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The Effect of Epimedium Extract on the Pharmacokinetics of Tadalafil in Beagle Dogs

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ABSTRACT

Objective: A sensitive high performance liquid chromatography with diode array detector (HPLC-DAD) method for determination of tadalafil in beagle dog plasma was developed, and to investigate the effect of Epimedium extract on the pharmacokinetics of tadalafil in beagle dogs. Methods: The beagle dog plasma was extracted with ethyl acetate under alkaline conditions. Tadalafil and internal standard (IS, carbamazepine) were separated on an XDB-C18 column, acetonitrile and 0.1% trifluoroacetic acid were used as the mobile phase. Tadalafil and IS were detected by DAD. This experiment adopts the experimental design of double cycle self-control. In the first cycle (group A), six beagle dogs were given Tadalafil 1 mg/kg orally in a single dose. In the second period (Group B), the same six beagle dogs were orally given Epimedium extract 0.6 g/kg once a day for 7 consecutive days, then Tadalafil was orally given. At the different time points after tadalafil was given in the two periods, the blood samples were collected. The concentration of tadalafil were detected by the developed HPLC method. DAS 2.0 was used to calculate the pharmacokinetic parameters of tadalafil. Results: Under the current experimental conditions, this HPLC-UV method showed good linearity in the detection of tadalafil. Inter-day and intra-day precision did not exceed 10%, the range of accuracy values were from -6.40% to 8.12%. The results of extraction recovery, and stability were met the requirements of FDA approval guidelines of bioanalytical method validation. Compared with the control group, the C_{max} of the experimental group decreased by 42.91%, the experimental group showed a significant increase in CLz/F, the $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ of tadalafil in the experimental group decreased by 24.60% and 29.57%, respectively. Conclusions: In this study, a HPLC-UV method for the determination of plasma tadalafil concentration was established. Epimedium extract could induce metabolism of tadalafil in beagle dogs and reduce plasma exposure to tadalafil.

KEYWORDS

Epimedium extract; Tadalafil; HPLC-UV; Pharmacokinetics; Herb-drug interacts

1. INTRODUCTION

Erectile dysfunction (ED) is a common disease of the male reproductive system, which seriously affects the life quality of patients and their partners [1]. ED is the most common sexual dysfunction disease in adult males. ED can be caused by many factors, such as vascular disease, neuropathy, metabolic disturbances, psychosocial causes, and side effects of medications [2]. At present, ED is considered as a social-psychological-physiological disease with complex etiology and various treatment methods, Oral PDE5I is the first-line treatment for erectile dysfunction with the advantages of high safety, good effect and non-invasiveness [1].

Tadalafil is classified as an oral phosphodiesterase type 5 (PDE5) inhibitor. Tadalafil was approved by the US Food and Drug Administration (FDA) in 2003 and is primarily used as first-line therapy for erectile dysfunction (ED) and benign prostatic hyperplasia (BPH) [3]. Tadalafil 5 mg should be considered a primary treatment option for patients with LUTS/BPH and ED [4].

Tadalafil was rapidly absorbed after oral administration and reaches C_{max} 2 hours after taking the drug. The absorption rate and degree of tadalafil are not affected by food, so this product can be taken with or without food. Tadalafil is classified as a cytochrome P450 (CYP)3A4 substrate and is mainly metabolized by CYP3A4 to catechol, which is extensively bound to form methyl catechol glucuronide, a major circulating metabolite of tadalafil through methylation[5].

Traditional Chinese medicine (TCM) has been used in clinical practice for the treatment of ED in men for hundreds of years. With the development of modern TCM, TCM comprehensive therapy plays an important role in male diseases. Syndrome differentiation and treatment is the most representative feature of TCM syndrome differentiation and treatment. Modern TCM scientists believe that damp-heat and blood stasis are important causes of ED [6]. TCM, including acupuncture and Chinese herbs, is used as an alternative therapy to increase the curative effect for ED. A large number of studies have been conducted to investigate the effect and mechanism of TCM for treating ED [7].

Epimedium koreanum Nakai, a member of the genus *Epimedium* in the family Berberidaceae, is a well-known and well-liked traditional herb used as a "kidney tonic". For thousands of years, it has been utilized for renal yang deficiency, impotence, spermatorrhea, impotence, weakness of tendons and bones, rheumatic paralysis and discomfort, numbness, and constriction. In traditional uses, *Epimedium* is frequently used to treat various diseases like ED, infertility, rheumatoid arthritis, osteoporosis, asthma, kidney-yang deficiency syndrome, etc [8]. *Epimedium* has functions of tonifying kidney and yang, strengthening tendons and bones, dispelling wind and emoving dampness. It is mainly used for the treatment of impotence and spermatorrhea, osteoporosis, Parkinson's, Alzheimer's, and cardiovascular diseases [9].

Several studies have revealed that Herb *Epimedium* extract can modulate liver cytochrome P450 enzymes, mainly CYP1A2, CYP3A4, and CYP2E1 [10]. Because tadalafil is mainly metabolized by CYP3A4, therefore, there is reason to hypothesize that *Epimedium* might have potential synergistic or antagonistic effect on the pharmacokinetics of tadalafil. A method for the determination of the concentration of tadalafil in beagle dog plasma by HPLC was established. By measuring the concentration of tadalafil in the plasma. the effect of *Epimedium* extract on the pharmacokinetics of tadalafil was studied, and a theoretical basis for herd-drug interaction was provided.

2. MATERIALS AND METHODS

2.1. Chemicals Materials

Tadalafil (purity > 98%, CAS:171596-29-5) and carbamazepine (purity > 98%, CAS:100142-199503) were purchased from Sigma (St. Louis, MO, USA). Tadalafil tablets (5 mg, CAS: C791509) were purchased from Qilu Pharmaceutical (Hainan) Co., Ltd. Epimedium Extract were purchased from Xi'an Wanfang Biotechnology Co., Ltd. (Xi'an, China). Methanol and acetonitrile and were HPLC grade and provided by Merck Company (Darmstadt, Germany).

2.2. Instrumentation and Conditions

The Agilent 1100 series HPLC system was used for liquid chromatography experiments. The sample was injected into the chromatography system through the G1313A automatic sampler, and the mobile phase was degassed through the G1379A vacuum degassing device before flowing into the Agilent ZORBAX Eclipse XDB-C18 chromatography column (4.6mm × 250mm, 5 μm, USA). The XDB-

C18 protection column (4.6×12.5mm, 5 μm, USA) was used as the protection column, and acetonitrile water 0.2% TFA=48:42:10 (V/V/V) was used as the mobile phase. The flow rate was set to 1.0 mL/min, and the column temperature was maintained at 35 ℃. The peak areas of tadalafil and carbamazepine were detected at a wavelength of 286 nm..

2.3. Preparation of Standard and Quality Control (QC) Samples

A stock solution of 1 mg/mL was prepared by weighing 10 mg of tadalafil and dissolving in 10 ml of methanol. The standard application solution is diluted with methanol to the concentrations of 100 μg/mL, 10 μg/mL, and 1 μg/mL. A stock solution of 1 mg/mL was prepared by weighing 1 mg of carbamazepine and dissolving in 1 ml of methanol, and then dilute to 200 ng/mL standard solution. All of the solutions were stored in a refrigerator at 4 °C.

Calibration curve standards were prepared by adding appropriate amounts of the working solutions in blank beagle dog plasma. The final concentrations of tadalafil were 1, 2.5, 5, 10, 25, 50, 100, 200 ng/mL. The preparation of QC samples was the same, with the three levels of plasma concentrations (2.5, 50 and 150 ng/mL).

2.4. Sample Preparation

Thaw the refrigerated sample at room temperature before the experiment. Transfer 200 μ L of the beagle plasma sample to a 2.0 mL EP tube, add 20 μ L of 1 μ g/mL carbamazepine internal standard working solution, and add 200 μ L of 10% sodium carbonate. Vortex and mix for 2.0 minutes. Add 1.0 mL of ethyl acetate, vortex and mix for 2 minutes, centrifuge at 10000 r/min for 10 minutes, then transfer 800 μ L of the upper organic phase into a 2.0 mL EP tube and dry under a nitrogen flow at 37 ℃. Dissolve with 100 μ L of mobile phase, vortex and mix for 2 minutes, then take the complex solution into the sample bottle of the automatic sampler and set 20 μ L for HPLC analysis and detection.

2.5. Method Validation

2.5.1. Specificity

Take blank plasma from (A), pure standard plasma with added tadalafil and internal standard from (B), and plasma samples from beagle dogs after 30 minutes of administration. After processing according to the plasma pretreatment method, take 20 μ L for injection detection. Analyze the spectrum by HPLC-DAD method to investigate its specificity under experimental conditions.

2.5.2. Accuracy and precision

Accuracy and precision were assessed by the determination of QC samples at three concentration levels (2.5, 50 and 150 ng/mL) in six replicates. In the same day, the Intra-day were calculated, and the Inter-day were calculated by continuous measurement within 3 days.

2.5.3. Recoveries

The recoveries of tadalafil at three QC levels (2.5, 50 and 150 ng/mL, n=6) were determined by comparing peak area of the analytes in the sample with the analyte added before extraction and the sample with the corresponding solution after extraction. The RSD of each concentration recovery should be within 15%.

2.5.4. Stability

The stabilities of tadalafil in beagle dog plasma were tested by analyzing six replicates of plasma samples at three concentration levels (2.5, 50 and 150 ng/mL) in different conditions. The short-term stability was determined after the exposure of the spiked samples at room temperature for 24 h. The freeze-thaw stability was evaluated after three complete freeze-thaw cycles (-20 °C) on consecutive

days. The long-term stability was assessed after storage of the standard spiked plasma samples at -20 °C for 21 days.

2.6. Animals

Six healthy beagle dogs weighing (8 ± 2) kg were purchased from Hubei Yizhicheng Biotechnology Co., Ltd., with production license number SCXK (Hubei) 2021-0020. All experimental procedures and protocols were approved by the Animal Laboratory of Henan University of Science and Technology and are consistent with the guidelines for the protection and use of laboratory animals.

2.7. Study Design

On the day before administration, beagle dogs were fasted but allowed to drink water freely. Six beagle dogs were orally administered tadalafil tablets 1 mg/kg (control group), followed by immediate gavage of a small amount of physiological saline (10-20 mL) to ensure complete oral administration of the tablets. After administration, plasma was collected from the medial cephalic vein of the forelimbs and the lateral saphenous vein of the hind limbs of beagle dogs at different time points. After a one week medication cleaning period, beagle dogs were orally administered 0.6 g/kg of Epimedium extract solution every morning. Immediately after administration, a small amount of physiological saline (10-20 mL) was given to ensure complete oral administration of Epimedium extract solution for 6 consecutive days. On the morning of the 7th day, after 0.5 hours of administration of Epimedium extract solution, 1 mg/kg tadalafil tablets were orally administered (experimental group). Plasma was collected at 12 different time points from the anterior limb medial head vein and posterior limb lateral saphenous vein of beagle dogs.

2.8. Plasma Specimen Collection

At 12 different time points of 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 36 hours after administration of tadalafil (1 mg/kg), 1.0 mL of venous blood was collected from the medial cephalic vein of the forelimb and the lateral saphenous vein of the hind limb in beagle dogs. The blood samples were collected in EP tubes containing heparin, gently mixed, centrifuged at 10000 r/min for 10 minutes, and the supernatant was collected in another EP tube and frozen at -20 ℃ for testing.

2.9. Statistical Analysis

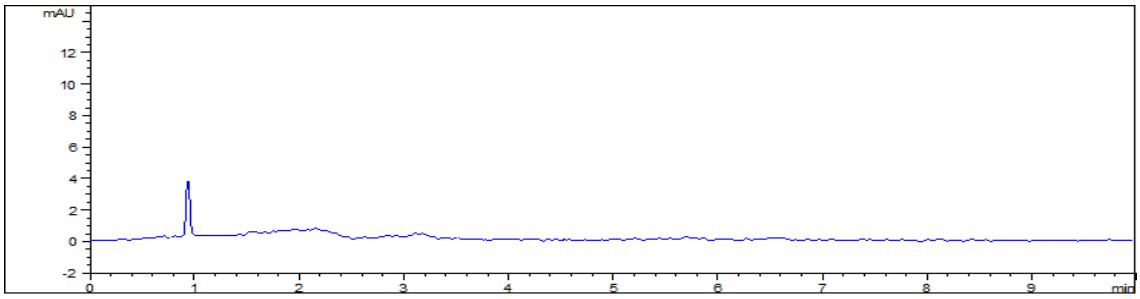
The mean and standard deviation (SD) were used for the results. The compartmental analysis was used to calculate the pharmacokinetic parameters by DAS 2.0. The statistical analyses were evaluated by unpaired t-test (SPSS 16.0, Chicago, IL). A value of $P < 0.05$ was considered to be statistically significant.

3. RESULTS

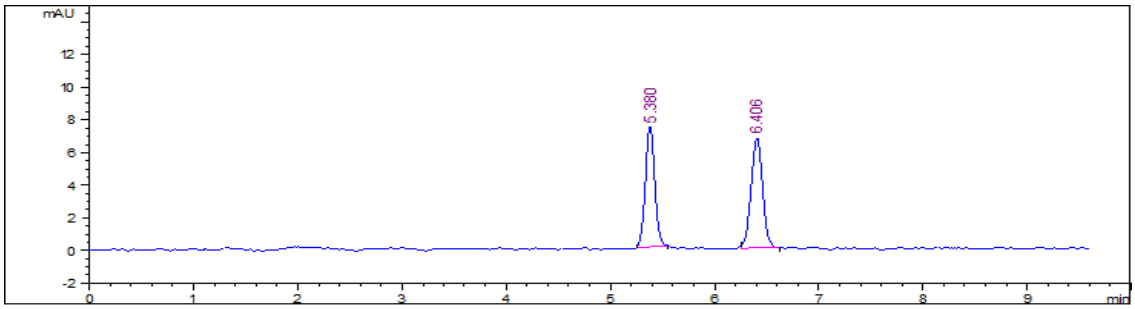
3.1. Methodology Results

3.1.1. Sensitivity

Under the experimental conditions described, tadalafil and IS were well separated from endogenous materials. Representative chromatograms of a blank plasma sample, a plasma sample spiked with tadalafil and IS, and a beagle dog sample obtained 0.5 h after oral administration of tadalafil were shown in Figure 1. The retention time of tadalafil and IS were 6.64 and 5.54 min, respectively.



(a)



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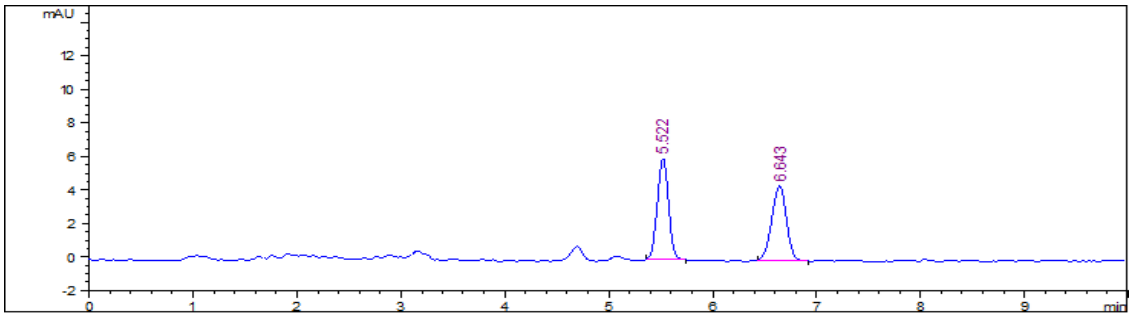

(c)

Figure 1. Representative HPLC for tadalafil and carbamazepine (IS). (a) blank plasma sample; (b) blank plasma sample spiked with tadalafil and IS; (c) beagle dog plasma sample obtained 0.5 h after oral administration of tadalafil.

3.1.2. Linearity of Calibration Curve

A linear relationship was established for Tadalafil within the concentration range of 1 to 200 ng/mL. The standard curve for Tadalafil plasma was determined to be y=0.0067x -0.0038, with a correlation coefficient of r=0.999 8. During the calibration curve construction process, the LLOQ was estimated, and the lower limit of quantification for Tadalafil was determined to be 1 ng/mL.

3.1.3. Precision and Accuracy

The precision of the method was evaluated by calculating RSD for QCs at three concentration levels (2.5, 50 and 150 ng/mL) over three validation days. Assay performance data is presented in Table 1. The results demonstrated that the values were within the acceptable range and the method was accurate and precise.

Table 1. Precision and accuracy for tadalafil of QC samples in rat plasma (n = 6).

Added	intra-day			inter-day		
(ng/mL)	Found (ng/mL)	RSD%	RE%	Found (ng/mL)	RSD%	RE%
2.5	2.38 ± 0.13	5.46	-4.80	2.34 ± 0.19	8.12	-6.40
50	52.49 ± 3.41	6.49	4.98	47.23 ± 2.17	4.59	-5.54
150	146.26 ± 7.32	4.50	-2.49	155.24 ± 7.21	4.64	3.49

3.1.4. Recovery

Mean extraction recoveries of tadalafil were (84.23 ± 3.21) %, (79.37 ± 5.49) % and (82.49 ± 2.41) % (n = 6) at the concentrations of 2.5, 50 and 150 ng/mL, respectively.

3.1.5. Stability

The RSDs of three quality control plasma samples (2.5, 50 and 150 ng/mL) spiked tadalafil were less than 10%, and tadalafil have shown good stability in plasma for 24 h at room temperature, during three freeze-thaw cycles, and for 21 days at -20 °C (Table 2).

Table 2. Stability results (RSD) for tadalafil in beagle plasma (n = 6)

Added (ng/mL)	Processed samples	Freeze thaw samples	Room temperature storage	Frozen preservation
2.5	4.57	3.67	3.27	3.57
50	6.23	7.24	4.46	4.23
150	3.25	3.26	6.35	5.53

3.2. The Effect of Epimedium on the Metabolism of Tadalafil in Beagle Dogs

After oral administration of 1 mg/kg tadalafil to the experimental and control groups of beagle dogs, blood samples were collected at different time points. The plasma samples were processed according to the plasma pretreatment method, and the concentration of tadalafil in the plasma was detected by the established HPLC-DAD method. The results were analyzed and analyzed using the DAS 2.0 program, and pharmacokinetic parameters were estimated using a non-compartmental model. The comparison of average pharmacokinetic parameters between the experimental group and the control group is shown in Table 3.

From the results, it can be seen that the Cmax of Tadalafil in the experimental group decreased by 42.91% compared to the control group, the AUC (0-t) decreased by 24.60%, the AUC (0- ∞) decreased by 29.57%, and the half-life was shortened by 3.33 hours. After the combination of Epimedium, the Cmax and AUC of Tadalafil can be reduced, the half-life slightly shortened, and the clearance rate increased. Epimedium can reduce the exposure of Tadalafil. Epimedium can induce the metabolism of tadalafil.

After oral administration of 1 mg/kg tadalafil to the experimental and control groups of beagle dogs, blood samples were collected at different time points. The plasma samples were processed according to the plasma pretreatment method, and the concentration of tadalafil in the plasma was detected by the established HPLC-DAD method. The average plasma drug concentration time curve of tadalafil in plasma is shown in Figure 2.

Table 3. The main pharmacokinetic parameters of tadalafil after oral administration of 1 mg/kg in control group and experimental group (Means ± SD, n=6).

Parameters	Control group	experimental group
$t_{1/2}$ (h)	12.39 ± 5.63	9.06 ± 2.08**
T_{max} (h)	1.25 ± 0.27	1.25 ± 0.27
$MRT_{(0-t)}$ (h)	10.9 ± 1.13	10.04 ± 1.21
$MRT_{(0-\infty)}$ (h)	15.12 ± 3.69	12.75 ± 1.86
C_{max} (ng/mL)	127.16± 17.93	72.59 ± 16.16**
CL_z/F (L/h/kg)	0.94 ± 0.29	1.26 ± 0.12
$AUC_{(0-t)}$ (ng·h/mL)	980.57 ± 197.58	739.34 ± 69.26*
$AUC_{(0-\infty)}$ (ng·h/mL)	1133.91 ± 271.70	798.63 ± 73.89*
Note: Compared with the control group, * P<0.05, * * P<0.01.		

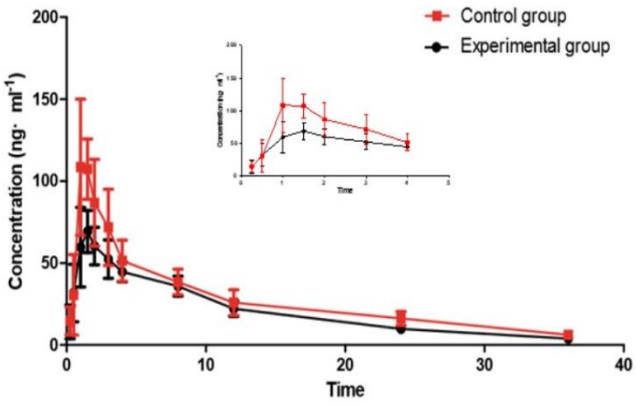

Figure 2. Mean plasma concentration time profiles of tadalafil in 2 groups after oral administration of 1 mg/kg tadalafiil (Means ± SD, n=6).

4. DISCUSSION

This study established a novel HPLC-DAD method for detecting the concentration of tadalafil in beagle dog plasma. The method has high sensitivity, suitable analysis time (10 min), and is suitable for rapid determination of large quantities of samples. In terms of the selection of plasma processing methods, we use acetonitrile extraction method, which is simpler in operation and more reliable in detection results compared to solid-phase extraction and liquid-liquid extraction methods. After experimental screening, carbamazepine was selected as the internal standard. Carbamazepine has stable properties, is easy to obtain, and has good separation and peak shape from tadalafil. At the same time, endogenous substances in beagle plasma samples do not interfere with the determination of their content, indicating high specificity. The RSD values of intraday precision and intra-day precision of Tadalafil are both less than 15%, indicating good precision results, high absolute and relative recovery rates, and good stability of plasma samples.

Epimedium, belonging to the Berberidaceae family, has a wide range of clinical applications. It has been recorded that Epimedium has been used for about 2000 years, and its branches and leaves have long been used to enhance reproductive function and skeletal system, with high health and medicinal value [11]. It has been used as a nourishing Chinese herbal medicine since ancient times. Now it is mainly used for the treatment of reproductive system diseases, breast cancer diseases, osteoporosis, etc. The main medicinal components of Epimedium are flavonol glycosides with 8-isoprenyl groups (and their derivative groups), such as icariin, baohuo glycoside I (icariin II), icariin I, chaohuoding A, chaohuoding B, chaohuoding C, etc. Research has shown that the activities of CYP450, CYP1A2, CYP3A4, and CYP2E1 in rat liver were significantly increased after treatment with total flavonoids of Epimedium. Epimedium total flavonoids induced the activities of CYP450 and major subtypes CYP1A2, CYP3A4, and CYP2E1 in rat liver [12]. Therefore, Epimedium may affect drug metabolism and cause adverse reactions by affecting the activity of enzymes such as CYP450.

Tadalafil is a substrate of CYP3A4 and is mainly metabolized by CYP3A4. Research has shown that drugs that inhibit CYP3A4 increase the exposure level of tadalafil. The selective strong inhibitor of CYP3A4, ketoconazole (400 mg/day), can increase the exposure (AUC) of Tadalafil (20 mg) by 312% and Cmax by 22% in a single dose; Compared to a single dose of tadalafil (20 mg), ketoconazole (200 mg/day) increased the exposure (AUC) of tadalafil (10 mg) by 107% and Cmax by 15%. Icagrelor reduces a CYP3A-mediated tadalafil metabolism and that tadalafil and a combination regimen with tadalafil and ticagrelor requires dose control and specific pharmacotherapy [13]. Drug-drug interaction between donepezil and tadalafil is primarily due to time-dependent inhibition against CYP3A4 by tadalafil [14].

In the experiment, continuous administration of Epimedium extract to beagle dogs for 7 days was sufficient to achieve a steady-state blood concentration of Epimedium and exert effects on enzymes and proteins in the body. Tadalafil is a substrate of CYP3A4 and is mainly metabolized by CYP3A4. Research has shown that drugs that induce CYP3A4 can reduce the exposure level of tadalafil. The tl/2 of the experimental group was (9.06 ± 2.08) hours, while that of the control group was (12.39 ± 5.63) hours, indicating that the combination of Epimedium and Tadalafil shortened the time for Tadalafil prototype drug to exert its therapeutic effect in vivo and induced its metabolism. The Cmax of the experimental group was (72.59 ± 16.16) ng/mL, while the Cmax of the control group was (127.16 ± 17.93) ng/mL. Compared with the control group, the Cmax of the experimental group decreased by 42.91%, significantly reducing the drug concentration of tadalafil in beagle dogs. Compared with the control group, the experimental group showed a significant increase in CLz/F, indicating a significant acceleration in the metabolism of tadalafil. Compared with the control group, the AUC(0-t) and AUC(0-∞) of tadalafil in the experimental group decreased by 24.60% and 29.57%, respectively. The difference between the two groups was statistically significant ($P<0.05$). The above results indicate that simultaneous administration of Epimedium and Tadalafil significantly reduces plasma exposure, accelerates metabolism, and shortens half-life of Tadalafil. This indicates that Epimedium induces the metabolism of Tadalafil in beagle dogs.

5. CONCLUSIONS

The HPLC-DAD method established in this study was used to detect tadalafil in beagle dog plasma, which had high specificity, complete separation and fast detection time, and was suitable for the pharmacokinetics and drug interaction studies of tadalafil. Epimedium extract could induce metabolism of tadalafil in beagle dogs and reduce plasma exposure to tadalafil.

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