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Short communication

Development of a gradient reversed-phase HPLC method for the determination of sodium ferulate in beagle dog plasma

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Abstract

A gradient reversed-phase HPLC assay has been developed to determine sodium ferulate (SF) in beagle dog plasma with tinidazole as an internal standard. Chromatographic separation was made on a C_{18} column using 0.5% acetic acid and acetonitrile (80:20, v/v) as mobile phase. UV detection was performed at 320 nm. The calibration curve for SF was linear in the range of 0.05–10 μg/ml, and the achieved limit of quantification (LOQ) was 51.4 ng/ml. The results of linearity, within- and between-day precision, and accuracy demonstrate that this method is reliable, sensitive and sufficient for *in vivo* beagle dog pharmacokinetic (PK) studies of SF.

Keywords: Sodium ferulate; HPLC

1. Introduction

Some Chinese crude drugs, such as Angelica sinensis, Cimicifuga heracleifolia, Ligusticum chuanxiong, etc., contain the effective antioxidant ferulic acid (3-hydroxy-4-methoxycinnamic acid). For the labilization of ferulic acid [1], synthesized sodium ferulate (SF, Fig. 1) or 3-methoxy-4-hydroxycinamate sodium has been approved by State Food & Drugs Administration of China and is used as a drug for patients with cardiovascular and cerebrovascular diseases [2]. Several studies have been published showing the quantifications of ferulic acid in some Chinese crude drug or patent medicines performed by HPLC [3–7], and quite a few studies employed the HPLC method to measure ferulic acid in plasma samples [8–12]. However, few reports concern the determination of SF in biological fluids. According to our knowledge, little information about sufficient method was available for the pharmacokinetic (PK) studies on SF.

In our preliminary trials, two endogenous substances in beagle dog plasma were extremely difficult to be removed under the optimized extraction condition and were, thus, present in HPLC chromatogram at retention times much longer than SF. These two substances were also observed in blank plasma and might interfere with the analysis of SF when encountered with multitude plasma samples. However, no reported analytical method had mentioned this phenomenon.

In this paper, a gradient reversed-phase HPLC method was developed and validated for quantitative analysis of SF in beagle dog plasma. Furthermore, the method was successfully applied to the plasma pharmacokinetic study of SF in beagle dogs after oral administration.

2. Experimental

2.1. Chemicals, reagents and animals

Sodium ferulate was obtained from the Institute for the Control of Pharmaceutical and Biological Products of China. The internal standard tinidazole (Fig. 1) was from Jichuan Pharmaceutical Ltd. Co. (Jiangsu, China). Sodium ferulate tablets (50 mg sodium ferulate per tablet) were purchased from Hengda Pharmaceutical factory (Chengdu, China). Methanol and acetonitrile (gradient grade) were purchased from Merck (Darmstadt, Germany). HPLC grade aether and acetic acid came from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade.

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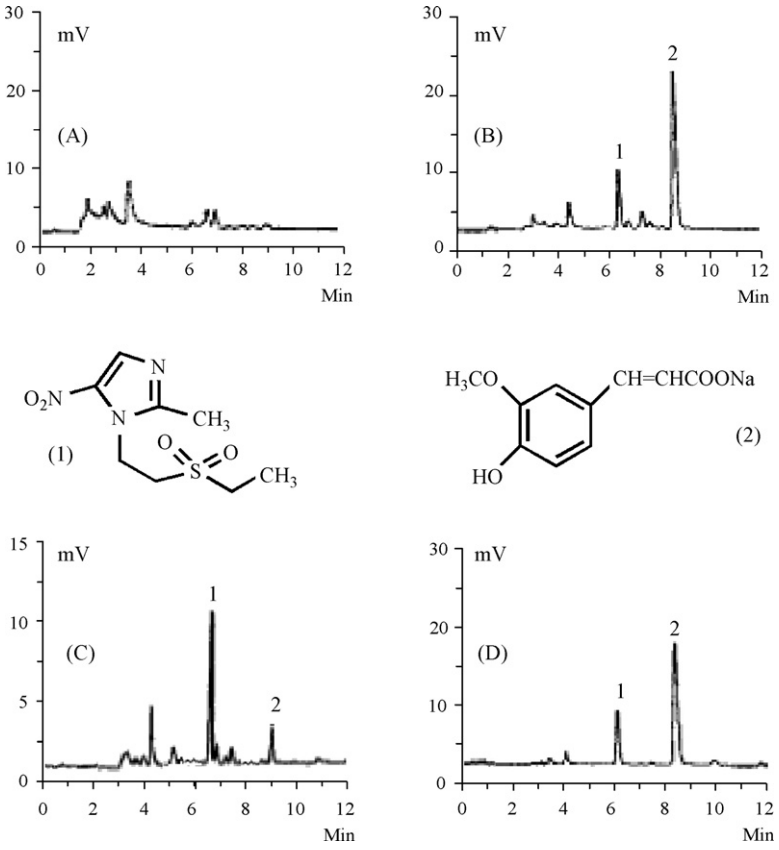

Fig. 1. Representative chromatograms of internal standard tinidazole (1) (2.5 μg/ml) and SF (2). A: blank plasma; B: quality control sample (0.2 μg/ml); C: the lower calibration sample (0.05 μg/ml); D: plasma sample (0.18 μg/ml).

Adult beagle dogs (10 ± 2 kg) were obtained from Zhenhe animal center for scientific research (Fuzhou, China). All procedures described in this study were approved by the Animal Care and Supply Committee of Second Military Medical University.

2.2. Chromatographic and detection conditions

The analyses were carried out using a LC-10A HPLC system (Shimadzu, Japan) consisting of two LC-10AT pumps (Shimadzu), a Rheodyne 7125 manual injection valve with 20 μl sample loop, an SPD-10A UV detector (Shimadzu) set at 320 nm, and a C-R8A data processor (Shimadzu). The chromatographic separation was accomplished on a C_{18} column (150 mm × 4.6 mm, 5 μm, Kromasil, Bohus, Sweden) protected by a guard column (15 mm × 2 mm, PelliguardTM LC-18, Supelco, Bellefonte, PA) at ambient temperature.

A gradient method was worked out, with two parameters changing linearly during analysis: acetonitrile content and flow rate. The flow of the mobile phase which consisted of 0.5% acetic acid and acetonitrile (80:20, v/v) was set at a rate of 0.8 ml/min until the peaks of SF and tinidazole were detected. Then 100% acetonitrile and 1.3 ml/min flow rate were adjusted instantly to elute the less polar endogenous substances. After this, mobile phase of 20% acetonitrile was regained to stabilize the chromatographic system at 0.8 ml/min flow rate. The analyses were repeated according to the gradient elution at every analytical cycle.

2.3. Preparation of stock solutions, working solutions and quality control (QC) samples

Protected from light, a stock solution was prepared by dissolving 40 mg of SF in water in a 10 ml brown volumetric flask. This stock solution was diluted in water to obtain working solutions of 0.2, 0.4, 0.8, 2, 4 and 40 μg/ml. Another stock solution of tinidazole was also prepared in methanol at a concentration of 20 μg/ml and used as the internal standard solution. The QC samples in the SF concentration range of 0.05–10 μg/ml were prepared by mixing working solutions with blank plasma, and three freeze–thaw (−20 °C/room temperature) cycles were adopted to investigate the stability of SF in beagle dog plasma.

2.4. Sample preparation and extraction

The calibration standards were obtained by mixing 0.4 ml blank plasma with appropriate dilutions of the SF working solution in a 10 ml glass centrifuge tube. The samples were vortexed to achieve final SF concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 10.0 μg/ml, respectively. Fifty microlitre of tinidazole solution (20 μg/ml) and 200 μl of 0.6 N hydrochloric acid were added into the calibration samples. After 5 s of vortex, 3 ml

of aether was added to extract SF and tinidazole from plasma. Subsequently, the samples were vortexed for 5 min and then centrifuged at 3000 × *g* for 10 min. The combined supernatants were transferred into a new glass tube, and the organic phase was evaporated at 40 °C under a stream of nitrogen. The dried residue was reconstituted with 200 μl of mobile phase, and an aliquot of the extract was injected into the chromatographic system.

2.5. Validation procedures

After chromatographic analysis of the standard calibration samples, the peak heights of SF and internal standard were calculated. The least square linear regression was used to fit the peak height ratio of SF to internal standard versus SF concentration. The precision and accuracy of the measurements were determined from the QC samples for the linearity-established method. Within-day precision was defined as relative standard deviation (R.S.D.) calculated from the values measured from five QC samples at concentrations of 0.05, 1.0 and 10.0 μg/ml, respectively, in one day. Within-day accuracy was defined as relative value on the same measurements of within-day precision. Between-day precision and accuracy were calculated using the values measured from the same QC samples on five consecutive days.

2.6. Application of the analytical method

In order to evaluate the applicability of the method for *in vivo* pharmacokinetic studies, plasma SF concentrations were assayed as followed by oral administration of SF tablets to beagle dogs. After a single oral administration of 150 mg SF, 2 ml of blood sample via the foreleg vein was taken and placed into centrifuge tube containing heparin. Then, the sample was centrifuged at 3000 × *g* for 10 min to get plasma, and 400 μl of plasma was collected and analyzed by the method described above. To provide an estimate of pharmacokinetics for SF, blood samples were obtained at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h after drench.

3. Results and discussion

3.1. Chromatographic separation and detection

As the easily water-soluble SF is usually hard to be retained in chromatogram behaviors, samples were acidified for convenient chromatographic analysis. As shown in Fig. 1, the internal standard and the assay compound have specific and independent chromatographic spikes when measured at the wavelength of 320 nm. The separation was complete with a baseline resolution. Less interferential peak information has been found among SF, the internal standard and previous endogenous substances. Assay of the plasma samples resulted in two quantifiable chromatographic peaks with retention times of 6.3 min for the internal standard and 8.9 min for SF (Fig. 1 B, C and D).

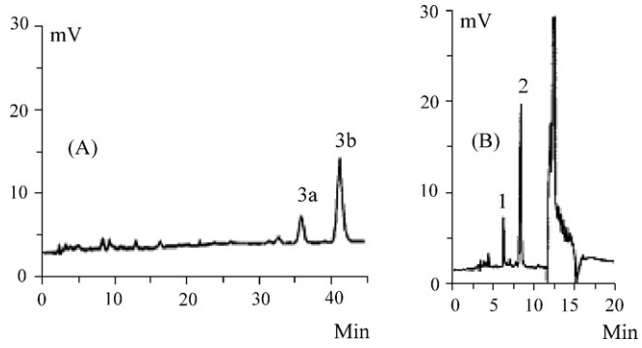

Fig. 2. Chromatograms of a blank plasma sample (A) without gradient elution and a plasma sample (B) eluted in gradient method. 1: internal standard (2.5 μg/ml); 2: SF (0.27 μg/ml); 3a, 3b: two endogenous substances.

Although no interferences were observed before 10 min in blank plasma samples, two unwanted peaks were observed to appear at retention times after 30 min (Fig. 2A), and their retention times remained largely unaffected even by using extraction solvents (methonal, acetonitrile, chloroform and acetic ether) and changing the mobile phase. The used double gradients of flow rate and acetonitrile content in mobile phase could successfully accelerate the outflow of the latter two endogenous substances after the aimed peaks appeared, and the two uninteresting peaks were retained between10 and 13 min (Fig. 2B). So, their chromatographic behaviors would not overlay and cause interferences to the next injected samples during the followed analysis circle of 16 min.

3.2. Method validation

The integrated peak height ratio of SF to internal standard increased linearly as the concentration of SF raised. Over the plasma concentration range of 0.05–10.0 μg/ml, regression analysis indicated that there was an excellent linearity between UV

Table 1
Within- and between-day precision and accuracy of quantifying SF in beagle dog plasma samples using the described HPLC method

Actual concentration (μg/ml)	Detected concentration (mean ± S.D., $n = 5$)	Precision (R.S.D.) (%)	Accuracy (%)
Within-day			
0.05	0.052 ± 0.004	7.51	104.00
1.0	1.02 ± 0.04	4.03	102.00
10.0	9.75 ± 0.24	2.45	97.50
Between-day			
0.05	0.051 ± 0.005	9.52	102.00
1.0	1.01 ± 0.007	6.67	101.00
10.0	9.79 ± 0.23	2.35	97.90

S.D.: standard deviation; R.S.D.: relative standard deviation.

absorption and SF concentrations, and the average correlation coefficient was 0.9995 ($n = 5$). The slope and the intercept were 0.166 and −0.239, respectively.

The acceptable within- and between-day precisions were set <10%, and the accuracy should be controlled between 95 and 105%, respectively [13]. Method reproducibility and repeatability were evaluated by five-replicated analysis of standard plasma samples. The calculated R.S.D. and accuracy values are summarized in Table 1.

It can be seen from Table 1 that the within-day precision, expressed by the coefficient of variation of observed concentrations of the routine QC samples, was less than 7.51%, whereas the within-day accuracy, expressed by the calculated bias between observed and theoretical concentrations, ranged from 4.0 to −2.5%, and the between-day precision and accuracy were, less than 9.52% and between 97.9 and 102%, respectively.

The limit of detection (LOD), defined as the amount of SF corresponding to a signal-to-noise ratio of 3:1 [14], was 6.15 ng/ml. The limit of quantification (LOQ), determined as the lowest concentration of the analyte in plasma with an accuracy of ± 20% and a coefficient of variation <20% [15], was 51.4 ng/ml for the determination and quantification of SF.

Extraction recovery of SF was examined by comparing the peak heights obtained from extracted plasma samples with those found in blank plasma, spiked with a known amount of SF, through the extraction procedure. The results showed that the mean extraction recoveries of SF were 95.98 ± 1.7, 97.6 ± 2.3 and 99.12 ± 0.9% at concentrations of 0.1, 1.0 and 10.0 μg/ml, respectively. The mean extraction recovery of the internal standard was 98.2 ± 1.7%.

SF in plasma samples was found to be stable (R.S.D. <7%) after three freeze–thaw cycles. The analytes were also shown to be stable within 24 h of storage in reconstitution solutions at room temperature (R.S.D. <6%). The good stability of SF simplified the handling of plasma samples during the analytical procedures.

3.3. Application of the analytical method in pharmacokinetic studies

SF was nearly detected at all the planned sampling times. The retention times of SF and internal standard were changing with narrow range during day 1 and the consecutive days. The method was found to be suitable for the generation of plasma concentration–time curves and the subsequent determination of pharmacokinetic parameters for SF. The mean plasma concentration for nine dogs each receiving 150 mg of SF tablets was shown in Fig. 3.

As deduced from Fig. 3, SF could be absorbed quickly from gastrointestinal tract. The peak concentration of 2.97 μg/ml was arrived at about 40 min, and the $t_{1/2}$ of SF in beagle dog was 19 min. Drug was eliminated quickly, and the plasma level of SF was extremely low after 2 h.

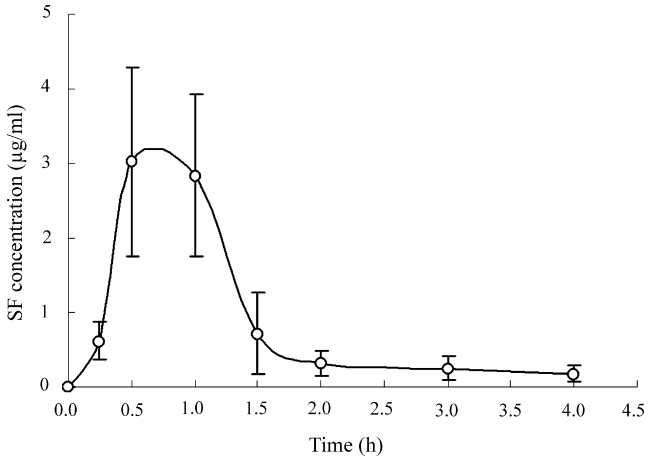

Fig. 3. Mean plasma concentration–time curve for SF following administration of 150 mg SF tablets to beagle dogs. The data shown as the mean ± S.D.

4. Conclusion

The described gradient reversed-phase HPLC method could offer a short chromatographic run to determine SF in plasma samples with advantageous sensitivity and selectivity, and it is suitable for carrying out PK-studies of SF in beagle dog. Further method validation is still needed for future PK-studies of SF-loaded drug delivery system in other animals or clinical patients.

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