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Stability-indicating RP-HPLC method for the determination of 6-gingerol in polyherbal formulations

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Abstract

Background: Among different systems medicine practiced worldwide, Unani medicine is the classical one and still commonly practiced in India and abroad for centuries. As it is widely used by a majority of population, it is necessary to come up with a systematic approach to develop well-designed methodologies for the quality control of different polyherbal formulations which are used for treatment of various diseases in this system of medicine.

Methods: A reverse phase stability indicating HPLC method was developed for the determination of 6-gingerol in polyherbal formulations. Separation of 6-gingerol was achieved on reverse phase C18 (250 × 4.6 mm) column with a mobile phase containing methanol: 0.05% orthophosphoric acid in water (60:40, *v/v*) at 280 nm using UV-visible detector. The flow rate was kept as 1 mL/min. The proposed method was validated according to ICH guidelines for accuracy, precision, robustness, LOD, and LOQ, and statistical analysis proved method was accurate, precise, and robust.

Results: The linear regression analysis data showed a good linear relationship ($r^2 = 0.9989 \pm 0.0010$) for 6-gingerol in the concentration range of 0.5 μg to 500 μg/mL. This proved the method can be employed for the determination 6-gingerol even in nanogram levels. The detection (LOD) and quantification (LOQ) limits were found to be 0.05 μg and 0.18 μg/mL, respectively. Satisfactory recovery results were observed from the herbal compound formulations (98.5 to 101%). Intra- and inter-day precisions of the method were found convincing, with relative standard deviation (%RSD) values in the range of 0.5 to 1.9%. Forced degradation studies of 6-gingerol was also carried out under different stress conditions which showed that the drug is stable in acidic and basic conditions whereas unstable against dry heat.

Conclusions: Hence, newly validated HPLC method can be used for the quality control and standardization of different herbal formulations which contain *Zingiber officianalis* as one of the ingredients.

Keywords: HPLC; 6-Gingerol; Stability; Validation; Herbal compound formulation

Background

The Unani system of medicine is one of the commonly practiced treatment methods, which was followed by India and many other countries. As the name indicates, it originated from Greece. This system is based on the belief that there are four humors present in the human body - blood, phlegm, yellow bile, and black bile. Every person has a unique humoral constitution, which represents his healthy state. Different types of herbal formulations are used in Unani system such as solid (Qurs, Habbs, Shafoof), semi-solid (Khamira, Itrifal, Majoon), and liquid (Sharbat) preparations (Bilal and Jamal, 2007).

Habb-e-Mastagi is a tablet formulation which has been commonly used in Unani as stomachic, carminative, and treatment for other problems associated with the stomach. It contains seven ingredients, viz. *Terminalia chebula, Zingiber officinale, Pistacia lentiscus, Aloe barbadensis, Allium ascalonicum, Nardostachys jatamansi* and Indian salt (Anonymous 2001).

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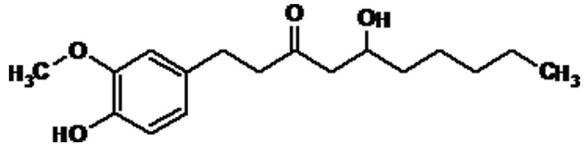

Figure 1 Chemical structure 6-gingerol.

Ginger, the rhizome of *Zingiber officinale*, is one of the most popular spices and has been frequently used in traditional medicines like Ayurveda and Unani both in fresh and dried forms. The rhizome contains essential oils and resins that give ginger its characteristic odor and spicy flavor and are responsible for its medicinal uses. Ginger is used primarily to treat nausea, upset stomach, and other gastric disturbances. Numerous chemical investigations of this material have led to the isolation and identification of a large number of biologically active compounds, such as 6-, 8-, and 10-gingerols, shogaols, and zingerones (Jian Ping et al. 2004).

6-Gingerol (5-hydroxy-1-(42hydroxy-32-methoxyphenyl)-3-decanone) (Figure 1), a major pungent ingredient of ginger, also has potent antioxidant activity. 6-Gingerol is the most abundant constituent of fresh ginger, but it decreases during postharvest storage and processing, especially thermal processing (Amir et al. 2004).

A thorough literature survey revealed that only a limited number of researches has been done on the quality control of Unani medicines, even though these drugs have been used for many decades and no relevant information exists about their quality, safety, and efficacy. Literature study revealed that there are publications on the quantification of gingerols by different analytical techniques like HPLC (Lee et al. 2007; Wang et al. 2002; Schwertner, and Rios 2007), LC-MS (Wang et al. 2009), and HPTLC (Rai et al. 2006). But all these methods lack complete validation parameters which include stability-indicating parameter as well. As far as we know, no chromatographic methods have been reported on the stability-indicating studies of this molecule even though it is one of the important parameters of validation. Hence, it was thought worthwhile to develop and validate a stability-indicating HPLC method for quantification of 6-gingerol in polyherbal formulation as per ICH guidelines (ICH, Q2 R1 2005). Moreover, the proposed HPLC method is superior to other reported methods on its low retention time, high sensitivity, and accuracy. Still, there are several chromatographic HPLC and HPTLC methods that have been developed and validated by an author, which are in use for the quality control of herbal drugs (Ansari et al. 2005; Ahmad et al. 2008). The developed HPLC method will help in the quality control and standardization of many herbal compound formulations which contain *Zingiber officinale* as one of the active ingredients.

Methods

Reagents and chemicals

6-Gingerol (98.5%) was obtained as gift sample from Sami Labs Ltd., Bangalore, India. HPLC grade methanol was purchased from Merck, Mumbai, Maharashtra, India. Milli-Q water was used throughout the experiment which was prepared using Millipore water purification System (New Orleans, USA). Orthophosphoric acid used for the experiment was of analytical grade and was purchased from SD Fine-Chem Ltd., Mumbai, India.

HPLC instrumentation

Chromatographic experiments were conducted on Shimadzu HPLC instrument comprising of quaternary LC-10A VP pumps, a variable wavelength programmable UV-visible

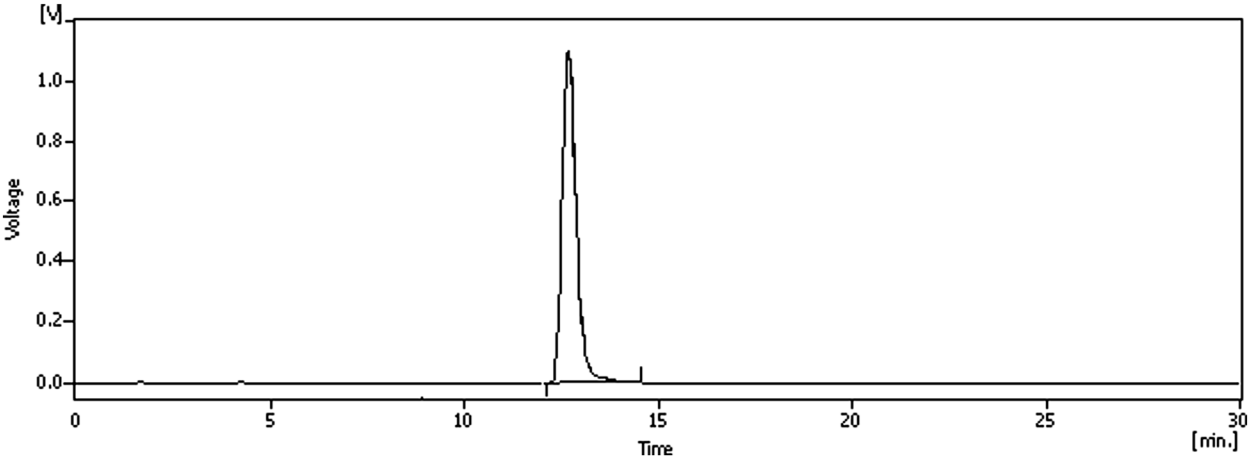

Figure 2 HPLC chromatogram of standard 6-gingerol at 280 nm showing R_t 13.48 min.

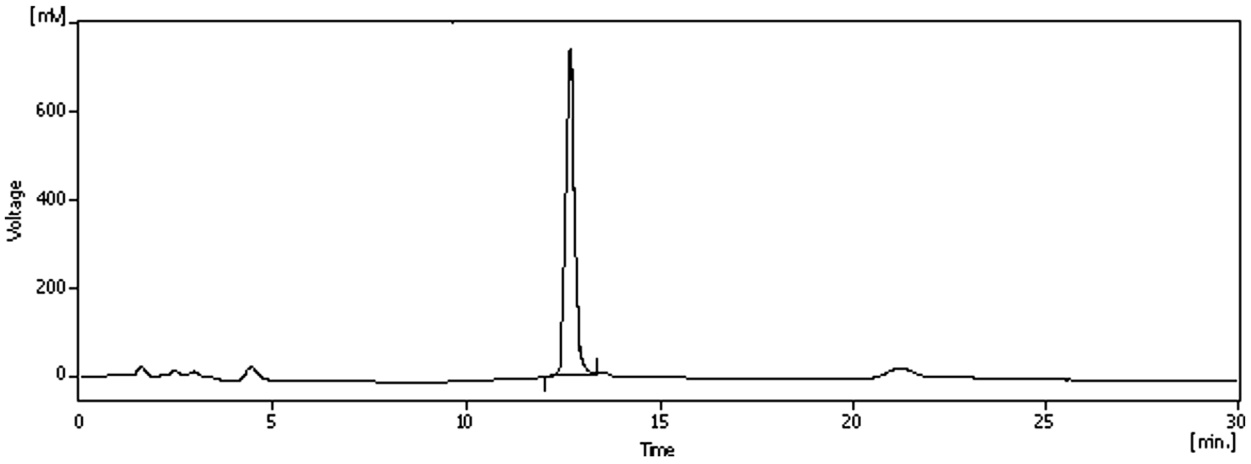

Figure 3 HPLC chromatogram of Habb-e-Mastagi tablet formulation at 280 nm showing 6-gingerol at R_t 13.59 min.

detector, SPD-10A VP column oven, and a SCL 10A VP system controller. The instrument was controlled by use of Class VP 5.032 software. Samples were injected by using a rheodyne injector fitted with a 20-μl fixed loop. Standard and sample solutions were filtered through 0.22-μm syringe filter (nylon) before injection. Compounds were separated by using C18 reverse phase column (25 × 4.6 mm, particle size 5 μm). >The mobile phase used was methanol: 0.05% orthophosphoric acid in water (60:40 *v*/*v*) with a flow rate of 1 mL/min. Analysis was performed at room temperature. Detection was carried out at a wavelength of 280 nm with UV-visible detector.

Preparation of mobile phase

Orthophosphoric acid (0.05%) was prepared by dissolving 0.25 mL of orthophosphoric acid in 500 mL of milli-Q water and sonicated the mixture for 10 min. Mobile phase was prepared by mixing HPLC grade methanol and 0.05% orthophosphoric acid in the ratio of 60:40, filtered the solution through 0.22-μm filter, sonicated and degassed the mobile phase.

Calibration curve for 6-gingerol

A stock solution of 6-gingerol having a known concentration of 1,000 μg/mL was prepared in methanol. From this, different aliquots were prepared to get known concentrations from 1 to 500 μg/mL. The calibration graph was plotted using peak area versus drug concentration to obtain linearity, the least square regression equation, and correlation coefficient.

Preparation of sample solution

Twenty tablets were taken randomly from the formulation and weighed it accurately to find out the average

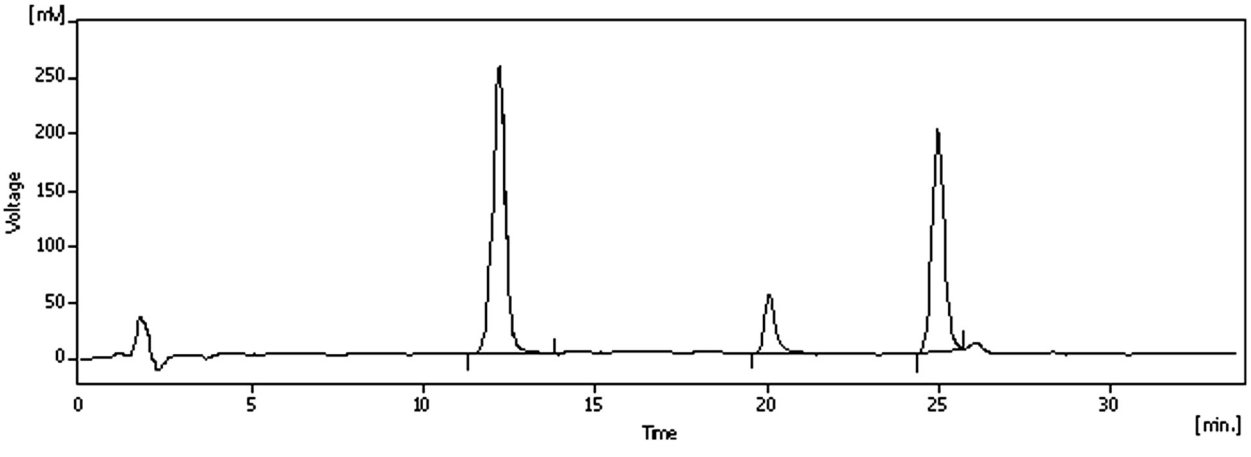

Figure 4 HPLC chromatogram of standard 6-gingerol after treating at 60°C for 1 h at 280 nm showing 6-gingerol at R_t 13.78 min with degradation products eluting at the R_t 20.47 and at R_t 26.52.

Table 1 Calibration data for 6-gingerol ($n = 3$)

Concentration (μg/mL)	Mean area ± SD ($n = 3$)	%RSD	SE
1	98,047 ± 621.4	0.63	358
10	995,776 ± 4,249.1	0.43	2,453
20	206,976 ± 4,164.9	2.0	2,404
50	4,958,881 ± 58,021.4	1.2	33,499
100	9,815,383 ± 53,584.9	0.55	33,499
200	19,835,527 ± 91,336.1	0.46	52,734
300	29,746,240 ± 553,656.1	1.9	319,662
500	49,588,818 ± 730,049.7	1.5	421,506

SD, standard deviation; RSD, relative standard deviation; SE, standard error.

weight. The tablet was triturated into fine powder to get a uniform sample. Two grams of powdered sample was transferred into a 50-mL conical flask having about 20 mL of methanol and sonicated for 20 minutes at room temperature. It was filtered using Millipore water purification System (New Orleans, USA) and filtrate was made up to volume 10 mL in volumetric flask with HPLC grade methanol. This was again filtered through 0.22-μm syringe filter prior to injection.

Validation of methodology

Accuracy as recovery

The accuracy of the method was determined as recovery by standard addition method. For this, pre-analyzed samples were spiked with standard drug at three different concentration levels, i.e., 50, 100, and 150%, and the mixtures were reanalyzed by the proposed method.

Precision

The precision of the method was carried out by doing repeatability and intermediate precision. In repeatability, six different injections of the same standard (three concentrations) were injected and calculated the assay. The %RSD of area and R_t were calculated. In intermediate precision, intra-day, inter-day, and inter-system precisions were carried out. Intra-day and inter-day precisions were done by preparing and applying three different concentrations of standard in triplicate six times a day and similarly on six different days, respectively. Inter-system precision was done by repeating the same procedure in different HPLC system. Assay for each analysis was calculated, and %RSD was determined.

Table 2 Linear regression data for calibration plot ($n = 3$)

Parameters	Observations
Linearity range	0.5 to 500 (μg/mL)
Regression equation	$y = 100{,}017x - 319{,}621$
Correlation coefficient	0.9989
Slope ± SD	100,017 ± 999.8
Intercept ± SD	319,621 ± 3,339.8

y, peak area; *x*, concentration of standard (μg/mL).

Table 3 Accuracy of the method ($n = 3$)

% of standard spiked to the sample	Theoretical content (μg/mL)	Amount of drug recovered (μg ± SD)	% of drug recovered	%RSD
0	18	17.7 ± 0.21	98.2	1.17
50	27	29.3 ± 0.3	108.5	1.02
100	36	37.5 ± 0.64	104.3	1.7
150	45	46.6 ± 0.15	103.6	0.32

Robustness of the method

Robustness of the proposed method was determined at single concentration level (100 μg/mL) in three different ways, i.e., by changing the composition of mobile phase, making deliberate change in the flow rate, and changing the detecting wavelength. The %RSD of the experiment was calculated to assess the robustness of the method.

Specificity

The specificity of the method was determined by doing stability studies by exposing the sample solution (100 μg/mL) in accelerated conditions like 0.1 M formic acid, 0.1 M NaOH, and dry heat. The resulting solutions were analyzed, and the analyte peak was evaluated both for peak purity and for resolution from the nearest eluting peak. All the samples were filtered before injecting to HPLC system.

Limit of quantification and limit of detection

The limit of quantification and limit of detection were determined based on the technique of signal-to-noise ratio. The concentration of sample giving a signal-to-noise ratio of three was fixed as the LOD. The concentration of the sample giving a signal-to-noise ratio of ten was fixed as LOQ. Once the LOD and LOQ were determined, six replicates of blank and the standard solution at the level of LOD and LOQ were applied and the %RSD calculated.

Table 4 Repeatability of the method ($n = 3$)

Conc (μg/mL)	Peak area		Retention time	
	Mean peak area ± S.D	%RSD	Mean R_t ± S.D	%RSD
50	4,963,874 ± 67,912.6	1.4	13.69 ± 0.20	1.5
100	9,842,050 ± 110,151.4	1.1	13.56 ± 0.07	0.56
200	19,471,334 ± 356,784.7	1.8	13.62 ± 0.16	1.2

Table 5 Precision of the method ($n = 3$)

Conc (μg/mL)	Inter-day precision		Intra-day precision		Inter-system precision	
	Mean area ± S.D	%RSD	Mean area ± S.D	%RSD	Mean area ± S.D	%RSD
50	4,960,041 ± 43,252.2	0.87	4,986,741 ± 6,581.8	0.13	4,890,871 ± 89,279	1.7
100	9,940,953 ± 49,519.8	0.50	9,964,553 ± 30,550.5	0.31	9,846,981 ± 100,596	1.0
200	19,607,079 ± 200,575	1.02	19,540,413 ± 113,271	0.58	19,507,079 ± 395,006	2.02

Conc, concentration of standard in μg/mL.

Acid-induced degradation study

Freshly prepared solution of 6-gingerol (1,000 μg/mL) in 0.1 M methanolic formic acid solution was transferred to a 100-mL volumetric flask. Warmed the solution at 30°C for 5 min and allowed to stand for 1 h for the completion of degradation reaction at room temperature, neutralized the solution using dilute NaOH solution and made up the volume with methanol (100 μg/mL solution). The blank and sample solutions were injected, and the chromatograms were analyzed for peak purity and resolution between the peaks.

Base-induced degradation study

Freshly prepared solution of 6-gingerol (1,000 μg/mL) in 0.1 M methanolic NaOH was transferred to a 100-mL volumetric flask, warmed at 30°C for 5 min, and allowed to stand for 1 h for the completion of degradation reaction at room temperature, neutralized using dilute HCl solution, and make up the volume with methanol (100 μg/mL solution). The blank and sample solutions were injected, and the chromatogram was analyzed for peak purity and resolution between the peaks.

Heat degradation study

The standard 6-gingerol (10 mg) was stored under dry heat condition in hot air oven at 60°C for 1 h. The sample was taken out after 1 h, transferred to a 100-mL volumetric flask, and diluted up to the volume with methanol to get a known concentration of 100 μg/mL. The solution was injected in HPLC along with the blank solution, and the chromatogram was analyzed for peak purity and resolution between the peaks.

Results and discussion

Optimization of method

The mobile phase was chosen after several trials with methanol and water in various proportions and at different pH values. In methanol and water, when used in the composition of 50:50 *v*/*v*, standard has given a sharp peak with RT around 10 min, but in sample, separation of 6-gingerol from the immediate impurities was unsatisfactory. After several trials, mobile phase changed to methanol and 0.05% of orthophosphoric acid in water in the ratio of 60:40 *v*/*v*. This mobile phase helped in achieving optimal separation with good resolution and sharp and well-defined peak in both standard and samples showing R_t of 6-gingerol at 13.5 ± 0.4 min, (Figures 2, 3, and 4). The mobile phase was also found good enough to separate the dry heat degradation products of 6-gingerol (Figure 4).

Calibration of 6-gingerol

The calibration curve was plotted by using peak area against concentration and was found linear in the range of 1 to 500 μg/mL with a good linear relationship of 0.9989. Calibration data, with their relative standard deviation, %RSD, slope, and intercept with their standard deviation, were given in Tables 1 and 2. The low values of %RSD and standard deviation indicated that the method is precise and reproducible. The linear regression data for the calibration plot are indicative of a good linear relationship between peak area and concentration over a wide range.

Analysis of tablet formulation

All sample solutions were prepared in methanol. Sample peaks were distinct and observed at R_t 13.5 ± 0.4 min which found to be corresponding to R_t of standard

Table 6 Robustness of the method by changing mobile phase composition ($n = 3$) (concentration 100μg/mL)

Mobile phase composition methanol:H_3PO_4 in H_2O (60:40 *v*/*v*)			Mean area ± SD	Mean R_t ± SD	%RSD of area	%RSD of R_t
Actual	Used	Level				
60:40	58:42	−2	9,867,378 ± 92,101	13.4 ± 0.26	0.93	1.9
	60:40	0	9,870,953 ± 64,002	13.4 ± 0.1	0.65	0.75
	62:38	+2	9,871,953 ± 108,701	13.6 ± 0.36	1.10	2.6

R_t, retention time.

Table 7 Robustness of the method by changing flow rate ($n = 3$) (concentration 100μg/mL)

Flow rate (mL/min) methanol:H_3PO_3 in H_2O			Mean area ± SD	Mean R_t ± SD	%RSD of area	%RSD of R_t
Actual	Used	Level				
1	0.8	−2	9,811,472 ± 83,932	14.1 ± 0.1	0.84	0.70
	1	0	9,929,473 ± 51,018	13.4 ± 0.05	0.51	0.43
	1.2	+2	9,827,873 ± 74,959	13.1 ± 0.15	0.75	1.1

H_3PO_4 in H_2O, solution orthophosphoric acid in water.

6-gingerol. The 6-gingerol content in the formulation Habb-e-Mastagi, from the analysis of three batches, was found to lie between the range of 0.033 and 0.045 mg/tablet with a %RSD of 1.1%, showing reproducibility of the developed method and can be employed by any of the lab for the analysis of 6-gingerol in tablet formulation.

Validation parameters

Accuracy as recovery

Recovery study for the proposed method was conducted by spiking previously analyzed test solution with the addition of standard drug solution. The recovery of the particular method was found (98.2 to 108.5%). The values of % recovery, standard deviation, and %RSD are listed in Table 3.

Precision

For the proposed analytical method, repeatability and intermediate precisions were calculated and reported in terms of %RSD in Tables 4 and 5. Intermediate precision included data of intra-day, inter-day, and inter-system precisions. The low values of %RSD indicated the reproducibility of the method. Satisfactory result of precision indicated that the method can be adopted in any lab which keeps standard and by any qualified person for the routine analysis of 6-gingerol in different formulations.

Robustness

For the method, robustness was studied by changing the composition of mobile phase, flow rate, and detection wavelength. Changes in the R_t and peak area were monitored. The standard deviation and %RSD were calculated and listed in Tables 6, 7, and 8. The low values of the %RSD show the robustness of the method.

Limit of quantification and limit of detection

The LOD and LOQ were determined by signal-to-noise ratio method and found to be 0.35 and 1 μg/mL, respectively.

Specificity

The specificity of the method was determined by exposing the drug in different stress conditions like 0.1 M formic acid, 0.1 M NaOH, and dry heat. There was no degradation observed after treatment with 0.1 M formic acid and 0.1 M NaOH, as the chromatograms show no significant difference in area and retention time of 6-gingerol, whereas a significant change in the area was found on exposing drug to dry heat condition, while retention time remained same (Table 9).

An increase in demand in the usage of traditional medicine is found worldwide in recent years. By considering this fact in mind, a RP-HPLC method reported here represents a simple, accurate, and rapid technique for the quantification of 6-gingerol in traditional formulation. The efficiency of the method was evaluated from conducting recovery experiments and the results found to be promising, which indicates that the method can be executed successively in various multi-component herbal formulations with high accuracy and precision. The linearity experiments conducted proved the method can be applied for the samples which contain these components in a wide range. The quantification limits were found to be low enough for the successful employment of the method in different polyherbal formulation which contains 6-gingerol even in very minute quantities.

The forced degradation studies also successfully carried out, and it was found that there was no degradation observed after treatment with 0.1 M formic acid and 0.1 M NaOH, at 30°C for 5 min, whereas a significant degradation was observed (21.7%) when the sample is kept under dry heat condition in hot air oven at 60°C for

Table 8 Robustness of the method by changing wavelength ($n = 3$) (concentration 100μg/mL)

Detection wavelength (nm)			Mean area ± SD	Mean R_t ± SD	%RSD of area	%RSD of R_t
Actual	Used	Level				
280	278	−2	9,823,452 ± 77,546	13.1 ± 0.26	0.78	1.9
	280	0	9,921,953 ± 65,391	13.3 ± 0.20	0.89	0.66
	282	+2	9,895,383 ± 100,166	13.6 ± 0.23	1.01	1.7

Table 9 Forced degradation studies of 6-gingerol

Stress conditions	Mean area ± SD	Number of degradation products with retention time in minutes	% of 6-gingerol degraded
0.1 M HCOOH	9,803,858 ± 96,712	0	0
0.1 M NaOH	9,789,674 ± 111,636	0	0
Dry heat	7,712,810 ± 40,050	2 (20.475, 25.525)	21.7

0.1 M HCOOH, 0.1 molarity formic acid; 0.1 M NaOH, 0.1 molarity sodium hydroxide.

1 h. From the chromatogram of heat degradation, study confirmed that the developed HPLC method is capable for separating the impurities from the 6-gingerol peak. The proposed method is useful as a reliable, fast, and effective tool for the quality control as well as standardization of different formulations, especially traditional polyherbal formulations of Unani and Ayurvedic systems of medicines.

Conclusion

A HPLC method with UV detection has been developed for the quantification of 6-gingerol in herbal compound formulations. Results obtained in the validation process and in drug analysis were encouraging and indicated suitability for routine tests. The method proposed was found to be easier, economic, sensitive, and less time consuming as compared with the reported HPLC and LC-MS methods with stability-indicating and applicable in general laboratory conditions. The method can be adopted for the determination of 6-gingerol in different herbal drugs and formulations containing *Zingiber officinale* as one of the ingredients.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KYT and SA developed the HPLC analytical method. PA, MS & SS actively participated in stability studies and validation of the method. All authors read and approved the final manuscript

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