International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.3, pp 1286-1293, July-Sept 2013

Validation Aspect In Multicomponent Fraction From Gokshur (Tribulus terrestris Linn.) By RP- HPLC Method

Renuka[1]*, V.J.Shukla[1], P.K.Prajapati[2]

[1]Department of Pharmaceutical chemistry, Institute of Post graduate teaching and research in Ayurveda, Gujarat Ayurved University, Jamnagar, India.

[2]Department of Rasashastra and Bhaishjyakalpana, Institute of Post graduate teaching and research in Ayurveda, Gujarat Ayurved University, Jamnagar, India.

***Corres.author: renuka.kalyani@gmail.com**

Abstract

Context: Analysis of botanicals is a great challenge because they are highly complex mixtures of compounds covering a broad range of substance classes and exhibit natural variability. These complex mixtures behave as matrix and may have an impact over the response factor of target analyte.
Aim: In the present study, we have concentrated on detailed description of matrix effects in different Botanical reference material (BRM) samples of *Tribulus terrestris* in a view of liquid chromatography.
Material method: As per the objective of work a HPLC method was developed by using phenomenex RPC18 column (250 x4.6mm, 5µm),with mobile phase of Acetonitrile: Water (90:10 v/v) at the flow rate 0.5ml/min with 20µl injection volume and UV detection at 203 nm.
Results: The retention time for Diosgenin was found to be 9.8 min. The peak obtained was well resolved by this particular method, Hence this method was selected for the study. The validation was carried out in different matrix environment of Gokshur (*Tribulus terrestris* Linn.*)* taking Diosgenin as target moiety.
Conclusion: The results confirm that there is practically an interference of matrix over the response factor of target analyte. All the findings for *Gokshur* show that, for plant samples little purification is required. Although analysis can be carried out without purification but results will not be much accurate reliable and repetitive. Since matrix effects may exert a negative effect on important method performance parameters, they have to be tested for and evaluated during method development/validation.
Keywords: Validation, matrix effect, multiple components, HPLC.

Introduction

In traditional medicine group of components is responsible for therapeutic activity. Hence this group is considered as analyte. The component other then target analyte collectively taken as matrix. The presence of matrix may alter the response factor of target analyte and may interfere in analysis of such active constituent. In this situation it is very important to reduce the matrix and for this purpose semi purified sample should be taken for study.

The presence of unwanted chemicals i.e., impurities even in small amounts may influence the efficacy and safety of the pharmaceutical products. Impurity profiling (i.e. the identity as well as the quantity of impurity in

the pharmaceuticals), is now getting important for critical attention from regulatory authorities. Due to the complex nature and inherent variability of the chemical constituents of the plant based drugs, it is difficult to establish quality control parameters and modern analytical techniques like HPLC and HPTLC are expected to help in circumventing this problem.[1] The different pharmacopoeias, such as the British Pharmacopoeia, United States Pharmacopoeia (USP) are slowly incorporating limits to allowable levels of impurities present in the API's or formulation. Hence, there is a need to develop method for the identification of these impurities in the drug substances. [2]

Matrix effects (ME) result from co-eluting residual matrix components affecting the separation of target analytes and can lead to erroneous results. Matrix can affects the separation of components, retention time, peak shape etc. in liquid chromatography. These can be caused by compounds of various origins. Since matrix effects may exert a negative effect on important method performance parameters, they have to be tested for and evaluated during method development/validation. No validation methods could be accepted without a thorough evaluation of ME and possible strategies to minimize or to correct their influence should be addressed. If possible, matrix effects should be reduced or eliminated by the optimization of chromatographic conditions and improving sample clean-up. After reduction of clustering of components (matrix) one can get clean CRF (chromatographic response factor).**[3]**

Once the analytical method is developed it should be validated so as to check the reliability, accuracy and specificity of the method. Method validation is the process of providing that an analytical method is acceptable for its intended purpose.[3] For pharmaceutical methods Guidelines from the United States Pharmacopeia (USP) are described steps for validation: Accuracy, Precision Specificity, Linearity, Detection limit, Quantification limit, Robustness.[4]

ICH guidelines The international conference for harmonization was initiated in 1990 to bring together the regulatory authorities of Europe, Japan and The United States and expert from pharmaceutical industries in the three regions to discuss scientific and technical aspects of product registration.ICH publishes guidelines that are either signed into law by member countries or recommended guidelines by national authorities such as the US FDA. ICH guidelines also provide analytical process validation.[5]

The single selected reactive moiety or the standard reference material was taken as certified reference material (CRM) while the samples containing SRM and other components were considered as Botanical reference material (BRM). The matrix reduction was followed by purification techniques. For *Gokshur* selective reaction method that is acid hydrolysis was adopted as method of purification. So in order to find out the response of SRM in BRM sample, HPLC method was designed and validated according to ICH guideline.

Methodology

Sample (BRM) preparation

Methanolic Extract (GM)

Methanolic extract was prepared by continuous extraction method, soxhlet Extraction for 48 h. For this 50 gm of coarse powder was taken and extracted with methanol in soxhlet apparatus. Solvent was distilled off and the extract was concentrated and dried under reduced pressure, which yielded a greenish brownish mass.[6] This crude extract was used for further investigation.

Gokshur Hydrolyzed extract I (GH1)

The powdered material was first extracted with alcohol. The alcohol extract was acid hydrolyzed to liberate the sapogenins from glycoside. The hydrolyzed extract was dried up to residue. The residue was dissolved in solvent of desired solubility and subjected for further analysis.**[7,8]**

Hydrolyzed extract (GH2)

One hundred milliliters of the *Gokshur* aqueous extract was treated with 15 ml chloridric acid (10 %).The mixture was refluxed for 2 h. The sapogenins were extracted with 50 ml chloroform. The extraction was repeated four times. The whole chloroform fraction was evaporated to dryness and the residue dissolved and

made up to 50 ml with methanol From this solution,1 ml was diluted to 10 ml with methanol. This solution was filtered through a 0.45 µm membrane (Millipore, HVHP) and analyzed by HPLC. The evaluation was repeated three times.[9]

Method development

Experimental conditions and requirements

Instrumental specification

Instrument	chromatograph (Shimadzu, model LC-20AD),
Injector	Rheodyne
Column	Phenomenex
Detector	PDA
Wavelength	203nm
Injection volume	20 µl
Flow rate	0.5ml/min

Chemicals and reagents

- Methanol HPLC grade
- Acetonitrile HPLC grade
- Millipore water

Samples for analysis

- Gokshur Methanolic extract GM (Matrix 1)
- Column purified Punarnava extract GH1 (Matrix 2)
- Gokshur hydrolyzed extract extract GH2
- Diosgenin sigma Aldrich

Procedural development

Selection of mobile phase

The proposed method of validation for the determination method of Boerhavinone B in matrix environment required adequate resolution of target moiety in the chromatogram. For the desired purpose ACN: Water in ratio of 90:10 v/v was used as mobile phase. The well resolved and sharp peak was obtained in this solvent system. The detection wavelength was 203 nm and flow rate was maintained at 0.5ml/min.

Preparation of mobile phase

The mobile phase was prepared with Acetonitrile and Water in the ratio of 90:10 v/v. The mobile phase was filtered by Millipore filtration assembly using nylon membrane filter paper of 0.45 mm diameter. The solvent mixture was degassed and sonicated in ultrasonic bath for 10min.

Preparation of sample solutions

Preparation of sample solution

10 gm of samples (GM,GH1,GH2) were extracted with 5 ml of Methanol. The material was refluxed for half an hour. The extract was filtered and volume was made up to 10 ml to get solution conc.1mg/ml.

Preparation of standard solution

10 gm of standard Diosgenin was extracted with 5 ml of Methanol. The material was refluxed for half an hour. The extract was filtered and volume was made up to 10 ml to get solution of conc.1mg/ml.

Method validation

As per the objective of work a HPLC method was developed by using phenomenex RP C18 column (250 x4.6mm, 5µm), with mobile phase of Acetonitrile: Water (90:10 v/v) at the flow rate 0.5ml/min with 20µl injection volume and UV detection at 203 nm. The retention time for Diosgenin was found to be 9.8 min. The peak obtained was well resolved in this particular method; hence this was selected for the study. The HPLC method developed was validated in different matrix environments of Punarnava as per ICH guidelines using various parameters. Following parameters were used.

Linearity

Five different concentrations of Diosgenin were analyzed and their calibration curve was constructed in the concentration range (0.2-1.0 µg/mL).The calibration curve plots were generated by replicate analysis (n = 3) at all concentration. The peak areas were plotted against the concentration of Diosgenin. The sample peak was identified by comparison of Retention time (tR) and UV absorption spectrum for standard.

Limit of detection and limit of quantification

The LOD and LOQ were determined by using numerical equation kD /S where k is constant (3.3 for LOD and 10 for LOQ), where SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

Precision

The precision was examined by performing the intra-day and inter- day assays of three replicate injections of standard Diosgenin solution, sample GM,GH1 and sample GH2 separately at three concentration levels (10, 20 and 30 µg/ml). The intra-day assay precision was performed with the interval of 3 h in 1 day, while the inter-day assay precision was performed over 3 days.

Accuracy

The accuracy of the method was evaluated by calculating the recoveries of Diosgenin by standard addition method. A known amount of standard (80%, 100% and 120%) was added to pre analyzed samples GM,GH1 and GH2 solution, and the amount of the standard was estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

Robustness

The robustness of the method was determine by varying the system suitability parameter like HPLC pump flow rate (± 0.1), wavelength (± 1) and mobile phase composition (± 2). Solution was injected 3 times for each change. Mean and SD were calculated for each peak. % RSD was calculated for each component during each change.

Result and discussion

Specificity

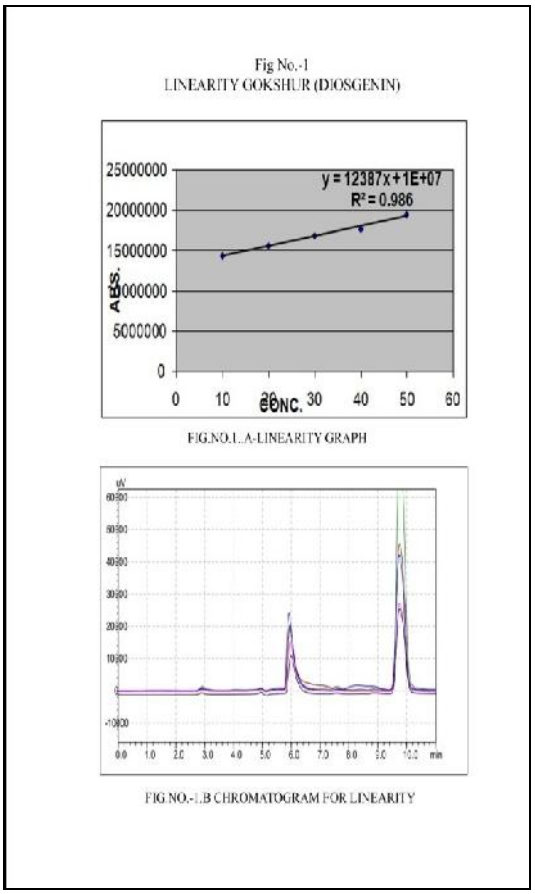
Specificity of the method was determined to study the ability to assist. There were no visible peaks in the retention time up to duration of 30 min in the sample matrix, indicating a high degree of specificity for the proposed method.

Linearity

Linearity for the Diosgenin by the proposed method was determined to study its ability to elicit test results which are directly proportional to concentration of the analyte in the sample. The response was found to be linear in the concentration range of 0.2 – 1.0 mcg/ml for Diosgenin. The correlation coefficient was 0.998. [Table no 1 and fig. no 1 & 2].

Table 1-Linearity data for Diosgenin

S.No.	Concentration (µg/ml)	Peak Area
1	0.2	527425
2	0.4	550206
3	0.6	827467
4	0.8	910413
5	1.0	1454813
Correlation coefficient	0.998	



Limit of detection and Limit of quantification

The Limit of detection was determined to find out the lowest amount of Diosgenin that can be detected which was found to be 0.05 mcg/ml. The limit of quantification was found to be 0.08 mcg/m. [Table no 2]

Precision

For Precision, the Interday and intraday analysis was carried out. The intraday precision was determined by replicate injection of standard and sample solution by an interval of 3 hrs in the same day. From the results, the % RSD of peak area for GM, GH1, GH2 and Diosgenin was calculated and found to be 10%, 1.8%, 1.8% and 0.7% respectively. The Interday precision of the method was determined by performing the assay on different days to check the reproducibility. The test result was found to be satisfactory for standard and sample PC with respect to relative standard deviation (%RSD). For set of analysis on the same day being less than 2.0 % for standard and sample GH1 and GH2. Hence the proposed method was found to provide high degree of precision and reproducibility. [Table no 3 and fig. no. 2A-2C].

Accuracy

Accuracy was determined through recovery studies of the Diosgenin in Gokshur samples GM, GH1 and GH2. Known amount of standard 80%, 100% and 120% was added to previously analyzed sample solutions. The % recovery was determined on the basis of obtained results. The results reveals that sample GH1 and GH2 showed 95-98 % recovery in 10 µg/ml sample solution while sample PM showed 72 % recovery in 10 µg/ml .The proposed HPLC method indicates that although the results obtained were well within the acceptance limit of 95-100 %, but for sample GM recovery was observed in sample of higher concentration indicating practically the interference of the matrix with the target moiety in sample. [Table no 4]

Robustness

Robustness of the HPLC method developed was determined by deliberately changing slightly the mobile phase ratio, flow rate and column of the mobile phase during the experiment. The percentage RSD of peak area, tailing factor and theoretical plates were found to be well within the acceptance criteria of NMT 2 %.The results shows that there was no deviation in the results during a small change in experimental conditions**.** [Table no 5]

Table 2-Limit of detection and Limit of quantification for Diosgenin

Parameter	Diosgenin
Limit detection	0.03µg/ml
Limit of quantification	0.05 µg/ml

Table 3-Results of precision of Diosgenin and Gokshur samples

Sample	Concentration (µg/ml)	Intraday precision		Interday precision	
		Peak area (% RSD)	Rt (% RSD)	Peak area (% RSD)	Rt (% RSD
Methanol extract *Gokshur*	10-30	10%	2.9 %	24%	3.1 %
Hydrolyzed extract I *Gokshur*	10-30	1.8%	0.15 %	0.9%	0.198 %
Hydrolyzed extract II	10-30	1.8%	0.13 %	1.8%	0.12 %
Standard Diosgenin	10-30	0.6%	0.09%	0.7%	0.04 %

Fig.No 2
SPECTRAL COMPARISION

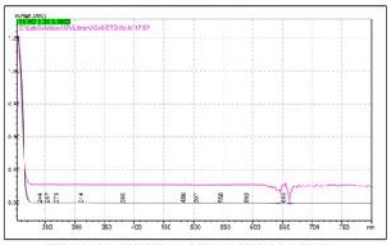


FIG.NO -2.A-SPECTRAL COMPARISION SAMPLE GM WITH STANDARD DIOSGENIN

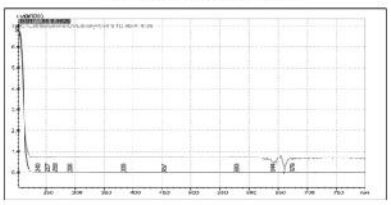


FIG NO.-2.B-SPECTRAL COMPARISION SAMPLE GH1 WITH STANDARD DIOSGENIN

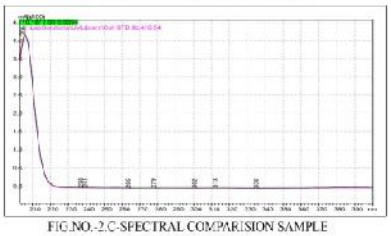


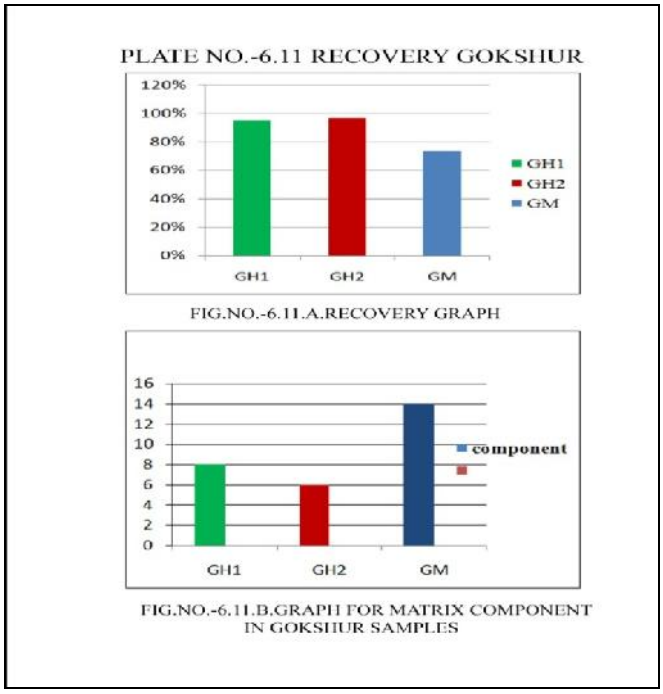
FIG.NO.-2.C-SPECTRAL COMPARISION SAMPLE GH2 WITH STANDARD DIOSGENIN

Table 4 -Results for recovery of Diosgenin in Gokshur samples

Sample	Concentration(µg/ml)	% recovery
GM	10	72
GH1	10	97
GH2	10	98

Table 5-Results for robustness of Diosgenin

Parameter	Diosgenin
Retention time	9.8 min
Theoretical plate number	5695.5
Tailing factor	1.3
% RSD	0.8 %



Conclusion

HPLC method was developed and validated successfully for determining the behavior of matrix environment on the response factor of target analyte. The phenomenon of separation of components in Liquid chromatography (LC) depends mainly on the sample matrix, sample preparation procedure, quality of chromatographic separation, mobile phase etc. It may be concluded that Matrix effects, i.e. variation in Rt (retention time), distorted peaks, unsatisfactory separation are well known phenomena in liquid chromatography (HPLC).They can be caused by compounds of various origins.

The severity of matrix effects is directly dependent upon chromatographic performance Matrix effects caused by co-eluting compounds can negatively affect method performance. Therefore, the evaluation of possible matrix effects is an essential part of method development/validation for HPLC.

If relevant matrix effects are found, they should be reduced or eliminated by the optimization of chromatographic conditions, improving the sample clean-up and/or by changing the type of ionization employed. Approaches to addressing matrix effects include reducing matrix co extractives by optimizing extraction, cleanup and chromatography methods as well as employing corrective calibration methods.

The present study was aimed to evaluate the behavior of matrix containing multicomponent environment over the response factor of target analyte. When there are number of components in the matrix system, there will be many variables. These variables can deviate the results. So in order to reduce the deviation it is proposed to reduce the matrix which can be achieve by using semi purified sample. Results justify the results obtained from validation that for *Gokshur* sample GH2 and sample GH1 shows better response for target moiety as compare to sample GM and are found to be nearer to certified reference material Diosgenin.

References

1. Patel MG, Patel VR, Patel RK, Development and Validation of Improved RP-HPLC method for Identification and Estimation of Ellagic and Gallic acid in Triphala churna. Inter. J. Chem. Tech. Res. 2010: 2 (3).
2. Interpretation and Treatment of Analytical Data: USP Pharmacopoeial Forum, United States Pharmacopoeial Convention, Inc.1994: 24; 5:7051-56.
3. Dharmendra Patel, Matrix Effect In A View Of LC-MS/MS: An Overview. IJPBS. : 2011; 2(1).
4. Interpretation and Treatment of Analytical Data: USP Pharmacopoeial Forum, United States Pharmacopoeial Convention, Inc.1994: 24; 5:7051-56.
5. Validation of Analytical Procedure Methodology, ICH Guidelines 1996:1-8.
6. M. Angela A. Meireles. Extracting Bioactive Compounds for Food Products 2009.
7. Jiangsu New Medical College, Dictionary of Chinese Materia Medica, Shanghai, Shanghai Science and Technology Publisher: 1976.
8. Ushijima M, Komoto N, Sugizono Y, MizunoI, Sumhiro M, Ichikawa M et al. Triterpene glycosides from the roots of Codonopsis lanceolata. Chem Pharm Bull:2008; 56(3): 308–314.
9. J. Braz. Chem. Soc. vol.16 no.4 São Paulo July/Aug. 2005,http:// dx.doi.org/ 10.1590/ S0103-50532005000500007).

*****