Original Article

Validation of HPLC Method for the Determination of Anti-allergic Compounds in Ethanolic Extract of Prasaprohyai remedy, a Thai Traditional Medicine

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Abstract

Introduction: Prasaprohyai remedy (PY) is a Thai traditional medicine that has long been used to treat the common cold and fever. Previous studies revealed that 3,000 mg/day of powdered PY capsule were able to relieve allergic rhinitis symptoms as effectively as 10 mg/day of Loratadine. PY consists of 21 herbs and there is a need for quality control to ascertain their standard before being used in patients. Earlier studies showed that Ethyl-*p*-methoxycinnamate (EPMC) and eugenol had the potential as markers for quality control of PY.

Objective: To develop a validated method for Prasaprohyai remedy extract.

Methods: HPLC methodology was performed to assess validation parameters.

Results: The validation parameters addressed were specificity, limit of detection, limit of quantification, linearity, accuracy and precision. The developed HPLC system was specific for the detection of EPMC and eugenol content in Prasaprohyai remedy. All other parameters for analysis complied with standard requirements. For EPMC the analytical range was 25-450 µg/ml with linearity (r^2=0.9999), LOD 0.1 µg/ml, LOQ 0.5 µg/ml. The analytical range of eugenol was 2.5-30 µg/ml with linearity (r^2= 0.9998), LOD 0.25 µg/ml, LOQ 0.5 µg/ml.

Conclusion: The developed HPLC method can be used to standardize the Prasaprohyai remedy extract.

Key words: HPLC, Quality control, Prasaprohyai remedy, Validated method, Ethyl-p-methoxy- cinnamate, Eugenol

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Introduction

Prasaprohyai remedy (PY), a Thai traditional medicine, has long been used to treat common cold and fever.[1] It consists of 21 herbs. The major component of PY is rhizome of *Kaempferia galanga* L. (KG) and other 20 herbs in equal weights. Previous *in vitro* studies showed that the 95% ethanolic extract of PY has notable anti-allergic effects in the RBL-2H3 cell line model (IC_{50} = 16.59 ± 1.68 µg/ml).[2] It also demonstrated anti-inflammatory effect, by inhibiting nitric oxide release (IC_{50} = 18.40 ± 0.43 µg/ml).[3] A previous clinical study comparing the efficacy and safety of 3,000 mg/day of powdered PY capsules and 10 mg/day of Loratadine in allergic rhinitis patients showed PY to be as safe and effective as Loratadine. In this study, patients received PY (3 x 1,000 mg/day) for six weeks. The patients showed improvement in total nasal symptoms. The raising of quality of life scores was as effective as the treatment with Loratadine.[4] Moreover, other reported biological activities of PY were analgesic,[5] antibacterial,[6] anticancer,[5] antimalarial,[7] antipyretic,[5] antioxidant[3] and cytotoxic activities.[8]

A previous quality control study showed that ethyl-p-methoxycinnamate (EPMC) and eugenol were the main chemical components of PY detected by GC-MS.[9] EPMC was the main constituent in KG,[10] and eugenol was the main compound in *Syzygium aromaticum* (L.).[11] Previous *in vitro* and *in vivo* studies showed that both EPMC and eugenol exhibited anti-allergic activity.[12] Eugenol could inhibit the systemic anaphylaxis induced by compound 48/80 at a dose of 10 µg/g BW and showed significant reduction in serum histamine levels.[13]

Quality control is an important process to ascertain the quality and consistency of the products for confirming their quality prior to be used in patients. The present study was aimed at developing a validated HPLC method for the determination of EPMC and eugenol in PY extract.

Methods

Plant materials

All PY component plant materials were purchased from various sources (Table 1). The voucher specimens were deposited at the Herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla province, Thailand (Table 1).

Chemicals and reagents

Standard EPMC was previously isolated from KG purity >98% as checked by HPLC.[12] Standard eugenol (Purity > 98%) was purchased from Sigma-Aldrich (Bangkok, Thailand), HPLC reagents such as methanol were purchased from RCI Labscan (Bangkok, Thailand), and purified water was prepared by Milli Q® system from Millipore (Bedford, MA, USA).

Instruments

The HPLC system (Agilent® 1200; Agilent Technologies, USA) consisted of a solvent degasser (G1322A), a quaternary solvent pump (G1311A), an autosampler (G1329A), a column oven (G1316A) and a photodiode array detector (G1315D). The chromatographic data were processed by the Chemstation® software revision B.04.01 SP1. The reversed-phase C18 column was Eclipse® XDB-C18, 4.6x250 mm, 5 micron.

Preparation of PY extract

Each plant material was cleaned, cut into small pieces, and dried at 50°C for 24 h. Dried plant material was powdered using an electric grinder (40 mesh). Each powdered plant material was weighed according to its proportion described in Table 1 and was homogenously mixed. The mixed powder was macerated with 95% ethanol (2:1) for 3 days, filtered with Whatman number 1 paper and the solvent removed using a rotary evaporator under reduced pressure at 40°C to obtain the dry ethanolic extract. The residue of PY was remacerated twice and the same treatment was applied. These three extracts were combined and further dried to constant weight in a hot air oven at 45 ± 5 °C. The extract was kept at -20 °C until use. The extractive yield was 5.05 %w/w.

Table 1 Medicinal plant ingredients in Prasapraohyai remedy formulation (for 1,000 g.)

Scientific name	Family	Voucher specimen	Part used	Weight (g)	Source
Amomum testaceum Ridl.	Zingiberaceae	SKP206011101	Fruit	25	Thailand
Anethum graveolens L.	Umbelliferae	SKP199010701	Fruit	25	India
Angelica dahurica Benth.	Umbelliferae	SKP199010401	Root	25	China
Angelica sinensis (Oliv.) Diels	Umbelliferae	SKP199010901	Root	25	China
Artemisia annua L.	Compositae	SKP051010101	All parts	25	China
Atractylodes lancea (Thunb.) DC.	Compositae	SKP051011201	Rhizome	25	China
Cuminum cyminum L.	Umbelliferae	SKP199030301	Fruit	25	India
Dracaena loureiri Gagnep.	Dracaenaceae	SKP065041201	Hart wood	25	Thailand
Foeniculum vulgare Mill. var. *dulce* (Mill.) Thell.	Umbelliferae	SKP199062201	Fruit	25	India
Kaempferia galanga L.	Zingiberaceae	SKP206110701	Rhizome	500	Thailand
Lepidium sativum L.	Brassicaceae	SKP057121901	Seed	25	India
Ligusticum sinense Olive. Cv. Chuanxiong	Umbelliferae	SKP199121901	Rhizome	25	China
Mammea siamensis Kosterm.	Guittiferae	SKP083131901	Flower	25	Thailand
Mesua ferrea L.	Guittiferae	SKP08313060	Flower	25	Thailand
Mimusops elengi L.	Sapotaceae	SKP171130501	Flower	25	Thailand
Myristica fragrans Houtt.	Myristicaceae	SKP121130601	Nutmeg/aril	25	Thailand
Myristica fragrans Houtt.	Myristicaceae	SKP121130601	Seed	25	Thailand
Myristica fragrans Houtt.	Myristicaceae	SKP121130601	Hart wood	25	Thailand
Nelumbo nucifera Gaertn.	Nelumboceae	SKP125141401	Pollen	25	Thailand
Nigella sativa L.	Ranunculaceae	SKP160141901	Seed	25	India
Syzygium aromaticum (L.) Merr. et Perry	Myristicaceae	SKP123190101	Flower-bud	25	Thailand

HPLC method for EPMC and eugenol determination in PY

Preparation of standard solutions

Stock standard solutions of EPMC and eugenol were prepared in methanol at a concentration of 1.0 mg/ml and stored at -20°C until use. Working standard solutions were diluted in methanol. Calibration curve was constructed by using serially diluted standard solution at concentrations 25, 50, 100, 200, 250 and 450 µg/ml for EPMC and 2.5, 5, 10, 15, 20 and 30 µg/ml for eugenol.

Preparation of PY extract sample solution

Ten milligrams of PY extract was dissolved in 1 ml methanol and mixed for 10 minutes by vortex mixer. This solution was filtered through a membrane filter (pore size 0.45 µm) prior to analysis.

HPLC conditions

The mobile phase gradient was a mixture of methanol (A) and purified water (B) programmed as follows: 0 - 23 min, 60%A, 40%B; 23 – 30 min, 90%A, 10%B; 30 – 35 min, 60%A, 40%B. Samples (10 µl) were injected into HPLC system and the flow rate was

set at 1 ml/min. The diode array detector was set at 227 nm. The operating temperature was maintained at room temperature (25°C). Data were analyzed by Agilent ChemStation® software.

Validation method

The validation of the developed HPLC method was conducted according to ICH guideline.[14] The validation parameters included specificity, limits of detection (LOD), limits of quantification (LOQ), linearity, accuracy and precision.

Specificity

The specificity is the ability of the method to assess the analyte unequivocally in the presence of other compounds. The specificity of the method was performed by comparing the chromatogram of blank (methanol), standard solutions and sample solution. The UV spectra of peaks in PY solution corresponding to EPMC and eugenol were identified by comparing with the peak of standard. UV spectra of peak were compared at peak start, peak apex and peak end for peak purity proof.

Linearity

Linearity of the method was conducted by preparing EPMC at different six concentrations (25, 50, 100, 200, 250 and 450 µg/ml) generating EPMC standard curve, and eugenol standard curve (2.5, 5, 10, 15, 20 and 30 µg/ml). The linearity was determined by a coefficient of determination (r^2) using the linear least-squares regression analysis.

LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) were determined by detecting the signal of the diluted standard solution of EPMC and eugenol against the noise based on signal-to-noise ratio of 3:1 and 10:1, respectively.

Precision

The precision of an analytic method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogenous sample. The precision of the analysis method was examined by intermediate evaluation method using measurements of the intra-day and inter-day variability. The standard solutions were prepared at three concentration levels and injected into HPLC system. The precision of the method was calculated by determining the relative standard deviation (RSD) of the mean concentration calculated from calibration curves. Intra-day variability was determined by analyzing three sets of standard solutions during one consecutive experiments. The inter-day variability was performed on three different days. The calculated RSD should not be greater than 2%.

Accuracy

The accuracy is the closeness of test results obtained by that method to the actual value. The standards of EPMC and eugenol were spiked into PY sample solution, of which the 3 concentrations of EPMC were 50, 150 and 300 µg/ml and eugenol were 5, 12 and 20 µg/ml. Intra-day and inter-day were calculated as RSD% of the concentration found from the subtracted spiked samples and calculated as percentage recovery of a standard compound.

Results

The system was specific to EPMC and eugenol as shown by good separation from other components in PY (Figure 1). Peak purity of each compound in the PY chromatogram was evaluated by comparing the UV spectra at peak start, peak apex and peak end which were superimposable (Figure 2).

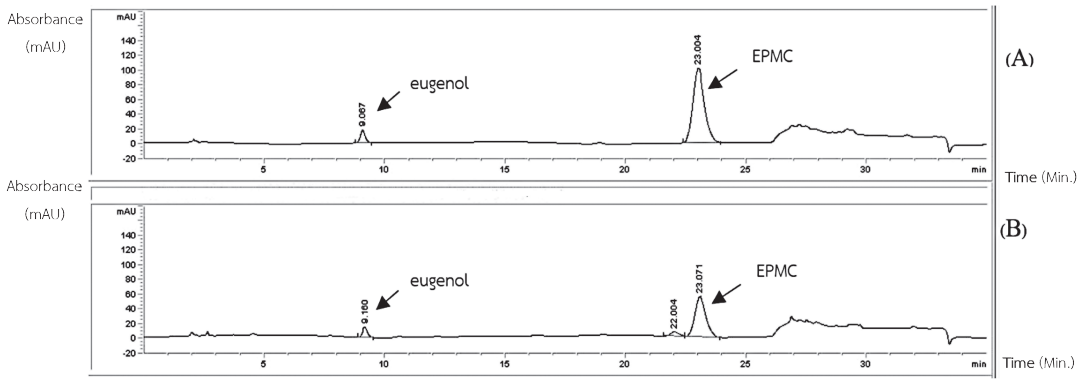

Figure 1 HPLC chromatograms of standard eugenol (9 min) and EPMC (23 min) (A) and PY extract (B) at UV detector 227 nm.

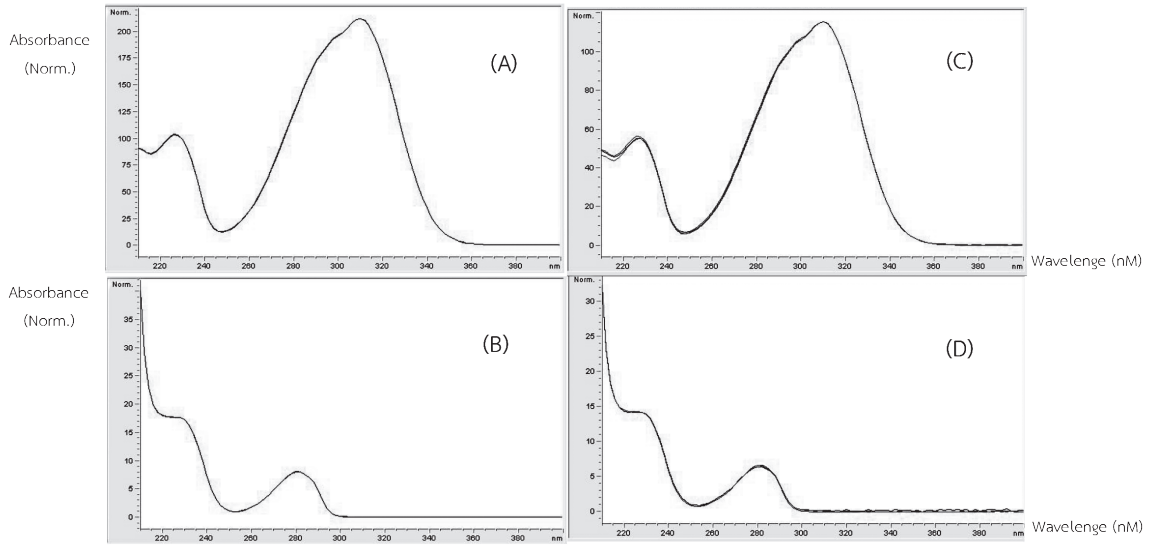

Figure 2 UV absorption spectra of EPMC (A), and eugenol (B), Superimposed layers of 3 UV spectra at peak start, peak apex and peak end of EPMC (C), and eugenol (D).

The linearity of EPMC was observed to be in the range of 25-450 µg/ml and that of eugenol was observed in the range 2.5-30 µg/ml (Table 2). The regression analysis for both EPMC and eugenol showed linear relationship between the peak area of markers and their concentration (r^2 >0.999) (Table 2).

Table 2 Linear ranges, coefficient of determination (r2), LOD and LOQ of calibration curves of ethyl-*p*-methoxycinnamate and eugenol

Parameters	Ethyl-p-methoxycinnamate	Eugenol
Range (µg/ml)	25 - 450	2.5 - 30
Linear equation (y = ax + b)	y = 31.833x - 123.16	y = 14.884x - 8.3727
Linearity (r^2)	0.9999	0.9998
LOD (µg/ml)	0.1	0.25
LOQ (µg/ml)	0.5	0.5

The limit of detection (LOD) and the limit of quantitation (LOQ) of EPMC were 0.1 and 0.5 µg/ml, respectively and LOD and LOQ of eugenol were 0.25 and 0.5 µg/ml, respectively (Table 2).

The Precision of the method was performed by repeatability (intra-day) and intermediate precision (inter-day). The RSD values were lower than 2% (between 0.40-1.73 %), indicating the high precision of this method (Table 3).

Table 3 Precision validation of the analytical method for ethyl-p-methoxycinnamate and eugenol

Compound	Concentration	Intra-day[a] (n = 3)		Inter-day[b] (n = 9)	
	(µg/ml)	Measured Conc. (µg/ml)	% RSD	Measured Conc. (µg/ml)	% RSD
Ethyl-*p*-methoxycinnamate	50	50.85 ± 0.74	1.45	50.57 ± 0.62	1.23
	150	153.18 ± 1.28	0.84	151.63 ± 1.62	1.07
	300	302.90 ± 4.21	1.39	300.25 ± 3.47	1.15
Eugenol	5	5.03 ± 0.09	1.73	4.99 ± 0.07	1.46
	12	12.15 ± 0.19	1.56	11.95 ± 0.21	1.72
	50	20.45 ± 0.08	0.40	20.27 ± 0.34	1.68

[a] All values are mean ± SD as obtained by triplicate analyses in a consecutive run.

[b] All values are mean ± SD, obtained by triplicate analyses per day over 3 different runs.

The accuracy of the method was determined by investigating the recovery of spiked EPMC and eugenol in PY extract and comparing the measured value to the actual value. The developed method exhibited good recovery in the range of 97.86-100.05% (Table 4).

Table 4 Accuracy validation of the analytical method for ethyl-p-methoxycinnamate and eugenol

Compound	Spiked level (µg/ml)	Recovery (%)[a]			Mean (%)	Relative standard deviation (RSD, %)
		N1	N2	N3		
Ethyl-*p*-methoxycinnamate	50	99.47	99.96	99.00	99.56± 0.48	0.48
	150	98.74	100.32	102.23	100.05 ± 1.75	1.75
	300	97.88	98.01	98.23	97.86 ± 0.17	0.18
Eugenol	5	99.64	97.80	99.59	99.01 ± 1.05	1.06
	12	101.31	99.46	97.75	99.51 ± 1.78	1.79
	20	99.04	100.81	98.09	99.31 ± 1.38	1.39

[a] All values are mean ± SD as obtained by triplicate analyses in a day.

The analysis of PY extract by this validated method revealed the presence of EPMC and eugenol contents to be 227.12 and 52.88mg/g, respectively (Table 5).

Table 5 Determination of EPMC and eugenol in PY extract

Sample	Observation conc. (mg/g)	
	EPMC	Eugenol
N1	227.13	52.91
N2	225.41	53.50
N3	228.83	52.22
Mean ± SD	227.12 ± 1.71	52.88 ± 0.64

Discussion

PY consists of 21 herbs with *K. galanga* L. (KG) as the major component (50%w/w). A previous study revealed the major compounds of PY as EPMC and eugenol.[2] These two compounds were previously shown to be the appropriate markers for the quality control of PY remedy. However, there was no validated quality control method for determining these two markers in PY extract. From this study the optimal condition could be obtained by gradient elution using a mixture of methanol (A) and purified water (B) with 35 minute analysis time. The EPMC peak (RT=23 min) was well separated from eugenol (RT=9 min) with a good resolution (R>1.5).[15] The developed HPLC system was specific for the detection of EPMC and eugenol contents in the PY remedy. The diode array detector was set at 227 nm where both EPMC and eugenol showed the highest absorbance and other peak were less conspicuous.

The developed HPLC method showed that all analytical parameters for EPMC and eugenol complied with standard requirements. The analytical range of EPMC was 25 - 450 µg/ml with good

linearity (r^2=0.9999), LOD 0.1 µg/ml, LOQ 0.5 µg/ml. For eugenol, the analytical range was 2.5-30 µg/ml with good linearity (r^2= 0.9998), LOD 0.25 µg/ml, LOQ 0.5 µg/ml.The accuracy and precision of the developed method were also good, with RSD lower than 2%.

In this study the HPLC instrument was chosen for the analysis because it was able to determine a wide range of chemical components in natural products, and it was a common analytical instrument in many laboratories and manufacturers. Moreover, valtdated HPLC methods were successfully used with other Thai traditional remedies i.e. Benjakul[16] and Benjalokawichien[17]. It also provided reduced time of PY analysis i.e. 35 minutes vs 48 minutes by GC-MS.[9]

In addition, this HPLC method can be used for the standardization of KG extract, which is the highest component of PY. By this method EPMC and ethyl cinnamate, the major KG components, can be separated with a good resolution value of more than 1.5 (Figure 1B.; Ethyl cinnamate: 22 min and EPMC: 23 min).

This is the first report on a validated HPLC method for PY quality control using the anti-allergic EPMC and eugenol as markers. The specified gradient HPLC system of methanol and purified water was optimal for the content determination of these two markers. This method gave sharp peaks of EPMC and eugenol; both peaks were well separated. All required parameters complied with the ICH guideline. This validated HPLC method is useful for the quality control of the PY extract and its preparations. This is the first time that the contents of EPMC and eugenol in PY were analyzed to be 227.12 and 52.88 mg/g respectively.

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บทคัดย่อ

การประเมินความถูกต้องของวิธีวิเคราะห์โดย HPLC ต่อสารที่มีฤทธิ์ต้านการแพ้ในสารสกัดจากเอธานอลของตำรับยาประสะเปราะใหญ่

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บทนำ: ตำรับยาประสะเปราะใหญ่เป็นยาไทย ที่ใช้ในการรักษาอาการหวัด น้ำมูกไหล และอาการไข้ งานวิจัยก่อนหน้าพบว่า ผงยาประสะเปราะใหญ่มีประสิทธิผลในการบรรเทาอาการจมูกอักเสบจากการแพ้ได้ดีเทียบเท่ายาลอราทาดีน โดยตำรับยาประสะเปราะใหญ่ เป็นยาที่มีส่วนประกอบของสมุนไพร 21 ชนิด จึงจำเป็นต้องควบคุมคุณภาพของยาก่อนที่จะนำไปใช้ในผู้ป่วย โดยงานวิจัยก่อนหน้าพบว่า สารเอทิล-พารา-เมท็อกซีซินนาเมต (EPMC) และยูจีนอล มีศักยภาพใช้เป็นสารควบคุมคุณภาพของตำรับยาประสะเปราะใหญ่ได้

วัตถุประสงค์: พัฒนาวิธีวิเคราะห์ที่ผ่านการตรวจสอบความถูกต้องสำหรับตำรับประสะเปราะใหญ่

วิธีการศึกษา: ใช้วิธีการทาง HPLC ในการตรวจสอบความถูกต้อง

ผลการศึกษา: พารามิเตอร์ของการตรวจสอบความถูกต้องของวิธีวิเคราะห์ ได้แก่ ความจำเพาะ, ขีดจำกัดของการตรวจพบ, ขีดจำกัดของการหาเชิงปริมาณ, ความเป็นเส้นตรง, ความเที่ยงและความแม่นยำ โดยวิธีการวิเคราะห์เฉพาะเจาะจงต่อการตรวจหาปริมาณ เอทิล-พารา-เมท็อกซีซินนาเมต และยูจีนอล ในตำรับประสะเปราะใหญ่ รวมทั้งพารามิเตอร์อื่นๆ เป็นไปตามข้อกำหนดมาตรฐาน สำหรับเอทิล-พารา-เมท็อกซีซินนาเมต ช่วงการวิเคราะห์อยู่ที่ 25-450 ไมโครกรัม / มิลลิลิตร และสมการมีความเป็นเส้นตรง (ค่าสัมประสิทธิ์ = 0.9999), ค่าขีดจำกัดของการตรวจพบ 0.1 ไมโครกรัม / มิลลิลิตร, ค่าขีดจำกัดของการหาเชิงปริมาณ 0.5 ไมโครกรัม / มิลลิลิตร สำหรับยูจีนอล ช่วงการวิเคราะห์อยู่ที่ 2.5-30 ไมโครกรัมต่อมิลลิลิตร สมการมีความเป็นเส้นตรง (ค่าสัมประสิทธิ์ = 0.9998), ค่าขีดจำกัดของการตรวจพบ 0.25 ไมโครกรัม / มิลลิลิตร, ค่าขีดจำกัดของการหาเชิงปริมาณ 0.5 ไมโครกรัม / มิลลิลิตร

สรุปผลการศึกษา: การพัฒนาวิธีวิเคราะห์ด้วย HPLC สามารถนำมาใช้ในการควบคุมคุณภาพของสารสกัดประสะเปราะใหญ่ได้

คำสำคัญ: HPLC, การควบคุมคุณภาพ, ยาประสะเปราะใหญ่, การทดสอบความถูกต้องของวิธีวิเคราะห์, เอทิล-พารา-เมท็อกซีซินนาเมต, ยูจีนอล