Research Article

Development and validation of a novel isocratic RP-HPLC method for simultaneous determination of 6-gingerol and thymol in herbal formulation NBIOTIC Premix

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ABSTRACT

A high-performance liquid chromatography method employing diode array detection was developed to determine levels of the 6-gingerol and thymol simultaneously, which are present in a natural complex matrices found in ayurvedic commercial product NBIOTIC Premix. Effective chromatographic separation of 6-gingerol and thymol was achieved using a Phenomenex Luna5μ (RP C18, 25 cm × 4.6 mm × 100Å) column with isocratic elution of the mobile phase composed of acetonitrile and water in 50:50 ratio. The proposed HPLC method was statistically validated with respect to linearity, range, precision, accuracy, selectivity, and robustness. Calibration curves were linear in the ranges of 6.0–48.0 μg/ml for 6-gingerol and 15.0–75.0 μg/ml thymol, respectively, with correlation coefficients >0.996. The HPLC method was applied to herbal formulation NBIOTIC Premix, in which the phytoconstituents were successfully quantified with good recovery values with no interfering peaks from the excipients.

Keywords: 6-Gingerol, HPLC, NBIOTIC Premix, phytoconstituents, polyherbal, simultaneous, thymol

Introduction

6-Gingerol chemically is (S)-5-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone and is main phytoconstituent of *Zingiber officinale* responsible for its biological activities. Thymol an antiseptic, eliminate internal parasites, used in hookworm infection and a phytoconstituent of *Trachyspermum ammi*, chemically, it is 5-Methyl-2-(propan-2-yl) phenol. Structural formulas of 6-gingerol and thymol are given in Figure 1.

NBIOTIC Premix is a proprietary polyherbal formulation of Ayurvet Limited, used as natural growth promoter for pigs and poultry. It is a perfect blend of essential oils and other secondary plant metabolites. This formulation is a premix of herbs such as *Z. officinale*, *Allium sativum*, and *Trigonella foenum-graecum* and essential oils like *T. ammi* as main constituents. These herbs contain number of secondary plant metabolites such as terpenoids, alkaloids, flavonoids, bitters, and tannins. These collectively with a synergistic effect through polyherbal formulation act as a natural growth promoter. The biggest advantage of the natural growth promoter over antibiotic growth promoter is that they do not bear any risk regarding the bacterial resistance or undesirable residue in animal products.[1-3]

The premix contains a variable amount of all ingredients due to their recommended pharmacological dose and activity. This variable amount of ingredients in such a polyherbal formulation makes the process of routine analysis difficult. Moreover, the active compounds have very different polarity and, therefore, chromatographic behavior. The literature reveals a number of analytical methods published for 6-gingerol and thymol. So far, no single HPLC method is reported to determine the mentioned ingredients quantitatively in this combination.[4-6]

To the best of our knowledge, the methods described in the literature do not cover the simultaneous analysis of these two analytes 6-gingerol and thymol in a single herbal formulation. Therefore, the main

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objective of this work was to develop a single separation method for quantifying these two analytes which are present in variable concentrations in NBIOTIC Premix. Within this context, a simple alternative methodology for the determination of these markers in herbal formulation using isocratic chromatographic mode in analysis time of 30 min was proposed. After validation of the method for various parameters, the method proved to be successful and was applied to the analysis of commercial product containing these phytoconstituents respective herbs as ingredients.[7,8]

Experimental

Chemicals and reagents

All the reagents and solvents were of AR or HPLC grade as per requirement. The active compound 6-gingerol was isolated in R&D Phytochemistry Lab Ayurvet Limited and structure was established by interpreting the ^{1}H, ^{13}C, and 2D NMR spectra. Thymol standard was procured from SD Fine Chemical Limited. Latest controlled samples of NBIOTIC Premix were obtained from the QA/QC department of AYURVET LIMITED, Baddi.

Instrumentation

The HPLC system consisted of WATERS, binary pump 515 with PDA 2996 detector, USA. Separation was obtained on Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 µm). The data were acquired on the Empower 2.0 controlling software (all equipment from Waters, Milford).

Preparation of standard stock solution

Preparation of standard stock solution of 6-gingerol: Accurately weighed around 5 mg of standard dissolved in 50 ml of methanol to obtain stock concentration of 100 µg/ml.

Preparation of standard stock solution of thymol: Accurately weighed around 5 mg of standard dissolved in 50 ml of methanol to obtain stock concentration of 100 µg/ml.

Preparation of mixed standard solution

A mixed standard solution was prepared from these stock solutions. Transfer 50 ml of each of the stock solution to a 100 ml volumetric flask to obtain mixed stock concentrations of 50 µg/ml each marker compound. Further dilutions were made with methanol to get desired concentration for linearity and quantification of 6-gingerol and thymol, respectively, in the formulation.

Preparation of sample solution of NBIOTIC premix

For sample preparation, 2 g NBIOTIC Premix was refluxed with 50 ml of methanol for 1 h and filtered, repeated the process twice. The final volume was made to 100 ml with methanol, filtered the solution through 0.45 µm membrane filter before injecting into HPLC.

Chromatographic conditions

Initial trials were carried by a gradient mode of analysis using the mobile phase, which consisted of a gradient solvent system of

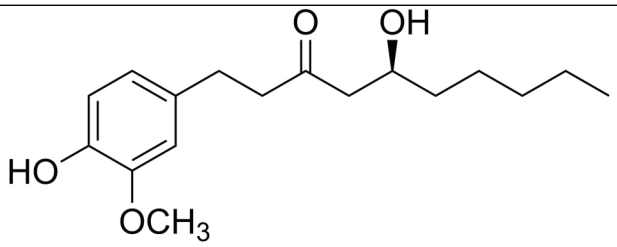
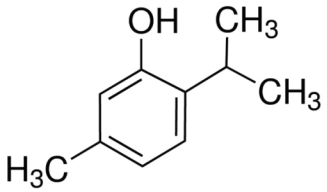
Phytoconstituent name	**Structural formula**
6-Gingerol	
Thymol	

Figure 1: The structures of 6-gingerol and thymol

water (containing 0.2% acetic acid) and acetonitrile (from 50:50 to 100:0 over 20 min). Experiments concluded lack of resolution of a complex mixture of different phytoconstituents and time consuming using the gradient approach of analysis. The simple isocratic mode was opted comprising water and acetonitrile in 50:50 ratio. The elution was clear and well-separated peaks of 6-gingerol and thymol with a flow rate of 1 ml/min over a runtime of 30 min. The both eluents were monitored at 280 nm. The mobile phase was filtered through 0.45 µm Millipore membrane filter and degassed before use. The injection volume was 20 µl and all analyses were performed at ambient temperature. Figure 2 shows the chromatogram for standard mixture and spectrum index plot

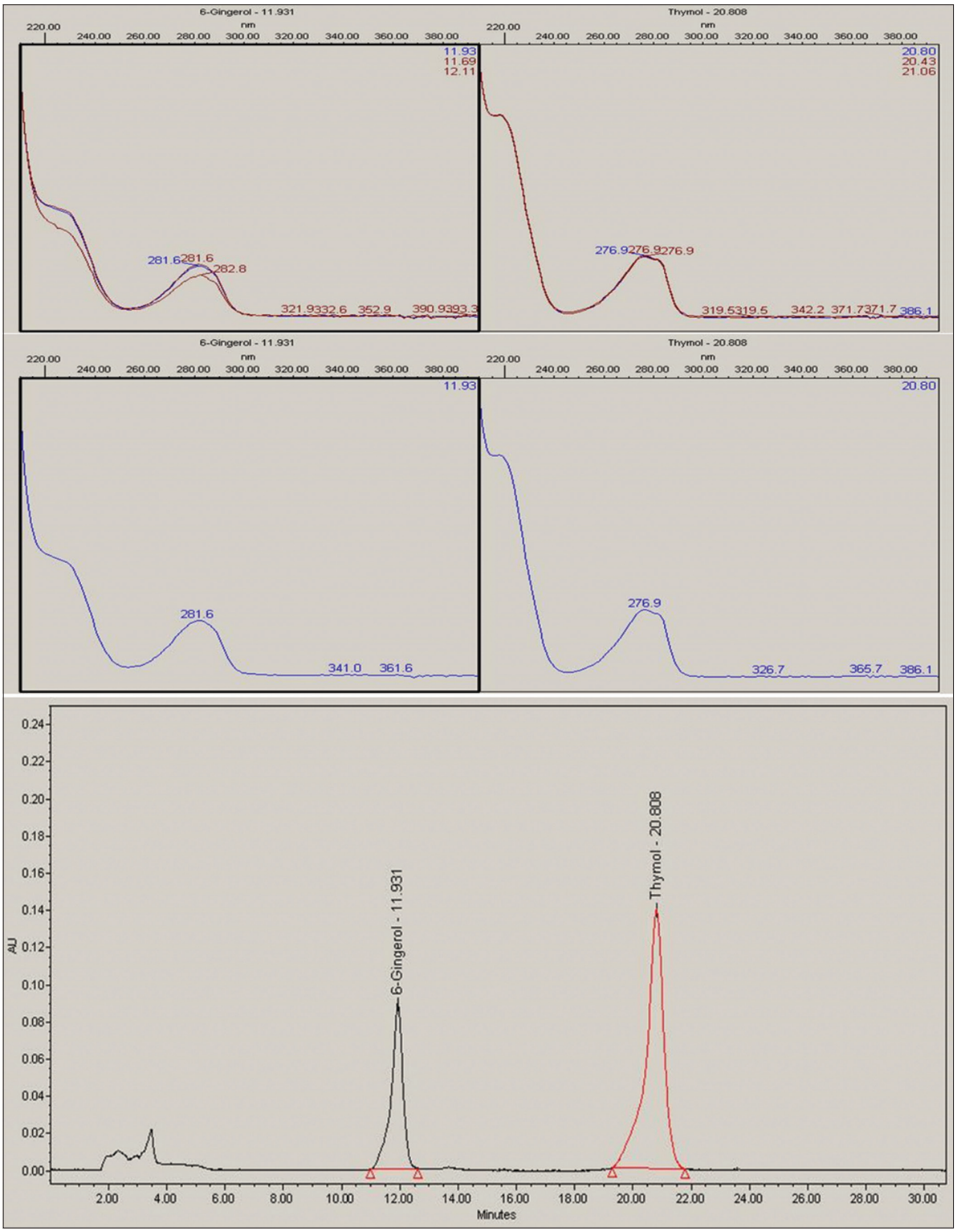


Figure 2: Spectrum index plot and chromatogram for the mixed standard solution of 6-gingerol and thymol

obtained through the optimized variables in accordance with the features described above.

RESULTS AND DISCUSSION

Method development and optimization of chromatographic conditions

The development of the method was based on the experience obtained from the HPLC method previously developed for the analysis of polyherbal formulations. The analytical results obtained by the method developed are only valid if the defined system suitability criteria are fulfilled. In this investigation, the experimental results indicate that the chromatographic system was suitable for intended analysis. Standard solution mixture containing known concentration of 6-gingerol and thymol was injected 7 times. Relative standard deviation (RSD) values for peak area and retention time of standard suggested the reproducibility for these parameters. The proposed method was validated for the determination of 6-gingerol and thymol simultaneously using following parameters as per International Council for Harmonization (ICH) guidelines.

Method validation

Selectivity and linearity

Method selectivity was assessed by the peak purity test (comparison between analyte peak and auto threshold in the purity plot) using photo diode array detector. The analyte chromatographic peak was not found to be attributable to more than 1 component indicating the method to be selective ICH. For linearity, an external method was used for the simultaneous determination of 6-gingerol and thymol ingredients. Five concentrations were chosen ranging from 50% to 150% of the target analyte concentrations in formulations. Hence, the linearity dilution concentrations were 6.0–48.0 µg/ml for 6-gingerol and 15.0–75.0 µg/ml for thymol, respectively. All the solutions were prepared by diluting in methanol. The linear regression equations for 6-gingerol, Y = 6462.x+3050 and for thymol, Y = 18,838x+8085, respectively. The regression coefficient values (R^2) were found to be 0.997 and 0.999, respectively, indicating an acceptable degree of linearity.

Specificity

The specificity of the method was accessed from the chromatogram where complete separation of 6-gingerol and thymol was achieved and against potential interferences in the presence of placebo (diluents, i.e., methanol). The peaks obtained were sharp and well separated at the baseline also excipients and matrices from formulation were not interfering with assay. No interferences were detected at retention times of 6-gingerol and thymol, also two well-defined spectral scans were observed in sample solution proving the method to be specific.

Precision

The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision is determined through the estimate of the RSD values. Precision studies were carried by carrying interday and intraday studies. The precision studies were done by injecting the prepared standard solution at three concentration levels in triplicate every day up to 3 consecutive days for interday studies. Intraday studies were done by injecting the standards at 3 different times on same day. %RSD values were measured, the low value of RSD (%) showed that the method is precise within the acceptance limit of 2%. The intra- and inter-day variability or precision data are given in Table 1. The results indicated good precision of the developed method.

Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the value, which is accepted reference value and the value found. Accuracy studies were done by the standard addition method. Accuracy is expressed as % recovery of the standard spiked to previously analyzed test sample of NBIOTIC Premix. The active standard phytoconstituents were spiked in previously analyzed NBIOTIC Premix sample at different concentrations and injected in developed chromatographic conditions in triplicate. The percentage recovery data for accuracy studies are shown in Table 2.

Table 1: Results of precision, LOD, LOQ, linear regression analysis, and their correlation coefficient for quantitative analysis of different marker compounds

S. No.	Parameters	6-Gingerol	Thymol
1.	Concentration range (µg/ml)	6–48	15–75
2.	Regression equation	y=6462.x+3050	y=18,838x+8085
3.	Correlation coefficient (r^2)	0.997	0.999
4.	Amount of marker compound in NBIOTIC Premix (%) (w/w) (mean, *n*=3)	0.05	1.72
5.	Method precision (repeatability) *n* – RSD %	1.7	0.5
	Intermediate precision (reproducibility) – RSD (%)		
6.	Intraday 1	1.21	0.79
	Interday 3	1.52	0.81
7.	LOD	0.04 µg/ml	0.062 µg/ml
8.	LOQ	0.12 µg/ml	0.186 µg/ml

y=Peak area response, x=Amount of marker compound. LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation

System suitability parameters

System suitability tests are an integral part of the analytical method, it is used to verify adequacy of the resolution and reproducibility of system. For study of system suitability parameter, seven replicate injections of mixed standard solution were injected and parameters such as peak area, retention time, asymmetry factor, and theoretical plates of the peaks were calculated.

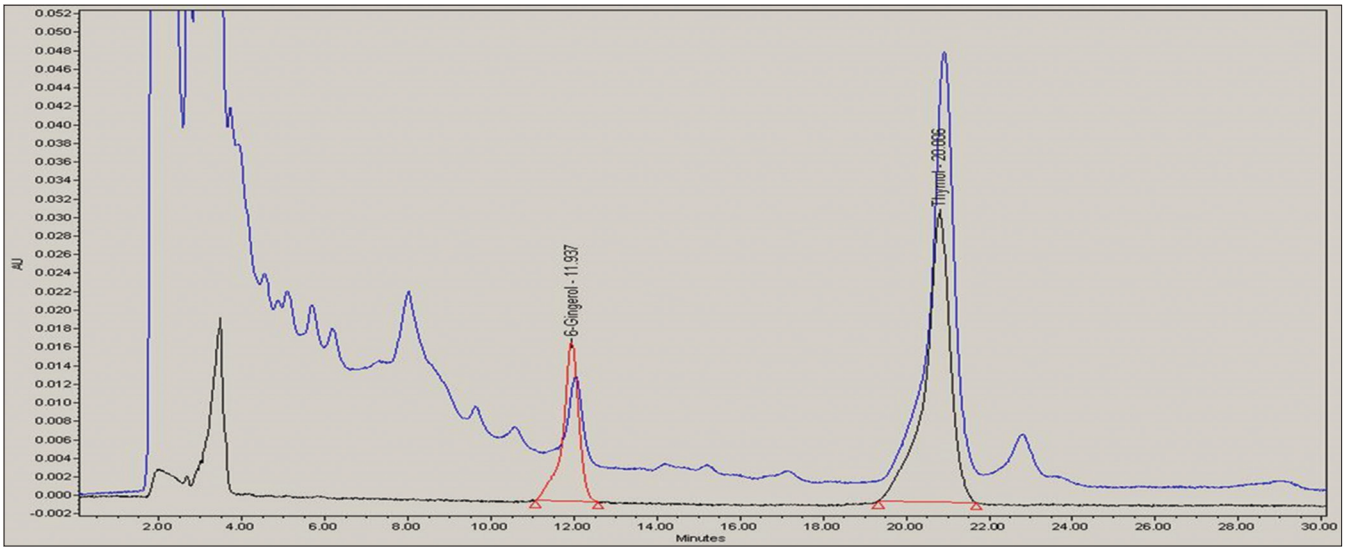


Figure 3: Comparison of standard and formulation chromatograms for simultaneous HPLC estimation of 6-gingerol and thymol

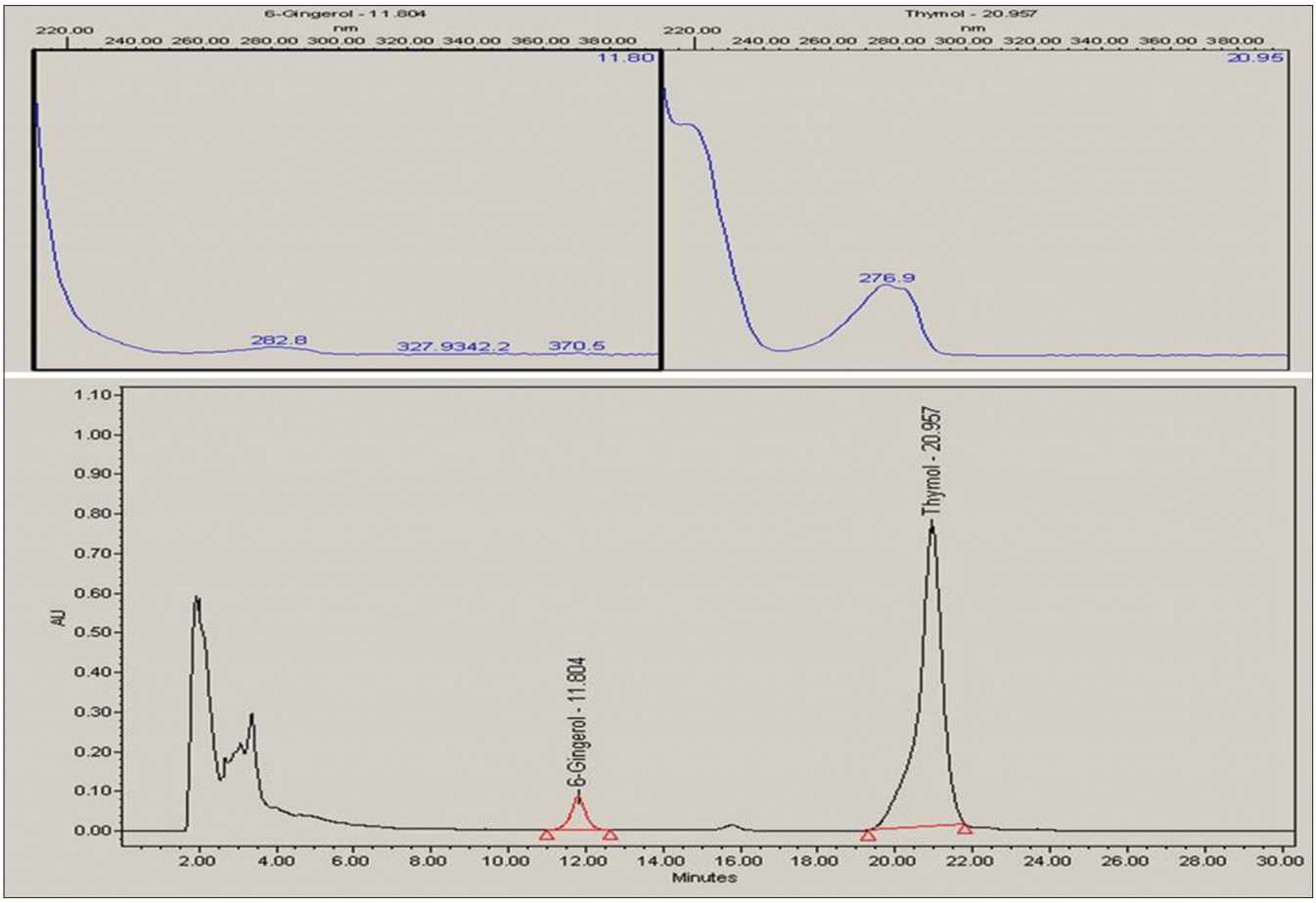


Figure 4: Spectrum index plot and chromatogram for simultaneous HPLC estimation of 6-gingerol and thymol in marketed formulation NBIOTIC Premix

Table 2: Results from determination of recovery

S. No.	Parameter	6-Gingerol			Thymol		
1.	Initial concentration in formulation (mg/g)	0.5	0.5	0.5	17.2	17.2	17.2
2.	Concentration added (mg/g)	0	2.0	4.0	0	40.0	80.0
3.	Total concentration (mg/g)	0.5	2.5	4.5	17.2	57.2	97.2
4.	Concentration found (mg/g)	0.48	2.41	4.38	16.80	54.20	94.68
5.	Relative standard deviation (%) (n=7)	0.98	0.95	0.95	0.97	0.96	0.98
6.	Recovery (%)	96.0	96.4	97.3	97.67	94.75	97.41
7.	Mean recovery (%)		96.57			96.61	

Limit of detection (LOD) and limit of quantification (LOQ)

For the determination of limits of detection and quantification, different dilutions of the markers were injected with mobile phase as blank and determined on the basis of signal-to-noise ratio 3:1 and 10:1, respectively. The LOD and LOQ for the standard marker compounds are calculated and tabulated in Table 1.

Analysis of formulation

The developed method was successfully applied to analyze 6-gingerol and thymol in marketed herbal formulation NBIOTIC Premix. No interferences of excipients were observed in analysis, a representative chromatogram for analysis of herbal formulation NBIOTIC Premix is shown in Figure 3. The chromatograms of standards and the test sample were then compared for the better understanding and selectivity of the method shown in Figure 4. The mean percentage recovery of phytoconstituents content in NBIOTIC Premix obtained by the proposed method was noted. The percentage recovery found was 96.57% for 6-gingerol and 96.61% for thymol. The results are given in Table 2.

Conclusion

In this study, a validated simple and reliable HPLC–DAD procedure was described for the simultaneous assay of a complex polyherbal formulation consisting secondary metabolites such as 6-gingerol and thymol which is indicated as natural growth promoters. To the bets of our present knowledge, this is the best attempt made for simultaneous assay of plant secondary metabolites in this polyherbal premix by any analytical methodology. Analytes (6-gingerol and thymol) were successfully resolved and quantified using a reverse-phase Phenomenex Luna5µ (C18, 25 cm × 4.6 mm × 100 Å) column in a relatively short run time with the last analyte eluting at 20.9 min the isocratic program contributed total run time of 30 min. Reliability was guaranteed as validation experiments proved that the HPLC method is linear in the proposed working range as well as accurate, precise, and specific. The good recovery percentage suggests that the excipients have no interference in the determination. The RSD (%) was also <2.0 showing a high degree of precision of the method. The proposed method was also found to be robust; hence, it can be recommended for the routine quality control of the studied phytoconstituents, either in bulk form or in their combination formulated in some other dosage form.

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