

DEVELOPMENT OF METHODS OF IDENTIFICATION AND QUANTITATIVE DETERMINATION OF ACTIVE SUBSTANCES IN SEMI-SOLID DOSAGE FORMS WITH SAPROPEL EXTRACTS

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Abstract

For use in veterinary medicine for the treatment of the wound process and the prevention of mastitis, the composition and technology of an emulsion-based cream, conventionally called «Saprocream», has been developed. Clinical trial of the drug «Saprocream» proved its effectiveness and safety for use in the healing of microtraumas, erosions and cracks (wounds) of the udders of cows.

The aim of the research were the standardization of the cream under the conventional name «Saprocream» for use in veterinary medicine as a wound healing agent, the development of methods of identification and quantification and their validation.

Materials and methods. Test samples of emulsion cream type o/v, containing 15 % aqueous extract of sapropel (VES), 10 % oil extract of sapropel (OES), 6 % emulsifier No. 1, 1 % cetylstearyl alcohol, preservatives – 0.01 % nisin, 0.1 % euxil K 100 and purified water up to 100 g were made by phase inversion.

The methods of pharmacopoeial articles of the European Pharmacopoeia of the 10th edition, SPH U 1.0, as well as industry standards were used to develop methods for identification and quantification of active pharmaceutical ingredients (API) in the developed cream and its standardization.

Results. The chromatographic profile of the acetone extract from the cream coincides with the chromatographic profile of the reference solution ((PRS) β -carotene), which indicates the presence of substances of carotenoid structure. The absorption spectrum of hexane extraction from the cream in the range from 500 nm to 750 nm has a maximum absorption at a wavelength of 670 ± 2 nm, which coincides with the maximum absorption of hexane solution OES, indicating the presence of chlorophyll.

A method for quantifying the total mass fraction of humic acids (HA) has been developed and it has been established that the total mass fraction of HA in cream samples with sapropel extracts is 0.828 %.

The results of the validation of the method show that there is a linear relationship between the concentration of the total mass fraction of HA and the mass of the AES sample with a correlation coefficient of 0.9981 (≥ 0.9981). The developed technique is precise, because the value of the relative confidence interval is less than the critical value for the convergence of the results: $\Delta \% = 1.34 \leq 1.60$ and the criterion of insignificance of systematic error $\delta = 0.51$ is fulfilled.

Conclusions. An emulsion-based cream under the conventional name «Saprocream» has been standardized, methods for identification and quantification of active substances have been developed and validation of the developed methods for use in veterinary medicine as a wound-healing and anti-inflammatory agent has been carried out.

Keywords: cream with sapropel extracts, carotenoids, identification, quantification, validation.

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1. Introduction

The production of pharmaceutical products intended for the needs of veterinary medicine, in particular for the treatment and prevention of diseases of farm animals, is a necessary prerequisite for the effective functioning of the livestock industry [1, 2].

Unfortunately, the competitiveness of domestically produced veterinary drugs (VD) is low, the technological level of Ukrainian producers compared to foreign companies is quite low, which explains the small volume of production of domestic VD [1, 3].

Bovine mastitis is an inflammatory disease that affects one or more of the four quarters of the bovine mammary gland or udder and can adversely affect the health of cows [4]. Mastitis is the most frequent disease of dairy cows and has well-recognized detrimental effects on animal wellbeing and dairy farm profitability [5]. Heifer mastitis is a disease that potentially threatens production and udder health in the first and subsequent lactations [6].

Taking into account that lesions on the nipples of the teats of dairy cows can lead to mastitis, which is the most common disease in cows, which, in addition to economic losses, poses a danger to dairy consumers, the problem of introducing available, effective and safe drugs for the treatment and prevention of microtraumas, erosions and cracks in the udder of cows, which reduce the risk of mastitis is relevant [7, 8].

The register of veterinary drugs on the website of the State Service of Ukraine for Food Safety and Consumer Protection as of June 2021 was analyzed [9]. It is established that according to ATCvet classification of group QD03AX the solution for external application Antiseptic stimulator «Road fraction 2» (LLC «AVZ SP», the Russian Federation), nutritious ointment (PJSC «GNP Ukrzoovet-prompostach», Ukraine), ointment «Zhyvosept» (Private enterprise «OL KAR-AgroZooVet-Ser-vice», Ukraine), ointment «Vetmekol» (PJSC NVC «Borshchahivsky HFZ», Ukraine) and suspension «Aluspray» (Vetokvinol SA, France) [10] (**Fig. 1**).

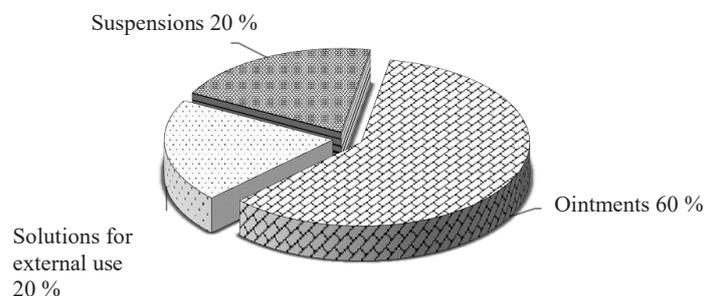


Fig. 1. Distribution of dermatological VD group QD03AH Other drugs that promote wound healing (scarring) by dosage forms

Semi-solid dosage forms (SDF) are widely used in veterinary practice, as 60 % of the market for veterinary drugs is made in the form of ointments [11].

Drugs for external use have certain advantages, including ease of use and monitoring of treatment. In addition, most of these drugs do not enter the systemic bloodstream and, accordingly, do not cause complications in the body. Interaction with other drugs is also infrequent [12, 13].

Given that lesions on the nipples of the teats of dairy cows can lead to mastitis, which is the most common disease in cows, which, in addition to economic losses, poses a danger to dairy consumers, the problem of introducing affordable, effective and safe drugs is relevant. treatment and prevention of microtraumas, erosions and cracks in the teats of cows, which reduce the risk of mastitis [1].

For use in veterinary medicine for the treatment of wound process and prevention of mastitis, we have developed an emulsion-based cream, tentatively called «Saprocream», containing 15 % sapropel oil extract (OES) and 10 % aqueous sapropel extract (AES) [14].

The cream technology was developed and the parameters of the technological process were investigated [15].

As a result of studying the anti-inflammatory and wound-healing effect of the cream with sapropel extracts in the model of UV erythema in rats, it was found that the study drug has

pronounced anti-inflammatory properties, reduces the intensity of acute photodynamic inflammation and shortens treatment time [16, 17].

Clinical trials of «Saprocream» have proven its effectiveness and safety for use in the healing of microtraumas, erosions and cracks (wounds) of teats of cows [18].

A prerequisite for the use of the cream in medicine and pharmacy is its standardization, which is a difficult task, as there is no analytical and regulatory documentation governing the quality of sapropel as a raw material for the production of drugs.

The aim of the work is to standardize the cream under the conditional name «Saprocream» for use in veterinary medicine as a wound healing agent, development of methods of identification and quantification and their validation.

2. Materials and methods

2.1. Materials

Production of test samples of cream. Test samples of emulsion cream type o/v, containing 15 % aqueous extract of sapropel (AES) and 10 % oily extract of sapropel (OES), 6 % emulsifier No. 1, 1 % cetylstearyl alcohol, preservatives – 0.01 % nisin, 0.1 % euskil K 100 and purified water up to 100 g [14], were made by phase inversion by the following technology: the aqueous phase (purified water, AES, preservatives) was heated to a temperature of 70 ± 5 °C and added to the oil phase (OES, emulsifier No. 1, cetylstearyl alcohol). The resulting mixture was emulsified using a Polytron PT 3100 D homogenizer («Kinematica AG», Switzerland) for 15 min at a speed of 5000 rpm. At a temperature of 70 ± 5 °C, the remaining aqueous phase was added and emulsified for 5 min at a speed of 5000 rpm. Cooled to room temperature [15].

Reagents. Preparation of 0.1 M alkaline solution of sodium diphosphate ($\text{Na}_4\text{P}_2\text{O}_7 \times 10\text{H}_2\text{O}$), 0.4 M solution of chromium mixture, 0.2 M solution of Mohr's salt ($(\text{NH}_4)_2\text{SO}_4 \times \text{FeSO}_4 \times 6\text{H}_2\text{O}$) and 0.2 % N-phenylanthranilic acid solution [19].

2.2. Methods

Description in accordance with the requirements of the pharmacopoeial article of the European Pharmacopoeia of the 10th edition «Semi-solid preparations for cutaneous application» p. 935 [20].

Odour. EP 10th ed., Vol. 1, 2.3.4., p. 133.

Homogeneity. The homogeneity of the cream and gel samples was determined by the method described in SPhU 1.0, p. 511 [21].

pH. The pH value was measured according to EP 10th ed., Vol. 1, 2.2.3., p. 24–25. – potentiometrically using a laboratory device pH-150-MI (LLC «Measuring Equipment», RF) [20].

Determination of thermal stability. To study the thermal stability of SDF for each sample used 5 glass tubes with a diameter of 15 mm and a height of 150 mm. The tubes were filled with (8–10) ml of test samples and placed in a thermostat brand TC-80 MG with a temperature of (42.5 ± 2.5) °C for 7 days. After that, the samples were transferred for 7 days in a refrigerator with a temperature of (6 ± 2) °C and then kept for 3 days at room temperature. Stability was determined visually: if no stratification was observed in any test tube, the sample was considered stable [22].

Determination of colloidal stability. For the test used a laboratory centrifuge T 62 with a set of tubes, a mercury thermometer with a range of measured temperatures from 0 to 100 °C and a partition price of 1 °C, as well as a stopwatch and a water bath. The tubes are filled to 2/3 volume (approximately 9 g) with the test samples so that the masses of the tubes with the drug do not differ by more than 0.02 g, and weighed to the nearest 0.01. at a temperature of (42.5 ± 2.5) °C for 20 min, then wiped dry on the outside and placed in the sockets of the centrifuge. Centrifuge for 5 min at 6000 rpm. The sample is considered stable if no stratification is observed in the tubes after centrifugation. If in at least one of the tubes there is a stratification of the sample or the separation of sediment, the analysis is repeated with new portions. If at least one test tube with stratification is detected during the repeated test, the sample is considered unstable [22].

Structural viscosity, mPa·s of the veterinary drug was determined on a rotary viscometer with a shear rate gradient of 18.0 s^{-1} and a temperature of (20.0 ± 0.1) °C according to the method of EP 10th ed., Vol. 1, 2.2.3., p. 28–30 [20].

Measurement of shear stress τ (PA or N/m²) and shear rate D_r (s⁻¹) is carried out by increasing the number of spindle revolutions from 20 to 100 rpm, achieving constant values at maximum rotation and further reducing the spindle speed

Microbiological purity. The studies were performed in accordance with the requirements of EP 10th ed., Vol. 1, 2.6.12. p. 201, 2.6.13 p. 205 [20].

To study the *validation characteristics* (AES) used the approach to the validation of BAS in herbal preparations, described in the literature [23]. The specificity of this analytical procedure was proved by determining the number of milligrams of HA in the studied AES by volumetric titration. To increase the specificity of the redox technique, a control experiment was performed in parallel.

The criterion for the acceptability of the proposed method is the linear relationship between the concentration of the total mass fraction of HA on the mass of the sample AES. When determining the linearity was performed titration of each sample in a concentration of from 80 % to 120 % of the selected at least three times.

Statistical analysis of the data was performed using the Shapiro-Wilk test using the software package GraphPad Prism 5.04 (GraphPad Software Inc., USA).

The result was calculated using two-way analysis of variance with the Bonferroni post hoc test. For each result obtained, the arithmetic mean (M) and the standard error of the arithmetic mean (m) were determined. The difference between the means was considered statistically significant if p was less than 0.05.

3. Experimental procedures

Methods of identification and quantification in the studied cream were developed – the presence of OES was determined by identifying substances of carotenoid structure and chlorophyll and AES by quantitative determination of the total mass fraction of HA.

3.1. Identification of sapropel oil extract in the cream

Thin layer chromatography (TLC) (EP 10th ed., Vol. 1, 2.2.27., p. 47–48). To identify substances of carotenoid structure used the method of TLC compared with a standard solution of β -carotene. The tests were performed using acetone extract from the cream on TLC plates with a layer of silica gel and a fluorescent indicator F254 (firm «Merk» Merck Silica gel F254), mobile phase petroleum ether-diethyl ether (10:3.5). Detection was performed after drying of chromatograms in UV radiation and treatment of chromatograms with 10 % solution of phosphoromolybdic acid and drying with heating in daylight.

Test solution: to 1.0 g of cream was added 10 ml of acetone and mixed with a magnetic mixer in a conical flask with a capacity of 100 ml for 30 minutes. The solution was filtered through a blue tape paper filter.

Comparison solution: 5 mg of PRS β -carotene was dissolved in 10 ml of hexane.

Plate: TLC plate with silica gel layer P and fluorescent indicator F₂₅₄ or identical.

Moving phase: petroleum ether P – diethyl ether P (10:3.5).

Sample volume: 5 μ l of the test solution and the comparison solution in strips, thoroughly dried the application of the strips.

The distance that the moving phase must pass: 10 cm from the starting line.

Drying: in the air for 20 minutes.

Detection A: viewed in UV radiation at a wavelength of 254 nm.

Results A: the chromatograms showed a zone of yellow fluorescence absorption in the upper part.

Detection B: sprayed with a 10 % solution of phosphorus-molybdic acid, heated in an oven at a temperature of 60 °C for at least 10 minutes.

Results B: blue spots were observed on a green-yellow background (carotenoids and their derivatives, chlorophyll).

To identify chlorophyll OES was used the method of absorption spectrophotometry in the visible area.

The absorption spectrum of hexane extract from the cream in the range from 500 nm to 750 nm should have a maximum absorption at a wavelength of 670 ± 2 nm, which coincides with the maximum absorption of hexane solution OES.

3.2. Quantitative determination of aqueous extract of sapropel in the cream.

Quantitative determination of the total mass fraction of humic acids in the cream. The total mass fraction of humic acids (HA) was determined by the method described in the literature, after oxidation of HA by the method of I. V. Tyurin in the modification of B. A. Nikitin and titration with Mohr's salt [14].

The total mass fraction of humic acids (HA) in the test sample of cream was determined after extraction of humic acids from a portion of the cream, their deposition and oxidation by the method of I. V. Tyurin in the modification of B. A. Nikitin and titration with Mohr's salt [19].

Extraction of humic acids from a sample of cream. 5,000 g of cream (exact portion) was placed in a 100 ml conical flask, 20 ml of hexane was added, stirred with a magnetic stirrer for 5 minutes, transferred to a separatory funnel, 2 ml of saturated potassium chloride solution was added, shaken and left to stratify. The aqueous layer (bottom) was poured into a 250 ml flask (flask A), 100 ml of alkaline sodium diphosphate solution was added and stirred for 1 h on a shaker.

The resulting suspension was transferred to a centrifuge tube and centrifuged for 15 min at 2000 rpm, decanted into a 1000 ml conical flask (flask B) and washed twice with 100 ml of 1 % sodium hydroxide solution, centrifuging each time and pouring the solution into flask B. The contents of flask B were filtered through a blue strip filter into a 1000.0 ml volumetric flask, made up to the mark with purified water P and mixed (solution to determine the total mass fraction of HA).

Precipitation of humic acids. From a 1000 ml flask containing an alkaline HA solution, 50.0 ml of the solution was taken, transferred to a 100 ml conical flask and, while stirring, sulfuric acid concentrated to a pH of 2.0 to 3.5 was added dropwise. The pH of the solution was measured potentiometrically. The flask was heated on a boiling water bath for 30 minutes, cooled and left for 16 hours for complete precipitation. HA was filtered through a blue strip filter. The filter cake and the flask in which the HA was precipitated were washed with 0.05 M sulfuric acid solution 3 times in 10 ml to give a clear filtrate.

The funnel with the precipitate on the filter is inserted into a volumetric flask with a capacity of 100.0 ml and dissolved the precipitate HA with hot 0.05 M sodium hydroxide solution. The filter was washed with hot sodium hydroxide solution until the precipitate was completely dissolved and a clear filtrate was obtained. The resulting alkaline HA solution was cooled to room temperature and the volume of the flask was adjusted to 0.05 M with sodium hydroxide solution.

Oxidation of humic acids by the method of I. V. Tyurin in the modification of B. A. Nikitin and titration with Mohr's salt. In a conical flask with a capacity of 100 ml was placed 20.0 ml of alkaline HA solution, the solution was evaporated in a boiling water bath to dryness without drying it.

To a conical flask with dry residue was added 10 ml of 0.4 M solution of chromium mixture. The solution was topped up slowly, washing the walls of the flask thoroughly. The flask was closed with a funnel and placed in an oven for 20 min at a temperature of 150–160 °C. After cooling, the flask was removed from the funnel, washed with purified water, 6 drops of 0.2 % N-phenylanthranilic acid solution were added and titrated with 0.2 M Mora salt solution until the color changed from cherry red to green.

A control study was performed in parallel.

The total mass fraction of HA(X), in milligrams, in 1 g of cream, was calculated by the formula:

$$X = \frac{(V_1 - V_0) \times K \times 41.36}{m}, \quad (1)$$

where V_1 – volume of Mohr's salt solution spent on titration of control experiment, ml; V_0 – volume of Mohr's salt solution spent on titration of analytical sample, ml; K – correction factor for Mohr's salt solution; M – the weight of the sample of the test sample, g.

The amount of HA in 1 g of cream should be at least 8.0 mg.

4. Results

Identification. The presence of OES was determined in the test cream by identifying carotenoids and chlorophylls and AES by quantifying the total mass fraction of HA.

To identify substances of carotenoid structure used the method of TLC compared with a standard solution of β -carotene.

The chromatographic profile of the acetone extract from the cream should coincide with the chromatographic profile of the reference solution (PRS β -caroten): two spots of yellow fluorescence with R_f about 0.60 and 0.80 (detection A); two gray spots with $R_f=0.09$ and $R_f=0.14$, blue spots $R_f=0.27$; $R_f=0.36$; $R_f=0.67$; $R_f=0.80$. Other zones are also observed on the chromatogram of the test solution (**Fig. 2**).

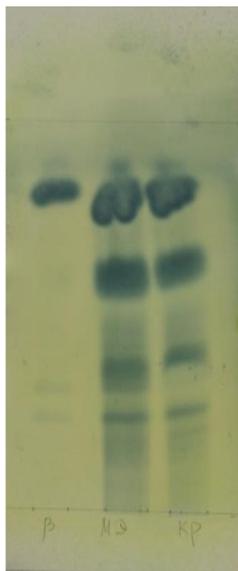


Fig. 2. Chromatogram of test solution and reference solution

The method of absorption spectrophotometry in the visible region was used to identify OES chlorophylls. The absorption spectrum of hexane extraction from the cream in the range from 500 nm to 750 nm should have a maximum absorption at wavelengths of 670 ± 2 nm (**Fig. 3, a**), which coincides with the maximum absorption of hexane solution OES (**Fig. 3, b**).

The results of quantification of the total mass fraction of HA in six samples of cream and metrological characteristics of the average result are given in **Table 1**.

The specificity of this analytical procedure was demonstrated by determining the number of milligrams of HA in the test dosage form with AES by volume titration. The results are given in **Table 2** and are shown in **Fig. 4**.

It was found that there is a linear relationship between the concentration of the total mass fraction of HA on the mass of the AES sample with a correlation coefficient of 0.9981 (≥ 0.9981), the angular coefficient of linear dependence (b) is 0.99, the free member of the linear dependence (a) – $0.30 \leq 2.60$.

Precision and accuracy studies were performed on nine determinations in the concentration range from 80 % to 120 % according to the selected method (**Table 3**).

The obtained results show that the method is precise, because the value of the relative confidence interval is less than the critical value for the convergence of the results: $\Delta \% = 1.34 \leq 1.60$ and the criterion of insignificance of systematic error $\delta = 0.51$ is fulfilled.

As a result of quantitative analysis, it was found that the total mass fraction of HA in cream samples with sapropel extracts is 0.828 %.

Based on the obtained data, it is proposed to introduce requirements for the quantitative content of the total mass fraction of HA of not less than 0.8 % to the project of MQC of the medicinal product (**Table 4**).

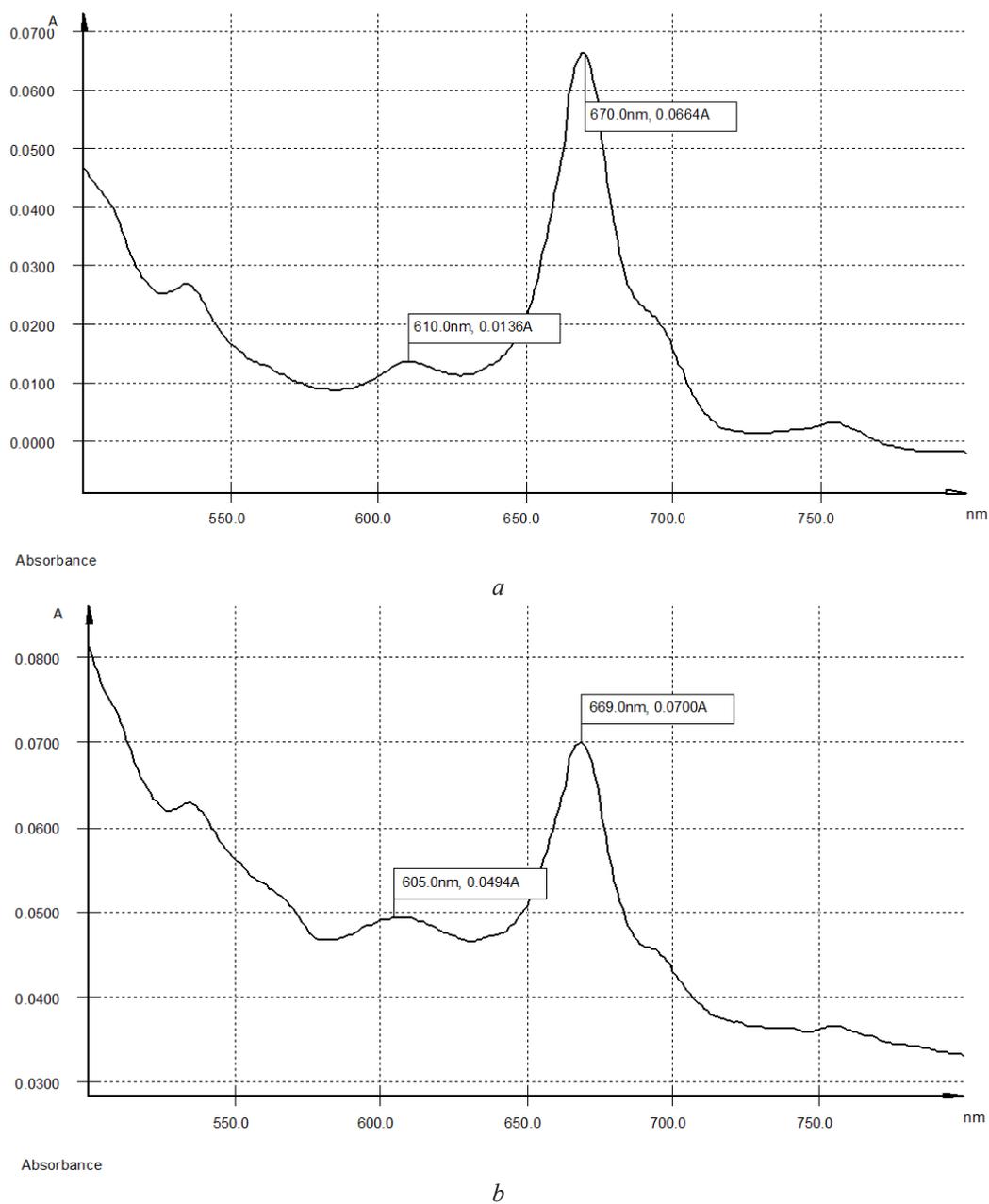


Fig. 3. Absorption spectrum: *a* – hexane extraction from the cream; *b* – hexane solution of OES

Table 1

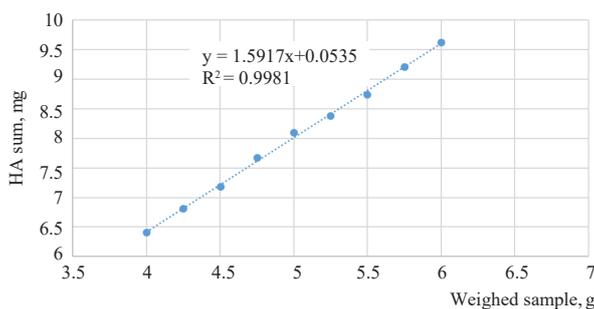
Metrological characteristics of the average result of quantitative determination of the total mass fraction of HA in the cream with sapropel extracts

Series No.	The content of the total mass fraction of humic acids, mg	S^2	S	$S\bar{x}$	Δx	$\Delta\bar{x}$	$\bar{\varepsilon}$, %	ε , %
1	8.10							
2	8.27							
3	8.03							
4	8.66	0.0495	0.2225	0.0908	0.5719	0.2335	2.82	6.90
5	8.37							
6	8.27							

Table 2

Calculation of linearity parameters of the method of quantitative determination of the mass fraction of HA

Working concentration, %	Weight sample of cream, g	Total mass fraction of HA, mg
80	4.0009	6.41
85	4.2513	6.81
90	4.5017	7.17
95	4.7501	7.67
100	5.0009	8.10
105	5.2518	8.35
110	5.5003	8.72
115	5.7511	9.21
120	6.0005	9.65

**Fig. 4.** The dependence of the concentration of the total mass fraction of HA on the mass of the sample of the test cream**Table 3**

The results of the study of the precision of the method of quantitative determination of the total mass fraction of HA in the cream

Parameter	Value	Criterion 1	Criterion 2	Conclusion
Precision	ΔZ	1.34	≤ 1.60	Sustained by the first criterion

Table 4

Cream quality indicators included in the MQC project

Indicator	Acceptable limits	The results of the analysis
Description	Cream light brown, without visible inclusions, odorless, creamy consistency	Corresponds
Identification	TLC. The chromatographic profile of the acetone extract from the cream must coincide with the chromatographic profile of the reference solution (PRS β -carotene).	Corresponds
Carotenoids	Method of absorption spectrophotometry in the visible area.	Corresponds
Chlorophyll	The absorption spectrum of hexane extraction shall have a maximum absorption at wavelengths of 670 ± 2 nm in the range from 500 nm to 750 nm	Corresponds
Homogeneity	Must be homogeneous	Homogeneous
pH	6.0–7.0	6.6 ± 0.1
Colloidal stability	Must be stable	Stable
Thermal stability	Must be stable	Stable
Structural viscosity, mPa·s.	6990 ± 30 mPa·s.	Corresponds
Microbiological purity	The total number of aerobic microorganisms (TAMS) is 10^2 CFU/ml. Total pure yeast and mold (TYMC) – 10^1 CFU/ml. Absence of <i>Staphylococcus aureus</i> in 1 ml. Absence of <i>Pseudomonas aeruginosa</i> in 1 ml	Corresponds
Quantitative determination Total mass fraction of HA, mg/g	The content of the amount of HA should be at least 8.0 mg/g of the drug.	8.28 ± 0.1

It is experimentally confirmed that the indicator of microbiological purity of the drug «Saprocream» meets the requirements of EP 10.0 which are put forward to drugs for dermal use.

The cream is standardized according to the following indicators: description, identification of active substances, homogeneity, colloidal and thermal stability, pH, structural viscosity, quantitative determination of active substances, microbiological purity.

4. Discussion

To determine carotenoids and chlorophylls in medicinal plant raw materials use the method of liquid chromatography-mass spectrometry [24], high performance liquid chromatography (HPLC) [25]. For simultaneous detection of carotenoids and chlorophylls in sapropels use the method of high-performance liquid chromatography (HPLC) (HPLC-UV-VIS) [26], as well as the method of HPLC with photodiodes with UV – detection in the visible region and atmospheric pressure (High performance liquid chromatography with photodiode array UV – Vis detection and atmospheric pressure chemical ionization mass spectrometry) [27] and Raman spectroscopy [28].

The most reliable methods of standardization of total drugs, drugs in which biological activity is associated with a large number of compounds, many of which are of unknown structure, concentrations of which can vary widely, are the determination of conditional concentrations by spectrophotometry and control of signal components by chromatographic methods [23].

The advantage of the proposed methods of identification and quantification of biologically active compounds contained in the veterinary cream and provide its biological effect is a separate determination of carotenoids, chlorophyll and HA available methods, which allows to standardize the veterinary agent.

Study limitations. As a disadvantage, it should be noted that the amount of HA may differ in the sapropels of different lakes, as their chemical composition depends on a number of factors [29, 30]. HAs have an indeterminate composition, which varies depending on the origin, production process and functional groups present in their structure [31], so the quantitative and qualitative profile of these compounds in the development of standardization methods may differ for different deposits.

Prospects for further research. According to the results of the research, analytical documentation has been developed for the cream under the conditional name «Saprocream», which makes it possible to introduce it into industrial production for the prevention and treatment of mastitis in cows.

In the future it is planned to develop «Saprocream» when used in lactating cows and determine its impact on milk quality, as well as testing other types of pharmacological activity and determining the prospects for the use of the cream for the treatment of other diseases.

5. Conclusions

Methods of identification of carotenoid substances in the cream with sapropel extracts by the TLC method in comparison with the standard solution of β -carotene and chlorophylls by the method of absorption spectrophotometry in the visible area have been developed.

A method for quantitative determination of the total mass fraction of HA after their extraction from the cream, precipitation and oxidation has been developed and this method has been validated for linearity and precision.

To the specification for the cream with sapropel extracts under the conditional name «Saprocream» are added sections: «Description», «Identification», «pH», «Quantitative determination», «Microbiological purity», «Structural viscosity», as well as sections that regulate requirements for packaging, storage conditions and shelf life of the developed drug (homogeneity, colloidal and thermal stability).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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