ORIGINAL ARTICLE

Comparison between high performance liquid chromatography and high performance thin layer chromatography determination of Diosgenin from fenugreek seeds

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Background: Fenugreek is used in the treatment of many diseases as reported in Ayurveda. The chemical like Diosgenin is one of the important chemicals of fenugreek. **Aim:** To develop simple, rapid, accurate methods for estimation of Diosgenin from fenugreek using high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC). **Materials and Methods:** The two chromatographic methods, HPTLC and HPLC, were developed and compared to quantitative determination of Diosgenin in fenugreek seed extract. For both the analyses, the scanning wavelength was set at 205 nm. The Diosgenin content of seven different extracts collected from different locations was compared by HPLC and HPTLC. **Statistical Analysis:** The relative standard deviation and correlation coefficient (r^2) were calculated by standard Formulae. **Results:** The comparison was done in HPTLC and HPLC results and all the results are found to be within the limits and showed no significant difference. **Conclusion:** Both the methods were accurate and specific for the analysis of Diosgenin.

Key words: Diosgenin, fenugreek, high performance liquid chromatography, high performance thin layer chromatography, quantitation

INTRODUCTION

Trigonella foenum-graecum Linn. (Family: *Leguminosae*) is native to the area from the eastern India. In Ayurvedic and Unani systems of medicine, fenugreek is used for the treatment of epilepsy, paralysis, gout, dropsy, chronic cough and piles. There are numerous other uses of fenugreek, including treatment of indigestion and baldness.[1,2]

Seeds are known for their pungent aromatic properties.[3] The seeds, available in whole and ground forms, are used to flavour many foods including curry powders, spice blends and teas. In recent years, legumes are becoming an important protein source, for use as both functional food ingredients and nutritional supplements together with green vegetables, which have been recognised as the cheapest and most abundant potential source of protein.[4]

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In recent years, laboratory studies and clinical trials have focused on fenugreek as a potential nutraceutical. Fenugreek is reported to have anti-diabetic, anti-fertility, anti-cancer, anti-microbial and anti-parasitic properties.[5] Seeds of fenugreek contain tannic acid; fixed and volatile oils and bitter extractives; alkaloids such as trigonelline, trigocoumarin and trigomethyl coumarin; steroidal saponins such as gitogenin; and traces of trigogenin and vitamin A. Fenugreek is rich in flavonoids such as apigenin, luteolin, orientin, quercetin, vitexin and isovitexin.[6]

Literature survey reviews that the seeds mainly contains Diosgenin (25R)-spirost-5-en-3 β-ol), a plant-derived sapogenin, shown to suppress inflammation, inhibit proliferation and induce apoptosis in a variety of tumour cells. It also has anti-rheumatic anti-viral properties.[7,8] Diosgenin is often used as a raw precursor for the production of steroidal drugs and hormones such as testosterone, glucocorticoids and progesterone.[9] In Ayurvedic Pharmacopoeia, couple of formulation like Mustakarista and Mrusanivari Sura is reported to contain fenugreek seeds.[10] The literature search showed that few analytical methods have been published for fenugreek such as high performance thin layer chromatography (HPTLC) determination of trigonelline and 4-hydroxyisoleucine from fenugreek seed.[2] But

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there is no analytical method available for the estimation of Diosgenin from fenugreek seeds.

With the growing need for safer drugs, attention has been drawn to their quality, efficacy and standards of the herbal raw material and formulation.[11]

Key bioactive compounds found in fenugreek need to be quantified in order to ensure that the plants being used possess the bioactive compounds essential to produce the desired effects in consumers. Once plants with these attributes have been identified, they can be used for selection of suitable genotypes that can be further developed into cultivars specific for the natural health product processing industry.

Diosgenin is one of the important compositions of fenugreek; therefore, there is a need to develop method for estimation of same using sophisticated instruments like high performance liquid chromatography (HPLC) and (HPTLC).

The purpose of this work was to optimise the conditions for separation and quantitative determination of Diosgenin using the HPTLC and HPLC methods and to compare results obtained by HPTLC and HPLC methods.

MATERIALS AND METHODS

Plant Materials

The seeds of fenugreek were collected from Pune regions of Maharashtra. The powdered seeds were passed through Sieve No. 85, weighed and then used for extraction.

Preparation of Extract

Powdered fenugreek seeds were extracted with water: Ethanol mixture (1:1) for 72 h at 70°C using soxhlet apparatus. This extract was then concentrated to dryness by removing the solvent in the rotary evaporator under reduced pressure.

Determination of Plant Extract Yield

The percentage yield of extract obtained from fenugreek seeds was calculated from the following equation:

% yield = (W1 × 100)/W2,

Where W1 was the weight of the extract after the solvent evaporation and W2 was the weight of powdered fenugreek seeds taken.

Chemicals

Analytical grade solvents such as toluene, ethyl acetate and methanol were obtained from Merck Ltd, India. Standard Diosgenin was purchased from Sigma, India. HPTLC aluminium plates pre-coated with silica gel $60F_{254}$ (20 cm × 20 cm, 0.2 mm thickness) were obtained from Merck, India.

Chromatographic Conditions for High Performance Thin Layer Chromatography

The samples were spotted in the form of band of width 6 mm with CAMAG 100-μL syringe pre-coated with silica gel $60F_{254}$ aluminium plate (20 cm × 10 cm with 0.2 mm thickness) using Linomat 5 applicator CAMAG (Switzerland) fitted with a CAMAG 100-μL syringe. The linear ascending development was carried out in the solvent system, Toluene: Ethyl acetate:Methanol (15:2:2 v/v/v) in a CAMAG twin trough chamber (20 cm × 10 cm) previously saturated with solvent system for 25 min. The volume applied on each track was 5 μL. The plate was allowed to run approximately 80 mm from the point of application. After development, plates were dried under current of air at room temperature. The densitometric scanning was performed using TLC scanner-3 CAMAG (Switzerland) operated by win CATS software V 1.4.3.6336 at 205 nm for Diosgenin. The slit dimension was 5 cm × 0.45 mm with the scanning speed of 20 mm/s. Evaluation was done via peak area with linear regression.

Preparation of Standard Solution of Diosgenin

A stock solution of Diosgenin was prepared by dissolving 10 mg of standard in 100 mL methanol.

Preparation of Sample for Analysis

One gram extract of fenugreek seeds was accurately weighed. To this, 80 mL 3 N Hydrochloric acid AR grade was added and it was kept on reflux for 1 h on water bath at 100°C. Mixture was allowed to cool at room temperature and diluted up to the mark with water. The mixture was extracted with diethyl ether AR grade (50 × 3). Ether layer was separated and allowed to evaporate. The residue was dissolved in 25 mL methanol. This resulting solution was used as test solution.

Preparation of Calibration Graph

The stock solution of Diosgenin was diluted to seven different concentrations (2-6 μg spot-1) and they were spotted in duplicate on HPTLC plate for the preparation of calibration graph. The calibration graph was plotted using the concentrations versus average peak area at 205 nm for Diosgenin.

Validation of High Performance Thin Layer Chromatography Method

The proposed method was validated in terms of specificity, linearity, accuracy and repeatability of measurement and

sample application as per international conference on harmonization (ICH) guidelines.[12]

Specificity

The specificity of the method was studied by analysing standard, blank and sample solutions by simultaneously spotting on the HPTLC plate. The bands for the Diosgenin in the sample solutions were confirmed by comparing R_F values and spectra of standards.

System Precision

The repeatability of the sample application and measurement of peak area were carried out by spotting seven replicate tracks of standard and was expressed in terms of percent relative standard deviation (% RSD) [Table 1].

Method Precision

Seven samples of extract were prepared separately and analysed as per the method. The percent assay was calculated and the precision was expressed in terms of % RSD [Table 1].

Robustness

The robustness of the method was studied by deliberately making small changes (±0.1 mL) in each solvent of the solvent system. The robustness also studied by small changes in mobile phase volume, saturation time and developing distance [Table 1].

Recovery Studies

The accuracy was determined by adding known amounts of standards at 80%, 100% and 120% level to the pre-analysed extract sample. The percent recovery was calculated by the proposed method [Table 1].

Chromatographic Conditions for High Performance Liquid Chromatography

High-performance liquid chromatography was performed with Waters 2695 Alliance system with a 2996 photodiode array detector (PDA). Diosgenin was separated on a reverse-phase 250 mm × 4.6 mm, 5 µ, Symmetry C8 column (waters). The mobile phase was prepared from water (solvent A) and acetonitrile (solvent B). The mobile phase was degassed and filtered through 0.45-µm filter before use. The gradient programme used was: Initial 0-20 min, from A to B (10:90 v/v); 20-21 min, linear change from A-B (10:90 v/v) to A-B (2:98 v/v); 21-25 min, constant change from A-B (2:98 v/v); 25-26 min, linear changes from A-B (2:98 v/v) to A-B (10-90 v/v) and 26-30 min, constant change from A to B (10-90 v/v). The mobile phase flow rate was 1 mL min^{-1}. Before the first injection, the column was saturated for 30 min with the initial mobile phase. The column temperature was maintained at 30°C. The injection volume was kept 5 µL. The PDA was set at 205 nm to acquire the chromatogram. The Diosgenin was identified by comparing the retention time and spectra obtained from sample and standard solutions. The present work was performed in an air-conditioned room maintained at 25°C.

Table 1: Method validation parameters for quantitation of diosgenin

Parameters	HPLC	HPTLC
Specificity	Specific	Specific
Linearity (correlation coefficient)	0.999	0.9985
System precision (% RSD) (*n*=6)	0.23	0.25
Method precision (% RSD) (*n*=6)	0.33	0.32
Intermediate precision (% RSD) (*n*=6)	0.59	0.57
% recovery	99.24	99.20
Solution stability	Stable	Stable

HPLC – High performance liquid chromatography; HPTLC – High performance thin layer chromatography; RSD – Relative standard deviation

Preparation of Calibration Graph

The stock solution of the standard was diluted to obtain different concentrations and these were injected in the system. Calibration plot was constructed by plotting concentration against peak area for Diosgenin. From the calibration graph, it was found that the response for Diosgenin was linear.

Validation of High Performance Liquid Chromatography Method

The proposed HPLC method was validated in terms of precision, specificity, linearity, accuracy, solution stability and robustness as per the ICH guidelines.

Precision

The system precision was carried out by six replicate injections from the same vial of standard and was expressed in terms of (% RSD). Six different samples of the same formulation were analysed for method precision. The percent assay of each analyte was calculated and % RSD is presented in Table 1. The intermediate precision was performed on different systems for six different samples by different analysts. The analyte content was calculated and the % RSD is presented in Table 1.

Solution Stability

The sample solution was prepared as per the proposed method and subjected to stability study at room temperature for 24 h. The sample solution was analysed at initial and at different time intervals up to 24 h. The change in response of Diosgenin in sample solution with respect to time is calculated as absolute percent difference against initial response [Table 1].

Robustness

The robustness of the method was determined by slight deviation in the method parameters. The parameters

selected were deviation in the wavelength, column temperature, flow rate and mobile phase gradient. The retention time of Diosgenin was determined and % RSD with system suitability parameters was observed [Table 1].

Specificity

The specificity of the method was studied by assessment of peak purity of Diosgenin using Waters empower software and diode array detector [Figures 1 and 2].

Recovery Studies

The accuracy of the method was determined from recovery studies by adding known amount of standards at 80%, 100% and 120% level to the pre-analysed sample followed by replicate quantitative analyses by the proposed method [Table 1].

Analysis of Extract

The content of Diosgenin in hydroalcholic extract was determined as per the method described under chromatographic conditions by HPLC and HPTLC. All the analyses were repeated 3 times.

RESULTS AND DISCUSSION

Estimation of Yield of Extract

The percent yield of extract of fenugreek seeds was found to be 54.23% w/w.

Chromatographic Study

The composition of solvent systems in HPTLC and HPLC was optimised by testing different solvent compositions of varying polarity, and the best results were obtained using present method which produces highly symmetrical peaks showing good resolution between Diosgenin and other peaks [Figures 3 and 4]. The scanning wavelength selected was 205 nm for Diosgenin for both the instruments to provide comparable results. At this wave length, the Diosgenin showed optimum response. Peak purity was assessed by comparison of overlay spectra of standard and test peak at the start, apex and end was found satisfactory [Figures 1 and 2]. The Diosgenin was satisfactorily resolved with R_F value of 0.47±0.05 on HPTLC and Diosgenin was resolved at retention time around 10 min on HPLC.

The precision of sample application and scanning of peak area were expressed in terms of % RSD and were found to be 0.23 and 0.25 for HPLC and HPTLC, respectively. The % RSD values are depicted in Table 1, which showed that the proposed method provides acceptable system precision.

The peak purity of Diosgenin was assessed by comparing their respective spectra at peak start, peak apex and

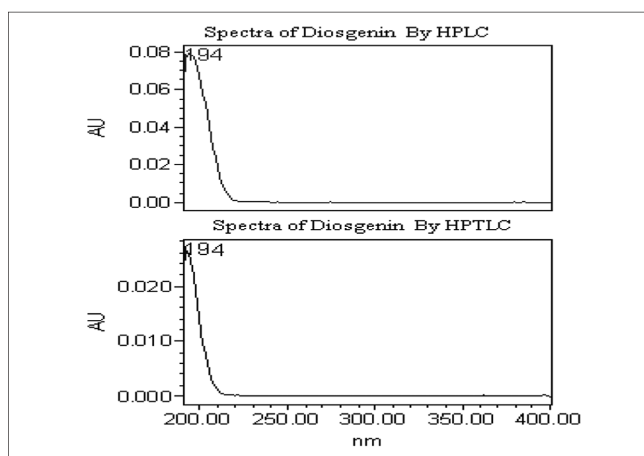

Figure 1: HPTLC and HPLC spectra of diosgenin

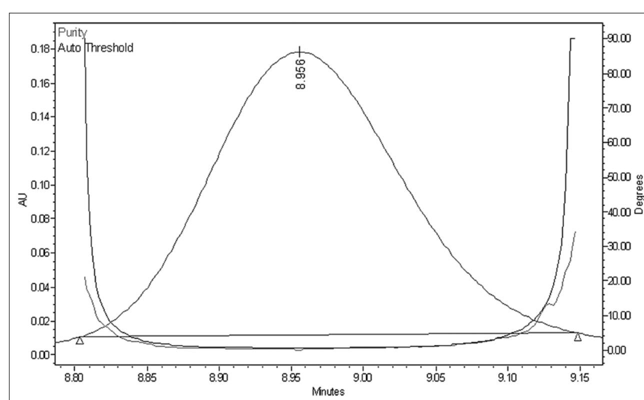

Figure 2: HPLC match plot for specificity

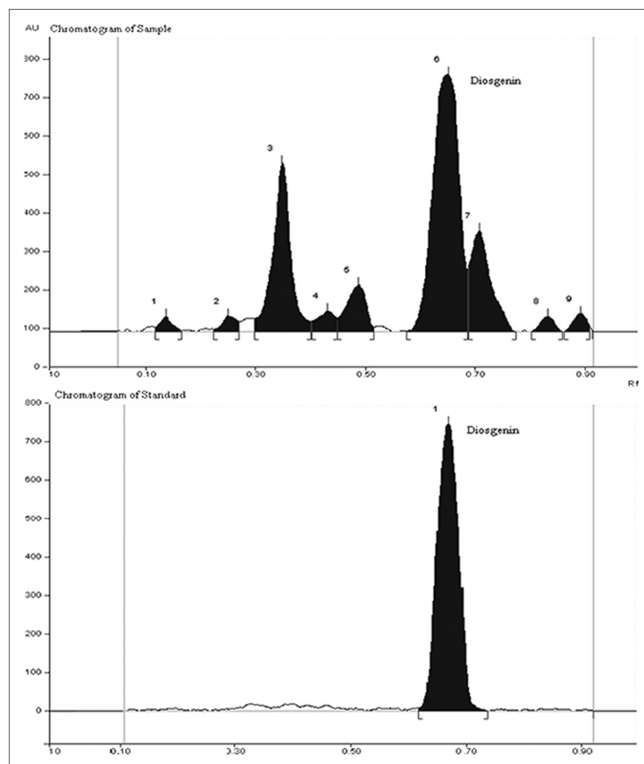

Figure 3: HPTLC chromatogram of sample and standard

peak end positions of the spot in HPTLC and peak in HPLC [Figures 1 and 2].

Both the methods were optimised by doing robustness. The values of % RSD as shown in Table 2 indicated better robustness of the method.

The proposed method was used for estimation of Diosgenin from extract after spiking with 80%, 100% and 120% of additional standard of Diosgenin to pre-analysed sample. The recovery percent of Diosgenin was found to be 99.18, 99.19 and 99.23 on HPTLC and 99.26, 99.22 and 99.23 on HPLC for 80%, 100% and 120%, respectively, in fenugreek extract [Table 1].

HPTLC is a powerful analytical technique. This method is visual, rapid and economical as it utilises smaller amounts of solvents with minimum sample clean up. Also, in a short duration, a large number of samples are simultaneously analysed. However, HPTLC has some limitations such as it is open system of analysis having less resolution, accuracy and sensitivity as compared to HPLC. On the other hand, HPLC has more accuracy, sensitivity reproducibility, resolution and automation than HPTLC.[13]

The HPLC method provides nanogram sensitivity and adequate linearity and repeatability, but HPTLC shows lower accuracy and precision in comparison with HPLC method, but the differences are not significant. Especially for herbal drug preparations, being multicomponent systems, HPTLC seems to have some advantages over HPLC. The sample preparation is simple, the detection by dipping reagents enables specific colour reactions and the consumption of organic solvents as well as the analysis time is less.

Consequently, both HPTLC densitometry and the HPLC method were found to be equal and could be used for the routine determination of Diosgenin from fenugreek seeds.

The method reported here is simple, rapid and suitable for the quantitation of Diosgenin from the extract of seeds of fenugreek. Diosgenin can easily be quantified in the presence of other constituents from the extract of fenugreek seed without compromising the accuracy.

CONCLUSION

According to statistical interpretation of results obtained from validation, the developed HPTLC and HPLC techniques are precise, specific, accurate and robust for the determination of Diosgenin.

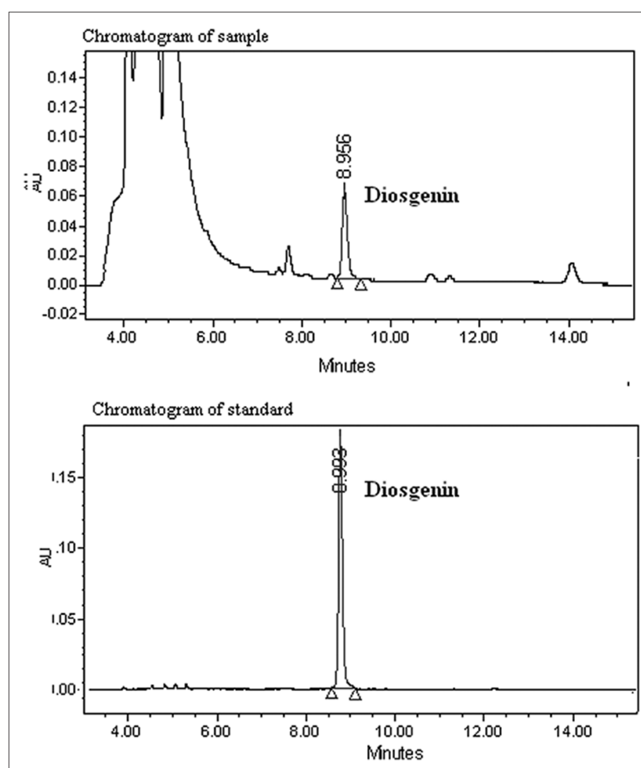

Figure 4: HPLC chromatogram of sample and standard

Table 2: Robustness of the method

	% RSD
Parameters for HPTLC	
Solvent of the mobile phase	0.10
Mobile phase volume	0.01
Saturation time	0.58
Developing distance	0.23
Parameters for HPLC	
Wavelength	0.01
Column temperature	0.30
Flow rate	0.35
Mobile phase gradient	0.12

HPLC – High performance liquid chromatography; HPTLC – High performance thin layer chromatography; RSD – Relative standard deviation

Therefore, the proposed method can be used for qualitative as well as quantitative analyses of Diosgenin in herbal extracts which may be useful for standardisation purposes in pharmaceutical industries. A statistical comparison of the quantitative Diosgenin determination from fenugreek seeds did not show any statistical significance between HPLC and HPTLC.

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