01	0.02	0.02	0.03	0.03
	R.S.D. (%)	4.43	3.13	5.29	7.39	8.97	7.75
	Bias (%)	11.35	5.75	5.97	−6.43	6.92	5.18
1.50	Mean	1.63	1.60	1.60	1.37	1.56	1.56
	S.D.	0.05	0.06	0.02	0.03	0.05	0.10
	R.S.D. (%)	3.10	3.51	1.12	1.80	3.43	6.18
	Bias (%)	8.58	6.35	6.64	−8.41	4.22	3.97
7.50	Mean	8.30	7.83	8.02	7.93	7.74	7.97
	S.D.	0.34	0.13	0.35	0.29	0.17	0.32
	R.S.D. (%)	4.12	1.63	4.40	3.60	2.17	3.98
	Bias (%)	10.66	4.39	6.92	5.78	3.21	6.21

Table 2
Stability of TA in plasma

Nominal concentration (μg/ml)	Determined concentration of stability sample (Mean ± S.D., μg/ml)		
	Storage stability (−80 °C, 35 days) ($n = 4$)	Freeze-thaw stability (−80 to −23 °C) ($n = 5$)	Autosampler stability (23 °C, 24 h) ($n = 5$)
0.035	0.033 ± 0.001	0.031 ± 0.001	0.031 ± 0.003
0.35	0.34 ± 0.03	0.33 ± 0.04	0.35 ± 0.01
1.50	1.41 ± 0.02	1.46 ± 0.03	1.42 ± 0.04
7.50	7.08 ± 0.26	7.31 ± 0.16	7.32 ± 0.43

3.5. Application to clinical pharmacokinetic study

The assay method was used in a clinical pharmacokinetic study of TA in 12 healthy subjects. The study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong. All subjects provided written informed consent prior to participating in the study. The subject received a 500 mg oral dose of TA after an overnight fast of 10 h and 14 blood samples were collected over a period of 12 h. After centrifugation at 1000 × g for 10 min, the separated plasma were stored at −80 °C and analyzed within 1 month.

The mean plasma concentrations versus time profile of TA following a single 500 mg oral dose in 12 subjects are shown in Fig. 4. Peak plasma concentrations of TA averaged 7.4 μg/ml, occurring around 2.5 h post-dosing. The mean apparent clearance of TA was estimated to be 0.2 l/(h kg) and its elimination half-life 2.4 h. These pharmacokinetic parameters were generally in agreement with those reported previously [8,9].

4. Discussion

The existing published analytical methods for TA (i.e. HPLC, GC) are inadequate and/or inconvenient for clinical pharmacokinetics studies, due to tedious pre- or post-column derivatization procedures. In the present study, we developed a reliable and sensitive new LC–MS–MS method to determine TA in human plasma. The advantages of this new method are simple extraction procedure, short turn-around time and good sensitivity.

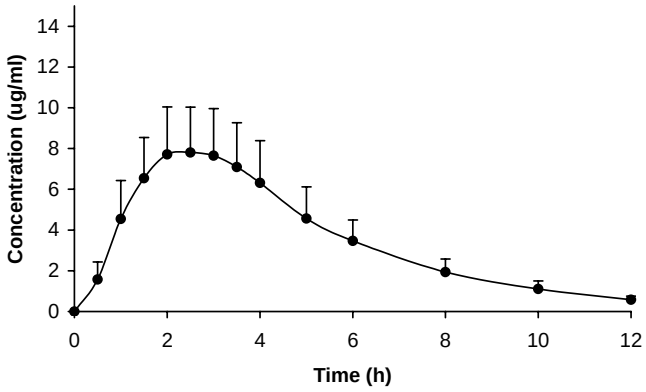

Fig. 4. Mean plasma concentration-time profile of tranexamic acid in 12 subjects following an oral 500 mg dose.

Since TA is a synthetic amino acid with large polarity, it is very difficult to separate TA from plasma by the liquid–liquid or solid phase extraction methods. In this study we utilized a simple one-step protein precipitation method, which provided satisfactory extraction efficiency. Among the various precipitating reagents tested (i.e. methanol, acetonitrile, 10% perchloric acid, 10% sulphosalicylic acid, and 10% trichloroacetic acid), methanol and acetonitrile were found to cause wide chromatographic peaks of TA and IS (peak width close to 2 min). However, clean and sharp peaks of TA and IS were observed when using 10% perchloric acid as the precipitating reagent. When compared to sulphosalicylic acid and trichloroacetic acid, perchloric acid resulted in much cleaner aqueous supernatant after deproteination.

The previously reported HPLC and GC methods have LOD in the range of 0.1–0.6 μg/ml [3,4,7]. In the present assay, the LOD is 0.01 μg/ml and the assay only requires 0.2 ml of plasma. These features are important for clinical situations when blood volume is restricted and yet high assay sensitivity is required.

The commercially available substance methyldopa worked well as IS in this study. One potential disadvantage of using methyldopa as IS is its instability. The 3, 4-dihydroxypenyl group of methyldopa is liable to oxidative reaction under neutral or basic pH conditions. However, with the addition of 0.1% formic acid to its stock solution, we found that methyldopa was stable for at least 6 months when stored at −20 °C.

Due to the large polarity of the analytes, high aqueous mobile phase is needed for proper retention of the analytes on reversed-phase column. In this study, various mobile phase composition with different flow rates were investigated for optimum ionization of the analytes. A mobile phase consisting 10% acetonitrile in 2 mM ammonium acetate buffer eluted at a relatively low flow rate (0.15 ml/min) was found to be satisfactory for this assay. With increased flow rate (i.e. greater than 0.15 ml/min), vaporization of the mobile phase into fine drops became less efficient and led to decreased ion formation. This is not surprising since high proportion of aqueous phase were present when TA and IS were eluting. The increased radius of the droplet could increase the

surface tension of the mobile phase and adversely affect the desolvation process into the ion source as well as the ionization of the analytes.

The mismatch between mobile phase and MS detection is unavoidable for polar drugs with reversed-phase LC–MS quantitation. Recently, LC–MS using hydrophilic interaction chromatography (HILIC) has been advocated as an alternative approach overcoming these limitations [10]. Nevertheless, our current reversed-phase LC–MS method has been demonstrated to be reliable and satisfactory for the determination of TA in the human plasma. Whether a HILIC method can provide a better solution for TA needs to be further investigated.

5. Conclusion

A reliable and sensitive new LC–MS–MS method has been developed for the determination of TA in human plasma. The method is suitable for clinical pharmacokinetic studies and is advantageous over existing methods due to its simplicity, short turn-around time and good sensitivity.

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