A validated RP-HPLC method for quantitation of trigonelline from herbal formulations containing *Trigonella foenum-graecum* (L.) seeds

Background: *Trigonella foenum-graecum* (L.) (Fabaceae, Fenugreek) is an important ingredient of Ayurvedic and other marketed herbal formulations. Fenugreek seeds are employed in many traditional systems as an antibacterial and antidiabetic agent, gastric stimulant and galactogogue. Trigonelline, a major phytoconstituent found in fenugreek seeds, shows estrogenic, anti-diabetic and anti-invasive activity. Therefore, it is a suitable bioactive marker to establish the quality of crude drug and its formulations. **Objective:** To develop an efficient and effective RP-HPLC method for estimation of trigonelline from *Trigonella foenum-graecum* seeds and its marketed herbal formulations. **Materials and Methods:** Separation and detection of trigonelline was carried out on a Cosmosil CN-MS column eluted with methanol:distilled water [95:5, v/v; pH 3.5 using hydrochloric acid]. Detection was carried out at 267 nm using a Photo Diode Array detector. Fenugreek seeds and two marketed herbal formulations were subjected for HPLC analysis of Trigonelline. **Results:** The RP-HPLC method was validated as per ICH guidelines and the content of trigonelline in marketed polyherbal formulations such as Dibet powder and Amyron syrup was determined. The LOD and LOQ were found to be 5.00 ng/mL and 50.00 ng/mL, respectively. Detector response was linear from 100.00 to 8000.00 ng/mL. The method was found to be simple, sensitive, accurate, reproducible and rugged. **Conclusion:** This work can be recommended for quality assurance and marker-based standardization of formulations containing fenugreek seeds.

Key words: Amyron syrup, dibet powder, RP-HPLC, *Trigonella foenum-graecum* (L.), trigonelline

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INTRODUCTION

Trigonella foenum-graecum (L.) (Fabaceae), commonly known as Fenugreek, is an aromatic and annual herb cultivated throughout the country. Fenugreek seeds are sharp bitter in taste and possess antipyretic, anthelmentic, antileprotic, antibronchitic, carminative and aphrodisiac properties. Several confections made with the seeds are used as a remedy for dyspepsia, loss of appetite, diarrhea of puerperal women and in rheumatism.[1,2]

The main chemical constituents of *Trigonella foenum-graecum* are fibers, flavonoids, polysaccharides, saponins, flavonoids, fixed oils, and some identified alkaloids namely, trigonelline and choline.[2] Trigonelline [Figure 1] is an important bioactive marker with estrogenic, anti-diabetic, and anti-invasive properties.[3-5] There are several chromatographic methods reported for quantification of trigonelline from pumpkin fruit, coffee seed etc. Most of these methods have issues such as the use of no organic phase, varied flow rate, costly columns (polymer based), and gradient elution which make them cumbersome for implementation in QC laboratories.[6-12]

This study aims at optimizing and validating a simple and reliable HPLC

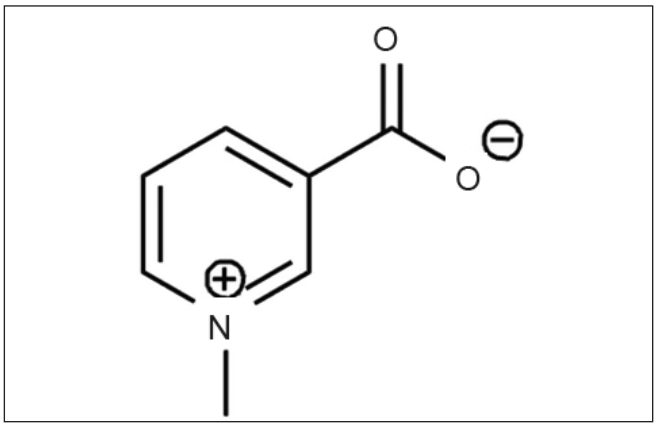

Figure 1: Structure of trigonelline

method on a reversed-phase Cosmosil CN-MS (250 mm × 4.6 mm) column with photodiode array (PDA) detection for monitoring the quality of *Trigonella foenum-graecum* seed.[13-15] The developed method was also successfully applied to commercially available polyherbal formulations (Dibet powder and Amyron syrup) to estimate relative trigonelline content.

MATERIALS AND METHODS

Materials and reagents

Seeds of *Trigonella foenum-graecum* were procured from a local market of Mumbai (India), authenticated by a Taxonomist and voucher specimens were deposited in the Herbal Research Lab, Ramnarain Ruia College.

Plant material was dried in an oven maintained at 35 °C for 7 days. It was then powdered, sieved through mesh (B.S.S.-85) and preserved in an airtight container. Two herbal formulations, Dibet powder and Amyron syrup, manufactured by Sheetal Medicare Products Pvt. Ltd., batch no. 906 and Aimil Pharmaceuticals (India) Ltd., batch no. ARS-1443, respectively, was procured from the local market.

Reference standard Trigonelline was procured from Natural Remedies (Bangalore, India). HPLC-grade methanol was procured from Merck (Darmstadt, Germany), ultra-pure water was obtained using a Milli-Q purification system (Millipore, USA).

Sample preparation

The methanolic extract of seeds was prepared by mixing seed powder and methanol in the ratio 1:10 (w/v). It was vortexed for 60 s and kept on a rotary shaker at 150 rpm for 12 h. The resultant mixture was filtered through a Whatmann paper no. 1 and stored at 4 ± 1 °C till further analysis. A similar procedure was applied for the trigonelline extraction from Dibet powder formulation. The Amyron syrup sample was mixed with methanol in the ratio of 1:9 (v/v). The mixture was then vortexed for 60 s and kept on a rotary shaker at 10 rpm for 12 h. The resultant mixture was centrifuged at 3000 rpm for 10 min and the upper organic layer was separated and stored at 4 ± 1 °C till further analysis.

HPLC analytical conditions

HPLC analysis was performed on a JASCO's HPLC system equipped with a PU-980 pump unit, a reversed-phase Cosmosil (Nacalai Tesque, INC. Japan) CN-MS (250 mm × 4.6 mm) column, an autosampler (AS-1555-10), and a Photo diode array detector (MD-910). Samples were eluted using the mobile phase of methanol:distilled water (95:5, v/v), adjusted to pH 3.5 with hydrochloric acid and delivered at a flow rate of 1.0 ml/min. Detection was carried out at 267 nm at room temperature (27 ± 1 °C). The injection volume was 20 μL for all runs. Data acquisition and analysis were carried out using Borwin Integrator Software, version 1.21, chromatography analysis software.

Standard solution

Trigonelline (10 mg) was dissolved in 10 mL of methanol to prepare a stock solution of 1000 μg/mL. Working standard solution was prepared by serial dilution of the standard stock solution.

Method validation

The developed RP-HPLC method was validated as per ICH guidelines in terms of its sensitivity (LOD and LOQ), linearity, assay, recovery, precision, stability, and ruggedness/robustness.

RESULTS AND DISCUSSION

Currently chemical markers or pharmacologically active components in polyherbal formulations are employed for evaluating the quality, consistency, and authenticity of polyherbal formulations.[16,17]

Based on the experiments carried out during the course of validation, the intended method has been validated for the estimation of trigonelline from seeds of *Trigonella foenum-graecum*. The precision and accuracy were within the acceptance of limits. Consistent recoveries were observed for LQC, MQC, and HQC (lower, middle and higher quality control samples respectively). Stability of stock and working standard were checked for short term (6 h) as well

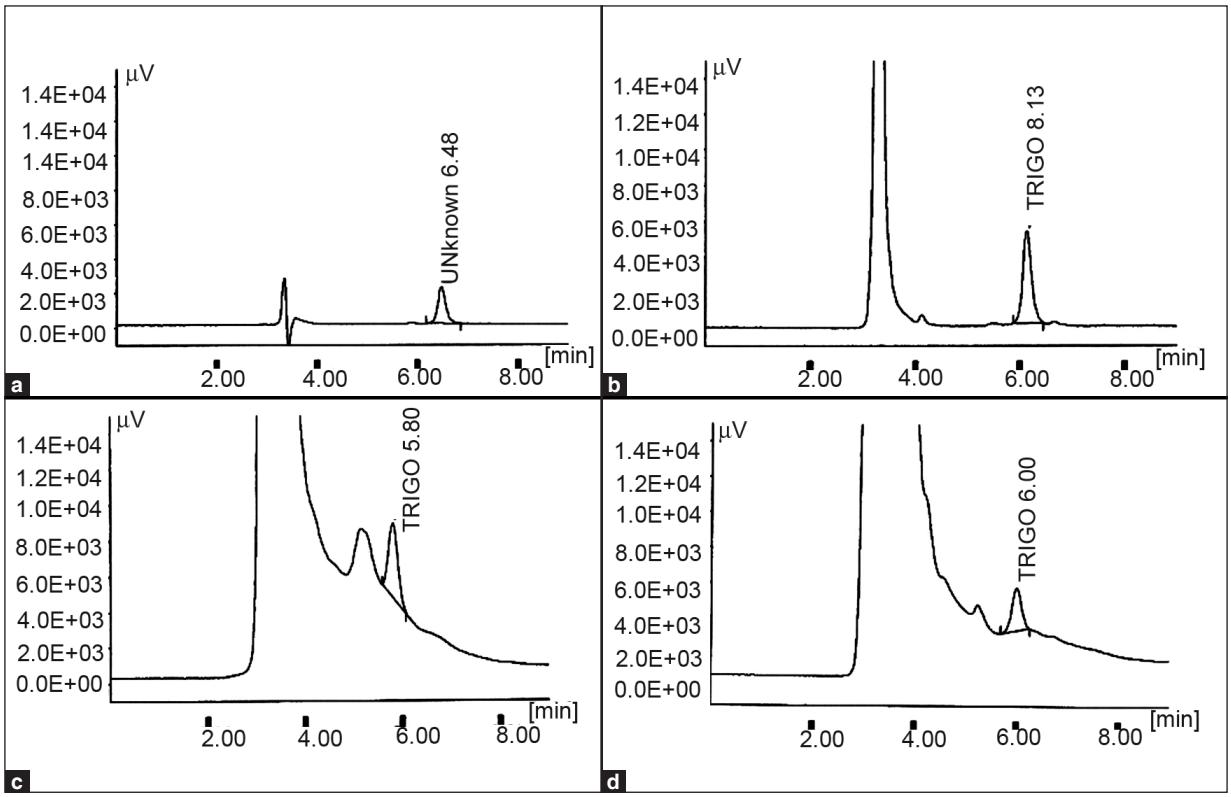

Figure 2: Representative HPLC chromatograms of (a) standard trigonelline 1000.0 ng/mL, (b) *Trigonella foenum-graecum* (L.) seeds, (c) Dibet powder and (d) Amyron syrup.

Table 1: Method validation parameters

Parameters	Results
LOD (ng/mL)	5.0
LOQ (ng/mL)	50
Linear range (ng/mL)	100–8000
Mean correlation coefficient (r^2)	0.9967
Mean slope	22.412
System suitability (% CV, n = 5)	
Retention time	0.13
Area	1.05
Precision (% CV, n = 3)	
Within-batch	1.14–1.73
Between-batch	1.69–1.96
Recovery (%, n = 7)	
LQC	101.80
MQC	99.24
HQC	98.67
Stability	
Long-term stability	
Standard stock solution stability (for 10 days)	Stable at (4 ± 1°C)
Short-term stability	
Bench top stability (For 6.00 h)	Stable at (25 ± 2°C)
Autosampler stability (For 12.00 h)	Stable at (4 ± 1°C)

as for long term (10 days). The stock standard was found to be stable at 4 °C for 10 days [Table 1]. The bench top stability and autosampler stability for the working standard showed stability for 6.00 h

Table 2: Robustness/ruggedness of the method during method validation

Parameters	Results	
	% CV (n = 3)	% Difference
Change in column		
Column 1	0.05–1.62	–1.40–1.02
Column 2	0.56–1.53	
Change in analyst		
Analyst 1	0.46–0.90	−0.94-1.09
Analyst 2	0.18–1.06	
Change in day		
Day 1	0.58–1.85	−1.44–0.84
Day 2	0.44–1.30	
Change in instrument		
Instrument 1	0.77–1.36	−0.07–0.39
Instrument 2	0.39–1.04	
Change in flow rate		
0.95 ml/min	0.92–1.82	−0.07–1.96
1.00 ml/min	0.40–1.70	
1.05 ml/min	0.61–1.48	−0.04–2.05
Change in injection volume		
15 μL	0.93–1.90	16.07–31.07
20 μL	0.51–1.07	
25 μL	0.26–1.86	−21.72–29.88
Change in mobile phase composition		
94.5:5.5	0.69–1.29	−2.01–3.27
95:5	0.48–1.07	
95.5:4.5	0.28–1.42	−2.76–2.49

Table 3: Assay results and method application

Sample	Concentration of trigonelline in mg/ml or mg/g (mean ± SD)
Trigonella foenum-graecum	98.36 ± 0.0154
Amyron syrup	0.025 ± 0.0011
Dibet powder	0.015 ± 0.0026

(25 ± 20°C) and for 12.00 h (4 ± 10°C) respectively [Table 1]. The validated method was also applied for quantification of trigonelline from two marketed herbal formulations Dibet powder and Amyron syrup containing seeds of *Trigonella foenum-graecum* [Figure 2]. The method has been found to be rugged for different columns, different analysts, different days, change in the instrument (PEV Jasco), change in the flow rate, change in injection volume, and variation in the mobile phase composition under specified conditions [Table 2]. The representative chromatograms and content of trigonelline in seeds of *Trigonella foenum-graecum*, Amyron syrup and Dibet powder are given in Figure 2 and Table 3 respectively.

CONCLUSION

As herbal preparations have chemical complexities, it is very difficult to identify and determine all of their chemical components. Single marker-based quantitative methods would be a complementary approach for the quality control and stability assessment of the herbal preparations. Our results provide a fully validated RP-HPLC method for quality control of plant extracts and phytopharmaceuticals containing seeds of *Trigonella foenum-graecum* using trigonelline as a chemical marker. Validation of the method as per ICH guidelines showed that the method is in compliance with the current guideline. The method was found to be robust. Moreover, the solvent consumption along with the short analytical run time of 8.0 min leads to a cost-effective and eco-friendly chromatographic procedure. Thus, the proposed methodology is rapid, selective, requires a simple sample preparation procedure and represents a good procedure for quantitation of trigonelline.

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