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The RP-HPLC method for analysis of usnic acid as potential marker of herbal drugs-based formulations containing *Usnea barbata*

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Abstract: The aim of this study was to develop and validate a sensitive RP--HPLC method for the determination of usnic acid, as a potential marker substance of the herbal product for oromucosal use being a complex mixture of plant extracts and an essential oil, including the extract of *Usnea barbata*. Analysis of usnic acid in the tested formulation was performed through an extraction with methanol, prior to injection onto the HPLC column (Zorbax Eclipse XDB-C18 600 Bar (4.6 mm×100 mm, 1.8 μm)). The employed gradient procedure of the solvents (phosphoric acid (pH 2.5) and acetonitrile), at flow rate of 0.1 mL min-1 allowed for the efficient and reproducible separation of usnic acid from the other compounds present in the investigated complex mixture. The established suitability, linearity, precision, accuracy and selectivity/specificity of this assay implied its applicability for the reliable examination in the stability study of the investigated multi-ingredient herbal preparation.

Keywords: usnic acid; marker substance; method development; method validation; herbal product.

INTRODUCTION

Nowadays, there is a growing world trend in the utilization of preparations based on medicinal plants, both in therapeutic and preventive applications. In addition to teas, as standard herbal products, this trend is particularly pronounced in the development of final dosage forms containing herbal preparations as the

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active ingredients.[1,2] This has led to stringent requirements regarding the production and quality of these preparations made by regulatory authorities, causing their development to be a challenging job that necessitates a number of pre-formulation and formulation studies, as well as investigations assembling scientific data on their stability, safety and efficiency.[3–6]

In this connection, the aim of this study was to develop and validate a reversed phase high performance liquid chromatographic (RP-HPLC) method, for the determination of usnic acid in herbal products in the form of compressed lozenges based on extracts/essential oils of: lichen Old Man's Beard (*Usnea barbata* (L.) Weber ex F. H. Wigg., Parmeliaceae, extract (obtained by supercritical CO_2 extraction), Greek oregano (*Origanum heracleoticum* L., Lamiaceae, water–alcohol extract (obtained by conventional continual extraction), Mountain tea (*Sideritis scardica* L., Lamiaceae, water–alcohol extract (obtained by conventional continual extraction) and Winter savory (*Satureja montana* L., Lamiaceae, essential oil). The stated preparation was intended for topical application, *i.e.*, local treatment of an inflammation of oropharyngeal mucosa comprising a combination of herbal preparations with antimicrobial activity against causative agents of these processes, which is registered as PCT (Patent Cooperation Treaty) application.[7]

Thereby, the supercritical CO_2 (SCO_2) extract of *U. barbata* was designated as the carrier of antibacterial activity of the formulated herbal product with strong antimicrobial potential. Usage of SCO_2 extract of *U. barbata* is also in accordance to the use of this lichen proposed in the monograph of German Commission E, *i.e.*, its utilization as an antimicrobial agent intended for local treatment of mild inflammation of the oral and pharyngeal mucosa,[8] while rationale for the inclusion of other herbal isolates was based on their high antioxidant activity (Supplementary material to this paper, Table S-I), as this feature is regarded as one of the proposed mechanisms of the anti-inflammatory effect of the chemical constituents of medicinal plants.[7]

Taking into account that usnic acid represents the most abundant secondary metabolite present in the SCO_2 extract of *U. barbata* and, at the same time, the mediator of its biological activities,[9–15] this substance was the compound of interest for determination in the tested herbal product. Namely, considering the appropriate guidelines of the European Medicines Agency (EMA), *i.e.*, Guideline on the quality of herbal medicinal products/traditional herbal medicinal products[3] and the Guideline on specifications: test procedures and acceptance criteria for herbal substances, herbal preparations and herbal medicinal products/traditional herbal medicinal products,[4] usnic acid was chosen as a potential marker substance of the investigated tablet formulation.

When it comes to the methods used for usnic acid analysis, several nonconventional techniques, such as capillary electrophoresis or reverse polarity capil-

lary zone electrophoresis were reported in the literature. However, conventional methods, such as spectroscopy, gas and especially high-performance liquid chromatography (HPLC) are widely used for the identification and quantification of this lichen metabolite in various materials.[16] Among the different proposed HPLC methods, most of them focused on the analysis of usnic acid in lichens,[17,18] investigations of its solubility[19] or encapsulation efficiency in nanocarriers,[20,21] as well as the determination and quantification in pharmacokinetic studies.[22,23] In fact, the only HPLC method described for the determination of usnic acid both in raw materials and market products was proposed by Ji and Khan.[24] However, the stated method conducted under the same conditions (admittedly employing Agilent 1200 series HPLC system instead of Waters 2695 Alliance Separation Module and the HPLC column Zorbax Eclipse XDB-C18 (4.6 mm×150 mm, 5 μm) that is an Agilent parallel for the column Xterra RP_{18} column (4.6 mm×150 mm, 5 μm), used in the study of Ji and Khan[24]) was not applicable for the analysis of usnic acid in the lozenges investigated in the present study.

Taking into account the abovementioned, the aim herein was to apply an appropriate analytical method, enabling the determination of usnic acid in a complex mixture containing three plant extracts (including an SCO_2 extract of *U. barbata*) and one essential oil. In addition, guidelines of the International Conference on Harmonization (ICH) have been used for validation of the developed HPLC method (ICH guideline for validation of analytical procedures: text and methodology[25]), as well as a stability study of the investigated lozenges (ICH guidelines for stability testing of drug substances and products[26]).

EXPERIMENTAL

Materials

Analytical grade methanol and H_3PO_4 were purchased from Sigma Aldrich, St. Louis, MO, USA. Acetonitrile (HPLC grade) was purchased from Merck, Darmstadt, Germany. Water (HPLC grade) was produced from double distilled water using a Simplicity 1 UV water purification system (Millipore, France). Reference HPLC standard usnic acid (purity > 98 %) was purchased from Santa Cruz Biotechnology Inc., Dallas, TX, USA.

Formulation

The investigated formulation was compressed lozenge, *i.e.*, tablet for oromucosal use.[7] Briefly, the lozenge was a complex formulation consisting of glucose monohydrate and saccharose, used as fillers; poly(vinylpyrrolidone) (PVP K-25) aqueous solution used as binder; microcrystalline cellulose, used as disintegrant; magnesium stearate, used as lubricant and aroma of peppermint, used as a flavour, combined with the extracts/essential oils in defined proportion – *U. barbata* extract (0.01 %), *O. heracleaoticum* extract (0.5 %), *S. scardica* extract (0.7 %), and *S. montana* essential oil (0.1 %). The physicochemical characteristics of the lozenges were determined and the theoretical mass was 1.1000 g, with diameter of 15 mm,

thickness being 3.30–3.50 mm and strength 8–15 kp*, while allowed friability was max. 1 % and dry weight loss defined to be 1.5–2.5 %.

Instrumentation and chromatographic conditions

HPLC analysis of the investigated product was performed using an Agilent HPLC model 1200 instrument (Agilent Technologies, Santa Clara, CA, USA); column Zorbax Eclipse XDB-C18 600 Bar (4.6 mm×100 mm, 1.8 μm). The mobile phase A consisted of 500.0 mL of water and 9.8 mL of 85 % phosphoric acid (pH 2.5), while B was pure acetonitrile. The flow rate was 0.10 mL min^{-1}, and the elution was as follows: 11–55 % B, 0–5 min; 55–80 % B, 5––10 min; 80 % B, 10–12 min; 80–100 % B, 12–20 min; 100 % B, 20–35 min; 100–11 % B, 35–40 min; 11 % B, 40–55 min. The temperature of the column was held constant at 25 °C and the injection volume was 4 μL, while the measuring wavelength was 280 nm.

Sample preparation

Investigated lozenges were crushed using a mortar and pestle. Thereafter, 2.0 g of this homogenous powder was accurately weighed in a beaker (analytical balance AG 104 Mettler Toledo, Germany), covered with 10.0 mL of methanol and then subjected to sonication (ultrasonic bath Sonorex RK100H, Bandelin, Germany) for 10 min. Thereafter, this mixture was filtered through a filter funnel into a 25 mL volumetric flask. Then, the filter paper was put back into the same beaker, covered with another 10 mL of methanol and submitted to sonication (ultrasonic bath Sonorex RK100H, Bandelin, Germany) for 10 min. This mixture was filtered through a filter funnel into the same volumetric flask, while the beaker and the filter funnel were flushed with portions of methanol to obtain a final amount of 25.0 mL of the analyte, which was then filtered through a 0.45 μm PTFE filter into a 2 mL glass HPLC vial prior to injection.

Preparation of standards

Usnic acid was used for a gradual dissolution in methanol in the appropriate volumetric flasks, followed by methanol dilutions in order to prepare final chemical standard solutions. The actual concentrations were expressed as percentage contents, corrected only for the impurities of the standards, as declared by the chemical supplier.

System suitability

The HPLC system was equilibrated with the initial mobile phase composition, and then system suitability parameters with respect to repeatability, number of theoretical plates and resolution were assessed by injecting a blank mobile phase followed by five replicates of investigated samples solution (80 mg ml^{-1}). Thereafter, five consecutive 4-μL portions of the usnic acid standard solution were injected into the instrument, recording the chromatograms and reading the usnic acid peak areas.

Stability study of the investigated lozenges

The developed and validated HPLC method for identification and quantification of usnic acid was used in the stability study of the investigated lozenges. As mentioned earlier, stability experiments were conducted in accordance to ICH guidelines for stability testing of drug substances and products.[26] Thus, for the purpose of long-term stability testing, lozenges from three different batches were stored in a plastic bottle (primary packaging) and carton box (secondary packaging) at 25±2 °C/60±5 % RH, and their usnic acid content was analysed by

* 1 kp = 9.807 N

means of the stated HPLC method initially (after preparation), and then after 3, 6, 9, 12 and 18 months.

RESULTS AND DISCUSSIONS

Method development

As outlined in the Introduction, in accordance to the appropriate EMA guidelines,[3,4] and the evaluated antibacterial potential (Table S-II of the Supplementary material), as well, usnic acid (Fig. 1) was chosen as substance selected for identification and quantification in the investigated lozenges for oromucosal use containing the extract of *U. barbata*, as an antimicrobial agent intended for the treatment of mild inflammation of the oral and pharyngeal mucosa, stated by the monograph of German Commission E.[8]

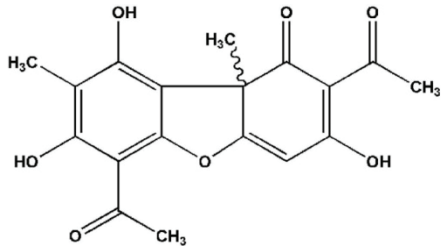

Fig. 1. Chemical structure of usnic acid ((9*bR*)-2,6-diacetyl-3,7,9-trihydroxy-8,9*b*-dimethyl-dibenzofuran-1-one).

The reason for this lies in the fact that usnic acid is the most abundant secondary metabolite determined in the SCO_2 extract used in the formulation (see Supplementary material), but at the same time, it represents mediator of its biological activities,[9,10] thus representing a substance of interest for analysis purposes of the tested herbal product.

According to the available literature, the only HPLC method for usnic acid determination in complex plant mixture was described in the paper of Ji and Khan.[24] However, the precise information on composition of the numerous herbal products used in the mentioned paper was lacking, and it was revealed that the suggested method could not be applied in analysis of our herbal product. Namely, the overlapping of the peaks, most probably caused by the complexity of composition of the lozenges (polyphenolic components co-eluting with usnic acid), imposed the need for the development of new HPLC method.

Initial assay parameters were based on physicochemical parameters of usnic acid found in literature. In accordance to the high partition coefficient (log *P*) of usnic acid (theoretical value = 2.679±0.631; value determined by direct measurement in the octanol/water system (25 °C) = 2.88), a C_{18} column was selected for the method development. Additionally, due to the very low water-solubility of usnic acid (less than 0.1 mg mL^{-1}),[16] a reverse-phase assay was chosen for the initial testing. Moreover, a preliminary study was performed with the aim of choosing an adequate solvent/solvent mixture that would be selective enough to

allow for appropriate peak separation. Furthermore, instead of using a wavelength of 233 nm used in the experiments performed by Ji and Khan,[24] in the present study, the chromatograms were recorded at 280 nm. In addition, taking into account the complexity of the formulation of the lozenges, the method duration of 20 min set in the Ji and Khan paper did not provide the expected separation of the components in the present samples. A time frame of 55 min was necessary to obtain the satisfactory separation of the usnic acid from the other substances present in this complex mixture. In accordance with the data found in the literature,[17,18,20,27] methanol, acetone, acetonitrile and mixture of acetonitrile and methanol (1:5.25) were investigated for the preparation of the tablet samples. Moreover, aside the solvent selection, the procedure of sample preparation itself, including the time needed for sonication, was varied, *i.e.*, optimized in the final experiment (data not shown). Selection of appropriate chromatographic conditions and preparation procedure for the investigated tablet-samples allowed efficient and reproducible separation of the compound of interest – usnic acid, from the other compounds present in the investigated preparation (example chromatograms shown in Fig. 2).

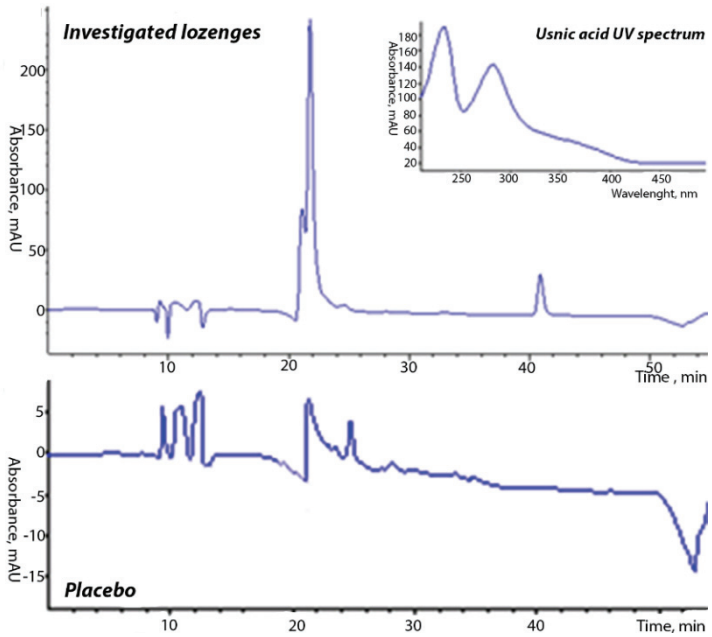

Fig. 2. Chromatograms of the investigated lozenges and the placebo sample with the spectrum of usnic acid.

Method validation

Selectivity of the developed HPLC method was investigated throughout by visual inspection of the chromatograms of the tested samples (Fig. 2). Excipients

from either the formulation or other active ingredients showed a detector response near retention time of usnic acid (≈41 min). Moreover, the peak corresponding to usnic acid was baseline resolved from any interference. The specificity of this assay was confirmed by library spectra matching using a photo diode array detector, which substantiated peak identity and the absence of any impurity that possess a UV spectrum to that of usnic acid.

The obtained results of 5 replicate injections showed that the parameters tested were within the acceptable range. Usnic acid, as well as a peak at 22.9 min, were repeatedly retained and well separated expressing the acceptable resolution (>3) between both peaks, with the number of theoretical plates >2000 in all chromatographic runs to ensure good column efficacy throughout the method (Table I). In addition, small value of relative standard deviation (0.58 %) determined from five consecutive usnic acid injections from the same methanolic solution (in the concentration corresponding to its declared content in the investigated formulation) indicated satisfactory system suitability.

TABLE I. System suitability results

No.	Retention time, min		Number of theoretical plates		Resolution between peaks
	Peak at 22.9 min	Usnic acid	Peak at 22.9 min	Usnic acid	
1	22.876	41.051	11723	6655	3.22
2	22.888	41.037	11822	6695	3.14
3	22.879	41.019	11858	6690	3.22
4	22.875	40.989	11668	6756	3.16
5	22.877	40.925	11877	6706	3.15
Mean	22.879	41.0042	11789.6	6700.4	3.18
SD	0.00469	0.045	80.73	32.62	0.035

In order to determine linearity, a series of usnic standard solutions in increasing concentrations were analysed (in triplicate) by means of the established HPLC method. A calibration curve was created by plotting the response area (AUC) *versus* usnic acid concentration. To ensure a complete linearity, the calibration curve was always visually inspected as well. The linearity over the concentration range of 3.7–11.2 μg mL^{-1} (encompassing the expected usnic acid concentration in the analysed lozenges) was established since the correlation coefficient of the resultant calibration curve (Table II) was greater than 0.9995.

The precision of the method was studied by having three persons on three different days engaged to collect the data for every tested sample (Table II). Intraday and inter-day recoveries of 99.44 and 100.04 %, respectively, suggested that the method had the required precision.

The accuracy of the method was studied by determining recovery values. Three different concentrations of usnic acid standard solution were added to

blank. Average recovery values 99.75.–100.54 % (Table II) suggested high accuracy of the developed method.

TABLE II. Validation parameters of the investigated HPLC method

Validation parameter	Obtained values		
Linearity			
Range, μg mL^{-1}	3.7–11.2		
Calibration equation (y=ax+b; R^2)	y = 50388x + 10.122; 0.9995		
Precision			
Declared content of usnic acid per tablet, μg	70.0 (59.5–80.5)		
Intraday precision (found content of usnic acid per tablet, μg)	69.1		
Interday precision (found content of usnic acid per tablet, μg)	68.9		
Accuracy			
Concentration of usnic acid added, μg mL^{-1}	6.0	7.5	9.0
Concentration of usnic acid found, μg mL^{-1}	5.9	7.4	8.8
Spike recovery, %	100.54	99.75	100.06
LOD / μg mL^{-1}	0.5		
LOQ / μg mL^{-1}	1.8		

In accordance to the appropriate guidelines,[25] the limit of detection (*LOD*) for this assay was calculated as three times signal-to-noise ratio (*S*/*N*), while *LOQ* for this assay was calculated as ten times *S*/*N* level. The *LOD* of usnic acid was calculated to be 0.5 μg mL^{-1}. The *LOQ* of usnic acid showed that it could be quantified at a concentration of 1.8 μg mL^{-1} (Table II).

Applicability of the developed and validated HPLC method was investigated by a long-term stability study of the tested tablets, declared to contain 0.1000±15 % mg of dry extract of *U. barbata* (Old Man's Beard), *i.e.*, 0.0700±15 % mg of usnic acid.[7]

The content of usnic acid was in the range 65.9±2.6 to 73.5±2.5 μg per tablet. The initial content of usnic acid was 73.5±2.5 μg per tablet, and in the measurements repeated after 3, 6 and 9 months of storage under the conditions prescribed by the appropriate ICH guideline,[26] these values were 71.5±2.2, 73.4±0.5 and 70.3±1.7 μg per tablet, respectively. A certain decrease of usnic acid content was detected in the measurements performed 12 and 18 months after the preparation of the lozenges. However, the measured values were in the declared range,[28] suggesting satisfactory long-term stability of the examined preparations.

CONCLUSIONS

An optimized RP-HPLC method was validated for identification and quantification of usnic acid, as marker of the investigated lozenge being the complex

mixture of several herbal extracts and an essential oil. The established suitability, linearity, precision, accuracy, selectivity/specificity as well as stability, indicating the nature of this assay, imply its applicability as a reliable examination for the multicomponent herbal preparation, as the basis for further investigations enabling potential clinical relevance of such formulation in the local treatment of oral/ /pharyngeal mucosa impairments.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/10152, or from the corresponding author on request.

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ИЗВОД

RP-HPLC МЕТОДА ЗА АНАЛИЗУ УСНИНСКЕ КИСЕЛИНЕ КАО МОГУЋЕГ МАРКЕР ЈЕДИЊЕЊА ФОРМУЛАЦИЈЕ НА БАЗИ ЛЕКОВИТОГ БИЉА КОЈА САДРЖИ ЛИШАЈ *Usnea barbata*

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Циљ овог истраживања био је развој и валидација осетљиве методе високо ефикасне течне хроматографије обрнутих фаза (reversed phase high performance liquid chromatography, RP-HPLC) за одређивање уснинске киселине, као могућег маркер једињења биљног производа за употребу на оралној слузокожи, који представља сложену смешу биљних екстраката и етарског уља, укључујући и екстракт лишаја *Usnea barbata*. Анализа уснинске киселине у тестираној формулацији изведена је екстракцијом метанолом пре инјектовања на HPLC колону (Zorbax Eclipse XDB-C18 600 Bar (4,6 mm×100 mm, 1,8 μm)). Примењени градијентни поступак растварача (фосфорне киселине (pH 2,5) и ацетонитрила), при брзини протока од 0,1 ml min^{-1}, омогућио је ефикасно и поновљиво раздвајање уснинске киселине од осталих једињења присутних у испитиваној сложеној смеши. Утврђена линеарност, прецизност, тачност и селективност/специфичност ове методе имплицирали су њену применљивост као поузданог теста у испитивању стабилности испитиваног вишекомпонентног биљног препарата.

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