Department of Pharmacy, Medical College of Nanchang University, Nanchang, China

Lappaconitine-loaded microspheres for parenteral sustained release: effects of formulation variables and *in vitro* characterization

HeLin Xu, HaiJun Zhong, MinMin Liu, ChunLian XU, Yuan Gao

Received March 27, 2011, accepted April 27, 2011

HaiJun Zhong, Department of Pharmacy, Medical College of Nanchang University, NO.461, Bayi Road, Nanchang, Jiangxi 330006, PR China
zhonghj@ncu.edu.cn

Pharmazie 66: 654–661 (2011) *doi: 10.1691/ph.2011.1042*

Lappaconitine instead of its hydrobromide salts has been encapsulated in poly (lactide-co-glycolide) acid (PLGA) microspheres by the simple o/w emulsion solvent evaporation technique. The effects of several variables including emulsifier (polyvinyl alcohol, PVA) concentration, stirring speed, PLGA concentration and drug/polymer mass ratios on quality of microspheres have been investigated. The particle size and size distribution can be controlled by PVA concentration, stirring speed and PLGA concentration. The entrapment efficiency and the burst release of lappaconitine from drug-loaded microspheres were dominantly affected by the drug/polymer mass ratio and PVA concentration. The best parameters of formulation were 1.5% PVA, the PLGA concentration of 50 g/L, and the stirring speed of 800 rpm and drug/polymer of 1:5. The optimized formulation has a mean particle size of 19.3 ± 0.93 μm, mean entrapment efficiency of 70.77 ± 3.23% and mean drug loading of 11.45 ± 0.47%. Based on the optimized parameters of formulation, the effects of oil/aqueous solubility partition ratio of drug on entrapment efficiency of drug-loaded microspheres prepared by o/w emulsion solvent evaporation were further studied. A good linear relation existed between the partition ratio and entrapment efficiency. The optimized microspheres were characterized by SEM, FT-IR, DSC and XRD. SEM shows spherical and smooth surface and uniform size distribution. The results of DSC, FT-IR study reveal no interaction between drug and polymer. The results of the XRD study indicate lappaconitine trapped in microsphere exists in form of an amorphous or disordered crystalline status in polymer matrix. The *in vitro* release models were evaluated with two different groups of drug-loaded microspheres including microspheres washed with distilled water and 0.01N HCL, respectively. The drug release profile of lappaconitine-loaded microspheres washed with distilled water agreed with zero order equation and that of the latter better agreed with first order equation.

1. Introduction

Lappaconitine, a diterpenoid alkaloid extracted from the roots of *Aconitum sinomontanum Nakai*, possesses strong central analgesic, local anesthetic, antifebric and anti-inflammatory effect. The analgesic effect of lappoconitine is 7 times as much as that of aminopyrine, and equivalent to pethidine (Ameri 1998). Lappaconitine have neither serious side effects associated with the opioid drugs, such as respiratory depression, addiction, etc (Hassett et al. 2008; Stein et al. 2000), nor the upper gastrointestinal symptoms for nonsteroidal antiinflammatory drugs (NSAIDs) (Eisenberg et al. 1994). Owing to its strong antinociceptive property and no addiction, lappoconitine is widely used to the treatment of moderate to severe pain, such as cancer and postoperative pain in China (Tang et al. 1983). As a clinic drug for the relieving of cancer pain, lappoconitine has been listed in the guideline for cancer pain management published by the National Ministry of Health of China (Myers et al. 2010).

The conventional preparations of lappoconitine on the market, tablets and injection solution, need to be administered frequently, which is inconvenient and leads to poor compliance for patients. For example, intramusclar injection needs to be given once or twice per day for conventional lappoconitine injection solution. To reduce the frequency of administration, afford patient compliance, increase antinociceptive effect of lappoconitine and thereby improve the life quality of patients, it is imperative to develop a sustained drug delivery system for lappaconitine which releases drug continuously. In the present study, biodegradable poly(lactide-co-glycolide) (PLGA) microspheres were used as drug delivery vehicles for the sustained release of lappaconitine.

Microspheres with PLGA as polymer matrix offer several prominent advantages. Firstly, PLGA is biodegradable and biocompatible. It finally degrades into lactic and glycolic acids *in vivo* through cleavage of its backbone ester linkages (Shive and Anderson 1997). Thus, PLGA is thought to be safe *in vivo* and has been approved by the FDA as pharmaceutical excipient. Secondly, PLGA microspheres can be tailored for desired drug release profiles. Depending on the lactide/glycolide ratio and molecular weight of the PLGA polymer, drug release from microspheres can be modified from several days to several months, and even several years (Freiberg and Zhu 2004).

Therefore, PLGA microspheres have been widely studied as injectable sustained release systems to prolong the release of therapeutic agents. Various therapeutic agents, including analgesic drugs, antibiotics, anti-inflammatory drugs, anticancer

drugs, steroids, peptides and proteins have been incorporated in the system (McGinity and O'Donnell 1997). Some products based on PLGA microspheres, such as Lupron Depot®, Nutropin Depot®, Zoladex®, Risperidal Consta®, etc., have been approved for clinical use.

The hydrobromide salt of lappaconitine is used in the commercial lappaconitine tablets and injection. Accordingly, in our pre-study, lappaconitine hydrobromide was entrapped into PLGA microspheres by various emulsification-solvent evaporation methods, including o/w, s/o/w, w/o/w, et al. Results have shown that the drug entrapment efficiencies of obtained microspheres were quite low. However, when the free base of lappaconitine was used instead of its hydrobromide salt, the drug entrapment efficiency of microspheres formulated by a simple o/w emulsion solvent evaporation technique was greatly improved. Preparation and characteristics of microspheres containing lappaconitine are firstly reported in this paper. Additionally, the correlation between the drug entrapment efficiency of microspheres and the drug solubility ratio in the oil to water phase was studied. To our knowledge, there is no report on the correlation between the drug entrapment efficiency of microspheres and the drug solubility ratio in the oil to water phase, although the influence of the drug partition between oil and water on drug entrapment efficiency has been widely realized (Benelli et al. 1998; Bodmeier and McGinity 1987).

2. Investigations, results and discussion

2.1. Solubility of lappaconitine and its hydrobromide salts

The choice of a particular method of microencapsulation is usually dependent on drug solubility characteristics. Table 1 shows the solubility of lappaconitine and its hydrobromide salt in various solvents. The free base of lappaconitine was much better soluble in dichloromethane than its hydrobromide salt. Conversely, although both of lappaconitine and its hydrobromide salt were poorly soluble in water, the water solubility of lappaconitine was significantly lower than that of its hydrobromide salt. The solubility of the free base of lappconitine was pH dependent, which resulted in a corresponding variation of the oil/aqueous solubility partition ratio (Table 1).

2.2. Preparation of microspheres and optimization of formulation

2.2.1. Effect of hydrobromide salt and neutral forms of lappaconitine

Lappaconitine hydrobromide was incorporated into PLGA microspheres since the hydrobromide salt of lappaconitine is used in commercial lappaconitine tablets and injection. Firstly, the o/w emulsification-solvent evaporation method was used to prepare PLGA microspheres containing lappaconitine hydrobromide. Due to the poor solubility of lappaconitine hydrobromide in dichloromethane, methanol was used as a cosolvent. The results have shown all of drug entrapment efficiencies of the microspheres containing lappaconitine hydrobromide obtained by the o/w method were 0% approximately, though various preparation process parameter were selected. The results can be attributed to the poor solubility of lappaconitine hydrobromide. The solubility of lappaconitine hydrobromide in water was remarkablely higher than that in dichloromethane. This caused facilitating the drug to the external water phase. After the oil phase consisted of dichloromethane and small amount of methanol as the cosolvent was mixed with the water phase, a large portion of the drug would follow the cosolvent and be lost to the continuous phase, which leads to the low drug entrapment efficiency (Jovanovic et al. 2008). With other emulsification-solvent evaporation methods the obtained drug entrapment efficiencies were below 10% for s/o/w and w/o/w method. Although the drug entrapment efficiencies increased to 20% approximately for o/o method, the sphericity of the obtained microspheres was poor and conglomeration occurred in the microsphere products. Overall it was difficult to achieve satisfactory results for the incorporation of the hydrobromide salt of lappaconitine into PLGA microspheres. Thus, the free form of lappaconitine was tried and comparatively high drug entrapment efficiency was obtained by a simple o/w emulsion solvent evaporation method. Therefore, the free form of lappaconitine was selected as the form of the drug and o/w emulsion solvent evaporation method was used in the in further study on the microspheres containing lappaconitine.

2.2.2. Effect of emulsifier concentration

As shown in Table 2, the emulsifier (PVA) concentrations in the aqueous phase had significant influence on entrapment efficiency and particle size. As the amount of PVA in the aqueous phase increased, both of the mean particle diameter and the value of span decreased. One of the reasons for this phenomenon may be that the more of PVA molecules are absorbed on the surfaces between organic phase and aqueous phase, the lower is the interfacial tension between the oil and aqueous phase (Ito et al. 2007). Another reason may be the increase in viscosity of the outer aqueous phase with increasing concentration of PVA, which prevents the emulsion droplets from coalescence (Cui et al. 2005). Both of these resulted in smaller and more stable emulsion droplets. As for entrapment efficiency, it decreased with increasing PVA concentration in aqueous phase. Especially, the entrapment efficiency decreased drastically when the PVA concentration increased from 1.5% to 3.0%. A similar result was obtained by Zidan et al. (2006).

2.2.3. Effect of stirring speed

Keeping the other conditions constant, the effect of stirring speed on entrapment efficiency and particle size of microspheres was investigated (Table 2). It was found that entrapment efficiency decreased slightly and the mean particle size was reduced with increase of stirring speed. The explanation for this phenomenon may be that a higher rate of agitation would be liable to subdivide the globules into smaller ones, which increase the surface facilitating the drug diffusing into aqueous phase (Alex and Bodmeier 1990; Zidan et al. 2006). Interestingly, the increase in stirring speed from 800 to 1000 rpm reduced particle size very slightly, resulting in similar mean particle size and size distribution (Fig. 1B, C). A similar result was reported by Nilkumhang and Basit (2009).

2.2.4. Effect of the ratio of the drug to PLGA

Keeping PLGA concentration and other conditions constant, and varying the drug concentration, the effect of drug/PLGA ratio on entrapment efficiency and particle size of microspheres was investigated. The results are listed in Table 2. No remarkable difference was observed for the drug entrapment efficiencies of the microspheres prepared with the polymer/drug ratios from 10:1 to 3:1. However, when the ratio of drug/PLGA was increased from 3:1 to 1:1, the entrapment efficiency showed a significant decrease. The increase in drug/PLGA ratio means the decrease of the relative amount of PLGA that could encapsulate the drug. The amount of PLGA was not enough to encapsulate the drug

Table 1: Solubility of lappaconitine and its hydrobromide salt in selected solvents and corresponding partition ratio (n = 3)

Solvents	Solubility in different solvents (Mean ± SD, g/L)		Partition ratio	
	Lappaconitine	Hydrobromide lappaconitine	Lappaconitine	Hydrobromide lappaconitine
Dichloromethane	25.70 ± 1.02	0.85 ± 0.03		
Water	0.44 ± 0.01	11.96 ± 0.48	58.50	0.07
pH 2.00	48.3 ± 1.20	–	0.53	–
pH 3.00	25.7 ± 1.12	–	0.99	–
pH 5.00	12.8 ± 0.45	–	2.00	–
pH 6.00	6.48 ± 0.32	–	3.96	–
pH 7.00	2.61 ± 0.12	–	9.84	–

Table 2: Characteristics of lappaconitine-loaded PLGA microspheres prepared at different conditions

Variables	Parameters	Actual drug loading (%)	EE (%)	Mean diameter (μm)	Span	Drug burst (%)	Yield (%)
PVA concentration	0.5%	12.73 ± 0.51	75.86 ± 1.51	37.9 ± 1.5	2.00 ± 0.08	4.03 ± 0.15	66.75 ± 2.67
	1.5%	11.34 ± 0.45	73.26 ± 1.47	20.3 ± 0.8	1.35 ± 0.05	8.04 ± 0.34	64.40 ± 2.56
	3.0%	10.09 ± 0.50	60.39 ± 1.81	16.4 ± 0.7	1.33 ± 0.06	16.63 ± 0.56	66.34 ± 2.45
	600 rpm	11.85 ± 0.47	73.64 ± 2.20	27.8 ± 1.2	2.00 ± 0.07	6.43 ± 0.25	63.25 ± 2.34
Stirring speed	800 rpm	11.96 ± 0.48	71.94 ± 1.43	18.8 ± 0.7	1.67 ± 0.07	7.85 ± 0.46	66.34 ± 2.54
	1000 rpm	11.40 ± 0.51	69.02 ± 1.38	17.5 ± 0.9	1.66 ± 0.08	16.11 ± 0.62	71.59 ± 2.87
Drug/polymer Ratio	1:10	4.72 ± 0.21	42.86 ± 1.71	22.5 ± 1.7	1.28 ± 0.06	8.04 ± 0.40	71.04 ± 2.76
	1:5	11.04 ± 0.44	68.73 ± 2.06	17.5 ± 0.7	1.39 ± 0.05	7.61 ± 0.30	64.40 ± 2.43
	1:3	17.50 ± 0.52	67.12 ± 2.01	18.0 ± 1.3	1.36 ± 0.04	8.71 ± 0.34	64.10 ± 2.32
	1:1	34.65 ± 1.39	63.90 ± 2.55	35.0 ± 2.1	1.45 ± 0.07	16.94 ± 0.67	69.08 ± 2.48
PLGA concentration	40 g/L	13.54 ± 0.54	67.95 ± 2.71	19.5 ± 1.0	1.37 ± 0.04	9.51 ± 0.28	63.85 ± 2.21
	50 g/L	11.06 ± 0.33	67.12 ± 2.01	18.7 ± 0.7	1.30 ± 0.03	9.61 ± 0.28	64.50 ± 2.12
	60 g/L	9.62 ± 0.38	67.24 ± 2.01	26.8 ± 1.8	1.27 ± 0.05	10.09 ± 0.40	58.68 ± 2.31

when the polymer/drug ratio was 1:1, which resulted in the low entrapment efficiency.

In order to evolve a formulation with higher drug loading and low burst release, the initial burst of microspheres prepared by different drug/polymer ratios within the first 24 h was detected. As shown in Fig. 2, the initial burst release was remarkable for the formulation prepared by a 1:1 drug/polymer ratio compared with the formulations prepared with a drug/polymer ratio lower than 1:1. Among the formulations prepared with drug/polymer ratio lower than 1:1, there was no significant difference in the initial release. Generally, the initial burst drug release of PLGA microspheres was caused by unencapsulated drug absorbed on the surface or localized near surface of microspheres (Lecorre et al. 1994). As mentioned above, when the polymer/drug ratio was 1:1, the amount of PLGA was not enough to encapsulate the drug, and thereby larger amounts of drug were adsorbed on the surfaces or localized near the surface of microspheres, which resulted in a higher burst release.

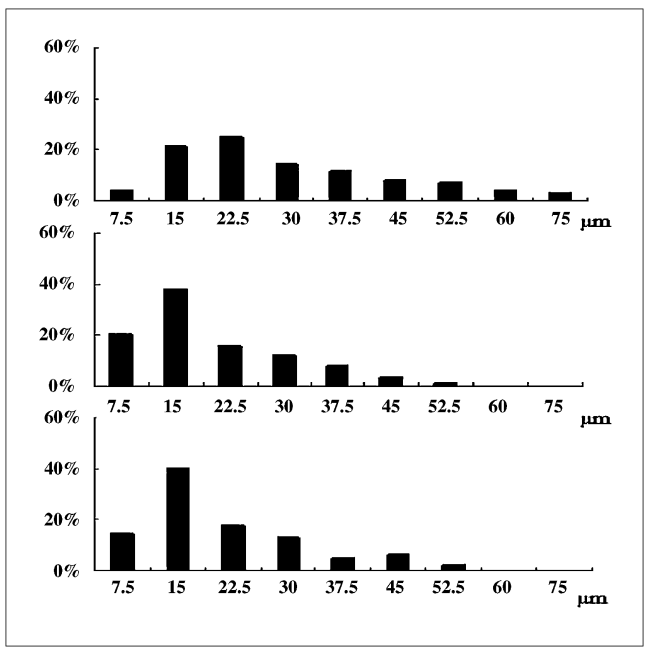


Fig. 1: Effect of stirring speed on size distribution of lappaconitine-loaded microspheres. A: 600 rpm, B: 800 rpm, C: 1000 rpm

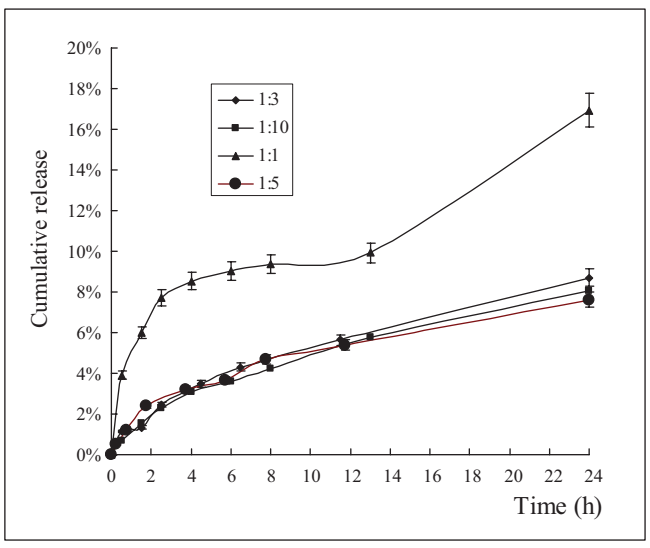


Fig. 2: Effect of drug/polymer ratio on initial burst of the microspheres containing lappaconitine

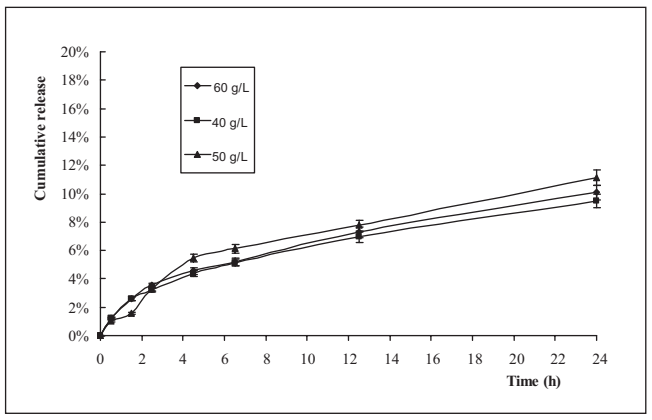


Fig. 3: Effect of PLGA concentration in organic phase on initial burst of the microspheres containing lappaconitine

2.2.5. *Effect of PLGA concentration in organic phase*

It has been reported that entrapment efficiency and particle size were significantly influenced by the polymer concentration in the organic phase (Zhang et al. 2008). However, as listed in Table 2, our results showed that PLGA concentration has a significant effect on particle size, while the entrapment efficiency was almost independent of the polymer concentration. Particle size of the microspheres increased with the increase of PLGA concentration and the size increase remarkably when the polymer concentration varied from 50 to 60 mg/ml. This may be explained by the fact that increasing polymer concentration resulted in higher viscosity of the organic phase, higher viscous resistance against the shear force during the emulsification process, and thereby larger particle size (Mundargi et al. 2007). Span value of particle size did not significantly change when PLGA concentration increased, implying that no aggregation of the microspheres did occur.

It has been reported that PLGA concentration in organic phase had great influence on the initial burst of the microspheres (Mao et al. 2007). Therefore, the effect of the polymer concentration on initial burst was detected. As shown in Fig. 3, the results indicated that the effect of PLGA concentration in organic phase on burst release of drug-loaded microspheres could be neglected at the tested range of the polymer concentration.

2.3. *Correlation between entrapment efficiency and oil/water solubility ratio*

As lappaconitine is an organic base, its aqueous solubility should depend on pH. During the process of o/w emulsion solvent evaporation method, modifying the pH of the outer aqueous phase means to vary the oil/water solubility partition ratio of drug under constant oil phase (dichloromethane). In order to investigate the effects of the oil/water solubility partition ratio of lappaconitine on entrapment efficiency of drug-loaded microspheres, a series of drug-loaded microspheres were prepared with different pH values of the outer aqueous phase (Table 3). It was found that the determined entrapment efficiency of the microspheres increased linearly with the partition ratio of lappaconitine ($R^2 = 0.9979$). It is concluded that the partition ratio was the determinant factor for high entrapment efficiency of drug-loaded microspheres, which could explain why lappaconitine hydrobromide could not be successfully incorporated into microspheres. The oil/water solubility partition ratio of lappaconitine hydrobromide was so small that large amoūnt of drug partitioned into the aqueous phase. However, when the pH of the outer aqueous phase was beyond 8, which means a higher partition ratio for lappaconitine and a higher entrapment efficiency, the poly(lactide-co-glycolide) acid was significantly degraded, resulting in irregular morphology of microspheres. Similar results were reported by Xu et al. (2010).

Table 3: Effects of oil/water solubility of lappaconitine on entrapment efficiency

pH	Solubility (g/L)	Partition coefficient	Entrapment efficiency (%)
2.00	48.3	0.53	2.52
3.00	25.7	0.99	3.10
5.00	12.8	2.00	11.57
6.00	6.48	3.96	30.64
7.00	2.61	9.84	79.31

2.4. *Scanning electronic microscopy (SEM)*

The surface morphology and particle size distribution could be observed by scanning electronic microscopy for all the formulations. The SEM of microspheres prepared by optimized formulation are shown in Fig. 4A. They are spherical in shape, have a smooth and slightly porous surface. Moreover, the uniform particle size distribution is evident from Fig. 4B.

2.5. *X-ray diffraction studies (XRD)*

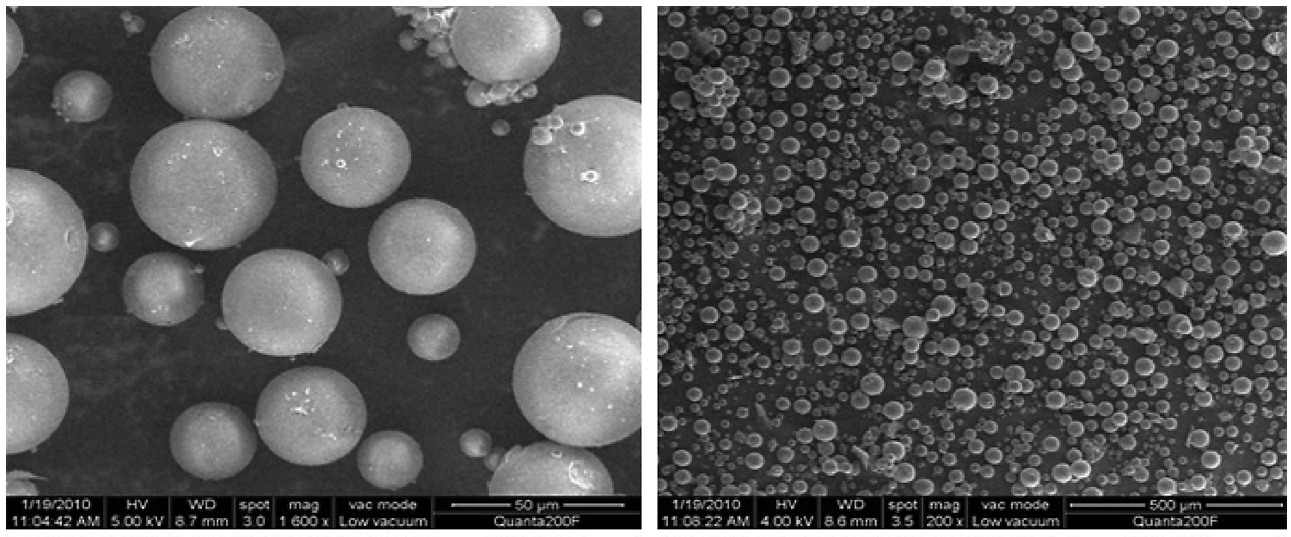
The XRD spectra of lappaconitine**,** the simple physical mixture, lappaconitine-loaded microspheres, and blank microspheres were shown in Fig. 5. It appeared that blank microspheres (Fig. 5D) were in amorphous state and lappaconitine powder (Fig. 5A) was crystalline. The crystalline diffraction peaks of lappaconitine were still visible for the physical mixture of lappaconitine and blank microspheres (1:5). Nevertheless, no crystalline diffraction patterns were founded in the graphs of lappaconitine-loaded microspheres (Fig. 5C), indicating that the drug entrapped in microspheres was an amorphous or disordered crystalline phase of molecular dispersion (Mu and Feng 2003). The similar transformation of crystalline for aconitine-loaded poly(D,L-lactide–Co-Glycolide) acid nanoparticles was reported by Xu et al. (2010).

2.6. *Differential scanning calorimetry (DSC)*

The DSC thermographs of the same samples used for XRD study are shown in Fig. 6. The DSC curve of lappaconitine (LP) shows a sharp endothermic peak at 221.3 °C, its melting peak (Fig. 6B) . The endothermic peaks at 42.8 °C for blank microspheres, lappaconitine and blank microspheres physical mixture, and lappaconitine-loaded microspheres was the Tg value of the polymer (PLGA) (Fig. 6A, D, C), which was consistent with the raw PLGA polymers (Martinez-Sancho et al. 2004). The simple physical mixture of lappaconitine and blank microsphere showed nearly the same thermal behavior as the individual components, indicating that there was no interaction between the drug and the blank microspheres (Fig. 6D). The absence of endothermic peak of the drug at 221.3 °C in the DSC curve of drug-loaded PLGA microspheres suggested that the drug existed in amorphous or disordered-crystalline phase as a molecular dispersion or a solid solution state in polymeric matrix (Fig. 6C) (Corrigan 1995). These results were consistent with the results of the XRD study.

2.7. *Fourier Transform Infra-red Spectroscopy (FT-IR)*

The FT-IR spectra of LP, blank MP, simple physical mixture (1:5) and LP-MP were shown in Fig. 7. Table 4 shows the assignment of characteristic peaks for blank microsphere and



(A) (B)

Fig. 4: SEM of LP-loaded microspheres prepared by optimized formulation: A 200 × magnification; B 1600 × magnification

Table 4: Assignment for characteristic peaks of FT-IR for blank microsphere and lappaconitine

	Peaks (cm^{-1})	Groups
Blank PLGA microsphere		
	1759.0	ester C=O
	2950.9, 1396.4, 1423.4	CH_3/CH_2
	1091.6, 1172.6	C–O–C
Lappaconitine		
	1685.7	amide C=O
	1523.7	amide N–H bending vibration
	1589.2, 1446.5, 759.9	benzene ring
	1087.8, 1265.2	ester or ether C–O–C

lappaconitine. The characteristic adsorption peaks for the benzene ring at 1589.2, 1446.5, 756 cm^{-1} could be detected in the FT-IR spectra of the simple physical mixture and lappaconitine-loaded microspheres. In addition, the peaks for amide C = O and amide N-H stretching vibration for lappaconitine in drug-loaded microsphere was slightly shifted to 1681.8 and 1527.5 cm^{-1}, respectively. Comparing with the blank microspheres, no significant shift for both of C = O stretching vibration of the PLGA ester in the physical mixture and lappaconitine-loaded microspheres was observed. The results may indicate that no obvious interaction between lappaconitine and the PLGA polymer occur in the physical mixtures and drug-loaded microspheres. Although, the peaks at 1685.7,1523.7, 759.9 cm^{-1}corresponding to amide C = O, amide N-H vibration and benzene ring of lappaconitine slightly shift to 1681.8, 1527.5 and 756 cm^{-1} in drug-loaded microspheres, respectively, the slight shift was attributed to the alternation of crystalline of lappaconitine in drug-loaded microspheres. The alternation of crystalline of drug in drug-loaded microspheres, which could result in the shift of peaks for FT-IR spectrums, has been reported (Rahman et al. 2010).

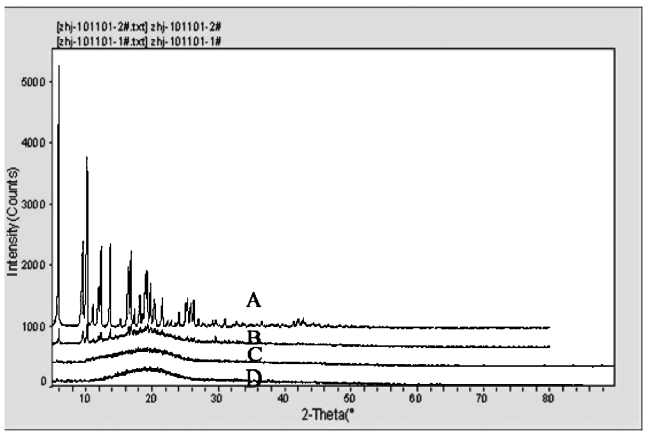


Fig. 5: X-ray diffraction parterns of raw lappaconitine (A), the simple physical mixture of lappaconitine and the blank microspheres (1:5) (B), the lappaconitine-loaded microspheres (C), the blank microspheres (D)

2.8. In vitro release

In order to investigate the release kinetics of lappaconitine from microspheres, two different particle groups including particles washed with 0.01N dilute hydrochloric acid and particles washed with distilled water were prepared. The drug release behavior was determined according to section 3.10. Fig. 8 shows the *in vitro* release profile of lappaconitine from the two particles groups. Compared to the raw lappaconitine, the sustained release from the two groups of drug-loaded microspheres was observed for three weeks. The burst release within the initial 24 h for microspheres washed with 0.01N HCl and distilled water was 16.1% and 7.6% of total drug in lappaconitine-loaded microspheres, respectively. The burst release was smaller than seen in many previous reports. For example, Kyo et al. prepared cisplatin-loaded microspheres and found that all the microspheres showed a burst release of 25–55% of loaded drug (Li et al. 1994). Le Corre et al. (1994) prepared bupivacaine-loaded microspheres and reported a burst release of 51.3% drug dur-

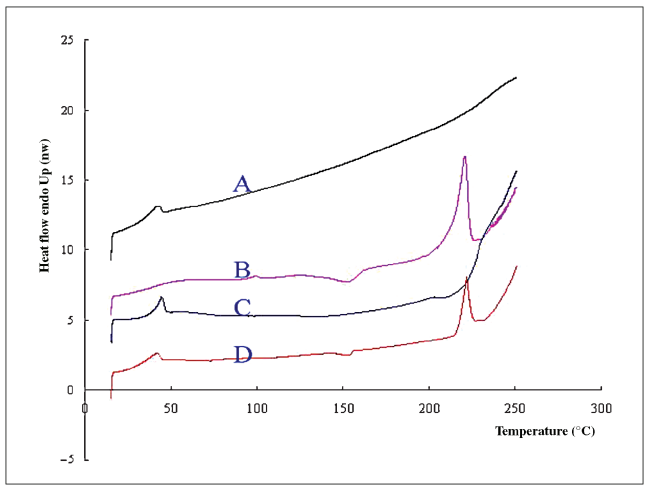


Fig. 6: DSC curves of the blank microspheres (A), the raw lappaconitine (B), the lappaconitine-loaded microspheres(C), (D) the simple physical mixture of lappaconitine and the blank microspheres (1:5)

ing the first 3 h. The burst release was believed to due to drug molecules adsorbed on the surface of microspheres (loosely trapped in particles) which were not removed by the washing procedure (Cheng et al. 1998). Because lappaconitne was easier to dissolve in 0.01N HCl solution (pH 2.0), the drug molecules adsorbed on the surface of microspheres could be completely removed by washing the drug-loaded microspheres with 0.01N HCl, resulting in lower burst release. Interestingly, in the study, comparing the burst effect profiles of the two groups of particles, it was found that 16.1% of the total drug in particles washed with 0.01N HCl was released, obviously higher than the latter, suggesting that no drug was adsorbed on the surface of microspheres and the drug incorporated in microspheres was inversely liable to transfer toward the surface of microspheres washed with 0.01N HCl during washing or desiccation.

Further, the two groups of release profiles were fitted to Higuchi equation, zero and first order kinetics models, respectively. The kinetic models and calculated coefficients are summarized in Table 5 . It was found that the drug release from particles washed with 0.01N dilute hydrochloric acid could be better agreed with the first-order kinetics model ($R^2 = 0.9811$), which indicated that drug release from microspheres was controlled by drug diffusion from microspheres and degradation of PLGA (Mollica et al. 2008). Whereas drug release from particles washed with distilled water was mainly dominated by zero-order kinetics ($R^2 = 0.9476$), independent on the concentration of drug in microspheres, which suggested that swelling and degradation of PLGA largely determined the drug release (Mundargi et al. 2008; Su et al. 2009). Thus, it was obvious that the washing methods affected the release models. The alternation of drug release model from microspheres washed with 0.01N HCl may be due to the following reasons: (1) ionization of lappaconitine in microspheres after washing with 0.01HCl may occur, which increases the solubility of lappaconitine in release mediums because of higher solubility of its hydrochloric acid salts; (2) the ionized lappaconitine may be liable to migrate from the core of microspheres to the surface during the desiccation of microspheres, which increases the concentration of drug near the surface of microspheres and reduces the diffusion pathway from which lappaconitine leached out; (3) there may be some dilute HCl remaining in the microspheres after desiccation, which resulted in a local acid micro-environments inside the microspheres, catalyzing hydrolysis of the polymer (Siepmann et al. 2005; Zolnik and Burgess 2007). However, drug in microspheres washed with distilled water was released as a constant rate, independent of drug concentration, which could be due to the presence of the drug in a relatively more localized way in the core of the microspheres (Obeidat et al. 2009). The drug localized in the core of the microsphere could be further confirmed by comparing the results of drug loading and entrapment efficiency for the two groups particles (Table 6). There was no significantly difference between the two groups particles, which showed that washing with 0.01 HCl could not lose the lappaconitine in the core of microsphere, otherwise resulting in decreasing the drug loading and entrapment efficiency.

In conclusion, lappaconitine instead of its hydrobromide salt was incorporated in PLGA microspheres prepared by o/w emulsion solvents evaporation techniques. No drug was absorbed on the surface of the microspheres. The *in vitro* release of lappaconitine from drug-loaded microsphere sustained about 3 weeks, which could satisfy the rules of drug therapy of carcinomatous pain (Myers et al. 2010).

Table 5: Release kinetics of microspheres Washed with 0.01N HCl and pure water

Mathematical model		R^2 HCl	R^2 Wats
Zero order	$Q(t) = kt$	0.9583	0.9476
First order	$In[1–Q(t)] = –kt$	0.9811	0.7818
Higuchi equation	$Q(t) = \sqrt{t}$	0.9755	0.8272

3. Experimental

3.1. Materials

Poly(lactide-co-glycolide) acid (lactide/glycolide, 50:/50; M_n, 15000) was supplied by Shandong Institute of Medical Instruments (Shandong, China). Lappaconitine and its hydrobromide salt was provided by The River Pharmaceutical Co.LTD (Shanxi, China). Polyvinyl alcohol (PVA-124, Japan) was purchased from Guangzhou Chang Fu Trade Co. Ltd., China. The other reagents and solvents were analytical-grade.

3.2. Saturated solubility and oil/water solubility partition ratio of lappaconitine and its hydrobromide salt in different solvents

To design the method of formulating microspheres and investigate the effect of partition coefficient on entrapment efficiency, we first detected the saturate solubility (S) of lappaconitine and its hydrobromide salt in different solvents such as dichloromethane, distilled water, different pH phosphate buffer. For the accurate partition ratio, the solvent was pre-saturated each other. The excess lappaconitine or its hydrobromide salt was added to the test vial containing 5 ml solvents. The vials were placed into shake bath thematated 25 °C at 150 rpm for 24 h. The saturated solution (S) was prepared by centrifugation, diluted with corresponding solvent, and analyzed by TU-1900 UV spectrophotometer (Peking, China) at 252 nm. The partition ratio was calculated by Eq. (1)

$$\text{Partition ratio} = \frac{\text{S(dichloromethane)}}{\text{S(aqueous phase)}} \quad (1)$$

3.3. Preparation of microspheres and formulation optimization

Drug-loaded and blank microspheres were prepared by modifying slightly the emulsification-solvent evaporation technique as described by Sinha and Trehan (2005). In short, 100 mg of poly(lactide-co-glycolide) acid were dissolved in 2 ml dichloromethane. 20 mg of lappaconitine was dissolved in the solution, while equivalent of its hydrobromide salt was first dissolved in 0.5 ml methanol, and then mixed with the polymer solution. Under stirring, the resulting solution was emulsified in 8 ml aqueous phase containing 1.5% PVA by adding slowly the polymer solution to the aqueous phase at 15 °C. The formed emulsion (o/w) was agitated for another hour. Subsequently, the resulting emulsion was poured to 20 ml thrice distilled water, and stirred continually for 4 h at 25 °C to allow solvent evaporation. The microspheres were collected by centrifugation at 5000 rpm for 20 min and washed three times with acid water (0.01N HCl) or distilled water before desiccation. The microspheres were dried in vacuum desiccators at 20 °C for 48 h.

In order to investigate the effect of formulation parameters formulation, batches of microspheres were prepared by modifying stirring speed, polymer

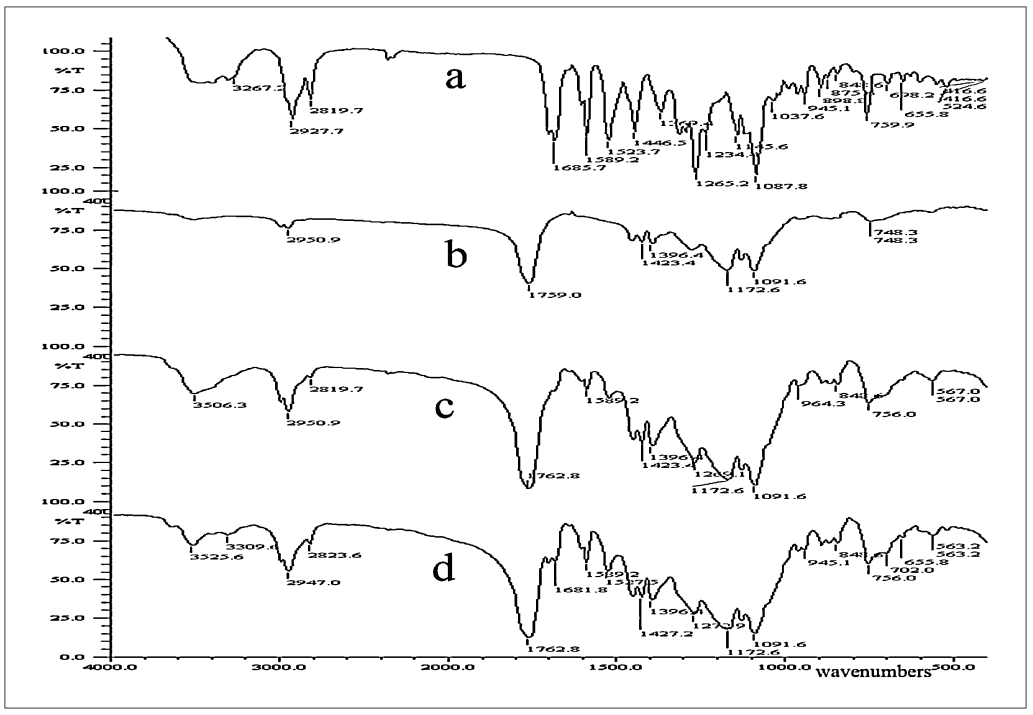


Fig. 7: The FT-IR spectrograms of the raw lappaconitine (a), the blank microspheres (b), the simple mixture of lappaconitine and blank microspheres (1:5) (c), the lappaconitine-loaded microspheres (d)

Table 6: Effects of different washing methods on quality of microsphere

Washing methods	Drug loading	Entrapment efficiency	Mean size(μm)	Span
Distilled water	10.97%	67.67%	19.6	1.38
0.01N HCl	11.04%	67.12%	18.6	1.42

concentration, surfactant concentration drug/polymer ratio and pH of outer aqueous phase (Table 2, Table 3).

3.4. Determination of drug content in the microspheres

The dried microspheres were dissolved in dichloromethane, filtered through 0.45 μm Millipore membranes and analyzed at 252 nm by a TU-1900 UV spectrophotometer. The drug loading (DL) and entrapment efficiency (EE) were calculated according to Eq. (2) and Eq. (3)

$$\mathrm{DL} = \frac{\text{the detected drug contents in microsphere}}{\text{the weight of microspheres}} \times 100\% \qquad (2)$$

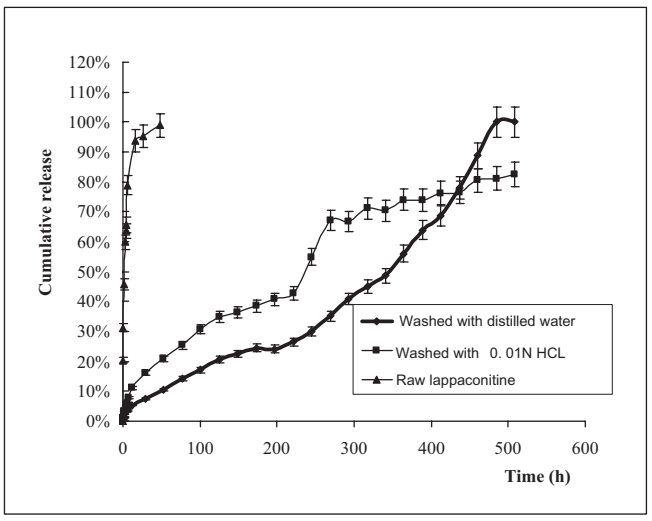


Fig. 8: *In vitro* release behavior of the raw lappaconitne and the two groups' particles in pH7.4 PBS

$$\mathrm{EE} = \frac{\text{the theoretical drug loading}}{\text{the pratical drug loading}} \times 100\% \qquad (3)$$

3.5. Size and sphericity of microspheres

Sphericity and particle size were determined by measuring the diameter of individual particles using an optical microscope with micrometer (2XC, Shanghai, China). The diameters of 500 particles were measured and the mean particle size determined. The size distribution was calculated by SPSS13.0 statics software. Thereafter, the polydisperity of microspheres was expressed by the span value (Eq. 4)

$$\mathrm{Span} = \frac{D_{90} - D_{10}}{D_{50}} \qquad (4)$$

where D_{90}, D_{10}, D_{50} represent the diameter under which there are 90%, 10%, 50% of the population distribution, respectively.

3.6. Scanning electronic microscopy (SEM)

The size distribution and surface morphology of microspheres were further examined on a scanning electronic microscope (Hitachi, Japan). The drug-loaded microspheres were positioned on a mental stud, which was coated with an adhesive label. The different magnification images were captured by adjusting the voltage.

3.7. Fourier Transform Infra-red Spectroscopy (FT-IR)

Fourier transform infra-red spectroscopy (FT-IR) spectra were taken for lappaconitine, blank microspheres, a physical mixture of lappaconitine and blank microspheres (1:10 ratio), and lappaconitine-loaded microspheres. The aim was to detect any possible interaction between drug and the polymer. Fourier transform infra-red spectroscopy measurements (FT-IR) were performed with FT-IR 8400 spectrophotometer (Shimadu, Japan) using the potassium bromide disk.

3.8. Differential scanning calorimetry

Differential scanning calorimetry thermograms were taken on a DSC PYRIS DIAMOND (USA) in standard aluminum pan. Nitrogen gas was sweeping gas, and the heating rate was 5 °C/min. Samples (12.5 mg) were loaded in a pan without further treatment. The initial and the end temperature were 15 °C and 250 °C, respectively. Aluminum was used as the standard reference material to calibrate the temperature and energy scales of DSC instrument.

3.9. X-ray diffraction studies

The XRD patterns of lappacontine, blank microspheres, the physical mixture of lappaconitine and blank microspheres and lappaconitine-loaded microspheres were performed with D8 Focus Bruker AXS. The X-ray source was Cu Kα radiation (40 kV, 40 mA).The diffractograms were recorded covering an angular interval between $2\theta = 5^\circ$ and 80° and using a step size of 0.02° with time per step of 1 s.

3.10. In vitro release behavior of lappaconitine-loaded microspheres

The *in vitro* release behavior of lappaconitine-loaded microsphere was evaluated by the dynamic dialysis method. Approximately, the amount of microspheres equivalent to 5 mg of lappaconitine were accurately weighed into 4 cm × 5 cm dialysis bag. 3 ml pH 7.4 PBS was added to the dialysis bag, then the bag was sealed with clamps and placed into amber jar containing 75 ml release medium. The jar was placed on the water bath shaker at 37 °C and shaken at 120 rpm. At various sample points, 2 ml release mediums was withdrawn, filtered by 0.45 μm Millipore filter membranes and determined by high performance liquid chromatography (HPLC, Shimadzu, class 10AVP). At the same time, 2 ml fresh release medium was supplemented to satisfy the leak condition after every sampling. The initial drug burst of microspheres was investigated by determining drug release from microspheres within 24 h.

Acknowledgements: This work was partly supported by the National Nature Sciences Foundation of China (Grant Number: 30960466) and the Research Foundation of Education Bureau of Jiangxi Province of China (Grant Number: GJJ08067).

References

Alex R, Bodmeier R (1990) Encapsulation of water-soluble drugs by a modified solvent evaporation method. I. Effect of process and formulation variables on drug entrapment. J Microencapsul 7: 347–355.

Ameri A (1998) The effects of Aconitum alkaloids on the central nervous system. Prog Neurobiol 56: 211–235.

Benelli P, Conti B, Genta I, Costantini M, Montanari L (1998) Clonazepam microencapsulation in poly-D,L-lactide-co-glycolide microspheres. J Microencapsul 15: 431–443.

Bodmeier R, McGinity JW (1987) The preparation and evaluation of drug-containing poly(dl-lactide) microspheres formed by the solvent evaporation method. Pharm Res 4: 465–471.

Cheng YH, Illum L,Davis SS (1998) A poly(D,L-lactide-co-glycolide) microsphere depot system for delivery of haloperidol. J Control Release 55: 203–212.

Corrigan OI (1995) Thermal-analysis of spray-dried products. Thermochim Acta 248: 245–258.

Cui F, Cun DM, Tao AJ, Yang MS, Shi K, Zhao M, Guan Y (2005) Preparation and characterization of melittin-loaded poly (DL-lactic acid) or poly (DL-lactic-co-glycolic acid) microspheres made by the double emulsion method. J Control Release 107: 310–319.

Eisenberg E, Berkey CS, Carr DB, Mosteller F, Chalmers TC (1994) Efficacy and safety of nonsteroidal antiinflammatory drugs for cancer pain: a meta-analysis. J Clin Oncol 12: 2756–2765.

Freiberg S, Zhu XX (2004) Polymer microspheres for controlled drug release. Int J Pharm 282: 1–18.

Hassett P, Ansari B, Gnanamoorthy P, Kinirons B, Laffey JG (2008) Determination of the efficacy and side-effect profile of lower doses of intrathecal morphine in patients undergoing total knee arthroplasty. BMC Anesthesiol 8: 5.

Ito F, Fujimori H, Makino K (2007) Incorporation of water-soluble drugs in PLGA microspheres. Colloids Surf B Biointerfaces 54: 173–178.

Jovanovic I, Jordovic B, Petkovic M, Ignjatovic N, Uskokovic D (2008) Preparation of smallest microparticles of poly-D,L-lactide by modified precipitation method: influence of the process parameters. Microsc Res Tech 71: 86–92.

Lecorre P, Leguevello P, Gajan V, Chevanne F, Leverge R (1994) Preparation and characterization of bupivacaine-loaded polylactide and polylactide-co-glycolide microspheres. Int J Pharm 107: 41–49.

Li C, Yang DJ, Nikiforow S, Tansey W, Kuang LR, Wright KC, Wallace S (1994) Formation and characterization of cisplatin-loaded poly(benzyl l-glutamate) microspheres for chemoembolization. Pharm Res 11: 1792–1799.

Mao S R, Xu J, Cai C F, Germershaus O, Schaper A, Kissel T (2007) Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres. Int J Pharm 334: 137–148.

Martinez-Sancho C, Herrero-Vanrell R, Negro S (2004) Optimisation of aciclovir poly(D,L-lactide-co-glycolide) microspheres for intravitreal administration using a factorial design study. Int J Pharm 273: 45–56.

McGinity JW, O'Donnell PB (1997) Preparation of microspheres by the solvent evaporation technique. Adv Drug Deliv Rev 28: 25–42.

Mollica F, Biondi M, Muzzi S, Ungaro F, Quaglia F, La Rotonda MI, Netti PA (2008) Mathematical modelling of the evolution of protein distribution within single PLGA microspheres: prediction of local concentration profiles and release kinetics. J Mater Sci-Mater M 19: 1587–1593.

Mu L,Feng SS (2003) PLGA/TPGS nanoparticles for controlled release of paclitaxel: Effects of the emulsifier and drug loading ratio. Pharm Res 20: 1864–1872.

Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM (2008) Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J Control Release 125: 193–209.

Mundargi RC, Srirangarajan S, Agnihotri SA, Patil SA, Ravindra S, Setty SB,Aminabhavi T M (2007) Development and evaluation of novel biodegradable microspheres based on poly(D,L-lactide-co-glycolide) and poly(epsilon-caprolactone) for controlled delivery of doxycycline in the treatment of human periodontal pocket: *In vitro* and *in vivo* studies. J Control Release 119: 59–68.

Myers J, Chan V, Jarvis V, Walker-Dilks C (2010) Intraspinal techniques for pain management in cancer patients: a systematic review. Support Care Cancer 18: 137–149.

Nilkumhang S, Basit AW (2009) The robustness and flexibility of an emulsion solvent evaporation method to prepare pH-responsive microparticles. Int J Pharm 377: 135–141.

Obeidat W M, Obeidat S M,Alzoubi N M(2009) Investigations on the physical structure and the mechanism of drug release from an enteric matrix microspheres with a near-zero-order release kinetics using SEM and quantitative FTIR. AAPS Pharmscitech 10: 615–623.

Rahman Z, Zidan AS, Habib MJ, Khan MA (2010) Understanding the quality of protein loaded PLGA nanoparticles variability by Plackett-Burman design. Int J Pharm 389: 186–194.

Shive MS, Anderson JM (1997) Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev 28: 5–24.

Siepmann J, Elkharraz K, Siepmann F, Klose D (2005) How autocatalysis accelerates drug release from PLGA-based microparticles: A quantitative treatment. Biomacromolecules 6: 2312–2319.

Sinha VR,Trehan A (2005) Formulation, characterization, and evaluation of ketorolac tromethamine-loaded biodegradable microspheres. Drug Deliv 12: 133–139.

Stein C, Schafer M, Machelska H (2000) Why is morphine not the ultimate analgesic and what can be done to improve it? J Pain 1(3 Suppl): 51–56.

Su Z, Sun F, Shi Y, Jiang C, Meng Q, Teng L, Li Y (2009) Effects of formulation parameters on encapsulation efficiency and release behavior of risperidone poly(D,L-lactide-co-glycolide) microsphere. Chem Pharm Bull (Tokyo) 57: 1251–1256.

Tang XC, Zhu MY, Feng J (1983) Study on pharmacological action of lappaconitine hydrobromide. J Pharm 18: 579.

Xu X, Xiang Q, He Z, Liu Y, Zhou D, Qin X, Fan T, Zhang Z, Huang Y (2010) Crystalline drug aconitine-loaded poly(d,l-lactide-coglycolide) nanoparticles: preparation and *in vitro* release. Yakugaku Zasshi 130: 409–418.

Zhang H, Lu Y, Zhang GQ, Gao S, Sun DX, Zhong YQ (2008) Bupivacaine-loaded biodegradable poly(lactic-co-glycolic) acid microspheres - I. Optimization of the drug incorporation into the polymer matrix and modelling of drug release. Int J Pharm 351: 244–249.

Zidan AS, Sammour OA, Hammad MA, Megrab NA, Hussain MD, Khan MA, Habib M J (2006)Formulation of anastrozole microparticles as biodegradable anticancer drug carriers. AAPS Pharmscitech 7(3).

Zolnik BS, Burgess DJ (2007) Effect of acidic pH on PLGA microsphere degradation and release. J Control Release 122: 338–344.