Determination of Melatonin in Plasma by Liquid-liquid Extraction and High Performance Liquid Chromatography Coupled with Fluorescence Detection and Its Application for Melatonin Pharmacokinetic Study in Humans

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Abstract

Melatonin is a neurohormone secreted from the pineal gland which regulates the circadian rhythm in human. To date, this compound has been used as a food supplement as well as a chemotherapy adjuvant for several types of cancer. However, data on the pharmacokinetics of melatonin at therapeutic doses is still limited. This study aimed to develop and validate a high performance liquid chromatography method for determination of melatonin in human plasma for purposes of its application to pharmacokinetic study after a therapeutic dose. Plasma melatonin was extracted with dichloromethane under alkaline conditions. A mobile phase consisting of 10 mM phosphate buffer: acetonitrile (80:20), pH 7.2 and a reverse phase, C18 column as well as a fluorescence detector were used. The excitation and emission wavelength were set at 286 and 346 nm and the flow rate of the mobile phase was set at 1 mL/min. Under these chromatographic conditions, the retention times for 5-methoxytryptamine, the internal standard, and melatonin, were 3.5 and 5.5 min, respectively. No interferences from endogenous plasma compounds were recorded at the retention times of the analytes. The lowest limit of quantitation of melatonin was 0.1 ng/mL. The good linearity of the calibration at the range 0.1-35 ng/mL was obtained with the correlation coefficient of 0.99. The inter-day and intra-day accuracy as well as precision were within the acceptance limits. This assay method was successfully applied for pharmacokinetic study of 20 mg oral melatonin dose in male healthy Thai volunteers fasting condition.

Keywords: melatonin, high performance liquid chromatography, plasma, pharmacokinetics, liquid-liquid extraction
วิธีการวิเคราะห์เมลาโทนินในพลาสมาโดยการสกัดด้วยของเหลวและโครมาโตกราฟฟีของเหลวประสิทธิภาพสูงควบคู่กับการตรวจวัดสัญญาณเรืองแสงและการประยุกต์ใช้เพื่อการศึกษาเภสัชจลนศาสตร์ของเมลาโทนินในมนุษย์

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บทคัดย่อ

เมลาโทนิน เป็นฮอร์โมนที่สังเคราะห์จากต่อมไพเนียลที่ทำหน้าที่ควบคุมวงจรของการหลับและตื่นของมนุษย์ ปัจจุบันสารนี้ถูกนำมาใช้เป็นผลิตภัณฑ์เสริมอาหารและใช้ร่วมกับยาเคมีบำบัดในการรักษามะเร็งหลายชนิด อย่างไรก็ตาม ข้อมูลเกี่ยวกับเภสัชจลนศาสตร์ของเมลาโทนินในขนาดที่ใช้รักษาไม่มีค่อนข้างมาก การศึกษาครั้งนี้เป็นการพัฒนาวิธีการวิเคราะห์เมลาโทนินในพลาสมาของมนุษย์โดยใช้วิธีโครมาโตกราฟฟีของเหลวประสิทธิภาพสูง ทั้งนี้เพื่อใช้ในการศึกษาเภสัชจลนศาสตร์ของเมลาโทนินหลังได้รับยาในขนาดที่ใช้รักษา โดยการนำพลาสมาที่มีเมลาโทนินมาสกัดด้วยโครมาโทกราฟผ่านได้สำราญที่ดีในต่าง โดยใช้ 10 mM phosphate buffer: acetonitrile (80:20), pH 7.2 เป็นวัสดุเคลื่อนที่ คอลัมน์ C18 เป็นวัสดุที่นิ่ง และวัดสัญญาณการเรืองแสงที่ความยาวคลื่น excitation และ emission ที่ 286 และ 346 นาโนเมตร ค่าคั่นกรดกรดของวัสดุคลีนที่ให้ 1 ลบ.นาที ภาวะการผลิตของเครื่องโครมาโตกราฟฟี สาร 5-เมทอกซีเมลาโทนิน (สารควบคุมภายใน) และเมลาโทนินจะปรากฏที่เวลา 3.5 และ 5.5 นาทีตามลำดับ ไม่พบสารภายในในช่วงเวลาที่มากกว่าตัวอย่างที่ต้องการวัดเวลา ค่าคั่นสุดของเมลาโทนินในวัสดุเครื่องที่ได้ค่า 0.1 นาโนกรัม/มล. ความแม่นยำความเข้มข้นระหว่าง 0.1-35 นาโนกรัม/มล. มีค่าเป็นต้นที่ดีและมีค่าสัมประสิทธิ์สหสัมพันธ์ 0.99 สำหรับความถูกต้องและความแม่นยำในวันเดียวกันและระหว่างวันใหม่ไม่แตกต่างกัน วิธีวินิจฉัยนี้สามารถนำไปใช้ศึกษาเภสัชจลนศาสตร์ของเมลาโทนินในสารระดับสูงของเมลาโทนินในเวลา 20 นาที ภาวะการผลิตอาหารได้เป็นอย่างดี

คำสำคัญ: เมลาโทนิน, โครมาโตกราฟฟีของเหลวประสิทธิภาพสูง, พลาสมา, เภสัชจลนศาสตร์
Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone secreted from the pineal gland which regulates the sleep-wake cycle or circadian rhythm in humans and is mediated by light. Melatonin is synthesized from 5-hydroxytryptamine by N-acetylation and followed by methylation. This compound has been used as sleep-inducer to treat insomnia or jet lag (with usual dose range of 0.5-10 mg) as food supplement in several countries. Apart from circadian control, several biological effects of melatonin have been reported including antioxidant, immunomodulator, and neuroprotective agent. Moreover, melatonin has been investigated as a chemotherapy adjuvant for treatment of several cancers. Results from meta-analysis reveal that high dose of melatonin as a single therapy or combination with chemotherapeutic agents can reduce both side effects of chemotherapeutic agents and 1 year mortality in several cancers.

Accurate and precise analytical methods for melatonin are needed for pharmacokinetic study of this compound in human. Several analytical methods have been developed for determination of melatonin in human serum or plasma including radioimmunoassay, gas-chromatography mass spectrometry, ELISA, LC/MS and high performance liquid chromatography (HPLC) with ECD or fluorescent detection. However, many of them are costly, require derivatization, or have been developed to measure low endogenous levels of melatonin, and are therefore not suitable for large numbers of samples from exogenous pharmacokinetic studies. Due to the fluorescence property of melatonin, HPLC coupled with fluorescence detection method permits the analysis of melatonin without derivatization and is suitable for detection in complex samples (i.e., plasma, central and peripheral tissues). The aim of the present study was to develop a simple, robust and reproducible HPLC with fluorescence detection method that can be used for determination of exogenous melatonin in human plasma for pharmacokinetic study.

Materials and Methods

Reagents

Melatonin was obtained from Huang gang Saikang Pharmaceutical Co., Ltd (Hubei, China) and 5-methoxytryptamine was purchased from Sigma (Zwijndrecht, Netherlands). Other chemicals used in this study were of analytical grade. Melatonin and 5-methoxytryptamine (internal standard, IS) stock solutions were dissolved in methanol and then adjusted with water to final concentration of 1 mg/mL.

Instrumentation

An autosampling HPLC system (Alliance Waters separation module e2695, Waters, Manchester, UK), was coupled with a fluorescence detector (Model 2475, Waters, Manchester, UK) with Empower 2 software (Waters, Manchester, UK) for data collection and data handling.
Chromatographic conditions

Optimized chromatographic conditions were a stationary phase was a Novapak® C18 (3.9 × 150mm), 4 µm particle size (Waters corporation, Manchester, UK) at room temperature. The mobile phase consisted of 10 mM potassium phosphate buffer and acetonitrile (80:20 v/v), final pH 7.2 with a flow rate 1.0 mL/min. The excitation and emission wavelengths of fluorescence detection were set at 286 nm and 346 nm, respectively.

Sample preparation

Prior to extraction, 1 mL of each plasma sample (for calibration curves, quality control or study subject samples) was pipetted into a screw capped tube and spiked with 50 µL of the IS, 5-methoxytryptamine (500 ng/mL) and 100 µL of 4 M NaOH solution. The sample was extracted with 5 mL of dichloromethane by vortexing for 1 min. The organic phase was separated by centrifugation at 2,091 x g for 10 min. The organic phase was then transferred to the new tube with a Pasteur pipette and evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 100 µL of HPLC mobile phase by vortexing for 30 seconds and subsequently transferred a microvial. An aliquot of 10 µL was injected onto the HPLC column using the HPLC autosampler. Blood samples obtained from subjects with melatonin concentrations more than 35 ng/mL were diluted 2- or 10-fold with blank plasma and consequently re-analyzed.

Assay method validation

The bioanalytical method developed for determination of melatonin in human plasma was validated as described below:

Specificity and selectivity

For assessment of the interfering endogenous compounds, human plasma samples obtained from five different blood donors were tested. Samples were prepared according to the sample preparation as described above with and without IS. A human plasma samples containing melatonin and IS in plasma were prepared and the chromatograms were compared with those of plasma blank samples.

Limit of quantitation

The lower limit of quantification (LLOQ) was defined as the lowest concentration of melatonin yielding an assay precision with CV ≤20% and accuracy within the range of 80-120% of the actual value.

Linearity

A calibration curve was prepared by spiking melatonin stock solution in human plasma samples. A calibration curve consisted of 7 plasma samples spiked to concentrations of 0.1, 0.25, 0.5, 2.5, 10, 25 and 35 ng/mL of melatonin. The range of melatonin concentration used in the calibration curve was chosen based on the expected concentration of melatonin in plasma after oral administration of 25 mg melatonin. The ratios between the areas of the melatonin peaks and the IS peaks were plotted against the melatonin concentrations.

Precision and accuracy

Three sets of quality control (QC) samples at low (0.3 ng/mL), medium (17 ng/mL) and high (25 ng/mL) melatonin concentrations were prepared from the same working standard solution of melatonin by spiking into 25 mL of human plasma. For intra-day precision and accuracy, each
concentration of QC sample was assayed in six replicates while the inter-day accuracy and precision were determined by the analysis of six replicates of each QC sets for three consecutive days. The accuracy of these QC samples should be within the range of 100±15% of the actual value with CV ≤15%.

Recovery of extraction Two sets of QC samples at low (0.3 ng/mL) and high (25 ng/mL) concentrations of melatonin were prepared by spiking melatonin working standard solution into human plasma. Five replicates of each melatonin concentration were then extracted by dichloromethane after adding an IS as described above in the sample preparation section. The percentage recovery of melatonin and IS was determined by measuring the mean peak area response of melatonin and IS obtained from the extracted QC samples and compared with the mean peak area response of five replicates of spiked melatonin in the mobile phase containing the same concentrations of analytes.

Dilution integrity Two set of melatonin concentration at 70 and 350 ng/mL (2- and 10-fold of upper limit concentration of calibration curve) were prepared in plasma. Four replicates of each concentration were diluted 2-fold for 70 ng/mL and 10-fold for 350 ng/mL with plasma blank before extraction. The extraction was performed as described above in the sample preparation section.

Pharmacokinetics of melatonin in healthy Thai volunteers

The validated assay method was applied to a pharmacokinetics study of melatonin in healthy male volunteers. The enrolled subjects were housed one night before the pharmacokinetic study day at the housing site, Arkarn KwanMor, Khon Kaen University near Srinagarind Hospital. The dinners provided for all subjects in the housing day of both periods were identical. After an overnight fast of at least 10 h (except water was allowed as desired until 1 h before drug administration), all subjects were transferred to Srinagarind Hospital and vital signs were monitored after resting for a while. Each subject took one 20 mg capsule of melatonin (General Drug House, Lt. Co., Thailand) with 240 mL water (room temperature). Administration of the drug was carried out while the subjects were in the sitting position. The subjects were allowed to have normal activities and avoid physical exertion, and lunch was provided to the subjects at 4 h after melatonin dosing. Blood samples were collected through an indwelling cannula placed in a forearm of the subject before drug administration (0 h) and at 0.5, 0.75, 1.0, 1.25, 1.5, 2, 3, 4, 5, 6 and 8 h after melatonin administration. For blood collection at each time point, the patency of indwelling cannula was maintained by 1 mL of saline and 2 mL of blood were withdrawn from the indwelling cannula and discharged at each time point prior subsequent collection of about 7-8 mL blood into a K$_3$EDTA coated tube. Tubes were then kept on ice before separation of plasma samples. Plasma samples were separated by centrifugation at approximately 2,091 × g for 10 min at 4°C. Plasma samples were kept at -80°C in a freezer until analysis. Melatonin concentrations were determined by the validated method described above.

The protocol for pharmacokinetic study of melatonin had been approved by the Khon Kaen University Ethics Committee for Human Research (HE561318).
**Pharmacokinetic analysis**

The pharmacokinetic parameters were calculated using a non-compartmental model using Kinetica 2.0 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Values for peak concentration (C$_{\text{max}}$) and time to C$_{\text{max}}$ (T$_{\text{max}}$) were taken directly from the observed data. Individual concentration versus time profiles were plotted and the terminal disposition rate constant (K$_e$) was determined by a log-linear regression of at least three data points judged to be in the terminal phase. The terminal-phase half-life (t$_{1/2}$) was calculated by dividing 0.693 by K$_e$. AUC$_{0-\text{t}_{\text{last}}}$ determined by the linear trapezoidal method from time zero to the time of the last observed concentration (C$_{t}$). Total AUC was determined as AUC$_{0-\text{t}_{\text{last}}}$ + Ct/K$_e$. The apparent volume of distribution (V$_d$) was calculated using Dose/AUC$_{\text{tot}}$·K$_e$.

**Results**

**Chromatography**

Under the chromatographic conditions used, melatonin and IS peaks were clearly separated and the retention times of melatonin and IS were 3.6 and 6.1 min, respectively (Figure 1).

![Figure 1](image1.png)

**Figure 1.** Representative chromatogram for an extract of plasma containing melatonin at 25 ng/mL and 5-methoxytryptamine; IS.

**Specific and selectivity**

Specificity of the assay with respect to endogenous compounds in human plasma was determined by analyzing five different plasma samples obtained from blood donors. No endogenous compounds with signal to noise ratio (S/N) of more than 5 were seen at the retention times of melatonin and IS (Figure 2). Representative of chromatograms of extracts from plasma samples containing various concentrations of melatonin are shown in Figure 3.
Figure 2. Representative chromatogram of an extract from a blank plasma sample.

Figure 3. Representative chromatograms of extracts from LLOQ (A), LQC (B), MQC (C) and HQC (D) samples.
Calibration and Linearity

Three sets of matrix-based calibration curve samples were assayed as described above. The typical chromatograms of calibration curve samples are shown in Figure 3. The melatonin concentrations were plotted against the peak area ratios. By using $1/X^2$ weighting mode, these three calibration curves were linear with the correlation coefficients of 0.9901 to 0.9918 (Table 1). The mean accuracy of each melatonin concentration obtained from back calculation using the calibration curve equation was within the acceptance limit of 100±15%.

Accuracy and precision

The limits of quantitation (LLOQ) of melatonin was 0.1 ng/mL and intra-day and between-day accuracy of the LLOQ were 103.50±0.01 and 105.31±0.01 respectively while the intra-day and between-day precision (%CV) were 8.66% and 8.43%, respectively (Table 2).

Six replicates of 3 sets of QC samples (0.30, 17.00 and 25.00 ng/mL) were determined during a 3-day validation period. The intra-day and inter-day accuracy were within 100±15% while the intra-day and inter-day precisions (%CV) were less than 15% (Table 2 and 3).

Table 1. Linearity of calibration curves.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Concentration calculated from calibration curve (ng/mL)</th>
<th>Accuracy</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Curve 1</td>
<td>Curve 2</td>
<td>Curve 3</td>
</tr>
<tr>
<td>35.00</td>
<td>39.36</td>
<td>39.46</td>
<td>32.39</td>
</tr>
<tr>
<td>25.00</td>
<td>21.28</td>
<td>22.66</td>
<td>22.21</td>
</tr>
<tr>
<td>10.00</td>
<td>10.94</td>
<td>9.68</td>
<td>10.75</td>
</tr>
<tr>
<td>5.00</td>
<td>4.66</td>
<td>4.80</td>
<td>5.42</td>
</tr>
<tr>
<td>2.50</td>
<td>2.41</td>
<td>2.51</td>
<td>2.70</td>
</tr>
<tr>
<td>0.50</td>
<td>0.52</td>
<td>0.55</td>
<td>0.51</td>
</tr>
<tr>
<td>0.25</td>
<td>0.19</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

$r^2$ = 0.9901, 0.9918, 0.9915, 0.99, 0.00

Equation

\[ y = 0.0055x + 0.0580 \]

\[ y = 0.0062x + 0.0708 \]

\[ y = 0.0061x + 0.0612 \]

\[ y = (0.0059 \pm 0.0003)x + (0.0633 \pm 0.0054) \]

Table 2. Intra-day and inter-day precision and accuracy of the validated assay method at 4 different concentrations of quality control samples.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Intra-day (n = 6)</th>
<th>Inter-day (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean measured concentration (ng/mL)</td>
<td>SD</td>
</tr>
<tr>
<td>0.10</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>0.30</td>
<td>0.32</td>
<td>0.01</td>
</tr>
<tr>
<td>17.00</td>
<td>18.31</td>
<td>1.57</td>
</tr>
<tr>
<td>25.00</td>
<td>25.24</td>
<td>1.29</td>
</tr>
</tbody>
</table>

* Mean±SD
Table 3. Summary of pharmacokinetic parameters of melatonin in healthy volunteers after an oral dose of melatonin 20 mg.

| Pharmacokinetic parameters | Mean ± SD  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>$C_{\text{max}}$, ng/mL</td>
<td>70.5±52.6</td>
<td>27.8-188.1</td>
<td>50.3</td>
</tr>
<tr>
<td>AUC$_{0-t}$, ng·h/mL</td>
<td>104.5±69.3</td>
<td>29.3-230.4</td>
<td>89.9</td>
</tr>
<tr>
<td>AUC$_{0-inf}$,ng·h/mL</td>
<td>106.0±69.6</td>
<td>29.8-230.8</td>
<td>90.4</td>
</tr>
<tr>
<td>$T_{\text{max}}$, h</td>
<td>0.65±0.2</td>
<td>0.5-1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Vd/F, L</td>
<td>331.5±190.8</td>
<td>106.9-730.0</td>
<td>297.2</td>
</tr>
<tr>
<td>$t_{1/2}$, h</td>
<td>0.81±0.2</td>
<td>0.33-1.3</td>
<td>0.79</td>
</tr>
<tr>
<td>Cl/F, L/h·kg</td>
<td>4.5±2.6</td>
<td>1.3-8.4</td>
<td>3.9</td>
</tr>
<tr>
<td>Ke, h$^{-1}$</td>
<td>0.81±0.1</td>
<td>0.53-1.2</td>
<td>0.81</td>
</tr>
</tbody>
</table>

**Recovery**

Percentage recoveries of melatonin and IS were determined by comparing the peak areas obtained after extraction of 5 replicates of each melatonin concentration and 5 replicates of IS of mobile phase analytes spiked at the same concentrations. Mean percentage recovery of melatonin was 109.91±6.96% and 102.38±0.98% at concentration of 0.30 and 25.00 ng/mL, respectively. Whereas the percentage recovery of an IS was 76.62±1.73%.

**Dilution integrity**

The accuracy and precision (%CV) of 2-fold and 10-fold dilution was 104.46±1.36%, 109.08±2.79% and 1.30% and 3.56%, respectively.

**Pharmacokinetics of melatonin in healthy Thai male volunteers**

This validated assay was suitable for study of the pharmacokinetics of melatonin after a single oral dose of 20 mg melatonin capsule. Mean age, weight and BMI (range) of these subjects were 23.4±2.7 (21-29) years, 64.4±8.7 (53-80) kg and 21.52±2.0 (18.5-21.2) kg/m$^2$.

The mean plasma profile of melatonin in 10 healthy Thai male volunteers is shown in Figure 4 and 5. The pharmacokinetics of melatonin in these 10 volunteers was calculated using a non-compartment model. Melatonin was rapidly absorbed into the blood circulation. The mean $C_{\text{max}}$ was 70.5±52.6 ng/mL (range from 27.8-188.1 ng/mL) and the mean $T_{\text{max}}$ was 0.65±0.2 (Table 3). The AUC$_{0-t}$ and AUC$_{0-inf}$ were 104.5±69.3 and 106.0±69.6 ng·h/mL. The mean $V_d$ of melatonin obtained from these subjects was 331.5±190.8 L. Melatonin was rapidly clear from the body with the mean clearance of 4.5±2.6 L/h·kg. The mean elimination rate constant (Ke) of melatonin calculated from these subjects was 0.81±0.1 h$^{-1}$ while the mean half-life ($t_{1/2}$) was 0.81±0.2 h.
**Figure 4.** Mean plasma concentrations (±SE) of melatonin after an oral dose of 20 mg in 10 healthy Thai male volunteers.

**Figure 5.** Semi-logarithmic plot of the mean plasma concentrations of melatonin after an oral dose of 20 mg in 10 healthy Thai male volunteers.
Discussions and Conclusions

An HPLC method coupled with a fluorescence detector was developed and validated to quantify the concentration of melatonin in human plasma. This method demonstrated high accuracy and high precision and was successfully applied to study the pharmacokinetics of melatonin after oral administration of 20 mg capsule in healthy Thai male volunteers.

Various organic solvents such as dichloromethane\textsuperscript{25}, chloroform\textsuperscript{26} and ethyl acetate\textsuperscript{27} have previously been used for extraction of melatonin. Dichloromethane was chosen as an extraction solvent in this study because higher recovery of melatonin has been reported in previous study.\textsuperscript{25} As shown from the present study, extraction with dichloromethane gave very high recovery of melatonin (109.91\%-102.38\%). However, the percentage recovery of 5-methoxytryptamine which used as IS, in the present study was less (76.62\%±1.73\%) than that obtained from melatonin. This may be due to the differences in pKa of these two compounds (15.8 for melatonin compared to 9.76 for 5-methoxytryptamine).

The assay method developed in the present study is selective. The limit of quantitation of the method was 0.10 ng/mL. No endogenous compounds with signal to noise ratio (S/N) of more than 5 were seen at the retention times of melatonin and IS. It should be noted that endogenous melatonin and other endogenous compounds with similar structure maybe detected in some human plasma sources but their S/N ratios were less than 5 and did not significantly interfere with the assay as demonstrated by the accuracy and precision of the LLOQ sample, which were within the acceptance limit of 80-120 \% and less than 20\%, respectively. The intra-day and between-day accuracy and precision of the assay at melatonin concentrations of 0.3, 17 and 35 ng/mL were within the acceptance limits according to the U.S. FDA Guidance for Industry on Bioanalytical Method Validation.\textsuperscript{27}

The pharmacokinetics of melatonin in healthy male Thai subjects fits well with a one-compartmental model. Under fasting conditions, melatonin was rapidly absorbed into the blood circulation with mean $T_{\text{max}}$ of 0.65±0.24 (range from 0.5-1.25) h. The $C_{\text{max}}$ and AUC$_{0\text{-}t}$ of melatonin observed in the present study ranged from 28.0-188.1 ng/mL and 29.3-230.4 ng·h/mL. These high variations in the $C_{\text{max}}$ and AUC$_{0\text{-}t}$ may due to the inter-individual differences in the absorption and metabolism of melatonin. Melatonin was rapidly cleared from the body with the mean clearance (Cl/F) of 4.5±2.6 (range 1.2-8.4) L/h·kg and the mean $t_{1/2}$ of 0.81±0.2 (range 0.33-1.30) h. The $T_{\text{max}}$ and $t_{1/2}$ obtained from these Thai healthy volunteers were not much different from those observed in healthy Swedish subjects after oral administration of 25 mg melatonin (1.58±0.79 h; range 0.5-2.5 h for $T_{\text{max}}$ and 0.93±0.37 h; range 0.4-1.7 h for $t_{1/2}$).\textsuperscript{24} However, the mean Cl/F value obtained from this study was about 2.7 fold lower than those reported in Swedish subjects 12.26±9.3 (range 3.3-34.3 L/h·kg).\textsuperscript{24}

In conclusion, the assay method for quantitation of melatonin in human plasma developed in the present study is simple, accurate and reliable and can be applied for pharmacokinetic study of melatonin in human.
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