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Original Article

BIOANALYTICAL METHOD VALIDATION FOR DETERMINATION OF ROSMARINIC ACID IN SIMULATED BIOLOGICAL MEDIA USING HPLC

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ABSTRACT

Objective: This study aims to develop and validate a simple, rapid and accurate HPLC method for the determination of rosmarinic acid (RA), an active marker of *Thunbergia laurifolia* (TL) tea, in Hank's Balanced Salt Solution (HBSS) using HPLC.

Methods: The separation was performed using a Xterra® C_{18} reversed-phase column (150 × 3.9 mm, 5 µm). The mobile phase consisted of 0.5% (v/v) glacial acetic acid (A) and acetonitrile (B). The flow rate was 1 ml/min and detection was carried out at 330 nm with UV-visible spectrophotometer. The method was validated according to the US FDA guidance on bioanalytical method validation in 2018.

Results: The method was successfully validated in the range of 50-500 ng/ml of RA. Intra-and inter-day precision ranged from 1.6 to 2.1% and 1.4 to 4.5%, respectively. Intra-and inter-day relative errors (% bias) were less than 7.6 and 3.6%, respectively. In addition, it was found that the stability of RA in HBSS could be dependent on the pH and temperature.

Conclusion: The developed method met the validation requirements and could be further applied to the permeability study of RA using an *in vitro* Caco-2 cell monolayer model.

Keywords: HPLC, Liquid chromatography, Rosmarinic acid, *Thunbergia laurifolia*, Validation

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INTRODUCTION

Thunbergia laurifolia (TL) or "Rang Cheud" is a Thai medicinal plant, which has been traditionally used as antipyretic and detoxifying herbal medicines [1]. Furthermore, TL alone or in combinations have been clinically used for treatment of drug addiction and smoking cessation [2]. Several biological activities of aqueous TL extract such as antioxidant, anti-inflammatory and anti-depressive-like have been published. It was found that the major active marker in the TL extract was rosmarinic acid (RA) [3, 4].

Since the contents of markers or active compounds in plant material may be varied greatly due to different growing and processing conditions, the alternative use of standardized extracts could minimize the variations and would be benefits in terms of therapeutic efficacy and quality consistency [5]. To improve ease of administration and patient compliance, the TL extract should be developed as an oral dosage form such as tablet, capsule and lozenge. In general, pharmaceutical excipients are the major component of a drug product with the active compound present in relatively small amounts [6]. Most excipient employed in oral dosage forms have been considered as inert. However, it was reported that some excipients such as PEG 400 and mannitol, etc., can interact with active compounds and physiological factors at the site of absorption. As a result, the bioavailability of active compounds, especially for the low-permeability compounds, may vary considerably among different formulations [7, 8]. RA is a hydrophilic polyphenolic compound and its absorption efficiency was reported to be low [9, 10]. Therefore, selection of excipients might be critical for therapeutic efficacy of TL.

The Caco-2 cells are one of the potential alternatives for investigation of the intestinal transportation of active compounds [11]. In order to investigate the threshold level of common excipients affecting RA permeability using an *in vitro* Caco-2 cell monolayer model, a bioanalytical method was needed. Previously, bioanalytical methods for the determination of RA in rat plasma, porcine skin and nasal mucosa were published [12, 13], and the permeability study of RA in Caco-2 cell monolayers was reported [14, 15]. However, the method validation and stability data of RA in the simulated biological media was limited. Therefore, this study aimed to develop and validate a high-performance liquid chromatography (HPLC) method for the determination of RA in the Hanks' Balance Salt Solution (HBSS) which has been used as a biological media in the permeability study.

MATERIALS AND METHODS

Chemicals and reagents

The reference standard of RA was purchased from Sigma-Aldrich (MO, USA). HBSS was obtained from Gibco® by life technologies (NY, USA). HPLC grade of acetonitrile and methanol were supplied by Honeywell (NJ, USA) and Fisher chemical (Loughborough, UK), respectively. Analytical grade of orthophosphoric acid and glacial acetic acid were provided by Ajax Finechem (MA, USA). The deionized water was obtained from Thai Nakorn Patana (Nonthaburi, Thailand).

Preparation of calibration and quality control samples

A stock standard solution of RA was prepared in methanol at a concentration of 1 mg/ml and stored at-20 °C for a maximum of 1 mo. The calibration standard solutions were prepared by diluting the stock solution with HBSS to obtain final RA concentrations of 50, 75, 100, 150, 250, 375 and 500 ng/ml. Similarly, the lower quality control (LQC) concentration (75 ng/ml), middle quality control (MQC) concentration (250 ng/ml) and high quality control (HQC) concentration (375 ng/ml) were prepared.

A 500 µl aliquot of calibration standard and QC sample was transferred to a 2 ml glass vial. Then, 500 µl of 0.5% glacial acetic acid/acetonitrile (85:15, %v/v) was added into the vial. The mixture was vortex for 30 s and then analyzed for RA contents by HPLC.

Instrumentation

The HPLC (Waters Corporation, MA, USA) instrument was equipped with a binary pump (model 1525), a dual wavelength UV detector (model 2487), an autosampler (model 717) and a column compartment. Samples were separated on a Xterra® C_{18} reversed-phase column (150 × 3.9 mm, 5 µm, Waters, USA). The detection wavelength was set at 330 nm. The mobile phase consisted of 0.5% (v/v) glacial acetic acid (A) and acetonitrile (B). The gradient elution was as follows: 0.0-15.0 min (15%B), 15.1-19.0 min (15-25%B), 19.1-23.0 min (25-15%B), and 23.1-30.0 min (15%B). The flow rate

was maintained at 1 ml/min with a total run time of 30 min. The column temperature was controlled at 30±2 °C and the injection volume was 100 µl. The integrations were performed using Empower 2 software (Waters, MA, USA).

Analytical method validation

The analytical method was validated according to the US FDA guidance on bioanalytical method validation [16]. The specificity was evaluated by comparison of the HPLC chromatograms of blank HBSS with the blank mobile phase (0.5% glacial acetic acid/acetonitrile (85:15, %v/v)) and standard samples. The absence of interfering peaks at the same retention time of the analyte was verified. Sensitivity was assessed by determining the lower limit of quantification (LLOQ) of RA. The LLOQ was established as the lowest concentration of RA used in the calibration curve with accuracy and precision of 100±20%. Bias and relative standard deviation (%RSD) were used as measures of accuracy and precision, respectively. Linearity was assessed by plotting RA peak areas versus concentrations of calibration standards. All calibration curves were required to have a correlation value (r^2) of at least 0.995. Precision and accuracy were evaluated by injecting QC samples (75, 250 and 375 ng/ml) in pentuplicate on three different days. Precision was measured by inter-and intra-day RSD (%). The accuracy was evaluated by the deviation or bias (%) of the observed concentration from the expected concentration. The potential for carryover was investigated by injecting blank solvent immediately after analysis of the highest concentration point of the calibration curve.

The stability of RA in HBSS samples was assessed by analyzing QC samples kept at-20 and-80 °C for 3, 5 and 7 d. For bench-top stability, QC samples were kept at ambient temperature (30 °C) for 2 and 6 h before analysis. To determine the post-preparative stability, three concentrations of QC samples from the first day were kept on the autosampler at 30 °C for 12 h and then analyzed again.

RESULTS AND DISCUSSION

Several mobile phases, including acetonitrile-water in combination with glacial acetic acid or orthophosphoric acid, were evaluated. It was found that a water-acetonitrile containing glacial acetic acid, as described above, gave the better system suitability parameters. The peak tailing factors were less than 0.85 and the numbers of theoretical plates were more than 5200. The injection volume was increased from 20 µl to 100 µl for better chromatography and sensitivity.

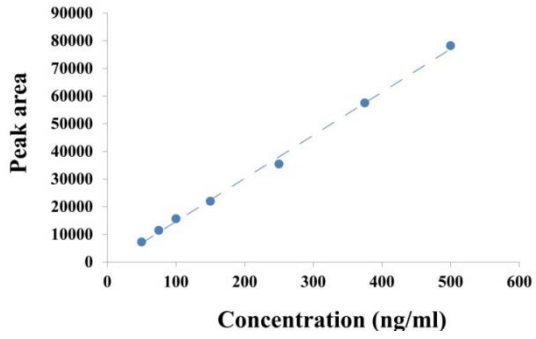

Fig. 1: Calibration curve of rosmarinic acid

The specificity of the method was evaluated as lack of interference by analyzing blank mobile phase and HBSS. No significant interfering peaks from blank samples were observed. The calibration curves showed good linearity within the range of 50-500 ng/ml. The correlation coefficient was >0.995 for all validation batches (fig. 1). The LLOQ was established as 50 ng/ml with accuracy and precision of 0.2 and 5.2%, respectively. In addition, there was no evidence of carry-over effect for the matrix. Representative chromatograms of blank mobile phase, blank HBSS and HBSS spiked with RA at LLOQ and HQC are shown in fig. 2.

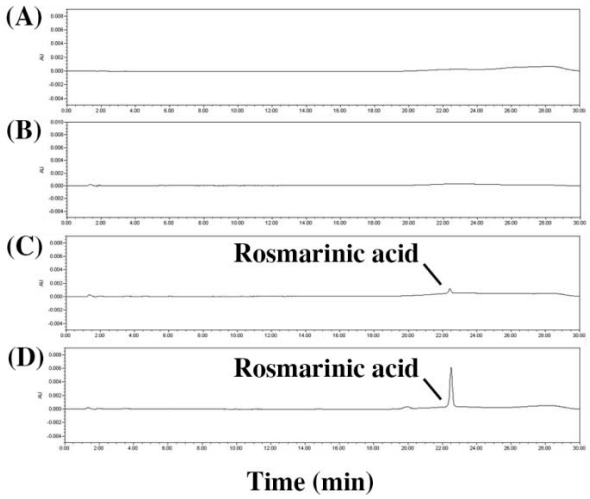

Fig. 2: Representative chromatograms of blank mobile phase (A); blank HBSS (B); LLOQ of RA in HBSS (50 ng/ml) (C) and HQC of RA in HBSS (375 ng/ml) (D). Peak of 22.5 min is RA

Table 1 summarizes the intra-and inter-day precision and accuracy of the method, which were within the acceptable ranges for bioanalytical purposes. Intra-and inter-day precision ranged from 1.6 to 2.1% and 1.4 to 4.5%, respectively. Intra-and inter-day relative errors (%bias) were less than 7.6 and 3.6%, respectively. Table 2 summarizes the stability results of RA in HBSS carried out under various conditions. For bench-top stability, the %bias of RA content was within acceptable range at ambient temperature for at least 2 h, after which an approximately 56-61% reduction in the RA content was observed at the storage time of 6 h. This implied that the permeability study of RA using HBSS as simulated biological media must be carried out in a short time, and the collected samples should be immediately analyzed or frozen until analysis. For frozen stability, it was found that after storing at-80 °C for 7 d, the accuracy at HQC levels was-17.4% which was beyond the acceptance criteria. Therefore, in order to obtain reliable data, the content of RA should be assessed within 7 and 5 d after storing at-20 and-80 °C, respectively. Although the bench-top stability of RA was low, the post-processed samples, in which the samples were diluted with 0.5% glacial acetic acid/acetonitrile (85:15, %v/v) at the ratio of 1:1 and kept at 25 °C, were shown to be stable for at least 12 h.

Table 1: Precision and accuracy of the HPLC assay for RA in HBSS media

Added (ng/ml)	Found (ng/ml)[a]	% RSD	% Bias
Intra-day (*n* = 5)			
75	69.3±1.4	2.0	-7.6
250	245.7±4.0	1.6	-1.7
375	370.2±6.6	1.8	-1.3
Inter-day (*n* = 15)			
75	72.3±3.3	4.5	-3.6
250	246.4±3.3	1.4	-1.4
375	372.3±5.1	1.4	-0.7

Data expressed as mean±SD; *n* = 5.

Table 2: Stability results of RA in HBSS media

Added (ng/ml)	Added (ng/ml)	Found (ng/ml)[a]	% RSD	% Bias
Bench-top stability (30 °C)				
0 h	75	75.2±0.5	0.7	-0.6
	375	374.5±2.3	0.6	0.1
2 h	75	68.2±0.4	0.6	-9.1
	375	365.8±3.1	0.9	-2.5
6 h	75	32.9±2.0	6.2	-56.1[b]
	375	144.23±5.6	3.4	-61.5[b]
Frozen-matrix (-20 °C)				
3 d	75	68.8±0.3	0.4	-8.3
	375	373.1±2.5	0.7	-0.5
5 d	75	70.9±1.2	1.6	-5.4
	375	340.8±1.0	0.3	-9.1
7 d	75	69.1±0.7	1.0	-7.9
	375	319.7±0.8	0.3	-14.8
Frozen-matrix (-80 °C)				
3 d	75	67.9±1.3	1.9	-9.4
	375	356.0±6.1	1.7	-5.1
5 d	75	68.1±2.1	3.0	-9.2
	375	333.2±5.6	1.7	-11.1
7 d	75	66.9±1.5	2.2	-10.9
	375	309.9±3.8	1.2	-17.4[b]
Autosampler (25 °C, 12 h)	75	67.1±0.6	0.9	-10.5
	250	244.7±3.2	1.3	-2.1
	375	372.4±2.1	0.6	-0.7

[a]Data expressed as mean±SD; *n* = 3, [b]Data failed to meet acceptance criteria

The results of this study implied that the stability of RA could be dependent on the pH and temperature. These results were in accordance with the gastrointestinal stability study of Dinis *et al.*, which reported that RA in *Mentha* species was stable at acidic pH of simulated gastrointestinal conditions [17]. However, the opposite result was also reported that acidic medium greatly reduced stability of RA. More interestingly, there were differences in the stability of pure RA and RA contained in plant extracts [18]. In order to avoid data misinterpretation, the factors affecting stabilities of RA as pure compound and in TL extract, such as incubation time and temperature should be further verified before performing the Caco-2 permeability assay.

CONCLUSION

In this study, an accurate, simple, and reproducible HPLC method was successfully developed and validated for the determination of RA in HBSS. This developed method could be applied to investigate the threshold level of common excipients, such as mannitol and sorbitol, in affecting RA permeability using an *in vitro* Caco-2 cell monolayer model. In addition, according to our best knowledge, this is the first report on the stability of RA in HBSS media. This information would help to design an appropriate experimental setup for the permeability study of RA.

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Nil

AUTHORS CONTRIBUTIONS

Dr. Kotchaphan Chooluck has generated the research plan, prepared and revised the manuscript. Dr. Piyanuch Rojsanga and Dr. Chutima Phechkrajang have given guidance and supervision to carry out this study. Dr. Montree Jaturanpinyo has supported in data analysis.

CONFLICTS OF INTERESTS

All authors have none to declare.

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