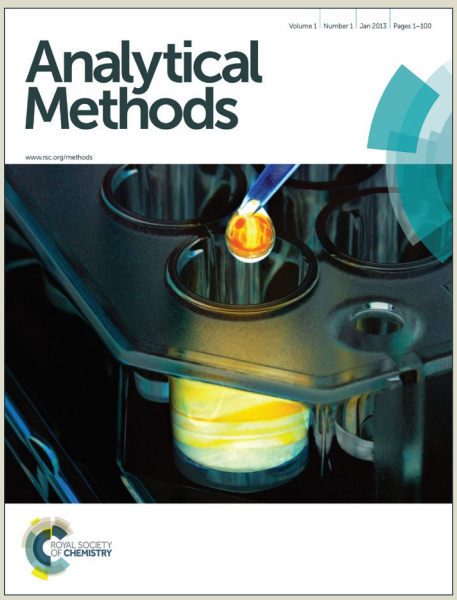
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Application of a sensitive liquid chromatographic-mass spectrometric method to a pharmacokinetic study of nerolidol in rat plasma

Yi-Sheng He, Wei Sun, Bi-Ying Zhang, Ling-Hui Xu, Jie Yang,
Wen Gao, Lian-Wen Qi, Ping Li, Xiao-Dong Wen*

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China

* Corresponding author. Tel.: +86 25 8618 5045; fax: +86 25 8618 5045.
E-mail addresses: xiaodongwen@cpu.edu.cn or cpuwxd@126.com (X.-D. Wen).

Abstract

Nerolidol is a dominant volatile compound isolated from *Oplopanax horridus*. Liquid chromatography-electrospray ionization-mass spectrometry (LC-MS) method was used to evaluate the pharmacokinetics determination of nerolidol in rat. A series of extracting and concentrating methods were optimized for the extraction efficiency of biosamples. Chromatographic separation was performed on a reverse LC column within a total run time of 14.5 min. This method was linear over 10-10,000 ng/mL and the limit of quantification for nerolidol was 10 ng/mL, with both inter-day and intra-day (CV) precision < 8% and accuracy (RE) ranged from -6% to 6%. Stabilizing determination of the plasma concentration of nerolidol was supported over a period of 27 h.

Keywords: nerolidol, pharmacokinetics, LC-MS, extraction optimization

Abbreviations used: IS, internal standard; IP, intraperitoneal; AUC, area under curve; C_{max}, area maximum concentration; T_{max}, time to maximum concentration; $T_{1/2}$, elimination half-life; MRT, mean residence time; Cl/F, clearance.

Introduction

Oplopanax horridus, commonly known as Devil's club, has been used in folk medicine by the native tribes of Alaska and Northwest China for centuries[1]. Traditionally, the inner bark of roots and stems were applied to medical usage[2]. Nerolidol, a natural aliphatic sesquiterpene alcohol, is the major sesquiterpene constituent extracted from stems and roots of *Oplopanax horridus*[3]. It also could be extracted from various plants and used in many fragrance compounds as a fragrance ingredient and flavor[4]. Moreover, nerolidol has exhibited plenty of medicinal benefits such as antioxidant activity, antibacterial properties, antitumor effects, antiulcer functions, and inhibiting the growth of parasite[5-9]. However, apart from these pharmacological studies, there is only one newly published paper reported an in-vivo analysis of nerolidol in mice by GC/MS method[14]. Meanwhile, the stability of samples and the extraction method did not investigated in this published paper[14]. As LC/MS is also a sensitive and widely used method，in this study, a valid and efficient LC/MS method was developed for determination of nerolidol in rat plasma. After that, this method was successfully applied to investigate the *in vivo* pharmacokinetics of nerolidol in rats.

Experimental

Chemicals, material and reagents

Nerolidol (*cis/trans* : 40/60) and Tween 80 were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). Diazepam was obtained from National Institute for the Control of Pharmaceutical & Biological Products (NICPBP, Beijing, China). Methanol and formic acid (HPLC grade) were obtained from ROE Scientific Inc. (Newcastle, PA, USA). Purified water prepared by the Millipore system (Millipore, Bedford, MA, USA) was employed for all the preparations. Other reagents were of analytical grade and purchased from Jiangsu Hanbon Sci. & Tech. Co. Ltd (Nanjing, Jiangsu, China).

Equipment and LC/MS condition

An Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a dual pump and a Rheodyne7125i injection valve with a 20 μL sample loop was employed to performance the chromatographic analysis. Data were acquired and processed by a HP ChemStation. Chromatographic separation was performed with a Thermo Hypersil BDS C18 column (4.6 mm×255 mm i.d., 5 μm) with an Agilent ZORBAX ODS C18 as the guard column (4.6 mm×12.5 mm i.d., 5μm). Throughout the procedure, columns were maintained at room temperature. The mobile phase consisted of (A) 0.1% aqueous formic acid and (B) methanol. The isocratic elution condition was 80% B (A : B, *v* / *v*) kept for 14.5 min. The injection volume was 5 μL and the flow rate was 1 mL/min.

An Agilent MSD SL-G2710BA with an electrospray ionization source (Agilent Corp, Santa Clara, CA, USA) was employed to perform detection by monitoring the ions. The analysis parameters were as follows: fragmentor voltage, 120 V; drying gas (N_2) flow rate, 10 L/min; gas temperature, 320 °C; nebulizer gas (N_2) pressure, 40 psig; capillary voltage, 3500 V. Analysis for diazepam and nerolidol was done in a positive ion mode with single ion monitoring (SIM) m/z values restricted to specific retention time windows 0-7.5 min, m/z 285.00 $[M + H]^+$; 7.5-14.5 min, m/z 205.00 $[M - H2O + H]^+$.

Preparation of standard solutions and quality control samples

The stock solution of nerolidol was prepared in mobile phase solution at the concentration of 100 μg/mL, while the stock solution of internal standard (diazepam) was prepared in methanol at the concentration of 1 μg/mL. All solutions were kept in volumetric flasks at 4 ºC before subsequent analysis.

Quality control (QC) samples were prepared in blank rat plasma by spiking appropriate aliquots from the stock solutions to obtain final concentrations at 20 ng/mL (low), 2,000 ng/mL (medium) and 10,000 ng/mL (high), respectively. All the spiked plasma samples were then processed according to optimized biosample preparation procedure.

Biosample preparation

Each 0.1 mL of plasma spiked with 0.01 mL of internal standard (IS: diazepam) solution was combined with 1.0 mL of precooled ethyl acetate/n-butyl alcohol (*v: v* = 90: 10) in a centrifuge tube. Then the solution was vortex-mixed for 1 min and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was transferred to another tube. This process was repeated once again and the supernatants were combined and dried by an EZ-2 evaporator with precise temperature control (Genevac Inc., Suffolk, UK) in very low BP mode. The dried residue was reconstituted in 100 μL mobile phase solution and then centrifuged. The supernatant was used for analysis. All the preparation was conducted within ice-bath and all tubes were wrapped with tinfoil in order to keep the samples at relatively low temperature and away from light.

Method validation

Linearity, accuracy, precision and lower limit of quantification

Linearity was evaluated by developing and assaying plasma calibration curves from 10-10,000 ng/mL, respectively. Plasma samples were quantified through the ratio of the sum peak area of *cis*-nerolidol and trans-nerolidol to that of IS. A linear regression function after $1/x^2$ weighting of the nerolidol/IS peak area ratio versus nerolidol concentration relationship was established. Intra-day and inter-day accuracy and precision were determined by assaying QC sample at three concentration levels. Accuracy was calculated as percent deviation from the nominal concentration (RE) while precision was determined by the coefficient of variation (CV). The lower limit of quantification (LLOQ) was determined as the lowest concentration with a signal-to-noise (S/N) ratio of 10, and both the precision and accuracy less than 20% by analyzing six replicates of analyte.

Recovery and matrix effect

The recoveries of nerolidol at three QC levels (n= 5) were determined by comparing the responses of the analytes from QC samples with the responses of analytes spiked in post-extracted samples at equivalent concentrations. Matrix effects

on the ionization of nerolidol were evaluated by comparing the responses of analytes added into pre-extracted plasma from untreated rats with those of analytes dissolved in the same volume of initial mobile phase.

Stability

QC samples at three concentrations stored at -80 °C were analyzed to evaluate the stability of analytes. Two freeze-thaw cycles of the QC samples were performed at 4 and 27 h (n = 5). Stock solution and post-preparative stability were tested at 24 h (n = 5).

Animal experiment

Normal male Sprague-Dawley rats weighing 250-300 g were obtained from BiKai Co., Ltd. (Shanghai, China) and kept at 25 ± 2 °C and 65 ± 5% relative humidity on a 12 h light-12 h dark cycle. All animals had free access to food and water until 12 h prior to the experiment. All animal experimental procedures were in accordance with protocols that approved by the Review Committee of Animal care and Use of China Pharmaceutical University.

To obtain stabilized drug solution, nerolidol was mixed with a pre-heated medium containing Tween 80, ethanol (nerolidol: Tween: ethanol = 1 : 2 : 2). After vortex-mixed for 0.5 min, the medium containing nerolidol was dissolved in the physiological saline solution to obtain the final solvent containing 90% saline, 0.5% Tween 80 and 0.5% ethanol. After two weeks' accommodation, the rats were fasted for 12 h before the experiment. Nerolidol (25 mg/kg; *cis/trans*: 40/60) was administered by intraperitoneal (IP) injection. Under light ether anaesthesia, blood samples were collected from retinal venous plexus into heparinized tubes at time points 0, 5, 10, 20, 30, 60, 90, 120, 240 and 360 min after dosing. The samples were immediately centrifuged at 13,000 rpm for 10 min at 4 °C. The plasma was finally obtained and stored at -80 °C until analysis.

Plasma concentration-time data were analyzed by Drug and Statistics 2.0 (DAS 2.0) software package (Mathematical Pharmacology Professional Committee of China,

Shanghai, China) to obtain pharmacokinetic parameters.

Results and Discussion

Quantitation target

Considering that little evidence could support the single isomer's bioactivity or toxicity because they were often administrated as a whole (*cis/trans* = 40/60) [10]. Therefore, we temporarily established a valid method to determine the pharmacokinetics of total *cis-trans* isomers of nerolidol due to the undisputable bioactivity of the total nerolidol isomers according to a series of preclinical and clinical study[11, 12].

Optimization of biosample preparation

During the development of preparation method, different extraction approaches including protein precipitation, SPE and liquid-liquid extraction were investigated. Though protein precipitation is relatively simple, the recoveries for nerolidol were low either using methanol or acetonitrile as protein precipitation agents. SPE is a common technique for sample preparation as it often offers higher sensitivities. However, the recoveries for nerolidol were not satisfactory. Alternatively, liquid-liquid extraction was used. Several extraction solvent such as ethyl acetate, ethyl acetate/n-butyl alcohol (*v: v* = 95 : 5), ethyl acetate/n-butyl alcohol (*v: v* = 90 : 10), ethyl acetate/n-hexane (*v: v* = 95 : 5), ethyl acetate/n-hexane (*v: v* = 90 : 10) were tested, after which ethyl acetate/ n-butyl alcohol (*v: v* = 90 : 10) was selected as extraction solvent because of its high extract efficiency. Considering the instability and volatility of nerolidol, two comparative concentrating approaches including pressured nitrogen blowing concentration and EZ-2 evaporator under controlled low temperature were tested to optimize the concentration process. As shown in Table 1, the EZ-2 evaporator exhibited better recovery efficiencies. Thus, ethyl acetate/ n-butyl alcohol (*v: v* = 90 : 10) extraction system and evaporator station were the final optimized method for biosample extraction.

Method validation

The approximate retention times for internal standard (IS), *cis*-nerolidol and *trans*-nerolidol (Figure1) were 5.1 min, 11.9 min and 13.1 min (Figure1), respectively. No interfering peak from endogenous substances was observed at the times of the analyte and IS, suggesting a good selectivity of the developed method.

The linear regression of nerolidol in the plasma of rats demonstrated good linear relationships over the range from 10 to 10,000 ng/mL. The regression equation of the calibration curve for nerolidol in the plasma was: y = 0.9081x+0.0266 (r^2 = 0.9985).

As shown in Table 2, both inter-day and intra-day precision of all the QC samples were within the acceptable range (< 8%) and accuracy (% bias) ranged from -6% to 6%. The LLOQ was 10 ng/mL, suggesting this method is sensitive.

As shown in Table 3, the extraction recoveries at three concentration levels of the analyte were all within the acceptable limit (~ 90%). Moderate matrix effects (< 10%) as shown in Table 2, indicated no significant interference from endogenous compounds occurred. The stability of the nerolidol could be affected by many enzymes in the biological matrix and the ambient temperature. As presented in Table 4, QC samples were found to be stable in the plasma through two freeze-thaw cycles. Relative errors (RE) for nerolidol were lower than 11% after 4 h and 16% after 27 h freeze-thaw cycles, indicating approved stability for subsequent study conditions. For stability of stock solution and post-preparative solution, RE were both lower than 4% after 24 h.

Pharmacokinetics analysis

The highest nerolidol level was observed in the plasma at 20 min after single intraperitoneal injection. The peak plasma concentration (C_{max}) for the nerolidol was 8.30 ± 1.07 (mean ± S.E.) mg/L. The area under the concentration curves (AUC) was 307.81 ± 42.90 mg•min/L. The corresponding pharmacokinetic parameters including the mean residence time (MRT), elimination half-life ($T_{1/2}$) and clearance (Cl/F), were summarized in Table 5. The plasma concentration-time curve after a single

intraperitoneal injection of nerolidol of was shown in Figure 2.

Discussion

Nowadays essential oils are not only used as phytotherapy treatments, but also extensively used in decorative cosmetics, toilet products and detergents. However, little information about *in vivo* pharmacokinetics of essential oils or their constituents are available.

Usually gas chromatography was adopted as the analysis method of essential oil in Oplopanax horridus [13]. Recently, a newly published paper reported an in-vivo analysis of nerolidol in mice by GC/MS method [14]. The report validated the adopted method, and for the first time conducted the quantitation of nerolidol in-vivo. However, this report did not investigate the stability of sample and extraction methods , nor did it resolve the quantitation of single isomer of neroliodol. As essential oil including nerolidol often exhibits instability and was formidable to extract from biosample, LC/MS, with convenience and stability features, could also be taken into account to evaluate the *in vivo* pharmacokinetics of nerolidol. Moreover, there were several researches on the LC analysis of essential oil, and they manifested valid and stable quantitation avenues towards those instable compounds[15].

In this study, some distinct yet a series of frequently-used extraction and concentration method were tested. Based on the extraction efficiency, we obtain the best extraction condition which could benefit the subsequent LC/MS analysis. Furthermore, compared with the previous GC-MS method, the matrix effect is greatly improved in our studies. In addition, samples were found to be stable in the plasma through two freeze-thaw cycles. Thus we could determine the pharmacokinetics parameters on the basis of relatively high and stable extraction efficiency of nerolidol. Further, this combinatorial optimized method could be developed as a general reference for the in-vivo volatile compound analysis.

However, there is an inevitable question that the compound has *cis*-trans

isomerism (*cis/trans* = 40/60). In this study, we have merely determined the total pharmacokinetic parameters of the isomers instead of each and every single isomer. The major concern was that there was little evidence to support the single isomer's bioactivity or toxicity because they were often administrated as a whole [11,16]. Therefore, we temporarily established a valid method to determine the pharmacokinetics of total *cis-trans* isomers of nerolidol. There was a report using LC method to separate essential oils in Devil's Club. Compared to this report, we optimized mobile phase and elution speed in order to provide a distinct separation between cis and trans nerolidol. Despite we have not quantitate single isomer of nerolidol, the distinct separation of isomers could reveal the compound profile and provide analysis regime for further analysis if stable standard isomer is available. Further research was needed, if necessary, to confirm the pharmacokinetics of each specific isomer.

Conclusion

In this project, we studied the *in vivo* pharmacokinetics of nerolidol in rats. The extraction and concentration methods were optimized in order to minimize the decomposition effect and avoid other factors that would impact the nerolidol extraction efficiency. An optimized method for extracted nerolidol from biosample and a sensitive, accurate and reproducible LC-MS method were developed and validated for the quantification of nerolidol. Moreover, because of its broad distribution in herbal products, our results could provide some suggestion in regards to the dose safety and regime of nerolidol during further clinical administration or civil employment of this perspective compound.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 81373957, 81102763 and 81373919), the Natural Science Foundation of Jiangsu

Province (no. BK20131306) and Jiangsu “Shuang Chuang” team.

Reference:

1 T. C. Lantz and J. A. Antos, Can J Bot., 2002, 80, 1052.

2 C. Z. Wang, H. H. Aung, S. R. Mehendale, Y. Shoyama and C. S. Yuan, Fitoterapia, 2010, 81, 132.

3 T. Inui, Y. H. Wang, D. Nikolic, D. C. Smith, S. G. Franzblau and G. F. Pauli, J Nat Prod., 2010, 73, 563.

4 R. Vila, M. Mundina, L. Muschietti, H. A. Priestap, A. L. Bandoni, T. Adzet and S. Canigueral, Phytochemistry, 1997, 46, 1127.

5 L. W. Wattenberg, Carcinogenesis, 1991, 12, 151.

6 B. F. Brehm-Stecher and E. A. Johnson, Antimicrob Agents Chemother., 2003, 47, 3357.

7 F. C. Klopell, M. Lemos, J. P. B. Sousa, E. Comunello, E. L. Maistro, J. K. Bastos and S. F. de Andrade, Z Naturforsch C., 2007, 62, 537.

8 J. D. N. Neto, A. A. C. de Almeida, J. D. Oliveira, P. S. dos Santos, D. P. de Sousa and R. M. de Freitas, Neurochem Res., 2013, 38, 1861.

9 D. C. Arruda, F. L. D'Alexandri, A. M. Katzin and S. R. B. Uliana, Antimicrob Agents Chemother., 2005, 49, 1679.

10 B. M. Hausen, Am J Contact Dermat., 2001, 12, 93.

11 A. J. King and J. R. Dickinson, FEMS Yeast Res., 2003, 3, 53.

12 G. Klecak, Curr Probl Dermatol., 1985, 14, 152.

13 F. X. Garneau, G. Collin, H. Gagnon, F. I. Jean, H. Strobl and A. Pichette, Flavour Frag J., 2006, 21, 792.

14 A. Y. Saito, R. A. C. Sussmann, E. A. Kimura, M. B. Cassera, A. M. Katzin, J Pharm Biomed Anal., 2015, 111, 100.

15 L. H. Wang and J. X. Chen, Microchim Acta., 2010, 168, 93.

16 S. Y. Li, Y. Yu and S. P. Li, J Agr Food Chem., 2007, 55, 3358.

Figure Caption

Figure 1. Chemical structures of (1) diazepam, molecular weight 284.74 Da, (2) cis-nerolidol, molecular weight 222.37 Da and (3) trans-nerolidol, molecular weight 222.37 Da.

Representative chromatograms for internal standard diazepam (peak 1), cis-nerolidol (peak 2) and trans-nerolidol (peak 3) in (a) blank plasma without exogenous compounds; (b) standard solution; (c) blank plasma with standard solution; (d) plasma sample after single administration of nerolidol (25mg/kg, ip) for 60 min.

Figure 2. Mean concentration-time profiles for nerolidol in rats' plasma (25mg/kg, ip). All values are represented as means ± SD, with $n = 8$.

Table 1

Comparison of extraction efficiencies of nerolidol in plasma by SPE, liquid-liquid extraction and protein and concentration approaches under the optimal conditions (mean ± S.D., n = 6).

Extraction Method	Theoretical （ng/mL）	Observed （ng/mL）	CV (100%)
Solid Phase Extraction			
C18 packing (water/methanol)	1,000	367 ± 34	9.16
C18 packing (water/acetonitrile)	1,000	505 ± 69	13.69
HLB packing (water/methanol)	1,000	497 ± 43	8.70
HLB packing (water/acetonitrile)	1,000	618 ± 30	4.84
Liquid-Liquid Extraction			
ethyl acetate	1,000	706 ±52	7.39
ethyl acetate/n-butyl alcohol (*v: v* = 95:5)	1,000	741 ± 20	2.73
ethyl acetate/n-butyl alcohol (*v: v* = 90:10)	1,000	809 ± 33	4.14
ethyl acetate/n-hexane (*v: v* = 95:5)	1,000	646 ± 49	7.53
ethyl acetate/n-hexane (*v: v* = 90:10)	1,000	627 ± 34	5.41
Protein Precipitation			
methanol	1,000	315 ± 58	18.30
acetonitrile	1,000	297 ± 63	21.12

Concentration Method	Theoretical （ng/ml）	Observed （ng/mL）	CV (100%)
Nitrogen Concentration	1,000	809 ± 33	4.14
EZ-2 evaporator	1,000	924 ± 19	3.14

Table 2

Method validation for nerolidol in rat plasma (accuracy and precision)

Inter-day	Theoretical (ng/mL)	Observed (ng/mL)	CV(100%)	RE(100%)
	10	9.48 ± 0.7	7.47	-5.20
	20	19.8 ± 1.1	5.18	-1.24
	2,000	2083 ± 109	5.26	4.20
	10,000	9,992 ± 657	4.58	-1.08
Intra-day	Theoretical (ng/mL)	Observed (ng/mL)	CV(100%)	RE(100%)
	10	10.4 ± 0.6	7.05	5.63
	20	20.7 ± 1.2	5.73	3.70
	2,000	2,116 ± 142	6.69	5.84
	10,000	10,182 ± 701	5.89	2.87

Concentrations measured are reported as mean ± SD; n = 5 for both inter-day and intra-day determination.

Table 3

Extraction efficiency and matrix effect for nerolidol in rat plasma

Added（ng/mL）	Extraction Efficiency (% mean, *n* = 5)	Matrix Efficiency (% mean, *n* = 5)
20	88.81 ± 2.75	5.86 ± 0.45
2,000	90.58 ± 1.50	9.19 ± 1.14
10,000	91.61 ± 1.71	8.62 ± 3.35

Concentrations measured are reported as mean ± SD; *n* = 5 for both Extraction efficiency and Matrix efficiency determination.

Table 4

Short-Term Temperature Stability, Long-Term Stability, Stock Solution Stability and Post-Preparative Stability

Added（ng/mL）	RE% (4 h)	RE% (27 h)
20	-2.22	-8.67
2,000	-6.57	-9.18
10,000	-10.92	-15.78
Concentration（ng/mL）	RE% (Stock solution)	RE% (Post-preparative solution)
100,000	-2.10	-3.47

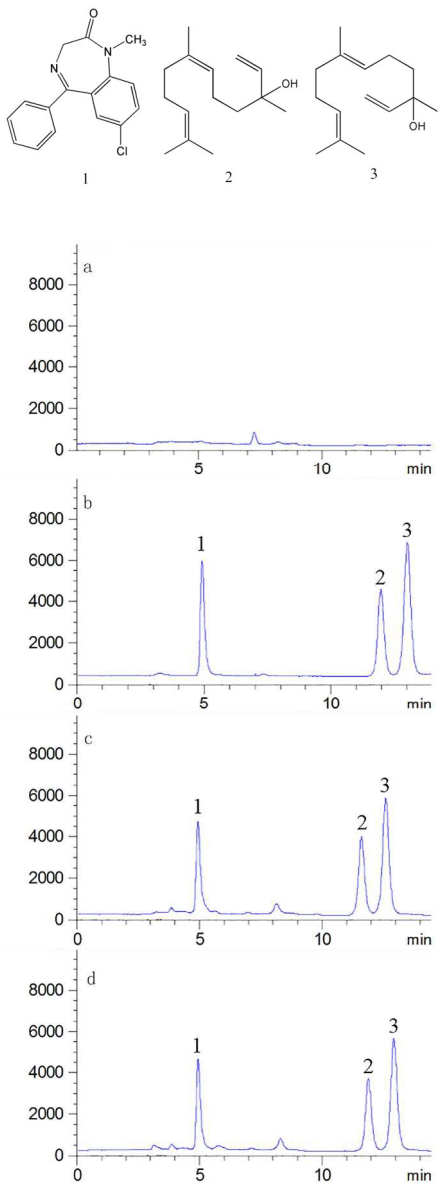
Concentrations measured are reported as mean ± SD; n = 5 for both Extraction efficiency and Matrix efficiency determination.

Table 5

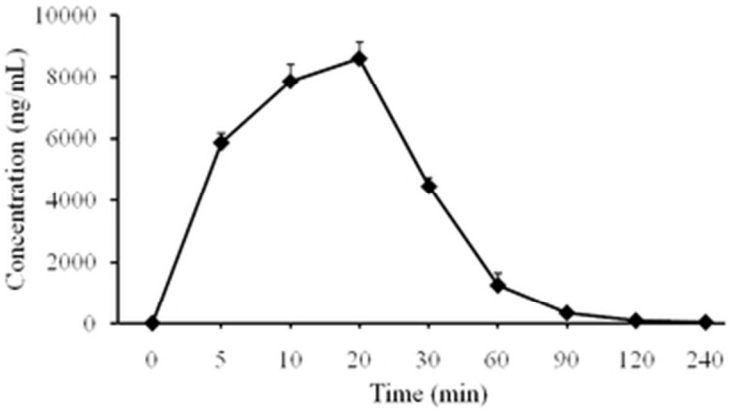
Mean pharmacokinetic parameters of nerolidol in rat serum (n = 5) after administration nerolidol (25 mg/kg, ip).

PK Parameters	Nerolidol
AUC (mg•min/L)	307.81 ± 42.90
MRT (min)	27.72 ± 2.14
$T_{1/2}$ (min)	20.98 ± 7.71
Cl/F (L/min/kg)	0.082 ± 0.012
T_{max} (min)	20
C_{max} (mg/L)	8.30 ± 1.07

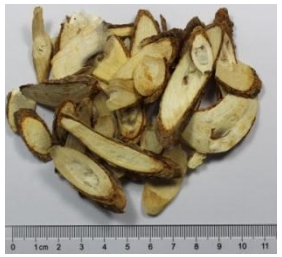
Data expressed as mean ± S.E. (n = 5). AUC: area under the concentration vs. time curve; MRT: mean residence time; $T_{1/2}$: elimination half-life; Cl/F: clearance; T_{max}: time point of maximum blood concentration of drug; C_{max}: maximum concentration.



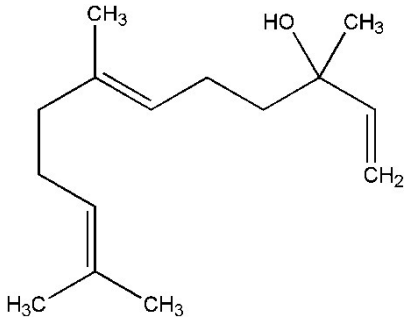
73x203mm (600 x 600 DPI)



16x9mm (600 x 600 DPI)



Root of *Oplopanax elatus*



cis and trans nerolidol