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Development and Validation of a HPLC Method for 6-Gingerol and 6-Shogaol in Joint Pain Relief Gel Containing Ginger (*Zingiber officinale*)

Tanwarat Kajsongkram, Saowalux Rotamporn, Sirinat Limbunruang, Sirinan Thubthimthed

***Abstract*—**High Performance Liquid Chromatography (HPLC) method was developed and validated for simultaneous estimation of 6-Gingerol(6G) and 6-Shogaol(6S) in joint pain relief gel containing ginger extract. The chromatographic separation was achieved by using C_{18} column, 150 x 4.6mm i.d., 5µ Luna, mobile phase containing acetonitrile and water (gradient elution). The flow rate was 1.0 ml/min and the absorbance was monitored at 282 nm. The proposed method was validated in terms of the analytical parameters such as specificity, accuracy, precision, linearity, range, limit of detection (LOD), limit of quantification (LOQ), and determined based on the International Conference on Harmonization (ICH) guidelines. The linearity ranges of 6G and 6S were obtained over 20-60 and 6-18 µg/ml respectively. Good linearity was observed over the above-mentioned range with linear regression equation Y= 11016x-23778 for 6G and Y = 19276x-19604 for 6S (x is concentration of analytes in µg/ml and Y is peak area). The value of correlation coefficient was found to be 0.9994 for both markers. The limit of detection (LOD) and limit of quantification (LOQ) for 6G were 0.8567 and 2.8555 µg/ml and for 6S were 0.3672 and 1.2238 µg/ml respectively. The recovery range for 6G and 6S were found to be 91.57 to 102.36 % and 84.73 to 92.85 % for all three spiked levels. The RSD values from repeated extractions for 6G and 6S were 3.43 and 3.09% respectively. The validation of developed method on precision, accuracy, specificity, linearity, and range were also performed with well-accepted results.

***Keywords*—**Ginger, 6-gingerol, HPLC, 6-shogaol.

I. Introduction

GINGER rhizome (*Zingiber officinale* Roscoe) is an herb of the *Zingiberaceae* family that has been extensively used in the traditional medicine [3], [11]. Ginger was first cultivated in Asia, and has been used as a medicinal herb for at least 2,000 years [2]. .Gingerols are the most abundant compounds in fresh roots. There are several chain-lengths of gingerols compounds and the most abundant form is 6-gingerol. However, the dehydrated form of gingerols: shogaols are only found in small amounts of the fresh root. The shogaols are mainly found in the dried and thermally treated roots. The most abundant form of shogaols is 6-shogaol [1], [6], [10]. In addition, the major active components, 6-gingerol (6G) and 6-shogaol (6S) are chosen as marker substances which have been shown to have a number of pharmacological activities including: carminative, antiemetic, antinauseant, and anti-inflammatory [4], [5]. The joint pain relief gel is a topical preparation developed in our research using ginger extract. It is tend to use for topical application to relieve muscle pain and inflammation also to emphasize on joint pain relief for osteoarthritis. The osteoarthritis is the most common chronic disease causing joint pain in adults over the age of 40 years. The pain relief gel revealed good efficacy and safety in pre-clinical and clinical studies. The objective of this research is to develop and validate the analytical method of these markers (6G, 6S) for quality control of this product. The chemical structures of 6G (a) and 6S (b) are shown in Fig. 1.

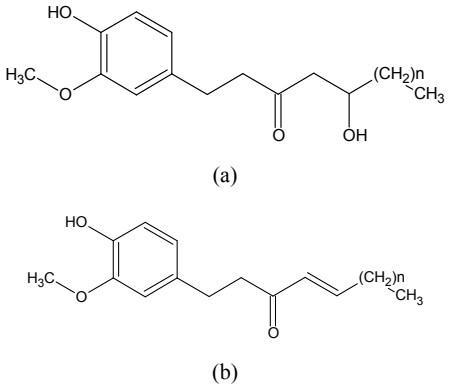

Fig. 1 Chemical Structures of (a) 6-Gingerol (b) 6-Shogaol (n=4)

II. Material and Method

A. Reagents and Samples

6G and 6S were purchased from Sigma-Aldrich, Germany. Acetonitrile and methanol were of HPLC grade from Merck, Germany. All the water used in this study was Ultrapure, obtained from a Milli-Q RO system (Milipore Corporation, France). The joint pain relief gel was developed in our research using ginger extract.

B. Preparation of Sample Solution

Topical gel 0.25 g was weighted and extracted with 20 ml methanol by sonicator for 30 minutes. The solvent was reduced at 45 ºC under reduced pressure in a rotary evaporator to less than 10 ml. and allowed to cool at room temperature. The solution was filtered through a Whatman No.1. Then the filtrate was transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol. After filtering through a 0.2 µm syringe filter, the final sample was

Tanwarat Kajsongkram, Saowalux Rotamporn, Sirinat Limbunruang, Sirinan Thubthimthed are with the Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research 35 Moo 3 Technopolis Tambon Khlong 5, Amphoe Khlong Luang, Pathum Thani 12120, Thailand (phone: 662-577-9105; e-mail: tanwarat@tistr.or.th, saowalux@tistr.or.th, sirinat@tistr.or.th, sirinan@tistr.or.th).

injected directly.

C. Preparation of Standard Solution

Standard stock solutions of 6G and 6S were prepared by dissolving 10 mg of 6G and 6S up to 10 ml of methanol, to get stock solution containing 1000 µg/ml of 6G and 6S.

The stock solutions were diluted to create two five-point standard curves (20-60 µg/ml for 6G and 6-18 µg/ml for 6S).

D. Instrumentation and Chromatographic Conditions

The analytical method of two markers was performed on a Waters Alliance 2695 LC system connected with a Waters model 2996 photodiode-array detector. Data collection and processing were carried out using an Empower workstation. The optimum HPLC system was comprised of a C_{18} reverse phase column (Luna C_{18}, 150x4.6 mm i.d., 5 µm particle size). The gradient was eluted with acetonitrile and water at a flow rate of 1.0 ml/min and PDA detection at 282 nm. The mobile phase consisted of two different solutions, solution A and solution B. Both solutions consisted of water (A) and acetonitrile (B). All solutions were degassed and filtered through a 0.45 µm pore size filter (Millipore, USA). Separations were effected by a gradient elution program as follows: from 0 to 25 min, B was isocratic at 33%; from 25 to 35 min, solution B followed a linear change from 33% to 55%; from 35 to 60 min, B was linearly changed from 55% to 90%; from 60 to 65 min, B was linearly changed from 90% to 33%; and from 65 to 70 min, B was isocratic at 33% [4], [7], [8].

E. Method Validation

The analytical method was validated on specificity, precision, accuracy, linearity, range, and limits of detection and quantification.

1. 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 [12]. In this study, the specificity was demonstrated by running a procedural blank, standard, and sample and placebo solutions.

2. Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample [12]. The precision was investigated with respect to repeatability by determination of sample solution. To assess the intra-day precision (repeatability) of the method, the sample was injected six times within a day. Precision was expressed as the relative standard deviations (% RSD) of the concentrations of each compound 6G and 6S.

3. Accuracy

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the 6G and 6S standard solutions to the placebo samples, at three different levels (80%, 100%, and 120%) of the initial concentration of the sample. Standards, 6G and 6S were added to the samples at 30, 40, 50 µg/mL and 10, 12, 14 µg/mL of these compound. Then the sample was prepared according to the sample preparation in triplicate. Average recoveries were calibrated by the formula recovery (%) = {(amount found - original amount)/amount spiked} x 100.

4. Linearity and Range

The linearity between peak area and concentration was analyzed using two calibration curves. The concentration of the two compounds in the solution specified at 100% was 40 µg/mL (6G) and 12 µg/mL (6S). The other concentrations used to generate calibration curves were 20, 30, 50, 60 µg/mL (6G) and 6,10,14,18 µg/mL (6S). The calibration standard solution 20 µl of each concentration was injected into the HPLC system to get the chromatograms. The average peak area and retention time were recorded. Linearity curve was constructed by plotting concentration of 6G and 6S on X-axis and average peak areas of standard 6G and 6S on Y-axis and regression equations were computed.

5. Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curves of 6G and 6S standards. LOD was calculated according to the expression 3.3 x SD/S. The SD is the standard deviation of the response and S is the slope of the calibration curve. LOQ was established by using the expression 10 x SD/S [9].

F. Statistical Calculations

Standard regression curve analysis was performed by using Micro-soft Office Excel 2007 software (Microsoft, USA), without forcing through zero. Means and standard deviations were calculated by using SPSS software version 9.5 (SPSS, Cary, NC, USA).

III. Results and Discussions

A. Specificity of the Developed Method

The specificity of this method was determined by analysis of the blank, placebo, standard and sample solution chromatograms (Figs. 2-5). Good separation between the peaks of 6G and 6S was achieved, with the retention times, 29.376 min for 6G, 41.354 min for 6S.The comparison of chromatograms among blank, placebo, standard and sample, there was no interference observed from the peaks of the blank, placebo. It shows that the method is high specificity.

B. Linearity and Range of the Developed Method

For linearity studied, five solutions in the range of 20-60 µg/ml for 6G and 6-18 µg/ml for 6S 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 equation as well as determined value of correlation coefficient (Table I). Good linearity was observed over the above - mentioned range with linear regression equation Y = 11016x - 23777 for 6G and Y = 19276x + 19605 for 6S (x is concentration of analytes in

µg/ml and Y is peak area). The value of correlation coefficient was 0.9994 for 6G and 6S.

C. Accuracy of the Developed Method

This study was performed by adding known amounts of 6G and 6S to the placebo samples. Three level of solutions were made and having concentrations at 30, 40, 50 µg/ml for 6G and 10, 12, 14 µg/mL for 6S. The recovery ranges for 6G and 6S were 91.57 to 102.36 % and 84.73 to 92.85 % respectively (limit 80 to 110%). The relative standard deviation ranged from 0.518 to 1.455 % for 6G and from 0.484 to 1.544 % for 6S.

D. Precision of the Developed Method

Repeatability was studied by calculating the relative standard deviation (RSD) from six determinations of the 100% concentration of sample. The studied was performed on the same day and under same experimental conditions. The concentrations of 6G and 6S determinations in the sample solution with the relative standard deviation were calculated (Table III). The RSD values obtained for 6G and 6S were 3.43 and 3.09% respectively (limit not less than 3.7%). The result showed that the developed method was precise.

E. Sensitivity of the Developed Method

LOD were calculated by using the following equations. LOD = 3.3 x SD/S and LOQ = 10 x SD/S, where SD = the standard deviation of the response, S = Slope of the calibration curve. The LOD values were 0.8567 and 0.3672 µg/ml and the LOQ values were 2.8555 and 1.2238 µg/ml for the simultaneous estimation of 6G and 6S. Method validation following ICH guidelines indicated that the developed method had high sensitivity.

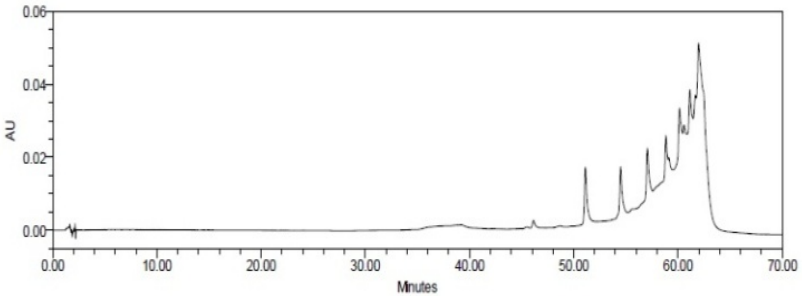

Fig. 2 HPLC Chromatogram of Blank Solutions

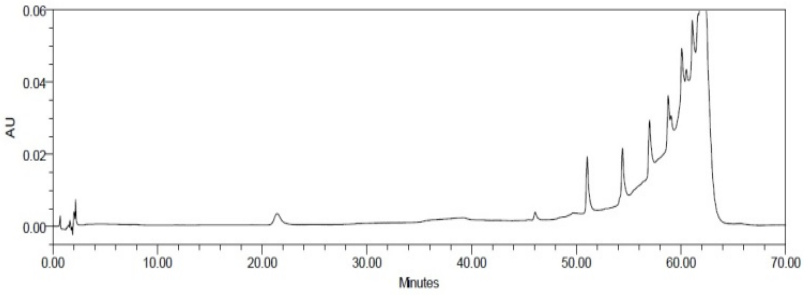

Fig. 3 HPLC Chromatogram of Placebo Solutions

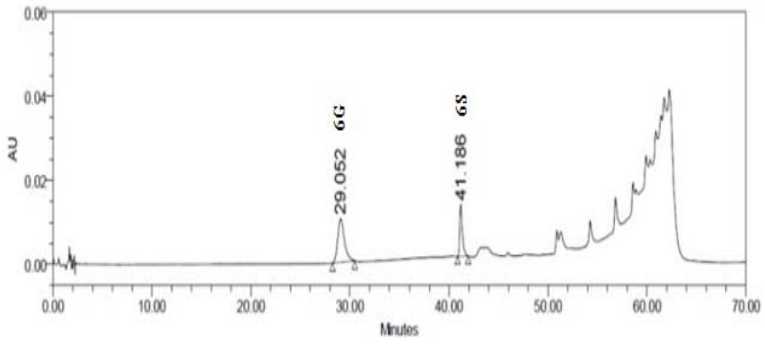

Fig. 4 HPLC Chromatogram of 6G and 6S Standard Solutions

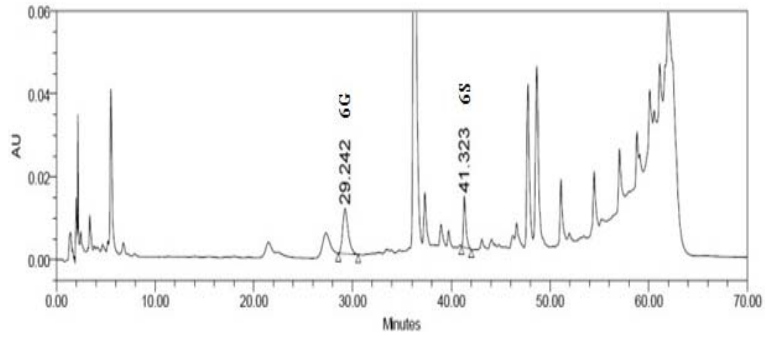

Fig. 5 HPLC Chromatogram of Sample Solutions

TABLE I
LINEARITY AND RANGE FOR 6G AND 6S BY HPLC

Sample number	Concentration of 6G (µg/mL)	Concentration of 6S (µg/mL)	Peak area	
			6G	6S
1	20	6	195,694	97,582
2	30	10	305,628	171,351
3	40	12	416,549	209,441
4	50	14	534,173	252,698
5	60	18	632,205	327,484

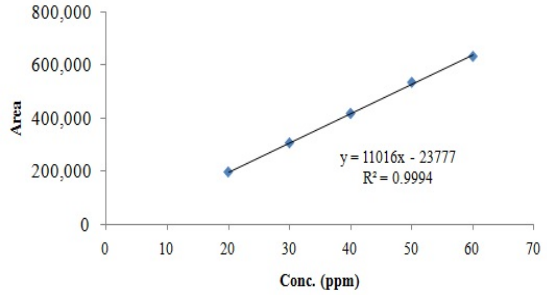

Fig. 6 Calibration Curve of 6G by HPLC

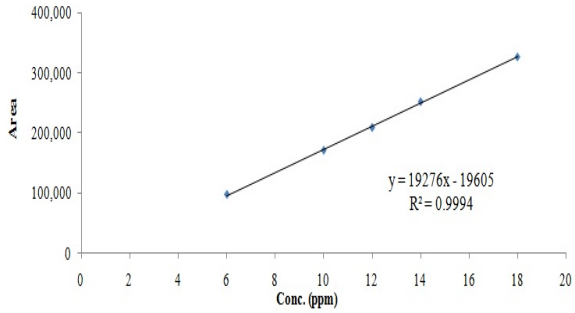

Fig. 7 Calibration Curve of 6S by HPLC

TABLE II
ACCURACY DATA FOR 6G AND 6S BY HPLC

Compounds	Amounted (µg/mL)	% Recovery	% RSD
6G	30	91.57	0.52
	40	97.00	1.46
	50	102.36	1.38
6S	10	84.73	1.10
	12	92.85	1.54
	14	92.69	0.48

TABLE III
PRECISION STUDIES FOR 6G AND 6S BY HPLC

N	%W/W	
	6G	6S
1	0.15038	0.04676
2	0.15104	0.05005
3	0.15960	0.05062
4	0.15870	0.04821
5	0.16060	0.04826
6	0.16364	0.05026
% RSD	3.43	3.09

IV. CONCLUSION

Using this method, 6G and 6S could be determined simultaneously, and the validity of the method was also verified. The proposed analytical method for simultaneous estimation of 6G and 6S in the topical gel is accurate, precise, linear, robust, reproducible and within the range.

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