SUPPLEMENTARY MATERIAL

Simultaneous quantification of six phenylethanoid glycosides in some Turkish Scutellaria species by a new HPLC-DAD method

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Simultaneous quantification of six phenylethanoid glycosides in some Turkish *Scutellaria* species by a new HPLC-DAD method

**Abstract**

A new HPLC-DAD method was developed and validated for simultaneous determination of six main phenylethanoid glycosides (calceolarioside D, neocalceolarioside D, verbascoside, isovertascoside, leucoseptoside A and martynoside) in the aerial parts of four *Scutellaria* L. taxa from flora of Turkey. All standard compounds showed a good linearity ($R^2>0.999$) in a relatively wide concentration range (1-120 µg/mL). The LOD of the compounds was in the range of 0.104-1.295 µg/mL and the LOQ was in the range of 0.450-2.536 µg/mL. The recoveries of the selected compounds were calculated in the range of 97.46-117.85%. The amounts of the phenylethanoid glycosides showed variation in the extracts. The developed method was found to be accurate, precise and reproducible, and successfully applied to identify and quantify the phenylethanoid glycoside composition of *Scutellaria* species.

**Keywords:** *Scutellaria hastifolia*, *S. velenovskyi*, *S. albida* ssp. *albida*, *S. orientalis* ssp. *pinnatifida*, HPLC-DAD, Phenylethanoid glycosides
Experimental

Chemicals and Reagents

Solvents used for HPLC analysis were methanol (MeOH) from Sigma-Aldrich Chemical Co., acetic acid (AA) from Riedel de Haen, acetonitrile (MeCN) from Merck and ultrapure water which was obtained from Milli-Q Plus system (Millipore, Bedford, MA, USA). The reference compounds, calceolarioside D (1), neocalceolarioside D (2), verbascoside (3), isoverbascoside (4), leucoseptoside A (5) and martynoside (6) were previously isolated mainly from *S. hastifolia* (Bardakci et al. 2015) and from some other Lamiaceae plant species in our lab. Their purities were determined to be more than 95% by HPLC analysis. Membrane filters (Sartorius model 0.45 µm PTFE filter) were used for the filtration of the mobile phase, standards and samples prior to working with HPLC system.

Plant materials

The localities where *Scutellaria* specimens were collected are as follows; *S. hastifolia* (YEF 11005) from Sakarya, Turkey in June 2011, *S. velenovskyi* (YEF 11006) from Karabük, Turkey in July 2011, *S. albida* ssp. *albida* (YEF 13008) from Istanbul, Turkey in June 2013, *S. orientalis* ssp. *pinnatifida* (YEF 13009) from Eskişehir, Turkey in June 2013. Plant materials were authenticated by Prof. Dr. Hasan Kırmızibekmez before any process. Voucher herbarium specimens were deposited at the herbarium of the Faculty of Pharmacy, Yeditepe University, Istanbul, Turkey. The plant materials were dried at dark and fresh air ventilated rooms and stored at 25 °C in airtight containers till further use.

Preparation of Standard Solutions

Stock solutions of the standard phenylethanoid glycosides were prepared as 1000 µg/mL by dissolving in MeOH. Standard mixture of the substances at 400 µg/mL was prepared by dilution of the stock solution with MeOH. This mixture was used to prepare standard and quality control solutions at different concentrations (1-120 µg/mL). Working concentrations were 3, 5, 15, 20, 40, 75 and 120 µg/mL for calceolarioside D and neocalceolarioside D, 3, 5, 15, 40, 75, 80 and 120 µg/mL for verbascoside, 3, 5, 15, 20, 40, 60, 75 and 120 µg/mL for isoverbascoside, 1, 3, 5, 15, 20, 40, 60, 75 and 120 µg/mL for leucoseptoside A, and finally 3, 5, 15, 20, 60, 75 and 120 µg/mL for martynoside. 10, 50 and 100 µg/mL concentrations of each
standard phenylethanoid solution were selected as quality control concentrations during validation studies of the method developed. All standard solutions were stored protected from light at -20°C during the study.

**Preparation of Sample Solutions**

Plant samples were powdered to a homogenous size by a mill. 1 g of each *Scutellaria* species were accurately weighed and extracted twice with 20 mL of HPLC grade MeOH in ultrasonic bath for 30 min at 30°C for each time. The pooled extracts were concentrated by rotary evaporator at 45°C. The amounts of each extract and the yields of extractions were calculated. Each sample was dissolved in 5 mL MeOH and filtrated through 0.45 µm Sartorius filters. Appropriate dilution processes were applied with MeOH when necessary. The working solutions were kept protected from light at -20°C until use.

**Instrumentation and Method Optimization**

Analysis was performed on an Agilent High Performance Liquid Chromatographic system (Series 1100, Agilent Technology, Palo Alto, CA, USA) which consisted of a G1311A quaternary pump, G1379A online degasser, A1313A automatic sampler and G1315B DAD detector. All data were acquired and processed using Agilent Chem Station software.

A variety of methods, solvents, times and column types were used in order to obtain an optimal separation of each standard in the extracts. MeOH:H₂O and MeCN:H₂O combinations with different buffer agents (FA, AA) and different concentrations, various flow rates (0.5-1.5 mL/min.), different column temperatures (10°C to 30°C) and column types (zorbax cyan column, zorbax C2 column, zorbax C8 column and zorbax phenyl column) were employed in order to obtain best resolution and the shortest analysis time. AA was preferred as a buffer agent due to eliminate the peak tailing and for the high resolution of peaks. In order to obtain the best separation of analytes various columns having different polarity degree were used and obtained chromatograms were evaluated. The less polar column supporting material, the best separation was procured. Thus, two types of C₁₈ columns were investigated with the particle sizes of 5 µm and 3.5 µm. Finally, the best separation was achieved on a Zorbax XDB-C18 column (4.6×150 mm, 3.5 µm) with the flow rate of 0.8 mL/min. The injection volume was set at 10 µL and three repetitive
injections were performed for samples and standard solutions. Wavelength was selected as 330 nm due to the maximum absorption value in the spectra obtained by DAD detector of HPLC system for phenylethananoid glycosides. Chromatographic column was thermostated at 25°C. The mobile phase was filtered from 0.45 µm pore sized filter and ultrasonically degassed before use. For the simultaneous analysis of six phenylethanoid glycosides gradient elution program was applied. The mobile phase A was H₂O, the mobile phase B was MeCN both containing 0.02% AA and 15-20% B from 0.0 to 20 min, 20-45% B from 25 to 35 min. Identification of the peaks were based on the comparison of retention times and the UV spectra presented in the chromatogram with those of standard compounds. In total, qualitative and quantitative analysis of six phenylethananoid glycosides in four Scutellaria extracts, was concluded in 31 min. Under the proposed analytical conditions, good baseline resolution was obtained for all analytes. Typical chromatograms of the authentic standard solutions are presented in Figure S1.

Method validation

System Suitability

The system suitability is used to verify that the resolution and repeatability of the system are adequate for the analysis to be performed. System suitability results are compared with the values given in the FDA guidelines (Shabir 2003). The capacity factor (k’>2), resolution (Rs >2), tailing factor (T≤2) and theoretical plates (N>2000) are evaluated in this respect and compared with the theoretical data. For this purpose standard mixture was analysed six times and obtained values given in S S1. According to these results, all parameters were in the appropriate range to show the system suitability.

Linearity, LOD and LOQ

The calibration curves were constructed from peak areas of the reference compounds versus their concentrations. The correlation coefficients (r²) of all the calibration curves were consistently greater than 0.999 in the relatively wide concentration range (1-120 µg/mL). The limit of detection (LOD) was defined as the lowest concentration level that can be detected and limit of quantification (LOQ) was defined as the lowest concentration level that can be quantified. LOD and LOQ were
calculated by using standard deviation of calibration curve intercept values (Sa) according to the ICH guidelines and following equations were used: LOD=3Sa/ m
LOQ=10Sa/m. In these equations "m " was the slope of calibration curve. LOD values were calculated between 0.104-1.295 µg/mL and the LOQ values were found in the range of 0.450-2.536 µg/mL. All detailed analysis performance characteristics were given in the Table S2.

**Precision**

Instrument precision was evaluated by analyzing the standard solutions in nine replicate injections. The retention times of the analytes were utilized to determine the intra- and inter-day variability of the method. Three different concentrations (10, 50 and 100 µg/mL) were prepared for each standard compound as quality control (QC) samples. These levels were selected as low level (10 µg/mL), medium level (50 µg/mL) and high level (100 µg/mL) QC samples. The intra-day precision was calculated using nine replications prepared from the QC solutions within the day, while inter-day precision was performed over three consecutive days. The relative standard deviation (RSD) was taken as a measure of precision. The obtained data showed that intra-day RSD values were in the range of 0.010-0.711% and the inter-day RSD values were found in the range of 0.018-0.884% as shown in the Table S3. In precision studies, standard deviation of results should be less or equal than 2%. All values obtained provided this situation. It shows that, the developed method is a precise method.

**Accuracy**

Accuracy of the method was calculated for both intra- and inter-day variations using at least triplicate analysis. In order to check the accuracy of the developed method, recovery values were calculated by using quality control concentrations of standard substances. Calibration curve was used for this purpose. Triplicate experiments of three independent concentrations were performed for each substance. The intra- and the inter-day recoveries of the analytes were found between 97.46-117.85% and 96.49-114.84%, respectively. In addition, RSD values were found between 0.088-3.907% and 0.232-4.165%, respectively and given in the Table S4. In recovery studies, recovery values should be between 90-110%. All results obtained
are in this range. It shows that, this method is appropriate at determined concentration range.

**Robustness**

Robustness studies of the developed method were evaluated according to the ICH guidelines (ICH Guidelines 2005). Temperature (23-27°C), acetic acid percentage (0.015%-0.025%) and wavelength (320–340 nm) were changed deliberately. Recovery values were again calculated for new conditions and compared. For this purpose, temperature (23-27°C), acetic acid percentage (0.015%-0.025%) and wavelength (320–340 nm) were deliberately changed and the effects of the changes were investigated via recovery values. Obtained values were presented on Table S5 which are between the range 90-110%.

**Quantification**

Chromatographic investigations of phenylethanoid glycosides were held by comparing the UV spectra of the corresponding peaks in the plant extracts and the standards (Figure S2-S5). The contents of the each Scutellaria sp. were determined from the corresponding calibration curve and the calculated amounts of the analysed phenylethanoid glycosides were given in Table S6.

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