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LC-MS/MS Method for Simultaneous Determination of Pteryxin and Bergapten in Rat Plasma for Pharmacokinetic Study

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A sensitive LC-MS/MS method for simultaneous determination of pteryxin and bergapten in rat plasma was established to analyze the pharmacokinetics. Plasma samples were extracted using methanol with isoimperatorin as an internal standard (IS). Separation was carried out on a C_{18} column using a mixture of water and methanol (30:70, v/v) as the mobile phase. The effluent was monitored by UV detector at 300 nm and at a flow rate of 1 mL/min and 30 ºC. The linearity ranges of proposed method were 0.132 5-53.00 μg/L for pteryxin and 8.12-162.4 μg/L for bergapten. The intra-day and inter-day RSD of the assay method for the two components were less than 10 % and mean recovery was within the 83.24-91.27 % range. The method was found to be precise, accurate and specific during the study and was successfully used to analyze the pharmacokinetic of pteryxin and bergapten. Pharmacokinetic data of pteryxin and its metabolites were obtained with this method after oral administration.

Key Words: LC-MS-MS, Pteryxin, Bergapten, Pharmacokinetic.

INTRODUCTION

Coumarin and its derivatives are widely distribution in the plant kingdom, especially in umbrelliferae, leguminosae, rutaceae and compositae. Coumarin has many aspects of physiological activity, such as antitumor[1], fight cell hyperplasia[2], fight blood-vessel sclerosis[3,4] and enhance immunity. Coumarin compounds not only can directly suppresses offered compounds cancer cells and can be produced by enhancing immunity in medicine anticarcinogenic properties, thus applications very extensive. *Peucedanum harry-smithii var subglabrum* is the main variety in Gansu, are mian product in Longnan, Tianshui and Longdong. *Peucedanum harry-smithii var subglabrum* is regarded as Radix Peucedani has a long history, is the local full-grown varieties as Radix Peucedani[5]. Pteryxin is the highest content in *Peucedanum harry-smithii var subglabrum*[6]. And as one of its bioactive component[7], the latest research results show that pteryxin have anticancer and anti immunodeficiency virus function, tip is expected to become the new cancer and AIDS prevention and treatment[8,9]. Bergapten is the furocoumarin monomer compounds extracted from *Peucedanum harry-smithii var subglabrum*. Bergapten exists in various plants, but bioactive research is less. Yin and Chen[10] reported that bergapten have the inhibition on human stomach cancer cells and colon cancer cells for 31 and 34 %, respectively. Bergapten can significantly inhibit xylene-induced ear edema and hind paw edema, can restrain the number of body torsion caused by acetic acid[11], bergapten have apparent analgesic effect. To elucidate the pharmacokinetics of pteryxin and bergapten, a rapid, simple and accurate method is required for their simultaneous determination. The pharmacokinetic process belongs to two-compartment model, pteryxin after gastrointestinal absorption into metabolite 2-methyl butyric acid, these results provide a firm basis for evaluating the clinical efficacy of pteryxin and bergapten and for the further research and development.

EXPERIMENTAL

Pteryxin (purity > 97 %, by HPLC) and bergapten (purity > 90 %, by HPLC) for pharmacokinetic study were isolated from the *Peucedunum harry-smithii var subglabrum* in a prior study. The structures of pteryxin and bergapten were assayed by NMR and MS and the structures are given in Fig. 1. The reference substance of 2-methyl butyric acid and bergapten were purchased from Alfa Aesar Company and the internal standard isoimperatorin was obtained from Sigma Chemical Co (St. Louis, MO, USA), the chemical structures are shown in Fig. 1. Methanol was of HPLC grade (Merck, Germany). All other reagents used were of analytical grade. The double-distilled water provided by the First Appendix Hospital of Lanzhou University was used during the entire HPLC procedure.

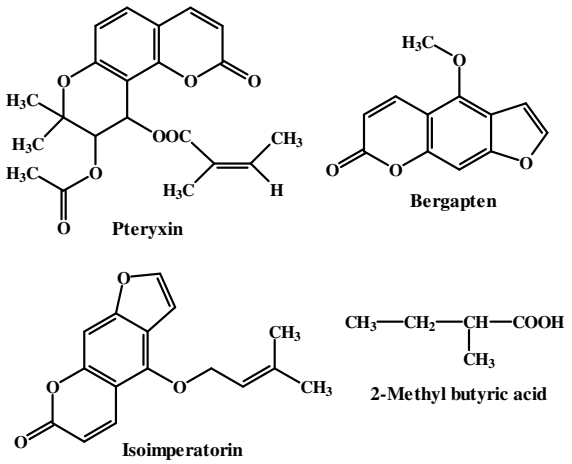

Fig. 1. Chemical structure of pteryxin, bergapten, isoimperatorin and 2-methyl butyric acid

Chromatographic condition: The HPLC system (Waters Alliance 2487 HPLC, USA) consisted of 2996 photodiode array detection and Waters 717 automatic injector. The analytical column used was Hypersial ODS C_{18} column (250 mm × 4.6 mm i.d., particle size 5 μm) at a temperature of 30 ºC. The mobile phase consists of methanol and water 70:30 (V/V). The effluent was monitored at 300 nm, at a flow rate of 1 mL/min.

Mass spectrometry: The MS instrument consisted of a Waters Micromass Quattro MicroTM triple-quadrupole system (Manchester, UK). The MS system was controlled by version 4.0 of MassLynx software. Ionization was performed in the positive electrospray mode. MS conditions were the following: capillary voltage 3.5 kV, cone voltage 15 V, extractor voltage 2 V, RF lens voltage 0.1 V. The source and desolvation temperatures were 100 and 400 ºC, respectively and the desolvation and cone gas flows were 500 and 50 L/h, respectively.

Plasma sample preparation: SD rats (Laboratory Animals Center, Lanzhou University, Lanzhou, China) weighing 200-220 g were housed under standard conditions (20 ºC, 12 h light-dark cycle) with unlimited access to standard food and water. The experimental procedures were carried out in accordance with the guidelines of the European Community, local laws and policies and were approved by Lanzhou Animal Protection Ethical Committee. Pteryxin and bergapten were suspended in water and singly administered orally at a dose of 160 mg/kg (n = 8) (containing 80 mg pteryxin and 80 mg bergapten) to rat. Blood was collected from each animal in heparinized tubes by orbit venous plexus at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24, after administration and plasma was obtained by centrifuging at 6000 rpm for 20 min at 4 ºC. Plasma samples were stored at -20 ºC until analyzed.

An aliquot of 500 μL of plasma in a centrifugation tube was spiked with 1 mL of methanol (when preparing calibration and quality control samples, standard solution was added instead of methanol) and 500 μL of internal standard solution (10 μg/mL). After mixing by UW at 400 w and 25 kHz for 10 min, vortexed to mix for 1 min, centrifugation at 6000 rpm for 10 min, the supernatant was transferred to another tube and 10 μL of the sample was injected into the LC-MS-MS system for analysis.

Preparation of the calibration standards and quality control (QC) samples: Standard stock solutions of pteryxin and bergapten were prepared with methanol at concentrations of 1.325 and 50 g/L, respectively. Working solutions for pteryxin standards were prepared by dilution of the stock solution with methanol to cover the concentration range 1.325-530.0 μg/L for pteryxin and 81.2-1624 μg/L for bergapten. Spiked samples for method validation were prepared by adding 10 μL of the working standard solution to 100 μL of plasma obtain concentration levels of pteryxin and bergapten of 0.1325-53.00 and 8.12-162.4 μg/L, respectively. Quality control samples at three different levels within linear concentration ranges. All the stock solutions were refrigerated at 4 ºC when not in use. To prepare samples for analysis, frozen plasma samples were thawed at room temperature and stirred thoroughly. A 500 μL aliquot of the plasma sample was extracted with 1 mL of methanol to mixing by UW at 400 w and 25 kHz for 10 min. After vortexed to mix for 1 min, centrifugation at 6000 rpm for 10 min, the supernatant was transferred to another tube and 10 μL of the sample was injected into the LC-MS-MS system for analysis (representative chromatograms were shown in Fig. 2).

Assay validation: The intra- and inter-day precision and accuracy were assessed by the determination of spiked samples of rat plasma extract at three concentration levels in five replicates on days 1, 2, 3, 5 and 7. The precision was expressed by percentage coefficient of variation (CV) and the accuracy by percentage relative error (RE). The intra- and inter-day precisions were required to be below15 % and the accuracy to be within 15 % for both of the two components except at the lower limit of quantification (LLOQ), where precision should be below 20 % and accuracy within 20 %.

The LLOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. The LOD was determined using the signal-to-noise ratio (S/N) of 3:1 by comparing test results from samples with known concentrations of analyte with blank samples.

The recoveries of pteryxin and bergapten were determined for quality control samples at three different concentration levels. Five replicates of each sample were spiked with the analyte prior to extraction and injected into the HPLC system. The extraction recovery at each concentration of pteryxin and bergapten was calculated using the following equation: recovery = (peak area after extraction/peak area after direct injection) × 100.

Stability studies: To ensure the reliability of the results in relation to handling and storing of plasma samples and stock standard solutions, stability studies were carried out at three different concentration levels. Freeze and thaw stability was determined at three different concentration levels too. The samples were obtained by thawing at room temperature for 1-12 h and then refreezing at -20 ºC for 1 month. The stability of spiked mice plasma and tissue samples stored at room temperature (bench top stability) was evaluated for 12 h and compared with freshly prepared extracted samples. The long-

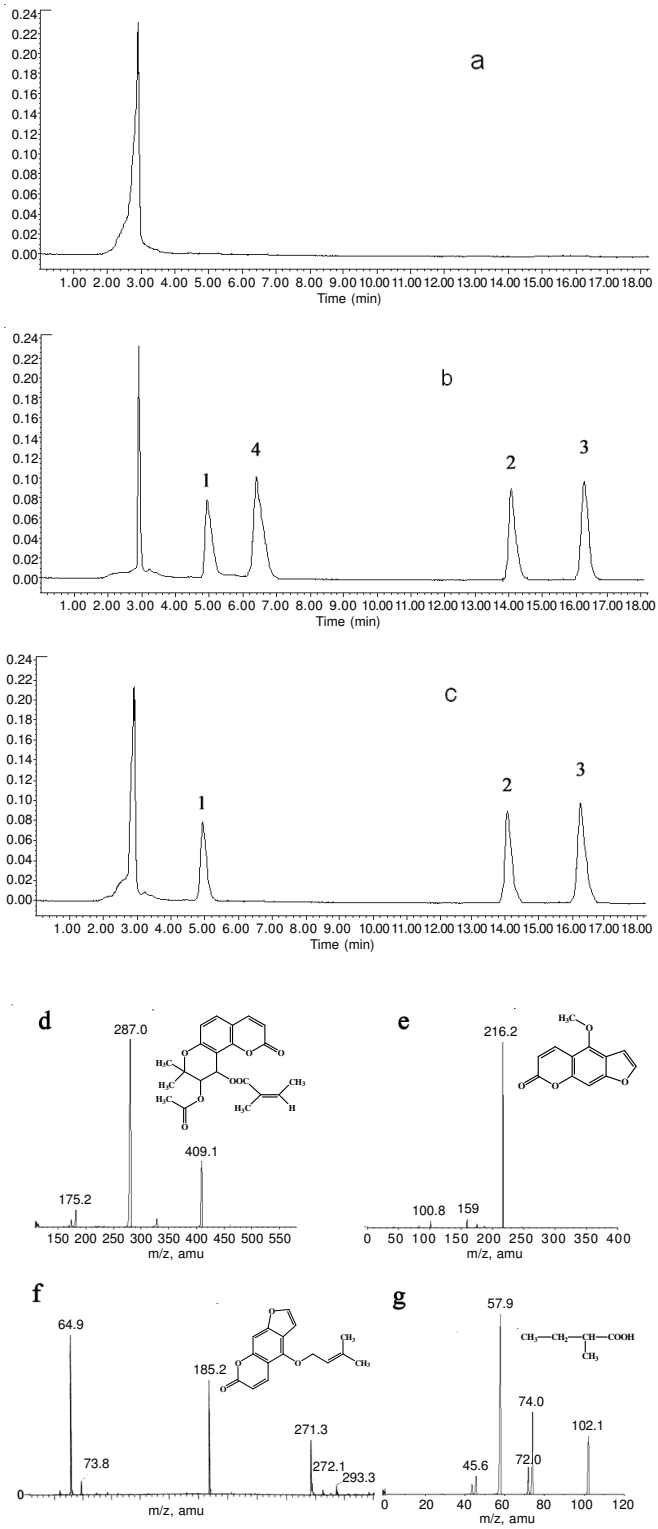

Fig. 2. Representative chromatograms of (a) blank (drug free) plasma, (b) a rat plasma sample 3 h after a single oral administration of a mix of pteryxin and bergapten, (c) blank plasma spiked with pteryxin, bergapten and internal standard; full-scan mass spectra of pteryxin (d), bergapten (e), isoimperatorin (f) and 2-methyl butyric acid (g). (1-bergapten; 2-pteryxin; 3-IS isoimperatorin; 4-metabolite: 2-methyl butyric acid)

term stability of pteryxin and bergapten in mice plasma was assessed by carrying out the experiment after 1 month of storage at -20 ºC. The stock solution stability of pteryxin and bergapten were determined at room temperature for 12 h and upon refrigeration (4 ºC) for 1 month. The concentrations of pteryxin and bergapten after each storage period were related to the initial concentration as determined for the samples that were freshly prepared.

RESULTS AND DISCUSSION

Quantification and calibration curve: The peak-areas of pteryxin and bergapten in the plasma were used for the quantification of pteryxin and bergapten in samples. The calibration curves of pteryxin and bergapten in plasma were shown in Table-1. The correlation coefficients obtained were higher than 0.9992.

TABLE-1
DATA ON CALIBRATION CURVES FOR PTERYXIN AND BERGAPTEN IN DIFFERENT BIOLOGICAL SPECIMEN (n = 6)

Compound	Calibration curve	Concentration range (μg L^{-1})	r
Pteryxin	Y = 664.5X–0.992	0.1325–53.00	0.9992
Bergapten	Y = 912.0X–0.5204	8.12-162.4	0.9999

Accuracy and precision: The accuracy and precision of the method were evaluated with quality control samples at three different concentration levels for pteryxin and bergapten, respectively. The intra-day precision (expressed as RSD) and accuracy were determined by analysis of five replicates of quality control samples at three different concentrations. The inter-day accuracy and precision were determined on five different days and the results are shown in Table-2. The inter-day and intra-day precisions of the quality control samples were satisfactory with RSD less than 10 %. The accuracy was within 97.2-103.4 % and RSD less than 6 % too.

Low limit of quantity (LLOQ) and limit of detection (LOD): LLOQ was established by determining the concentrations of three spiked calibration standards. The LLOQ of the method was found to be 0.1325 μg/L for pteryxin and 4.06 μg/L for bergapten in mice plasma with coefficient of variation less than 20 % and accuracy of 80-110 %. The LOD was determined to be 0.062 μg/L for pteryxin and 0.89 μg/L for bergapten based on a signal to noise (s/n) ratio of 3:1.

Recovery: The extraction recovery was determined by standard addition at three different concentrations for pteryxin and bergapten, respectively. The extraction recovery was calculated by comparing the peak areas of the prepared standard samples with those of the standard solutions; the results are shown in Table-3. The extraction recovery of pteryxin at 1.325, 26.5, 53.0 μg/L was (83.24 ± 8.31), (85.46 ± 7.17) and (91.27 ± 2.34) %, respectively. The extraction recovery of bergapten at 8.12, 81.2, 162.4 μg/L was (85.59 ± 6.51), (88.67 ± 4.33) and (86.33 ± 7.87) %, respectively. The mean recovery of pteryxin and bergapten was found to be (86.66 ± 5.9) and (86.86 ± 6.24) %, respectively. The recovery of pteryxin and bergapten using the described procedure was consistent and efficient.

Stability: Analysis of the working solution was performed at 53.0 and 162.4 μg/L of pteryxin and bergapten. After storage for 1 month at 4 ºC and at room temperature for 12 h, more than 99 % of pteryxin and bergapten remained unchanged, based on peak areas in comparison with freshly prepared

TABLE-2
INTRA-DAY AND INTER-DAY PRECISION AND ACCURACY DATA OF PTERYXIN AND BERGAPTEN IN SPIKED PLASMA SAMPLE (n = 5)

Compound	Spiked concentration (μg/L)	Observed concentration (μg/L)	Accuracy (% n = 5)	RSD (%)	Precision (RSD %)	
					Intra-day (n = 5)	Inter-day (n = 5)
Pteryxin	1.325	1.357	102.4	3.36	3.71	8.31
	26.5	26.3	99.2	2.58	5.36	7.17
	53.0	51.49	97.2	5.47	3.82	2.34
Bergapten	8.12	8.05	99.1	3.66	5.21	5.03
	81.2	84.0	103.4	4.01	6.87	8.02
	162.4	159.2	98.0	5.31	8.28	9.49

TABLE-3
RECOVERY OF PTERYXIN AND BERGAPTEN FROM PLASMA

Compound	Spiked conc. (μg/L)	Observed conc. (μg/L)	Recovery (% n = 5)	RSD (%)
Pteryxin	1.325	1.103	83.24	8.31
	26.5	22.6	85.46	7.17
	53.0	48.4	91.27	2.34
Bergapten	8.12	6.95	85.59	6.51
	81.2	72.0	88.67	4.33
	162.4	140.2	86.33	7.87

solution of pteryxin and bergapten working solution. This suggests that pteryxin and bergapten in standard solution were stable for at least 1 month when stored at 4 ºC and for 12 h at room temperature. Bench top stability of pteryxin and bergapten in mice plasma was investigated at the concentrations of 1.325, 26.5, 53.0 μg/L and 8.12, 81.2, 162.4 μg/L for pteryxin and bergapten, respectively and the results revealed that both of them in plasma were stable for at least 12 h at room temperature with an average percentage of above 98 %. Long-term stability of the pteryxin and bergapten in plasma at -20 ºC was also performed after 30 d of storage at three levels (1.325, 26.5, 53.0 μg/L and 8.12, 81.2, 162.4 μg/L), which showed mean percentage concentration of them above 98 %. The results of the stability studies indicated that both of the components were stable in the studied conditions.

Identification of metabolite: In the determination of blood samples collected at different time points, we found there was one peak before the peak of pteryxin, vary regularly with time. When scanned by Q1, the quasi-molecular ion peak (m/z) was 102.1. When scanned its daughter ion, the major fragment ion peak (m/z) was 57.9. Compared the quasi-molecular ion peak with pteryxin which was 386, we found this unknown compound is the fracture fragments on the 10th position of pteryxin. So we guess it is the fragment ion of 2-methyl butyric acid. The mass-spectrogram of standard preparation of 2-methyl butyric acid is consistent with this metabolite on the molecular weight and the major fragment ions, so we made sure it is 2-methyl butyric acid.

Application of the method to a pharmacokinetic study in SD rats: Pharmacokinetic of the mix concentration-time data was performed using DAS Version 2.0. The pharmacokinetic parameters were analyzed based on statistical moment theory for the plasma concentration-time data of pteryxin and bergapten. The validated analytical method was applied to the assay of pteryxin and bergapten in rat plasma after a single oral administration of 160 mg/kg mixed solution (containing 80 mg pteryxin and 80 mg bergapten) to rat. The plasma samples were processed based on the proposed extraction protocol for the quantification of pteryxin and bergapten. Mean plasma concentration *vs.* time profile is presented in Fig. 3. The pharmacokinetic parameters estimated were shown in Table-4. After oral administration of the mix at a single dose in SD rats, peak concentrations of pteryxin and bergapten were 142.78 and 128.80 μg/L (C_{max}) reached 2.0 and 3.0 h (T_{max}), respectively. The half-life and area under plasma concentration (AUC_{0-t}) were found to be 4527.251 and 1093.216 μg/L/h, respectively. Blood drug concentration vs time profiles were all fitted in a two-compartment model and the pharmacokinetic results were reasonable.

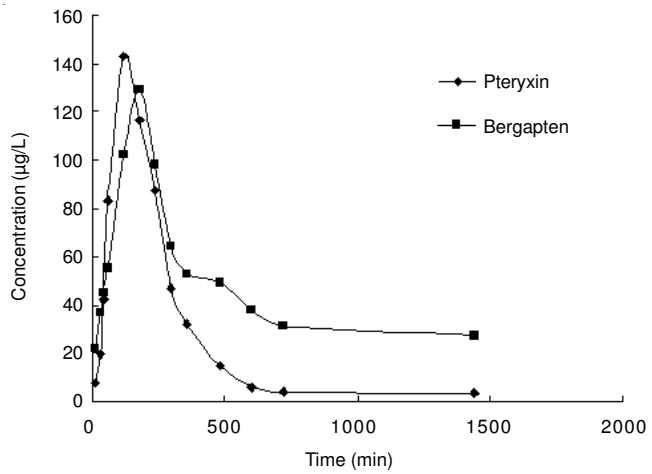

Fig. 3. Mean plasma concentration-time curve of pteryxin and bergapten with oral administration (n = 6)

TABLE-4
MAIN PHARMACOKINETIC PARAMETERS OF PTERYXIN AND BERGAPTEN FOLLOWING SINGLE ORAL DOSES OF 160 mg/kg (n = 6)

Parameter (unit)	Parameter value	
	Pteryxin	Bergapten
C_{max}(μg/L)	142.78	128.80
T_{max} (h)	2.00	3.00
AUC_{0-t} (μg/L)	4527.251	1093.216
$AUC_{0-\infty}$ (μg/L)	4527.477	1306.015
MRT_{0-t} (h)	6.685	9.072
$MRT_{0-\infty}$ (h)	36.566	10.449
$T_{1/2}$ (h)	1.012	1.698
CL/F (L/h/kg)	2618.832	61.255

According to our research, the pharmacokinetic properties of pteryxin and bergapten in rats were linear within the concentration range. As illustrated in the time-concentration profile, pteryxin and bergapten could be detected at 0.5 h and only trace amount of pteryxin and bergapten could be detected

at 8 h after oral administration of the mix, suggesting that elimination or biotransformation of pteryxin and bergapten was relatively quick in rats. After oral administration of the mix, the metabolites of pteryxin could be detected at 1 h and the peak concentrations reached at 4 h, indicating that metabolism of pteryxin rapidly occurred.

Conclusion

The pharmacological property of pteryxin and bergapten is realized. The pteryxin in the *Peucedunum harry-smithii var subglabrum* up to 0.8-1.4 %[3], but has not been reported in the pharmacokinetic study. A rapid, simple and accurate LC-MS/MS method had been developed and validated for the determination of pteryxin and bergapten in plasma. The main advantages of this method are: (1) small blood volume (10 μL); (2) rapid analysis (15.0 min); (3) simple sample preparation procedure and good recovery. The application of this method was demonstrated for the quantitative analysis of two analytes in plasma of rat after oral administration.

Pharmacokinetic data of pteryxin and bergapten were obtained after oral administration and showed that their pharmacokinetic process all belong to two-compartment model. And pteryxin after gastrointestinal absorption become metabolite 2-methyl butyric acid. The pharmacokinetic results provide a firm basis for evaluating the clinical efficacy of pteryxin and bergapten and for the further research and development.

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