An alternative high performance liquid chromatographic method for determination of piperine in serum: Application to pharmacokinetic studies


Abstract

Introduction: Piperine, a major alkaloid found in Benjakul remedy of the spice black and long peppers *Piper longum* Linn. and *Piper nigrum* Linn., is used as a traditional medicine in several tropical countries particularly Asia. The aim of the study was to establish an alternative high performance liquid chromatography (HPLC) with ultraviolet (UV) detection method, which is relatively simple, rapid, sensitive, accurate and reproducible method for determination of piperine in human serum.

Methods: Piperine and the internal standard β-17-estradiol acetate were separated from serum on a ZORBAX Eclipse XDB-C18 (4.6 x 250 mm, 5 μm particle size) column, with the mobile phase consisting of a mixture of 25 mM KH$_2$PO$_4$ and acetonitrile at the ratio of 35:65 (v:v) running at a flow rate of 1.0 mL/min. Ultraviolet detection was at the wavelengths of 340 and 280 nm for piperine and β-17-estradiol acetate, respectively. Retention times of piperine and β-17-estradiol acetate were 4.6 and 10.0 min, respectively. Sample preparation was done by extraction of protein with the mixture of 9:1 (v:v) ethylacetate and propanol. The developed method was validated according to guidance for industry bioanalytical method validation.

Results: Good precision and accuracy were obtained for both within-day repeatability and day-to-day reproducibility. Limit of quantification (LOQ) for piperine was 25 ng/mL using 500 μL sample. The mean recoveries for piperine were between 86.44-99.30%. The method was successfully applied for a pharmacokinetic study of piperine in healthy Thai males.

Discussion and Conclusion: An alternative analytical method for the determination of piperine in serum using HPLC with UV detection established in this study meets the criteria for application to routine clinical drug level monitoring or pharmacokinetic study. The developed method is relatively rapid, simple, and sensitive.

Key words: Piperine, HPLC-UV, Pharmacokinetics, Benjakul

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In the present report, we describe an alternative HPLC with UV detection method, which is relatively simple, rapid, sensitive, accurate and reproducible method for determination of piperine in human serum. The method has been applied successfully for pharmacokinetic study of piperine in healthy Thai males following a single oral dose of 200 mg Benjakul formulation, an ethanolic extract of parts from five plants, i.e., *Piper chaba* Hunter. (fruit), *Piper sarmentosum* Roxb. (root), *Piper interruptum* Opiz. (stem), *Plumbago indica* Linn. (root), and *Zingiber officinale* Roscoe. (rhizome).

### Materials and methods

**Chemicals**

Piperaquine [1-[5-[1,3-benzodioxol-5-yl]-1-oxo-2,4, pentadienyl] piperidine], (Figure 1a) and the internal standard β-17-estradiol acetate (Figure 1b) (99% pure) were purchased from Merck Co. Ltd. (Darmstadt, Germany) and Sigma-Aldrich (CA, USA). Acetonitrile, methanol, ethylacetate, and propanol were of HPLC grade, which were purchased from Labscan Co. Ltd., Bangkok, Thailand. The following chemicals and solvents were obtained in the highest purity available: phosphoric acid, and dipotassium hydrogen phosphate-3 hydrate were obtained from Analyticalxal Sciences Co. Ltd., Bangkok, Thailand. Deionized double distilled water was used for the preparation of working nicotine standard solutions. Serum from healthy volunteers used for standard curves was provided from the blood bank of Thammasat Chalermprakiet Hospital.

![Figure 1](image-url)  
**Figure 1** Chemical structures of piperine (a) and β-17-estradiol acetate (b).
Preparations of standards

Stock solutions of piperine and β-17-estradiol acetate at the concentration of 1,000 ng/μL by dissolving 5 mg of the compound in 5,000 μL methanol and stored at -20°C until used. Working standard solutions were prepared by diluting the stock standard solutions with methanol. Standard solutions were stored at -20°C until analysis.

Seven aliquots of blank control serum were spiked with piperine working solution in a serial dilution to obtain the standard calibration at the concentrations of 25, 50, 100, 250, 500, and 1,000 ng/mL, with 3,000 ng/μL internal standard (3,000 ng/μL β-17-estradiol acetate).

Chromatography

Piperine and the internal standard were separated on a ZORBAX Eclipse XDB-C18 (4.6 x 250 mm, 5 μm particle size) (Agilent Technologies®, CA, USA. The HPLC system was operated under an isocratic mode at a flow-rate of 1 mL/min. The mobile phase was a mixture of 25 mM KH₂PO₄ (pH 4.5, adjusted with orthophosphoric acid) and acetonitrile at the ratio of 35:65 (v:v). The HPLC system consisted of a solvent delivery system (SpectraSystem™ P4000 Quaternary solvent delivery/controller: Thermo fisher scientific, CA, USA) equipped with solvent degasser (SpectraSystem SCM1000 solvent degasser: Thermo fisher scientific, CA, USA), and an autosampler (SpectraSystem AS3500: Thermo fisher scientific, CA, USA) and a UV detector (SpectraSystem UV/Vis 3000: Thermo fisher scientific, CA, USA). The wavelength of UV-Vis detector was operated at 340 and 280 nm for piperine and β-17-estradiol acetate, respectively.

Sample preparation

Five hundred microliters (μL) of unknown serum samples, or quality control samples were transferred to polypropylene tubes, and 100 μL internal standard (3,000 ng/μL) was added to each tube. After thoroughly mixing, distilled water (500 μL) and 12 mM phosphate buffer pH 3.4 (100 μL) were added. After vortex mixing, the mixture was then extracted with 6 mL of the mixture of 9:1 (v:v) ethylacetate and propanol by vortex mixing for 10 minutes. The upper organic phase was separated through centrifugation at 2,000 x g for 5 min. Organic solvents were evaporated to dryness at 40°C in a parallel evaporator. The residue was reconstituted with 100 μL of methanol, and 40 μL portion was injected onto the column.

Detector linearity

Solutions of piperine in distilled water at concentrations ranging from 25 to 2,000 ng/mL were injected into the HPLC system in order to assess detector linearity. Peak heights were plotted against the quantities of all standards injected. All were found to be linear (r² > 0.999) over the concentration range observed.

Calibration curves

Calibration curves of piperine were prepared by replicate analysis of six serum samples (500 μL each) spiked with varying concentrations of piperine (25, 50, 100, 250, 500, and 1,000 ng/mL) and a fixed concentration of the internal standard (3,000 ng/μL). Samples were analyzed as described above.

Data analysis

Concentrations of piperine were determined from the peak height ratios (peak heights of piperine/peak heights of internal standard), which corresponded to the known piperine concentrations in a calibration curve as described above. Peak detection, peak height integration, peak height ratio calculation were performed by the Millennium 2000 Chromatograph™ software (Waters, Milford, MA, USA). Peak height ratios of piperine/internal standard were calculated. Concentrations of piperine in serum samples were determined by matching peak height responses against a calibration curve of response ratio (height of piperine/height of internal standard) vs concentration, obtained from standard sample injection.

Method validation

The developed method was validated according to guidance for industry bioanalytical method validation. Precision: The precision of the method based on within-day repeatability was determined by replicate analysis of six sets of serum samples spiked at six different concentrations of piperine (25, 50, 100, 250, 500, and 1,000 ng/mL). The reproducibility (day-to-day
variation) of the method was validated using the same concentration range of serum as described above, but only a single determination of each concentration was made on six different days. Coefficient of variation (CV) were calculated from the ratios of standard deviation (SD) to the mean and expressed as percentage.

Accuracy: The accuracy of the method was determined by replicate analysis of six sets of serum samples spiked at four different levels of piperine (25, 50, 100, 250, 500, and 1,000 ng/mL) and comparing the difference between the spiked value and that actually found (theoretical value).

Recovery: The analytical recovery of sample preparation procedure for piperine was estimated by comparing the peak heights obtained from serum samples prepared as described above, with those measured with equivalent amounts of piperine in methanol. Triplicate analysis was performed at piperine and internal standard concentrations of 50, 500, and 1,000 ng/mL, respectively.

Selectivity: The selectivity of the assay was demonstrated by checking for the absence of endogenous interferences at the retention times of piperine in human blank serum obtained from six different lots, after subjecting them to sample preparation procedures.

Limit of quantification: The limit of quantification (LOQ) of the assay procedure was determined from the lowest concentration of piperine (in spiked serum sample) that produced a peak height ten times the baseline noise (absorbance unit full scale) in a 500 μL sample.

Quality control: Quality control (QC) samples for piperine were made up in serum using a stock solution separated from that used to prepare the calibration curve, at the concentrations of with 50 lowl, 500 (medium), and 1,000 ng/mL (high) piperine and 3,000 ng/mL internal standard. Samples were aliquoted into cryovials, and stored frozen at -20°C for use with each analytical run. The results of the QC samples provided the basis of accepting or rejecting the run. At least four of the six QC samples had to be within ±20% of their respective nominal values. Two of the six QC samples could be outside the ±20% of their respective nominal value, but not at the same concentration.

Application of the method to biological samples

The method was applied to the investigation of the pharmacokinetics of piperine in serum samples in a total of 5 Thai males (aged 20-25 yr, weighing 55-74 kg) following a single dose of 200 Benjakul formulation (1 tablet, 12 mg piperine). This part of the study was approved by the Human ethics committee of faculty of medicine, Thammasat University, Thailand. Written informed consents for study participation were obtained from all patients. Venous blood samples (5 mL) were collected into plastic tubes at the following time points: 0 (before dose), and at 0.5, 1, 2, 4, 6, 9, 12, 18, 24 and 48 hr after drug administration. All samples were stored immediately after collection at -80°C until analysis.

Results

Chromatographic separation

Under the chromatographic condition described above, the chromatograms of piperine and internal standard (β-17-estradiol acetate) were free from any interference peak, with good resolution and sharp peaks. Blank serum sample showed little noise fluctuation. The retention times of piperine and β-17-estradiol acetate were 4.6 and 10.0 min, respectively (Figure 2).

Sample preparation

A number of sample preparation procedures were investigated to optimize the extraction of piperine and the internal standard β-17-estradiol acetate from serum samples. The optimal sample preparation procedure used in this study was simple involving only a single step liquid-liquid extraction by organic solvents (ethylacetate: propanol = 9:1 v:v), which resulted in clean chromatograms (Figure 2).
Figure 2 Chromatogram of (a) blank serum, (b) serum spiked with 500 ng/mL piperine and 3,000 ng/mL internal standard, and (c) serum sample collected from one subject after one hour of the administration of a single oral dose of 200 mg Benjakul formulation (spiked with 3,000 ng/mL internal standard).

Calibration curves

Serum analysis was calibrated using concentration range of 25-1,000 ng/mL. All calibration ranges yielded linear relationships with correlation coefficients ($r^2 > 0.999$) or better (Figure 3). The linear regression equation obtained from the mean of the six calibration curves was $y = 0.25x + 3.201$, where $y$ is the peak height ratio and $x$ is the analyte concentration in ng.
Method validation

**Precision** Little variation of piperine assay in serum samples was observed; coefficients of variation (CV) for six analysis at the concentration range of 25-1,000 ng/mL were all below 15%. The intra-day assay CV of piperine in serum samples varied between 3.0 and 14.07%. The inter-day assay CV varied between 1.18 and 19.11%. (Table 1).

**Accuracy** Good accuracy was observed from both the intra-day or inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added). For intra-day assay validation, the mean deviation from the theoretical values (MDV) varied between -14.96 and +19.68%. The inter-day assay MDV varied between -14.22 and +11.98% (Table 1).

**Recovery** The mean recoveries for piperine at 50, 500 and 1,000 ng/mL were 90.13, 90.06 and 97.18%, respectively. The recovery of β-17-estradiol acetate at the concentration of 3,000 ng/mL was 99.47 ± 0.15% (Table 2). The results reflect essentially high recovery for all compounds from the spiked serum and indicate lack of interference from sample preparation procedure.

**Selectivity** Selectivity of the chromatographic separation was demonstrated by the absence of interferences from endogenous peaks and samples collected from one healthy male following a single oral dose of 200 mg Benjakul formulation (Figure 2).

**Limit of quantification** The limit of quantification (LOQ) in human serum samples for piperine was accepted as 25 ng/mL using 500 μL blood.

![Figure 3](image_url) Calibration curve of piperine standard at concentration 25-1,000 ng/mL.

### Table 1 Intra-day and inter-day precision and accuracy of piperine assay

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Precision (%CV)</th>
<th>Accuracy (%DMV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>19.11</td>
</tr>
<tr>
<td>50</td>
<td>2.23</td>
<td>1.18</td>
</tr>
<tr>
<td>100</td>
<td>14.07</td>
<td>3.41</td>
</tr>
<tr>
<td>250</td>
<td>13.59</td>
<td>4.79</td>
</tr>
<tr>
<td>500</td>
<td>9.31</td>
<td>2.40</td>
</tr>
<tr>
<td>1,000</td>
<td>10.78</td>
<td>1.28</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>8.83 ± 5.13</td>
<td>5.36 ± 6.87</td>
</tr>
</tbody>
</table>

1CV : coefficient of variation (%)

2DMV : deviation of mean value from theoretical value (%)
Application of the method to biological samples

The method appears to be robust and has been applied to the investigation of the pharmacokinetics of piperine in serum samples in a total of 5 Thai males following a single dose of 200 Benjakul formulation (1 tablet, 12 mg piperine). Figure 4 showed mean and each individual concentration-time profiles of piperine in all subjects. Median C<sub>max-1st</sub>(first peak of maximum concentration) and AUC<sub>0-48hr</sub> (area under the concentration time curve from 1 to 48 hr) values were 1,078 ng/mL and 10,216 ng.hr/mL, respectively.

Table 2 Recovery of piperine in serum at different concentrations and internal standard (IS).

<table>
<thead>
<tr>
<th></th>
<th>% Recovery of piperine</th>
<th>% Recovery of IS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 ng/mL</td>
<td>500 ng/mL</td>
</tr>
<tr>
<td>1</td>
<td>90.97</td>
<td>86.44</td>
</tr>
<tr>
<td>2</td>
<td>86.48</td>
<td>86.01</td>
</tr>
<tr>
<td>3</td>
<td>92.92</td>
<td>97.72</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>90.13 ± 3.30</td>
<td>90.06 ± 6.64</td>
</tr>
</tbody>
</table>

Figure 4 Serum concentration-time profiles of piperine in serum samples in a total of 5 Thai males following a single dose of 200 Benjakul formulation (1 tablet, 12 mg piperine). Data were presented as median (circle) and individual (line) values.

Conclusion and discussion

An alternative analytical method for the determination of piperine in serum using HPLC with UV detection established in this study meets the criteria for application to routine clinical drug level monitoring or pharmacokinetic study. The developed method is relatively rapid, simple, and sensitive. It is noted however that the ubiquitousness of piperine in the human diet and the presence of lipids in the plasma of humans may interfere with piperine assay in human serum.

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References


บทคัดย่อ
การใช้เทคนิค HPLC ในการหาปริมาณ piperine ในซีรัม และการประยุกต์ใช้ในการศึกษาทางเภสัชจลนศาสตร์

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บทนำ:
Piperine เป็นสารออลคาลอยด์ที่พบมากที่สุดในตำารับยาเบญจกูล ซึ่งพบในพริกไทย, ดีปลี และช้าพลู และมีการใช้ในการแพทย์พื้นบ้านในประเทศเขตร้อน โดยเฉพาะในเอเชีย. วัตถุประสงค์ของการศึกษาคือเพื่อพัฒนาวิธีการหาปริมาณ piperine ในซีรัมของมนุษย์ด้วยเทคนิค HPLC-UV ซึ่งเป็นวิธีที่ดี รวดเร็ว ถูกต้อง และมีประสิทธิภาพ.

วิธีการศึกษา:
แยกสาร piperine และ internal standard (β-17-estradiol acetate) ในซีรัมโดยใช้คอลัมน์ชนิด ZORBAX Eclipse XDB-C18 (4.6 x 250 mm, 5 µm particle size) และสารละลายเคลื่อนที่ซึ่งประกอบด้วย 25 มิลลิโมล่าร์โปแทสเซียมไดฮัยโดรเจนฟอสเฟต และอะซิโตไนไตร ในอัตราส่วน 35:65 (ปริมาตร/ปริมาตร) ด้วยอัตราเร็ว 1 มิลลิลิตร/นาที โดยตั้งเครื่องวัด UV-VIS ที่ความยาวคลื่น 340 นาโนเมตร สำาหรับ piperine และ 280 นาโนเมตร สำาหรับ internal standard ซึ่งสามารถแยกสาร piperine และ internal standard ที่เวลา (retention time) 4.6 และ 10.0 นาที ตามลำดับ การเตรียมตัวอย่างเข้มข้นของ piperine และ internal standard ที่ใช้ในการวัด ซึ่งสามารถทำได้ตามหลักเกณฑ์ของ guidance for industry bioanalytical method validation.

ผลการศึกษา:
วิธีที่ใช้ในการหาปริมาณ piperine ในซีรัมมีความถูกต้อง เที่ยงตรง และมีผลที่มั่นคง การวัดปริมาณ piperine ได้อย่างแม่นยำ โดยใช้ตัวอย่างซีรัม 500 ไมโครลิตร และมีประสิทธิภาพในการวัดการเจริญเติบโตของนักเรียนได้ดี ร้อยละ 86.44-99.30 ซึ่งวิธีนี้สามารถนำมาประยุกต์ใช้ในการศึกษาทางเภสัชจลนศาสตร์ของ piperine.

วิจารณ์และสรุปผลการศึกษา:
วิธีการวัดที่พัฒนาขึ้นนี้ รวดเร็ว ง่าย และมีความแม่นยำในการวัด piperine ซึ่งจะเป็นพื้นฐานในการวิจัยทางเภสัชจลนศาสตร์.

คำสำคัญ: Piperine, HPLC-UV, เภสัชจลนศาสตร์, เบญจกูล