Determination of clemastine by the HPLC method in the blood

Topicality. Clemastine fumarate (tavegil)-1-methyl-2-[2-α-methyl-p-chlorobenzhydryloxy]-ethyl-pyrrolidine fumarate is the first generation H1-histamine receptor blocker. Clemastine fumarate selectively inhibits histamine H1 receptors and reduces capillary permeability. The drug has a pronounced anti-allergic and antipruritic effect. Clemastine prevents the development of vasodilation and the smooth muscle contraction induced by histamine. Clemastine fumarate has an insignificant anticholinergic activity, causes sedation. The drug is used to treat pruritus in psoriasis, multiple sclerosis and optic neuritis. Clemastine is characterized by the following side effects: increased fatigue, drowsiness, sedation, weakness, lethargy, impaired coordination of movements; nausea, vomiting, decreased blood pressure, palpitations, hemo-lytic anemia, skin rash, anaphylactic shock. In case of an overdose, the drug has a neurotoxic effect, which manifests itself in impaired consciousness with the development of generalized anticholinergic convulsive syndrome. The urgent task for monitoring the treatment effectiveness of the population with clemastine fumarate and diagnosis of drug intoxication is the choice of highly sensitive and selective research methods of its analysis in pharmaceuticals and biological matrices during the treatment.

Aim. To develop an algorithm for directed analysis of clemastine in biological extracts from the blood using a unified method of the HPLC research.

Materials and methods. The extraction of clemastine was performed with chloroform at pH 9.0. The extracts were purified from impurities by a combination of TLC and extraction with hexane. The TLC purification and identification of clemastine were carried out under optimal conditions: the system of organic solvents - methanol - 25 % solution of ammonium hydroxide (100 : 1.5) and chromatographic plates - Sorbfil PTLC-AF-A, Rf сlemastine = 0.60 ± 0.03. To detect clemastine, the most sensitive location reagents were used - UV light (λ = 254 nm) and Dragendorff’s reagent modified by Mounier. The chromatographic analysis was performed on a “Mîlîchrome A-02” microcolumn liquid chromatograph (EkoNova, Closed Joint-Stock Company, Russia) under standardized HPLC conditions: the reversed-phase variant using a metal column with a non-polar absorbent Prontosil 120-SC 18 AQ, 5 μm; the mobile phase in the linear gradient mode - from eluent A (5 % acetonitrile and 95 % buffer solution - 0.2 M solution of lithium perchlorate in 0.005 M solution of perchloric acid) to eluent B (100 % acetonitrile) for 40 min. Regeneration of the column was conducted for 2 min with the mixture of solvents; the flow rate of the mobile phase was 100 μl/min, the injection volume - 4 μl. The multichannel detection of the substance was performed using a two-beam multi-wave UV spectrophotometer at 8 wavelengths of 210, 220, 230, 240, 250, 260, 280, and 300 nm; the optimal value of the column temperature - 37-40 °C and the pump pressure - 2.6-3.2 MPa.

Results and discussion. Isolation of clemastine from the blood was performed according to the method developed, including the extraction with chloroform at pH 9.0; the extraction purification of extracts with hexane from impurities; the TLC purification and identification of clemastine. Using the unified HPLC method clemastine was identified by retention parameters and spectral ratios. For the quantitative determination, a calibration graph or the straight line equation corresponding to this graph were used. The results obtained indicated the reliability and reproducibility of the method. It was found that the relative uncertainty of the average result in the analysis of clemastine in the blood was ε = ±4.63 %, the relative standard deviation of the average result was RSDε = 1.67 %.

Conclusions. Clemastine was extracted with chloroform at pH 9.0 from the blood. Purification of extracts from co-extractive compounds was performed by combining TLC and extraction with hexane. It has been found that when isolating clemastine from the blood according to the methods developed it is possible to determine 36.05-39.55 % of the substance (ε = ±4.63 %, RSDε = 1.67 %). The method of TLC purification and identification of clemastine in biogenic extracts was tested under the optimal conditions: the system of organic solvents - methanol - 25 % solution of ammonium hydroxide (100 : 1.5), the use of reagents - UV light, Dragendorff’s reagent modified by Mounier, Rf сlemastine = 0.60 ± 0.03 (Sorbfil PTLC-AF-A). The unified HPLC method for identification and quantification of clemastine was tested in biogenic extracts from the blood according to the algorithm of the directed analysis developed. It has been found that clemastine can be identified by the retention time - 25.997-26.011 min; the retention volume - 2599.7-2601.1 μl; spectral ratios - 0.741; 0.536; 0.096; 0.023; 0.027; 0.005; 0.003. The clemastine content was determined by the equation $S = 0.15 \cdot 10^{-3} \cdot C + 0.14 \cdot 10^{-3}$; the correlation coefficient was equal to 0.9998. Chromatographic methods can be recommended for implementation in practice of the Bureau of Forensic Medical Examination, poison control centers, clinical laboratories regarding the study of medicinal substances in biological objects.

Key words: clemastine fumarate (tavegil); extraction with chloroform; purification of extracts from impurities by the TLC method and extraction with hexane; identification and quantitative determination by HPLC; blood.
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Визначення клемастину ВЕРХ-методом у крові.

Актуальність. Клемастина фумарат (тавегіл) – і-метил-2 [2-а-метил-п-хлорбензгідрокси) етил] пріоро-дину фумарат є блокатором H1-гістамінових рецепторів першого покоління. Клемастина фумарат вибірково інгібуват гістамінові H1-рецептори та зменшує проникність капілярів. Препарат має вирівнювательну активність та проти-свербіжну дію. Клемастина збільшує розвиток вазодилатації та скорочення гладких м'язів, викликаних гістаміном. Клемастина фумарат має незначну антихолінергічну активність, викликає седацію. Препарат застосовують для лікування алергічних реакцій, розширювання капілярів та трофічних якостей.

Мета дослідження. Розробка алгоритму спрямованого аналізу клемастину в біологічних екстрактах крові за допомогою уніфікованого методу дослідження ВЕРХ.

Матеріали та методи. Використані методи включають ідентифікацію та ідентифікацію клемастину в біологічних екстрактах крові з допомогою уніфікованого методу дослідження ВЕРХ.

Висновки. Метод ВЕРХ може бути рекомендован для використання у практиці бюро судово-медичної експертизи, центрів контролю за отруєннями, клінічних лабораторій. Він є надійним і точним методом для визначення клемастину.

Ключові слова: клемастина фумарат, тавегіл, екстракція, уніфікований метод ВЕРХ.

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Целью исследования является разработка алгоритма направленного анализа клемастина в биологических экстрактах крови с помощью унифицированного метода исследования ВЭЖХ.

Материалы и методы. Экстракцию клемастина проводили хлороформом при рН 9,0. Экстракты очищали от примесей комбинацией TСХ и экстракцией гексаном. Очистка TСХ и идентификация клемастина проводили в оптимальных условиях: система органических растворителей метанол – 25 % раствор гидроксида аммония (100 : 1,5) и хроматографические пластинки Sorbfil PTLC-AF-A, \( Rf_{\text{kлемастина}} = 0,60 \pm 0,03 \). Для обнаружения клемастина использовали наиболее чувствительные реагенты – УФ-спектр (\( \lambda = 254 \) нм) и реагент Драгендорфа в модификации Мунье. Хроматографический анализ проводили на микропланочном жидкостном хроматографе «Mİluchromе A-02» (ЭкоНова, ЗАО, Россия) с применением унифицированных условий ВЭЖХ: в асвинг с обращенной фазой с использованием металлической колонки с неполярным сорбентом Prontosil 120-5С 18 АQ, 5 мм; подвижная фаза в режиме линейного градиента – от элюента А (5 % ацетонитрила и 95 % буферного раствора – 0,2 M раствора перхлората натрия) до элюента В (100 % ацетонитрила) в течение 40 мин. Регенерацию колонки проводили в течение 2 мин смесью растворителей; скорость потока подвижной фазы составляла 100 мл/мин, объем пробы – 4 мл. Многокanalное детектирование вещества проводили с помощью двухлучевого УФ-спектрофотометра при 8 длинах волн от 210, 220, 230, 240, 250, 260, 280 и 300 нм; оптимальное значение температуры колонки – 37–40 °С; давление насоса – 2,8-3,2 МПа.

Результаты и их обсуждение. Выделение клемастина из крови проводили по разработанной методике, включая экстракцию хлороформом при рН 9,0; экстракционную очистку экстракта гексаном от примесей; TСХ-очистку и идентификацию клемастина. С помощью унифицированного метода ВЭЖХ клемастина идентифицировали по параметрам удерживания и спектральным соотношениям. Для количественного определения использовали калибровочный график или уравнение прямой линии, соответствующее этому графику. Полученные результаты свидетельствовали о надежности и воспроизводимости метода. Установлено, что относительная неопределенность среднего результата при анализе клемастина в крови составляла \( \varepsilon \geq 4,63 \% \), относительное стандартное отклонение среднего результата было равным \( RSD \geq 1,67 \% \).

Выводы. Клемастина экстрагировали хлороформом при рН 9,0 из крови. Очистку экстрактов от нежелательных веществ проводили путем комбинирования TСХ и экстракции гексаном. Установлено, что при выделении клемастина из крови по разработанным методикам можно определить 36,05-39,55 % вещества (\( \varepsilon \leq 4,63 \% \), \( RSD \leq 1,67 \% \)). Метод очистки TСХ и идентификации клемастина в биогенных экстрактах апробирован в оптимальных условиях: система органических растворителей метанол – 25 % раствор гидроксида аммония (100 : 1,5), применение реагентов – УФ-спектра и реагента Драгендорфа в модификации Мунье, \( Rf_{\text{kлемастина}} = 0,60 \pm 0,03 \) (Sorbfil PTLC-AF-A). Унифицированный метод ВЭЖХ для идентификации и количественной оценки клемастина был апробирован в биогенных экстрактах крови с помощью унифицированного алгоритма направленного анализа. Установлено, что клемастина можно идентифицировать по времени удерживания 25,997-26,011 мин; объему колонки – 30,0-30,1 мл.

Ключевые слова: клемастина фумарат (тавегил); экстракция хлороформом; очистка экстракта от примесей методом TСХ и экстракции гексаном; идентификация клемастина.

INTRODUCTION

Clemastine fumarate (tavegil)-1-methyl-[2-α-methyl-p-chlorobenzhydryloxy]-ethyl-pyrrolidine fumarate is the first generation H\(_1\)-histamine receptor blocker. Clemastine fumarate selectively inhibits histamine H\(_1\) receptors and reduces capillary permeability. The drug has a pronounced anti-allergic and antipruritic effect, which reaches its maximum in 5-7 hours and lasts for 10-12 hours. Clemastine prevents the development of vasodilation and the smooth muscle contraction induced by histamine. The drug reduces the permeability of blood vessels, capillaries, inhibits exudation and formation of edema, reduces itching, and has the anti-cholinergic effect. Clemastine fumarate has an insignificant anticholinergic activity, causes sedation [1, 2]. The drug is used to treat pruritus in psoriasis [3], multiple sclerosis [4], and optic neuritis [5]. Clemastine is characterized by the following side effects: increased fatigue, drowsiness, sedation, weakness, lethargy, impaired coordination of movements; nausea, vomiting, decreased blood pressure, palpitations, hemolytic anemia, skin rash, anaphylactic shock. In case of an overdose, the drug has a neurotoxic effect, which manifests itself in impaired consciousness with the development of generalized anticholinergic convulsive syndrome [6, 7].

The urgent task for monitoring the treatment effectiveness of the population with clemastine fumarate and diagnosis of drug intoxication is the choice of highly sensitive and selective research methods of analysis in pharmaceuticals and biological matrices during the treatment.

According to the toxicological studies, one of the leading places among drug poisonings is occupied by intoxication with antihistamines due to the multi-vector pharmacological effects, the uncontrolled use of many combined drugs for the treatment of seasonal diseases. According to the literature, systematic studies of most antihistamines of the first generation are absent, the methods of their isolation, identification, quantification in biological objects have been insufficiently studied. All these determines the relevance of such studies using modern highly sensitive and selective methods of analysis [6, 7].

The development of the analytical service in the directions of monitoring the treatment effectiveness of the population with antihistamines and diagnosis of intoxications with the use of these drugs is based on the creation
of databases of effective, economical and express methods of
the drug analysis in biological objects using a unified
HPLC method [8, 9].

Clemastine is determined in drugs and biological objects
by such highly sensitive methods as spectrophotometry [10],
thin layer chromatography (TLC) [11, 12], high perfor-
man ce liquid chromatography (HPLC) [9, 13, 14].

The most common highly sensitive and selective chro-
matographic methods of chemical and toxicological analysis
of the first-generation antihistamines are HPLC and TLC.
These methods are widely used for the separation of drug
mixtures, detection of impurities and purification from
them.

The literature presents HPLC methods for the study of
clemastine using different conditions (variants for de-
tecting the test substance, the use of isocratic and grad-
uate modes of elution, the use of different compositions of
mobile phases, sorbents, buffer solutions).

According to the European Pharmacopoeia, the HPLC
analysis of the methanol solution of clemastine was per-
formed using the reversed-phase chromatography with
an UV-detector. Detection of substances was carried out
at a wavelength of 220 nm; the column (0.1 m x 4.6 mm)
with octadecyl silica gel C18, 5 μm. The mobile phase was
orthophosphoric acid – acetone – 1 % solution of ammo-
nium dihydrophosphate (0.1 : 45 : 55). The flow rate of
the eluent in the column was 1.0 ml/min [13].

For HPLC separation of antihistamines, the use of dif-
ferent conditions is recommended: the column: C18 end-
capped LiChrospher 100 RP-18, (125 x 4.0 mm, 5 μm)
with the pre-column LiChrocart; the mobile phase: addi-
tion of 146 μl of triethylamine and about 750 μl of phos-
phoric acid to 530 ml of water. The pH was adjusted
to 3.3 using 10 % potassium hydroxide solution, then 470 ml
acetonitrile was added. The flow rate was 0.6 ml/min;
detection – UV diode-array; the retention time of clem-
astine – 14 min [9].

The disadvantages of the above HPLC methods are
the use of isocratic elution regime [9, 13], which limits
the possibility of all sample components leaving the column
in the form of narrow zones and effective separation of
drug mixtures. Detection at the same wavelength [13]
reduces the reliability of the results obtained in the identifi-
cation and study of mixtures with other drugs since it al-
lows using only the retention parameters without the use
of spectral ratios.

Modern HPLC methods for the analysis of clemastine
indicate the absence of systematic studies, which does not
allow the selection of the optimal conditions for the ana-
lysis of the drug in biological objects and pharmaceuticals.

The aim of the study is to develop an algorithm for
directed analysis of clemastine in biological extracts from
the blood using a unified method of the HPLC research.

To achieve these aims, the following tasks should be
solved:

1. to carry out extraction of clemastine according to
the method developed for isolation of organic substanc-
es of the basic character from biological objects;
2. to select the optimal conditions for TLC purification
and identification of clemastine in biogenic extracts
(thin layers of sorbents, organic solvent systems, substan-
cce detection agent);
3. to approve a unified HPLC method for identifica-
tion and quantification of clemastine in biogenic extracts
from the blood according to the algorithm of directed
analysis developed in biological extracts using a unified
HPLC method.

MATERIALS AND METHODS

Clemastine fumarate (taevgil) was isolated from “Taevgil”
tables (10 pcs), 1 mg (Novartis AG, pharmaceutical com-
pany, Basel, Switzerland) as follows: the number of tab-
lets containing 200 mg was transferred to a porcelain
mortar and triturated to a homogeneous state. 100.0 ml
of methanol was added to the mixture and mixed thor-
oughly. The resulting mixture was filtered through a pa-
per filter in a porcelain cup and evaporated on a water
bath at a temperature not higher than 40° C to remove
the organic solvent; the residue was dried.

0.1000 g of the test substance was added to a 100.0 ml
volumetric flask, dissolved in methanol, and the solution
was diluted to the volume with the solvent (the standard
solution with the concentration of 1000.0 μg/ml).

Organic solvents – chloroform, methanol, hexane –
were of analytical grade (Sigma-Aldrich, USA). Reagents –
10 % solution of trichloroacetic acid, 25 % solution of
ammonium hydroxide, 0.1 M solution of sodium hydroxide
were also of analytical grade (Chimmed, Russia).

According to the algorithm for the study of biological
objects for the presence of drugs, the following steps were
performed: isolation of the substance from the biologi-
ical object, purification from nutrients, identification and
quantification. Purification of extracts from co-extractive
compounds was performed by combining TLC and hexane
extraction [16, 17].

A model mixture of the blood with clemastine fuma-
rate was used for the research. To 10 ml of the ap-
propriate biological fluid, 1000.0 μg of clemastine fumarate
using the methanol solution of the drug substance con-
taining 1000.0 μg/ml, as well as control samples, were
added. The samples were left for 24 h at room tempera-
ture. A day later, the studies were performed according
to the extraction procedures developed.

Methods of isolation of clemastine from the blood
and extraction purification by hexane. To 10.0 ml of a mo-
del mixture of the blood with clemastine fumarate, 5.0 ml
of 10 % solution of trichloroacetic acid was added for
breaking bonds with proteins, mixed and checked with
an universal indicator of the mixture pH of 2.0-2.5, allowed
to stand for 2 h with constant stirring at room tempera-
ture.

The mixture was centrifuged at 3000-5000 rpm for
10 min, the liquid over the precipitate was separated.
Lipid impurities were extracted three times with hexane
in portions of 5 ml. The hexane phases were not studied.

The aqueous layer was alkalinized with 0.1 M solu-
tion of sodium hydroxide to pH 9.0, and the clemastine
The purified chloroform solutions were evaporated at room temperature to dryness, dissolved in 2.0-3.0 ml of methanol, and then quantitatively transferred to a 5.0 ml volumetric flask, and diluted to the volume with methanol.

**Methods of TLC purification of biogenic extracts.**

The purified chloroform solutions were evaporated at room temperature to dryness, dissolved in 2.0-3.0 ml of methanol, and then quantitatively transferred to a 5.0 ml volumetric flask, and diluted to the volume with methanol.

The TLC purification and identification of clemastine were performed under conditions: chromatographic plates – Sorbil PTLC-AF-A, the system of organic solvents – methanol – 25 % solution of ammonium hydroxide (100 : 1.5), \( R_f \) clemastine, 0.60 ± 0.03, impurities were located at the start line or at the finish line. The detection of clemastine was performed under uniform conditions using the most sensitive developers: UV light (\( \lambda = 254 \) nm) – a purple color of spots, sensitivity in the sample – 0.3-0.5 μg. Drangendorff’s reagent modified by Mounier – an orange color of spots, sensitivity of the developer – 1.0-3.0 μg of the substance in the sample [12].

The TLC purification of clemastine in the extracts was performed according to the following method: 1.0 ml of the methanol solution of clemastine after extraction purification was evaporated to 0.3-0.5 ml. At the starting line of the chromatographic plate at a distance of 1-2 cm from the edge to the point, 0.01 % methanol solution of clemastine was applied using a calibrated capillary witness. At a distance of 1-2 cm from the witness, the extract from the control sample was applied, the diameter of the stain should not exceed 0.5 cm. The methanol solution of clemastine after extraction purification was applied in the form of a strip of 1.0-1.5 cm long.

The chromatographic plate was placed in a chromatography chamber, which was a glass vessel with a ground lid with a volume of 500 cm³; the system of solvents for chromatography (50 ml) was introduced into it. The chamber was carefully closed, and there was the solvent vapor saturation for at least 30-60 min. The length of the mobile phase front was 7 cm. The chromatography was completed when the solvent reached the finish line. The chromatographic plate was dried at room temperature, after that its part with spots of the witness and extracts from the control sample was developed using UV light and Drangendorff’s reagent modified by Mounier.

At the level of the spot of the standard methanol, 0.01 % solution of clemastine from the part of the plate that was not treated with the developer removed a layer of the sorbent with an area of 4-5 cm², and was transferred to the filter. The substance was eluted three times with 5.0 ml of methanol, and the resulting solution was filtered through a filter (“red tape”).

The resulting solutions were evaporated at room temperature to dryness, dissolved in 2.0-3.0 ml of methanol, and then quantitatively transferred to a 5.0 ml volumetric flask, and diluted to the volume with methanol.

**Methods of studying clemastine by HPLC.**

The chromatographic analysis was performed on a “Milichrome A-92” microcolumn liquid chromatograph (EkoNova, Closed Joint-Stock Company, Russia) according to the unified HPLC method developed by Baram G.Y. [14]: the reversed-phase variant using a metal column with a non-polar absorbent Prontosil 120-5C 18 AQ, 5 μm; the mobile phase in the linear gradient mode – from eluent A (5 % acetonitrile and 95 % buffer solution – 0.2 M solution of lithium perchlorate in 0.005 M solution of perchloric acid) to eluent B (100 % acetonitrile) for 40 min. Regeneration of the column was conducted for 2 min with the mixture of solvents; the flow rate of the mobile phase was 100 μl/min, the injection volume – 4 μl.

The multichannel detection of the substance was performed using a two-beam multi-wave UV spectrophotometer at 8 wavelengths of 210, 220, 230, 240, 250, 260, 280, and 300 nm; the optimal value of the column temperature – 37-40 °C and the pump pressure – 2.8-3.2 MPa.

**RESULTS AND DISCUSSION**

Identification of clemastine was performed by retention parameters and spectral ratios. It was found that the absolute retention time of clemastine was 25.997-26.011 min, and the absolute retention volume was 2599.7-2601.1 μl.

To obtain reliable identification results, the spectral ratios of the optical density values at wavelengths from 220 to 280 nm to the optical density values at 210 nm were determined – 0.741 ± 0.005; 0.536 ± 0.005; 0.969 ± 0.006; 0.023 ± 0.006; 0.027 ± 0.006; 0.005 ± 0.005; 0.003 ± 0.008.

Peak symmetry coefficients and capacitance coefficients were determined to verify the choice of chromatography conditions. It was found that the values of the coefficients of symmetry of the peaks were equal to 0.92 ± 0.07 (less than 2.0-2.5), and the coefficients of capacity – 16.34 ± 0.02 (more than 0.5-2.0) showed the suitability of the chromatographic system HPLC analysis [15].

The method of the clemastine determination by HPLC was validated by the following parameters: the range of linearity, limits of the quantitative determination (LOQ), accuracy and precision in the areas of low, medium and high concentrations of the test substance [18]. To quantify clemastine, the absolute calibration method was applied using the area of the peaks of the substances.

The concentration of clemastine in the methanol solution (C, μg/ml) obtained after purification of biogenic extracts by TLC and extraction methods was calculated using a calibration graph or the equation of the line corresponding to the calibration graph.

The linearity of the calibration graph was observed in the concentration ranges of 5.0-100.0 μg/ml, which corresponded to the content of clemastine in the sample (4 μl) from 20.0 ng to 400.0 ng. The lower limit of the clemastine determination by HPLC was 5.0 μg/ml (20.0 ng in the sample).
**THE RESULTS OF THE HPLC ANALYSIS OF CLEMASTINE EXTRACTS IN THE BLOOD**

| The value of the area of the peaks, S, mm² | The clemastine isolated (µg) | Metrological characteristics, % | \( \bar{X} \) | \( S \) | RSD\( \bar{X} \) | Sx | Δx | \( \bar{e} \) |
|--------------------------------------------|-----------------------------|---------------------------------|----------------|------|----------------|----|----|------|
| 0.00186                                    | 11.46                       | 38.4                            | 37.8          | 1.41 | 1.67           | 0.63 | 1.75 | 4.63 |
| 0.00183                                    | 11.28                       | 37.8                            | 10.81         | 36.2 | 36.8           | 11.99 | 39.8 | 11.99 |

**Table 1**

**THE ALGORITHM FOR THE DIRECTED ANALYSIS OF CLEMASTINE IN THE BLOOD**

| Stage of the algorithm | Conditions of performance |
|------------------------|----------------------------|
| Destruction binding of clemastine to proteins | To 10.0 ml of a model mixture of the blood with clemastine fumarate, 5.0 ml of 10 % solution of trichloroacetic acid was added, mixed and checked with an universal indicator of the mixture \( \text{pH} = 2.0-2.5 \), allowed to stand for 2 h with a constant stirring at room temperature |
| Extraction purification by hexane | The mixture was centrifuged at 3000-5000 rpm for 10 min, and the liquid over the precipitate was separated. Lipid impurities were extracted three times with hexane in portions of 5 ml. The hexane phases were not studied |
| Chloroform extraction of the clemastine base | The aqueous layer was alkalinized with 0.1 M solution of sodium hydroxide to \( \text{pH} 9.0 \), and the clemastine base was extracted twice with chloroform in portions of 10.0 ml followed by centrifugation at 3000-5000 rpm for 10 min to destruct water-chloroform emulsions |
| TLC-purification and the preliminary identification of clemastine in extracts | The system of organic solvents – methanol – 25 % solution of ammonium hydroxide (100 : 1.5), developers – UV light, Dragendorff’s reagent modified by Mounier; \( \Delta \text{Rf base} = 0.60 \pm 0.02 \) (Sorbfil PTLC-AF-A) |
| Confirming the HPLC-identification of clemastine in extracts | Clemastine was identified with the retention time of 25.997-26.011 min; the retention volume was 25.997-26.011 µl; spectral relations: 0.741; 0.536; 0.096; 0.023; 0.027; 0.005; 0.003 |
| Quantitative determination of clemastine in extracts by the HPLC-method | The range of linearity of the calibration graph was 5.0-100.0 µg/ml corresponding to the content of clemastine in the sample from 20.0 ng to 400.0 ng, respectively. To determine the content of clemastine, the equation \( S = 0.15 \cdot 10^{-3} C + 0.14 \cdot 10^{-3} \) was used, the correlation coefficient was 0.9998 |

The equation of the linear dependence of the area of clemastine peaks \( (S, \text{mm}^2) \) on its concentration \( (C, \mu \text{g/ml}) \) had the form of \( S = 0.15 \cdot 10^{-3} C + 0.14 \cdot 10^{-4} \). The correlation coefficient was 0.9998 [18].

When performing the HPLC analysis of clemastine in model solutions using the method proposed the relative uncertainty of the average results did not exceed \( \pm 1.90 \% \), indicating the suitability of HPLC conditions for analysis in biological objects.

The amount of clemastine in the blood samples was calculated by the formula:

\[
C_\bar{x} = \frac{C \cdot V_1 \cdot V_3 \cdot k \cdot 100}{a \cdot V_2},
\]

where: \( C_\bar{x} \% \) – is the mass fraction of clemastine in 10 ml of the blood, \( % \); \( V_1 \) – is the volume of a volumetric flask with the methanol extract of the substance from the blood after extraction purification, 5 ml; \( V_2 \) – is the volume of the methanol extract of the substance from the blood taken for TLC purification, 1 ml; \( V_3 \) – is the volume of the volumetric flask with the extract after TLC purification using methanol, 5 ml; \( k \) – is the coefficient of recount equal to the ratio of molecular weights of salt/base; \( a \) – is the weight of the sample of clemastine fumarate in 10.0 ml of the blood, 1000.0 µg.

The results of the study are shown in Tab. 1. According to the results of research, it has been found that when isolating clemastine from the blood according to the methods developed it is possible to determine 36.05-39.55 % of the substance \( (\pm 4.63 \%, \text{RSD} = 1.67 \%) \).

According to the results of HPLC and TLC studies, an algorithm for the directed blood analysis for clemastine was developed (Tab. 2).

The algorithm developed as a result of the present research had a number of advantages. They were:

- the use of a unified HPLC method made it possible to identify clemastine by retention parameters and spectral ratios, which made the results accurate, reliable and reproducible;
- the use of the linear gradient mode of clemastine elution allowed to obtain symmetrical, sharp chromatographic peaks (the peak symmetry coefficient did not exceed 2-2.5) and perform their reliable processing. The results of the identification and quantification by HPLC were calculated using a "Multichrome" computer program (CJSC "Ampersend", Russia) included in the chromatograph;
- a high degree of purification of extracts from impurities allowed to obtain reliable and reproducible results in accordance with metrological characteristics.
The disadvantage of the analysis algorithm developed was the low degree of clemastine extraction from the blood – 36.05–39.55 % of the substance. These results were due to losses in the multi-stage purification of extracts from impurities. Another drawback was the use of a unified method of extraction of the substance without taking into account the individual properties of clemastine.

CONCLUSIONS

1. Clemastine was extracted with chloroform at pH 9.0 from the blood. Purification of extracts from co-extractive compounds was performed by combining TLC and extraction with hexane. It has been found that when isolating clemastine from the blood according to the methods developed it is possible to determine 36.05–39.55 % of the substance (E = ± 4.63 %, RSDR = 1.67 %).

2. The method of TLC purification and identification of clemastine in biogenic extracts was tested under the optimal conditions: the system of organic solvents – methanol – 25 % solution of ammonium hydroxide (100 : 1.5), the use of reagents – UV light, Dragendorff’s reagent modified by Mounier, Rf clemastine = 0.60 ± 0.03 (Sorbfil PTLC-AF-A).

3. The unified HPLC method for identification and quantification of clemastine was tested in biogenic extracts from the blood according to the algorithm of directed analysis developed. It has been found that clemastine can be identified by the retention time – 25.997–26.011 min; the retention volume – 2599.7–2601.1 μl; spectral ratios – 0.741; 0.536; 0.096; 0.023; 0.027; 0.005; 0.003. The clemastine content was determined by the equation S = 0.15 · 10–3 C + 0.14 · 10–2; the correlation coefficient was equal to 0.9998.

4. Chromatographic methods can be recommended for implementation in practice of the Bureau of Forensic Medical Examination, poison control centers, clinical laboratories regarding the study of medicinal substances in biological objects.

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