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Development of Methods of Quality Control for the complex Herbal Extract of "Cholophyt" Syrup

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ABSTRACT

At present there is a trend of increasing drugs containing Biologically Active Substances (BAS) of the plant origin in their composition. Standardization of the medicinal plant raw material and development of methods of quality control for multi-component drugs of the plant origin are one of the topical problems of modern pharmaceutical science. The work in development of methods of the quality control for biologically active substances of the complex herbal extract in the composition of "Cholophyt" syrup has been conducted in order to create the corresponding sections of the analytical normative documentation for the drug. The presence of the amount of hydroxycinnamic acids, being similar with the reference sample by the structure, has been proven in the herbal extract and in the syrup by the methods of absorption spectrophotometry and thin-layer chromatography compared to the reference standard of chlorogenic acid. The quantitative content of BAS was studied in the dosage form under research by the method of absorption spectrophotometry in the ultraviolet and visible region in 20% solution of ethanol at the wavelength of 237 nm. The content of the amount of hydroxycinnamic acids was calculated by the method of standard and specific absorbance calculated with reference to chlorogenic acid. According to the results of studying the validation characteristics it has been found that the method for quantitative determination of the amount of hydroxycinnamic acids in the syrup calculated with reference to chlorogenic acid is accurate, precise and linear ($\Delta_z = 1.02 \leq max\ \Delta_z = 1.60$, $\delta = 0.47 \leq max\ \delta = 0.51$, $a = |0.695| \leq max\ a = 2.60$, $r = |0.9999| \geq min\ r = |0.9981|$).

Keywords: Syrup, TLC, Validation of analytical methods, Quantitative determination, Spectrophotometry

INTRODUCTION

At the Chemist's Technology of Drugs Department of the National University of Pharmacy (Kharkiv, Ukraine) the research is underway to develop the technology of the syrup with the hepatoprotective and choleretic action on the basis of natural low-calorie sugar substitutes–the herb of stevia and sorbitol [1].

Taking into account the approach to standardization of drugs containing plant components it is expedient to determine Biologically Active Substances (BAS) or groups that are common to all components when identifying these drugs [2,3]. If a drug contains plant components with the similar composition or there is a very large number of components, then in the first case identification of BAS or their groups that are characteristic for several components is carried out, and in the second case the typical components, which are included in the herbal drug in a larger amount or have the greatest activity, are determined. The systematic analysis of different approaches to quantitative determination of the medicinal plant raw material and complex drugs in the State Pharmacopoeia of Ukraine (SPhU) has shown that the most reliable methods of standardization are determination of conditional concentrations by spectrophotometry and control of the signal components using chromatographic methods [4].

The aim of our work was to develop the methods of the quality control for BAS of the complex herbal extract in the composition of the syrup.

MATERIALS AND METHODS

The study object is dosage form syrup containing the complex herbal extract and such excipients as sorbitol, glycerine, Hydroxyethylcellulose (HEC), citric acid and sorbic acid. To prepare the complex herbal extract the standardized medicinal plant raw material of *Cynarae folium* (batch 001, manufacturer–PJSC "Liktravy"), *Rosae pseudo-fructus* (batch 0010216, manufacturer–PJSC "Liktravy"), *Stevia herba* (batch 002, manufacturer–PJSC "Liktravy"), *Helichrysi arenarii flores* (batch 160916, PJSC "Viola"), *Zeae maydis styli cum stigmatis* (batch 10215, manufacturer–PJSC "Liktravy") was used.

The analytical studies were carried out by thin-layer chromatography on TLC plates with Silica gel 60 F_{254}, 25 Aluminium sheets of 20 × 20 cm ("Merk", Germany), and by spectrophotometry using an Evolution 60S spectrophotometer ("Thermo Fischer Scientific", USA). The sample preparation was performed using AXIS ANG200 electronic balance (Poland) and the measuring glassware of class A.

As a standard the reference standard of chlorogenic acid (RS, SPhU, batch 03), as well as excipients and reagents meeting the requirements of the SPhU, were used.

Thin Layer Chromatography (TLC)

Test solution a. To 0.5 ml of the herbal extract add 10 ml of alcohol (60% v/v) R and mix.
Test solution b. To 0.5 g of the syrup add 10 ml of alcohol (60% v/v) R and mix.

Reference solution a. Dissolve 5 mg of chlorogenic acid RS and 5 mg of caffeic acid RS in methanol R and dilute the volume of the solution to 10 ml with the same solvent.

Plate: TLC plate with the layer of silica gel F_{254} (25 μm).

Mobile phase: Anhydrous formic acid R-glacial acetic acid R-water R-ethyl acetate R (11:11:27:100). *Spotting volume*: 10 μl, 10 mm bands. Distance covered by the mobile phase 13 cm from the start line.
Drying: In the air.
Development: Heat the plate at 100°C for 5 min; spray the warm plate with the solution of 10 g/L of diphenylboric acid aminoethyl ester R in methanol R, then with the solution of 50 g/L of macrogol 400 R in methanol R, observe in UV light at the wavelength of 365 nm.

Assay: Place 0.500 g of the syrup studied into a 50 ml volumetric flask and dilute to the volume with 20% ethyl alcohol.

Reference solution: Place approximately 0.05 g (accurate weight) of chlorogenic acid (*RS*) into a 100 ml volumetric flask, dilute in 20% solution of ethyl alcohol, then dilute the solution to the volume with the same solvent and mix. Place 1.0 ml of the solution obtained into a 50 ml volumetric flask, dilute to the volume with 20% ethyl alcohol and mix.

The optical density of the test solution and the reference solution was measured using a spectrophotometer at the wavelength of 327 nm.

Compensation solution: 20% ethanol.

The content of the amount of hydroxycinnamic acids calculated with reference to chlorogenic acid (*X*, %) was calculated by the method of standard according to the formula:

$$x, \% = \frac{A \cdot m_{st} \cdot 50 \cdot 100\%}{A_{st} \cdot m \cdot 100 \cdot 50}$$

And by the method of specific absorbance:

$$\mathrm{X}, \% = \frac{A \cdot V}{m \cdot A_{1\%}^{1cm}}$$

Where, *A*–is the optical density of the test solution; A_{st}–is the optical density of the reference solution; *m*–is the sample weight of the syrup studied, g; m_{st}–is the sample weight of the chlorogenic acid *RS*, g; 50 and 100–are the volumes of dilution, ml; $\mathrm{A}_{1\%}^{1cm}$ – is the specific absorbance of chlorogenic acid at the wavelength of 327 nm that equals 531 [5]. In the syrup the content of the amount of hydroxycinnamic acids calculated with reference to chlorogenic acid should be from 0.10 to 0.15%.

RESULTS AND DISCUSSION

To identify biologically active substances in the syrup under research the method of absorption spectrophotometry in the ultraviolet region and the method of thin-layer chromatography was used. According to the literature data almost all medicinal plant raw material (the artichoke leaves, rose hips, the herb of stevia, the immortelle flowers, and corn silk) in the composition of the herbal extract of the syrup contains the complex of substances of the polyphenol structure [6-11].

The previous content of BAS of the complex herbal extract was determined using absorption spectrophotometry in the ultraviolet region. The aqueous extracts of the artichoke leaves, rose hips, the herb of stevia, the immortelle flowers, and corn silk, the complex herbal extract were prepared and their UV absorption spectra were studied in 20% ethanol (Figure 1).

The results obtained show that the absorption spectrum of the aqueous-alcoholic solution of *Rosae pseudo-fructus* is characterized by the absorption maximum at the wavelength of 280 nm and has a plateau at 327-330 nm. In the absorption spectrum of the aqueous-alcoholic solution of *Helichrysi arenarii flores* two absorption maxima at the wavelengths of 290 nm and 326 nm, as well as a plateau at 250-252 nm are observed. In the absorption spectrum of the aqueous-alcoholic solution of *Cynarae folium* two rather flat maxima are observed at the wavelengths of 298 nm and 326 nm. In its turn, the absorption spectrum of the aqueous-alcoholic solution of *Stevia herba* is characterized by a wide plateau in the region from 296 nm to 307 nm and the absorption maximum at the wavelength of 327 nm.

In the absorption spectrum of the aqueous-alcoholic solution of *Zeae maydis styli cum stigmatis* the wide plateau in the region from 263 nm to 269 nm and in the region from 327 nm to 331 nm there is a rather flat shoulder. The absorption spectrum of the complex herbal extract is characterized by two quite intense maxima at the wavelengths of 287 nm and 324 nm. Therefore, in all absorption spectra of the test solutions there are characteristic absorption bands in the region of 290-327 nm, they indicate the presence of substances of the aromatic nature [12]. Moreover, the absorption spectrum of 0.001% chlorogenic acid solution in the region of 220-400 nm is characterized by two peaks at wavelengths of 234 nm and 327 nm and two shoulders in the areas of 239-242 nm and 280-285 nm (Figure 1).

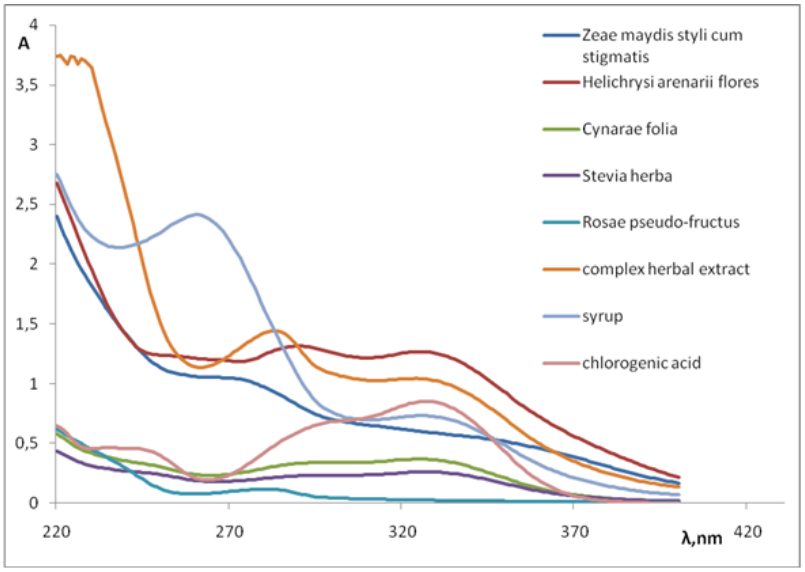

Figure 1: The UV absorption spectra in 20% solution of ethanol

The next stage of our studies was to use thin-layer chromatography for identification of BAS in the complex herbal extract introduced to the syrup composition, as well as in the syrup itself. With this purpose the following chromatographic conditions were used. The tests were carried out on TLC plates with Silica gel 60 F_{254}; the samples were diluted with 60% ethanol; such mixture of solvents as anhydrous formic acid–glacial acetic acid–water–ethyl acetate in the ratio of 11:11:27:100 was used as a mobile phase; detection was performed by viewing chromatographs in UV light at the wavelength of 365 nm after complete drying and spraying the plates with the solution of diphenylboric acid aminoethyl ester in methanol and the solution of Macrogol 400 in methanol. The samples were compared with the reference standards of chlorogenic acid, caffeic acid and rutin (Figure 2).

The sequence of areas on the chromatograms of the test solutions *a*, *b* and the reference solution is given below. On the chromatogram of the test solution other fluorescent areas can appear.

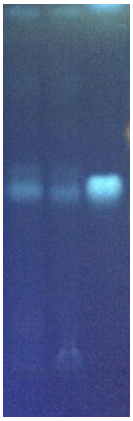

Figure 2: The chromatogram obtained when identifying phenolic compounds in "Cholophyt" syrup for: (1) the test solution of the syrup; (2) the solution of the complex herbal extract; (3) the reference solution

On the chromatogram of the reference solution there should be the areas (in the order of increasing R_f) with chlorogenic acid and caffeic acid. Specificity of the method was confirmed by comparing the chromatograms of the test solution of the syrup and the test solution of the complex herbal extract. On the chromatogram of the syrup studied there were zones of chlorogenic acid with a blue fluorescence, which by retention values coincided with the zones of chlorogenic acid on the chromatogram of the test solution of the complex herbal extract (Figure 2).

Therefore, the validation characteristics studied (specificity, chromatographic system suitability) correspond to the specified acceptance criteria. Based on the data obtained by thin-layer chromatography, which results are given in Figure 2, it can be argued about the presence of the amount of hydroxycinnamic acids, which are mainly similar in structure to chlorogenic acid, in the complex herbal extract and in the syrup. Quantitative determination of the amount of hydroxycinnamic acids calculated with reference to chlorogenic acid was conducted by absorption spectrophotometry in the UV region in 20% ethanol at the wavelength of 327 nm.

To develop the method for quantitative determination it was necessary to study the nature of the absorption spectrum of the complex herbal extract, compare it with of the absorption spectrum of the alcoholic extract from the syrup and the spectrum of placebo (Figure 3).

The results (Figure 3) indicate that in the absorption spectrum of the aqueous-alcoholic solution of the complex herbal extract, the solution of the syrup and chlorogenic acid there was a rather flat maximum at the wavelength of 327 nm, and it was chosen for quantitative determination of the amount of hydroxycinnamic acids in the dosage form under research.

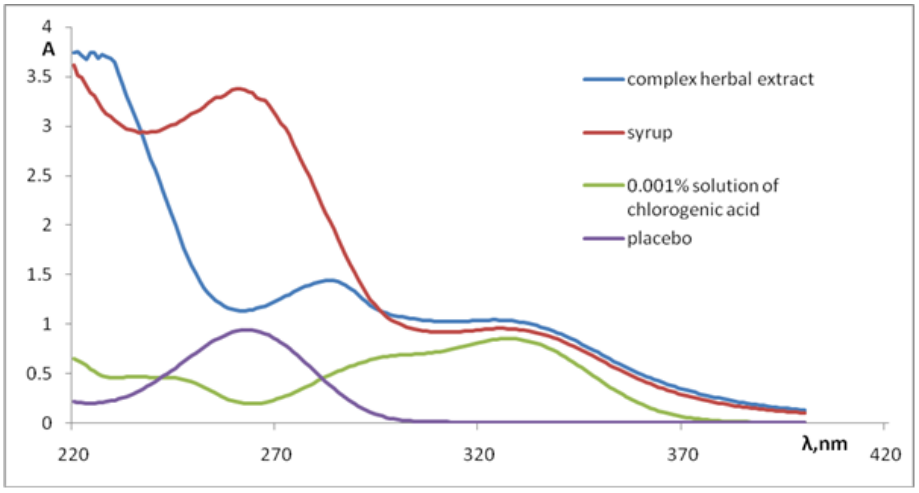

Figure 3: The absorption spectrum in 20% solution of ethanol

According to the requirements of the SPhU the validation of the method for determining the amount of hydroxycinnamic acids in the syrup calculated with reference to chlorogenic acid was carried out by absorption spectrophotometry for inclusion in the analytical documentation (max Δ_{As} 1.60%). The validation of the method was performed by the method of mathematical statistics according to the following parameters: specificity, linearity, accuracy and precision [13].

Specificity of the method was confirmed by the presence of the absorption maxima at the wavelength of 327 ± 2 nm in the absorption spectra of the complex herbal extract and the syrup studied in 20% ethanol. The position of the absorption maxima coincided with the position of the absorption maximum of the reference solution of chlorogenic acid (Figure 3), and it became a prerequisite for standardization of the dosage form by the amount of hydroxycinnamic acids calculated with reference to chlorogenic acid.

When developing the method, first of all, the placebo effect of the dosage form on the optical density of the amount of BAS studied was tested. It was found that the maximum of the analytical length of 327 nm was absent on the spectrum of the placebo solution (Figure 3). Moreover, the optical density of the background absorption obtained was not more than 0.002 (0.32% ≤ Δ_{As} 1.60%). Therefore, the background absorption in the method is statistically insignificant, and the requirements for specificity are met.

Linearity, accuracy and precision were studied on 9 model mixtures simultaneously in the range of the analytical method application for quantitative determination of chlorogenic acid within concentrations from 20 to 180% taking into account the nominal amount of hydroxycinnamic acids in the syrup. When determining linearity 9 model solutions were prepared with the range of concentrations from 20 to 180%, and their optical density was measured at the wavelength of 327 nm (Tables 1 and 2).

Table 1: The initial data for calculation of the validation characteristics of the method for quantitative determination

No. of the test solution	Introduced (Xi, %)	Optical densities Ai	Optical density Ast	Found (Yi, %)	Found in % to the introduced Zi=100(Yi/Xi)
1	20.16	0.107	0.525	20.38	101.10
2	40.32	0.214		40.76	101.10
3	59.54	0.315		60.00	100.77
4	80.56	0.424		80.76	100.25
5	100.96	0.533		101.52	100.56
6	119.38	0.630		120.00	100.52
7	140.36	0.739		140.76	100.29
8	159.42	0.840		160.00	100.36
9	180.16	0.939		178.86	99.28

Table 2: The results of studying linearity of the method for quantitative determination

Parameter of linearity	Value of the parameter	Criterion	Conclusion
b	0.9955	-	-
S_b	0.0038	-	-
a	0.6950	≤ |2.6|	Satisfied
S_a	0.4241	-	-
r	0.9999	> |0.9981|	Satisfsied
RSD_{range}	54.6726	-	-

The graphical depiction of the regression line for the test sample is the alternative of the mathematical evaluation of linearity (Figure 4).

According to the results obtained it has been found that in the range of the method application selected there is a directly-proportional ratio between the concentration of hydroxycinnamic acids in the sample determined and the optical density.

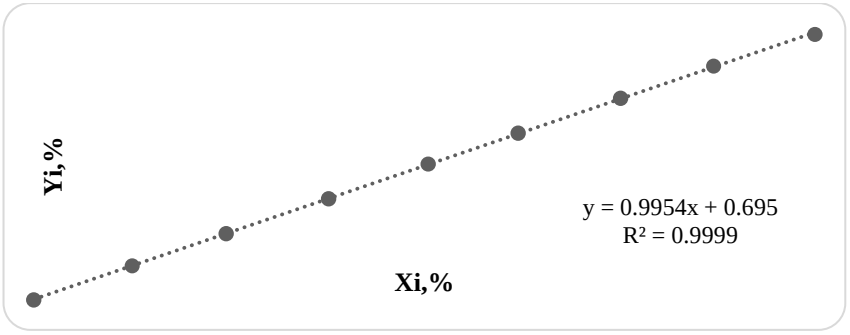

Figure 4: The plot of the linear dependence of the optical density on the concentration of the amount of hydroxycinnamic acids in the normalized coordinates

All linear dependencies are characterized by high correlation coefficients (r>0.9981) (Table 2 and Figure 4), i.e., linearity of the method is confirmed within the range of 20-180% of the concentrations selected.

To check the range of the method application (accuracy and precision) the amount of hydroxycinnamic acids in the syrup calculated with reference to chlorogenic acid (20-180% of the nominal concentration) was determined (Table 3).

Table 3: The results of studying precision and accuracy of the method for quantitative determination

Parameters		Values	Criterion 1	Criterion 2	Conclusion
Precision	ΔZ	1.02	≤ 1.60	-	-
Accuracy	$\|Z_{av}$ -100|	0.47	≤ 0.51	-	Satisfied according to Criterion 1

The experimental results for determining precision of the method are characterized by tolerance of values relative to the mean, as well as the relatively low standard deviation within the range of the concentrations studied. During our studies stability of the syrup solutions and the reference solution of chlorogenic acid was checked. The optical density was measured in 15, 30, 45 and 60 min after preparation. The results of the studies conducted are given in Table 4.

Table 4: The study of stability of the analytical solution

Model solution	Time of the stability studies t, min					Average	Sr	RSDt	Δt	δ_{max}, %
	0	15	30	45	60					
Test solution	0.525	0.526	0.526	0.525	0.525	0.525	0.0010	0.1042	0.22	0.51
Reference solution	0.537	0.536	0.538	0.536	0.536	0.537	0.0017	0.1667	0.36	

Reproducibility of the method was determined on six sample weights of the dosage form. The metrological characteristics obtained and calculated by the method of standard are presented in Table 5 and calculations using the absorbance method are given in Table 6.

Table 5: The metrological characteristics of the method for determining the amount of hydroxycinnamic acids in the syrup calculated with reference to chlorogenic acid by the method of standard, P(t,ν)=2.5706

n	The amount of biologically active substances found, %	x_{avg}	S^2	S	S_{Xavg}	Δ_x	ε,%
6	0.1011	0.1025	$6.79 \cdot 10^{-7}$	$8.24 \cdot 10^{-4}$	$3.36 \cdot 10^{-4}$	$3.53 \cdot 10^{-4}$	0.84
	0.1025						
	0.1029						
	0.1020						
	0.1035						
	0.1027						

Table 6: The metrological characteristics of the method for determining the amount of hydroxycinnamic acids in the syrup calculated with reference to chlorogenic acid by the absorbance method, P(t,ν)=2.5706

n	The amount of biologically active substances found, %	x_{avg}	S^2	S	S_{Xavg}	Δ_x	ε,%
6	0.0999	0.1013	$7.34 \cdot 10^{-7}$	$8.57 \cdot 10^{-4}$	$3.50 \cdot 10^{-4}$	$3.67 \cdot 10^{-4}$	0.89
	0.1013						
	0.1017						
	0.1008						
	0.1024						
	0.1016						

The validation characteristics studied indicate that the given method for quantitative determination of the amount of hydroxycinnamic acids calculated with reference to chlorogenic acid can be used to study biologically active substances of the complex herbal extract in the syrup.

CONCLUSION

The method for quantitative determination of the content of the amount of hydroxycinnamic acids calculated with reference to chlorogenic acid in the syrup has been developed. The main validation characteristics of the given method for quantitative determination such as specificity, linearity, accuracy and precision have been studied. They confirm correctness and prove suitability of the method developed.

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