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Selection of "Green" Conditions for Identifying Components in a Combined Medicine by TLC/HPTLC Methods

Abstract

Aim. To select "green conditions" for identifying components in a combined medicine for the treatment of alcohol intoxication.

Materials and methods. Thin-layer chromatography and high-performance thin-layer chromatography methods were used. An analytical GREEnness calculator was applied to assess the environmental friendliness of the analytical procedure.

Results and discussion. The choice of mobile and stationary phases that comply with the principles of "green chemistry" and can be used to detect glutamic acid and glycine in the composition of a combined medicine has been substantiated. It has been determined that by the indicators R_f, R_s, ΔR_f, α, N, H the most effective for the division is the *ethanol (96%) – water* (70:30) mobile phase (the length of the solvent front is 10 cm, the application volume is 5 µL), which allows, in addition to amino acids, to determine another prescription component – ascorbic acid. The conditions for identification of substances by the high-performance thin-layer chromatography method (the length of the solvent front is 7 cm, the application volume is 2 µL) have been selected. It has been found that to detect chromatographic zones, it is optimal to use *ninhydrin solution R1* with further heating of the plate at a temperature of 100–105 °C for 5 min. The specificity of determination of glutamic acid, glycine and ascorbic acid in comparison with solutions of standard substances has been proven. While studying the robustness of the method the influence of chromatographic conditions on the final result (influence of plate materials of different manufacturers, chamber saturation, application volume, distance from the "start line" to "finish line", influence of the detection solution, the stability of analyte in solution and on the plate) has been researched. The precision of the method on one and three plates of the same type has been studied; the intermediate precision has been researched. The calculated assessment of greenness of the analytical procedure is 0.66.

Conclusions. As a result of the studies conducted, "green conditions" for identifying amino acids (glutamic acid, glycine), as well as ascorbic acid in a combined medicine by thin-layer chromatography and high-performance thin-layer chromatography methods have been selected. The validation characteristics of the method (specificity, robustness and precision) have been studied.

Keywords: "green" chemistry; thin-layer chromatography; high-performance thin-layer chromatography; glycine; glutamic acid; ascorbic acid

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Добір «зелених» умов для ідентифікації компонентів у комбінованому лікарському засобі методами ТШХ/ВЕТШХ

Анотація

Мета. Добрати «зелені» умови для ідентифікації компонентів у складі комбінованого лікарського засобу, призначеного для лікування алкогольної інтоксикації.

Матеріали та методи. Під час дослідження використано методи тонкошарової та високоефективної тонкошарової хроматографії. Для оцінювання екологічності аналітичної методики було застосовано аналітичний калькулятор «GREEnness».

Результати та їх обговорення. Обґрунтовано вибір рухомих фаз, які відповідають принципам «зеленої хімії» та можуть бути використані для виявлення глутамінової кислоти та гліцину у складі комбінованого лікарського засобу. Визначено, що за показниками R_f, R_s, ΔR_f, α, N, H найефективнішою для розділення є рухома фаза *етанол (96%) – вода* (70:30) (довжина фронту розчинника – 10 см, об'єм для нанесення – 5 мкл), яка дозволяє, окрім амінокислот, також визначити ще один компонент пропису – кислоту аскорбінову. Дібрано умови для визначення речовин методом

високоефективної тонкошарової хроматографії (довжина фронту розчинника – 7 см, об'єм для нанесення – 2 мкл). З'ясовано, що для виявлення хроматографічних зон оптимальним є використання *нінгідрину розчину Р1* із подальшим нагріванням пластини за температури 100–105 °С протягом 5 хвилин. Доведено специфічність визначення глутамінової кислоти, гліцину та аскорбінової кислоти проти розчинів стандартних речовин. Під час вивчення робасності методики досліджено вплив умов проведення хроматографування на кінцевий результат (тип нерухомої фази, насиченість камери, об'єм нанесення, відстань від лінії старту до фінішу, розчин для виявлення, стабільність розчинів для нанесення). Вивчено прецизійність методики на одній та на 3-х пластинах одного типу, досліджено внутрішньолабораторну прецизійність. Розрахункова оцінка «зеленості» аналітичної методики становить 0,66.

Висновки. У результаті проведених досліджень було дібрано «зелені» умови для ідентифікації амінокислот (глутамінової кислоти, гліцину) та аскорбінової кислоти в комбінованому лікарському засобі методами тонкошарової і високоефективної тонкошарової хроматографії, а також вивчено валідаційні характеристики методики (специфічність, робасність та прецизійність).

***Ключові слова**:* «зелена хімія»; тонкошарова хроматографія; високоефективна тонкошарова хроматографія; гліцин; глутамінова кислота; аскорбінова кислота

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■ Introduction

Nowadays pharmaceutical analysis of medicines presupposes the use of chemical substances, solvents and reagents, which, of course, have a negative impact on both the laboratory personnel and the environment. Quite often, a significant volume of waste, including toxic organic solvents, which require further processing and disposal, can also appear after carrying out the analysis.

Taking into consideration the increasing concern of the society for the environment, modern approaches to the pharmaceutical development and research foresee the implementation of the concept "*Quality by design*", which means not the use of the correct methods of drug quality control, but also taking into account modern demands to these methods from the point of view of "green chemistry". The "green chemistry" principles are aimed at diminishing the resources, energy costs, time minimization, actions and operations during the analysis, waste minimization and, where possible, replacement of toxic chemicals with less harmful and safer for people [1–5].

Amino acids used in medicine for treating metabolic disorders, diseases of the gastrointestinal tract, nervous system, etc., are common active pharmaceutical ingredients of both mono- and combined medicines. A considerable arsenal of analytical methods is used to control the quality of such medicines in modern pharmaceutical analysis. However, chromatographic methods of research, which are known to allow solving several issues of drug quality control at the same time, play the main role [6, 7].

For example, thin-layer chromatography method (TLC) is an important pharmacopoeial express method for the identification and semi-quantitative determination of amino acids [8–13]. Due to differences in the chemical structure of the compounds analyzed, their solubility and polarity, they enter into a specific interaction with the stationary and mobile phases, which causes different speed of the substance transport [12]. This allows the simultaneous separation of both amino acid mixtures, including enantiomers and medicines, which are combinations of amino acids with other active pharmaceutical ingredients. In addition, the method is convenient and economical.

High-performance thin-layer chromatography method (HPTLC) is becoming more and more common nowadays as an alternative and more "green" method for studying amino acids. Due to the use of plates with a smaller particle size of the sorbent (from 2 to 10 μm), the effective chromatographic separation is achieved using a smaller plate size, application volume, and, accordingly, spending less mobile phase volume [12, 13].

However, the procedure of carrying out the research by TLC/HPTLC methods quite often requires the use of toxic solvents both for the

moving phase, the samples under study, and for the identification of the chromatography zones obtained [1, 3, 4]. The amino acid analysis is most often performed in the system of the normal-phase chromatography where combinations of silica gel with two- or three-component mobile phases are used for the research. One of the solvents is often acetone, methanol, acetic and formic acid, sometimes with the addition of ammonia, pyridine, chloroform, etc. [7, 12, 13].

One of the twelve principles of "green chemistry" supported by *P. T. Anastas* and *J. C. Warner* in 1998 is the use of safer solvents and excipients. As a result, there has been a growing trend in recent decades to use more environmentally friendly alternative solvents in analytical research [14].

An important issue of choosing a solvent is the assessment of its environmental friendliness. To date, there are numerous guidelines in the scientific literature on the choice of solvents, which provide recommendations for the choice of solvents, taking into account their impact on the environment, stability, flammability and explosiveness [3–5, 14, 15]. However, the use of environmental assessment criteria requires special tools that will allow obtaining an easily interpreted and informative result, especially for comparing analysis methods. For this purpose, a GREEnness analytical calculator is used; its evaluation criteria are taken from the twelve principles of "green chemistry", transformed into a unified scale 0–1, and the final score is calculated based on the principles of importance. The result is an icon indicating the final score, the effectiveness of the analytical procedure for each criterion and the user-assigned weights that are convenient to use both when developing a new "green" method of analysis and comparing it with the existing methods [16].

In the modern scientific literature, scientific publications that offer more "green" mixtures of mobile phases for the amino acid analysis, including pure water, aqueous solutions of surfactants, ethyl acetate, *n*-butanol, *n*-butyl acetate, ethylene glycol, have been described. They may become the dominant "green eluents" in the chromatographic analysis [7, 14].

However, taking into account the significant variety of medicines with amino acids, the possible effects of active substances and excipients, this issue still remains open.

Therefore, the selection of conditions for the efficient separation of amino acids included into the composition of combined medicines, which will allow us to identify specifically the substances studied and which will meet the principles of "green chemistry", is an urgent task of the research.

■ Materials and methods

The study object was a combined original medicine intended for the pharmacotherapy of alcohol intoxication. By its chemical composition it is a mixture of amino acids with other substances in the form of an effervescent powder (two sachets) for the preparation of oral solution (*sachet bag 1*: glutamic, acetylsalicylic, ascorbic acids and anhydrous citric acid, sorbitol; *sachet bag 2*: glycine, sodium bicarbonate, sorbitol) [17, 18].

The research was carried out according to the requirements of the European Pharmacopoeia (EP)/the State Pharmacopoeia of Ukraine (SPhU), 2.2.27 "Thin-layer chromatography" [19, 20]. The identification procedure was performed simultaneously with the determination of its validation characteristics [19, 20].

A GREEnness analytical calculator for the assessment of the analytical procedure's greenness was also used [16].

Stationary phase

TLC-plates: TLC Silica gel (Supelco), aluminum plates; TLC Silica gel 60 (Merck), aluminum plates; Silica gel 60 (Merck), glass plates; *HPTLC-plates:* HPTLC Silica gel 60 (Merck), glass plates.

Verification of the separation ability of the stationary phase for the identification was performed as required by the EP/SPhU, 4.1.1 [19, 20].

Before using the plates were activated by means of heating in an electric drying cabinet (model "2 Ш-0-01") at a temperature of 120°C for 20 min [19, 20] to remove residual moisture, which could reduce the sorbent activity.

Solvents and reagents

Water for chromatography and solvents of the appropriate quality and purity meeting the requirements of the EP/SPhU were used for the study.

Reference standards (RS)

The substances of glutamic acid (c. SLBS0553V, manufactured by Sigma Aldrich), glycine (c. LRAA8813, manufactured by Sigma Aldrich) and ascorbic acid (c. DYD2622000008, manufactured by Northeast Pharmaceutical Group Co., Ltd) were used as reference standards.

Test solutions (TS)

Test solution 1 (TS-1). To 200 mg of powder from sachet bag 1, equivalent to 25 mg of glutamic

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acid and 5 mg of ascorbic acid, add 15 mL of *water R*. Sonicate in an ultrasonic bath at 50°C for 12 min and cool. Dilute to 25.0 mL with *water R* and mix.

Test solution 2 (TS-2). To 130 mg of powder from sachet bag 2, equivalent to 10 mg of glycine, add 15 mL of *water R*. Sonicate in an ultrasonic bath at 50°C for 12 min and cool. Dilute to 25.0 mL with *water R* and mix.

Reference solutions (RS)

Glutamic acid reference solution (RS-1). To 25 mg of *glutamic acid RS*, add 15.0 mL of *water R*. Sonicate in an ultrasonic bath at 50°C for 12 min, cool. Dilute to 25.0 mL with *water R* and mix.

Glycine reference solution (RS-2). Dissolve 10 mg of *glycine RS* in *water R* and dilute to 25.0 mL with the same solvent.

The mixture of glutamic acid and glycine reference solutions (RS-3). To 25 mg of *glutamic acid RS* and 10 mg of *glycine RS,* add 15.0 mL of *water R*. Sonicate in an ultrasonic bath at 50°C for 12 min, cool. Dilute to 25.0 mL with *water R* and mix.

Ascorbic acid reference solution (RS-4). Dissolve 5 mg of *ascorbic acid RS* in *water R* and dilute to 25.0 mL with the same solvent.

The type and configuration of the chromatographic chambers

To conduct the research, the chambers with the split-performance of the firms “Sorbfill” (19×19.5×6.5 cm) and “Camag” (27×7×26 cm) were used.

Drying the plates after the elution and detection of chromatographic zones (derivatization)

Drying the plates after the elution was performed in the air. The detection of chromatographic zones was carried out by sprinkling with a solution for detection and further heating in a drying electric cabinet (model “2Ш-0-01”). The results were evaluated in the daylight.

For derivatization of chromatographic zones, *ninhydrin solution R* and *ninhydrin solution R1* prepared according to the requirements of the EP/SPhU were used [19, 20].

The sample application

A Hamilton Bonaduz AG microsyringe (Via Crusch 8, CH-7402 Bonaduz, Switzerland) with the volume of 10.0 µL was used for the sample application.

Documentation

After detection, chromatographs were documented using a Camag® TLC Visualizer 2 and a winCATS® software.

Temperature and humidity

To get the results, the experiment was conducted at the temperature of 25°C and the air relative humidity of not more than 75% [19, 20].

Calculation of criteria

The retardation factor (retention factor) (R_f), ΔR_f, resolution (R_s), selectivity (α) was calculated using the following formulas (1–4) [9, 19–22]:

$$R_f = \frac{l}{L}, \quad (1)$$

$$\Delta R_f = R_{f1} - R_{f2}, \quad (2)$$

$$R_s = \Delta X/[\frac{(W_1 + W_2)}{2}], \quad (3)$$

$$\alpha = \frac{\left(\frac{1}{R_{f2}} - 1\right)}{\left(\frac{1}{R_{f1}} - 1\right)} \quad (4)$$

where, l is the distance from the centre of the chromatographic zone to the “start line”, mm;
L is the distance of the solvent front, mm;
ΔX is the distance between the centers of chromatographic zones, mm;
W is the height of a chromatographic zone, mm.

The separation efficiency was calculated by the quantity of theoretical plates (N) and their height (H) using the following formulas (5–6) [9, 19–22]:

$$N = 16\left(\frac{L \times R_f}{W}\right)^2, \quad (5)$$

$$H = \frac{L}{N}. \quad (6)$$

The study of validation characteristics

Specificity. It was determined on one plate by comparing the chromatographic zones of *Test* and *Reference solutions*. The method is specific if the chromatographic zones obtained with *Test solutions* are similar to the chromatographic zones of *Reference solutions* with respect to position, color, and intensity of bands.

Robustness. The influence of chromatographic conditions on the final result (the impact of the stationary phase type, chamber saturation, application volume, distance from the “start line” to the “finish line”, the effect of the detection solution, stability of solutions for application) was assessed.

The influence of the chamber saturation. The study was conducted in a chromatographic chamber pre-saturated (for 1 h) and unsaturated by an eluent [11, 23].

The influence of the distance that the mobile phase should overcome. Such distances of solvents front as 10, 12 and 15 cm were compared [11].

The influence of plate materials of different manufacturers. The impact of the plate materials of different manufacturers on the result of the separation capacity of the mixture components and the fluctuation of R_f values for the corresponding zones was assessed.

The influence of the volume of application solutions. The results obtained during application of *Test solutions* and *Reference solutions* of different volumes were compared.

The stability of the analyte in the solution and on the plate. The stability of the solutions studied was assessed simultaneously on one TLC plate. *Test solutions* and *Reference solutions* were applied 3 hours earlier and immediately before chromatography.

The stability of derivatization results. The impact of heating conditions of the plate after the elution at a temperature from 100°C to 105°C for 5–15 min was studied.

The stability of chromatographic results. The stability results were assessed in 5, 15, 30 and 60 min after chromatography.

Precision. R_f values for chromatographic zones with testing on one TLC plate, on 3 different plates of the same type were calculated. The analysis was performed on different days by different analysts (the intermediate precision) [7, 12, 19, 20, 23].

Results and discussion

1. The TLC-method development

To select conditions for the identification of amino acids (glutamic acid and glycine) in a combined medicine for the treatment of alcohol intoxication by TLC/HPTLC methods, the primary task of the research was the choice of the mobile phase, which would meet the principles of "green chemistry" and was suitable for separation of amino acids in a combined medicine.

A great number of mobile phases for the identification of amino acids by the TLC method have been described in the scientific literature. For example, in the EP/SPhU, glycine and glutamic acid are determined in the mobile phase of *glacial acetic acid – water – butanol* (20:20:60) [10, 24]; among other mobile phases *isopropyl alcohol – concentrated ammonia* (70:30), *acetonitrile – water* (10:90), *chloroform – methanol* (1:1), etc., are the most common [7, 12, 13]. However, based on the work of scientists in defining the criteria of solvents according to their safety, the impact on human health and environment [1–5, 24], it was found that not all combinations of solvents used met the principles of "green chemistry".

Thus, in the theoretical evaluation of these mobile phases using a GREEnness analytical calculator [16] (Figure 1) it was found that under the same conditions of determination (the number of test samples, type and amount of a derivatizing reagent), but with different types and volumes of solvents and the total amount of waste, the methods analyzed had similar values of the degree of environmental friendliness.

In accordance with the principles of "green chemistry" in the development of "green" methods of research the choice of solvents should avoid the formation of large volumes of waste and give preference to reagents derived from renewable sources. Commonly recommended analytical solvents (after water) are alcohols (ethanol, isopropyl alcohol, butanol, etc.) [1, 4, 5].

When evaluating the existing mobile phases for the amino acid analysis using a GREEnness analytical calculator it was found that the combinations "alcohol – water" had values close to 1, indicating that the evaluated procedure was

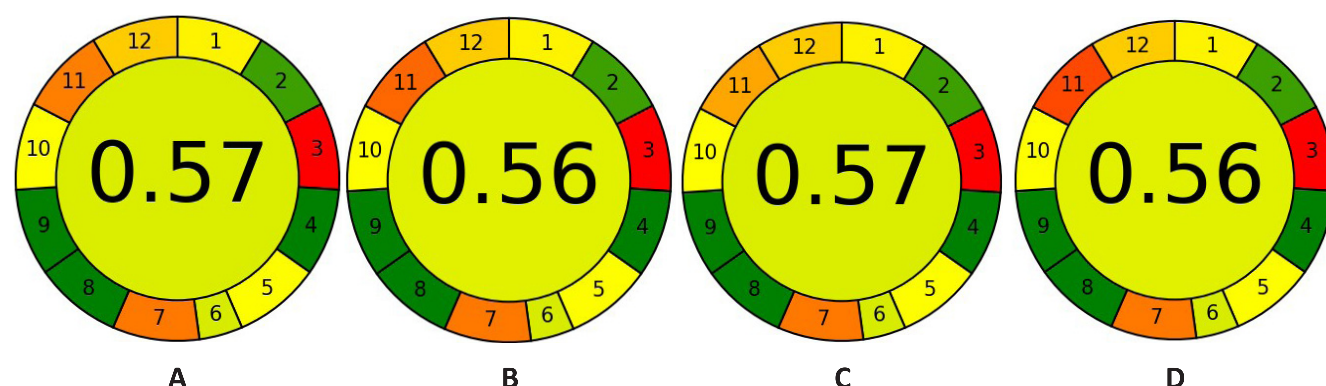

Figure 1. The results of the assessment of analytical procedures of greenness using different mobile phases: **A** *glacial acetic acid – water – butanol* (20:20:60); **B** *isopropyl alcohol – concentrated ammonia* (70:30)*;* **C** *acetonitrile – water* (10:90)*;* **D** *chloroform – methanol* (1:1)

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Table 1. Chromatographic parameters R_f, ΔR_f, R_s, α, *N, H* on the TLC plate Supelco, aluminum plate

Mobile phase/analyte		R_f	ΔR_f	R_s	α	*N*	*H*
Mobile phase 2: *isopropyl alcohol – water* (70:30)	Glutamic acid	0.40 ± 0.01	0.04	1.00	1.19	1024	0.098
	Glycine	0.36 ± 0.01				829	0.121
Mobile phase 3: *ethanol (96 %) – water* (70:30)	Glutamic acid	0.58 ± 0.01	0.10	2.50	1.50	3364	0.030
	Glycine	0.48 ± 0.01				2304	0.043

“greener” [16]. Therefore, the following mobile phases were selected for further study:

- mobile phase 1: *isopropyl alcohol – water* (10:90) [7, 13];
- mobile phase 2: *isopropyl alcohol – water* (70:30) [7, 13];
- mobile phase 3: *ethanol (96 %) – water* (70:30) [7, 13, 25].

To select the most optimal mobile phase, the mixture of glutamic acid and glycine, *Reference solution* (RS-3), was used. To standardize the analysis conditions, the distance travelled by the solvent front from the point of application to the “finish line” was 10 cm.

According to the results of the study it was found that mobile phase 1 was not suitable for the separation of glutamic acid and glycine mixture, and unseparated chromatographic zones concentrated near the “finish line”. Mobile phases 2 and 3 seemed to be more effective for the implementation of the task set. According to the classification of solvents by *L. Snaider*, isopropyl alcohol and ethanol have close polarity values. In mobile phase 1 the quantity of even more polar solvent – water – three times exceeds its content compared to mobile phases 2 and 3. From literary sources, it is known that with a decrease of the organic solvent concentration, the stronger hydrogen bonds in the amino group with alcohol molecules, which are on the surface of a sorbent, can be formed, and thus, we can see the predominance of the process of sorption on the surface of sorbent over association in the solution [26].

In this regard, further research was conducted using mobile phases 2 and 3; their suitability was assessed with the values R_f, R_s, ΔR_f, *α, N, H* (Table 1).

The chromatographic system must be selective for the separation of substances, which means that the compounds must be retained in different ways. The separation of two substances is practically possible if $R_{f1} > R_{f2}$ and $\Delta R_f \geq 0.1$ [22]. According to the results of the analysis (Table 1), R_f of the first component (glutamic acid) exceeds R_f of the second component (glycine) in each of the given mobile phases. However, when comparing ΔR_f of chromatographic zones it was found that mobile phase 3 had the optimal value. According to the resolution (R_s) and selectivity (α), mobile phase 3 was characterized by a more complete separation of amino acid chromatographic zones. While assessing separation efficiency parameters (*H*, *N*) it was concluded that in mobile phase 3 the balance between the phases was more often achieved, and the separation of the components of the mixture analyzed went on more effectively.

Therefore, mobile phase 3 among tested ones is the most optimal for the separation of glutamic acid and glycine.

It was also concluded that a mixture of glutamic acid and glycine (RS-3) should be used to test the suitability of the chromatographic system for analysis: two clearly separated chromatographic zones should be identified.

The next stage of the research was to select the optimal reagent for detecting chromatographic zones of amino acids. It is known that the effectiveness of the derivatizing reagent is determined by the ability to color the compounds analyzed in a specific way [9]. *Ninhydrin solution R* and *ninhydrin solution R1* are used to identify amino acids according to the EP/SPhU.

When evaluating them according to the principles of “green chemistry” it was found that, by the amount of waste, *ninhydrin solution R* had a higher value of the indicator approaching 1, i.e. was “greener” than *ninhydrin solution R1*.

However, while performing chromatography of the mixture of glycine and glutamic acid (RS-3) in the *ethanol (96 %) – water* (70:30) mobile phase it was found that *ninhydrin solution R1* providing a clearer and more intense coloring of the chromatographic zones was the most optimal one (Figure 2).

The research also showed that the processing of chromatograms consumed less volume of the derivative reagent *ninhydrin solution R1* and reduced the duration of heating (chromatographic zones had intense color after 5 minutes of heating at 100–105°C). Therefore, from the point of view of the efficiency of detection of analytical zones and the most optimal one principles of “green

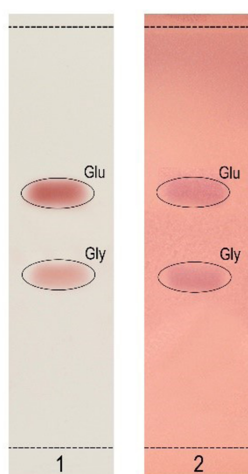

Figure 2. The chromatogram of the mixture of *Reference solution* of glutamic acid (Glu) and glycine (Gly), RS-3, in the *ethanol (96 %) – water* (70:30) mobile phase*:* **1** – using *ninhydrin solution R1*; **2** – using *ninhydrin solution R* on the TLC Silica gel (Supelco), aluminum plate

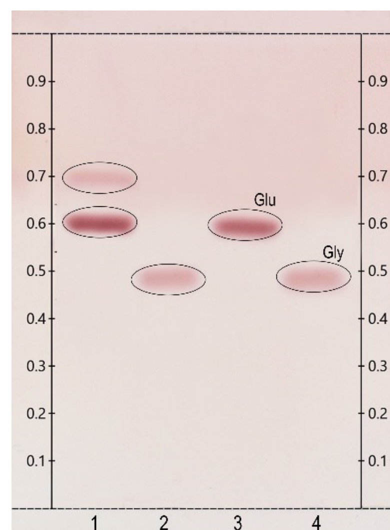

Figure 3. The chromatogram of *Test solution* 1, TS-1 (1); *Test solution* 2, TS-2 (2); *Reference solution* of glutamic acid (Glu), RS-1 (3); *Reference solution* of glycine (Gly), RS-2 (4) on the TLC Silica gel (Supelco), aluminum plate

chemistry", the use of *ninhydrin solution R1* is the optimal for analysis.

In the given conditions the chromatographic study of sachet bag 1 (TS-1) and sachet bag 2 (TS-2) compared to glutamic acid *RS* (RS-1) and glycine *RS* (RS-2) was carried out (Figure 3).

In the above mobile phase, *ethanol (96%) – water* (70:30), for TS-1 and TS-2, chromatographic zones were detected at the level of the zones of glutamic acid *RS* (RS-1) and glycine *RS* (RS-2). The zones of *Reference solutions* and *Test solutions* have a complete separation, coincided in color and location, indicating the specificity of the separation of the mixture components.

After considering the results of chromatography it was found (Figure 3) that in addition to glutamic acid zones in TS-1 there was another unidentified zone. This indicates that another component of sachet bag 1 entered the derivatization reaction with *ninhydrin solution R1*. Taking into account the composition of the sachet bag studied we can assume that ascorbic acid reacts with *ninhydrin* since it has the strong reducing properties (E_o = +0.18 V) [27, 28]. After conducting the study for the second time with ascorbic acid *RS* (RS-4), it was proven that the unidentified zone corresponded to ascorbic acid (Figure 4).

Thus, under the given conditions, it is possible to simultaneously identify three components of the dosage form – glycine, glutamic acid and ascorbic acid. This approach is optimal in terms of the principles of "green chemistry" as it allows reducing the number of analytical operations during the medicine quality control.

2. The TLC-method validation

2.1. Specificity

The method is specific, which is confirmed by the chromatogram (Figure 4). The chromatographic zones obtained with *Test solutions* are similar to the chromatographic zones of *Reference solutions* with respect to the position, color, and intensity of bands.

2.2. Robustness

The next stage of the research was to study the procedure robustness.

The influence of the distance that the mobile phase should overcome

Such distances of the solvent front as 10, 12 and 15 cm were compared (Figure 5).

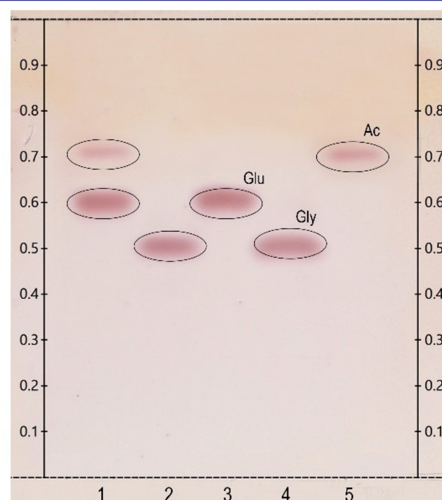

Figure 4. The chromatogram of *Test solution* 1, TS-1 (**1**); *Test solution* 2, TS-2 (**2**); *Reference solution* of glutamic acid (Glu), RS-1 (**3**); *Reference solution* of glycine (Gly), RS-2 (**4**); ascorbic acid RS (Ac), RS-4 (**5**) on the TLC Silica gel (Supelco), aluminum plate

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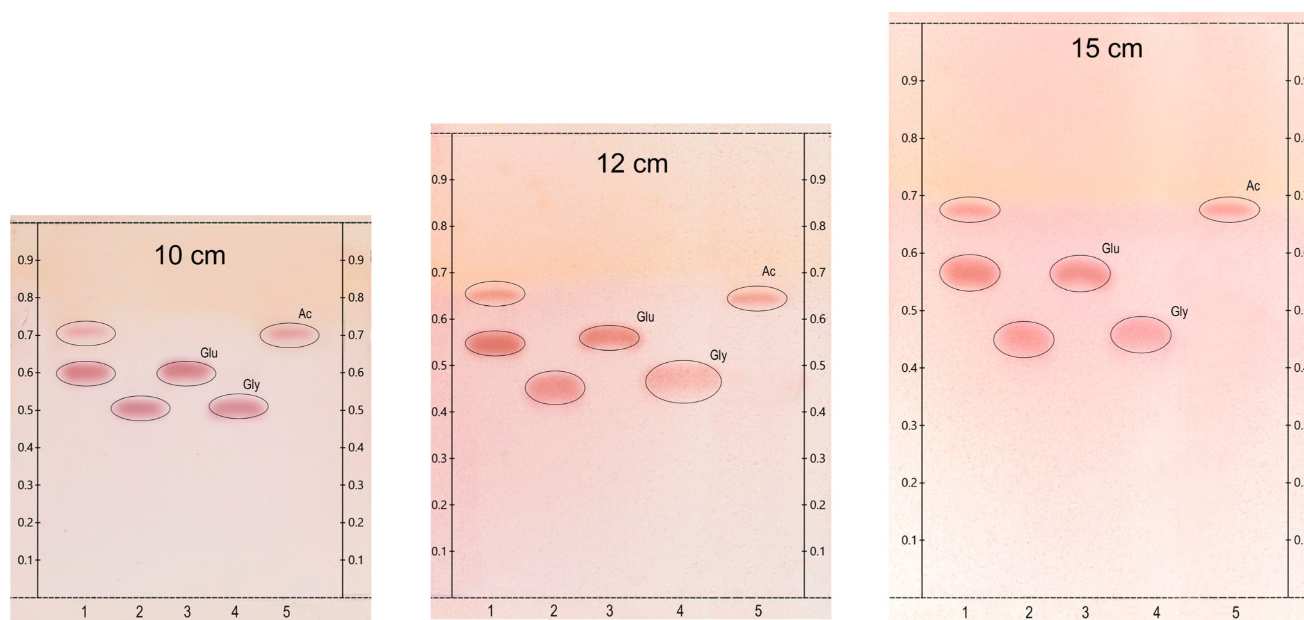

Figure 5. The influence of the distance for the mobile phase to overcome: Test solution 1, TS-1 (**1**); Test solution 2, TS-2 (**2**); glutamic acid RS (Glu), RS-1 (**3**); glycine RS (Gly), RS-2 (**4**); ascorbic acid RS (Ac), RS-4 (**5**) on the TLC Silica gel (Supelco), aluminum plate

A value of 10 cm is optimal as increasing the distance from the "start line" to the "finish line" leads to blurring, lengthening of the zones obtained and, in general, increases the duration of the analysis and reduces the "greenness" of the method [16].

The influence of plate materials of different manufactures

The separation of the mixture components was achieved on all the plates analyzed (Figure 6, Table 2).

However, evaluating the results obtained on the plates of different manufacturers with aluminum and glass plates (Table 2) it was found that a more complete and efficient separation of chromatographic zones was achieved on the plates produced by "Merck".

The influence of the chamber saturation

As a result of the study, it was concluded that in the pre-saturated chromatographic chamber, due to the formation of the gas phase of the eluent, there was a better separation of chromatographic zones. The elution process in these conditions was faster, so the chromatographic time was reduced.

The influence of the volume of the application solution

While conducting the research, the results obtained when applying *Test solutions* and *Reference solutions*, which sample volumes were 5, 10, 15 μL, were compared (Figure 7).

As we can see, the optimal application volume is 5 μL, including 5 μg of glutamic acid, 3.25 μg of glycine and 1.95 μg of ascorbic acid.

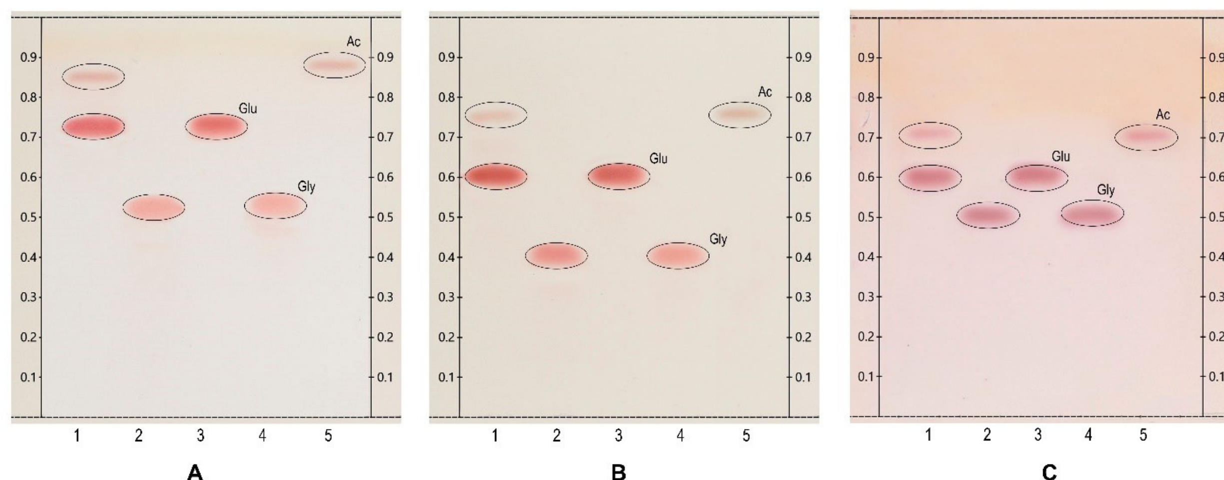

Figure 6. The influence of plate materials of different manufactures: **A** – TLC Silica gel 60 (Merck), glass plate; **B** – TLC Silica gel 60 (Merck), aluminum plate; **C** – TLC Silica gel (Supelco), aluminum plate; **1** – *Test solution* 1, TS-1; **2** – *Test solution* 2, TS-2; **3** – RS-1; **4** – RS-2; **5** – RS-4

Table 2. Chromatographic parameters *Rf*, ΔR_f, R_s, α, *N*, *H* on different TLC plates

Mobile phase/analyte		R_f	ΔR_f	R_s	α	*N*	*H*
TLC Silica gel 60 (Merck), glass plate	Glutamic acid	0.73 ± 0.01	0.21	5.25	2.50	5329	0.019
	Glycine	0.52 ± 0.01				2704	0.037
TLC Silica gel 60 (Merck), aluminum plate	Glutamic acid	0.61 ± 0.01	0.21	5.25	2.35	3721	0.027
	Glycine	0.40 ± 0.01				1600	0.063
TLC Silica gel (Supelco), aluminum plate	Glutamic acid	0.58 ± 0.01	0.10	2.50	1.50	3364	0.030
	Glutamic acid	0.48 ± 0.01				2304	0.043

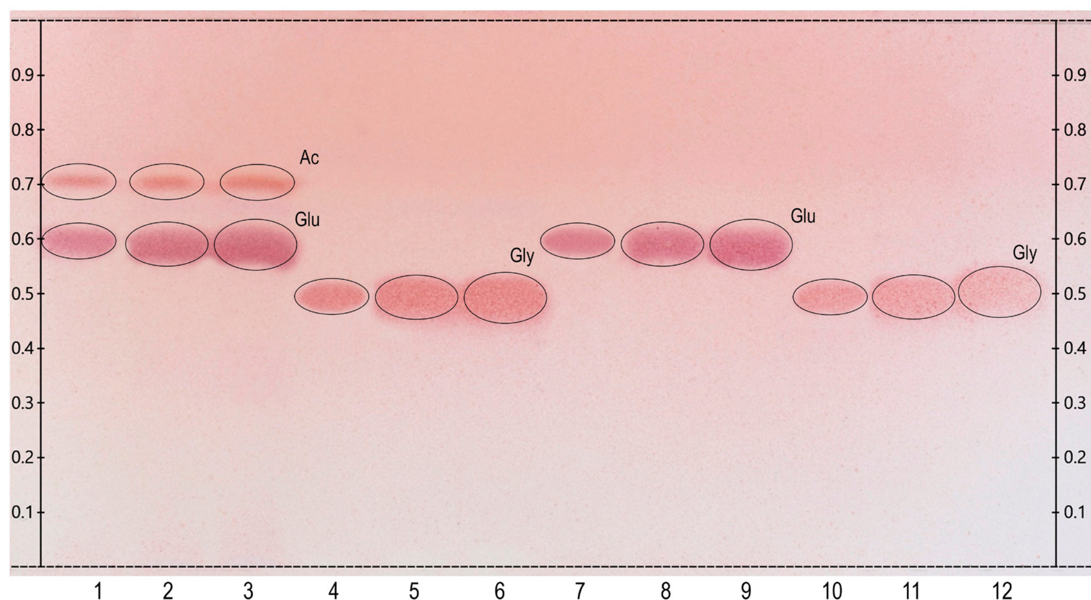

Figure 7. The influence of the volume of the application solution: **1**–**3** – Test solution 1, TS-1; **4**–**6** – Test solution 2, TS-2; **7**—**9** – glutamic acid RS (Glu), RS-1; **10**–**12** – glycine RS (Gly), RS-2: **1**, **4**, **7**, **10** – 5 µL; **2**, **5**, **8**, **11** – 10 µL; **3**, **6**, **9**, **12** – 15 µL on the TLC Silica gel (Supelco), aluminum plate

While increasing the application volume the blurring of chromatographic zones and changing their form can be seen. The size of the chromatographic zone samples allowing to achieve reproducible results is 10×2 mm.

The stability of an analyte in the solution and on the plate

The results are shown in Figure 8. It was found that the individual zones obtained did not differ in location, color, intensity and shape, indicating the stability of the solutions analyzed within the specified period of time.

The stability of derivatization results

As mentioned earlier (Figure 3, 4), during the research it was concluded that heating the plate at a temperature of 100–105°C for 5 min was sufficient for the derivatization process.

The stability of chromatographic results

The stability of the results was evaluated at 5, 15, 30 and 60 min after chromatography. Within the given time, no difference was found between the areas studied.

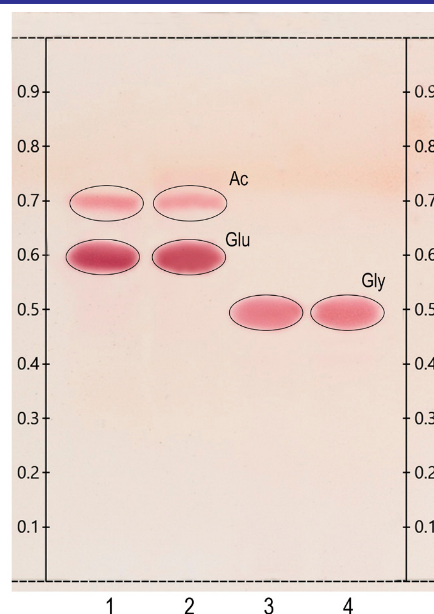

Figure 8. The stability of *Test solution* 1, TS-1 (**1**, **2**) and *Test solution* 2, TS-2 (**3**, **4**): **1**, **3** – samples applied 3 hours before chromatography; **2**, **4** – samples applied just before chromatography on the TLC Silica gel (Supelco), aluminum plate

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2.3. Precision

When studying the procedure precision the values of R_f for the zones of glutamic and ascorbic acids (TS-1) and glycine (TS-2) were calculated:

- on one TLC plate;
- on 3 plates of the same type;
- in the analysis on different days by different analysts (the intermediate precision).

The analysis was performed on the TLC (Supelco) aluminum plate [29].

It was summed up that the chromatographic zones obtained were clearly separated, identical in their location and color. Metrological characteristics of R_f values for chromatographic zones corresponding to glutamic acid, glycine and ascorbic acid are as follows:

- on one plate:

$R_f(Ac) = 0.71$; RSD,% = 0.81%; $R_{f\,max} - R_{f\,min} = 0.01$
$R_f(Glu) = 0.58$; RSD,% = 1.00%; $R_{f\,max} - R_{f\,min} = 0.01$
$R_f(Gly) = 0.48$; RSD,% = 1.21%; $R_{f\,max} - R_{f\,min} = 0.01$

- on 3 plates of the same type:

$R_f(Ac) = 0.72$; RSD,% = 1.61%; $R_{f\,max} - R_{f\,min} = 0.02$
$R_f(Glu) = 0.58$; RSD,% = 1.72%; $R_{f\,max} - R_{f\,min} = 0.02$
$R_f(Gly) = 0.48$; RSD,% = 2.08%; $R_{f\,max} - R_{f\,min} = 0.02$

- the intermediate precision:

$R_f(Ac) = 0.72$; RSD,% = 2.90%; $R_{f\,max} - R_{f\,min} = 0.04$
$R_f(Glu) = 0.58$; RSD,% = 3.45%; $R_{f\,max} - R_{f\,min} = 0.04$
$R_f(Gly) = 0.48$; RSD,% = 4.31%; $R_{f\,max} - R_{f\,min} = 0.04$

For the TLC method developed for the analysis of amino acids and ascorbic acid using a "GREEnness" analytical calculator the greenness of the analytical procedure was calculated (Figure 9). As we see, it exceeds the results when using other mobile phases (Figure 1). Thus, we can conclude that the method developed is greener.

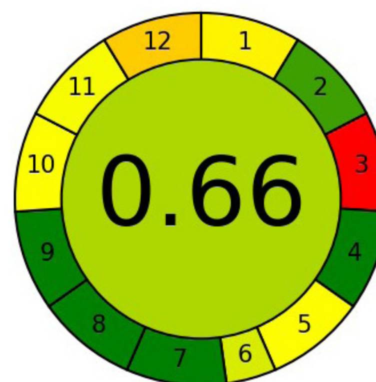

Figure 9. The results of assessing the greenness of the analytical procedure for the method developed

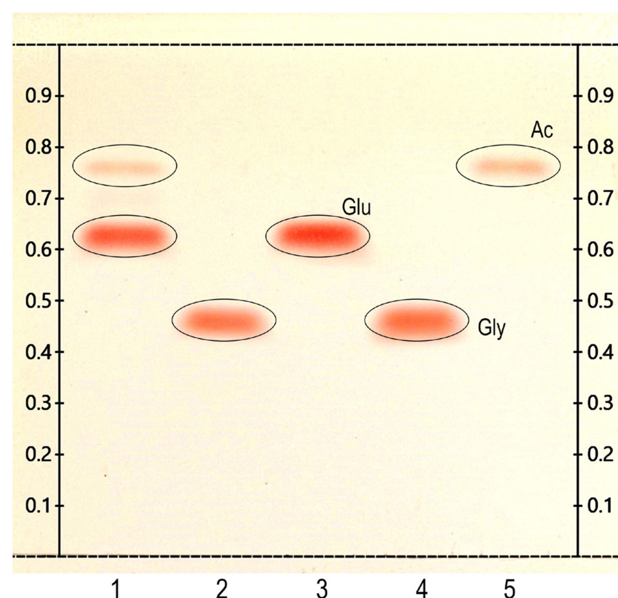

Figure 10. The chromatogram of *Test solution* 1, TS-1 (**1**); *Test solution* 2, TS-2 (**2**); glutamic acid (Glu) *RS*, RS-1 (**3**); glycine *RS* (Gly), RS-2 (**4**); ascorbic acid *RS* (Ac), RS-4 (**5**) on the HPTLC Silica gel (Merck), glass plate

3. Development of the HPTLC method

At the same time, a procedure was developed for its use by the HPTLC method. The research was conducted at the stage of studying robustness. The optimal sample volume for plate application was 2 µL; the solvent front distance was 7 cm. The chromatographic results are shown in Figure 10.

4. Method of analysis

The validated TLC/HPTLC method for analyzing glutamic acid, glycine, ascorbic acid is given below.

Thin-layer chromatography (EP/SPhU, 2.2.27).

Test solution (a): To 200 mg of powder from sachet bag 1, equivalent to 25 mg of glutamic acid and 5 mg of ascorbic acid, add 15 mL of *water R*. Sonicate in an ultrasonic bath at 50°C for 12 min, cool. Dilute to 25.0 mL with *water R* and mix.

Test solution (b): To 130 mg of powder from sachet bag 2, equivalent to 10 mg of glycine, add 15 mL of *water R*. Sonicate in an ultrasonic bath at 50°C for 12 min, cool. Dilute to 25.0 mL with *water R* and mix.

Reference solution (a): To 25 mg of *glutamic acid RS*, add 15 mL of *water R*. Sonicate in an ultrasonic bath at 50°C for 12 min, cool. Dilute to 25.0 mL with *water R* and mix.

Reference solution (b): Dissolve 10 mg of *glycine RS* in *water R* and dilute to 25.0 mL with the same solvent.

Reference solution (c): To 25 mg of *glutamic acid RS* and 10 mg of *glycine RS*, add 15 mL of *water R*. Sonicate in an ultrasonic bath at 50°C

for 12 min, cool. Dilute to 25.0 mL with *water R* and mix.

Reference solution (d): Dissolve 5 mg of *ascorbic acid RS* in *water R* and dilute to 25.0 mL with the same solvent.

Plate: TLC silica gel plate R (HPTLC silica gel plate R).

Mobile phase: ethanol (96%) R – water R (70:30).

Application: 5 µL (2 µL).

Development: 10 cm (7 cm).

Drying: in air.

Detection: spray with *ninhydrin solution R1* and heat at 100–105°C for 5 min.

System suitability: *Reference solution (c)*:

– the chromatogram shows 2 clearly separated spots.

Results: the principal spots in the chromatogram obtained with *Test solutions* (*a, b*) are similar in position, color and size to the principal spots on the chromatogram obtained with *Reference solutions* (*a, b, c, d*).

Conclusions

As a result of the studies conducted, "green conditions" for identifying amino acids (glutamic acid, glycine), as well as ascorbic acid in a combined medicine by thin-layer chromatography and high-performance thin-layer chromatography methods have been selected. The validation characteristics of the method (specificity, robustness and precision) have been studied.

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