Determination of lotaustralin in *Rhodiola* species

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Summary

In our research, the concentration of lotaustralin in the roots of two species *Rhodiola kirilowii* and *Rhodiola rosea* were compared. Aqueous and hydroalcoholic extracts from those plants were analyzed too. To determine the content of this compound the ultra performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS, Waters) was used. The obtained results showed that the content of measured lotaustralin depends on the species of *Rhodiola*. *R. rosea* roots are the richer source of lotaustralin then *R. kirilowii*. The same situation was observed in the extracts. A hydroalcoholic extract from *R. rosea* contains up to 135.276 mg of lotaustralin in 100 g of dry powdered material. In the case of *R. kirilowii* extracts, an aqueous extract contained more lotaustralin (74.791 mg/100 g of dry powdered material) then a hydroalcoholic extract.

Key words: *Rhodiola kirilowii, Rhodiola rosea, cyanogenic glucoside, lotaustralin, UPLC-MS/MS*

INTRODUCTION

The phenomenon of cyanogenesis, the ability to release a hydrogen cyanide (HCN) from plant tissue, was been found in more than 2650 species of higher plants [1]. The most important source of HCN in the plants are mainly cyanogenic glycosides, cyanogenic lipids as well as processes of transformation of glyoxylate, hydroxylamine and ethylene [2,3]. It is well known that hydrogen cyanide is an effective inhibitor of cytochrome oxidase activity and is toxic to animals. Therefore, it is considered that cyanogenic glycosides are responsible for the protective function against herbivores and pathogens. Some of them, for example linamarin and lotaustralin are synthesized from valine and isoleucine, respectively and were found in various higher plants [4]. The research over the content of these compounds are mainly focused on the plants consumed in human diet as well as others used as herbal medicines, for example of *Rhodiola* species. *Rhodiola* has been used for centuries in the traditional folk medicine of Russia, Scandinavia and other countries [5,6].

Review of the literature indicates that *R. rosea*, *R. kirilowii* and *R. quadrifida* are used as common medications. *R. rosea* has biological activities: antiallergenic and anti-inflammatory effects, enhanced mental alertness, stress-protective effects, lifespan increasing effects [7, 8]. The main pharmacologically active chemical substances that are responsible for the beneficial properties of *R. rosea*, are polyphenolic compounds (phenylpropanoids, flavonoids, proanthocyanidins and tannins). The best-known active ingredients of anti-stress and antidepressant effects are rosavins, rosin, rhodiolin, salidroside and p-tyrosol. Rhizomes from *R. rosea* contain also essential oils (0.05%) with monoterpene hydrocarbons, monoterpene alcohols and straight chain aliphatic alcohols [6,9]. In addition, the raw material and its preparations have significant antioxidant, antitumor and immunostimulatory activities. Moreover, it seems that *Rhodiola rosea* has a beneficial effect on the heart muscle and increases the intellectual functions [4].

R. kirilowii is used in traditional Chinese medicine as a source of compounds which have anti-anoxia and anticoagulative activity, decrease a saccharides level in blood and influence on cardiopulmonary disorders. Roots contain salidroside, p-tyrosol, daucosterol, β-sitosterol, lotaustralin, epigallocatechin gallate [10-15]. Peng and et al. found an toxic cyanogenic glucoside namely lotaustralin in *R. kirilowii* rhizome [14]. It was indicated that lotaustralin is also present in *R. rosea* [6]. According to Kang and Wang, this compound is present on the highest level in *R. kirilowii* among 10 analyzed *Rhodiola* species [16]. Lotaustralin can also be detected in cassava (*Manihot esculenta* Crantz), white clover (*Trifolium repens*), lima beans, *Phaseolus lunatus* L., *Tetranychus urticae*, *Phytoseiulus persimilis* [17]. During processing and chewing, lotaustralin can set free toxic hydrogen cyanide (HCN). Consumption of plant product containing this compound can cause acute poisoning, progression of Kenzo disease in women and detrimental effect on the development of central nervous system during pregnancy [18]. High content of cyanide in the human body causes dizziness, weakness, nausea, vomiting, diarrhoea and sometimes death [19]. In our experiment, we used two well-known species of *Rhodiola*: *R. kirilowii* and *R. rosea,* which were cultivated in the Institute of Natural Fibres and Medicinal Plants in Poznań. In these plants the content of lotaustralin was determined because this compound can be dangerous for the human health.

MATERIAL AND METHODS

Plant material

Rhodiola kirilowii and *Rhodiola rosea* used in the study were collected in October 2009 from field crops of the Institute of Natural Fibres and Medicinal Plants in Poznań. Roots were dried at a room temperature (22–24ºC).

Preparation of plant extracts

Subsequently, dry roots were powdered (0.315) and two kinds of extract were prepared: aqueous extract and 50% *(v/v)* ethanol extract.

Preparation of aqueous extract

The powdered dry roots were extracted with purified water for 3 hours at 90ºC (material to solvent ratio 1:10). After filtering, the extracts were frozen at –55ºC and than lyophilised [2]. The dry plant extracts were stored at a temperature of 20–25ºC.

Preparation of 50% *(v/v)* ethanol extract

The powdered dry roots were extracted with 50% (*v/v)* ethanol using the percolation method at plant material to solvent ratio 1:10. After the evaporation of the alcohol in reduced pressure at a temperature of 40–45ºC the extracts were frozen at –55ºC and than lyophilised [2]. Dry plant extracts were stored at a temperature of 20–25ºC.

Standards substances

The following comparison substances were used in the experiment: lotaustralin (TRC) and linustain (SIGMA).

Stock solutions

Calibrate curves of lotaustralin as implemented standard from TRC and linustatin as a internal standard (Sigma Aldrich) were prepared. All substances were dissolved in methanol and different concentration of stock solutions were prepared. Calibration curves were prepared as 5 different levels of concentration at a range of 100–1000 ng/ml.

Sample preparation

Roots of *Rhodiola*

About 2.0 g of dried powdered root from *Rhodiola kirilowii* or 1.5 g of dried powdered root from *Rhodiola rosea* was weighed out and placed in a 100 ml round-bottom flask. To 20 ml of 70% (*v/v*) methanol 0.5 ml of methanolic solution of linustatin (IS) was added. That sample was heated under a reflux condenser in the boiling point of the solvent for 45 min and after cooling, the sample was filtrated and extraction was repeated two more times. All extracts were connected to evaporate the methanol up to dry in a rotary evaporator in vacuum. A dry residue was dissolved in 3 ml of 70% (*v/v*) methanol. Sample was transferred quantitatively to 5 ml volumetric flask for *Rhodiola kirilowii* and to 10 ml for *Rhodiola rosea*. Subsequently, the solution was then made up to the mark with the 70% (*v/v)* methanol. The sample was filtered through a membrane filter with a diameter of 0.20 μm.

Extracts from *Rhodiola*

About 0.1 g of *Rhodiola kirilowii* or *Rhodiola rosea* extract was weighed out and placed in a 100 ml round-bottom flask. To 20 ml of 70% (*v/v*) methanol 0.5 ml of methanolic solution of linustatin (IS) was added. That sample was heated under a reflux

condenser in the boiling point of the solvent for 45 min and after cooling, the sample was filtrated and extraction was repeated two more times. All extracts were connected to evaporate the methanol up to dry in a rotary evaporator in vacuum. A dry residue was dissolved in 3 ml of 70 % (*v/v*) methanol. Sample was transferred quantitatively to 5 ml volumetric flask for *Rhodiola kirilowii* and to 10 ml for *Rhodiola rosea*. Subsequently, the solution was made up to the mark with the 70% (*v/v)* methanol. The sample was filtered through a membrane filter with a diameter of 0.20 μm.

LC-MS/MS assay

For determining concentration of lotaustarlin in the *Rhodiola* species the ultra performance liquid chromatography - tandem mass spectrometry (UPLC-ESI MS/MS; Waters) was used. The separation of lotaustralin was performed on an Acquity UPLC BEH C18 column, 1.7 μm, 2.1 × 50 mm (Waters). Mobile phase: phase A: methanol, phase B: water. Mobile phase flow rate was: 0.25 ml/min. The assay was performed in isocratic elution: 80% of phase B. Column temperature was 24ºC; ion source temperature: 100ºC; desolvation temperature: 300ºC. Gas flow rate: desolvation gas: 700 L/h; cone gas: 10L/h. That method of extraction was prepared to determine the concentration of lotaustralin in *Rhodiola* species. Fragmentation of lotaustralin m/z 260→161 Da and internal standard linustatin m/z 408→323 Da. Lotaustralin and linustatin (IS) were analyzed in the negative-ions source.

Validation

Validation of this method of extraction and detection was conducted in accordance with the requirements of ICH. The calibrations curves were prepared on 5 different concentrations of substances. Retention time, peak area and regression coefficient were checked to accept recurrence and linearity of calibration curves. The calibration curves were prepared in the range 100-1000 ng/ml. Then, the precision of extraction was done for six samples. The accurancy of this method was conducted on three different levels. The percentage of recovery ranged from 88.2 to 91.6%. All validated parameters allowed to indicate that the extraction and detection methods are validated.

Statistical method

For lotaustralin and internal standard, the regression analysis was performed at 5 concentration levels. Concentration of lotaustralin was carried out for all samples in 6 repeats. The average and relative standard deviations (RSD) for those results were determined.

RESULTS

The basic analytical and statistical parameters designed during the preparation of method are summarized in table 1. For the analytical method used in the study the precision, linearity and accuracy were determined. Precision and linearity were evaluated by using regression analysis for each of the comparison substances (tab. 2). The recovery of analytical method was analysed for *R. kirilowii* roots using the enrichment method by the addition of dry hydroalcoholic extract of *R. rosea* with the determined level of lotaustralin to the sample. Figure 1 presents the MRM chromatogram showing fragmentation of cyanogenic glucoside in *Rhodiola kirilowii* and figure 2 concerns *Rhodiola rosea* roots. Detection of every analysed compound was conducted in negative ions source. The signals visible in the chromatogram came from the parent ion fragmentation $[M\text{-}H]^-$. The content of lotaustralin in 2 different species of *Rhodiola* was presented in the table 3. Comparing the results of the roots, *R. rosea* contained a higher quantity of lotaustralin (10.880 mg/100g of dry powdered material) then *R. kirilowii* (3.226 mg/100 g of dry powdered material). In the both cases, concentration of lotaustralin in the extracts was higher then in the roots. As shown, the aqueous extract from *R. kirilowii* contained 74.791 mg/100 g of dry powdered material of lotaustralin and was richer in this compound then hydroalcoholic extract. In *R. rosea*, richer source of cyanogenic glucoside was hydroalcoholic extract (135.276 mg/100g of dry powdered material).

Table 1.

Characteristic parameters of cyanogenic glucoside detection

Compound	Retention time [min]	RSD_t (n=15) [%]	RSD_p (n=5) [%]	Fragmentation m/z [Da]
Lotaustralin	1.44	0.34	4.88	260→188
Linustatin (IS)	0.82	0.43	1.62	408→323

RSD_t – relative standard deviation of retention time
RSD_p – relative standard deviation of peak area

Table 2.

Method recovery (n=3)

Sample	Lotaustralin [%±RSD]
20%	88.2±4.3
40%	91.6±6.8
80%	90.0±3.7

RSD – relative standard deviation

Table 3.

Content of cyanogenic glucoside in *Rhodiola kirilowii* and *Rhodiola rosea* roots and extracts

Sample	Lotaustralin	
	Content [mg/100 g of dry powdered material]	RSD [%]*
Rhodiola kirilowii root	3.226	1.57
50% ethanol extract	53.773	2.03
aqueous extract	**74.791**	2.83
Rhodiola rosea root	10.880	3.03
50% ethanol extract	**135.276**	1.74
aqueous extract	106.582	2.42

* – RSD – relative standard deviation (n=6)

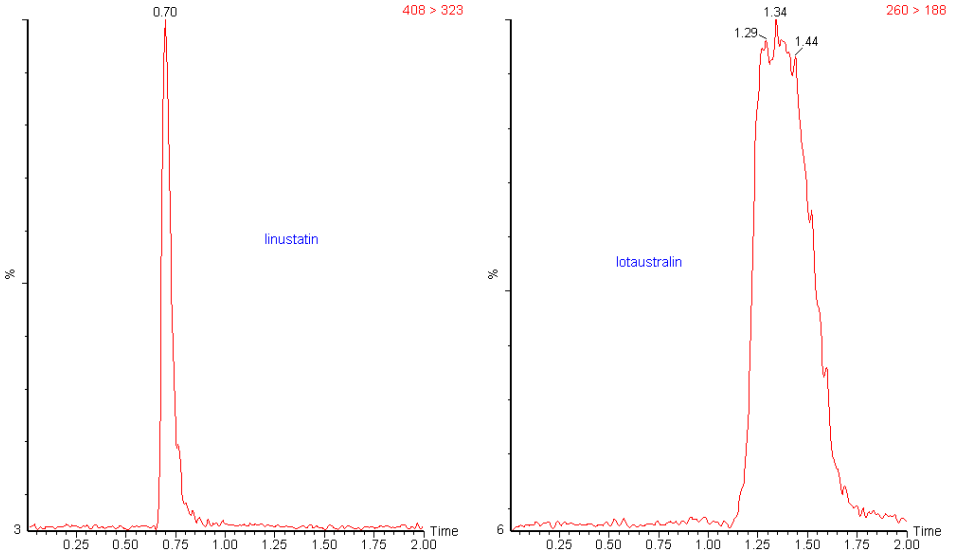

Figure 1.
The MRM chromatogram showing fragmentation of cyanogenic glucoside from *Rhodiola kirilowii* roots.

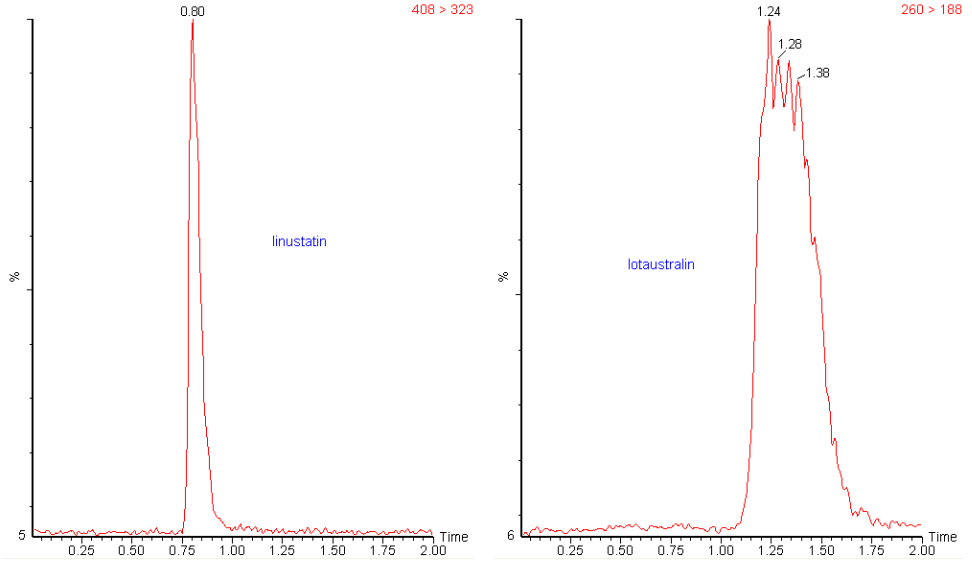

Figure 2.
The MRM chromatogram showing fragmentation of cyanogenic glucoside from *Rhodiola rosea* roots.

DISCUSSION

In recent years, modern and sensitive methods of the detection of plant compounds including those with toxic activity are used. Therefore, we have made attempts in our Department to analyze one of these types of compounds - lotaustralin. The structure of lotaustralin was determined by Akgul et al. using spectroscopic methods [20]. It has been shown that this component is present in the methanol extract from the roots of *Rhodiola rosea* as a mixture of two diastereoisomers form [20]. So far, the research on the lotaustralin content in *Rhodiola rosea* and *Rhodiola kilirowii* using HPLC techniques conducted Xiao (2005) and Wiedenfeld et al. (2007), respectively [21,10]. We used the ultra performance liquid chromatography - tandem mass spectrometry (UPLC-ESI MS/MS; Waters) to determine the concentration of lotaustralin in *Rhodiola* root extracts. This method of detection allows the identification of lotaustralin using the fragmentation of parent ion. Among the herbal materials used in this study, the roots of *R. rosea* contained a higher amount of lotaustralin then *R. kirilowii*. In both cases, concentration of lotaustralin in the extracts was higher then in the roots. The results of our study showed that the highest concentration of cyanogenic glucoside was found in hydroalcoholic extract of *R. rosea* (135.276 mg/100 g of dry powdered material). Aqueous extracts of *R. rosea* and *R. kirilowii* contain much less lotaustralin than the hydroalcoholic one. It is recognized that content of this toxic ingredient depends

on the species and the habitat of the plant [16]. Moreover, according to several authors, the concentration of cyanogenic compounds in plants varies significantly duringgrowing season depending on the type of mineral nutrition and a number of environmental factors [1, 22-24].

CONCLUSION

In summary, we showed that the method the ultra performance liquid chromatography – tandem mass spectrometry (UPLC-ESI MS/MS) method can be successfully used for the determination of lotaustralin in the extracts of roots of *Rhodiola* species. Due to the fact that *Rhodiola rosea* is an endangered medicinal species having a important pharmacological properties, it is necessary to conduct further studies regarding the content of beneficial and undesirable metabolites in root extracts.

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OZNACZANIE ZAWARTOŚCI LOTAUSTRALINY W GATUNKACH *RHODIOLA*

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S t r e s z c z e n i e

W badaniach przeprowadzonych w Instytucie Włókien Naturalnych i Roślin Zielarskich porównano zawartość lotaustraliny w dwóch gatunkach roślin: *Rhodiola kirilowii* i *Rhodiola rosea*, jak również w przygotowanych z tych roślin dwóch wyciągach: wodnym oraz wodnoalkoholowym (50% etanolowy wyciąg). W celu wyznaczenia zawartości lotaustraliny wykorzystano ultrasprawny chromatograf cieczowy sprzężony z tandemowym spektrometrem mas (UPLC-MS/MS, Waters). Wykazano, że zawartość cyjanogennego glukozydu zależna jest od gatunku. Przeprowadzona walidacja metody pozwala na satysfakcjonujące wykorzystanie tej metody w badaniach zawartości lotaustraliny.

Słowa kluczowe: *Rhodiola kirilowii, Rhodiola rosea, cyjanogenny glukozyd, lotaustralina, UPLC-MS/MS*