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QUANTIFICATION OF DIOSGENIN IN EXTRACTS AND FORMULATIONS CONTAINING *SOLANUM NIGRUM*

Sonal Desai [1], Pratima Tatke *[1] and S. Y. Gabhe [2]

Department of Pharmaceutical Chemistry [1], C. U. Shah College of Pharmacy, S. N. D. T. Women's University, Santacruz (W), Mumbai-400 049, India
Department of Pharmaceutical Chemistry [2], Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune-411 038, India

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Correspondence to Author:

Dr. Pratima A. Tatke

Professor of Pharm.Chem.
C. U. Shah College Of Pharmacy,
S. N. D. T. Women's University,
Sir Vithaldas Vidhyavihar, Juhu Road, Santacruz (W), Mumbai-400 049, India.

E-mail:patatke@gmail.com

ABSTRACT: A reversed phase high performance liquid chromatography coupled with diode array detector (HPLC–DAD) was developed and validated to quantitate diosgenin in berries of *Solanum nigrum* using C18 analytical column as stationary phase and acetonitrile: water (92: 08, v/v) as mobile phase. The flow rate of mobile phase was kept at 1.0 ml/min. Column oven temperature was set at 25 ^{0}C. The method was found to be linear over the range 1.0-60 µg/ml. The limit of detection and limit of quantitation of the developed method were found to be 0.33µg/ml and 1.0 µg/ml, respectively. The recoveries for the spiked samples ranged 96.85 - 103.68 %. The RSD values for intraday and interday precision studies of the method were all less than 1.11 %. The proposed method was found to be robust in terms of % w/w of diosgenin. The developed and validated method was applied for quantitative determination of diosgenin in extracts and formulations containing *Solanum nigrum.*

INTRODUCTION: Many plant saponins have been used in traditional system of medicine for the treatment of different ailments including several cancers. Diosgenin is a naturally-occurring steroidal saponin present in *Solanum nigrum* (Solanaceae). Diosgenin serves as important starting material for the production of corticosteroids, sexual hormones, oral contraceptives as well as other steroidal drugs.[1] It is also used in the treatment of diseases such as diabetes, leukemia, hypercholesterolemia, climacteric syndrome and colon cancer.[2]

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Many HPLC methods have been reported for determination of diosgenin in various species of genus *Dioscorea*. [3-8] However, no HPLC method is reported for determination of diosgenin in berries of *Solanum nigrum.* Thus, the present work describes development and validation of a new, simple and sensitive reversed phase HPLC method for determination of diosgenin of *Solanum nigrum* and standardization of extracts and marketed Ayurvedic formulations containing *Solanum nigrum* in terms of diosgenin.

MATERIALS AND METHODS:

Plant materials, chemicals and reagents

Dried berries of *Solanum nigrum* were procured from three different geographical sources of India namely Valsad district of Gujarat (sample 1), Tirunelvelli district of Tamilnadu (sample 2) and local market of Mumbai, Maharashtra (sample 3) in February 2012. Different herbaria of sample 1,

sample 2 and sample 3 were deposited at Botanical Survey of India (BSI), Pune, India under names SNGSOD16, SNTSOD17 and SNMSOD18, respectively and authenticated. Standard of doisgenin was procured from Sigma-Aldrich. Other chemicals were purchased from S.D. fine-chem limited, Mumbai, India. Acetonitrile, purified water and hexane were of HPLC grade. Concentrated sulphuric acid was of AR grade.

Instrumentation and chromatographic conditions

HPLC analysis was carried out using Dionex-UltiMate 3000 HPLC system (Germany), consisting of auto-sampler, quaternary pumps, column oven and diode array detector (DAD). All data were analysed using Chromeleon software version 6.80. Separation was carried out using Thermo Hypersil C18 column (250 mm X 4.6 mm, 5 µm) with a mobile phase consisting of acetonitrile: water (92: 08, v/v) at the flow rate of 1.0 ml/min. Detection was carried out at 203 nm. Column was equilibrated at 25 ^{0}C. Injection volume was 20 µl.

Preparation of extracts and samples

Preparation of extracts [9]

Different extracts were prepared by refluxing dried and powdered berries of *Solanum nigrum* (20 g) with 200 ml of 20 % of H_2SO_4 in 70 % IPA under stirring for 8 h. The solution was filtered and marc was discarded. The filtrate thus obtained was extracted with hexane. Hexane was evaporated to dryness.

Preparation of standard solution

Diosgenin (100 mg) was dissolved in 100 ml of methanol by sonication for 10 min to prepare a stock solution of 1000 µg/ml. From this, various aliquots were taken and diluted with appropriate volume of methanol to produce different concentrations which were used to validate the method.

Preparation of sample solution

Hexane extract obtained as discussed above was evaporated to dryness. The hexane extract thus obtained (10 mg) was dissolved in 10 ml of methanol, sonicated for 10 min and filtered through 0.2 µ PTFE filter.

Method validation[10]

Specificity and system suitability

To assess specificity and system suitability of the proposed method, two injections of blank (methanol), six individual injections of standard solution (100 µg/ml) and two injections of sample solution were applied before all measurements. Any interference from blank or sample was checked and system suitability parameters such as resolution, theoretical plates, asymmetry and repeatability of the peak area were evaluated.

Linearity, limit of detection and limit of quantitation

To determine the linear relationship, appropriate aliquots of diosgenin stock solution were taken in 10 ml volumetric flasks and diluted up to mark with methanol to obtain final concentrations of 1.0, 10, 20, 30, 40, 50 and 60 µg/ml. Duplicate injections using 20µl loop were applied and chromatograms were recorded at 203nm. Quantitation was carried out by keeping peak area and concentrations of compound to straight line equation and correlation coefficient (r^2) was determined.

The LOD and LOQ were calculated based on signal to noise ratio method. Diosgenin solutions in increasing concentrations were injected until signal to noise ratio of 3:1 and 10:1 were obtained for determination of LOD and LOQ, respectively.

Accuracy

The accuracy of the developed method was evaluated through the analyte recovery test at three concentration levels. Known amount of sample was spiked with 32, 40 and 48 µg/ml of the standard solutions of diosgenin and % recovery was calculated by following formula:

% Recovery= Measured value /True value X 100.

Precision

Intraday precision and interday precision studies were carried out by repeating injections twice in a day and on two different days. Precision studies were carried out at three concentration levels of sample that is 80 %, 100 % and 120 %. For each concentration level, triplicate injections of sample were applied and % RSD was calculated.

Robustness

Robustness was evaluated by deliberately changing method parameters and their effects on peak area and retention time was observed. Only one parameter was altered at a time keeping the other parameters constant.

The robustness of the method, related to the variation in retention time, area, and % w/w of diosgenin in the sample was evaluated by changing the detection wavelength (202, 203 and 204 nm), mobile phase flow rate (0.9, 1.0 and 1.1 ml/ min), column oven temperature (24, 25 and 26 ^{0}C) and mobile phase composition (acetonitrile: water, 91.5: 8.5, 92:08 and 92.5: 7.5, v/v). For each condition, six injections of the standard solution (40 µg/ml) and two injections of sample (5 mg of semipurified extract in 10 ml of methanol) were applied.

Standardization of extracts and marketed Ayurvedic formulations

The developed and validated method was used for quantitative determination of diosgenin in three different extracts and different marketed Ayurvedic formulations such as tablets and capsule containing *Solanum nigrum*. Different hexane extracts were prepared from berries of *Solanum nigrum* procured from three different geographical regions of India (Gujarat, Tamilnadu and Maharashtra).

Marketed formulations containing *Solanum nigrum* are polyherbal formulations mainly used for treatment of liver problems. To determine the content of diosgenin present in these formulations, samples were prepared as given below:

Tablets: Twenty tablets were individually weighed; their mean weight was determined and the tablets were triturated. Accurately weighed 49.598 g of tablet triturate was taken and treated in similar manner as given in section preparation of extracts.

The precipitates thus obtained were filtered, dried and 0.079 mg of precipitates was dissolved in 2.0 ml of methanol. The solution was sonicated and 0.05 ml of above solution was diluted to 10 ml with methanol. The resulting solution was filtered and injected in HPLC.

Capsules: Twenty capsules were individually weighed; their mean weight was determined. Accurately weighed 21.093 g of capsule triturate was taken and treated in similar manner as given in section preparation of extracts. The precipitates thus obtained were filtered, dried and 0.023 mg of precipitates was dissolved in 2.0 ml of methanol. The solution was sonicated and 1.0 ml of above solution was diluted to 5.0 ml with methanol. The resulting solution was filtered and injected in HPLC.

RESULTS AND DISCUSSION:

HPLC method optimization

The goal of chromatographic separation conditions was to obtain chromatograms with optimum resolution of critical pair of peaks within a short analysis time. All the chromatograms were recorded at 203 nm. Column selected was C18 Thermo Hypersil (250 mm X 4.6 mm, 5µm). Injection volume was kept 20µl.

Initially, methanol was tried in combination water in ratio of 90:10, v/v and 95:5, v/v. In this case, components of extract could not be separated. Also, lower detection wavelength produced interference.

Therefore, further modification in mobile phase composition was done by replacing methanol with acetonitrile. 50 % of acetonitrile could not resolve the components present in extract. When the proportion of acetonitrile was increased from 50 % to 75 %, optimum resolution was achieved but the number of theoretical plates were low. Therefore, the proportion of acetonitrile was further increased to 92 %. Acetonitrile: water in the ratio of 92:08, v/v produced well resolved peaks.

As the proportion of acetonitrile was increased to 95 %, retention time of diosgenin was reduced but it also reduced the resolution between the peaks in extract. So 92 % of acetonitrile was selected for analysis. Analysis was carried out at column oven temperature of 25 ^{0}C and at flow rate of 1.0 ml/min which gave lower retention time of diosgenin and desired for resolution of peaks. Diosgenin was eluted with mean retention time of 6.343 min (**Fig. 1** and **Fig. 2**).

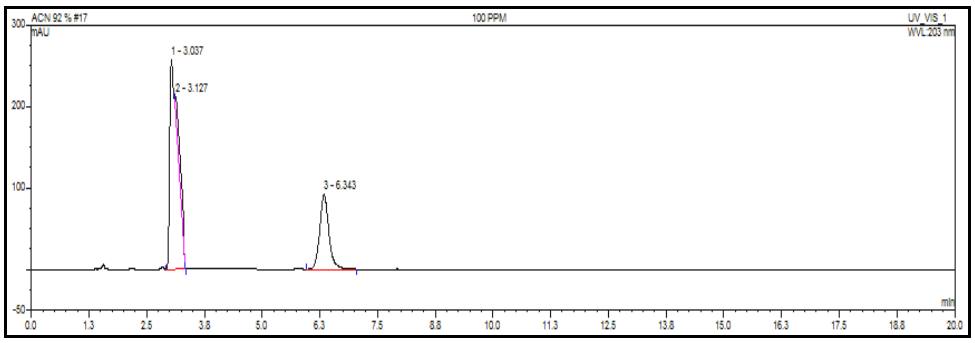

FIG. 1: HPLC CHROMATOGRAM OF STANDARD DIOSGENIN

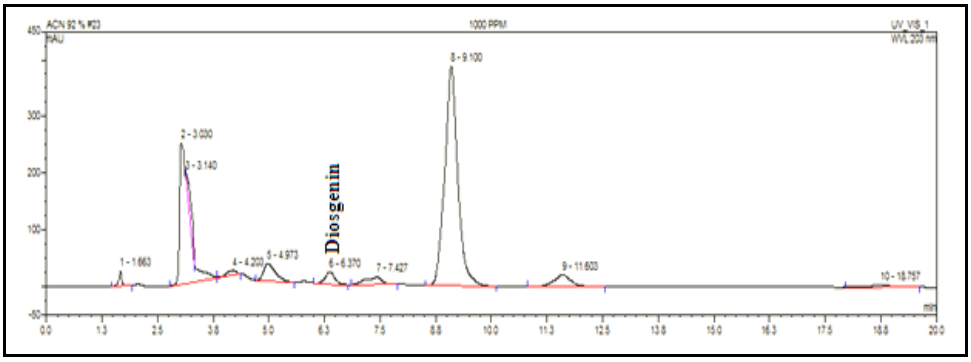

FIG. 2: HPLC CHROMATOGRAM OF SEMIPURIFIED EXTRACT OF *S. NIGRUM*

Method validation

The developed method was validated for various parameters such as linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, robustness and system suitability as per ICH guidelines.

Specificity and system suitability

No interference from blank or other components of sample was observed indicating that the developed method was specific. Results of system suitability revealed that number of theoretical plates (N) were more than 5000, resolution (Rs) was 1.85, peak asymmetry was less than 2.0 and % relative standard deviation (% RSD) obtained for six injections of standard solution was less than 1.0 indicating that the method and instrument were suitable for carrying out analysis (**Table 1**).

TABLE 1: RESULTS OF SYSTEM SUITABILITY PARAMETERS FOR DIOSGENIN

Compound	Number of plates (N)	Peak asymmetry (As)	R.S.D. of area
Diosgenin	5419	1.18	0.0565
Standard limits	> 2000	< 2.0	< 2.0 %

a n=6, six injections

Linearity, limit of detection and limit of quantitation

The developed method was found to be linear over the range 1.0-60 µg/ml with correlation coefficient (r^2) of 0.9984. LOD and LOQ were found to be 0.33µg/ml and 1.0 µg/ml, respectively (**Table 2**).

TABLE 2: RESULTS OF QUANTITATIVE DETERMINATION OF DIOSGENIN

Parameters	Results
Concentration range (µg/ml)	1.0-60
Regression equation	y = 0.2259x−0.0205
Correlation coefficient (r^2)	0.9984
Limit of Detection (LOD)	0.33 µg/ml
Limit of Quantitation (LOQ)	1.0 µg/ml

Accuracy

The mean recoveries were found be 103.68 %, 100.64 % and 96.85 % at 80 %, 100 % and 120 % level, respectively (**Table 3**). The recovery was found within the range 96.85-103.68 % which is largely within the 90-110 % range that is considered acceptable. So the proposed method was found to be accurate.

TABLE 3: RESULTS OF ACCURACY STUDIES FOR DIOSGENIN

Component	Amount Present[a] (µg/ml)±S.D.	Amount Found[a] (µg/ml)±S.D.	% Recovery[a]±S.D.
Diosgenin	30.4311±0.12	31.5529±0.01	103.6872±0.38
	42.5601±0.07	42.8338±0.04	100.6433±0.21
	47.5796±0.20	46.0814±0.11	96.8531±0.66

[a] n=3, triplicate injections

Precision: Values of % RSD obtained during precision studies at all level were less than 2.0, it indicates that the proposed method was precise (**Table 4**).

TABLE 4: RESULTS OF INTRADAY AND INTERDAY PRECISION STUDIES FOR DIOSGENIN

Component	Amount level (mg/10 ml)	Intraday (% RSD)[a]	Interday (% RSD)[a]	
		Day 1	Day 1	Day 2
Diosgenin	4	0.0748	0.0610	0.1032
	5	0.0678	0.0566	0.1284
	6	1.1188	0.0630	0.0960

[a] n=3, triplicate injections

Robustness The robustness was estimated using the overall mean, standard deviation and % RSD for each variable. % RSD was lower than 2.0 for the variables such as detection wavelength, flow rate, column oven temperature and mobile phase composition. The developed method was found to be robust for the % w/w of diosgenin present in the sample (**Table 5**).

Standardization of extracts and marketed Ayurvedic formulations The % w/w of diosgenin was found to be higher in Extract-III (sample obtained from Maharashtra) than the other two extracts. In the analysis of formulations, tablets contained higher amount of diosgenin as compared to capsules (**Table 6**).

TABLE 5: RESULTS OF ROBUSTNESS STUDY FOR DIOSGENIN

Parameters	Mean Retention time[a]	Mean Area[a]	% RSD[a]	% w/w of diosgenin
Detection wavelength (nm)				
202	6.372	10.5509	0.4302	3.2311
203	6.372	9.6246	0.4665	3.2669
204	6.372	8.7549	0.4169	3.2802
Flow rate (ml/min)				
0.9	7.077	10.6995	0.2018	3.2844
1.0	6.372	9.6246	0.4665	3.2669
1.1	5.803	8.7779	0.1013	3.2581
Column oven temperature (^{0}C)				
24	6.460	9.6756	0.1932	3.2971
25	6.372	9.6246	0.4665	3.2669
26	6.295	9.6995	0.1463	3.2768
Mobile phase composition (v/v)				
Acetonitrile: water (91.5: 8.5)	6.440	9.7204	0.1378	3.2221
Acetonitrile: water (92: 08)	6.372	9.6246	0.4665	3.2669
Acetonitrile: water (92.5: 7.5)	6.232	9.7370	0.2084	3.4818

[a] n=6, six injections

TABLE 6: % W/W OF DIOSGENIN IN EXTRACTS AND FORMULATIONS CONTAINING *SOLANUM NIGRUM*

Formulations/Extracts	% w/w of diosgenin [a] ± S.D.	mg of diosgenin[a] ± S.D.
Tablets	1.5556 ±0.06	0.0124±0.00/Tablet
Capsules	0.3564 ±0.06	0.0013±0.00/Capsule
Extract-I (Gujarat)	1.7104±0.00	-
Extract-II (Tamilnadu)	3. 0761±0.00	-
Extract-III (Maharashtra)	3.5255±0.00	-

[a] n=3, triplicate injections

CONCLUSIONS: A simple, rapid, accurate and convenient HPLC method was developed using diosgenin and validated as per ICH guidelines. The content of diosgenin was estimated in three hexane extracts and marketed formulations containing *Solanum nigrum.* This sensitive, accurate and precise validated method can be very well used to determine batch to batch variations and routine analysis of formulations containing *Solanum nigrum* by herbal manufacturers.

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