JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Paraquat Detoxification with *p*-Sulfonatocalix-[4]arene by a Pharmacokinetic Study

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ABSTRACT: The *p*-sulfonatocalix[n]arenes are supposed to show potential application in the clinical treatment of viologen poisoning. In the present study, *p*-sulfonatocalix[4]arene (C4AS), the most common derivative of *p*-sulfonatocalix[n]arenes, is used to study the antidotic mechanism for paraquat (PQ) by pharmacokinetics in vivo. A high-performance liquid chromatography (HPLC) method was established to determine the concentration of PQ in rat plasma. The results showed that the peak plasma concentration (C_{max}) and area under the plasma concentration−time curve ($AUC_{0\text{-}t}$) were significantly lower after C4AS intervention than in the PQ intoxication group. It was considered that C4AS has great effective detoxication to PQ poisoning, and the results of in vitro intestinal absorption studies showed that C4AS can inhibit the absorption of PQ via oral administration by forming a stable inclusion constant.

KEYWORDS: Paraquat, *p*-sulfonatocalix[4]arene, pharmacokinetics, in vitro intestinal absorption, detoxication

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, PQ, Figure 1a) is widely used as an effective and quick acting herbicide for about 50 years because it is nonselective and is rapidly deactivated upon soil contact.[1,2] However, in the past decades, there have been numerous PQ poisonings mainly caused by accidental or voluntary ingestion. Many studies have reported that PQ was absorbed through the digestive tract, respiratory tract, and skin and thus resulted in various diseases or even death from inflammatory reaction of tissues, multiorgan failure, and pulmonary fibrosis with respiratory failure.[3−7] The clinical treatments to PQ poisoning are mainly symptomatic such as gastric lavage, hemoperfusion, forced diuresis, and antioxidant treatment, but no antidote or efficient treatment to PQ poisoning has been identified.[8−11]

In this study, a new therapeutic protocol, using the concept of host−guest chemistry, was suggested to develop an efficient treatment to PQ poisoning. Calixarenes, made up of phenol and methylene units, are macrocyclic molecules, like crown ethers and cyclodextrins, which represent the third generation of supramolecular host molecules.[12] Calixarenes are reported to possess an astounding selectivity, especially to cations, when substituted with appropriate tethers.[13−15] Moreover, water-soluble calixarenes have become increasingly important in supramolecular chemistry because of their high water solubility, good stability, and potential bioactivities,[16−18] among which *p*-sulfonatocalix[4]arene (C4AS, Figure 1b) is perhaps the most common derivative. It possesses three-dimensional, flexible, π-rich cavities that can selectively bind numerous guest molecules, such as metal ions, neutral molecules, organic cations, and pharmaceutical molecules[19−21] in aqueous solution. Surprisingly, C4AS was demonstrated to be safe and with no toxicity in vivo.[22−24]

In previous studies, Professor Liu and co-workers conceptually proposed a new therapeutic protocol for the treatment of viologen poisoning. They had demonstrated that *p*-sulfonatocalix[n]arene by oral administration could significantly decrease the mortality rate of PQ poisoning and mitigate the damage in the lung and liver.[25] Many antidotes were explored to study the detoxication on PQ poisoning through survival rate and histological analysis.[26−30] In the present work, we introduced a protocol based on pharmacokinetics, aimed to macroscopically clarify the detoxication of C4AS through studying the physiological disposition of PQ in rats. This would provide a powerful proof to explain the detoxification model of PQ by C4AS.

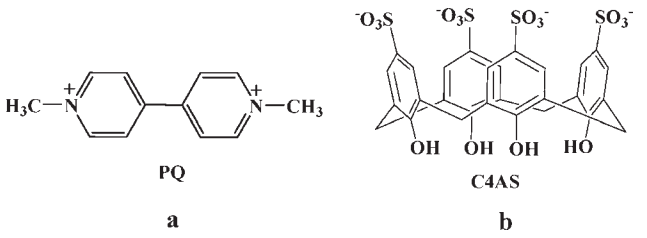

Figure 1. Structural illustration of PQ (a) and C4AS (b).

MATERIALS AND METHODS

Chemicals and Reagents. PQ (1,1′-dimethyl-4,4′-bipyridinium dichloride; molecular mass = 257.2 g/mol) was purchased from Dr. Ehrenstorfer (Germany, batch number: 71012). Sodium octane sulfonate was purchased from Tianjin Chemical Reagent Research Institute (Tianjin, China). C4AS was provided by Nankai University (Tianjin, China), which identified by a ^{1}HNMR spectrum on a Varian 300 spectrometer and elemental analysis on a Perkin-Elmer-2400C instrument, and the found elemental analysis values for C4AS were within ±0.4% of the calculated values, confirming ≥95% purity. Acetonitrile of

Received: December 18, 2010
Accepted: March 16, 2011
Revised: March 15, 2011
Published: March 16, 2011

HPLC grade and perchloric acid of analytical grade were obtained from Tianjin Concord Technology Co., Ltd. (Tianjin, China).

Sample Preparation. *Preparation of PQ Working Standard Solutions.* PQ stock solution was prepared in double distilled water at a concentration of 1 mg/mL. Working standard solutions of PQ were prepared by dilution with double distilled water. The solutions were stored at 4 °C.

Pretreatment of Plasma Samples. An aliquot of 100 μL of plasma sample was transferred to a clean centrifuge tube, and 10% perchloric acid of 50 μL was added to precipitate proteins. The solution was vortexed for 1 min (XW-80A Microvortex mixed Miriam) and centrifuged at 14000 rpm for 10 min to obtain a supernatant. Then the supernatant was transferred into a clean centrifuge tube for injection into the HPLC system.

Determination of PQ by HPLC. The analysis of PQ in plasma samples was performed on the Waters 600E system equipped with a 2487 UV detector (Waters Corporation, USA). The plasma samples of 10 μL were injected onto a HPLC reverse phase column (Elite, 250 mm × 4.6 mm, 5 μm) at 25 °C. All samples were detected with a UV detector at 256 nm. The mobile phase was a mixture of 0.07% aqueous phosphate solution−acetonitrile (72:28, V/V) containing 6.0 mmol/L sodium octane sulfonate, and the flow rate was 1 mL/min.

Pharmacokinetic Study. PQ solution (20% w/v) with vomit, purchased from the market, was used in the study. Eighteen healthy male Wistar rats with weights of 250 ± 10 g were obtained from Tianjin Shanchuanhong Laboratory Animal Technology Co., Ltd. (SCXK (Jin)2009−2010, Tianjin, China). The rats were housed under standard conditions and fasted overnight with free access to water until experiment.

Blood samples (0.5 mL) were collected into heparinized centrifuge tubes from the retinal venous plexus and immediately placed on ice pending further processing. For the pharmacokinetic study, the blood samples were centrifuged at 5000 rpm for 10 min (3K15 Sigma low-temperature centrifuge) to obtain plasma, and the plasma samples were stored at −20 °C before analysis.

All experimental procedures were approved by Experimental Animal Care and carried out in accordance with the corresponding guidelines. The rats were randomly divided into three groups (six rats in each group) and were treated as follows.

Intoxication Group. Six rats were orally dosed with PQ at 25 mg/kg (10 mg/mL PQ; 2.5 mL/kg of body weight) according to the published LD_{50} value in rats.[31] Then, serial blood samples (0.5 mL) were collected at 0, 10, 30, 45, 60, 90, 120, 180, 270, 360, 480, and 600 min after PQ oral administration. Blood samples were processed as described above to analyze the physiological disposition of PQ.

Inclusion Complex Group. Six rats were orally administrated with the PQ/C4AS inclusion complex (at a molar ratio of 1:1), and serial blood samples (0.5 mL) were collected at 0, 5, 10, 20, 30, 60, 120, 180, 270, 360, 480, and 600 min. Blood samples were processed as described above to analyze the physiological disposition of PQ after treatment with the inclusion complex.

Treatment Group. Six rats were treated orally with C4AS at a molar ratio of 1:1 (20 mg/mL, 4 mL/kg) 30 min later after PQ administration at the same dose as the intoxication group. Then serial blood samples (0.5 mL) were collected at 0, 10, 30, 45, 60, 90, 120, 180, 270, 360, 480, and 600 min after PQ administration. Blood samples were processed as described above to analyze the physiological disposition of PQ after late C4AS treatment.

Pharmacokinetic Data Analysis. The PQ concentration data were processed by Drug and Statistics, version 1.0 (DAS ver1.0), to generate pharmacokinetic parameters of each rat with a noncompartmental model. The peak concentration (C_{max}) and peak time (T_{max}) were observed values, and the area under the concentration−time curve ($AUC_{0\text{-}t}$) was calculated by the trapezoidal rule with extrapolation. The results were demonstrated as the mean ± standard deviation (SD).

In Vitro Intestinal Absorption Studies. Male Wistar rats weighing 250 ± 10 g were fasted overnight with free access to water until the rats were anesthetized with chloral hydrate. A middle abdominal incision was made, and the jejunum was taken quickly. After thoroughly washing both the inside and outside with 0.9% saline (0.9% NaCl solution), the jejunum was cut into segments (5 cm long each). Each of the sacs was carefully everted with a glass rod (2.5 mm diameter). One end of the sac was ligated, and the other end was ligated with a conical rubber stopper for the removal and addition of the serosal fluid. After filling with the Krebs−Ringer solution as serosal fluid (inner compartment), we placed the intestinal sacs in three glass tubes with a Krebs−Ringer solution of 10 mL containing 6 mg/mL PQ, 19.4 mg/mL C4AS, and the PQ/C4AS inclusion complex (1:1) of the same concentration. They were kept in a 37 °C water bath and continuously supplied with 5% CO_2 and 95% O_2 throughout the experiment. The 100 μL sample of solution was collected from inside the sac every 0.5, 1, 1.5, and 2 h. The volume of the collected sample was replaced by injecting blank oxygenated Krebs−Ringer solution into the sac. Then the samples were kept at −20 °C until HPLC analysis.

Statistical Analysis. One-way ANOVA or the *t*-test was used for determining the statistically significant differences between the values of various experimental groups. A significant *F*-value was found by ANOVA. Data were expressed as the mean ± SD, and a P value <0.01 was considered statistically significant.

RESULTS AND DISCUSSIONS

Method Validation. Under optimized HPLC conditions, PQ was completely separated from endogenous interferences of rat plasma (Figure 2). No significant interference from endogenous substances of rat plasma with PQ was detected. A typical equation of the calibration curve was as follows: $Y = 17262X - 5048.5$ ($r = 0.9999$, $n = 6$) for plasma samples. The linear range of PQ in rat plasma was from 0.5 to 16.0 μg/mL. The results showed that an excellent correlation existed between the peak area and concentration of PQ. The data of intra- and interday precision and accuracy, recovery, and stability are summarized in Tables 1 and 2, respectively. The results demonstrated that the values were within the acceptable range.[32] Thus, the method was successfully applied to the determination of PQ plasma concentration levels in rats following oral administration of a single 25 mg/kg dose.

Pharmacokinetic Study. Pharmacokinetic studies were performed on 18 male rats after oral administration of PQ (25 mg/kg), inclusion complex, and treatments with C4AS 30 min later. Figure 3 shows the mean plasma concentration−time profiles of PQ in the three groups. The pharmacokinetic parameters are summarized in Table 3. In the inclusion complex group and treatment group, PQ cannot be detected after 480 min. The time-to-peak (T_{max}) was shortened after intervention with C4AS. The result showed that the intervention of C4AS could accelerate the absorption of PQ. This seemed disadvantageous, however, in fact, both the area under the concentration−time curve ($AUC_{0\text{-}t}$) and maximum concentration (C_{max}) of PQ significantly decreased after treatment with C4AS. These results were most important because $AUC_{0\text{-}t}$ and C_{max} represent total PQ absorption within a certain period and a peak plasma concentration of PQ after an oral administration, respectively.

The results may indicate that C4AS had the effective detoxication to PQ by reducing the $AUC_{0\text{-}t}$ and C_{max} of PQ. To further validate the effective detoxication to PQ by C4AS, a survival rate and pathological study was performed in this work. The results demonstrated that rats exposed only to PQ (intoxication group)

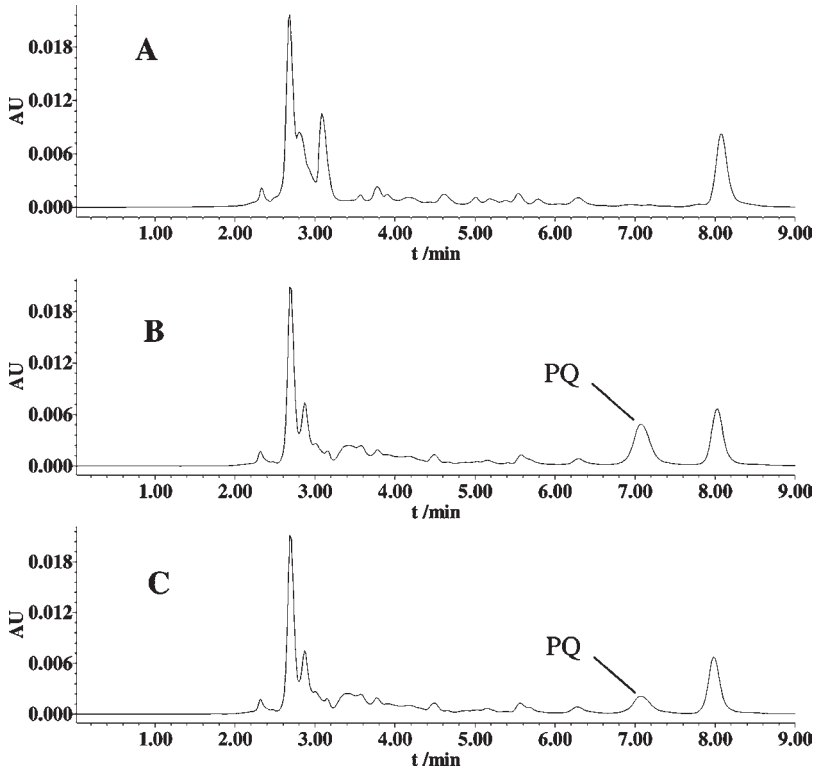

Figure 2. Representative chromatograms of PQ in rat plasma were determined by a HPLC−UV method. A is blank rat plasma; B is blank rat plasma spiked with PQ (8 μg/mL); C is a rat plasma sample collected at 10 min after an oral administration of 25 mg/kg PQ to Wistar rats.

Table 1. Precision, Accuracy, and Recovery of the Study Results (n = 6)

	intra-assay (%) (n = 6)		interassay (%) (3days)		
concentration (μg/mL)	accuracy (%)	precision *RSD* (%)	accuracy (%)	precision *RSD* (%)	recovery (%) (n = 6)
1.0	107.6 ± 2.6	4.4	113.4 ± 2.0	4.5	95.4 ± 4.2
4.0	95.0 ± 1.8	2.2	93.0 ± 1.8	2.9	95.2 ± 2.7
16.0	100.4 ± 1.6	1.6	101.4 ± 1.3	1.7	97.3 ± 1.6

Table 2. Stability Study Result (Detected at 0, 2, 4, 6, and 8 h)

numeration	1	2	3	mean *RSD* %
protein precipitation (*RSD* %)	0.6	1.1	1.4	1.0 ± 0.4
without protein precipitation (*RSD* %)	3.5	3.1	1.6	2.7 ± 1.0

displayed 50% of mortality at the end of 7 days. Deaths were verified between 24 and 48 h after oral administration of PQ. The inclusion complex group and treatment group elicited different results. An increase in the survival rate to 100% was achieved for an existence of C4AS at the molar ratios of 1:1. Meanwhile, the tissues were fixed and sectioned for microscopic examination of the structure (Figure 4). No appreciable pathological changes were observed in the lungs of rats in the control group. In contrast, the microscopic examination of lungs of the surviving rats in the intoxication group showed a massive destruction of structures of the tissues; moreover, several macrophage-like cells within the alveolar space were also noticed. The intoxication group showed a marked alveolar collapse and the enlargement of alveolar walls compared to those of the control group. The inclusion complex group and treatment group showed little enlargement of alveolar walls compared to that of the intoxication group. The results of the histological effect of C4AS were as good as those of the other studies,[26−30] which further validated the great effective treatment of PQ poisoning by C4AS.

In Vitro Intestinal Absorption Studies. To find the reason for the decrease of $AUC_{0\text{-}t}$ and C_{max}, an in vitro intestinal absorption study was performed in this work. Two rapid and sensitive HPLC systems for PQ and C4AS in Krebs−Ringer solution were developed. Under optimized HPLC conditions, the calibration curve of PQ in Krebs−Ringer solution was linear in the range of 1.0 to 100.0 μg/mL, and the equation of the calibration curve was Y = 37791X − 5420.4 (r = 0.9999, n = 6); the calibration curve of C4AS in Krebs−Ringer solution was linear in the range of 10.0 to 200.0 μg/mL, and the equation of the calibration curve was Y = 2835.1X − 34627 (r = 0.9990, n = 5). The results of precisions and recoveries satisfied the requirement of the determination. In the experiment, all the samples were diluted (1→100, v/v) with blank Krebs−Ringer solution before HPLC analysis. A comparison of the serosal content of the everted gut sac between the PQ group and inclusion complex group is demonstrated in Figure 5.

As shown in Figure 5, PQ could be detected in the everted gut sac model which indicated that it can be absorbed from the mucosal side to the serosal side, and the absorption of PQ

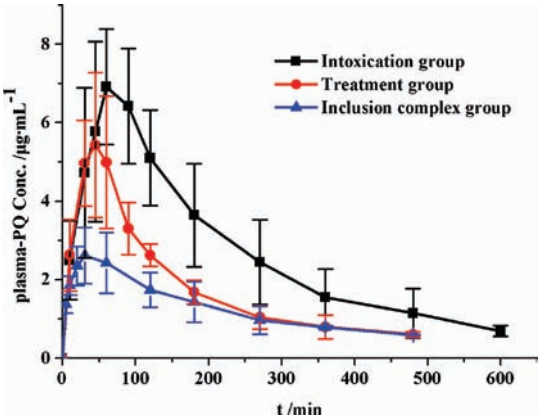

Figure 3. Mean plasma concentration−time profiles of PQ after oral administrations of PQ, inclusion complex, and treatment with C4AS 30 min later. Each point (obs) is the experimental data (mean ± SD, n = 6); vertical bars represent each standard error of the mean; and the lines (cal) are the pharmacokinetic model fitted curves.

Table 3. Pharmacokinetic Parameters of PQ for Three Groups (Mean ± SD, n = 6)

parameters	inclusion complex group	treatment group	intoxication group
T_{max} (min)	38.3 ± 17.2	39.0 ± 8.2	65.0 ± 20.5
C_{max} (mg/L)	2.8 ± 0.7	5.6 ± 1.8	7.7 ± 1.3
$MRT_{0\text{-}t}$ (min)	153.1 ± 31.7	151.7 ± 22.9	185.0 ± 23.6
$AUC_{0\text{-}t}$ (mg/L·min)	572.8 ± 208.1	876.7 ± 127.9	1577.0 ± 346.4

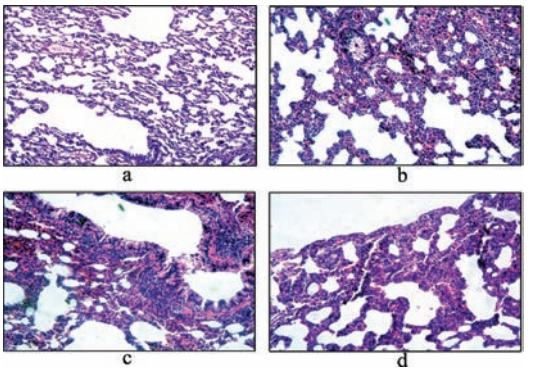

Figure 4. Light micrographs of lungs from the control group (a), intoxication group (b), inclusion complex group (c), and treatment group (d) stained with hematoxylin-eosin.

increased with incubation time in both the PQ group and inclusion complex group. In the inclusion complex group, the absorption of PQ was reduced by 78.4% ± 11.2 (mean ± SD, n = 4), which demonstrated that the absorption of PQ was efficiently impeded after C4AS intervention. To further validate the detoxication path of C4AS, another comparison of the serosal content of the everted gut sac between the C4AS group and inclusion complex group was performed (Figure 6).

As shown in Figure 6, C4AS could also be absorbed into the serosal side, and the absorption of C4AS increased with incubation time in both the C4AS group and inclusion complex group.

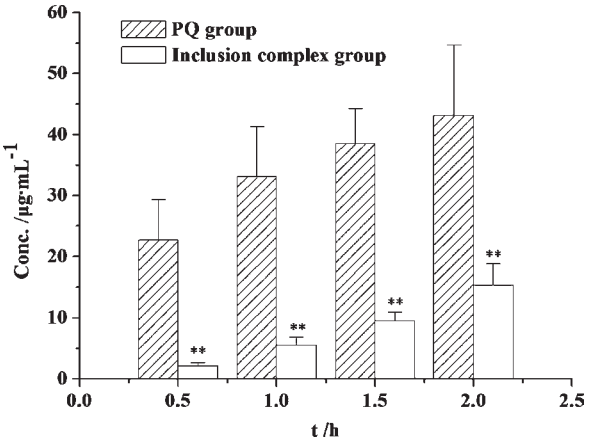

Figure 5. Comparison of the serosal content between the PQ group and inclusion complex group. *p < 0.05 and **p < 0.01 (n = 3) comparing the PQ group with the inclusion complex group.

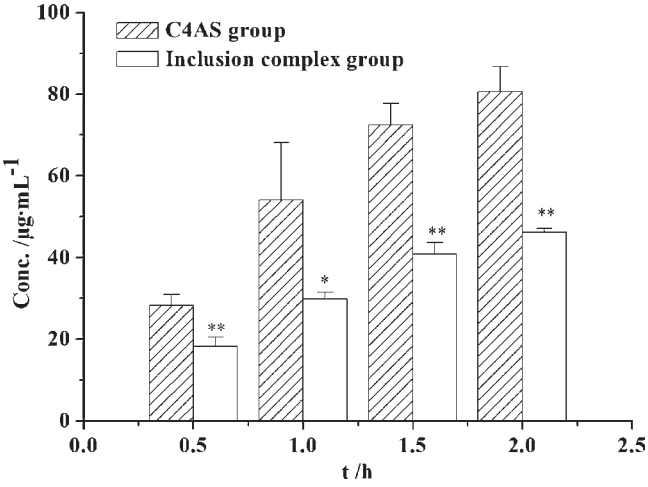

Figure 6. Comparison of the serosal content between the C4AS group and inclusion complex group. *p < 0.05 and **p < 0.01 (n = 3) comparing the C4AS group with the inclusion complex group.

In the inclusion complex group, the absorption of C4AS was also reduced, which further demonstrated that not only the absorption of PQ was impeded but also the absorption of C4AS was impeded. The detoxication path was presumed as follows: Both free molecules PQ and C4AS and PQ/C4AS inclusion complex can be absorbed through the biomembrane (such as the mucous membrane of the small intestine), but the inclusion complex is more difficult to pass through the biomembrane than free molecules owing to their large molecular volume and their strongly hydrophilic and three-dimensional molecular structure.[33] The inclusion constant, K, is given by the equation of the inclusion reaction:

$$\mathrm{PQ} + \mathrm{C4AS} = \mathrm{PQ/C4AS} \quad (1)$$

The concentration of free PQ molecules is as follows:

$$[\mathrm{PQ}] = \frac{[\mathrm{PQ/C4AS}]}{K \cdot [\mathrm{C4AS}]} \quad (2)$$

In the experiment, the stable inclusion complex was formed between PQ and C4AS (the inclusion constant was 7.3×10^4 L/mol,

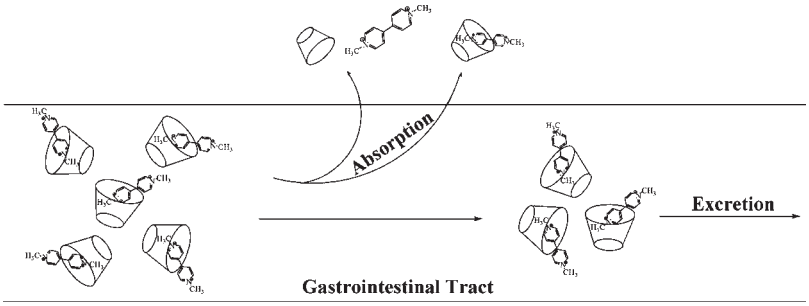

Figure 7. Schematic diagram of the absorption process.

which was obtained by double-reciprocal measurements). The larger the inclusion constant, the more stable was the inclusion complex and the fewer the free molecules. When the inclusion complex pass through the gastrointestinal tract, few free molecules and the inclusion complex can be absorbed into blood, the other molecules were excreted from gastrointestinal tract mainly in the form of the inclusion complex. Therefore, the concentration of free PQ decreased in the gastrointestinal tract after treatment with C4AS, which resulted in the absorption of PQ being efficiently impeded. The schematic diagram of the absorption process is demonstrated in Figure 7.

In conclusion, the rapid and sensitive high-performance liquid chromatography systems for the determination of PQ and C4AS in biological specimen were developed. The pharmacokinetic data of PQ in rats presented here is important in the expectation; furthermore, C4AS has great effective detoxication to PQ poisoning by impeding the absorption of PQ. It could be inferred that macrocyclic molecules with a larger inclusion constant to PQ may have great effective detoxication because complexation can impede PQ absorption via oral administration.

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Funding Sources

This work was supported by the Doctoral Fund of Ministry of Education of the People's Republic of China (No. 20070063003).

ACKNOWLEDGMENT

We thank Professor Liu Yu at Nankai University for assistance in the preparation and revision of this manuscript.

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