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Determination of palonosetron in human plasma by ultra performance liquid chromatography–tandem mass spectrometry and its application to a pharmacokinetic study

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ABSTRACT

A rapid, sensitive and selective ultra performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method was developed for the determination of palonosetron (PALO) in human plasma. Verapamil was used as the internal standard (I.S.). Sample pretreatment involved liquid–liquid extraction with diethyl ether under alkaline condition. Chromatographic separation was carried out on an ACQUITY UPLC™ HSS T_3 column with mobile phase consisting of methanol–water containing 0.1% formic acid (80:20, v/v) at a flow-rate of 0.20 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source. The ion transitions of PALO and I.S. were m/z 297.3 → 109.8 and m/z 455.1 → 164.9, respectively. Each plasma sample was chromatographed within 1.2 min. The linear calibration curves were obtained in the concentration range of 0.0190–3.80 ng/mL ($r^2 \geq 0.99$) with a lower limit of quantification (LLOQ) of 0.0190 ng/mL. The intra- and inter-day precision (relative standard deviation, R.S.D.) values were all less than 11% and accuracy (relative error, R.E.) was from 4.3% to 6.1% at all quality control (QC) levels. The method has been successfully applied to determine the plasma concentration of PALO in healthy Chinese volunteers after intravenous administration of a single dose of 0.125 mg palonosetron hydrochloride.

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1. Introduction

Palonosetron (PALO) is a potent, highly selective second-generation 5-hydroxytryptamine-3 (5-HT_3) receptor antagonist with strong binding affinity, which was developed for the prevention of acute and delayed chemotherapy-induced nausea and vomiting (CINV) [1]. Due to a low intravenous dosage of 0.125 mg and long plasma elimination half-life [2,3], the concentration of PALO in human plasma is very low. Therefore, the development of a rapid, sensitive and specific method to determine PALO in human plasma is necessary and valuable.

Few analytical methods for PALO in biological samples were available. The radioimmunoassays [2,3] was high sensitive and selective but radiation was harmful to operators and environment. A LC–MS method [4] developed by Ding et al. had a sensitivity of 0.0212 ng/mL. Some LC–MS/MS methods [5,6] were developed to determine PALO in human plasma with LLOQs of 0.0300 and 0.0435 ng/mL, respectively, but the product ion was same as the precursor ion in the mass monitoring mode which had low selectivity in the assay [5]. And these methods [4–6] needed long chromatographic run time (longer than 5.5 min), which was not suitable for the high throughput of biological samples.

This paper describes an ultra performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method, which enables quantitative determination of PALO with high speed and good accuracy at concentrations in human plasma as low as 0.0190 ng/mL. The total run time of 1.2 min per sample was shorter than those reported [4–6], which promised the high throughput analysis of biological samples. The method was fully validated and successfully applied to a pharmacokinetic study in healthy Chinese volunteers after intravenous administration of a single dose of 0.125 mg palonosetron hydrochloride.

2. Experimental

2.1. Chemicals and reagents

Reference standard of palonosetron hydrochloride (99.7% purity) was provided by Changzhou Pharmaceutical Co., Ltd. IV (Jiangsu, China). Verapamil hydrochloride (I.S., 99.5% purity) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). The structures of palonosetron and verapamil are given in Fig. 1. Methanol (HPLC grade) was obtained from Tedia (Fairfield, OH, USA). Formic acid

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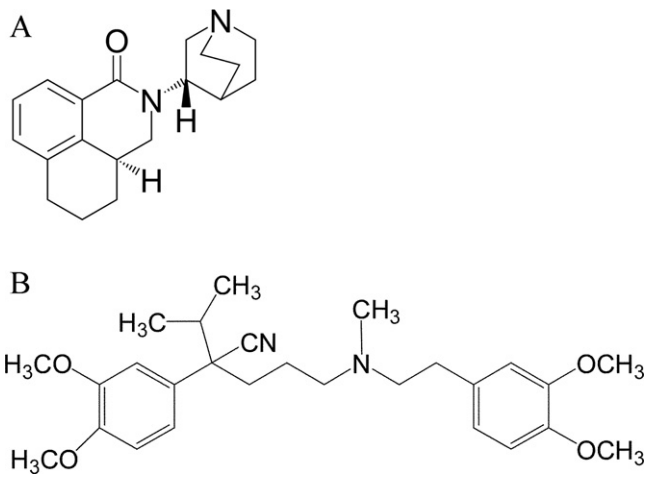

Fig. 1. Structures of PALO (A) and verapamil (I.S.) (B).

(HPLC grade) was purchased from Dikma (Richmond Hill, NY, USA). Sodium hydroxide and diethyl ether of analytical grade were purchased from Yuwang (Chemical Reagent Plant, Shandong, China). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

2.2. Apparatus and operating conditions

2.2.1. Liquid chromatographic conditions

The chromatography was performed on an ACQUITY Ultra Performance LC™ system (Waters Corp., Milford, MA, USA). An ACQUITY UPLC™ HSS T_3 column (50 mm × 2.1 mm I.D, 1.8 μm) was employed for the separation maintained at 45 °C. The mobile phase was composed of menthanol–water containing 0.1% formic acid (80:20, v/v). The flow rate was set at 0.20 mL/min. The autosample temperature was kept at 4 °C and 10 μL of sample solution was injected.

2.2.2. Mass spectrometric conditions

A triple quadrupole tandem mass spectrometer (Micromass® Quattro micro™ API mass spectrometer, Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytical detection. The ESI source was set in positive ionization mode. Quantification was performed using MRM of the transitions of m/z 297.3 → 109.8 for PALO and m/z 455.1 → 164.9 for I.S., respectively, with scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage of 2.5 kV, cone voltage of 40 V, source temperature 110 °C and desolvation temperature 450 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 550 and 30 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 2.6×10^{-3} mbar. The optimized collision energy for PALO and I.S. both was 30 eV. All data collected in centroid mode were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Primary stock solutions of PALO and I.S. were prepared by dissolving the accurately weighed reference standard of palonosetron hydrochloride and verapamil hydrochloride in methanol to yield concentrations of 950 and 960 ng/mL, respectively. The internal standard (I.S.) working solution of 9.60 ng/mL was prepared by dilution with water. The PALO stock solution was then serially diluted with water to provide working standard solutions of desired concentrations. All the solutions were stored at 4 °C and brought to room temperature before use.

Calibration standards were prepared daily by spiking 500 μL of the blank plasma with 50 μL of the appropriate working standard solutions and 50 μL I.S. solution (9.60 ng/mL) to yield final concentrations of 0.0190, 0.0475, 0.0950, 0.190, 0.475, 0.950 and 3.80 ng/mL. One calibration curve was constructed on each analysis run using freshly prepared calibration standards. The quality control (QC) samples were prepared in blank plasma at low, mid and high concentrations of 0.0380, 0.380 and 2.85 ng/mL and stored at −20 °C after preparation. The standards and QC samples were extracted on each analysis run along with the unknown samples with the same procedure.

2.4. Plasma sample preparation

A 50 μL aliquot of I.S. working solution (9.60 ng/mL) and 500 μL 50 mM sodium hydroxide solution were added to 500 μL of collected plasma sample in a 10-mL glass tube and the mixture was vortex-mixed for 30 s. The sample was extracted with 3 mL of diethyl ether by vortex mixing for 60 s and centrifuged for 10 min at 3500 rpm. The upper organic layer was then transferred into another clean glass tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 100 μL of methanol–water (80:20, v/v), followed by vortexing for 30 s. The supernatant was transferred to an autosampler vial and an aliquot of 10 μL was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, extraction recovery and stability according to FDA guidance for validation of bioanalytical methods [7]. Validation runs were conducted on three consecutive days. The peak area ratios of PALO to the I.S. of QC samples were interpolated from the calibration curve on the same run to give the concentration of PALO. The results from QC samples in three runs were used to evaluate the precision and accuracy of the method developed.

2.5.1. Selectivity

The selectivity was checked by comparing the chromatograms of different batches of blank human plasma from six subjects to those of corresponding standard plasma samples spiked with PALO and I.S. (9.60 ng/mL) and plasma sample after intravenous administration of palonosetron hydrochloride. Each blank plasma sample was tested using the proposed extraction procedure and UPLC–MS/MS conditions to ensure no interference of PALO and I.S. from plasma.

2.5.2. Linearity and lower limit of quantification

Calibration curves were constructed by assaying standard plasma samples at seven concentration levels in the range of 0.0190–3.80 ng/mL with weighted ($1/x^2$) least squares linear regression.

According to FDA guidance, the LLOQ is defined as the lowest concentration of the calibration curve that can be quantitatively determined with acceptable precision and accuracy, at which the relative standard deviation (R.S.D.) was below 20% and the relative error (R.E.) was within ±20% [7].

2.5.3. Precision and accuracy

The precision and accuracy were evaluated by using replicate analysis of QC samples on three consecutive days (one run per day). The accuracy as well as the intra- and inter-run precision of the method was determined by analyzing six replicates of LLOQ and QC samples at three concentrations along with two standard curves on

each of three runs. The precision was expressed as the R.S.D. and the accuracy as the R.E.

2.5.4. Extraction recovery and matrix effect

The extraction recovery was determined by dividing the peak areas of PALO added into blank plasma and extracted using LLE procedure with those obtained from the compound spiked into equivalent volume of post-extraction supernatant. This procedure was repeated for six replicates at three QC concentration levels of 0.0380, 0.380, and 2.85 ng/mL. Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus, the evaluation of matrix effect from the influence of co-eluting components on analyte ionization is necessary for an UPLC–MS/MS method. The matrix effect was measured by comparing the peak areas of sample spiked post-extraction (A) with that of pure standard solution containing equivalent amount of the compound (B). The ratio ($A/B \times 100\%$) was used to evaluate the matrix effect. The extraction recovery and matrix effect of I.S. were also evaluated using the same procedure.

2.5.5. Stability

To evaluate the stock solution stability of PALO and I.S., five aliquots of the stock solutions of PALO (950 ng/mL) and I.S. (960 ng/mL) were kept at 4 °C for 7 days. The mean peak areas of PALO and I.S. from five replicate chromatographic runs were compared with those from freshly prepared solutions at the same concentration.

The stability of PALO in human plasma was assessed by analyzing three replicates of low and high QC samples under different temperature and time conditions. Freeze–thaw stability was performed by subjecting unextracted QC samples to three freeze (−20 °C)–thaw (room temperature) cycles. QC samples were stored at −20 °C for 30 days and at ambient temperature for 4 h to determine long-term and short-term stability, respectively. The post-preparative stability was studied by analyzing the extracted QC samples kept in the autosampler at 4 °C for 12 h. All stability testing QC samples were determined by using calibration curve of freshly prepared standards. The concentrations obtained were compared with the nominal values.

2.6. Application to pharmacokinetic study

The validated method was applied to determine the plasma concentration of PALO from 6 healthy volunteers selected strictly. The pharmacokinetic study was approved by the local Ethics Committee and carried out in the hospital. All volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected before and 1, 5, 15, 30 min and 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144, 168 h after intravenous administration of a single dose of 0.125 mg palonosetron hydrochloride. Plasma was separated by centrifugation and stored at −20 °C until analyzed.

The maximum plasma concentration (C_{max}) and the time of the maximum plasma concentration (T_{max}) were noted directly from the measured data. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

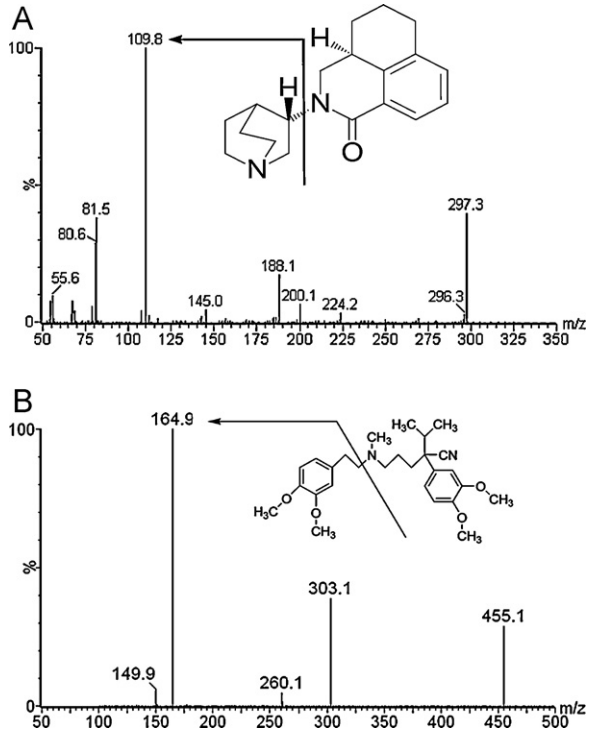

Fig. 2. Full-scan product-ion mass spectra of $[M+H]^+$ of PALO (A) and I.S. (B.)

3. Results and discussion

3.1. Optimization of mass spectrometry

In this assay, UPLC–MS/MS operation parameters were carefully optimized for the determination of PALO. Standard solutions of PALO and I.S. were directly infused along with the mobile phase into the mass spectrometer with ESI as the ionization source. And the mass spectrometer was tuned in both positive and negative ionization modes for optimum response of PALO. It was found that the signal intensity of positive ion was higher than negative ion. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+$ m/z 297.3 and 455.1 for PALO and I.S., respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain highest intensity of protonated molecule of PALO. The product ion scan spectra showed high abundance fragment ions at m/z 109.8 and 164.9 for PALO and I.S., respectively. The product-ion spectra of the two compounds are shown in Fig. 2. The collision gas pressure and collision energy of collision-induced decomposition (CID) were optimized for maximum response of the fragmentation of m/z 109.8 for PALO. Multiple reaction monitoring (MRM) using the precursor → product ion transition of m/z 297.3 → m/z 109.8 and m/z 455.1 → m/z 164.9 was employed for quantification of PALO and I.S., respectively. In the previous paper [5] the transition at m/z 297.0 → m/z 110.2 showed unsatisfactory sensitivity. However, the positive ions were detected in the MRM mode with precursor to product ion pairs as m/z 297.3 → m/z 109.95 for PALO in the reported paper [6], which had a good sensitivity with the LLOQ of 0.0435 ng/mL. In our experiment it was found that the fragmentation of m/z 109.8 showed highest MS response in the product ion scan spectra of PALO. Therefore, ion transition of m/z 297.3 → m/z 109.8 for PALO was also adopted in this assay.

3.2. Optimization of chromatography

The chromatographic conditions were modified to obtain high sensitivity, short retention time and a symmetrical peak shape. The separation and ionization of PALO and I.S. were affected by the composition of mobile phase. Methanol and acetonitrile were both attempted as the organic modifier of mobile phase. It was found that the peaks were more symmetric and the signal-to-noise (S/N) ratio of PALO was obviously higher with methanol than those with acetonitrile. The ionization of PALO and I.S. was increased by adding formic acid in the mobile phase. The effect of formic acid of 0.1%, 0.2% and 0.5% in aqueous phase on the response was investigated. Both PALO and I.S. were found to have higher response in the mobile phase with 0.1% formic acid. Even if the analyte and I.S. can be separately detected, the choice of the analytical column is still important. ACQUITY UPLC™ BEH C_{18} column and HSS T_3 column were both tested during the method development. Ion suppression was observed on BEH C_{18} column, which was attributed to the early elution of PALO. In order to improve the retention behavior of PALO on BEH C_{18} column, different proportions of methanol was tested in the following experiment. High proportion of aqueous phase was investigated, which showed no significant matrix effect, but the response of PALO could not meet the requirement of this experiment. To improve the response of PALO, HSS T_3 column was used and high proportion of organic phase was investigated in our experiment. As a result, a better sensitivity and a shorter analytical time without interference of endogenous substance were achieved on HSS T_3 column with 80% of methanol in the mobile phase.

Finally, a mobile phase consisting of methanol–water containing 0.1% formic acid (80:20, v/v) was used. The use of T_3 chromatographic column benefited the retention of analyte and the separation from endogenous interference. The total run time was as short as 1.2 min per sample, which was shorter than those reported [4–6]. Under the optimal conditions, the short total run time for each sample promised the high sample throughput of requirement of bioanalysis in pharmacokinetic study of PALO.

Two channels were used for recording the response, channel 1 for PALO with a retention time of 0.81 min, and channel 2 for the I.S. with a retention time of 0.79 min. No interference was observed for both PALO and I.S. (Fig. 3).

3.3. Selection of internal standard

According to FDA guidance, an internal standard in the analysis of biological sample could be a structurally similar analog of analyte or a stable labeled compound [7]. Deuterated standard would be a preferred I.S. in LC–MS assay. However, considering the radiation and the high cost of the deuterated I.S., we did not use deuterated palonosetron as I.S., although it would have been the best and most logical choice. Moreover, a compound that has similar structure, extraction recovery, chromatographic and mass spectrometric behavior to the analyte also may be considered. In this experiment, diazepam, diphenhydramine and verapamil with some structural similarity to PALO were tested as I.S. respectively. Finally, verapamil was chosen as I.S. due to its similarity to PALO in retention, ionization and extraction efficiency.

3.4. Plasma sample preparation

Sample preparation was an important step for accurate and reliable LC–MS/MS assay. The most widely employed biological sample preparation techniques currently are liquid–liquid extraction (LLE), protein precipitation (PPT), and solid-phase extraction (SPE). SPE

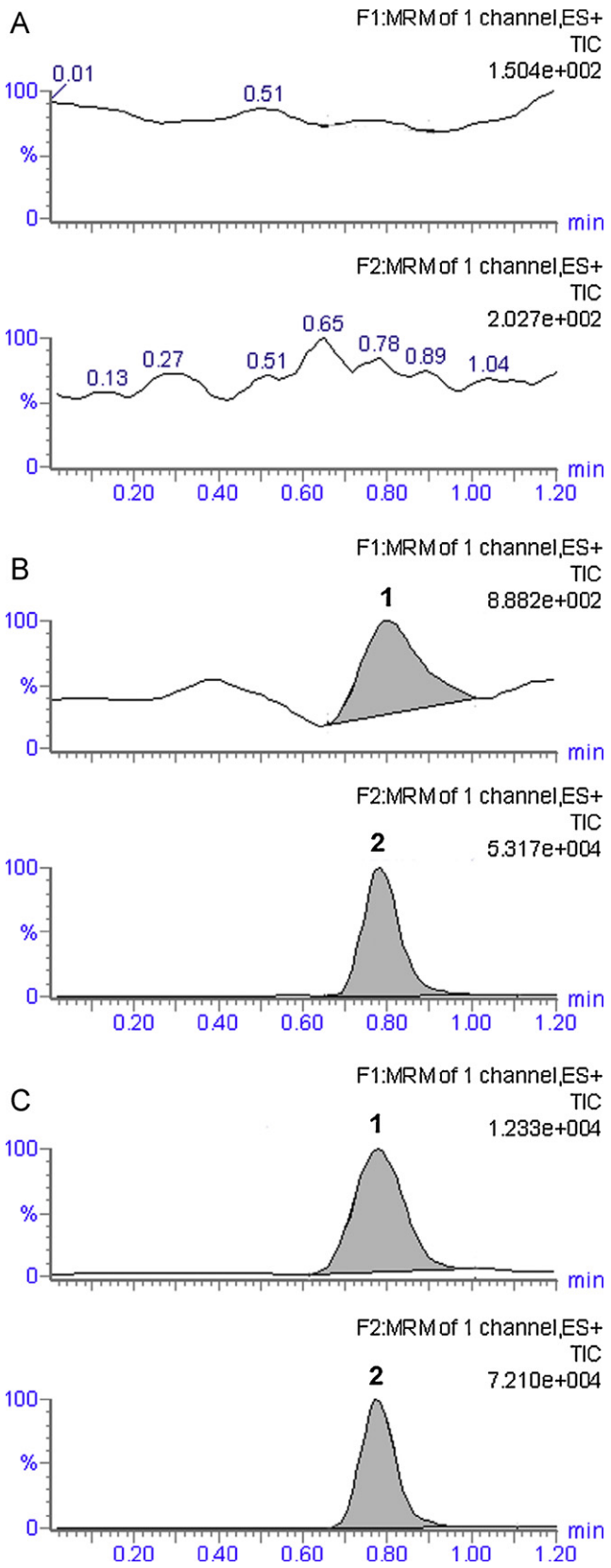

Fig. 3. Representative MRM chromatograms of PALO (peak 1, channel 1) and I.S. (peak 2, channel 2) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with PALO at the LLOQ of 0.0190 ng/mL and I.S.; (C) a plasma sample from a volunteer 1 h after intravenous administration of a single dose of 0.125 mg palonosetron hydrochloride. The retention time of PALO and I.S. were 0.81 and 0.79 min, respectively.

Table 1
Precision and accuracy for the determination of PALO in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days).

Concentrations (ng/mL)		Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Accuracy R.E. (%)
Added	Found			
0.0190	0.0198 ± 0.0016	8.2	5.5	4.3
0.0380	0.0398 ± 0.0040	9.7	10	4.7
0.380	0.401 ± 0.020	3.9	9.3	5.6
2.85	3.02 ± 0.21	6.1	11	6.1

was limited by the cost of the apparatus and instrumentation. Compared with PPT, LLE can not only purify but also concentrate the sample. Therefore, LLE procedure was used to extract PALO from plasma samples, which was also reported in the literatures [4,5]. Initially, diethyl ether and ethyl acetate were chosen as candidate extraction solvents. Further test results proved that diethyl ether showed more stable extraction recovery to PALO than ethyl acetate. Moreover, the boiling point of diethyl ether is lower, and it was evaporated to dryness more quickly. PALO and I.S. were both basic compounds with an amide group, and the pK_a values were 9.77 and 8.97, respectively, so alkaline condition was very important. Therefore, 500 μL 50 mM sodium hydroxide solution was added as alkaline reagent which benefited the extraction of PALO and I.S. from plasma samples.

3.5. Method validation

3.5.1. Selectivity

The selectivity of the assay was checked by comparing the chromatograms of six individual human plasma with the corresponding spiked plasma. As shown in Fig. 3, there was no interference from endogenous substances at the retention time of PALO and I.S.

PALO is converted *in vivo* to an N-oxide palonosteron [2]. N-oxide palonosteron in general may be unstable and may convert back to the parent drug during storage and/or analysis, especially under acidic and basic conditions and at high temperature, which could lead to an overestimation of PALO levels in samples from dosed subjects. An additional validation experiment with the reference standard of N-oxide palonosetron was performed to demonstrate that the method did not suffer from interference of N-oxide palonosetron. The result indicated that the method was sufficiently selective towards PALO in our assay.

3.5.2. Linearity and LLOQ

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 0.0190–3.80 ng/mL for PALO in human plasma. A typical regression equation for the calibration curve was $y = 5.14 \times 10^{-1}x + 1.03 \times 10^{-3}$, $r = 0.9968$, where y is the peak area ratio of PALO to I.S., and x is the concentration of PALO in human plasma.

The LLOQ for PALO was 0.0190 ng/mL with precision below 20% and accuracy within ±20% (Table 1), and a corresponding chromatogram was given in Fig. 2B.

3.5.3. Precision and accuracy

The data of intra- and inter-day precision and accuracy of the method were given in Table 1. The intra- and inter-day R.S.D.s were not more than 9.7% and 11%, and R.E.s were from 4.7% to 6.1% at three QC levels, indicating acceptable precision and accuracy of the present method.

3.5.4. Extraction recovery and matrix effect

The extract recoveries of PALO from human plasma were 75.1 ± 6.9%, 74.9 ± 9.8%, and 76.3 ± 10.9% at concentrations of 0.0380, 0.380, and 2.85 ng/mL, respectively. The mean extraction recovery of I.S. was 75.7 ± 8.7%. Thus, the consistency in recoveries of PALO and I.S. supported the extraction procedure for its application to routine sample analysis. All the ratios ($A/B \times 100\%$) mentioned in Section 2 were between 93.0% and 106%. No significant matrix effect for PALO and I.S. was observed, indicating that no co-eluting substance influenced the ionization of the analyte and I.S.

Table 2
Stability of PALO in human plasma at two QC levels ($n = 3$).

Nominal concentration (ng/mL)	Concentration found (ng/mL; mean ± S.D.)	R.S.D. (%)	R.E. (%)
Short term (room temperature for 4 h)			
0.0380	0.0370 ± 0.0030	8.6	−4.3
2.85	2.68 ± 0.37	14	−9.3
Long term (−20 °C for 30 days)			
0.0380	0.0370 ± 0.0030	7.2	3.7
2.85	2.82 ± 0.13	4.6	−2.5
Three freeze–thaw cycles			
0.0380	0.0360 ± 0.0020	4.3	−13
2.85	2.75 ± 0.21	7.5	−3.6
Post-preparative (4 °C for 12 h)			
0.0380	0.0400 ± 0.0020	5.7	3.4
2.85	2.70 ± 0.26	9.6	−8.7

3.5.5. Stability

The R.E. between the stocked and the freshly prepared solutions for PALO and I.S. were 3.2% and 4.7%, respectively, which indicated the stock solutions of PALO and I.S. were stable for at least 7 days.

The results of stability studies were shown in Table 2, which indicated a good stability of PALO in plasma stored at room temperature for 4 h, at −20 °C for 30 days and during three freeze–thaw cycles, and in prepared samples at 4 °C for 12 h. The method is therefore proved to be applicable for routine analysis.

3.6. Application to a pharmacokinetic study

This validated UPLC–MS/MS method was successfully applied to the pharmacokinetic study of PALO in 6 Chinese healthy volunteers following intravenous administration of a single dose of 0.125 mg palonosetron hydrochloride. The profile of the mean plasma concentration of PALO versus time was shown in Fig. 4, which demonstrated the applicability of the developed method with a

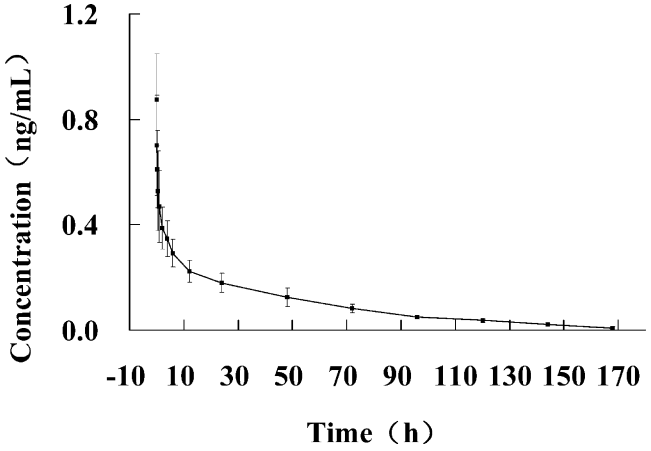

Fig. 4. Mean plasma concentration–time profile of PALO after intravenous administration of palonosetron hydrochloride injection (containing 0.125 mg palonosetron hydrochloride) to 6 healthy volunteers.

high sensitivity to pharmacokinetic study with low dosage. The maximum plasma concentration (C_{max}) was 0.875 ± 0.173 ng/mL, the area under the plasma concentration–time curve from 0 h to the time of last measurable concentration (AUC_{0-t}) was 15.7 ± 3.0 ng h/mL, area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$) was 16.6 ± 3.0 ng h/mL, the half-life of drug elimination at the terminal phase ($t_{1/2}$) was 39.5 ± 4.8 h. These pharmacokinetic parameters were in accordance with those reported in the literatures [4,5], indicating the applicability of this method to the pharmacokinetic study of PALO.

4. Conclusions

A rapid, sensitive and selective UPLC–MS/MS method for quantification of PALO in human plasma was developed and validated. Compared with the analytical methods reported in the literatures, the method offered satisfactory selectivity, good sensitivity with an LLOQ of 0.0190 ng/mL and short run time of 1.2 min by using special UPLC system, which are of particular advantage to high sample throughput of bioanalysis. The method was proved superior in speed and selectivity to previously reported methods and was successfully applied to the pharmacokinetic study of palonosetron hydrochloride.

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