Drug Development and Industrial Pharmacy, 2009; 35(6): 639–645

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healthcare

RESEARCH ARTICLE

Preparation and characteristics of erythromycin microspheres for lung targeting

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Abstract

Background: If erythromycin is micronized into microspheres with suitable particle size, it can improve pulmonary drug concentration to maximize its effectiveness and minimize the adverse side effects. *Aim*: In this study, erythromycin gelatin microspheres (EM-GMS) were prepared and some characteristics of EM-GMS were investigated. The drug-targeting index (DTI) of EM-GMS was evaluated to predict their potential as a targeted delivery system. *Method*: Erythromycin was microencapsulated with gelatin by a double emulsion solvent evaporation method. Some characteristics of EM-GMS, including morphology, particle size, in vitro release, and safety were researched. *Results*: EM-GMS had a spherical shape and smooth surface morphology. The drug loading and encapsulation efficiency of EM-GMS were 13.56 ± 0.25% and 55.82 ± 2.23%, respectively. The release of erythromycin from EM-GMS showed an initial burst and following a sustained release, with an accumulate release of 80% at 4 hours. The EM-GMS was safe since there was no vein irritation and no hemolysis on the erythrocyte of rabbit at 3.5 mg/mL and a LD50 of 173.07 mg/kg. After administering EM-GMS to rabbits, the concentration of erythromycin in lung was 15.92 times higher than that in plasma and the DTI of EM-GMS in lung was 6.65 as compared with erythromycin lactobionate. *Conclusions*: The preparation technology of EM-GMS for lung targeting was successful and the quality of microspheres was good.

Key words: *Character; erythromycin; lung-targeting; microspheres; preparation*

Introduction

Micronization of drugs plays an important role in improving the drug dosage form and therapeutic efficiency today. If an antibiotic is micronized into microspheres with suitable particle size, it can be addressed directly to the lung by the mechanical interception of capillary bed in the lungs[1].

Erythromycin is a macrolide antibiotic used for the treatment of bacterial infections caused by gram-positive bacteria and some gram-negative bacteria, anaerobes, legionella bacteria, Chlamydia, and mycoplasma. Erythromycin is widely used clinically in respiratory infectious diseases[2]. However, erythromycin has a short half-life ($T_{1/2}$, about 1.4–2 hours) and distributes all over the body, which is likely to affect unwanted sites[3]. If erythromycin is prepared as microspheres in the size range of 7–25 μm, the microspheres can be concentrated in lung through i.v. administration[4]. This technique can improve pulmonary drug concentration to maximize its effectiveness against some pulmonary infections such as mycoplasmal pneumonia and minimize the adverse side effects.

Microparticulate delivery systems are generally produced with polymeric materials of synthetic or natural origin. Among natural polymers, gelatin represents a good raw material since it easily forms films and particles. Gelatin is also a nontoxic, biodegradable, biocompatible, noncarcinogenic, nonimmunogenic, and inexpensive natural polymer, which has been largely investigated for the preparation of nanoparticles and microparticles[5]. Gelatin is approved to be injected by *Pharmacopoeia of the People's Republic of Chinese*, 2005 edition[6]. The gelatin-prepared EM-GMS was injectable, and the purity, molecular weight, and impurity levels of gelatin conformed to injection standards.

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(Received 28 Jun 2008; accepted 28 Sept 2008)

ISSN 0363-9045 print/ISSN 1520-5762 online © Informa UK, Ltd.
DOI: 10.1080/03639040802512243

In the present study, erythromycin gelatin microspheres (EM-GMS) were prepared, and some characteristics of EM-GMS, including morphology, particle size, in vitro release, and safety, were investigated. The drug-targeting index (DTI) of EM-GMS was evaluated to predict their potential as a targeted delivery system for intravenous drug administration.

Materials and methods

Erythromycin was purchased from Guangdong Taishan City Chemical Pharmaceutical Co., Ltd., China. Gelatin was purchased from Shanghai Gelatin Factory. Erythromycin lactobionate (EL) for injection was purchased from Dalian Merro Pharmaceutical Co., Ltd., China. Erythromycin standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. All other chemicals were of analytical grade.

Kunming mice weighing 20–22 g (half of them male and the others female) and New Zealand rabbits weighing 2–2.5 kg were purchased from the Center for Laboratory Animal Science of Guangdong. The use of rabbits in this study was approved and had ethical clearance.

Instrumental analysis and measurements

A spectrophotometer, UV-1700 (ShiMadZu corporation, Japan), was used for determination of drug content in the microspheres. DSC-204 (Germany NETZSCH company, a differential scanning calorimeter), was used for differential scanning analysis of microspheres. An optical microscope with camera (Japan Olympus corporation, Japan) and a scanning electron microscope (SEM) (400F, Germany Zeiss company, Germany) were used for observing the morphology of the microspheres. High-performance liquid chromatograph consisted of a constant flow pump (LC-10ATVP, ShiMadZu corporation, Japan), and a variable-wavelength UV detector (SPD-10Avp, ShiMadZu corporation, Japan) was used for determination of drug content in rabbit tissues.

Preparation of erythromycin gelatin microspheres

Erythromycin gelatin microspheres (EM-GMS) were prepared according to the method described by Rita Cortesia et al.[7] To begin with, 10 mL of 10% gelatin solution containing 0.5 g erythromycin was gently stirred to form a suspension and then the suspension was added dropwise to paraffin at 50°C under mechanical stirring (1000 rpm) to form a W/O emulsion for 10 minutes. The W/O emulsion was rapidly cooled to 5°C in an ice bath and then the gelatin in the emulsion was cross-linked with 5 mL glutaraldehyde under continuous stirring for 60 minutes. The EM-GMS were recovered by filtration through sintered glass filter. The removal of residual oil was performed by washing the microspheres with ethyl ether and isopropanol. EM-GMS were vacuum-dried for 3 days and maintained in a vacuum drier at room temperature.

The morphology and particle size of EM-GMS

The morphology of EM-GMS was observed by scanning electron microscopy (SEM) after the microspheres coating with gold palladium and optical microscopy. After vaccum-drying, the suspension (10 mg of EM-GMS in 10 mL of water) was placed in the glass cell of a laser diffraction particle size analyzer and size distribution of EM-GMS was plotted.

Drug loading and encapsulation efficiencies of EM-GMS

The drug loading of EM-GMS was determined by UV spectroscopy at 482 nm[6]. The erythromycin contained in EM-GMS was calculated by drug loading and then the EM-GMS with equivalent doses of erythromycin was used for injection. An amount of 11.0 mg of erythromycin was dissolved in 2 mL ethanol, and adequate amounts of phosphate buffer (pH 7.4) were added to make 100 mL stock solution. Stock solutions of 1, 2, 3, 4, and 5 mL were mixed with PBS of pH 7.4 to make working solutions of 5 mL each. H_2SO_4 (5 mL) (75%, v/v) was added to each working solution to obtain a test solution. The test solution was cooled to room temperature, and was analyzed at the wavelength of 482 nm. UV absorption and erythromycin concentration showed good linearity when the concentration of erythromycin in the solution was between 0.0112 and 0.0560 mg/mL.

$$C = 0.0557A + 0.0003\,\text{mg/mL},\ r = 0.9999 \qquad (1)$$

A quantity of 10 mg of EM-GMS was digested with 5 mL phosphate buffer solution (pH 7.4, 0.1 M) and 5 mL H_2SO_4. The solution was filtered through a 0.45 μm millipore filter and then analyzed by UV spectroscopy at 482 nm. The solution of blank gelatin microspheres treated by the same method showed no UV absorbance at 482 nm. The amount of erythromycin in microspheres was calculated using the standard curve equation shown earlier.

The drug-loading efficiency of EM-GMS = (the amount of erythromycin in microspheres/the amount of erythromycin used) × 100%.

The encapsulation efficiency of EM-GMS = (the amount of erythromycin in microspheres/the amount of erythromycin used) × 100%.

Differential scanning calorimetry profile of EM-GMS

Differential scanning calorimetry (DSC) measurements were carried out using ultrasensitive differential scanning calorimeter. Samples of 10 mg of EM-GMS, blank gelatin microspheres (GMS), EM, and a physical mixture of EM and GMS were heated in an open aluminum pan using dry nitrogen as the effluent gas. Thermograms were obtained at a scanning rate of 10°C/min with a heating range of 30°C–250°C.

In vitro release of EM-GMS

In vitro release of EM-GMS was carried out in test tubes by using phosphate buffer (pH 7.4, 0.1 M) as release medium. EM-GMS containing 7.5 mg of erythromycin were suspended in 10 mL of phosphate buffer (pH 7.4) and the suspension was shaken at 100 rpm in an air-bath incubator at 37°C. At predetermined time intervals, the samples were centrifuged at 2500 rpm for 5 minutes and clear supernatant of the release medium was withdrawn and replaced with fresh release medium. To 5 mL of each of the collected solutions 5 mL of H_2SO_4 was added and then mixed, filtered, and analyzed by UV spectroscopy at 482 nm. The amounts of erythromycin were calculated from the standard curve [Equation (1)]. Data obtained were used to prepare the curve of accumulated drug release time.

The vein irritation test of EM-GMS

For vein irritation study, injections of EM-GMS and EA were prepared by suspending 35 mg EM-GMS in 10 mL of saline and dissolving 8.75 mg EA in 10 mL saline. New Zealand rabbits were divided into two groups (Group A and Group B), with three in each group. The rabbits were injected 2 mg/kg of erythromycin. Every rabbit in group A was given an injection of EM-GMS suspension into the right ear marginal vein and an injection of the same volume of saline into the left ear marginal vein. Every rabbit in group B was given an injection of EA solution and the same volume of saline into the left ear marginal vein. All injections were made at a rate of 1 mL/min, and injections were administered once a day for three consecutive days.

The appearance of the veins and the surrounding tissue was observed at intervals after the injection. At 24 hours after the last injection, the rabbits were killed and then the appearance of the veins and surrounding tissues was examined by an experienced unbiased observer. A part of the injection sites was excised and pathological sections were prepared.

In vitro hemolysis of EM-GMS

In vitro hemolysis of EM-GMS was evaluated using the method described by Qian Zhiyu et al.[8] A sample of 10 mL of rabbit blood was collected into tubes and the fibrinogen was removed by stirring with glass rod. All samples did not cause hemolysis and erythrocyte agglutination at 37°C. Erythrocytes were washed three times with 10 mL saline by removing the supernatant each time after centrifugation (1000 rpm, 5 minutes). The erythrocytes were suspended in saline to make 2% (v/v) erythrocyte suspension. Suspensions with 5 mg/mL of EM-GMS were prepared by suspending EM-GMS in saline.

Seven test tubes were numbered and 2.5 mL of erythrocyte suspension was added to each test tube. Different amounts of EM-GMS suspension with volume of 0.1, 0.2, 0.3, 0.4, and 0.5 mL were added into test tubes numbered 1–5, respectively. Adequate amounts of saline was added to every tube to obtain a final volume of 5 mL. Tube no. 6 was treated as a negative control tube by adding 2.5 mL of saline, and tube no. 7 was considered as a complete hemolysis control tube (a positive control tube) by adding 2.5 mL of distilled water instead of saline. All the tubes were incubated for 3 hours at 37°C. The hemolytic activities were observed with naked eye at 15, 30, 45, 60, 120, and 180 minutes.

The acute toxicity of EM-GMS

The median lethal dose (LD_{50}) was measured to determine the acute toxicity of EM-GMS. Mice were injected with various doses of EM-GMS, GMS, or EA via the lateral vein tail. Kunming mice were housed under normal conditions with free access to food and water. One hundred and fifty Kunming mice (half male and half female, 18–22 g) were divided into 15 groups randomly, each with 10 mice. Groups 1–5 were administrated i.v. EM-GMS suspensions at doses of 120.0, 144.0, 173.0, 208.0, and 250.0 mg/kg in 0.3 mL saline, respectively. Groups 6–10 as control groups were administrated i.v. GMS at doses of 130.0, 157.2, 190.6, 231.0, and 280.0 mg/kg in 0.3 mL saline, respectively. Groups 11–15 as another set of control groups were administrated i.v. EA solutions at doses of 224.0, 272.2, 331.1, 402.8, and 490.0 mg/kg in 0.3 mL saline, respectively.

The toxic response such as anxiety, piloerection, catatonia, and respiratory distress were observed by an experienced unbiased observer for 2 weeks. The number of dead mice was recorded and the LD_{50} was calculated using the Bliss method. The main organs of dead mice, such as heart, liver, spleen, lung, and kidney, were subject to macroscopic examination.

The lung-targeting effect tests of EM-GMS

A modification of the method described by Stubbs et al.[9] was used to detect the erythromycin content in

rabbit tissues. Chromatographic analysis was performed with a DIKMA (ODS, 250 × 4.6 mm, 5 µm, USA) column. The mobile phase was acetonitrile-ammonium dihydrogen phosphate buffer, 0.1 M (33:67, v/v) at 1.0 mL/min. The UV detector was used at a wavelength of 210 nm. Samples of 20 µL were injected into the HPLC column. The areas of the chromatographic peak of samples were used to quantify the erythromycin.

EM-GMS were suspended in saline and EA was dissolved in saline for injection. New Zealand rabbits of the control group were injected. EM-GMS suspensions and EA solution into their ear marginal vein. The dose for rabbits was 3.5 mg/kg of erythromycin. Rabbits were sacrificed at 2 hours after drug administration and the heart, liver, spleen, lung, kidney, and blood were collected. Tissue samples were washed in icecold saline and blotted with filter paper to remove excess fluid. Blood and tissue samples were stored at −20°C until assessment for drug concentration by HPLC.

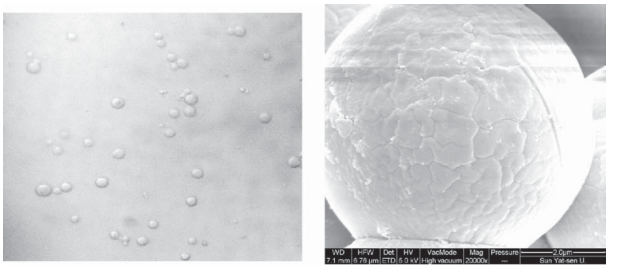
Tissue samples were homogenized with two volumes of phosphate buffer (pH 7.8, 0.1 M) to make homogenates. A volume of 50 µL of 1 mol/L sodium hydroxide was added to 1 mL of blood or tissue homogenates in a centrifuge tube (10 mL capacity) and vortexed for 5 minutes. Then 6 mL of dichloromethane-methanol solvent mixture (3:1, v/v) was added to the solution and vortexed for 5 minutes and centrifuged for 25 minutes at 3000 rpm. The organic layer was transferred into another centrifuge tube and dried by vacuum drying. The residue was resolved with 100 µL mobile phase, and then the solution was washed two times with n-Hexane as described subsequently. *n*-Hexane, 0.5 mL, was added to the solution and vortexed for 5 minutes, and then centrifuged at 2000 rpm for 3 minutes. The supernatant *n*-Hexane was discarded. DTI was calculated using the following formula[10]:

$$\mathrm{DTI} = \frac{\begin{array}{c}\text{Drug concentration in lung at time ‘t’ after}\\ \text{administration of EM-GMS}\end{array}}{\begin{array}{c}\text{Drug concentration in lung at time ‘t’ after}\\ \text{administration of EA solution}\end{array}}.$$

Results and discussion

The morphology and particle size of EM-GMS

Under optical microscopy and SEM, surface morphology of EM-GMS showed a spherical shape with smooth and porous surface, as shown in Figure 1. EM-GMS microspheres were less aggregated and readily dispersed in saline for intravenous injection. Particle size distributions of EM-GMS showed that more than 90% of



(A) (B)

Figure 1. The surface morphology of EM-GMS (A) optical photomicrograph (100×), (B) scanning electron micrograph (20000×).

microspheres fell within the size range of 7–28 µm, and the mean diameter of microspheres was 15.62 µm. The average drug-loading efficiency and average encapsulation efficiency of EM-GMS were 13.56 ± 0.25% (n = 3) and 55.82 ± 2.23% (n = 3), respectively.

DSC profile of EM-GMS

DSC analysis of EM-GMS was performed to find out the physical nature of erythromycin entrapped in the gelatin microspheres. The thermogram of erythromycin showed a peak at approximately its melting point (198.0°C), and the thermogram of GMS showed a peak at its melting point (240.10°C), as shown in Figure 2. The thermogram of the physical mixture of erythromycin and GMS (1:5) produced two peaks at approximately 198.0°C and 240.10°C, which were the melting points of erythromycin and GMS, respectively. The thermogram of EM-GMS showed a peak at its melting point, 234.5°C, which was lower than the thermogram of GMS by 5.6°C. The peak of erythromycin at 198.0°C was absent in the thermogram of EM-GMS, which revealed that erythromycin

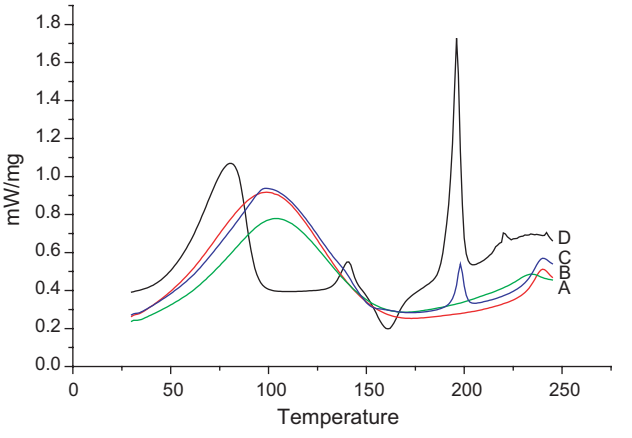


Figure 2. (A) Thermogram of EM-GMS, (B) GMS, (C) the physical mixture of erythromycin and GMS, and (D) erythromycin.

was entrapped in microspheres but did not form a physical mixture.

In vitro release of EM-GMS

In vitro release profile of EM-GMS is illustrated in Figure 3. The release of erythromycin from EM-GMS in pH 7.4 phosphate buffer showed an obvious burst effect, with an accumulated release of 48.10% and 80.00% within 30 minutes and 4 hours, respectively. The EM concentration was too low to be detected after the release of more than 80%, indicating that the release rate of erythromycin from EM-GMS was very fast, which was conducive to rapidly reaching effective treatment concentrations of erythromycin. The release behavior of the drug from EM-GMS showed a biphasic pattern that was characterized by an initial burst, followed by a slower sustained release. The biexponential equation of EM-GMS was $Q/100 = 0.8047 - 0.17\exp(-128.9t) - 0.6347\exp(-1.378t)$, $R^2 = 0.9995$, and fitted well.

The irritation of EM-GMS

After administration of EM-GMS suspension, EA solution, and saline injection once a day for three consecutive days, erythema, edema, and visible damage were not observed at the injected sites and the surrounding tissues of rabbits. Histopathologic examination of the rabbit ear marginal vein indicated that the vessel wall and endothelial cell structures were unimpaired. Furthermore, angiectasia and thrombus were not observed in the lumen of the vein. There were no significant pathological changes—inflammatory cell infiltrate, hemorrhage apomorphosis, and necrosis in the vessel wall and surrounding tissues. The results demonstrated that irritation with EM-GMS suspension was similar to that with saline and EA solution, and that EM-GMS suspension was suitable for intravenous injection (Figure 4).

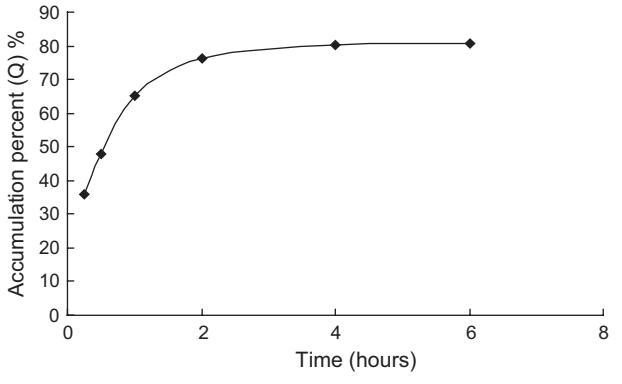


Figure 3. In vitro release profile of EM-GMS.

In vitro hemolysis of EM-GMS

Complete hemolysis was observed in the tube of positive control at 15 minutes, the solution was red clear, diaphanous, and no erythrocyte survived at the bottom of the tube. Hemolysis was not observed in the tube of negative control and in the tubes numbered 1–5. These results demonstrated that the EM-GMS suspension at a concentration of 3.5 mg/mL did not cause hemolysis and erythrocyte agglutination at 37°C.

Acute toxicity of EM-GMS

After administration of EM-GMS and GMS, the toxic response of mice such as anxiety, piloerection, catatonia, and respiratory distress were observed. Several mice died within 12 hours (Table 1) and the main organs of dead mice were subjected to macroscopic examination. Pulmonary congestion was observed in dead mice and the heart, liver, spleen, lung, and kidney of dead mice showed no obvious changes. The survivors gradually recovered after 14 days.

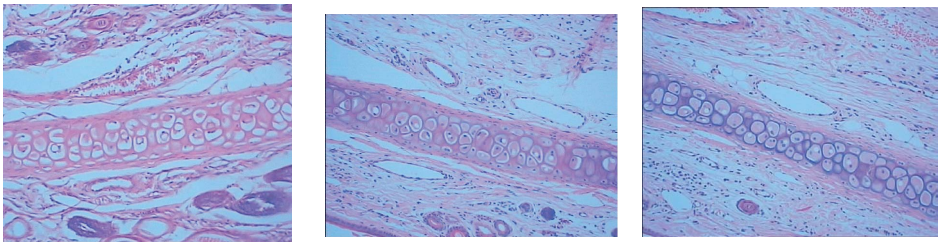
After administration of EA, the toxic response of mice such as anxiety and catatonia were observed. Several mice died within 24 hours (Table 1) and the main organs of dead mice were subject to macroscopic examination. The heart, liver, spleen, lung, and kidney of dead mice had no obvious changes. The survivors gradually recovered after 7 days. As reported by other researchers, the microspheres with suitable size were mechanically entrapped by pulmonary capillaries and then ingested into lung tissue or lung bubble by mononuclear leukocytes[11]. Therefore, the drug was accumulated more in the lung than in any other organ. The results of our experiment were also similar.

The LD_{50} of EM-GMS, GMS, and EA administered by intravenous injection were 173.07, 191.01, and 337.70 mg/kg, respectively. The acute toxicities of EM-GMS and GMS were more than that of EA and this might be due to the mechanical trapping effect of microspheres in pulmonary capillary vessel. For decreasing the toxicity of EM-GMS in lung, efforts should be made to increase the drug loading to decrease the doses of microspheres, control the particle size of EM-GMS, and decrease the degree of cross-linking for rapid degradation.

The lung-targeting effect test of EM-GMS

As the results in Figure 5 show, retention time of erythromycin under the chromatographic conditions of this assay was 13.2 minutes, without any interfering peaks of the tissue homogenates.

The tissue distribution of erythromycin at 2 hours after administration of EM-GMS or EA into rabbit ear marginal vein is shown in Figure 6. After administration



(A) (B) (C)

Figure 4. (A) The pathological sections of rabbit ear vein after injecting EM-GMS suspension, (B) EA solution, and (C) saline.

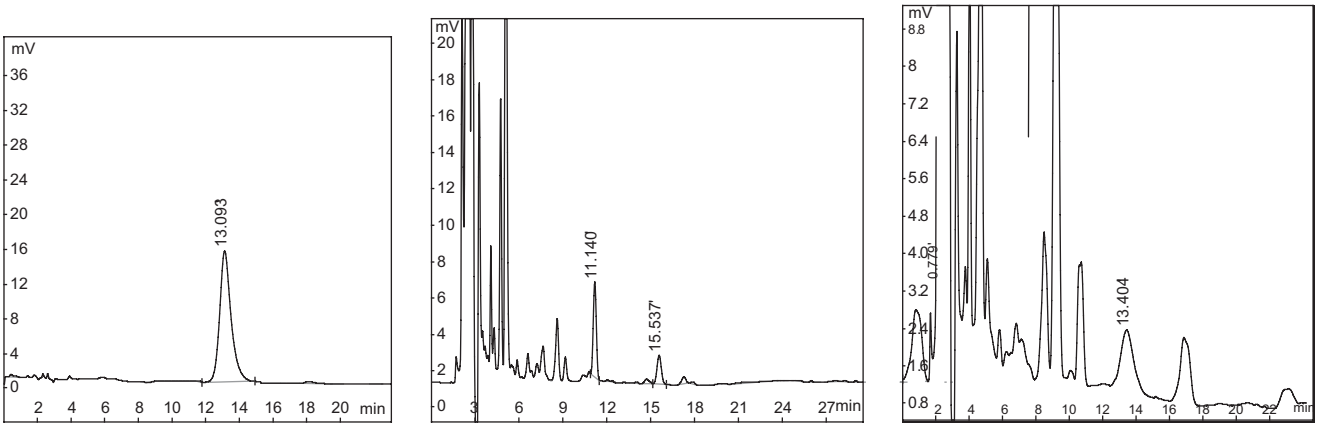
Table 1. The LD_{50} of EM-GMS, GMS, and EA after i.v. in mice.

Drug	Dose (mg/kg)	Number of dead mice	LD_{50} (mg/kg)
EM-GMS	250.0	10	173.07
	208.0	8	
	173.0	5	
	144.0	2	
	120.0	0	
GMS	280.0	10	191.01
	231.0	7	
	190.6	6	
	157.2	2	
	130.0	1	
EA	490.0	10	337.70
	402.8	7	
	331.1	5	
	272.2	2	
	224.0	0	

of EM-GMS, the concentration of erythromycin in the lung was significantly higher than that in the liver, kidney, spleen, and heart, and was 15.92 times higher than that in plasma. However, the concentration of erythromycin in lung was similar to that in liver and kidney and was 3.51 times higher than that in plasma, after administration of EA. As compared with EA, the DTI of EM-GMS in lung was 6.65, so the lung-targeting effect of EM-GMS was proved to be remarkable.

Conclusions

EM-GMS were prepared with gelatin by a double emulsion solvent evaporation method for lung targeting. Under optical microscopy and scanning electron microscopy, the surface morphology of EM-GMS showed a spherical shape with smooth and porous surface. The drug loading and encapsulation efficiencies of EM-GMS were 13.56 ± 0.25% and 55.82 ± 2.23%, respectively. The mean diameter of EM-GMS was 15.62 ± 1.73% μm with over 90% of the microspheres ranging from 7.0 to 28.0 μm. Differential scanning calorimetric analysis of EM-GMS confirmed that erythromycin was entrapped in microspheres but did not form a physical mixture. The in vitro release proile of EM-GMS fitted a biexponential equation, $Q/100 = 0.8047 - 0.17\exp(-128.9t) - 0.6347\exp(-1.378t)$. To evaluate the safety of EM-GMS irritation test, hemolysis test in vitro, and acute toxicity test of EM-GMS were



(A) (B) (C)

Figure 5. (A) Chromatograms of erythromycin standard, (B) extract from blank rabbit lung, and (C) extract from rabbit lung administrated drug.

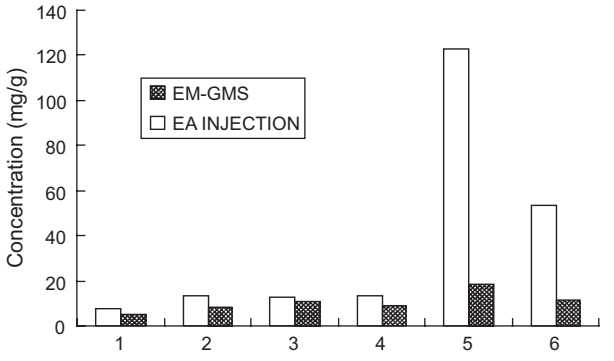


Figure 6. The concentration of erythromycin in different tissues (1) plasma, (2) heart, (3) liver, (4) spleen, (5) lung, (6) kidney.

performed. The rabbit vein irritation profile of EM-GMS was similar to that of EL solution. There was no visible damage at the injected sites and no significant pathological changes in the vessel wall after administering EM-GMS. The EM-GMS suspension at a concentration of 3.5 mg/mL did not cause hemolysis and erythrocyte agglutination. The LD_{50} of EM-GMS, blank gelatin microspheres, and EL administered by intravenous injection were 173.07, 191.01, and 337.70 mg/kg, respectively. After administering EM-GMS to rabbits, the concentration of erythromycin in lung was significantly higher than that in liver, kidney, spleen, and heart and was 15.92 times higher than that in plasma. As compared with EL, the drug-targeting index of EM-GMS in lung was 6.65 so the lung-targeting effect of EM-GMS was significant.

Declaration of interest: The authors report no conflicts of interest.

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