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A HPLC method for the analysis of germacrone in rabbit plasma and its application to a pharmacokinetic study of germacrone after administration of zedoary turmeric oil

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Abstract

A validated new, simple and highly sensitive reversed-phase HPLC method is developed for studying the pharmacokinetics of germacrone after intravenous administration of zedoary turmeric oil (ZTO) oil-in-water microemulsion. The method did not require a complex and expensive equipment. A high extraction recovery (>80%) of germacrone was obtained. Linear calibration curves obtained with the peak-area ratio (y) of germacrone to internal standard (tanshinoneIIA) versus drug concentration (x) were found to be linear between 8.08 and 808 ng/ml. The limit of quantitation was 8.08 ng/ml.The monitored compounds were completely separated from others in ZTO and from endogenous species in plasma by HPLC. Pharmacokinetic investigations were performed on 18 male rabbits after intravenous administration of ZTO microemulsion via the ear vein at germacrone doses of 3.2, 6.4 and 9.6 mg/kg. The plasma concentration–time data fit to a two-compartment intravenous model with a weight of $1/C^2$ (C: germacrone concentration in plasma). Germacrone exhibited linear pharmacokinetics after intravenous administration of ZTO microemulsion to rabbits over the germacrone dose range 3.2–9.6 mg/ml.
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1. Introduction

Curcuma zedoaria (Berg.) Rose. (Zingiberaceate), also called Er-zhu in Chinese, has long been used as a folk medicine. The essential oil, zedoary turmeric oil (ZTO), is extracted from the dry rhizoma of this plant. In Japanese and Chinese traditional medicines, ZTO is prescribed as a stomachic and emmenagague in various traditional preparations [1,2]. From ZTO, some hepatoprotective sesquiterpene compounds were isolated [3–5], including furanodiene, germacrone, curdione, neocurdione, curcumenol, isocurcumenol, aerugidiol and zedoarondiol. A few studies on germacrone (8.012%, w/w, in ZTO) [3,7-cyclodecadien-1-one,3,7-dimethyl-10-(1-methylethylidence)-(E, E), A in Fig. 1] showed its potent protective effects on D-galactosamine/lipopolysaccharide-induced liver injury in mice and potent inhibitory effects on contractions induced by high concentration of K^+ in isolated rat aortic strips [6,7]. GC–MS [8] and HPLC methods [9] were developed for the determination of the active components (e.g. germacrone) in ZTO to evaluate the quality of Rhizoma Curcumae. However, no information is available about the pharmacokinetics of germacrone as yet. Three difficulties restrict the further study of in vivo absorption and pharmacokinetics of germacrone, such as instability [10,11], strong volatility and low UV absorption. In this study, we develop a validated new, simple and highly sensitive reversed-phase HPLC method for studying the pharmacokinetics of germacrone after intravenous administration of ZTO oil-in-water microemulsion. The monitored compounds were completely separated from the

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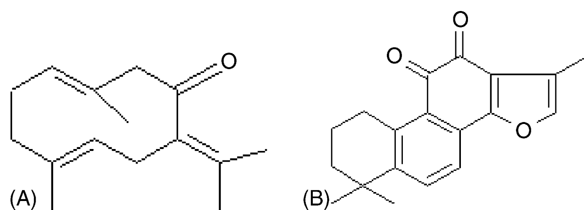

Fig. 1. The structure of germacrone (A) and tanshinone IIA (B).

others in ZTO and endogenous species in plasma by HPLC. The study of the pharmacokinetic parameters of germacrone will provide useful information for its clinical development.

2. Materials and methods

2.1. Chemicals and reagents

ZTO was provided by Jiangxi Tongren Natural Perfume Co. (Jiangxi, China); the standards (germacrone and tanshinoneIIA (B in Fig. 1); the purities: 98.3 and 97.9%, respectively) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol, tetrahydrofuran and acetonitrile of HPLC-grade were obtained from Concord Tech. Co. (Tianjin, China). All other chemicals, such as zinc sulfate heptahydrate solution, anhydrous sodium sulfate were of analytical grade. Water was deionized and distilled.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a pump (Hitachi L-7110, Japan), a μBondapak C18 column (Diamonsil^{TM}, 200 mm × 4.6 mm, 5 μm particle size), a guard column (10 mm × 4.6 mm) packed with C18 material, a column oven (Shimadzu CTO-10AS, Japan) and a UV–vis detector (Hitachi L-7420, Japan). The mobile phase was prepared by mixing 800 ml methanol with 50 ml tetrahydrofuran and 200 ml 0.1 mol/L sodium acetate solution adjusted to pH6.8 with acetic acid. The solvent was filtered through a 0.45 μm filter and degassed. The concentration of germacrone in the samples was determined at 245 nm by injecting 20 μl volume onto the column with a flow rate of 0.8 ml/min. All chromatography was performed at 30 °C.

2.3. Preparation of the microemulsion of ZTO

To prepare microemulsions of ZTO, oil phase was composed as follows: propanediol (0.82 ml) and poloxamer188 (0.28 g) were melted down and mixed with polysorbate 80 (0.38 g). 0.11, 0.22 and 0.33 g of ZTO dissolved in ethanol (0.73 ml) were respectively added into above the mixture under vigorous agitation to obtain the emulsions with three concentration levels of ZTO. The obtained oil phase was added into the distilled water. The resultant microemulsions (O/W) with three concentration levels of ZTO were obtained after agitation (FA 25, FLUKO Equipment Shanghai Co. Ltd., China) at 10000 rpm for 5 min, in which the contents of germacrone were 2.0, 4.0 and 6.0 mg/ml, respectively.

2.4. Animal study

Male rabbits weighing 2.5 ± 0.3 kg were provided by the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The rabbits were fasted overnight but were allowed free access to water. The animals were divided into three groups (six animals each), receiving germacrone dose of 3.2, 6.4 and 9.6 mg/kg via the ear vein. The blood samples (about 1.0 ml) were withdrawn via the other ear vein at predetermined time intervals up to 24 h after the drug administration and were collected in heparin tubes. The plasma was separated by centrifuging at 10000 × g for 5 min and stored at −20 °C until used for assay.

2.5. Sample preparation and assay

Thawed specimens (0.5 ml) were mixed by vortexing with 20 μl internal standard (3.3 μg/ml tanshinoneIIA in methanol) for 30 s, and 100 μl zinc sulfate heptahydrate solution (0.5 g/ml) was added in order to precipitate proteins. The solution was centrifuged at 12000 × g for 5 min to obtain a clean supernatant. After the supernatant was separated, 80 μl of acetonitrile and 80 mg of anhydrous sodium sulfate (as the dehydrating agent) were sequently added to the precipitate and the mixture was vigorously agitated by vortexing for 10 min. The resultant solution was centrifuged at 12000 × g for 10 min again. The obtained clean supernatant was transferred into a clean glass tube for injection onto the HPLC system.

Peak area ratios of the compound compared to the internal standard were taken. Using the slope and intercept calculated by linear regression analysis of the calibration curve data, made on each day of analysis, the concentration of germacrone was calculated.

2.6. Data analysis

Pharmacokinetic parameters were calculated from plasma concentration–time data using a single-dose, two-compartment intravenous model with 3P97, a practical pharmacokinetic program (the Chinese Society of Mathematical Pharmacology). The data were best fit with a weight of $1/C^2$.

3. Results and discussion

TanshinoneIIA was chosen as the internal standard because of its similar property with germacrone and the satisfying resolution between the two compounds (Fig. 2). As can be seen, no interference was found in the region of investigated compounds. Germacrone and the internal standard (tanshinoneIIA) were well separated under the experimental conditions as described above, with retention times of 13.87

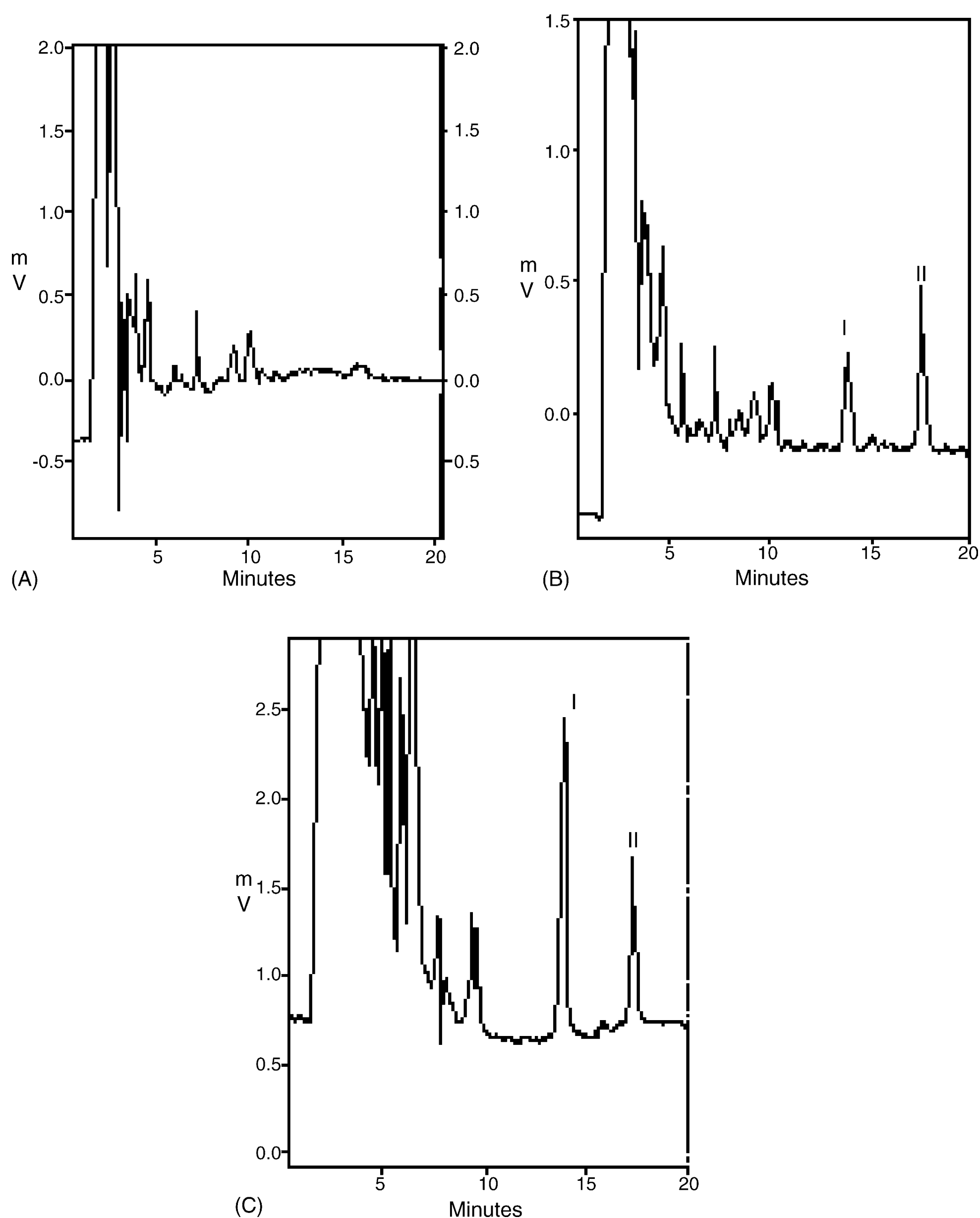

Fig. 2. Chromatograms of germacrone and tanshinoneIIA. (A) Blank plasma; (B) blank plasma spiked with germacrone (40.4 ng/ml, peak I) and tanshinoneIIA (132 ng/ml, peak II); (C) plasma sample of a rabbit, 20 min after intravenous administration of the ZTO microemulsions at germacrone dose of 6.4 mg/kg (determined germacrone concentration: 628.8 ng/ml, peak I).

and 17.47 min, respectively. The chromatographic mobile phase composition is a critical factor for the separation of monitored compounds from the others in ZTO and the endogenous in plasma. The resolution between germacrone and the internal standard depended strongly on the amount of tetrahydrofuran. With more tetrahydrofuran the resolution became smaller than 1.5. Contrarily, a scarcity of tetrahydrofuran produced greater resolution between the two peaks. Complete separation between monitored compounds and the others in ZTO was obtained at the methanol/water ratio (800:200), while the determination of germacrone and the internal standard was interfered with by the endogenous compounds in plasma when pH of the mobile phase was lower than 6.0. The final separation of samples was performed at pH 6.8.

Germacrone, as a sesquiterpene compound, has high volatility. A liquid–liquid extraction procedure could not be employed here, since germacrone would be lost during extraction due to its volatility. Protein-precipitation procedure, as an alternative preparing-sample method, was not on its own adequate because of the incomplete removal of protein, and moreover, the sensitivity became lower when the samples were diluted with the precipitating agent. To determine the amount of germacrone in plasma, a novel method of sample preparation was employed, which was composed of two processes, protein precipitation followed

by solvent extraction. It was found that the types of precipitating agent and extracting-solvent as well as the amounts of them were critical factors for protein-precipitation and extract recovery. Three steps were involved in the preparation of samples, using zinc sulfate heptahydrate solution, acetonitrile and anhydrous sodium sulfate, after which the sample cleanup was complete. In the first step, protein precipitation, the water-soluble precipitating agent zinc sulfate heptahydrate was added, and then liquid was separated from the mixture by centrifugation and removed. The denatured protein and monitored compounds were coprecipitated because of their water-insoluble nature. From the precipitate, the monitored compounds were extracted by addition of acetonitrile, which has the capacity to dissolve them while maintaining the majority of proteins in a precipitated state. In addition, acetonitrile is suitable for injection onto the HPLC system, and a high extraction recovery of germacrone was obtained when acetonitrile was used as the extracting-solvent. The extraction recovery was improved on the increase of acetonitrile. But, the sensitivity will become low because of diluted samples with excessive addition of acetonitrile. As a drying and precipitating agent, the residual moisture and protein were completely removed from the clean supernatant by addition of anhydrous sodium sulfate and then centrifugation. Sufficient time of extraction is required to obtain the high recovery achieved in this method.

For the calibration curves, pools of plasma were spiked with germacrone working standards, which were obtained using serial dilution from a stock solution, to obtain concentrations of: 8.08, 20.2, 40.4, 80.8, 202.0, 404.0 and 808.0 ng/ml. The calibration curves were obtained with peak-area ratio of germacrone to internal standard from plasma samples after analytical procedure versus drug concentration. Within- and between-day precision were calculated for three concentrations (10.1, 101.0 and 707.0 ng/ml), corresponding to quality control samples of the method. The samples, which were prepared before experiment and were stored in the same conditions as samples taken from animals, were assessed over the current made calibration curve. The within- and between-day precisions (R.S.D.) ranged from 4.8 to 7.7% and from 3.5 to 8.9%, respectively (Table 1). Method and extract recoveries were quantified at three different concentrations (10.1, 101.0 and 707.0 ng/ml). The recovery of internal standard, in concentration used in experimental procedure, has also been carried out. The mean method recoveries (accuracy) were

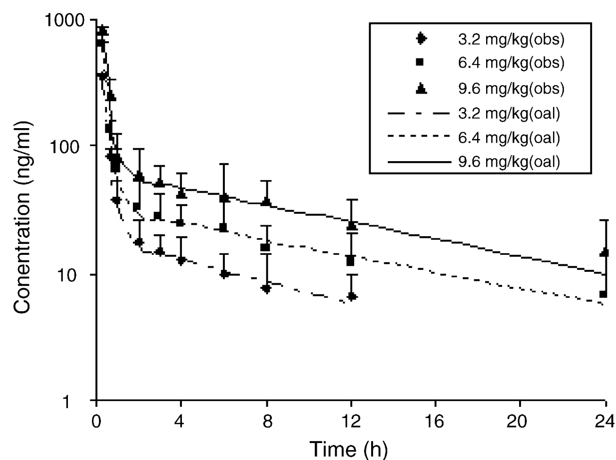

Fig. 3. The mean plasma concentration–time profiles of germacrone after intravenous administrations of the microemulsion, the points (obs) are the experimental data (mean + S.D.), the lines (cal) are the pharmacokinetic model fitted curves.

103.5, 96.6 and 95.8% at three different concentrations (10.1, 101.0 and 707.0 ng/ml), respectively. The extract recovery of germacrone was within the range of 81.3–90.2%. The internal standard showed a mean extraction recovery of 67.5% with R.S.D. < 5% ($n = 5$). The limit of quantitation, defined as the lowest concentration level which was measured precisely and accurately with R.S.D. and bias less than 10%, was calculated from the five independent made replications and was confirmed to be 8.08 ng/ml. The stability tests of germacrone in plasma, stored in three concentrations (10.1, 101.0 and 404.0 ng/ml), after each of the three freeze–thaw cycles, during 1 month, confer stability of germacrone during these operations.

Pharmacokinetic investigations were performed on 18 male rabbits after intravenous administration via the ear vein at germacrone dose of 3.2, 6.4 and 9.6 mg/kg. Fig. 3 shows the mean plasma concentration–time profiles of Germacrone after intravenous administration of the ZTO microemulsion. The plasma concentration–time data fit to a two-compartment intravenous model with a weight of $1/C^2$. The pharmacokinetic parameters are summarized in Table 2. The distribution half-lives were very short (0.16, 0.15 and 0.15 h for germacrone dose of 3.2, 6.4 and 9.6, respectively), but the elimination half-lives were longer (6.7, 9.5 and 8.9 h for germacrone dose of 3.2, 6.4 and 9.6, respectively), which

Table 1
Recovery and precision of the method in rabbit plasma ($n = 5$, mean ± S.D.)

Conc. (ng/ml)	Method recovery (%)	Extract recovery (%)	Within-day[a] R.S.D. (%)	Between-day[b] R.S.D. (%)
10.1	103.5 ± 5.5	90.2 ± 3.4	4.8	5.5
101.0	96.6 ± 9.3	82.5 ± 7.2	7.7	8.9
707.0	95.8 ± 6.5	81.3 ± 5.7	5.2	3.5

[a] Reference calibration curve ($n = 7$); linear regression equation; x-coefficient, 0.005199; intercept, 0.002287; correlation, 0.9994.

[b] Reference calibration curve; linear regression equation; Parameters: 1. x-coefficient, 0.005423; intercept, 0.002619; correlation, 0.9989; 2. x-coefficient, 0.005231; intercept, 0.002306; correlation, 0.9986; 3. x-coefficient, 0.005222; intercept, 0.002413; correlation, 0.9991; 4. x-coefficient, 0.005121; intercept, 0.002139; correlation, 0.9982; and 5. x-coefficient, 0.005168; intercept, 0.002369; correlation, 0.9995.

Table 2
Pharmacokinetic parameters of germacrone (mean ± S.D., $n = 6$)

Parameters*	Dose of germacrone (mg/kg)		
	3.2	6.4	9.6
V_c (L/kg)	2.4 ± 0.7	2.5 ± 1.4	2.6 ± 1.8
$T_{1/2\alpha}$ (h)	0.16 ± 0.092	0.15 ± 0.045	0.15 ± 0.066
$T_{1/2\beta}$ (h)	6.7 ± 2.4	9.5 ± 5.8	8.9 ± 3.0
K_{21} (1/h)	0.17 ± 0.10	0.31 ± 0.23	0.16 ± 0.07
K_{10} (1/h)	2.7 ± 1.5	2.5 ± 1.1	2.3 ± 1.4
K_{12} (1/h)	1.6 ± 1.3	1.9 ± 1.6	2.3 ± 1.8
AUC (ng h/ml)	498.4 ± 255.1	1002.2 ± 534.9**	1606.5 ± 944.2***
CL (L/kg h)	6.4 ± 2.6	6.2 ± 2.5	6.0 ± 3.1

* V_c, center compartment volume of distribution; $T_{1/2\alpha}$, half life of distribution; $T_{1/2\beta}$, half life of elimination; K_{21} and K_{12}, transportation constants; K_{10}, elimination constant; AUC, area under the blood concentration–time curve; CL, clearance.
** $p < 0.01$, compared with those at germacrone dose of 3.2 mg/kg.
*** $p < 0.01$, compared with those at germacrone dose of 3.2 or 6.4 mg/kg.

showed that germacrone was rapidly transported to tissues or organs from blood, then cleared slowly from blood. The plasma concentration increased with dose level; accordingly the area under the concentration–time curve (AUC_{0-t}) values also increased with dose. The AUC_{0-t} values were 498.4, 1002.2 and 1606.5 ng h/ml for 3.2, 6.4 and 9.6 mg/kg of germacrone dose, respectively. The AUC values versus dosage was linear (correlation coefficient $r = 0.998\,6$).

4. Conclusion

In this study, a validated new, simple and highly sensitive reversed-phase HPLC method is developed for studying the pharmacokinetics of germacrone after intravenous administration of ZTO oil-in-water microemulsion. A high extraction recovery of germacrone was obtained. The monitored compounds were completely separated from the others in ZTO and endogenous species in plasma by HPLC. Pharmacokinetic investigations were performed on male rabbits and the plasma concentration–time data fit to a two-compartment intravenous model. Germacrone exhibited linear pharmacokinetics after intravenous administration to rabbits over the germacrone dose range 3.2–9.6 mg/ml.

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