Department of Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, P.R. China

Evaluation of different methods to determine the loading of proteins in PLGA microspheres

C. H. ZHENG, W. Q. LIANG, H. Y. YU, H. L. CHEN

Received August 27, 2003, accepted October 26, 2003

Prof. Wenquan Liang, College of Pharmaceutical Sciences, Zhejiang University, 353 Yan'an Road, Hangzhou 310031, P.R. China
wqliang@cmm.zju.edu.cn

Pharmazie 59: 232–233 (2004)

Various determination methods for protein encapsulation efficiency of PLGA microspheres were compared. Acetonitrile is recommended as an extraction solvent.

Biodegradable microspheres loaded with proteins have gained importance as one of preferred dosage forms in the field of protein delivery (Diwan and Park 2001). Especially microspheres composed of poly(lactic-co-glycolic acid) (PLGA) have been studied extensively. An accurate, simple, reliable, and reproducible method to determine the encapsulation efficiency (EE%) of proteins in PLGA microspheres is indispensable for the evaluation of the quality or for the characterization of microspheres. Several methods have been employed to determine loading of proteins in microspheres. These mainly include extraction and hydrolysis or a combination of both. However, the results may vary due to different sample processing as well as the different conditions during the process.

In this paper, bovine serum albumin (BSA) was selected as a model protein and BSA loaded PLGA microspheres were prepared. The total protein was analyzed by a Coomassie Brilliant Blue (CBB) staining method. Various determination methods of the BSA content in PLGA microspheres referred to in the literature were performed in this work. These methods include: (I) extraction (Yang et al. 2001; Prior et al. 2000; Ravivarapu et al. 2000); (II) surface-associated method (Rosas et al. 2001; Tuncay et al. 2000); (III) centrifugation-extraction method (Castellanos et al. 2002); (IV) hydrolysis (Lam et al. 2000; Kang and Singh 2001; Gutierro et al. 2002); (V) and (VI) combined extraction and hydrolysis (Diwan and Park 2001); and (VII) an indirect method (Li et al. 2001). The results of BSA EE% determined by these methods are listed in the Table.

Of these methods, method VII is the reference method and the results calculated by the difference between the total amount and the amount of non-entrapped protein can be regarded as the actual EE%. However, the indirect method can only be used during preparation processing; it cannot be used in the analysis of dried microspheres. The drawback of method I is the incomplete extraction of BSA. The most likely reason is that continuous vortex and shaking may create more aggregated protein, leading to difficulties in extraction. In addition, methylene chloride possesses 2% solubility in water. In method II, the surface-associated BSA was firstly separated and the remaining BSA in microspheres was further extracted as described in method I. The amount of surface-associated BSA was more than the total entrapped protein as measured by method I. The surface-associated protein is responsible for the burst effect. We have determined that the amount of the initial released protein within 24 h was close to that of the surface-associated protein in microspheres. Method III is a modification of method I. The soluble protein can be withdrawn after discarding the organic solvent that contains the dissolved PLGA. FTIR spectroscopy verified that the non-soluble protein aggregate during the preparation procedure (Castellanos et al. 2002). Consequently, the EE% is lower than the actual value. Method IV as reported in the literature did not provide a detail process, especially regarding the conditions of hydrolysis. Our experiments indicate that the EE% results may greatly depend on the ratio of the weight of microspheres to the volume of NaOH and the hydrolysis time. Usually, incomplete hydrolysis leads to an underestimation of EE%.

In the view of the limitations of method III, it is straightforward considered that most non-soluble protein aggregates can be recovered by further dissolving the pellet in 0.1 N NaOH. So the result may get close to the accurate EE%. However, the selection of organic solvents in the extraction step is very important. Although methylene chloride (method V) is widely used as an extraction solvent, we found acetonitrile (method VI) to be superior to methylene chloride. In our experiment, after the microspheres were dissolved and centrifuged, the acetonitrile organic phase is clear while for methylene chloride it is turbid. The EE% determined by method VI is nearly the same as for method VII and the procedure is simple and rapid.

Table: BSA encapsulation efficiency (EE%) in two types of PLGA microspheres determined by various methods (n = 3)

Methods	Encapsulation efficiency (%)	
	PLGA (50 : 50) microspheres	PLGA (85 : 15) microspheres
I	4.17 ± 0.72	15.67 ± 1.65
II	27.82 ± 0.45	30.12 ± 0.60
III	35.45 ± 1.03	48.28 ± 1.14
IV	38.21 ± 0.58	44.76 ± 0.05
V	40.62 ± 0.68	49.85 ± 0.54
VI	59.94 ± 0.04	70.06 ± 0.21
VII	64.54 ± 0.18	70.71 ± 1.04

Experimental

1. Materials

PLGA, lactic to glycolic acid molar ratio of 50 : 50 and 85 : 15, were purchased from Shandong Medical Instrumental Institute. Bovine serum albumin (BSA, 0108) was provided by the Sino-American Biotechnology Co., Poly (vinyl alcohol) (PVA, average MW 30000–70000, 20010730) and CBB G250 (20020324) were procured from Shanghai Chemical Reagent Company of Chinese Medicin (Group). The methylene chloride, acetonitrile and all other reagents were of analytical grade supplied by Huadong Medical Company.

2. Determination methods

PLGA microspheres containing BSA were made by double-emulsion solvent evaporation. The mean size of microspheres was about 4 ~ 5 μm. The methods of determining EE% were the following: Method I – Twenty milligrams of microspheres were dissolved in 1 ml dichloromethane and 1 ml of phosphate buffer solution (PBS) was added, the mixture was then vortexed for 2 min to extract BSA. The step was repeated four times, the total aqueous solution was analyzed. Method II – One milliliter PBS was first used to extract BSA by incubating 20 mg microspheres at 37 °C under continuous shaking. After centrifugation, the surface-associated protein

was determined and the precipitate was vacuum dried and then the procedure of method I was repeated. Method III – Twenty milligrams of microspheres were added to 1 ml dichloromethane with stirring and the supernatant containing the dissolved polymer was discarded after centrifugation. The pellet was vacuum dried and extracted with 1 ml PBS. This procedure was performed twice. Method IV – Two milliliters of 1 N NaOH was used to digest 20 mg microspheres by sonication for 15 min and incubation at 37 °C for 2 days. The solution was analyzed after neutralization with HCl. Method V and VI – Twenty milligrams of microspheres were dissolved in 1 ml dichloromethane (V) or acetonitrile (VI). After centrifugation, the precipitated protein was centrifuged and supernatant containing polymer was discarded. After being vacuum dried, the pellet was re-dispersed in 1 ml PBS and centrifuged again. The supernatant was retained and the residue was dissolved in 1 ml of 0.1 N NaOH, then vortexed thoroughly and centrifuged. The protein in the PBS and alkaline extractions was neutralized with HCl before estimation. Method VII – The non-entrapped protein in the aqueous phase was collected during the preparation and the protein in the supernatant was determined.

Acknowledgement: This work was supported by National Natural Science Foundation of China (project no. 30171113).

References

Castellanos IJ, Cruz G, Crespo R et al. (2002) Encapsulation-induced aggregation and loss in activity of γ-chymotrypsin and their prevention. J Control Release 81: 307–319.

Diwan M, Park TG (2001) Pegylation enhances protein stability during encapsulation in PLGA microspheres. J Control Release 73: 233–244.

Gutierro I, Hemandez RM, Igartua M et al. (2002) Influence of dose and immunization route on the serum IgG antibody response to BSA loaded PLGA microspheres. Vaccine 20: 2181–2190.

Kang F, Singh J (2001) Effect of additives on the release of a model protein from PLGA microspheres. AAPS Pharm Sci Tech 2: 230–239.

Lam XM, Duenas ET, Daugherty AL et al. (2000) Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes. J Control Release 67: 281–292.

Li YP, Pei YY, Zhang XY et al. (2001) PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. J Control Release 71: 203–211.

Prior S, Gamazo C, Irache JM et al. (2000) Gentamicin encapsulation in PLA/PLGA microspheres in view of treating Brucella infections. Int J Pharm 196: 115–125.

Ravivarapu HB, Burton K, Deluca PP (2000) Polymer and microsphere blending to alter the release of a peptide from PLGA microspheres. Eur J Pharm Biopharm 50: 263–270.

Rosas JE, Hernandez RM, Gascon AR et al. (2001) Biodegradable PLGA microspheres as a delivery system for malaria synthetic peptide SPf 66. Vaccine 19: 4445–4451.

Tuncay M, Calis S, Kas HS et al. (2000) Diclofenac sodium incorporated PLGA (50 : 50) microspheres: formulation considerations and in vitro/in vivo evaluation. Int J Pharm 195: 179–188.

Yang YY, Chung TS, Ng NP (2001) Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 22: 231–241.

Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, (Hamdard University), New Delhi, India

Molecular inclusion of rofecoxib with cyclodextrin: pharmacological properties in laboratory animals

S. Baboota, M. Dhaliwal, K. Kohli, J. Ali

Received July 30, 2003, accepted October 10, 2003

Dr. Sanjula Baboota, Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, New Delhi-110062 India
sbaboota@rediffmail.com

Pharmazie 59: 233–234 (2004)

Rofecoxib is practically insoluble in water and its prolonged use is associated with the incidence of side effects like gastro intestinal perforations, ulcerations and bleeding. Therefore, an attempt has been made to improve the aqueous solubility of the drug by making an inclusion complex using dimethyl-β-cyclodextrin (DIMEB). The complexes were prepared by kneading and by the spray drying method. The prepared complexes showed better anti-inflammatory activity and decreased ulcerogenic potential than the pure drug.

Water insoluble drugs are usually characterized by a low bioavailability, because their absorption is dissolution rate limited and consequently slow (Kamada et al. 2002; Fernandes et al. 2002; Peeters et al. 2002; Baboota et al. 2003). The potential use of cyclodextrins as a novel drug carrier material is to control the drug release at the desired level.

Rofecoxib, a selective COX-2 inhibitor with strong anti-inflammatory activity, is practically insoluble in water and has a longer onset of action. Its prolonged use is associated with side effects like gastro intestinal (GI) perforations, ulcerations and bleeding (McEvoy 2001; Schrefer 2000). Therefore the aim of the present study was to investigate the effect of inclusion of rofecoxib with dimethyl-β-cyclodextrin (DIMEB) upon anti-inflammatory activity and GI mucosal toxicity.

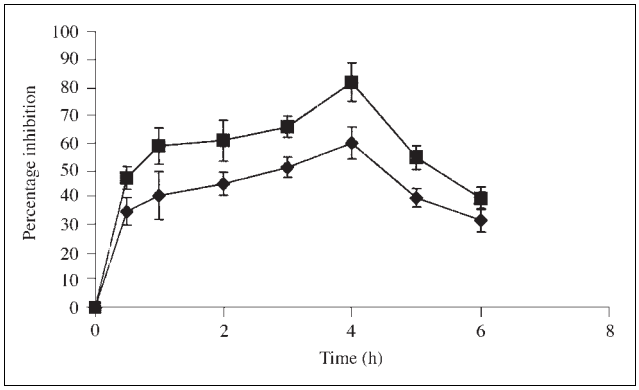

Fig. Anti-inflammatory studies of rofecoxib and rofecoxib DIMEB complex by rat hind paw odema method (mean ± SD; n = 4).
—◆— Pure rofecoxib, —■— Rofecoxib-DIMEB SD complex