METHOD DEVELOPMENT AND VALIDATION ON HPLC-RP HPLC ON GYMNEMIC ACID

M.SWETHA[1], Dr. N. SARITHA[2], Dr. N. DEVANNA[3]

M.SWETHA[1,] RESEARCH SCHOLAR , JNTUA ANATHAPURAMU

Dr. N. SARITHA[2], ASSTISTANT PROFESSOR, DEPT OF CHEMISTRY, JNTUACEK

Dr. N. DEVANNA[3] PROFESSOR, Director of OTRI, JNTUA ANATHAPURAMU

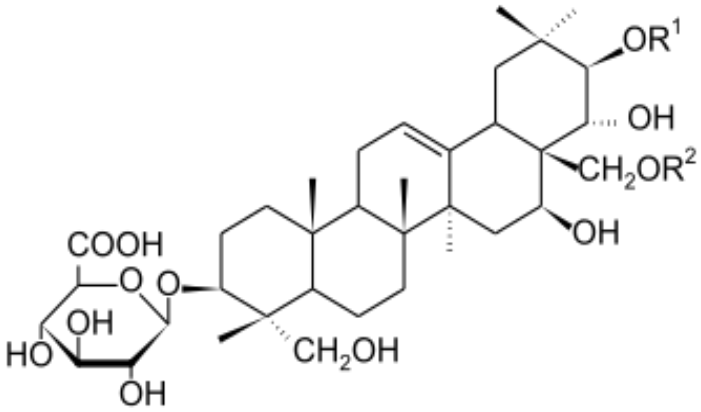
Abstract:

The aim of present study is to develop and validate a simple, precise and rapid HPLC method for the quantification of gymnemic acid in Gymnema sylvestre extracts and formulations. The analysis was performed by reverse-phase on HPLC chromatography on a Phenomenex Gemini-NX-5 µm C18with isocratic elution of Methanol: Phosphate buffer 0.005M pH 3.5 (70:30) at a flow rate of 1.0mL/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 02- 10µg/ml with correlation coefficient of 0.9994. 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**.**

KEY WORDS: gymnemic acid, gymnema slyvestrae

INTRODUCTION:

Gynmema slyvestrae is a potent anti- diabetic activity. It is used as acrid, anti-inflammatory, diuretic, liver tonic, emetic, refrigerant, astringent. It is also used in hepato splenomegaly , constipation , jaundice, dyspepsia, helminthiasis , amenorrhea.in addition to this it is also used as anti-cancer agent, anti-microbial, anti-inflammatory activities. The leaves of gynmema slyvestrae belongs to triterpenes saponins which includes oleananes and dammarane classes. The active important ingredient in G.slyvestre is gymnemicacid. Gymnemicacid contain several ingredients in it. The structure of gynmemic acid is as follows



In the present study a modified method was tried to quantify gynmemic acid by using HPLC-RP HPLC method. According to the literature survey a standard method and technique was implemented for development of compound. A number of mobile phases has been performed with buffer solutions for gynmemic acid -I by using isocratic run. The method was tried to give best peak and run time for the validation of gynmemic acid.

MATERIALS AND METHODS:

Standard gynmemic acid was pocured from the sigma Aldric ,Bangalore, India . Gynmema Slyvestre extract was collected from SV university, Tirupathi ,India. Market formulations of G. slyvestrae i.e. Meshashringi Capsules (Himalaya, Bangalore,India)were used as marketed formulations for the estimation. Water obtained from the Milli-Q water purification system. All other reagents were HPLC grade or AG grade.

PREPARATION OF STANDARD SOLUTION:

10mg of Gymnemic acid was taken in 10ml volumetric flask and make up the volume to 10 ml with methanol (the concentration of this solution is **1**mg/ml). From this 1ml of the solution is pipetted in 10ml of volumetric flask and the volume was made up with methanol. The concentration of this solution is 100µg/ml. From this 1ml of the solution is pipetted in 10ml of volumetric flask and the volume was made up with methanol. The concentration of this solution is 10µg/ml. Different concentration ranging from 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml of Gymnemic acid was prepared by transferring required aliquotes of solution to 10ml volumetric flask and make up the volume up to the mark by methanol. This was sonicated for 8 mins then the solution was filtered using 0.45 micron Millipore filters.

CHROMATOGRAPHIC CONDITIONS:

Chromatographic measurements were made on Shimadzu integrated liquid chromatographic system which consist of a solvent delivery pump (model spd10A uv-vis, Japan, Shimadzu LC-10ATVP, Japan) injector (model7725i Rheodyne) UV visible absorbance detector (Model spd 10A) and the instrument was connected to the computer –VP software.

HPLC was performed on a lichosorb (Phenomenex Gemini-NX-5 µm C 18(2) 110 Å, LC Column 250 x 4.6 mm, Ea) .The Mobile phase was consisted of Phosphate buffer 0.005M: Methanol and pumped at a flow rate of 1ml/min. The Mobile phase was filtered through 0.45 micron Millipore filter paper and degassed by sonication for 10 mins. The detection was carried out at 210nm. An injection volume of sample was 20µl. The temperature was maintained at ambient conditions.

PREPARATION OF SAMPLE SOLUTIONS:

An accurately weighed quantity of extract form of gynmena slyvestrae was saponified for 1hr with phosphate buffer: methanol .The saponified mixture was concentrated . The residue was dissolved in 1:1 mixture of methanol and HPLC grade water, followed by the acidifying with theconcentrated hydrochloric acid. The acidified sample was transferred to a 10ml volumetric flask and the volume was made up to the mark with methanol and filtered through whatman filter paper and used for further analysis of HPLC

The HPLC estimation was carried out by injecting 20µl of the sample solution. Percentage of gynmemic acid was estimated using the area under the curve obtained from the sample by comparing the same with the standard.

CALIBRATION CURVE:

Five different concentrations of SS after dilution up to 10 ml (2µg/ml-10µg/ml) with mobile phase were injected in triplicates.

Regression equation with slope, intercept and co-efficient of correlation (r^2) was derived (table 1)

METHOD VALIDATION

The developed RP HPLC method was validated by the determination of precision, specificity, selectivity, linearity, limit of detection and 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 100µg/ml of gymnemicacid.The repeatability of sample application and measurement of peak area for gymnemic acid 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 , f the determination of gynmemic acid was carried out at three different concentration levels 80,100,120 µg mL-1.

Limit of detection (LOD) and Limit of Quantification (LOQ):

LOD and LOQ were experimentally verified by diluting known concentrations of SS until the average response s were approximately three or ten times the standard deviation of the responses for six replicates determinations. In order to find LOD and LOQ, the solution was prepared and injected six times following the proposed method. LOD was considered as3:1 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.

RECOVERYSTUDIES:

Accuracy of the method was ascertained by spiking the pre-analysed samples with known amount o standard

Drug solution and then analyzing by HPLC .the spiking was done at three different concentration levels i.e. 80, 100 ,120 % of the assay concentration level of sample (10 µg mL-1.) in triplicates. The average percentage recovery at each concentrations level was evaluated.

Validation parameters	Results
Linearity range (µg mL-1)	2-10(µg mL-1)
Correlation coefficient (r2)	0.9994
Regression equation	y = 27,960.1011x - 2,479.8606
LOD (µg mL-1)a (Limit of detection)	**0.024504511**
LOQ (µg mL-1)b (Limit of quantification)	**0.245045112**
System precision (RSD)c	1.56
Method precision(RSD)d	1.53
Intermediate precision (RSD)e inter day Intraday	1.41 0.023
RSD (Linearity of the method)	0.75
Recovery f	98.13-99.027
Stability g (Recovery % µ SD) Room Temperature Refrigeration Temperature	99.85 98.23% µ2.51

Stability:

The stability of the sample was ascertainted by storing them at room temperature (4 C) for 24 hrs(short time)and 72 hrs (long time) for analyzing of samples.

Results and Discussions:

Total gynmemic acid can be determined and quantified by gravimetrical analysis; which is a crude method of study. Different scientists have developed methods for the quantification of gynmemic acid by HPLC method .

This method is complicated method as it involves two steps of processing acid hydrolysis followed by the basic hydrolysis .this method was modified and made an attempt to develop a simplified method which is reproducible results and the method was validated.

The method was standardized with C18 column with Phosphate buffer 0.005M: Methanol (70:30) as the solvent system at the λ max of 210nm. The flow rate was standardized to 1.0 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 10ml 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 80 to 12 µg mL-1 (Table1). The calibration curve of standard revealed that they had similar pattern are shown in Figure 1 and 2. Table 1: Validation parameters of the developed HPLC method for gives linear regression of the data points with the equation $y = 27{,}960.1011x - 2{,}479.8606$ regression co-efficient (r2) $R^2 = 0.9994$ and RSD 0.015%. Retention time was 8.15 min with flow rate of 1 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. 80, 100 and 120 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 gynmemic acid. 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 µ 0.3 min. The standard Retention time (RT-8.15 min) of gynmemic acid 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 gynmemic acid standard and Gymnema extract revealed that they had similar pattern are shown in Figure 1 and 2.

Method validation

Linearity

The method was found to be linear in the concentration range of 2to 10 µg mL-1 (Table1). The calibration curve of standard gynmemic acid gives linear regression of the data points with the equation $y = 27{,}960.1011x - 2{,}479.8606$ regression co-efficient (r2) 0.994 and RSD 0.75%. Retention time was 8.15 min with flow rate of 1 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. 80, 100 and 120 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 gymnemic acid

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 μ 0.3 min. The standard Retention time (RT-8.15 min) of gynmemic acid 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 gynmemic acid 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

Recovery studies

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

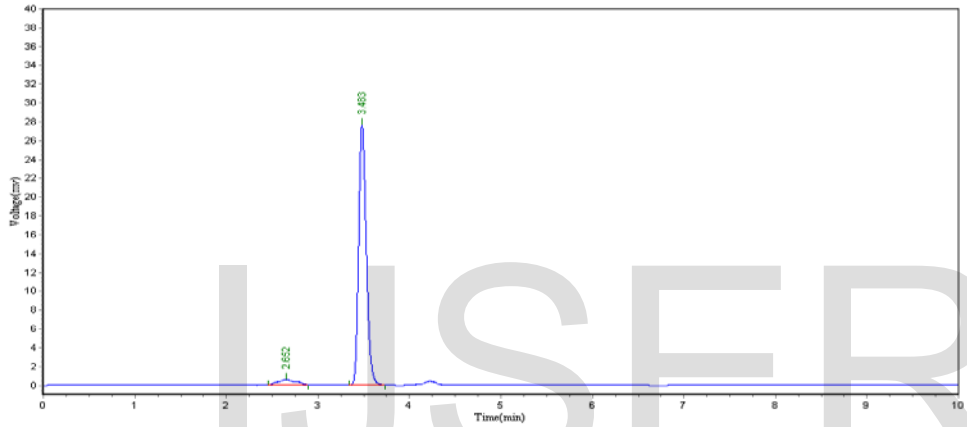


Figure 1 chromatogram of standard gynmemic acid

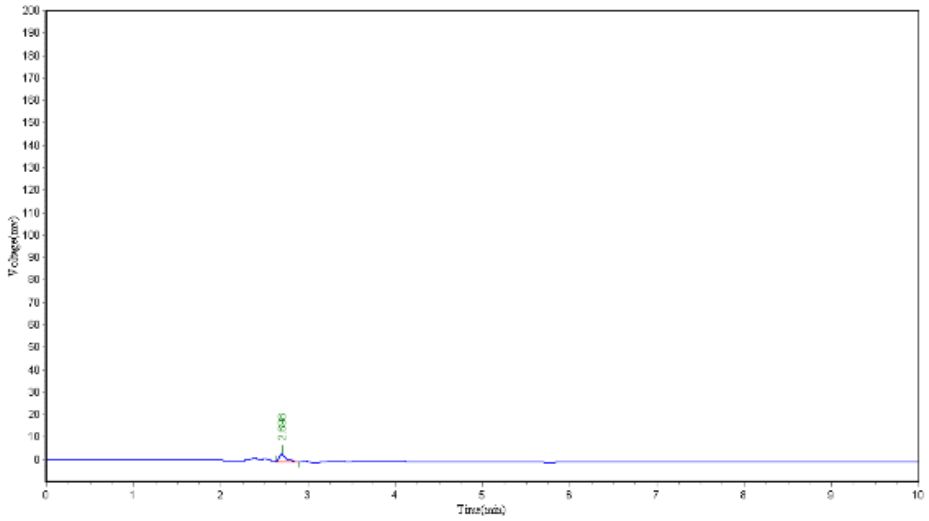


Figure 2 chromatogram showing the blank compound

References:

[1]. Matthew J Leach. Gymnema sylvestre for Diabetes Mellitus: A Systematic Review. The Journal of Alternative and Complementary Medicine 2007; 3(9): 77ă983.

[2]. K Patel, M Gadewar and R Tripathi, DK Patel. Pharmacological and analytical aspects of gymnemic acid: a concise report. Asian Pac J Trop Dis 2012; 2(5): 414-416.

[3]. Sinsheimer JE and Rao GS. Constituents from gymnema sylvestre leaves VI: acylated genins of gymnemic acid ă isolation and preliminary characterization. Journal of pharmaceutical sciences 1970; 59: 622-628.

[4]. Tsuda Y, Kiuchi F and Liu HM. Establishment of the structure of gymnemagenin by X-ray analysis and the structure of deacyl gymnemic acid. Tetrahedron letters 1989; 30; 361-362.

[5]. Suzuki K, Ishihara S, Uchida M and Komoda Y. Quantitative analysis of deacyl gymnemic acid by HPLC. Yakugaku Zasshi 1993; 113: 316-320.

[6]. Puratchimani V and Jha S. Standardization of Gymnema sylvestre R. Br. with reference to gymnemagenin by high-performance thin-layer chromatography. Phytochemical Analysis 2004; 15: 164-166.

[7]. Valvirathi SR, Raju Kannababu S, and Subba Raju G.. Standardization of Gymnema sylvestre R. Br. By high performance Thin Layer Chromatography-An Improved method. Phytochemical Analysis 2006; 17: 192- 196.

[8]. Toshiaki I, Yamamoto F, Miyasaka A and Hatano H. High-performance liquid chromatography-atmospheric pressure ionization mass spectrometry of gymnemic acids. Journal of Chromatography 1991; 557: 383-389.

[9]. Kanetkar PV, Singhal RS, Laddha KS and Kamat MY. Extraction and quantification of gymnemic acids through gymnemagenin from callus cultures of Gymnema sylvestre. Phytochemical Anal 2006; 17:409-413.

[10] Kusum devi, Nimisha Jain, A validated HPLC method for the estimation of gymnemic acid as deacyl gymnemic acid in various extracts and formulations of gynema slyvestrae. International Journal of Phytomedicine 6 (2) 165-169.