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A validated HPLC method for estimation of Gymnemic acids as Deacyl gymnemic acid in various extracts and formulations of *Gymnema sylvestre*

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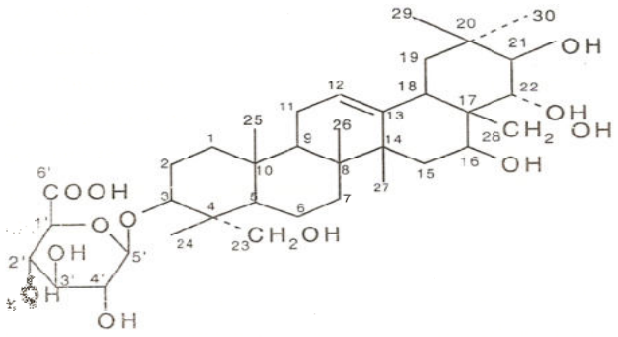
Abstract

The aim of present study is to develop and validate a simple, precise and rapid HPLC method for the quantification of gymnemic acid as Deacyl gymnemic acid in *Gymnema sylvestre* extracts and formulations. The analysis was performed by reverse-phase chromatography on a phenomenex C18 column with isocratic elution of acetonitrile: buffer (23:77 v/v) at a flow rate of 2.0mL/min. The method was validated in terms of precision, specificity, selectivity, linearity, limit of quantitation and detection, accuracy, recovery, and stability as per the ICH guidelines. The linear range of method was found to be 50- 800µg/ml with correlation coefficient of 0.9998. The developed method was found to be a relatively simple, precise and reproducible for the quantification of gymnemic acids in *gymnema sylvestre* extracts and formulations.

Keywords: *Gymnema sylvestre*, Deacyl gymenemic acid.

Introduction

Gymnema sylvestre is a potent antidiabetic plant and used in folk, ayurvedic and homeopathic systems of medicine. It is also used in the treatment of asthma, eye complaints, family planning, snakebite, urinary complaints, stomach problems, piles, chronic cough, breathing troubles, colic pain, cardiopathy, constipation, dyspepsia and hemorrhoids, hepatosplenomegally etc.[1,2] In addition, it also possesses antimicrobial, antihypercholesterolemic, anti- inflammatory and sweet suppressing activities. The leaves of *G. sylvestre* contain triterpene saponins belonging to oleanane and dammarene classes.[3] The active ingredient has been identified as group of gymnemic acids (saponins) including gymnemic acids I-VII, gymnemosides A-F and gymnemasaponins. The gymnemic acids contain several acylated (tiglolyl, methylbutyroyl etc.) derivatives of deacylgymnemic acid (DAGA) which is a 3-O-β-glucouronide of gymnemagenin (3β, 16β, 21β, 22 , 23, 28-hexahydroxy-olean-12-ene).[3,4] The structure of DAGA is [4] :



The total gymnemic acids present in the leaves or extract can be quantified by gravimetric analysis; which is a crude method of study [1]. Estimation of total Gymnemic acid by HPLC gives erratic results, due to its complex nature. Deacyl Gymnemic acid (DAGA) is the basic frame work of various gymnemic acids and hence this plant can be standardized with reference to this marker.[5] Quantification of DAGA by HPLC has been reported. Suzuki et. al., 1993 have reported the estimation of gymnemic acids content as DAGA. [5]

In the present study, a modified method was tried to quantify gymnemic acid as DAGA by HPLC. The method described in literature was modified to develop standard technique for estimation of DAGA. A number of mobile phases like different combinations of acetonitrile, methanol and ethanol with various buffers were tried for the estimation of DAGA using isocratic run. The method giving the best peak and run time was optimized and validated.

Materials and Methods

Standard Deacyl gymnemic acid was procured from Natural Remedies, Bangalore, India. *Gymnema sylvestre* dried powdered extracts (25% and75%) were gifted from Sami labs, Bangalore. Market formulation i.e. Meshashringi capsules (Himalaya, Bangalore, India) were used as marketed formulations for estimation. Water was obtained from Milli-Q (Millipore, Beford, MA, USA) water purification system. All other reagents were of HPLC grade or AR grade as per the requirement.

Preparation of standard solution

0.01 g of standard Deacyl gymnemic acid was dissolved in 10 ml methanol (1.0 mg mL-1) (Stock solution SS). The solution was stored in refrigerator and found to be stable for more than one month.

Chromatographic system

Chromatographic measurements were made on Shimadzu integrated liquid chromatographic system which consisted of a solvent delivery pump (model LC-10AT), injector (Model SC), UV–visible absorbance detector (model SPD-10A) and the instrument was connected to the computer with Class-VP software.
HPLC analysis was performed on a lichrosorb (Phenomenex) Luna 5 μ C 18, 250 X 4.6 mm column. The mobile phase was consisted of acetonitrile: 0.1% ortho Phosphoric acid (23:77 v/v) and pumped at a flow rate of 2.0 ml/min. The mobile phase was filtered through 0.45 μm Millipore filter and degassed by sonication for 30 min. The detection was carried out at 205 nm. An injection volume of the sample was 20μl. The temperature in laboratory was maintained at ambient conditions.

Preparation of sample solutions

An accurately weighed quantity of various extracts of Gymnema was saponified for 1 hr with 3.0% potassium hydroxide in methanol. The saponified mixture was concentrated. The residue was dissolved in 1:1 mixture of methanol and HPLC grade water, followed by acidifying with concentrated hydrochloric acid. The acidified sample was transferred to a 10 ml volumetric flask and the volume was made up to 10 ml with methanol (50%) and filtered through Whatman filter paper and used for further HPLC analysis.
The HPLC estimation was carried out by injecting 20 μl of the sample solution. Percentage of deacyl gymnemic acid was estimated using the area under the curve obtained from the sample by comparing the same with standard.

Calibration curve

Eight different concentrations of SS after dilution upto one mL (50 to 800 μg mL-1) with mobile phase were injected in triplicates. Regression equation with slope, intercept and co-efficient of correlation (*r2*) was derived. (Table 1).

Method validation

The developed RP-HPLC method was validated by determination of precision, specificity, selectivity, linearity, limit of quantitation and detection, accuracy, recovery, and stability as per the ICH guidelines.

Precision

System repeatability was determined by six replicate applications and six times measurement of sample solution at the analytical concentration of 400 μg mL-1 of DAGA. The repeatability of sample application and measurement of peak area for DAGA were expressed in terms of relative standard deviation (RSD). Method repeatability was obtained from RSD value by repeating the assay six times on the same day for intra-day precision. Intermediate precision was assessed by the assay of three, six sample sets on different days (inter-day precision). The intra- and inter-day variation for determination of DAGA was carried out at three different concentration levels 100, 400 and 600 μg mL-1.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were experimentally verified by diluting known concentrations of SS until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations. In order to find LOD and LOQ, the solution was prepared and injected six times following the proposed method. The signal to noise ratio was determined. LOD was considered as 3: 1 and LOQ as 10: 1.

Specificity (selectivity)

The specificity of the method was ascertained by analyzing standard drug and sample and comparing the retention time of the standard solution with that of sample solution.

Recovery studies

Accuracy of the method was ascertained by spiking the pre-analysed samples with known amount of standard drug solution and then analyzing by HPLC. The spiking was done at three different concentration levels i.e 80, 100 and 120% of the assay concentration level of sample (400 μg mL-1) in triplicates. The average percentage recovery at each concentration level was evaluated.

Stability

The stability of the samples was ascertained by storing them at room temperature and refrigeration temperature (4 C) for 24hr (short time) and 72hr (long time) and then analyzing.

Results and Discussion

Total Gymnemic acids can be quantified only by gravimetric analysis; which is a crude method of study. Different scientists have developed methods for quantification of gymnemic acid by HPLC by conversion of gymnemic acids to gymnemagenin.[6-9] This method is a complicated method as it involves two steps of processing acid hydrolysis followed by basic hydrolysis. In our study we have proposed a simple HPLC method of estimation of Gymnemic acid as DAGA. Suzuki et al have described estimation of Gymnemic acids by a similar HPLC method.[5] This method was modified and an attempt was made to develop a simplified method which gave reproducible results and the method was also validated.

The method was standardized with C18 column with Acetonitrile: Buffer (23% Acetonitrile + 67% Buffer) as the solvent system at the λ_{max} of 205nm. The flow rate was standardized to 2.0 ml/minute. This method was chosen because of its best suitability, good resolution and a clear peak was obtained between 7-9 minutes. The uniqueness of this analysis is that all unwanted peaks were eluted within 6 minutes, which ensures that analysis is over in 12 minutes with not more than 25ml of mobile phase. The chromatogram is shown in figure 1.

Method validation

Linearity

The method was found to be linear in the concentration range of 50 to 800 µg mL-1 (Table1). The calibration curve of standard DAGA gives linear regression of the data points with the equation y = 2672 x + 20.12, regression co-efficient ($r2$) 0.998 and RSD 0.015%. Retention time was 8.15 min with flow rate of 2 mL min-1.

Precision

i) System precision
System precision was evaluated by analyzing SS for six times and RSD was found to be less than 2% (Table 1).
ii) Method precision (Repeatability)
Method precision experiment was performed by preparing the same sample for six times and analyzing as per the method. The assay values were evaluated for RSD. It was found to be less than 2% (Table 1).
iii) Intermediate precision (Reproducibility)
The same sample was prepared and analyzed for three times on different days. The data was generated in three sets for each case (n=2 x 3). The resultant six assay values in each different condition were evaluated for RSD. The assay was carried out at three different concentrations i.e. 100, 400 and 600 µg mL-1. It was found to be less than 2% (Table 1). The results depicted in Table 1 showed that no significant intra- and inter-day variation was observed in the analysis of DAGA.

LOD and LOQ

The LOD with S/N ratio of 3: 1 was found to be 6.5 µg mL-1 and LOQ with S/N ratio of 10: 1 was found to be 12.5 µg mL-1 (n = 6)(Table 1).

Specificity

The difference in the retention time of standard and sample was compared and found to be ± 0.3 min. The standard Retention time (RT-8.15 min) of DAGA is corresponding to the sample chromatogram (RT-8.18 min) of the Gymnema extract along with other components. There was no interference from other component present in chromatogram. Chromatograms obtained for the DAGA standard and Gymnema extract revealed that they had similar pattern are shown in Figure 1 and 2.

Table 1: Validation parameters of the developed HPLC method for quantification of DAGA

Validation parameters	Results
Linearity range (µg mL-1)	50 - 1000
Correlation coefficient (r2)	0.9978
Regression equation	y = 2672 x + 20.12
LOD (µg mL-1)[a] (Limit of detection)	6.5
LOQ (µg mL-1)[b] (Limit of quantification)	12.5
System precision (RSD)[c]	0.78
Method precision(RSD)[d]	1. 53
Intermediate precision (RSD)[e] Interday Intraday	0. 37 0. 59
RSD (Linearity of the method)	0.015
Recovery [f]	97.5 - 98.4%
Stability [g] (Recovery % ± SD) Room Temperature Refrigeration Temperature	97.45% ±2.16 98.23% ±2.51

[a] n = 6, [b] n = 6, [c] n = 6, [d] n = 6,[e] n = 2 x 3, [f] n = 3, [g] n = 6
y = Peak Area, x = Concentration (µg mL-1)

Recovery studies

The method when used for extraction and subsequent estimation of DAGA from extract after spiking with 80, 100 and 120% of additional standard DAGA, yielded average recovery of 98.2 ± 0.74 (assay concentration 461.73µg mL-1).

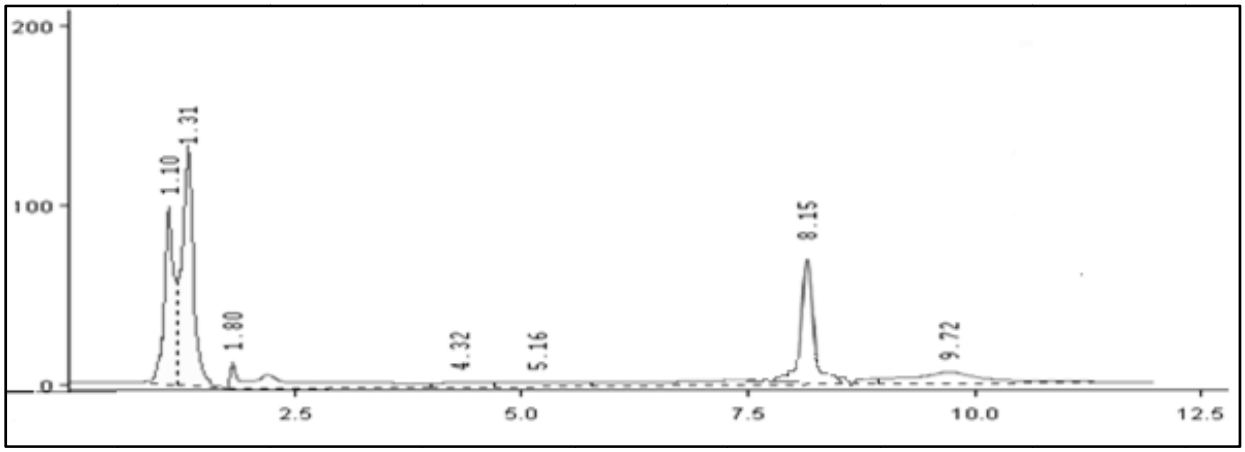


Figure 1: HPLC Chromatogram of Deacyl gymnemic acid (DAGA).

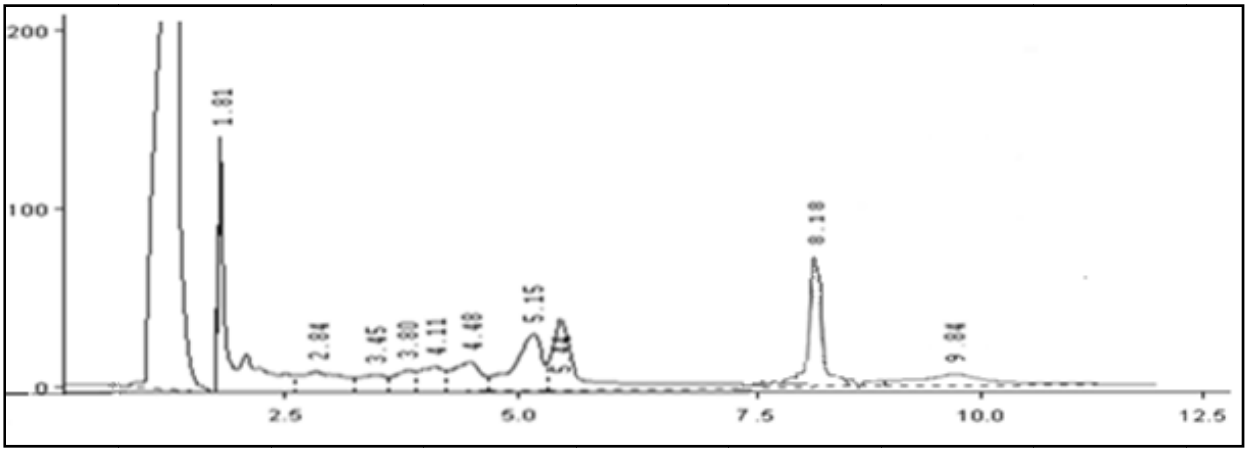


Figure 2: HPLC chromatograph of gymnemic acid converted to DAGA in the procured extract

Stability

The stability study indicated that the sample was quite stable when kept at room temperature and
refrigeration temperature (4 C) for 24hr (short time) and 72hr (long time). The results of the studies found that the percentage ratio are within the acceptance range of 90- 110%.
To quantify gymnemic acid in the extracts and formulations, the total gymnemic acids were converted to DAGA by saponification. The saponified sample was then analyzed by the developed HPLC method and the quantity of gymnemic acid in the procured extracts was found to be 24.12% and 73.4%. The same method was used for quantification of gymnemic acids in marketed formulations Meshashringi capsules. The concentration of gymnemic acid in the marketed formulation was found to be 103 ± 2.67% of the reported value.

Conclusion

The developed HPLC method is rapid, specific, accurate and precise for determination of gymnemic acids in *Gymnema sylvestre* extract and formulations. The method was validated and it can be adopted for the routine quantification of gymnemic acids in *Gymnema sylvestre* extracts and formulations because of its short run time.

Author's contribution

Nimisha jain is the research fellow responsible for experimental work and writing manuscript. Dr. V Kusum Devi is the research supervisor and coordinator for the project. She also advised and edited manuscript.

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Conflict of interest

No

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