

# A Validated Reverse Phase Liquid Chromatographic Method for Quantification of Gymnemagenin in the *Gymnema Sylvestre* R. Br. Leaf Samples, Extract and Market Formulation

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**Abstract:** A simple liquid chromatographic method was developed for the determination of gymnemagenin in leaves of a renowned anti-diabetic herb, *Gymnema sylvestre*. Gymnemagenin was obtained after acidic hydrolysis followed by basic hydrolysis of the sample and extraction into ethyl acetate. Analyte separation and quantitation were achieved by isocratic reversed-phase liquid chromatography and UV detection at 220 nm. The method involves the use of an RP-18e Lichrocart reversed-phase column (5  $\mu\text{m}$ , 75 x 4 mm id) and a binary isocratic mobile-phase profile. Linearity was observed in the range of 9.18 to 720  $\mu\text{g mL}^{-1}$  with correlation coefficient of 0.998. Relative standard deviation of linearity of the method was found to be 0.015%. Detection limit was 5.5  $\mu\text{g mL}^{-1}$  and quantitation limit was 7.5  $\mu\text{g mL}^{-1}$ . Average recovery of 99.2  $\pm$  0.54, was obtained by spiking pre-analyzed samples with standard solution at 3 different concentration levels (80, 100, and 120%). Three leaf samples of *G. sylvestre* from three different regions, one marketed *G. sylvestre* extract and an anti-diabetic polyherbal formulation containing *G. sylvestre* leaf powder were analyzed by this method. They were found to contain 0.30-0.34, 5.9 and 0.125% w/w gymnemagenin respectively. The new method is comparatively simpler, reproducible and sensitive than the other reported methods.

**Keywords:** Liquid chromatography; Gymnemagenin; *Gymnema sylvestre*; Standardization.

## 1. Introduction

Leaves of *Gymnema sylvestre* have been used in India for the treatment of diabetes for over 2000 years [1]. Studies have shown that *Gymnema* reduces blood sugar levels by stimulation of insulin release (and possibly by pancreatic regeneration or repair) and stimu-

lation of enzymes responsible for glucose uptake and utilization [2, 3]. Antimicrobial, hypolipidaemic and antiatherosclerotic effect of leaf extract of *Gymnema* are also reported recently [4, 5].

Gymnemic acid, a group of complex

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triterpenic glycosides were reported to be responsible for the antidiabetic action [6].

Gymnemagenin (Figure 1) is a common aglycone of gymnemic acids which can be produced after acidic and basic hydrolysis. Quantitative analysis of gymnemagenin by HPLC [7] and HPTLC [8, 9] has been reported. One HPLC method reported involving ion-spray mass detector for qualitative analysis of gymnemic acid [10].

This study was undertaken to develop a simple HPLC method using an isocratic mobile phase and UV detector for quantification of gymnemagenin in herb samples and formulations. The method could be successfully employed for routine analysis of *Gymnema* for quantification of gymnemagenin.

## 2. Experimental

### 2.1. Herbal materials and chemicals

Standard gymnemagenin (90%) was procured from Natural Remedies, Bangalore, India. It was used as such without any further purification. Dried powdered samples from the leaves of gurmar (*G. sylvestre* R. Br., Family: Asclepiadaceae) were procured from three different geographical regions of India i.e. Ahmedabad (Gujarat), Neemach (Madhya Pradesh) and Mumbai (Maharashtra). *G. sylvestre* extract was gifted from Garlico herbal concentrates (Mandsaur, India). Market formulation i.e. Mersina capsules (J & J De Chane Laboratories, Hyderabad, India) were used for estimation of gymnemagenin. Ammonium acetate buffer (100mM) of pH 6.74 was prepared as per Indian Pharmacopoeia [11]. Water obtained from Milli-Q (Millipore, Bedford, MA, USA) water purification system. All other reagents were of HPLC grade or AR grade as per the requirement.

### 2.2. Preparation of standard solution

0.0100 g of standard gymnemagenin (90%) was dissolved in 10 ml methanol (0.9 mg

mL<sup>-1</sup>) (Stock solution SS). The solution was stored in refrigerator and found to be stable for one month.

### 2.3. Chromatographic system

LC system Lachrom Merck Hitachi (Darmstadt, Germany) with isocratic pumps L-7110, Interface D-7000, Autosampler L-7200, and U.V Detector L-7400 was used. The data were acquired on the HSM administrator data system (Merck Hitachi, Japan). Lichrocart® 60 RP C18 e column (5µm, 4 x 75mm)(Merck, Darmstadt, Germany) and a mobile phase consisting of 100 mM ammonium acetate buffer: acetonitrile (75: 25, v/v) were employed. pH of the buffer was adjusted to 6.7 with ortho phosphoric acid. The mobile phase was filtered through 0.45 µm Millipore filter and degassed by sonication for 30 min. The flow rate was adjusted to 1.2 mL min<sup>-1</sup> with run time of ten minutes. Injection volume was adjusted to 100 µL and detection was made at 220 nm.

### 2.4. Preparation of sample solutions

An accurately weighed sample was refluxed for two hours in 2.5 N 50 % methanolic HCl, filtered and filtrate was added in ice cold water to obtain precipitate which was refluxed for 2 h in 50 ml of 2% methanolic KOH. The mixture is cooled, diluted with water and extracted with ethyl acetate. Ethyl acetate layer was evaporated and residue was reconstituted in 10 mL HPLC grade methanol.

### 2.5. Calibration curve

Eight different concentrations of SS after dilution upto one mL (9.18 to 720 µg mL<sup>-1</sup>) with mobile phase were injected in triplicates. Regression equation with slope, intercept and co-efficient of correlation ( $r^2$ ) was derived (Table 1).

### 2.6. Method validation

### *Precision*

System repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentration of  $400 \mu\text{g mL}^{-1}$  of gymnemagenin. The repeatability of sample application and measurement of peak area for gymnemagenin 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 gymnemagenin was carried out at three different concentration levels 100, 400 and  $600 \mu\text{g mL}^{-1}$ .

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

In order to find LOD and LOQ, blank 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. 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.

### *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 LC. The drug spiking was done at three different concentration levels i.e 80, 100 and 120% of the assay concentration level of sample ( $450 \mu\text{g mL}^{-1}$ ) in triplicates. The average percentage recovery at each concentration level was evaluated.

## **3. Results and Discussion**

### **3.1. Method validation**

#### *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 analysing 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 ( $n=2 \times 3$ ). 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  $600 \mu\text{g mL}^{-1}$ . 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 gymnemagenin

#### *LOD and LOQ*

The LOD with S/N ratio of 3: 1 was found to be  $5.5 \mu\text{g mL}^{-1}$  and LOQ with S/N ratio of 10: 1 was found to be  $7.5 \mu\text{g mL}^{-1}$  ( $n = 6$ )(Table 1).

### Specificity

The difference in the retention time of standard and sample was compared and found to be  $\pm 0.3$  min.

### Recovery studies

The method when used for extraction and subsequent estimation of gymnemagenin from poly-herbal formulation after spiking with 80, 100 and 120% of additional standard gymnemagenin, yielded average recovery of  $99.2 \pm 0.54$  (assay concentration  $671.43 \mu\text{g mL}^{-1}$ ).

Chromatograms of standard gymnemagenin and that of a leaf powder sample of *G. sylvestre* are shown in Figure 2 A and B respectively. For leaf powder sample of *G. sylvestre* two major peaks were observed with RT of 3.3 min and 7.3 min(gymnemagenin).

The method was found to be linear in the concentration range of 9.18 to  $720 \mu\text{g mL}^{-1}$  (Table1). The calibration curve of standard gymnemagenin gives linear regression of the data points with the equation  $A = 0.038 C - 0.6384$ , regression co-efficient ( $r^2$ ) 0.998 and RSD 0.015%. Retention time was 7.3 min with flow rate of  $1.2 \text{ mL min}^{-1}$ .

Analyses of dried leaf powder samples of *G. sylvestre* plant procured from three different geographical regions of India, dried powder extract, and a poly-herbal formulation containing *G. sylvestre* leaf powder showed gymnemagenin in the concentration range of 0.3 – 0.34 %w/w on dry weight basis, 5.9% w/w and 0.125% w/w on a dry weight basis in extract and formulation respectively (Table 2).

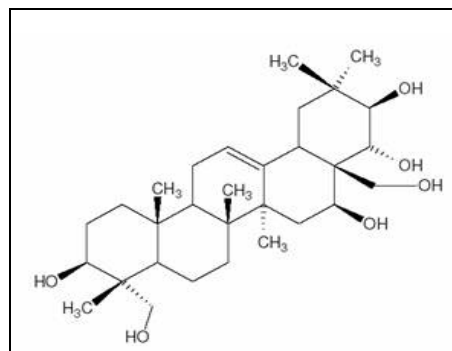


Figure 1. Structure of gymnemagenin

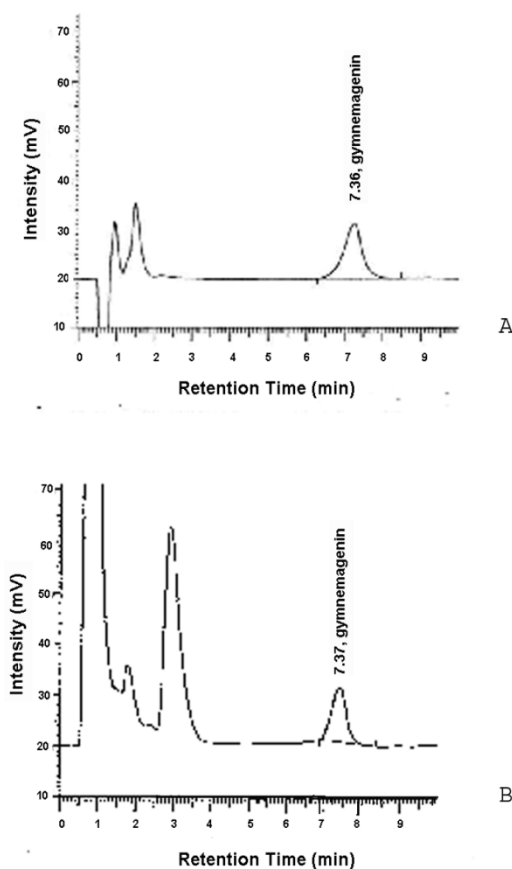


Figure 2. A) Chromatogram of estimation of standard gymnemagenin( $670 \mu\text{g mL}^{-1}$ ) B) Chromatogram of estimation of gymnemagenin leaf powder sample obtained from ahmedabad (Assay concentration is  $671.43 \mu\text{g mL}^{-1}$ )

Table 1. Validation parameters of the developed HPLC method for quantification of gymnemagenin

Validation parameters	Results
Linearity range ( $\mu\text{g mL}^{-1}$ )	9.18 - 720
Correlation coefficient ( $r^2$ )	0.9978
Regression equation	$A = 0.038 C - 0.6384$
LOD ( $\mu\text{g mL}^{-1}$ ) <sup>a</sup> (Limit of detection)	5.5
LOQ ( $\mu\text{g mL}^{-1}$ ) <sup>b</sup> (Limit of quantification)	7.5
System precision (RSD) <sup>c</sup>	0.04
Method precision(RSD) <sup>d</sup>	0.07
Intermediate precision (RSD) <sup>e</sup>	
Interday	0.15
Intraday	0.09
RSD (Linearity of the method)	0.015

<sup>a</sup> $n = 6$ , <sup>b</sup> $n = 6$ , <sup>c</sup> $n = 6$ , <sup>d</sup> $n = 6$ , <sup>e</sup> $n = 2 \times 3$

A = Peak Area, C = Concentration ( $\mu\text{g mL}^{-1}$ )

Table 2. Amount of gymnemagenin found in plant samples by the proposed method

Sample Name	Concentration <sup>a</sup> (%w/w)
GYA (d.w.b) <sup>b</sup>	0.335
GYN (d.w.b) <sup>b</sup>	0.301
GYM (d.w.b) <sup>b</sup>	0.328
GYext (d.w.b) <sup>b</sup>	5.956
GYcap (d.w.b) <sup>b</sup>	0.125

GYA=*sylvestre* leaf powder from Ahmedabad, GYN=*sylvestre* leaf powder from Neemach, GYM=*sylvestre* leaf powder from Mumbai, GYext=*sylvestre* market extract, GYcap=Market formulation (Capsule)

In HPLC method reported in literature [7] isocratic as well as gradient elutions were used and detection was made by LC-MS connected to a Hitachi M80 Mass spectrophotometer. The method was more of a qualitative study for identification rather than a routine analytical study. For a routine analysis this method may not be feasible. Only one HPLC method has so far been reported for quantification of gymnemagenin [7]. In reported HPLC method [7] gradient elution program is employed i.e 25%(0 min)→75% (20 min)→ 100%(21-25 min)→25% (26 min),

with mobile phase of acetonitrile/water/phosphoric acid 800:200:1v/v/v and water/phosphoric acid 1000:1 v/v while in the proposed method a simple binary isocratic mobile phase system is used which yielded good separation. Moreover the reported HPLC method required column temperature of 40 °C and runtime was around 26 minutes whereas our method provides good separation at room temperature (25 °C) with runtime of 10 minutes. In the proposed method detection of gymnemagenin was done at 220 nm compared to 213 nm in the reported HPLC meth-

od [7].

Puratchimani, V. and Jha, S.[8] and Valvirathi et al.,[9] reported HPTLC method for estimation of gymnemagenin. In the method reported in literature [8] there are many flaws which are highlighted and corrected in the method reported by Valvirathi et al. In literature [8] concentration of gymnemagenin in various plant samples has been reported without acidic or basic hydrolysis of the sample. Gymnemagenin is not present in free form in plant, it is a common genin of gymnemic acids which can be produced only after acidic and basic hydrolysis. The method reported in literature [9] reports properly validated HPTLC method for gymnemagenin estimation. Precision and reproducibility of our method is better compare to the method reported in literature [9]. But the reported method has better sensitivity compare to our method as it reports detection of gymnemagenin at nanogram level.

As HPLC is most widely used tool for quantification of analytes with good reproducibility we attempted to develop a reliable and reproducible validated method for gymnemagenin estimation using HPLC.

As gymnemagenin happens to be a common genin for most of the gymnemic acids its quantification reflects the quantity of gymnemic acids. The method is properly validated and found to be simple, reproducible, accurate and reliable.

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