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Preparation of regulatory documents for sodium heparin

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Annotation

Heparin is a direct-acting anticoagulant that regulates many biochemical and physiological processes. The quality of each drug or substance entering the market to the consumer, its packaging, storage conditions and shelf life, as well as quality control methods are established in accordance with the State Pharmacopoeia of the Russian Federation. However, despite the widespread use of heparin in medical practice, the State Pharmacopoeia of the Russian Federation XIV 2018 does not prescribe articles on the substance and drugs containing it as an active substance. Quality control of medicines is undoubtedly relevant. Thus, a negligent attitude in 2008 in China led to the fact that at the early stages of production, the drug was added to the sulfated chondroitin sulfate, which, like heparin, has anticoagulant properties, but is 100 times cheaper. As a result of the use of this drug, there have been more than 100 deaths in the United States and hundreds of serious adverse reactions in patients in Europe. In this regard, the purpose of this study is to determine the quality of heparin-containing injectable drugs.

Keywords: Sodium heparin, spectrophotometry, toluylene blue, Pharmacopoeia article, physical and chemical methods of analysis;

1. Introduction

Heparin is an acidic sulfur-containing glycosaminoglycan, a biopolymer consisting of polysaccharide chains linked to a common protein core. It is a direct-acting anticoagulant and a regulator of many biochemical and physiological processes.

Despite the fact that heparin is widely used in medicine, in the State Pharmacopoeia of the Russian Federation XIV 2018, there are no articles on either the substance or the drug heparin. Today, the issue of the quality of medicines, the need for its provision and strict control is widely discussed [1, 2, 3].

The effectiveness and safety of anticoagulant therapy largely depends on the correct selection of doses of used heparin, as well as on its quality. In 2008, the use of low-quality Chinese-made heparin led to the death of more than 200 patients in the United States and Europe. In this regard, the need for research and determination of heparin, both in pharmaceuticals and in the blood of patients is not in doubt.

2. Materials and methods

2.1. Equipment

Infrared absorption spectra of heparin were recorded on an Agilent 660 Fourier-infrared spectrometer.

The capillary electrophoresis system "KAPEL-105M" with an unmodified quartz capillary with an internal diameter of 60 microns, an effective length of 50 cm, and a total length of 75 cm was used to detect impurities in the object under study.

To study the optical properties of heparin, the method of ultraviolet spectroscopy was chosen. The research was carried out on A cary60 spectrophotometer.

The pH of heparin solutions was determined using the PH meter "ITAN" Reagents.

2.2. Reagents:

- One mixed sodium phosphate, monohydrate.
- Phosphoric acid.
- Distilled water.

The search for the analytical signal of heparin was performed using background solutions with different pH. For the preparation of aqueous background solutions with a given pH value, a set of standard titers of working standards of the third category was used:

- pH 1.65-potassium tetraoxalate, 0.05 M $KH_3C_4O_8 \cdot 2H_2O$;
- pH 4.01-potassium phthalic acid, 0.05 M $KC_8H_5O_4$;
- pH 6.86-potassium phosphoric acid single-substituted, 0.025 M KH_2PO_4, sodium phosphoric acid double-substituted, 0.025 M Na_2HPO_4;
- pH 9.18-sodium tetraborate, 0.01 M $Na_2B_4O_7 \cdot 10H2O$.

All background solutions were prepared by dissolving the contents of an ampoule or precisely weighted suspension with bidistilled water in a flask at 1000.0 cm3.

- Hydrazine sulfate-CH.
- Hexamethylentetramine-CH.
- Toluene blue C15H19ClN4-CH.
- Hydrochloric acid, HCl. - CH.
- Sodium hydroxide, NaOH-CH.
- Potassium bromide, KBr. - CH.
- Cobalt chloride, CoCl2-CH.
- Copper sulfate, CuSO4-CH.
- Iron chloride, FeCl3-CH.

2.3. Object of research

The sources of heparin for the study were:

Standard of sodium heparin salt (≥180 u/mg) produced by SigmaAldrich;

- Pharmacological solution of sodium heparin for injection, produced by JSC "Bryntsalov-A" Russia, Moscow, Nagatinskaya street, 1, each milliliter of such a solution contains 5000 UNITS (1 UNIT=0.0077 mg of heparin) or 38.5 mg of salt;
- Pharmacological solution of heparin for intravenous and subcutaneous administration, produced by FSUE "Moscow endocrine plant", each milliliter of such solution contains 5000 UNITS (1 UNIT=0.0077 mg of heparin) or 38.5 mg of salt.

3. Results and discussion

3.1. Verification of the authenticity of the tested sodium heparin

To verify the authenticity of heparin, the method of IR spectroscopy was chosen. In this paper, a sample of standard heparin was studied by IR spectroscopy in potassium bromide. A small amount of the sample (1 mg of heparin) was crushed in an agate mortar until it glistened. KVG (0.18 g) was added to the sample and the grinding was continued while thoroughly mixing. Then the sample was quantitatively transferred to a tablet press. The resulting tablet was loaded into an Agilent 660 IR Fourier spectrometer, and the absorption spectrum was recorded in KBr (Fig.1).

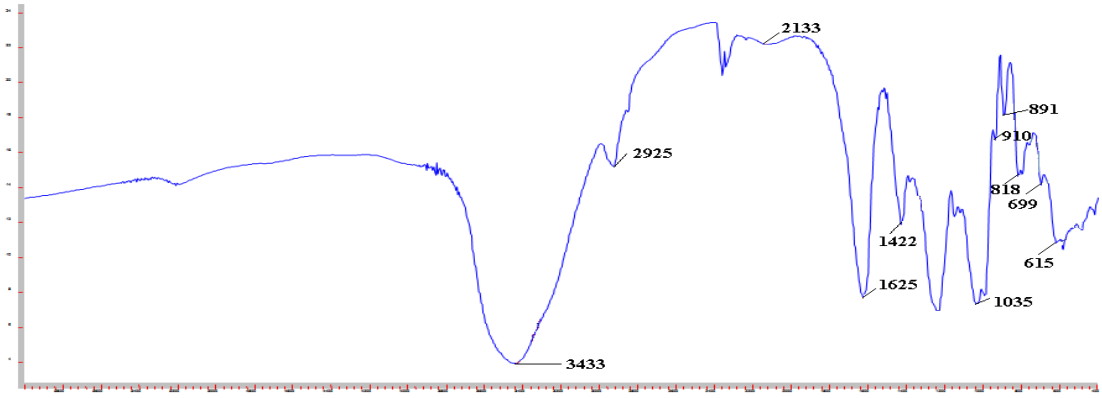

Fig.1-IR spectrum of heparin in potassium bromide

Based on the IR spectral analysis data, the following spectral characteristics of heparin were obtained in Table 1.

Table 1-Spectral characteristics of heparin in the IR region

Groups and types of vibrations	Frequency range (cm $^{-1}$)
O-H valence	3434 (3550-3200)
C-O valence	1025 (1075-1000)
O-H deformational	1421-1375 (1450-1250)
O=S=O deformational	1238 (1280-1215)
C-O-C valence	1147 (1150-1095).
N-H valence	3500-3300
C-H valence	2975-2860
N-H deformational	800

This study confirms the structure of heparin.

3.2. Quality control of the studied heparin by capillary electrophoresis

In connection with the detection of serious side effects when using injectable heparin preparations associated with the presence of an impurity of hypersulfated chondroitin sulfate in the heparin substance, a method for identifying this impurity by capillary electrophoresis in capillary solution has been developed [4]

Requirement: The electrophoregram of the test solution should not contain a sharp peak of hypersulfated chondroitin sulfate before the peak of heparin. The presence of dermatan sulfate peak is allowed after the peak of the heparin.

Fig. 2 shows the resulting electrophoregram of a sodium heparin solution.

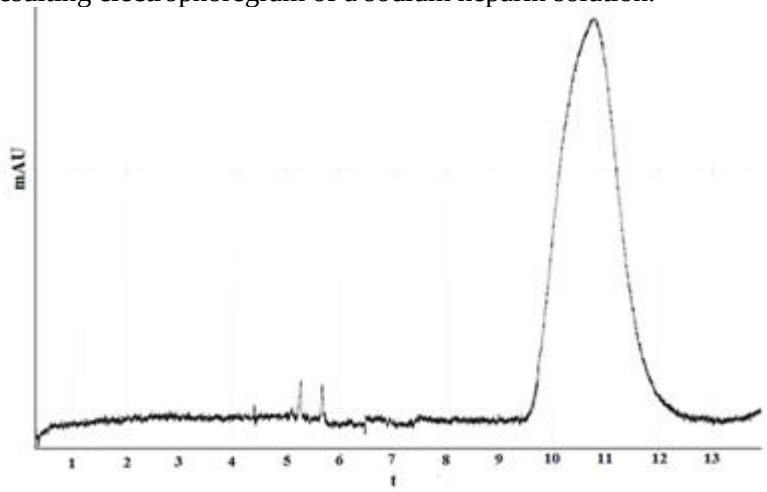

Fig. 2-Electrophoregram of heparin C solution C = 1 mg / ml

According to the results of capillary electrophoresis, it was found that the heparin used in research does not contain impurities.

3.3. Determination of a number of indicators of heparin according to the SF RF XIV [5]

Solubility. It is easily soluble in water, but not soluble in organic solvents (ethyl alcohol, acetonitrile) (SF RF XIV according to the OFS method.1.2.1.0005.18).

Transparency. The solution is transparent, corresponds to 1 standard (SF RF XIV according to the OFS method.1.2.1.0007.18).

Chromaticity. The intensity of staining of the heparin solution corresponds to the intensity of staining of the reference solution Y6 (SF RF XIV according to the OFS method.1.2.1.0006.18).

Determination of pH (SF RF XIV by the OFS method.1.2.1.0004.18).

pH of heparin (C=1000 mg / cm3) = 7.2;

pH of heparin (C=100 mg / cm3) = 6.8;

Heparin solution for intravenous and subcutaneous administration (5000 U/cm3): pH= 6.4.

Density determination.

(SF RF XIV according to the OFS method.1.2.1.0014.18).

The density of the standard heparin (C=1000 mg / cm3) = 0.998 (g/cm3). The density of the dosage form of heparin (5000 U /cm3) = 1.1(g / cm3).

Melting point. The melting point of heparin was determined using the OFS method.1.2.1.0011.18. In the course of research, it was found that heparin does not melt, but decomposes at high temperatures. The decomposition temperature of heparin was = 240.3 °C.

Refractive index (SF RF XIV according to the OFS method.1.2.1.0017.18).

- heparin solution (1%) = 1.3325
- heparin solution (1.5%) = 1.334
- heparin solution (2%) = 1.3355
- heparin solution for intravenous and subcutaneous administration (5000 U /cm^3): = 1,338

Rotation angle (SF RF XIV according to the OFS method.1.2.1.0018.18).

- heparin solution (C=1000 mg / cm^3) = 0.02
- heparin solution (C=100 mg / cm^3) = 0.02
- heparin solution for intravenous and subcutaneous administration (5000 U /cm^3): = 1,2.

3.4. Determination of microbiological purity

A study of the microbiological purity of the standard of sodium heparin salt and the injectable drug heparin was conducted in accordance with OFS 1.2.4.0002.18 of the State Pharmacopoeia of the Russian Federation XIV 2018. During the experiment, no bacteria or fungi were found in the standard sodium heparin salt. That meets the requirements presented in the SF RF XIV.

The presence of bacteria and fungi in the pharmacological solution of sodium heparin for injection was not detected. The pharmacological solution meets the requirements of the Pharmacopoeia articles in the Pharmacopoeia of Europe and the United States.

3.5. UV-visible spectroscopy

Initially, the study recorded the UV spectrum of heparin. Figure 3.

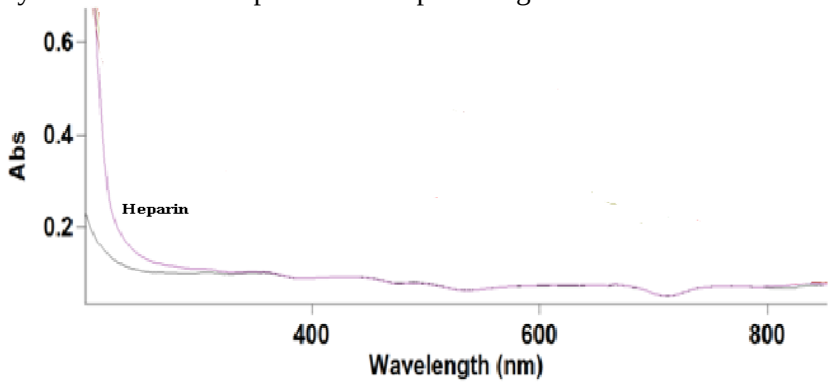

Fig. 3-Heparin absorption Spectrum

Figure 3 shows that heparin itself does not absorb in the UV region. In some cases, the metachromatic effect of basic dyes in the presence of chromotropic substances is used for spectrophotometric detection and quantitative determination of heparin. Resonance in the dye molecule is accompanied by a change in the electron density and the formation of an excess positive charge at the end of the dimer, whose electrostatic interaction with negatively charged heparin groups leads to delocalization of the π-electrons of the conjugated chromophore system. Therefore, it was proposed to investigate its complex, with the previously unused cationic dye toluylene blue. Toluylene blue base dye of the indamine class, which is a pH and redox indicator [6].

Initially, the spectral characteristics of toluylene blue were studied (figure 4), the maximum absorption of TB was 656 nm.

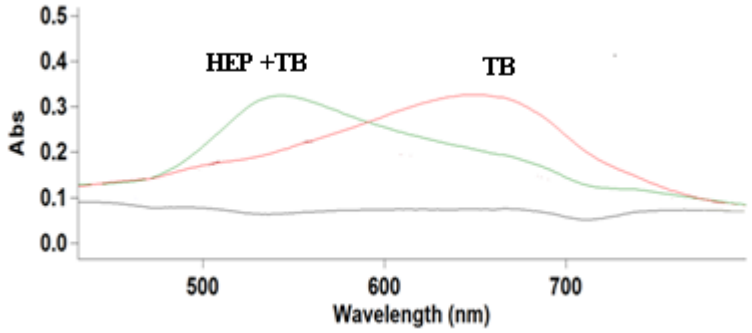

Fig. 4 -absorption Spectrum of the complex compound of the Hep-TB system

When adding Hep to the CCC, a new spectral pattern with an absorption band of 542 nm is observed, corresponding to the absorption spectrum of the complex compound of the Hep - TB system (figure 4).

3.6. Establishing the composition of the Hep - TB complex

The molar ratio method was used to determine the composition of the Hep – TB complex [7].

Using the data obtained by the molar ratio method, a graph of the dependence of the optical density of solution A on the molar concentration of TB at a constant concentration of heparin equal to $1.12*10^{-5}$ M was constructed.

The intersection point of the tangents is the equivalence point of the composition of the complexes Hep: TB = 1: 2.

3.7. Identification of the influence of auxiliary components of the medicinal form of heparin on the spectrum of TB

After the Hep-TB system was studied on standard samples, this approach was applied to real objects, namely a pharmacological solution of heparin for injection.

In accordance with the instructions for the use of injectable heparin, the following components are included in the dosage form:

- Sodium heparin 5000000 IU
- Benzyl alcohol 9 g
- Water for injection up to 1 l.

Due to the presence of auxiliary components in the LF, it is logical to assume that they can have an interfering effect on the absorption band of the Hep - TB complex, therefore, the effect of benzyl alcohol on the received signal was studied (figure 5).

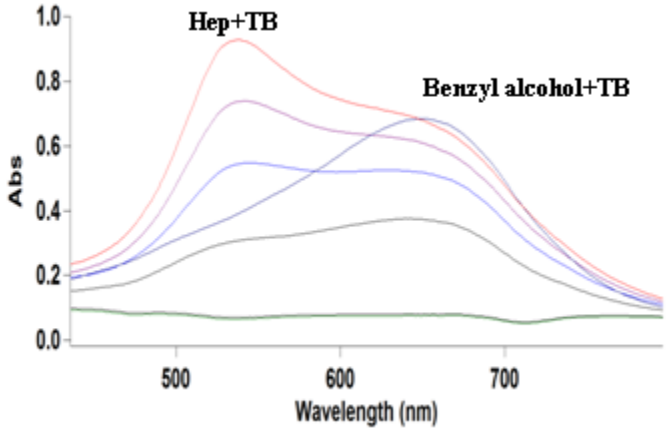

Fig. 5 -absorption Spectrum of a complex system connection Hep – $(TB)_2$

When increasing the concentration of heparin in the system, there was a decrease in the intensity of the absorption band corresponding to the dye (wavelength=668 nm) and an increase in the absorption band at (wavelength=535 nm), which corresponds to the binding of the dye with heparin.

Figure 5 shows that benzyl alcohol does not interfere with the absorption band of the Hep – $(TB)_2$ complex.

3.8. Research on the effect of pH

It is known from the literature [8] that at high pH values (< 2, >9,5), the oxidizing particles of the indamine dye toluylene blue decompose to form first phenolic blue and then indophenol. Changes in the pH of the medium affect the state of redox interaction and can even cause complete destruction of colored compounds. As a result, research was conducted on the influence of pH on the spectral behavior of the dye. Initially, the properties of toluylene blue from pH in buffer solutions were studied (figure 6).

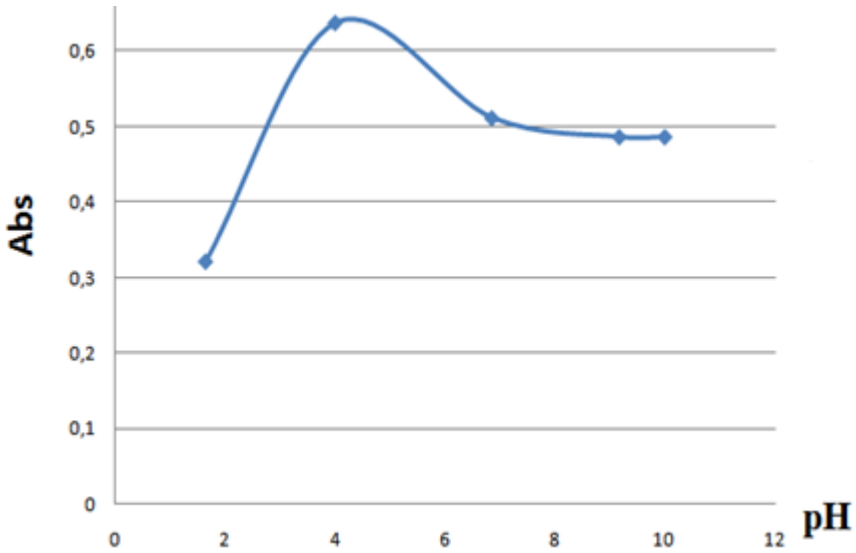

Fig. 6 - Dependence of the optical density of toluene blue dye on pH in buffer solutions

It was found that using background solutions with a pH from 1.65 to 10.01 does not change the wavelength of the absorption band of toluene blue, the color of the solutions has a blue color. Since buffer solutions are salts of different nature (potassium tetraoxalate, potassium phthalate, potassium phosphoric acid, sodium tetraboronic acid), it was decided to investigate the optical properties of toluene blue in background solutions of the same composition. To establish the pH from 1 to 6, hydrochloric acid (HCl) was used, and for pH from 9 to 13, a solution of sodium hydroxide (NaOH) was used.

Figure 7 (curve, a) shows the dependence of the optical density of toluene blue dye on the pH of solutions of different nature. The dependence shows that the most intense signal is at pH=4. In addition, it was noted that the offset of the absorption band does not depend on the pH in the long-wave or short-wave region. In HCl with a pH from 1 to 6, the color of the solution changed from orange-pink to blue, and in NaOH in the pH range from 9 to 13, from blue to orange. This study confirmed the literature data that the vehicle is a redox indicator.

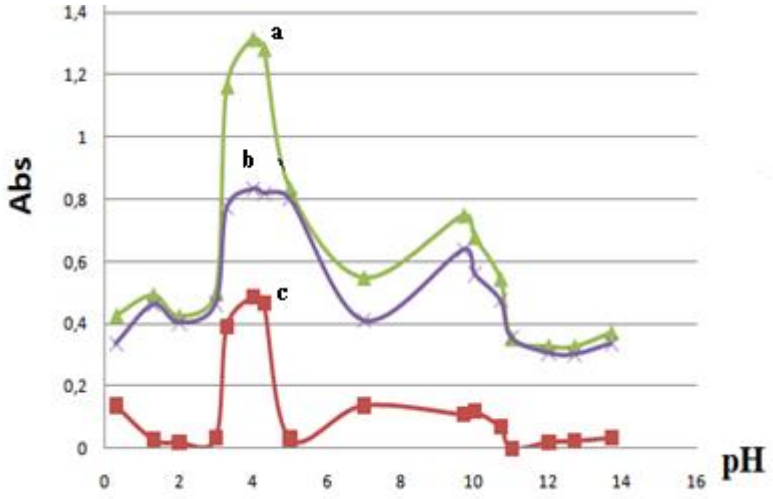

Fig. 7 -Dependence of the optical density of toluene blue dye, Hep complex: TB from pH solutions of different nature

The next stage of the experiment was the study of the Hep – TB complex from pH using the previously announced solutions.

The effect of the pH of the solution on the colored complexes is expressed in various forms, but in most cases it is reduced to a change in the composition of the colored complex. Changing the pH of the medium contributes to the formation of colored complexes with foreign ions present in the solution, causes a change in the solubility of colored compounds.

The Hep —TB complex (aqueous solution) has a maximum absorption at 535 nm, but at pH<6, due to the high degree of complexation, the value of light absorption is relatively greater. At pH>9, the maximum light absorption value of the complex decreases sharply at 535 nm. The amount of light absorption by the dye itself at this wavelength also decreases (figure 7 curve b). Figure 7 (curve b) shows that the most intense signal of the Hep —TB complex is observed in the pH range from 3 to 5.

Figure 7 (curve c) shows the difference in the absorption spectra of the TB, Hep —TB complex in different pH, which shows that the most intense signal is observed in the pH range from 3 to 5.

In water, HCl, and NaOH, when Hep is added to the vehicle, the absorption band is shifted. The dependence of this shift on pH was studied from the difference in the absorption bands of the vehicle and the Hep - vehicle complex. When using 1 mm HCl, the largest offset of the absorption band of the Hep - TB complex occurs relative to the absorption band of the TB (figure 8).

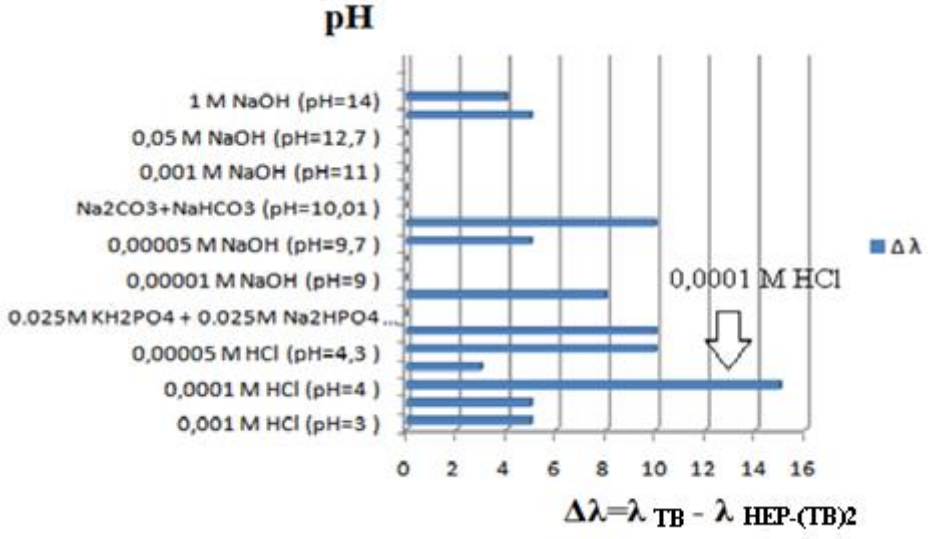

Fig. 8-Graph of the dependence of the absorption band shift of the Hep - TB complex relative to the absorption band of the vehicle

As a result, for subsequent experiments and quantitative determination of heparin in combination with toluylene blue, the analyzed solutions were proposed to be prepared in 1 mm HCl.

3.9. Evaluation of the influence of matrix components and impurity compounds on the spectrophotometric method for determining heparin using toluylene blue dye

An analytical technique is considered specific if it can be used to reliably determine a medicinal substance, even with auxiliary and impurity compounds present in it. It is possible to reliably assess the effect of matrix components and impurity compounds when adding a proportional amount of impurities or auxiliary components to the drug to confirm that their presence in the drug does not affect the analysis result.

According to the instructions for medical use, the pharmacological solution of sodium heparin for injection contains sodium heparin as the main component, benzyl alcohol, sodium chloride, water for injection as auxiliary compounds. However, in addition to the auxiliary components prescribed in the instructions, the drug may contain micro-mixtures such as hypersulfated chondroitin sulfate, dermatansulfate, as well as various amino acids, which often remain in the substance of the drug even after isolation of heparin from biological objects and subsequent purification.

In order to assess the specificity of the method, this paper examines the influence of impurity compounds, such as dermatansulfate, benzyl alcohol, as well as valine, lysine and glutamic acid, amino acids contained in a large percentage of human globulin see table 2.

Table 2 -Estimation of the influence of matrix components and impurity compounds (t table = 4.303; n=2; P=0.95)

№	Component	Heparin concentration in solution, g / dm^3		t calculated time
		Before the post component was introduced	After the introduction of the post component	
1	Benzyl alcohol	14,4±1,4	14,9 ±1,6	2,43
2	Valine		14,8±1,9	2,75
3	Lysine		14,9±1,8	3
4	Glutamic acid		14,4±1,6	1,67
5	Dermatan sulfate		28,6±3,2	7,76

This study showed that dermatan sulfate interferes with the determination of heparin by the developed spectrophotometric method, so it was proposed to conduct a preliminary analysis of heparin samples by capillary electrophoresis.

The presence of amino acids (leucine, valine, glutamic acid), as well as benzyl alcohol, does not affect the results obtained during the development of a spectrophotometric method for determining heparin using a TB dye, so this analysis is performed without preliminary sample preparation.

3.10. Method of quantitative determination of heparin

To develop a method for determining heparin in combination with indamine dye toluene blue in a background solution of 1mm HCl with a concentration of toluene blue in a $5*10^{-5}$ M system, the choice of the dye concentration is due to the composition of the Hep – (TB) complex, the absorption spectra in the region from 400 to 800 nm depending on the concentration of heparin figure 9.

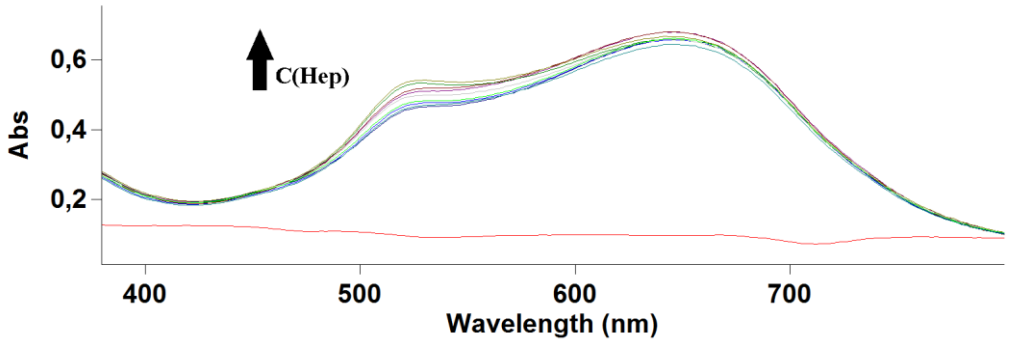

Fig. 9 -absorption Spectra of the Hep – $(TB)_2$ complex with different concentrations of heparin in the system, the concentration of TB $5*10^{-5}$ M C (Hep) from 6 to 24 mg/l

Based on the experimental data obtained (from figure 9), a graph of the dependence of the intensity of the optical density on the concentration of heparin is constructed (figure 10).

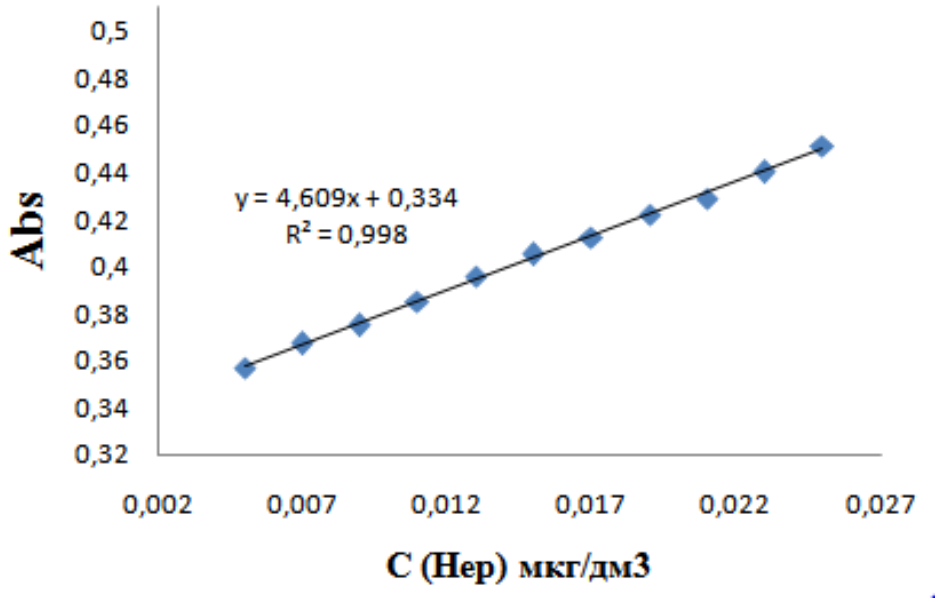

Fig. 10 - Dependence of the optical density intensity on the concentration of heparin

Figure 10 shows that the graph is linear in the range of concentrations of heparin from 6 to 24 mg / l with R^2=0.998. The correctness of the method of quantitative determination of heparin in model solutions was checked by the "introduced-found" method, table 3.

Table 3 Quantitative determination of heparin in model solutions (n=3; P=0.95)

№	Introduced (mg / l)	Found (mg / l)	S_r	Δ,%
1	6	6,02	0,01	0,33
2	10	10,01	0,005	0,1
3	18	18,03	0,015	0,17
4	22	22,02	0,01	0,09

Table 3 shows that the data obtained by the spectral method using toluylene blue are satisfactory. Based on this, the developed method can be used for quantitative determination of heparin in medicinal products (table 4).

Table 4 Results of quantitative determination of heparin in medicinal products (n=3; P=0.95)

№	Investigational drug	The heparin content stated in the instructions for use in terms of mg / ml	Results of spectral determination of heparin using TB, mg / ml
1	Pharmacological solution of sodium heparin for injection, produced by JSC "Bryntsalov-A", contains 5000 UNITS in 1 ml.	38,5	38,3±0,53
2	Pharmacological solution of heparin for intravenous and subcutaneous administration, produced by FSUE "Moscow endocrine plant", contains 5000 UNITS in 1 ml	38,5	38,4±0,3

According to the conducted research, the quantitative content of heparin in medicines corresponds to that stated in the instructions.

4. Conclusion

1. The structure of heparin was confirmed by methods of physicochemical analysis, in particular by infrared spectrometry. According to the results of IR spectroscopy, it was found that heparin contains the following functional groups: O-H**,** C-O, O=S=O, C-O-C, N-H, C-H.

2. The analysis for the presence of foreign impurities by capillary electrophoresis was performed. According to the results of capillary electrophoresis, the heparin used in research does not contain impurities.

A number of indicators were determined in accordance with the SF RF XIV (solubility, color, density, turbidity, pH, decomposition temperature, refractive index (refractive index), rotation angle.

3. According to the results of UV visible spectroscopy, heparin does not absorb in the UV region. Due to its complexing properties, the polyanion heparin molecule forms a complex with the cationic dye toluylene blue (TB) in the ratio 1:2 (Hep $(TB)_2$).

4. Operating conditions were selected for the determination of heparin in model solutions. A method for its quantitative determination in the range of heparin concentrations from 6 to 24 mg/l with R^2=0.99 is proposed.

5. Determination of heparin in medicinal products by the developed spectral method using toluene blue dye was performed.

6. Draft pharmacopoeia article on sodium heparin is proposed.

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