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Poly(D,L-Lactide-Co-Glycolide) Microspheres Containing 5-Fluorouracil: Optimization of Process Parameters

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Rajesh H. Parikh[1], Jolly R. Parikh[1], Rajesh R. Dubey[1], Heena N. Soni[1], and Kishor N. Kapadia[2]

[1]Department of Pharmaceutics and Pharmaceutical Technology, AR College of Pharmacy and GH Patel Institute of Pharmacy, Gujarat, India

[2]Institute of Science and Technology for Advanced Studies and Research, Vallabh Vidyanagar-388120, Gujarat, India

ABSTRACT

The objective of this research was to optimize the processing parameters for poly(D,L-lactide-co-glycolide) (PLGA) microspheres of 5-fluorouracil (5-FU) and to mathematically relate the process parameters and properties of microspheres. Microspheres were prepared by a water-in-oil-in-water emulsion solvent evaporation technique. A 3^2 factorial design was employed to study the effect of the volume of the internal phase of the primary emulsion and the volume of the external phase of the secondary emulsion on yield, particle size, and encapsulation efficiency of microspheres. An increase in the volume of the internal phase of the primary emulsion resulted in a decrease in yield and encapsulation efficiency and an increase in particle size of microspheres. When the volume of the external phase of the secondary emulsion was increased, a decrease in yield, particle size, and encapsulation efficiency was observed. Microspheres with good batch-to-batch reproducibility could be produced. Scanning electron microscopic study indicated that microspheres existed as aggregates.

KEYWORDS: 5-fluorouracil, microspheres, PLGA microspheres, optimization

Corresponding Author: Rajesh R. Parikh, Department of Pharmaceutics and Pharmaceutical Technology, AR College of Pharmacy and GH Patel Institute of Pharmacy, Gujarat, India. Phone: 91-2692-230788; Fax: 91-2692-230788; Email: rhp59@rediffmail.com.

INTRODUCTION

Poly(D,L-lactide-co-glycolide) (PLGA) is a biocompatible, bioabsorbable, and biodegradable polymer that is used to formulate many types of implantable and injectable drug delivery systems for humans and other animals. PLGA microspheres have been reported as carriers for site-specific delivery of various drugs like adapalene[1] and tetracycline.[2]

In the present study, an attempt was made to prepare PLGA microspheres of 5-fluorouracil (5-FU) for site-specific delivery of 5-FU through passive targeting. The pyrimidine analogue 5-FU is an antimetabolite and immunosuppressive agent. It is used in the treatment of breast, colon, stomach, pancreas, ovary, head and neck, urinary bladder and lung cancer.[3] It has a rapid plasma clearance and a short half-life ($t_{1/2}$:10-20 minutes). The drug can be encapsulated in microspheres to prolong its residence time and thereby its action.

The 5-FU is water soluble. Several reports are available for encapsulation of hydrophilic drugs into PLGA microspheres by a water-in-oil-in-water (w/o/w) emulsification solvent evaporation technique.[4,5] Hence, we prepared PLGA microspheres containing 5-FU by this technique. We could prepare microspheres with good batch to batch reproducibility.

Several researchers have tried to study the effect of process parameters on properties of microspheres and to derive mathematical models that can be used to predict process kinetics. Sato et al[6] reported that the sphericity, size, and yield of microspheres were influenced by the preparation procedure, surfactant type and concentration, temperature of the continuous phase, polymer concentration in the dispersed phase, and ratio of marker to polymer. Jeyanthi et al[2] have shown the effect of the solvent removal technique, the dispersed phase composition, and the ratio of dispersed to continuous phase volume (D/C ratio) on matrix character-

Table 1. Levels of Independent Variables for 3^2 Factorial Design for Optimization of Process Parameters*

Independent Variables	Levels		
	1	2	3
1 Volume of internal phase of primary emulsion	0.75 mL	1.00 mL	1.25 mL
2 Volume of external phase of secondary emulsion	50 mL	65 mL	80 mL

*Other parameters such as stirring speed, polymer concentration, and stirring time were kept constant. Every batch was prepared in triplicate.

istics and physicochemical properties of salmon calcitonin-loaded PLGA microspheres. Li et al[9,10] developed mathematical models to predict the kinetics of solvent removal, the dispersed phase composition profile, and the effects of D/C ratio and initial dispersed phase composition in the preparation of microspheres by the solvent extraction/evaporation method. The authors tested the models using peptide-loaded PLGA microspheres, and good agreement was found between predicted and experimental data.

The main objective of this study was to optimize some process parameters in the preparation of PLGA microspheres containing 5-FU by a w/o/w emulsification solvent evaporation technique and to derive mathematical equations describing the relationship between process parameters and properties of microspheres.

MATERIALS AND METHODS

Materials

PLGA (75:25) (Resomer® RG 752, molecular weight: 20 000; intrinsic viscosity: 0.185-0.205 dL/g) was from Boehringer Ingelheim (Ingelheim, Germany). The 5-FU was a gift from Biochem Pharmaceutical Industries (Mumbai, India) and was of pharmacopeial grade. Polyvinyl alcohol (PVA) was obtained from SD Fine-Chemicals Ltd (Mumbai, India), and dichloromethane was obtained from Allied Chemical Corporation (Vadodara, India). Both PVA and dichloromethane were of analytical reagent grade.

Preparation of Microspheres

PLGA microspheres were prepared using a w/o/w multiple emulsion solvent evaporation technique as reported by Leo et al.[11] Briefly, PLGA (150 mg) was dissolved in dichloromethane, and 5-FU was dissolved in distilled water. The 5-FU solution was added to the PLGA solution, and the mixture was sonicated for 2 minutes using a 250-W probe-type sonicator (MAGNA-PAK-250, Libra Ultrasonic, Kolkata, India) to prepare a primary (w/o) emulsion. Subsequently, the resulting primary emulsion was added to an aqueous solution of PVA (1% wt/vol).

The resulting w/o/w emulsion was stirred at maximum speed with a magnetic stirrer (MS-500, Remi Equipments, Mumbai, India) for 1.5 hours at room temperature to allow solvent evaporation and microsphere formation. The microspheres were separated by vacuum filtration. Most of the nonencapsulated 5-FU remained in the continuous aqueous phase and was removed in the filterate. The remaining loose drug and the PVA on the surface of the microspheres was removed by washing the microspheres 3 times, using 100 mL of distilled water each time. The microspheres were collected from the filter paper and dried at 37°C for 16 hours. Microspheres were preserved in a desiccator kept in a refrigerator until the time of evaluation.

Study Design for Optimization of Process Parameters (3^2 Factorial Design)

Batches were prepared to optimize process parameters for preparation of microspheres, according to a 3^2 factorial design as follows: 2 independent variables (volume of internal phase of primary emulsion and volume of external phase of secondary emulsion) and 3 levels of study, as indicated in **Table 1**.

Statistical Analysis

Each batch was evaluated for the following parameters: yield of microspheres; particle size and morphology; and encapsulation efficiency.

The effect of independent variables on the above-mentioned parameters was evaluated by paired *t* test.

Evaluation of PLGA Microspheres Containing 5-FU

Yield of Microspheres

Microspheres recovered at the end of preparation were weighed and the yield was calculated as a percentage of the total amounts of polymer and drug added during the preparation of microspheres.

Particle Size and Morphology

Approximately 25 mg of microspheres were taken from Batch 1(a), Batch 1(b) and Batch 1(c) [Triplicate of Batch 1] and mixed. Carbon paste was applied on aluminum stubs and was allowed to dry overnight at room temperature. The powder sample of microspheres was sprinkled on the dried carbon paste. Aluminum stubs were placed in the vacuum chamber of a scanning electron microscope (XL 30 ESEM TMP+EDAX, Philips, (Eindhoven, Netherlands). The samples were observed for morphological characterization using a gaseous secondary electron detector (working pressure: 2-5 mm Hg, acceleration voltage: 10.00 kV (XL 30, Philips, (Eindhoven, Netherlands)The particle size of 25 particles was measured from the electron micrographs. A similar procedure was followed for remaining 8 batches.

Encapsulation Efficiency

The encapsulation efficiency of 5-FU in the microspheres was calculated after determining the actual drug loading of microspheres. The actual drug loading of microspheres of each batch was determined by the following procedure: 25 mg of microspheres was dissolved in dichloromethane to prepare a 10-mL solution. The 5-FU was extracted 3 times from dichloromethane using 25 mL of N-saline each time. Extraction was carried out using a separating funnel. Each time, the separating funnel was hand-shaken for 15 minutes and then allowed to equilibrate for 10 minutes. The absorbance of each aqueous extract was measured at 266 nm using a spectrophotometer against a blank of N-saline.

RESULTS AND DISCUSSION

Table 2 shows the effect of formulation factors on characteristics of 5-FU-loaded PLGA microspheres. The effect of independent variables on yield, particle size, and encapsulation efficiency was evaluated by paired *t* test. In each comparison, the calculated value of *t* was compared with the table value of *t* at a 95% confidence level. The effect was considered significant when the calculated value of *t* was greater than the table value of *t*.

Morphology

Observed using a scanning electron microscope (**Figure 1**), the microspheres appeared spherical. When the volume of the internal phase of the primary emulsion was 1.25 mL, a little change in spherical shape was observed. A surface structure with some troughs and small pores was seen scattered over the microspheres. Our observation is in agreement with the observation of Crotts and Gwan Park,[12] who reported that with an increase in the volume of the inner aqueous phase in the primary emulsion, the porosity of the microspheres increases.

Microspheres existed as aggregates. Aggregation of microspheres may be due to incomplete removal of dichloromethane during preparation of microspheres. It may also be due to softening of microspheres during drying at the elevated temperature of 37ºC. Aggregation of microspheres increased as the volume of the internal phase of the primary emulsion increased, which means that as the ratio of dichloromethane:water decreased, the degree of aggregation increased. Li et al[10] have reported that a low dichloromethane:water ratio results in a higher residual solvent. Thus, the increase in degree of aggregation associated with a low dichloromethane:water ratio may be attributed to higher residual solvent. However, the volume of the external phase of the secondary emulsion had no apparent effect on the aggregation of microspheres.

It is evident from the micrograph that in a given batch, there is variation in the particle size of microspheres. This may be due to formation of globules of different sizes during the primary and secondary emulsification process, resulting in formation of microspheres of different particle sizes on evaporation of dichloromethane from the globules of the secondary emulsion.

Yield

The effect of the volume of the internal phase of the primary emulsion and the volume of the external phase of the secondary emulsion on yield of microspheres is shown in **Figure 2**. An increase in the volume of the internal phase of the primary emulsion resulted in a decrease in the yield of microspheres. The effect was significant when the volume of the internal phase of the primary emulsion increased from 0.75 to 1.00 mL

Table 2. Formulation Factors and Characteristics of 5-FU-Loaded PLGA Microspheres*

	Formulation Factors†		Characteristics of Microspheres					
	Volume of Internal Phase of Primary Emulsion‡ (mL)	Volume of External Phase of Secondary Emulsion§ (mL)	% Yield ‖	Size¶	Drug Loading#	Encapsulation Efficiency**	SEM Observation	
							Shape	Aggregation
1	0.75	50	82.28 ± 1.81	29.65 ± 10.73	19.6 ± 0.64	34.80 ± 1.57	Spherical	Low
2	1.00	50	81.01 ± 2.31	32.50 ± 16.45	26.2 ± 2.34	33.77 ± 0.33	Spherical	Medium
3	1.25	50	79.11 ± 1.27	34.22 ± 14.77	25.2 ± 0.40	27.77 ± 0.47	Slight deviation from spherical shape	High
4	0.75	65	77.32 ± 2.08	27.25 ± 15.05	17.2 ± 0.31	30.61 ± 0.51	Spherical	Low
5	1.00	65	75.06 ± 1.42	28.63 ± 12.01	18.5 ± 0.65	25.40 ± 0.61	Spherical	Medium
6	1.25	65	73.10 ± 2.88	31.08 ± 13.41	20.8 ± 0.62	22.79 ± 0.55	Slight deviation from spherical shape	High
7	0.75	80	71.00 ± 2.54	23.40 ± 12.48	09.8 ± 0.41	17.48 ± 0.74	Spherical	Low
8	1.00	80	69.06 ± 3.76	26.43 ± 16.21	09.3 ± 0.50	12.70 ± 0.68	Spherical	Medium
9	1.25	80	68.04 ± 2.63	28.83 ± 15.80	09.1 ± 0.22	10.13 ± 0.26	Slight deviation from spherical shape	High

* 5-FU indicates 5-fluorouracil; PLGA, poly(D,L-lactide-co-glycolide); SEM, scanning electron microscopy; PVA, polyvinyl alcohol.
† Exactly 150 mg of PLGA dissolved in 10-mL dichloromethane was used as the external phase for preparation of primary emulsion.
‡ A 12 mg/mL solution of 5-FU in distilled water.
§ A 1% wt/vol solution of PVA in distilled water
‖ Mean ± SD of 3 experiments.
% yield = 100 * c/a + b
a = weight of PLGA in mg taken for preparation of microspheres
b = weight of 5-FU (present in internal phase of primary emulsion)
c = weight in mg of microspheres obtained
¶ Size in microns, mean ± SD for 25 particles.
# Micrograms of 5-FU per mg of microspheres, mean ± SD of 3 experiments.
** Mean ± SD of 3 experiments.
Encapsulation efficiency (%) = 100 * b/a, b = c*d/25
a = theoretical drug loading in mg
b = actual drug loading in mg
c = amount of drug present in 25 mg of microspheres
d = total weight of drug and polymer in mg taken for microspheres preparation

(volume of the external phase of the secondary emulsion equal to 50 mL and 65 mL) and from 0.75 mL to 1.25 mL (volume of the external phase of the secondary emulsion equal to 50 mL, 65 mL, and 80 mL). An increase in the volume of the external phase of the secondary emulsion resulted in a decrease in the yield of microspheres. The effect was significant when the volume of the external phase of the secondary emulsion increased from 50 mL to 65 mL and from 50 mL to 80 mL at the 0.75-mL, 1.00-mL, and 1.25-mL levels of the volume of the internal phase of the primary emulsion, and from 65 mL to 80 mL at the 0.75-mL and 1.25-mL levels of the volume of the internal phase of the primary emulsion. The decrease in yield associated with an increase in the volume of the internal phase of the primary emulsion as well as the volume of the ex-

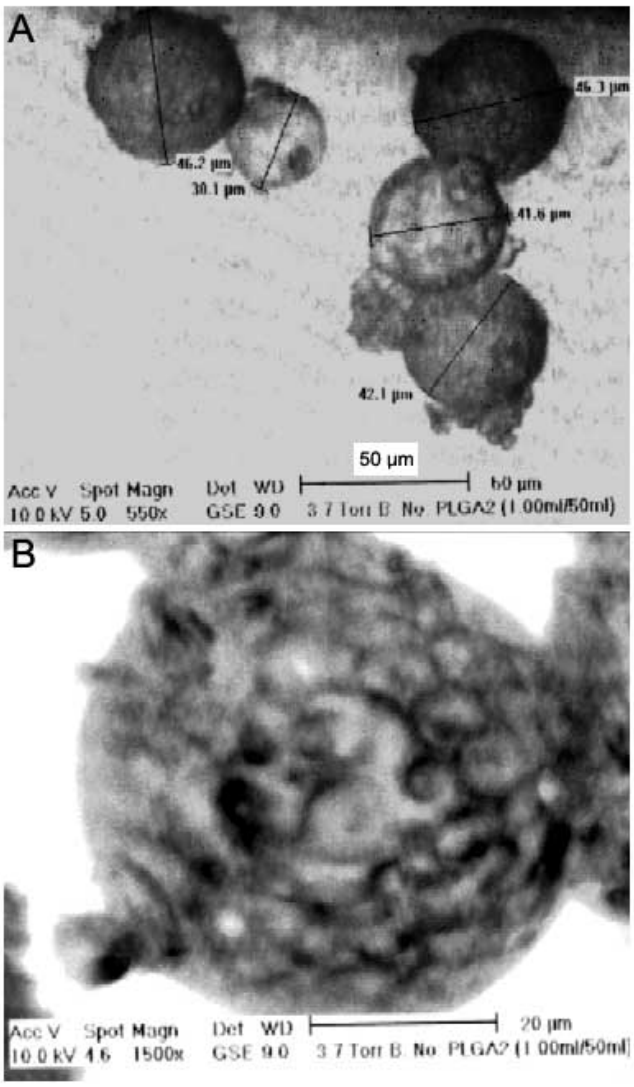

Figure 1. Scanning electron micrographs of 5-FU-loaded PLGA microspheres: (A) indicates aggregation and variation in size of microspheres; (B) indicates surface characteristics of microspheres.

ternal phase of the secondary emulsion may be attributed to a higher rate of leaching of drug from the internal phase of the primary emulsion to the external phase of the secondary emulsion during the preparation of microspheres. Factors such as the amount of drug present in the internal phase of the primary emulsion, and the globule size of the primary and the secondary emulsion, may affect the rate of leaching of the drug.

The following equation was derived by the best-fit method to describe the relationship between the yield of microspheres (z), the volume of the internal phase of the primary emulsion (x), and the volume of the external phase of the secondary emulsion (y).

$$z = 157.6 - 2.92y + 2.028y^2 + (-6 + 1.32y - 0.022y^2)x + (-53.85 + 1.3y - 0.0067y^2)x^2 \quad (1)$$

Particle Size

The effect of the volume of the internal phase of the primary emulsion and the volume of the external phase of the secondary emulsion on the particle size of microspheres is shown in **Figure 3**. An increase in the volume of the internal phase of the primary emulsion led to an increase in particle size of microspheres. The effect was significant when the volume of the internal phase of the primary emulsion increased from 0.75 mL to 1.00 mL (volume of the external phase of the secondary emulsion equal to 65 mL and 80 mL) and from 0.75 mL to 1.25 mL (volume of the external phase of the secondary emulsion equal to 50 mL and 65 mL and 80 mL). The droplet size of the primary emulsion may increase with an increase in the volume of the internal phase of the primary emulsion, which in turn may be responsible for the increase in the size of microspheres. Schlicher et al[13] and Jeffery et al[14] have reported similar observations.

An increase in the volume of the external phase of the secondary emulsion led to a decrease in the particle size of microspheres. The effect was significant when the volume of the external phase of the secondary emulsion increased from 50 mL to 65 mL (volume of the internal phase of the primary emulsion equal to 1.00 mL and 1.25 mL), from 50 mL to 80 mL (volume of the internal phase of the primary emulsion equal to 0.75 mL and 1.00 mL and 1.25 mL), and from 65 mL to 80 mL (volume of the internal phase of the primary emulsion equal to 1.00 mL and 1.25 mL). The droplet size of the secondary emulsion may decrease because of a decrease in the frequency of collision of droplets with an increase in the volume of the external phase of the secondary emulsion. The decrease in the particle size of microspheres associated with an increase in the volume of the external phase of the secondary emulsion may be attributed to a decrease in the droplet size of the secondary emulsion. Castellanos et al[15] have reported similar observations.

The following equation was derived by the best-fit method to describe the relationship between the particle size of microspheres (z), the volume of the internal phase of the primary emulsion (x), and the volume of the external phase of the secondary emulsion (y):

$$z = 191.66 - 4.93y + 0.36y^2 + (158.7 - 4.91y + 0.0373y^2)x + (711.46 - 10.96y - 0.069y^2)x^2 \quad (2)$$

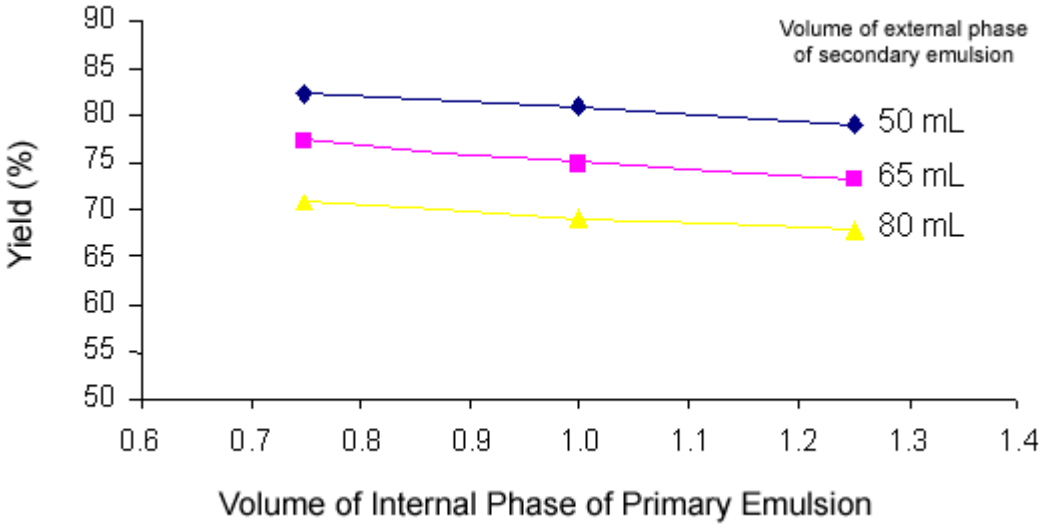

Figure 2. Effect of volume of internal phase of primary emulsion and volume of external phase of secondary emulsion on yield of 5-FU-loaded PLGA microspheres.

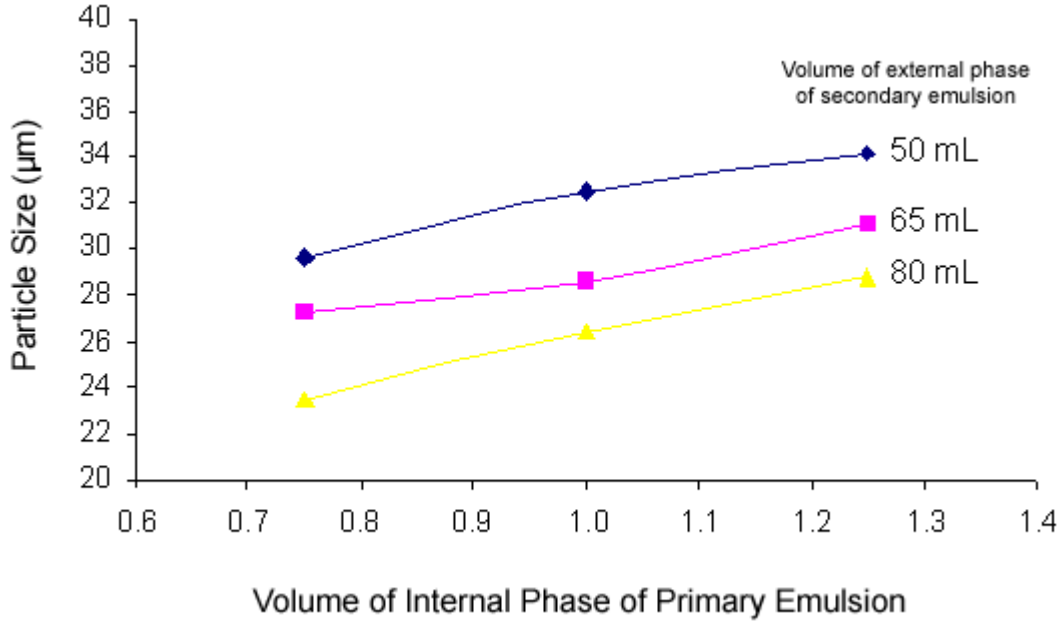

Figure 3. Effect of volume of internal phase of primary emulsion and volume of external phase of secondary emulsion on particle size of 5-FU-loaded PLGA microspheres.

Encapsulation Efficiency

The effect of the volume of the internal phase of the primary emulsion and the volume of the external phase of the secondary emulsion on encapsulation efficiency is shown in **Figure 4**. Encapsulation efficiency decreased as the volume of the internal phase of the primary emulsion increased. The effect was significant when the volume of the internal phase of the primary emulsion increased from 0.75 mL to 1.00 mL at 65-mL and 80-mL levels of the volume of the external phase of the secondary emulsion. The effect was also significant when the volume of the internal phase of the primary emulsion was increased from 0.75 mL to 1.25 mL and from 1.00 mL to 1.25 mL at 50-mL, 65-mL, and 80-mL levels of the volume of the external phase

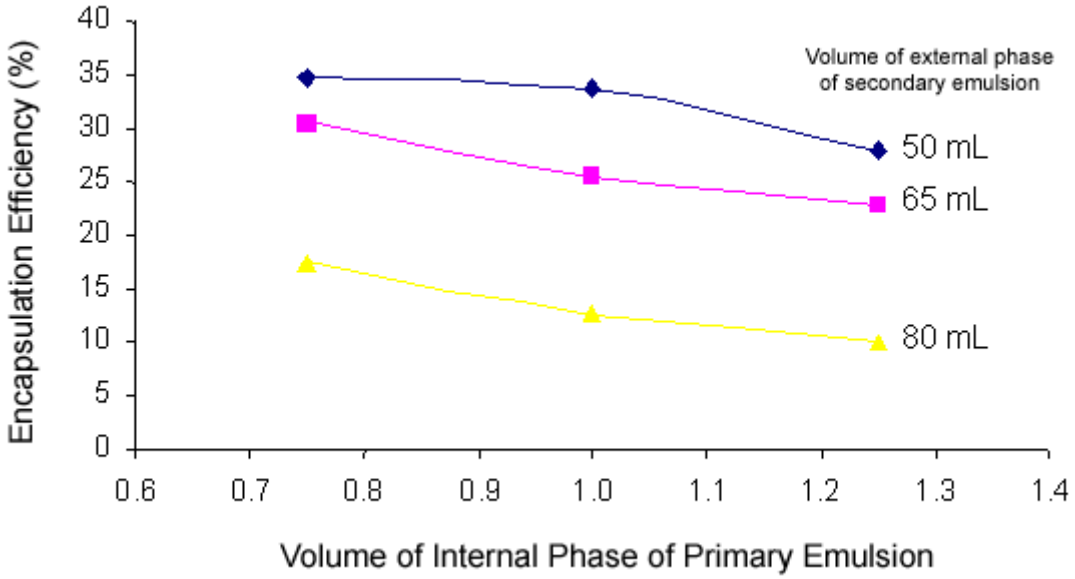

Figure 4. Effect of volume of internal phase of primary emulsion and volume of external phase of secondary emulsion on encapsulation efficiency of 5-FU-loaded PLGA microspheres.

of the secondary emulsion. Higher rate of leaching, poor physical stability of primary emulsion, and increase in number of pores on the surface of the microspheres may be responsible for the decrease in encapsulation efficiency associated with the increase in volume of the internal phase of the primary emulsion. Yeh et al[16], Leo et al[11], and Schügens et al[18] also reported similar observations.

Encapsulation efficiency decreased significantly at all levels as the volume of the external phase of the secondary emulsion increased from 50 mL to 65 mL, from 50 mL to 80 mL, and from 65 mL to 80 mL. An Increase in the rate of drug extraction, increased the demixing of the primary emulsion during the formation of the secondary emulsion, and an increase in the number of pores on the surface of the microspheres may be associated with an increase in the volume of the external phase of the secondary emulsion, which in turn may decrease encapsulation efficiency. Schlicher et al[13] and Jeffery et al[14] reported a decrease in encapsulation efficiency and core loading for a water-soluble drug. Alex and Bodmeier[18] reported similar results for the water-soluble drug pseudoephedrine hydrochloride.

The following equation was derived by the best-fit method to describe the relationship between encapsulation efficiency (z), the volume of the internal phase of the primary emulsion (x), and the volume of the external phase of the secondary emulsion (y):

$$z = -676.93 + 21.50y - 0.156y^2 + (-2720 + 41.30y + 0.288y^2)x + (-698.8 + 20.23y - 0.141y^2)x^2 \quad (3)$$

CONCLUSION

In conclusion, 5-FU solution can be encapsulated into PLGA microspheres at laboratory scale by a w/o/w emulsification solvent evaporation technique with good batch-to-batch reproducibility with respect to yield, particle size, and encapsulation efficiency of microspheres. The volume of the internal phase of the primary emulsion and the volume of the external phase of the secondary emulsion affect, sometimes significantly, the above-mentioned characteristics of microspheres. However, microspheres exist as aggregates and not as single entities. Mathematical equations describing the relationship between process parameters and properties of microspheres can provide useful guidelines for experimental designs, thereby reducing the number of trial-and-error experiments.

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