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Original Article

VALIDATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS DETERMINATION OF 6-, 8-, 10-GINGEROLS AND 6-SHOGAOL FROM GINGER EXTRACTS

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ABSTRACT

Objective: Development and validation of a High-Performance Liquid Chromatography (HPLC) method for the simultaneous estimation of 6-, 8-, 10-Gingerols and 6-Shogaol in ginger extract using authentic standards.

Methods: The chromatographic separation was achieved by using a C18 column and a mobile phase composed of acetonitrile, ortho-phospohoric acid in water and methanol. The proposed method was validated in terms of the analytical parameters such as specificity, accuracy, precision, linearity, range, the limit of detection (LOD) and limit of quantification (LOQ) according to ICH guidelines.

Results: Linear calibration curves were obtained over concentration ranges of 10-250 µg/ml for 6-, 8-, 10-gingerols and 6-shogaol with determination coefficients more than 0.99 for each analyte. Intra and inter-day precisions of the method were found to be below 2% for each analyte, with relative standard deviation (% RSD) values in the range of 0.47 to 1.55% for 6-gingerol, 0.44 to 1.51% for 8-gingerol, 0.24 to 1.90% for 10-gingerol and 0.25 to 1.67% for 6-shogaol. The percentage recovery of gingerols and shogaol was obtained with an average of 99.53%, 99.97%, 100.13% and 100.53% respectively, which was well within acceptance range.

Conclusion: Simple, accurate, precise and rapid HPLC method was developed for the simultaneous analysis of 6-, 8-, 10-gingerols and 6-shogaol and validated in accordance with ICH guidelines. The developed method was found to be suitable for the standardization of herbal extracts and polyherbal formulations for the content of 6-, 8-, 10-gingerols and 6-shogaol.

Keywords: 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, Validation

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INTRODUCTION

Ginger (*Zingiber officinale* Roscoe), a member of the family *Zingiberaceae,* that has been extensively used in traditional medicines [1-2]. The major constituents in ginger rhizomes are phenolic compounds, terpenes, polysaccharides, lipids, organic acids, and raw fibers. Terpene components of ginger include zingiberene, β-bisabolene, α-farnesene, β-sesquiphellandrene, and α-curcumene, while phenolic compounds include gingerol, paradols, and shogaol [3-5]. Gingerols are the most abundant compounds present in the fresh and dry roots of ginger. Gingerol compounds with varying chain lengths were reported from the species of which 6-gingerol is the most abundant form [6]. Gingerols possesses diverse biological activities including anti-inflammatory, antioxidant, anticancer, analgesic, gastroprotective, cardiotonic, antipyretic, anti-angiogenic, anti-platelet aggregation effects and anti-hyperglycemia [7-20]. There is great demand for 6-gingerol due to its low toxicity and attractive medicinal potential [21]. Shogaols, the dehydrated form of gingerols, are found only small quantities in fresh root and are mainly found in the dried and thermally treated roots [22, 23]. The most abundant form of shogaols is 6-shogaol and has many biological effects such as antibacterial, antifouling and antioxidant [23, 24].

USP method is widely used for the estimation of gingerols from ginger rhizome in which the reference standard used is capsaicin [25]. Several analytical methods including high-performance liquid chromatography (HPLC) have been employed for the estimation of different gingerols where 6-gingerol was used as a reference standard [1, 2, 26, 27]. Gingerols, being the major active components possessing various pharmacological and physiological effects, an accurate optimization of total gingerol is highly essential for formulating products using ginger extracts.

Hence, the present study was aimed at the development of a validated High-Performance Liquid Chromatographic method for the fast and simultaneous determination of 6-gingerol (6-G), 8-gingerol (8-G), 10-gingerol (10-G) and 6-shogaol (6-S) in ginger extracts using authentic standard compounds.

MATERIALS AND METHODS

Reagents and samples

The reference standards of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol were purchased from Sigma-Aldrich, Germany. Acetonitrile and methanol were HPLC grade purchased from Merck, Germany. The water used in this study was ultrapure, obtained from a Milli-Q RO system (Millipore Corporation, France). Ortho-phosphoric acid used for the experiment was of analytical grade and was purchased from Merck Specialties Private Limited, Mumbai, India. Ginger extracts were obtained from the commercial batch manufactured at Arjuna Natural Pvt. Limited, Aluva, Kerala.

Instrumentation and chromatographic conditions

Liquid chromatographic separations were performed on a binary HPLC (Waters) separation 2998 series, variable wavelength photodiode array (PDA) detector module equipped with autosampler 2707 with injection volume 20 µl, 1525 pump, column used was C_{18} Sunfire (150 × 4.6 mm, 5 µ particle size) and data recorded using Empower 3 software. The separation was carried out with the mobile phase consisting of acetonitrile, (0.1%) ortho-phospohoric acid in water and methanol (55:44:1, v/v/v) at a flow-rate of 1.0 ml/min and chromatograms were monitored at 282 nm with PDA detector.

Preparation of standard solution

Standard stock solutions of 6-G,8-G,10-G and 6-S were prepared by dissolving 10 mg of 6-, 8-, 10-gingerols and 6-shogaol up to 10 ml of methanol, to get stock solution containing 1000 µg/ml of 6-, 8-, 10--gingerols and 6-shogaol. From this, different aliquots were prepared to get known concentrations from 10-250 µg/ml.

Preparation of sample solution

Approximately, 125 mg of Ginger extract-GOR-015 was weighted into a 25 ml volumetric flask and dissolved in methanol by sonication for 1 minute. The solution was filtered through 0.2 µm syringe filter and 20µl of the final sample was injected directly.

Method validation

The analytical method was validated as per ICH guidelines [28] for specificity, precision, accuracy, linearity, range, and limits of detection and quantification.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurity, degradation products, and matrix components. In this study, the specificity was demonstrated by running blank, standard and sample solutions.

Precision

The precision of the method was investigated with respect to repeatability and intermediate precision. The repeatability (intraday precision) of the method was evaluated by assaying three replicate injections of the gingerols and shogaol standard at concentrations of 15, 30, 75 and 200 µg/ml on the same day at different times. The percentage relative standard deviation (%RSD) of the peak area and retention time was calculated. The intermediate precision (inter-day precision) was demonstrated by evaluating the relative peak area at four different concentration levels as taken in an intraday study that cover the assay method. The precision was expressed as % RSD of the system and the samples analyzed in triplicate.

Accuracy

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the 6-G, 8-G, 10-G and 6-S standard solutions to the placebo samples, at three different levels (80%, 100%, and 120%) of the initial concentration of the sample. Standards, 6-G, 8-G, 10-G and 6-S were added to the samples at 50, 75 and 100 µg/ml of these compounds. Then the sample was prepared according to the sample preparation in triplicate. Average recoveries were calibrated by the formula recovery (%) = $\left\{\frac{(\text{amount found}-\text{original amount})}{\text{amount spiked}}\right\} \times 100$

Linearity

The linearity of measurement was evaluated by analyzing different concentrations (10-250 µg/ml) of the standard solutions. Calibration curve was constructed for gingerols and shogaol by plotting average peak area against concentration and regression equation. The correlation coefficient and the slope of the peak were also computed. All the samples were analyzed in triplicate.

Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curves of 6-G, 8-G, 10-G and 6-S standards. LOD was calculated according to the expression Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the formula $LOD = (3.3 \times \sigma) \div s$ in which σ is intercept standard deviation and s is the slope of the calibration curve. LOQ was established by using the expression $LOQ = (10 \times \sigma) \div s$.

Statistical analysis

The data were analyzed by one-way analyses of variance (ANOVA) software. The data is presented as the mean±SD (standard deviation) and p value<0.05 was considered as significant.

RESULTS

Liquid chromatographic separation of gingerols was carried out on a binary HPLC system (Waters 2998 series). Samples were separated on a Sunfire C18 column by using an isocratic solvent system composed of acetonitrile, (0.1%) ortho-phospohoric acid in water and methanol (55:44:1, v/v/v). The flow rate was constant at 1 ml/min and the detection wavelength was set at 282 nm. There is no interference was observed from diluents, impurities, or excipients present in the gingerol samples at 282 nm. The developed method precisely separates the constituents with well-resolved peaks for gingerols and shogaol under these conditions.

Specificity of the developed method

The specificity of this method was determined by analysis of the blank, standard and sample solution chromatograms. Good separation between the peaks of 6-G, 8-G, 10-G and 6-S was achieved, with the retention times, 4.553 min for 6-G, 9.754 min for 8-G, 23.947 min for 10-G and 12.227 min for 6-S. No interference was observed at the retention time when chromatograms of blank, standard and sample were monitored (fig. 1).

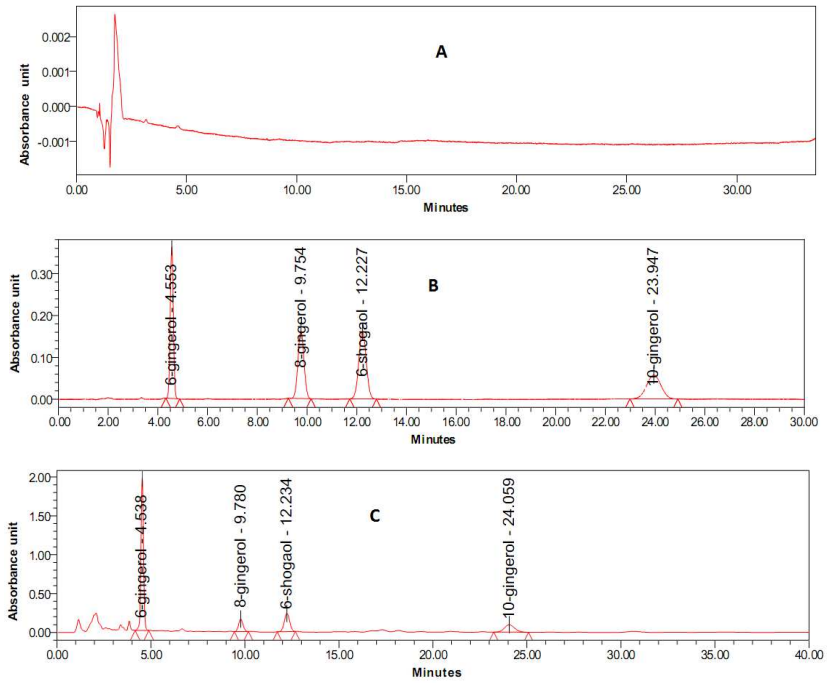

Fig. 1: Chromatograms of Blank (A), Standard (B) and Sample (C)

Linearity and range of the developed method

For linearity studies, eight solutions in the range of 10-250 µg/ml for 6-G 8-G, 10-G and 6-S were analyzed. Each concentration was made and analyzed in triplicate. The peak areas obtained against each concentration of the analytes were used to build a linear regression. Good linearity was observed over the above-mentioned range with linear regression equations $Y = 1.52e^4X - 3.06e^4$ for 6-G, $Y = 1.36e^4X - 8.8e^4$ for 8-G, $Y = 1.01e^4X - 1.52e^4$ for 10-G and $Y = 1.09e^4X + 3.67e^4$ for 6-S (X is the concentration of analyte in µg/ml and Y is peak area). The value of the correlation coefficient was 0.998 for 6-G, 0.998 for 8-G, 0.998 for 10-G and 0.995 for 6-S.

Accuracy of the developed method

This study was performed by adding known amounts of 6-G, 8-G, 10-G and 6-S to the placebo samples. Three-level of solutions were made and having concentrations at 50, 75 and 100 µg/ml for 6-G, 8-G, 10-G and 6-S. The recovery ranges for 6-G 8-G, 10-G and 6-S were 99.54-99.88%, 98.78-100.12%, 99.37-100.82% and 99.87-101.10% respectively for all three spiked levels. The relative standard deviation ranged from 0.471-1.808% for 6-G, 0.275-1.346% for 8-G, 0.855-1.543% for 10-G and from 0.553-1.670 % for 6-S.

Precision of the developed method

For the proposed analytical method, repeatability and intermediate precisions were calculated and reported in terms of relative standard deviation (%RSD) in table 2. Intermediate precision and repeatability included data of interday and intraday analysis. The relative standard deviation ranged from 0.47-1.55% for 6-G, 0.92-1.51% for 8-G, 0.24-1.90% for 10-G and 0.45-1.67 % for 6-S for intraday analysis and 0.50-0.78% for 6-G, 0.44-1.06% for 8-G, 0.34-0.95% for 10-G and from 0.25-1.00 % for 6-S for inter-day analysis. The low values of %RSD indicated the reproducibility of the method. The result showed that the developed method was precise.

Table 1: Accuracy data for 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol

Compound name	Amount added (µg/ml)	Mean recovered amount±SD (µg/ml)	Mean recovery %	% RSD
6-Gingerol	50	49.77±0.900	99.54	1.808
	75	74.907±0.353	99.36	0.471
	100	99.698±1.503	99.7	1.508
8-Gingerol	*50*	49.390±0.665	98.78	1.346
	75	75.087±0.206	100.12	0.275
	100	100.014±1.129	101	1.129
10-Gingerol	50	50.412±0.778	100.82	1.543
	75	74.531±0.637	99.37	0.855
	100	100.209±0.947	100.21	0.945
6-Shogaol	50	50.550±0.844	101.1	1.67
	75	74.904±0.414	99.87	0.553
	100	100.626±0.903	100.63	0.898

Values are expressed as mean±SD, n=3

Table 2: Repeatability and intermediate precision of the proposed method as determined after three intraday and inter day replicate injections of samples at four different concentrations

Compound name	Nominal concentration (µg/ml)	Intraday precision		Inter day precision	
		Measured concentration (µg/ml)		Measured concentration (µg/ml)	
		Mean Conc.±SD	% RSD	Mean Conc.±SD	% RSD
6-Gingerol	14.7	14.73±0.07	0.47	14.76±0.11	0.77
	29.4	29.51±0.15	0.52	29.48±0.23	0.78
	73.5	73.19±0.59	0.81	73.75±0.54	0.73
	196	194.39±3.01	1.55	195.48±0.97	0.50
8-Gingerol	14.25	14.14±0.13	0.92	14.27±0.15	1.06
	28.5	28.28±0.28	0.99	28.42±0.12	0.44
	71.25	71.03±0.88	1.24	71.27±0.34	0.47
	190	188.47±2.85	1.51	189.74±1.11	0.59
10-Gingerol	14.25	14.40±0.27	1.90	14.25±0.14	0.95
	28.5	28.30±0.32	1.11	28.46±0.15	0.54
	71.25	71.19±1.11	1.56	71.58±0.38	0.53
	190	189.97±0.45	0.24	190.69±0.64	0.34
6-Shogaol	13.5	13.39±0.14	1.05	13.53±0.12	0.92
	27	27.12±0.12	0.45	27.01±0.27	1.00
	67.5	67.04±1.12	1.67	67.15±0.17	0.25
	180	180.28±0.31	0.31	180.30±0.45	0.25

Values are expressed as mean±SD, n=3

Sensitivity of the developed method

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the formula $LOD = (3.3 \times \sigma) \div s$ and $LOQ = (10 \times \sigma) \div s$, in which σ is intercepted standard deviation and s is the slope of the calibration curve. The LOD values were 3.34, 2.43, 1.31 and 1.14 µg/ml and the LOQ values were 10.12, 7.39, 3.99 and 3.45 µg/ml for the simultaneous estimation of 6-G, 8-G, 10-G and 6-S respectively.

DISCUSSION

The HPLC method developed could achieved good separation and simultaneous estimation of gingerols and shogaols using Sunfire C18 column with a mobile phase composed of acetonitrile, (0.1%) ortho-phospohoric acid in water and methanol (55:44:1, v/v/v). The method is highly specific as there was no interference observed between chromatograms of blank, standard and sample. Good linearity with the coefficient of correlation 0.999 indicated that the proposed method was linear within the range 10-250 µg/ml. The percentage recovery of gingerols and shogaol was obtained with an average of 99.53%, 99.97%, 100.13% and 100.53% respectively, which was well within the range of 98-102%. The % RSD for intraday and interday precisions of gingerols and shogaol were 0.77, 0.93, 0.89 and 0.74 respectively. As per USP method, the percentage of total gingerol and 6-shogaol in ginger extract GOR-015 were found to be 16.26% and 1.12% respectively using capsaicin as

standard [25]. According to the method described herein, the percentage of gingerols and 6-shogaol were determined to be 20.68% and 1.56% respectively in the same extract. The results showed acceptable precision of the method, with RSD values much lower than 2%. The LOD and LOQ values showed the method was sensitive for the simultaneous estimation of 6-, 8-, 10-gingerols and 6-shogaol at low concentrations also.

CONCLUSION

Gingerols are the most important pharmacologically active components of ginger rhizomes. The present study is unique for the estimation of different gingerols, 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol using corresponding standards. The proposed analytical method is accurate, precise, linear, reproducible and within the range of acceptance as per ICH guidelines. The HPLC method developed can be adopted for the estimation of total gingerols present in the ginger extract and polyherbal formulations containing ginger.

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AUTHORS CONTRIBUTIONS

Benny Antony and Merina Benny conceived the study and design the experiments. Mary Reshma performed the analysis and wrote the manuscript with input from all authors.

CONFLICT OF INTERESTS

Declared none

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