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Preparation and characterization of Pingyangmycin-loaded bovine serum albumin microspheres for embolization therapy

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Abstract

Bovine serum albumin microspheres (BSA-MSs) containing Pingyangmycin hydrochloride (PYM) were prepared for the interventional embolization by an emulsification-crosslinking method. The average diameter of the MSs was 83.6 μm with 80% ranging from 35 to 200 μm. The MSs showed a rather high entrapment efficiency (EE%) of 87.6 ± 5.7% and the drug loading efficacy (DL%) was 20.2 ± 1.3%. The drug release behaviors were evaluated both *in vitro* and *in vivo*, using UV-spectroscopy and HPLC, respectively. The *in vitro* results showed a significantly delayed release of drug for 10 days. The central auricular artery of rabbit was chosen as an embolization sites to study the *in vivo* drug release and the pharmacokinetics of the MSs compared with PYM injections. Experiments performed by artery perfusion and embolization in rabbits central auricular artery revealed that the PYM loaded BSA-MSs (PYM-BSA-MSs) could obviously prolong *in vivo* drug release, extend the mean residence time (MRT) and had equal bioavailability compared with plain PYM injections. These results demonstrated that by embolization of the central auricular artery with PYM-BSA-MSs, the local drug concentration could maintain at a relative high level for a longer time, thus achieve the aim of tumor targeting therapy. PYM-BSA-MSs are excellent potential alternatives of interventional embolization materials for the treatment of maxillofacial region tumors.

Keywords: Microspheres; Pingyangmycin; Bovine serum albumin; Emulsification-crosslinking method; Pharmacokinetics

1. Introduction

Head and neck tumors are serious diseases with a high attack rate of nearly 10% of the total incidence of malignant tumors in China (Li, 1993). The therapy of head and neck tumors mainly depended on surgery for a long time, however, there are so many challenges for conventional surgery such as high risk, heavy trauma and high tumor relapse rate due to the complex blood supply and anatomic structure in the oral and maxillofacial regions. And further, the potential of deformation of facial features after surgery also have significant effects on the patients' quality of life (Wang and Zhu, 2004). Embolization has been used extensively to occlude vessels in the last few decades as an alternative to such diseases, especially when traditional therapy failed. Recently, embolization techniques have been employed more broadly and with greater precision and convenience (Bendszus et al., 2000; Yamamoto et al., 2003). As a result, this "last resort" technique has quickly evolved into first-line therapy for many complex clinical conditions (Robert et al., 2004). Nowadays, as technological advancements allow easier and safer embolization access to small and remote lesions, transcatheter arterial embolization (TAE) offers a new approach for the therapy of oral and maxillofacial malignant tumors (Chen et al., 2005). An ideal embolic agent is a prerequisite for successful TAE therapy in clinical applications, it must be biodegradable, spherical, smooth surfaced and cannot be absorbed quickly (Yutaka et al., 2000). BSA has a good biocompatibility, the degradation products of BSA were nontoxic, which was found many applications in diagnosis and treatment in recent years and more than 100 diagnostic agents and drugs have been incorporated into albumin microparticles (Dandagi et al., 2006), which suggests its suitability in the drug delivery field and artery embolization.

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PYM, as also called Bleomycine A5, is a new type of cytotoxic glycopeptide antitumor antibiotic, which was developed in China in the 1980s. Besides its benefit of wide antitumor spectrum and lower toxicity in chemotherapy of malignant tumors, PYM plays a particular curative role in head and neck tumors therapies (Wan et al., 1999; Li et al., 2000). But it has obvious defects such as short half-life and lung-toxicity (Li et al., 1995). In the present study, we employed BSA as a matrix for PYM-MSs through an emulsification-crosslinking method, for the sake of developing a new embolic agent for interventional chemoembolization therapy, which cannot only occlude vessels to cut off the nutrition supply of tumor tissues but also control the drug release to maintain an effective therapeutic concentration at local tissues. For this purpose, the PYM-BSA-MSs were prepared and characterized, the pharmacokinetics and embolic effect of drug-loaded MSs were investigated as well.

2. Materials and methods

2.1. Materials

PYM was supplied by Taihe pharmaceutical Co. Ltd. (Tianjin, China) and BSA was purchased from Boao Biotechnology Co. Ltd. (Shanghai, China); purified rapeseed oil was purchased from Lijia corn and oil Co. Ltd (Sichuan, China). All other chemicals used were of analytical grade.

2.2. Preparation of PYM-MSs

PYM-BSA-MPs were prepared by an emulsification-crosslinking method. Briefly, 75 mg of PYM was dissolved in 0.5 ml BSA solution (25%) in phosphate buffer solution (pH 7.4) (PBS) and used as the aqueous phase. The oil phase comprised of 25 ml rapeseed oil with 1% Span-80 (as stabilizer and emulsifier). The aqueous phase was added dropwisely to the oil phase and stirred at the 250 rpm for 10 min to form a primary emulsion. Then 50 ml ether was added to dilute the primary emulsion, and soon 0.2 ml formaldehyde (25%) were added to solidify the MSs and the mixture was kept stirring for 1 h. The suspension containing the MSs was centrifuged at 3500 rpm for 30 min and the sedimented MSs were washed for three times with ether to remove traces of oil on MSs surfaces. The obtained MSs were then dried in vacuum overnight and stored at 4 °C.

This experimental method has been published elsewhere in details (Wang et al., 2003). The main factors influencing the particle size of MSs, *i.e.* water/oil ratio; viscosity of oil phase; BSA concentration; pH; stirring rate; emulsification time; dose of emulsifier; crosslinking agent and amount; crosslinking time, *etc.*, were tested by single-factor studies. Subsequently, three factors (stirring rate; emulsification time; dose of emulsifier) among the above mentioned variables which influence the size distribution considerably were chosen and divided into seven levels. An U_7 (7^6) uniform design table was used to optimize the experiment. Recovery rate and size distribution were regarded as the indices and the results were statistical analyzed using a uniform-design data-processing program.

2.3. Particle size distribution and morphology

Particle size analysis was performed by Malvern-2000 particle size analyzer and each sample was detected in triplicate. The morphology was observed by the light microscopy (Olympus, Japan).

2.4. EE% and DL%

Ten milligram of PYM-BSA-MSs was transferred to a 25 ml volumetric flask. A quantum of PBS (pH 7.4) was added to a total amount of 25 ml and then ultrasonicated for 30 s to dissolve PYM absorbed on the surface of MSs. After centrifugation, the supernatant was collected for further determination. The washed MSs were digested by 5 ml of 5% pepsase solutions and metered volume by PBS (pH 7.4). All the samples were filtered (PTFE 0.45 μm) before determination by a UV spectrophotometer (Shimadzu spectrophotometer U-2201 Instruments, Japan) at the wavelength of 293 nm. Triplicate measurements were performed for each sample. The concentrations of washed solution and the digested sample were referred as C_1 and C_2, respectively, the EE% and the DL% of the MSs can be calculated according to the Eqs. (1) and (2):

$$\mathrm{EE\%} = \frac{C_2}{C_1 + C_2} \times 100\% \quad (1)$$

$$\mathrm{DL\%} = \frac{C_2}{\text{the amount of MSs}} \times 100\% \quad (2)$$

2.5. In vitro release studies

The drug release study was carried out using the small beaker method, as described in the Chinese Pharmacopoeia 2000, PBS (pH 7.4) (100 ml) was used as the release medium at 37 °C and at a stroke speed of 100 rpm. A dynamic dialysis technique was employed. Typically, fifty milligram of PYM-BSA-MSs was dispersed in a pretreated dialysis bag with 5 ml PBS (pH 7.4). The dialysis bag was sealed and suspended in 95 ml PBS (pH 7.4) to ensure a sink condition. Samples of 5 ml were removed at predetermined time intervals, filtered through a Millipore filter (PTFE 0.45 μm), and assayed spectrophotometrically at a wavelength of 293 nm according to the previously reported method (Chen et al., 2000). Five milliliter of PBS (pH 7.4) was added to the release medium immediately after sampling. All the *in vitro* release tests were carried out in triplicate, and the accumulation release amount of drug from PYM-BSA-MSs was calculated.

2.6. Pharmacokinetics studies

2.6.1. Chromatography

An ion-pairs-HPLC was employed for determination of PYM concentrations in plasma samples. A Hanbang C_{18} column (150 mm × 4.6 mm, 5 μm) was used. The mobile phase

was a mixture of methanol:acetonitrile:water (15:8:50), the water phases contains: 0.186% ethylenediamine tetraacetic acid (EDTA), 0.098% sodium pentanesulfonate, 0.44% (v/v) tetramethyl ethylenediamine (TEMED), 0.9% (v/v) glacial acetic acid and was adjusted to pH 4.0 with phosphoric acid. The flow rate was l.0 ml/min. The effluent was monitored at 293 nm at 40 °C and Caffeine was selected as internal standard. All measurements were performed in triplicate.

2.6.2. Plasma sample preparation and validity

Plasma (500 μl) was obtained after centrifugation (15 min, 4000 rpm) and stored at −20 °C until analyzed. When the plasma sample thawed, 25 μl of caffeine (2 mg/ml) solution and 475 μl of 10% trichloroacetic acid (TCA) were added, and agitated for 30 s. This mixture was shaken for 3 min and then centrifuged at 12,000 rpm for 10 min. Aliquots (20 μl) of the supernatant were injected for HPLC analysis. The method was validated by adding various quantities of PYM and the same amount of caffeine to blank rabbit plasmas. The resulting concentrations of PYM were 0.5, 1.0, 5.0, 10.0, 20.0 and 50.0 μg/ml. These calibrations were subjected to the entire analytical procedure, so as to test the linearity, precision and accuracy of the method.

2.6.3. Pharmacokinetic study of PYM injections and PYM-BSA-MSs in rabbits

Twelve rabbits (2.5 ± 0.11 kg) were used for pharmacokinetics studies which were divided randomly into two groups, and fasted for 12 h but water was available *ad lib*. In the experiments, PYM-BSA-MSs formulations equivalent to 8.0 mg/kg (calculated by PYM) suspended in soybean oil were administered to one group of rabbits through auricular central artery. The PYM injections equivalent to 8.0 mg/kg of PYM were administered in the same way to another group. Blood specimens were collected from another auricular brim veins of rabbits at different predetermined time intervals (0, 5, 10, 15, 25, 35, 45, 55, 75, 95, 115, 190, 250, 310 min) in heparinized tubes.

Pharmacokinetic values such as the area under the curve (AUC), half-life, and mean residence time (MRT) were calculated with pharmacokinetics program Drug And Statistics (DAS2.0, Anhui, China) and compartment models were based on AIC variance and *F*-values.

2.7. Statistical analysis

The results were expressed as mean values ± S.D. the Student's *t*-test was applied to examine the significance of differences when analysis of normality and equal variance were passed. In all cases $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Preparation of MSs

In previous studies, Bahukudumbi found that particle size was significantly affected by the stirring rate and duration (Bahukudumbi et al., 2004). In this experiment, once the stirring rate exceeds 500 rpm, the particle size decreased rapidly to an average of 20 μm, and the stirring time can be controlled under 10 min to form a suitable particle size, but the duration should be longer than 5 min in order to provide sufficient energy to form the primary emulsion.

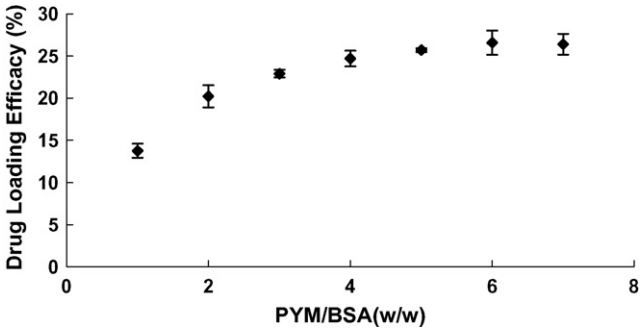

Fig. 1. The effect of drug/BSA ratio on DL% of PYM-BSA-MSs ($n = 5$).

Emulsifier is also extremely important for maintain the stability of the primary emulsion when the ether was poured into the system. If the amount of emulsifier is above 2%, the particle size decreased dramatically to form a large amount of MSs which unfit for embolization. After preliminary studies, 1% Span-80 was chosen as a stabilizer and emulsifier.

The concentration of BSA solution was also investigated, the optimum concentration was 25% (g/ml), with such a concentration, the MSs with optimum flexibility as a rigid spherical entity can be obtained, while those produced from lower concentration were not suitable for embolization therapy due to the high swelling rate and soft textures.

3.2. EE% and DL%

The drug/BSA ratio has a significant influence in EE% and DL%, which is summarized in Table 1. DL% increased with the initial drug amount (Fig. 1), while the EE% kept at still level or even decreased with the initial drug amount to a certain point. When the ratio exceeds 15/50, a rapidly decrease of EE% was observed (Fig. 2). It reveals that with the increase of drug/BSA ratio, the initial added drugs were mainly attached and absorbed at the surface of MSs, however, when rashed with PBS, the drug loosely absorbed at the surface was removed, which led to the decrease of EE%. So in present study, a high DL% and

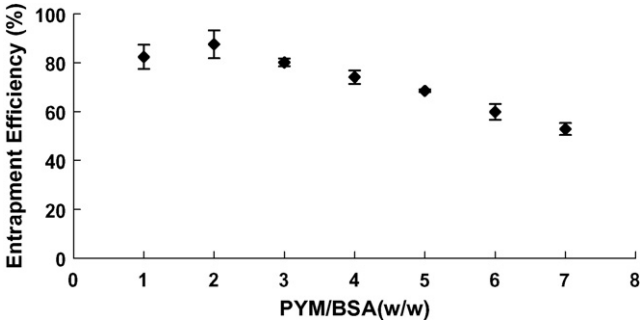

Fig. 2. The effect of drug/BSA ratio on EE% of PYM-BSA-MSs ($n = 5$).

Table 1
DL% and EE% of formulations with different drug/BSA ratios ($n = 5$)

No.	0	1	2	3	4	5	6	7
PYM/BSA	0/50	10/50	15/50	20/50	25/50	30/50	40/50	50/50
EE ± S.D.%	–	82.4 ± 5.0	87.6 ± 5.7	80.2 ± 1.6	74.1 ± 2.8	68.5 ± 0.6	59.8 ± 3.3	52.8 ± 2.5
DL ± S.D.%	–	13.7 ± 0.8	20.2 ± 1.3	22.9 ± 0.4	24.7 ± 0.9	25.7 ± 0.2	26.6 ± 1.4	26.4 ± 1.2

(–), the concentration is below the LOD.

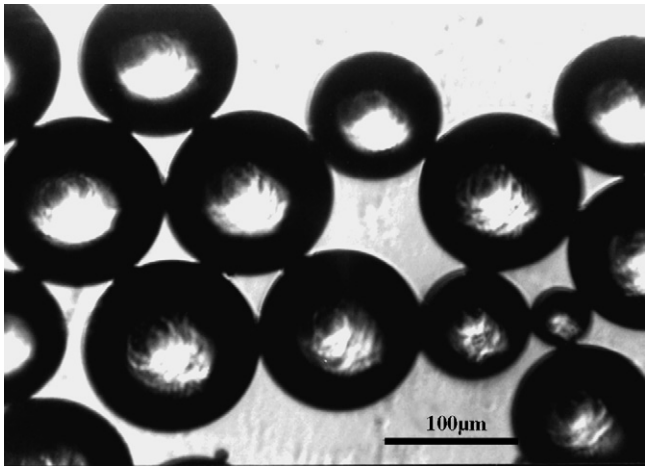

Fig. 3. Light microscopy of PYM-BSA-MSs, scale bar: 100 μm.

EE% was obtained simultaneously when the drug/BSA ratio was 15/50. The DL% and EE% are 20.2 ± 1.3% and 87.6 ± 5.7%, respectively.

3.3. Morphology and particle size of MSs

MSs prepared under optimum conditions were spherical rigid entities with smooth surface under the light microscopy, which are shown in Fig. 3. The average diameter of the MSs was 83.6 μm with 80% within 35–200 μm (Fig. 4 shows one of the size distribution profiles).

MSs could be separated with a multilevel sieve and sized for intervention embolization therapy in various tumors and at different anatomic sites. Particles with an average diameter larger than 40 μm are preferable for embolization of neck and head tumor artery and the blood vessel of angioma (Bastian et al., 1998), and the minimum diameter limitation of MSs is 10 μm, avoiding embolization of some other important organs such as brain and lung. In our experiments, the average diameter of sieved microparticles was around 80 μm with stirring rate of approximately 250 rpm, which fits the need of embolization. Whereas, when the stirring rate was raised to 500 rpm, the average diameter of MSs became smaller than 30 μm. There were so many critical preparation parameters which determine the size of the MSs, such as BSA content, stability of emulsion, stirring rate, and types of solidification reagents (Ugwoke and Kinget, 1998).

3.4. In vitro release studies

The characteristics of drug release were investigated *in vitro* by UV spectroscopy. The cumulative percentage of drug release of crude drug was found to be 88.28% at 4 h. The release profiles of PYM from BSA-MSs prepared by emulsification-crosslinking method are shown in Fig. 5, which shows the influence of formaldehyde treating time of BSA on the sustained-release behavior of PYM-BSA-MSs. Extension of the treating time would cause a decrease of ultimate-release rate and the "burst effect" could be controlled also, in other word, the drug release rate would slow down. The total release amount can reach 80% after 240 h (solidified for 1 h) and 480 h (solidified for 6 h and 12 h) (data not shown).

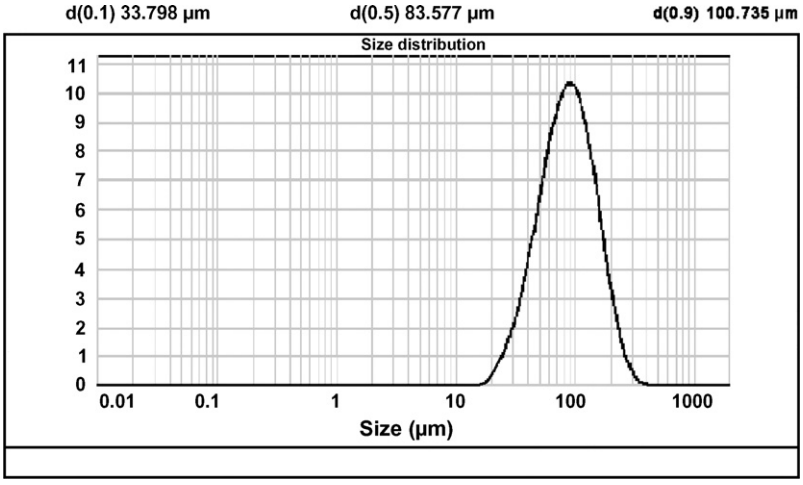

Fig. 4. Size distribution of PYM-BSA-MSs detected by laser scattering.

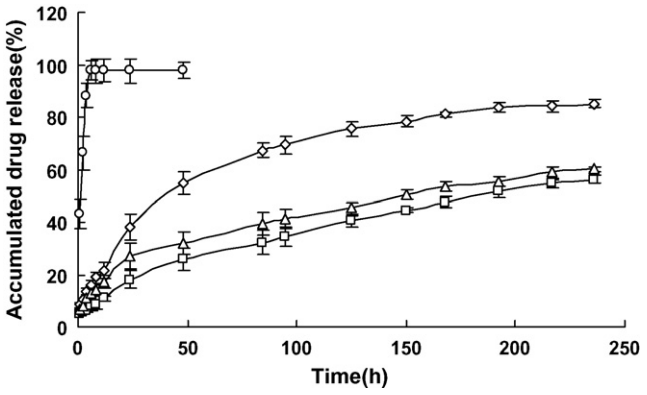

Fig. 5. The release profiles of PYM-BSA-MSs prepared by chemical-crosslink method *in vitro* ($n = 6$). (◊) The release curve of PYM-BSA-MSs solidified for 1 h; (△) the release curve of PYM-BSA-MSs solidified for 6 h; (□) the release curve of PYM-BSA-MSs solidified for12 h; (◯) the release curve of PYM.

The addition of enzyme (0.5% trypsin) can cause a rapid collapse and erosion of the MSs, and the drug release rate was accelerated obviously, 80% of entrapped drugs were released after 24 h.

The *in vitro* drug release behaviors of MSs made by emulsification-heat-denatured method were also tested according to dialysis method described above (Fig. 6). MSs denatured for different heated time showed slight difference in drug release profiles. The drug release rate was more rapid than that solidificated by formaldehyde. This fact indicates that PYM was not only encapsulated into the MSs but also covalently combined with the surface active amino residue of BSA by the formaldehyde, which cause the sustained drug release during 10 days, this result is in accordance with previous research result (Merodio et al., 2001).

According to these figures, we find that two batches of MSs prepared by different method have same release pattern except the ultimate release rate. First, there is a slow release phase caused by removing the free drugs on the surface of MSs, then a rapid release occurs with the diffusion of the inner drugs through the BSA skeleton and the erosion of MSs and finally enter a smooth release phase. This drug release procedures might be the possible explanation for the absorption phase of *in vivo* drug release curve.

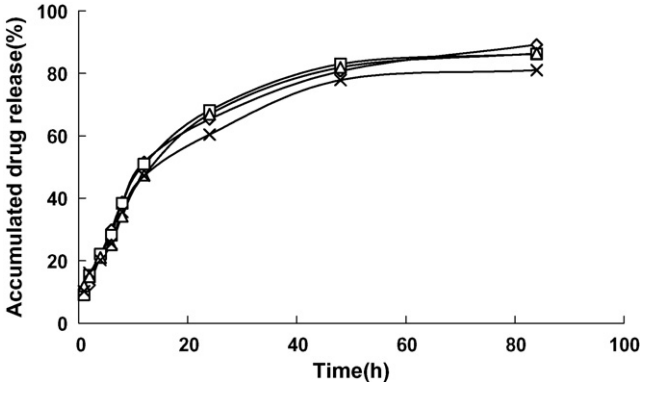

Fig. 6. The drug release profiles of PYM-BSA-MSs prepared by heat-denatured method ($n = 6$).

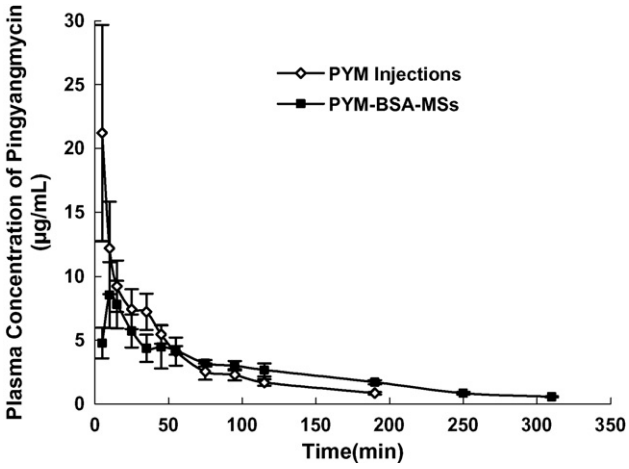

Fig. 7. The plasma concentration-time profiles of PYM in rabbits after intravenous administration PYM-BSA-MSs and PYM solution ($n = 6$).

3.5. Pharmacokinetics studies

3.5.1. Chromatography

PYM in plasma was completely separated under analytical conditions, and standard curves ranging from 0.5 to 50 μg/ml were linear ($r = 0.9985$). The results attained from the method recoveries of high, middle and low concentrations were 96.9 ± 2.1, 98.4 ± 3.7 and 97.2 ± 3.0%, respectively. The relative standard deviation (R.S.D.) in days were 4.89, 3.31and 5.07%, respectively, the intra-days precision were 5.36, 4.55 and 5.78%, respectively, which showed recoveries and R.S.D. in days or intra-days were satisfactory, and the limit of detection (LOD) was 100 ng/ml.

3.5.2. Pharmacokinetics

The mean plasma concentrion-time profiles of PYM-BSA-MSs and plain PYM solutions (8 mg/kg) were shown in Fig. 7. And the calculated pharmacokinetic parameters were given in Table 2, consequently, the parameters were calculated according to compartment models (Table 3). The mean retention

Table 2
The plasma level after a single-dose of PYM solution and PYM-BSA-MSs after intravenous administration in rabbits ($n = 6$)

Time (min)	PYM (μg/ml)	PYM-BSA-MS (μg/ml)
5	21.20 ± 8.48	4.77 ± 1.20
10	12.21 ± 3.60	8.53 ± 2.59
15	9.22 ± 2.02	7.79 ± 1.86
25	7.43 ± 1.55	5.72 ± 1.30
35	7.22 ± 1.42	4.36 ± 1.07
45	5.46 ± 0.73	4.46 ± 1.68
55	4.11 ± 1.10	4.20 ± 0.34
75	2.53 ± 0.64	3.18 ± 0.25
95	2.28 ± 0.44	2.99 ± 0.37
115	1.67 ± 0.25	2.66 ± 0.52
190	0.83 ± 0.10	2.10 ± 0.16
250	–	0.94 ± 0.10
310	–	0.55 ± 0.04

(–), the concentration is below the LOD.

Table 3
Pharmacokinetic parameters of PYM and PYM-BSA-MSs in rabbits after intravenous administration ($n = 6$)

Pharmacokenitic parameters	PYM injections	PYM-BSA-MS
C_{max} (mg/l)	21.20 ± 8.48	8.53 ± 2.59
$t_{1/2\alpha}$ (min)	1.64 ± 1.41	47.99 ± 8.54
$t_{1/2\beta}$ (min)	25.84 ± 4.38	48.18 ± 6.42
Vc (l/kg)	0.09 ± 0.01	1.075 ± 0.21
Cl (l/min kg)	0.008 ± 0.002	0.009 ± 0.001
$AUC_{(0-t)}$ (mg/l min)	831.22 ± 123.37	774.81 ± 101.24

time (MRT) of the drug was calculated using the statistical square method, whereas the area under concentration–time curve (AUC) was obtained using the trapezoidal rule.

The parameters shown that the artery administration of PYM solution could be metabolized more rapidly than MSs with the approximate same AUC ($P > 0.05$). The C_{max} was reduced to 40% ($P < 0.01$) of the injection group; $t_{1/2\beta}$ was prolonged to 1.86-folds ($P < 0.01$); clearance (Cl) was 1.12-folds ($P > 0.05$); apparent volume of distribution (Vc) was 11.94-folds ($P < 0.01$). The $MRT_{0-310\,min}$ of injection and MSs was 45.51 min and 95.92 min, respectively. In conclusion, the half elinimination time of MSs was prolonged compared with PYM injections, which reflects higher stability in the embolized artery than the free drugs.

4. Conclusion

To improve the effect of chemoembolization therapy, we choose BSA-MSs as sustained release carriers for interventional embolization. With formaldehyde as crosslinkers, BSA-MSs were prepared by improved emulsified-crosslinking methods. The results indicated that by incorporation into the BSA-MSs, a sustained release of PYM was achieved both *in vitro* and *in vivo*. The pharmacokinetics of PYM-BSA-MSs was evaluated and plain PYM injections was adopted as control. The results demonstrated that a small dosage of PYM-BSA-MSs by artery embolization could produce higher drug concentrations at locally tissues for a longer time and reduce the drug level of body circulation, which achieve the goal of targeted tumor therapy. Compared with other embolization materials, the ready-for-use BSA-MSs are excellent alternatives to superselective embolization materials for arterioles. This drug delivery systems play a potential role for the administration of antitumor drugs by chemoembolization procedures.

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