Development of a New Approach for Standardization of the Herb *Centaurium erythraea* Rafn. by High Performance Liquid Chromatography

*Centaurium erythraea* Rafn. Bitkisinin Yüksek Basınçlı Sıvı Kromatografisi Yöntemi ile Standardizasyonu İçin Yeni Bir Yaklaşımın Geliştirilmesi

**Objectives:** The aim of this study was the development a new, fully validated high performance liquid chromatography (HPLC) method for the quantitative analysis of secoiridoid glycosides by an active marker swertiamarin in the herb *Centaurium erythraea* Rafn. The article describes a new approach to the standardization of *C. erythraea* and more specifically the development of a new validated HPLC method for the quantitative determination of secoiridoid glycosides by swertiamarin.

**Materials and Methods:** The quantitative determination of swertiamarin was performed in isocratic mode on a Symmetry C18 column using water and acetonitrile as solvents for the mobile phase.

**Results:** Validation characteristics of the developed method showed that it was linear in the whole range of concentrations from 0.01 mg/mL to 0.05 mg/mL swertiamarin. All validation characteristics met the established acceptance criteria.

**Conclusion:** This method can be used in the standardization of raw materials, as well as in the analysis of medicinal products and dietary supplements that include *C. erythraea*. The established chromatographic method was successfully applied for the analysis of raw materials of *C. erythraea* with the quantitative content determination of swertiamarin in the analyzed samples.

**Key words:** Common centaury herb, method development, HPLC, swertiamarin, validation

**ABSTRACT**

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**ÖZ**

Amaç: Bu çalışmanın amacı, *Centaurium erythraea* Rafn. bitkisinde bulunan sekoiridoidleri glikozitlerinin aktif göstergesi olan swertiamarinin yeniden, tam valide bir yüksek basınçlı sıvı kromatografisi (HPLC) yöntemiyle quantitatively analizir. Bu makale *C. erythraea*’nin standardizasyonunun ve daha spesifik olarak da swertiamarin ile sekoiridoid glikozitlerini yenide HPLC yöntemi geliştirerek quantitativa olarak belirlenmesinden söz etmektedir.

Gereç ve Yöntemler: Swertiamarinin quantitativa belirlenmesi mobil faz için su ve asetonitrile bileşenleriyle performed in isocratic mode on a Symmetry C18 column using water and acetonitrile as solvents for the mobile phase.

Bulgular: Geliştirilen yöntemin validasyon karakteristikleri 0,01 mg/mL swertiamarinden 0,05 mg/mL swertiamarine dek olan geniş bir konsantrasyon aralığında yöntemin doğruluk ve güvenilirlik kriterlerini dikkate alındığında kabul edilebilir olarak belirlenmiştir. Tüm validasyon karakteristikleri kabul edilebilir kriterlere uymaktadır.

Sonuç: Bu yöntem, *C. erythraea* içerisindeki ham hammadde ve medisinal ürünler ve diyetel suplemenlerin analizinde standardizasyon için kullanılabilir. Bu kromatografik yöntem analiz edilen *C. erythraea*’nin hammaddelemelerde swertiamarinin kalitativa içeriğini başarılı bir şekilde belirlenmesi için uygulanmıştır.

Anahtar kelimeler: Kantaron bitkisi, yöntem geliştirme, HPLC, swertiamarin, validasyon

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INTRODUCTION

Centaurium erythraea Rafn. is a valuable source of various biologically active compounds (BACs), including bitters.1 Due to the presence of this particular group of BACs, the plant is widely used to treat diseases of the gastrointestinal tract and is included in the composition of drugs and dietary supplements.2 The main representatives of secoiridoid glycosides, which determine the pharmacological action of this raw material, are swertiamarin, sweroside, and gentiopicroside (Figure 1).

Bitters improve intestinal motility, increase the reduced secretory function of the stomach, and are used in the treatment of hypoacidic and chronic atrophic gastritis as well. In addition, secoiridoid glycosides show anti-inflammatory and antibacterial activity.3

The results of studies on the biological action of a secoiridoid glycoside of centaury, namely swertiamarin, are widely described in the literature. It has antihyperlipidemic,4 hypoglycemic,5 insulinotropic,6,7 and antinociceptive8 actions. In addition, swertiamarin exhibits an anticholinergic effect9 and depressant effect, inhibits human DNA lipase, and has a central depressant effect, inhibits human DNA lipase, and has a central nervous system depressant effect10 as well as inhibiting the growth of Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Escherichia coli, Proteus mirabilis, and Serratia marcescens.2

It is important to mention that there are only a few publications regarding the description of the methods of C. erythraea analysis. Kaluzova et al.11 described the analysis of gentiopicroside by high performance liquid chromatography (HPLC) in C. erythraea. Valentao et al.12 analyzed xanthones (validation is not described). Glatz et al.13 described the method for determining gentiopicroside in extracts of C. erythraea using micellar electrokinetic capillary chromatography, and Nikolova-Damyanova and Handjieva14 carried out a quantitative determination of swertiamarin and gentiopicroside in C. erythraea using thin-layer chromatography method. Based on this, as well as on the results of the study of its biological activity, we suggested choosing swertiamarin as an active marker in the development of methods for standardization of C. erythraea.

The European Pharmacopoeia16 suggests the use of swertiamarin as a marker compound during the test “Identification” by the thin-layer chromatography method. Based on this, as well as on the results of the study of its biological activity, we suggested choosing swertiamarin as an active marker in the development of methods for standardization of C. erythraea. The literature describes approaches for the quantitative determination of swertiamarin in other types of raw materials, such as its estimation in Enicostemma littorale17 and its analysis in different Swertia species.18 When developing the methodology, all the described approaches to the analysis of swertiamarin were studied and a new selective, sensitive, and accurate HPLC method for its quantitative determination in C. erythraea was developed. The most optimal conditions for the quantitative analysis of swertiamarin in the common centaury herb by the HPLC method are proposed: isocratic elution mode and more acceptable chromatographic time, based on the characteristics of this raw material in order to ensure maximum specificity (exclusion of influence on the analysis of ballast substances and other BAS groups of this raw material).

During the course of the experiment, a new, fully validated method was developed for the quantitative analysis of secoiridoid glycosides by the active marker swertiamarin in C. erythraea by HPLC method. This method can be used in the standardization of raw materials, as well as in the analysis of medicinal products and dietary supplements that include C. erythraea.

MATERIALS AND METHODS

HPLC

Quantitative analysis of swertiamarin in C. erythraea was carried out on a ProStar liquid chromatograph equipped with an autosampler 410 and two detectors, spectrophotometric PDA 325 and photodiode array detector PDA 330, made by Varian (USA). A Symmetry C18 column (150x4.6 mm, particle size 3.5 µm) with a precolumn was also used. A mixture of water and acetonitrile at the ratio of 91:9 was used as the mobile phase. The separation was carried out in isocratic mode. The flow rate of the mobile phase was 1 mL/min, the injection volume was 20 µL, the detection was carried out at a wavelength of 238 nm, and the column temperature was 40°C.

Figure 1. Structural formulas of secoiridoid glycosides of centaury

Swertiamarin  Sweroside  Gentiopicroside
The sampling was carried out on an analytical balance, Ohaus Adventurer brand AR2140 (USA), by standard procedure for raw materials, according to the European Pharmacopoeia (Eur. Ph. 2.9.12). During the sample preparation, the following items were also used: a 355 sieve (SL-200) and a medical laboratory centrifuge with a rotor RU-180 OPN-12 “OAO TNK DASTAN”.

**Raw materials and reagents**

To conduct the research, 20 series of *C. erythraea* plants were collected in various regions of Ukraine during the flowering period. Macroscopic and microscopic identification of raw materials was carried out at the Department of Botany of the National University of Pharmacy, Ukraine. The plant species were deposited in the herbarium section of the same department (code - BDC 12703).

The following reagents were used: acetonitrile (Sigma Aldrich, gradient grade, for HPLC), methanol (Sigma Aldrich, gradient grade, for HPLC), and water for chromatography (Millipore).

A standard sample of swertiamarin (purity 99.5%), series OS10475 (Carbosynth, UK), was also used.

Solutions for the analysis were prepared according to the following methods:

**Test solution:** 0.500 g (accurately weighed) of the powdered raw material (355 µm, Eur.Ph. 2.9.12) was supplemented with 20 mL of methanol, shaken for 15 min, and centrifuged and the supernatant was removed into a 50-mL volumetric flask. The extraction was repeated with a further 20 mL of methanol, with collection of the supernatant as before. The volume of the solution was made up to the mark with methanol and mixed. Then 10 mL of the obtained solution was diluted to 50 mL with water. The solution was filtered through a 0.45 µm membrane filter.

**Reference solution:** 0.010 g of swertiamarin (accurately weighed) was placed into a 100 mL volumetric flask, dissolved in 50 mL of methanol, and then the volume was made up to the mark with the same solvent and mixed. Then 10 mL of the obtained solution was diluted to 50 mL with water, mixed, and filtered through a 0.45 µm membrane filter.

**Validation**

Validation of the developed method was carried out in accordance with the recommendations of the ICH,\(^9\) the requirements of article 2.2.N.2 of SPhU,\(^20\) and the standard procedure of quantitative methods validation using an external standard by studying its linearity, as well as its accuracy, robustness, and precision.

To study the specificity, the following solutions were prepared: a blank solution, a reference solution (a solution of a standard sample of swertiamarin), and a test solution.

To confirm the linearity of the method, five model solutions were prepared, the concentration of which varied uniformly within the application range to the extent of 50-250% (step 50%).

To determine the accuracy and precision within the range of use of the analytical method, five test solutions were prepared, in compliance with all the stages of the analytical procedure. The concentration of swertiamarin in the prepared solutions ranged from 0.01 mg/mL to 0.05 mg/mL.

In order to determine the intra-laboratorial precision, one sample was examined six times by two analysts on different days during one working week using various measuring glassware.

**Statistical analysis**

The analytical performance of the HPLC method was verified for compliance with the requirements. All tests were performed on three replicate injections and standard deviations for each analysis were calculated.

**RESULTS AND DISCUSSION**

**Analysis of medicinal plant raw materials**

An HPLC method was developed for analyzing the quality control of the medicinal plant raw material, *C. erythraea*. Swertiamarin was chosen as the active marker. It is suggested for the standardization of the raw material, as it was previously established that centaury contained the highest amount of swertiamarin and other secoiridoid glycosides, such as sweroside and gentiopicroside, in smaller amounts.\(^21\)

The HPLC method for analyzing secoiridoid glycosides in centaury was developed on the basis of the State Research Laboratory for Quality Control of Medicines of the NUPh.

The results of quantitative determination of the swertiamarin content in the analyzed samples of the medicinal plant raw materials are shown in Table 1.

**Method validation**

When choosing the criterion for rationing the quantitative content of swertiamarin in the centaury, we used the results of the analysis of the raw materials for all indicators applicable to medicinal plant raw materials. It was found that the raw materials, in which the content of swertiamarin was less than 3%, did not meet the requirements of the Pharmacopoeia for such parameters as “foreign matter” and “total ash”. On this basis, the quantitative content of swertiamarin in the centaury was not less than 3% in terms of dried raw materials. The results obtained during the analysis showed that 15 series of raw materials met these requirements.

The total uncertainty of the developed method was calculated, which in this case is related to the limits of the analyte content in the medicinal plant raw materials. For the centaury, the established content of swertiamarin is normalized at a level of at least 3%. In accordance with the requirements of SPhU 2.0 for quantitative determination (one-sided rationing “no more”), the maximum permissible total uncertainty of the analysis method is \(\max \Delta_{AS} < 6.4\%\).\(^17\)

The criterion of insignificance compared with the maximum permissible uncertainty of the results is \(\Delta_{AS, inug} ≤ \Delta_{AS, max} \sqrt{\Delta_{AS}} \times 0.32 = 6.4\% \times 0.32 = 2.048\%\).
The calculation of the uncertainty of the final analytical operation $\Delta_{FAO}$ was carried out for the test solution and the reference solution. When calculating the intervals, Student’s one-sided coefficient was used for a probability of 95% and the corresponding number of freedom degrees. Confidence intervals for the reference solution and the testing solution were calculated for an average of five results.

According to the requirements of suitability of the chromatographic system in the determination procedure, the relative standard deviation for five parallel determinations should be no more than 2.0%.

When $n=5$, $t(95\%, n-1)=2.1318$:

$$\Delta_{FAO} = \frac{t \times \text{S.E.}}{\sqrt{n}} = 2.1318 \times 2.0\% = 1,907\%$$

The total uncertainty of the final analytical operation:

$$\Delta_{FAO} = \sqrt{\Delta_{FAO}^2 + \Delta_{FAO}^2} = 2.70\%$$

Complete uncertainty of the analysis techniques $\Delta_{AS}\%$:

$$\Delta_{AS} = \sqrt{\Delta_{FAO}^2 + \Delta_{AS}^2} = 3.39\%$$

Thus, the calculated total uncertainty of the analysis $\Delta_{AS}\%$ is less than max $\Delta_{AS}$ (3.39% < max $\Delta_{AS}$=6.4%), which meets the requirements for this parameter.

**Specificity**

Under the conditions of the developed method, the determination of the active substance of swertiamarin was not interfered with by the solvent or the mobile phase, or other co-eluting impurities from the raw material at a detection wavelength of 238 nm, which indicates the specificity of the developed method.

Chromatograms of the blank solution, the test solution, and the reference solution are shown in Figure 2 in order to confirm the specificity of the method.

**Linearity**

The method of quantification must be linear within the application range and must cover the possible values of the active substance concentrations. According to the requirements of the State Pharmacopoeia of Ukraine, the application range of the method of quantitative determination of swertiamarin in the medicinal plant raw materials must be from 50% to 250%.

Chromatograms of the solutions studied are shown in Figure 3. The linearity curve is presented in Figure 4.

The linearity parameters, which are presented in Table 2, indicate the linearity of the method within the test range.

The results obtained confirm that the method developed for the quantitative determination of swertiamarin by HPLC in the concentration range from 0.01 mg/mL to 0.05 mg/mL is linear.

**Accuracy, precision, and intermediate precision**

Accuracy is characterized by two criteria:

- Criterion of statistical insignificance: $\delta\% = | Z - 100 | \leq \frac{\Delta z}{\sqrt{5}}$

| No series | The region of collection of raw materials | Quantitative content of swertiamarin, % |
|-----------|-----------------------------------------|---------------------------------------|
| 1         | 2                                       | 3                                     |
| 1         | Dnipropetrovsk region                    | 3.6                                   |
| 2         | Dnipropetrovsk region                    | 3.9                                   |
| 3         | Dnipropetrovsk region                    | 1.7                                   |
| 4         | Ivano-Frankivsk region                   | 7.7                                   |
| 5         | Kharkov region                           | 4.5                                   |
| 6         | Kharkov region                           | 6.4                                   |
| 7         | Kharkov region                           | 2.8                                   |
| 8         | Kiev region                             | 8.3                                   |
| 9         | Lviv region                             | 7.3                                   |
| 10        | Lviv region                             | 4.8                                   |
| 11        | Lviv region                             | 2.4                                   |
| 12        | Poltava region                           | 6.6                                   |
| 13        | Poltava region                           | 7.1                                   |
| 14        | Poltava region                           | 1.5                                   |
| 15        | Rovenyskaya region                       | 9.1                                   |
| 16        | Sumy region                             | 7.8                                   |
| 17        | Sumy region                             | 8.4                                   |
| 18        | Sumy region                             | 2.2                                   |
| 19        | Volyn region                            | 7.8                                   |
| 20        | Volyn region                            | 6.0                                   |

HPLC: High performance liquid chromatography

Table 1. The results of experimental studies of the swertiamarin content in centaury by HPLC method

![Figure 2. Chromatograms of the blank solution 1), the test solution 2), and reference solution 3)](image-url)
δ% - criterion of practical insignificance - in the case the above ratio is not satisfied, we must use the criterion of insignificance of this systematic error compared with the maximum allowable uncertainty of the analysis:

\[
|Z - 100| \leq \Delta, \text{insignificance} = 2.048% 
\]

The fulfillments of the criteria of accuracy, precision, and intermediate precision for determining swertiamarin in the common centaury herb by HPLC are given in Table 3.

The method of determining swertiamarin in centaury satisfies the criteria for acceptability of the validity indicators accuracy, precision, and intermediate precision.

![Figure 3. Chromatogram of swertiamarin solutions for linearity determination in the concentration range from 0.01 mg/mL to 0.05 mg/mL](image)

![Figure 4. The linearity curve of swertiamarin concentration by HPLC method](image)

HPLC: High performance liquid chromatography

| Table 2. The linearity parameters of the quantitative determination method |
| --- | --- | --- | --- | --- |
| No | Parameter | Requirements | Received value | Criterion fulfillment |
| 1 | \( |a| \) | \( \leq 5.1 \) | 1.0625 | Performing |
| 2 | \( S_0 \) | \( \leq 3.4 \) | 0.67 | Performing |
| 3 | \( r \) | \( \geq 0.9691 \) | 0.9999 | Performing |

| Table 3. The results of the evaluation of the accuracy, precision, and intermediate precision of the HPLC method |
| --- | --- | --- | --- | --- |
| Parameter | Index | Requirements for statistical insignificance | Requirements for practical insignificance | Criterion fulfillment |
| \( |Z| - 100| \) | 1.73 | \( \leq 0.64\% \) | \( \leq 2.048\% \) | Performing by the second criteria |
| \( \Delta Z \) | 1.428 | \( \leq 6.4\% \) | \( \leq 6.4\% \) | Performing |
| \( \Delta \text{intra} \) | 1.22 | \( \leq 6.4\% \) | \( \leq 6.4\% \) | Performing |

**Stability**

The study of the stability of the reference solution was carried out immediately after the preparation and 12 h and 24 h later. The results are presented in Table 4.

Differences between the obtained values of the swertiamarin content must not exceed the criterion of insignificance in comparison with the maximum permissible uncertainty of the analysis results \( (\Delta_{\text{max}, \text{insignificance}}) \), that is 2.048%. According to the results of the determination, for optimal chromatographic
conditions it is necessary to use a freshly prepared comparison solution within 12 h, which means in one working day.

| No | model solution | Parameter change, % |
|----|----------------|---------------------|
|    |                | 12 h later | 24 h later |
| 1  |                | 1.0214    | 6.1820    |
| 2  |                | 1.0296    | 5.8819    |
| 3  |                | 1.0762    | 6.4831    |
| 4  |                | 1.1395    | 6.1431    |
| 5  |                | 1.6306    | 6.3427    |
| ∆_ser |            | 1.18      | 6.21      |

CONCLUSION

This article presents a new method for the quantitative determination of swertiamarin as an active marker for the standardization of raw materials, i.e. *C. erythraea*. The method developed was fully validated and can be used to control the quality of both the raw material (*C. erythraea*) and in the analysis of medicinal products and dietary additives that include this plant.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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