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A Validated High Performance Thin - layer Chromatographic method for the estimation of gymnemic acids through gymnemagenin in Gymnema sylvestre, materials, extracts and formulations

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Abstract: A reproducible and reliable HPTLC method for the indirect determination of gymnemic acids as gymnemagenin in *Gymnema sylvestre* plant has been reported. As gymnemagenin, a common aglycone of gymnemic acids, lacks in conjugation, very poor UV absorption is observed. So post-derivatzation method was used for quantification of gymnemagenin. Linearity was observed in the range of 180 – 1440 ng/spot. Thus the method was found to be more sensitive where gymnemagenin was quantified at nanogram level. The method was validated as per ICH guidelines and successfully applied for quantification of gymnemagenin from plant leaf powder, extract and poly herbal marketed formulation. Percentage recovery was found to be 98.4 ± 1.0%.

Keywords: High Performance Thin-Layer Chromatography; Gymnemagenin, Standardizatio; *Gymnema sylvestre.*

1. Introduction

Leaves of *Gymnema sylvestre* have been used in India for the treatment of diabetes for over 2000 years [1]. Many studies have shown that oral administration of *Gymnema* extract reduces serum glucose level and improves glucose tolerance in mildly diabetic rats [2]. Administration of water extract of *Gymnema sylvestre* leaves was found to increase serum insulin level suggesting its insulin releasing effect [3]. Number of beta cells within pancreatic tissue were increased which suggests a restorative effect of the *Gymnema* extract on pancreatic tissue [4].

Gymnema leaf contains more than 20 saponin glycosides. The major saponin fraction comprises of gymnemic acid (the anti-sweet principle) which is a complex mixture of at least nine closely related acidic glycosides [5]. Thus it is difficult to separate gymnemic acid in pure form. A quantitative analysis of gymnemic acids by HPLC analysis of gymnemagenin, the common aglycone obtained on hydrolysis has been reported by Toshihiro et al. [6]. Puratchimani and Jha [7] reported a HPTLC method for analysis of gymnemagenin. Valivarthi et. al. [8] also

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reported an improved comparative method for HPTLC analysis of Gymnemagenin in UV range. These authors adopted a basic hydrolysis (l h) followed by acid hydrolysis (1 h) and passed the hydrolysate through polyamide C-200. Subba Rao and Sinsheimer [9] reported a process for isolation of gymnemagenin involving initial acid hydrolysis followed by basic hydrolysis.

Gymnemagenin lacks conjugation in its structure (Figure. 1). No maxima is obtained between 200 to 340 nm. Thus it has very poor UV absorption. Both the afore mentioned HPTLC methods quantified gymnemagenin at 210 nm / 290 nm.

The present work was undertaken with a view to standardize hydrolysis process and develop a sensitive validated HPTLC method for quantification of gymnemagenin in the visible range after derivitization.

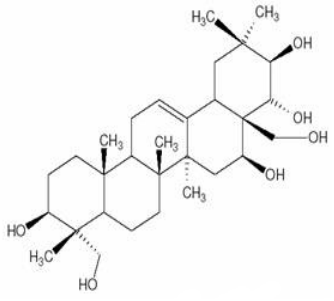

Figure 1. Structure of gymnemagenin

2. Experimental

Plant materials and chemicals

Standard gymnemagenin (90%) was procured from Natural Remedies, Banglore. Dried powdered samples from the leaves of Gurmar (*Gymnema sylvestre* R.br. Ex Schult*)*, (Family: Asclepiadaceae) were procured from Ahmedabad (Gujarat, India), Neemach (M.P, India) and Mumbai (Maharashtra, India). *G. sylvestre* extract was gifted from Garlico herbal concentrates (Mandsaur, M.P, India). Mersina capsules (J & J De Chane) were used for estimation of gymnemagenin. All other reagents were of analytical-reagent grade. Triple distilled water was used for all the dilutions and reagent preparations. Modified vanillin-sulfuric acid reagent was prepared by preparing 0.5% vanillin in sulfuric acid-ethanol (4: 1) v/v (stock solution). 25 mL of stock solution was taken and diluted up to 100 mL with ethanol. The final spray reagent contains 0.125 g of vanillin in 20% sulfuric acid in ethanol.

Preparation of standard solution

Standard gymnemagenin solution was prepared by dissolving 10 mg (± 0.1 mg) of standard gymnemagenin (90%) in 10 ml methanol (0.9 mg mL^{-1}). One mL of aliquote from this solution was taken and diluted upto 10 mL using methanol (90 μg mL^{-1}).

HPTLC instrumentation and experimental conditions

Sample solutions were applied onto the plates with automated TLC sampler Linomat V (Camag, Muttenz, Switzerland) and were controlled by WinCATS software 1.3.3 (Camag, Muttenz, Switzerland). Plates were developed in 20 x 10 cm twin trough glass chambers (Camag, Muttenz, Switzerland). A TLC scanner III with WinCATS software was used for scanning the TLC plates. Pre-coated silica gel aluminum plates 60 F_{254} (20 x 10) with 250 μm thicknesses (Merck, Darmstadt, Germany), were used for all determinations. The plates were pre-washed with methanol and activated at 60° for 5 minutes prior to chromatography. Five different aliquots (2, 4, 8, 12 and 16 μl) of standard solution were applied in triplicates on 20 x 10 cm TLC plates for the preparation of calibration curve. Six such plates were prepared. A constant application rate of 0.1 μL S^{-1} was employed with a bandwidth of 6 mm. The slit dimension was kept at 6.0 mm x 0.45 mm and scanning speed

of 10 mm S^{-1} was employed. The monochromatic bandwidth was set at 20 nm. The mobile phase consisted of toluene: chloroform: methanol (5: 8: 3) v/v. 15 ml mobile phase was used per plate. The optimized chamber saturation time for mobile phase was 15 min at room temperature (25° ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 7.0 cm. After development, chromatographic plates were dipped into derivatization reagent i.e. modified vanillin-sulfuric acid reagent and again dried for 10 minutes using hair dryer on hot mode. After drying, the plates were heated at 110° for 15 min in a pre-heated oven. The bluish-black colored spot corresponding to gymnemagenin was observed at R_f 0.30 ± 0.05. The chromophore was found to be stable for 20 min. The plates were scanned within 10 min using densitometric scanner III in the remission mode at 610 nm. The source of radiation was tungsten lamp emitting a continuous visible radiation between 400-900 nm. Evaluation was done by measuring peak areas with linear regression.

Preparation and estimation of gymnemagenin in sample solution

An accurately weighed quantity (1 g) of samples were taken and refluxed for two hours in 2.5 N 50 % methanolic hydrochloric acid separately. The solution was filtered and added in ice cold water with stirring. The resulting precipitate were collected, washed and dried below 60° and dissolved in 50 ml methanol. 1 g KOH was added into the solution and refluxed for 2 h, diluted with water and extracted with ethyl acetate. Ethyl acetate layer was evaporated and residue was reconstituted in 10 mL methanol. 5 µL of aliquot of these sample solutions were applied on TLC plates in triplicates and analyzed as described above.

3. Method validation

Precision

Precision of the method was determined with the product. The samples containing *Gymnema* leaf powder were accurately weighed (1 g) and assayed. System repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentration of 450 ng/spot of gymnemagenin. The repeatability of sample application and measurement of peak area for active compound 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 (Table 1). 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 200, 450 and 800 ng $spot^{-1}$ (Table 2).

Table 1. Validation parameters of the proposed HPTLC method for estimation of gymnemagenin.

Validation Parameters	Results
Linearity range (ng $spot^{-1}$)	180 - 1440
Correlation coefficient (r^2)	0.994
Regression equation	Y = 6.062X + 2363.559
LOD (ng $spot^{-1}$) (Limit of detection)	50
LOQ (ng $spot^{-1}$) (Limit of quantification)	100
RSD of linearity of the method (n = 3 x 6)	1.96

RSD- Relative Standard Deviation

Table 2. Intermediate precision data of proposed HPTLC method.

Intermediate precision (Reproducibility) Sample (n = 3 x 2)		
Day-to-day (n = 6)		
Theoretical amount (ng/spot)	**Experimental amount (ng/spot)**	**RSD**
200.0	199.9 ± 0.21	0.98
450.0	449.2 ± 0.36	0.87
800.0	798.2 ± 0.25	1.16

RSD- Relative Standard Deviation

Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like toluene: chloroform: methanol (5.5:7.5:3) v/v and toluene: chloroform: methanol (5: 7.5:2.5) v/v were tried and chromatograms were run. The amount of mobile phase was varied in the range of ±5%. The plates were pre-washed by methanol and activated at 60° ± 5 for 5, 10, 12 min prior to chromatography. Robustness of the method was done at three different concentration levels 200, 450 and 800 ng/spot. Amount of mobile phase was varied and plates were developed in 8, 10 and 12 ml mobile phase. Time from spotting to chromatography and chromatography to scanning were also varied and RSD were determined and found to be less than 2 %.

Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank solution (methanol) was spotted six times following the same method as explained above. 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 reference solution until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spots for gymnemagenin in sample were confirmed by comparing R_f and spectra of spot with that of standard. The peak purity (90%) of gymnemagenin was assessed by comparing the spectra at three different levels i.e. peak start, peak apex and peak end positions of the spot. Purity of sample spot corresponding to gymnemagenin was determined by taking the spectra and by comparing it with that of standard.

Recovery studies (Accuracy)

The pre-analyzed samples were spiked with 80, 100 and 120% of the standard solution and the mixtures were analyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the drug at different levels in the formulations. Recovery study was carried out for the powder sample of *Gymnema* leaf powder from Ahmedabad.

4. Results

Calibration curve of standard gymnemagenin

Linearity was found between the concentra-

tion range of 180 to 1440 ng spot^{-1} with r^2 ± S.D = 0.994 ± 0.005. Linearity was evaluated by spotting each concentration (180 to 1440 ng) in triplicates per TLC plate and six such plates were evaluated (n= 3 x 6).

Peak areas and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. Relative standard deviation for determination of linearity of method was found to be 1.96 (Table 1). The validation parameters of the proposed method are shown in Table 1. Chromatogram of standard gymnemagenin is shown in Figure. 2.

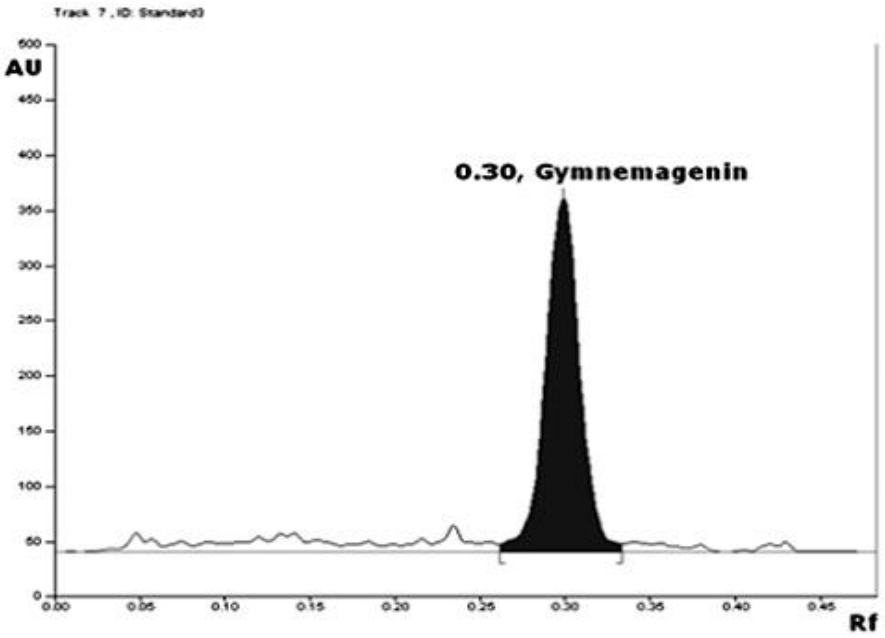

Figure 2. HPTLC chromatogram of gymnemagenin reference standard.

5. Validation of method

Precision

The relative standard deviation for repeatability of sample application, intra- and inter-day analysis was found to be less than 2%. The results depicted in Table 2 showed that no significant intra- and inter-day variation was observed in the analysis of gymnemagenin at three different concentration levels 200, 450 and 800 ng spot$^{-1.}$.

Robustness of the method

The standard deviation of peak areas was calculated for each parameter and RSD was found to be less than 2%. The low values of RSD as shown in Table 3 indicated robustness of the method.

LOD and LOQ

The LOD with signal/noise ratio of 3: 1 was found to be 50 ng spot^{-1}and LOQ with signal/noise ratio of 10:1 was found to be 100 ng spot^{-1} (Table 1).

Specificity

The peak purity (95%) of gymnemagenin was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., r (start, middle) = 0.9999 and r (middle, end) = 0.9998 and the overlaid spectra is shown in Fig.3. Good correlation (r^2

= 0.9998) was also obtained between standard and sample overlaid spectra of gymnemagenin.

Recovery studies

The method when used for extraction and subsequent estimation of gymnemagenin from powder sample from Ahmedabad after spiking with 80, 100 and 120% of additional standard gymnemagenin, yielded recovery of 98.4 ± 1.0% (Table 4).

Estimation of gymnemagenin in samples

Amount of gymnemagenin found in different samples containing *Gymnema* leaf powder is given in Table 5. Chromatogram of sample of *Gymnema* leaf powder from Ahmedabad, India is shown in Figure. 4.

Table 3. Robustness testing parameters of the proposed HPTLC method.

Parameter	SD[a] (%) of peak area	RSD[a] (%)
Mobile phase composition Toluene: Chloroform: Methanol (5: 8: 3) v/v		
i. 5:8:3	1.45	0.98
ii. 5.5:7.5:3	1.57	1.10
iii. 5: 7.5:2.5	1.61	1.15
Amount of mobile phase		
i.8 ml	1.36	1.12
ii.10 ml	1.28	0.97
iii.12 ml	1.25	1.10
Time from spotting to chromatography		
i.1 min.	0.98	0.87
ii. 5 min.	1.12	0.96
iii. 8 min.	1.18	0.98
Time from chromatography to scanning		
i. 1 min.	1.11	0.79
ii. 5 min.	1.14	0.83
iii.10 min	1.20	0.95
Plate pretreatment Pre-washed with methanol and dried at 60^{o} for:		
i. 5 min.	0.87	0.58
ii. 10 min	0.92	0.54
iii. 12 min.	0.98	0.62

[a]Average of three concentrations 200, 450 and 800 ng/spot.
SD- Standard Deviation
RSD- Relative Standard Deviation

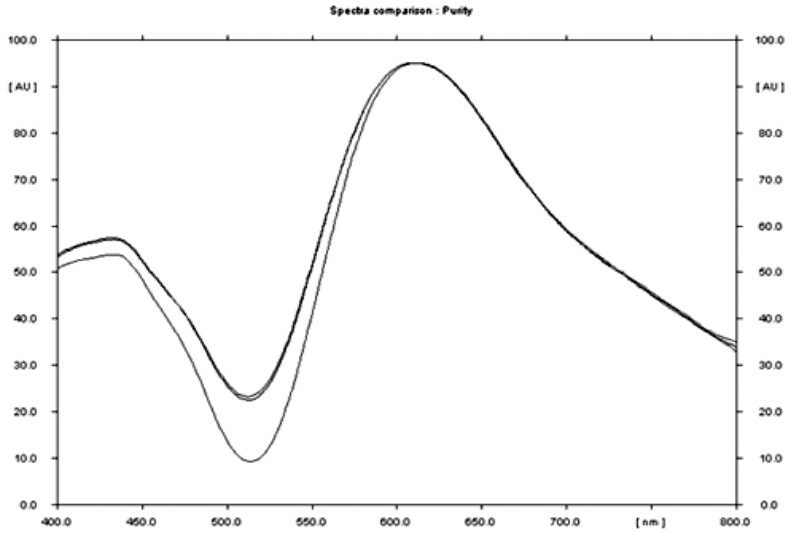

Figure 3. Overlaid spectra for peak purity of gymnemagenin standard and sample.

Table 4. Recovery of gymnemagenin from the leaf sample of *Gymnema sylvestre* from Ahmedabad.

Excess standard added to the analyte (%)	**Estimated content (ng/spot)**	**Amount of gymnemagenin found (ng/spot), (SD%)**	**% Recovery**
0	185.1	----	-
80	333.2	325.9 (1.85)	97.81
100	371.4	366.9 (0.91)	98.79
120	408.2	402.2 (0.85)	98.52
		Mean ± SD	98.4 ± 1.0

SD- Standard Deviation

Table 5. Amount of gymnemagenin found in plant samples by the proposed method.

Sample Name	**RSD(%)**	**Concentration[a] (%w/w)**
GYA (d.w.b)[b]	0.25	0.370
GYN (d.w.b)[b]	0.48	0.299
GYM (d.w.b)[b]	0.57	0.390
GYext (d.w.b)[b]	0.79	6.248
GYcap (d.w.b)[b]	0.34	0.101

[a] n = 6

[b] Dry weight basis

RSD- Relative Standard Deviation

GYA- *Gymnema* leaf powder from Ahmedabad, **GYN**- *Gymnema* leaf powder from Neemach, **GYM**- *Gymnema* leaf powder from Mumbai, **GYext**- *G. sylvestre* extract, **GYcap**- Capsule containing *Gymnema* leaf powder.

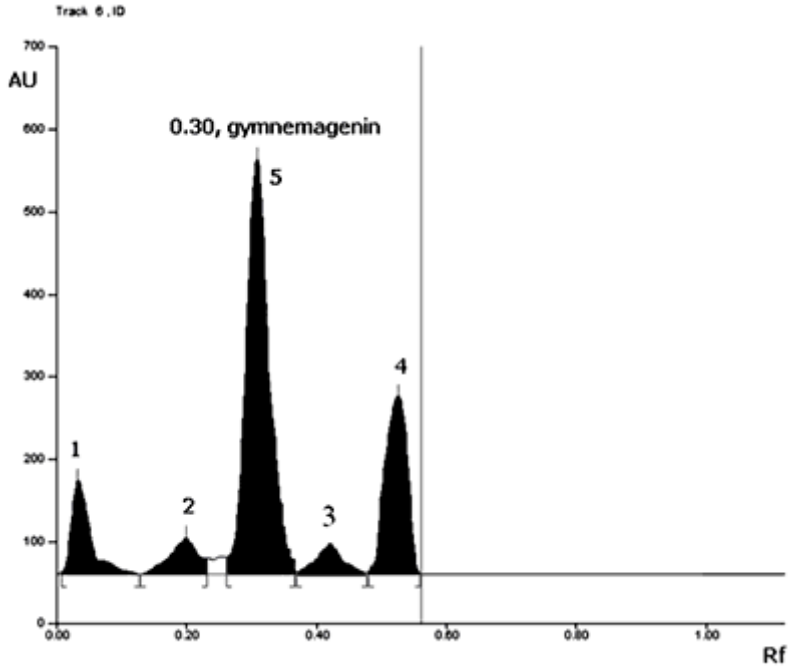

Figure 4. HPTLC chromatogram of *Gymnema sylvestre* sample containing gymnemagenin.

6. Discussion

Linearity of the method was found to be in the range of 180 to 1440 ng/spot which indicates that the method has better sensitivity compared to the recently reported methods [7, 8]. Modification of vanillin-sulfuric acid in our process has a strong advantage compared to the use of conventional vanillin-sulfuric acid. Lot of background interference and charring were observed with 1% vanillin and 50% sulfuric acid. By reducing the percentage of vanillin to 0.125% and sulfuric acid to 20%, we were able to obtain a clear chromophore without charring and background interference. Thus use of modified vanillin-sulfuric acid reagent removes the background interference problem commonly observed in post-derivatization HPTLC measurements.

Peak purity data of standard and that of sample shows the specificity of the method for estimation of gymnemagenin in plant samples successfully.

Gymnemagenin usually does not contain conjugated system and hence it gives poor absorption in UV range. Hence we took this approach of post derivatization of gymnemagenin using modified vanillin-sulfuric acid reagent in our method. In the methods reported by Valivarthi et. al.[8] and Puratchimani and Jha, [7] quantification of gymnemagenin was done at 210 nm and 290 nm. The chromophore in our system is sharp with a λ_{max} of 610 nm. Estimation of gymnemagenin at λ_{max} by generation of a chromophore ensures complete detection of the compound. The method was found to be robust and able to withstand small changes.

There is no uniformity in the extraction process of gymnemagenin. We found that the method originally described by Subba Rao and Sinsheimer [9] with 2 hour heating is sufficient to hydrolyse gymnemic acid and form gymnemagenin. During our experiments we found that gymnemagenin can not be generated without acidic as well as basic hydrolysis. Valivarthi et. al. [8] have also highlighted this aspect in the publication. They employed base hydrolysis first followed by acid hydrolysis with hydrolysis time of one hour each. It has been noted that both acid and base hydrolysis are absolutely necessary to generate

gymnemagenin. We adhered to Subba Rao and Sinsheimer's sequence of acid hydrolysis followed by base hydrolysis. The liberated gymnemagenin was selectively extracted with ethyl acetate by us. The various market samples were found to contain gymnemagenin between 0.29 to 0.390% w/w. The method was also applied for quantification of one of the standardized extracts which contained around 6% of gymnemagenin. One of the market formulations for diabeties i.e. Mersina capsules, was also analyzed for gymnemagenin content. We did not find any matrix interference from other herbal ingredients of the formulation. The contents of gymnemagenin in the leaf samples are less than the ones reported by Valivarthi et. al. [8]. However, we checked the exhausted powders and aqueous hydrolysate for gymnemagenin. They did not show any traces of gymnemagenin. All the plant samples including a poly-herbal formulation could be successfully analyzed by the method without any background interferences, matrix interferences and with good specificity (Figure. 4). Thus the described method should have reliable application in quantitative estimation of gymnemagenin, not only in plant samples and extracts but also in poly-herbal formulations containing *Gymnema* as one of the ingredients.

We employed a modified vanillin-sulfuric acid reagent to generate a sharp chromophore, after trying various derivatization reagents used for detection of aglycons. We found that most of the reagents form colored spots upon reaction with aglycons but very few produce sharp chromophore .The method was properly validated as per ICH guidelines [10] and could be successfully employed for quantification of gymnemagenin from market formulation and extract.

Acknowledgements

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