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The identification and quantitative determination of amitriptyline by the HPLC-MWD method

Aim. To develop the sensitive and specific HPLC-MWD method for the determination of amitriptyline suitable for use in bioanalytical studies in clinical and forensic toxicology.

Materials and methods. The analysis was performed on a "MiLiChrome A-02" microcolumn liquid chromatograph attached to a dual-beam multi-wave UV spectrophotometer under the following conditions: 2×75 mm column ProntoSIL 120-5-C_{18} AQ, 5 μm; the gradient elution mode with a linear gradient, eluent A (5 % acetonitrile and 95 % perchlorate buffer) to eluent B (100 % acetonitrile) for 40 min, then 100 % eluent B for 3 min; the mobile phase flow rate – 100 μL/min; the thermostat temperature – 40°C; detection was carried out at 8 wavelengths (210, 220, 230, 240, 250, 260, 280, 300 nm).

Results. The retention time of amitriptyline was 22.80 min; the absorbance ratios S_λ/S_{210} were also set as additional option to increase the reliability of identification. The calibration curve was represented by the dependence of a peak area (*S*, mm^2) against concentration (*c*, μg/μL) and described by the regression equation $y = 0.00514 \times x$. The method showed linearity in the range of 1.0–100.0 μg/mL. The correlation coefficient was lower than 0.99. The LOD and LOQ values were calculated from the parameters of the calibration curve, they were 0.3 μg/mlL and 0.9 μg/mL, respectively. The accuracy and precision of the method developed were determined at three concentration levels (low, middle, high) within one day (*intraday*); *RSD* did not exceed 1.0 % across the full concentration range studied when analyzing model solutions.

Conclusions. The sensitive and specific method for the quantitative determination of amitriptyline using the HPLC-MWD method has been developed. The method meets the requirements set to techniques recommended for use in clinical and forensic toxicology, and it has been confirmed by a number of the validation parameters. The HPLC-MWD method developed can be recommended for implementation into the practice of regional bureaus of forensic medical examination and clinical and diagnostic laboratories of regional drug addiction centers.

Key words: *amitriptyline; high performance liquid chromatography-multi-wavelength detector; identification; quantitative determination.*

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Ідентифікація та кількісне визначення амітриптиліну методом ВЕРХ-MWD

Мета – розроблення чутливої та специфічної методики ВЕРХ-MWD визначення амітриптиліну, придатної для застосування в біоаналітичних дослідженнях у клінічній та судово-медичній токсикології.

Матеріали та методи. Аналіз проводили на мікроколонковому рідинному хроматографі «MiLiChrome A-02», з'єднаному з двопроменевим багатохвильовим УФ-спектрофотометром, за таких умов: колонка 2×75 мм ProntoSIL 120-5-C18 AQ, 5 мкм; градієнтний режим елюювання з лінійним градієнтом, елюент А (5 % ацетонітрилу та 95 % перхлоратного буферу) до елюенту В (100 % ацетонітрилу) протягом 40 хв, потім 100 % елюент В протягом 3 хв; швидкість потоку рухомої фази – 100 мкл/хв; температура термостату – 40°С; детектування проводилося на 8 довжинах хвиль (210, 220, 230, 240, 250, 260, 280, 300 нм).

Результати та їхнє обговорення. Час утримування амітриптиліну становив 22,80 хв; також було визначено спектральні відношення S_λ/S_{210} як додатковий параметр для підвищення надійності ідентифікації. Калібрувальний графік був представлений залежністю площі піка (S, мм^2) від концентрації (*c*, мкг/мкл) та описувався рівнянням регресії $y = 0{,}00514 \cdot x$. Діапазон лінійності становив 1,0–100,0 мкг/мл. Коефіцієнт кореляції був нижчим за 0,99. Значення LOD та LOQ були розраховані за параметрами калібрувального графіка, вони становили 0,3 та 0,9 мкг/мл відповідно. Точність та прецизійність розробленої методики було визначено на трьох концентраційних рівнях (низькому, середньому, високому) протягом одного дня (*intraday*); RSD не перевищував 1,0 % у всьому досліджуваному діапазоні концентрацій під час аналізу модельних розчинів.

Висновки. Розроблено чутливу та специфічну методику кількісного визначення амітриптиліну за методом ВЕРХ-MWD. Методика відповідає вимогам, установленим до методів, рекомендованим для використання в клінічній та судово-медичній токсикології, що було підтверджено низкою валідаційних параметрів. Розроблена методика ВЕРХ-MWD може бути рекомендована для упровадження в практику обласних бюро судово-медичної експертизи та клініко-діагностичних лабораторій обласних наркологічних диспансерів.

Ключові слова: *амітриптилін; високоефективна рідинна хроматографія з мультихвильовим детектором; ідентифікація; кількісне визначення.*

Introduction. Tricyclic antidepressants (TCAs), especially amitriptyline, are among the most frequent drugs involved in intoxications. The amitriptyline ingestion was found to be an important cause of poisoning, morbidity and mortality in many countries over the world [1, 2]. Thus, TCAs are now considered second-line options after selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs) [3]. However, amitriptyline is still commonly used to treat major depressive disorder (MDD) and a number of somatic diseases even in cases not approved by the US Food and Drug Administration (FDA) [4, 5] due to its highest efficiency out of 21 antidepressants; hence, it is superior to SSRIs and SNRIs [6].

Amitriptyline has been reported to cause various adverse effects, particularly anticholinergic drug reactions [7], hepatotoxicity [8], and unusual serious complications, such as irreversible central nervous system disability and lethal arrhythmia [9]. Over the past decade, many cases of acute and fatal amitriptyline intoxication have been reported [1–3, 10–12]. Amitriptyline was also misused, resulting in severe acute poisoning [9]. It was reported that about 20 tablets of amitriptyline (25 mg) caused acute toxicity [3]. The amitriptyline toxic levels in the blood were 186 μg/L (case 1) and 844 μg/L (case 2) [11].

In recent years, a number of modern instrumental methods for the detection and quantitative determination of amitriptyline have been developed for bioanalytical testing. They are as follows: liquid chromatography-tandem mass spectrometry (LC-MS/MS) [10, 13] ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [14], high-performance liquid chromatography-ultraviolet detection (HPLC-UV) [15], gas-chromatography-mass spectrometry [16], differential pulse voltammetry at novel carbon/cellulose nanomaterial [17].

The increased flexibility of HPLC-system can be provided when it is attached to multi-wavelength detector (MWD). It is a sensitive instrument which is enable to monitor simultaneously multiple wavelengths (e.g., 2 to 8 or more) in UV/Visible light (190–950 nm). HPLC-MWD provides superior selectivity and sensitivity compared to single-wavelength detectors by capturing full spectral information for better peak identification and the quantitative assessment of trace target analyte.

The aim of this study was to develop the sensitive and specific HPLC-MWD method for the determination of amitriptyline suitable for use in bioanalytical studies in clinical and forensic toxicology.

Materials and methods. Amitriptyline hydrochloride was extracted from commercially available tablets *Amitriptyline* (25 mg) ("Experimental Plant DNCLZ", Ukraine) as follows. 25 Tablets were weighted, placed into a porcelain mortar and triturated, 50 mL of chloroform was added to the tablet mass, and the resulting mixture was filtered into a porcelain cup. The organic solvent was evaporated on a water bath at a temperature not higher than 40°C to dry a residue. The residue on the filter was dried and weighed. The quality was assessed in accordance with the requirements of the pharmacopoeial monograph [18, P. 352–353]. A standard methanol solution of amitriptyline hydrochloride with the concentration of 1.0 mg/mL was obtained from the Kharkiv Regional Bureaus of Forensic Medical Examination.

Acetonitrile (Sigma-Aldrich Laborchemikallen, GmbH), methanol (Merk, Germany), and double-distilled water (Merk, Germany) were of the HPLC grade, chloroform was of analytical grade, lithium perchlorate trihydrate and acid perchloric 70 % were purchased from Sigma-Aldrich (USA). The following measuring tools were used: 10 mL, 25.00 mL, 50.00 mL volumetric flasks, volumetric pipettes, Class A (Simax, Czech Republic).

A "MiLiChrome A-02" high pressure liquid chromatograph was equipped with a double syringe gradient pumping system, an autosampler, a column oven, a multiwave UV-detector. 2×75 mm column filled with a reversed-phase sorbent Bischoff ProntoSIL 120-5-C18 A Q 5 μm (Bischoff, Analysentechnik und Geräte GmbH, Germany). The gradient elution mode was used with a linear gradient: eluent A (5 % acetonitrile and 95 % perchlorate buffer) to eluent B (100 % acetonitrile) for 40 min, then 100 % eluent B for 3 min. The column was regenerated by the mixture of 2 % acetonitrile and 98 % perchlorate buffer for 2 min. To prepare the perchlorate buffer, 0.2 M lithium perchlorate solution in 0.005 M perchloric acid was 25 times diluted by adding 0.005 M perchloric acid to pH 3 solution, which was determined potentiometrically. The flow rate of the mobile phase was maintained at 100 μL/min; the thermostat temperature was set at 40 °C. During the identification and quantitative determination, the injected volume was 1 μL and 10 μL, respectively.

The detection was carried out at 8 wavelengths (210, 220, 230, 240, 250, 260, 280, 300 nm) by means of a dual-beam multi-wave UV spectrophotometer in the wavelength range of 190–360 nm with an accuracy of 0.5 nm. The data acquisition and integration were performed using the "MultiChrom" software (Ampersand LTD).

To optimize the conditions of the HPLC analysis, a standard methanol solution of amitriptyline with the concentration of 0.75 mg/mL was prepared.

Construction of the calibration curve. The *Stock solution (SS)* with the concentration of amitriptyline hydrochloride of 100 μg/mL was prepared as follows: 0.00565 g of amitriptyline hydrochloride was dissolved in 50.00 mL of methanol using a 50.00 mL-volumetric flask. To prepare calibration standards, a range of *SS* aliquots of 0.10; 0.30; 0.50; 1.00; 3.00; 4.00; 6.00 and 8.00 mL were placed into 10 mL volumetric flasks and diluted with methanol to the appropriate volume. The resulting concentrations of the calibration standards were of 1.0; 3.0; 5.0; 10.0; 30.0; 40.0; 60.0 and 80.0 μg/mL. Using an autosampler, 10 μL aliquots of *SS* and 8 calibration standards were injected, the chromatographic analysis was performed under the optimized conditions given above. The detection was performed at a wavelength of 240 nm, which corresponded to the maximum absorbance of amitriptyline in the UV region. A peak area was used as a quantitative parameter. The data obtained were processed by

the linear regression model described in the general form as $y = b \times x + a$.

Results and discussion. Amitriptyline exhibited sharp and symmetrical peaks under the chromatographic conditions developed (Fig. 1). The symmetry coefficient of the peaks *(Ks)* did not exceed the optimal values of 0.8–1.5, and the number of theoretical plates (*N*) were not less than 2,000–10,000 (Table 1). These criteria confirmed that the chromatographic system was suitable for the HPLC analysis and the method could produce reliable results [18].

The retention time, retention volume of amitriptyline and the absorbance ratios S_λ/S_{210} were determined for the purpose of the qualitative analysis (Table 1, Table 2). Combining the retention parameters and the spectral characteristics provides more reliable identification, and makes available to perform identification even in the case when retention parameters coincide.

The absolute calibration method was developed for the quantitative determination of amitriptyline. The method was validated by a range of parameters, namely linearity region, limit of detection (LOD), limit of the

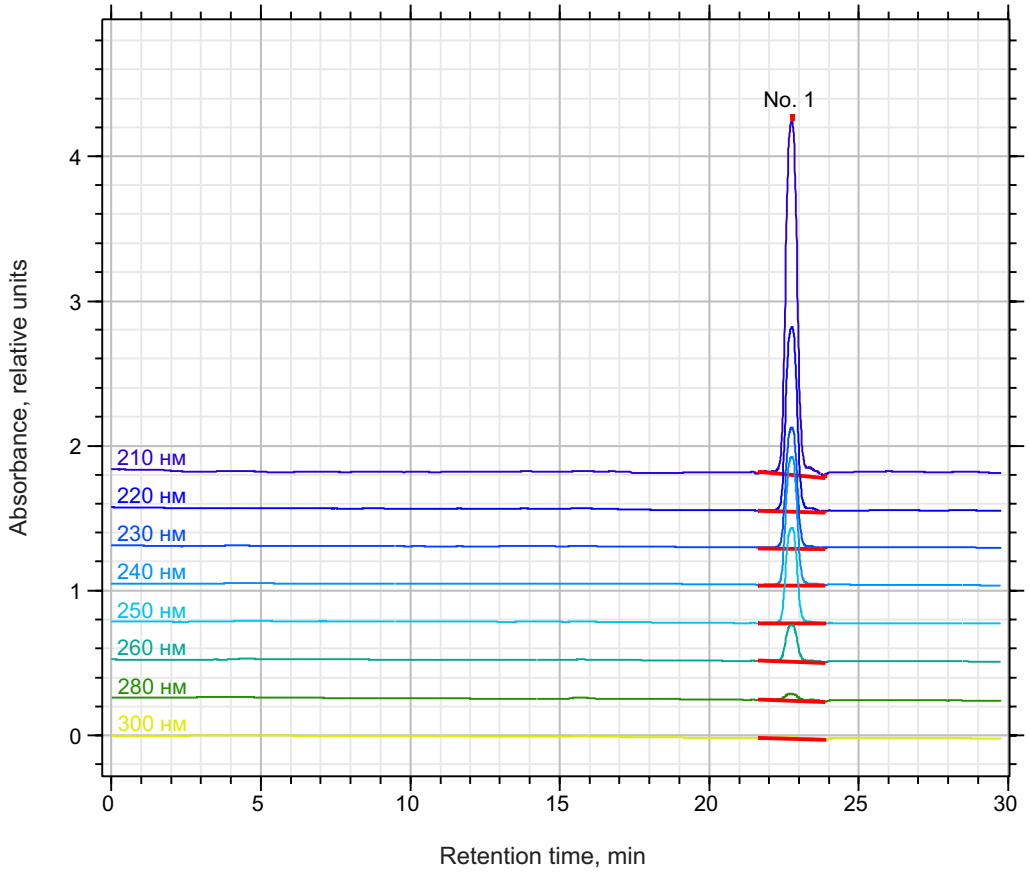

Fig. 1. The chromatogram of the amitriptyline methanol solution (*c* = 0.75 mg/mL)

Table 1

Retention parameters of amitriptyline (*n* = 5; *P* = 0.95 %)

Parameter	Parameter range	Metrological characteristics					
		$\bar{X}$	*S*	$S_{\bar{X}}$	$\Delta\bar{X}$	RSD, %	ε, %
t_R, min	22.80 ± 0.02	22.80	0.007	0.004	0.02	0.03	0.09
V_R, µL	2.280 ± 2	2.280	0.70	0.404	2	0.03	0.09
The symmetry factor, *Ks*	0.74 ± 0.01	0.74	0.0144	0.0065	0.01	1.95	1.87
The number of theoretical plates, *N*	18.288 ± 181	18.288	208.2	84.99	181	1.1	1.0

Table 2

The absorbance ratios (S_λ/S_{210}) of amitriptyline (*n* = 5; *P* = 0.95 %)

	S_{220}/S_{210}	S_{230}/S_{210}	S_{240}/S_{210}	S_{250}/S_{210}	S_{260}/S_{210}	S_{280}/S_{210}	S_{300}/S_{210}
$\bar{X} \pm \Delta\bar{X}$	0.521 ± 0.009	0.339 ± 0.009	0.358 ± 0.001	0.268 ± 0.004	0.105 ± 0.015	0.02 ± 0.01	0.011 ± 0.009
RSD, %	0.77	1.18	0.16	0.75	5.71	18.52	27.3
ε, %	1.65	2.54	0.36	1.61	14.75	47.5	78.2

Table 3

The parameters of the calibration curve $y = b' \times x$ of the quantitative determination of amitriptyline by the HPLC method ($n = 5$; $P = 0.95$ %)

Regression coefficient (b')	Dispersion (S_o^2)	Standard deviations of y-intercept S_a	Confidence interval of the regression coefficient ($\Delta b'$)	Correlation coefficient (r)	Linearity range, μg/μL
0.00514	9.5×10^{-7}	4.3×10^{-4}	2×10^{-5}	0.999	1.0–100.0

Table 4

Accuracy and precision of the method of the quantitative determination of amitriptyline in the model solutions by the HPLC ($n = 5$; $P = 0.95$ %)

Concentration of the solution, μg/μL	Metrological characteristics					
	$\bar{X}$	S	$S_{\bar{X}}$	$\Delta\bar{X}$	RSD, %	ε, %
3.0	102	0.9	0.4	1	0.9	1.1
50.0	98	0.9	0.4	1	0.9	1.1
100.0	100	1.0	0.4	1	1.0	1.1

quantitative determination (LOQ), accuracy and precision. The calibration curve was represented by the dependance of a peak area (S, mm^2) against concentration (C, μg/μL) and described by the regression equation $y = 0.00514 \times x$. The significance of the regression coefficient in the regression model was checked using the F-test. The conclusion was drawn that it was impossible to have the equation in the form of $y = b' \times x$. The key parameters of the calibration curve are given in Table 3.

The method showed linearity in the range of 1.0–100.0 μg/mL. The correlation coefficient was lower than 0.99, meeting the requirements for the bioanalytical methods used in forensic toxicology [19].

The LOD and LOQ values were calculated from the parameters of the calibration curve according to the equations: $LOD = 3.3 \times S_a/b$ and $LOQ = 10 \times S_a/b$ where S_a was the standard deviation of the y-intercept, b was the slope [18]. They were 0.3 μg/mlL and 0.9 μg/mL, respectively. These results showed that the method developed provided the reliable detection and the quantitative determination of toxic and lethal concentrations of amitriptyline in the biological matrixes [11].

The accuracy and precision of the method developed were determined across three concentration ranges (low, middle, high) within one day (*intraday*) (Table 4).

The number of replicates per a concentration range was three. As can be seen from Table 4, the precision (RSD, %) did not exceed 20 %, which met the requirements for the validation of analytical methods in forensic toxicology [19].

Conclusions and prospects for further research

1. The sensitive and specific method for the quantitative determination of amitriptyline using the HPLC-MWD method has been developed.

2. It has been shown that the method developed meets the requirements set to techniques recommended for use in clinical and forensic toxicology. This has been confirmed by a number of the validation characteristics, such as linearity region, limit of detection (LOD), limit of the quantitative determination (LOQ), *intraday* accuracy and *intraday* precision.

3. Further research on combining the analytical method developed with adequate methods for preparing samples for various biological matrices is of practical interest, which will allow this HPLC-MWD method to be implemented into the practice of regional bureaus of forensic medical examination and clinical and diagnostic laboratories of regional drug addiction centers.

Conflict of interests: authors have no conflict of interest to declare.

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