



## Development and validation of a HPLC-UV method for determination of Proscillaridin A in *Drimia maritima*

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### Abstract

**Background and objectives:** *Drimia maritima* (L.) Stearn also known as squill is a medicinally important plant that has been used for various ailments such as dropsy, respiratory disorders, jaundice and epilepsy from ancient times. Proscillaridin A is identified as one of the most effective compounds in the plant with remarkable pharmacological features including efficacy against congestive heart failure, antitumor, t-cell suppressive and analgesic activities. In the present study, a reliable high performance liquid chromatography (HPLC) method has been developed for quantification of proscillaridin A in *D. maritima*. **Methods:** The separation of compounds was performed using gradient elution (methanol: water) on a reversed phase ACE C<sub>18</sub> with flow rate of 1 mL/min and UV detection at 300 nm for 50 min. The method was evaluated using validation parameter such as selectivity, linearity, precision, recovery, limit of detection (LOD) and limit of quantization (LOQ). **Results:** The separation technique was selective for quantification of proscillaridin A. The calibration graph was linear with  $r^2 > 0.998$ . The intra and inter-day precision (RSD%, 3.8-4.16 and 7.5) were satisfactory. LOD and LOQ were calculated as 0.6 and 1.8 µg/mL respectively. The recovery average was 93.7%. **Conclusion:** Due to precision, accuracy and speed, the proposed HPLC-UV method could be applied for determination of proscillaridin A in *Drimia maritima* samples.

**Keywords:** *Drimia maritima*, high performance liquid chromatography (HPLC), proscillaridin A, validation

### Introduction

*Drimia* (syn: *Urginea*) species are including bulbous plants which are taxonomically classified in the family Asparagaceae [1,2]. Different *Drimia* species are distributed in Mediterranean area, Africa and India [3-5]. *Drimia maritima* (L.) Stearn (squill) is the only native species reported in Iran and has been widely used in folk

medicine since ancient time [1,3]. Two varieties of *D. maritima* exist with same morphology and bulbs in white or red colors. White variety is commonly used for medicinal purposes [1]. In summary, main applications of squill in traditional medicine are including dropsy, respiratory ailments, bone and joint

complications, jaundice, cancer and epilepsy [6-8]. This plant also applied as a poison for rat and insect elimination during the ages [9]. Bufadienolides identified as the main phytochemical constituents of *D. maritima*. Scillaren A was the first bufadienolide compound isolated from this plant by Stoll and his co-workers in 1933 [10]. Proscillaridin A (figure 1) is one of the most important constituents identified in *D. maritima* and has been clinically applied for treatment of cardiac disorders [11]. Antitumor activity of this compound has been investigated against various cell lines such as: human lymphoma [12], breast cancer [13-14], human fibroblasts [15], human multiple myeloma [16] and advanced adrenocortical carcinoma [17]. Analgesic, T-cell suppressive and insecticidal activities have been also reported for proscillaridin A [18-20]. Many researchers applied different chromatographic methods for isolation and identification of bufadienolide compounds [21]. There are also limited studies which applied high performance liquid chromatography (HPLC) for quantitative analysis of bufadienolides in mostly explored red variety of this plant [22-25]. The important pharmacological effects of proscillaridin A and the lack of data about its content in Iranian source of plant (white variety) are the two main reasons for developing a validated ultraviolet high performance liquid chromatography method for determination of this compound.

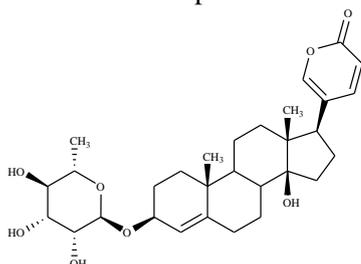


Figure 1. Chemical structure of proscillaridin A

## Experimental

### Instrumentation

The HPLC-UV experiment was performed using a Waters Alliance equipped with a vacuum

degasser and quaternary detector. The UV spectra were collected across the range of 200-900 nm, extracting 300 nm for chromatograms. Empower software was applied for instrument control, data collection and data processing. The column was an ACE C<sub>18</sub> (4.6 × 250 mm, 5 μm). The mobile phase was a linear gradient system with methanol (A)- water (B) for 50 min starting with A:B (50:50) for 15 min, changing to A:B (80:20) for 20 min, A:B (50:50) for 5 min with equilibrating with ten min. The flow rate was 1 mL/min. The injection volume for all samples and standard solutions was 20 μL.

### Plant material

*D. maritima* was collected In September 2013 from Chenarshahijan, Kazerun, Fars province, Iran at the altitude of approximately 1050 m. The collected plant was authenticated by Dr. Gh. Amin (Department of pharmacognosy, Faculty of pharmacy, Tehran University of Medical Sciences, Tehran, Iran), and a voucher specimen (No. 6622-TEH) was deposited at the Herbarium of faculty of pharmacy. Fresh squill bulbs were cleaned thoroughly to remove any soil or debris. Bulbs were cut into small pieces and dried at room temperature.

### Instrumentation

The HPLC-UV method was carried out using a Waters Alliance equipped with a vacuum degasser and quaternary detector. The applied wavelength for chromatograms (300 nm) was extracted from UV spectra collected across the range of 200-900 nm. Empower software was used for programming instrument. The column (ACE C<sub>18</sub> (4.6 × 250 mm, 5μm) was eluted with a flow rate of 1 mL/min by a linear gradient of the mobile phase (A methanol: B water) for 50 min starting with A:B (50:50) for 15 min, changing to A:B (80:20) for 20 min, A:B (50:50) for 5 min with equilibrating with ten min. The injection volume of samples and standards was 20μL.

#### *Chemicals*

Proscillaridin A was purchased from Sigma-Aldrich chemical Co. (Germany). All solvents were prepared from Merck Co. (Darmstadt, Germany). Deionized water used in all experiments was prepared by Purelab UHQ Elga (Germany).

#### *Proscillaridin A determination*

##### *Solvent and extraction method effects*

For exploring the solvent and method effects on the extraction efficacy, powdered samples were suspended in different solvents including methanol, acetone, chloroform and ethanol-water 80:20 and extracted with different methods such as heat- reflux extraction (2.5 g, 100 mL solvent, 60 min), maceration (2.5 g, 100 mL solvent, 72 h) and ultrasonic radiation (0.25 g, 10 mL solvent, 60 min, 40 °C).

##### *Sample preparation*

With respect to obtained results from the above experiments, methanol (10 mL) was added to the powdered samples (300 mesh, 0.25 g) and sonicated in ultrasonic bath for 1 h at 40 °C. The mixture was filtered and the extraction procedure was repeated two more time using 10 mL methanol. The filtrate was diluted to 100 mL in a volumetric flask. The obtained solution was filtered via a membrane filter with a pore size of 0.45µm prior to injection.

##### *Preparation of the standard solution*

The stock standard solution was prepared by dissolving 1 mg of proscillaridin A, reference standard, in 10 mL methanol. Daily prepared working standards in the range of 4-14 µg/mL were obtained from the stock solution.

#### *Validation*

The method for analysis of proscillaridin A was evaluated regarding the validation parameters including selectivity, linearity, precision, recovery, limit of detection and limit of quantization [26].

#### *Selectivity*

The selectivity of the chromatographic method is the ability of the method to exactly measure the analyte response in the presence of other compounds in the matrix of sample; therefore, two factors of peak purity and resolution from neighbor peaks were analyzed.

#### *Linearity*

The linearity of the method was evaluated through the relationship between the concentration of proscillaridin A and the area of the peaks obtained from the analysis of the standard solutions. The determination coefficient ( $r^2$ ) was calculated by means of the least-square analysis [27-29]. The linear regression modes was applied to obtain the calibration line at the range of (4, 6, 8, 10, 12 and 14 µg/mL), to identify the extent of the total variability of the response.

#### *Precision*

The dispersion within a series on the determination of the same sample revealed the precision of the analytical method. Six real samples were analyzed on the same day (intra-day) and three on consecutive days (inter-day), and then the relative standard deviations (RSDs%) were calculated. Each sample was analyzed by three time injection to HPLC.

#### *Recovery*

This parameter reveals the closeness between the obtained values and the real ones. It ensures that no loss or absorption has occurred during the extraction procedure [27-28]. The determination of this parameter was performed by checking the recovery after preparation of spike samples in two different levels. Three replicate amounts of plant powder (9×0.25g) were weighted. One part was allocated as the real sample and others had been spiked with proscillaridin A reference standard solution to obtain desire concentrations (2 and 4 µg/mL). Three determinations were performed at each level and the recovery

percentage was calculated. Each sample was analyzed by three time injection to HPLC.

#### Limit of detection and quantization

Limit of detection and quantization were defined as the concentration of analyte giving a signal to noise 3:1 and 10:1, respectively.

### Results and Discussion

Methanol, ethanol, acetone and chloroform are some of the main solvents used for extraction of bufadienolides. In the present study, methanol was the best choice for extraction of proscillaridin A from *D. maritima*. Due to higher recovery and lower interfering in comparison with other solvents, methanol was selected as the best choice for the extraction of proscillaridin A and preparation of the standard solutions. Comparison between ultrasonic extraction, reflux and maceration showed that ultrasonic technique was the best choice. As it has been shown in table 1, ultrasonic radiation of the plant (0.25 g, 60 min, 40 °C, three times) with methanol resulted in the best recovery of proscillaridin A in extraction procedure (table 1).

**Table 1.** Effect of different solvents and extraction procedures on recovery of proscillaridin A

Method	Solvent			
	Acetone	Chloroform	Ethanol 80%	Methanol
Ultrasonication	1.5	0.8	1.5	8.4
Reflux	0.8	0.5	0.9	6.4
Maceration	0.5	0.3	0.4	2.1

All results represented as (µg/mL)

The structures of main bufadienolides of *D. maritima* are similar. So, it is hard to separate proscillaridin A from all of these compounds. The chromatograms obtained by different solid phase such as C<sub>8</sub>, C<sub>18</sub>, CN and phenyl showed that C<sub>18</sub> column resulted the best separation and efficacy. Selecting different mobile phases revealed that the ratio of organic modifier (methanol) played an important role in well separation. In order to achieve the best peak separation and the shortest run time, different percentage of the mobile phase was tested.

Proscillaridin A peak was separated from the nearest peaks and exhibited well peak symmetry and separation efficiency as seen in figure 2. The obtained results regarding to validation parameters including linearity, selectivity, accuracy and precision showed that the current method was suitable for the determination of proscillaridin A. The comparison between the purity threshold and purity angle obtained from HPLC software (Empower) revealed that the method was selective for proscillaridin A. The relationship between detector response and different concentrations of proscillaridin A was linear as shown in table 2. The precision of the analytical method was studied by relative standard deviation (RSD%) of the intra-day and inter-day experiments as reported in table 3.

**Table 2.** Linearity, LOD and LOQ parameters of proscillaridin A analysis in *Drimia maritima*

Linear range *	Equation	r square	LOD *	LOQ*
4-14	Y=19200x-13863	0.998	0.6	1.8

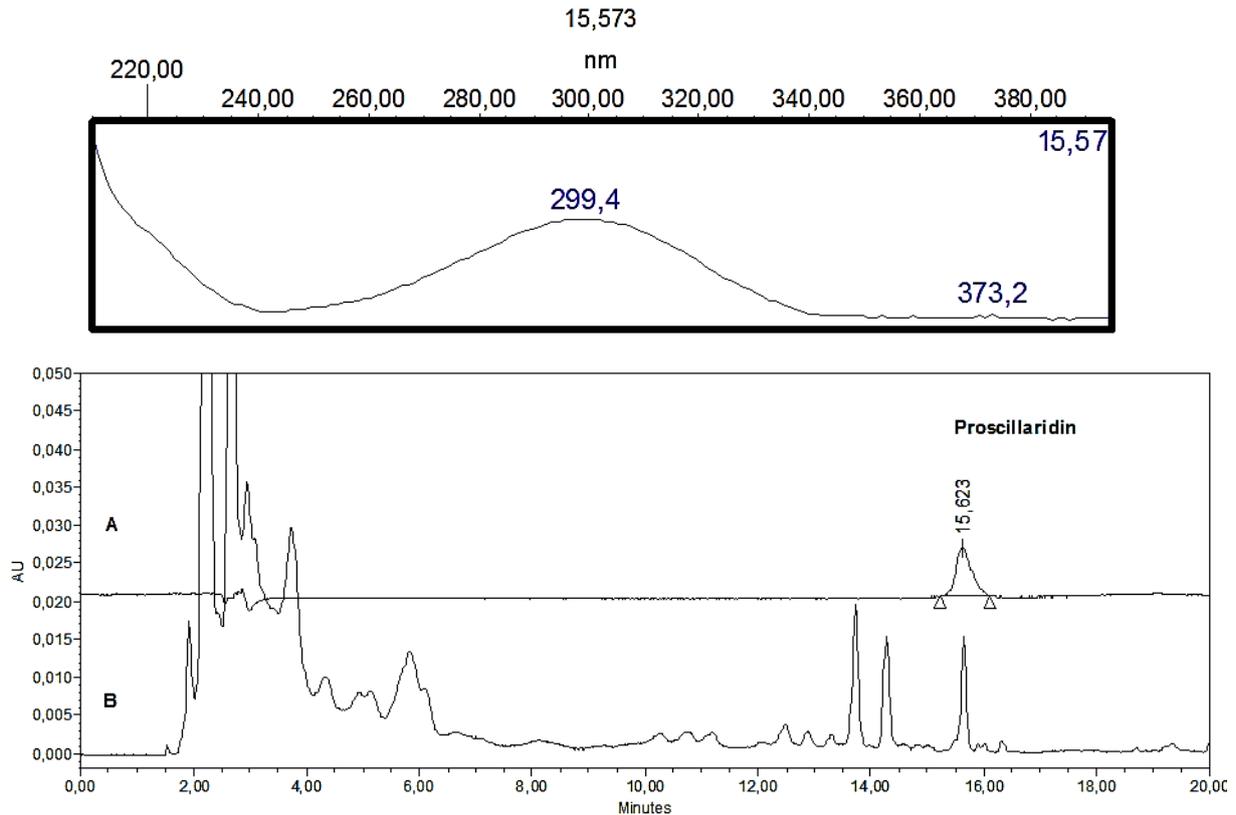
\*Represented as (µg/mL)

The results of intermediate precision (change in analyst and instruments) showed that these parameters did not have any remarkable effect on the results (data not shown). The evaluation of reproducibility of the method which focused more on the bias in the results, as inter-laboratory crossover studies, would be our next purpose. The results of recovery (accuracy), after spiking the plant samples with standards at two concentration levels have been shown in table 4 which was satisfactory. The calculated LOD and LOQ (0.6 and 1.8 µg/mL) were sufficient for determination of trace amount of proscillaridin in plant samples. Tittle *et al.* reported a quantitative HPLC with UV detection for the determination of bufadienolides in squill samples [22]. Limit of quantification (LOQ) and run time for the analysis were 200 µg/mL and 40 min, respectively. This method was not validated regarding to validation parameters. Knittel and coworkers described a method for identification and determination of bufadienolides and phenolic compounds in red squill samples with

**Table 3.** Repeatability of proscillaridin A analysis in *Drimia maritima*

Day/Sample	Proscillaridin A content *						Mean± SD (Intra-day)	RSD% (Intra-day)	RSD% (Inter-day)
	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>	A <sub>4</sub>	A <sub>5</sub>	A <sub>6</sub>			
1	3.58	3.64	3.44	3.52	3.66	3.88	3.62±0.015	4.16	7.5
2	3.98	4.33	4.32	4.14	4.23	4.11	4.18±0.13	3.22	
3	4.07	4.19	3.91	3.84	4.22	4.17	4.10±0.16	3.89	

\*Represented as (µg/mL)



**Figure 2.** HPLC chromatogram of A: Proscillaridin standard solution and B: *Drimia maritima* sample with chromatographic UV spectra at 200-400 nm

HPLC- diodarray-mass. LOD and LOQ were 0.19 and 0.64 µg/mL, respectively with 100 min retention time for proscillaridin [25]. In comparison with previous reports which mainly analyzed the bufadenolids in red squill, the resulting method in this study is fast, simple and reproducible which permit separation of proscillaridin and compounds in matrix in a run time of less than 18 min. Therefore the presented method can be recommended for the quantification

of proscillaridin A in *D. maritima*. due to gaining suitable precision, recovery and shorter run time

**Table 4.** Recovery of proscillaridin A analysis in *Drimia maritima*

Spiked *	Found*	Mean recovery%	Total recovery±SD
0.0	4.1	-	93.7±2.4
2.0	5.8	93.5±1.8	
4.0	7.9	94±3.1	

\*Represented as (µg/mL)

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### Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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**List of abbreviations:**

HPLC: high performance liquid chromatography

LOD: limit of detection

LOQ: limit of quantization

RSD%: relative standard deviation

