Comparative Evaluation Of RP-HPLC And TLC-Densitometric Method For Determination Of Barakol Content in *Senna Siamea* Leaves

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Abstract

Using RP-HPLC and TLC-densitometric for quantitative determination of barakol in *Senna siamea* leaves was validated and compared. The HPLC method utilized a ZORBAX Eclipse Plus C18 column (3.5 µm, 100 mm × 4.6 mm i.d.) with a mixture of methanol and water (68:32 v/v) as the mobile phase at a flow rate of 1 ml/min, and UV detection at 245 nm. TLC-densitometric method was performed on precoated TLC plates, coated with silica gel 60F-254, with the CAMAG TLC scanner controlled by CAT software. A mixture of ethyl acetate, hexane and chloroform (40:40:20 v/v/v) were used as mobile phases and were also detected with UV at 372 nm. The parameters of linearity, intra-day and inter-day precision, accuracy, specificity, sensitivity LOD and LOQ of both methods were evaluated. Both assays provided good linearity, accuracy, reproducibility, and selectivity for determination of barakol. The quantitative results of both analytical methods did not show any statistically significant differences between them.

Abbreviations: RP-HPLC = Reversed phase high performance liquid chromatography

TLC-densitometric method = Thin layer chromatography densitometric method

Keywords: RP-HPLC; TLC-densitometric method; *Senna siamea*; barakol

1. Introduction

Senna siamea Lam. is a plant belonging to the Leguminosae family. It is a small to medium sized tree, in Thailand called "Khi-Lek." The fresh young leaves and flowers of Khi-Lek are used in Thai cuisine. They are edible when cooked as a curry and can also consumed as a health food as the plant has medicinal value. A decoction of the bark is given as a diabetic treatment; the roots are used as an antipyretic and the leaves for treating constipation, hypertension and insomnia (Kaur 2006). It contains many compounds including emodin, luteolin, chromone alkaloids, flavanoid glycoside and barakol (Ahe 1987; Shafiullah 1995). Barakol (3,4-dihydroxy-2,5-dimethyl-1,4-dioxyphenalene) is the major compound was found in *S. siamea* leaves (Fig. 1) and is biological active. Pharmacological studies imply that barakol possessed anxiolytic activity on the elevated plus maze (EPM) behavioral model and decreased spontaneous locomotor action in rats (Thongsaard 2001). According to solid scientific support with regard to therapeutic efficacy, commercially available tablets of *S. siamea* powdered leaves have been very popular in Thailand for producing natural sleep. However use for this purpose is now discouraged due to hepatotoxicity (Hongsirinirachorn 2003). Acute hepatitis was reported in patients after taking a daily dose of 2-4 *S. siamea* tablets, containing 20-40 mg of barakol or approximately 0.3-0.6 mg/kg/day of barakol. This might imply that barakol at the recommended dose, which is at least 40-fold lower than the toxic dose in rat model, could induce toxicity. Consumers of *S. siamea* as a herbal medicine should therefore be wary of its barakol content in order to avoid any risk of toxicity (Wongtongtair 2011). Therefore, the

amount of barakol in *S. siamea* leaves should be detected and controlled.

There are few techniques for the quantification of barakol content in *S. siamea* leaves, apart from the HPLC and TLC-densitometric methods (Padumanonda 2007). So far, there is no report to compare the result with the established RP-HPLC and TLC-densitometric methods for determination of barakol content. Therefore, the purpose of research is to develop RP-HPLC and TLC-densitometric methods for the rapid identification and quantitation of barakol in *S. siamea* leaves, and to compare the results obtained by these two different quantification methods.

2. Materials and Methods

2.1 Plant material

S. siamea leaves were collected from Pathum Thani Province, Thailand, in January 2013. The plant was authenticated at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, where herbarium specimen (Voucher SKP 098 19 19 01) is kept. These plants were dried at 50 ºC for 24 hours in a hot air oven and were reduced to a coarse powder using a grinder.

2.2 Chemicals

All solvents used for chromatography were methanol (HPLC grade), water (HPLC grade), hexane (analytical grade), and ethyl acetate (analytical grade) obtained from Merck (Darnstadt, Germany).

2.3 Purification of barakol from *S. siamea* leaves

Barakol was isolated from *S. siamea* leaves by the method used by Padumanonda 2007. The young leaves (300 g) were reduced into small pieces and boiled with 3% sulfuric acid 600 ml for 15 minutes. The extract was then passed through filter paper. The marcs were subsequently extracted again under the same conditions. The extracts were combined and alkalinized with sodium carbonate to pH 8. The basic filtered was partition with chloroform which was filtered and concentrated under reduced pressure until the volume was a quarter of the starting volume. After that, an equal volume of cold water was added and the mixture was cooled for 30 minutes in the refrigerator to produce crude barakol. The crude barakol was then purified by recrystallization from ethanol. Purified barakol (≥98% HPLC) was identified by comparing its spectroscopic data with an authentic sample.

2.4 Preparations of plant extracts

The dried leaf powder of *S. siamea* (200 mg) was extracted with methanol (20 ml) using sonication method for 1 hour. The extract was then filtered and adjusted to 50 ml with methanol. Samples were filtered through a 0.45 µm membrane filter and analyzed immediately after extraction in order to avoid possible chemical degradation. All assays of samples were performed in triplicate.

2.5 Standard solutions

A stock solution of the reference standard (barakol) was made in methanol, and subsequently diluted to provide a series of the standard ranging from 84-2.625 ng/ml for use in constructing a calibration curve.

2.6 RP-HPLC analysis

Analysis was carried out using an Agilent 1260 series HPLC equipped with a Photodiode-array detector (PDA). Data analysis was performed using OpenLAB CDS EZChrom software (Agilent, USA). Separation was achieved at 25 ºC on a ZORBAX Eclipse Plus C18, 3.5 µm, 100 mm × 4.6 mm i.d. (Agilent, USA). The mobile phase consisted of methanol and water (68:32, v/v). The mobile phase flow rate was 1 ml/min. Sample injection volumes were 10 µl, and detection was by UV at a wavelength of 245 nm.

2.7 TLC-densitometric method

A TLC precoated silica gel 60 F_{254} plate measuring 20 × 10 cm (Merck, Darmstadt, Germany) was used. Samples were applied with a 100 µl sample syringe using a Linomat V system (Camag, Muttenz, Switzerland). TLC scanner and CAT 4 software were used for sample application and quantitative evaluation. Samples of 10 µl were applied as 8 mm bands with a 15 mm distance between the bands. Chromatography was developed in a pre-saturated for 30 minutes in a vertical twin trough glass chamber (Camag, Muttenz, Switzerland), using ethyl acetate, hexane and methanol (40:40:10 v/v/v) as mobile phases. After

development the plate was dried at room temperature for 10 minutes and was visualized by UV at 254 and 366 nm. The photo documented TLC plate taken at UV light is show in figure 2. Barakol was quantified by direct densitometric scanning of a developed plate at 245 nm without derivertization.

2.8 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). Calculating data for the linearity, accuracy, intra-day and inter-day precision, specificity, limit of determination (LOD) and limit of quantitation (LOQ) were used to validate the RP-HPLC and TLC-densitometric methods.

2.8.1 HPLC assay

Calibration curves were constructed on three consecutive days by analysis of the standard solutions at six different concentrations, and by plotting peak areas against the concentration of the reference standard. The linearity of the detector response for the standard was determined by means of linear regression. In order to evaluate the intermediate precision (interday-precision) and repeatability (intraday-precision) the same stock solutions with three different barakol concentrations were injected into the HPLC system six times on the same day. The data were used to calculate % RSD (relative standard deviation) for intraday-precision. The interday-precision was validated by repeating the extraction procedure on the same sample of *S. siamea* leaves. 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 use to calculate % RSD for interday-precision. Accuracy was evaluated by means of recovery assays carried out by adding known amounts of the reference compounds to the sample solutions. LOD and LOQ were the concentrations that gave a signal to noise ratio equal to 3:1 and 10:1, respectively.

2.8.2 TLC-densitometric method

Various amounts of the stock solution (84-2.625 ng/band) were analyzed by TLC-densitometric method exactly as described above, and calibration curves were prepared by plotting peak areas against concentration. The precision of the instrumentation was checked by repeated scanning of the same bands of barakol six times each, and the % RSD was calculated. The repeatability of the method was tested by replicate scanning six times of standard barakol after application to a TLC plate. The variability of the method was studied by analyzing aliquots of different concentrations of standard solutions of barakol (42, 21, and 10.5 µg/ml) on the same day (intraday-precision) and on different days (interday-precision) and %RSD values were calculated (Table 2). The accuracy of the method was tested in a similar fashion to the HPLC method at three levels. In order to obtain estimates of LOD and LOQ a series of concentrations of barakol were spotted on TLC plates. LOD and LOQ were determined by considering the signal to noise ratio (S/N). LOD was considered as S/N 3:1, while LOQ was S/N 10:1.

2.9 Statistics

Values are expressed as a mean ± SD. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Tukey's range test ($P<0.05$).

3. Results and discussion

3.1 HPLC analysis

HPLC chromatograms of standard barakol and *S. siamea* extract are shown in figure 3. The linearity, accuracy, intraday- and interday-precision, specificity and limits of detection and quantitation were determined to validate the RP-HPLC method. The calibration curve for barakol was linear over the concentration rang 84-2.625 µg/ml with a linear equation of $Y = 154554X-27869$ ($R^2 = 0.9999$). The relative standard deviation values for both intraday and interday analysis of barakol were less than 1 and 2%, respectively. Recoveries in the range of 98-102% were observed for barakol. 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 diode-array detector. The spectra were taken at three points of the peak for barakol. When it was compared with the barakol 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 barakol with LOD and LOQ

values of 0.04 and 0.26 µg/ml, respectively. All of the validated data is shown in Table 1.

3.2 TLC-densitometric method

This TLC-densitometric method is simple and fast. In addition, the method does not require many steps for sample preparation. A mixture of ethyl acetate, hexane and methanol (40:40:10) used as mobile phase gave a good resolution of barakol. The TLC-chromatograms and 3D-densitograms of standard barakol and methanol extract of *S. siamea* leaves with an approximate range of Rf value of 0.17 are given in figure 4 and 5. Multiple wavelength detector (MWD) were used to produce UV absorption spectra for identity of the barakol band in *S. siamea* leaves extract, which were in good agreement with the spectrum of pure barakol (Fig. 6). The calibration curve for barakol was linear over the range of 84-2.625 ng/band. The regression equation was $Y = 119653X + 523.21$ ($R^2 = 0.9995$). The low value of standard deviation showed that the method was precise. The relative standard deviation values for both intraday and interday analysis of barakol were found to be less than 2% ensuring repeatability of the procedure. The recovery rate was determined to be 98-102%. The LOD and LOQ were found to be 13 ng/band and 20 ng/band, respectively. All of the validated data is shown in Table 1.

3.3 Comparison between HPLC and TLC-densitometric methods

Five samples of *S. siamea* leaves were used for the quantitative determination of barakol content by RP-HPLC and TLC-densitometric method. Each sample was analyzed in triplicate. The result of both analysis methods were compared with the reported UV spectrometric method by performing one way ANOVA studies. F Test value at p value less than 0.05 was found to be less than the table F value. The result indicated that there was not significant difference between the mean values of barakol content (Table 2). Therefore, both RP-HPLC and TLC-densitometric methods were found to be equal and could be used for the determination of barakol in *S. siamea* leaves.

4. Conclusion

The HPLC and TLC-densitometric methods provided a similar reproducibility, accuracy and selectivity for the quantitative determination of barakol in methanol extract of *S. siamea* leaves. The advantage of the TLC-densitometric method compared with the HPLC method is that it show less time consuming, and does not require many steps for sample preparation. A statistical comparison of the quantitative determinations of barakol in six different methanol extract samples did not show any statistical significant difference between RP-HPLC and TLC-densitometric methods.

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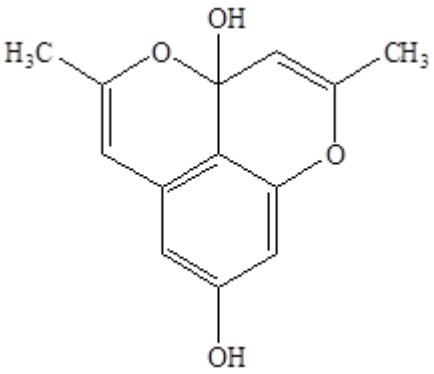

Fig. 1 Structure of barakol

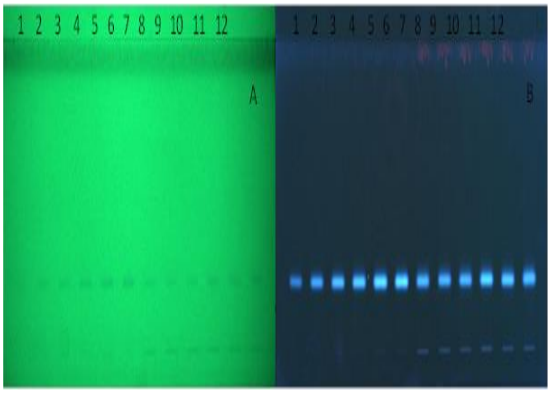

Fig.2 Photo-documentation at 254 nm (A) and 366 nm (B): lanes 1-6 were standard barakol of 84-2.625 ng/band; lanes 7-12 were methanol extract samples of *S. siamea* leaves

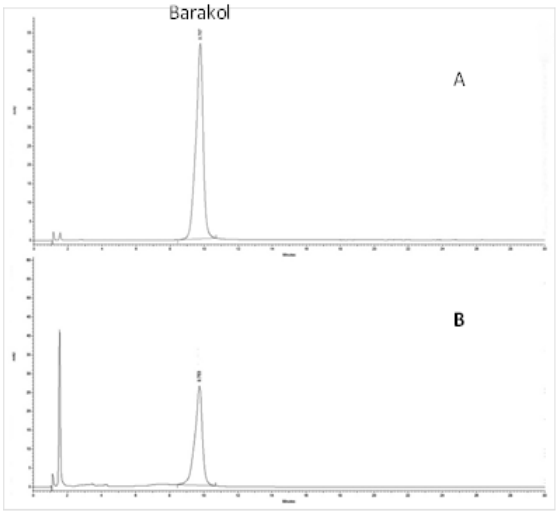

Fig. 3 RP-HPLC chromatograms of barakol standard (A) and methanol extract of *S. siamea* leaves (B)

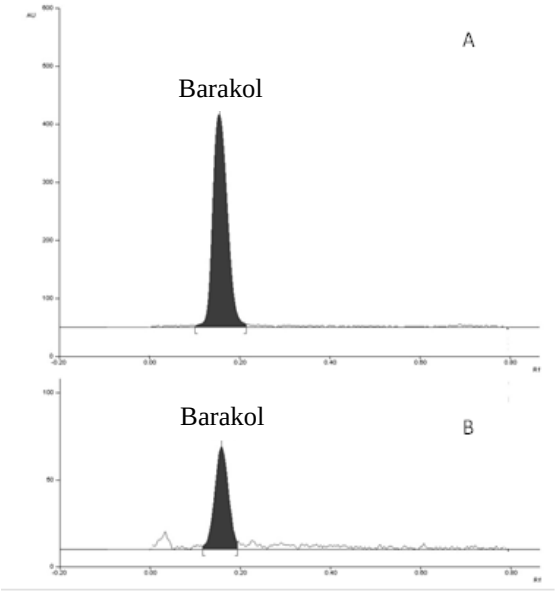

Fig. 4 The TLC-chromatograms of standard barakol (A) and the methanol extracts of *S. siamea* (B)

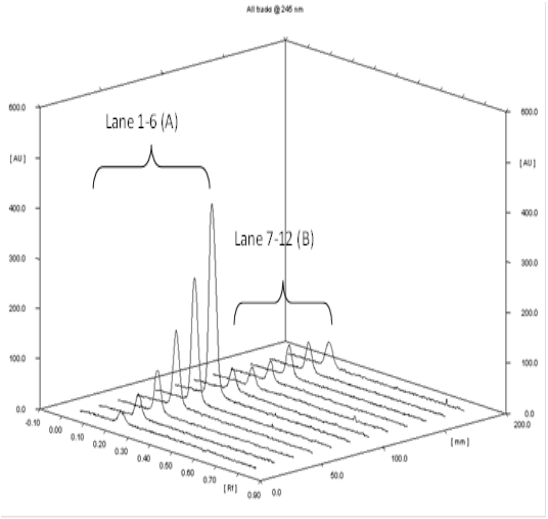

Fig.5 TLC-densitograms of various concentrations of standard barakol, and the scan area with barakol content in methanol extract sample of *S. siamea* leaves

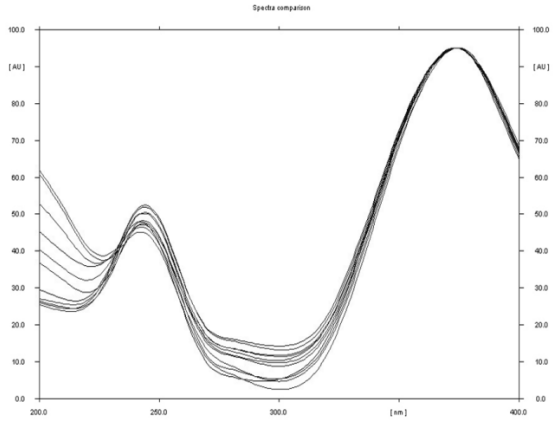

Fig. 6 UV spectra of various concentrations of standard barakol overlayed with barakol extracts (maximum absorption at 247 and 375 nm).

Table 1. Validation data of RP-HPLC and TLC-densitometry assay

Parameters	RP-HPLC	TLC-densitrometry
Linear range	84-2.625 µg/ml	84-2.625 g/band
Linear equations	Y = 154554X - 27869	Y = 119653X + 523.21
R^2	0.9999	0.9995
Precision (%RSD)		
Intraday	0.48	0.72
Interday	1.62	1.83
LOQ	0.265 µg/ml	20 ng/band
LOD	0.038 µg/ml	13 ng/band

Accuracy reference value	RP-HPLC		TLC-densitrometry	
(µg/ml)	Recovery(%)	RSD(%)	Recovery(%)	RSD (%)
10.5	98.1	0.84	98.5	1.07
21	101.2	0.65	101.8	1.13
42	102.4	0.72	102.9	1.34

Table 2. Barakol content in methanol extract of *S. siamea* leaves determined by RP-HPLC and TLC-densitometric methods

Samples	Content of barakol (% w/w) HPLC (Mean ± SD)	TLC-desitometry (Mean± SD)
1	2.57 ± 0.09*	2.64 ± 0.11*
2	2.57 ± 0.08*	2.62 ± 0.10*
3	2.62 ± 0.07*	2.68 ± 0.08*
4	2.65 ± 0.11*	2.71 ± 0.12*
5	2.54 ± 0.09*	2.60 ± 0.13*

* No Significant difference ($P<0.05$) when RP-HPLC and TLC-densitometric methods were compared