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Original Article

DEVELOPMENT AND VALIDATION OF LC-MS METHOD FOR QUANTITATIVE ANALYSIS OF A TRADITIONAL THAI ANTIHYPERTENSIVE HERBAL RECIPE

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ABSTRACT

Objective: To develop a new validated LC-MS method in which can be used for identification and quantification of the active pharmaceutical compounds in a traditional Thai antihypertensive herbal recipe (TTAH).

Methods: First, a quadrupole-ion trap MS was used to identify the active pharmaceutical compounds to facilitate a set of markers in the TTAH using multiple reactions monitoring (MRM) method. Coupling to LC in gradients mode, the chromatogram of the TTAH was established. Second, the method validation was conducted to observe several parameters, such as, carryover, linearity, a limit of detection, a limit of quantification, precision, accuracy, robustness, specificity, and system suitability.

Results: Piperine, imperatorin, and pinostrobin were identified using the quadrupole-ion trap MS by comparison the mass spectrum of the TTAH samples and reference standards. The parent and product ions were optimized using MRM method. Good chromatographic peaks were achieved along with a simple and fast analysis. All parameters were validated and found that the method reported in this article can be used to quantify the amount of piperine, imperatorin, and pinostrobin in the TTAH.

Conclusion: Piperine, imperatorin, and pinostrobin were selected as the markers in LC-MS analysis of the TTAH. This new validated LC-MS method is readily to use in a quality assurance of the TTAH.

Keywords: Traditional Thai antihypertensive herbal recipe, LC-MS, Method validation.

INTRODUCTION

Since traditional Thai medicine (TTM) becomes an important part of health care in Thailand, the government has recognized the need of proving its efficacy, safety, and quality following to the World Health Organization (WHO) Traditional Medicine Strategy [1-2]. Beside the general quality control topics of plant materials [3], it is accepted that analysis of an active ingredient is a requirement in the quality control process of TTM [4-5].

Moreover, it is known that the basis of analytical procedure contains both quantification and method validation [6]. In this article a traditional Thai antihypertensive herbal recipe (TTAH), which its ingredients including *Acanthus ebracteatus*, *Aegle marmelos*, *Boesenbergia pandurata*, *Cyperus rotundus*, *Piper nigrum*, and *Tinospora crispa*, is selected. Recently, piperine, imperatorin, and pinostrobin (Fig. 1), the active ingredients in the TTAH, were previously used as the markers for the TTAH analysis by liquid chromatography-mass spectrometry (LC-MS) [7]. However, long time for analysis was required and the method validation had not yet been reported. In this article, those three compounds in the TTAH were identified and quantified by a new developed LC-MS method. This method was also validated to prove its specificity, precision, accuracy, robustness, and the other parameters.

MATERIALS AND METHODS

Materials

All raw materials including *A. ebracteatus*, *A. marmelos*, *B. pandurata*, *C. rotundus*, *P. nigrum*, and *T. crispa* were collected from plants located in Nakhon Si Thammarat province, Thailand. Species were identified by Mr. Nirun Wipunngeun, specialist in botany, from faculty of Pharmacy, Rangsit University. All the materials were dried at 45°C in a hot air oven and grinded to obtain the herbal powder. The TTAH was then produced by mixing all ingredients in the exact ratio according to the recipe.

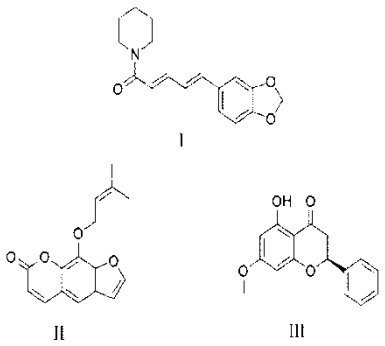

Fig. 1: Chemical structure of piperine (I), imperatorin (II), and pinostrobin (III)

HPLC grade acetonitrile, water, and formic acid were purchased from B&J (Korea). Piperine, imperatorin, and pinostrobin standards were purchased from Sigma Aldrich (USA).

Individual stock solutions (1.0 mg/ml) of three standards were prepared in acetonitrile-water (80:20) and filtered through 0.45 µm membrane filter. Working standards, piperine (0.25, 0.5, 2.5, 5, and 10 µg/ml), imperatorin (0.01, 0.05, 0.1, 0.5, and 1 µg/ml), and pinostrobin (0.04, 0.2, 0.4, 2, and 4 µg/ml) were prepared by diluting the corresponding stock solution with acetonitrile-water (80:20) for LC-MS analysis.

Sample preparation

To prepare the extract, 5 g of the TTAH samples was sonicated 5 times in 50 ml hexane for 20 min, pooled, and evaporated. The extract was accurately weighed to 10 mg. The volume was adjusted to 5 ml in a volumetric flask with acetonitrile-water (80:20). The

sample was sonicated for 5 min and filtered through 0.45 µm membrane filter. The experiments were conducted in triplicate.

LC-MS analysis

The extracts were analyzed using a Dionex Ultimate TM3000 HPLC coupling with a Bruker Amazon SL (LC-MS). Acclaim® 120 C18 chromatography column (3 µm, 2.1 mm x 150 mm) was used as a stationary phase. Analyses were performed in the gradients mode using aqueous 0.2% formic acid-acetonitrile from 60:40 to 20:80 (0-15 min), an isocratic at a ratio of 20:80 (15-20 min), and re-equilibrate at 60:40 (20-25 min). The column was maintained at 25°C with a flow rate of 0.15 ml/min. A 10 µl aliquot sample was then injected onto the column. The mass spectrometer was equipped with an ESI ion source.

The system was tuned for optimum sensitivity and resolution using a Bruker ESI tuning mix in both positive and negative electrospray ionization modes. ESI-MS evaluation was performed using a full scan in positive mode recorded on a mass range of *m/z* 100-1,000. Capillary voltage was set at 4,500 V and drying gas temperature was 200°C with a flow rate of 7.0 l/min. Nebulizer pressure was set at 2 bars. Piperine, imperatorin, pinostrobin were identified with a high capacity 3D quadrupole ion trap by comparison the parent and product ions with the reference standards using multiple reactions monitoring (MRM) method. The constituent amount in the TTAH was quantified. Data were processed by Compass 1.3 SR2.

Method validation

The analytical procedure was validated in accordance with the guidelines of the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use.

Prior to validation, the highest concentration of working standard solution was injected into the column until a response was obtained. A blank mobile phase was injected in triplicates to verify the carryover effect.

Linearity

Calibration curves of piperine, imperatorin, and pinostrobin were constructed by linear regression by plotting peak areas against five concentrations of each reference standards. The calibration curves should show a coefficient of correlation (R^2) ≥ 0.9995.

Limit of detection and limit of quantification

The Limit of detection (LOD) and limit of quantification (LOQ) were determined by means of a serial dilution based on signal-to-noise ratios of 3:1 and 10:1, respectively. Moreover, the LOQ concentration was evaluated by precision of six replicate injections.

Precision

The precision was conducted for intra-day and inter-day analyses. A sample solution was used to achieve the intra-day experiment. The data was the content of six injections done separately on the same day. The inter-day precision was studied by comparing the assay on three different days. The precision was calculated as the relative standard deviation (RSD). The RSD of intra-day precision should be not more than 2% while the value should not be above 5% for inter-day precision.

Accuracy

The sample solutions were fortified with three concentrations of known quantities of the standards in order to optimize the accuracy of the data. Prior to fortification, the background levels of piperine, imperatorin, and pinostrobin in the TTAH were determined so as to calculate actual recoveries. The amounts of each standard were determined in triplicate and the percentage recoveries were calculated.

Robustness

The robustness was assessed by introducing small variations from the optimum condition. These variations included column temperature and flow rate.

Specificity

The specificity was investigated by monitoring the mass of each reference standard using multiple reactions monitoring (MRM) method.

System suitability

System suitability was performed using the working standard solution of pinostrobin. Theoretical plate number (N) and precision (RSD) were measured.

RESULTS AND DISCUSSION

MRM transition

The transition was observed both in the reference and sample solutions for each piperine ([*M*+H]$^+$ 286.1), imperatorin ([*M*+H]$^+$ 271.0), and pinostrobin ([*M*+H]$^+$ 271.0) parent ions to guarantee selectivity and specificity. Each transition had its specific reaction amplitude. First, the piperine transition was [*M*+H]$^+$ 286.1 to 200.9 with the reaction amplitude of 0.7. Second, the imperatorin transition was [*M*+H]$^+$ 271.0 to 202.9 with the reaction amplitude of 0.6. Third, the pinostrobin transition was [*M*+H]$^+$ 271.0 to 166.9, 130.9, and 103.1 with the reaction amplitude of 0.6 (Fig. 2).

HPLC method development

Several parameters were observed for the optimization of LC-MS analysis. The first challenge was to figure out the appropriate mobile phase. Different composition of aqueous 0.2% formic acid and acetonitrile were tried. The most suitable peaks were showed when the gradient was aqueous 0.2% formic acid-acetonitrile from 60:40 to 20:80 in 0-15 min following with the isocratic at a ratio of 20:80 for 5 min. The elution order was piperine (tR = 13.0 min), imperatorin (tR = 14.3 min), and pinostrobin (tR = 15.8), the chromatograms were shown in Fig. 3.

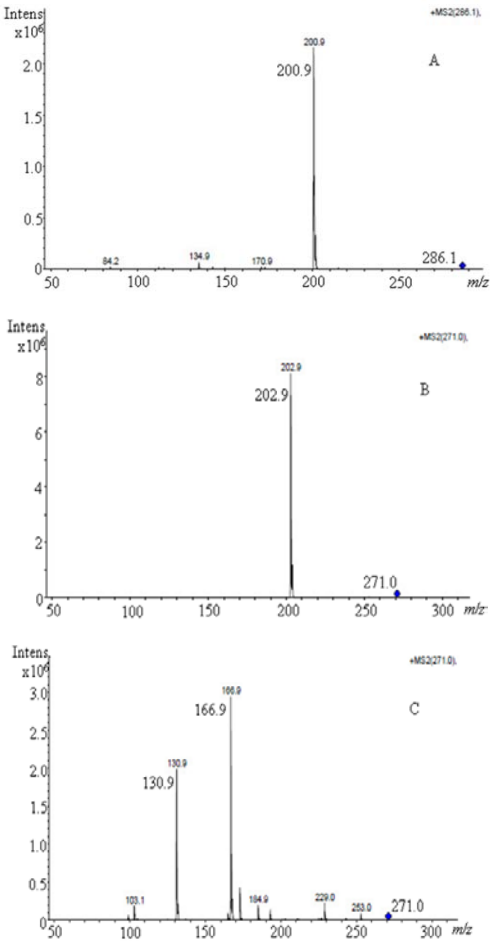

Fig. 2: MS/MS spectra of piperine (A), imperatorin (B), and pinostrobin (C)

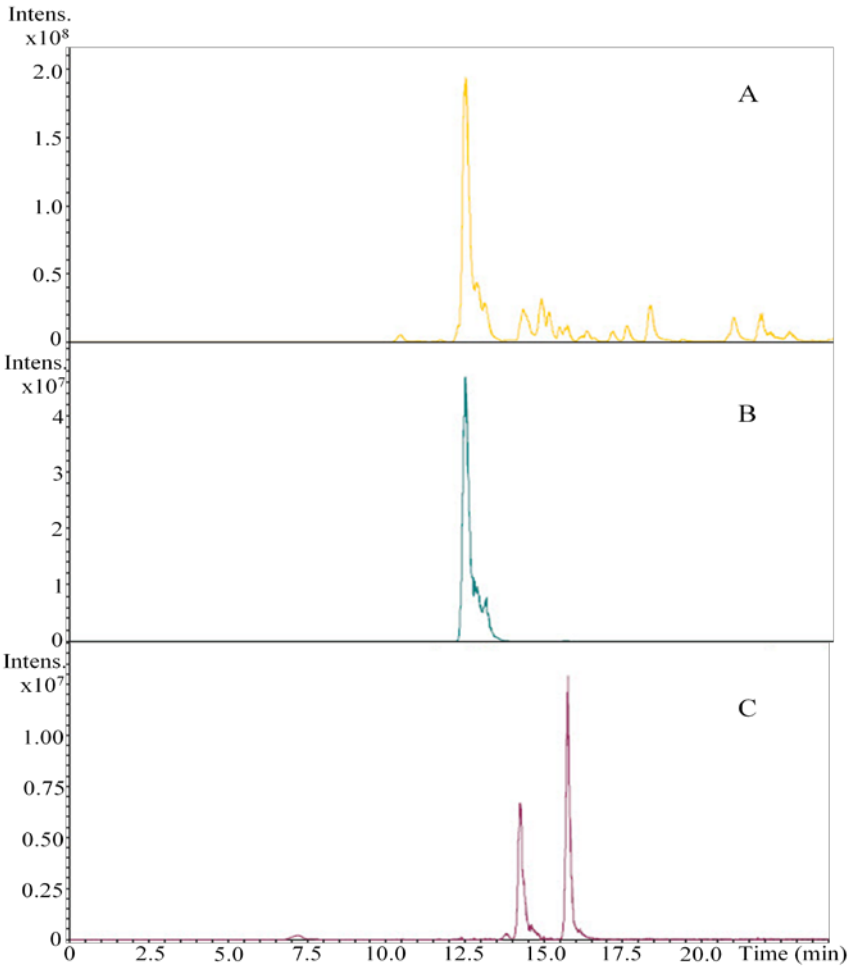

Fig. 3: LC-MS chromatograms of TTAH hexane extract (A), standard piperine at tR 13.0 min (B), standard imperatorin at tR 14.3 min, and standard pinostrobin at tR 15.8 min (C)

Method validation

Carryover

The carryover effect was assessed in triplicate runs of blank as describe previously. The noise signals at retention times of all markers in the blank runs were negligible when compare with the LOQ.

Linearity

Three calibration curves of the reference standards were constructed. First, the calibration curve for piperine was linear over the concentration range of 0.25-10 µg/ml (0.25, 0.5, 2.5, 5, and 10 µg/ml) with a correlation coefficient of 0.9998.

Second, the calibration curve for imperatorin was linear over the concentration range of 0.01-1 µg/ml (0.01, 0.05, 0.1, 0.5, and 1 µg/ml) with a correlation coefficient of 0.9998. Third, the calibration curve for pinostrobin was linear over the concentration range of 0.04-4 µg/ml (0.04, 0.2, 0.4, 2, and 4 µg/ml) with a correlation coefficient of 1 (Table 1).

LOD and LOQ

It was found that the method was very sensitive to detect all three markers. The LOD values of piperine, imperatorin, and pinostrobin were 0.050 ng/ml, 0.10 ng/ml, and 4.0 ng/ml, respectively.

Under the developed method, LOQ was determined to be 0.20 ng/ml, 0.40 ng/ml, and 20.0 ng/ml for piperine, imperatorin, and pinostrobin, respectively (Table 1). The precision of six replications at LOQ levels of each standard was calculated as RSD (%) in which the values were 0.29, 0.27, and 0.18 for piperine, imperatorin, and pinostrobin, respectively.

Precision

The intra-day and inter-day precisions expressed as RSD were found to be 0.11-0.16% and 0.96-1.30%. It was agreed that the method was considerably precise, which guaranteed that reliable results were achieved (Table 1).

Accuracy

Accuracy of method was evaluated by analyzing the TTAH extracts spiked with known concentrations of the standards. Prior to spiking, the background levels of piperine, imperatorin, and pinostrobin in the extracts were analyzed so as to calculate actual recoveries. Mean recoveries in the range of 97-99% were observed for all markers (Table 1).

Robustness

The results showed good reliability of an analysis with respect to deliberate variations in method parameters, including column temperature and flow rate (Table 2).

Specificity

Using MRM method, the transition was observed both in reference and sample solutions for all reference markers as described in MRM transition section. Good specificity was achieved for piperine, imperatorin, and pinostrobin determining by their specific molecular mass and retention time. In addition, by combination the MS/MS technique, imperatorin and pinostrobin which present same

molecular mass but different structures can be identified individually.

System suitability

According to ICH guidelines, the system is suitable when N is more than 2,000 and variation of standard response (RSD) does not go beyond 1%. The values obtained for this method (N = 22,168 and RSD = 0.11%) were within the criteria.

Application

The proposed method was applied to the determination of piperine, imperatorin, and pinostrobin in TTAH.

A relative determination of the same samples was also examined by a previous method [7]. Statistical evaluation of the results by the Student's t-test at the 95% confidence level showed no significant different between the precision of the two methods.

Table 1: Validation data of the developed LC-MS method

Markers	Linear equation	%Recovery	RSD (%)		LOQ (ng/ml)	LOD (ng/ml)
			Intra-day	Inter-day		
Piperine	Y=5E+08X-5E+06	99.72 ± 0.41	0.16	1.30	0.20	0.05
Imperatorin	Y=3E+08X+394557	97.54 ± 0.57	0.12	1.19	0.40	0.10
Pinostrobin	Y=7E+06X-31491	98.07 ± 0.30	0.11	0.96	20.0	4.0

Table 2: Robustness data for the developed LC-MS method

Parameter	Modification	Markers	Mean ± SD (ng/ml)	RSD (%)
Column temperature (°C)	25	Piperine	250.12 ± 0.43	0.17
	26		249.98 ± 0.37	0.15
	25	Imperatorin	10.02 ± 0.013	0.13
	26		9.96 ± 0.011	0.11
	25	Pinostrobin	40.03 ± 0.045	0.11
	26		39.99 ± 0.040	0.10
Flow rate	0.15	Piperine	250.07 ± 0.45	0.18
(ml/min)	0.17		249.95 ± 0.42	0.17
	0.15	Imperatorin	10.04 ± 0.013	0.13
	0.17		9.97 ± 0.010	0.10
	0.15	Pinostrobin	40.02 ± 0.052	0.13
	0.17		39.97 ± 0.048	0.12

CONCLUSIONS

This new validated method proposes the development of a fast, simple, sensitive, and reliable LC-MS method for the determination of three important markers, piperine, imperatorin, and pinostrobin, in the TTAH. In addition, the new developed method is more practical in the quality control process since the analysis time is shorter than the previous method for 40 min. Hence, the validated LC-MS method can be used as a tool to prove the TTAH quality, which is one of the most important criteria in TTM evaluation.

CONFLICT OF INTERESTS

Declared none

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