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Research Article

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Development and validation of a method for the determination of rosmarinic acid in *Mentha piperita* L. using solid-phase extraction and RP-HPLC with photodiode array detection

Andriy Gudzenko

State Laboratory for Quality Control of Medicines, State Institution "Institute of Pharmacology and Toxicology of National Medical Academy of Science of Ukraine", Kiyv, Ukraine. 14, Eugene Potie str., 03680, Kyiv, Ukraine

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ABSTRACT

An analytical method based on an optimized solid-phase extraction procedure and followed by high-performance liquid chromatography (HPLC) separation with diode array detection was developed and validated for the determination of rosmarinic acid in leaves of Mentha piperita L. The chromatographic determination of rosmarinic acid was carried out with a Phenomenex Synergi 4u Fusion-RP C18 (250 x 4.6 mm i.d., 4 μm particle size), as stationary phase, with a flow rate of 1 mL/min and detection at a wavelength of 330 nm. The proposed method was validated by ICH Harmonised Tripartite Guidelines "Validation of analytical procedures: Text and Methodology Q2(R1)". In this study, an excellent linearity was obtained with r higher than 0.99. With other validation data, including precision, specificity, accuracy and robustness, this method demonstrated good reliability and sensitivity, and can be conveniently used for the quantification of rosmarinic acid in leaves of Mentha piperita L. In summary, the method above can be considered specific, exact, precise, linear, robust and easy to perform. Further this method can be applied to a standardization of multicomponent herbal remedies, that incorporate leaves of Mentha piperita L.

Keywords: leaves of *Mentha piperita* L.; HPLC; rosmarinic acid; solid-phase extraction;

INTRODUCTION

Peppermint (*Mentha piperita* L.) is currently one of the most economically important aromatic and medicinal crops. Peppermint leaf and oil are used for folk medicine, as flavoring agents, and in cosmetic a pharmaceutical products throughout the world [1-3].

Clinical investigations and other research suggest that extracts of leaves of *Mentha piperita* L. have multiple health effects including spasmolytic, carminative, cholagogue, antiinflammatory, anticancerogenic, antiallergic, antibacterial , antioxidant and radical scavenging activities [2, 4, 5].

The diversity and complexity of the phytochemical composition of *Mentha piperita* L. species may explain their polyvalent pharmacological activity. The raw material of leaves of *Mentha piperita* L. contains volatile oil, flavonoid glycosides, caffeic acid derivatives, tocopherols and tannins [4-6].

Caffeic acid derivatives constitute one of the most important groups of pharmacologically active principles in *Mentha piperita* L. It is suggested that anti-inflammatory, free-radical-scavenging, and antibacterial activities of leaves of *Mentha piperita* L. are attributed to the caffeic acid derivatives complex [6-8].

The main components of the caffeic acid derivatives fraction of leaves of *Mentha piperita* L. is rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid [6, 9, 10]. Structure of rosmarinic acid shown in the Fig.1.

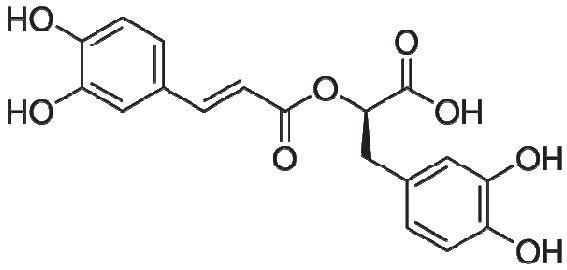

Figure 1: Chemical structure of Rosmarinic acid

RA has been reported to have some biological activities *in vitro* such as antiviral properties including anti-HIV-1, antibacterial, antioxidant, anti-carcinogenic, and anti-allergic activities [11-14]. *In vivo* studies have shown that RA exhibit anti-allergic, anti-thrombotic, and anti-carcinogenic properties as well [15-17].

Thus, the objective of this study was to develop and validate a method for the separation and quantitative analysis of rosmarinic acid using solid-phase extraction and RP-HPLC with photodiode array detection, obtained from an extract of leaves of *Mentha piperita* L.

The method was validated according to ICH Harmonised Tripartite Guidelines "Validation of analytical procedures: Text and Methodology Q2(R1)" [18].

The following validation characteristics were assessed: specificity, linearity, limit of detection and quantification, accuracy, precision and robustness.

EXPERIMENTAL SECTION

Plant material.
The leaves of *Mentha piperita* L. was collected in the region of Borispol (Kyiv region, Ukraine) in June 2011. Identification of the species was confirmed in State Laboratory for Quality Control of Medicines, State Institution "Institute of pharmacology and toxicology National Academy of Medical Sciences of Ukraine" (Ukraine). A voucher specimen (M011-5) was deposited at the herbarium in this laboratory.

Chemicals and reagents.
All reagents and solvents were analytical and HPLC grades (Fluka, USA). Ultra-pure water obtained using a Simplicity® apparatus (Millipore, USA) with conductivity of 0.60 µS/cm was used in all experiments. Rosmarinic acid (Fluka, USA) of the highest grade (purity>97.0%) were used as the external standards.

Instrumentation and chromatographic conditions.
The analyses were carried out using an HPLC system (Shimadzu, Japan) consisting of a solvent delivery pump (Model LC-20 AD), a diode array detector (Model SPD-20A), an auto-injector (Model SIL-20A) and system controller (Model CBM-20A). Data collection and analyses were performed using LCsolution (ver. 1.22SP1). A gradient elution was performed on a Synergi 4u Fusion-RP C18 (250 x 4.6 mm i.d., 4 µm particle size) (Phenomenex, USA). The mobile phase consisted of two different solutions, solution A and solution B. Solution A: 0.05% trifluoroacetic acid solution in acetonitrile. Solution B: 0.05% trifluoroacetic acid in water. All solutions were degassed and filtered through a 0.45 µm pore size filter (Millipore, USA). Separations were effected by a gradient elution program as follows: from 0 to 5 min, B was isocratic at 95%; from 5 to 30 min, solution B followed a linear change from 95% to 60%; from 30 to 40 min, B linearly changed from 60% to 50%, from 40 to 45 min, B was isocratic at 50%; and from 45 to 60 min, B was isocratic at 95%. The mobile phase flow rate was 1 mL/min and the injection volume was 5 µL. UV detection was performed at 330 nm.

Using these chromatographic conditions, it was possible to confirm the retention time of rosmarinic acid by injection of standard.

Sample preparation
Plant samples (1.0 g) were extracted two times, each time for 45 min at 100°C, with a 50 % ethanol, by use of a hot reflux equipment, and the extracts were combined in a 100 ml flask with 50 % ethanol.

To 10 ml the obtained extract was added 20 ml of water and mixed (sample extract).

The SPE cartridge, namely Superclean lc-18 SPE (100 mg/1 mL) from Supelco (USA) was preconditioned with 5 mL of methanol and equilibrated with 3mL of deionized water. Then 15 mL of the sample extract was poured into the Superclean lc-18 SPE cartridge. The cartridge washed with 10 ml of 15% ethanol. Then eluted with 4 ml of methanol. Final fraction were collected and diluted with methanol to a volume 5 ml.

Aliquots of 5 µL were injected into the HPLC column.

Preparation of standard solution.
Accurately weighed appropriate amounts of the reference compound (rosmarinic acid) were mixed and dissolved in methanol in a 100-mL volumetric flask, to obtain a stock solution. The concentration of compound in this solution was 1038 µg/mL. Besides, external standards were established at seven data points covering the concentration range of rosmarinic acid according to the level estimated in the plant sample. Working solutions were prepared by stepwise dilution of the stock solution with methanol.

Method validation.
In the validation of the analytical method used for the quantification of rosmarinic acid in leaves of *Mentha piperita* L., the following parameters were determined: specificity, linearity, sensitivity, accuracy, precision and robustness.

Specificity.
Specificity is the ability of a method to discriminate between the study analyte(s) and other components in the sample. The specificity was demonstrated by running a procedural blank.

Linearity.
The linearity between peak area and concentration was analyzed using calibration curve obtained with standard solutions at seven different concentrations of standard RA. The concentrations of the compound in the solution that was considered 100% was 51.9 µg/mL. The other concentration levels used to construct calibration curves were 10%, 50%, 75%, 100%, 125%, 150 % and 200% of the concentration mentioned above. The data for peak area versus drug concentration were treated by linear regression analysis.

Sensitivity.
The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were determined from the calibration curves of the rosmarinic acid standards. *LOD* was calculated according to the expression DPx3/IC, where DP is the standard deviation of the response and IC is the slope of the calibration curve. LOQ was established by using the expression DP x10/IC [18].

Accuracy
The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the RA to the sample, at three different levels (5%, 10% and 15%) of the initial concentration of the sample. Each solution was injected in triplicate. Average recoveries were calibrated by the formula recovery (%) = {(amount found - original amount)/amount spiked} x 100.

Precision
The precision of the method was investigated with respect to repeatability, intermediate precision (inter-day variation) and reproducibility by determination of standard solution at 100% of the test concentration. To assess the intra-day precision (repeatability) of the method, the sample was injected six times within a day. The inter-day precision was determined with the sample assayed on different days and by another analyst. Precision was expressed as the relative standard deviations (% RSD) of the concentrations of rosmarinic acid.

Robustness.
Three sample solutions were prepared and analyzed under the conditions established and by changing the wavelength parameter from 328 nm to 332 nm, by using columns from different suppliers and by changing the mobile phase composition (±5% change organic solvent and ±5% change trifluoroacetic acid concentration) [18].

Statistical analysis.
The data were submitted to statistical analysis using Excel® software.

RESULTS AND DISCUSSION

The HPLC method carried out in this study was aimed at developing a chromatographic system, capable of eluting and resolving rosmarinic acid in leaves of *Mentha piperita* L. In the development of the HPLC method for determination of rosmarinic acid in leaves of *Mentha piperita* L., several solvent systems (methanol-water-trifluoroacetic acid, acetonitrile-water-trifluoroacetic acid, tetrahydrofuran-water-trifluoroacetic acid) and separation columns Phenomenex Synergi 4u Fusion-RP C18 (250 x 4.6 mm i.d., 4 µm particle size), Waters X-Terra C18 column (250 x 4.6 mm i.d., 5 µm particle size), Macherey_Nagel Nucleosil 100-5 C18 (250 x 4.6 mm i.d., 5 µm particle size) were evaluated and compared. The Phenomenex Synergi 4u Fusion-RP C18 column provided better separation of the plant extract than with other specifications or brands of columns.

The choice of detection wavelength was determined by performing a screening with 10 ppm of rosmarinic acid in methanol in a spectrophotometer UV/VIS. The UV spectra were recorded from 220 to 380 nm and exhibited maximum wavelengths at 330 nm.

The results for quantification of the investigated component in the sample were 51,752 µg/mL of rosmarinic acid, which means, 0.2888% of compound contained in leaves of *Mentha piperita* L., based on the dried raw, respectively.

System suitability test showed that critical parameters such as retention time, area, number of theoretical plates and asymmetry factor met the acceptance criteria on all the experimental days (Table 1).

Table 1: System suitability test

Compound	Parameter	Acceptance	Average	%RSD	Status
Rosmarinic acid	Retention time	% RSD < 2	25.46	0.75	Passed
	Peak area	% RSD < 2	590654	1.33	Passed
	No. of Plates	> 100000	244356	4.21	Passed
	Asymmetry factor	< 2	1.070	1.01	Passed

The specificity of the method was evaluated by analysis of blank, standard and sample solution chromatograms (Figure 2). Under the conditions of this method the retention time for rosmarinic acid was 25.46 min. In relation to asymetry, the peaks RA showed values 1.070.

Linearity was evaluated by the correlation coefficient r, and all values for the two compounds were greater than 0.999, showing that responses for the standard in the concentration ranges examined (from 10 to 200%) were linear. Besides, according to (23), the minimum acceptable correlation coefficient is 0.990.

As shown in Table 2, the LOD values was 0.01 µg/mL for the RA, while the LOQ values was 0.04 µg/mL.

Table 2: Calibration curve parameters, limit of detection (LOD), limit of quantification (LOQ) for rosmarinic acid

Compound	Calibration curve equation	Correlation coefficient (r)	Linear range (µg/mL)	LOD(µg/mL)	LOQ(µg/mL)
Rosmarinic acid	y = 12233x + 0.89	0.9998	5.19-103.80	0.01	0.04

The recovery of the compounds RA was determined by spiking the extracts of leaves of *Mentha piperita* L. with known amounts of RA standards. Recovery of RA was obtained from the calculated amount found and original amount. The results are presented in Table 3 and conform with the recommendations of [18].

The data of the precision are shown in Tables 4. The results display a coefficient of variation less than that recommended by [18] whose limit is 5%.

Also, there were no significant differences between assay results, indicating that the precision of the proposed method was satisfactory.

Robustness was evaluated to ensure that the HPLC method is insensitive to small changes in the experimental conditions. In this study, the wavelength, column supplier and composition of the mobile phase were changed. None of the modifications caused any significant change in the response of the RA peaks.

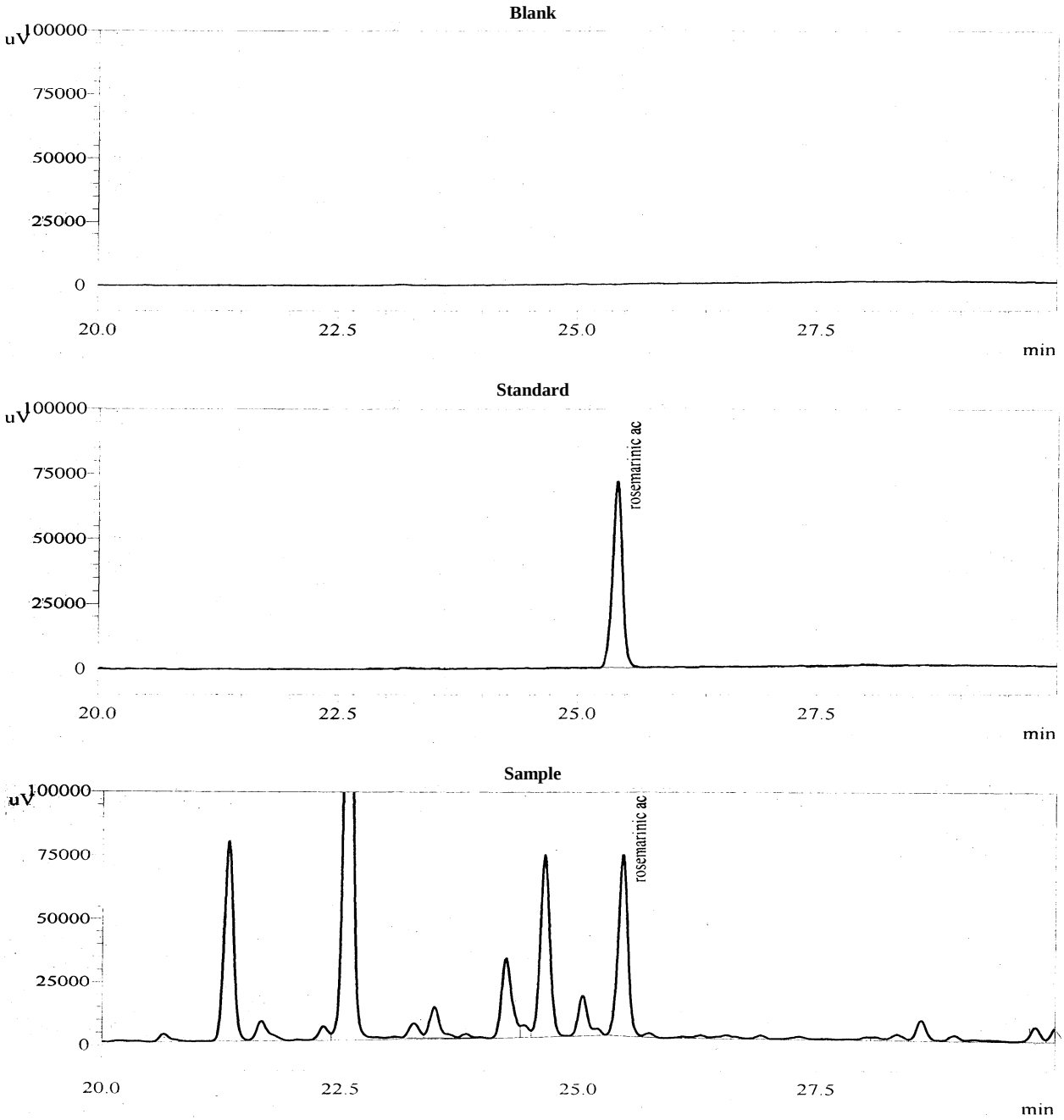

Figure 2: Chromatogram of the blank standard and sample solution performed on Phenomenex Synergi 4u Fusion-RP C18 (250 x 4.6 mm i.d., 4 µm particle size) at 330 nm

Table 3: Results of accuracy determination by analyzing of the Rosmarinic acid of known concentrations

Compound/Initial concentration	Theoretical concentration after dilution added in the extract (µg/mL)	Amount recovered (µg/mL)	Recovery (%)	Mean (%)	RSD (%)
Rosmarinic acid (Concentration measured in the sample = 51.752 µg/mL)	2.610	52.938	97.38	99.33	1.78
		54.242	99.78		
		54.813	100.83		
	5.220	55.850	98.03	99.17	1.27
		56.379	98.96		
		57.490	100.53		
	7.830	57.818	97.04	98.32	1.19
		58.742	98.59		
		59.183	99.33		

Table 4: Results of the repeatability and the intermediate precision

Repeatability		
Compound	Mean (μg/mL)±standard deviation (n=6)	RSD (%)
Rosmarinic acid	51.752±0.717	0.54
Intermediate precision		
Compound	Mean (μg/mL)±standard deviation (n=18)	RSD (%)
Rosmarinic acid	51.681±1.253	1.33

All results were displayed according to the ICH Harmonised Tripartite Guidelines "Validation of analytical procedures: Text and Methodology Q2(R1)" [18].

CONCLUSION

An analytical method based on an optimized solid-phase extraction procedure and followed by high-performance liquid chromatography (HPLC) separation with diode array detection was developed and validated for the determination of rosmarinic acid in leaves of *Mentha piperita* L.

The proposed method demonstrated high specificity at 330 nm detection for the extracts of leaves of *Mentha piperita* L. showing reliability in the quantification of RA. Further, this method can be applied to a standardization of multicomponent herbal remedies, that incorporate herb of leaves of *Mentha piperita* L.

In summary, the method above can be considered specific, exact, precise, linear, robust and easy to perform.

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