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Determination of Lappaconitin, Diterpene Alkaloide Obtained from Plants *Aconitum Leucostomum*, and its Active Metabolite N-desacetyllappaconitin in Human Plasma and Blood

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Abstract

Introduction. Lappaconitine is an alkaloid, contained into *Aconitum leucostomum* Vorosh. roots and herbs. The alkaloid is indicated to arrhythmia. The lappaconitine drugs are metabolized into eight pharmacologically active substances, but N-desacetyllappaconitine is the most effective. Drugs based on a lappaconitine has narrow therapeutic range and many kinds of side effects. Pharmacokinetics of lappaconitine should be more studied for safety medical use of lappaconitine drugs.

Aim. The aim of this study is to develop method for the quantitative determination of lappaconitine and its active metabolite N-desacetyllappaconitine in human plasma and blood by high performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS).

Materials and methods. Determination of lappaconitine and N-desacetyllappaconitine in plasma and blood was carried out 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, spike recovery, lower limit of quantification, carry-over effect and stability.

Conclusion. The method of the quantitative determination of lappaconitine and N-desacetyllappaconitine in human plasma and blood was developed and validated by HPLC-MS/MS. The analytical range of the was 0.50–50.00 ng/ml for lappaconitine and 0.50–100.00 ng/ml for N-desacetyllappaconitine in biological matrix. Method could be applied to determination of lappaconitine and N-desacetyllappaconitine for PK studies.

Keywords: lappaconitine, N-desacetyllappaconitine, plasma, blood, HPLC-MS/MS, validation, pharmacokinetics

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, Dana S. Shchelgacheva, Alexandra A. Aleshina have developed and validated an analytical method. Natalia S. Bagaeva carried out statistical processing of the obtained results. Anton V. Rogov and Igor E. Shohin 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 лаппаконитина, дитерпенового алкалоида, получаемого из растений *Aconitum leucostomum*, и его активного метаболита N-дезацетиллаппаконитина в плазме крови и в цельной крови человека

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Резюме

Введение. Лаппаконитин представляет собой алкалоид, содержащийся в корнях и надземной части аконита белоустого (*Aconitum leucostomum* Vorosh.) и обладающий антиаритмическим действием. После приема препаратов лаппаконитина в организме человека образуется 8 изученных фармакологически активных метаболитов, наибольшую активность среди которых проявляет N-дезацетиллаппаконитин. Фармакокинетика препаратов лаппаконитина изучена недостаточно. Препараты лаппаконитина имеют узкий терапевтический диапазон и обладают большим количеством побочных эффектов, поэтому для оценки безопасности применения препаратов лаппаконитина возникает необходимость полноценного изучения его фармакокинетики.

Цель. Целью исследования является разработка методики определения лаппаконитина и его активного метаболита N-дезацетиллаппаконитина в плазме крови и в цельной крови человека методом высокоэффективной жидкостной хроматографии с тандемным масс-селективным детектированием (ВЭЖХ-МС/МС).

Материалы и методы. Определение лаппаконитина и N-дезацетиллаппаконитина в плазме крови и в цельной крови человека проводили методом ВЭЖХ-МС/МС. В качестве пробоподготовки был использован способ осаждения ацетонитрилом.

Результаты и обсуждение. Разработанная методика определения лаппаконитина и N-дезацетиллаппаконитина в плазме крови и в цельной крови человека 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 лаппаконитина и N-дезацетиллаппаконитина в плазме крови и в цельной крови человека методом ВЭЖХ-МС/МС. Подтвержденные аналитические диапазоны методики составили 0,50–50,00 нг/мл в биологической матрице для лаппаконитина и 0,50–100,00 нг/мл в биологической матрице для N-дезацетиллаппаконитина. Полученные аналитические диапазоны позволяют применять разработанную методику для проведения фармакокинетических исследований препаратов лаппаконитина.

Ключевые слова: лаппаконитин, N-дезацетиллаппаконитин, плазма, кровь, ВЭЖХ-МС/МС, валидация, фармакокинетика

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

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

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INTRODUCTION

According to the WHO, cardiovascular diseases take the first position in the list of the leading mortality causes. Cardiac diseases have remained the leading mortality cause worldwide already for 20 years. Since 2000, the number of deaths due to cardiovascular diseases has increased for over 2 million and achieved almost 9 million in 2019 [1]. Cardiac rhythm disorders take not the last position in development of the medical conditions and their sequalae [2]. Cardiac arrhythmia characterized with irregular cardiac cycles affects over 33 million of people and is a serious burden on healthcare systems of many countries. Ventricular arrhythmia is the cause of about 80 % of sudden heart attacks [3].

One of the causes of developing arrhythmias is viral infections. There are over 20 viruses involved in myocardial inflammation, the most common are parvovirus B19, human herpes virus, adenovirus and Coxsackievirus B3. Myocardial inflammation induced by a viral infection leads to ion canal dysfunction or electrophysiological and structural remodeling as the mechanism of arrhythmia [4]. There is also the data indication that new coronavirus infection SARSCoV2 (Severe acute respiratory syndrome related to coronavirus 2) plays an important role in cardiovascular disease complications, in particular, arrhythmia [5].

Arrhythmias may lead to other cardiovascular diseases sometimes causing heart failure or sudden death. Severe ventricular tachycardia or fibrillation may be fatal, and it occurs when the heart cannot pump blood in a normal rate for effective cardiac output [6].

On the territory of the Russian Federation, lappaconitine hydrobromide is actively used in clinical practice for treatment of ventricular and supraventricilar extrasystole, paroxysmal supraventricular taxycardia including Wolf-Parkinson-White syndrome, palpitation atrial fibrillation, paroxysmal ventricular tachycardia in the absence of organic myocardial changes [7].

Lappaconitine is a C18-diterpene alkaloid [8] of *Ranunculaceae* family [9] contained in the roots and epiterranean part of *Aconitum leucostomum* and having anti-arrhythmic action [10]. Following administration

of lappaconitine products, 8 known pharmacologically active metabolites are formed in humans, however, N-desacetyl lappaconitine shows the greatest activity among them [7, 11]. Structural formulae of lappaconitine and N desacetyl lappaconitine are given on figures 1–2.

Therapy of heart arrhythmias with anti-arrhythmic drugs, in particular, lappaconitine products, is often accompanied with adverse events predominantly related to arrhythomogenic action. It may be due to large drug doses having a toxic action on human body, increase of its elimination rate, interaction with other medicinal products, liver and renal diseases, electrolyte balance disorders [12, 13]. As pharmacokinetics of lappaconitine products is not well-known, then its full investigation becomes challenging for provision of the drug safety [14].

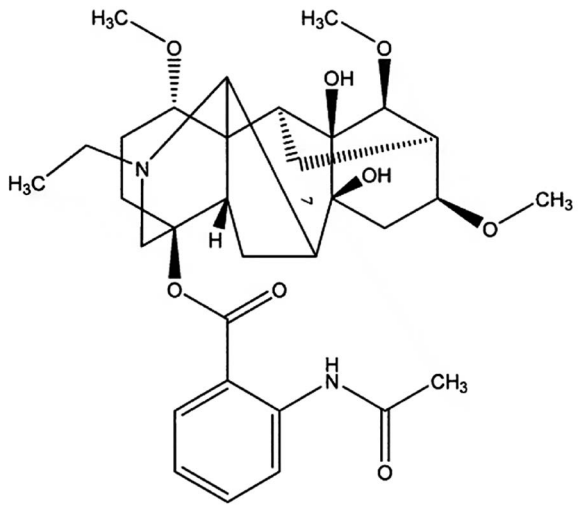

Figure 1. **Chemical structure of lappaconitine**

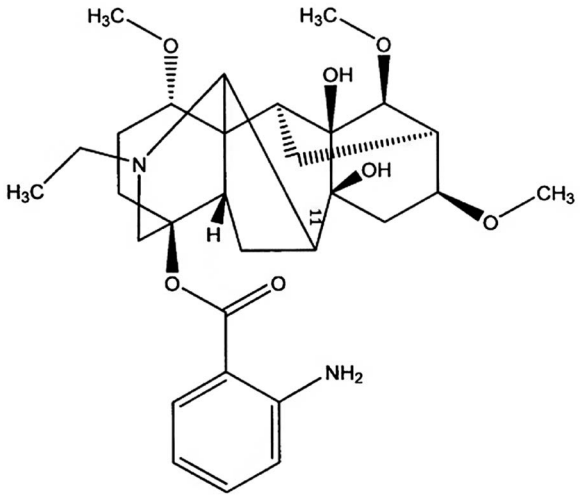

Figure 2. **Chemical structure of N-desacetyllappaconitine**

Nowadays, peer-reviewed journal provides only methods for determination of lappaconitine (LAP) in biological animal matrixes with the method of high performance liquid chromatography coupled with tandem mass selective detection (HPLCMS/MS) and sample preparation with acetonitrile precipitation and liquid-liquid extraction (LLE) (table 1).

As literature sources do not contain any data on determination of lappaconitine and N-desacetyl lappaconitine in human biological matrixes, it was decided to develop the method for determination of the substances in human blood plasma and whole blood with the method of HPLC MS/MS.

MATERIALS AND METHODS

Equipment

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

Reagents and solutions

The following reagents were used in the work: methanol (class UHPLC, J.T. Baker, Netherlands), acetonitrile (class LCMS grade, Biosolve, France), formic acid (class 98 % pure, PanReac, Spain), aqueous ammonium (class for analysis, PanReac, Spain), MilliQ water. For preparation of stock working solutions, reference samples of lappaconitine hydrobromide (assay 100.07 %, JSC "Pharmcenter VILAR", Russia), N-desacetyl lappaconitine (assay 99.37 %,

Table 1. **Methods of lappaconitine quantitative determination in biological matrix**

Analytical method	Object	Sample preparation	Analytical range, ng/ml	Reference
HPLC-MS/MS	Mouse blood	Protein precipitation by acetonitrile	Lappaconitine: 0.100–500.000	[15]
HPLC-MS/MS	Rabbit plasma	Liquid-liquid extraction	Lappaconitine: 13.125–1050.000	[16]

JSC "Pharmcenter VILAR" Russia) and trimebutine (assay 99.37 %, LGC, Great Britain) were used.

Stock reference solutions of lappaconitine, N-desacetyl lappaconitine (DAL) [N-desacetyl lappaconitine (DAL)] and internal standard (IS) of trimebutine (TRI) were prepared with dissolution of substance weighs in methanol. Mixed working reference solutions of lappaconitine and N-desacetyl lappaconitine were prepared with the dilution of stock solutions with the same solvent till the following concentrations were obtained in the biological matrix: 0.50 ng/ml [level LLOQ (lower limit of quantification)], 1.00 ng/ml, 3.00 ng/ml, 9.00 ng/ml, 15.00 ng/ml, 25.00 ng/ml, 35.00 ng/ml, 50.00 ng/ml, 1.50 ng/ml [level L (low)], 15.00 ng/ml [level M (medium)], 40 ng/ml [level H (high)] – for lappaconitine; and 0.50 ng/ml (level LLOQ), 1.00 ng/ml, 5.00 ng/ml, 10.00 ng/ml, 25.00 ng/ml, 50.00 ng/ml, 75.00 ng/ml, 100.00 ng/ml, 1.50 ng/ml (level L), 30.00 ng/ml (level M), 80 ng/ml (level H) – for N-desacetyl lappaconitine. IS working solution was prepared with dilution of IS stock solution with the same solvent up to concentration in the biological matrix 8.00 ng/ml.

Stock and working reference solutions, as well as intact matrix samples were kept in the refrigerator chamber at temperature –45 °C.

Sample preparation

To 200 µl of a calibration sample placed to Eppendorf centrifuge microvials 2 ml, 10 µl of working solution of trimebutine IS was added, then 400 µl of acetonitrile was added, mixed on vortex for 10 seconds, then centrifuged for 15 min with the rate 13 500 rot/min. Then the supernatant was transferred to chromatographic vials and transferred to the chromatograph autosampler.

Samples of chromatographic separation and detection

- Column: YMCPack Pro C18, 100 × 2,0 mm, 3 µm.
- Thermostate temperature: 40 °C.
- Mobile phase: eluent A: 0.1 % formic acid solution in water with the addition of 0.08 % of ammonium (by volume); eluent B: 0.1 % of formic acid in methanol with the addition of 0,08 % of ammonium (by volume).
- Gradient by composition of mobile phase (MP) is given in table 2.
- Injection volume: 6 µl.
- Run time of the mass-spectrometry detector: 0.00–7.00 min.
- Parameters of ionization source (electrospray): spraying gas 3 l/min, drying gas 20 l/min, heating unit 400 °C, desolvation unit 200 °C, capillary voltage +4.25 kV.
- Ionization mode: positive.
- Detection conditions of lappaconitine: 585.30 → 324.15 m/z; 585.30 → 162.15 m/z.
- Detection conditions of N-desacetyl lappaconitine: 544.25 → 120.00 m/z; 544.25 → 325.25 m/z; 543.25 → 324.10 m/z; 543.25 → 120.10 m/z.
- Detection conditions of trimebutine: 388.20 → 198.05 m/z; 388.20 → 195.10 m/z; 388.20 → 131.05 m/z.

Table 2. Gradient elution

Time, min	Eluent A, %	Eluent B, %	Mobil phase flow rate, ml/min
0.00	77.00	23.00	0.70
1.25	77.00	23.00	
4.00	0.00	100.00	
5.00	0.00	100.00	
5.50	77.00	23.00	
7.00	77.00	23.00	

RESULTS AND DISCUSSION

Method development

As while developing methods, we cannot fully rely exclusively on a high selectivity of the mass-spectrometric detector to avoid false positive results, the conditions for chromatographic separation of analytes not only with plasma components but also with whole blood components are selected which allows to use the method for pharmacokinetic studies of lappaconitine and N-desacetyl lappaconitine in various biological objects. Based on physical-chemical properties of the analyzed substance, chromatographic column YMCPack Pro C18 providing a good retention of the compounds was selected.

Method validation

The bioanalytical method was validated in human plasma and whole blood based on the Rules o bioequivalence studies of drugs within the Eurasian Economic Union [17], as well as the FDA [18] and EMA [19] guidance by the following parameters: selectivity, matrix effect, calibration curve, accuracy (intra-, intercycle levels),

precision (intra-, intercycle levels), recovery, lower limit of quantification, sample carry-over, stability [stability of stock and working reference solutions; short-term stability (bench and post-preparative); stability within three-fold analyte freezing-thawing; long-term stability of the analyte in the matrix].

Selectivity

6 samples of blank plasma (BP), 2 samples of hyperlipemic blank plasma (LBP), 2 samples of hemolyzed blank plasma (HBP) obtained from various sources were analyzed for evaluation of parameter "Selectivity" in human plasma, as well as 6 samples of blank blood (BB) obtained from various sources for evaluation of parameter "Selectivity" in human whole blood, and samples of blank plasma, hemolyzed blank plasma, hyperlipemic blank plasma and whole blood with the addition of the mixed working reference solution up to concentrations 0.50 ng/ml for lappaconitine, 0.50 ng/ml for N-desacetyl lappaconitine and IS solution up to concentration 8.00 ng/ml. On chromatograms due to blank plasma samples, peak signals with retention times corresponding to retention times of the test samples and IS do not exceed 20 % of the signal on the level of the lower limit of quantification (LLOQ) and 5 % of IS signal, respectively. Corresponding chromatograms are given below on figures 3, 4.

Matrix effect

To evaluate matrix effect, the samples with the addition of mixed working reference solutions of lappaconitine, N-desacetyl lappaconitine and solution of trimebutine IS not influenced by biological matrix were analyzed, as well as samples prepared on the blank matrix, without regards to recovery of lappaconitine, N-desacetyl lappaconitine and trimebutine from the biological matrix.

The matrix effect was evaluated on levels L и H. Matrix level for trimebutine IS was calculated on the level of 8.00 ng/ml. The data is given in tables 3, 4.

Calibration curve

Eight samples of blank plasma and eight samples of blank blood with the addition of IS working solution and mixed working solutions of the analytes were analyzed. Based on the obtained data, calibration plots were made in coordinates of ratio of peak area of lappaconitine to peak area of trimebutine from the ratio of lappaconitine concentration to trimebutine concentration, as well as calibration plots in coordinates ratio of peak area of

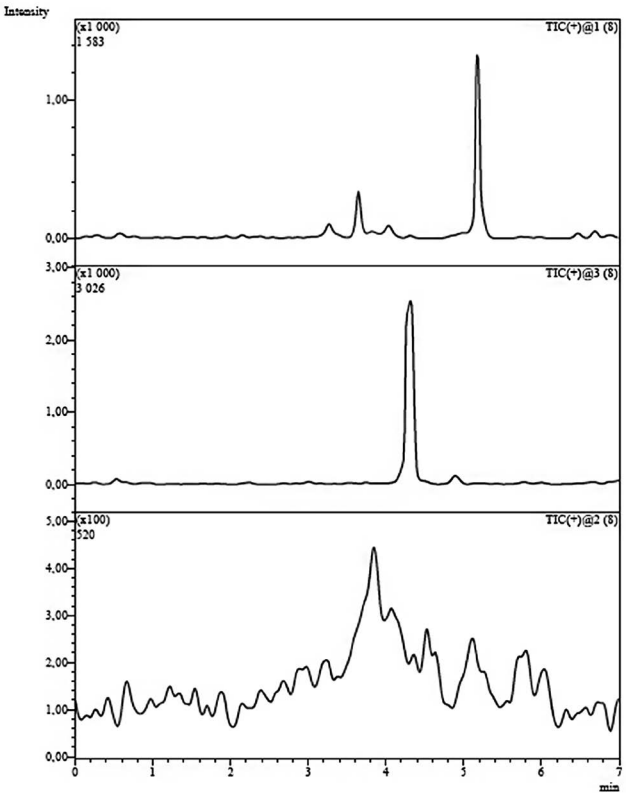

Figure 3. **Blank plasma sample chromatogram**

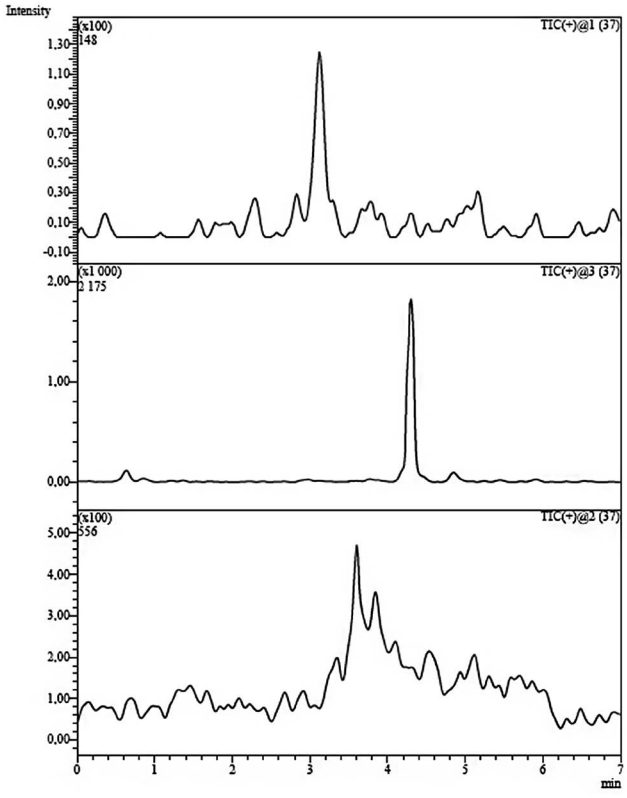

Figure 4. **Blank blood sample chromatogram**

N-desacetyl lappaconitine to the peak area of trimebutine from ratio of N-desacetyl lappaconitine concentration to trimebutine concentration given on figures 5–8. The obtained correlation coefficients correspond to the normal. The examples of chromatograms are given on figures 9–12.

Table 3. The matrix factor of LAP calculations, normalized by the internal standards matrix factor

	Plasma		Blood	
	Normalized Mf (L)	Normalized Mf (H)	Normalized Mf (L)	Normalized Mf (H)
Average	0.93	1.16	1.14	1.15
CV, %	11.87	3.53	10.93	3.37

Table 4. The matrix factor of DAL calculations, normalized by the internal standards matrix factor

	Plasma		Blood	
	Normalized Mf (L)	Normalized Mf (H)	Normalized Mf (L)	Normalized Mf (H)
Average	0.84	1.09	0.98	1.12
CV, %	6.94	3.93	9.68	4.54

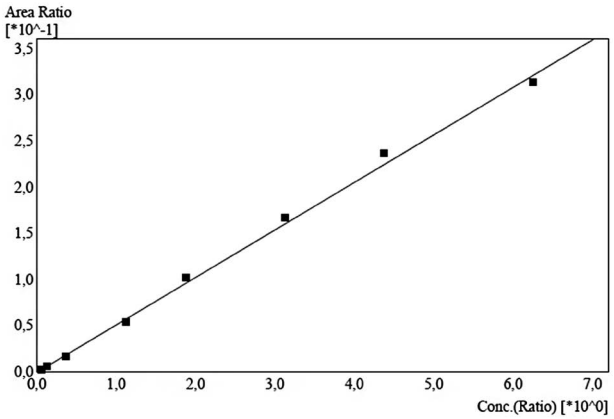

Figure 5. The calibration curve representing dependence of the ratio area peak of LAP to the TRI on the concentration ratio of LAP to the TRI in plasma

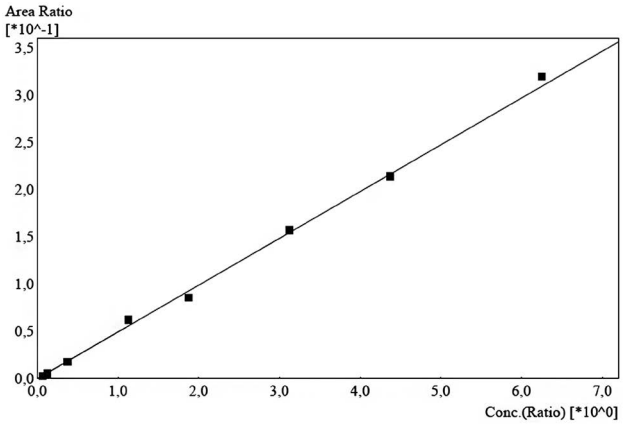

Figure 6. The calibration curve representing dependence of the ratio area peak of LAP to the TRI on the concentration ratio of LAP to the TRI in blood

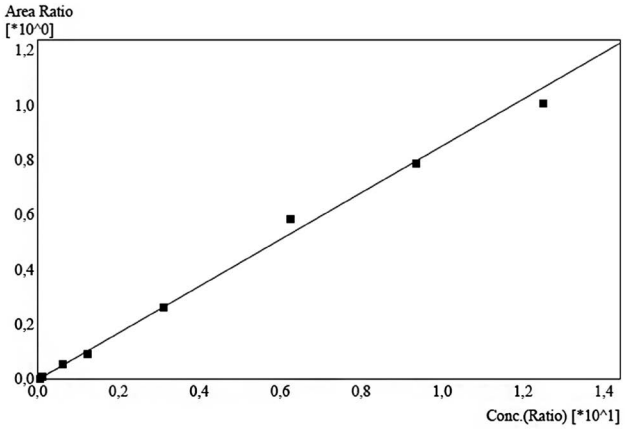

Figure 7. The calibration curve representing dependence of the ratio area peak of DAL to the TRI on the concentration ratio of DAL to the TRI in plasma

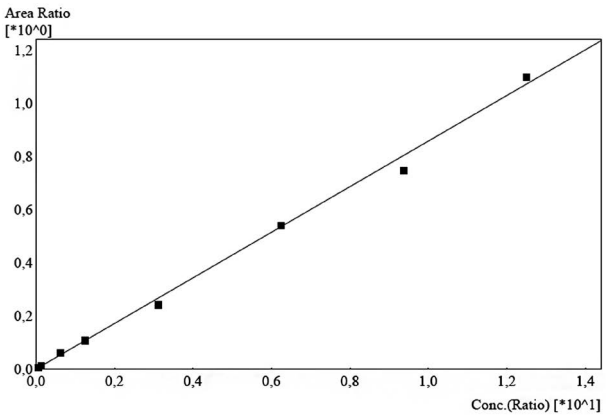

Figure 8. The calibration curve representing dependence of the ratio area peak of DAL to the TRI on the concentration ratio of DAL to the TRI in blood

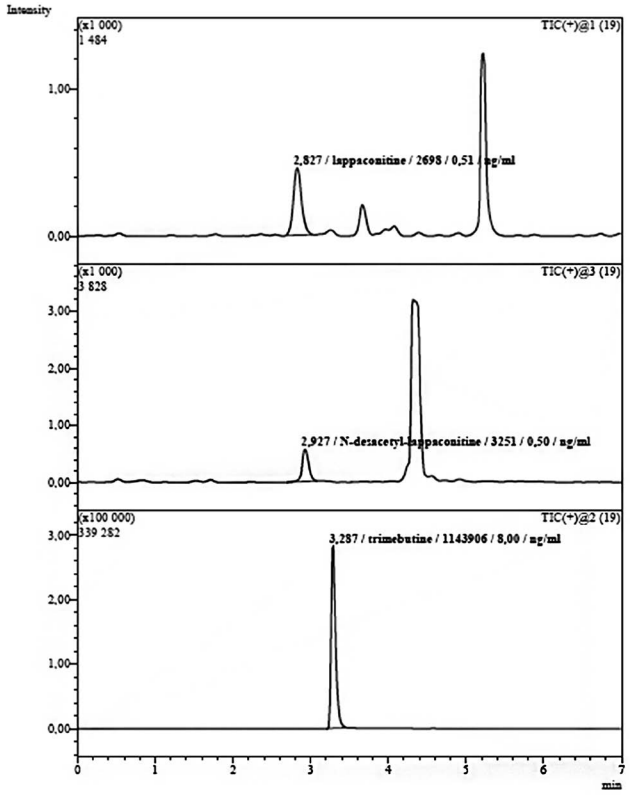

Figure 9. **Chromatogram of plasma sample (LAP – 0.50 ng/ml, DAL – 0.50 ng/ml)**

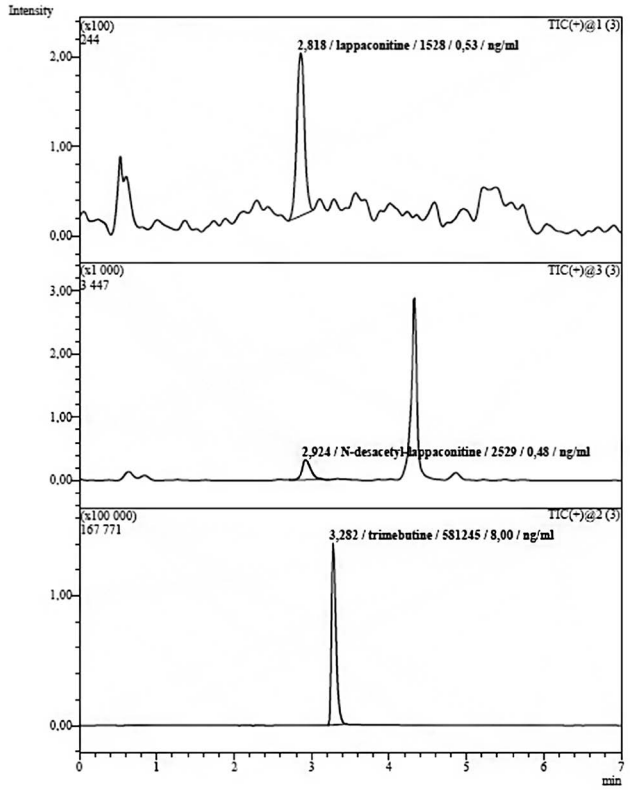

Figure 10. **Chromatogram of blood sample (LAP – 0.50 ng/ml, DAL – 0.50 ng/ml)**

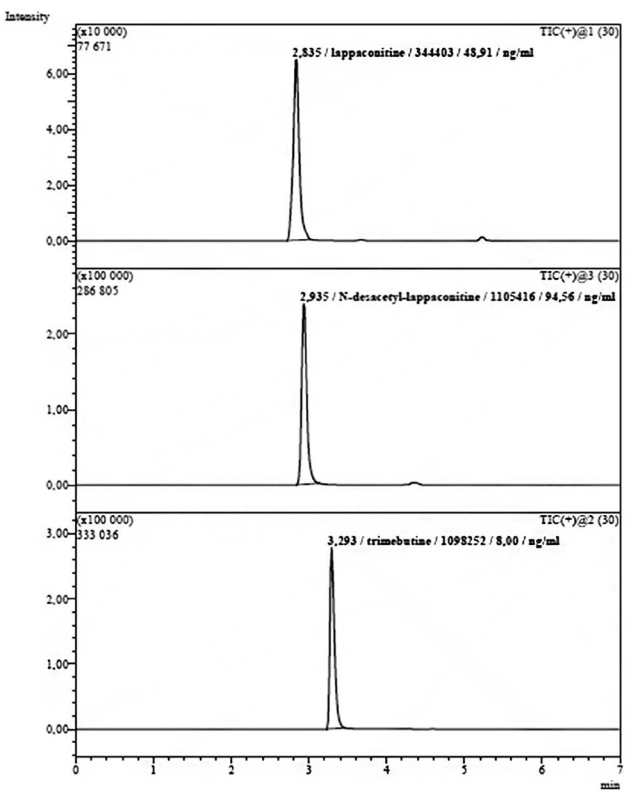

Figure 11. **Chromatogram of plasma sample (LAP – 50.00 ng/ml, DAL – 100.00 ng/ml)**

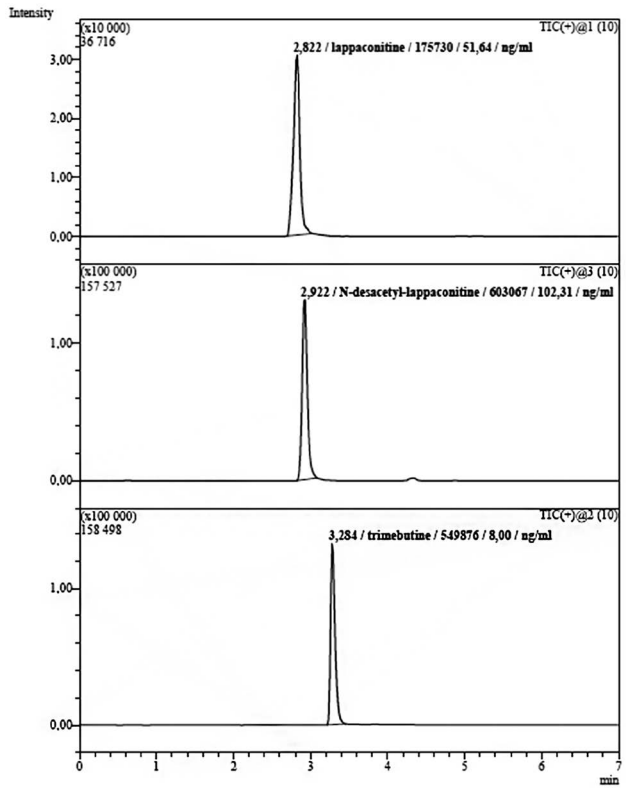

Figure 12. **Chromatogram of blood sample (LAP – 50.00 ng/ml, DAL – 100.00 ng/ml)**

Accuracy and precision

Calibration samples of plasma and whole blood corresponding to the level of LLOQ, level L, level M and level H were analyzed. The samples were analyzed within 3 sequences of 5 samples for each level. Accuracy and precision were evaluated in the cycle, between two and three cycles. The obtained values correspond to the normal in relation to standard deviation (precision) and relative error (accuracy).

Recovery

To evaluate recovery (R), 3 samples prepared from blank plasma, hemolyzed plasma, hyperlipemic blank plasma and whole blood not influenced by the recovery on the low (level L), medium (level M) and high (level H) levels were analyzed, as well as samples for quality control prepared on various blank matrixes for evaluation of recovery. The data is given in tables 5, 6.

Table 5. Calculation of LAP recovery at L, M, H levels for biological matrix

Biological matrix	Recovery LAP (L), %	Recovery LAP (M), %	Recovery LAP (H), %
BP	123.03	76.35	91.22
	120.71	79.49	91.42
	123.19	85.89	84.62
HBP	130.29	95.25	120.72
	146.42	101.47	122.88
	128.52	103.06	122.88
LBP	89.53	104.27	103.59
	99.30	101.86	99.76
	118.40	103.79	98.08
Average	119.93	94.60	103.91
SD	16.79	11.11	14.76
RSD	14.00	11.74	14.20
Biological matrix	Recovery LAP (L), %	Recovery LAP (M), %	Recovery LAP (H), %
BB	90.04	122.95	99.60
	97.04	127.90	102.09
	99.78	117.41	105.74
Average	95.62	122.76	102.47
SD	5.02	5.25	3.09
RSD	5.25	4.27	3.01

Table 6. Calculation of DAL recovery at L, M, H levels for biological matrix

Biological matrix	Recovery DAL (L), %	Recovery DAL (M), %	Recovery DAL (H), %
BP	124.21	86.25	101.95
	112.16	85.79	98.39
	118.16	86.13	92.13
HBP	105.63	100.71	118.42
	115.79	98.34	129.24
	130.56	105.97	122.35
LBP	97.15	104.33	105.19
	102.72	105.53	106.13
	116.17	109.69	97.97
Average	113.62	98.08	107.97
SD	10.53	9.57	12.54
RSD	9.27	9.75	11.61
Biological matrix	Recovery DAL (L), %	Recovery DAL (M), %	Recovery DAL (H), %
BB	115.63	132.83	97.71
	138.65	123.24	103.27
	105.83	118.90	109.98
Average	120.04	124.99	103.65
SD	16.85	7.13	6.14
RSD	14.04	5.71	5.93

Stability

The short-term stability (bench and post-preparative), stability in 3-fold freezing-thawing, stability of reference and working reference solutions (when kept for 20 days at temperature –45 °C), long-term stability (when kept for 60 days at temperature –45 °C) of the test substances were confirmed on the low and upper concentration levels.

Sample carry-over

The successive analysis of the calibration sample with the greatest concentration and blank matrix sample in the chromatogram due to a blank matrix sample did not show any peaks with retention times corresponding to peaks of test substances and IS.

CONCLUSION

The method for determination of lappaconitine and N-desacetyl lappaconitine in human plasma and whole blood with HPLC MS/MS was developed and validated. The confirmed analytical ranges of the method were 0.50–50.00 ng/ml in the biological matrix for lappaconitine and 0.50–100.00 ng/ml in the biological matrix for N-desacetyl lappaconitine. The obtained analytical ranges allow to use the developed method for pharmacokinetic studies of lappaconitine products.

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