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Bioanalysis and pharmacokinetics of chitosan ester in rabbit serum by HPLC with postcolumn fluorescence derivatization

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Abstract

Interest in antiatherosclerotic activity of chitosan ester (PS916) with a new form of sulfate amino polysaccharide derived from marine chitin has necessitated the development of a sensitive and specific method to study its pharmacokinetics. A sensitive and reproducible high-performance liquid chromatography (HPLC) with postcolumn fluorescence derivatization method was developed and validated for the determination of PS916 in rabbit serum. Chromatography was carried out using a C8 reversed-phase column with an isocratic mobile phase consisting of methanol–water (20:80, v/v) at a flow rate of 0.2 ml/min. The derivatization procedure involved postcolumn reaction with guanidine hydrochloride in an alkaline medium at 110 °C. The fluorometric detector was operated at 250 nm (excitation) and 435 nm (emission). The assay was linear over the concentration range of 5–100 μg/ml. The lower limit of detection (LLOD) was found to be 1.0 μg/ml. The proposed method was successfully applied for a pharmacokinetic study of PS916 in rabbits.

Keywords: Polysaccharide; PS916; High-performance liquid chromatography; Fluorescence derivatization; Pharmacokinetic study

1. Introduction

Atherosclerosis is the main cause of coronary heart disease, brain stroke and limb necrosis [1] and it is a complex disease, encompassing both a chronic process characterized by vascular inflammatory reactions, and having acute features, such as plaque rupture and endothelial injury [2–4]. Drugs applied to antiatherosclerotic therapies have different acting mechanisms because of the different pathological processes of atherosclerosis [5–8]. Chitosan ester (PS916) is a new form of sulfated amino polysaccharide that is derived from marine chitin by ways of molecular modifications. It is characterized by 1, 4-linked β-D-glucosamine with an average of 1.0 sulfate and 0.5 carboxylmethyl groups per unit monosaccharide (Fig. 1). The average molecular weight of PS916 is 7000 Da and the distribution width of molecular weight is less than 1.5. Systematic pharmacodynamic research shows that PS916 has good antiatherosclerotic activity and was authorized to enter clinical trial in China. The microdetermination of PS916 is very important to study its pharmacokinetics and to monitor the drug clinically. However, PS916, similar to other polysaccharides, such as glucosan, possesses few chromophoric or fluorophoric groups and usually has been detected by measuring its refractive, scattering indexes or using colorimetric assays. These methods are simple to operate, but their sensitivities are relatively poor. Currently, the determination of polysaccharides in biological materials has been carried out mainly by prelabeling methods [9,10] or bioassay methods [11,12]. Although these prelabeling methods have high sensitivity, the polysaccharides may undergo isomerization, degradation, or conformation change during the labeling procedure. Bioassay methods are easily influenced by sensitive biochemical reactions that limit their applications to pharmaceutical analysis. An HPLC method with postcolumn fluorescence derivatization using guanidine or 2-cyanoacetamide as a fluorometric detection reagent was developed to determine sulfate, oversulfated dermatan and chondroitin sulfate in plasma, and the limit of detection of these polysaccharides is 10–20 ng [13,14]. PS916 is a kind of polysaccharide, and it can be also derivatized by guanidine hydrochloride. So an HPLC method with postcolumn fluorescence derivatization was developed in this study for the microdetermination of PS916 in rabbit serum.

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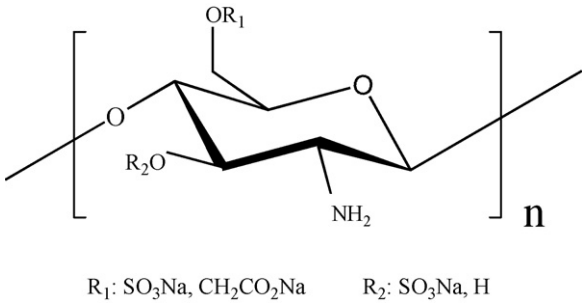

Fig. 1. The general structure of PS916.

2. Experimental

2.1. Materials

PS916 was provided by Marine Drug and Food Institute (Ocean University of China) and the purity was at least 99%. Guanidine hydrochloride was purchased from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from TEDIA Company (China). All other chemicals were of analytical-reagent grade.

Drug-free serum was obtained from healthy male or female rabbit (1.5–2.5 kg) purchased from Qingdao Institute for Drug Control (China).

2.2. Instrumental

The postcolumn HPLC analyses system was assembled with an Agilent 1100 binary pump, a Rheodyne 7725i injector, a PCX 5200 postcolumn derivatization instrument (Pickering Laboratories) and an Agilent 1100 fluorescence detector set at λ_{Ex} 250 nm and λ_{Em} 435 nm. Separations were achieved with a Zorbax Eclipse XDB-C8 column (150 mm × 4.6 mm i.d., 5 μm particle size) purchased from Agilent Technologies Company. The mobile phase was methanol/water (20:80, v/v) at a flow rate of 0.2 ml/min. In the postcolumn procedure, a 0.5 M NaOH solution containing 0.05 M guanidine hydrochloride was added to the column eluting at a flow rate of 0.3 ml/min, which was passed through polytetrafluaroethylene (PTFE) tube (0.5 mm i.d., 10 m) thermostated at 110 °C. Subsequently, a 0.5 M NaOH solution at a flow rate of 0.3 ml/min was added to cool down the mixture in another PTFE tube (0.25 mm i.d., 3 m) at ambient temperature and detected.

2.3. Preparation of stocks, calibration standards and quality control samples

The stock solution of PS916 (1.0 mg/ml) was prepared in water and keep at 4 °C with light protection. Fresh calibration standards of PS916 in concentrations ranging from 5 to 100 μg/ml were prepared in drug-free serum. Quality control (QC) samples (5, 10, 50 and 75 μg/ml) were also prepared in a similar manner.

2.4. Sample preparations

All serum samples, spiked serum calibration standards, and spiked serum quality control samples were treated in the same manner described below. To a 100 μl portion of serum sample, 150 μl acetonitrile was added, and the mixture was centrifuged at 12600 × *g* for 20 min at 4 °C to precipitate the denatured serum proteins. An aliquot (10 μl) of the supernatant was submitted to the HPLC system.

2.5. Assay validation

2.5.1. Quantifications

To quantify the concentration of PS916 in QC or unknown samples, peak-area of PS916 was related to standard curves of PS916 in drug-free serum. Lower limits of detection (LLOD) and quantitation (LLOQ) were determined from signal-to-noise ratios. The LLOD is defined as the lowest concentration level resulting to a signal-to-noise ratio of 3:1, while the signal-to-noise ratio should be 5:1 for the LLOQ.

2.5.2. Specificity

Control rabbit serum, obtained from four rabbits, was assessed by procedure as described above and compared with the serum sample spiked with PS916 to evaluate specificity of the method.

2.5.3. Recovery

The absolute recovery of PS916 from serum was determined for different standard concentrations by spiking the drug into the drug-free serum. The percentage recovery was calculated by comparing the peak-areas of extracted samples with samples in which the compound was spiked directly in mobile phase. Recoveries at three QC concentration levels for serum were examined at least five times. According to the acceptance criteria, the recovery of the analyte does not need to be 100%, but should be consistent, precise and reproducible.

2.5.4. Linearity

For the construction of calibration curves in serum, six different calibration standards of PS916 were prepared and processed as described above. Calibration curves were constructed by plotting area of PS916 against the known amounts PS916. Linear regression analysis of the calibration data was performed using the equation $A = aC + b$ where A is the peak-area, C is the concentration of PS916; unknown concentrations were computed from the linear regression equation of the peak-area against concentration for the calibration curve.

2.5.5. Precision and accuracy

Intra-day accuracy and precision were evaluated from replicate analysis ($n = 5$) of QC samples at different concentrations on the same day. Inter-day accuracy and precision were also assessed from the analysis of the same QC samples on four separate occasions in replicate ($n = 5$). QC samples were

analyzed against calibration curves. The evaluation of precision was based on criteria that the relative standard deviation should not more than ±15% for higher concentrations and below ±20% for lower concentrations. Similarly for accuracy, the mean value should not deviate by ±15% for the higher concentration and below ±20% for lower concentrations.

2.6. Stability studies

To evaluate long-term stability of PS916 in serum samples, three QC samples of serum (10, 50 and 75 μg/ml) were stored at 4 or −20 °C for five days. Thereafter, the samples were processed and analyzed using freshly prepared calibration standards. The concentrations thus obtained were compared with the theoretical value of QC samples to determine the long-term stability of PS916 in rabbit serum. The samples were to be considered stable if the percentage change (bias) in the concentration of the stability samples was not more than ±15% for higher concentrations and below ±20% for lower concentrations.

2.7. Pharmacokinetics of PS916

The animal handling fully complied with our institutional policies. The rabbits were fasted overnight but were allowed free access to water. Each of three animals received a PS916 dose of 50 mg/kg via the ear vein. They were anesthesized with 30 mg/kg pentobarbital sodium and equipped with a cannula in the carotid artery for blood sampling. The animals remained under anesthesia during the course of the pharmacokinetic study. The blood samples (about 0.5 ml) were withdrawn via the carotid artery at predetermined time intervals up to 24 h after the drug administration. Serum was obtained by allowing the blood samples to stand for 1 h on ice, after which the samples were centrifuged at 12600 × *g* for 20 min at 4 °C. Serum samples were stored at −20 °C until assayed.

Pharmacokinetic analysis of PS916 concentrations in serum was performed using the Practical pharmacokinetic program (3p87) (Chinese society of mathematical pharmacology). Goodness-of-it was evaluated from visual inspection of the measured and calculated data points and of the residuals plotted against time and against concentration. The choice between 1, 2 and 3 compartment models was based on the lowest value of the Akaike's Information Criterion (AIC) [15,16].

3. Results and discussion

3.1. Method development

In the present study, we have developed a straightforward and accurate HPLC method to detect PS916 in serum. The extracted samples of PS916 were successfully separated on a C8 analytical column. We tested several mobile phases and found methanol: water (20:80, v/v) as an appropriate mobile phase for the separation of the compounds within a run-time of 15 min. We also found the appropriate flow rate was 0.2 ml/min. If the flow rate >0.2 ml/min, PS916 couldn't be separated from endogenous compounds. Typical HPLC chromatograms of PS916 after extraction from serum are shown in Fig. 2. No interfering peaks of endogenous compounds were found at the retention time of PS916.

3.2. Method validation

3.2.1. Linearity, LLOD and LLOQ

All calibration curves were found to be linear over the calibration range of 5–100 μg/ml. The mean (±S.D.) regression equation for calibration curves in serum was $A = (12.725 \pm 0.001)C + (136.4 \pm 0.021)$, $r^2 = 0.9943 \pm 0.0011$. The coefficient of variations of slope for PS916 in serum was found to be <15%, which indicates a high precision of the assay. The LLOD and LLOQ of PS916 were found to be 1.0 and 2.5 μg/ml.

3.2.2. Recovery

The mean absolute recoveries of PS916 from serum at 10, 50 and 75 μg/ml were 65.6 ± 2.0%, 65.9 ± 1.9% and 67.2 ± 2.0%, respectively. Although absolute recoveries were low because of the strong binding between serum protein and PS916, they were consistent, precise and reproducible and still able to assure the accuracy and precision of measurement of serum PS916.

3.2.3. Accuracy and precision

The intra- and inter-day variability of the assay for serum is listed in Table 1. The intra-day CV% at 5, 10, 50 and 75 μg/ml of PS916 were 3.91%, 3.72%, 3.22% and 1.97% ($n = 5$), respectively. The inter-day CV% at the above concentrations were 5.16%, 4.57%, 4.34% and 5.59% ($n = 5$), respectively. One-way analysis of variance (ANOVA) was carried out with grouping variable "day" to assess precision at 95% level. The result was non-significant when data for each day was compared with other days and within the same day.

Table 1
Intra- and inter-day precision and accuracy of PS916 in rabbit serum ($n = 5$)

Concentration (μg/ml)	Intra-day			Inter-day		
	Mean ± S.D. (μg/ml)	Precision (% CV)	Accuracy (% bias)	Mean ± S.D. (μg/ml)	Precision (% CV)	Accuracy (% bias)
5	4.13 ± 0.16	3.91	−17.4	4.02 ± 0.21	5.16	−19.6
10	8.71 ± 0.32	3.72	−12.9	8.59 ± 0.39	4.57	−14.1
50	43.60 ± 1.40	3.22	−12.8	43.00 ± 1.87	4.34	−14.0
75	69.97 ± 1.38	1.97	−6.71	69.65 ± 3.89	5.59	−7.13

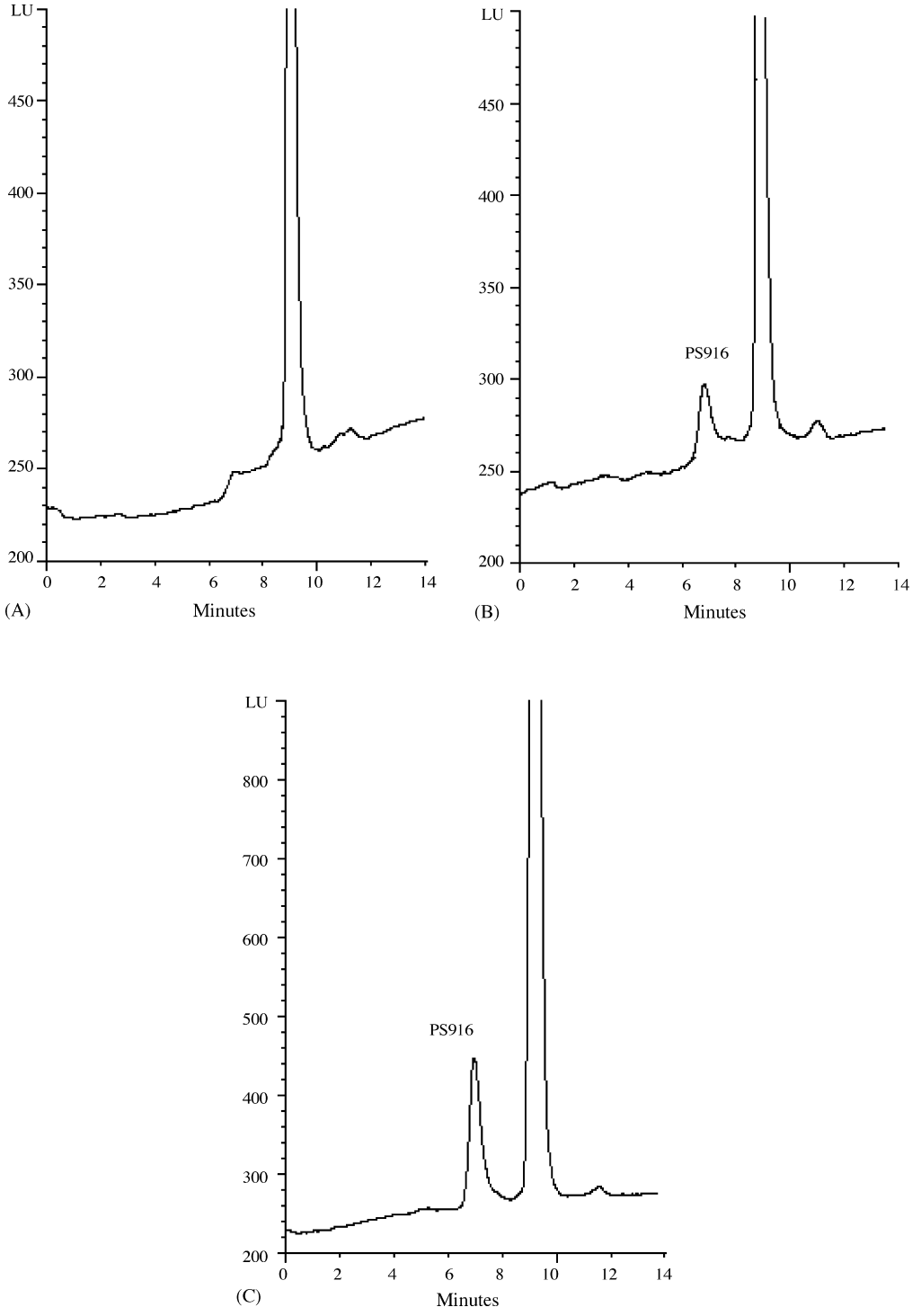

Fig. 2. Chromatograms of (A) blank rabbit serum, (B) serum spiked with 2.5 μg/ml PS916, (C) serum sample obtained at 30 min after i.v. administration of 50 mg/kg of PS916 from a rabbit.

3.3. Stability

Long-term stability studies showed no significant degradation of PS916 in serum samples stored at 4 and −20 °C since the % CV and % bias were below ±15% for higher concentrations and below ±20% for lower concentrations (Table 2).

3.4. Pharmacokinetic study of PS 916 in rabbits

We applied the method for a pharmacokinetic study in which PS916 was administered to healthy rabbits. After a single intravenous bolus injection of 50 mg/kg of PS916, we monitored the drug concentrations in serum until 1440 min after administration (Fig. 3). According to the lowest value of the AIC

Table 2
Stability of PS916 in rabbit serum at different temperature ($n = 5$)

T (°C)	Concentration (μg/ml)	Mean ± S.D.	% CV	% Bias
4	10	8.42 ± 0.38	4.69	−15.8
	50	43.00 ± 1.86	4.34	−14.0
	75	64.34 ± 1.65	2.60	−14.2
−20	10	8.59 ± 0.39	4.57	−14.1
	50	44.69 ± 2.12	4.74	−10.7
	75	65.85 ± 0.96	1.46	−12.2

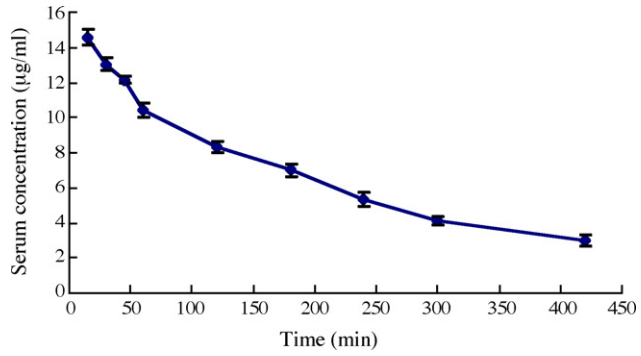

Fig. 3. Serum concentration–time disappearance curve of PS916 in rabbits following i.v. administration of 50 mg/kg. Each data point represents the mean ± standard deviation ($n = 3$).

Table 3
Pharmacokinetic parameters (two-compartment model) derived from serum concentration–time curves after a single intravenous dose of 50 mg/kg of PS916 in rabbits

Parameters	Values (mean ± S.E.M.)
V_c (ml/kg)	2820 ± 323
V_{ss} (ml/kg)	4163 ± 358
CL (ml/kg/min)	13.4 ± 3.4
$AUC_{(0-\infty)}$(min μg/ml)	3736.2 ± 4.3
$t_{1/2}$ α (min)	21.1 ± 0.7
$t_{1/2}$ β (min)	222.9 ± 4.5
MRT (min)	311.0 ± 7.6

V_c: volume of distribution in central compartment; V_{ss}: volume of distribution at steady state level; CL: serum clearance; $AUC_{(0-\infty)}$: area under the curve from zero to infinity; $t_{1/2}$ α: distribution half life; $t_{1/2}$ β: elimination half life; MRT: mean residence time.

implemented in 3p87, PS916 serum concentration-time date in rabbits fitted to a two compartment open model. The calculated pharmacokinetic parameters are listed in Table 3. The area under the curve ($AUC_{(0-\infty)}$) of PS916 in present study was observed to be 3736.2 ± 4.3 min μg/ml. The value of mean residence time (MRT) was 311.0 ± 7.6 min. The distribution half life ($t_{1/2}$ α) of PS916 was concluded to be 21.1 ± 0.7 min. PS916 appears adequately absorbed, and well distributed to body fluids. The pharmacokinetic parameters of PS916 have not been previously reported.

4. Conclusion

An HPLC method with postcolumn fluorescence detection provides a new simple, reproducible and validated assay for the determination of PS916 in serum. There is no doubt that it can be used for pharmacokinetic study on PS916. This method also gives the potential for quantitative determination of other polysaccharides in biological materials during a single analytical run.

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