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EXPERIMENTAL RESEARCH ON DEVELOPMENT AND VALIDATION OF METHODS OF QUANTITATIVE DETERMINATION OF FLAVONOIDS AND ESSENTIAL OIL IN SOLID MULTI-COMPONENT CAPSULES "UROHOLUM"

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Мета. *Розробка і валідація методик кількісного визначення флавоноїдів та ефірної олії в твердих багатокомпонентних капсулах, призначених для застосування в урології.*
Методи. *Для кількісного визначення суми флавоноїдів було розроблено та валідовано спектрофотометричну методику з використанням спектрофотометра Evolution 60 s фірми Thermo Fisher Scientific, USA, з метою визначення ефірної олії – метод гравіметрії.*
Результати. *У результаті проведеного дослідження було розроблено спосіб кількісного визначення суми речовин флавоноїдної будови сумарного фітоекстракту у складі готового лікарського засобу у вигляді капсул спектрофотометричним методом і суми ефірних олій після попереднього відгону з водяною парою і екстрагування органічним розчинником методом гравіметрії. Спектрофотометричне кількісне визначення речовин флавоноїдної будови проводили після реакції комплексоутворення з солями алюмінію (III) в оцтовокислому середовищі при аналітичній довжині хвилі 410 нм. Розрахунок вмісту біологічно активних речовин проводили методом стандарту, в якості котрого використовували рутин. Для методик кількісного визначення суми речовин флавоноїдної будови і ефірних олій були вивчені такі валідаційні параметри як специфічність, лінійність, і прецизійність.*
Висновки. *Розроблено та валідовано доступні методики кількісного визначення суми біологічно активних речовин сухого багатокомпонентного екстракту рослинного походження у твердих капсулах. Вивчені валідаційні параметри відповідають критеріям прийнятності*
Ключові слова: *стандартизація, багатокомпонентний склад, сухий екстракт, спектрофотометрія, валідація, тверді капсули*

1. Introduction

Drugs derived from medicinal herbal raw materials have a polytropic pharmacological effect at low toxicity, an important reserve for improving the therapy of kidney disease, which often occur chronically [1, 2]. The polytropic nature of herbal remedies often eliminates complications from other organs and systems, normalizes the general condition of the patient, can increase the effectiveness of treatment of acute inflammatory diseases of the kidneys and urinary tract, provide primary prevention of chronic kidney diseases, slow their progression, and reduce harmful effects. Phytopreparations in the treatment of kidney disease can prevent their chronicity [3, 4].

Considering certain inconveniences at preparation of herbal remedies at home (complexity of obtaining standard concentration, storage of finished extracts, non-guarantee of safety of purchased medicinal plants), which reduce the addiction of patients to long-term treatment, in modern clinical practice rational use of ready-to-use herbal remedies with , standardized for the main active substances [5–7].

Drops "Uroholum" is a multicomponent preparation of plant origin, the active substances of which is the BAS of such medicinal herbal raw materials: mint pepper and Java tea leaves; hop stems; wild carrot seeds; birch buds; corn columns with receptacles; elder flowers; common horsetail, knotgrass and St John's wort herbs extracted with ethanol 40 % [6–8].

One of our aim was to research on pharmaceutical development in capsule creation, without changing the quality of the composition compared with the "Uroholum" drops, to determine the critical indicators of the quality of the medicinal substance and excipients that are crucial for the process of its manufacture.

2. Planning (methodology) of research

Within the framework of pharmaceutical development, the task was to develop a drug in form of capsules.

On the pharmaceutical market of Ukraine are presented “Uroholum” drops, obtained by extraction of medicinal plant material with 40 % ethanol.

The composition of the “Uroholum” oral drops, based on multicomponent liquid extract (1: 1): Dauci carotae fructus, Orthosiphonis staminei folia, Polygoni avicularis herba, Zeae maydis styli cum stigmatis, Sambuci nigrae flores, Equisulieemlosemba herba, herb, Menthae piperitae folia.

The composition of a multicomponent preparation in a dosage form, where the active substance - liquid extract (1:1) Urocholum was substantiated and developed by previous studies [3]. It was established that the medicinal plant raw materials included in the combined phyto-

composition, mainly contains substances of flavonoid structure and essential oils. The problem of quantifying the biologically active substances of the total dry extract to further standardize the finished drug in the form of capsules remained unresolved.

3. Materials and methods

An experimental batch of hard gelatine capsules containing dry extract "Uroholum" and excipients was selected as the object of study.

Reagents, solvents, and measuring vessels that meet the requirements of SPhU were used for analytical studies. Evolution 60 s spectrophotometer (Thermo Fisher Scientific, USA), and Axis analytical scales (SARTORIUS, Poland) was used for analytical studies. To quantify the amount of flavonoids in the obtained dry extract "Uroholum" we used the method of absorption spectrophotometry in the visible area using a spectrophotometer [10].

Method of quantitative determination of the sum of flavonoids.

Test solution: the contents of the capsule equivalent to 50 mg of the dry extract are placed in a 25.0 ml volumetric flask, 2.0 ml of water P is added, shaken, volume is adjusted with ethanol (70 % v / v) P and stirred (original solution).

5.0 ml of the original solution is placed in a 25.0 ml volumetric flask, add 2 ml of aluminum chloride reagent P, and brought 5 % glacial acetic acid solution in ethanol (70 %) P to the label and mix.

Comparison solution: 0.050 g of rutin standard sample (SS) rutin (PSS SPhU, cat. No. R0366) was dissolved in 50 ml of 96 % ethanol when heated in a water bath, cooled and the volume of the solution is adjusted to 100.0 ml with the same solvent. To 1.0 ml of the resulting solution was added 2.0 ml of aluminum chloride reagent P and bring the volume of the solution to a solution of 5 % acetic glacial acid in ethanol P to 25.0 ml.

Compensation solution: 5.0 ml of the original solution was placed in a 25.0 ml volumetric flask, adjusted with 5 % glacial acetic acid in ethanol (70 %) P to the mark and stirred.

After 30 min, measure the optical density of the test solution and the comparison solution at a wavelength of 410 nm relative to the compensation solutions.

The content of flavonoids, in milligrams (x) in terms of rutin and dry extract, was calculated by the formula:

$$x = \frac{A \cdot m_o \cdot V_2 \cdot V_4 \cdot V_6 \cdot \%C3 \cdot 100 \cdot 100}{A_o \cdot m \cdot V_1 \cdot V_3 \cdot V_5 \cdot 100 \cdot (100 - \%_{вол})},$$

where A – the optical density of the test solution; A_0 – the optical density of the rutin SS solution; m_0 – mass of rutin SS sample, r; m – the weight of the sample content of the capsules; V_1, V_2, V_3, V_4, V_5, V_6– dilution volumes of the test solution and comparison solution, ml.

The content of flavonoids in the preparation, in milligrams, in terms of rutin and water content, should be at least 1.5 mg per average weight of capsule content.

Method of quantitative determination of essential oil. The contents of the capsule mass, equivalent to 2.000 g of dry extract, quantitatively transfer 50 ml of 20 % ethanol P into a 250 ml round bottom flask and distil, using a refrigerator, into a separating funnel, which was prelabelled with 25 ml.

The distillation was stopped when the distillate level reaches 25 ml. A sufficient amount of sodium chloride P was dissolved in the distillate to obtain a saturated solution. The system is disconnected and after cooling, the refrigerator was rinsed with 10 ml of petroleum ether P, which is combined with the contents of the separating funnel. The contents of the separating funnel are shaken with 3 portions, 20 ml each, of petroleum ether R. The combined layers of the organic solvent were filtered through a paper filter of 6 g of anhydrous sodium sulphate P into a pre-weighed beaker. Sodium sulphate was washed with 10 ml of petroleum ether P, which was added to the extract. The solvent was removed at a temperature not higher than 40 °C. The residue was dried in a desiccator over phosphorus (V) oxide P and paraffin at room temperature for 3 h and weighed.

The content of essential oil in capsules (x), in milligrams, calculated on the average weight of the capsule, was calculated by the formula:

$$X = \frac{(m_2 - m_1) \cdot m_{cep} \cdot 1000}{m},$$

where m – mass of sample of drug, r; m_1 – the mass of an empty weighting cup, r; m_2 – the mass of the flask with essential oil, r; m_{cep} – the average weight of the capsule, g.

The content of the essential oil in the preparation should be at least 0.05 mg, based on the average weight of the capsule content.

4. Results of the research

To carry out experimental studies on the development of methods for the quantitative analysis of the main active substances of a multicomponent total plant extract that is part of the capsules, we have made model samples.

In appearance, the investigated dosage form is a hard gelatine capsule with a straw case and a dark green cap. Capsule content is a powder from light brown to brown with small particles ranging from light to dark in colour.

To quantify the amount of flavonoids, a spectrophotometric technique was used, based on the reaction of the formation of complex compounds of polyphenols with aluminum chloride [9].

The absorption spectrum of the test solution of the dry extract in 70 % ethanol after reaction with a solution of aluminum chloride in the medium of acetate acid at the position of the maximum absorption and in the course of the absorption curve coincides with the maximum absorption of the standard solution of rutin obtained under the same conditions.

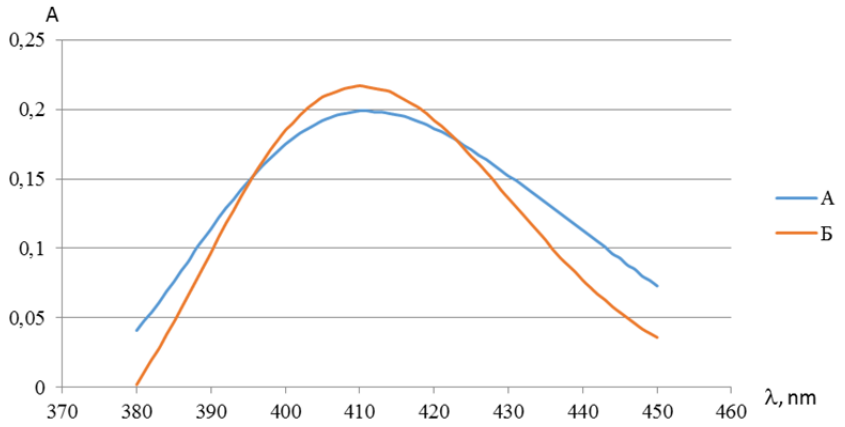

Fig. 1. Absorption spectra of the test solution (A) and rutin standard sample (B) after reaction with aluminum chloride

The quantitative content of the sum of the substances of the flavonoid structure in the dry extract was performed by absorption spectrophotometry at a wavelength of 410 nm. The most common approach to the quantitative analysis of total herbal preparations is the quantitative control of the conditional concentrations of the active substances, which was carried out in terms of the target compound, for which we have chosen a rutin. For further application of the methodology in the analysis of the finished medicinal product, such validation characteristics as specificity, linearity and precision were studied.

To study the validation characteristics of the method for quantifying the sum of substances of the flavonoid structure, we used the approach to validate the total herbal preparations, described in the literature [11]. The specificity of this analytical procedure was performed by comparing the position of the absorption maxima and the intensity of the optical density of the test solution and the comparison solution when determined by spectrophotometric method after reaction with a solution of aluminum chloride. Placebo solutions (all capsules excipients) were prepared; model mixture (extract dry with all the excipients of the capsules); of the dry extract according to the above procedure and the absorption spectra of the obtained solutions were recorded (Fig. 2).

The placebo effect was found to be 0.86 %, so the capsule excipients had little effect on the quantification results. The deviations are insignificant, that is, the specificity of the methodology was confirmed.

The selectivity of the spectrophotometric analysis of the biologically active substances of the extract was ensured by the use of a group reagent for substances of flavonoid nature (aluminum chloride solution) for isolation of the analytical signal.

When determining linearity, measurements of optical density (three times for each solution with the removal of the cuvette) routine SS solution in a concentration of from 80 % to 120 % of the selected. In relation to the average values of the optical density for each of the 9 solutions to the selected concentration, a calibration graph of dependence was constructed (Fig. 3).

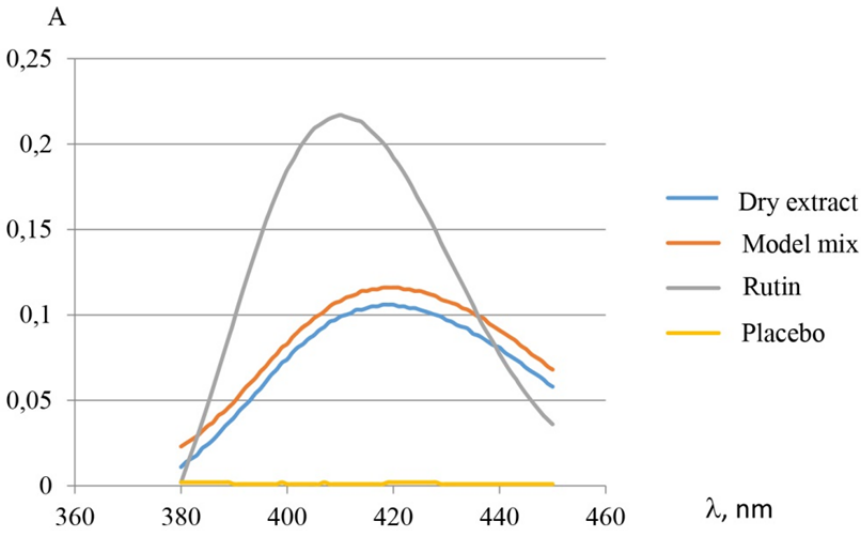

Fig. 2. Absorption spectra after reaction with aluminum chloride solution

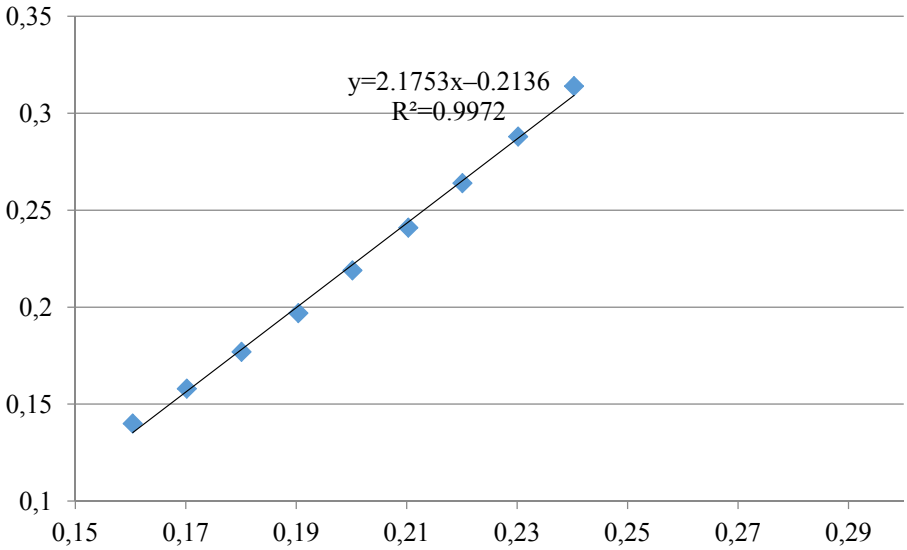

Fig. 3. Calibration graph of optical density versus rutin concentration after complexation reaction with aluminum chloride

The optical density of the obtained model solutions was determined on a spectrophotometer at a wavelength of 410 nm in a cuvet with a layer thickness of 10 mm (the results are shown in Table 1 and Fig. 4). There is a linear relationship between the concentration of the sum of substances of the flavonoid structure of the total dry phytoextract in capsules and optical density with a correlation coefficient of 0.9999 (≤ 0.9995), the angular coefficient of linear dependence (b) is equal to 0.9971, free member of linear dependence (a) 1.59≤5.10.

Table 1

The results of determining the optical density of model solutions

% from work concentration	Weight of sample extract, g	Optical density
80	0.0401	0.094
85	0.0425	0.099
90	0.0451	0.105
95	0.0475	0.111
100	0.0501	0.117
105	0.0525	0.122
110	0.0551	0.129
115	0.0575	0.134
120	0.0601	0.139
Mean=100.42 %	RSD=0.78 %	

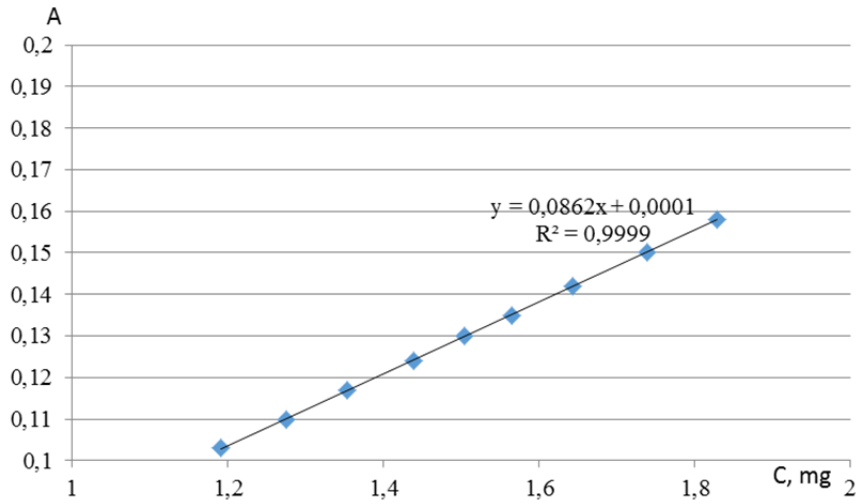

Fig. 4. Calibration graph of optical density versus flavonoid content in a capsule mixture after complexing reaction with aluminum chloride

Precision studies were performed on nine determinations in the concentration range from 80 to 120 % according to the chosen method (Tab. 1). The obtained results (Table 2) showed that the method is precise, since the value of the relative confidence interval is less than the critical value for the convergence of results: Δ %=0.41≤2.60 and the criterion of insignificance of systematic error δ=0.32 is fulfilled.

The results of experimental studies of 6 series of the drug "Uroholum, capsules" and metrological characteristics of the average result are shown in Table 3.

Table 2

Results of the study of the precision of the method of quantitative determination of flavonoids

Parameters		Value	Criterion 1	Criterion 2	Conclusion
Precision	ΔZ	0.78	≤2.60		

Table 3

Metrological characteristics of the average result of quantitative determination of the amount of flavonoids in capsules, in terms of rutin

Series No.	The content of flavonoids, mg	S^2	S	$S_{\bar{x}}$	Δx	$\Delta\bar{x}$	$\bar{\varepsilon},\%$	$\varepsilon,\%$
1	1.49	0.0074	0.0862	0.0352	0.0709	0.0289	1.87	4.59
2	1.45							
3	1.69							
4	1.55							
5	1.50							
6	1.59							

The control of the content of essential oils is obligatory for the vegetable raw materials containing them. For proper standardization, quality control techniques must be validated.

Study of the validation characteristics of the method of quantitative determination of essential oils. The specificity of the gravimetric analytical procedure was proved by determining the mass of the weight form: solvent; placebo (all capsules excipients); model mixture (dry extract with all the excipients of the capsules); the test extract.

Research has shown that neither the solvent, nor the placebo, nor the excipients of the capsules, affect the results of the quantitative determination of the essential oil. The placebo effect was δ=0.02 % ≤ max δ=1.02 %. Therefore, the deviations are insignificant and therefore the specificity of the technique was confirmed.

The eligibility criterion is a linear relationship between the concentration of the essential oils in the capsules and the mass of the weight form with a correlation coefficient of not less than 0.9924.

The results of the studies are given in Table 4 and Fig. 9 and showed that the method is linear, since the magnitude of the correlation coefficient (r) is 0.9995≥0.99957, the angular coefficient of linear dependence (b) is 0.9538, the free term of linear dependence (a) is 4.55≤5.10.

Precision studies were performed on nine determinations in the concentration range of 80 to 120 % according to the method chosen. The obtained results (Table 4 and 5) show that the method is precise because the value of the relative confidence interval is less than the critical value for the convergence of results: Δ %=1.43≤2.60 and the criterion of insignificance of systematic error δ=0.02 is fulfilled.

The results of experimental studies of 6 series of the drug "Uroholum, capsules" are given in Table 6.

Table 4

The results of the study of the dependence of the concentration of essential oil in the capsule by weight of the form

% from working concentration	Weight of the capsule mass, g	Weight of the form, mg
80	1.6001	1.71
85	1.7003	1.80
90	1.7992	1.90
95	1.9004	2.02
100	2.0002	2.11
105	2.1003	2.20
110	2.2008	2.32
115	2.3006	2.42
120	2.3997	2.50

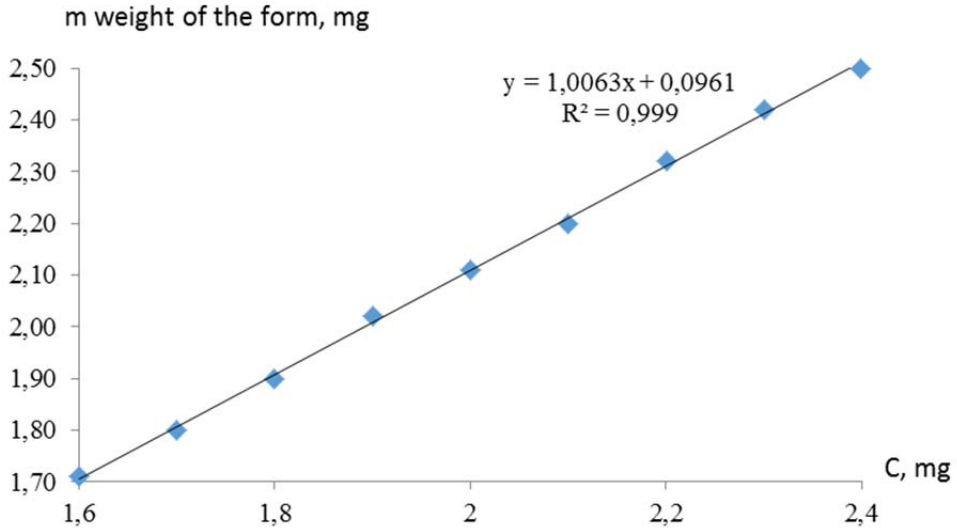

Fig. 9. Calibration graph of the concentration of essential oils in the capsule by weight of the form

Table 5

Results of the study of precision and correctness of the method of quantitative determination of essential oil

Parameters		Value	Criterion 1	Criterion 2	Conclusion
Precision	ΔZ	1.43	≤2.60		

Table 6

Metrological characteristics of the result of quantitative determination of the amount of essential oil in capsules

Series No.	Content of essential oil, mg	S^2	S	$S_{\bar{x}}$	Δx	$\Delta\bar{x}$	$\bar{\varepsilon}$,%	ε,%
1	0.0687	0.00003	0.0055	0.0022	0.0111	0.0045	7.37	18.05
2	0.0552							
3	0.0606							
4	0.0551							
5	0.0628							
6	0.0655							

5. Discussion of the results

It is proposed to standardize the dry total phytoextract in capsules by the sum of substances of flavonoid structure and the sum of essential oils. When studying the validation characteristics of the spectrophotometric quantitative determination of the amount of flavonoids in terms of rutin, it was found that the effect of background absorption is negligible δ_{noise}=0.86 %≤max δ=1.02 %, the linear dependence of the amount of flavonoids in terms of rutin in the concentration range of the total dry extract in capsules is observed from 80 to 120 %, since the value of the correlation coefficient (r) is 0.9999 ≥ 0.9995. The technique is precise because the relative confidence interval is less than the critical value for the convergence of results: Δ %=0.41≤2.60.

We studied the validation characteristics of the gravimetric method of quantitative determination of the sum of essential oils - linearity (correlation coefficient (r) is 0.9995≥0.9995), precision (Δ %=1.43≤2.60), testify to the correctness of the method.

Study limitations. The multicomponent nature of the studied phytoextract and, at the same time, the limitations of quantitative determination methods in standardization of products with plant components (the presence of various biologically active substances in each medicinal product leads to the same number of quality control procedures) leads to the determination of the sum of the basic BAS that are responsible for the pharmacological action of the proposed dosage form.

Prospects for further research. The results of studies have shown that sensitive and specific methods for quantifying the amount of biologically active compounds of dry multicomponent phytoextract in capsule formulation have been developed. Since the total extract includes 10 medicinal plants, further methods of analysis will also be applied in the future.

6. Conclusions

Available and validated methods for the quantitative determination of the amount of biologically active substances of a dry multicomponent herbal extract in solid capsules have been developed and validated. The determination of the quantitative content of dry total phytoextract in capsules by the sum of substances of flavonoid structure by the method of absorption spectrophotometry and the sum of essential oils were substantiated, for the quantitative estimation of which we used the method of gravimetry. Validation characteristics, such as specificity, linearity, and precision were studied, and they meet the eligibility criteria.

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