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## DEVELOPMENT OF A UNIFIED APPROACH TO THE METHOD OF IDENTIFICATION, QUANTITATIVE DETERMINATION OF ACTIVE SUBSTANCES AND ACCOMPANYING IMPURITIES IN A COMBINED DRUG BY HPLC METHOD

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**The aim** of the work is to develop a method of identification, quantification of acetylsalicylic and ascorbic acids in the combined presence and concomitant impurities in the combined drug in the form of effervescent powder for preparation of oral solution by liquid chromatography and study of validation characteristics.

**Materials and methods.** ProStar liquid chromatograph with "Varian" spectrophotometric detector. Chromatographic column with a size of  $150 \times 4.6$  mm, filled with aminopropylsilyl silica gel for chromatography (Supelcosil LC-NH<sub>2</sub>, "Supelco") with a precolumn (particle size 3  $\mu$ m), mobile phase – buffer solution pH 3.2 – acetonitrile P (80:20), elution mode – isocratic; mobile phase velocity – 1.2 ml/min; the detection wavelength is 240 nm.

**Results.** To determine acetylsalicylic and ascorbic acids by high-performance liquid chromatography with UV detection, the optimal chromatographic conditions were selected considering the influence of other active and excipients in the drug. To prove the possibility of applying the proposed technique in the subsequent analysis of the effervescent powder, its validation was performed. The obtained validation characteristics indicate that the method of quantitative determination of acetylsalicylic acid in the studied dosage form corresponds to the parameters: accuracy, precision, linearity ( $\Delta z = 0.92 \leq \max \Delta z = 1.60$ ,  $\delta = 0.19 \leq \max \delta = 0.51$ ,  $a = 0.17 \leq \max a = 2.60$ ,  $r = 0.9994 \geq \min r = 0.9981$ ). In the quantitative determination of ascorbic acid in the combined effervescent powder it is established that the correctness, precision, linearity are performed ( $\Delta z = 0.86 \leq \max \Delta z = 1.60$ ,  $\delta = 0.02 \leq \max \delta = 0.51$ ,  $a = 1.99 \leq \max a = 2.60$ ,  $r = 0.9997 \geq \min r = 0.9981$ ).

**Conclusions.** A new method for the identification, quantification of acetylsalicylic and ascorbic acids in the combined presence and concomitant impurities in the effervescent powder using high performance liquid chromatography has been developed. The validation of the proposed method is carried out and its acceptability for use in pharmaceutical analysis is proved

**Keywords:** liquid chromatography, acetylsalicylic acid, ascorbic acid, effervescent powder, validation, accompanying impurities

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## 1. Introduction

In recent years, the approach to the development of chemical process technologies based on the principles of green chemistry has been improved. This approach applies not only to chemical synthesis and production in all sectors of the economy, but also quality control methods [1, 2].

The problems of "green chemistry" competencies are related to various areas (approaches). The introduction of one of them dramatically reduces the burden of chemical production on the environment and is associated with the processing, disposal and destruction of environmentally hazardous by-products and waste products of the chemical industry. The second, more promising approach involves the development of new industrial processes that would do without environmentally harmful products (including by-products) or reduce their use and formation [3–5].

The second approach could also include analytical methods that are constantly changing and improving. Re-

searchers around the world are developing and proposing new pharmaceutical research methods based on the principles of "green chemistry", which would be characterized by reducing the impact of toxic substances on the environment and production staff, the least waste and the lowest cost. This applies to chromatographic research methods that allow to determine the analytes in the sample without pre-treatment or sample preparation, reduce time and energy consumption for sample preparation, the amount of solvent for analysis, the need for re-analysis, integration of multi-stage analytical procedures in one step. Thus, from the well-known principles of green chemistry, three main principles of green high-performance chromatography emerged: reduce, replace, and recycle [6, 7].

During pharmaceutical development, the principles of standardization and quality control of medicines (drugs) are improved, which ensure the effectiveness and safety of quality control methods, determines the devel-

opment, unification and validation of methods of analysis. In accordance with the recommendations of the International Conference on Harmonization of Technical Requirements for the Registration of Medicinal Products for Human Use ICH Q6 «*Specifications: Test procedures and acceptance criteria for new drug substances and new drug products: chemical substances*» during pharmaceutical development should establish indicators that characterize drug quality. According to the pharmacopoeial requirements to determine the quality of the combined drug it is necessary to conduct tests on indicators: identification, quantification, the content of concomitant impurities using mainly physico-chemical methods.

The object of the study was selected a new combined dosage form for use for alcohol intoxication in the form of effervescent powder for the preparation of oral solution in two sachets. The first package consists of glutamic acid (GA), acetylsalicylic acid (ACA), ascorbic acid (AA), citric acid anhydrous (CAA) and sorbitol, the second package contains glycine, sodium bicarbonate and sorbitol.

One of the universal, highly sensitive and specific methods of analysis, which allows the simultaneous identification and control of the content of several active pharmaceutical ingredients (API) and possible impurities, is the method of high-performance liquid chromatography (HPLC) [8, 9]. Given that the study drug for the treatment of alcohol intoxication is combined [10], the simultaneous determination of several indicators by HPLC will reduce the duration of the analysis, its cost and ensure high accuracy and reproducibility of measurement results.

Methods of chromatographic determination of ACA and its impurities [11], AA and its metabolite dehydroascorbic acid [12], ACA in combination with other drugs [13] and AA in multivitamins [14] are known in the literature.

The aim of this study was to develop a unified chromatographic technique with its subsequent validation, which will be suitable to simultaneously identify, quantify the content of acetylsalicylic and ascorbic acids and their impurities in the new combined drug.

## 2. Planning (methodology) of the research

The main stages of the study were the development of chromatographic methods for identification and quantification of active substances of multicomponent dosage forms and its validation.

The planning of studies considered the peculiarities of the HPLC method to find the optimal chromatographic separation conditions and justify the choice of unified mobile phase: type of stationary phase (sorbent), chromatographic behaviour API considering the activity of unprotected silanols sorbent depending on  $pK_a$  API.

The selection and study of validation characteristics were carried out in accordance with the requirements of the SPhU for chromatographic methods.

## 3. Materials and methods

The object of the study was a combined original drug for the treatment of alcohol intoxication, in the form

of effervescent powder for oral solution, pharmacologically sound component composition of which is given in the publication [10] and consists in a study sachet of 0.500 g of glutamic acid; 0.325 g of acetylsalicylic acid; 0.100 g of ascorbic acid; 0.700 g of anhydrous citric acid and 2.375 g of sorbitol.

Experimental studies were performed on a ProStar liquid chromatograph from "VARIAN" using a ProStar 325 spectrophotometric detector and a ProStar 330 spectrophotometric diode array detector. Analytical scales of "Kern & Sohn GmbH" ABT 120-5DNM, pH meter "Seven Easy" of "Mettler Toledo", measuring vessels of class A and reagents meeting the requirements of EP/SPhU were used in the work.

The tests were performed by HPLC in accordance with the requirements of SPhU, 2.2.29 "Liquid chromatography" by the following method.

Chromatography is performed under the following conditions:

- 150×4.6 mm column filled with aminopropylsilyl silica gel for chromatography *P* (Supelcosil LC-NH2, 150×4.6 mm, Supelco) with a pre-column with a particle size of 3  $\mu$ m, for which the conditions of suitability of the chromatographic system are met ;

- speed of the mobile phase – 1.2 ml/min;

- column temperature – 45 °C;

- detection at wavelengths – 240 nm;

- injection volume – 20  $\mu$ l;

- mobile phase: buffer solution pH 3.2 – acetonitrile *P* (80:20), degassed in any convenient way;

- elution mode – isocratic.

The chromatography time is 10 minutes

*Preparation of buffer solution pH 3.2.* Place 1.00 g of potassium dihydrogen phosphate *P* in a 1000.0 ml volumetric flask, dissolve in 950 ml of water *P* and adjust to pH 3.2±0.05 with dilute phosphoric acid *P*, make up to volume with water *P* and stir.

*Quantitative determination of AA and ACA.* Standard substances AA and ACA of the European Pharmacopoeia (EP CRS) were used as reference substances. API: ACA (p. 21787, manufacturer Shandong Xinhua Pharmaceutical Co., Ltd), AA (p. DYD2622000008, manufacturer Northeast Pharmaceutical Group Co., Ltd).

*Test solution.* Place 4,000 g (exact weight) of the contents of the package in a 200.0 ml volumetric flask, add 40 ml of acetonitrile *P* and shake for one minute, make up to volume with water *P*, mix and filter through a "blue ribbon" filter paper. Place 10.0 ml of the resulting solution in a 250.0 ml volumetric flask, make up to volume with water *P* and mix. The solutions are used freshly prepared.

*Comparison solution (a).* Place 100.0 mg (exact portion) of EP CRS AA in a 100.0 ml volumetric flask, dissolve in 70 ml of 20 % acetonitrile solution *P*, make up to volume with the same solvent and mix.

*Comparison solution (b).* Place 65.0 mg (exact portion) of EP CRS ACA in a 100.0 ml volumetric flask, dissolve in 75 ml of 20 % acetonitrile solution *P*, make up to the mark with the same solvent and mix.

*Comparison solution (c).* Place 10.0 ml of reference solution (b) in a 100.0 ml volumetric flask, add

2.0 ml of reference solution (a), make up to volume with water *P* and mix.

The solutions are used freshly prepared.

Prior to chromatography, test and reference solutions were filtered through a membrane filter with a pore size of not more than 0.45 µm.

*Calculation of quantitative content.* The content of AA ( $X_1$ ) in one package, in milligrams, based on the average weight of the contents of the sachet, is calculated by the formula:

$$X_1 = \frac{S_1 \times m_0 \times 200 \times 250 \times 2 \times P \times b}{S_0 \times m \times 10 \times 100 \times 100 \times 100} =$$

$$= \frac{S_1 \times m_0 \times P \times b}{S_0 \times m \times 100},$$

where  $S_1$  – the average value of the areas of the AA peaks, calculated from the chromatograms of the test solution;

$S_0$  – the average value of the areas of the AA peaks calculated from the chromatograms of the comparison solution (c);

$m$  – the weight of the portion of the package taken for the preparation of the test solution, in milligrams;

$m_0$  – the mass of the portion of the EP CRS AA taken to prepare the reference solution (a), in milligrams;

$P$  – the content of the basic substance in EP CRS AA, taken to prepare the solution of comparison (a), in percent.

$b$  – the average weight of the contents of the package, in milligrams.

The ACA content ( $X_2$ ) in one package, in milligrams, based on the average weight of the sachet, is calculated by the formula:

$$X_2 = \frac{S_1 \times m_0 \times 10 \times 250 \times 200 \times P \times b}{S_0 \times m \times 10 \times 100 \times 100 \times 100} =$$

$$= \frac{S_1 \times m_0 \times P \times b}{S_0 \times m \times 20},$$

where  $S_1$  – the average value of the ACA peak areas calculated from the chromatograms of the test solution;

$S_0$  – the average value of the ACA peak areas calculated from the chromatograms of the comparison solution (c);

$m$  – the weight of the portion of the package No. 1, taken for the preparation of the test solution, in milligrams;

$m_0$  – the mass of the portion of the EP CRS ACA taken to prepare the reference solution (a), in milligrams;

$P$  – the content of the basic substance in the EP CRS ACA, taken to prepare the solution of comparison (a), in percent;

$b$  – the average weight of the contents of the package, in milligrams.

The content of C<sub>6</sub>H<sub>8</sub>O<sub>6</sub> (AA) in the package in terms of the average weight of the contents of one package should be:

– at release: from 95.0 mg to 105.0 mg in a package.

The content of C<sub>9</sub>H<sub>8</sub>O<sub>4</sub> (ACA) in the package in terms of the average weight of the contents of one package should be:

– at release: from 308.75 mg to 341.25 mg in a package.

*Accompanying impurities.* As a reference substance EP CRS SA was used (c. 3).

*Test solution.* Place 4,000 g (exact weight) of the contents of the package in a 200.0 ml volumetric flask, add 40 ml of acetonitrile *P* and shake for one minute, make up to volume with water *P*, mix and filter through a “blue ribbon” filter paper. Place 10.0 ml of the resulting solution in a 25.0 ml volumetric flask, make up to volume with water *P* and mix.

*Comparison solution (a).* Place 13.0 mg (exact portion) of EP CRS SA in a 20.0 ml volumetric flask, dissolve in 4 ml of acetonitrile *P*, make up to volume with water *P* and mix.

*Comparison solution (b).* Place 1.0 ml of reference solution (a) in a 100.0 ml volumetric flask, make up to volume with water *P* and mix.

*Comparison solution (c).* Place 1. ml of reference solution (b) in a 10.0 ml volumetric flask, make up to the mark with water *P* and mix.

*Solution for checking the suitability of the chromatographic system.* Place 1.0 ml of the test solution and 1.0 ml of reference solution (a) in a 100.0 ml volumetric flask, make up to the mark with water *P* and mix.

The solutions are used freshly prepared.

Prior to chromatography, test and reference solutions were filtered through a membrane filter with a pore size of not more than 0.45 µm.

Chromatography is performed under the conditions of the method “Quantitative determination of AA and ACA”.

Chromatograph the solution to check the suitability of the chromatographic system, comparison solution (c), comparison solution (b) and test solution at least 3 times.

*Standardization of the content of accompanying impurities.*

At the time of release:

– SA: on the chromatogram of the test solution the area of the SA peak should not exceed 0.5 of the area of the main peak on the chromatogram of the comparison solution (b) (0.50 %);

– any other impurity: on the chromatogram of the test solution, the peak area should not exceed 1.0 area of the main peak on the chromatogram of the reference solution (c) (0.10 %);

– sum of impurities: on the chromatogram of the test solution the sum of the areas of all peaks should not exceed 1.0 areas of the main peak on the chromatogram of the comparison solution (b) (1.00 %);

– do not consider: peaks with an area of less than 0.5 of the area of the main peak on the chromatogram of the comparison solution (c) (0.05 %). Peaks that coincide with the corresponding peaks on the blank chromatogram and AA and ACA peaks are also not considered.

During the shelf life:

– SA: on the chromatogram of the test solution, the area of the SA peak should not exceed 1.0 of the area of the main peak on the chromatogram of the reference solution (b) (1.0 %);

– any other impurity: on the chromatogram of the test solution, the peak area should not exceed 1.0 area of the main peak on the chromatogram of the reference solution (*c*) (0.1 %);

– sum of impurities: on the chromatogram of the test solution, the sum of the areas of all peaks should not exceed 1.5 areas of the main peak on the chromatogram of the reference solution (*b*) (1.5 %);

– do not consider: peaks with an area of less than 0.5 of the area of the main peak on the chromatogram of the comparison solution (*c*) (0.05 %). Peaks that coincide with the corresponding peaks on the blank chromatogram and AA and ACA peaks are also not considered.

Validation of the developed methods of quantitative determination of AA and ACA and accompanying

impurities was carried out in accordance with the requirements of Article 5.3.N.2 of SPhU [15].

#### 4. Research results

According to the recommendations of ICH Q8 in pharmaceutical development, the quality, efficacy, and safety of the drug are based [16]. Experimental studies are aimed at determining the target quality profile of the drug. To establish the quality of the drug, guided by the pharmacopeial requirements and recommendations of ICH Q6 [17].

The identification of AA and ACA is proposed to be performed by HPLC in the conditions of the method of quantification of the retention time of the peaks obtained for the test solution and standard samples (SS). Solvent (blank chromatogram) and placebo solutions were chromatographed in parallel (Fig. 1, a–g).

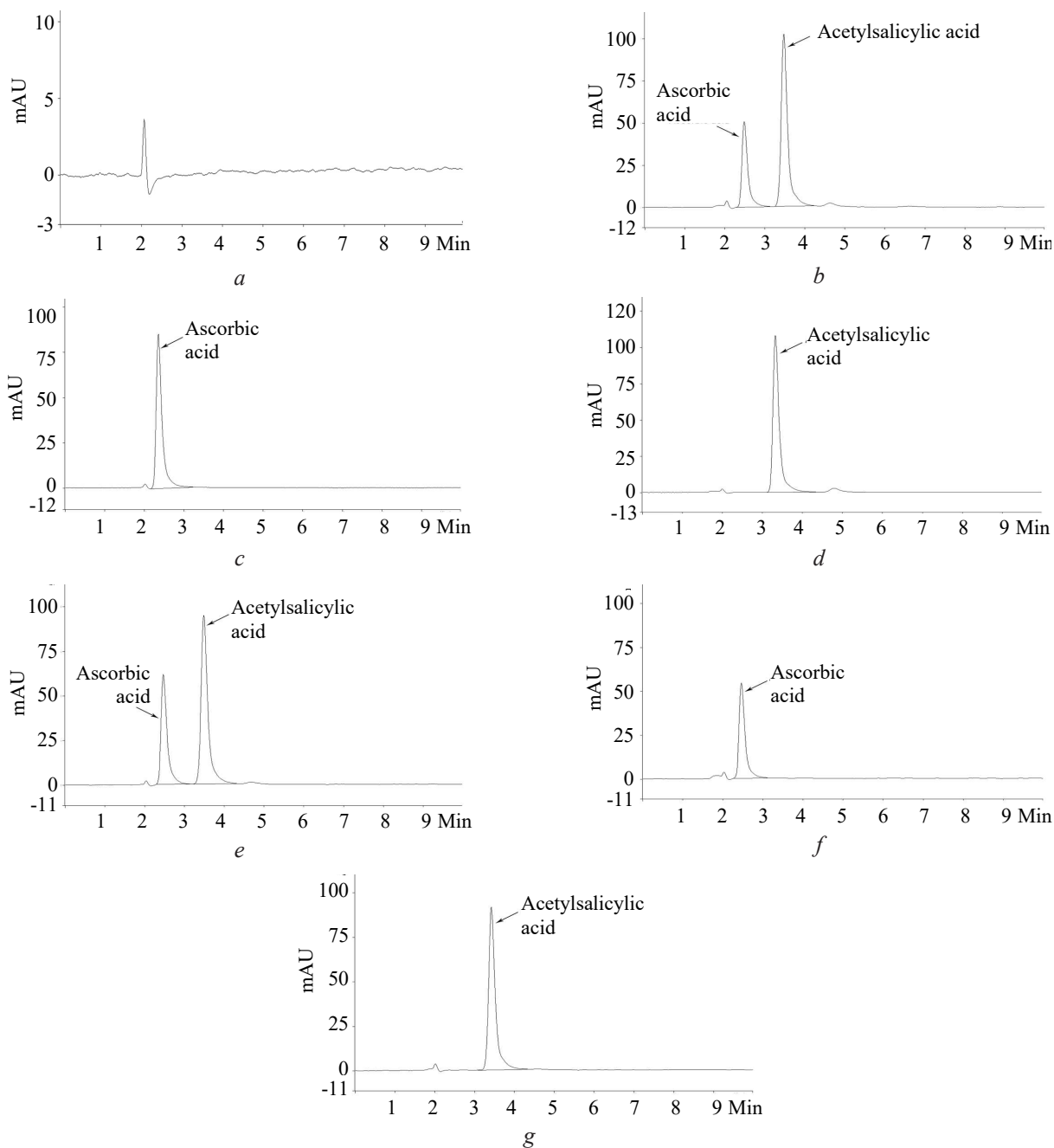


Fig. 1. Chromatograms: *a* – the blank solution; *b* – test solution; *c* – SS of AA solution; *d* – SS of ACA solution; *e* – comparison solution; *f* – placebo solution without ACA; *g* – placebo solution without AA

Under the proposed unified chromatographic conditions, the accompanying impurities were determined – salicylic acid (SA), which is the main product of ACA decomposition, which could be formed during storage of drugs. To confirm the possibility of identification of SA impurities and other impurities under the conditions of quantification of AA and ACA, solvent chromatography (blank chromatogram), comparison solution (c), comparison solution (b), placebo solution, solution for checking the suitability of the chromatographic system and test solution (Fig. 2, a–f).

To identify impurities, a chromatogram of the solution is used to check the suitability of the chromatographic system. The retention time of the SA peak is about 4.4 minutes. Peak exit order and relative retention times: AA (0.71), ACA (1.00), SA (1.41).

Comparison of chromatograms shows that in the conditions of the method of quantitative determination of AA and ACA the determination of impurities is not hindered by the solvent, mobile phase, or basic substances, which indicates the specificity of the method.

Verification of linearity, correctness and precision of the method was carried out by the method of “introduced-found”. The permissible concentration of AA and ACA at the time of issue is within  $\pm 5\%$  of the nominal values, for the study of linearity, accuracy and convergence, the range of

concentrations was chosen from 80 to 120 %, in increments of 5 %. Eligibility criteria were chosen for  $B=5\%$ , therefore, the maximum uncertainty of the analysis should not exceed 1.6 %. Quantitative determination of AA in model solutions in the range of  $\sim 16\text{--}24\text{ }\mu\text{g/ml}$ , and ACA –  $\sim 52\text{--}78\text{ }\mu\text{g/ml}$ .

Validation parameters for quantification of AA and ACA were evaluated (Table 2).

In Fig. 3 there is the dependence of the analytical signal on the actual concentration of AA and ACA solutions in normalized coordinates, which are linear.

In the study of robustness studied the stability of the test solutions (*test*) and comparison solutions (*rsd*) in time immediately after preparation, after 1 h and 6 h (Table 3).

To determine the limit of detection of accompanying impurities, the values of the signals obtained for the control experiment (blank solution) and for samples with low concentrations of the compound to be determined (determination of the signal-to-noise ratio) were compared. The limit of detection (*LD*) is sufficient for analysis and does not significantly affect the quality decisions for the limit tests if it is insignificant compared to the limit content of the impurity *ImL*:

$$LD \leq \max MB = \\ = 0.32 \times \text{Im } L \text{ (or } 32\% \text{ from Im } L).$$

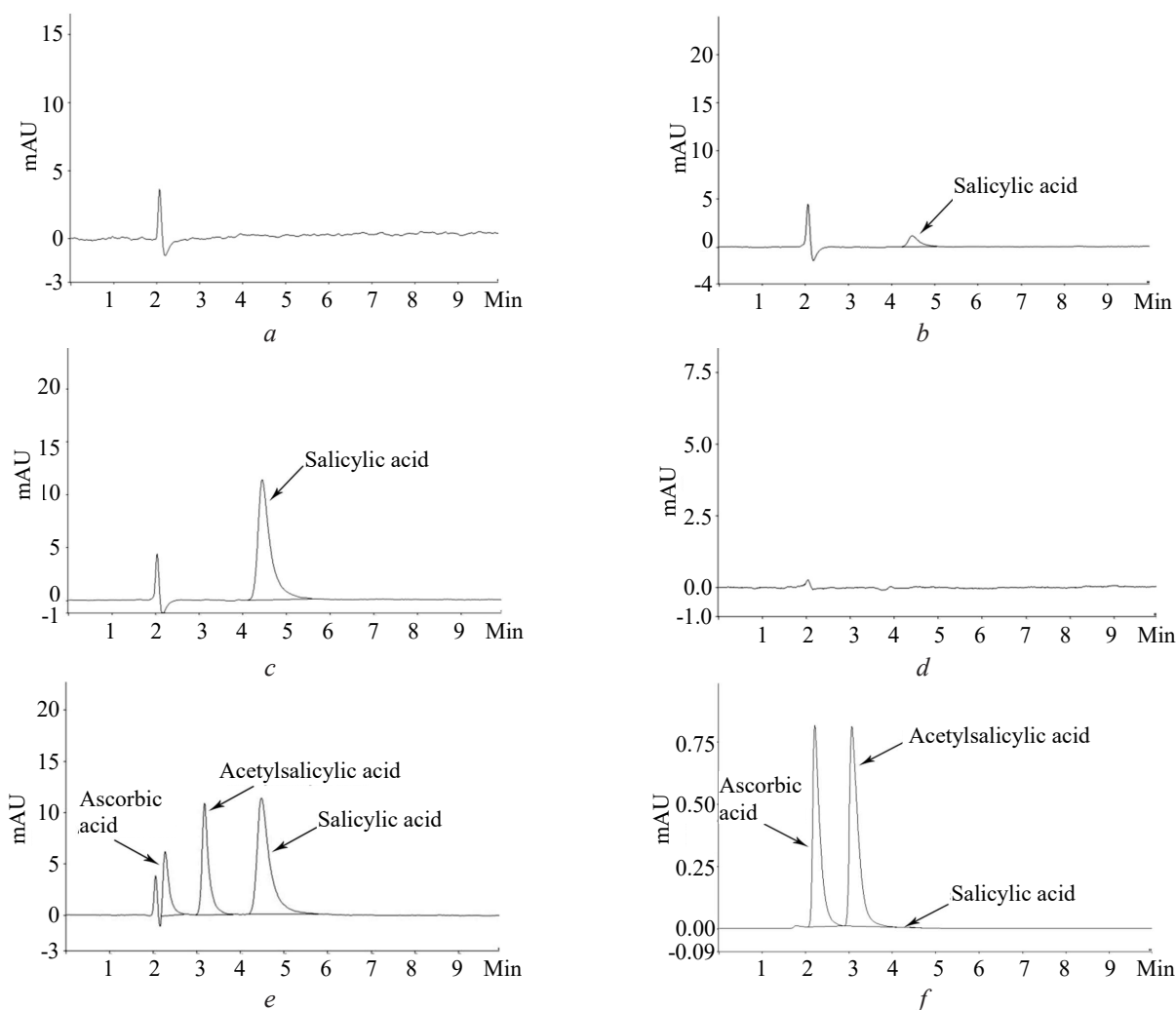


Fig. 2. Chromatograms: a – the blank solution; b, c – comparison solution; d – placebo solution without AA and ACA; e – a solution for checking the suitability of the chromatographic system; f – test solution



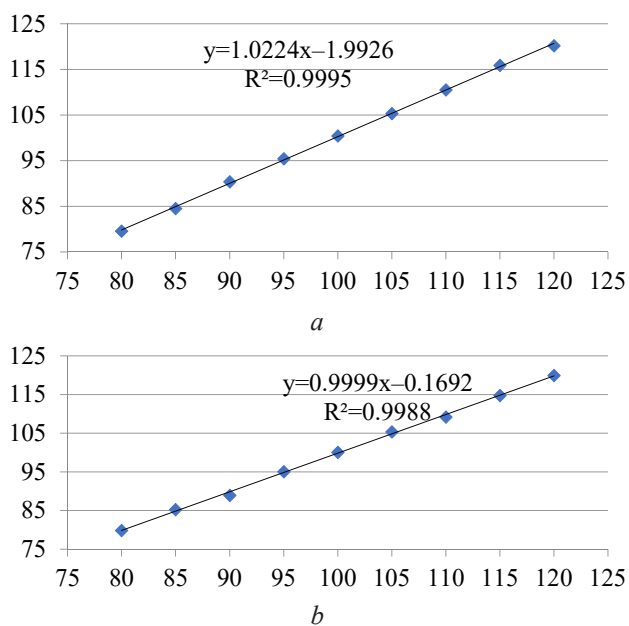


Fig. 3. Graphs of the linear dependence of the analytical signal on the actual concentration of solutions: *a* – AA; *b* – ACA, built in normalized coordinates

The maximum content of impurities in accordance with the method of determination should not exceed 0.5 %.

$LD = 0.32 \times 0.5 \% = 0.16 \%$  from the ACA content in the drug.

Under the conditions of the method, the concentration of the test solution relative to the substance is about 0.65 mg/ml. Thus, the estimated

$$MB_{imp} \leq 0.16 \% \times 0.65 \approx 0.00104 \text{ mg/ml} \approx 1.04 \text{ mg/ml.}$$

To determine the LD, the signal-to-noise ratio was used by comparing the magnitude of the signals obtained for the control experiment (blank solution) and for the sample with a low concentration of the substance. Based on the obtained data, the minimum concentration is set, for which the signal-to-noise ratio is 3:1.

Chromatograms of dilute solutions (0.65  $\mu\text{g/ml}$ , 0.26  $\mu\text{g/ml}$ , 0.13  $\mu\text{g/ml}$ ) in comparison with the blank solution are shown in Fig. 4, *a–d*.

Table 2

Results of evaluation of validation parameters for quantification of AA and ACA

Parameter	Requirements, %		Obtained value, %		Compliance with the criterion
	AA	ACA	AA	ACA	
$\Delta \%$	$\leq 1.6$		1.51	1.44	Executed
<i>a</i>	$\leq 2.6$		$ -1.9926 $	$ -0.1692 $	Executed
$S_r \%$	$\leq 0.84$		0.602	0.514	Executed
<i>r</i>	$> 0.9981$		0.9997	0.9994	Executed
$\delta$	$\leq 0.2$	$\leq 0.31$	0.02	0.19	Executed according to two criteria
	$\leq 0.51$	$\leq 0.51$			
$\Delta Z$	$\leq 1.6$		0.60	0.92	Executed
$\Delta_{int} \%$	$\leq 1.6$		1.41	1.07	Executed

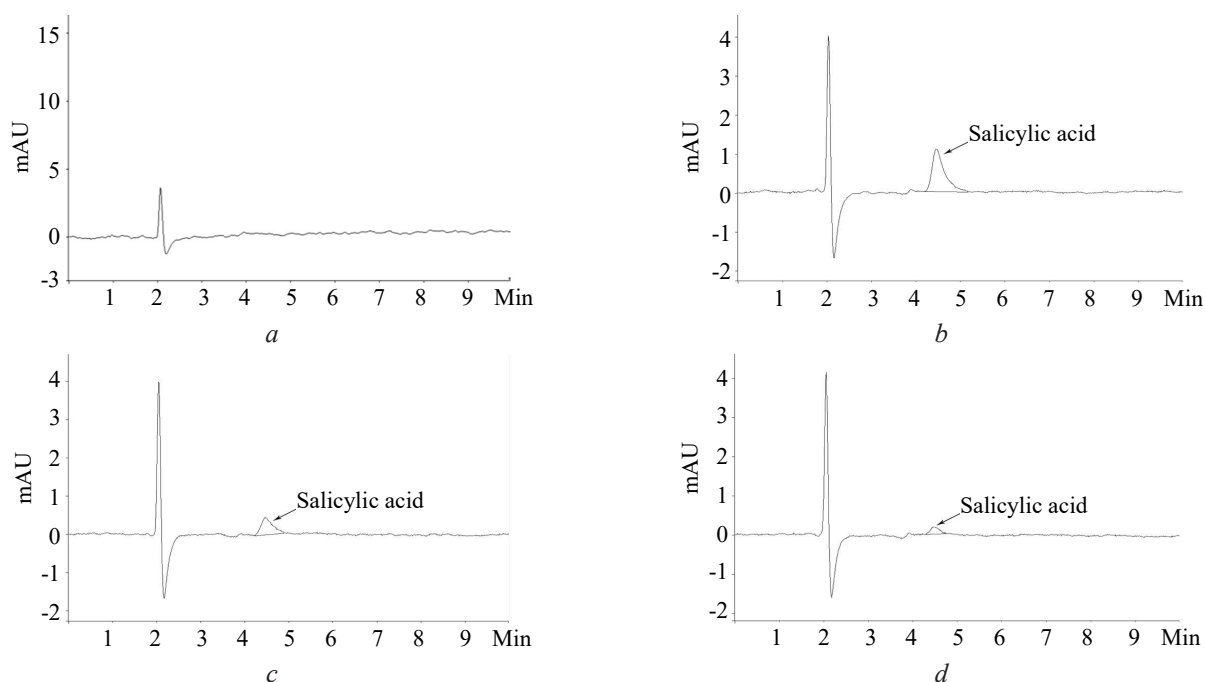


Fig. 4. Chromatograms: *a* – the blank solution; *b* – dilute solution of 0.65  $\mu\text{g/ml}$ ; *c* – dilute solution 0.26  $\mu\text{g/ml}$ ; *d* – dilute solution of 0.13  $\mu\text{g/ml}$

Table 3

The results of determining the stability of solutions in quantitative determination

–	0 min	In 1 hour	Changes, γ %	In 6 hours	Changes, γ %
AA					
<i>rso</i>	3,479,235	3,456,116	0.665	3,375,006	2,996
<i>test</i>	3,667,086	3,613,385	1.464	3,465,119	5,508
$\Delta_{aver}$	–	–	1.06	–	4.25
ACA					
<i>rso</i>	5,618,698	5,540,459	1.392	5,476,476	2,531
<i>test</i>	6,661,629	6,595,630	0.991	6,553,711	1,620
$\Delta_{aver}$	–	–	1.19	–	2.08

## 5. Discussion of research results

When developing the methodology, one of the key tasks is the choice of sorbent and mobile phase, under which the optimal chromatographic separation of test substances (AA, ACA) and impurities (SA and others) is achieved.

An effective method for changing the selectivity of separation and simultaneous control of the ionization state of the analyte in reversed phase HPLC conditions is to adjust the pH values. Ionized functional groups are capable of ionic interaction with unprotected and active silanols of the sorbent, which affects the shape of the peak and its reproducibility. In addition, the high value of the polarity of these compounds reduces their retention on the sorbent.

In the pH range of the mobile phase near the  $pK_a$  values, APIs are present in solution in both neutral and ionized forms. Silanols of the sorbent in a neutral medium are ionized, which increases the degree of their interaction with polar functional groups, leading to the formation of a strong tail factor and reduce the efficiency of the column. At pH below their  $pK_a$  by 1–1.5 units, they are in the neutral state, so the acidification of the mobile phase is a common approach to the establishment of secondary interactions.

Given the operating range of the column (pH 2.0 to 7.5),  $pK_a$  AA – 4.10,  $pK_a$  ACA – 3.5,  $pK_a$  SA – 2.97 and the properties of free silanols sorbent, the most acceptable for determining the studied API is mobile phase with pH 2–3. Therefore, mobile phases based on phosphoric, trifluoroacetic acids and their buffer solutions were used.

It was possible to obtain the separation of API with the most acceptable selectivity in a solution of phosphate buffer with a pH of 3.2.

Three different chromatographic columns were used in the sorbent type selection studies:

1. Hydrosphere C18 column company YMC size 150×4.6 mm with a front column with a particle size of 3 μm.

2. Symmetry C18 column from Waters with a size of 150×4.6 mm with a front column with a particle size of 3.5 μm.

3. Supelcosil LC-NH2 column from “Supelco” with a size of 150×4.6 mm with a front column with a particle size of 3 μm.

When using the first two chromatographic columns, it was found that these sorbent types were not

suitable for chromatographic separation of AA, ACA and SA, because the AA peak came out in the dead volume of the column, i.e., AA is not retained on these sorbents. Only using a 150×4.6 mm column filled with aminopropylsilyl silica gel for *chromatography P* (Supelcosil LC-NH2, “Supelco”) with a precolumn (particle size 3 μm) and elution in the mobile phase buffer solution pH 3.2 – *acetonitrile P* (80:20) in isocratic mode achieved efficient chromatographic separation of AA, ACA and SA (peak yield order: 1 – AA, 2 – ACA, 3 – SA). Detection of substances was performed at a wavelength of 240 nm; the speed of the mobile phase was 1.2 ml/min; column temperature – 45 °C; injection volume – 20 μl. The chromatography time was only 10 minutes, which is optimal in the HPLC method.

In accordance with the defined conditions of chromatography, a test of the suitability of the chromatographic system was developed for each quality indicator separately, both for the quantitative determination of active substances and accompanying impurities.

Under the conditions of quantification of AA and ACA, the chromatographic system is considered suitable if the following conditions are met for reference solution (c):

- chromatographic column efficiency calculated on AA and ACA peaks of at least 2500;
- the coefficient of symmetry of the AA and ACA peaks is not more than 1.8;
- the separation coefficient between the AA and ACA peaks is at least 2.0.

For the quantification of AA and ACA, the efficiency of the chromatographic column was for the AA peak – 2646, and for the ACA peak – 2802; the symmetry coefficient of the AA peak is 1.71, for the ACA peak is 1.66; the separation coefficient between AA and ACA peaks is 3.8, which satisfies the requirements of this test.

In the conditions of determination of accompanying impurities, the chromatographic system is considered suitable if the following conditions are met for the solution for checking the suitability of the chromatographic system:

- the symmetry coefficient of the SA peak is not more than 2.0;
- the separation coefficient between the ACA and SA peaks is at least 3.0.

To determine the accompanying impurities, the symmetry coefficient of the SA peak was 1.83; the separation coefficient between the ACA and SA peaks is 3.6, which also satisfies the requirements of this test.

When studying the validation characteristics of the method of quantification of AA and ACA, it was found that the complete uncertainty of the analysis method  $\Delta_{AS}\%$  for AA 1.44 % and ACA 1.51 %, which is less than  $\Delta_{AS}\%$  for AA 1.44 % and ACA 1.51 %, which is less than  $\max\Delta_{AS}$  1.60 % [15]. Thus, the uncertainty of sample preparation and analysis in general provide sufficient measurement accuracy.

The specificity of the method was confirmed by comparing the chromatograms of the comparison solutions, test solution, blank solution and placebo solution (Fig. 1, *a–g*). As could be seen from Fig. 1, *a–g* on the chromatograms are clearly separated peaks AA and ACA, which coincide in retention time with the peaks of the respective SS. Approximate retention times of peaks: AA – 2.45 minutes, ACA – 3.50 minutes. This indicates the possibility of simultaneous identification of AA and ACA during the quantification of AA and ACA. No chromatograms were found on the chromatogram of the blank solution and the placebo solution, the retention time of which would coincide with the retention time of the AA and ACA peaks. The obtained results confirm that the method of quantitative determination of AA and ACA in the tested sachet is specific.

In the study of linearity, it was found that the values of the correlation coefficients  $r=0.9995$  (AA) and  $r=0.9988$  (ACA) meet the requirements of the criterion of acceptability ( $r>0.9981$ ) (Table 2, Fig. 3). Thus, in the whole range of concentrations from 80 % to 120 % relative to the nominal amount of AA and ACA in drugs, the method is linear.

It is established that the method is characterized by sufficient convergence and correctness in the whole range of investigated concentrations (Table 2). The found values of  $\Delta Z$  for AA – 0.86 % and ACA – 0.92 % less than the critical value for the convergence of results (1.6 %) and satisfies the criteria of acceptability of the validation indicator “Precision”. The systematic error of the method  $\delta=0.02\%$  (for AA) and  $\delta=0.19\%$  (for ACA) satisfies the requirements of the validation indicator “Correctness” for two criteria, statistical insignificance (for  $AA\leq 0.2$  and for  $ACA\leq 0.31$ ) and practical insignificance (for AA and  $ACA\leq 0.51$ ).

Intralaboratory precision studies were performed on 4 samples of one drug sample on different days by different analysts using different measuring vessels. It was found that the value of the relative confidence interval ( $\Delta_{intra}\%$ ) for AA=1.41 %, and for ACA=1.07 % satisfies the criterion of acceptability ( $\leq 1.6\%$ ) (Table 2).

When studying the robustness of the method of quantitative determination of ACA and AA studied the stability of solutions over time (Table 3). It is established that the criterion of insignificance in comparison with the maximum allowable uncertainty of the analysis results ( $\Delta AS=1.6\%$ ) is fulfilled within 1 hour, i.e., the solutions must be used freshly prepared (Table 3).

All calculated validation parameters meet the established criteria; therefore, the method is correct and could be used to quantify AA and ACA in drugs.

During the validation of the method for determining the accompanying impurities, such validation parameters as specificity and detection limit were determined.

The specificity of the method was confirmed by comparing the chromatograms of the comparison solutions, the solution for checking the suitability of the chromatographic system, the test solution, the blank solution, and the placebo solution (Fig. 2, *a–f*).

Chromatography of the blank solution and dilute SA solutions with a concentration of 0.65  $\mu\text{g/ml}$ , 0.26  $\mu\text{g/ml}$  and 0.13  $\mu\text{g/ml}$  was performed by determining LD. Under the conditions of the proposed method, the LD is less than 0.13  $\mu\text{g/ml}$ , which is confirmed by comparison of the obtained chromatograms (Fig. 4, *a–d*).

When comparing chromatograms (Fig. 4, *a–d*), even for a solution with a concentration of 0.13  $\mu\text{g/ml}$ , the signal-to-noise ratio exceeds 3:1, so the LD is less than 0.13  $\mu\text{g/ml}$ , which meets the acceptance criterion ( $\leq 1.04\text{ }\mu\text{g/ml}$ ). The method of quantifying the API content is suitable and correct for the determination of concomitant impurities.

Study limitations. These studies are limited to the developed composition of drugs.

Prospects for further research. Choose the device and conditions that allow you to quantify the content of all components of the proposed dosage form.

## 6. Conclusions

A unified approach to the methods of identification, quantification of AA and ACA and accompanying impurities by HPLC in effervescent powder for the preparation of oral solution has been developed. During the validation of the AA and ACA quantification method, validation parameters such as specificity, linearity, precision, accuracy, intralaboratory accuracy, robustness, values of which do not exceed the established acceptance criteria, i.e., that is, the method of quantifying the API in sachets is validated and suitable for analysis. During the validation of the method of determination of accompanying impurities, such validation parameters as specificity and limit of detection are determined, the values of which meet the requirements for the established acceptability criteria. The method of quantifying the API content is also suitable and correct for determining the accompanying impurities.

## Conflict of interests

The authors declare that they have no conflicts of interest.

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