The Thai Journal of Pharmaceutical Sciences

Volume 38
Issue 0 *supplement issue*

Article 35

1-1-2013

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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR QUANTITATIVE ANALYSIS OF INDIVIDUAL γ-ORYZANOL IN COLD PRESSED RICE BRAN OIL

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KEYWORDS: rice bran oil, oryzanol, RP-HPLC, validation

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INTRODUCTION

The people of Thailand have been using the brown outer layer of the rice kernel, known as rice bran, for generations. Rice bran is rich in oil and frequently sold as a dietary supplement. It is a plentiful source of many bioactive compounds, including γ-oryzanol, phytosterols, ferulic acid and phytic acid. γ-Oryzanol (steryl ferulates) has been shown to be a major bioactive compound in rice. The four major γ-oryzanol constituents in rice were determined to be cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate. They have been previously shown to display various biological activities including anti-inflammatory, antioxidant and anti-tumor activities [1]. The reported values of γ-oryzanol range from 0.2 to 2.72%, depending on the method of extraction, rice variety, weather, and area of cultivation [2, 3]. Nowadays, HPLC methods for quantitative determination of γ-oryzanol in rice bran oil have been reported and determining the total content of γ-oryzanol in rice products rather than on analyzing the content of individual steryl ferulate [4, 5]. Recently, the individual steryl ferulates in conventional and organic brown rice were determined by HPLC [6]. However, long time for analysis was required and RP-HPLC validation methods have not yet been conducted. In this study four major of γ-oryzanol in cold pressed rice bran oil were identified by mass spectroscopy and RP-HPLC method was developed for determination of γ-oryzanol content. The RP-HPLC method was also validated.

MATERIALS AND METHODS

Materials Solvents used for chromatography were acetonitrile (HPLC grade), methanol (HPLC grade), isopropanol (analytical grade) and water (HPLC grade). All solvent were obtained from B&J (Korea). A standard mixture of γ-oryzanol (analytical grade) was purchased from TCI, Tokyo, Japan. Hom-Pathum rice bran samples were provided by a local milling company in Thailand. The rice bran samples were passed though sieve number 20 and immediately extracted under cold press conditions.

Isolation of individual γ-oryzanol The four major of steryl ferulates were isolated from standard γ-oryzanol mixture by HPLC. A 50 mg of γ-oryzanol mixture was dissolved in isopropanol 10 ml. An aliquot of 30 µl was separated on HPLC (Agilent Technologies, USA) with a Poroshell 120 EC-C18 column (3.0×150 mm, 2.7µm). Elution was achieved by using a solvent mixture of acetonitrile and methanol (60:40 v/v), with a flow rate of 0.8 ml/min. Four peak were collected (peak 1 t_R 10.6 min; peak 2 t_R 12.2 min; peak 3 t_R 13.8; peak 4 t_R 15.9 min). The isolated compounds were used as an external standard for HPLC quantitative analysis.

Identification of individual γ-oryzanol LC-MS was carried out using Dionex Ultimate TM3000 equipped. The sample was separated at 25 °C on a Poroshell 120 SB-C18 (2.1×150 mm, 2.7 µm) using a mobile phase consisting of acetonitrile and methanol (60:40 v/v). Injection volume was 10 µl and the UV detector was at 325 nm (variable wavelength detector). Individual γ-oryzanol was identified with high capacity 3D quadrupole ion trap (Bruker Amazon SL). The mass spectrometer was equipped with an ESI ion source. The ESI-MS spectra were acquired in negative ionization mode recorded on a mass range of

m/z 100-800. Capillary voltage was 4500 V. Drying gas temperature was set at 200 °C with a flow rate of 7.0 L/min and nebulizing pressure was of 2 bar. Data were processed by Compass 1.3 SR2.

HPLC conditions HPLC analysis was carried out using the Agilent 1200 series equipped with an Agilent 1200 series photodiode-array detector (PDA) and autosampler. Data analysis was performed using OpenLAB CDS EZChrom software (Agilent, USA). Separation was achieved at 25 °C on a Poroshell 120 EC-C18, 3.0×150 mm, 2.7 µm (Agilent Technologies, USA). The mobile phase consisted of acetonitrile-methanol (60:40 v/v) and was pumped at a flow rate of 0.8 ml/min. The injection volume was 10 µl. The quantitation wavelength was set at 325 nm.

Preparation of standard solutions γ-Oryzanol was accurately weighed to 25 mg in a volumetric flask (size 25 ml) and the volume was adjusted to 25 ml with isopropanol. The stock solution was serial two-fold diluted to six concentrations and filtered through a membrane filter (0.45 µm) before HPLC analysis.

Preparation of samples Rice bran oils were accurately weighed to 25 mg in a volumetric flask (size 25 ml) and the volume was adjusted to 25 ml with isopropanol. The samples were filtered through a membrane filter (0.45 µm) before HPLC analysis. The experiments were carried out in triplicate.

Method validation For validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use were followed (ICH, 2005). Calculated data were checked for their linearity, accuracy, intraday and interday precision specificity, LOD and LOQ to validate the HPLC method.

Calibration curve and linearity The calibration curves were analysis of a mixture containing each of the standard γ-oryzanol at six concentrations and plotting peak areas against the concentration of each reference standard. The linearity of the detector response for the standards was determined by means of linear regression analysis. The calibration curve should show a coefficient of correlation (R^2) $\geq$ 0.9995.

Accuracy Rice bran oil sample solutions were fortified with three concentrations of known quantities of the standard γ-oryzanol in order to check the accuracy of the data. Prior to fortification with standard γ-oryzanol the background levels of standard γ-oryzanol in the rice bran oil were determined so as to calculate actual recoveries. The amounts of γ-oryzanol were determined in triplicate and the percentage recoveries were then calculated.

Precision Precision experiments were conducted to ascertain any intraday and inter-day variability. The solution of one rice bran oil sample was used to check the intra-day precision. Six separate injections of this sample were carried out on the same day. The data were used to calculate the % R.S.D. (not more than 2%) for intraday precision. The inter-day precisions were validated by repeating the extraction procedure on the same sample. An aliquot of each extract was then injected and quantified. This parameter was evaluated by repeating the extraction in triplicate on three different days with a freshly prepared mobile phase and sample. The data was used to calculate % R.S.D. (not more than 5%) for inter-day precision.

Specificity Peak identification was carried out using authentic standards and scanning the UV spectrum of each peak using the photodiode-array detector. The UV spectra were taken at various points of the peaks to check peak homogeneity.

Limits of detection (LOD) and quantification (LOQ) Serial dilutions of a reference standard were carried out with isopropanol and were then analyzed by the HPLC method. LOD and LOQ were the concentrations that give a signal to noise ratio equal to 3 and 10, respectively.

RESULTS AND DISCUSSION

Peak identification and assignment Peak identification and assignment in HPLC fingerprints of rice bran oil were based on the comparison mass spectral data with published data. Four characteristic peaks were identified as cycloartenyl ferulate (peak 1 t_R 10.6 min, $[M-H]^-$ (*m/z*) 601.5; MS/MS (*m/z*) 586.5), 24-methylcycloartenyl ferulate (peak 2, t_R 12.2 min, $[M-H]^-$ (*m/z*) 615.6; MS/MS (*m/z*) 600.5), campesteryl ferulate (peak 3, t_R 13.8 min, $[M-H]^-$ (*m/z*) 575.5; MS/MS (*m/z*) 560.5), β-sitosteryl ferulate (peak 4, t_R 15.9 min, $[M-H]^-$ (*m/z*) 589.6; MS/MS (*m/z*) 574.5). The chemical structures and MS spectra of individual γ-oryzanol are shown in figure 1 and 2, respectively.

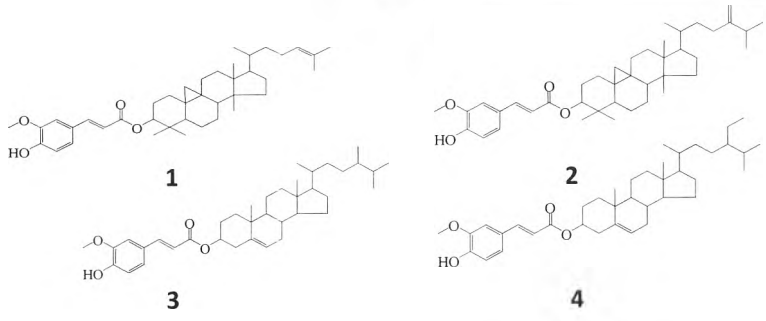

Figure 1 Chemical structures of the steryl ferulates identified from the commercial standard γ-oryzanol mixture (1) cycloartenyl ferulate; (2) 24-methylenecycloartanyl ferulate; (3) campesteryl ferulate; (4) and β-sitosteryl ferulate.

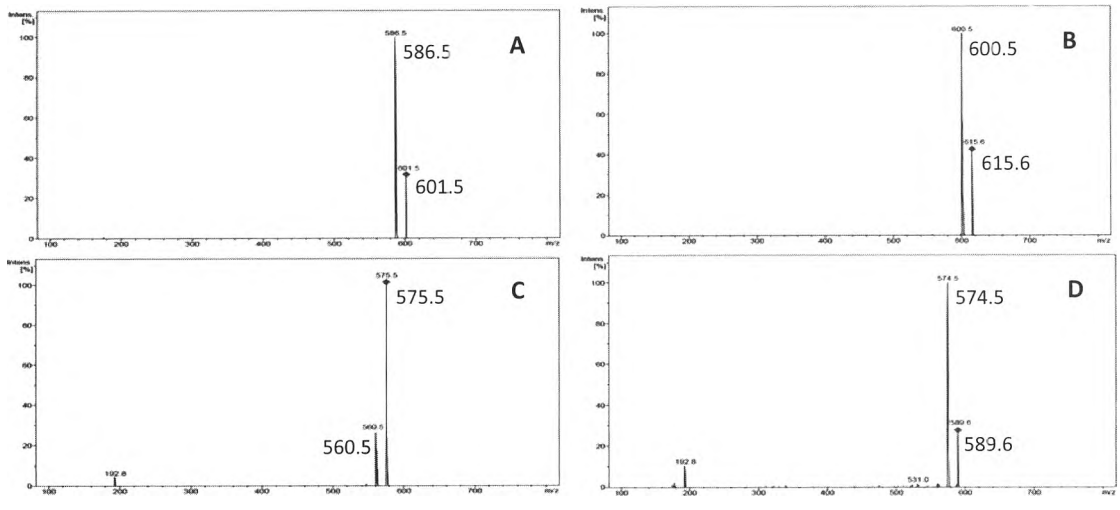

Figure 2 MS/MS spectra of cycloartenyl ferulate (A), 24-methylenecycloartanyl ferulate (B), campesteryl ferulate (C), and β-sitosteryl ferulate (D)

Validation method The optimal conditions for the simultaneous quantitative determination of cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate in cold pressed rice bran oil were examined using an isocratic RP-HPLC system. As all four compounds have good absorption at 325 nm, this wavelength was used for quantitation. Mixtures of acetonitrile and methanol (60:40 v/v) were examined as the mobile phase. This mobile phase and ratio obtained a good resolution. All four compounds were eluted within 17 min with satisfactory resolution (Figure 3).

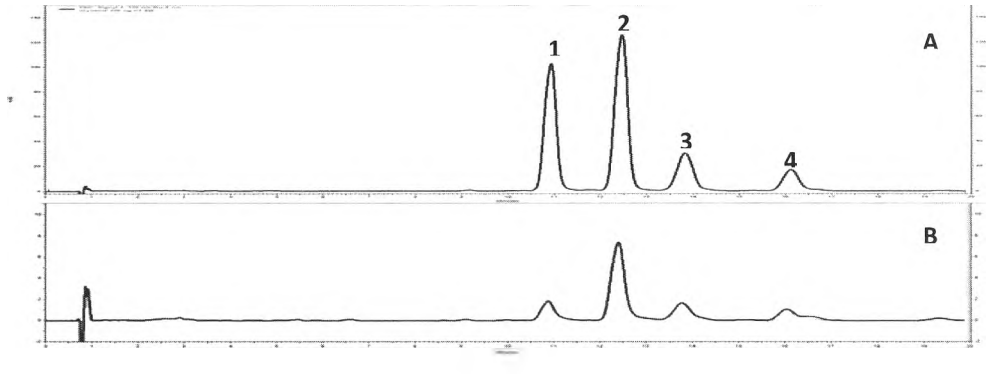

Figure 3 HPLC chromatograms of standard γ-oryzanol (cycloartenyl ferulate (1), 4-methylenecycloartanyl ferulate (2), campesteryl ferulate (3), and β-sitosteryl ferulate (4)) (A) and Hom-Pathum cold pressed rice bran oil (B)

The linearity, accuracy, intra-day and inter-day precision, specificity and limits of detection and quantitation were determined to validate the RP-HPLC method. The calibration curve for was linear over the concentration range 500-15.125 μg/ml. Cycloartenyl ferulate, 4-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate exhibited linearity over the evaluated ranges with correlation coefficients 0.9996. Both intra-day and inter-day precission were estimated by the relative standard deviation were less than 2% and 5%, respectively. Recoveries in the range of 100.1-101.9% were observed for all compounds. Utilising the photodiode array (PDA) makes it possible to obtain the UV spectra. Specificity of the method was evaluated using the UV-absorption spectra produced by the photo diode-array detector. The spectra were taken at three points of the peak for cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate. When it was compared with the standard, the spectra of the peak were observed to be homogenous. Finally, it was found that the RP-HPLC method was very sensitive for detecting cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate with LOD and LOQ values of 0.156 and 0.312 μg/ml. The LOD and LOQ of β-sitosteryl ferulate were 0.781 and 1.562 μg/ml, respectively. All of validated data were shown in Table 1.

Table 1 Validation data of RP-HPLC method

Substances	Linear equations	%Recovery	%RSD		LOQ	LOD
			Intra-day	Inter-day	(μg/ml)	(μg/ml)
R1	y = 44978x - 38615	101.15 ± 1.02	0.06	0.52	0.312	0.156
R2	y = 44989x - 54136	101.97 ± 1.25	0.42	0.60	0.312	0.156
R3	y = 45040x - 19571	101.35 ± 1.15	1.12	4.00	0.312	0.156
R4	y = 44983x - 9316	100.10 ± 1.43	0.02	0.58	1.562	0.781

R1, cycloartenyl ferulate; R2, 24-methylenecycloartanyl ferulate; R3, campesteryl ferulate; R4, β-sitosteryl ferulate

Individual γ-oryzanol content in Hom-Pathum cold pressed rice bran oil was determined using HPLC. In the present study, the content of individual γ-oryzanol were as follows cycloartenyl ferulate (0.53±0.08%w/w), 24-methylenecycloartanyl ferulate (0.87±0.13%w/w), campesteryl ferulate (0.45±0.06%w/w), and β-sitosteryl ferulate (0.31±0.11%w/w). Of these four compounds, 24-methylenecycloartanyl ferulate show the highest content in Hom-Pathum cold pressed rice bran oil (Table 2).

Table 2 Individual γ-oryzanol content in Hom-Pathum cold pressed rice bran oil

Hom-Pathum rice bran/Lot Number	% Content; Mean ± SD (%w/w)				
	R1	R2	R3	R4	Total
1	0.45 ± 0.01	0.73± 0.02	0.35 ± 0.01	0.30 ± 0.01	1.83 ± 0.04
2	0.61 ± 0.01	0.89 ± 0.01	0.48 ± 0.01	0.43 ± 0.01	2.41 ± 0.01
3	0.61 ± 0.01	0.89 ± 0.01	0.49 ± 0.01	0.42 ± 0.01	2.40 ± 0.01
4	0.55 ± 0.00	1.07 ± 0.03	0.50 ± 0.00	0.19 ± 0.00	2.31 ± 0.05
5	0.45 ± 0.01	0.79 ± 0.01	0.43 ± 0.01	0.23 ± 0.00	1.90 ± 0.03
Average	0.53 ± 0.08	0.87 ± 0.13	0.45 ± 0.06	0.31 ± 0.11	2.17 ± 0.28

R1, cycloartenyl ferulate; R2, 24-methylenecycloartanyl ferulate; R3, campesteryl ferulate; R4, β-sitosteryl ferulate

CONCLUSIONS

A simple, specific, precise, accurate, rapid and reproducible RP-HPLC has been developed to quantify cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate in Hom-Pathum cold pressed rice bran oil. The separation was achieved on Agilent 1260 series with Poroshell 120 EC-C18 (3.0×150 mm, 2.7μm) column. A mixture of acetonitrile and methanol (60:40)

were use as mobile phase and was pumped at a flow rate of 0.8 ml/min. The injection volume was 10 µl. The quantitation wavelength was set at 325 nm.

ACKNOWLEDGEMENTS

The authors wish to thank the Faculty of Pharmacy and Sino-Thai Traditional Medicine Research Center (Cooperation between Rangsit University and Harbin Institute of Technology and Heilongjiang University of Chinese Medicine), Rangsit University, Pathum Thani, Thailand for all chemicals and instruments.

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