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ARTICLES

Simultaneous Determination of Wogonin, Scutellarin, Baicalin, and Baicalein in Extracts from *Scutellariae baicalensis* by High-Performance Liquid Chromatography with Tandem Mass Spectrometry

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Abstract—*Scutellariae baicalensis* is one of the most popular herb in the traditional Chinese medicine. It is used for the treatment of inflammatory process, hypertension, cardiovascular diseases, and also in the treatment of bacterial and virus infections because of such active components as wogonin, scutellarin, baicalin, and baicalein, which are contained in this herb. A sensitive and selective method of the simultaneous determination of these compounds in the extracts from plant raw materials using high-performance liquid chromatography–tandem mass spectrometry with electrospray ionization was developed. The proposed method has been successfully tested on commercially available samples of the *Scutellariae baicalensis* root. Analysis of samples was carried out using reversed-phase chromatography with an Acclaim RSLC C18 adsorbent. Using multiple reactions monitoring the following limits of detection were achieved: 1 ng/mL for wogonin and baicalin, 3 ng mL^{-1} for scutellarin, and 4 ng mL^{-1} for baicalein. It was found that the calibration curve was linear in the concentration range 20 ng mL^{-1} and 2000 ng mL^{-1} for scutellarin and 20 ng mL^{-1} and 500 ng mL^{-1} for wogonin, baicalin, and baicalein.

Keywords: high-performance liquid chromatography/mass spectrometry, electrospray ionization, extraction, *Scutellariae baicalensis*, wogonin, scutellarin, baicalin, baicalein

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INTRODUCTION

At present, special attention is paid to the investigation of various medicinal plants in order to identify their active components. The study of the properties of the known substances and the identification of previously unknown compounds open ways to the creation of new pharmaceuticals. Thus, e.g., a plant of the species *Scutellaria baicalensis* (Baikal skullcap) includes such flavonoid biologically active compounds as wogonin, scutellarin, baicalin, and baicalein (Fig. 1), which have various pharmacological functions, including anticancer, antibacterial, antiviral, antioxidant, etc. [1–4].

According to their chemical properties, these compounds are classified as medium-polarity organic substances with high hydrophobicity; they do not have strongly pronounced acid-base properties; and at intensive heating they decompose. Chromatography method plays a key role in solving the problem of the separation, determination, and identification of physiologically active compounds. Because of the large number of hydroxyl groups, these compounds are low-volatile and inconvenient for determination by gas chromatography; in this regard the most common method for separating these compounds is liquid chromatography [5].

Before analysis by liquid chromatography, the active components should be extracted from plant samples or medical materials. Solid-phase and liquid extraction are widely used methods of sample preparation of medicinal compounds in complex matrices [6]. Supercritical fluid extraction (SFE) is another widely used method of extracting active components from samples of plant raw materials. In [7], SFE was used to extract baicalin, baicalein, and wogonin from *Scutellaria baicalensis*. The authors of [8] proposed pressurized liquid extraction using methanol and hot water to extract baicalein from *Scutellaria radix*.

The literature describes the use of various chromatographic methods for determining the concentration of active components of *Scutellaria baicalensis* in various parts of the plant and preparations based on it. These methods include high-performance liquid chromatography (**HPLC**) [9–13], high-speed countercurrent chromatography [14], capillary electrophoresis [15, 16], and micellar electrokinetic capillary

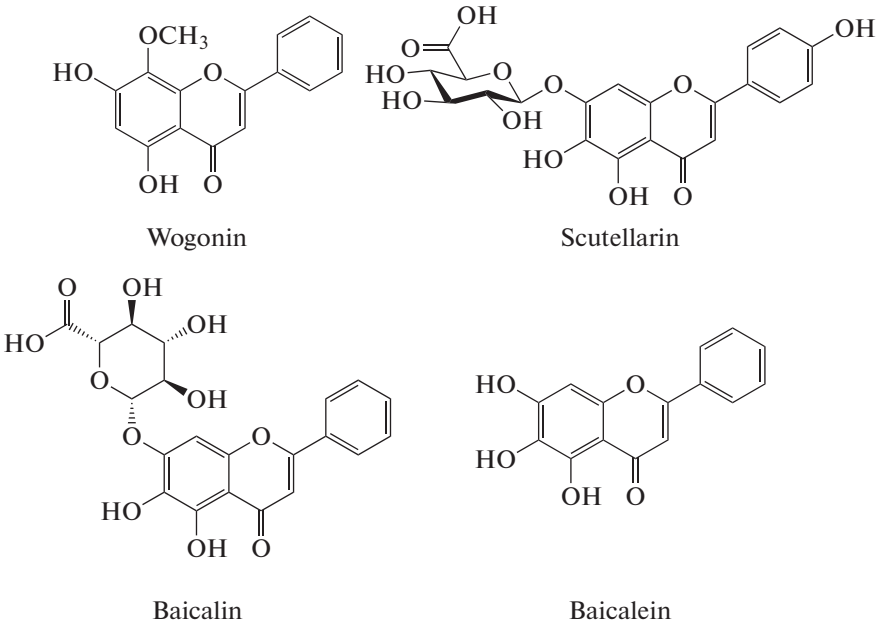

Fig. 1. Structural formulas of the investigated flavonoid biologically active compounds.

chromatography [17, 18]. A large number of works devoted to the determination of these substances by the method of liquid chromatography with tandem mass spectrometric detection (**HPLC–MS/MS**) have also been published [9–13]. They ensure the identification and determination of organic compounds in complex multicomponent samples with high fidelity and sensitivity.

For example, in [9] a simultaneous determination of wogonin, baicalin, baicalein, berberine, palmatine, and gatrorkhizin in rat plasma was carried out using HPLC–MS/MS. The mobile phase consisted of 0.1% formic acid in water and acetonitrile. Detection was accomplished with a mass spectrometer using electrospray ionization in the multiple reaction monitoring mode for positive ions. For wogonin, the transition m/z 285→270 was chosen. The limit of detection by this method was 24 ng/mL.

In [10], a method was proposed that was distinguished by high sensitivity and selectivity for the determination of scutellarin in rat plasma by the HPLC–MS/MS. This method has also been successfully applied to assess pharmacokinetic profiles after taking "*scutellarin guttate*" tablets in 20 healthy volunteers. Mass spectrometric determination was carried out in the version of electrospray ionization in the mode of positive ion detection. Quantitatively, the compounds were determined in the multiple reaction monitoring mode. For scutellarin, the m/z 463→287 transition was chosen, and for the internal baicalin standard, m/z 447→271. The linear range of the calibration curve was 0.2–20 ng/mL. The lowest detectable concentration of this method is 0.2 ng/mL.

In [11], baicalin, wogonoside, baicalein, wogonin, oroxylin A, and chrysin were simultaneously determined by HPLC–MS/MS in plasma of rats using naringin as an internal standard with very low limits of detection, The developed method was successfully used to study the pharmacokinetics of the main flavonoids of the *Radix scutellariae* extract after oral administration to rats. The mass spectrometric determination was carried out by electrospray ionization in the positive ion detection mode. The concentrations of the components were found from the ion transitions that were chosen for baicalin: m/z 447→271 and 273→153 for the naringin internal standard. The limit of detection was 0.5 ng/mL.

Thus, HPLC–MS/MS is the optimal method for the simultaneous determination of wogonin, scutellarin, baicalin, and baicalein. The development of a procedure includes the optimization of mass spectrometric detection conditions, the selection of suitable chromatographic separation conditions providing the rapid and effective separation, the assessment of performance characteristics (limit of detection, linearity range of the calibration curve), and approbation of the developed procedure on real samples of extracts.

EXPERIMENTAL

Solutions and reagents. The following reagents were used in the work: wogonin, scutellarin, baicalin, baicalein (>98%, Phytolab, Germany), acetonitrile for gradient chromatography (Panreac, Spain), formic acid of reagent grade (Khimmed, Russia), methanol for liquid chromatography (B&J, Honeywell, Germany). Deionized water (electrical conductivity of

18.2 μS cm^{-1}) was obtained with a Milli-Q water preparation system (Millipore, USA) from distilled water.

Equipment. A HPLC–MS/MS system consisting of a QTrap 3200 tandem mass spectrometer detector (AB Sciex, Canada) equipped with a Turbo Spray^{tm} electrospray ionization source and an Ultimate 3000 HPLC system (Dionex, USA) was used.

The column used was a reversed-phase C18-column (3 μm, 2.1 × 150 mm, RSLC Acclaim) (Thermo Scientific, USA). The experimental data were recorded and processed using a personal computer and the "Analyst" software package (AB Sciex, Canada).

Preparation of standard solutions and construction of calibration dependencies. Weighed portions of wogonin, scutellarin, baicalin, and baicalein (1 mg) were dissolved in 1 mL of methanol. The solutions obtained were used to prepare series of calibration solutions with the concentrations 0.02, 0.05, 0.2, 0.5, and 2 μg/mL.

Detection of the wogonin, scutellarin, baicalin, and baicalein in plant extracts. Sample preparation for the determination of the concentrations of wogonin, scutellarin, baicalin, and baicalein in dry samples of plant material "1" and "2" (OOO "Zabaikal'e", OOO "Belovod'e") was carried out as follows. Ten milliliters of methanol were added to a weighed portion of a sample (0.1 g), and ultrasonic extraction was carried out for 30 min. The supernatant was filtered, the resulting solution was diluted by 100 and 1000 times with the mobile phase, and a chromatographic determination of wogonin, scutellarin, baicalin, and baicalein concentrations was carried out.

Determination by chromatography—mass spectrometry was performed using an ion source for electrospray ionization in the negative mode of recording selected ion transitions. The temperature of the ion source was 250°C, the voltage on the capillary was −4.5 kV; the pressure of the gas curtain was 15 bar; and pressure of spraying gas was 40 bar. Separation was carried out in a gradient mode (Table 1) of the eluent feed; the flow rate of the eluent was 0.4 mL/min. The temperature of the column thermostat was 25°C; the injected sample volume was 0.020 mL.

RESULTS AND DISCUSSION

Optimization of the conditions of mass spectrometric detection. To select the optimal conditions for tandem mass spectrometric detection, the effect of the mass spectrometer operation parameters (declustering potential, input potential on the zero quadrupole of the mass analyzer, collision energy, etc.) on the intensity of the recorded signals of characteristic ion transitions for wogonin, scutellarin, baicalin, and baicalein was studied.

Table 1. Chromatographic separation conditions in determining wogonin, scutellarin, baicalin, and baicalein

Volume of the injected sample	0.020 mL
Column thermostat temperature	25°C
Eluent flow rate	0.400 mL min^{-1}
Composition of the mobile phase	A–0.5% formic acid solution in water; B–acetonitrile
Gradient elution program	0.00–2.00 min 20% B; 2.01–11.00 min 20–90% B; 11.01–14.00 min 90% B; 14.01–15.00 min 90–20% B; 15.01–19.00 min 20% B

The work was carried out in the direct injection mode using a syringe pump (water–acetonitrile solutions of the determined compounds with the concentration 5–10 μg/mL were used). The formation of a deprotonated molecule was, most likely, due to one of alcohol groups of the test compound skeletons (aglycons).

The criterion for choosing the optimal value of the declustering potential and the input potential on the zero quadrupole of the mass spectrometric detector is the maximum intensity of the peak of the precursor ion in the mass spectrum. Thus, in studying the influence of the declustering potential and the input potential on the intensity of the peaks of precursor ions of each of the investigated compounds, the optimum values were selected, which are presented in Table 2.

The next step in optimizing the mass spectrometry detection of wogonin, scutellarin, baicalin, and baicalein was the selection of suitable ion transitions. For this purpose, the effect of collision energy (**CE**) in the collision chamber of the mass spectrometer on the character of the spectrum of ion products formed during the decomposition of the deprotonated molecules of wogonin, scutellarin, baicalin, and baicalein, and the intensity of their peaks was studied. Figure 2 shows mass spectra of ion products of the determined compounds obtained in the mode of recording of negatively charged ions in the version of electrospray ionization. The most intense signals of fragment ions in the case of scutellarin and baicalin formed as a result of the elimination of the glucuronic acid. The ion products related to the precursor ions of wogonin and baicalein formed by the cleavage of carbon bonds at elevated EF. Thus, in order to obtain the maximum analytical signal in the mode of detecting negatively charged ions, it was necessary to use the selected parameters of the mass spectrometer (Table 2).

Selection of conditions for the chromatographic separation of components. The gradient elution program was used for the chromatographic determination of

Table 2. Optimal parameters of mass-spectrometric detection of wogonin, scutellarin, baicalin, and baicalein using electrospray ionization in the mode of registration of negatively charged ions

Parameters	Optimized values			
	wogonin	scutellarin	baicalin	baicalein
Declustering potential	32 V	32 V	26 V	45 V
Input potential on the zero quadrupole	10 V	10 V	10 V	10 V
Collision energy	30 V	25 V	23 V	38 V
Selected ion trans no. 1	283→268	461→285	445→269	269→195
Selected ion trans no. 2	283→163	461→175	445→175	269→139
Polarity of the recorded ions	negative			

Table 3. Chromatographic parameters for the separation of wogonin, scutellarin, baicalin, and baicalein on the Acclaim RSLC column (150 × 2.1 mm, adsorbent grain size 3 μm) at a flow rate of 0.4 mL min^{-1}

Compound	Retention time, min	Apparent capacity factor	Apparent number of theoretical plates, TP m^{-1}	Equation of the calibration dependence	Correlation coefficient
Wogonin	8.62	9.0	198145	$y = 659.1x + 1544.6$	0.9997
Scutellarin	3.58	3.2	21146	$y = 71.0x + 3913.3$	0.9990
Baicalin	5.65	5.6	151336	$y = 205.0x - 1238.5$	0.9996
Baicalein	7.46	7.7	263829	$y = 19.6x - 576.8$	0.9989

the test compounds (Table 1) in the version of reversed phase chromatography on the Acclaim RSLC C18 adsorbent, under the conditions of which the apparent capacity factors (k') of wogonin, scutellarin, baicalin, and baicalein were 9.0, 3.2, 5.6, and 7.7, respectively, and were acceptable for the method of chromatographic analysis. The terms "apparent capacity factors" and "apparent number of theoretical plates" were introduced to describe the chromatographic parameters of the compounds to be determined in the gradient elution mode. Table 3 shows the chromatographic separation parameters calculated for the compounds under study. In the calculations, a dead time value of 0.86 min was used.

A typical chromatogram obtained under the selected separation conditions (Fig. 3) shows the values of the analytical signal for each of the selected ion transitions.

Assessment of the limits of detection and approbation of the developed method. Under the selected conditions of chromatographic–mass spectrometric determination of wogonin, scutellarin, baicalin, and baicalein, an analysis of the investigated samples of plant materials was carried out. The retention time and the coincidence of two pairs of selected ion transitions corresponding to the component being determined were selected the criterion for the presence of active components in the investigated samples.

The evaluation of the characteristics of the developed approach for the determination of wogonin, scutellarin, baicalin, and baicalein was carried out in the analysis of extracts from the *Scutellaria baicalensis* plant. The limits of detection by the developed method were found as the minimum concentration of substances in the sample that can be reliably recorded (the signal-to-noise ratio for the chromatographic peak is not less than 3). The limits of detection for wogonin, scutellarin, baicalin, and baicalein in the samples of plant extracts were 1, 3, 1, and 4 ng/mL, respectively.

A linear calibration dependence was observed at the concentrations of the determined compounds in the samples in the range 20–2000 ng/mL. Calculations were carried out according to the equation of the calibration curve obtained in the day of analysis (Table 3).

Figure 3 shows chromatograms of samples of the studied extracts "1" and "2," obtained in the analysis (the sample preparation procedure was indicated in the Experimental section).

From the data presented (Table 4), one can see that the concentration of scutellarin and baicalin in sample "1" is much larger than in sample "2." Consequently, the concentration of active components may differ depending on the series and the producing company, and the control of the composition of the plant material used for the production of pharmaceuticals should be carried out using such reliable methods of analysis, such as HPLC–MS. Also, chromatograms (Fig. 3) contain peaks with retention times of 6.2 and 6.4 min, which are related to the glycoside derivative of wogonin. As these compounds have lower hydrophobicity

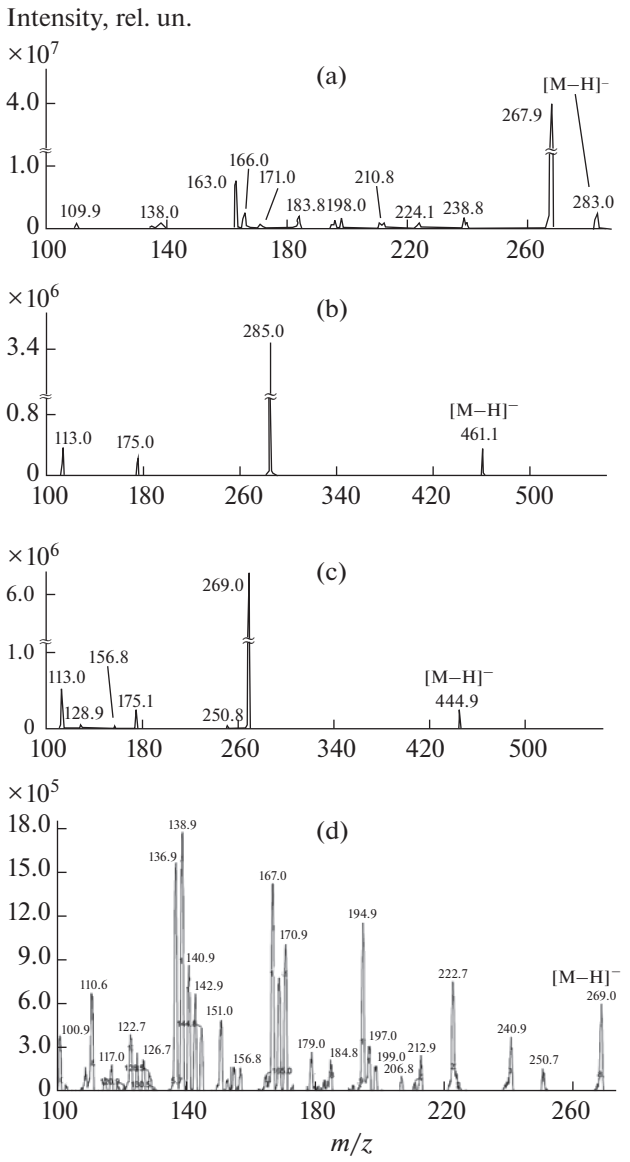

Fig. 2. Ion product spectra of deprotonated molecules: (a) wogonin with m/z 283; (b) scutellarin with m/z 461; (c) baicalin with m/z 445; and (d) baicalein with m/z 269. Electrospray ionization mode in the version of registration of negatively charged ions.

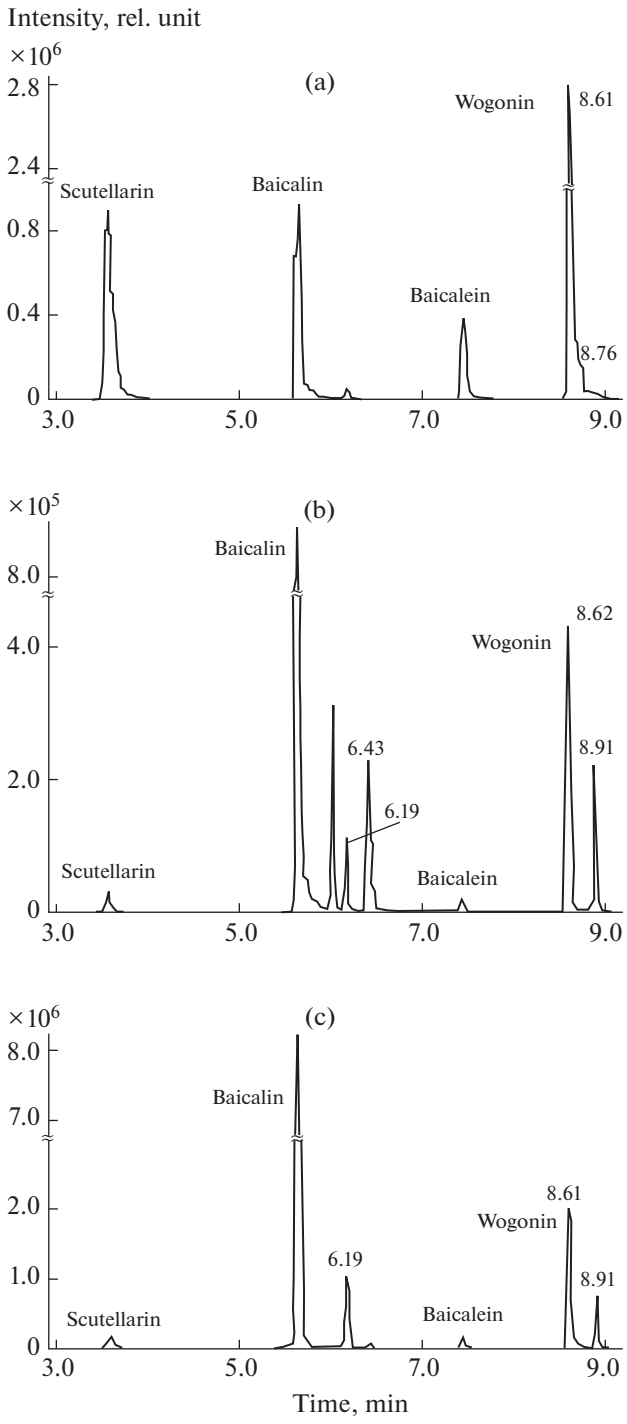

Fig. 3. (a) Chromatogram of a standard aqueous solution containing a mixture of 10000 ng/mL of wogonin, scutellarin, baicalin, and baicalein; chromatograms of extracts of (b) "1" and (c) "2" samples diluted 100-fold, obtained in the determination of wogonin, scutellarin, baicalin, and baicalein.

due to the presence of saccharide substituents, their peaks were completely separated and did not interfere with the determination of the main components.

CONCLUSIONS

Within the framework of this work, an approach to the simultaneous determination of wogonin, scutellarin, baicalin, and baicalein by HPLC–MS/MS was developed and tested for the first time on extracts of commercial samples of the herb Baikal skullcap. In determining each of the compounds, the following limits of detection were achieved in the mode of registration of selected ion transitions: for wogonin and baicalin—1 ng/mL, for scutellarin—3 ng/mL, and for baicalein—4 ng/mL.

Table 4. Concentrations of wogonin, scutellarin, baicalin, and baicalein in dry samples (the results were obtained in the analysis of extracts)

Sample	Concentration of the determined component in the sample, mg g^{-1}			
	wogonin	scutellarin	scutellarin	baicalein
1	3.0 ± 0.5	2.3 ± 0.5	200 ± 11	4 ± 1
2	1.8 ± 0.5	1.6 ± 0.3	120 ± 10	3 ± 1

The linearity range of the calibration curve of standard solutions was 20–2000 ng/mL for scutellarin and 20–500 ng/mL for wogonin, baicalin, and baicalein. A possibility of using the developed method for analyzing samples of plant materials on an example of two commercially available samples of the *Scutellaria baicalensis* herb was demonstrated. Owing to the selected chromatographic separation conditions, the present peaks of wogonin derivatives do not interfere with the determination of the main active components. According to the results obtained, the concentration of active components may vary depending on the series and the producing company; therefore, the introduction of more modern and selective methods for assessing the composition of plant materials in the near future is promising.

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