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# Determination of palonosetron in human plasma by liquid chromatography–electrospray ionization-mass spectrometry

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**Abstract**

A high performance liquid chromatography–electrospray ionization-mass spectrometry (HPLC–ESI-MS) method for the determination of palonosetron (PALO) in human plasma using naloxone as the internal standard (IS) was established. After adjustment to a weakly basic pH with saturated sodium bicarbonate, plasma samples were extracted with ethyl acetate and separated on a Hanbon Lichrospher 5-$C_{18}$ column with a mobile phase of 40 mM ammonium acetate buffer solution containing 0.04% formic acid–methanol (46:54, v/v). PALO was determined with electrospray ionization-mass spectrometry (ESI-MS). HPLC–ESI-MS was performed in the selected-ion monitoring (SIM) mode using target ions at $[M+H]^+$ *m/z* 297.2 for PALO and $[M+H]^+$ *m/z* 328.2 for the IS. Calibration curve was linear over the range of 0.02124–10.62 ng/ml. The lower limit of quantification (LLOQ) was 0.02124 ng/ml. The intra- and inter-run variability values were all less than 10.4%. The method has been successfully applied to determine the plasma concentration of PALO in healthy Chinese volunteers.


*Keywords:* Palonosetron; HPLC–ESI-MS; Pharmacokinetics

## 1. Introduction

Palonosetron (PALO, Fig. 1) is a potent and highly selective 5-$HT_3$ receptor antagonist recently approved by the US Food and Drug Administration (FDA) for the prevention of acute and delayed chemotherapy-induced nausea and vomiting. Compared with available 5-$HT_3$ receptor antagonists, PALO with a prolonged plasma elimination half-life may provide a longer duration of action than other approved agents [1,2]. The pharmacokinetic profile of PALO in Chinese volunteers has not been reported yet. Recently, a new formulation of PALO hydrochloride injection has been developed by Jiangsu Chia-tai Tianqing Pharmaceutical Co., Ltd. (Jiangsu, China) and approved by State Food and Drug Administration of China to be put into clinical trial. As entrusted by Jiangsu Chia-tai Tianqing Pharmaceutical Co., Ltd., the investigation of the pharmacokinetics of the drug in Chinese volunteers was carried out. To evaluate the pharmacokinetics of PALO hydrochloride injection in humans, a sensitive method for the determination of PALO in human plasma is required. However the assay of PALO in human plasma has not been reported. This paper describes the development and validation of a sensitive LC–ESI-MS method with an LLOQ of 0.02124 ng/ml for the quantification of PALO in human plasma. The assay is validated over the range of 0.02124–10.62 ng/ml, and has been successfully applied to study pharmacokinetics of PALO in Chinese volunteers.

## 2. Experimental

### 2.1. Materials and reagents

PALO was obtained from Jiangsu Chia-tai Pharmaceutical Co., Ltd. (Jiangsu, China). Naloxone hydrochloride (IS) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The test drug was PALO hydrochloride injection containing 0.25 mg of PALO per vial, which was provided by Jiangsu Chia-tai Pharmaceutical Co., Ltd. (Jiangsu, China). Methanol was of HPLC grade (Merck KGaA). Ethyl acetate, ammonium acetate, sodium bicarbonate and formic acid were of analytical grade purity and purchased

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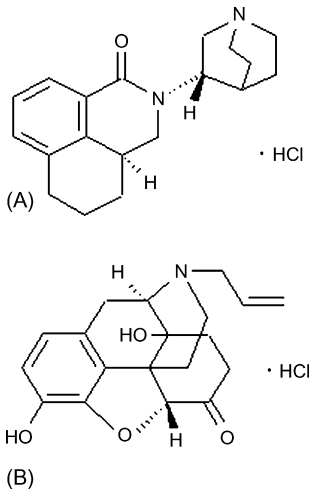


Fig. 1. Chemical structures of PALO hydrochloride (A) and naloxone hydrochloride (B).

from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Distilled water, prepared from demineralized water, was used throughout the study.

### 2.2. Instrument and conditions

HPLC–ESI-MS analyses were performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA) with a Hanbon Lichrospher 5-$C_{18}$ column, 5 μm, 250 mm × 4.6 mm i.d. (Jiangsu Hanbon Science & Technology Co., Ltd., China). The HPLC–ESI-MS was controlled by a computer employing the HP Chemstation software (10.02 A) supplied by Agilent.

### 2.3. HPLC–ESI-MS condition

The mobile phase was 40 mM ammonium acetate buffer solution containing 0.04% formic acid–methanol (46:54, v/v) at a flow rate of 1.0 ml/min. The column temperature was maintained at 28 °C. A quadrupole mass spectrometer equipped with an electrospray ionization source was set with a drying gas ($N_2$) flow of 12 l/min, nebulizer pressure of 40 psi, drying gas temperature of 350 °C, capillary voltage of 3 kV and the positive ion mode. The fragmentor voltage was 150 V. HPLC–ESI-MS was performed in selected-ion monitoring (SIM) mode using target ions at $[M+H]^+$ *m/z* 297.2 for PALO and $[M+H]^+$ *m/z* 328.2 for IS. Fig. 2 shows the typical full-scan ESI mass spectrum of PALO and IS.

### 2.4. Preparation of working solutions

The stock solution of PALO (1.062 mg/ml) and internal standard (991.0 ng/ml) were prepared in methanol and stored at −20 °C. Standard solution of PALO with concentrations of 106.2 μg/ml, 10.62 μg/ml, 1.062 μg/ml, 106.2 ng/ml and 10.62 ng/ml, were made by serial dilution of PALO stock solution with methanol in separate 10 ml volumetric flasks. A solution containing 99.1 ng/ml internal standard was also

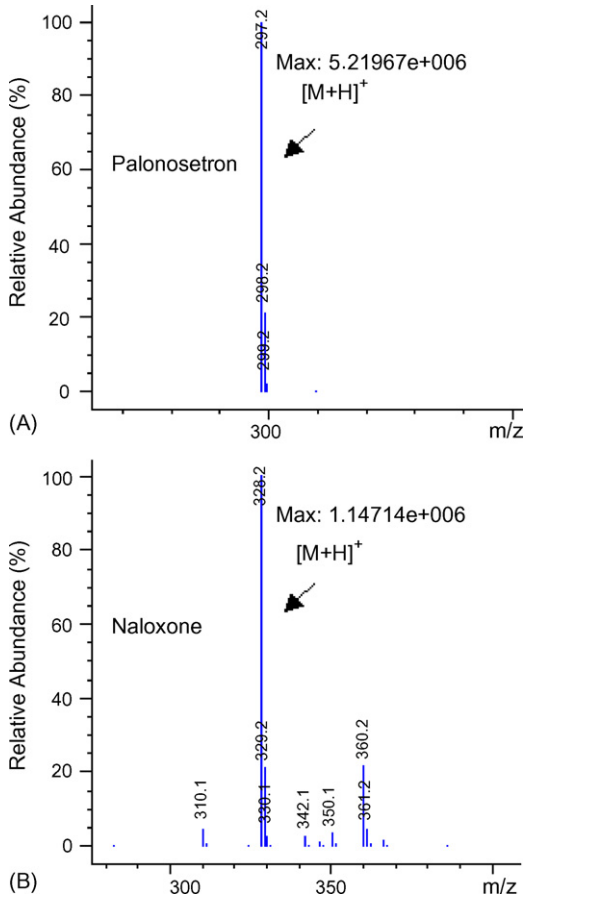


Fig. 2. Mass spectra of the positive ions of PALO (A) and IS (B) at 150 V fragmentor voltage.

obtained by further dilution of IS stock solution with methanol. All the solutions were stored at −20 °C.

### 2.5. Sample preparation

A 0.5-ml aliquot plasma sample was extracted with 5 ml ethyl acetate after addition of 50 μl IS (99.1 ng/ml) solution and 100 μl saturated sodium bicarbonate solution. Following centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen in a water bath of 30 °C. The residue was reconstituted in 120 μl of mobile phase and a 30-μl aliquot was injected onto the HPLC–ESI-MS for analysis.

### 2.6. Preparation of the calibration curves and quality control samples

Calibration standards of PALO were prepared by spiking appropriate amounts of the working solutions in 0.5 ml blank plasma obtained from healthy volunteers. Standard curves were prepared in the range of 0.02124–10.62 ng/ml for PALO at concentrations of 0.02124, 0.05310, 0.1062, 0.3186, 1.062, 3.186, 10.62 ng/ml. The calibration curves were prepared and assayed along with quality control (QC) samples and each run of clinical plasma samples. The QC samples were prepared in

blank plasma at concentrations of 0.05, 0.50 and 8.0 ng/ml for PALO and stored at −20 °C. The stock solution of PALO for preparation of QC was prepared at 1 mg/ml and stored at −20 °C. The QC samples were prepared independently of the calibration standards, and analyzed with processed test samples at intervals in each run. The results of the QC samples provided the basis of accepting or rejecting the run.

### 2.7. Assay validation

#### 2.7.1. Selectivity

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and HPLC–ESI-MS conditions to ensure no interference of PALO and IS from plasma.

#### 2.7.2. Linearity of calibration curve and lower limit of quantification

Calibration standards of seven PALO concentration levels at 0.02124, 0.05310, 0.1062, 0.3186, 1.062, 3.186 and 10.62 ng/ml were extracted and assayed. The PALO calibration curve was constructed by plotting the peak-area ratios of PALO to the IS versus the concentrations of PALO, using weighed least squares linear regression (the weighing factor was $1/C$).

The LLOQ was defined as the lowest concentration on the calibration curve at which the standard deviation was within 20% and accuracy was within ±20% [3], and it was established using five samples independent of standards.

#### 2.7.3. Precision and accuracy

Validation samples were prepared and analyzed on 3 consecutive days (one run per day) to evaluate the accuracy and the intra- and inter-run precision of the analytical method. The accuracy as well as the intra- and inter-run precision of the method was determined by analyzing five replicates at 0.05310, 0.5310, and 8.496 ng/ml of PALO along with one standard curve on each of 3 days. Assay precision was calculated using the relative standard deviation (R.S.D. (%)). The accuracy is the degree of closeness of the determined value to the nominal true value under prescribed conditions. Accuracy is defined as the relative deviation in the calculated value ($E$) of a standard from that of its true value ($T$) expressed as a percentage (R.E. (%)). It was calculated by using the formula: R.E. (%) = $(E - T)/T \times 100$.

These QC samples were assayed along with clinical samples in each run to monitor the performance of the assay and to assess the integrity and validity of the results of the unknown clinical samples analyzed.

#### 2.7.4. Extraction recovery

The extraction recovery of PALO was evaluated by analyzing five replicates at 0.05310, 0.5310, and 8.496 ng/ml of PALO. Recovery was calculated by comparison of the peak areas of PALO extracted from plasma samples with those of injected standards.

#### 2.7.5. Stability

The stability of PALO in plasma was studied under a variety of storage and handling conditions at low (0.05310 ng/ml) and high (8.496 ng/ml) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low- and high-concentration samples that were thawed at room temperature and kept at this temperature for 6 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. Three aliquots at each of the low and high concentrations were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low and high concentrations stored at −20 °C for 6 weeks.

#### 2.7.6. System suitability test

Prior to running each run of clinical plasma samples, the instrument performance (e.g., sensitivity, reproducibility of chromatographic retention and separation, plate number and tailing factor) was determined by the analysis of the reference standard of PALO, IS, blank plasma and plasma spiked with PALO and IS.

#### 2.7.7. Application of the method to a pharmacokinetic study in healthy Chinese volunteers

The method described above was applied to the pharmacokinetic study. Ten healthy Chinese volunteers included five men and five women participated in the study. Each volunteer received a single 0.25 mg intravenous dose of PALO hydrochloride injection. The intravenous infusion of the PALO hydrochloride injection was designated to finish within 1 min. Blood was sampled pre-dose and at 1, 5, 15, 30 min and 1, 2, 4, 6, 12, 24, 48, 72, 120, 144, 168 h following dosing for determination of plasma concentration of PALO.

## 3. Results and discussion

### 3.1. Sample preparation

Sample preparation is an important step for accurate and reliable LC–MS assays. The most widely employed biological sample preparation techniques currently are liquid–liquid extraction (LLE), protein precipitation (PPT), and solid-phase extraction (SPE). SPE is limited by the cost of the apparatus and instrumentation. LLE cannot only purify but also concentrate the sample. The plasma samples containing PALO were prepared by LLE procedure. PALO and IS are all alkaline compounds. The experiment results showed that adjustment of the plasma pH to a weakly basic value with saturated sodium bicarbonate could improve the extraction efficiency of PALO and IS. Further test results proved that ethyl acetate shows higher extraction recovery to PALO and IS than diethyl ether, cyclohexane or their mixture solutions. The ethyl acetate with 0.1 ml saturated sodium bicarbonate was finally adapted because of its high extraction efficiency.

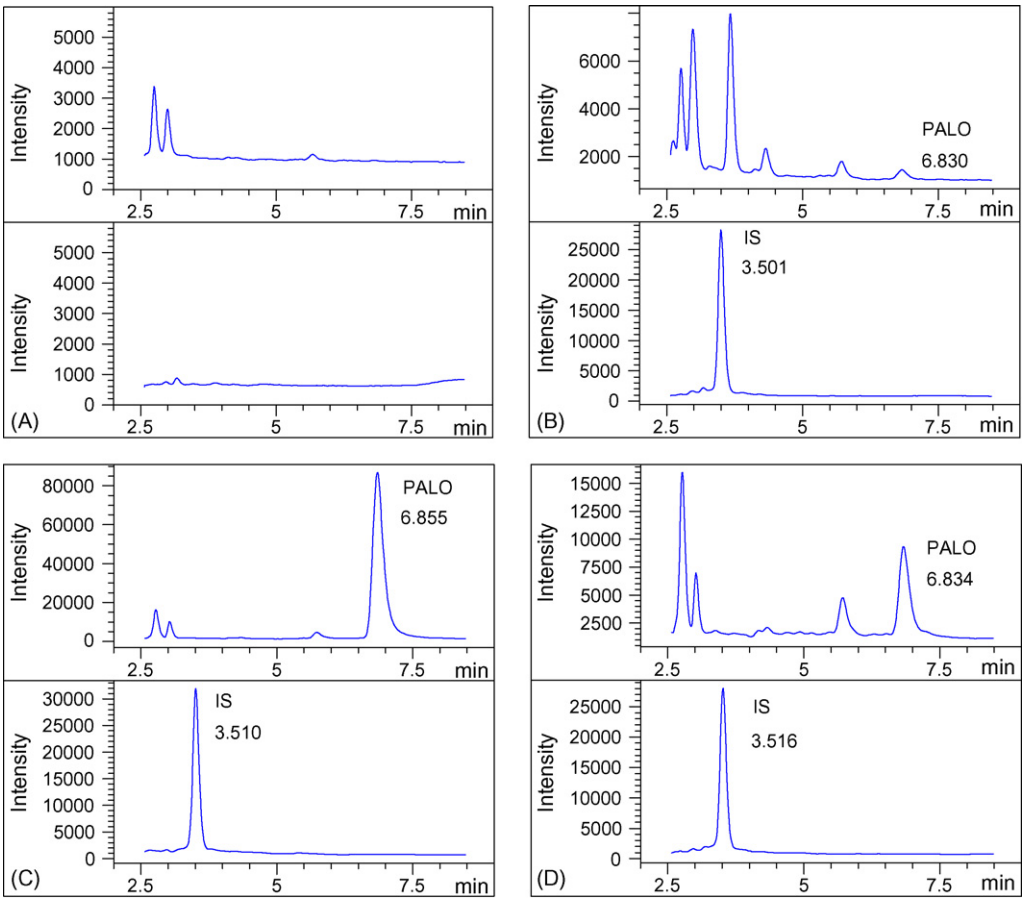


Fig. 3. Typical SIM chromatograms of blank plasma (A), LLOQ for PALO in plasma (0.02124 ng/ml) and IS (B), plasma spiked with PALO (10.62 ng/ml) and IS (C), plasma obtained from a patient at 15 min after a single 0.25 mg intravenous dose of the PALO injection, the plasma concentration of PALO was estimated to be 1.011 ng/ml (D).

### 3.2. Conditions of chromatography

Several experiments showed that the employment of an appropriate ratio of ammonium acetate buffer solution in the mobile phase might improve the chromatographic peak shapes [4–9]. To suppress the tailing phenomena of chromatographic peaks of PALO and IS, the ammonium acetate buffer solution was adopted in the mobile phase of HPLC. The test results showed that the mobile phase of 40 mM ammonium acetate solution could improve the peak shapes and suppress the tailing phenomena of chromatographic peaks of PALO and IS. Further experiment results showed that acidifying the mobile phase with formic acid could not only improve peak shapes of PALO and IS, but also increase the MS sensitivity to PALO and IS. Good separation of target compounds was obtained using an elution system of 40 mM ammonium acetate buffer solution containing 0.04% formic acid–methanol (46:54, v/v). Representative chromatograms are shown in Fig. 3 in which the retention times were about 6.8 min for PALO and 3.5 min for IS.

### 3.3. Conditions for ESI-MS

Because PALO is an alkaline compound, a positive ion-monitoring mode was adopted in the HPLC–MS assay. In order to minimize the undesirable fragmentation of PALO, the fragmentor voltage was set at a lower value. At lower fragmentor voltages, ESI produced an abundant positive molecular ion at $[M+H]^+$ *m/z* 297.2 for PALO with less fragmentation. In order to determine the optimal fragmentor voltage, the intensities of the positive molecular ion $[M+H]^+$ of PALO at *m/z* 297.2 were compared at fragmentor voltages of 30, 50, 70, 90, 100, 120, 150, 170, 190, 220 and 250 V. The results showed that while selecting the positive molecular ion $[M+H]^+$ at *m/z* 297.2 as the target ion of PALO, the highest sensitivity of the assay could be achieved by using a 150 V fragmentor voltage. Therefore, a fragmentor voltage of 150 V was used to carry out the ESI-MS in the assay. Fig. 2A shows a full-scan ESI-positive mass spectrum of PALO at a 150 V fragmentor voltage. At this fragmentor voltage, the base peak in the mass spectrum of IS was the positive molecular ion $[M+H]^+$ of IS at *m/z* 328.2 (see Fig. 2B). Therefore, the positive molecular ion at $[M+H]^+$ *m/z* 328.2 was selected as the target ion of IS in the SIM.

### 3.4. Method validation

#### 3.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corre-

Table 1
Accuracy and precision for the analysis of LLOQ ($n = 5$)

| Added C (ng/ml) | Found C (ng/ml) | Mean (ng/ml) | R.S.D. (%) | R.E. (%) |
|---|---|---|---|---|
| 0.02124 | 0.02369 | 0.02171 | 7.15 | 11.3 |
| 0.02124 | 0.01978 | | | −6.7 |
| 0.02124 | 0.02090 | | | −1.6 |
| 0.02124 | 0.02282 | | | 7.3 |
| 0.02124 | 0.02134 | | | 0.5 |

*Note:* R.S.D., relative standard deviation; R.E., relative error; *n*, number of replicates.

sponding spiked plasma. Fig. 3 shows the typical chromatograms of a blank, a spiked plasma sample with PALO at LLOQ level and IS, a spiked plasma sample with PALO (10.62 ng/ml) and IS, and a plasma sample from a healthy volunteer. There was no significant interference or ion suppression from endogenous substances observed at the retention times of the analytes. Typical retention times for PALO and IS were 6.8 and 3.5 min, respectively.

### 3.4.2. Calibration curve and sensitivity

The calibration curves, which related the concentrations of PALO to the area ratio of PALO to IS, showed good linearity over the range of 0.02124–10.62 ng/ml. The typical calibration curve for PALO had a slope of 0.3917, an intercept of 0.002875 and $R = 0.9997$. Calibration curves were prepared and analyzed with each run of clinical samples and QC samples. The LLOQ for PALO in plasma was 0.02124 ng/ml (Fig. 3; Table 1). Those data show that the assay is sensitive enough for pharmacokinetic study of PALO.

### 3.4.3. Matrix effect

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [3]. The matrix effect of the assay was determined by comparing the peak areas of analytes resolved in the blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution) (*A*) with those resolved in mobile phase (*B*). ME was calculated by using the formula: ME (%) = $A/B \times 100$. The matrix effect of the assay was evaluated at three PALO concentration levels of 0.05310, 0.5310 and 8.496 ng/ml, and five samples at each level were analyzed. The blank plasma samples used in this study were five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an exogenous matrix effect is implied. The results showed there was no matrix effect of the analytes observed in this study.

Table 2
Accuracy and precision for the analysis of PALO in human plasma (in pre-study validation, three runs, five replicates per run)

| Added to plasma (ng/ml) | Mean measured concentration (ng/ml) | R.E. (%) | Intra-assay R.S.D. (%) | Inter-assay R.S.D. (%) |
|---|---|---|---|---|
| 0.05310 | 0.05138 | −3.3 | 10.4 | 10.4 |
| 0.5310 | 0.5288 | −0.4 | 5.3 | 9.3 |
| 8.496 | 8.642 | 1.7 | 2.6 | 5.9 |

### 3.4.4. Assay precision and accuracy

The intra- and inter-run precision and accuracy are summarized in Table 2. The standard deviation was calculated by using one-way-ANOVA. The results in Table 2 demonstrate that the precision and accuracy of this assay are within the acceptable range and the method is accurate and precise.

### 3.4.5. Extraction recovery

Extraction efficiency of different extraction solvents is compared. The extraction recovery values of PALO from human plasma with cyclohexane, diethyl ether or the mixture of cyclohexane and diethyl ether were all less than 70%. Ethyl acetate was chosen as the extraction solvent for its higher extraction efficiency to the two target compounds. The recovery values of PALO from human plasma with ethyl acetate, determined at three concentrations of 0.05310, 0.5310, and 8.496 ng/ml were $87.0 \pm 5.1$, $87.2 \pm 7.1$ and $89.5 \pm 4.4\%$ ($n = 5$), respectively.

### 3.4.6. Stability

The stability of PALO was studied under a variety of storage and handling conditions. The results in Table 3 showed that no significant degradation occurred after being kept at room temperature for 6 h and during the three freeze–thaw cycles for the PALO plasma samples. PALO in plasma at −20 °C was stable for at least 6 weeks. The stock solutions of PALO and IS at −20 °C were stable for 1 month at least.

## 3.5. Application

The method described above was successfully applied to the pharmacokinetic study in which plasma concentrations of

Table 3
Stability data of PALO in human plasma under various storage conditions ($n = 3$)

| Storage conditions | Added C (ng/ml) | Found C (ng/ml) | Inter-run R.S.D. (%) | R.E. (%) |
|---|---|---|---|---|
| Room temperature for 6 h | 0.05310 | 0.05144 | 10.5 | −3.1 |
| | 8.496 | 8.292 | 2.4 | −2.4 |
| Three freeze–thaw cycles | 0.05310 | 0.05170 | 3.1 | −2.6 |
| | 8.496 | 8.716 | 1.5 | 2.6 |
| 6 weeks at −20 °C | 0.05310 | 0.04970 | 8.2 | −6.4 |
| | 8.496 | 8.646 | 3.0 | 1.8 |

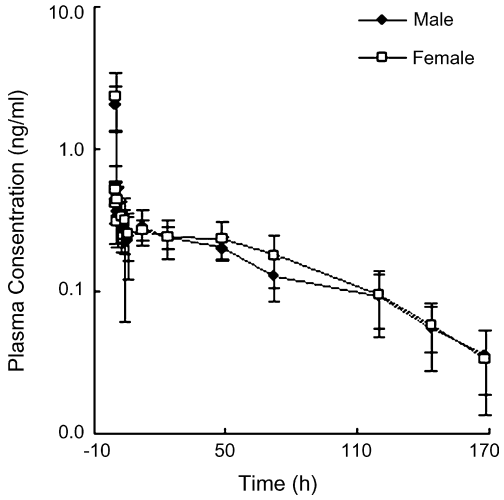


Fig. 4. Mean PALO plasma concentration–time profiles in male and female Chinese volunteers after a single intravenous PALO dose of 0.25 mg ($n = 5$).

Table 4
Main pharmacokinetic parameters of PALO in male and female Chinese volunteers after a single intravenous dose of 0.25 mg PALO ($n = 5$)

| Parameters | Mean ± S.D. | |
|---|---|---|
| | Male | Female |
| $C_{max}$ (ng/ml) | 2.07 ± 0.74 | 2.37 ± 1.03 |
| $t_{1/2}$ (h) | 52.2 ± 16.8 | 42.3 ± 4.3 |
| $V_d$ (l) | 711 ± 208 | 557 ± 158 |
| CL/F (l/h) | 9.7 ± 2.2 | 9.2 ± 2.6 |
| $AUC_{0–168}$ (h ng/ml) | 23.8 ± 4.3 | 27.0 ± 7.1 |
| $AUC_{0–\infty}$ (h ng/ml) | 26.8 ± 5.4 | 29.1 ± 8.3 |

PALO in 10 healthy Chinese volunteers (5 male and 5 female) were determined up to 168 h after receiving a single intravenous PALO dose of 0.25 mg. The mean plasma concentration–time curves of PALO in male and female Chinese volunteers are shown in Fig. 4. The main pharmacokinetic parameter values in male and female Chinese volunteers are calculated and summarized in Table 4. Stoltz et al. [10] described the pharmacokinetic profiles of PALO in US and Japanese healthy volunteers administered the intravenous doses ranging from 0.3 to 90 μg/kg of PALO. The pharmacokinetic parameters of PALO were generally similar in US, Japanese and Chinese volunteers.

## 4. Conclusions

The method had a good sensitivity and specificity for the determination of PALO in human plasma. No significant interferences and matrix effect caused by endogenous compounds were observed. The assay is suitable for pharmacokinetic study of PALO in human subjects.

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