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Development and Validation of Tranexamic Acid Determination in Human Plasma by HPLC-MS/MS method

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Abstract

Introduction. Tranexamic acid is one of the most common drugs used to stop bleeding after trauma, in surgery and gynecology. The most common analytical method for the determination of this compound is reversed-phase high-performance liquid chromatography (HPLC). However, this compound belongs to the group of so-called poorly retained compounds due to its chemical structure. It is necessary to develop an analytical method that will allow the determination of tranexamic acid in human blood plasma with the least time, resource costs and without the use of specialized columns.

Aim. The aim of this study is to develop a method for tranexamic acid in human plasma by high performance liquid chromatography with tandem mass-spectrometry (HPLC-MS/MS) for pharmacokinetic studies.

Materials and methods. Determination of tranexamic acid in plasma by HPLC-MS/MS. The samples were processed by acetonitrile protein precipitation.

Results and discussion. This method was validated by next parameters: selectivity, matrix effect, calibration curve, accuracy, precision, recovery, lower limit of quantification, carry-over effect and stability.

Conclusion. The method of the determination of tranexamic acid in human plasma was developed and validated by HPLC-MS/MS. The linearity in plasma sample was achieved in the concentration range of 100.00–15000.00 ng/ml. Method could be applied to tranexamic acid determination in plasma for pharmacokinetics and bioequivalence studies.

Keywords: tranexamic acid, plasma, HPLC-MS/MS, validation, bioequivalence

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Timofey N. Komarov, Olga A. Archakova, Alexandra V. Aleshina have developed an analytical method. Dana S. Shchelgacheva, Veronika V. Davydanova have validated an analytical method. Natalia S. Bagaeva carried out statistical processing of the obtained results. Igor E. Shohin and Alla Yu. Savchenko carried out the organization of work in this direction. All the above authors participated in the discussion of the results in the format of scientific discussion.

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Определение транексамовой кислоты в плазме крови человека методом высокоэффективной жидкостной хроматографии с масс-спектрометрическим детектированием

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Резюме

Введение. Транексамовая кислота является одним из наиболее распространенных препаратов, применяемых для остановки кровотечений после травм, хирургических вмешательств и в гинекологии. Наиболее распространенным аналитическим методом определения данного соединения является обращенно-фазовая высокоэффективная жидкостная хроматография (ВЭЖХ), однако из-за своей химической

структуры данное соединение относится к группе так называемых слабоудерживаемых соединений, в связи с чем возникает необходимость разработки методики, позволяющей с наименьшими временными и ресурсными затратами, без использования специализированных колонок провести определение транексамовой кислоты в плазме крови человека.

Цель. Целью исследования является разработка и валидация методики определения транексамовой кислоты в плазме крови человека методом высокоэффективной жидкостной хроматографии с тандемным масс-селективным детектированием (ВЭЖХ-МС/МС) для проведения фармакокинетических исследований.

Материалы и методы. Определение транексамовой кислоты в плазме крови человека проводили методом ВЭЖХ-МС/МС. В качестве пробоподготовки использовали способ осаждения ацетонитрилом.

Результаты и обсуждение. Разработанная методика была валидирована по следующим валидационным параметрам: селективность, эффект матрицы, калибровочная кривая, точность, прецизионность, степень извлечения, нижний предел количественного определения, перенос пробы, стабильность.

Заключение. Разработана и валидирована методика определения транексамовой кислоты в плазме крови человека методом ВЭЖХ-МС/МС. Подтвержденный аналитический диапазон методики составил 100,00–15000,00 нг/мл в плазме крови. Аналитический диапазон позволяет применять разработанную методику для проведения исследований фармакокинетики препаратов транексамовой кислоты.

Ключевые слова: транексамовая кислота, плазма, ВЭЖХ-МС/МС, валидация, биоэквивалентность

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. Т. Н. Комаров, О. А. Арчакова, А. В. Алешина участвовали в разработке биоаналитической методики. Д. С. Щелгачева и В. В. Давыданова принимали участие в проведении валидации биоаналитической методики. Н. С. Багаева проводила статистическую обработку данных. И. Е. Шохин и А. Ю. Савченко отвечали за организационную часть исследования. Все вышеуказанные авторы участвовали в обсуждении полученных результатов в форме научной дискуссии.

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INTRODUCTION

Tranexamic acid (TA) (figure 1) is a product with antifibrinolytic action reversibly blocking lysine binding sites on plasminogen hereby preventing plasmin interaction (which is formed as a result of plasminogen activation) with lysine residues and subsequent fibrin degradation [1]. The therapeutic indications for tranexamic acid are conditions with abnormal bleeding or bleeding trends in which local or systemic hyperfibronolysis are thought to be involved [2]. Tranexamic acid has been also widely used in gynecology as a non-hormonal product for treatment of abundant menstrual bleedings, for decrease of a bleeding during gynecological surgeries such as abdominal myomectomy or hysterectomy [3]. There is the data on the possible use of tranexamic acid as the product against COVID-19 (coronavirus disease 2019) for some groups of patients. The antiviral effect is associated with the theory according to which SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus-2), virus causing COVID-19 becomes more virulent when the virus S-protein is cleaved by plasmin. Therefore plasmin inhibition may prevent COVID-19 progression. Tranexamic acid, in its turn, as a typical antifibrinolytic product decreases plasmin production [4]. In the Russian Federation, tranexamic acid is included to the list of vital and essential which indicates the significance of the drug for national healthcare [5].

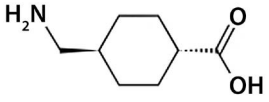

Figure 1. Chemical structure of tranexamic acid

By its structure, tranexamic acid is trans-4-(aminomethly)-cyclohexanecarboxylic acid and represents a synthetic analogues of lysine amino acid. Owing to the presence of carboxylic acid, the compound has hydrophilic properties which is illustrated by octanol-water partition coefficient (log P) for the substance (table 1).

Table 1. Log P and pKa for tranexamic acid

	Tranexamic acid
Log *P*	–1.6
pKa	4.56
Reference	[6]

It is known that physical-chemical properties of drug compounds adequately influence pharmacokinetics. Such characteristics as hydrophility/lypophility influence bioavailability of a drug substance, as well as, substance behavior in the chromatographic system. However, as for hydrophilic and polar compounds to which tranexamic acid belongs, when they are determined with the reversed-phase high performance liquid performance (HPLC), the difficulties occur which are related to the poor retention of the substances on classical octadecyl silica gel columns. To solve practical tasks on retention of poorly retainable components, there are various approaches such as introduction of ion-paired supplements to the mobile phase, the use of columns with low carbon percentage, the use of columns with grafted polar radicals, e.g. cyano-, amino modification of silicagel and the method of hydrophilic chromatography [Hydrophilic interaction liquid chromatography (HILIC)] [7]. However, not all approaches given above may be implemented in practice in conditions of everyday work. Moreover, often, analytical laboratories not always have a full variety of chromatographic columns at their disposal. Due to that, it becomes necessary to develop chromatographic columns for poorly retained substances with the selection of optimal conditions of chromatographic separation, detection, as well as the method for sample preparation.

Nowadays, peer-reviewed journals present various methods for determination of tranexamic acid with the method of high performance liquid chromatography with high performance liquid chromatography – triple quadrupole tandem mass spectrometry (HPLCMS/MS) in human plasma or serum (table 2).

However, it should be mentioned that the methods given above require labor consuming sample preparation or imply corrosion aggressive reagents for protein precipitation. The study presents the development and validation of the method for determination of tranexamic acid with mass selective detection and use of protein precipitation as sample preparation with acetonitrile which to simplify and accelerate the procedure for sample preparation thus obtaining the method meeting all the requirements of the regulatory documents.

MATERIALS AND METHODS

Equipment

Chromatographic separation and detection were performed on high performance liquid chromatograph Nexera XR equipped with a gradient pump, column and sample thermostat , autosampler and a tandem mass spectrometry detector (triple quadrupole). Source data was processed with software Lab Solutions (Ver. 5.91) (Shimadzu Corporation, Japan).

Reagents and solutions

The following reagents were used in the work: methanol ("UHPLC-grade" class, J.T. Baker, Netherlands), acetonitrile ("LC-MS grade" class, Biosolve, France/Netherlands/Israel), formic acid (class "98 % pure", PanReac, Spain), ammonia hydrate ("for analysis" class, PanReac, Spain), Milli-Q water. For preparation of stock working solutions, reference standard samples of tranexaminc acid were used (assay 99.3 %, Hunan Duntin Pharmaceuticals Co, Ltd., China) and vildaglyptin (assay 99.8 %, Sigma-Aldrich, Germany).

Stock reference solutions of tranexamic acid and internal standard (IS) of vildagliptin (VIL) were prepared by dissolution of weighs of the substances in methanol; working reference solutions of tranexamic acid and IS working solution was prepared by dilution of reference solutions with the same diluent up to necessary concentrations in plasma corresponding to levels 1–8, as well levels LLLOQ (lower limit of quantification), L (low), M (middle) and H (high) (table 3).

Table 2. Bioanalytical methods of quantitative determination of tranexamic acid

Analytical method	Sample preparation	Internal standart	Analytical range	Chromatography column	Reference
HPLC-MS/MS	Protein precipitation by perchloric acid	Methyldopa	0.02 – 10.00 μg/ml	XTerra™ MS C18 Column (100 × 2.1 mm, 3.5 μm)	[8]
HPLC-MS/MS	Protein precipitation by perchloric acid	cis-4-aminocyclohexanecarboxylic acid	1.00 – 200.00 μg/ml	HyPURITY C18 Thermo Hypersil column (150 ×2.1 mm, 5 μm)	[9]
HPLC-MS/MS	Phospholipid clean-up and protein precipitation by Ludox® AS-40 and lanthanum chloride	cis-4-aminocyclohexanecarboxylic acid	1.00 – 1000.00 μg/ml	Thermo Scientific™ Accucore™ Urea HILIC HPLC Columns (150 ×3 mm, 2. μm)	[10]

Table 3. **Concentrations of analyte in calibration samples and in quality control samples**

Level	Analityte concentration, ng/ml	IS concentration, ng/ml
	TA	VIL
1	100.00	500.00
2	250.00	500.00
3	500.00	500.00
4	1000.00	500.00
5	5000.00	500.00
6	8000.00	500.00
7	10000.00	500.00
8	15000.00	500.00
9	100.00	500.00
LLOQ	300.00	500.00
L	7500.00	500.00
M	12500.00	500.00
H	100.00	500.00

Stock and working reference solutions were kept in the freezer at temperature –40 °C. The samples of blank plasma were kept in the plasma freezer at temperature –40 °C.

Sample preparation

To 200 µl of a calibration sample, quality control sample, blank plasma sample transferred to Eppendorf centrifuge micro tube 2 ml, 10 ml of vildagliptin IS working solution was added, 400 µl of acetonitrile was added, mixed on vortex for 10 seconds, then centrifuged for 15 min with the rate 13500 rot/min. Then the supernatant was transferred to chromatographic vials and then to the chromatograph autosampler.

Conditions for chromatographic separation and detection

- Chromatographic column: Shim-pack GWS C18, 150 × 4.6 mm, 5 µm.
- Thermostat temperature: 40 °C.
- Mobile phase: Eluent A: 0.1 % formic acid solution in Milli-Q water with the addition of 0.08 % ammonium (by volume), eluent B: 0.1 % formic acid solution with the addition of 0.08 % ammonium in methanol (by volume).
- Gradient by mobile phase (MP) composition is given in table 4.

Table 4. **Gradient elution**

Time, min	Eluent A, %	Eluent B, %	Mobile phase flow rate, ml/min
0.00	12.5	87.5	1.00
0.50	12.5	87.5	1.00
2.50	12.5	87.5	1.00
2.60	12.5	87.5	1.30
3.00	100.0	0.0	1.30
4.00	100.0	0.0	1.30
4.20	12.5	87.5	1.30
4.70	12.5	87.5	1.00
7.50	12.5	87.5	1.00

- Injection volume: 5 µl.
- Run time of the mass spectrometry detector: 0.00–7.50 min.
- Ionization source parameters (electrospray): spraying gas 3 l/min, drying gas 20 l/min, heating block 400 °C, desolvation line 200 °C, capillary pressure +4.5 kV.
- Ionization mode: positive.

Table 5. **Detection conditions**

Analyte name	Detection conditions		
Tranexamic acid	158.10 → 55.10 m/z	158.10 → 67.10 m/z	158.00 → 95.20 m/z
Vildagliptin	304.10 → 107.05 m/z	304.10 → 151.10 m/z	

RESULTS AND DISCUSSION

Method development

The main particularity of the method development for determination of tranexamic acid is the necessary of retention of hydrophilic, polar substance in the conditions of reversed-phase chromatography. When selecting an optimal chromatographic column, the columns with grafted amino and cyano groups, octyl groups, as well as chromatographic columns with octadecyl modification of

silicagel containing various carbon percentage. Moreover, when the method was developed, various eluents were tested as mobile phase including buffer systems.

Based on the experimental data as stationary phase, chromatographic column Shim-pack GWS C18, 150 × 4.6 mm, 5 µm with carbon contents 9.5 % and low residual silanol activity was selected. The column used with ammonium formate allowed to achieve optimal retention on the column, to prevent washout and achieve optimal form of chromatographic peaks.

Method validation

The bioanalytical method was validated based on the Guidance on drug expertise, volume I [12] and FDA [13] and EMA guidelines [14] on the following parameters: selectivity, matrix effect, a standard curve, accuracy (at intracycle, intercycle levels), precision (at intracycle, intercycle levels), recovery, the lower limit of quantification, sample transfer, stability [stability of stock and working reference analyte and IS solutions; short-term stability ("bench-top" and "post-preparative" stability); stability in triple freeze-thaw cycles; long-term analyte in the matrix].

Selectivity

6 samples of blank blood plasma obtained from various sources, as well samples of blank plasma with the addition of working reference solutions up to concentrations corresponding to LLLOQ level (table 3) were analyzed. The separate analysis of hemolyzed blank plasma samples and samples with the increased level of lipids. On the chromatograms of blank plasma, peak signals with retention times corresponding to retention times of the analyte and IS do not exceed 20 % of the analyte signal at the level of the lower limit of quantification (LLQ) and 5 % of IS signal, respectively. The corresponding chromatogram is given below in figure 2.

Matrix effect

To evaluate matrix effect, samples with the addition of working reference solutions of tranexamic acid and vildagliptin IS working solution were analyzed without the effect of the biological matrix, as well as samples prepared on blank plasma, without regards to the effect of recovery of tranexamic acid from the biological matrix.

The matrix effect was evaluated at low (level L) and high (level H) levels of the analytical range of tranexamic acid concentrations (table 3). For vildagliptin IS, the matrix effect was calculated at the level of 500.00 ng/ml. The data is given in table 6.

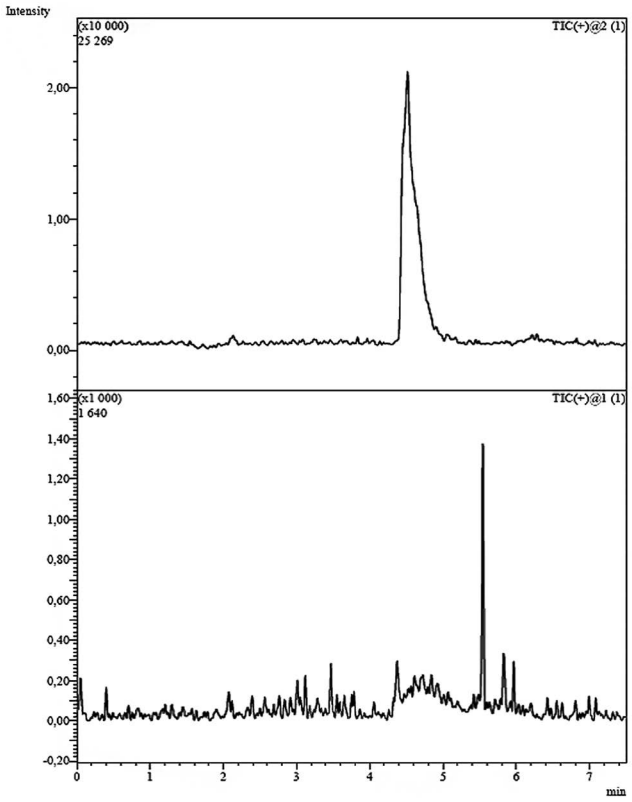

Figure 2. **Blank plasma sample chromatogram**

Table 6. **The matrix factor of TA, normalized by the IS matrix factor**

	Blank plasma		Hemolyzed blank plasma		Hyperlipedimic blank plasma	
	Normalized Mf (L)	Normalized Mf (H)	Normalized Mf (L)	Normalized Mf (H)	Normalized Mf (L)	Normalized Mf (H)
Average	1.25	0.92	1.45	1.03	1.37	1.04
CV, %	5.90	4.45	6.84	4.07	5.35	3.31

Calibration curve

Eight samples of blank plasma with the addition of vildagliptin IS working solution and working reference solutions of tranexamic acid up to the concentrations given in table 3 were analyzed (levels 1–8). Using the data, calibration plots representing ratio of peak area of tranexamic acid to peak area of vildaglip-

tin from the ratio of tranexamic acid concentration to vildagliptin concentration in plasma (figure 4). The chromatogram of the sample at level 8 (table 3) is given in figure 3.

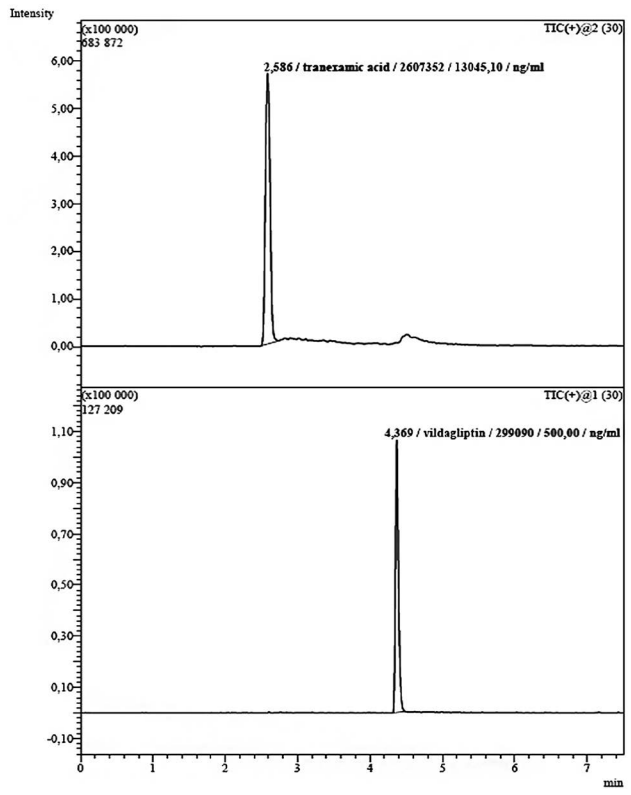

Figure 3. **Level 8 plasma sample chromatogram**

Accuracy and precision

Plasma samples corresponding to levels LLLOQ, L, M and H (table 3) were analyzed. The analysis was performed within 3 sequences of 5 samples for each level. Accuracy and precision were evaluated within one cycle, between two and thee cycles, the data is given in table 7.

The values of relative standard deviation (RSD, precision) and relative error (E, accuracy) correspond to the standard (not more than 20 % at LLLOQ level, not more 15 % – for other points).

Recovery

To evaluate recovery (R), 3 samples prepared from blank plasma, were analyzed without the effect of recovery at levels L, M, and H (table 3), as well as quality control samples for recovery evaluation. The separate analysis of hemolyzed blank plasma samples with the increased lipid levels. The data is given in table 8.

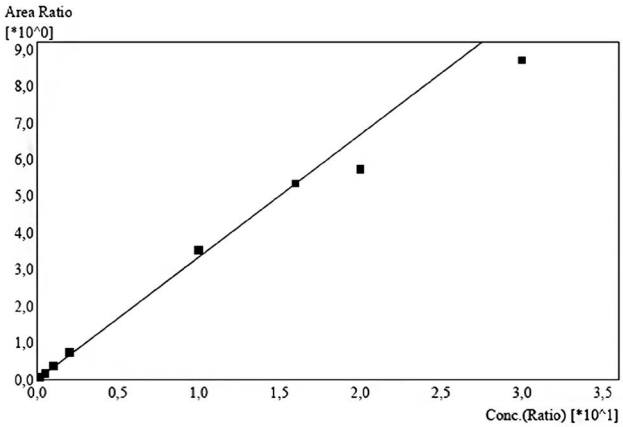

Figure 4. **Example of the calibration curve representing dependence of the ratio area peak of tranexamic acid to vildagliptin on the concentration ratio of tranexamic acid to the vildagliptin in plasma.**

Table 7. **Accuracy and precision of the method**

Level	RSD, %			*E*, %		
	(*n* = 5)	(*n* = 10)	(*n* = 15)	(*n* = 5)	(*n* = 10)	(*n* = 15)
LLOQ	3.17	6.05	3.75	–11.49	–6.77	–5.49
L	2.84	3.58	5.02	3.43	6.10	8.15
M	2.68	1.95	3.95	1.34	1.68	–0.30
H	0.79	2.20	4.50	–7.22	–5.97	–6.45

Table 8. **Calculation of tranexamic acid recovery at L, M, H levels from different types of biological matrix**

	Recovery (level L), %	Recovery (level M), %	Recovery (level H), %
Average	58.35	83.43	67.87
SD	6.88	2.23	6.24
RSD	11.80	2.67	9.19

The recovery should not be equal to 100 %, but efficient and reproducible substance recovery from the biological matrix should be provided. The relative standard deviation of estimated values of the analyte recovery from biological matrixes should not exceed 15 %.

Lower limit of quantification

LLOQ of the method was determined based on the linearity, accuracy and precision data. As LLOQ of the method, the minimal concentration of tranexamic acid in plasma in the analytical range in which tranexamic acid may be quantified with values RSD and E not more 20 %. LLOQ was 100.00 ng/ml. The chromatogram of plasma containing tranexamic acid at LLOQ level is given in figure 5. The signal/noise ration by the peak of tranexamic acid at LLOQ calculated with software LabSolutions (Ver. 5.91) (Shimadzu Corporation, Japan), is 28.3.

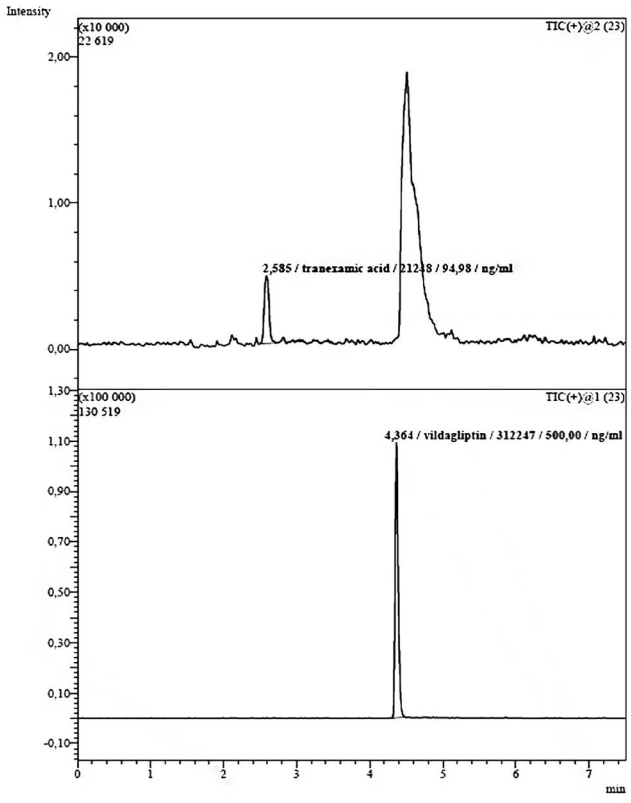

Figure 5. LLOQ plasma sample chromatogram

Stability

Short-term stability has been confirmed ("Tabletop" and "post-preparative"), stability with three times freezing and defrosting, stable stock and working standard solutions (when stored for 16 days at a temperature –40 °C), long-term stability (storage within 71 days at temperature –40 °C) of the test substances at low (L) and high (H) concentration levels (table 3).

Sample transfer

The successive analysis of the calibration sample corresponding to level 8 (see table 3), and blank plasma sample on the chromatogram of the blank plasma sample showed no peaks corresponding to the peak of the test substance and IS by retention times.

CONCLUSION

The method for determination of tranexamic acid in human plasma by HPLC-MS/MS was developed and validated. The confirmed analytical range of the method was 100.00–15000.00 ng/ml in human plasma. The obtained analytical range allows to use the developed method for the analytical part of pharmacokinetic studies of tranexamic acid products.

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