Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 852 (2007) 479–484
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Application of a sensitive liquid chromatographic/tandem mass spectrometric method to pharmacokinetic study of nalmefene in humans

Ping Li [a,b], Xiaoyan Chen [a], Xiaojian Dai [a], Aidong Wen [c], Yifan Zhang [a], Dafang Zhong [a,*]

[a] *Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, China*
[b] *Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China*
[c] *Xijing Hospital of the Fourth Military Medical University, Xi'an 710033, China*

Received 7 December 2006; accepted 4 February 2007
Available online 15 February 2007

Abstract

A sensitive, specific and rapid liquid chromatographic/tandem mass spectrometric (LC/MS/MS) method was developed and validated for quantification of nalmefene in human plasma. An aliquot of 200 μL plasma sample was simply precipitated by 400 μL methanol. Separation of nalmefene and the internal standard hydromorphone from the interferences was achieved on a C_{18} column followed by MS/MS detection. The analytes were monitored in the positive ionization mode with a TurboIonspray source. The method had a total chromatographic run time of 4.5 min and linear calibration curves over the concentration range of 10–5000 pg/mL. The lower limit of quantification (LLOQ) was 10 pg/mL. The intra- and inter-day precision was less than 10.1% determined from QC samples at concentrations of 30, 300 and 4500 pg/mL, and the accuracy was within ±3.4%. As the method was more sensitive (10 times higher) than those reported previously, we investigated the pharmacokinetics of nalmefene in healthy volunteers after a single intravenous injection of low dose (30 μg) of nalmefene hydrochloride for the first time.

Keywords: Nalmefene; LC/MS/MS; Pharmacokinetics

1. Introduction

Nalmefene is a specific opioid antagonist with actions similar to naloxone. Its pharmacodynamic activity is mediated via competitive inhibition of opioid receptor sites [1]. Nalmefene effectively reversed the respiratory depression produced following therapeutic or excessive opioid administration, when administered intravenously with a dose between 0.25 and 0.5 μg per kg as an emergency drug [2]. Considering that it has no agonist activity and no abuse potential, nalmefene has several applications in the treatment of dependence disorders, including alcoholism and pathological gambling [3,4]. Its advantages over naloxone are its longer duration of action and higher oral bioavailability [5–7].

Many analytical methods including radioimmunoassay [8], high performance liquid chromatography (HPLC) with electrochemical detection [7,9–11] and gas chromatography/mass spectrometry (GC/MS) [12] have been reported for determining the plasma concentration of nalmefene in biological matrices. Dixon et al. made use of radioimmunoassay with the lower limit of quantification (LLOQ) at 0.3 ng/mL to characterize the kinetics of nalmefene in human after single intravenous doses of 2 mg, 6 mg, 12 mg and 24 mg, respectively [10]. Recently, Fang et al. established a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method to quantify nalmefene in human and rabbit plasma with the LLOQ of 0.1 ng/mL [13]. However, the plasma concentration was expected to be less than 0.1 ng/mL after a single intravenous injection of 30 μg nalmefene after 0.5 h. Because of their limited sensitivity, all the methods above cannot satisfy the demand of pharmacokinetic (PK) study of nalmefene in human receiving a lower dose of intravenous injection.

On the basis of optimizing the mass spectrometric conditions, the chromatographic conditions and the sample preparation

* Corresponding author. Tel.: +86 21 50 800 738; fax: +86 21 50 800 738.
E-mail address: zhongdf@china.com (D. Zhong).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.02.013

procedures, we developed and validated a more sensitive LC/MS/MS method with the LLOQ down to 10 pg/mL to measure nalmefene concentration in human plasma. Then this assay method was successfully applied to a PK study of nalmefene after a single intravenous injection of 30 μg nalmefene hydrochloride (dose calculated as free-base) to nine healthy volunteers.

2. Experimental

2.1. Chemical and reagents

Nalmefene hydrochloride was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and hydromorphone hydrochloride (internal standard, IS) was kindly provided by Qinghai Pharmaceutical Factory Co. Ltd. (Xining, China). Methanol and ammonium acetate of HPLC grade were purchased from Merck (Darmstadt, Germany) and Tedia (Fairfeild, OH, USA), respectively. Heparinized blank (drug-free) human plasma was obtained from Xijing hospital (Xi'an, China). Distilled water was prepared from demineralized water and was used throughout the study.

2.2. Instrumentation and analytical conditions

Chromatographic analysis was performed using an Agilent 1100 series LC system (Agilent, Waldbronn, Germany) consisting of a quaternary pump, an autosampler, a column oven and a degasser. Separation of the analytes from plasma was achieved at 23 °C on a Zorbax Eclipse XDB C_{18} column (150 mm × 4.6 mm i.d., 5 μm; Agilent, Wilmington, DE, USA) with a Security-Guard C_{18} guard column (4 mm × 3.0 mm i.d.; Phenomenex, Torrance, CA, USA). A mobile phase of methanol/5 mM ammonium acetate (75:25, v/v) at a flow-rate of 0.5 mL/min was employed.

An API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) was operated with a TurboIonspray interface in positive ion mode. Analyst 1.4.1 software (Applied Biosystems) was used for the control of equipment, data acquisition and analysis. For the optimization of MS/MS parameters, we made use of the auto-optimization feature of the software and standard solutions of nalmefene and hydromorphone (IS) prepared in a mixture of methanol/water (50:50, v/v) were infused into the mobile phase (0.5 mL/min) at a flow rate of 30 μL/min using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Finally, the instrument was operated with an ion spray voltage at +4.2 kV, heater gas temperature at 450 °C, nebulizer gas (Gas 1) of 0.34 MPa, heater gas (Gas 2) of 0.41 MPa, curtain gas of 0.069 MPa and collision gas of 0.025 MPa. All gases used were nitrogen. Declustering potential (DP) was set at 50 V for both the analyte and IS. Multiple reaction monitoring (MRM) was employed for data acquisition. The optimized MRM fragmentation transitions (Fig. 1) were *m/z* 340 → *m/z* 268 with collision energy (CE) of 38 eV for nalmefene, and *m/z* 286 → *m/z* 185 with CE of 39 eV for hydromorphone (IS). The dwell time for each transition was 200 ms.

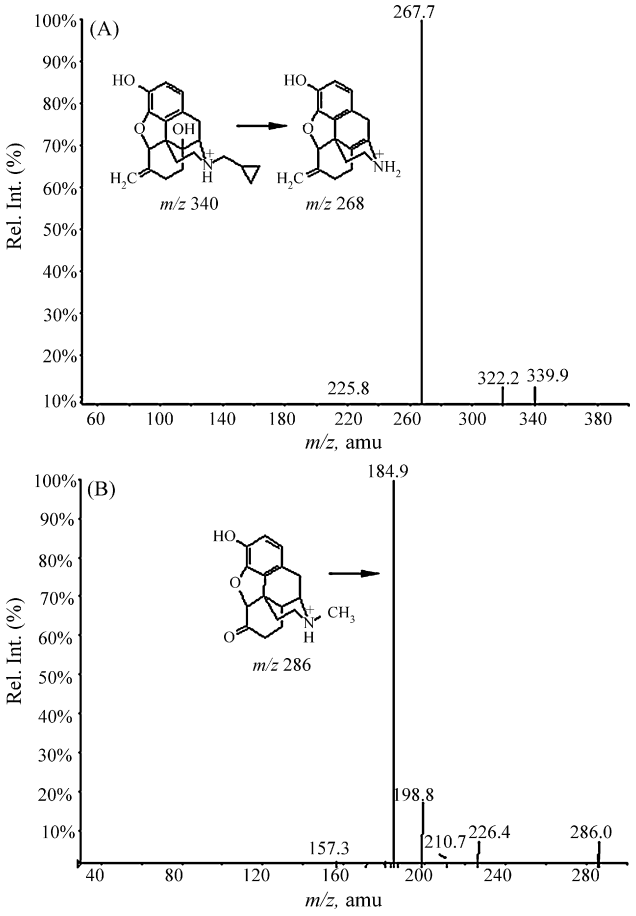

Fig. 1. Product ion mass spectra of $[M+H]^+$ of (A) nalmefene and (B) hydromorphone (internal standard).

2.3. Preparation of standard and quality control (QC) samples

Two stock solutions of nalmefene at a concentration of 400 μg/mL were prepared by dissolving the accurately weighed reference substance in methanol. One solution was then serially diluted with a mixed solution of methanol/water (50:50, v/v) to give working solutions at the following concentrations: 50, 150, 500, 1500, 4000, 10000 and 25000 pg/mL. The other stock solution was independently diluted in a similar way to achieve quality control (QC) solutions at concentrations of 150, 1500 and 22500 pg/mL. Internal standard working solution (100 ng/mL) was prepared by diluting the 400 μg/mL stock solution of hydromorphone with a mixture of methanol/water (50:50, v/v). All the solutions were kept at 4 °C and were brought to room temperature before use.

Both the calibration standard samples and the quality control samples, which were used in the pre-study validation and during the pharmacokinetic study were prepared by spiking 200 μL blank plasma with 40 μL working solutions correspondingly.

2.4. Sample preparation

To 200 μL of plasma sample in a 2.0 mL eppendorf tube, 40 μL of the IS (100 ng/mL), 40 μL of methanol/water (50:50,

v/v) and 400 μL of methanol were added. The mixture was vortexed for 1 min and then centrifuged at 6000 × *g* for 5 min. A 20 μL aliquot of the clean supernatant was directly injected onto the LC/MS/MS system.

2.5. Method validation

To investigate the selectivity of the method, human blank plasma samples from six different donors were pretreated and analyzed at LLOQ. While LLOQ was defined as the lowest concentration of analyte determined with acceptable precision and accuracy (six replicates with relative standard deviation below 20% and relative error within ±20%). Moreover, the analyte's response at this concentration level should be >5 times of the baseline noise.

Linearity was assessed by assaying calibration curves in human plasma in duplicate in three separate runs. And the curves were fitted by a linear weighted ($1/x^2$) least squares regression method through the measurement of the peak-area ratio of the analyte to IS.

To evaluate the precision and accuracy of the method, QC samples at three concentration levels (30, 300 and 4500 pg/mL) were analyzed in six replicates on three validation days. The assay precision was calculated by using the relative standard deviation (RSD) and a one-way analysis of variance (ANOVA). It separates out the sources of variance due to within- and between-run factors. The assay accuracy was expressed as relative error (RE), i.e. (observed concentration − nominal concentration)/(nominal concentration) × 100%. The accuracy was required to be within ±15%, and the intra- and inter-day precisions not to exceed 15%.

The recovery of nalmefene was determined by comparing the mean peak areas of the regularly pretreated QC samples at three concentration levels (six samples each) to those of spike-after-extraction samples, which represented the 100% recovery. To obtain the spike-after-extraction samples, protein of 200 μL blank human plasma was removed with an addition of 400 μL methanol. Then all supernatant was transferred to a tube containing 40 μL of QC solution and 40 μL of IS solution.

As far as the stability of nalmefene in human plasma was concerned, there was no significant degradation observed under the storage conditions described in the previous reports [12,13]. Herein we just investigated the stability of nalmefene in the post-extraction supernatant. The QC samples at low and high concentrations (three replicates each) were processed following the sample preparation procedure as described above. The ready-to-inject samples were left in the autosampler vials at 23 °C for 24 h.

According to the method described by Matuszewski et al. [14], we assessed the matrix effects (ME), i.e. whether the potential ion suppression or enhancement owing to co-eluting matrix components existed in the present experiment. The corresponding peak areas of the analyte from the spike-after-extraction samples at low and high concentration levels were then compared to those of the standard solution at the same concentration in mobile phase.

2.6. PK study

The validated method was applied to a pharmacokinetic study of injection containing 0.1 mg nalmefene hydrochloride per 1 mL. Nine male Chinese healthy volunteers (aged 19–24, body weight 64.5 ± 6.2 kg) took part in the study after a thorough medical, biochemical and physical examination. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved by the Human Investigation Ethical Committee at Xijing Hospital of the Fourth Military Medical University. Each volunteer was injected a single dose of 30 μg nalmefene intravenously over a period of 5 min. Venous blood samples about 4 mL were collected in heparin-containing tubes before drug administration and at 0.033, 0.083, 0.167, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12, 24 h after the end of the injection. Plasma samples were obtained by centrifugation at 2000 × *g* for 10 min and were frozen at −20 °C until analysis.

The plasma concentrations of nalmefene versus time profiles were acquired for each subject. Then major non-compartmental PK parameters of nalmefene were calculated. The elimination rate constant (k) was calculated as the slope of the linear regression fit of the logarithm scale plasma concentrations versus time data for the last four measurable points. Apparent elimination half-life ($t_{1/2}$) was obtained as $0.693/k$. The area under the plasma concentration–time curve (AUC) was calculated according to the linear trapezoidal rule to the last measurable point (AUC_{0-t}) or to infinity ($AUC_{0-\infty}$) by $AUC_{0-t} + C_t/k$, where C_t is the last measurable drug concentration. The mean residence time (MRT) was obtained by dividing the area under the first moment-time curve ($AUMC_{0-\infty}$) by the area under the curve ($AUC_{0-\infty}$). The total body clearance (CL) was calculated as Dose/$AUC_{0-\infty}$ and apparent volume of distribution (V) as Dose/C_0.

3. Results and discussion

3.1. MS conditions

Nalmefene is moderately polar and has a tertiary amino group in its structure, and so produces good mass spectrometric responses in positive electrospray ionization (ESI) mode. In the Q1 full scan mode $[M+H]^+$ ion with a high MS response was generated and no other additive ions were observed. Furthermore, the product spectrum of $[M+H]^+$ ion of nalmefene was dependent on the collision energy (CE). At low CEs (20–28 eV), a major fragment ion at m/z 322 was formed by neutral loss of one molecule of H_2O. Increasing the CE value to 38 eV, the most abundant fragment ion at m/z 268 was generated by the removal of cyclopropylmethyl on the basis of the cleavage of C–N bond of the fragment ion at m/z 322. The transition of m/z 340 → m/z 322 was once used in the quantification of nalmefene by Fang et al. to determine nalmefene in human and rabbit plasma with the LLOQ at 0.1 ng/mL [13]. At first, we also used the fragment ion at m/z 322 as product ion to measure nalmefene in human plasma, but high chemical background noise was observed. As a result, we were only able to quantify nalmefene in human plasma with the LLOQ of 0.1 ng/mL.

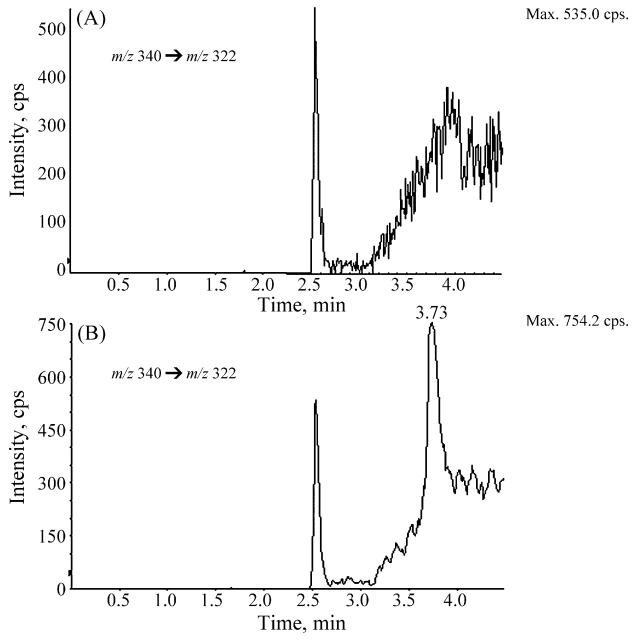

Fig. 2. Representative MRM chromatograms with the transition of *m/z* 340 → *m/z* 322 employed for nalmefene in human plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with nalmefene (10 pg/mL).

The chromatographic peaks of nalmefene at lower concentrations were submerged in the baseline noise (see Fig. 2). And the signal-to-noise ratio (S/N) was just about 2 at the concentration of 10 pg/mL. Consequently, the alternative was taken into consideration. As far as we observed, much lower background noise was obtained when monitoring the fragment ion at *m/z* 268 and the S/N was >5 at the concentration of 10 pg/mL. Because many endogenous interferences in plasma are easy to lose one molecule of H_2O in the MRM mode, the transition of $[M+H]^+$ to $[M+H-H_2O]^+$ is lack of selectivity for the analysis of biological samples. Therefore, the high chemical noise leads to poor sensitivity.

In our case, hydromorphone, a structural analogue of nalmefene was used as the internal standard. The base peak $[M+H]^+$ ion at *m/z* 286 in the Q1 full scan mode was selected as the precursor ion. Its fragment ion at *m/z* 185 proved to be steady and abundant which was chosen for the MRM acquisition. However, this method is not suitable for samples of subjects treated with hydromorphone or related drugs that are metabolized to hydromorphone.

3.2. Chromatographic conditions

Simple protein precipitation technique is often used for rapid sample clean-up in LC/MS/MS analysis. However, the occurrence of matrix effect (ME) usually restricts its application. Matrix effects can severely decrease or increase the response of the analyte, and so the sensitivity and accuracy of a biological method can be adversely affected. The usual mean to eliminate ME is to optimize the chromatographic conditions. In our study, we attempted different chromatographic conditions. As far as the mobile phase was concerned, that containing acetonitrile resulted in higher background noise than methanol. When the mobile phase consisting of methanol/5 mM ammonium acetate (90:10, v/v) was used, weak chromatographic peaks of nalmefene and the IS in the spiked plasma sample were observed with the retention times at about 2.0 min. Whilst in the chromatograms there was a collapse zone at about 2.0 min appearing in both channels, when analyzing the blank samples. Thus, we presumed that ME existed in the analysis process. In order to achieve the separation of analytes with the co-eluting interferences, we decreased the proportion of organic phase in the mobile phase to 70%. The absolute responses of both interest greatly increased and the retention times were about 4.0 min. The capacity factor of nalmefene increased to 1.5. The proportion of organic phase was then increased to three quarters, in order to avoid ME effectively and to shorten the run-time. As a result, the retention times were about 0.3 min earlier. Finally, methanol/5 mM ammonium acetate (75:25, v/v) was employed as the mobile phase in the experiment. We came to a conclusion that the proportion of organic phase in the mobile phase could significantly influence the retention time of nalmefene on the reverse-phase columns.

Matrix effects under the optimized mass spectrometric and chromatographic conditions were evaluated by comparing the peak areas of nalmefene from the spike-after-extraction samples to those obtained for the standards in mobile phase at equivalent concentrations. The ratios were $90.3 \pm 5.9\%$ at low concentration and $94.8 \pm 5.6\%$ at high concentration. The same assay was performed for the IS and the ratio was $96.3 \pm 3.8\%$. Then we came to a conclusion that ion suppression or enhancement from plasma matrix could be negligible in this experiment.

3.3. Method validation

3.3.1. Assay selectivity

Fig. 3 shows the typical MRM chromatograms of a blank plasma, a spiked plasma sample with nalmefene (10 pg/mL) and IS (20 ng/mL), and a plasma sample from a healthy volunteer 4.0 h after an intravenous injection of 30 μg of nalmefene. In virtual of the figure, thanks to the high selectivity of the MRM, there were no significant endogenous interferences observed at the retention times of the analyte and IS.

3.3.2. Linearity and LLOQ

The calculated peak area ratios of nalmefene to the internal standard versus the nominal concentration displayed a good linear relationship over the concentration range from 10 to 5000 pg/mL in human plasma.

Typical equation of the calibration curves was as follows:

$$y = 2.88 \times 10^{-4}x + 4.76 \times 10^{-3}, \quad r = 0.9972$$

where y represents the ratio of nalmefene peak area to that of the IS and x represents the plasma concentration of nalmefene.

The LLOQ was established at 10 pg/mL, which was sensitive enough to investigate the pharmacokinetics of low dose (30 μg) of nalmefene in human. The precision and accuracy at this concentration level were acceptable, with 6.2% of the RSD and 2.0% of the RE.

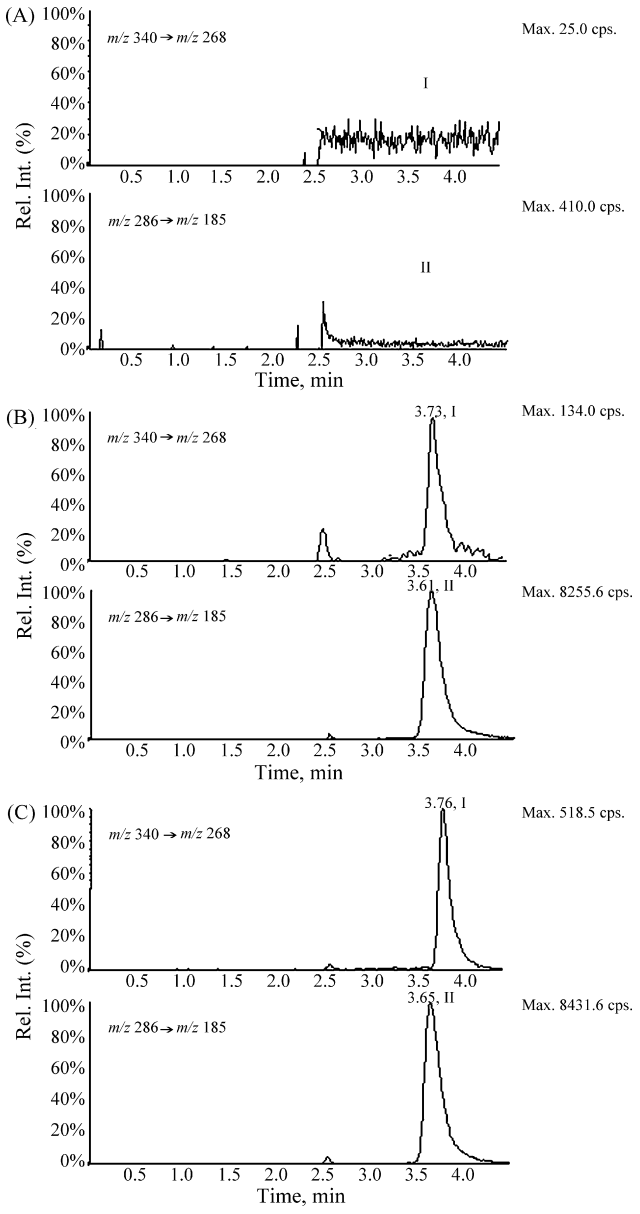

Fig. 3. Representative MRM chromatograms for nalmefene (I) and IS (hydromorphone, II) in human plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with nalmefene (10 pg/mL) and IS (20 ng/mL); and (C) a plasma sample from a volunteer 4.0 h after an intravenous injection of 30 μg nalmefene.

3.3.3. Precision and accuracy

The intra- and inter-day precision and accuracy of the assay were investigated by analyzing QC samples. All the values are summarized in Table 1. Intra-day RSD was below 6.0% and inter-day RSD was below 10.1%. Relative error was within ±3.4%. Hence, the method was proved to be accurate and precise.

Table 2
The main pharmacokinetic parameters of nalmefene after an intravenous injection of 30 μg nalmefene to nine healthy volunteers

Parameter	Mean ± SD
k (h^{-1})	0.206 ± 0.107
$t_{1/2}$ (h)	4.63 ± 2.91
AUC_{0-t} (pg h/mL)	399.87 ± 223.01
$AUC_{0-\infty}$ (pg h/mL)	478.07 ± 236.89
V (L)	443.3 ± 245.7
CL (mL/min)	1254 ± 511.5
MRT (h)	6.02 ± 3.22

3.3.4. Recovery and stability

The clean-up of the plasma samples was achieved through a simple one-step protein precipitation procedure with methanol. The recoveries of nalmefene were 87.5 ± 2.0%, 81.6 ± 2.5% and 81.1 ± 1.0% at three concentration levels individually. While the recovery of the IS was 94.4 ± 3.5%. This indicated that the recovery of nalmefene and the IS was consistent and was not concentration-dependent.

It was reported that nalmefene in human plasma was stable when stored at room temperature for 24 h, −25 °C for 30 days and after three freeze–thaw cycles [12,13]. For the RE values were within ±15% for both the low and high concentrations, it indicated that no significant degradation occurred in the ready-to-inject samples at 23 °C for 24 h. Take all the points into consideration, nalmefene can be stored and extracted under routine laboratory conditions without special attention.

3.4. PK study

Due to the poor sensitivity of the existing analytical methods [7,8,11–13], there was no report on the pharmacokinetic study of nalmefene in human administered intravenously with 30 μg nalmefene hydrochloride. Therefore, we developed the present method with the LLOQ down to 10 pg/mL to satisfy the demand of evaluating pharmacokinetics of the drug. Using this analytical method, we were able to measure the concentration of nalmefene up to 6 h for all subjects after a single intravenous administration of 30 μg nalmefene, up to 8 h for 90% of the subjects, up to 12 h for 40% of the subjects and up to 24 h for 10% of the subjects. Fig. 4 shows the profile of the mean nalmefene plasma concentration versus time. Meanwhile, the major pharmacokinetic parameters of nalmefene are listed in Table 2. Data demonstrates that there is significant individual difference between the subjects receiving 30 μg nalmefene intravenously with the AUC_{0-t}

Table 1
Accuracy and precision for the analysis of nalmefene in human plasma (in pre-study validation, $n = 3$ days, six replicates per day)

Added C (pg/mL)	Found C (pg/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Relative error (%)
30.0	30.4 ± 2.01	6.0	10.1	1.5
300	290 ± 15.8	5.8	0.9	−3.4
4500	4579 ± 247	5.4	5.5	1.8

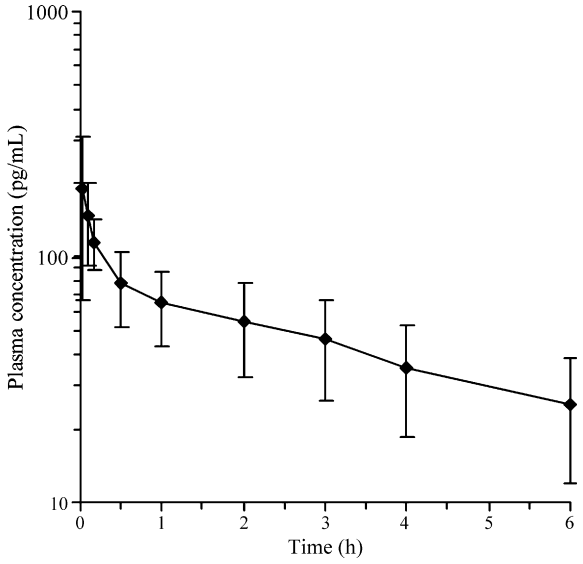

Fig. 4. Mean plasma concentration–time curve of nalmefene after an intravenous injection of 30 μg nalmefene hydrochloride to healthy volunteers ($n = 9$, mean ± SD).

ranging from 192.71 to 900.49 pg h/mL. As the method was more sensitive (10 times higher) than those reported previously, we presented the pharmacokinetic parameters of nalmefene in healthy volunteers after a single intravenous injection of low dose (30 μg) of nalmefene hydrochloride for the first time.

4. Conclusions

A sensitive and efficient LC/MS/MS method with high selectivity was developed and validated for the determination of nalmefene in human plasma. The sample pretreatment was a single step protein precipitation followed by the analysis of a total running time of 4.5 min per sample with the LLOQ of 10 pg/mL. The assay was applied to characterize the pharmacokinetics of nalmefene in healthy volunteers after an intravenous injection of 30 μg nalmefene hydrochloride.

References

[1] M.E. Michel, G. Bolger, B.A. Weissman, Methods Find. Exp. Clin. Pharmacol. 7 (1985) 175.
[2] C.S. Sean, et al., Martindale, vol. 33, The Pharmaceutical Press, Great Britian, 2002, p. 1014.
[3] B.J. Mason, L.D. Williams, E.C. Rivto, R.B. Cutler, Arch. Gen. Psychiatry 56 (1999) 719.
[4] J.E. Grant, M.N. Potenza, E. Hollander, R. Cunningham-Williams, T. Nurminen, G. Smits, A. Kallio, Am. J. Psychiatry 163 (2006) 303.
[5] P.S. Glass, R.M. Jhaveri, L.R. Smith, Anesth. Analg. 78 (1994) 536.
[6] J.P. Gonzalez, R.N. Brogden, Drugs 35 (1988) 192.
[7] R. Dixon, J. Gentile, H.B. Hsu, J. Hsiao, J. Howes, D. Garg, D. Weidler, J. Clin. Pharmacol. 27 (1987) 233.
[8] R. Dixon, J. Hsiao, W. Taaffe, E. Hahn, R. Tuttle, J. Pharm. Sci. 73 (1984) 1645.
[9] J. Hsiao, R. Dixon, Res. Commun. Chem. Pathol. Pharmacol. 42 (1983) 449.
[10] R. Dixon, J. Howes, J. Gentile, H.B. Hsu, J. Hsiao, D. Garg, D. Weidler, M. Meyer, R. Tuttle, Clin. Pharmacol. Ther. 39 (1986) 49.
[11] J.Z. Chou, H. Albeck, M.J. Kreek, J. Chromatogr. 613 (1993) 359.
[12] S. Xie, R.F. Suckow, B.J. Mason, D. Allen, T.B. Cooper, J. Chromatogr. B 773 (2002) 143.
[13] W.B. Fang, D.M. Andrenyak, D.E. Moody, E.S. Nuwayser, J. Anal. Toxicol. 29 (2005) 169.
[14] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.