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DETERMINATION OF MAIN ACTIVE COMPONENTS IN LIQUID EXTRACT «HEPAPHYT»

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ABSTRACT

Methods have been developed for the qualitative and quantitative content of main biologically active substances in the liquid extract "Hepaphyt", which is recommended as a hepatoprotective agent. The quantitation methods were validated in accordance with the ICH (International Council on Harmonization) Topic Q 2 (R1) "Validation of Analitical Procedures: Text and Methodology" recommendations. Validation parameters for the analytical procedure, such as accuracy, specificity, linearity and reproducibility, were used.

KEYWORDS: liquid extract, validation, total flavonoids, glycyrrhizic acid (GA), specificity, linearity, precision and accuracy.

INTRODUCTION

The developed extract is obtained on the basis of Corn stigmas, Sant Wort herb and Licorice roots.[1] Preliminary pharmacological studies,[2] showed the presence of hepatoprotective and anti-inflammatory effects. According to literature data and the composition of objects, the indicated pharmacological properties are possessed by substances of the flavonoid group (Corn stigmas and Sant Wort herb) and glycyrrhizic acid (Licorice roots).

The aim of the study was to develop a method for controlling the quality of a liquid extract based on the content of the total flavonoids content (TFC) and glycyrrhizic acid (GA).

EXPERIMENTAL
MATERIALS AND METHODS

The samples of the liquid extract were used as materials.
Apparatus. UV spectrophotometer UV-1800 (Shimadzu, Japan) and high-performance liquid chromatograph Agilent 1200 Series (Agilent, USA) were used in the study.

Chemicals and Materials. $AlCl_3$ (Sigma-Aldrich), Glacial Acetic acid were analytical grade, Acetonitrile HPLC grade were used. Quercetin was obtained from Sigma-Aldrich. Purified water was obtained by bi-distillation method at the laboratory. 3 batches of liquid extract were used for analysis.

The detection of the main groups of biologically active substances were carried out by using UV spectrophotometer and high-performance liquid chromatography (HPLC).[3]

TFC was determined by UV spectrophotometric method. 1.0 ml of the extract was placed into a 25 ml volumetric flask, 3 ml of 2% ethanolic solution of aluminum chloride, one drop of diluted acetic acid was added and diluted to volume with 96% ethanol. The solution was stirred and placed in a dark place. After 40 minutes, the solution is filtered through a paper filter. Finally, the absorbance of the resulting mixture was measured with spectrophotometer in a 1^{-cm} path length quartz cell at 422 nm against blank solution. As a blank solution is used a solution, which is prepared in the same manner, but without adding a solution of aluminum chloride. Each sample was measured in parallel three times.

At the same time, the optical density of a working standard sample solution of quercetin, which is prepared similar to the test solution, was measured.

TFC(X), in%, in terms of quercetin, is calculated by the following formula:

$$X = \frac{D_1 \times a_0 \times 1 \times 25 \times 100 \times P}{D_0 \times 1 \times 100 \times 25 \times 100} = \frac{D_1 \times a_0 \times P}{D_0 \times 100},$$

Where
D_1– the optical density of the test solution;
D_0– the optical density of a quercetin standard solution;
a_0– the weight of a standard sample of quercetin, g;

P – the content of quercetin in a standard sample, %;
1–the volume of the test solution.

Preparation of a standard sample solution of quercetin. 0.05 g of a working standard sample of quercetin, pre-dried at a temperature of (130 – 135°C) for 3 hours, was placed into a 100 ml volumetric flask and dissolved at 85 ml of 96% ethanol while heating in a water-bath. After cooling till room temperature, dilute solution to volume with the same solvent and stirred. 1.0 ml of the prepared solution is placed into a 25 ml volumetric flask and then do in the same manner, when preparing the test solution, starting from the words "... add 3 ml of 2% aluminum chloride solution ..." to "... without adding aluminum chloride solution.

Preparation of 2% aluminum chloride solution. 2.0 g of aluminum chloride is placed into a 100 ml volumetric flask, dissolved in 96% ethanol and dilute to volume with the same solvent.

The GA was determined by HPLC method. 10.0 ml of the liquid extract was placed into a 50 ml volumetric flask, dilute to volume with the mobile phase and mixed. The resulting solution is filtered through 0.45 μm membrane filter.

20 μl of sample solution and working standard solution was injected into chromatograph Agilent 1200.The chromatograph was equipped with a UV detector and an isocratic pump. At result was obtain at least 3 chromatograms for each of the solutions, under the next conditions:
- Chromatographic column - 3.0 x 150 mm, packed with Zorbax Eclipse XDB C-18 sorbent, with a particle size of 3.5 microns;
- Detector wavelength - 254 nm;
- Mobile phase - acetonitrile: water: glacial acetic acid (190: 307: 3), previously degassed and filtered through 0.45 μm membrane filter;
- Flow rate - 0.75 ml / min;
- Injection volume - 20 μl;
- Analysis time - 15 minutes.

The content of GA(X) in 1 ml of the liquid extract, in mg, is calculated by the following formula:

$$X = \frac{S_t \times a_{std} \times 50 \times P \times 822{,}94}{S_{std} \times 10 \times 50 \times 100 \times 839{,}97} = \frac{S_t \times a_{std} \times P \times 822{,}94}{S_{std} \times 1000 \times 839{,}97}$$

Where:
S_t– the area of the peak of GA on the chromatogram of the test sample solution;
S_{std} – the area of the GA peak on the chromatogram of the working standard sample solution;
a_{std} – weight of a sample of working standard of monoammonium salt of GA, mg;
822.94 – the molecular weight of GA;
839.97 – molecular weight of GA monoammonium salt;
P – The content of the monoammonium salt of glycyrrhizic acid in the working standard sample, %.
*Preparation of a standard sample solution of GA monoammonium salt.*Weigh accurately about 20 mg of the working standard sample of the monoammonium salt of GA is placed into a 50 ml volumetric flask, dissolved in the mobile phase, dilute to volume and mixed. The resulting solution is filtered through 0.45 μm membrane filter.

RESULTS AND DISCUSSION

Qualitative and quantitative determination of TFC in the liquid extract was carried out by the spectrophotometry method.

It can be seen from Figure 1, the maximum absorption of spectrum of the liquid extract solution coincides with the maximum absorption of the working standard sample of quercetin at a wavelength of 422 ± 1 nm. This fact proves the presence of flavonoids in the liquid extract.

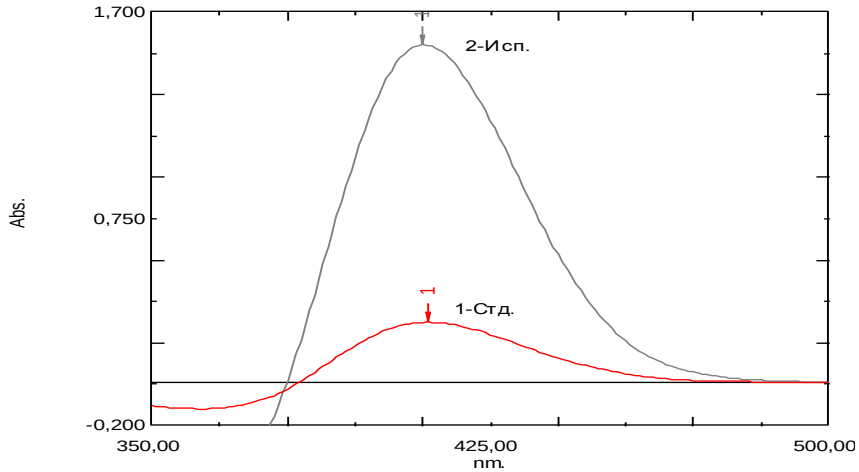

Fig. 1: Absorption spectrums of the extract solution and the quercetin standard sample solution after interaction with the $AlCl_3$. 1 - quercetin solution, 2 - test sample (extract).

HPLC was used for the qualitative and quantitative analysis of GA. The identification of the GA was proved

by comparing the retention time of the main peak of the test sample with the peak of the working standard sample of GA solution. The identity of the retention times is shown in Figures 1 - 4.

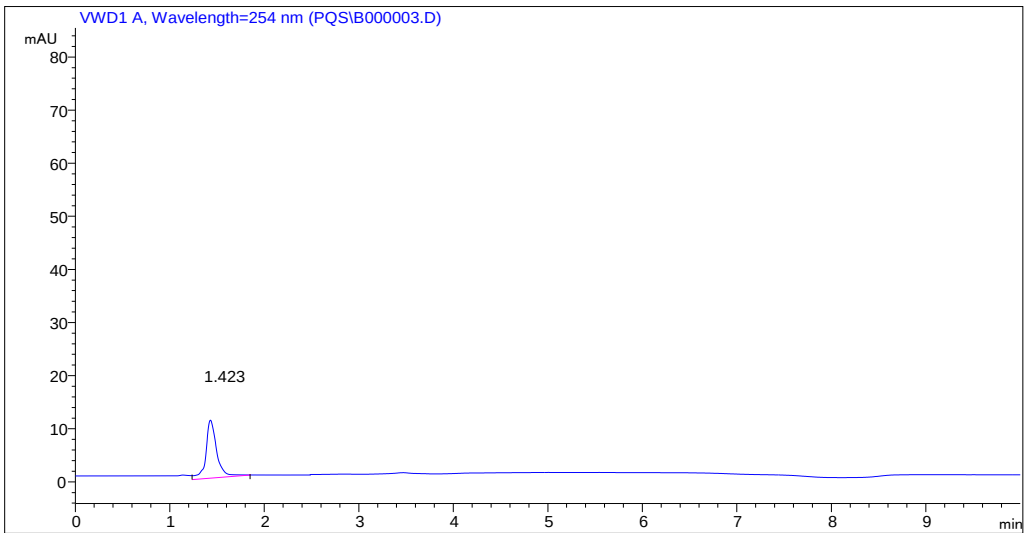

Fig. 2: HPLC chromatogram of the extractant solution.

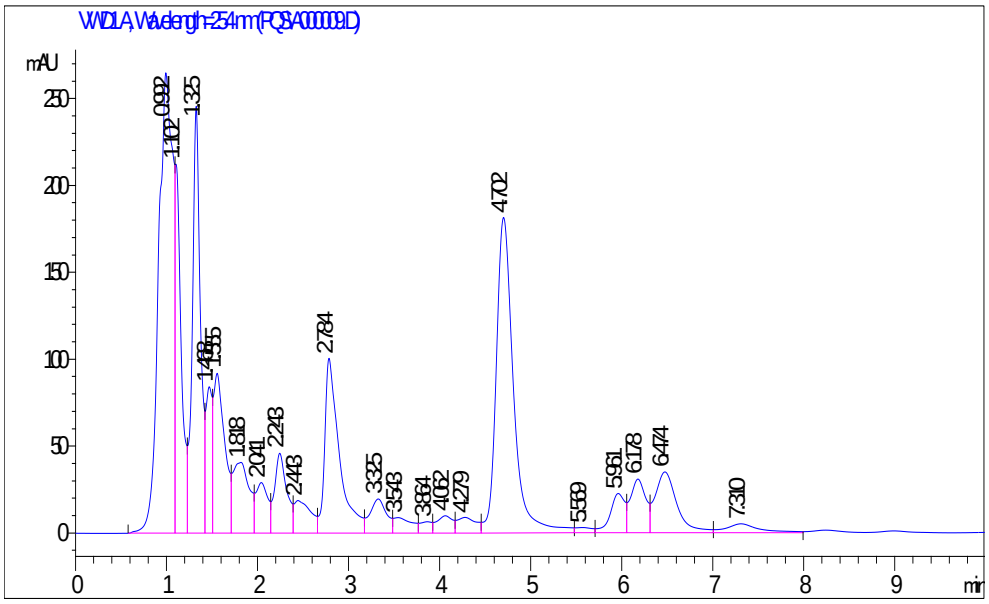

Fig. 3: HPLC chromatogram of the Hepaphyt extract.

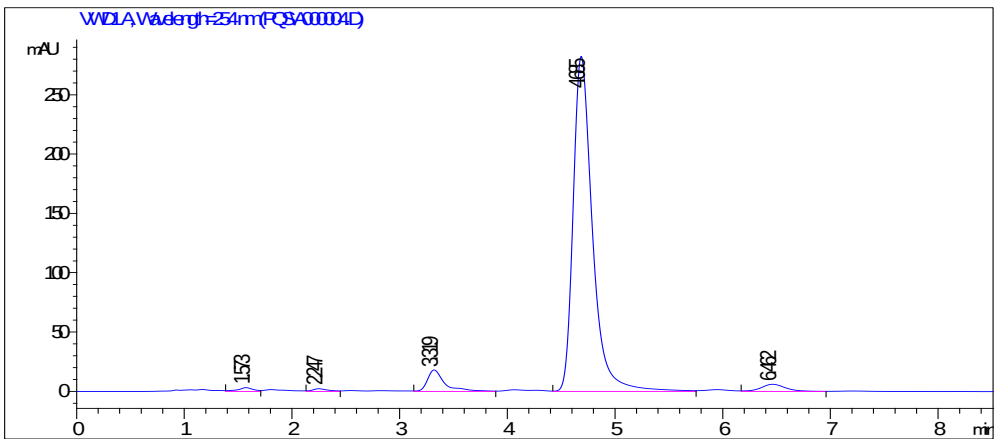

Fig. 4: HPLC chromatogram of the working standard sample of GA.

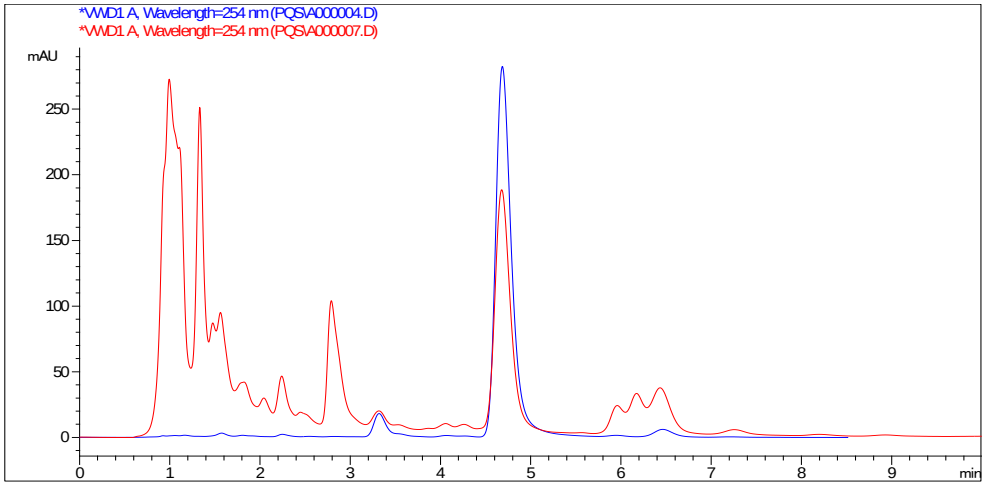

Fig. 5: Combined chromatogram of the working standard sample of GA (blue line) and Hepaphyt extract (red line).

The chromatograms of solutions of the extractant, extract and working standard sample, which is shown at the figures 1 - 4, show the degree of separation of substances in the research object. In particular, the main peak of GA with a retention time of 4.8 min (Fig. 3) corresponds to a similar peak in the chromatogram of the extract with a retention time of 4.7 min (Fig. 2). The combined chromatogram of both solutions (Fig. 4) shows that under developed chromatographic conditions, GA a clear separation from other related substances of the extract.

The obtained results for the quantitative determination of GA and TFC in the liquid extract were statistically processed and presented in Table 1.

Table 1: Metrological characteristics of quantitative determination of GA and TFC.

TFC determination results									
X_i	0,0569		0,0572	0,0572		0,0563		0,0576	
Metrological characteristics									
N	F	P (%)	t (P, f)	$\overline{X}$	S2	S	$\Delta\overline{X}$	$\overline{\varepsilon}$,%	RSD
5	4	95	2,78	0,057	$2,3*10^{-7}$	$4,83*10^{-4}$	$6*10^{-4}$	1,05	0,85
GA determination results									
X_i	0,3027		0,2979	0,3003		0,2958		0,2997	
Metrological features									
N	F	P (%)	t (P, f)	$\overline{X}$	S^2	S	$\Delta\overline{X}$	$\overline{\varepsilon}$,%	RSD
5	4	95	2,78	0,299	$6,7*10^{-6}$	$2,6*10^{-3}$	$3,2*10^{-3}$	1,07	0,87

During experiments validation tests were carried out on the developed methods for the quantitative determination of biologically active substances in the liquid extract "Hepaphyt" in accordance with the requirements of the ICH.[4,5] The obtained results are presented in tables № 2,3.

Table 2: Results of validation of the spectrophotometry method for determining the TFC in the liquid extract "Hepaphyt".

Criterion	**Norma**	**Results**
Specificity	The spectrum of the test solution should be identical to the spectrum of the standard sample and have a similar absorption maximum.	The spectrum of the test solution is identical to the spectrum of the standard sample and have a similar absorption maximum at 422 nm.
Linearity	Correlation coefficient | r | ≥0,99	y = 0,0038x + 0,0132 R^2 = 0,9989
Range	±20% from the declared value of the content	From 0,0125% to 0,2%
Accuracy, RSD	No more than 3%	±0,917%
Precision, RSD	No more than 3%	±1,521%

The presented results in the table show that the method for quantitative determination of TFC is accurate and reproducible.

Table 3: Results of validation of the HPLC method for determining GA in liquid extract "Hepaphyt.

Criterion	Norma	Results
Specificity	The retention time of the GA peak of the test sample solution should correspond to the retention time of the GA peak of the standard sample solution.	The retention time of the GA peak of the test sample solution corresponds to the retention time of the GA peak of the standard sample solution
Linearity	Correlation coefficient $\lvert r \rvert \geq 0,99$	$y = 0,0026x + 0,0025$ $R^2 = 0,9999$
Range	±20% from the declared value of the content	From 0,0625% to 1,0%
Accuracy, RSD	No more than 3%	±0,928%
Precision, RSD	No more than 3%	±1,746%

The presented results in the table showed that the method for quantitative determination of GA is accurate and reproducible.

Based on the obtained results, a specification was drawn up for the liquid extract. The resulted data are included in the relevant regulatory documents.

CONCLUSIONS

It is concluded from the results of the study that, the developed methods for the qualitative and quantitative determination of biologically active substances in the liquid extract "Hepaphyt" have been validated. The obtained results prove the accuracy and reproducibility of the methods, which allows them to be included in the monograph.

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