Chromatographic Fingerprint Development for Quality Assessment of "Ayurved Siriraj Prasachandaeng" Antipyretic Drug

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ABSTRACT

Quality assessment of traditional herbal medicines is of benefit not only in research but also in practice. The method of quality assessment of the Thai traditional medicine, Ayurved Siriraj Prasachandaeng, was established by using High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC). In HPTLC, the chromatographic fingerprints were developed; the color and the relative retardation factor (rR_f) of bands were compared with those of reference markers. Likewise, relative retention time (rR_t), and applied information content (ϕ) were evaluated in HPLC fingerprints. Reference markers, gallic acid, caffeic acid, p-coumaric acid, ferulic acid, vanillic acid and kojic acid were used as qualitative markers in HPTLC whereas gallic acid, caffeic acid and vanillic acid were used as qualitative and quantitative markers in HPLC. Similarity of the chromatographic pattern among batches was determined by the presence of stated mathematic parameters in the range of 80 to 125 percent of the average. The HPTLC and HPLC fingerprints of three batches of Ayurved Siriraj Prasachandaeng showed similar chromatographic patterns. Such similarity showed that the productions of different batches in the recipe were consistent. Moreover, it revealed that some markers found in the recipe certainly came from various medicinal herbal components of their own recipes. In conclusion, the combination of rR_f from HPTLC, and rR_t and ϕ from HPLC is the suitable method for identification and quality control of different batches of Ayurved Siriraj Prasachandaeng.

Keywords: High performance thin layer chromatography (HPTLC); high performance liquid chromatography (HPLC); fingerprint analysis; quantitative analysis; pattern recognition

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Traditional herbal medicines have been widely used for thousand of years in many oriental countries, for instance China, India and Thailand. The use of herbal medicines has obviously increased in the past decade. The recent herbal product development tends to use modern pharmaceutical techniques and the application of suitable standard and good manufacturing practices. These are not only for controlling the quality of herbal medicines which directly affects their efficacy and safety,[1-4] but also controlling the consistency of different batches. In general, one or two markers of pharmacologically active components in herbs or herbal compounds were currently employed for evaluating the quality and authenticity of herbal medicines, especially in the identification of single herb medicines[5-8]. Without doubt, quality control of medicines, consisting of more than two kinds of crude herbs, is even more complicated. If identification of an active principle is not possible, a characteristic substance or mixture of substances e.g. chromatographic fingerprint should be identified to ensure consistent quality of the preparation. Thin layer chromatography (TLC) including HPTLC is the common method of choice for chromatographic fingerprinting analysis. It is flexible, fast, and an inexpensive method. HPTLC can identify several samples at the same time and may enable separation of a crude plant

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extract without an earlier purification. Generally, it may be used as preliminary method preceding the other methods. HPLC is another chromatographic method of choice which is a high-resolution technique. HPLC shows excellent analytical power with more sensitivity and reproducibility for the quantitative determination as compared to HPTLC. However, these two techniques in combination or alone, can be successfully used to determine most of the phytochemical constituents of herbal products in order to (i) ensure the reliability and reproducibility of pharmacological and clinical researches in herbal medicine, (ii) understand bioactivities and possible side effects of herbal medicines, and (iii) enhance quality control of herbal products. The standardization in herbal drugs obtained from fingerprinting of HPTLC and HPLC will offer integral characterization of a complex system with a quantitative reliability.

Ayurved Siriraj Prasachandaeng is a Thai traditional medicine used extensively as an antipyretic for both children and adults. The formula, prepared in powder, requires the combination of twelve medicinal herbs, based on historical references and several lines of empirical evidence in Thai traditional medicine practice. Ayurved Siriraj Prasachandaeng belongs to the Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, which formerly was the Clinic of Thai Traditional Medicine established by Prof. Ouay Ketusingh in 1982. CATTM has many Thai traditional recipes which have been produced and used with satisfaction for a long time. However, some basic scientific information of the recipes are still lacking. Moreover, chromatographic fingerprints of each recipe have never yet been established. In this study, we firstly developed a simple, reliable and reproducible method to set up a characteristic fingerprint of Ayurved Siriraj Prasachandaeng and its twelve medicinal herbal components. This chromatographic profile should feature the fundamental attributions of "integrity" and "fuzziness"; in other words, "sameness" and "differences"[9] of different batch prepa rations. With HPTLC and HPLC, twelve medicinal herbal components and three batches of Ayurved Siriraj Prasachandaeng produced from different times were analyzed. The chromatograms from three batches were regarded as the original fingerprints of Ayurved Siriraj Prasachandaeng. Phenolic composition, probably found in some components such as gallic acid, caffeic acid, p-coumaric acid, ferulic acid, vanillic acid and kojic acid will be also determined. The chromatographic fingerprints and contents of such substances are necessary for investigating a proper condition for quality control of the recipe.

MATERIALS & METHODS

2.1 Reagents and sample materials

For HPLC analysis, all solvents were HPLC grade. Water was purified by a Milli-Q system (Millipore Corp., USA). Other chemicals and reagents were analytical grade purchased from Merck, Germany. Gallic acid, caffeic acid, p-coumaric acid, ferulic acid, vanillic acid and kojic acid were purchased from Sigma, USA.

Ayurved Siriraj Prasachandaeng and its twelve raw herbs* were obtained from the Herbal Medicines and Products Manufacturing Unit, Ayurved Siriraj, CATTM, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand. All of them were independently identified by at least two Thai traditional pharmacists of CATTM according to morphological characteristics and produced following the standard operating procedures (SOPs) of good manufacturing.

*Twelve plants were ordered for component no. 1-12 which were *Citrus aurantifolia* (Christm et. Panz.) Swing., *Bouea macrophylla* Griff., *Knema globularia* Warb., *Dracaena loureiri* Gagnep., *Myristica fragrans* Linn., *Caesalpinia sappan* Linn., *Conioselinum univitatum* Trucz., *Kaempferia galanga* Linn., *Mesua ferrea* Linn., Jasminum sambac (Linn.) Ait., *Mammea siamensis* Kosterm., and *Nelumbo nucifera* Gaertn, respectively.

2.2 Instrumentation

The HPTLC system (Camag, Muttenz, Switzerland) consisted of a Linomat 5 sample applicator equipped with a 100 µl syringe and a Scanner 3. Aluminum HPTLC plates (20 cm x 10 cm) pre-coated with silica gel 60, were purchased from Merck (Darm-stadt, Germany). HPLC system (Waters, Milford, MA, USA) consisted of a Waters 2695 Separation Module system equipped with a photodiode array detector. An X-Terra RP18 column 100 mm x 4.6 mm I.D., with 1.7 µm particle size was purchased from Waters (Ireland).

2.3 Preparation of sample and reference solutions

Three batches of Ayurved Siriraj Prasachandaeng and twelve herbal components were extracted separately in 80% ethanol and lyophilized to dry powder. Each powder was dissolved separately in 80% ethanol for HPTLC and 50% methanol for HPLC and then filtered through a 0.2 µm membrane filter before used.

For the HPTLC qualification of retardation factor (R_f) value, gallic acid, caffeic acid, p-coumaric acid, ferulic acid, vanillic acid and kojic acid (1 mg each) were dissolved separately in 80% ethanol. Gallic acid and caffeic acid solutions were prepared likewise for the HPTLC qualification of relative retardation factor (rR_f) value. For HPLC, gallic acid, caffeic acid and vanillic acid (1 mg each) were dissolved separately in 50% methanol. Each solution was vortexed for two minutes and filtered through a 0.2 µm membrane filter to obtain the filtrate as the reference marker.

2.4 Chromatographic conditions

2.4.1 HPTLC[10]

The mobile phase was composed of hexane: ethylene acetate: acetic acid (31:14:5). Each reference mixture (5 µl) and each sample (2 µl) were loaded on the HPTLC plate and developed in a saturated mobile phase to a distance of 90 mm. After plate drying by a stream of cold air for three minutes, each plate was visualized under 254 and 366 nm UV, and then sprayed with 0.5% Fast Blue B Salt (FBS) heated at 110^0C until the bands were clearly visible under visible light. Finally, R_f and rR_f values were calculated as follows.

$$R_f = \frac{\text{Distance of agent}}{\text{Distance of solvent}}$$

$$rR_f = \frac{R_f \text{ of sample band}}{R_f \text{ of reference band}}$$

Using reference markers, the fingerprints of samples were identified.

2.4.2 HPLC[11-14]

The mobile phase was composed of (A) O-phosphoric acid (0.1%, v/v) and (B) acetonitrile using an isocratic condition of 95% A, 5% B at 0-4 min; a gradient elution of 95-85% A, at 4-8 min; 85-0% A, at 8-13 min and 0-95% A, at 13-16 min. The flow rate was 1.0 ml/min, UV spectra were recorded over the range of 200-400 nm. The mathematic methods for HPLC analysis, relative retention time (rR_t) value and applied information content (ϕ) value were used to validate similarities among batches as follows.[15-16]

$$rR_t = \frac{R_t \text{ of sample peak}}{R_t \text{ of reference peak}}$$

$$\phi = -\sum \left(\frac{\chi_i}{\sum \chi_i} \log \left(\frac{\chi_i}{\sum \chi_i} \right) \right)$$

X_i = area under identified peak

2.5 Data analyses

The calculated factors, including R_f and rR_f from HPTLC, and rR_t and ϕ from HPLC, were applied for the identification and quality control of each batch of the recipe and each component was compared with the reference markers. Like bioequivalent (BE) assessment, coding a standard BE range for basic pharmacokinetic parameters 80-125% of mean has been generally accepted.[17-19] Moreover, the similarity among different batches of the recipe was indicated by the relative standard deviation (RSD) of the rR_t and ϕ values less than 15%.[20]

RESULTS

3.1 Validation of HPTLC[21]

The calibration curve derived from five dilutions of reference marker, gallic acid, at concentration range of 50 to 400 ng was performed by the relation of polynomial regression. System repeatability was determined by running known samples of 200 ng in triplicate on the same day for three times (intra-day precision) and on three different days (inter-day precision) and evaluated by calculating the RSD. The accuracy of the method was determined by analyzing the percentage of difference from actual. The RSD of intra-day, inter-day analyses and the accuracy of method were within the acceptance criteria (<15-20%)[20] which implied that the established method was sufficiently precise.

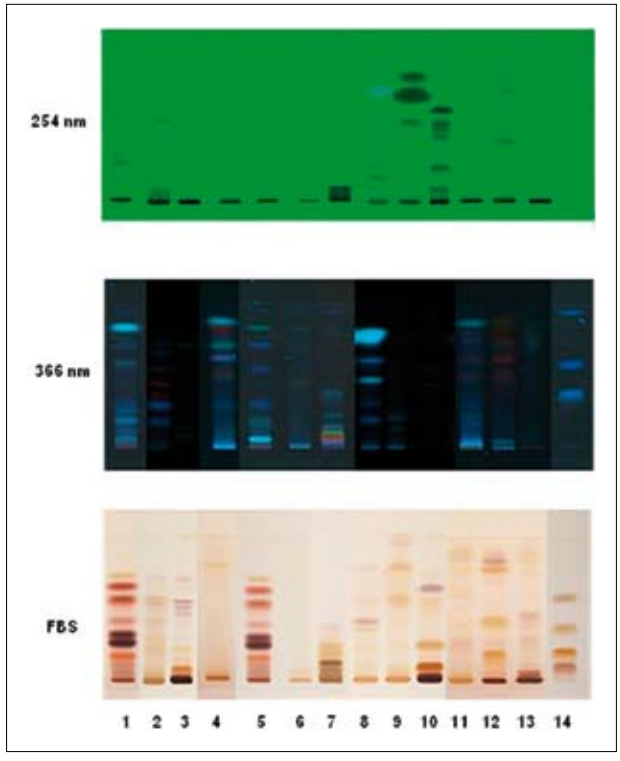

Fig 1. HPTLC fingerprints of Ayurved Siriraj Prasachandaeng and its twelve components visualized at 254 and 366 nm under UV and visible light, respectively (after spraying with fast blue salt: FBS). Lane 1: Prasachandaeng; lane 2-13: component no. 1-12; lane 14: phenolic substance mixture (from bottom to top): kojic acid, gallic acid, caffeic and p-coumaric acid, ferulic acid and vanillic acid.

3.2 Validation of HPLC[15]

The calibration curve derived from six dilutions of gallic, caffeic and vanillic acids was performed by the relation of linear regression. The limit of quantification (LOQ) was determined by calculating the signal-to-noise ratio (S/N =10) and taken experimentally as the lowest concentration on the calibration curve. The ranges of the method were then 0.2, 1, 5, 10, 15, and 20 µg/ml for gallic acid (R^2=0.9988); 0.2, 1, 5, 10, 15, and 20 µg/ml for caffeic acid (R^2=0.9999); and 0.3, 1, 5, 10, 15, and 20 µg/ml for vanillic acid (R^2=0.9999). The corresponding areas (n=3) generated from the reference markers were plotted against the concentrations of reference markers. Low, medium and high concentrations of the reference markers were used to achieve repeata-

TABLE 1. The rR_f to gallic acid marker and caffeic acid marker in HPTLC fingerprints of Ayurved Siriraj Prasachandaeng (3 batches).

Band No.	Batch 1		Batch 2		Batch 3		Mean		Accepted range	
	Gallic	Caffeic	Gallic	Caffeic	Gallic	Caffeic	Gallic	Caffeic	Gallic	Caffeic
1	0.54	0.29	0.47	0.25	0.53	0.28	0.51	0.27	0.43-0.68	0.22-0.34
2	0.95	0.50	0.84	0.44	0.84	0.44	0.88	0.46	0.76-1.19	0.37-0.58
3	1.47	0.78	1.47	0.78	1.44	0.76	1.46	0.77	1.18-1.84	0.62-0.96
4	1.82	0.96	1.79	0.94	1.75	0.93	1.79	0.94	1.46-2.28	0.76-1.18
5	2.28	1.20	2.32	1.22	2.26	1.19	2.29	1.21	1.82-2.85	0.96-1.51
6	3.21	1.69	3.18	1.68	3.16	1.67	3.18	1.68	2.57-4.01	1.34-2.10
7	3.67	1.94	3.74	1.97	3.74	1.97	3.71	1.96	2.94-4.59	1.57-2.45
8	3.96	2.09	0.00	0.00	0.00	0.00	3.96	2.09	3.17-4.95	1.67-2.61
9	4.23	2.23	4.12	2.18	4.11	2.17	4.15	2.19	3.38-5.29	1.75-2.74

TABLE 2. Matching of HPLC fingerprint profiles of Ayurved Siriraj Prasachandaeng or its components to those of phenolic reference markers (gallic acid, caffeic acid and vanillic acid).

Sample name	Gallic acid		Caffeic acid		Vanillic acid	
	R_t	PDA	R_t	PDA	R_t	PDA
Prasachandaeng	3.647	√	11.565	-	10.417	-
Component No. 1	-	-	11.650	-	10.417	-
Component No. 2	3.648	√	11.629	-	10.407	-
Component No. 3	3.581	-	11.530	-	10.415	-
Component No. 4	3.789	-	-	-	10.434	-
Component No. 5	-	-	11.541	-	10.412	√
Component No. 6	-	-	11.562	-	10.754	-
Component No. 7	-	-	11.407	√	-	-
Component No. 8	-	-	-	-	-	-
Component No. 9	3.659	√	11.649	-	10.448	-
Component No. 10	-	-	-	-	-	-
Component No. 11	3.632	√	11.636	-	10.385	-
Component No. 12	3.706	-	11.608	-	10.404	-

bility testing for intra-day (n=5), inter-day (n=3) and precision (n=15). The RSD of intra-day and inter-day analyses were within the acceptance criteria as well.

3.3 Determination of phenolic components using HPTLC

HPTLC chromatograms showed phenolic characteristics of Ayurved Siriraj Prasachandaeng, its twelve medicinal herbal components and reference markers captured at 254 and 366 nm under UV and visible light (after derivatization with FBS) (Fig 1). The chromatograms showed the possibility that the bands of phenolic reference markers were presented in Ayurved Siriraj Prasachandaeng and its herbal components nos. 1, 2, 4, 5, 6, 7, 9, 10, 11 and 12 (Fig 1), suggesting that the phenolic compounds in the recipe may be derived from these components. The bands of each Ayurved Siriraj Prasachandaeng batch were similarly identified by matching the color and R_f values (Fig 2). Gallic and caffeic acids were chosen to be the reference makers for rR_f analysis in Ayurved Siriraj Prasachandaeng batches (Table 1). All suspected bands of samples had rR_f in the acceptance 80-125% range of their means (Table 1), suggesting the similarities of the batches. Nevertheless, there were only three accepted ranges that covered 1, rR_f to gallic acid of band no. 2 and rR_f to caffeic acid of band no. 4 and 5 (Table 1), indicating the possibility of existence of gallic acid and caffeic acid. Thus, the present study exhibited the presence of phenolic components in Ayurved Siriraj Prasachandaeng and the similarity of HPTLC fingerprints among batches.

3.4 Fingerprinting and quantitative analysis using HPLC

Two-dimensional HPLC chromatogram fingerprints among three batches of Ayurved Siriraj Prasachandaeng recipe and its twelve herbal components were shown from bottom to top in Fig 3A and the three-dimension HPLC chromatogram fingerprint of the recipe was shown in Fig 3B. One peak in the HPLC fingerprint profile of the recipe was identified as gallic acid, by matching the R_t and photodiode array (PDA) to the gallic acid reference marker indicating the presence of gallic acid in the recipe. Likewise, gallic acid was found in components nos. 2, 9, and 11, and vanillic acid and caffeic acid were found in components nos. 5 and 7, respectively (Table 2). Quantitative determination showed that the recipe contained gallic acid about 0.00497±0.00014% w/w. After carefully analyzing the peak profiles of three batches of the recipe, five common peaks with acceptable area of more than 5% were selected for rR_t and ϕ analysis. Peak no. 2 of all batches was selected as a reference peak for calculation of rR_t variation (Table 3). The ϕ value of three batches (n=8) were 1.2265±0.0085, 1.2549±0.0162 and 1.2096±0.0287, respectively, and were in the acceptable range of 80-125% of the mean. These observations indicated the similarity among batches of the recipe.

DISCUSSION

HPTLC and HPLC were developed for fingerprinting and quantitative analysis of Ayurved Siriraj Prasachandaeng. The methods provided more chemical information used for the identification of crude drugs as well as for the quantification of phenolic compounds that were directly associated with the quality of the herbal medicine. The HPTLC fingerprinting method had the advantages of simplicity, rapidity and visuality, whereas, the HPLC fingerprinting method had the advantages of specificity, powerful separation ability and the ability to derive detailed chemical information. Both methods improved the reliability of identification of Thai traditional medicines.

TABLE 3. The rR_t in HPLC fingerprints of Ayurved Siriraj Prasachandaeng. Peak number 2 was selected as the reference peak.

Peak number	Batch 1	Batch 2	Batch 3	Average	RSD (%)
1	0.2378	0.2351	0.2351	0.2360	0.6576
2	1.0000	1.0000	1.0000	1.0000	0.0000
3	1.0442	1.0448	1.0453	1.0448	0.0542
4	1.1552	1.1569	1.1563	1.1561	0.0749
5	1.3779	1.3795	1.3784	1.3786	0.0588

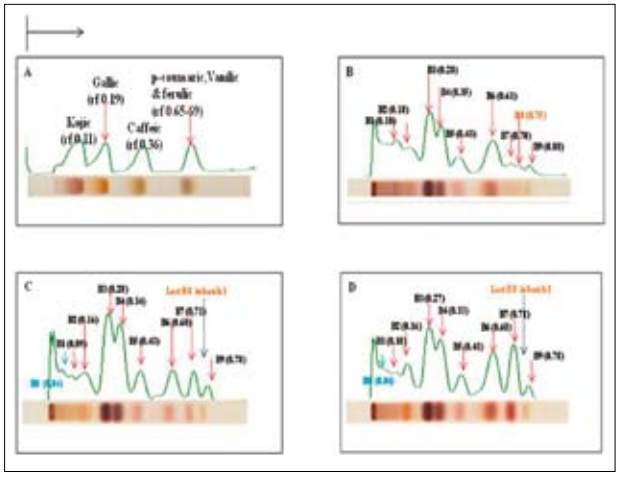

Fig 2. Typical HPTLC images and corresponding scanning profiles of phenolic mixture and Ayurved Siriraj Prasachandaeng recipe. A) Phenolic mixture: kojic, gallic, caffeic, p-coumaric, vanillic and ferulic acid. B) Ethanol extract of Prasachandaeng batch 1. C) Ethanol extract of Prasachandaeng batch 2. D) Ethanol extract of Prasachandaeng batch 3. All were visualized under visible light after derivatization with fast blue salt (FBS).

From the HPTLC chromatogram of Ayurved Siriraj Prasachandaeng, rR_f might not clearly provide corroborative evidence for the identification of complex plant samples because different compounds possibly have the identical R_f value. However, the chromatographic fingerprints with nine band expressions were similar among three batches (Fig 2). From the HPLC chromatogram, the chemical constituents were identified by matching of R_t and PDA to the reference compounds (Table 2). The expression of the chemical properties in the chromatogram was assessed as rR_t (Table 3). The rR_t and ϕ values of characteristic peaks were used in this study as parameters for quality assessment. The accepted range of 80-125% of mean and RSD of less than 10% were applied as the additional considerations. With the low percentage of RSD of rR_t as shown in Table 3 and of ϕ value (data not shown) the similarity among the three different batches of Ayurved Siriraj Prasachandaeng was confirmed more. Five peaks in the overlay fingerprint of three batches of Ayurved Siriraj Prasachandaeng (Fig 3) may represent the major constituents of this recipe.

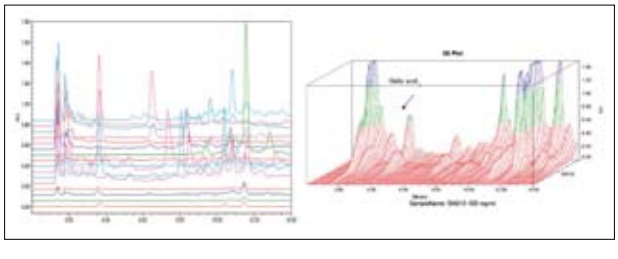

Fig 3.(A) Two-dimension HPLC overlay chromatogram of reference markers (gallic, caffeic and vanillic acid, from left to right), Ayurved Siriraj Prasachandaeng (batch 1-3, from bottom to top) and its twelve herbal components (no. 1-12, from bottom to top) at 280 nm under UV light. (B) Three-dimension HPLC-PDA chromatogram of Ayurved Siriraj Prasachandaeng (batch 1).

The HPTLC and HPLC procedures used in this study exhibited a satisfactory repeatability which potentially provides a reliable measure for quality control of the herbal products. The similarity of three batches of this recipe was considered by rR_f, rR_t and ϕ values. This study may suggest that (i) the chromatogram fingerprints should be used together with phenolic markers to identify and assess Ayurved Siriraj Prasachandaeng recipe and apply for its quality control and (ii) these methods are feasible for comprehensive quality evaluation of Thai traditional medical recipes.

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