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Study of the determination and pharmacokinetics of Compound Danshen Dripping Pills in human serum by column switching liquid chromatography electrospray ion trap mass spectrometry

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Abstract

Compound Danshen Dripping Pill (CDDP) is an important drug widely used for the treatment of cardiovascular diseases. An active component Danshensu (DS) of CDDP was separated by reversed-phase high performance liquid chromatography using column-switching system and analyzed by electrospray mass spectrometry. With this validated assay the pharmacokinetics of CDDP was studied in 10 healthy volunteers after a single oral administration of 250 mg. After trichloroacetic acid precipitation of serum proteins, the analytes were preconcentrated and black-flushed on a reversed-phase column for separation using a switching valve. The analytes were ionized using negative electrospray ionization (ESI) mode. The precursor ion of *m*/*z* 196.6 was used to quantify DS in serum. The linear calibration curve ranged from 1.25 to 175 μg/mL. The limit of quantification (LOQ) for DS was 0.15 μg/mL. The intra-day and inter-day precision (R.S.D.) was less than 7.4 and 7.9%, respectively.

Keyword: Compound Danshen Dripping Pill

1. Introduction

Compound Danshen Dripping Pill (CDDP) is an important drug used for the treatment of cardiovascular diseases in China [1,2]. Salvia miltiorrhiza Bunge, Danshen in Chinese, is one of the three materials of CDDP. Clinical studies demonstrate that it has many effects on increasing coronary flow rate and superoxide dismutase activity, expanding the blood vessel, promoting blood circulation, relieving blood stasis, improving the microcycle and changing the blood viscidity, also decreasing the consuming oxygen of the cardiac muscle, with the effects on anticancer, antibacterial, antiinflammation, etc. [3–5]. Danshensu (DS, 3,4-dihydroxyphenyllactic acid, Fig. 1) is a major active component of Danshen in CDDP [6,7]. DS can dilate isolated coronary artery, decrease the biosynthesis of cholesterol in cells and inhibits lipoprotein oxidation [8].

HPLC methods coupled with fluorescence detection or UV detection have been developed for the determination of the active components in Danshen or materia medica [6,9–12]. However, some of these methods were not sufficiently specific and sensitive, some were not validated and some were not directly applicable. The sensitivities of the

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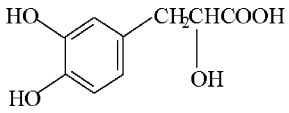

Fig. 1. Chemical structure of Danshensu (DS, 3,4-dihydroxyphenyllactic acid).

above methods are insufficient for determining active components of medicine or herb in large animal or human serum after oral administration.

This method utilizes column switching and high performance liquid chromatography tandem mass spectrometry (CS–HPLC–MS) with negative electrspray ionization (ESI) mode for the determination of DS in CCDP in human serum sample. The method is based on simple sample preparation procedures, following an oral dose of this Chinese medicine and fully automated LC–MS/MS. Mass spectrometry has been recognized to be a powerful technique for the determination of molecular masses and structural analysis for traditional Chinese medicine [13–16]. This analytical method was very useful for carrying out simultaneous studies of sample purification, enrichment and separation and was proved to be suitable for pharmacokinetic studies [17–22].

2. Experimental

2.1. Apparatus

The CS–HPLC–MS analysis system comprised two 1100 HPLC Binary Pumps (Agilent, Waldbronn, DE), a 1100 series Injector with 100M1 sample loop and mounting stand (Agilent), two 1100 Diode-array Detectors (DAD), a mass spectrometer including an ion trap SL LC/MS module, APIESI source, fast power supply, data system and ChemStation SW for data acquisition and data analysis (Agilent), two reversed-phase columns (100 mm × 2.1 mm i.d., 5.0 μm, Agilent SB-C18), and a system of column switching (Fig. 2, Agilent).

2.2. Chemicals and reagents

Compound Danshen Dripping Pills (25 mg/pill, No. 20020312, Tianjin, China) was supplied by Tianshili Pharmaceutical Company (Tianjin, China). DS was provided by Xi'an Jiaotong University (Xi'an, China). Methanol and water were of HPLC reagent grade (Sigma, MO, USA). Others were of analytical reagent grade. Prior to use, the sample solution was filtered through a 0.2-μm membrane filter (Dongkang, Tianjin, China, and 13 mm in diameter).

2.3. Chromatographic conditions

The present HPLC system consisted of two pumps for delivering the mobile phases for the pretreatment column (mobile phase A) and the analytical column (mobile phase B),

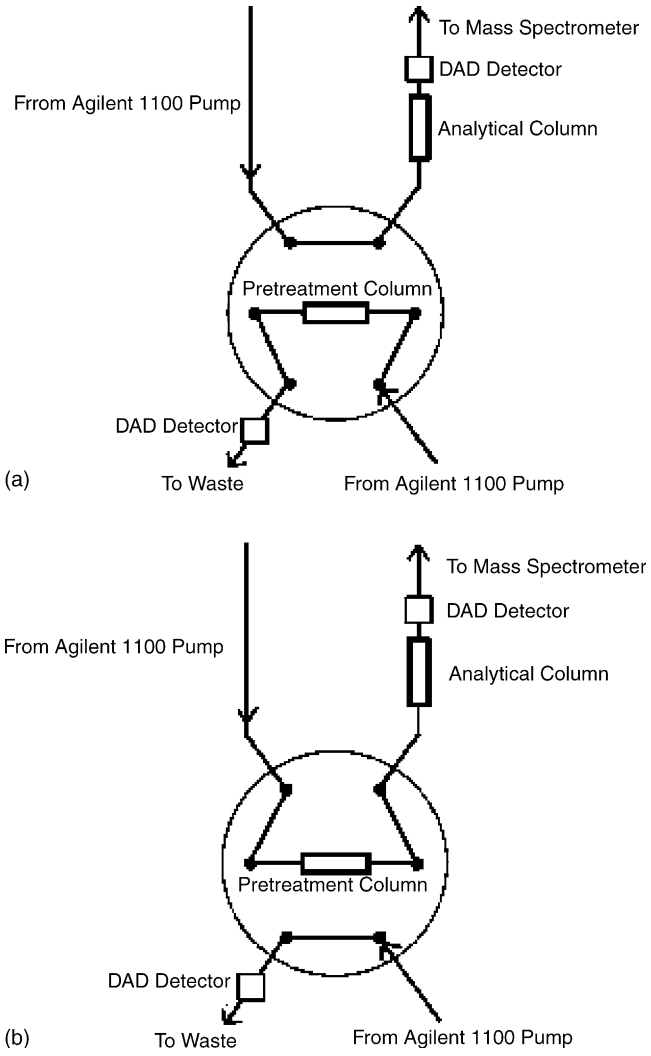

Fig. 2. Column switching system. The Fig. 2a shows the LOAD state flow circuit diagram; the Fig. 2b shows the INJECT state flow circuit diagram. The pretreatment mobile phase and analytical mobile phase flow through pump and were injected into pretreatment column and analytical column, respectively (a). After 5 min of operation, the LOAD state was changed into the INJECT state (b).

respectively. Mobile phase A (for the loading step) consisted of methanol and water (30:70, V/V). Mobile phase B (the injecting step, for elution of the sample to the analytical column and subsequent analysis) consisted of ammonium formate in a solution of methanol–water (ammonium formate, methanol and water, 1:15:85, V/V/V). Both mobile phases were filtered through 0.2-Mm membrane filters before use. The mobile phase A and B were pumped through the system at rate of 0.3 and 0.2 mL/min, separately. The two DAD detectors for columns were interfaced to the Agilent Chromatographic manager system loaded on a Compaq 6400X/CDS computer for deciding switching time of the two columns, data handling and chromatogram generation.

The connections to the switching valve were made in following way. The serum samples were injected into a pretreatment column after dilution. After injection, the samples were

loaded on to the precolumn with mobile phase A for 5 minutes (Fig. 2a). Then the valve was switched to the inject position and the concentrated serum samples were back-flushed into the analytical column for separation and detection by the mass spectrometer (Fig. 2b). After an elution time of 30 min the valve was switched back to the load position for next sample. While the analysis was proceeding the precolumn was equilibrated with mobile phase A and thus was ready for the next injection at the end of the analytical run. The eluate from HPLC was directly transferred into MS.

Mass spectra were recorded in the range *m/z* 50–400 using negative ion electrospray ionization (ESI) mode. The nitrogen nebulizing gas was set at 35 psi with the nitrogen dry gas adjusted to a constant flow rate of 7 L/min. The drying gas temperature was 325 °C.

Prior to each run, the CS–HPLC–MS system was allowed to warm up for 30–40 min. Using freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

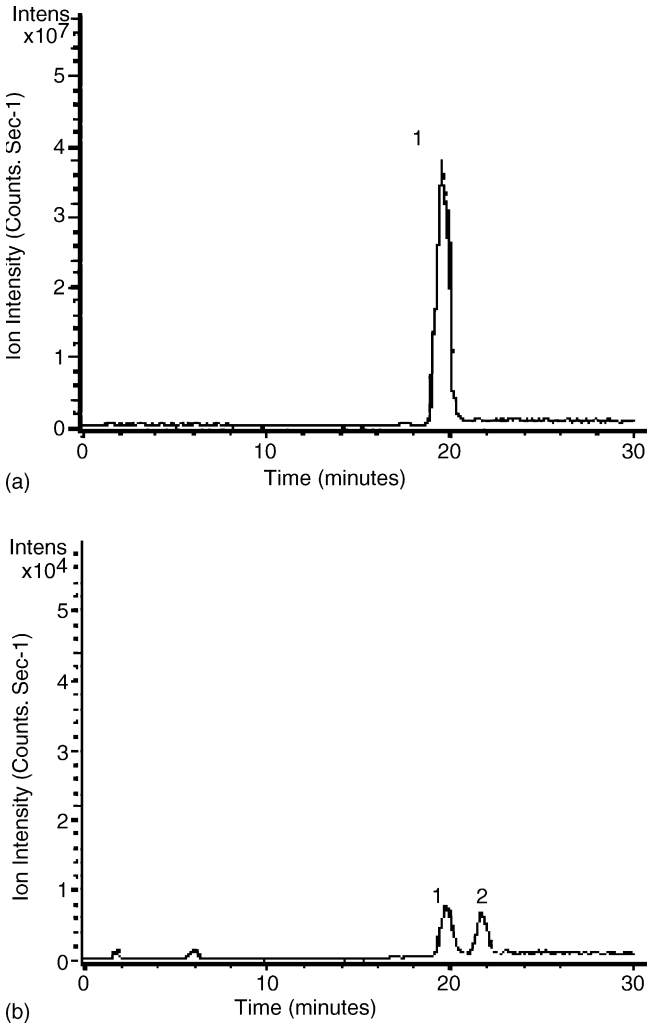

Fig. 3. The negative total ion chromatography (TIC) of DS (a) and serum sample (b). Peak 1 is DS, peak 2 is metabolite found in the serum sample.

2.4. Standard solutions and calibration standards

In a clean, dry 10-ml volumetric flask, reference standard DS 2.5 mg was accurately weighed and dissolved in methanol to make a stock solution. All working solutions were stored at −4 °C and brought to room temperature before use. Calibrators were prepared by diluting stock solution with the blank human serum to make a series calibration standard over the range 1.25–175 μg/mL. All chromatograms obtained were evaluated by peak area measurement. The calibration curves were plotting by peak area against concentration. The data provided a linear function for the compound DS following the equation: $Y = a + bX$ with Y being the log value of the peak area, X the log value of sample amount, a the intercept and b the slope.

2.5. Sample preparation

The blood serums (0.5 mL) were obtained from 10 healthy volunteers treated orally and centrifuged immediately after venipuncture and adding 0.1 mL trichloroacetic acid of 0.3 mol/L at 12,000 × *g* for 10 min; the serum layer was transferred into a tube and dried at 20 °C under a stream of

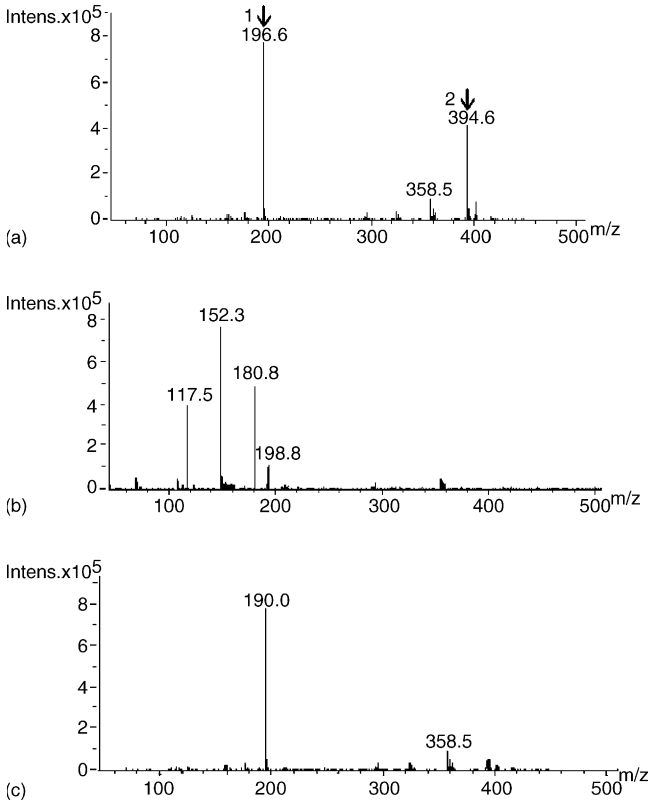

Fig. 4. The Negative-ion ESI-MS and MS/MS Spectra of DS. The two marked peaks (1, *m/z* 196.6; 2, *m/z* 394.6) can be found in spectrum a. The MS/MS spectra of *m/z* 196.6 and *m/z* 394.6 were submitted in b and c. (a) The negative-ion ESI-MS spectra of DS. (b) The negative-ion MS/MS spectra of marked peak 1 in DS. (c) The negative-ion MS/MS spectra of marked peak 2 in DS.

nitrogen. The residue was dissolved in 1 mL analytical mobile phase and stored in polypropylene tubes at −4 °C until the analysis, and 100 ML was injected into CS–HPLC–MS system.

2.6. Pharmacokinetic study

The CS–HPLC–MS method was used to investigate the serum profile of DS after single 250 mg oral dose of CCDP. Blood samples were collected in polypropylene tubes pre-dose (0 h) and at 1, 2, 4, 6, 8, 12, 16 and 18 h post-dose.

3. Results and discussion

3.1. LC–MS/MS analysis

HPLC–MS total ion chromatography obtained for blank serum spiked with DS and for volunteer samples are shown in Fig. 3. The retention time of DS was 19.8 min. Each chromatogram run required approximately 25 min. The ESI-MS spectra were measured in Figs. 4 and 5. The profile of DS concentration in serum against time in volunteers after oral administration of CCDP was presented in Fig. 6. The precursor ion of *m*/*z* 196.6 was used to quantify DS.

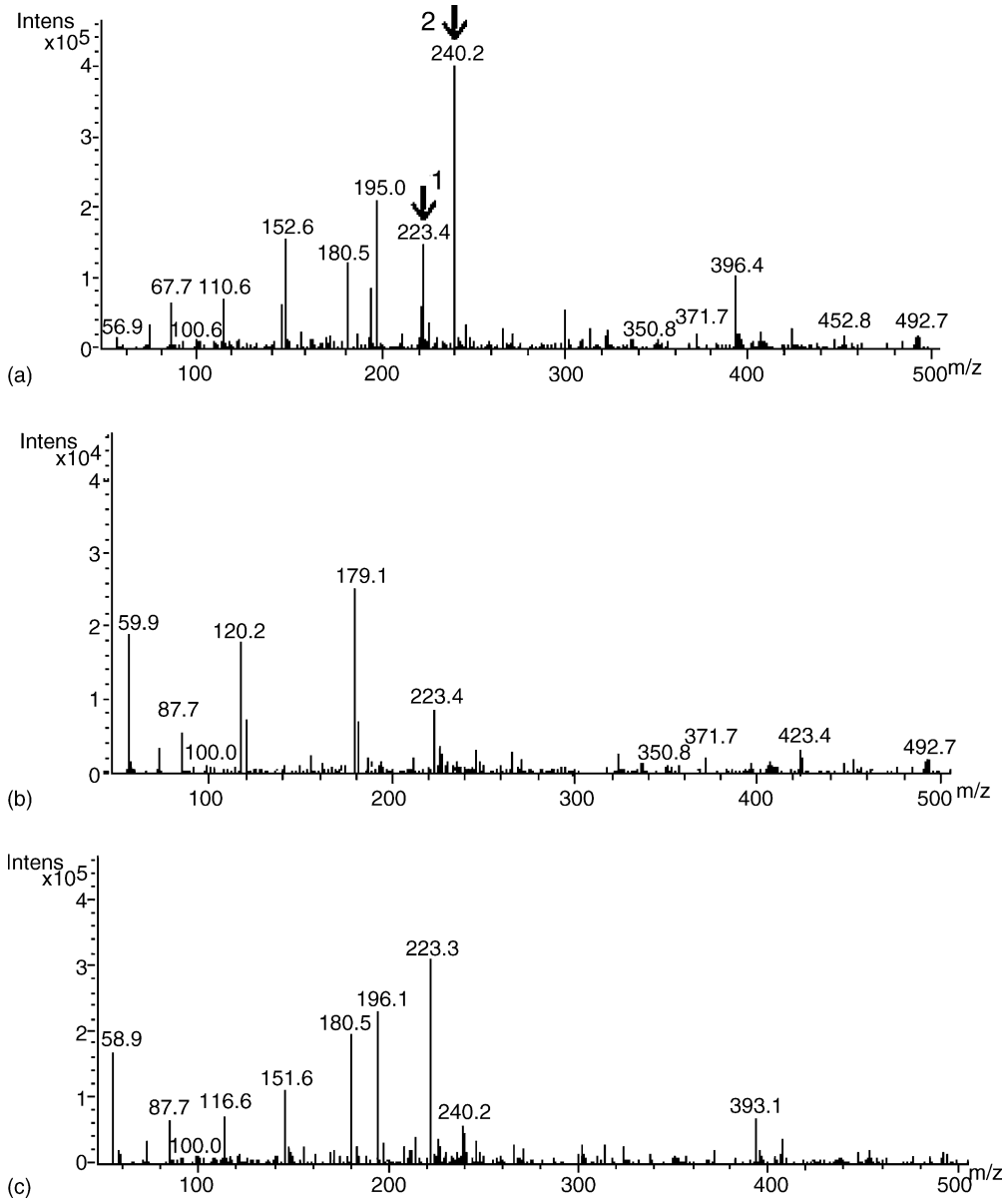

Fig. 5. The negative-ion ESI-MS and MS/MS spectra of serum sample. The two marked peaks (*m*/*z* 223.4, *m*/*z* 240.2, a) were chosen to produce MS/MS spectra (b and c). (a) The negative-ion ESI-MS spectra of serum sample. (b) The negative-ion MS/MS spectra of marked peak 1 in serum sample. (c) The negative-ion MS/MS spectra of marked peak 2 in serum sample.

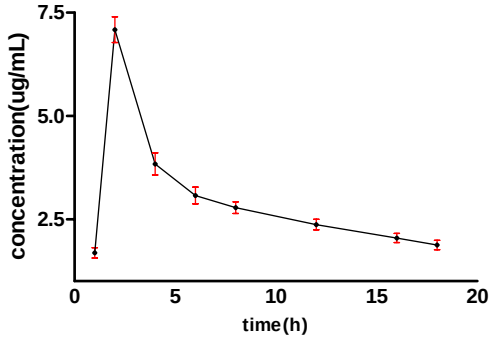

Fig. 6. The mean serum concentration–time curve of DS after oral administration of CDDP to volunteers (dose: 250 mg).

3.2. Linearity, precision and accuracy

The serum calibration curve was constructed of eight calibrators (1.25–175 μg/mL). The calibration curve was linear over the specified range. The calibration standards were prepared and analyzed following the procedures described above. The line equation was $Y = 429.09X - 84.507$. The correlation coefficients were better than 0.9995 ($n = 8$). The detection limit and the low limit of quantification (LOQ) for DS were 0.05 μg/mL (S/N = 3) and 0.15 μg/mL (S/N = 10), respectively (Table 1).

The reproducibility of the method was evaluated by analyzing five calibration samples on three consecutive days ($n = 6$) and calculating the R.S.D.% (precision) and accuracy. During the period of collecting the reproducibility data, the CS–HPLC–MS system was run non-stop for 3 days. The intra-day precision ranged from 3.6 to 7.4% R.S.D. and the accuracy was from 94.2 to 99.5%. The inter-day precision was less than 7.9% R.S.D. and the accuracy ranged from 94.0 to 99.8%. The large R.S.D. and RE were observed on day 3 though the controls were made on each day. The large R.S.D. and RE on the 3rd day might mean that there is instrument fatigue after 48 h of operation. The results of the intra-assay and inter-assay study are listed in Table 2. The results indicate that the method is reliable, reproducible and accurate.

Table 1
Elecrospray tandem mass data of DS standard and serum sample

Compound	MS ion data		MS^2 ion data	
	Fragment ion *m/z*	Relative abundance	Sub fragment ion *m/z*	Relative abundance (%)
DS standard	196.6 $[M - H]^-$	100	180.8 $[M - OH]^-$	65
			152.3 $[M - COOH]^-$	100
			117.5 $[M - COOH–2OH]^-$	54
	394.6 $[2M - H]^-$	54	196.6 $[M - H]^-$	100
			358.5 $[2M - H_2O]^-$	15
Serum sample	396.4 $[2M]^-$	26		
	240.2 $[M + CH_3CO]^-$	100	223.4 $[M + CH_3CO - OH]^-$	100
			196.1 $[M - H]^-$	76
			151.6 $[M - COOH]^-$	67
			116.8 $[M - COOH - 2OH]^-$	24
	223.4 $[M + CH_3CO - OH]^-$	37	179.1 $[M + CH_3CO - OH - COOH]^-$	100
			120.2 $[M + CH_3CO - OH - COOH - CH_3CO]^-$	71
			59.3 $[CH_3CO]^-$	75
	180.9 $[M - OH]^-$	32		
	152.6 $[M - COOH]^-$	39		

Table 2
Intra-day and inter-day precision and accruacy of DS in human serum predicted concentration (μg/mL)

Determined concentration (μg/mL)	Predicted concentration (μg/mL)	Precision R.S.D. (%)	Accuracy (%)
Intra-day precision and accuracy ($n = 6$ replicate samples)			
1.25	1.24 ± 0.04	3.65	98.80
25	23.55 ± 1.62	6.86	94.21
75	74.21 ± 2.98	4.02	98.94
150	149.24 ± 7.30	4.89	99.49
175	173.91 ± 12.84	7.38	99.38
Inter-day precision and accuracy ($n = 3$ days of replicate samples)			
1.25	1.23 ± 0.05	3.83	98.66
25	24.52 ± 1.94	7.89	98.07
75	70.52 ± 4.80	6.80	94.02
150	149.65 ± 5.34	3.57	99.76
175	168.50 ± 7.16	4.25	96.28

3.3. Pharmacokinetic study

The pharmacokinetic study was performed in ten volunteers after administration of CDDP at a single oral dose of 250 mg. Serum concentration–time curves of DS after oral administration of CDDP are shown in Fig. 6. The details of the pharmacokinetic data will be described in a separate publication.

4. Conclusions

The described high performance liquid chromatography mass spectrometry (LC–MS) method with online sample clean up by column switching is for the determination of DS in human serum. With the using of column switching system, sample was purified and enrichmented effectively on line. The identity of the compound was confirmed using ESI-MS analysis; it provides 0.05 μg/mL detection limit and 0.15 μg/mL low limit of quantification. The validated analytical method was applied to pharmacokinetic study. It has been to be linear, precise and accurate in the concentration range 1.25–175 (g/mL; therefore, it is suitable for pharmacokinetic study of DS in CDDP or other Danshen preparations.

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