Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 859 (2007) 52–61
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of thienorphine and its active metabolite thienorphine-glucuronide in rat plasma by liquid chromatography–tandem mass spectrometry and its application to pharmacokinetic studies

Qi Kong, Jianzhong Qiao, Xiaoying Wang, Shulan Yuan, Zhenqing Zhang *, Zehui Gong, Jinxiu Ruan

Key Laboratory of Drug Metabolism & Pharmacokinetics, Beijing Institute of Pharmacology and Toxicology, 27 Taiping Road, Beijing 100850, PR China

Received 31 January 2007; accepted 2 September 2007
Available online 29 September 2007

Abstract

A simple, sensitive and reliable method was developed to determine simultaneously the concentrations of thienorphine and its metabolite thienorphine glucuronide conjugate in rat plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The metabolite was identified by MS: thienorphine glucuronide conjugate. Sample preparation involved protein precipitation with methanol. Analytes were separated on Finnigan BetaBasic-18 column (150 mm × 2.1 mm i.d., 5 μm) using methanol: water: formic acid (56:44:0.1, v/v/v) as mobile phase at a flow rate of 0.2 ml/min. The method had a linear calibration curve over the concentration range of 0.1–50 ng/ml for thienorphine and 2–1000 ng/ml for thienorphine glucuronide conjugate, respectively. LOQ of thienorphine and thienorphine glucuronide conjugate was 0.1 and 2 ng/ml, respectively. The intra- and inter-batch precisions were less than 12% and their recoveries were greater than 80%. Pharmacokinetic data of thienorphine and its metabolite thienorphine glucuronide conjugate obtained with this method following a single oral dose of 3 mg/kg thienorphine to rats were also reported for the first time.

Keywords: Thienorphine; Thienorphine glucuronide conjugate; LC–MS/MS; Rat plasma; Pharmacokinetics

1. Introduction

Opioid abuse and dependence remains a serious worldwide health problem. The drugs currently in clinical use for treating opioid dependence are either full-opioid agonist, methadone, and LAAM or antagonist naltrexone. However, the agonist merely substitutes one addiction for another, and the antagonist is unable to retain patients in treatment due to a lack of desired positive subjective effects [1]. Buprenorphine, an oripavine derived from thebaine, has been widely shown to be a partial agonist and antagonist of the μ-opioid receptor, producing both μ-opioid receptor-mediated antinociception and blocking morphine-induced antinociception in laboratory animals [2–4]. It has been widely used in the treatment of acute and chronic pain, and recently for the treatment of heroin addicts [5,6]. The limited respiratory effects of high-dose buprenorphine are of utmost importance regarding the safety of this drug for use in substitution treatment. Numerous buprenorphine-related deaths have been reported by forensic toxicologists and other sources of information since 1996. These fatalities may result from misuse (intravenous injection of crushed tablets) or overdose with substitution treatment [7,8]. It has been shown that buprenorphine can cause dependence both in physical and psychological studies, probably because of its fairly strong agonist effect at the μ-opioid receptor [9]. Moreover, buprenorphine has been restricted by its very low oral bioavailability in the treatment of opioid dependence [10]. Therefore, there is an urgent requirement for developing new partial opioid agonists with higher oral bioavailability, longer duration of action and more safety than buprenorphine.

* Corresponding author. Tel.: +86 10 66930632; fax: +86 10 68211656.
E-mail address: zqzhang55@yahoo.com (Z. Zhang).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.09.028

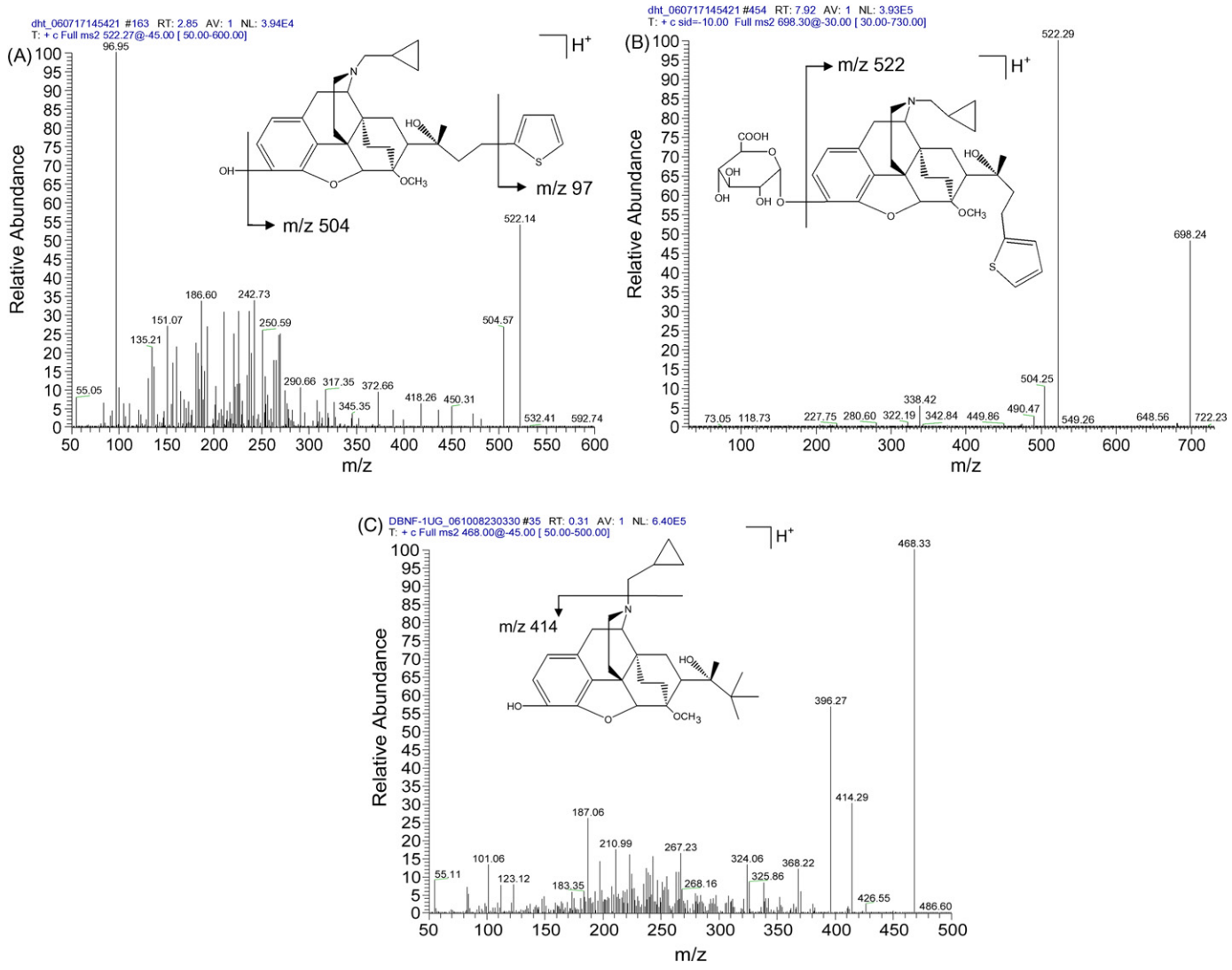

Fig. 1. Mass spectra and structures of the two analytes and I.S.: (A) thienorphine, mother ion: *m/z* 522; daughter ion: *m/z* 97 and 504; (B) thienorphine glucuronide conjugate, mother ion: *m/z* 698; daughter ion: *m/z* 522; (C) buprenorphine (internal standard), mother ion: *m/z* 468; daughter ion: *m/z* 396 and 414.

In a search for such compounds, thienorphine [*N*-cyclopropylmethyl-7(-[(*R*)-1-hydroxy-1-methyl-3-(thien-2-yl)-propyl]-6,14-*endo*-ethano-tetrahydronororipavine], a novel analog of buprenorphine, was synthesized by the chemists in our institute [11]. As buprenorphine, it has been reported that thienorphine bound potently and nonselectively to μ-, δ-, and κ-opioid receptors stably expressed in CHO (Chinese hamster ovary) cells and behaved as a partial agonist at μ-opioid receptor. However, some differences were observed between the pharmacological profiles of thienorphine and buprenorphine. In vitro, thienorphine was more potent than buprenorphine in inhibiting [^{3}H]diprenorphine and stimulating guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding to rat μ-opioid receptor stably expressed in CHO cells. In vivo, thienorphine exhibited more potent antimorphine effect compared with buprenorphine. Moreover, compared with buprenorphine, thienorphine showed a similar long-lasting antinociceptive effect but a much longer antagonism of morphine-induced lethality [12]. In contrast to common opioid agonists, it was observed that thienorphine induced hypoactivity in mice and that this effect declined after repeated administration. In addition, coadministration of thienorphine dose-dependently suppressed the development, transfer, and expression of behavioral sensitization to morphine in mice [13]. These results indicate that thienorphine is a potent, long-acting partial opioid agonist and may have possible application in treating addiction.

The plasma drug concentration in animal was very low according to preliminary experiment result. These HPLC/UV methods are not sensitive enough for detecting thienorphine in the plasma of rats within 2 h after an oral dose of 3 mg/kg thienorphine. In addition, the different polar character between thienorphine and thienorphine glucuronide conjugate makes it difficult to simultaneously extract from plasma. In this study, protein precipitant procedure adopted to condense supernatant was found to simultaneously, facilely extract both thienorphine and its metabolite from rat plasma. A sensitive, and selective liquid chromatography–mass spectrometry (LC–MS) method for the simultaneous determination of two analytes was devel-

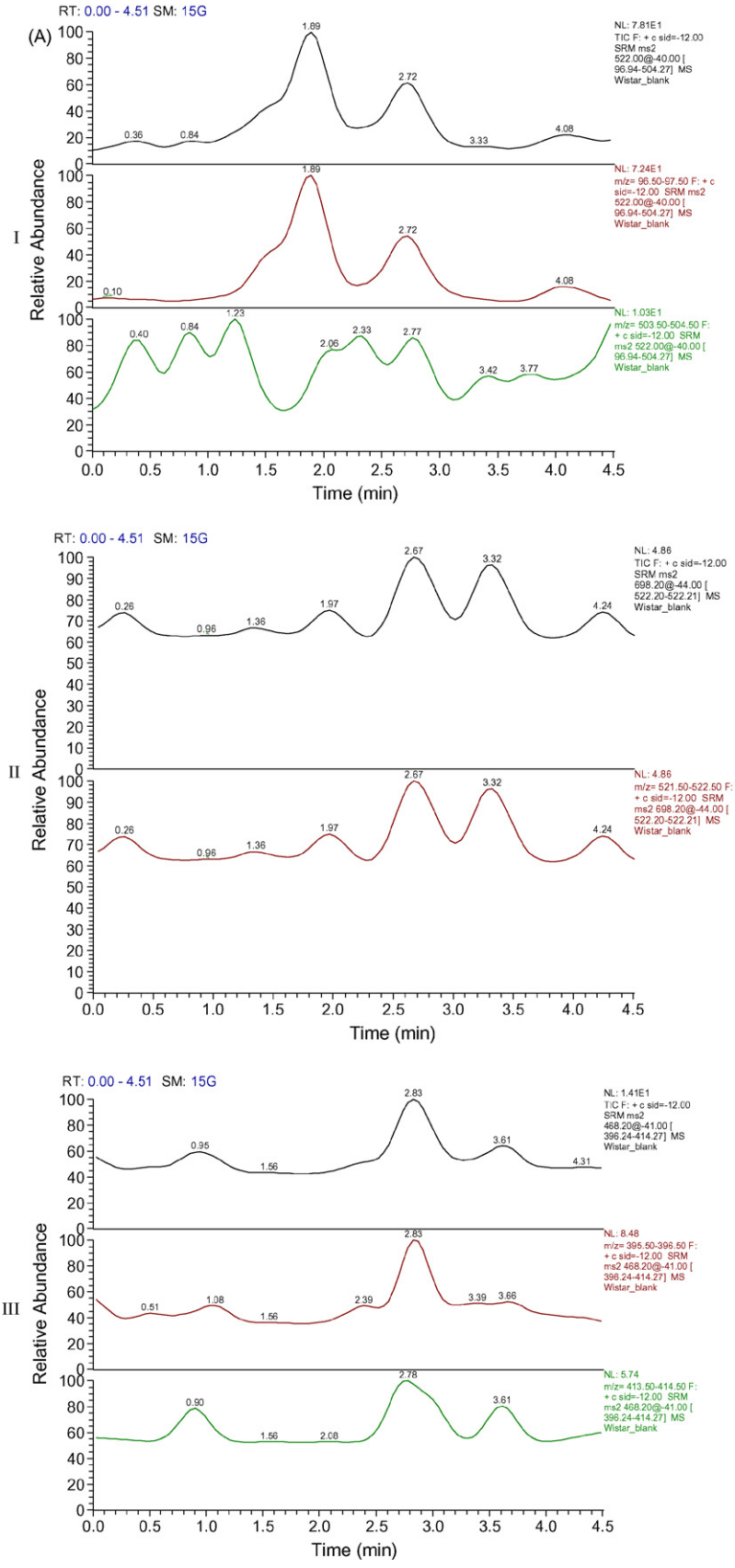

Fig. 2. Representative SRM chromatograms of thienorphine (I, *m/z* 522 → *m/z* 97 and 504), thienorphine glucuronide conjugate (II, *m/z* 698 → *m/z* 522) and I.S. (III, *m/z* 468 → *m/z* 396 and 414) in rat samples. (A) Blank plasma sample; (B) blank plasma sample spiked with thienorphine, thienorphine glucuronide conjugate at the LLOQ and I.S. (40 ng/ml); (C) plasma sample from a rat 3 h after an oral administration.

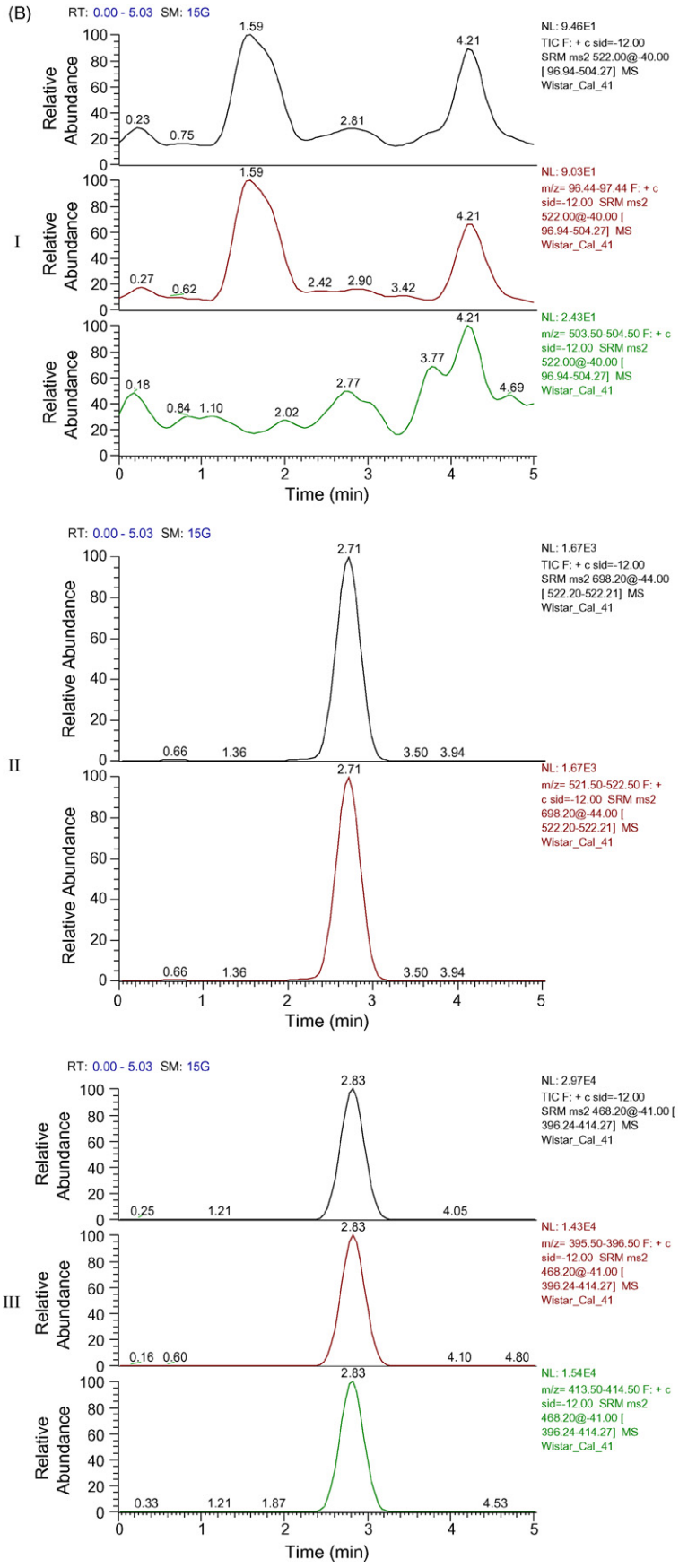

Fig. 2. (*Continued*)

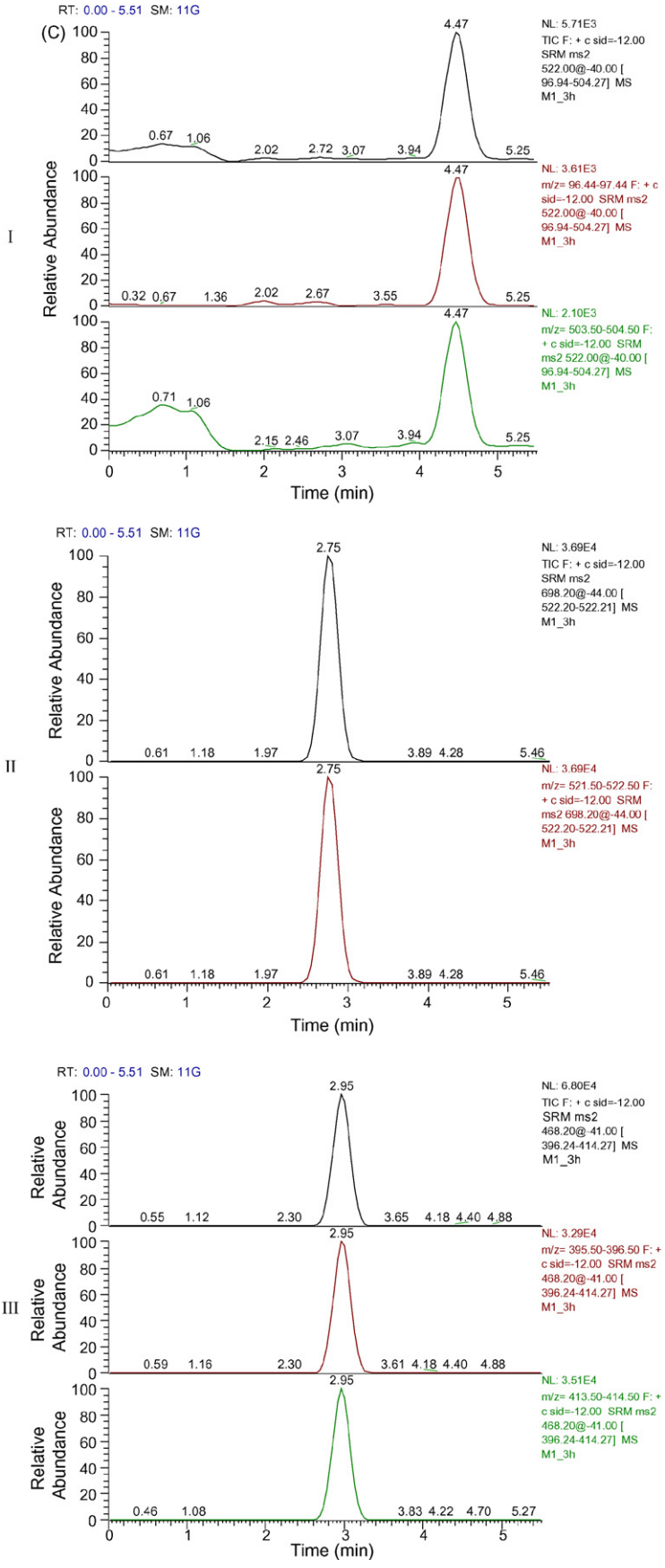

Fig. 2. (*Continued*).

Table 1
Intra-day assay variations of thienorphine and thienorphine glucuronide conjugate in rat plasma

Constituent	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Intra-day	
			RSD (%)	CV (%)
Thienorphine	0.30	0.30 ± 0.01	−0.1	2.9
	5.00	5.18 ± 0.22	3.5	4.2
	25.00	23.88 ± 2.08	−6.3	8.7
Thienorphine glucuronide conjugate	6.00	6.65 ± 0.10	10.8	1.5
	100.00	108.40 ± 1.47	8.4	1.4
	500.00	468.54 ± 21.83	−6.3	4.7

Mean ± SD ($n = 5$).

oped to provide LOQ lower than 0.1 ng/ml for thienorphine in rat plasma. The method with protein precipitant procedure is adapted for the analysis of the small plasma samples taken from rats and has been successfully applied to pharmacokinetic studies of thienorphine and thienorphine glucuronide conjugate in rats following oral administration of thienorphine for the first time.

2. Experimental

2.1. Reagents and materials

Thienorphine (99% purity) and thienorphine glucuronide conjugate (99% purity) were supplied by Beijing Institute of Pharmacology and Toxicology (Beijing, China) [11]. Buprenorphine [internal standard (I.S.)] (>99% purity) was obtained from the Sigma (St. Louis, MO, USA). Chemical structures of three constituents are shown in Fig. 1. Formic acid (analytical grade) was from Beijing Chemical Co. (Beijing, China). Methanol was of HPLC grade (J&K Chemical LTD). Ultrapure water, prepared by a Milli-Q Reagent Water System (Millipore, MA, USA), was used throughout the study.

2.2. Instrumentation

A Thermo Finnigan TSQ Quantum Discovery MAX triple–quadrupole tandem mass spectrometer equipped with electrospray ionization (ESI) source (Thermo Finnigan, USA), a Finnigan surveyor LC pump and an autosampler were used for LC–MS/MS analysis. Data acquisition was performed with Xcalibur 1.4 software (Thermo Finnigan, USA). Peak integration and calibration were carried out using LC Quan 2.0 software (Thermo-Finnigan). CentriVap Concentrator (LABCONCO, USA).

2.3. LC–MS–MS conditions

The chromatographic separation was achieved on a BetaBasic-18 column (150 mm × 2.1 mm i.d., 5 μm, Finnigan, Thermo, USA) using a mobile phase of methanol–water–formic acid (56:44:0.1, v/v/v), which was degassed by supersonic procession before use. The liquid flow rate was set at 0.2 ml/min. The column temperature was maintained at 22 °C.

Mass spectrometer was operated in the positive mode (ESI^+). Quantification was performed using selected reaction monitoring (SRM) of the transitions of m/z 522 → m/z 97 and 504 for thienorphine, m/z 698 → m/z 522 for thienorphine glucuronide conjugate, m/z 468 → m/z 396 and 414 for performed (I.S.), respectively, with a scan time of 0.5 s per transition. Quantification by choosing two daughter ions for analytes was more sensitive and selective than one daughter. In order to optimize all the MS parameters, a standard solution (1 μg/ml) of the analytes and I.S. was infused into the mass spectrometer. For thienorphine, thienorphine glucuronide conjugate and IS, the following optimized parameters were obtained. The spray voltage was set at 4.8 kV. Nitrogen was used as the sheath gas (16 psi) and auxiliary gas (5 psi). The capillary temperature was set to 300 °C. Collision induced dissociation (CID) studies were performed and argon was used as the collision gas with a collision cell gas pressure of 1.5 mTorr. The optimized collision energy of 12 eV was chosen for analytes and I.S.

Table 2
Inter-day assay variations of thienorphine and thienorphine glucuronide conjugate in rat plasma

Constituent	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Inter-day	
			RSD (%)	CV (%)
Thienorphine	0.300	0.28 ± 0.03	−6.0	11.2
	5.00	5.04 ± 0.14	0.8	2.7
	25.00	22.98 ± 0.66	−8.1	2.9
Thienorphine glucuronide conjugate	6.00	6.37 ± 0.33	6.1	5.3
	100.00	101.60 ± 3.47	1.6	3.4
	500.00	488.12 ± 47.18	−2.4	9.7

Mean ± SD ($n = 5$).

2.4. Standard and working solutions

Individual standard stock solutions of thienorphine (100 μg/ml) and thienorphine glucuronide conjugate (100 μg/ml) were prepared by accurately weighing 5 mg of each compound in volumetric flasks and volume was made up to 50 ml with methanol. Mixed working stock solution (MWS) of thienorphine (500 ng/ml) and thienorphine glucuronide conjugate (10 μg/ml) was prepared in methanol. MWS was used in the preparation of analytical and calibration standards. Analytical standards were prepared from MWS by diluting it with appropriate volumes of methanol to obtain a concentration range of 0.1–50 ng/ml for thienorphine and 2–1000 ng/ml for thienorphine glucuronide conjugate. A working internal standard solution was prepared by diluting the 1000 μg/ml stock solution of buprenorphine in methanol with methanol to provide a final concentration of 40 ng/ml. All stock and mixed working stock solutions were prepared prior to start of validation, stored at 4 °C, and brought to room temperature before use. These solutions were found to be stable over 6 months and used for the complete method validation programme.

2.5. Calibration and quality control samples

A series of standard working solutions were evaporated to dryness at 37 °C with CentriVap Concentrator. The residue was reconstituted in 0.1 ml of blank plasma to prepare the calibration standards. Effective concentrations in plasma samples were 0.1/2, 0.3/6, 1/20, 5/100, 10/200, 25/500, 50/1000 ng/ml for thienorphine/thienorphine glucuronide conjugate. Quality control (QC) samples, which were used both in pre-study validation and during the pharmacokinetics study, were prepared separately to give concentrations of 0.3/6, 5/100, 25/500 ng/ml of thienorphine/thienorphine glucuronide conjugate. The spiked plasma samples (standards and quality controls) were then treated following the protein precipitation procedure on each analytical batch along with the unknown samples.

2.6. Animals and blood sampling

Wistar rats (adult male), weighing 200 ± 20 g, were obtained from Academy of Military Medical Sciences Animals Center (Beijing, China). These animals were quarantined for 1 week prior to begin the study. The rats were housed in a windowless room, which was illuminated for 12 h each day at (22 ± 1) °C. All animals were weighed daily and observed twice daily, in order to assess their general health. Diet was prohibited for 12 h before the experiment while water was taken freely. The 3 mg/kg thienorphine dose of oral administration to rats was selected on the basis of pharmacodynamic action of thienorphine to rats. After oral administration of thienorphine, serial blood samples were collected at intervals of 0, 5, 20, 40 min and 1, 2, 3, 4, 5, 7, 10, 14, 24 h from the same rat ($n = 5$). The blood samples were collected about 0.25 ml in heparinized 1.5 ml polythene tubes by orbital bleeding, via heparinized capillary tubes, and centrifuged at 2000 × g for 15 min. Plasma sample was subsequently collected and stored at −20 °C until analysis.

2.7. Sample preparation

0.1 ml I.S. solution (40 ng/ml) was added to 100 μl plasma sample in a 1.5 ml test tube. The sample mixture was deproteinized with 0.8 ml of methanol and vortex-mixed for approximate 1 min, and the precipitate was removed by centrifugation at 12,000 rpm (revolutions per minute) for 10 min. Then 800 μl of supernatant was transferred to another clean test tube and evaporated to dryness at 37 °C with CentriVap Concentrator. The dry residue was reconstituted in 100 μl of the mobile phase, vortex-mixed, and centrifuged at 12,000 rpm for another 10 min. Twenty microlitres of the clean supernatant was injected onto the LC/MS/MS for analysis.

2.8. Validation of the method

2.8.1. Sensitivity and specificity

The limit of detection for thienorphine was approximately 0.03 ng/ml at a signal to noise ratio of 3:1 and limit of quantification corresponding with a coefficient of variation of less than 20% was 0.1 ng/ml at a signal to noise ratio of 10:1. The specificity was defined as non-interference in the regions of interest with the endogenous substances, in the determination of the concentration. Five different lots of blank plasma were tested for interference or matrix effects.

2.8.2. Linearity

Linearity of calibration was tested by extraction and assayed ($n = 5$). Calibration curves in the concentration range of 0.1–50 ng/ml for thienorphine and of 2–1000 ng/ml for thienorphine glucuronide conjugate were constructed by plotting the peak-area ratios of analyte/internal standard versus thienorphine or thienorphine glucuronide conjugate concentration in rat plasma. The data were best fit with a weight of $1/x^2$. Weighted ($1/x^2$) least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The concentration of thienorphine or thienorphine glucuronide conjugate in plasma was determined from the peak-area ratios by using the equations of linear regression obtained from the calibration curves.

2.8.3. Precision and accuracy

Intra-day and inter-day accuracy and precision for the assay were evaluated by analyzing rat plasma samples containing thienorphine/thienorphine glucuronide conjugate at 0.3/6, 5/100, 25/500 ng/ml, respectively. Intra-day accuracy and precision (each, $n = 5$) were evaluated by analysis of samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of samples over five consecutive days ($n = 1$ series per day). The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy of the method was determined by RSD (%), which was calculated by the equation: (mean of

determined concentration − actual concentration/actual concentration) × 100%. Precision was determined by the coefficients of variation (CV).

2.8.4. Absolute recoveries

Absolute recoveries of thienorphine and thienorphine glucuronide conjugate at three QC levels were determined by comparing the peak areas of the precipitated samples with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

2.8.5. Stability

The stability of the analytes in rat plasma under different temperature and timing conditions was evaluated at three QC levels ($n = 3$). The thaw–freeze stability was determined after three thaw–freeze cycles (−20 to 20 °C) on consecutive days. The long-term stability was assessed after storage of the test samples at −20 °C for 10 days, and short-term for 4 h at room temperature in plasma. The results were compared with the freshly prepared QC samples, and the percentage concentration deviation was calculated.

2.9. Assay application

The present method was used to determine concentration–time profiles of thienorphine and thienorphine glucuronide conjugate in rat plasma after oral administration of thienorphine. Pharmacokinetic parameters were calculated using the 3P97 pharmacokinetic software package (version 2.0 PK software; Chinese Pharmacological Association, Anhui, China). The compartment model could be a sum of exponential of the following: $C = \sum C_i \exp(\lambda_i t)$, where C is the predicted concentration, t is time, $C_i = (A, B, \ldots)$ and λ_i $(= \alpha, \beta, \ldots)$ are the pre-exponential and exponential coefficients, was fitted to blood concentration data using nonlinear regression analysis. The area under concentration versus time curve (AUCs) was calculated using the trapezoidal rule up to the last detectable concentration and extrapolated to infinite time using the terminal elimination; analogous method was used for the calculation of the area under the first moment curve (AUMC), but using the concentration versus time data. The elimination half-life ($t_{1/2\beta}$) was $0.693/k_{el}$, where k_{el}, the elimination rate constant, was calculated by linear regression from the terminal linear portion of plasma concentration–time curve. Mean residence time (MRT) was calculated as AUMC/AUC. Volume of distribution (Vc) of the central compartment was calculated as dose/C_0, where C_0 is the concentration measured just after the administration. Clearance (Cl) was calculated as dose/AUC.

3. Results and discussion

3.1. MS conditions selection

Analytical parameters of each of the analytes and I.S., such as sheath gas, the auxiliary gas, collision induced dissociation and the collision energy, were investigated to optimize MS conditions and increase the detection sensitivity of thienorphine, thienorphine glucuronide conjugate and I.S. Fig. 1 shows the product ion spectra of $[M+H]^+$ of thienorphine, thienorphine glucuronide conjugate and IS. It is clear that the analytes and I.S. formed predominantly protonated molecules $[M+H]^+$ in Q1 full scan spectra, and protonated molecules $[M+H]^+$ were detected at *m/z* 522 for thienorphine, *m/z* 698 for thienorphine glucuronide conjugate and 468 for I.S. No sodium or other solvent adducts or dimmers were observed. These fragment ions at *m/z* 97 and 504, *m/z* 522, *m/z* 414 and 396 were chosen in the SRM acquisition for thienorphine, thienorphine glucuronide conjugate and I.S., respectively.

3.2. Chromatographic conditions optimization

It is critical to optimize chromatographic conditions to obtain good selectivity, high sensitivity, quick speed and symmetrical peak shape. In positive ion mode, the presence of a low amount of formic acid in the mobile phase can improve the detection response of the analytes. With compositions of mobile phase methanol–water–formic acid (56:44:0.1, v/v/v), the symmetry of the peak was good while the analytes and internal standard were free of interference from endogenous substances. The overall chromatographic run time was finished within 5.5 min.

3.3. Preparation of plasma samples

Sample preparation is a critical step for accurate and reliable LC–MS/MS assays. The most widely employed biological sample preparation methodologies currently are liquid–liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation (PPT). The polar character of thienorphine glucuronide conjugate makes it difficult to extract from plasma by conventional techniques such as LLE, which moreover was complex and time-consuming. In this study, the selected protein precipitant was methanol because of satisfactory efficiency in precipitating. A one-step protein precipitation procedure was adopted to simplify the sample preparation and the treatment of condensing supernatant could provide LOQ lower than 0.1 ng/ml for thienorphine in 0.1 ml plasma. Moreover, the protein precipitant procedure was found to simultaneously, facilely extract both thienorphine and its metabolite from rat plasma.

3.4. Method validation

3.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of five different batches of blank human plasma with the spiked samples at LOQ levels. Endogenous peaks at the retention time of the analytes were not observed for any of the plasma batches evaluated. This indicated no significant direct interference in the SRM channel for the analytes at the expected retention time. Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with thienorphine, thienorphine glucuronide conjugate at the LOQ and I.S., and a plasma sample from a rat 3 h after an oral administration. The retention times of thienorphine, thienorphine glucuronide conjugate and I.S. were approximately 4.4,

Table 3
Summary of stability studies of thienorphine and thienorphine glucuronide conjugate in rat plasma under various storage conditions ($n = 3$)

	Thienorphine concentration (ng/ml)			Thienorphine glucuronide conjugate concentration (ng/ml)		
	0.30	5.0	25.0	6.0	100	500
Freshly prepared						
Measured concentration (ng/ml)	0.30 ± 0.03	5.23 ± 0.29	23.42 ± 0.34	6.20 ± 0.22	98.76 ± 7.18	458.83 ± 5.23
Three freeze–thaw cycles						
Measured concentration (ng/ml)	0.31 ± 0.04	5.32 ± 0.15	25.49 ± 0.67	6.08 ± 0.54	99.26 ± 1.72	450.51 ± 3.39
Concentration deviation (%)	3.3	1.7	8.8	−1.9	0.5	−1.8
Room temperature (4 h)						
Measured concentration (ng/ml)	0.32 ± 0.03	4.97 ± 0.20	24.48 ± 1.26	5.83 ± 0.26	107.10 ± 0.16	446.24 ± 2.84
Concentration deviation (%)	6.7	−5.0	4.5	−6.0	8.4	−2.7
Stored at −20 °C for 10 days						
Measured concentration (ng/ml)	0.30 ± 0.03	4.88 ± 0.46	24.81 ± 1.26	5.91 ± 0.27	105.72 ± 6.72	457.99 ± 6.46
Concentration deviation (%)	0.0	−6.7	5.9	−4.7	7.0	−0.2

Concentration deviation (%): (measured concentration − freshly prepared concentration)/freshly prepared concentration × 100%.

2.7 and 2.9 min, respectively, and most of the endogenous substances were eluted within 2 min.

3.4.2. Linearity of calibration curves and lower limits of quantification

The linear regression analysis of thienorphine or thienorphine glucuronide conjugate was constructed by plotting the peak-area ratio of thienorphine or thienorphine glucuronide conjugate to the internal standard (y) versus analyte concentration (ng/ml) in spiked plasma samples (x). The calibration curves were constructed in the range 0.1–50 ng/ml for thienorphine and 2–1000 ng/ml for thienorphine glucuronide conjugate. The calibration model for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a $1/x^2$ weighting factor for both the analytes. The regression equation of these curves and their correlation coefficients (r^2) were calculated as follows: thienorphine, $y = 0.028485x - 0.000638$ ($r^2 = 0.9943$); thienorphine glucuronide conjugate, $y = 0.0235603x + 0.008201$ ($r^2 = 0.9968$); it showed good linear relationships between the peak areas and the concentrations. The LOQ was 0.1 ng/ml for thienorphine and 2 ng/ml for thienorphine glucuronide conjugate, and an acceptable accuracy of ±15% and a precision below 15% were obtained ($n = 5$). Under present LOQ, the thienorphine and thienorphine glucuronide conjugate concentration can be determined in plasma samples until 24 h after a single oral dose of 3 mg/kg thienorphine, which is sensitive enough to investigate the pharmacokinetic behaviors of thienorphine and thienorphine glucuronide conjugate in rats.

3.4.3. Precision and accuracy

Intra- and inter-precision was assessed from the results of QC samples. The intra- and inter-batch precision and accuracy data for thienorphine and thienorphine glucuronide conjugate are summarized in Tables 1 and 2. The reproducibility of the method was defined by examining both intra- and inter-day variance. The intra-day and inter-day CV data of thienorphine and thienorphine glucuronide conjugate assays at low to high concentrations were less than 12% and 10%, respectively. Assay accuracy, assessed by RSD, was found to range from −8.1% to 10.8%. The results above demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.4.4. Recovery and stability

The recoveries (extraction efficiency) of thienorphine from rat plasma following methanol precipitation were 115.9 ± 12.0%, 101.5 ± 9.5% and 81.5 ± 7.1% at concentrations of 0.3, 5.0 and 25 ng/ml, respectively. The recoveries of thienorphine glucuronide conjugate were 101.2 ± 13.1%, 100.1 ± 7.2% and 83.8 ± 6.7% at concentrations of 6, 100 and 500 ng/ml, respectively. The mean recoveries of thienorphine and thienorphine glucuronide conjugate were more than 80% ($n = 5$). There was no significant degradation under the conditions described in this study. All stability results are shown in Table 3. Thienorphine and thienorphine glucuronide conjugate were stable for 4 h at room temperature in plasma samples; the mean percentage concentration deviations were 6.7%, −5.0% and 4.5%, respectively, and −6.0%, 8.4% and −2.7%, respectively, at three concentration levels of QC. Thienorphine and thienorphine glucuronide conjugate were also stable in plasma samples when stored at −20 °C for 10 days, and following three freeze–thaw cycles. Their mean percentage concentration deviations were found to range from −6.7% to 8.8%. In the preliminary test, extraction of spiked samples with only thienorphine glucuronide conjugate results in no signal in the thienorphine channel under these conditions. These results indicated that analytes were stable under routine laboratory conditions. These results indicated that the assay was simple, accurate, sensitive and reproducible.

3.5. Application to pharmacokinetic study

The pharmacokinetic profiles of thienorphine and thienorphine glucuronide conjugate were investigated by the described method following a single oral administration of 3 mg/kg thienorphine to rats. The mean plasma concentration–time

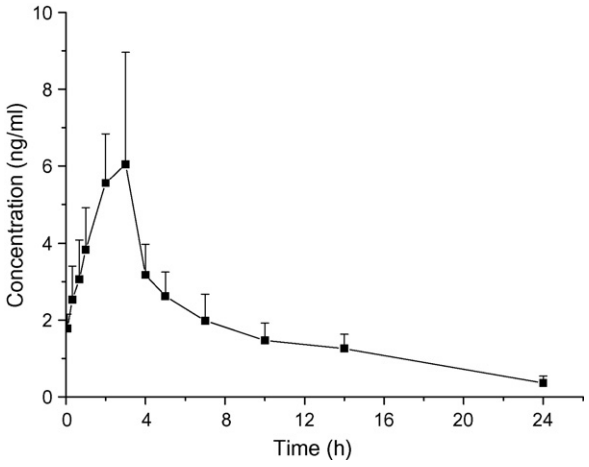

Fig. 3. Mean plasma concentration–time curve of thienorphine after a single oral dose of thienorphine in rats. Each point represents the mean ± SD of five experiments.

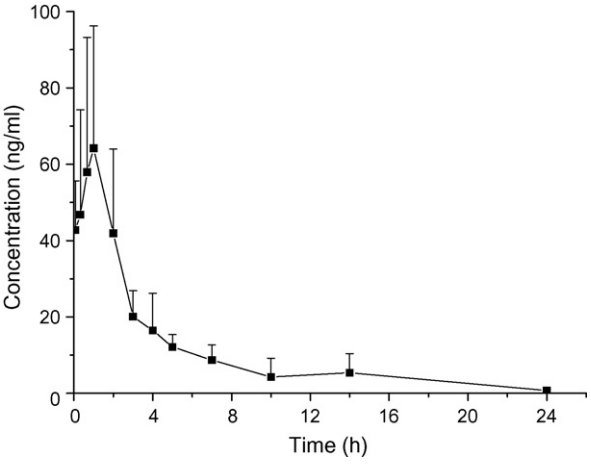

Fig. 4. Mean plasma concentration–time curve of thienorphine glucuronide conjugate after a single oral dose of thienorphine in rats. Each point represents the mean ± SD of five experiments.

Table 4
Pharmacokinetic parameters of thienorphine and its metabolites after single oral dose of thienorphine to rats

Parameter	Thienorphine	Thienorphine glucuronide conjugate
T_{max} (h)	2.60 ± 0.55	0.95 ± 0.70
C_{max} (ng/ml)	7.37 ± 1.62	69.83 ± 24.84
$t_{1/2}$ (h)	7.74 ± 2.32	6.97 ± 4.17
MRT_{0-t} (h)	7.18 ± 0.67	4.77 ± 1.39
AUC_{0-t} (ng h/ml)	43.99 ± 8.28	257.26 ± 92.91

Mean ± SD (n = 5).

profiles of thienorphine and thienorphine glucuronide conjugate are shown in Figs. 3 and 4, respectively. The mean estimated pharmacokinetic parameters are listed in Table 4. After oral administration of thienorphine, the mean values of C_{max} for thienorphine were found to be 7.37 ng/ml (range 5.75–8.99 ng/ml), 69.83 ng/ml (range 44.99–94.67 ng/ml) for thienorphine glucuronide conjugate, respectively. Moreover, the AUC_{0-t} value for metabolite thienorphine glucuronide conjugate (257.26 ng h/ml) was about 5.8 times as large as that for thienorphine (43.99 ng h/ml). It was suggested that most of thienorphine was transformed into thienorphine glucuronide conjugate in rats, and its active metabolite thienorphine glucuronide conjugate seems to play an important role in the pharmacological action of orally administered thienorphine. The mean residence time (MRT) of thienorphine glucuronide conjugate was shorter than those of thienorphine, indicating that elimination of thienorphine glucuronide conjugate was faster than parent drug in rats.

4. Conclusion

An LC–MS–MS method for simultaneous determination of thienorphine and thienorphine glucuronide conjugate was developed and validated in rat plasma. It was proved that the method was rapid, sensitive and selective and stable. The analytical procedure was applied as a routine analysis of large batches of biological samples to support a pharmacokinetic study. Following partial validation, the present method can be adopted for and extrapolated to dog plasma also.

Acknowledgements

The work is now supported by National Natural Science Foundation of China (30472059).

Contract/grant sponsor: National Natural Science Foundation of China, contract/grant number: 30472059.

References

[1] R.E. Johnson, E.C. Strain, L. Amass, Drug Alcohol Depend. 70 (2003) S59.
[2] A. Cowan, J.W. Lewis, I.R. McFarlane, Br. J. Pharmacol. 60 (1977) 537.
[3] J. Dum, A. Herz, Br. J. Pharmacol. 74 (1981) 627.
[4] J. Kamei, M. Sodeyama, M. Tsuda, T. Suzuki, H. Nagase, Life Sci. 60 (1997) PL333.
[5] P.J. Hoskin, G.W. Hanks, Drugs 30 (1991) 326.
[6] T.M. Tzschentke, Psychopharmacology 161 (2002) 1.
[7] A. Tracqui, P. Kintz, B. Ludes, J. Anal. Toxicol. 22 (1998) 430.
[8] G.M. Robinson, P.D. Dukes, B.J. Robinson, R.R. Cooke, G.N. Mahoney, N. Z. Drug Alcohol Depend. 33 (1993) 81.
[9] O.V. Lopatko, J.M. White, A. Huber, W. Ling, Drug Alcohol Depend. 69 (2003) 317.
[10] R.C. Heel, R.N. Brogden, T.M. Speight, G.S. Avery, Drugs 17 (1979) 81.
[11] C.H. Liu, H. Liu, X.Y. Han, B. Wu, B.H. Zhong, Z.H. Gong, Synth. Commun. 35 (2005) 701.
[12] Gang Yu, Yong-Juan Yue, Meng-Xun Cui, Ze-Hui Gong, JPET 318 (2006) 282.
[13] W.L. Zhao, Z.H. Gong, J.H. Liang, Acta Pharmacol. Sin. 25 (2004) 1413.