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Preparation of floating microspheres for fish farming

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Abstract

The aim of this study was to develop floating microspheres with practical applications to fish farming. Each microsphere with a central hollow cavity was prepared using a solvent diffusion and evaporation method with Eudragit® E100. Various manufacturing parameters were investigated by single factor method. The macrolide antibiotic josamycin was selected as a model drug. The loading efficiency of the drug in the microspheres was 64.7%. In the release study, virtually none of the drug was released into the fresh water whereas the entire drug was released from the josamycin-loaded microspheres into the simulated gastric fluid of rainbow trout (pH 2.7). The buoyancy was excellent with approximately 90% of the microspheres still floating after 24 h.

Keywords: Drug delivery; Floating microspheres; Eudragit® E100; Josamycin; Fish farming

1. Introduction

Aquaculture is one of the world's most rapidly growing sectors in animal production with fish farming occupying a major proportion. Public demand for cheaper food has increased the need to densely stock fish in aquaculture medium to cut cost. However, the fish can contract diseases more easily in this state. Some of the most serious infectious diseases of fish include mycobacteriosis (Belas et al., 1995), bacterial kidney disease, rickettsiae and rainbow trout specific diseases such as enteric redmouth (ERM) and rainbow trout fry syndrome (RTFS). Huge amounts of antibiotics are needed to keep the fish moderately healthy. Flumequine, fluorfenicol, sulfonamides, oxolinic acid, oxytetracycline hydrochloride, and amoxycillin trihydrate are the most frequently used antibiotics (Rangdale et al., 1997; Lalumera et al., 2004). The most commonly used methods for administering these antibiotics include the oral administration of drugs as food additives (Rogstad et al., 1991), bathing of fish inside an aquaculture medium containing drugs and the spraying of drugs that dissolve in the medium. However, these methods are inefficient and have undesirable effects. Some antibiotics precipitate in water (Costanzo et al., 2005). Therefore, overdoses of these drugs have been used to cover for the loss of precipitated antibiotics. This plethora of medication can aggravate the development of resistant pathogens in farmed fish or in the vicinity of farms. In addition, the presence of drug residues in market fish as well as drug-caused physiological impairment in fish can occur.

These conventional methods for administering drugs contaminate the whole medium. Moreover, it is virtually impossible to recover the unused drugs for reuse. Therefore, in this context, a floating drug carrier can be of interest from which drugs do not leach out in the medium but are released inside the body of the fish when engulfed. In addition, such unused floating dosage forms can be easily recovered from the surface water using a net and depending on the stability of the drug, can be dried and stored for further use. This can minimize the adverse effects of the extravagant use of drugs.

This study focused on the treatment of bacterial diseases in rainbow trout using floating microspheres due to their importance in aquaculture (Hardy et al., 2000). Eudragit® E100 was used to form the microspheres because of its water insolubility and ability to dissolve in acidic pH. Eudragit® E100 consists of a 1:2:1 ratio of methyl methacrylate, *N*,*N*-dimethylaminoethyl methacrylate, and butylmethacrylate monomers. The polymer is soluble in gastric fluid when pH < 5 due to the tertiary amino groups, which are ionized in an acidic pH (Chang and Shukla, 2000). The pH of the stomach of the rainbow trout is 2.7 (Dauble

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and Curtis, 1990), and the microspheres dissolve rapidly releasing the drug. Josamycin, a macrolide antibiotic, was chosen as the model drug for this study. It is a hydrophobic drug with poor solubility in fresh water. Its broad-spectrum activity does not induce resistance in susceptible microorganisms and it is relatively acid stable compared with erythromycin. The antibiotic is well tolerated and shows excellent tissue penetration and distribution after oral administration (Skinner and Skinner, 1992).

2. Materials and methods

2.1. Materials

Eudragit® E100 was obtained from Rohm GmbH and Co. KG (Darmstadt, Germany). Poly(vinyl alcohol), 87–89% hydrolyzed (typical molecular weight 13,000–23,000) was purchased from Sigma–Aldrich (St. Louis, USA). Josamycin was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). All other chemicals were of reagent grade and used as received without further purification.

2.2. Methods

2.2.1. Preparation of microspheres

The optimized microspheres were prepared as follows: 1.2 g of Eudragit® E100 granules (15%, w/v polymer concentration) was dissolved in a mixture of 6.55 ml of ethanol and 1.45 ml of dichloromethane (4.5:1 solvent ratio). The solution (4%, v/v internal phase volume fraction) was then introduced into 200 ml of a 0.4% (w/v) aqueous poly (vinyl alcohol) solution stirred at a speed of 250 rpm at 45 °C. The microspheres formed were further stirred for 10 min to allow hardening. The microspheres were separated by filtration and dried at room temperature for 12 h. The drug-loaded microspheres were prepared as mentioned above except that 63.2 mg of josamycin (5% w/w of total weight of non-volatile components) was dissolved in the solvent mixture together with 1.2 g of Eudragit® E100.

Each parameter was optimized one at a time. During optimization all other parameters were kept constant except the parameter to be optimized. The internal phase volume fraction was optimized using 2, 4, 6 and 8% of the internal phase. The polymer concentration was optimized using 11% (w/v), 13% (w/v), 15% (w/v) and 17% (w/v) of the polymer. Ethanol and dichloromethane ratios of 1.5:1, 2.5:1, 3.5:1, 4.5:1, 5.5:1 and 6.5:1 were used to optimize the solvent ratio. The stirring speed used was 150, 250 and 350 rpm. The microspheres were prepared at 25 °C (room temperature), 45 and 70 °C. Aqueous surfactant solutions at concentrations of 0.2% (w/v), 0.4% (w/v), 0.8% (w/v) and 1.2% (w/v) were used to optimize the concentration of the surfactant solution.

2.2.2. Yield of formed microspheres

The prepared microspheres were collected and weighed. The yield was calculated by dividing the measured weight by the total weight of all non-volatile components.

2.2.3. Particle size analysis

The particle size distribution of microspheres was measured using a particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK).

2.2.4. Scanning electron microscopy (SEM)

The morphology of the microspheres was examined by field emission scanning electron microscopy (FESEM, S-4700, Hitachi, Japan). The sample was mounted onto an aluminum stub and sputter-coated with platinum particles for 120 s in an argon atmosphere.

2.2.5. Differential scanning calorimetry (DSC)

Thermal analysis was carried out using a DSC unit (Pyris 6 DSC, Perkin-Elmer, Netherlands). Indium was used to calibrate the temperature scale and enthalpic response. Samples were placed in aluminum pans and heated at a scanning rate of 10 °C/min from 30 to 180 °C. Four samples i.e. pure josamycin, josamycin:Eudragit® E100 (5.26:94.74) physical mixture, josamycin-loaded microspheres and pure Eudragit® E100 were analyzed.

2.2.6. Analysis of josamycin

Josamycin was analyzed by HPLC (Shimadzu Scientific Instruments, MD, USA), consisting of a UV detector (SPD-10A), a pump (LC-10AD), and an automatic injector (SIL-10A). The mobile phase used was a phosphate buffer (adjusted to pH 4) and acetonitrile at a ratio of 60:40. The wavelength of the UV detector was 231 nm and a reversed-phase column (Luna C8, Phenomenex, USA) was used. The column temperature was maintained at 30 °C while the flow rate was 1 ml/min.

2.2.7. Loading content and loading efficiency

To measure the loading content and loading efficiency, the josamycin-loaded samples prepared by optimized method were used. The loading content and loading efficiency were determined using the following formulae:

$$\text{Loading content (\%)} = \frac{\text{weight of josamycin in the microspheres}}{\text{weight of the microspheres}} \times 100$$

$$\text{Loading efficiency (\%)} = \frac{\text{weight of josamycin in the microspheres}}{\text{weight of the fed josamycin}} \times 100$$

2.2.8. Dissolution study of josamycin

Fresh water was used as the dissolution medium for simulating the water in fish farms. The dissolution test was carried out in a dissolution tester (DS-600A, Labfine, Inc., Suwon, Korea). The quantity of the josamycin-loaded microspheres used was calculated to make a drug concentration of 5 μg/ml if the entire drug was released in the medium. The microspheres were spread over the surface of the 1000 ml medium. The paddle speed was set to 100 rpm and the test was carried out at room temperature.

A 2 ml aliquot was drawn at predetermined times: (0.5, 1, 2, 4, 8, 12 and 24 h) and equivalent amounts of fresh medium were added. The collected samples were analyzed by HPLC. After the dissolution test, the amount of drug remaining in the microspheres was measured by dissolving the microspheres in ethanol and analyzing the josamycin concentration by HPLC.

A pH 2.7 HCl solution was used to simulate the pH of the stomach of rainbow trout. One thousand milliters of the medium was used and the dissolution of josamycin-loaded microspheres was examined as described previously. A 2 ml aliquot was drawn at set times (5, 15, 30, 45, 60 and 120 min), and the josamycin concentration was analyzed by HPLC.

2.2.9. Floating test of microspheres

The floating test was carried out using a dissolution tester (DS-600A, Labfine, Inc., Suwon, Korea) with 900 ml of fresh water as the medium and a paddle speed of 100 rpm at room temperature. One hundred and fifty milligrams of the microspheres (prepared without loading of josamycin) were spread over the surface of the medium. The floating microspheres were collected after 24 h and filtered. The filter paper containing the microspheres was dried in an oven at 80 °C for 2 h. The percentage of floating microspheres was then determined. The change in weight of the filter papers was determined after wetting them with fresh water and drying in an oven at 80 °C for 2 h. The change in the weight of the filter papers was <5% and was considered to be insignificant.

3. Results and discussion

3.1. Formation of floating microspheres

Kawashima et al. reported the mechanism for the formation of floating microspheres made from an acrylic polymer (Kawashima et al., 1991, 1992). The microspheres prepared in this study, as observed under scanning electron microscopy (Fig. 1a and b), were spherical in shape with a smooth outer surface. The josamycin loading did not cause any significant change in morphology. A photograph of a broken half of a microsphere loaded with josamycin (Fig. 1c) showed that each microsphere contained a central hollow core surrounded by a thick and porous shell. No drug crystals were observed on the outer or inner surface of the microspheres. Differential scanning calorimetry (DSC) of the josamycin-loaded microspheres revealed that the drug in the microspheres was amorphous (Fig. 2).

Water insoluble Eudragit® E100 shows higher solubility in dichloromethane than ethanol. However, ethanol has higher solubility in water. As soon as the polymer solution was added to the aqueous medium, the ethanol diffused rapidly from the droplets of the polymer solution. Simultaneous diffusion of water inside the sphere further decreased the ethanol concentration and hence the polymer precipitated resulting in the formation of microspheres. Dichloromethane remaining as the central core diffused slowly due to its low water solubility. Therefore, the diffusion of ethanol played an important role in determining the size and shape of the microspheres. The inner porous structure was attributed to the inward diffusion of water, which resulted in the solidification of the polymer and the formation of several smaller pockets of dichloromethane rich entrapments which diffused out together with ethanol. The entrapped dichloromethane diffused slowly out of the pocket giving a porous structure to the wall of the microspheres. Due to the poor miscibility, water could not effectively invade the dichloromethane rich core. Therefore, the diffusion of dichloromethane began late, after the initial solidification, and formed a central hollow structure. During the

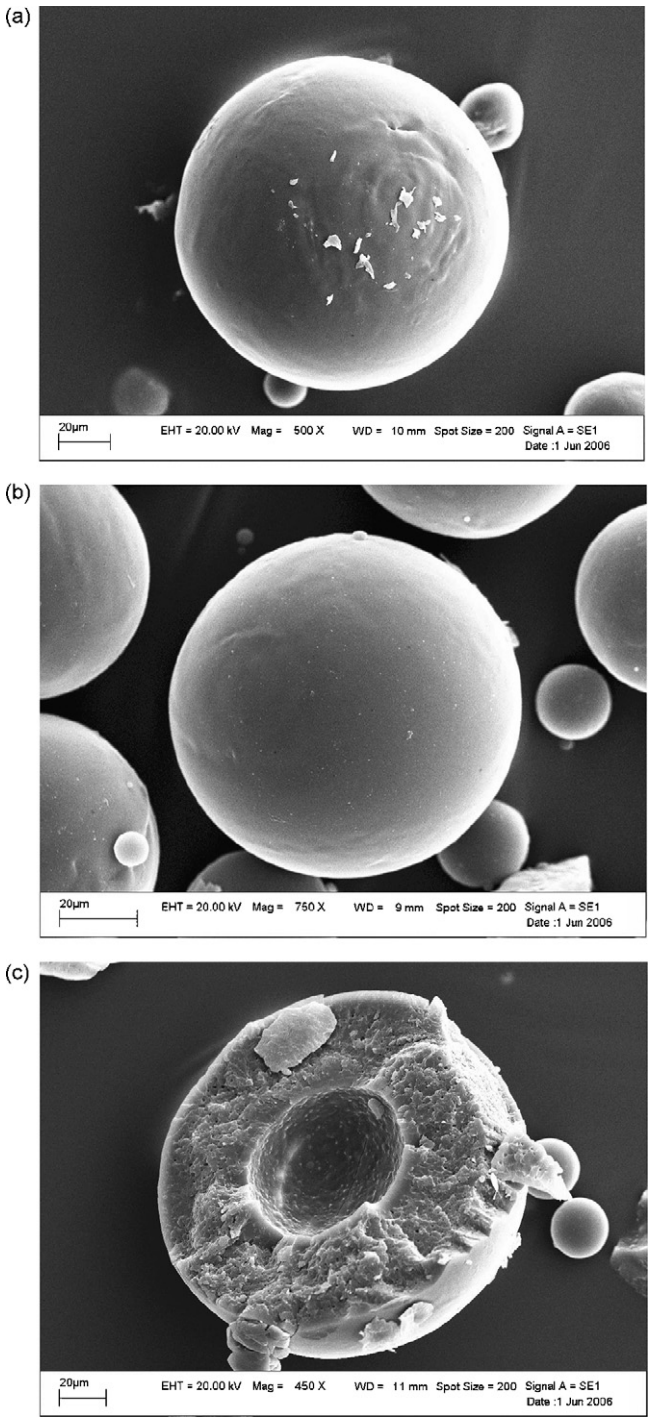

Fig. 1. SEM photographs of (a) the outer surface of a microsphere without josamycin; (b) outer surface of a microsphere with josamycin; (c) inner surface of a broken half of a microsphere with josamycin.

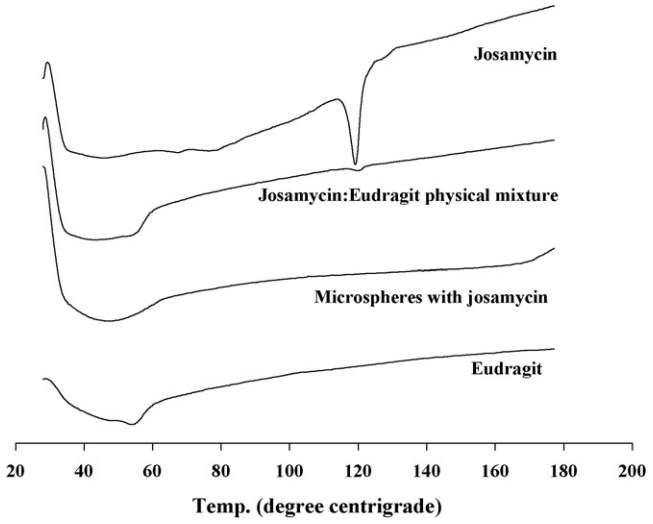

Fig. 2. DSC thermograms of josamycin, josamycin:Eudragit (5.26:94.74) physical mixture, microspheres containing josamycin, and Eudragit.

diffusion of the solvents, the polymer was pulled outward as a result of the dragging force of the solvents and thus the central void space emerged. The central cavity produced by the solvents was gradually filled with water due to the reduced internal pressure. Water escaped out of the cavity during the drying process ultimately forming hollow microspheres.

3.2. Size distribution and yield of microspheres

Table 1 summarizes the size distribution and yield of the microspheres at various manufacturing parameters. It was found that particle size decreased with increasing internal phase volume fraction. As the internal phase volume fraction increased, the diffusion of organic solvents decreased due to the higher concentration of solvents in the medium. Therefore, more shearing energy can be provided before solidification resulting in smaller microspheres. However, the yield did not show any proportionality relationship. The yield increased with increasing internal phase volume fraction up to 4% (v/v), and then decreased thereafter despite the increase in the internal phase. At 2% (v/v), the diffusion rate of the solvents was higher, and some of the polymer aggregated in a fiber like structure as it solidified before forming complete droplets. At a higher internal phase volume fraction, the slower diffusion rate led to some coalescence of the microspheres due to the slow hardening of the microspheres. In both cases, the yield of the microspheres decreased. However, at 4% (v/v), a minimum amount of film was formed indicating the optimum rate of solvent diffusion.

The particle size increased with increasing the polymer concentration. This is due to the increase in viscosity of the solution and the decrease in stirring efficiency. The best yield was obtained at a polymer concentration of 15% (w/v). This indicates that the optimum diffusion rate of the solvents was obtained at a polymer concentration of 15%.

Table 1
Size distribution and yield of the microspheres at various manufacturing parameters ($n = 3$)

Optimized parameter	Value of parameter	Diameter (μm) (mean ± S.D.[a])	Yield (%) (mean ± S.D.[a])
Internal phase volume fraction (IPVF)	2% (v/v)	187.0 ± 42.0	70.0 ± 4.4
	4% (v/v)	153.4 ± 76.2	72.5 ± 2.2
	6% (v/v)	123.7 ± 21.3	69.3 ± 1.7
	8% (v/v)	119.2 ± 7.2	67.1 ± 3.3
Polymer concentration	11% (w/v	100.3 ± 7.6	59.8 ± 8.6
	13% (w/v)	110.9 ± 8.2	65.1 ± 2.0
	15% (w/v)	131.3 ± 12.8	69.7 ± 1.8
	17% (w/v)	128.3 ± 4.5	63.2 ± 1.5
Solvent ratio[b]	1.5:1	123.0 ± 12.2	59.2 ± 4.3
	2.5:1	124.8 ± 8.1	64.2 ± 3.0
	3.5:1	119.5 ± 2.3	71.1 ± 4.8
	4.5:1	149.6 ± 2.7	77.5 ± 2.2
	5.5:1	157.6 ± 3.8	80.8 ± 2.2
	6.5:1	283.6 ± 13.1	81.9 ± 4.6
Stirring speed	150 rpm	164.6 ± 10.5	74.7 ± 1.3
	250 rpm	109.9 ± 3.9	77.2 ± 2.1
	350 rpm	91.7 ± 3.7	75.8 ± 2.5
Preparation temperature	25 °C	114.9 ± 1.2	78.6 ± 4.7
	45 °C	138.9 ± 6.7	77.5 ± 5.0
	70 °C	142.1 ± 1.7	73.6 ± 6.4
Surfactant concentration	0.2% (w/v)	193.9 ± 7.8	73.2 ± 0.8
	0.4% (w/v)	164.4 ± 9.5	77.1 ± 2.9
	0.8% (w/v)	126.0 ± 9.3	80.9 ± 1.5
	1.2% (w/v)	128.7 ± 2.8	78.9 ± 2.5

[a] Standard deviation.
[b] Ethanol:dichloromethane.

Both the particle size and yield increased with increasing proportion of ethanol in the solvent mixture. Ethanol, being more soluble in water than dichloromethane, diffused preferentially thereby minimizing the diffusion of dichloromethane. With increasing ethanol percentage, the rate of diffusion increased and the hardening time of the microspheres was shortened. Consequently, a shorter time was provided for the breakup of droplets and larger microspheres were formed. In addition, the polymer might have been dragged further by the presence of more ethanol in the droplets, resulting in a thicker water/ethanol mixture zone. The thicker water/ethanol mixture zone resulted in a thicker wall thickness and a larger particle size. The increasing percentage of ethanol not only shortened the hardening time of the microspheres by the increased diffusion rate but in turn reduced the stickiness of the microspheres and hence the formation of aggregates and process loss. Therefore, the yield of microspheres increased with increasing proportion of ethanol in the solvent mixture.

Smaller microspheres were generated with increasing stirring speed due to higher shear force. The polymer solution under high turbulence was finely divided into smaller droplets. When the stirring speed was too slow, the diffusion rate of the solvents decreased due to the formation of larger droplets. The subsequent slow hardening may decrease the yield of microspheres due to their sticking to the vessel and coalescing into aggregates. When the stirring speed was too fast, solidification of the polymer occurred before the formation of spherical microspheres due to the higher rate of diffusion. Too fast solidification may result in a fiber like structure. The decrease in yield was mainly due to aggregation at lower stirring speed and the fiber like structure at higher stirring speed.

The size of the microspheres increased with increasing preparation temperature. At higher temperatures, the rate of solvent diffusion increased. Hence, less time was provided to breakup the droplets. The increased diffusion rate of the solvents also enhanced the formation of fiber like aggregates as a result of polymer solidification before microsphere formation was complete.

With increasing surfactant concentration, the size of the microspheres decreased and the yield increased up to 0.8%. An increase in the surfactant concentration produced smaller and more stable droplets, thereby reducing the fusion of smaller droplets to form larger droplets or aggregates. Ultimately, small sized microspheres were generated. No further effect was observed when the surfactant concentration exceeded 0.8%.

3.3. Loading content and loading efficiency

Josamycin is a hydrophobic drug that is freely soluble in ethanol (Budavari et al., 1996) and dichloromethane. As ethanol is rapidly partitioned into an aqueous phase during emulsification, most of the josamycin molecules remain within the polymeric shell area and solidify together with the polymer. The thick wall of the microspheres provides a larger volume for loading the drug. Hence, the loading content and loading efficiency of josamycin was fairly high. Compared with the 5% theoretical loading content, the actual josamycin loading was $4.5 \pm 0.18\%$.

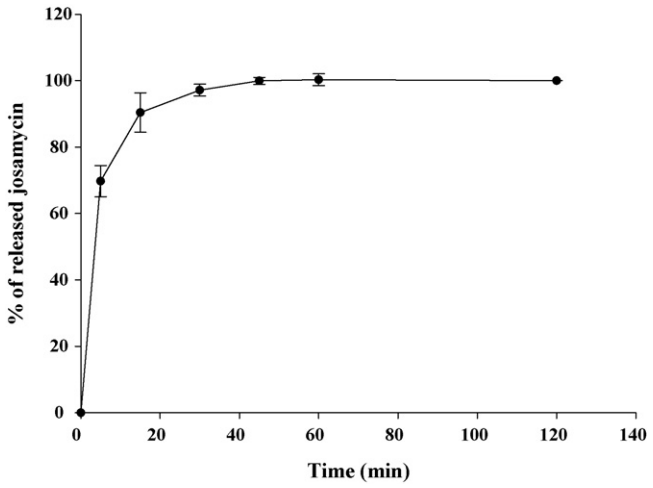

Fig. 3. Release profile of josamycin from the Eudragit microspheres in the pH 2.7 medium ($n = 3$).

However, the loading efficiency of josamycin was determined to be $64.7 \pm 2.7\%$. This lower loading efficiency was attributed to the moderate yield value of microspheres i.e. $71.8 \pm 3.1\%$.

3.4. Dissolution of josamycin-loaded microspheres

The prime objective in the development of the present dosage form was to prevent the release of the drug in farm water but allow the release of the drug in the body of rainbow trout. Therefore, it is necessary to examine the release characteristics of the microspheres in media simulating both conditions. Fresh water was used in the place of farm water, and a pH 2.7 HCl buffer was used to simulate the gastrointestinal tract of the fish. The stability study of josamycin in a pH 2.7 medium revealed that there was no significant degradation during the dissolution study.

HPLC analysis of the samples collected from the dissolution test in fresh water did not show any noticeable josamycin peak. To further confirm the finding, all the microspheres in the release medium were collected and analyzed for their josamycin content by HPLC. The result showed almost 100% of the drug to be present in the collected microspheres. Therefore, it was concluded that virtually none of the drug was released from the microspheres in fresh water for 24 h. On the other hand, almost all the microspheres had dissolved within 45 min during the dissolution test in the pH 2.7 medium (Fig. 3). Eudragit® E 100 is soluble in acidic pH. Therefore, all the drugs were released in the medium after dissolution of the polymer.

3.5. Floating behavior of Eudragit microspheres

The floating test showed that $89.6 \pm 0.63\%$ of the microspheres remained floating for 24 h. A central hollow core greatly reduces the density of the microspheres below than that of water. More importantly, the polymer forming the wall of the microspheres is water insoluble. The combined effects of these properties of the microspheres will prevent leaching of the drug in fresh water thus increasing its suitability in fish farming. In addition, the floating state of the microspheres will increase the

probability of the intake of a higher amount of microspheres by the fish. The recovery of unconsumed microspheres can be accomplished simply by using a small pore sized net.

4. Conclusion

The josamycin-loaded Eudragit® E100 microspheres, prepared using a solvent diffusion and evaporation method, have the qualities suitable for their successful use in fish farming. The floating test revealed that approximately 90% of the microspheres were still floating after 24 h. The loading efficiency of josamycin in the microspheres was fairly good. Virtually none of the drug was released in fresh water, thereby preventing the contamination of the entire farm water with drug. The microspheres dissolved in the stomach of the rainbow trout rapidly within 45 min to release the drug, as confirmed by the dissolution test at pH 2.7 medium. Therefore, the excess use of a drug can be minimized and all the adverse effects inherent to this overuse can be minimized. In addition, depending upon the stability of josamycin, the recollected unused microspheres can be dried and stored for future use making it more economical.

Acknowledgement

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