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# Analytical method development and validation for simultaneous estimation of monoammonium glycyrrhizinate and sennoside-B in polyherbal laxative tablet using RP-HPLC

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## Abstract

**Background:** Standardization of polyherbal medicine though being the need of the hour is a toilsome task. Among the various methods employed for quality control and standardization of polyherbal medicine, phytochemical profiling is of utmost importance as it signifies the quality as well as efficacy of the medicine. The work was aimed to develop and validate a simple, quick, and accurate RP-HPLC technique for simultaneous assessment of monoammonium glycyrrhizinate and sennoside-B in a polyherbal laxative tablet. The phytomarkers were effectively quantified by RP-HPLC system on C<sub>18</sub> analytical column using gradient mobile phase consisting of phosphate buffer to acetonitrile with the detector wavelength set at 254 nm. This developed method was validated by determination of parameters like accuracy, linearity, precision, limit of detection, and quantification as well as robustness according to ICH guidelines.

**Results:** Calibration curve of both phytomarkers showed excellent linear correlation coefficients. LOD and LOQ were also calculated by equation. Precision studies were carried out using intra-day and inter-day intervals and RSD values were found to be less than 2.00%. The method was found to be accurate, which was evident from 98.96 to 101.39% and 99.17 to 100.67% recovery of monoammonium glycyrrhizinate and sennoside-B, respectively, when the formulation was spiked with the respective phytomarkers.

**Conclusion:** The validated method can be employed as standardization tool for herbal formulations with accuracy and precision. The developed method will assist in maintaining the good quality and batch to batch uniformity of polyherbal formulations containing *Yashtimadhu*, *Swarnpatri*, and *Aragvadha* as active ingredients.

**Keywords:** RP-HPLC, Monoammonium glycyrrhizinate, Sennoside-B, Polyherbal tablet, Method development and validation

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## Background

Herbs and products containing herb(s) are marketed for several purposes. It is evident that moreover 80% of the entire world population relies on herbal medicines and products made from for healthy living [1]. Plant materials and preparations are manufactured and sold most commonly in the pharmaceutical market for treating diseases and promoting public health due to its virtues like minimum cost, potency and efficiency, improved tolerance, effectivity in chronic diseases, widespread availability, more protection, fewer side-effects, complete accessibility, and recyclable. Lack of quality in medicines leads to disappointment of consumers and also can lead to fatal consequences.

Standardization or quality control of herbal formulations is significant to determine its quality standards on basis of the concentration of actives along with phytochemical, chemical, physical, and in vivo and in vitro parameters. The acceptability in modern system of medicine can be justified by the quality assessment of herbal formulations as the scope for variation in different batches of herbal medicine is obvious [2–4]. To ensure that the consumers obtain the medication which give assurance of safety, purity and potency along with good efficacy are the primary responsibility of producer and such processes rationally would apply to all the medication types of allopathy or the traditional systems [5].

A commercial ayurvedic laxative formulation, Vasulax, was selected for development of simultaneous estimation of phytoconstituents present in it by reverse phase high-performance liquid chromatography (RP-HPLC). Vasulax is mainly composed of *Yashtimadhu*, *Swarnpatri*, *Aragvadha*, and *Sunthi* along with excipients. *Glycyrrhiza glabra* L. (Liquorice) is known as *yashtimadhu*, one of the widely known herbs from ancient time of Ayurveda as a medicine. Traditionally, *yashtimadhu* is reported as expectorant, demulcent, sweetener, anti-tussive, and laxative [6]. The chief sweet tasting and active constituent in the *yashtimadhu* is monoammonium glycyrrhizinate. *Swarnpatri* commonly known as *Cassia angustifolia* M. Vahl (Senna) is an important plant drug in traditional and modern medicine for the management of constipation [7–9]. Sennosides are the major anthraquinone glycosides accountable for purgative property of *Swarnpatri*. A range of poly-herbal formulations comprising *swarnpatri* leaves are existing in India for relief of constipation and allied troubles. *Aragvadha*, known as *Cassia fistula* L. (Amaltas), belongs to family Leguminosae reported to possess laxative effect [10, 11]. This plant is also reported to contain sennoside glycoside along with other chemical constituents.

For quantification purposes, monoammonium glycyrrhizinate was selected as a phytomarker of *Yashtimadhu*

**Table 1** Chromatographic conditions of developed method

Parameters	Value
Injection volume	20 $\mu$ L
Column temperature	27o C
Detection wavelength	254 nm
Flow rate	1.5 mL/min
Mobile phase	Phosphate buffer (pH 3):acetonitrile
Mode of separation	Gradient
Run time	45 min

and sennoside-B was selected as a phytomarker of *Swarnpatri* and *Aragvadha*.

There are reported methods available in literature for determining sennoside-B and monoammonium glycyrrhizinate independently and in blend with other drugs from *swarnpatri*, *yashtimadhu*, and *aragvadha* [12–17]. On the other hand, there is no RP-HPLC technique documented for the simultaneous estimation of mentioned combined phytomarkers in any dosage forms. Accordingly, the present work was aimed to develop a validated RP-HPLC method for simultaneous determination of sennoside-B and monoammonium glycyrrhizinate in Vasulax tablet.

## Methods

### Chemicals

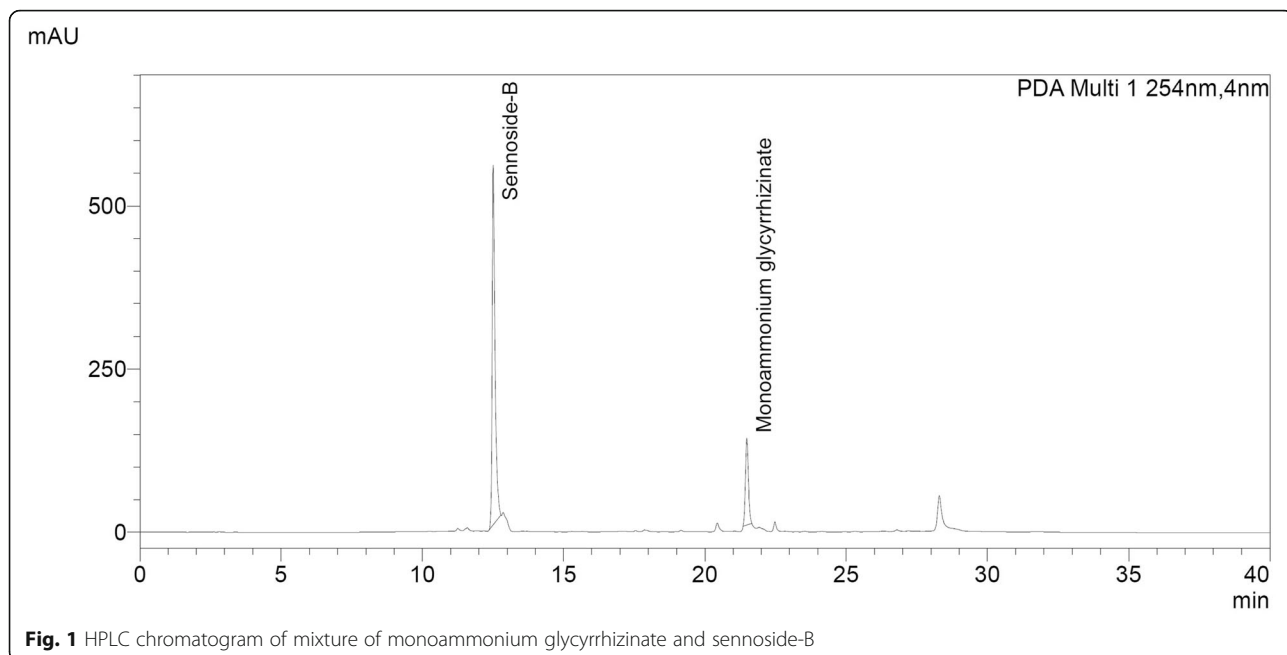
- Acetonitrile—gradient grade for liquid chromatography (Merck, India)
- Water—chromatography grade (Merck, India)
- Potassium dihydrogen orthophosphate—analytical grade (Merck, India)
- Ortho-phosphoric acid—chromatography grade (Merck, India)

### Reference standards

- Monoammonium glycyrrhizinate—purity > 99%, analytical standard (Natural Remedies, India)

**Table 2** Gradient program of optimized mobile phase

Time	% of mobile phase	
	Solvent A, phosphate buffer (pH 3)	Solvent B, acetonitrile
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5



**Fig. 1** HPLC chromatogram of mixture of monoammonium glycyrrhizinate and sennoside-B

(b) Sennoside-B—purity > 99%, analytical standard (Natural Remedies, India)

(d) Column oven—CTO 10 AS (Shimadzu, Japan)

(e) Column—C<sub>18</sub>, 100 Å, 5 µm, 4.6 × 250 mm (Shimadzu, Japan)

(f) Detector—PDA detector SPD M 20 A (Shimadzu, Japan)

**Instruments**

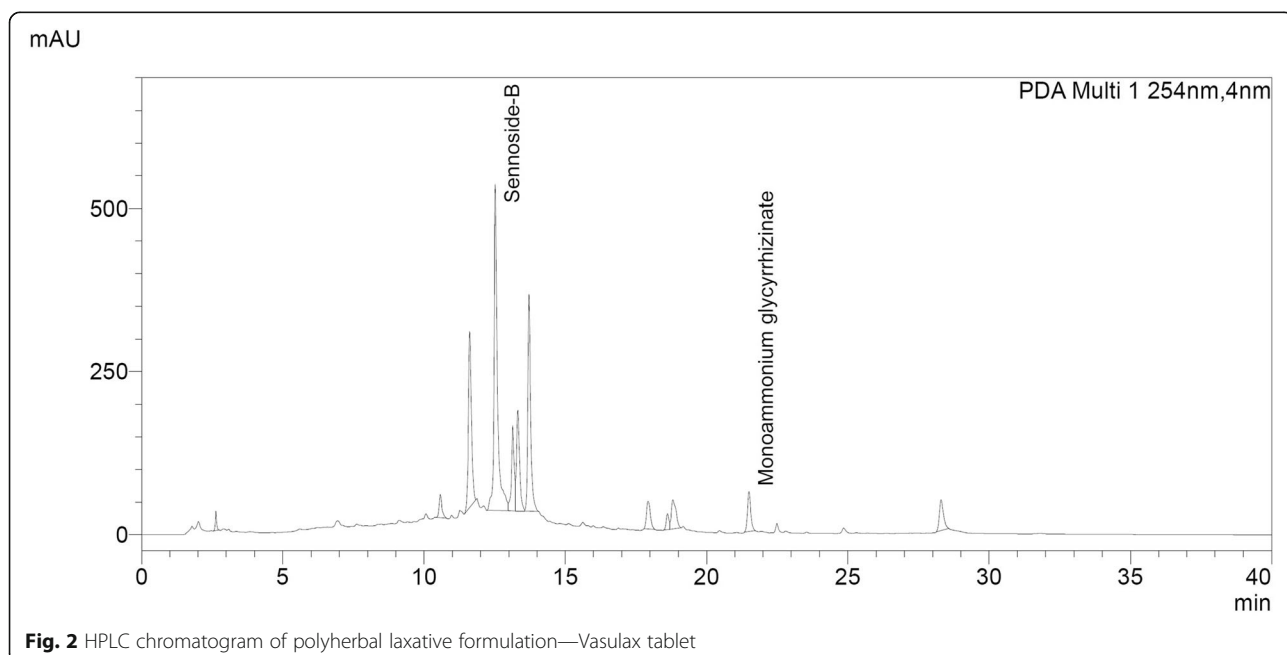
(a) HPLC—prominence UFLC (Shimadzu, Japan)

(b) Pump—quaternary gradient LC 20 AD (Shimadzu, Japan)

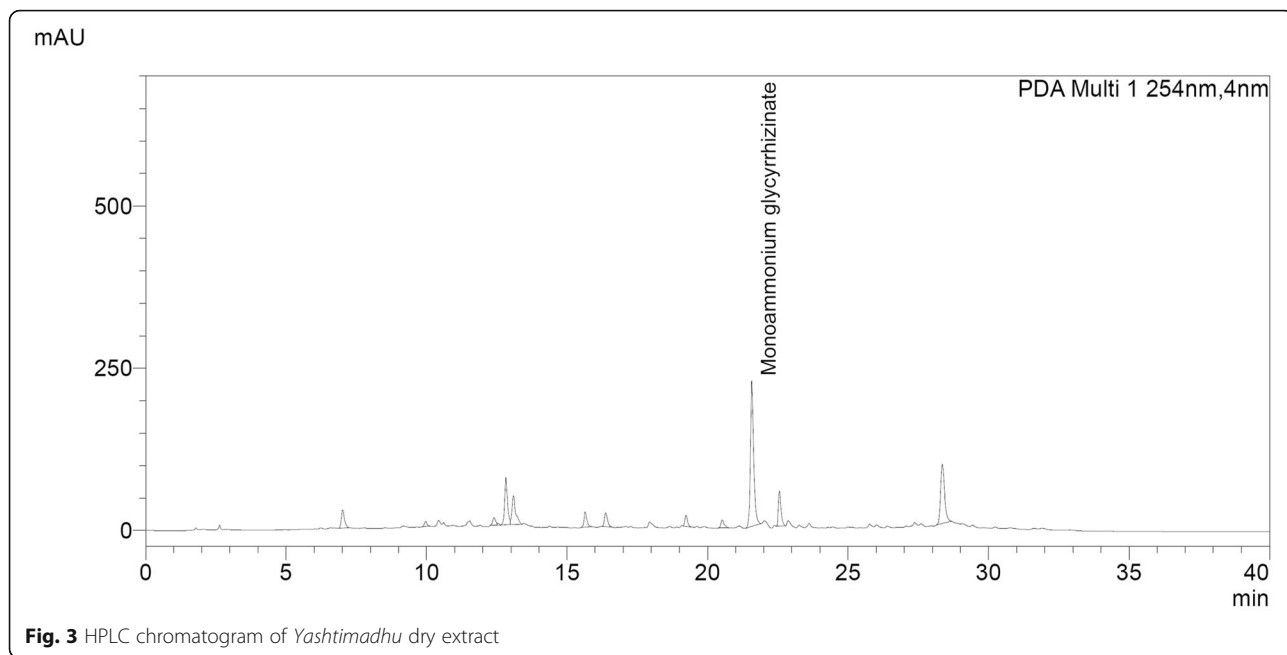
(c) Injector—auto sampler SIL 20 AC (Shimadzu, Japan)

**Preparation of phosphate buffer**

Accurately weighed 0.07 g of anhydrous potassium dihydrogen orthophosphate was transferred to 500 mL volumetric flask and approx. 450 mL



**Fig. 2** HPLC chromatogram of polyherbal laxative formulation—Vasulax tablet

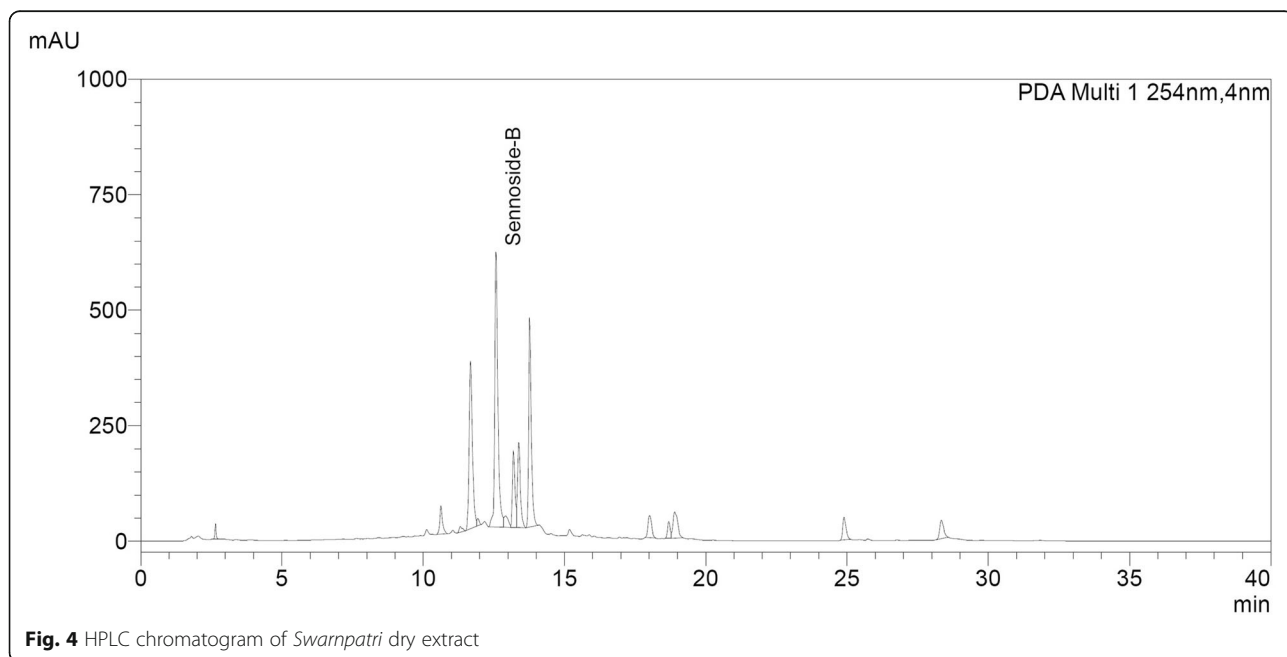


**Fig. 3** HPLC chromatogram of *Yashtimadhu* dry extract

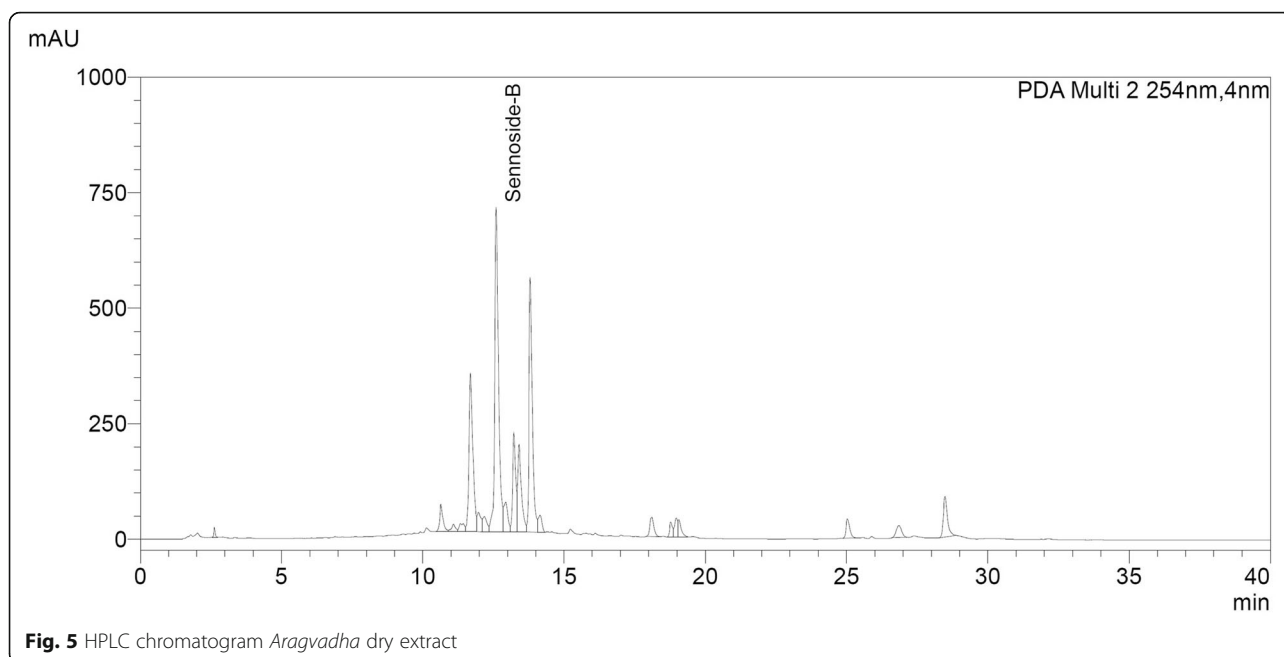
chromatography grade water was added into it. This mixture was kept for ultra-sonication for 10 min for complete dissolution. 0.25 mL of orthophosphoric acid was added to it and finally volume was made up to 500 mL using water. The resulting solution was filtered through 0.22- $\mu$ m nylon filter paper using vacuum filtration unit.

**Preparation of reference standards solution**

The solution of standard was made by dissolving accurately weighed 2.5 mg of monoammonium glycyrrhizinate and 2.5 mg of sennoside-B in a 5-mL volumetric flask and make up the volume to 5 mL using chromatography grade water. The resulting solution was sonicated for 5 min to aid dissolution and then filtered through 0.22- $\mu$ m nylon syringe filter assembly.



**Fig. 4** HPLC chromatogram of *Swampatri* dry extract



**Fig. 5** HPLC chromatogram *Aragvadha* dry extract

#### Preparation of sample solution

Accurately weighted and powdered 2 g tablet formulation was taken to 100 mL volumetric flask and volume was adjusted to 100 mL using water of chromatography grade. The resulting solution was sonicated for 5 min and then filtered through 0.22- $\mu$ m nylon syringe filter assembly.

#### Analytical method development

Various steps involved in the method development were optimized for accurate and precise separation of selected phytomarkers. Several parameters were studied to select optimal conditions for selection of extracting solvent, selection of column phase, selection of mobile phase, and selection of detecting wavelength. After taking several trials, the optimized chromatography method which shows optimum peak resolution and good symmetry for the simultaneous estimation of both phytomarkers is shown in Table 1.

#### Validation of developed method

Validation of the proposed method was performed as per ICH guideline [18] which includes specificity, precision, linearity, accuracy, robustness, limit of detection (LOD), limit of quantitation (LOQ), and system suitability.

#### Specificity

Specificity is the capability to review unequivocally the analyte in the existence of components that may be anticipated to be present. To evaluate specificity of the method, two samples were prepared of which one

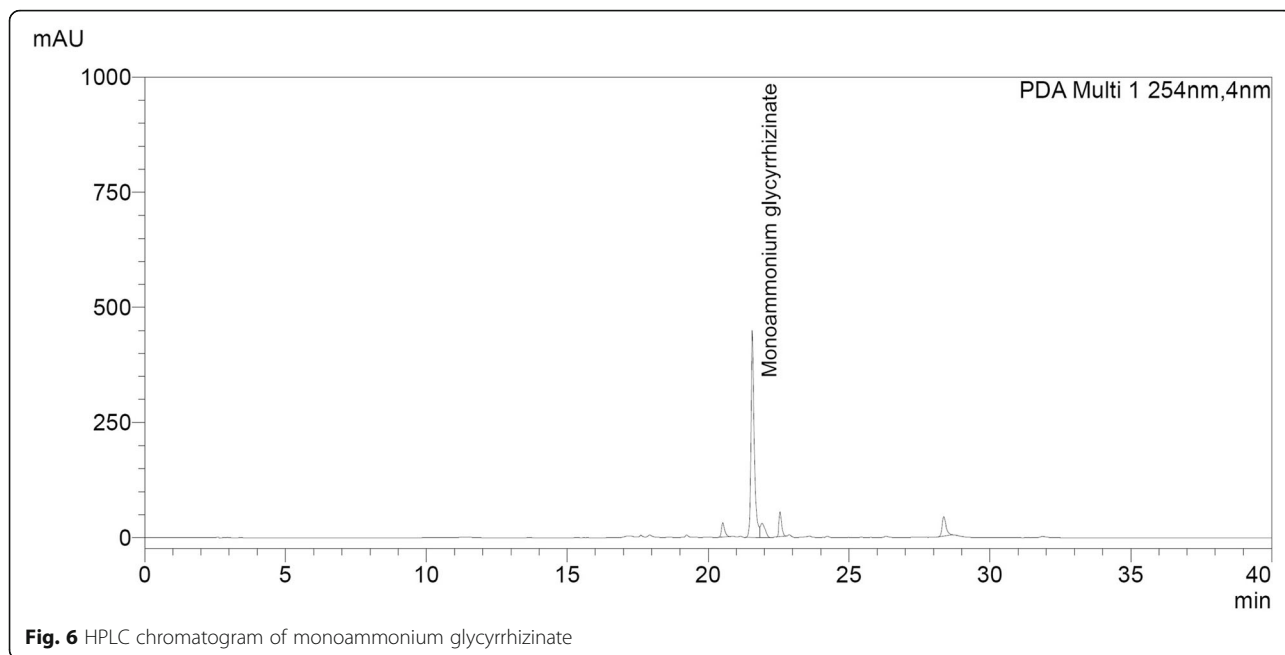
contain placebo formulation (unspiked) and in another sample; placebo were spiked with 2 phytomarkers. The specificity was evaluated by comparing the chromatograms obtained from unspiked samples with those from samples spiked with standards individually.

#### Linearity

Analytical procedure's ability to attain test results that are directly corresponding to the concentration of analyte present in test sample is known as linearity. One hundred micrograms per milliliter stock solution of both phytomarkers was prepared by dissolving accurately weighted 10 mg monoammonium glycyrrhizinate and 10 mg sennoside-B in 100 mL volumetric flask. Dilution was performed to achieve final concentration of 5–15  $\mu$ g/mL for both the phytomarkers. The linearity was obtained from calibration curves made by plotting average peak area against concentrations of the standards.

**Table 3** Application of developed method on polyherbal formulation and its raw materials

Name of sample	Phytomarkers assay (% w/w)	
	Monoammonium glycyrrhizinate	Sennoside-B
Vasulax tablet	0.623%	5.060%
<i>Yasthimadhudry</i> extract	7.11%	---
<i>Swarnpatri</i> dry extract	---	16.79%
<i>Aragvadha</i> dry extract	---	1.86%



**Fig. 6** HPLC chromatogram of monoammonium glycyrrhizinate

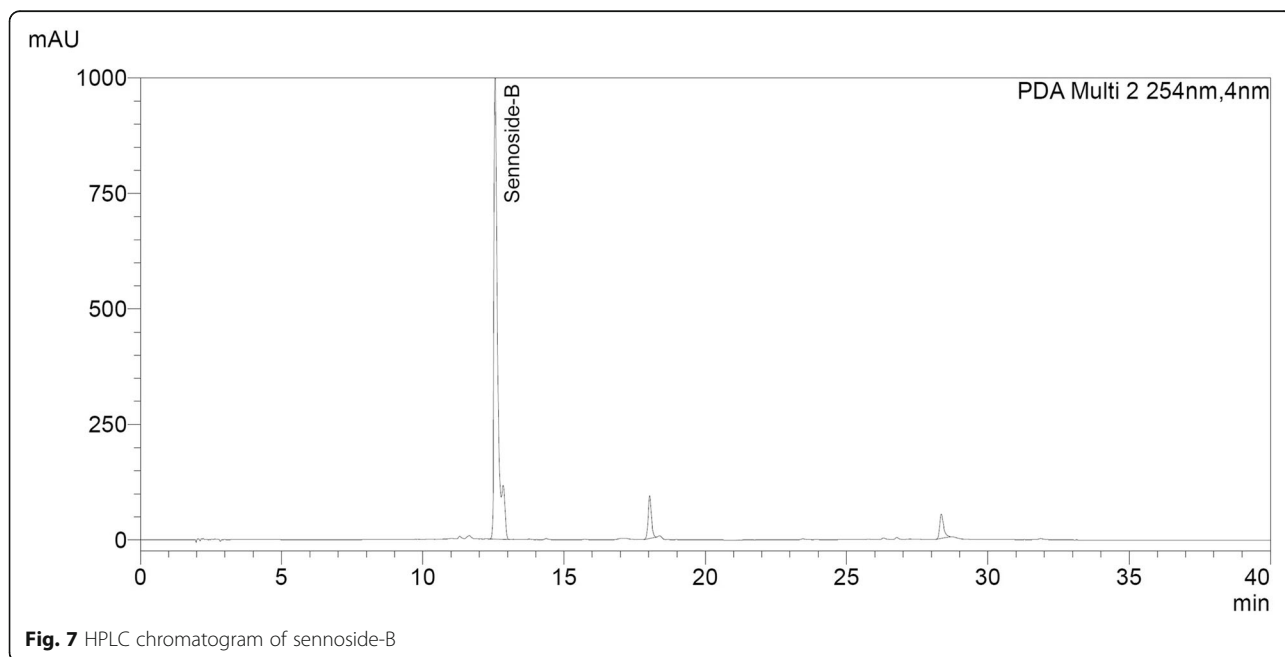
**Detection limit and quantitation limit**

The limits of detection (LOD) and limits of quantitation (LOQ) were used to evaluate the sensitivity of the developed method. For the determination of LOD and LOQ, linearity of the standards was performed three times to obtain standard deviation of the intercept (SD) and slope of the regression equation (*S*) value. LOD and LOQ were determined by the standard deviation method and calculated as follows:

Limits of detection =  $3.3 \times SD/S$  and Limits of quantitation =  $10 \times SD/S$

**Precision**

The nearness of conformity amid a series of measurements attained from multiple sampling of the similar homogeneous sample under the stipulated circumstances is called precision of the analytical method. Precision was evaluated in terms of intra-day precision and inter-day



**Fig. 7** HPLC chromatogram of sennoside-B

**Table 4** System suitability parameters of the formulation

Parameters	Monoammonium glycyrrhizinate	Sennoside-B
Rt	21.49	12.52
Theoretical plates (N)	920976	388668
Resolution (Rs)	9.739	2.325
Tailing factor ( $T_f$ )	1.844	1.911
Capacity factor ( $k'$ )	9.650	5.204

precision (ruggedness). The intra-day precision was performed by determining response of sample 6 times in a day. The inter-day precision analyses were performed by determining the subsequent responses on 6 dissimilar days. The samples were prepared accordingly the method described in the "Preparation of sample solution" section. The results of all data related to precision were documented by way of relative standard deviation.

#### Accuracy

Nearness of an agreement among the value which is considered either as a conventional true value or a conventional reference value as well the value found in analytical method is called accuracy. The accuracy was estimated as the relative recovery, which was measured in three replicates at three spiked concentrations (80%, 100%, and 120%) in the sample. The placebo formulation was spiked with 0.48%, 0.60%, and 0.72% of monoammonium glycyrrhizinate and 4%, 5%, and 6% of sennoside-B, respectively, and percent recovery calculated by comparing obtained value with spiked value.

#### Robustness

The measurement of analytical method's ability to stay unchanged by little but intentional variation in method parameters and give an indication of its consistency while regular usage is called robustness. Robustness of the method was studied by deliberately changing the experimental conditions like column temperature and flow rate. Flow rate was changed from 1 to 0.9 mL/min and 1.1 mL/min, and column temperature varied from 40 to 38 °C and 42 to check robustness of the method.

**Table 5** Linearity data of phytomarkers and their  $R^2$  value

Sr. No.	Phytomarkers	$R^2$ value	Equation
1	Monoammonium glycyrrhizinate	0.9993	$y = 118356.9829x - 25472$
2	Sennoside-B	0.9996	$y = 493618x + 251599$

**Table 6** LOD and LOQ data of developed method

Parameter	Monoammonium glycyrrhizinate	Sennoside-B
LOD	0.17 µg/mL	0.61 µg/mL
LOQ	0.51 µg/mL	1.85 µg/mL

## Results

#### Optimization of chromatographic conditions

Initial trials were conducted by using various mobile phase compositions containing methanol, water, and acetonitrile in different ratio but proper separation was not achieved. Various gradient methods were also tried containing these solvents but desired separation with peak shape was not achieved. Then, phosphate buffer was tried in different ratio with methanol and acetonitrile at different pH for better resolution of phytomarkers. Finalized gradient mobile phase program is shown in Table 2.

#### Quantification of phytomarkers

Optimized chromatographic conditions were applied to analyze sample solutions to quantify both the phytomarkers present in the formulation along with mixture of standards (Figs. 1 and 2). Individual raw materials used in the preparation of formulation, *Yashtimadhu* dry extract, *Swarnpatri* dry extract, and *Aragvadha* dry extract were also quantified with respective phytomarkers (Figs. 3, 4, and 5). The results calculated based on calibration curve of individual markers are summarized in Table 3.

#### Validation of the developed analytical method

##### Specificity

The specificity was demonstrated by comparing chromatograms of standard and sample solutions (Figs. 6 and 7). From this study, it is clear that both the phytomarkers were clearly resolved, with no interference from the sample matrix.

##### System suitability

The system suitability of the developed method was confirmed by calculating various chromatographic parameters such as number of theoretical plates (N), Resolution (Rs), tailing factor ( $T_f$ ), and capacity factor ( $k'$ ) from the chromatogram of standard solutions. Results obtained are shown in Table 4. System suitability parameters confirmed that the given chromatographic conditions were good for the method development and validation.

**Table 7** Intra-day and inter-day precision study

Precision	% RSD	
	Monoammonium glycyrrhizinate	Sennoside-B
Intra-day (n = 6)	1.5111	0.6078
Inter-day (n = 6)	1.1906	0.5602

**Table 8** Accuracy study of the developed method

Parameters	% Recovery	
	Monoammonium glycyrrhizinate	Sennoside-B
80%	98.96	99.17
100%	100.83	100.67
120%	101.39	100.28
Average	100.39	100.04

**Linearity**

The linearity of calibration curves for monoammonium glycyrrhizinate and sennoside-B was established in range of 5–25 µg/mL. Representation of results obtained is given in Table 5.

**Detection limit and quantitation limit**

The LOD and LOQ values were determined and results obtained are shown in Table 6.

**Precision**

The intra-day precisions were measured six times a day and % relative standard deviation (RSD) ranged from 0.6078% to 1.5111% and inter-day precisions were measured on six consecutive days and % RSD ranged from 0.5602 to 1.1906%. Results obtained of precision study are shown in Table 7.

**Accuracy**

The accuracy of the assay method, measured as relative recovery at three concentration levels, was 98.96–101.39%, with all RSD values ≤ 2%. Recovery study results are shown in Table 8.

**Robustness**

Robustness of the developed method was performed by deliberately altering the experimental conditions such as flow rate from 1 to 0.9 mL/min and 1.1 mL/min, and column temperature was varied from 40 to 38 °C and 42 °C. Results obtained of robustness study are shown in Table 9.

**Discussion**

The research involves new RP-HPLC method for the simultaneous estimation of monoammonium glycyrrhizinate and sennoside-B in polyherbal formulations and plant materials. A previous study has shown that there are several methods for quantification of

monoammonium glycyrrhizinate and sennoside-B individually [12–17]. However, there is no RP-HPLC method reported for the simultaneous estimation of these phyto-markers combined in any dosage forms. The main objective of the research work is to develop a fast, reliable, and cost-effective method for such estimation which requires minimal sample preparation.

On the basis of solubility data, it was clear that all the constituents were properly soluble in water. So water was selected as extracting solvent. Reverse phase column is widely used for separation of organic compounds, and all the phyto-markers present in tablets were effectively separated on reverse phase column such as C<sub>8</sub> and C<sub>18</sub>. Hence, reverse phase column was used for method development. For better resolution, C<sub>18</sub> column was preferred over C<sub>8</sub> column. During the development of the method, several combinations of mobile phase with different solvents were evaluated. Finally, phosphate buffer at pH 3 with acetonitrile in gradient mobile phase was finalized to quantify both phyto-markers in an accurate manner (Table 2). Quantification of the polyherbal formulations along with its individual plant materials was done using a developed method (Table 3). The results obtained for quantification of phyto-markers in the formulation were in equivalent with the theoretical values derived based on the amount of phyto-markers present in raw material used in the preparation of the formulation indicating the accuracy of the method for routine quality control of the formulation.

This developed method was validated by determination of parameters like accuracy, linearity, precision, limit of detection, and quantification as well as robustness according to ICH guidelines [18]. During the specificity study, both the phyto-markers were clearly resolved, with no interference from the sample matrix indicating the developed method usability for the analysis without interference (Figs. 6 and 7). System suitability were also checked using factor such as number of theoretical plates (*N*), Resolution (*R<sub>s</sub>*), tailing factor (*T<sub>f</sub>*), and capacity factor (*k'*). All the factors were observed in accordance with specified limits (Table 4). The system was found to be suitable for analyzing monoammonium glycyrrhizinate and sennoside-B by the described method. In linearity study, the obtained  $r^2 > 0.999$  for all standards indicate that the developed method is linear for both phyto-markers in tested range. LOD and LOQ were also calculated to get an idea about the minimum concentration required to detect and quantify the markers in the sample solution. Precision study was conducted via intra-day and inter-day precision study. Results obtained in both the study were in accordance with ICH guidelines, i.e., % RSD < 2% (Table 7). Accuracy study was performed by spiking the phyto-markers in placebo tablet at three different concentration levels. Full

**Table 9** Results of robustness study

Parameters	Deliberate changes	% RSD	
		Monoammonium glycyrrhizinate	Sennoside-B
Flow rate	1 ± 0.1 mL/min	1.83	2.02
Column Temperature	40 ± 2 °C	1.43	1.77



recovery was obtained for the compounds of interest which indicate that the method was able to completely recover markers from excipients in the formulation matrix (Table 8). A simple sample preparation method was the main reason for lesser variation of recovery. From the result of the robustness study, the method was found to be robust for routine analysis.

These results demonstrate that the developed method is linear, precise, and accurate. Moreover, the method consumes a small amount of sample and involves very simple sample preparation method for quantification of phytomarkers.

## Conclusion

Due to complex nature of polyherbal formulation, it is important to ensure the quality of finished product based on reliable scientific methods, which have not been reported earlier. The proposed research involved in development and validation of analytical method used in quality control of polyherbal laxative formulation. The developed and validated HPLC method will help in the quality control of polyherbal laxative formulation by means of biologically active phytomarkers. The HPLC method developed for simultaneous estimation of mono-ammonium glycyrrhizinate and sennoside-B is accurate, precise, reproducible, and repeatable. With the growing demand for herbal drugs and increased belief in the usage of herbal medicine, the development of a reliable standardization tool will help in maintaining the quality of such important polyherbal preparations.

## Abbreviations

HPLC: High-performance liquid chromatography; ICH: International Council for Harmonization; LOD: Limit of detection; LOQ: Limit of quantification; RP-HPLC: Reverse-phase high-performance liquid chromatography; RSD: Relative standard deviation; S: Slope; SD: Standard deviation

## Acknowledgements

Authors are thankful to Vasu Research Centre, a division of Vasu Healthcare Pvt. Ltd., Vadodara, Gujarat, for providing support to carry out this research work. Authors are also thankful to Parul University, Vadodara, Gujarat, for providing essential support throughout the work.

## Plant authentication

The authentic plant extracts and polyherbal formulation was obtained as gift samples from Vasu Healthcare Pvt. Ltd., Vadodara, Gujarat.

## Authors' contributions

MP and VP developed and designed the study. SC has performed all the experiments and was responsible for data acquisition. VP supervised the experiment and interpreted the data. MP and VP wrote the manuscript. HS and VT reviewed the data and supported for writing the manuscript. The authors have read and approved the manuscript.

## Funding

There is no funding source for this project.

## Availability of data and materials

All data and materials are available upon request.

## Ethics approval and consent to participate

Not applicable

## Consent for publication

Not applicable

## Competing interests

The authors declare no competing interests.

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Received: 2 November 2020 Accepted: 8 February 2021

Published online: 18 February 2021

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