

Pharmacokinetic Study of Lappaconitine Hydrobromide Transfersomes in Rats by LC-MS

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Abstract

A rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for determination of lappaconitine hydrobromide in rat plasma and study on the pharmacokinetics of lappaconitine hydrobromide transfersomes in rat. Analyses were performed on Altima™ HP C18 column (50 mm×2.1 mm, 3 μm) with internal standard of tetrahydropalmatine and a mobile phase of methanol-0.1% formic acid (80:20). Agilent™ LC/MSD QQQ mass spectrometer with the mode of multiple reactions monitoring (MRM) was applied in the detection. Pharmacokinetic parameters of isodose lappaconitine hydrobromide transfersomes in transdermal patch were analyzed and calculated by DASver 2.0 software. The results indicated that with the linear range of 2.0-2000.0 ng/ml, quantitative lower limit was 2.0 ng and both the inter-day and the intra-day precisions were less than 9.9%. Lappaconitine hydrobromide transfersomes could significantly increase AUC and extend the circulation time of in rats.

Keywords: Lappaconitine hydrobromide; Transfersomes; Pharmacokinetic study

Introduction

Transfersomes (TF), alias ultradeformable nano-liposomes, were obtained by adding surfactants (such as sodium cholate, etc) in phospholipid bilayers of conventional liposomes. Besides advantages of the good affinity with skin, innocuity and safety, the priorities associated with transfersomes were their high deformability and high skin penetration. The drugs transfer across the skin from the epidermis into the dermis and finally into lymphatic vessels and blood vessels, and possess a therapeutic effect. A research report on transfersomes system, which was first proposed by professor Cevc from Technical University of Munich, indicated that transfersomes membrane had high deformability and its composition was stable via the skin. It was reported by Zellmer that there were complete transfersomes in blood circulation, which had extraordinary superiority in transdermal administration [1-9].

Lappaconitine hydrobromide (LH) was an alkaloidal hydrobromate extracted from the roots of *Aconitum Sinomontanum* Nakai (Ranunculaceae). Its chemical name was (1α,14α,16β)-20-ethyl-1,14,16-trimethoxyaconitane-4,8,9-triol-4-[2-(acetyl amino) benzoate] hydrobromide-monohydrate with a molecular formula of C₃₂H₄₄N₂O₈·HBr·H₂O. The structural formula was illustrated in Figure 1. The molecular weight and melting point of LH were 683.64 Dalton and 217-221°C, respectively. LH was soluble in methanol and hexane, slightly soluble in water, very slightly soluble in ethanol, but practically insoluble in chloroform. Due to the relatively strong analgesic effect [10,11], LH was a good non-addictive anodyne and an effective therapy for various pain symptoms in clinic, such as postoperative pain, gastrointestinal ulcer, gastritis, hepatitis, rheumatoid arthritis, sciatica, headache caused by cold and so on. The applications show that LH indeed provide an good analgesia effect on patients with advanced cancer and certain chronic patients. As a surgical analgesic and an adjuvant drug for radiotherapy and chemotherapy, LH was used in the treatment of cancer pain and simultaneously in improving general symptoms of tumor patients to enhance immune function. Analgesic effect of LH was seven times as strong as aminopyrine, equivalent with dolantin [12]. Because the efficacy kept for a long time without

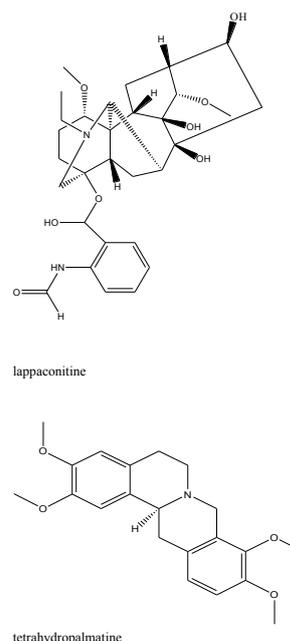


Figure 1: The chemical structures of lappaconitine (1) and tetrahydropalmatine (2).

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Received March 14, 2013; Accepted March 23, 2013; Published March 26, 2013

Citation: Zheng WS, Sheng YX, Zhang YJ, Fang XQ, Wang LL (2013) Pharmacokinetic Study of Lappaconitine Hydrobromide Transfersomes in Rats by LC-MS. Pharmaceut Anal Acta 4: 217. doi:10.4172/2153-2435.1000217

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teratogenic effect, mutagenesis or accumulative intoxication, LH was a good non-addictive analgesic.

LH was an insoluble drug taken in low dose. As an analgesic, there was a requirement in clinic that its preparations ought to have the characteristics of fast absorption and rapid action in order to relieve pain quickly. However, lappaconitine hydrobromide tablets sold in market belong to conventional tablet formulation. The properties of excipient employed in preparation result in long disintegration time, low dissolution rate, fast action and low bioavailability, which cannot satisfy the clinical requirements [13-15]. Therefore, a new preparation was urgently needed to be developed to improve the bioavailability and to reduce the times of administration for convenient administration. According to the WHO three ladder acesodyne principle, non-invasive administration was the first choice in treating cancer pain and thus LH was made into transdermal preparation with the advantages of convenient administration and non-invasion. It avoided gastrointestinal damage of drug and hepatic first pass effect, and reduces the toxicity and side effects and the times of administration. In addition, it led to steady-state plasma concentration and long effect, was convenient for long-term administration and rarely had psychological dependence (addiction). Based on its physicochemical properties, pharmacological effects and the requirement of fast absorption and rapid action in clinic, LH was made into transfersomes, which had small the particle size, fast percutaneous absorption, short onset time and good analgesia effect, to improve bioavailability.

Although lappaconitine had remarkable pharmacological activities and promising clinical application, pharmacokinetics of lappaconitine were rarely reported at present. In this study, lappaconitine hydrobromide transfersomes were prepared and the pharmacokinetic behavior in rat was investigated, which aim to establish the sensitive and specific determination method of lappaconitine hydrobromide in rat by LC-MS/MS and to investigate the pharmacokinetics quality of lappaconitine hydrobromide transfersomes in rat for providing theoretical basis for safe and rational medication and new drug design.

Experimental

Chemicals and reagent

Lappaconitine hydrobromide (99.0% purity) were purchased from Pharmaceutical Factory (Shanghai, China). Tetrahydropalmatine (IS, 98.0% Purity) were obtained from National Institute for the Control of Pharmaceutical and Biological Products. Methanol (HPLC grade) and n-hexane (HPLC grade) were purchased from Fisher Scientific (New Jersey, USA). Formic acid (analytical grade) was purchased from MREDA technology Inc (USA). Purified water was purchased from Wahaha Group Co., LTD (Hangzhou, China).

Apparatus and analytical conditions

Liquid chromatographic separation and mass spectrometric detection were achieved by employing the Agilent™ LC/MSD QQQ system consisted of a Agilent Surveyor LC pump, a Agilent Surveyor auto-sampler and a Triple Quad mass spectrometer (Agilent Technologies, Co. Ltd, Santa Clara, California, USA). Data acquisition, peak integration and calibration were performed with Agilent MassHunter software (Agilent Technologies, Co. Ltd, Santa Clara, California, USA). The chromatographic separation was achieved on a Grace Altima™ HP C¹⁸ (50 mm×2.1 mm, 3 μm) analytical column with a Security-Guard C18 guard column (12.5×2.1 mm, 5 μm; Agilent Technology, USA) at a temperature of 20°C. The system was run in

isocratic mode with mobile phase (methanol-water-formic acid, 80: 20: 0.1, v/v/v). Mobile phase was duly filtered through 0.45 μm Millipore filter and delivered at a flow rate of 0.2 ml/min.

The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in multiple reaction monitoring (MRM). The selected mass-to-charge (m/z) ratio of lappaconitine, m/z 585→m/z162; IS; m/z356→m/z192. The ESI conditions were: capillary temperature at 350°C; capillary voltage at 4 kV; and Nebulizer at 40 V.

Sample preparation

Preparation of transfersomes: Preparation of lappaconitine hydrobromide transfersomes: lappaconitine hydrobromide in methanol solution and soybean lecithin and sodium deoxycholate in ethanol solution were mixed and homogenized and subsequently the appearance of mixture was clarification and transparent. After most organic solvent was evaporated by vacuum, the left was removed by nitrogen. As hydration media the pellet in 100 ml distilled water was completely hydrated at room temperature. With high pressure homogenization pressure of 500-600 bar and cycle number of 3-4, emulsion color changed from turbidity to clarification and blue opalescence.

Sample preparation

Lappaconitine hydrobromide weighed 58.5 mg (equivalent to 50.0 mg free base of lappaconitine) was placed in a 50 ml volumetric flask. After adding appropriate amount of saline, the volumetric flask was shook to make solution homogenized and clarified. Saline was filled up to the mark and 1.0 mg/ml physic liquor was obtained.

Treatment of plasma samples

After precisely pipetting of 100 μl plasma sample into an EP tube, 20 μl internal standard solution (10.0 μg/ml tetrahydropalmatine), 100 μl ultrapure water and 60 μl sodium hydroxide solution (1.0 mol/l) were added and mixed, and then 1 ml extraction solvent (n-hexane : methanol, 95:5, v/v) was added. The mixture was homogenized for 10 min, shook for 10 min and centrifuged for 5 min (at 14000 rpm). Subsequently, 20 μl supernatant was analyzed by LC/MS/MS (Wang Qing et al. 2011).

Standard curve and quantitative limit of LH in plasma

After precisely pipetting of 100 μl rat plasma into an EP tube, various concentrations of reference substance solutions of LH and internal standard solution were added precisely. Plasma samples, in which LH concentrations were 2.0, 10.0, 20.0, 80.0, 400.0, 1000.0 and 2000.0 ng/ml respectively, were treated according to "Treatment of plasma samples" except adding 100 μl water to establish the standard curve. The linear regression equation, so called standard curve, was obtained with determine and as abscissa and the ratio of determined to internal standard peak area as ordinate by weighted least square.

Accuracy and precision of method

Three portion standard solutions of LH were treated according to "Treatment of plasma samples" to obtain quality control (QC) samples, which plasma concentrations were 10.0, 400.0 and 1000.0 ng/ml. Each concentration divided into six portions was analyzed for three days. Based on intraday standard curve, concentrations of quality control (QC) samples were calculated. Ultimately, accuracy and intra-day and inter-day precision of determination method were obtained in contrast with compound concentration.

Recovery

Three portion standard solutions of LH were treated according to "Treatment of plasma samples" to obtain quality control (QC) samples, which plasma concentrations were 10.0, 400.0 and 1000.0 ng/ml. Each concentration divided into six portions was analyzed for three days. The extraction recovery at low, middle and high concentrations was calculated using plasma standard calibration curve.

Stability investigation

The stabilities of treated plasma samples of lappaconitine placed at room temperature for 24 h and untreated plasma samples of lappaconitine placed at room temperature for 15 min, 30 min, 1 h and 2 h were investigated in this experiment. In addition, the stabilities of plasma samples with freezing-thawing cycle once and for three times and plasma samples with freezing at -20°C for 30 days were studied. In stability investigation, 100 µl blank plasma was treated according to "Standard curve and quantitative limit of LH in plasma" to obtain the plasma samples of lappaconitine at low and high concentrations (10.0 ng plus 1, 1000.0 ng/ml). Each concentration divided into three portions was analyzed.

Stability experiment

According to the treatment method of standard curve, the plasma samples at 10.0, 400.0 and 1000.0 ng/ml were treated and each concentration divided into three portions was analyzed. The stabilities of plasma samples with freezing-thawing cycle once and for three times and plasma samples with freezing at -20°C for 30 days and then thawing at room temperature were determined.

Method for animal experiment

All animal studies were performed in accordance with the experimental protocols approved by the Animal Care Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences. Male SD rats used in the present study were supplied by Institute of Materia Medica (Beijing, China). Rats were housed in standard cages and allowed free movement and access to water and standard laboratory diet throughout the experiments. 15 rats weighed 200 ± 20 g were randomly and equivalently divided into 3 groups, which were A group, B group and C group and were given different doses. During experiment period, rats were fed free with food and water. Then, transdermal administration at same dose of LH transfersomes was accomplished. 0.3 ml Blood was collected from postocular venous plexus at 1, 2, 4, 6, 8, 10, 12, 14 h and intervals of 1 hour, 72 hours in total, after administration. Plasma was obtained after centrifuging at 5000 rpm for 10 min. The supernatant was frozen for later use.

Data statistical method

Pharmacokinetic parameters of lappaconitine administration were analyzed and calculated by DASver 2.0 software. The compartmental model fitting of dynamic process in rat was accomplished.

Results and Discussion

HPLC-MS chromatograms

The chemical structures of lappaconitine and IS both contained nitrogen atom, so the abundance was strong in positive mode. Appropriate amount of standard solution of lappaconitine (2.0 ng/ml) and internal standard solution (10.0 µg/ml) were added to blank plasma and treated as the same process to obtain the corresponding chromatogram. The fragmentation process of lappaconitine (m/z

585→m/z162) and tetrahydropalmatine (m/z356→m/z192) were shown in Figure 2. The retention times of lappaconitine and IS were about 1.8 and 1.9 min, respectively. The overall chromatographic run time was finished within 4 min. The endogenous substance in plasma did not interfere with the determination of lappaconitine and internal standard tetrahydropalmatine. Under the mass spectrometer conditions, the plasma sample collected at 5 h after transdermal administration of 10 mg/ml lappaconitine hydrobromide transfersomes and added with internal standard was treated according to "Treatment of plasma samples". When secondary scanning was carried out under MRM mode, the endogenous substance and other impurities in plasma did not interfere with the separation and determination of sample.

Standard curve of LH in plasma

The linear regression equation was calculated according to area ratio of LH to internal standard in plasma with the standard curve of typical rat plasma sample as $y=3.8799E-005x+0.0027$. The standard curve in the range of 2.0-2000.0 ng/ml had good linear relation with weighted coefficient of $1/X^2$ ($r=0.9996$). The limit of quantification was 2 ng/ml. Data of the limit of quantification indicated that the determination of LH in rat was extremely sensitive and accurate.

Accuracy and precision

Three portion standard solutions of LH were treated according to "Treatment of plasma samples" to obtain quality control (QC) samples, which plasma concentrations were 10.0, 400.0 and 1000.0 ng/ml. Each concentration divided into six portions was analyzed for three days. Concentrations of quality control samples were calculated based on intraday standard curve. The accuracy (RE) was below $\pm 4.8\%$ and both intra-day precision and inter-day precision (RSD) were below 8.11 (Table 1).

Extraction recovery

Three portion standard solutions of LH were treated according to "Treatment of plasma samples" to obtain quality control (QC) samples, which plasma concentrations were 10.0, 400.0 and 1000.0 ng/ml. Each concentration divided into six portions was analyzed. The extraction recovery at low, middle and high concentrations was 81.6%, 94.4% and 92.3% by calculating peak area ratio by two treatment methods of one concentration (Table 2).

Stability investigation

The stabilities of treated LH plasma samples with freezing-thawing cycle once and for three times and LH plasma samples with freezing at -20°C for 30 days were investigated. Data of stability investigation illustrated that both treated LH plasma samples with freezing-thawing cycle once and for three times and LH plasma samples with freezing at -20°C for 30 days were steady (RSD% 4.87, RSD% 8.34) (Table 3).

Application of the method to pharmacokinetic study

The plasma concentration after transdermal administration of 10 mg/ml lappaconitine hydrobromide transfersomes in rat was determined by LC/MS and the pharmacokinetic behavior of lappaconitine hydrobromide transfersomes in rat was deeply investigated. The obtained plasma concentration-time curve was shown in Figure 3. The average plasma concentration after transdermal administration was fitted to two-compartment model by DASver2.0 software. The fitted pharmacokinetic parameters were listed in Table 4. The areas under plasma concentration-time curve (AUC_{0-4}) were 53.886 at transdermal administration of dose of 10.0 mg/kg in rats, respectively.

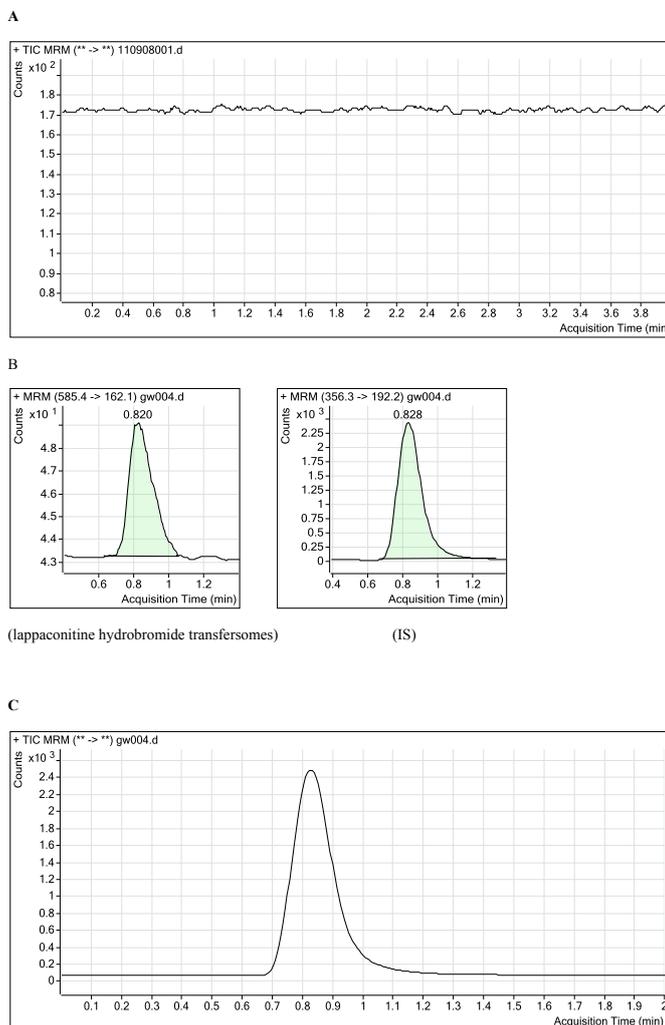


Figure 2: Typical chromatograms for the determination of lappaconitine and tetrahydropalmatine in samples: (A) chromatogram of a blank plasma sample; (B) chromatogram of a plasma sample spiked with lappaconitine hydrobromide transfersomes and internal standard (I.S.); (C) chromatogram of the plasma sample collected between 12 h and 24 h after transdermal administration of 10 mg/ml of hydrobromide transfersomes.

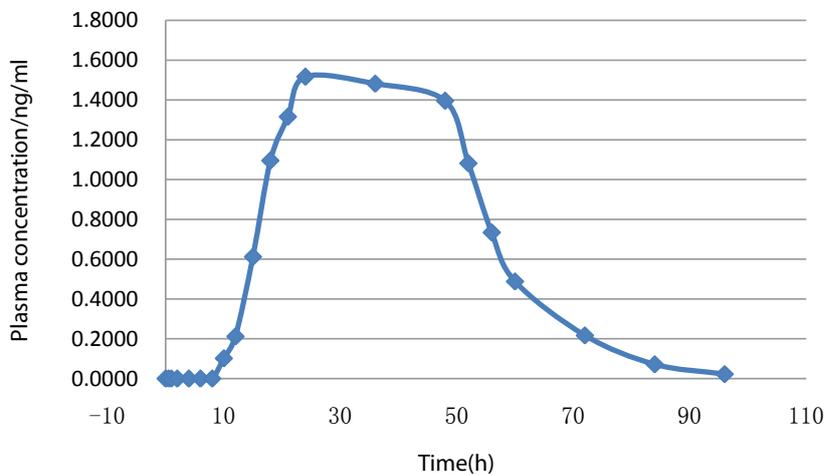


Figure 3: Pharmacokinetic parameters for lappaconitine after transdermal administration of 10 mg/ml in rats.

Concentration added to plasma (ng/ml)	Intra-day (n=6)			Inter-day (n=4)		
	Mean measured concentration (ng/ml)	Accuracy (%)	RSD (%)	Mean measured concentration (ng/ml)	Accuracy (%)	RSD (%)
10	8.88 ± 0.72	88.8	8.11	8.95 ± 0.61	89.5	6.82
400	372.5 ± 8.46	93.1	2.27	377.6 ± 11.67	94.4	3.09
1000	875.5 ± 5.78	87.5	0.66	901.0 ± 6.33	90.1	0.70

Table 1: Intra-day and inter-day precision and accuracy of LC/MS determination of lappaconitine in rats plasma.

Concentration added to plasma (ng/ml)	Mean measured concentration (ng/ml)	Recovery (%)	RSD (%)
10	8.16 ± 0.52	81.6	6.37
400	377.6 ± 7.88	94.4	2.09
1000	923.4 ± 15.11	92.3	1.64

Table 2: Recoveries of lappaconitine in rats plasma (n=6).

Concentration added to plasma (ng/ml)	Room temperature (8 h)			Four freeze-thaw circles		
	Mean measured concentration (ng/ml)	Accuracy (%)	RSD (%)	Mean measured concentration (ng/ml)	Accuracy (%)	RSD (%)
10	8.75 ± 0.73	87.5	8.34	8.42 ± 0.41	84.2	4.87
400	369.4 ± 11.32	92.4	3.06	355.6 ± 9.24	88.9	2.60
1000	934.6 ± 18.91	93.4	2.02	978.7 ± 15.34	97.9	1.57

Table 3: Stability of lappaconitine in rats plasma (n=6).

parameters			Statistical moment parameters		
t1/2 α	h	6.013	AUC(0-t)	ug/L*h	65.292
t1/2 β	h	10.108	AUC(0- ∞)	ug/L*h	65.531
V1/F	L/kg	209.773	AUMC(0-t)		2527.314
CL/F	L/h/kg	182.692	AUMC(0- ∞)		2552.735
AUC(0-t)	ug/L*h	53.886	MRT(0-t)	h	38.708
AUC(0- ∞)	ug/L*h	54.737	MRT(0- ∞)	h	38.955
K10	1/h	0.871	VRT(0-t)	h ²	254.471
K12	1/h	0.774	VRT(0- ∞)	h ²	270.65
K21	1/h	0.086	Zeta	1/h	0.095
Ka	1/h	0.28	Zeta		123
t1/2Ka	h	2.477	Cz	ug/L	0.023
Tlag	h	0	t1/2z	h	7.32
			Tmax	h	24
			Vz/F	L/kg	1611.844
			CLz/F	L/h/kg	152.601
			Cmax	ug/L	1.51673914

Table 4: Pharmacokinetic parameters for lappaconitine hydrobromide transfersomes after transdermal administration of 10 mg/ml of rats.

“Peak and valley” phenomenon is often observed in time-plasma concentration curve by oral or injection administration. The therapeutic effects are hardly exerted completely at valley plasma concentration, while drug overdose leads to toxicity at peak plasma concentration. In this study, after transdermal administration, that peak time increased, peak concentration decreased and the curve trended to smooth with mean retention time (MRT) of 38.708 avoided “peak and valley” phenomenon clearly shown in Table 4 and Figure 3. That the area under curve increased relatively indicated better absorption via transdermal administration.

Due to sustained drug release of patches, plasma concentration had a slight decline, which helped medicine with sustained efficacy. The constant speed release of drug avoided first pass effect and gastrointestinal stimulation.

Both the time of steady-state plasma concentration and half-life were long, which extended the time of dosing intervals.

Conclusions

The method for determination and pharmacokinetics of lappaconitine hydrobromide transfersomes in rat were established

in this study and it features high sensitivity, simple operation, good linearity and high precision. There is no obvious detected endogenous substance that might interfere with the determination. This method can be successfully applied in pharmacokinetic evaluations of lappaconitine hydrobromide transfersomes for transdermal administration and provides pharmacokinetic theoretical basis for clinical medication by offering pharmacokinetic parameters of lappaconitine hydrobromide transfersomes.

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