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Quantitation of alpha amyryn in *Scoparia dulcis* L. whole plant powder by high performance liquid chromatography

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ABSTRACT

A rapid and sensitive HPLC method with UV detection (206 nm) for routine analysis of alpha amyryn from *Scoparia dulcis* plant extract was developed. Chromatography was performed with mobile phase containing a mixture of acetonitrile and water (59:41) with a flow rate of 1.5 mL min⁻¹. The procedure was validated by linearity (correlation coefficient = 0.9997), accuracy, robustness and intermediate precision. Limit of quantitation and Limit of detection were found to be 1.2 µg and 0.4 µg respectively; this indicates the method is highly sensitive. Linearity was established by analyzing different concentration levels of alpha amyryn ranging from 0.008mg mL⁻¹ to 0.032 mg mL⁻¹. For method precision the percent relative standard deviation was 2.96 and for intermediate precision it was 2.05 (n=6) value indicated a good precision of the analytical method. The proposed method was simple, highly sensitive, precise, accurate indicating that the method is useful for routine quality control analysis of *Scoparia dulcis* Linn. whole plant powder and quantitative determination of alpha amyryn.

Keywords: *Scoparia dulcis*, Alpha amyryn, HPLC, Method validation.

INTRODUCTION

Alpha amyryn is a potential anti arthritic agent [1] having a chemical name (3β)-Urs-12-ene-3 ol; α-amyrenol Alpha amyryn is triterpene having an empirical formula C₃₀H₅₀O, a molecular weight of 426.73 and have structural formula of (Fig. 1). Alpha amyryn has the inhibitory activity against lipoxygenase enzyme [2] and the antinociceptive properties of alpha amyryn were reported [3]. *Scoparia dulcis* Linn (Family. Scrophulariaceae) commonly known as "Vassorinha" grows widely in open places through India, Brazil, China, Japan, and Taiwan. The plant aerial parts were traditionally used for the medicinal purpose. The literature survey reveals the pharmacological importance of the plant and the plant extracts have been reported to demonstrate antiviral activity in rats in addition to anti diarrhoeal activity and anti spasmodic activities [4].

In traditional medicine the plant powder has been used in the treatment of bronchitis, fever and kidney disorders [5-7]. The plant is also used as sweet broom and it is recognized by the Indian System of Medicine. Three chemotypes of plants have been identified based on the predominant type of diterpene produced in the plant [8-10]. This paper reports a rapid and sensitive HPLC determination method with UV detection, useful for the routine quality control of alpha amyryn in plant extracts. The method was validated by parameters such as linearity, accuracy, precision etc.

The same experimental conditions enable the quantitation of alpha amyryn in plant extracts and thereby enabling the confirmation of the label claim of alpha amyryn containing products.

MATERIALS AND METHODS

2.1. Chemicals and reagents

Standard alpha amyryn was obtained from Sigma Aldrich (USA). HPLC grade acetonitrile was obtained from Merck India. Ultra pure water was obtained using a Milli-Q UF-Plus apparatus (Millipore, MA, USA) was used to prepare all solutions for the method. All other chemicals used were of analytical grade unless otherwise stated.

2.2. Apparatus and chromatographic conditions

Hitachi L-2130, series chromatographic apparatus was used for this work. Data was collected and processed with Ezechrome Elite workstation. The HPLC system was equipped with an injection valve (Autosampler-L-2200), pumping system (VWR Hitachi pump L-2130) and with L-2400 UV dual λ absorbance detector. Chromatographic separation was performed on an Inertsil (phenyl) column (250 mm x 46 mm, 5 μ m). The mobile phase comprising a mixture of acetonitrile and water (59:41) was delivered at a flow rate of 1.5 mL min⁻¹. The mobile phase is filtered through 0.45 μ m filter (Pall Life Sciences) and degassed. Chromatography was performed at ambient temperature and detection was made at 206 nm. The injected volume was 20 μ L.

2.3. Preparation of standard solution

A stock solution of standard alpha amyryn was prepared in mobile phase. The prepared stock solution was diluted with mobile phase to give a solution containing alpha amyryn in the concentration of 20 μ g.

2.4. Preparation of plant extract

The plant of *Scoparia dulcis* was collected from the surroundings of Visakhapatnam and Bhubaneswar. The whole plant collected, was dried at room temperature and then ground to fine powder, which was passed through an ASTM BSS 85 mesh. 10 gm of plant powder was taken in a flask, 100 mL of methanol was added and kept on shaker for 2 hr. After shaking, the flask was left overnight at room temperature. The contents of the flask were filtered through a Whatman No. 1 filter paper. The resultant filtrate was passed through 0.45 μ filter and sealed to prevent loss of methanol.

RESULTS AND DISCUSSION

The applied chromatographic conditions permitted a good separation of alpha amyryn in the plant extract, no decomposition of alpha amyryn was observed during the analysis. The HPLC method was validated for the parameters reported below.

3.1. System suitability

The chromatographic separation was carried out by injecting standard solution five times, the peak area was considered, for five replicates the percent relative standard deviation was found to be less than 2.0. As explained above was carried out with HPLC to evaluate the chromatographic parameters (asymmetry 1.2 to 1.4 and theoretical plates 6000 to 8500). Figures 2 and 3 represent the chromatograms of the standard and sample (plant extract), which corresponds to the chromatographic separation of the compound. The system suitability test was performed by injecting a standard alpha amyryn five times. The % Relative Standard Deviation was found to be less than 2.0. It was concluded that the developed method is the optimum according to the studied parameters. Therefore this method can be applied to its intended purpose with no problems, its suitability being proved.

3.2. Specificity

Under the same chromatographic condition blank methanol was injected standard was injected, the peak purity of Alpha amyryn was studied by using PDA detector and found to be homogenous at set condition at peak coverage 95% the peak purity obtained was 0.999 for both standard as well as sample. The U.V spectrum of sample and standard are same. No placebo studies performed as we all know well it was plant powder sample. Reproducible results were observed

3.3. Stability of the solution

The experiment was conducted for 74 hr with the standard and sample solutions injected at hours 0, 2, 4, 8, 12, 24, 36, 48 and 72hr. The solutions of the standard and sample were found to be stable up to 72 hr at auto sampler chiller temperature configured at 25⁰C. The area percent difference with initial and 72 hr standard and sample solutions was found to be 98.9% and 98.1% respectively.

3.4. Linearity

Linearity was evaluated by different concentrations of standard solutions chromatographed using the methanol, analyzing each concentration twice. The peak area and concentration was subjected to least square linear regression analysis to calculate the correlation coefficients. The linearity of peak area responses versus concentrations was observed with in the range of 0.008 mg mL⁻¹ to 0.032 mg mL⁻¹ ($r = 0.9997$, $n = 7$). The linear response was observed over the examined concentration range. The results were tabulated table 1.

3.5. Accuracy and repeatability

Accuracy of the method was established by means of a recovery experiment. Accuracy was studied using three different solutions containing 120, 140 and 160% of Alpha amyirin in the whole plant powder (the external standard addition technique). The recovery data was reported in table 5. The obtained values were within the range of 100.56 to 102.33 %, which indicates the accuracy of the method. The system repeatability was calculated from seven replicate analysis and the low coefficient of variation values are indicative of precision of the method.

3.6. Robustness

The robustness of the method was studied, on the wavelength ± 2 nanometer and flow rate ± 0.2 ml/min during the study, where a slight variation in the retention time of alpha amyirin was observed but the percent assay in both the cases was found to be more or less the same indicating the robustness of the method.

3.7. Precision

The intermediate precision is a method of precision between repeatability and reproducibility and it should be established according to the circumstances under which the procedure was intended to be used [11]. The precision is usually expressed as variance, relative standard deviation or coefficient of variation of a series of measurements [12].

Method precision was carried out in one day at same experimental conditions using six different samples of assay concentration alpha amyirin with two repetitions each. The mean, standard deviation and coefficient of variation (RSD = 2.97%) were tabulated (Table.3). The low values of the coefficient of variation are indicative of the precision of the method.

The intermediate precision was obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory [11]. In order to study these effects simultaneously, a multivariate approach was used. The considered variables include analysts (1 and 2) and days (1 and 2). Each experiment was repeated three times in order to estimate the experimental error variance. The analysis was carried out in a randomized order according to the experimental plan reported (table 4). The content of alpha amyirin obtained were comparable to that of the method precision results. The percent relative standard deviation found was 2.05, indicating a good precision of the analytical procedure.

3.8. Limit of quantitation

Limit of quantitation and limit of detection of HPLC method for alpha amyirin in standard solution was established at a signal/noise (S/N) ratio of 10 and 3 respectively, which were experimentally verified by nine injections. The experiment was carried out up to 72 hr with the standard and sample solutions injected at 0, 2, 4, 8, 12, 24, 36, 48 and 72 hr. the solutions of the standard and sample were found to be stable up to 72 hr at auto sampler chiller temperature configured at 25⁰C. The area percent difference with initial and 72 hr for standard and sample was found to be 98.9% and 98.1% respectively, which indicate the sensitivity of the method. The limit of detection and limit of quantitation for alpha amyirin was found to be 0.4 $\mu\text{g mL}^{-1}$ and 1.2 $\mu\text{g mL}^{-1}$ respectively.

Fig. 1. Chemical Structure of Alpha amyirin

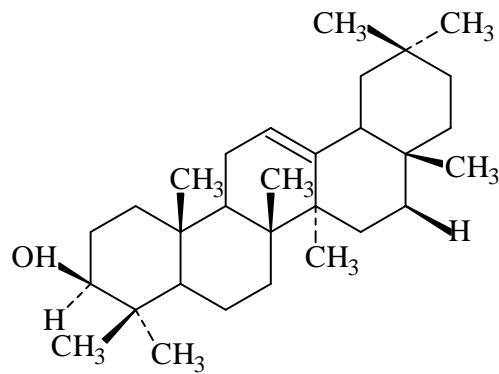


Fig. 2. Chromatogram of Standard Alpha amyirin

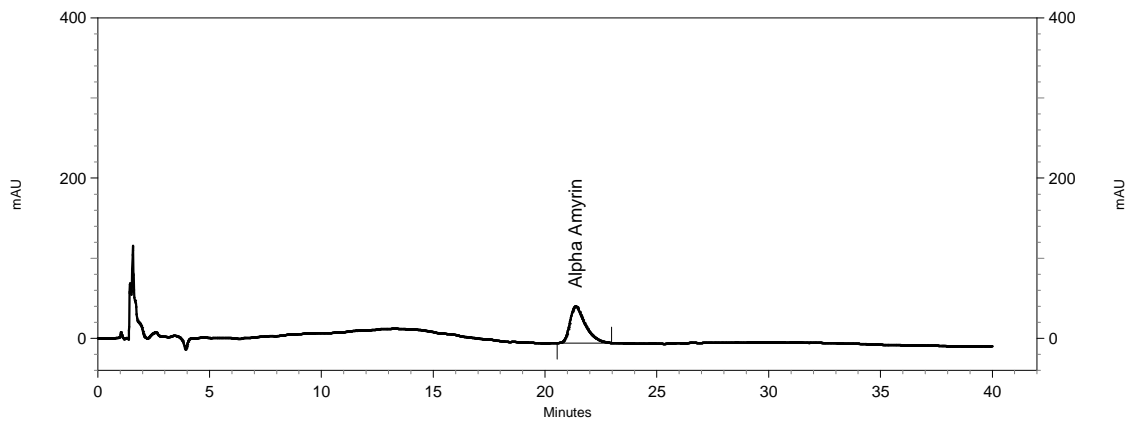


Fig. 3. Chromatogram of alpha amyirin in plant extract

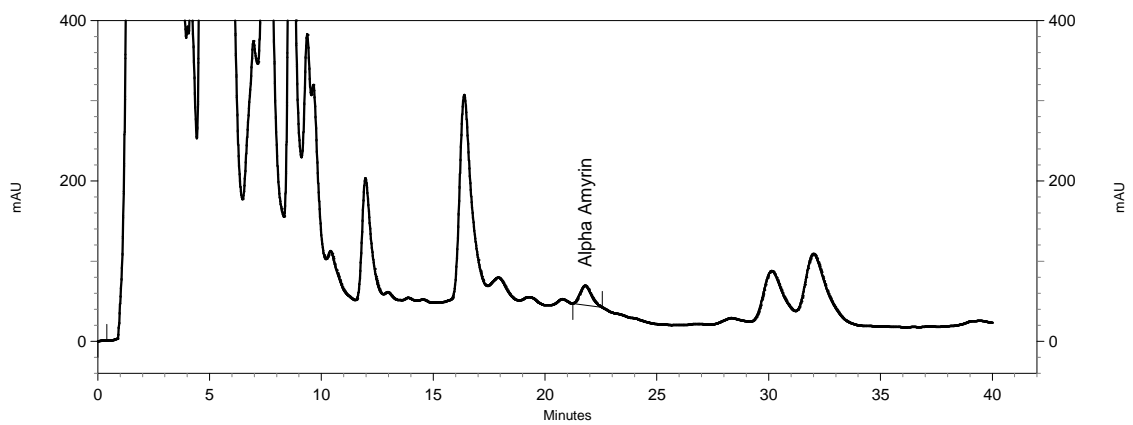


Table 1. Results of the data analysis for the quantitative determination of Alpha amyirin by the proposed method.(Linearity results)

Parameter	Value
Intercept	-8868.42
Standard error of y estimate	4912.15
r (Regression coefficient)	0.9997
Number of observations	7
Degrees of freedom	5
x coefficient (slope)	33936303.60
Standard error of coefficient	232077.55
Limit of detection (LOD), $\mu\text{g mL}^{-1}$	0.4
Limit of quantitation (LOQ), $\mu\text{g mL}^{-1}$	1.2

Table 2 Results from recovery experiment

% of Alpha amyirin	Powder taken (mg)	Std added ml stock	Rep-1	Rep-2	Mean	Found (mg)	%Recovery	Mean recovery
100%	I	10002.3	0.0	853853	853965	853909	0.64	101.4
	II	10003.5	0.0	866328	862526	864427	0.64	102.6
	III	10008.6	0.0	862536	872645	867591	0.65	103.0
120%	I	10003.6	1.0	1025695	1024690	1025193	0.76	121.7
	II	10008.5	1.0	1034791	1044685	1039738	0.77	123.4
	III	10003.8	1.0	1028780	1025680	1027230	0.76	122.0
140%	I	10009.3	2.0	1195473	1160378	1177926	0.88	139.8
	II	10004.5	2.0	1186378	1193586	1189982	0.89	141.3
	III	10005.8	2.0	1189453	1191591	1190522	0.89	141.3
160%	I	10005.3	3.0	1366255	1358296	1362276	1.01	161.7
	II	10000.5	3.0	1356284	1366578	1361431	1.01	161.7
	III	10008.8	3.0	1378652	1376385	1377519	1.03	163.5

Table 3 Results from Method precision

Method Precision							
Sample No	Quantity of powder (mg)	Volume (ml)	Peak Area			Alpha amyirin found (mg)	% Alpha amyirin present
			Rep-1	Rep-2	Avg		
1	10002.5	100	858659	859645	859152	5.28	0.053
2	10005.8	100	815698	821542	818620	5.03	0.050
3	10002.9	100	834152	834523	834338	5.13	0.051
4	10005.6	100	845689	839854	842772	5.18	0.052
5	10003.8	100	851235	854535	852885	5.24	0.052
6	10004.5	100	791562	792846	792204	4.87	0.049
Mean						5.12	0.05
SD						0.15	0.002
%RSD						2.96	2.97

*S.D: Standard Deviation**%R.S.D: Relative Standard Deviation***Table 4 Results from Intermediate precision**

Intermediate Precision(different day)							
Sample No	Quantity of powder (mg)	Volume (ml)	Peak Area			Alpha amyirin found (mg)	% Alpha amyirin present
			Rep-1	Rep-2	Avg		
1	10000.5	100	823456	835468	829462	4.99	0.050
2	10008.8	100	859235	861243	860239	5.17	0.052
3	10004.9	100	862358	859241	860800	5.17	0.052
4	10001.6	100	856234	852645	854440	5.14	0.051
5	10005.8	100	852365	849562	850964	5.11	0.051
6	10002.5	100	814278	823568	818923	4.92	0.049
Mean						5.08	0.05
SD						0.10	0.001
%RSD						2.06	2.05

*S.D: Standard Deviation**%R.S.D: Relative Standard Deviation*

Table 5 Results for Comparison

Comparison for Method precision and intermediate	
Method precision spl-1	5.28
Method precision spl-2	5.03
Method precision spl-3	5.13
Method precision spl-4	5.18
Method precision spl-5	5.24
Method precision spl-6	4.87
Intermediate precision spl-1	4.99
Intermediate precision spl-2	5.17
Intermediate precision spl-3	5.17
Intermediate precision spl-4	5.14
Intermediate precision spl-5	5.11
Intermediate precision spl-6	4.92
Mean	5.10
S.D	0.13
%RSD	2.47

Table 6 Results from assay experiment

Assay Experiment\Different instrument							
Sample No	Quantity of powder (mg)	Volume (ml)	Peak Area			Alpha amyryn found (mg)	%Alpha amyryn present
			1	2	Average		
1	10008.2	100	1935265	1935893	1935581	5.26	0.0526
2	10005.6	100	1952635	1962315	1957475	5.32	0.0532
3	10008.5	100	1942358	1945623	1943990	5.28	0.0528
4	10001.5	100	1882633	1896251	1889442	5.13	0.0513
5	10004.2	100	1923523	1915236	1919380	5.21	0.0521
6	10006.8	100	1953261	1942365	1947813	5.29	0.0529
7	10001.2	100	1942315	1912356	1927336	5.24	0.0524
					Mean	5.25	
					SD	0.006	
					%RSD	1.14	

CONCLUSION

The proposed high-performance liquid chromatographic method has been evaluated over the linearity, precision, accuracy and suitability and proved to be convenient and effective for the quality control of alpha amyryn in plant extracts. The measured signal was found to be precise, accurate and linear over the concentration of range tested (0.008-0.032 mg mL⁻¹) with a correlation coefficient of 0.9997. The proposed method on HPLC to determine alpha amyryn has been proved in a linear, precise, accurate, selective manner and encouraging its application in analysis applied in routine quality control.

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