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Short communication

High-performance liquid chromatographic method for the determination and pharmacokinetic study of dehydrotumulosic acid in the plasma of rats having taken the traditional chinese medicinal preparation Ling-Gui-Zhu-Gan decoction

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Abstract

A high-performance liquid chromatographic method for the determination of dehydrotumulosic acid in plasma of rats having been administrated orally with the traditional Chinese medicinal preparation Ling-Gui-Zhu-Gan decoction was developed. Plasma samples taken from rats were acidified with hydrochloric acid and extracted with ethyl acetate. Separation of the main effective constituent dehydrotumulosic acid was accomplished on a C_{18} stationary phase and a mobile phase of methanol–acetonitrile–2% glacial acetic acid (13:12:10, v/v), with a UV detector setting at 242 nm. After validation, the method was used for preliminary investigation of the pharmacokinetic profiles of dehydrotumulosic acid administrated in Ling-Gui-Zhu-Gan decoction.

Keywords: Ling-Gui-Zhu-Gan decoction; Dehydrotumulosic acid

1. Introduction

Traditional Chinese medicines (TCM) or more exactly Chinese materia medica, are the use of natural therapeutic agents under the guidance of the theory of traditional Chinese medical science, which has played an indispensable role in the prevention and treatment of diseases in China. Herbal medicines are used mostly in combinations in China and are made into certain preparations for easy and efficient use.

The Ling-Gui-Zhu-Gan decoction was reported originally in the Treatise on cold-induced febrile diseases and the Synopsis of the golden chamber, both of which were written by Zhang Zhongjing in the last years of the Han dynasty in China (B.C. 206~A.D. 200) [1]. The decoction is prepared by boiling *Poria*, *Ramulus Cinnamoni*, *Rhizoma Atractylodis Macrocephalae* and *Radix Glycyrrhizae* together. In the clinical practice of TCM, the decoction has been used to treat arrhythmia, cardiac failure, angina pectoris and other cardiovascular diseases over a very long period of time [2].

Poria is the main medicinal component of the decoction, with dehydrotumulosic acid as its the major effective constituent [3,4]. Thus dehydro-

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tumulosic acid is used as one of the characteristic marker compounds to the decoction [5–8]. There are no published methods for the quantitation of dehydrotumulosic acid in biosamples. Owing to the complexity of chemical constituents in traditional Chinese medicinal formulas, there is scarcely any report of their pharmacokinetic studies as well. This paper reports a HPLC method for analyzing the constituent pharmacokinetic study accomplished on the constituent ingredient of dehydrotumulosic acid found in Ling-Gui-Zhu-Gan decoction and the use of the method for preliminary pharmacokinetic study of dehydrotumulosic acid.

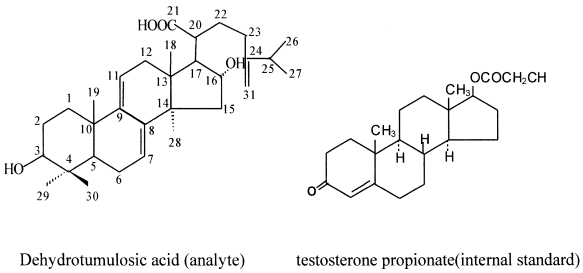
2. Experimental

2.1. Materials and reagents

Fuling (*Poria cocos* (Schw.) Wolf), Guizhi (*Cinnamomum cassia* Presl), Baizhu (*Atractylodes macrocephala* Koidz.) and Gancao (*Glycyrrhiza uralensis* Fisch.) were all purchased at Tianyitang TCM shop, Shenyang, China; dehydrotumulosic acid and testosterone propionate (structures see Fig. 1), were ordered from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; their purity both are more than 99%; ethanol, hydrochloric acid and ethyl acetate, all of analytical grade and methanol and acetonitrile (chromatographic grade) were from Yuwang chemical reagents company, Shandong, China.

2.2. Chromatographic system

The essential parts of the HPLC system consisted of a Shimadzu LC-10AD pump, a SPD-10A ultraviolet (UV)–visible detector set at 242 nm, a 20-μl injection loop, a LC workstation for data collection and a 200×4.6 mm I.D. column-with Hypersil ODS C_{18} (5 μm particle size) stationary phase and a mobile phase of methanol–acetonitrile–2% glacial acetic acid (13:12:10, v/v) at a flow rate of 1.0 ml/min, operated at room temperature.



Dehydrotumulosic acid (analyte) testosterone propionate(internal standard)

Fig. 1. Molecular structures of dehydrotumulosic acid and the internal standard.

2.3. Standard solutions

Stock solutions of standard dehydrotumulosic acid (0.1 mg ml^{-1}) and internal standard testosterone propionate (20 μg ml^{-1}) were prepared in methanol. These solutions were spiked into drug-free plasma samples of rats to determine the recovery, precision, accuracy and detection limit of the HPLC method. All standards were kept at 4 °C before use.

2.4. Sample preparation

Aliquots (2.0 ml) of plasma were acidified with 600 μl of 0.1 mol/l HCl, with addition of internal standard. Then each sample was shaken with 6 ml of ethyl acetate for 10 min and centrifuged at 2500 rpm for 10 min and the organic layer was transferred into an empty tube. This procedure was repeated four times and the organic layer pooled was dried at 40 °C under a stream of nitrogen. The residue was dissolved in 100 μl methanol to injection to the chromatographic system.

2.5. Calibration procedure

The calibration was accomplished via a standard curve by chromatographing known weight ratios of the sample constituent dehydrotumulosic acid (dt) and the internal standard testosterone propionate (tp). A plot was made of the ratio $A_{dt}A_{tp}$ against W_{dt}/W_{sp}, where A was peak-area and W, weight. By adding the same weight of internal standard to the sample and fixing the aliquot portion (2.0 ml in the case), concentration can be used as the abscissa of plot. Concentrations of dehydrotumulosic acid ranged from 0.20 to 20.0 μg/ml were studied.

2.6. Recovery, precision and accuracy

Recovery was determined by adding dehydrotumulosic acid at concentrations of 0.40, 4.00 and

20.0 μg/ml and the precision (within-day and day-to-day) of the method was calculated at the same three concentrations. The variability of peak-area ratio at each concentration was determined as a measure of the precision of the assay. The accuracy was determined by comparing the measured concentration with the spiked value.

3. Results and discussion

Typical chromatograms of the blank and spiked plasma are given in Fig. 2a and b, in which the retention time was 33 min for dehydrotumulosic acid and 35 min for testosterone propionate. There are no co-eluting disturbing peaks in the vicinity of the two peaks on the chromatogram of the blank plasma. A chromatogram of plasma sample of rat taken 1 h after oral administration of the Ling-Gui-Zhu-Gan decoction (18 ml/kg) is given in Fig. 2c.

The calibration curve for the determination of dehydrotumulosic acid in rat plasma ($Y = 1.165 \times 10^{-3} + 2.514 \times 10^{-1}x$) is linear over the range of 0.20~20.0 μg/ml with a coefficient of determination (r^2) of 0.9981($n = 7$). The linear range has showed adequate to the use of this method in the current pharmacokinetic studies of this drug. The quantitation limit is 0.20 μg/ml. The within-day precision (RSD%) is 3.4~6.0% ($n = 18$), and the day-to-day precision (RSD%) 1.1~5.8% ($n = 18$). The accuracy is 0.05~4.6% ($n = 18$) (see Table 1). The recovery of dehydrotumulosic acid was obtained through comparison of concentrations of its methanol extracts with those of the corresponding spiked plasma. The mean recovery was 89.8% ($n = 6$). In all instances, the accuracy, precision, and recovery showed satisfactory levels [9,10].

Ideally, an internal standard should display similar physico-chemical properties to the analyte. For this reason, testosterone propionate, which has similar chemical structure to dehydrotumulosic acid, was chosen as the internal standard showed adequate separation from the analyte. A mixture of acetonitrile and methanol in the mobile phase was required to achieve resolution of dehydrotumulosic acid and the internal standard. The plasma sample was acidified with hydrochloric acid and extracted into ethyl acetate. The type and amount of the added acid was

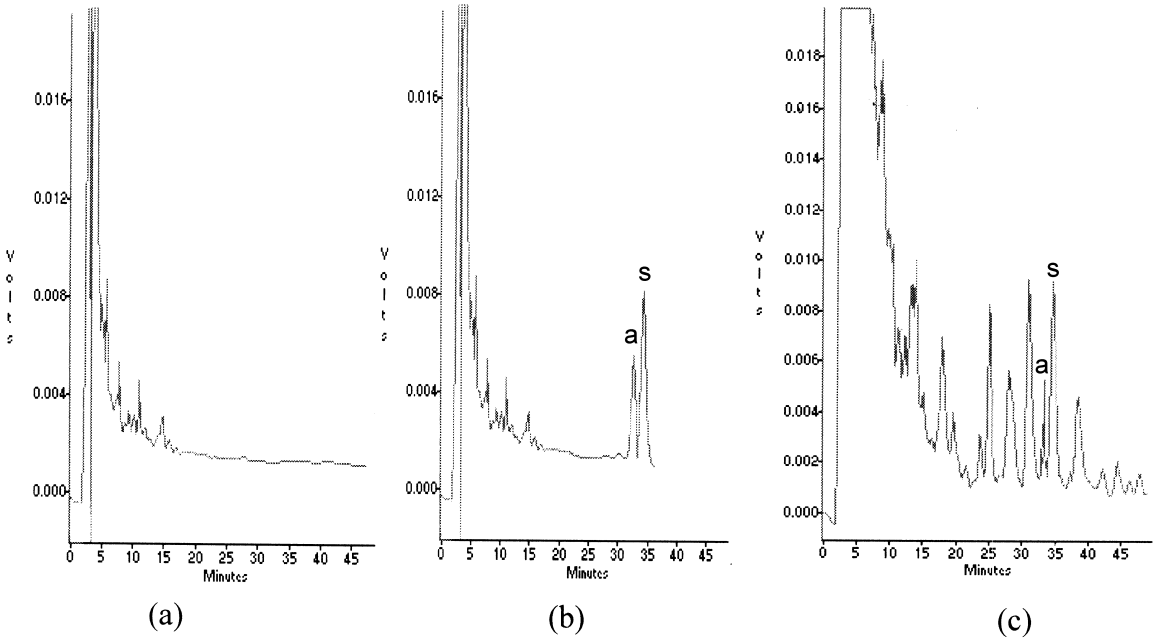


Fig. 2. Typical chromatograms for determination of dehydrotumulosic acid in plasma samples. (a) Chromatogram of a blank plasma sample; (b) chromatogram of a plasma sample spiked with dehydrotumulosic acid and internal standard; (c) chromatogram of a plasma sample of rat taken 1 h after oral administration of the Ling-Gui-Zhu-Gan decoction. s, internal standard; a, dehydrotumulosic acid.

Table 1
Precision and accuracy of HPLC method to determine the dehydrotumulosic acid in rat plasma samples

Run	Added concentration (μg ml^{-1})		
	0.40	4.0	20.0
1	0.4135	4.0293	20.07
	0.4051	4.2039	20.88
	0.4191	4.4160	19.94
	0.3590	4.2974	22.16
	0.3761	4.2314	20.15
	0.4354	4.3834	19.75
2	0.4270	4.8289	20.07
	0.3721	3.9394	20.01
	0.3797	4.2827	19.51
	0.4179	4.0169	21.34
	0.4008	4.2067	19.79
	0.3900	4.0786	19.84
3	0.4286	3.9457	19.76
	0.4123	3.8964	19.94
	0.4191	4.2688	19.95
	0.3574	3.8618	21.37
	0.4139	4.3046	19.44
	0.3773	4.1239	20.20
n	18	18	18
Mean (μg ml^{-1})	0.4002	4.1842	20.23
SD	0.025	0.234	0.726
Relative error (%)	0.05	4.6	1.2
Between day RSD (%)	1.1	5.8	2.5
Within day RSD (%)	6.0	5.2	3.4

investigated, using H_3PO_4 and HCl, where the amount of HCl used varied from 0.1, 0.3, 0.5 to 1.0 times of the volume of plasma samples. The pretreatment method adopted is able to remove excessive interference and efficiently extract the drug of interest from plasma samples.

The assay has been applied to the pharmacokinetic study of dehydrotumulosic acid in Ling-Gui-Zhu-Gan Decoction. Plasma samples from rats were taken at 0.0 (before administration), 0.25, 0.50, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 and 12.0 h after oral administration of the decoction (18 ml/kg). Fig. 3 is the mean plasma concentration–time plot of dehydrotumulosic acid, in which its pharmacokinetic parameters were obtained: $t_{max} \approx 2$ h, $C_{max} = 12.8 \pm 1.8$ μg/ml, and $t_{1/2} = 5.5$ h.

Several pharmacologic studies of Ling-Gui-Zhu-Gan decoction on myocardial ischemia and arrhythmia have been attempted [11]. There were some

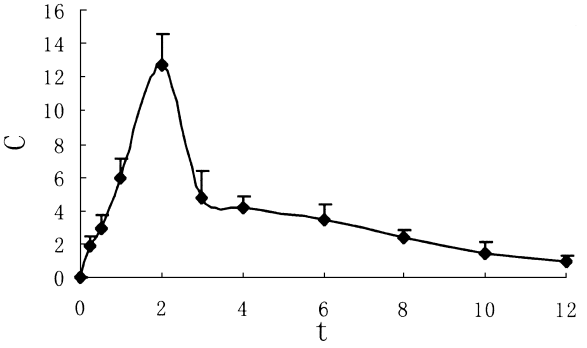


Fig. 3. Plot of the mean concentration of dehydrotumulosic acid in plasma of rats against time after oral administration of the Ling-Gui-Zhu-Gan decoction.

indications that it could reduce the myocardial consumption of oxygen and the preload of heart and increase heart blood perfusion flow. All these pharmacologic indices reached a maximum at about 60~180-min after oral administration of the Ling-Gui-Zhu-Gan decoction. This was consistent with the C_{max} which appeared at about 2 h in the pharmacokinetic curve. Traditionally the Ling-Gui-Zhu-Gan decoction has been taken orally 3 times a day since the ancient times of Zhang Zhongjing; according to the current study, $t_{1/2}$ was 5.5 h, so the decoction should be taken about 4 times a day. The pharmacokinetic parameters C_{max} and $t_{1/2}$ of dehydrotumulosic acid suggest that it may be used as a marker compound to characterize some profiles of a TCM formula.

This paper describes a HPLC method with UV detection suitable for the determination of dehydrotumulosic acid in rat plasma. This method has been demonstrated to be usable in pharmacokinetic studies of dehydrotumulosic acid in Ling-Gui-Zhu-Gan decoction.

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