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# Method for quantification of prazosin in dog plasma and its application to pharmacokinetic study

Zhang Niping[1] Yu Shuqin[2] Wang Hui[2] Shen Yanyan[1,3] Xu Shi[1] Zhang Ling[1] Xu Qian[1,3]

([1] Ministry of Education Key Laboratory of Environmental Medicine and Engineering, Southeast University, Nanjing 210009, China)
([2] Jiangsu Key Laboratory for Medical Supermolecule Material and Application, Nanjing Normal University, Nanjing 210046, China)
([3] Suzhou Key Laboratory of Environment and Biosafety, Suzhou Institute, Southeast University, Suzhou 215123, China)

**Abstract:** A simple and sensitive high performance liquid chromatography method using fluorescence detection (HPLC-FLD) and a one-step single solvent extraction for the determination of prazosin(PZS) in dog plasma is developed and validated. After extraction with ether, the chromatographic separation of PZS is carried out using a reverse phase C18 column (150 mm × 4.6 mm, 5 μm) with a mobile phase of 30% acetonitrile and 70% acetic acid-sodium acetate buffer solution (pH = 3.6) and quantified by fluorescence detection operated with an excitation wavelength of 258 nm and an emission wavelength of 387 nm. The flow rate of the mobile phase is 1.0 mL/min and the retention time of PZS and the internal standard is found to be 4.4 and 5.8 min, respectively. The calibration curve is linear within a concentration range from 1.0 to 1 000.0 ng/mL ($r^2 > 0.998$). The limit of detection is 0.4 ng/mL. The inter-day coefficient of variation (COV) of the calibration standards is below 5.0% and the mean accuracy is in the range from 92.7% to 104.2%. Moreover, by analyzing quality control plasma samples for three days, the results show that the method is precise and accurate, for the intra- and inter-day COV within 10% and the accuracy from 95.9% to 112.7%. The developed and validated method is successfully applied to pharmacokinetic study for the preclinical evaluation of a new peroral PZS-sulfobutyl ether beta-cyclodextrin (PZS-SBE-β-CD) inclusion complex tablets (test preparation), which demonstrates that the test preparation released PZS is conducted in a slow and controlled way, and the relative bioavailability of the test preparation is found to be 105.0%.

**Key words:** prazosin (PZS); PZS-sulfobutyl ether beta-cyclodextrin (PZS-SBE-β-CD) inclusion complex tablets; high performance liquid chromatography (HPLC); pharmacokinetics
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Prazosin(PZS), a selective $\alpha_1$-adrenoceptor antagonist disclosed in 1970, is one of the most commonly used antihypertensive agents, which has gained a widespread acceptance in the management of hypertension and in the treatment of congestive heart failure[1]. The initial dose of the drug may cause syncope with a sudden loss of consciousness, which is known as "first-dose response"[2]. In most cases this is believed to be due to an excessive postural hypotensive effect. This adverse effect is seriously self-limiting. Therefore, it is very important to introduce effective methods to enhance the safety and elevate compliance in patients. A sustained release dosage from which released PZS at a relatively low dose should be used.

Cyclodextrins (CDs) have hydrophobic central cavities capable of forming stable complexes with properly sized drug molecules. Complexation of drugs with CDs shows that it not only increases the solubility and stability in aqueous solutions, but also releases drugs in a slow and controlled way[3-8].

As one of the CDs derivatives, sulfobutyl ether beta-cyclodextrin (SBE-β-CD) has been widely investigated on account of its high solubility in water and minimal toxicity[9]. PZS-SBE-β-CD was synthesized to form inclusion complex tablets by our group with the labeled content of 1 mg/tablet. To comprehensively characterize the pharmacological profile and bioavailability of the new preparation in dogs, a sensitive and specific assay for PZS should be developed.

Based on the literature review, there were a few reports of the quantitative determination of PZS using electrochemical detection in human plasma and methods for sample pretreatment which mainly included liquid-liquid extraction and solid phase extraction[10-14]. Liquid-liquid extraction, a common sample pretreatment method, has been employed for extracting PZS in various biological matrices. We have developed a simple and sensitive high-performance liquid chromatography method with fluorescence detection (HPLC-FLD) and a one-step single solvent extraction method for the determination of PZS in dog plasma. The assay described here is fully validated and successfully used for studying the relative bioavailability of PZS-SBE-β-CD inclusion complex tablets (test preparation) after a single intragastric administration to the dogs, and the commercial PZS conventional tablets are chosen as the reference preparation.

## 1 Experiment

### 1.1 Standards and chemicals

PZS conventional tablets (1 mg/tablet), PZS (batch, 20080714) and the reference preparation were purchased from Changzhou No. 2 Pharmaceutical Factory (Jiangsu, China). PZS-SBE-β-CD inclusion compound tablets (1 mg/tablet) as test preparation was prepared by our research group. Quinine as an internal standard (I.S.), analytical grade acetic acid, ether, sodium acetate and sodium hydrate were obtained from Shanghai No. 2 Reagent Factory (Shanghai, China). Acetonitrile (HPLC grade) was supplied by Dikma

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**Biographies:** Zhang Niping (1987—), female, graduate; Xu Qian (corresponding author), female, doctor, associate professor, q_xu@163.com.
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Instrument Co., Ltd. (Beijing, China). Deionized water was used throughout the study.

### 1.2 Standard and QC solutions

Stock solutions of PZS and I. S. were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving them in a mobile phase to yield primary standard solutions with a concentration of 1.0 mg/mL.

Working solutions to obtain the standard points of the calibration curve were prepared by appropriate dilutions of the stock solution in a mobile phase to achieve PZS concentrations from 0.01 to 10.0 μg/mL. The I. S. working solution was prepared at a concentration of 2.0 μg/mL by diluting the stock solution with the mobile phase. The working solutions to prepare the plasma quality control(QC) samples (low, medium and high concentration) were prepared in the same way. All the solutions were stored at 4 ℃.

### 1.3 Calibration standard and QC samples

The calibration standard for PZS was prepared by spiking 450 μL of blank dog plasma with 50 μL of each working solution to obtain a final dilution of 1∶10, resulting in final concentrations of PZS being at 1.0, 2.0, 5.0, 10.0, 50.0, 200.0 and 1 000.0 ng/mL.

To prepare QC samples, three fractions of plasma were added with an appropriate amount of QC solutions, obtaining QC samples at final PZS concentrations of 1.0, 50.0 and 1 000.0 ng/mL (low, medium and high concentration).

Several aliquots of the three concentrations were stored at −20 ℃ as controls for future assays to check the short term stability under storage conditions. The analytes were considered stable at each concentration when the differences between the freshly prepared samples and the stability testing samples were found to be not exceeding 15% of the nominal concentration.

### 1.4 Sample preparation

Plasma samples (500 μL) were mixed with 100 μL (200 ng) of I. S. working solution and 100 μL of 2 mol sodium hydrate solution, followed by an addition of 3 mL ether extraction solvent. The mixture was vortex-mixed for 1 min and allowed to stand for 5 min. The organic layer was transferred into a new sample tube and evaporated to dryness at 30 ℃ under a gentle stream of nitrogen gas. The residue was reconstituted with 100 μL of a mobile phase and 20 μL was injected into the column.

### 1.5 Chromatographic conditions

Chromatographic analysis is performed using an LC-10AD pump and an RF-530 fluorescence detector (Shimadzu, Japan). Separation of the analytes from potentially interfering material is achieved at room temperature using Dikma Platisil C18 column (150 mm × 4.6 mm, 5 μm) equipped with a C18 precolumn, 4 mm × 3 mm (Dikma). The mobile phases for the chromatographic separation are composed of 30% acetonitrile and 70% acetic acid-sodium acetate buffer solution (pH = 3.6) at a flow rate of 1.0 mL/min. 20 μL of the sample was quantified by fluorescence detection operated with an excitation wavelength of 258 nm and an emission wavelength of 387 nm. Data collection and processing are performed using Shimadzu LC-Solution software.

The stationary phase can be equilibrated with the mobile phase for 30 min before analyzing the samples. And for long-term column efficiency and reproducibility of drug peaks, an overnight cleansing of the column after everyday use with methanol should be performed.

### 1.6 Validation of analysis method

#### 1.6.1 Calibration curve

A calibration curve is constructed from working standard solutions of PZS at concentration ranges from 1.0 to 1 000 ng/mL. The linearity of the calibration curves is validated over three days and calculated by the peak area ratios of PZS/I. S. to the nominal concentration of PZS in the sample. The linearity of the standard curves is determined by a regression analysis and a good regression fit is determined by calculating Pearson's coefficient $R^2$ and by comparison of the true and back-calculated concentrations of the calibration standards.

#### 1.6.2 Accuracy and precision

To assess the accuracy and precision of the method, intra- and inter-day measurements of PZS are determined by analyzing PZS in three replicates of three QC samples at the nominal concentrations of 1.0, 50.0 and 1 000.0 ng/mL (low, medium and high concentration) on the same day and on three different days. A standard calibration curve is prepared and processed each day to analyze the QC samples. The accuracy of the measure is expressed as a percentage value (COV = (measured concentration/nominal concentration) × 100%), while the precision of the method at each concentration is estimated as a percentage relative standard deviation (% RSD), expressing the standard deviation as a percentage of the mean calculated concentration.

#### 1.6.3 Limit of detection and limit of quality

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected. It is expressed as a concentration at which the signal-to-noise ratio is 3∶1. The limit of quality (LOQ) is defined as the lowest concentration of the analyte in plasma that can be determined with acceptable precision (±15%) and accuracy (range from 80% to 120%) under the stated operational conditions of the method.

#### 1.6.4 Extraction recovery

The extraction recoveries are determined in triplicate and at three QC levels of 1.0, 50.0 and 1 000.0 ng/mL for PZS from plasma and they are calculated by comparing the peak area ratios of PZS/I. S. from extracted plasma samples with those obtained by directly determining PZS external standard solutions spiked with 100 μL (200 ng) of the I. S. working solution at the same concentration, respectively.

#### 1.6.5 Stability

The stability of PZS in dog plasma is profiled with QC samples at three concentration levels (1.0, 50.0 and 1 000.0 ng/mL). These plasma samples were frozen at −20 ℃ and thawed at room temperature on 0 (immediately after preparation), 7, 14, 21, and 30 d. The post-prepara-

tive stability study is conducted by a freshly prepared calibration curve.

### 1.7 Pharmacokinetic application

A total of 18 healthy dogs ((8.0 ± 0.5) kg, half female and half male) were provided by the Laboratory Animal Center of Southeast University (Nanjing, China). Dogs were randomly divided into three groups (six dogs per group, half female and half male) and allowed free access to water in this study. A single dose 2 mg of PZS-SBE-β-CD was administered by intragastric administration to group 1 after an overnight fasting diet, while a corresponding dose of the reference preparation was administered in the same way to group 2. Group 3 offered blank plasma for establishment of the analysis method. Blood samples (2.0 mL) were drawn from the fore-leg saphenous vein into heparinized centrifuge tubes just before dosing (0 h) and at 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 16 h and 24 h after the administration. After centrifuging at 5 000 r/min for 5 min, the plasma samples were separated and stored frozen at −20 ℃ until analysis.

The pharmacokinetic model and the parameters are calculated by the practical pharmacokinetic Program Version 97, edited by the Committee of the Mathematic Pharmacology of the Chinese Society of Pharmacology. The parameters considered are: $C_{max}$ (maximum plasma concentration), AUC (area under the concentration-time curve from time 0 to the last detectable sample), $T_{1/2}$ (plasma elimination half-life in the terminal phase), and the relative bioavailability after administration ($T/R$ ratio, $F = AUC_{test}/AUC_{reference} \times 100$). All the parameters are reported as mean values ± standard deviation. A non-compartmental analysis was applied.

## 2 Results and Discussion

### 2.1 Assay validation

#### 2.1.1 Specificity

The specificity/selectivity of the method was investigated by analyzing blank dog plasma extract, an extract of the plasma spiked with 20 ng/mL of PZS and 2.0 μg/mL of I.S. and a plasma sample collected 24 h after intragastric administration of the test preparations.

As shown in Fig. 1, the retention times of PZS and I.S. are 4.4 and 5.8 min, respectively, with good resolution and without any interference from endogenous plasma constituents or PZS metabolites at or near the retention time of PZS and I.S., which indicates that the present sample preparation method removed the interfering substances in dog plasma efficiently. Therefore, satisfactory specificity of the proposed method is obtained.

#### 2.1.2 Calibration curve

The linear regression of PZS in dog plasma displays a good linear relationship between the peak-area ratios of PZS/I.S. vs. the nominal concentrations over the range of concentrations studied. The calibration curve, prepared on three different days, is linear over a wide range of PZS concentrations from 1.0 to 1 000.0 ng/mL with $R^2 \geqslant 0.998$. The accuracy and precision are determined each day. The mean accuracy is in the range from 92.7% to 104.2%. The precision expressed as COV ranges between 3.1 % and 4.6 %.

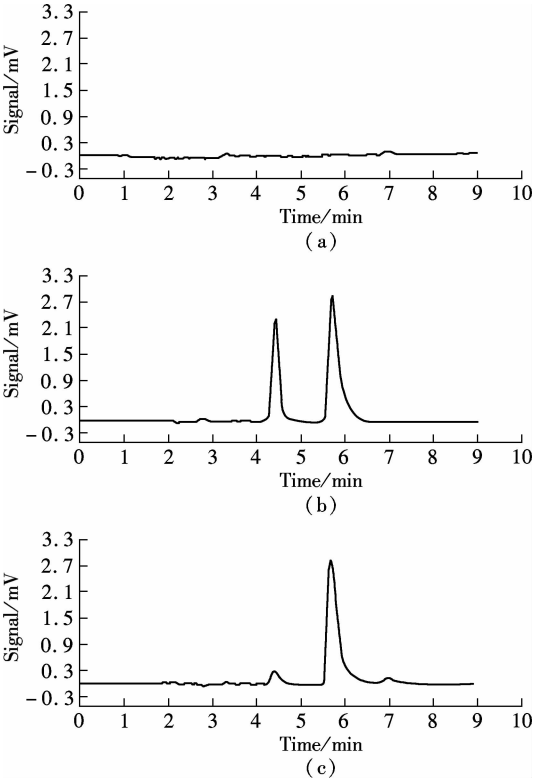


**Fig. 1** HPLC chromatograms. (a) Blank plasma; (b) Blank plasma spiked with PZS 20 ng/mL + I.S. 2.0 μg/mL; (c) Plasma collected from a dog 24 h after intragastric administration of the test preparation at a dosage of 2 mg PZS (PZS concentration is 2 ng/mL) + I.S. (2.0 μg/mL)

#### 2.1.3 Limit of detection and limit of quality

Sensitivity is evaluated by determining the LOD and the LOQ. To determine the LOD, the plasma samples are spiked to contain 0.4 ng/mL PZS and are analyzed each day. The peak area in chromatograms for the spiked plasma samples containing the above lowest concentrations is compared with the noise signals. The LOD should have a precision within 10% and the signal-to-noise ratio should be equal to 3. The LOQ is defined as the lowest concentration that can be measured with a precision within 15% and an accuracy between 80% and 120%. Consistent with our aims, we set the LOQ at 1.0 ng/mL, validated through three replicates. The intra-day COV and accuracy are 7.9% and 97.2%, respectively.

#### 2.1.4 Accuracy and precision

The intra-day and inter-day accuracy and precision are assessed by determining PZS in three replicates of three QC samples at low, medium and high concentrations for PZS on the same day and on three different days, respectively. The results are shown in Tab. 1. Tab. 1 gives a summary of the accuracy and precision obtained from three replicates of the QC samples at 1.0, 50.0 and 1 000.0 ng/mL. The method is precise and accurate, with intra- and inter-day COV < 10% and accuracy in the range of 95.9% to 112.7%. According to the requirements of Chinese Pharmacopoeia[15], these values are within the acceptable range. This suggests that the present method has satisfactory accuracy, precision and reproducibility.

**Tab. 1** Accuracy and precision of the method for determining PZS concentration in dog plasma samples ($n = 9$)

| | Concentration added/ ($ng \cdot mL^{-1}$) | Concentration measured / ($ng \cdot mL^{-1}$) | Accuracy/% | Precision/% |
|---|---|---|---|---|
| Intra-day | 1.0 | 0.959 ± 0.077 | 95.9 | 8.0 |
| | 50.0 | 49.7 ± 1.95 | 99.4 | 3.9 |
| | 1 000.0 | 1 007.9 ± 11.3 | 100.8 | 1.12 |
| Inter-day | 1.0 | 1.13 ± 0.094 | 112.7 | 8.3 |
| | 50.0 | 51.3 ± 2.27 | 102.6 | 4.4 |
| | 1 000.0 | 1 012.5 ± 15.9 | 101.2 | 1.57 |

### 2.1.5 Extraction recovery

The recovery is evaluated in triplicate and determined by analysis of QC samples at low, medium and high concentrations (1.0, 50.0 and 1 000.0 ng/mL). The extraction recoveries of PZS at the three QC levels are calculated by comparing the peak areas of PZS in extracted biological samples with those obtained by directly determining PZS standard solutions at the same concentration. We verify the absence of significant variations ( < 15% ) for the areas of both the analytes. Tab. 2 shows the recovery, expressed as a percentage. As a result, the mean extraction recovery for PZS at three QC levels is higher than 90% with reproducibility expressed as COV < 11%.

**Tab. 2** Extraction recovery of the method for determining PZS concentration in dog plasma samples ($n = 3$)

| Concentration added/($ng \cdot mL^{-1}$) | Extraction recovery/% | COV/% |
|---|---|---|
| 1.0 | 91.6 ± 9.7 | 10.6 |
| 50.0 | 93.9 ± 8.2 | 8.7 |
| 1 000.0 | 96.7 ± 7.5 | 7.8 |

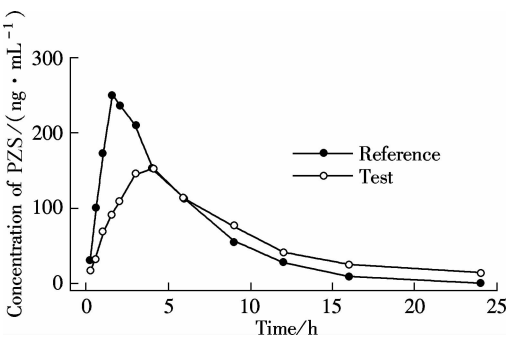


**Fig. 2** Mean plasma concentration-time profiles after single intragastric administration

### 2.1.6 Stability in frozen matrix

Plasma samples of PZS at three concentrations (1.0, 50.0 and 1 000.0 ng/mL, $n = 3$) are used for the stability experiments. The accuracy is in the range from 97.3% to 105.4% and the COV between 7.2% and 12.5%. Five freeze-thaw cycles of the quality control samples did not appear to affect the quantification of PZS. The quality control samples stored in a freezer at −20 ℃ remained stable for at least 1 month.

In summary, a simple and sensitive method has been developed and validated to quantify PZS in dog plasma. As this HPLC protocol has good linearity, sensitivity, precision, reproducibility and accuracy, it is subsequently used to investigate the relative bioavailability of the test preparation.

## 2.2 Pharmacokinetic application

This method is used to examine the pharmacokinetics of test preparation and reference preparation after a single intragastric administration of 2 mg PZS-SBE-β-CD in dogs. Fig. 2 reports the mean plasma concentration-time profiles after single intragastric administration of test preparation and reference preparation of PZS at a dosage of 0.25 mg/kg in dogs ($n = 6$). From Fig. 2, it can be seen that the reference drug concentration rapidly increases during the first 1 h, while the test drug concentration maintains a long and slow increasing tendency during the first 4 h. Moreover, the test drug concentration stays higher than the reference drug concentration for the rest of the time. Tab. 3 shows the comparative pharmacokinetic parameters after intragastric administration of test preparation and reference preparation at a dosage of 0.25 mg/kg in dogs ($n = 6$), and the main pharmacokinetic parameters, such as $AUC_{0\to\infty}$, $AUC_{0\to t}$, $C_{max}$, $T_{max}$ and $t_{1/2}$ are calculated from plasma concentrations for both preparations. The mean relative bioavailability between the test and the reference preparation is 105.0%. The test preparation has lower $C_{max}$ and longer $T_{max}$ than the reference preparation ($p < 0.05$), and $t_{1/2}$ of the test preparation are significantly longer than that of the reference preparation ($p < 0.01$), which indicates that the test preparation released PZS occurs in a slow and controlled way. From the results of the present study, it is concluded that the test preparation also has satisfactory relative bioavailability compared with the reference preparation and, therefore, it can be considered to be a good candidate for further preclinical and clinical development.

**Tab. 3** Pharmacokinetic parameters ($n = 6$)

| Samples | Reference | Test |
|---|---|---|
| $C_{max}$/($\mu g \cdot mL^{-1}$) | 270 ± 82 | 190 ± 46 * |
| $T_{max}$/min | 1.8 ± 0.3 | 3.2 ± 0.8 * |
| $t_{1/2}$/min | 2.83 ± 1.26 | 5.96 ± 0.86 ** |
| $AUC_{0\to t}$/($\mu g \cdot min \cdot mL^{-1}$) | 1 400 ± 490 | 1 400 ± 250 |
| $AUC_{0\to\infty}$/($\mu g \cdot min \cdot mL^{-1}$) | 1 500 ± 510 | 1 500 ± 260 |
| $F$/% | | 105.0 ± 13.7 |

Note: $C$ is the concentration; $T$ is the time; AUC is the area under the curve; $F$ is determined by dividing $AUC_{test}$ by $AUC_{reference}$; * means less than 0.05; and ** means less than 0.01.

## 3 Conclusion

A simple and sensitive HPLC method is developed and validated for the quantification of PZS in dog plasma. This reliable method has been successfully applied in the pharmacokinetic study of PZS in dogs. As the novel PZS preparation possesses good relative bioavailability and potent pharmacokinetics, future studies on preclinical and clinical application can be extended to PZS-sulfobutyl ether beta-cyclodextrin inclusion complex tablets.

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# 狗血浆中哌唑嗪的含量测定方法及其在药代动力学中的应用

张妮萍[1] 余书勤[2] 王 辉[2] 申艳艳[1,3] 徐 师[1] 张 玲[1] 许 茜[1,3]

([1] 东南大学环境医学工程教育部重点实验室,南京 210009)
([2] 南京师范大学江苏省医药超分子及应用重点实验室,南京 210046)
([3] 东南大学苏州研究院苏州市环境与生物安全重点实验室,苏州 215123)

**摘要**:建立并评价了一种简单灵敏的方法测定狗血浆中 PZS 的含量. 该方法利用 HPLC-FLD 结合乙醚萃取的前处理方法,采用 C18 色谱柱 (150 mm×4.6 mm, 5 μm),流动相为 30% 乙腈和 70% 乙酸-乙酸钠缓冲液(pH = 3.6),荧光检测器的激发波长为 258 nm,发射波长为 387 nm,在流动相流速为 1.0 mL/min 时,其 PZS 和内标物的保留时间分别为 4.4 和 5.8 min. 标准曲线在 1.0～1 000.0 ng/mL 的浓度范围内呈线性相关($r^2 > 0.998$),检出限为 0.4 ng/mL,标准曲线的日间变异系数低于 5.0%,准确度在 92.7%～104.2% 范围内. 在 3 d 的分析质量控制研究中,日内和日间精密度均小于 10%,准确度为 95.9%～112.7%. 该方法成功应用于一种新的口服制剂(PZS-磺丁基醚-β-环糊精包合物)的临床前期药代动力学评价,结果表明:该新型的包合药物具有缓释 PZS 的功效,且其相对生物利用率为 105.0%.

**关键词**:哌唑嗪;PZS-SBE-β-CD 包合物片剂;高效液相色谱;药物代谢动力学
**中图分类号**:O657