# Development of a Dialysis In Vitro Release Method for Biodegradable Microspheres

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## ABSTRACT

The purpose of this research was to develop a simple and convenient in vitro release method for biodegradable microspheres using a commercially available dialyzer. A 25 KD MWCO Float-a-Lyzer was used to evaluate peptide diffusion at 37°C and 55°C in different buffers and assess the effect of peptide concentration. In vitro release of Leuprolide from PLGA microspheres, having a 1-month duration of action, was assessed using the dialyzer and compared with the commonly used ''sample and separate'' method with and without agitation. Peptide diffusion through the dialysis membrane was rapid at 37°C and 55°C in all buffers and was independent of peptide concentration. There was no detectable binding to the membrane under the conditions of the study. In vitro release of Leuprolide from PLGA microspheres was tri-phasic and was complete in 28 days with the dialysis technique. With the sample and separate technique, linear release profiles were obtained with complete release occurring under conditions of agitation. Diffusion through the dialysis membrane was sufficiently rapid to qualify the Float-a-Lyzer for an in vitro release system for microparticulate dosage forms. Membrane characteristics render it useful to study drug release under real-time and accelerated conditions.

**KEYWORDS:** biodegradable microspheres, in vitro release methods, dialysis

## INTRODUCTION

Microspheres formulated from biodegradable polymers such as polylactide (PLA) and poly(lactide-co-glycolide) (PLGA) are being used for sustained release of drugs via subcutaneous or intramuscular administration.[1-3] These polymers have been approved for human use as surgical sutures, implantable devices, and drug delivery systems by the US Food and Drug Administration (FDA).[4,5] Some of the advantages of microspheres as drug delivery devices include enhanced stability of protein therapeutics, continuous and controlled drug release, reduced dosage, decrease in systemic side effects, reduced possibility of dose dumping, reduced frequency of administration, and, therefore, increased patient compliance.[6,7]

Assessment of drug release from these formulations requires long-term studies under in vivo or in vitro conditions. As a result of higher costs and labor associated with in vivo studies and the need for rapid screening techniques, a greater emphasis has been placed on performance under in vitro release conditions. In fact, in vitro testing of controlled release parenterals was the focus of a recent workshop sponsored by the American Association of Pharmaceutical Scientists (AAPS), the FDA, and the United States Pharmacopeia (USP).[8] The use of USP Apparatus 3 (reciprocating cylinder) and 4 (flow through cell), designed for oral extended release formulations, was considered relevant for in vitro testing of controlled release parenterals, after appropriate modification.

Currently, the most common methods used to study drug release from microspheres are the sample and separate and the dialysis methods. The more conventional method is the sample and separate method, often referred to as the tube method, in which drug-loaded microspheres are introduced into a sealed tube or vial or a stoppered Erlenmeyer flask containing buffer, and release is followed over a specified time.[9-14] Sampling is performed at predetermined intervals and isolation of the microspheres is achieved by filtration or by centrifugation. Advantages of the sample and separate method are accurate measurement of the initial burst of drug from microspheres and maintenance of sink conditions by replacement of the buffer. Prominent disadvantages, however, are the cumbersome sampling technique and undesirable withdrawal of microspheres from the media. To counter this, sampling is often performed by using a filter attached to a syringe. Another alternative is centrifugation to facilitate the withdrawal of supernatant. However, upon centrifugation, microspheres settle and must be resuspended by shaking or vortexing. Resuspension is often difficult because of aggregation of the microspheres. Total media replacement, often required for in vitro release studies with poorly soluble drugs, is difficult with the sample and separate technique.

An alternative method is dialysis, in which the loaded microspheres are separated from the bulk media by a dia-

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lyzing membrane. Passage of drug occurs through the membrane into the bulk media, a sample of which is withdrawn at intermittent intervals to assess drug release. Dialysis membranes having varying molecular weight exclusion cutoffs (MWCOs) and composition have been used in a variety of setups (eg, dialysis bag, diffusion cell).[15-20] Sampling and media replacement are convenient with the dialysis setup owing to a physical separation of the microspheres from the outer media. However, with all the reported techniques, an initial preparation step is required for the dialysis setup. In some cases, achievement of equilibration with the outer media was slow owing to the small membrane surface area available for drug passage. Slow equilibration limits an accurate analysis of initial drug levels in formulations where the burst release is high. These 2 disadvantages can be overcome by using a commercial dialysis setup with a large surface area to facilitate drug transport.

Hence, the goal of this study was to develop a simple and convenient in vitro release method using a commercially available dialyzer with a large membrane surface area. Further, the dialysis technique was compared with the conventional sample and separate method with and without agitation. A PLGA microsphere formulation of the peptide Leuprolide (LHRH agonist), intended for 1-month duration of action, was selected for evaluation of the dialyzer.

## MATERIALS AND METHODS

### *Materials*

Leuprolide acetate was obtained from Bachem (Torrance, CA) and 50:50 PLGA (RG503H, MW 30 KD) from Boehringer Ingelheim (Ingelheim, Germany). Float-a-Lyzer (25 KD MWCO, 5-mL capacity, regenerated cellulose membrane) was purchased from Spectrum Labs (Rancho Dominguez, CA). All other reagents used were of analytical grade.

### *Methods*

#### *Evaluation of Dialysis Device*

Figure 1 shows the Float-a-Lyzer dialyzer. The 25 KD MWCO membrane selected as the MWCO is sufficiently large to allow passage of peptide. For the experiment, 5 mL of a 1-mg/mL solution of leuprolide acetate in 0.1 M phosphate-buffered saline (PBS) pH 7.4 (release media) was introduced into the inner tube of the dialyzer. The peptide is freely soluble in this media. The dialyzer was placed into a 50-mL glass cylinder containing release media, which was continually stirred at 300 rpm using a small magnetic stir bar to prevent the formation of an unstirred water layer at the membrane/outer solution interface. Diffusion to the outer solution at 37°C and 55°C was assessed by sampling the contents of the outer solution at periodic intervals. Leuprolide solution stability was assessed at both 37°C and 55°C and found to be stable over the course of the experiment.

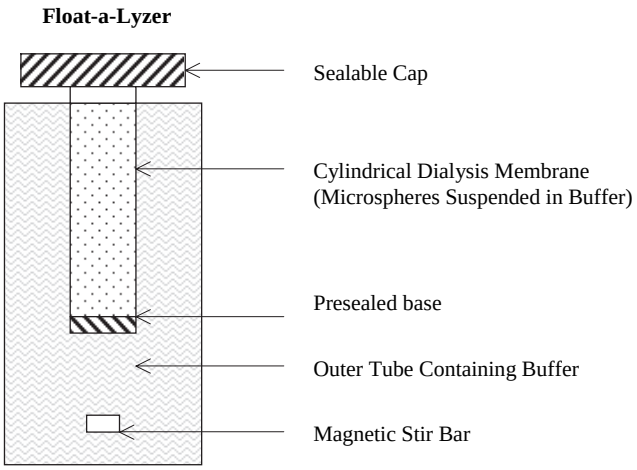


**Figure 1.** Illustration of set-up for in vitro release from microspheres using the Float-a-Lyzer.

#### *Membrane Stability*

Stability of the membrane was evaluated by soaking the dialyzer in 0.1 M PBS pH 7.4 at an elevated temperature. Since future accelerated release studies were planned at 55°C, membrane stability was assessed by soaking at this temperature for 10 days and then studying the peptide diffusion through the membrane and attainment of equilibrium at 37°C in 0.1 M PBS pH 7.4. In subsequent release studies with microspheres, the membrane was pretreated by soaking at the study temperature for 10 days.

#### *Preparation of Microspheres*

Leuprolide microspheres were prepared by a solvent extraction/evaporation method.[19] In brief, a solution of leuprolide in methanol was added to a 22% (wt/wt) solution of polymer in methylene chloride to form a clear solution (dispersed phase [DP]). The DP was added to an aqueous solution containing 0.35% polyvinyl alcohol (continuous phase [CP]) under stirring with a Silverson L4R mixer (Silverson Machines, East Longmeadow, MA) at a predetermined speed. The solvents were removed by stirring for 3 hours. The resulting microspheres were recovered by filtration, washed to remove traces of polyvinyl alcohol, and dried under vacuum at room temperature for at least 3 days.

#### *Determination of Peptide Content (Drug Loading)*

Approximately 10 mg of drug-loaded microspheres were dissolved in 2 mL methylene chloride. The peptide was

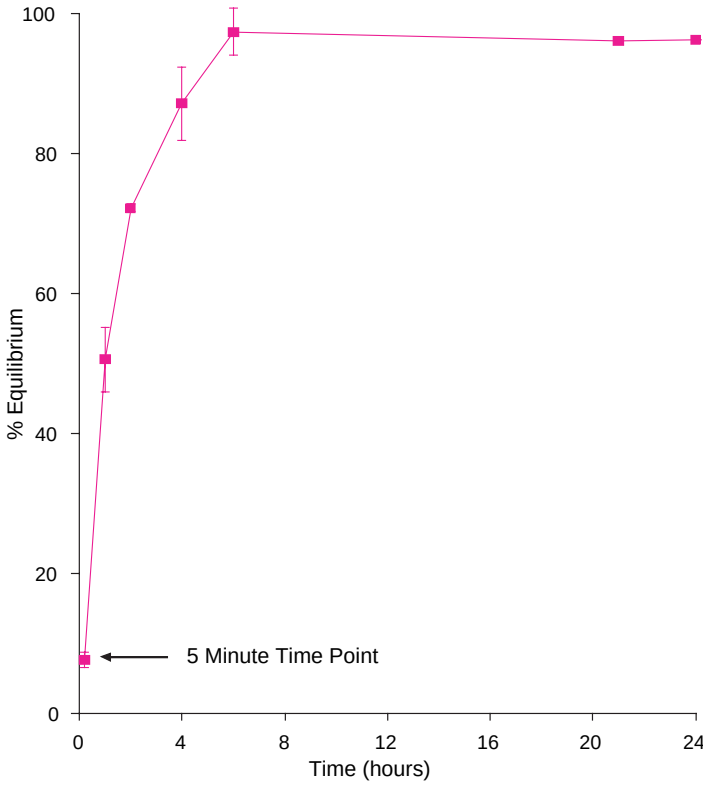


**Figure 2.** Diffusion of leuprolide through the Float-a-Lyzer at 37°C in 0.1 M PBS pH 7.4. Note: 100% equilibrium signifies equal concentration in the inner and outer solutions.

extracted by addition of 0.1 M acetate buffer (pH 4.0) to the organic phase followed by mixing for 1 hour using a wrist-shaker. A reverse phase high-performance liquid chromatography (HPLC) method was used to assay peptide content in the aqueous phase employing a Bondclone 10 C-18 column (150 × 3.9 mm, Phenomenex, Torrance, CA), mobile phase consisting of 27% (vol/vol) acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 1 mL/min, and UV detection of 215 nm.

### *In Vitro Release Using Sample and Separate Method*

About 50 mg of peptide-loaded microspheres were transferred to a 50-mL stoppered glass cylinder and suspended in release media (0.1 M PBS pH 7.4) at 37°C (n = 6). Effect of agitation was assessed by subjecting 1 set of samples to moderate agitation using a magnetic stir bar. At predetermined intervals, sampling (1 mL) was performed using a syringe attached to a single-use 0.22-μm filter. The microspheres that adhered to the filter were reintroduced into the bulk during buffer replacement. Peptide release was assessed by a reverse phase HPLC method using a C-18 column (3.9 × 300 mm, μBondapak, Waters, Milford, MA) using an acetonitrile-water mixture containing 0.1% trifluoroacetic acid at a flow rate of 1.1 mL/min and UV detection wavelength of 220 nm. Different HPLC conditions were used in the determination of peptide content and in vitro release. An extraction procedure was involved in the determination of peptide content, which necessitated the use of a gradient reverse phase HPLC method, while in vitro release from aqueous buffer could easily be assessed using isocratic conditions.

### *In Vitro Release Using Dialysis Method*

About 50 mg of peptide-loaded microspheres were transferred to the dialyzer and suspended in 5 mL release media (0.1 M PBS pH 7.4) at 37°C. The dialyzer was then introduced into a 50-mL glass cylinder containing release media, which was stirred at 300 rpm using a magnetic stir bar. Drug release was assessed by intermittently sampling the contents (1 mL) of the outer media. Replacement of the buffer occurred immediately after sampling. Peptide release was assessed in a manner similar to the sample and separate method.

## RESULTS AND DISCUSSION

Preliminary studies with the dialyzer showed that approximately 50% of peptide had diffused from the dialyzer into the outer cylinder within 1 hour at 37°C (Figure 2). Diffu-

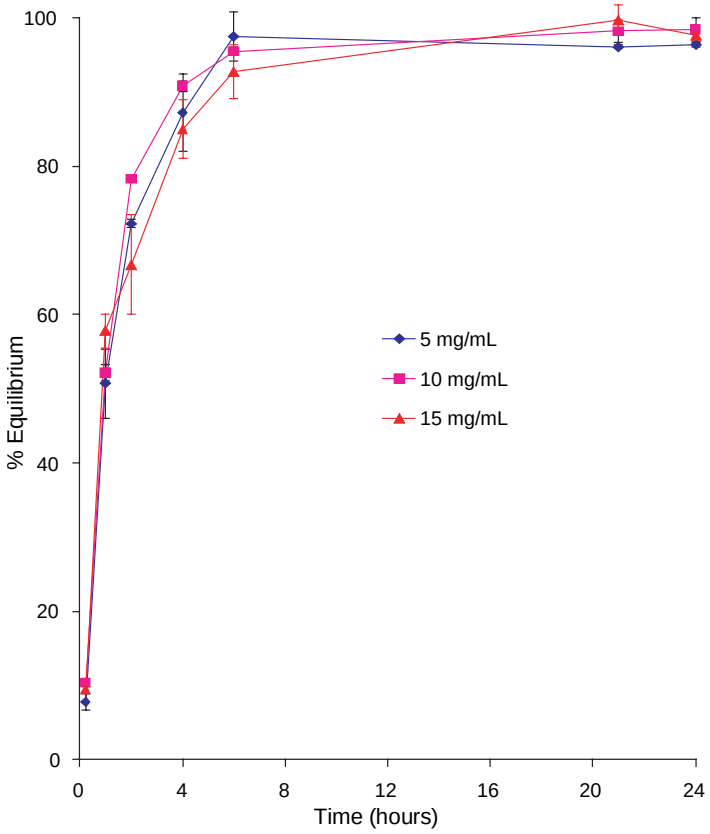


**Figure 3.** Effect of concentration on diffusion of leuprolide through the Float-a-Lyzer.

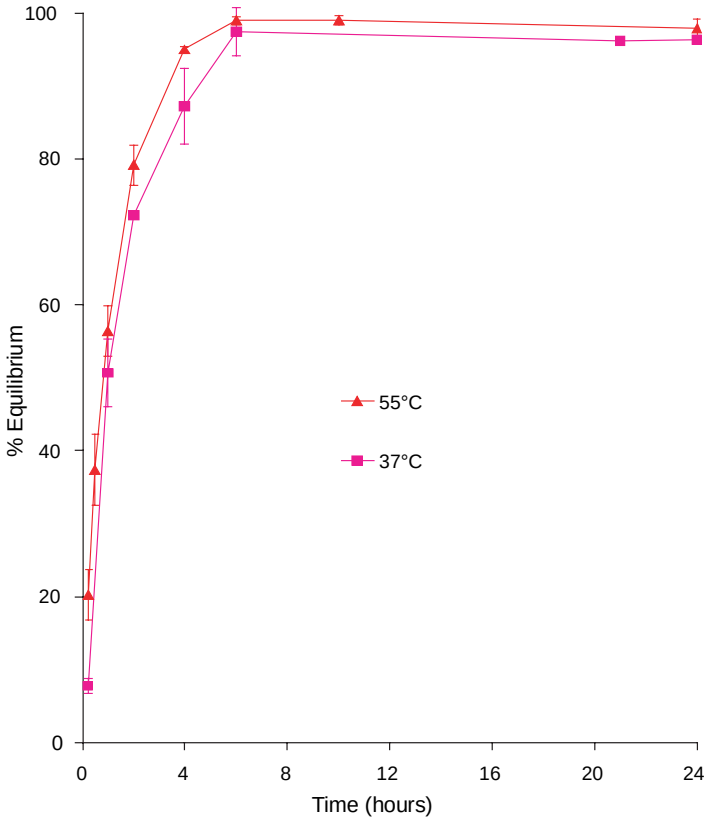


**Figure 4.** Effect of temperature on the diffusion of leuprolide through the Float-a-Lyzer.

sion was steady and complete (equilibrium) within 6 hours. In order to assess if drug passage through the membrane was a saturable process, varying concentrations of peptide were added to the dialyzer and diffusion was followed for a period of 24 hours. Figure 3 shows that passage of peptide through the dialyzer occurs independently of concentration and that diffusion was complete within 6 hours, suggesting that peptide binding was not a concern with this technique.

Peptide diffusion at an elevated temperature, 55°C, was studied with the dialyzer to determine the feasibility of using the method at higher temperatures for accelerated release conditions. Approximately 90% of the peptide diffused into the outer sink within 4 hours, a slightly faster rate than at 37°C, where similar diffusion took 6 hours. Figure 4 shows the profiles at both temperatures to be similar and not significantly different implying that temperature did not influence diffusion greatly between 37°C and 55°C. Also, a pretreatment study of the membrane at 55°C was performed. The membrane was subjected to the elevated temperature for 10 days, which was the maximum time anticipated for short-term release of a 1-month leuprolide PLGA formulation, and peptide diffusion at 37°C in 0.1 M PBS pH 7.4 was assessed. Pretreatment of the membrane did not affect peptide diffusion, suggesting that membrane integrity was maintained. These results suggested the membrane would lend itself to studies at higher temperatures. In recent years, in vitro drug release studies performed at elevated temperatures have been gaining popularity as a means of shortening long-term release studies that normally take several weeks or months.[21,22] Rapid peptide diffusion at the elevated temperatures shows that the regenerated cellulose membrane dialyzer may be used for accelerated release studies. The maximum temperature recommended by the manufacturer of the dialyzer is 60°C.

Figure 5 shows the diffusion of leuprolide at 55°C from different buffer systems at varying pH. Equilibrium with no binding was achieved within 4 hours, independent of the buffer and the pH of the media.

Prior to using the dialysis method to assess peptide release, in vitro studies were performed using the sample and separate method. This technique is frequently used with[10,23-25] or without agitation[26,27] of media. From the in vitro release study (Figure 6), the sample and separate method without agitation showed an initial burst of about 7% followed by slow but nearly linear release reaching 35% in 35 days. In contrast, release was much faster and nearly completed within 28 days when the release media was continuously agitated. About 60% of unreleased peptide was found in

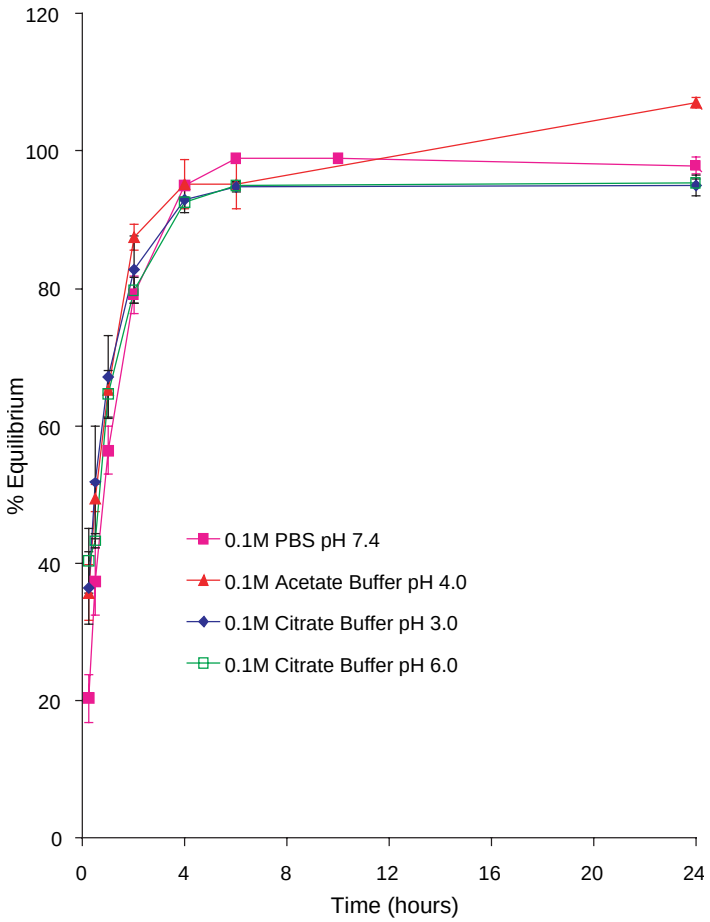


**Figure 5.** Diffusion of leuprolide through the Float-a-Lyzer in different buffers at 55°C.

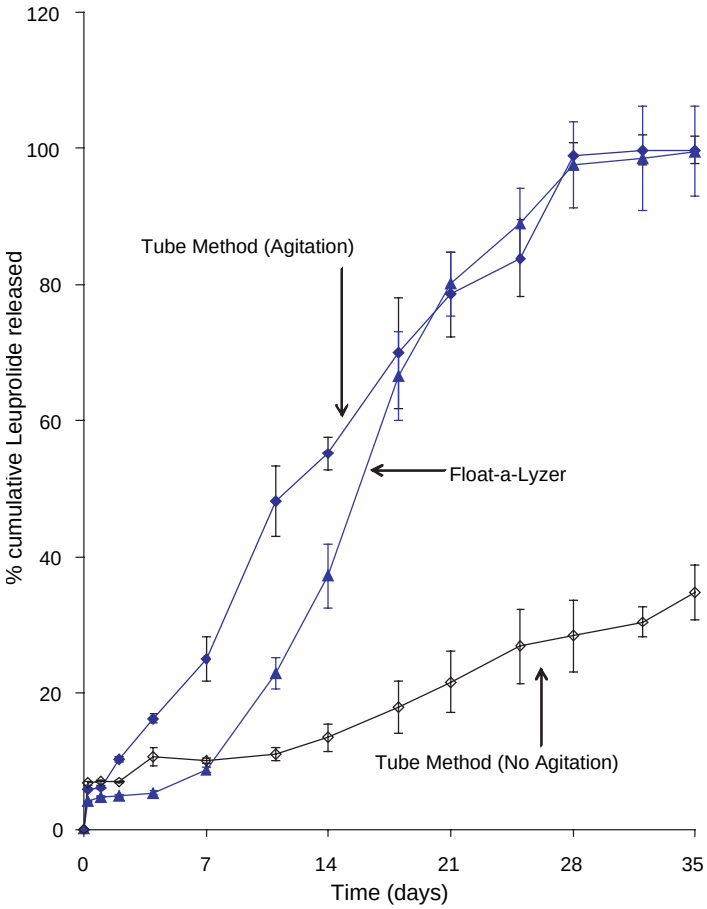


**Figure 6.** In vitro release of 1-month leuprolide microspheres at 37°C in 0.1 M PBS pH 7.4.

the microspheres from the nonagitated samples. The effect of agitation on dissolution of dosage forms has been published.[28-30] Increasing the agitation eliminates the unstirred water layer that is commonly attributed to the slow dissolution of insoluble drugs. Agitation of the microspheres was also thought to increase dissolution of the polymer resulting in faster release of peptide. However, the process may be more complicated considering the formation of a microenvironment within the microspheres,[31-33] the presence of a boundary layer in an unstirred system, and binding of dissolved drug within the microenvironment prior to diffusion. These issues are beyond the scope of this study.

With the novel ''dialysis'' technique, the microspheres were introduced into the dialyzer, and peptide release was assessed from the outer media, which was continuously stirred. This technique mimics the in vivo condition after subcutaneous or intramuscular administration, where the microspheres are immobilized in the tissue. From the in vitro studies, the initial burst at 6 hours was determined to be comparable to the sample and separate method. Following the initial period, drug release was steady up to 7 days after which release rate increased, presumably due to erosion of the polymer. Complete release was obtained in 28 days with the dialysis technique. Although peptide release from microspheres was assessed by analysis of the supernatant in this study, residual analysis of the drug from microspheres is an option in the case of drugs unstable in the release media. The dialysis method is less desirable if the drug binds or adsorbs to the membrane.

In vitro release profiles obtained from the sample and separate method and dialysis technique were different. With the former, release profiles were linear with different rates, the lower rates being attributed to the presence of a large unstirred water layer and slower polymer dissolution in the nonagitated samples. In the dialysis technique, the microspheres were not directly subjected to agitation, but the external media (sink) was continuously stirred. Peptide release inside the dialyzer is thought to be similar to its release in the nonagitated sample and separate method. However, there is a faster rate of diffusion through the membrane into the outer sink owing to continuous stirring of the outer media, thereby reducing the effects of the unstirred water layer. Such a situation does not exist with the nonagitated sample and separate method, thereby resulting in lower release. Aggregation of microspheres has been cited as a potential problem in assessing drug release at elevated temperatures.[21] However, in this study aggregation was not observed in the techniques employed so the different profiles observed with the sample and separate method cannot be attributed to aggregation.

Figure 6 shows the release profiles in which 3 separate runs were performed with the dialysis technique, each time with triplicate samples to assess reproducibility. The results were highly reproducible ($P > .05$) and showed the suitability of using the dialysis technique with the 25 KD membrane for in vitro release.

Although this research was focused on evaluating the dialysis method to study peptide release from biodegradable microspheres, this technique could be expanded to include currently marketed controlled release dosage forms like Zoladex, Eligard, Atridox, and Profact, all of which are formulated using PLGA polymers. This technique would also be useful for nonpolymeric dosage forms like implants, which release drugs for an extended period in the body.

## CONCLUSION

An in vitro release method using a regenerated cellulose membrane dialysis apparatus (Float-a-Lyzer) was suitable for studying in vitro release of peptide-loaded biodegradable microspheres since peptide diffusion through the dialysis membrane was rapid at the physiological and elevated temperature studied. The pretreatment study showed that the membrane was stable to elevated temperatures, which implied that it could be used for a short-term release study in which high temperatures are used. The in vitro release

method using the dialyzer was capable of accurately assessing a low initial burst release from the 1-month leuprolide PLGA microspheres. Complete peptide release with good reproducibility was obtained suggesting that the commercially available Float-a-Lyzer is reliable and suitable for drug release testing from biodegradable microspheres, even for accelerated release testing.

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