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MANUFACTURE AND IN-VITRO ASSESSMENT OF BROMSULPHTHALEIN AND PHENOLPHTHALEIN LOADED POLYESTER MICROSPHERES

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ABSTRACT

Poly(lactic acid) and Poly(glycolic acid) microspheres were manufactured by a solvent evaporation and precipitation technique respectively. Particle size and image analysis of the microspheres were performed to establish their morphology. Phenolphthalein and bromsulphthalein, were incorporated in the polymeric microspheres. The release of phenolphthalein in glycine buffer pH 10.8 and bromsulphthalein in phosphate buffer pH 7.4 at 25°C was delayed compared with dye alone. This data has been correlated with the in-vivo performance of the polymeric matrix dye delivery systems.

INTRODUCTION

The solvent evaporation (1) or precipitation (2) techniques are commonly used to prepare polyester microspheres. The porosity of the matrix, drug/dye loading and the release rate may be adjusted by varying parameters which are involved in the manufacturing processes (3). Parameters that may be varied are the ratio of drug or dye to polymer in the dispersed phase;

stirring rate; temperature and; solvent concentrations in the continuous phase and diluent phase.

Phenolphthalein and bromsulphthalein are eliminated as conjugated compounds in bile (4). Consequently the effects of preparing a formulation of these materials may be assessed in-vivo by collecting bile following their administration (5).

The aim of this study was to optimize the manufacturing process of poly(lactic acid) and poly(glycolic acid) microspheres containing phenolphthalein (PT) and bromsulphthalein (BSP) as model compounds. Particle size and image analysis were performed to observe the morphology of polymeric microparticulates (6).

MATERIALS AND METHODS

Microsphere Manufacture:

Microspheres were manufactured by the evaporation or precipitation technique using poly(lactic acid) (PLA) or poly(glycolic acid) (PGA) respectively as the matrix material into which PT or BSP were incorporated. Figure 1 illustrates the method employed to prepare PLA microspheres.

Table 1 shows the characteristics of continuous and diluent phases developed for the manufacture of PLA

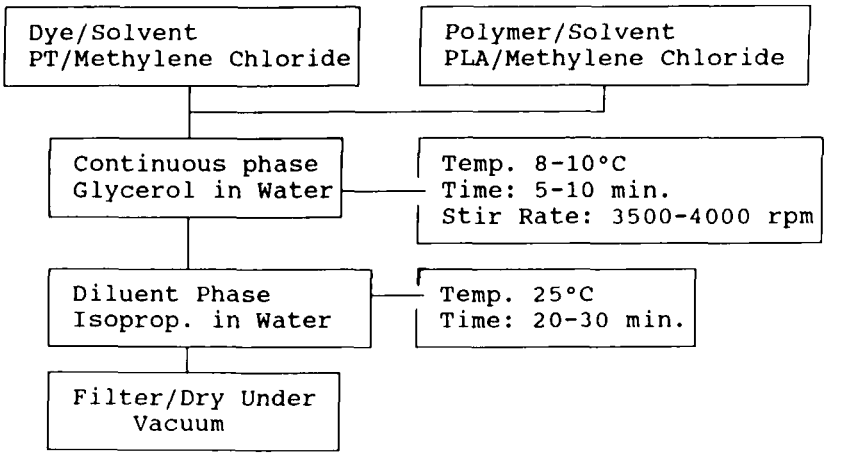

FIGURE 1. Method of Preparation of Phenolphthalein (PT) Loaded Poly(lactic acid) (PLA) Microspheres

TABLE 1.
Characteristics of the Continuous and Diluent Phase Employed in the Manufacture of Poly(lactic acid) Microspheres.

BATCHES	I	II	III
Conc. of Glycerol,v/v (Continuous Phase)	50%	70%	70%
Conc. of 2-Propanol,v/v (Diluent Phase)	50%	10%	5%

microspheres to illustrate the experimental approach to formulation. Glycerol was selected as a continuous phase component because of its immiscibility with the dispersed phase solvent, methylene chloride, and miscibility with water. The viscosity of glycerol may be employed to influence the particle size and shape of polymer

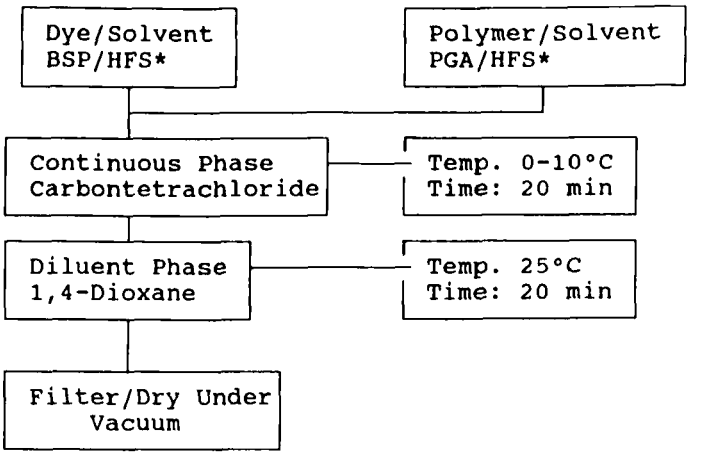

FIGURE 2. Method of Preparation of Bromsulphthalein (BSP) Loaded Poly(glycolic acid) (PGA) Microspheres. (*HFS=Hexafluoroacetone Sesquihydrate)

particles. Isopropanol was selected as a component of the diluent phase because of its miscibility with glycerol and water and its capacity to dissolve methylene chloride. The presence of isopropanol in the diluent phase aids in the extraction of methylene chloride during particle solidification.

The temperature, stirring rate and time were held constant in this procedure as shown in Figure 1. PGA microspheres were manufactured by a method shown in Figure 2.

Five batches of dye loaded microspheres were prepared. The percentages of PT loaded in PLA microspheres were 10, 25 or 33, respectively. BSP was

loaded at nominally 33% of the total mass of PGA microspheres.

Electron Microscopy:

A Jeol 35c scanning electron microscope (Jeol Ltd, Tokyo, Japan) was employed. Samples were mounted on aluminum stubs and gold-palladium coated using a Polaron sputter coater (Polaron, England).

Image Analysis:

Particle images were traced from electron micrographs. The digitized image of the particle gave the (x, y) co-ordinates of particle boundary (Research Resources Laboratory, University of Illinois at Chicago, Chicago, IL). A computer program was developed using SAS^R software (SAS Institute Inc., Cary, NC), to describe polar co-ordinates with relation to its centroid. These co-ordinates were subjected to Fourier analysis. Fourier analysis calls for the solution of the following expression of the fourier series (7):

$$f(X) = a_o + a_1 \cos x + a_2 \cos 2x + \ldots$$
$$b_1 \sin x + b_2 \sin 2x + \ldots$$

To derive the coefficient $A_n = (a_n^2 + b_n^2)^{1/2}$.

It has been noted the first coefficient (A1) relates to sphericity, the second to elongation (A2), the third triangularity (A3) and the fourth squareness (A4) (8,9).

Particle Size Analysis:

Particle size analyses were performed by light microscopy (AO Instruments, Div. Warner-Lambert Tech. Inc., Buffalo, NY) and light blockage (HIAC/ROYCO, Pacific Scientific Co., Silver Spring, MD) techniques. One hundred particles were examined by light microscopy and three particle populations were examined by light blockage technique. Samples were placed in aqueous 0.04% Tween 20 (Fluka Chemie AG, CH-9470 Buchs, Switzerland) solution to aid dispersion.

Total Dye Content:

The total dye content was estimated by following the release of dye from the microspheres into an appropriate dissolution medium (concentration x volume of reservoir) for an extended period, to exhaustion (see in-vitro dissolution section). The fraction of the total mass of microspheres attributable to dye was expressed as a percentage (fraction of the mass of microspheres x 100).

In-Vitro Dissolution:

Dissolution studies were performed using the apparatus illustrated in Figure 3.

Microspheres containing dye were placed in the outer chamber of a cartridge filter (IVEX-2, Abbott

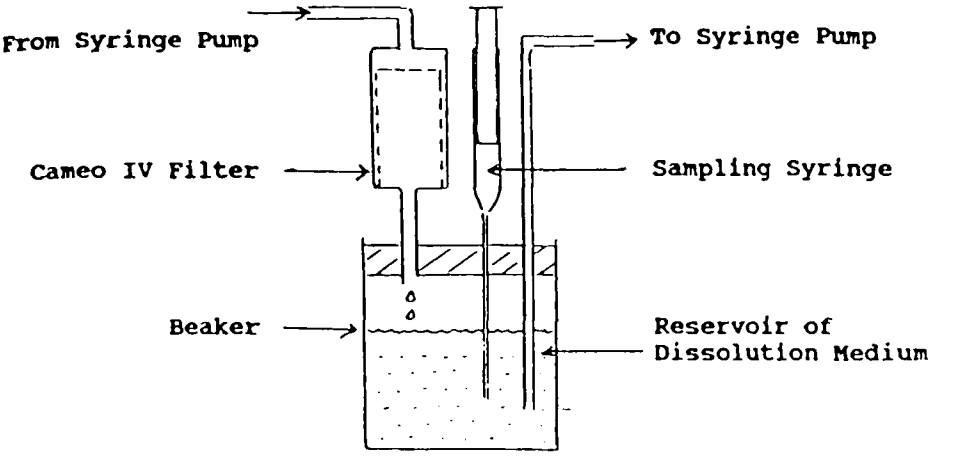

FIGURE 3. In vitro Dissolution Apparatus.

Laboratories, IL). Five percent of ethanol in glycine buffer, pH 10.8 for PLA microspheres, or phosphate buffer, pH 7.4 for PGA microspheres, was pumped (Multi-Staltic Pump, Buchler Instrument, Kansas City, MO) through the filter at 1 ml/min from a reservoir of 50 ml and at 25°C. One milliliter samples were taken at intervals and replaced with buffer to maintain the reservoir volume. The samples were subjected to visible spectrophotometry (Lambda2, Perkin Elmer, Oakbrook, IL) at 550 nm and 580 nm for PT and BSP, respectively (4). Calibration curves prepared at these wavelengths allowed the dye concentration to be calculated.

RESULTS AND DISCUSSION

Microsphere Manufacture:

Spherical microspheres appoximately 10 μm or less in diameter were produced and details of their characteristics are described in later sections.

Electron Microscopy and Image Analysis:

Scanning electron micrographs of particles were subjected to image analysis and the findings were used to assess the manufacturing process.

Initial preparations of PLA microspheres did not meet the requirement for sphericity. Figure 4 shows micrographs indicating the appearance of three preparations of PLA microspheres. The three stages correspond to the manufacturing parameters defined in Table 1. Fourier analyses of particle perimeters, described in polar co-ordinates, were performed. An example image of a particle perimeter is shown in Figure 5.

The sphericity of the product improves as the viscosity of the continuous phase is increased from 50% glycerol, sample I, to 70% glycerol, sample II. In addition the diluent phase has its capacity to dissolve dispersed phase solvent reduced by lowering the concentration of isopropanol from 50%, sample I, to 10%, sample II.

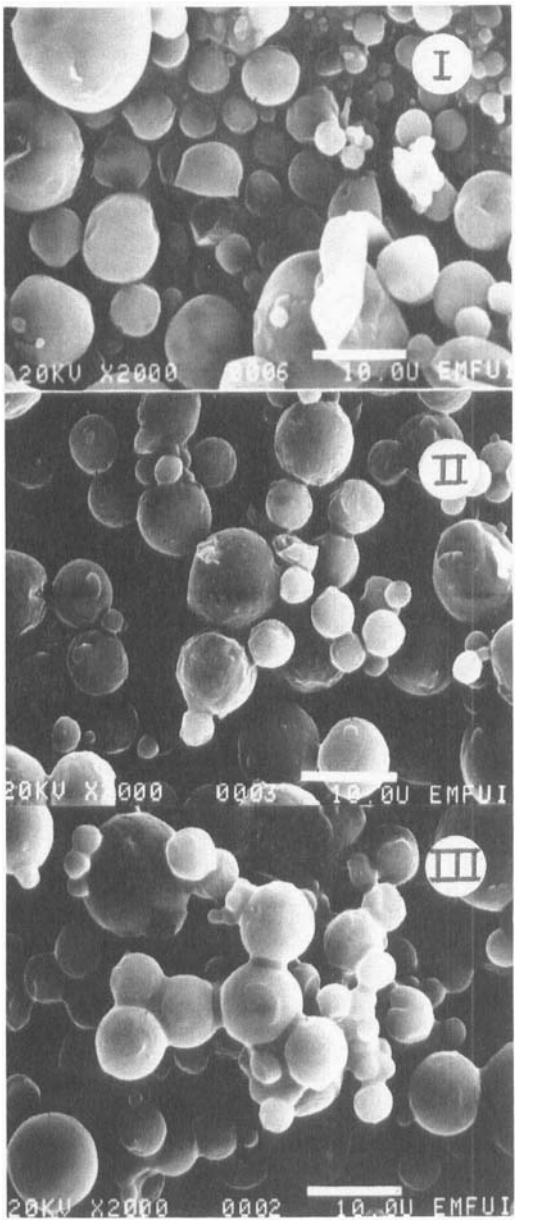

FIGURE 4. Scanning Electron Micrographs Showing Poly(lactic acid) Microspheres Prepared with I) 50%; II) and III) 70% of Glycerol in Continuous Phase and I) 50%; II) 10% and III) 5% Isopropanol in Diluent Phase.

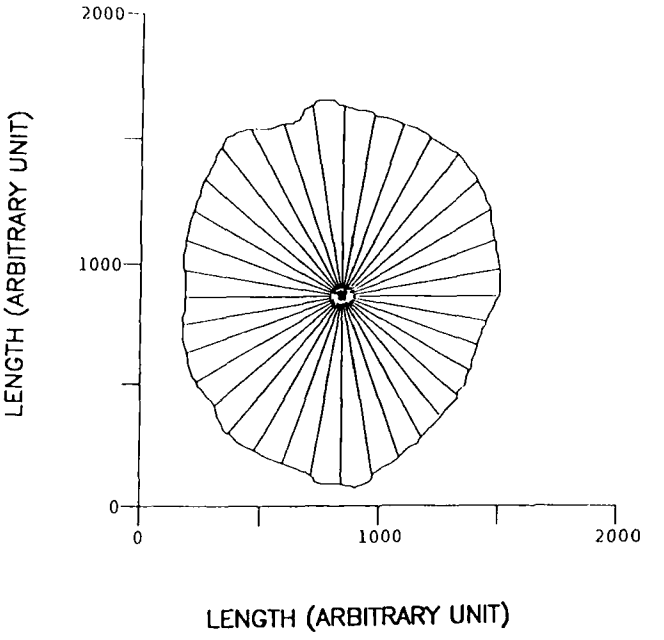

FIGURE 5. An Example Outline of a Microsphere Trace Showing the Centroid and Radii which may be Defined in Polar Co-ordinates.

Further reduction in the isopropanol concentration from 10%, sample II, to 5%, sample III, does not improve sphericity. Shape analysis was independent of particle size. Table 2 illustrates the progression from squareness to sphericity, as indicated by the tendency to the limit value for rotational symmetry of A1 and from the limit value of A4 as the products progress from I to III. Figure 6 shows two independently obtained micrographs of a final product of PLA microspheres manufactured using the same procedure as that for sample II. Table 3 shows Fourier coefficients for this final product.

TABLE 2.
Fourier Coefficients Representing Sphericity (A1), Elongation (A2), Triangularity (A3) and Squareness (A4) of Phenolphthalein (PT) and Various Samples of Microspheres.

SAMPLE*	SHAPE COEFFICIENTS			
	A1	A2	A3	A4
PT Alone	0.1037	0.2975	0.1274	0.0658
I	0.0354	0.0779	0.0697	0.0469
II	0.0010	0.0632	0.0284	0.0240
III	0.0185	0.0572	0.0352	0.0237
Limit+	0	1	0	1
	Spherical	Elongated	Triangular	Square

* Samples referred to appear in Figure 4.
\+ Limit value represents perfect geometrical symmetry.

The micrographs of the final product gave similar Fourier coefficients. The image analysis technique allowed assessment of the subtle differences in particle morphology. In addition, this method was employed to monitor the effectiveness of varying a particular processing parameter to achieve an optimal product. The range of dye loads employed in these studies did not affect the appearance of the product. Particle size analysis may also be performed by this method if the linear dimension of the micrograph is defined.

Initial preparations of PGA microspheres met the requirement for sphericity. Further optimization of the manufacturing process was not required. An electron micrograph of the product is presented elsewhere (5).

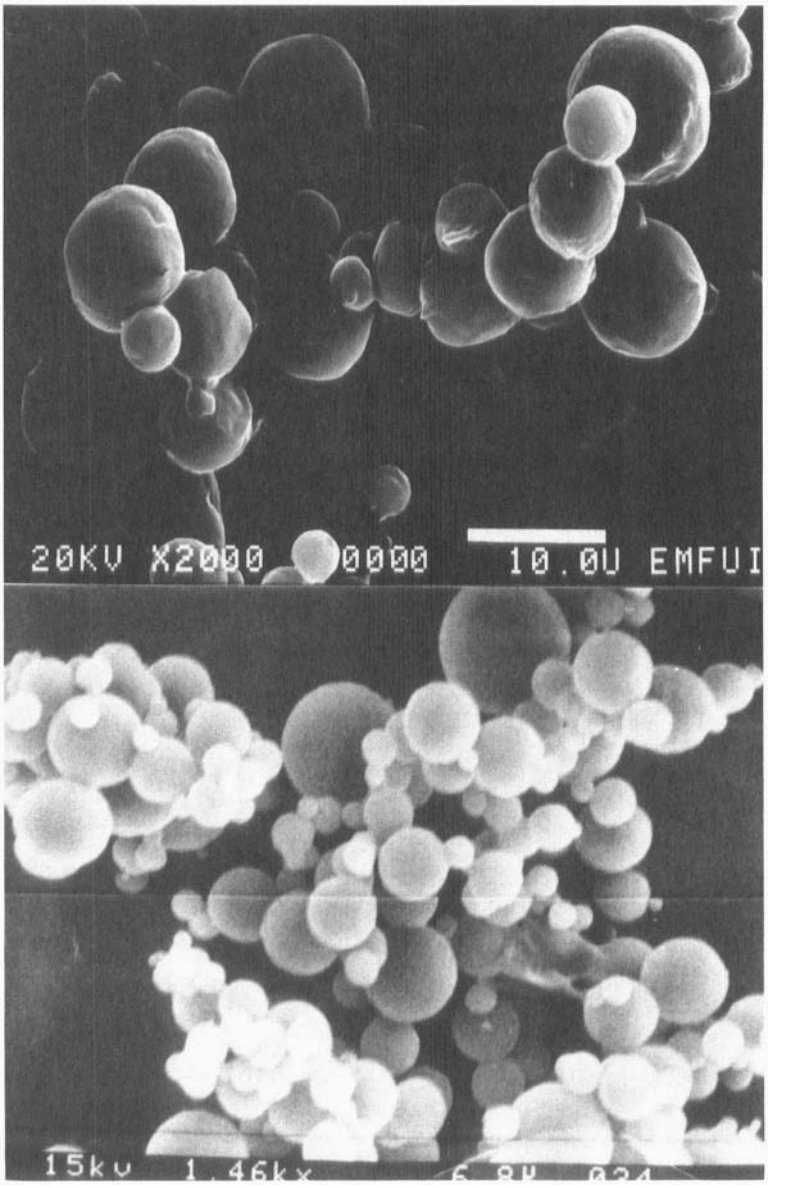

FIGURE 6. Scanning Electron Micrographs of 8.5% PT Loaded PLA Microspheres.

TABLE 3.
Fourier Coefficients Representing Sphericity (A1), Elongation (A2), Triangularity (A3) and Squareness (A4) of PLA Microspheres.

MICROGRAPH*	SHAPE COEFFICIENTS			
	A1	A2	A3	A4
1	0.0101	0.0931	0.0223	0.0345
2	0.0113	0.0375	0.0266	0.0482
Limit	0	1	0	1
	Spherical	Elongated	Triangular	Square

* The micrographs referred to appear in Figure 6.
+ Limit value represents perfect geometrical symmetry.

TABLE 4.
Median Diameters and [Geometric Standard Deviations] of Bromsulphthalein (BSP) and Phenolphthalein (PT) Microspheres as Estimated by Light Microscopy (M) and Light Blockage (B) Techniques.

SAMPLE	DYE	POLYMER	MEDIAN DIAMETERS (μm) [GSD]	
			M	B (n=3)
1	PT	PLA	1.75 [1.94]	6.7±0.4 [2.2±1.0]
2	PT	PLA	4.90 [1.96]	8.3±0.4 [1.8±0.0]
3	PT	PLA	10.40 [2.07]	10.7±3.0 [2.0±0.6]
4	BSP	PGA	2.05 [2.00]	5.6±0.0 [2.0±0.0]
5	BSP	PGA	2.05 [2.00]	5.5±0.7 [2.0±0.2]

Particle Size Analysis:

Estimates of median particle diameter are shown in Table 4. The particle size distributions were adequately described by log-normal functions and therefore, may be defined by geometric standard deviations (GSD).

The microsphere populations were moderately polydisperse with GDSs of approximately 2. The estimates of particle size by light blockage in some cases were larger than those by light microscopy. There are several explanations for this observation. Light microscopy involves the examination of a small number of particles and the potential for statistical error is large. Light blockage estimates the diameters of a large population of particles in suspension. The latter technique will count each unit structure, aggregate or individual particle, as a single particle. This is a valid method as it may be assumed that the suspension of particles for injection will exhibit similar dynamics, i.e. some particles existing in aggregates. The particle diameters estimated by the two techniques in most cases indicate some aggregation. Similar estimates of particle size were obtained for preparation 3 by both microscopy and light blockage technique. The most aggregated sample, preparation 1, exhibited a shift from a median diameter of 1.75 μm by microscopy to 6.7 μm by light blockage. This is approximately equivalent to the aggregation of 15 particles, based on volume and assuming uniform density.

Total Dye Content:

Table 5 shows the combinations of dyes and polymers employed and the anticipated and recovered BSP and PT

TABLE 5.
Phenolphthalein (PT) and Bromsulphthalein (BSP) Nominally and Actually Loaded in Poly(lactic acid) (PLA) and Poly(glycolic acid) (PGA) Microspheres.

SAMPLE	DYE	POLYMER	DYE LOAD (% TOTAL MASS OF MICROSPHERES) NOMINAL	ACTUAL
1	PT	PLA	10	8.5
2	PT	PLA	25	20.0
3	PT	PLA	33	30.0
4	BSP	PGA	33	30.0
5	BSP	PGA	33	30.0

loads expressed as percentages of the total mass of microspheres. The conditions employed for the manufacture of the PLA microsphere were those indicated by method II in Table 1.

The actual dye load falls short of the nominal amount (88±5% loaded), based on initial quantities used in manufacturing. The large quantities of solvent employed in both methods of preparation and the capacity of dyes to act as adsorbates contributes to the low actual dye content of the product. The magnitude of the loss of dye during the manufacturing process was accepted for these studies.

In-Vitro Dissolution:

Calibration curves of absorbance versus concentration were prepared for both dyes. Concentrations of PT up to

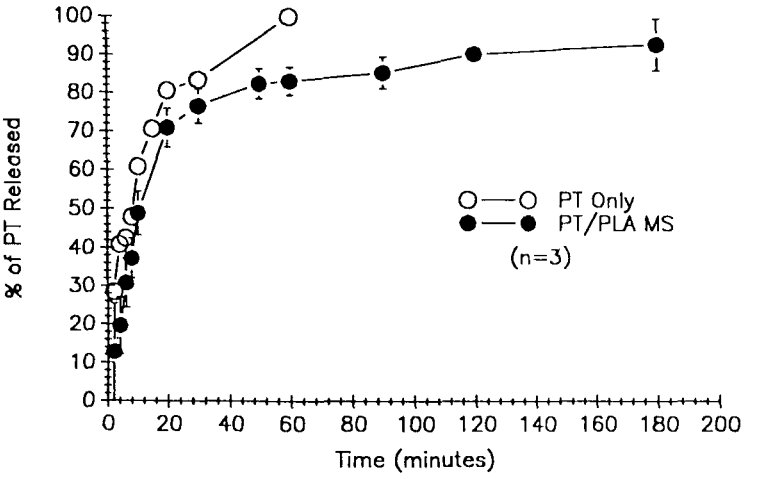

FIGURE 7. In vitro Release Profile of PT from 20% Dye Loaded PLA Microspheres Expressed as the Percentage of Total Dye Content Released Plotted Against Time.

50 (μg/ml) and BSP up to 20 (μg/ml) were analysed. The correlation coefficients for linear regression analyses of the calibration curves were 0.989 for PT and 0.999 for BSP (n=3).

Figure 7 and Figure 8 show the in vitro dissolution expressed as percentage of the total drug load released at times following initial exposure to glycine buffer, pH 10.8 for 20 % PT loaded PLA microspheres and phosphate buffer, pH 7.4 for 30 % BSP loaded PGA microspheres. Dissolution of phenolphthalein could not be performed at pH 7.4 as it would be solubility limited. Consequently sink conditions, required for dissolution, would not have been achieved. Logarithms of percent undissolved dye were

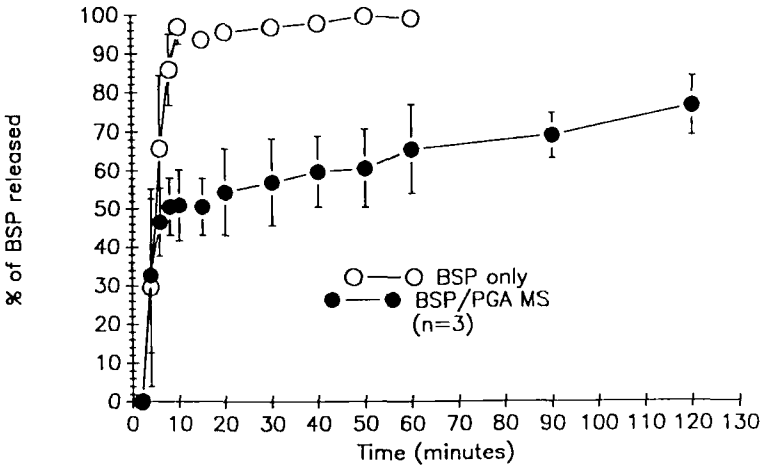

FIGURE 8. In vitro Release Profile of BSP from 30% Dye Loaded PGA Microspheres Expressed as the Percentage of Total Dye Content Released Plotted Against Time.

plotted against time, facilitating calculation of the dissolution rate constants. The dissolution profiles of PT and BSP alone were monoexponential with rate constants of 0.071 and 0.243 min^{-1}, respectively. The dissolution profiles of the dyes from microspheres were biexponential. The initial rate constant (α) of PT from PLA microspheres was 0.085 min^{-1}. After initial release, the rate constant (β) of PT was 0.007 min^{-1}. The initial rate constant was 0.129 min^{-1} for the BSP, and β was 0.008 min^{-1}. Following the rapid initial dissolution of dye twenty five percent of PT and forty percent of BSP were released slowly for approximately 3 or 5 hours, respectively.

TABLE 6.
Comparison of the Rate Constants (min^{-1}) of the Dyes, Phenolphthalein (PT) and Bromsulphthalein (BSP), Released from Polymeric Microspheres, Poly(lactic acid) (PLA) and Poly(glycolic acid) (PGA), in-vitro and in-vivo Studies.

Dyes		PT	BSP	PT/PLA	BSP/PGA
In-Vitro	α	0.071	0.243	0.085	0.129
	ß	---	---	0.007	0.008
In-Vivo[+]		0.021	0.139	0.008	0.009

[+] reference 5.

A small-sample t-test showed that there was no significant difference between the initial rate constants for release of dye from microspheres and the dissolution rate constants of dye alone ($p<0.05$). It may be inferred that the initial release of dye was from the surface of the microspheres where it was freely available. The remaining dye may be assumed to have been released from internal surfaces or locations that were not immediately accessible and from which dye molecules were not readily dissolved.

These microspheres have been used to assess the sensitivity of biliary elimination rate in an experimental animal, the rat, to the rate of dissolution of these dyes (5). There is a correlation between the rate of appearance of free dye in the dissolution medium and its appearance in bile as shown in Table 6. The rate of appearance of dye in bile after microsphere

administration was monoexponential with a rate constant equivalent to the slower rate observed in the dissolution experiments as shown in Table 6. The complex fluid environment in the rat is different from that in the dissolution medium. The presence of cells, enzymes, proteins, and other macromolecules, and the composition of the ionic environment may account for the absence of a rapid apparent dissolution rate of dye from microspheres in-vivo. A specific explanation for the mechanisms resulting in the absence of an initial rapid dissolution rate in the rat has yet to be elucidated.

CONCLUSION

Microspheres were prepared from poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) containing the dyes, bromsulphthalein (BSP) and phenolphthalein (PT). A proportion of each of the dyes loaded (25% of PT and 40% of BSP, respectively) was released from the microspheres at a slower dissolution rate than the dye alone. There was a correlation between the in-vitro dissolution rates and previously published in-vivo biliary elimination rates for these dyes.

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