Stabilization of Vinca Alkaloids Encapsulated in Poly(lactide-*co*-glycolide) Microspheres

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Purpose. The purpose of this study was to stabilize the vinca alkaloids, vincristine sulfate (VCR) and vinblastine sulfate (VBL), in poly(lactide-*co*-glycolide) (PLGA) microspheres and to release the drugs in a sustained manner for more than a month.

Methods. An oil-in-oil emulsion-solvent extraction method was used to encapsulate VCR and VBL in PLGA50/50 microspheres. Stability and release kinetics of the drugs during the incubation at 37°C in PBS/Tween 80 were assessed by HPLC. Degradation products were identified with HPLC-MS.

Results. VCR and VBL were encapsulated in PLGA microspheres unchanged. During the microsphere incubation, however, VCR degraded inside the particles with a $t_{1/2} \sim 7.5$ days. The degradation product was identified by LC-MS as the deformyl derivative, commonly formed at acidic pH. VBL, which differs only by a stable methyl group in place of the N-formyl group in VCR, was completely stable in the PLGA microclimate. The neutralization of acidic PLGA microclimate by addition of 3–10% $Mg(OH)_2$ completely inhibited deformylation of VCR during release, but introduced a new degradation product formed under the more alkaline conditions used during the preparation. The substitution of $Mg(OH)_2$ with a weaker base, $ZnCO_3$, inhibited the formation of both degradation products resulting in VCR stabilization of >92% for 4 weeks. The optimal formulations of VCR (containing $ZnCO_3$) and VBL (no additives) slowly and continuously released stable drugs for over a month.

Conclusions. VCR and VBL were successfully stabilized and released in a sustained manner from PLGA microspheres. Co-encapsulation of $ZnCO_3$ stabilizes VCR against acid-catalyzed degradation during release from the polymer and minimizes VCR decomposition during encapsulation.

KEY WORDS: vincristine sulfate; vinblastine sulfate; PLGA microspheres; microclimate pH; local drug delivery; chemotherapy; Kaposi's sarcoma; drug stability.

INTRODUCTION

Polymer implants containing chemotherapeutic agents are clinically useful for both site-specific and systemic drug therapies in cancer (1–3). Once injected in the body, the polymer implant slowly releases anti-tumor agent, providing desirable drug levels for over a month. By intratumoral administration of an implant, it is possible to maintain drug concentration in the tumor at cytotoxic levels while minimizing the systemic toxicity (1,2). Biodegradable poly(lactide-*co*-glycolide) (PLGA) polymers have been used to encapsulate, slowly release, and stabilize a variety of anticancer agents such as cisplatin (4), doxorubicin (5), mitomicin C (6), camptothecin (7) and several others. In this study, PLGA microspheres were used to stabilize and deliver two potent vinca alkaloids, vincristine (VCR) and vinblastine (VBL). The ultimate goal of this study was to design controlled release formulations of vinca alkaloids clinically useful for the local chemotherapy of mucocutaneous AIDS-related Kaposi's sarcoma (AIDS-KS) (8,9).

Vinca alkaloids have been used extensively for treatment of various cancers including AIDS-KS (9–11). The mechanism of drug action is to arrest cells at the metaphase by binding to tubulin, which inhibits tubulin polymerization (10). The systemic administration of vinca alkaloids is compromised by severe dose-limiting toxicity such as loss of reflexes, paraesthesia, and muscle weakness for VCR; and leucopenia and anemia for VBL (10). Because vinca alkaloids are cycle-dependent agents with narrow therapeutic windows, the importance of dosing schedule is well recognized. Although VBL and VCR are typically administered by i. v. bolus injection, an increase in anti-tumor activity has been reported when the drugs are continuously infused (12,13). In addition, *in vitro* data has indicated that the longer exposure of VCR above a critical threshold concentration induced more profound cytotoxicity (14). Hence, controlled release formulations of vinca alkaloids appear to be a generally attractive goal to pursue.

VCR and VBL are structurally identical with exception of the group attached to the nitrogen at position 1, at which VCR possesses a labile N-formyl group and VBL has a stable methyl group (Fig. 1). Both drugs undergo pH-dependant degradation in aqueous solution, the pH of maximum stability is ~2 for VBL and ~4.5 for VCR (15,16). We report that VCR becomes unstable in PLGA (50% D,L lactide content) microspheres, whereas encapsulated VBL is highly stabilized. Herein, we used a mechanistic analysis of VCR degradation to develop PLGA microspheres that stabilize VCR for over a month.

MATERIALS AND METHODS

Chemicals

Vincristine sulfate (98% purity) and vinblastine sulfate (97% purity) were obtained from Sigma (St. Louis, MO). PLGA with copolymer ratio of D,L-lactide to glycolide 50:50 and inherent viscosity of 0.23 dl/g was purchased from Birmingham Polymers (Birmingham, AL). $Mg(OH)_2$ was obtained from Aldrich Chemical Co. (St. Louis, MO) and $ZnCO_3$ was purchased from ICN Biopharmaceuticals (Aurora, OH). All other reagents and solvents were of analytical grade or purer and purchased from commercial suppliers.

Microspheres Preparation

Microspheres were prepared by a standard oil-in-oil emulsion-solvent extraction method (17). 150 mg PLGA were dissolved in 450 μl of acetonitrile (ACN) before addition of 15 μl of aqueous VCR or VBL solution (20 mg/ml). In some instances, $Mg(OH)_2$ or $ZnCO_3$ at 0.5, 3, and 10% (wt. base/wt. polymer) were suspended in the polymer solution to raise the microclimate pH inside the microspheres. The resulting

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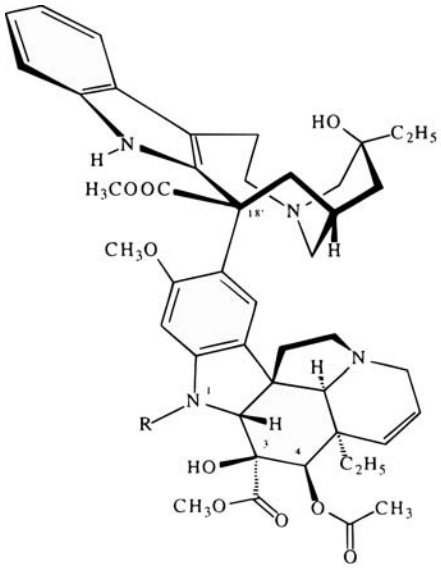

	VINCRISTINE	VINBLASTINE
R	CHO	CH_3

Fig. 1. Structures of vincristine and vinblastine. The most susceptible moieties for the chemical degradation are: the N-formyl group at position 1 (for VCR), the methylesters at positions 3 and 18′, and the acetate at position 4.

solution or suspension was added drop-wise to 25 ml of oil (95% cottonseed oil and 5% Span 85 emulsifier) stirred at 500 rpm and room temperature. After 2.5 h of microsphere hardening, 40 ml of petroleum ether (bp: 50 to 110°C) were added to the emulsion to extract ACN. The emulsion was stirred for additional 15 min, the particles were collected by filtration, and washed 3 times with petroleum ether. The hardened microspheres were flash-frozen with liquid nitrogen and lyophilized with a Labconco Freezone 6 system for 1 day.

Analysis of Drugs and Their Degradation Products by HPLC

VCR and VBL were examined by high performance liquid chromatography (HPLC), as described previously (15, 16, 18). The HPLC system consisted of the following: a 510 pump, a 717 Plus autosampler, and a 486 UV detector (Waters, Milford, MA). A C_{18} 3.9 × 150 mm reverse phase column (Waters Nova-Pak) was used at a flow rate of 1 ml/min. The mobile phase was composed of aqueous solution of sodium phosphate (10 mM) and methanol 40:60 (v/v) (pH 7.0). For UV detection, the wavelength was set to 298 nm.

Identification of VCR Degradation Product by LC-MS

For identification of VCR and its degradation products a LC/MS system was used. The system consisted of a Perkin-Elmer Sciex API 300 triple-quadruple mass spectrometer (Thornhill, Ontario, Canada) coupled to a Schimadzu HPLC system (Columbia, MD). The HPLC system was equipped with an SCL-1A system controller, a LC-10A pump, a GT-104 degasser, and an SIL-10A autosampler. The separation of the parent drug and the degradation products was performed in 10 mM ammonium formate (pH 4) and ACN (40/60 v/v) on a C_{18} reversed phase column.

Microscopic Evaluation of Microsphere Size Distribution and Morphology

Greater than one hundred particles for each preparation were sized by sight under Zeiss Axiolab light microscope equipped with a 10× objective and a sizing scale bar. Scanning electron microscopy (SEM) images of PLGA microspheres were obtained by using a Philips XL30 field emission gun scanning electron microscope. Samples were coated with conductive gold prior to analysis.

Evaluation of VCR and Its Degradation Products During Release

Drug release from microspheres was carried out in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4) containing Tween 80 (0.02% w/w) (PBST) at 37°C under perfect sink conditions. VCR and VBL were unstable in the release media so release kinetics was monitored indirectly from the drug remaining in the polymer. Microspheres were weighed and dissolved in a 50% (v/v) ACN/water solution. The precipitated polymer and salts were spun down by brief centrifugation. An aliquot of the supernatant containing drug was removed and analyzed by HPLC.

Non-aqueous Solvent pH Measurements

150 mg of PLGA were dissolved in 450 μl ACN and $ZnCO_3$, and $Mg(OH)_2$ was suspended in the polymer solution at 0.5, 3, and 10% (wt. base/wt. polymer). 15 μl of double distilled water were added to the suspension and vortexed for 20 s to simulate microsphere preparation conditions. The undissolved salts were spun down by a brief centrifugation and the supernatant was diluted in an ACN:H_2O mixture to make a 50 mg/ml polymer concentration. The final solvent composition was 80:20 (v/v) ACN:H_2O. The pH was measured with a Corning Semi-Micro Combination glass pH-electrode attached to a Corning pH meter (VWR scientific, PA). As described previously (19), the actual proton activity in the organic solution mixture (a_H^*) was calculated from the pH meter reading (pH) by $pa_H^* = pH - \delta$, where δ is a correction coefficient which equals 0.95 for an ACN:H_2O 80:20 (v/v) mixture.

RESULTS AND DISCUSSION

Degradation of VCR Encapsulated in PLGA Microspheres

Microspheres containing 0.22% (w/w) drug were obtained by an oil-in-oil emulsion-solvent extraction technique. The encapsulation efficiency was ~91% (Table 1, Protocol A). Microspheres were spherical in shape with the mean particle size of 46 μm (Fig. 2A).

All the encapsulated vincristine was originally preserved

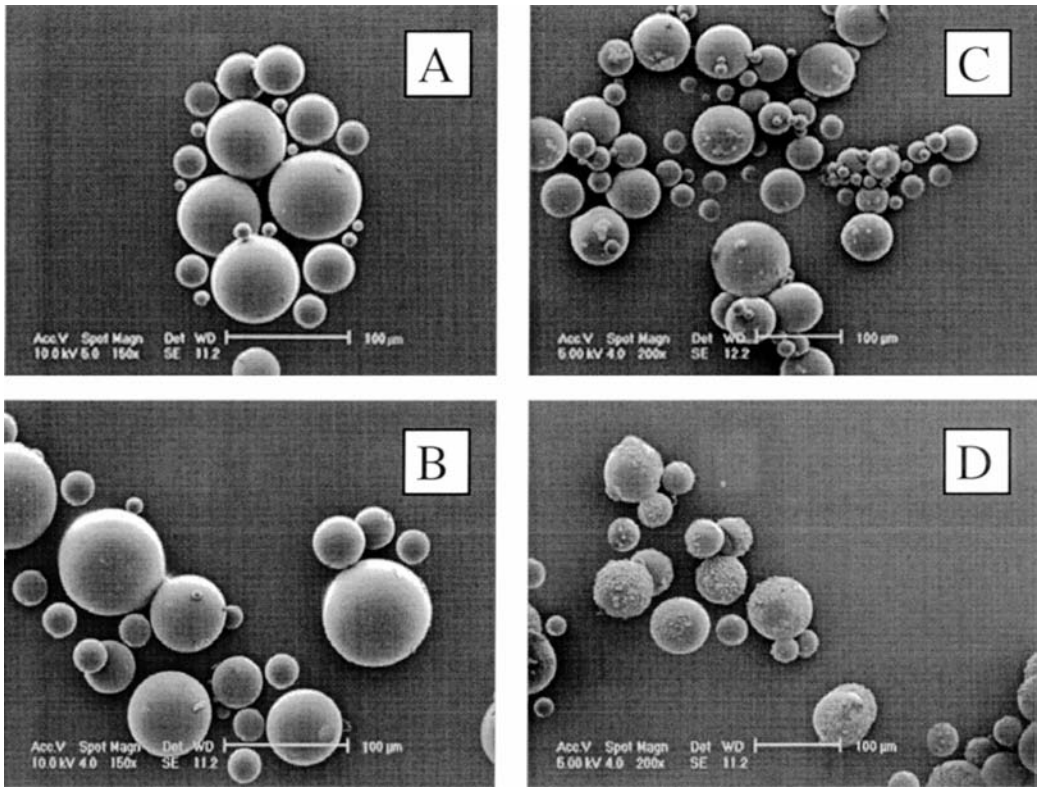

Fig. 2. SEM images of PLGA microspheres after preparation containing: (A) VCR and no additives, (B) VBL, (C) VCR and 10% $Mg(OH)_2$, (D) VCR and 10% $ZnCO_3$.

in its native form following encapsulation. During microsphere incubation, the drug degraded rapidly inside the particles (Fig. 3A). The appearance of a major degradation product was observed in the chromatogram (peak II, Fig. 4A). Only 23% of drug remained in its native form after 14 days of the incubation. Curve fitting assuming pseudo-first order kinetics for the degradation of encapsulated VCR gave a rate constant of $k = 1.07\ 10^{-6}\ s^{-1}$ and $t_{1/2} = 7.5$ days at 37°C.

In order to improve drug stability in the formulation, the following methodology was used (20): (a) identification of the degradation product, (b) elucidation of the cause and mechanism of VCR degradation in the PLGA, and (c) stabilization of VCR in PLGA microspheres by inhibiting or bypassing the cause and mechanism of VCR degradation.

Identification of the VCR Degradation Product

The degradation product was more hydrophobic relative to the parent drug since its retention time (peak II at 7.6 min) was longer than the retention time of VCR (peak I at 5.5 min). LC-MS analysis revealed the main molecular peaks of 797.5 Da for the degradation product and 825.5 Da for VCR (Fig. 5). The difference of 28 Da was likely due to the loss of N-formyl group at the position 1 (Fig. 1). Formation of the deformyl derivative of VCR was reported previously by Sethi *et al.* (18) and is favorable at acidic pH (21). The retention time of the degradation product formed in PLGA microspheres also corres-ponds to the retention time of VCR degradation product formed in solutions pH 1.5 (15). It is well established

Table 1. Characterization of Microspheres

Protocol code	Drug added	Base added	Base loading, % (w/w)	Drug loading, % (w/w)[a]	Encapsulation efficiency, %[a]	Particle size, μm[b]	Yield, %
A	VCR	—	—	0.22 ± 0.01	91 ± 1	46 ± 3	89
B	VBL	—	—	0.18 ± 0.01	88 ± 3	50 + 2	93
C	VCR	$Mg(OH)_2$	0.5	0.15 ± 0.02	76 ± 8	42 ± 3	91
D	VCR	$Mg(OH)_2$	3	0.27 ± 0.01	98 ± 1	59 ± 4	87
E	VCR	$Mg(OH)_2$	10	0.18 ± 0.01	94 ± 1	50 ± 3	94
F	VCR	$ZnCO_3$	3	0.15 ± 0.01	82 + 3	52 ± 3	89
G	VCR	$ZnCO_3$	10	0.19 ± 0.02	87 + 5	43 ± 5	92

[a] N = 3 ± SD.
[b] N = 100 ± SEM.

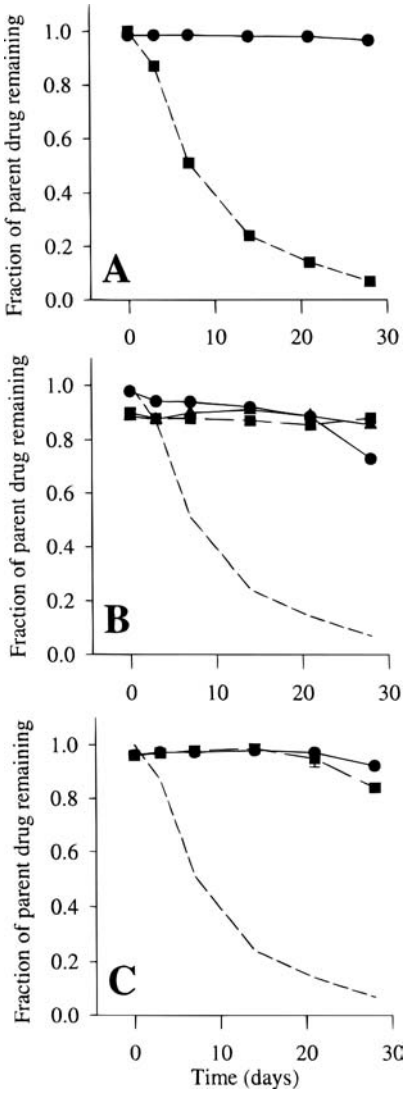

Fig. 3. (A) Rapid degradation of VCR (■) compared to the stability of VBL (●) inside PLGA microspheres. The VCR degradation half-life of ~7.5 days was obtained by assuming pseudo-first order kinetics. (B) Stabilization of VCR by the addition of 0.5 (●), 3(■), and 10% (▲) of $Mg(OH)_2$ compared to VCR degradation without additives (––). (C) Stabilization of VCR by the addition of 3 (●) and 10% (■) of $ZnCO_3$ compared to VCR degradation without additives (––). Drug stability was assessed during microsphere incubation in PBST at 37°C (Mean ± S. D., n = 2).

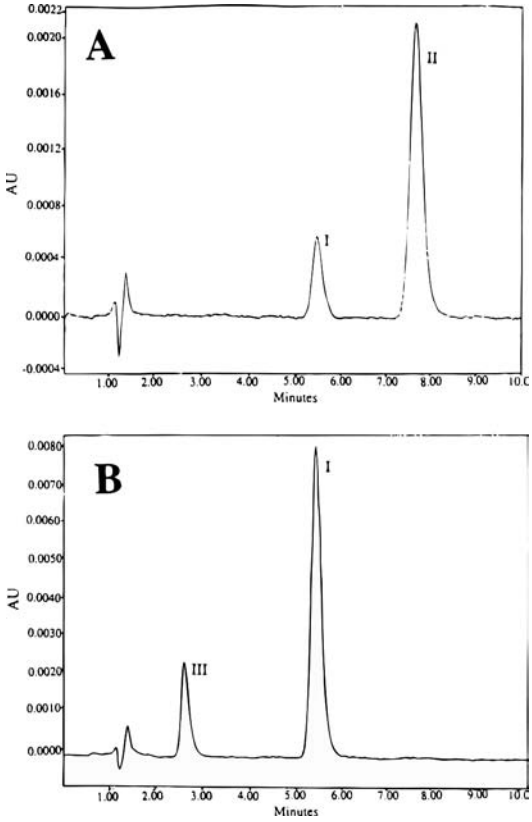

Fig. 4. HPLC chromatograms of VCR and its degradation products extracted from the microspheres containing (A) no additives after 14 days of incubation in PBST at 37°C and (B) 10% $Mg(OH)_2$ after preparation. Peak I is VCR, peak II is the acidic degradation product formed in PLGA microspheres during incubation, and peak III is the alkaline degradation product formed during encapsulation in the presence of $Mg(OH)_2$.

that PLGA 50/50 microspheres made by standard emulsion-solvent evaporation techniques can develop acidic microclimate pH in the range of 1.5 to 3.5 (19,22). Hence, we hypothesized that the mechanism of VCR degradation in PLGA microspheres is the acid-catalyzed loss of the N-formyl group.

Acid-stable Vinblastine Exhibits Negligible Degradation in PLGA Microspheres

Vinblastine is structurally very similar to vincristine. The only chemical difference between these drugs is that the VCR formyl group at position 1 is substituted with a methyl group in VBL (Fig. 1). This results in a superior stability of VBL compared to VCR under acidic conditions (15,16). If acid catalyzed deformylation were the main source of VCR instability in microspheres, then VBL with no formyl group would be expected to be stable in the microspheres.

VBL microspheres were prepared by the same technique used to encapsulate VCR. The obtained VBL microspheres had similar properties and appearance to VCR microspheres (Table 1, Protocol B, Fig. 2B). As expected, VBL was more than 98% stable in PLGA microspheres (Fig. 3A). The retention time of VBL extracted from PLGA microspheres was unmodified compared with the non-encapsulated drug and no additional peaks were detected after microsphere preparation and during the 4-week incubation at 37°C (Fig. 3A). This confirms the hypothesis that acid-catalyzed VCR deformylation occurs in the PLGA microclimate.

Co-encapsulation of $Mg(OH)_2$ in PLGA Microspheres Stabilizes VCR

The encapsulation of insoluble bases in PLGA microspheres causes an increase in the microclimate pH (19) and an inhibition of acid-induced instability of encapsulated proteins (20,23). To inhibit acidic degradation of VCR, $Mg(OH)_2$ was co-encapsulated in PLGA microspheres at 0.5, 3 and 10% (wt.

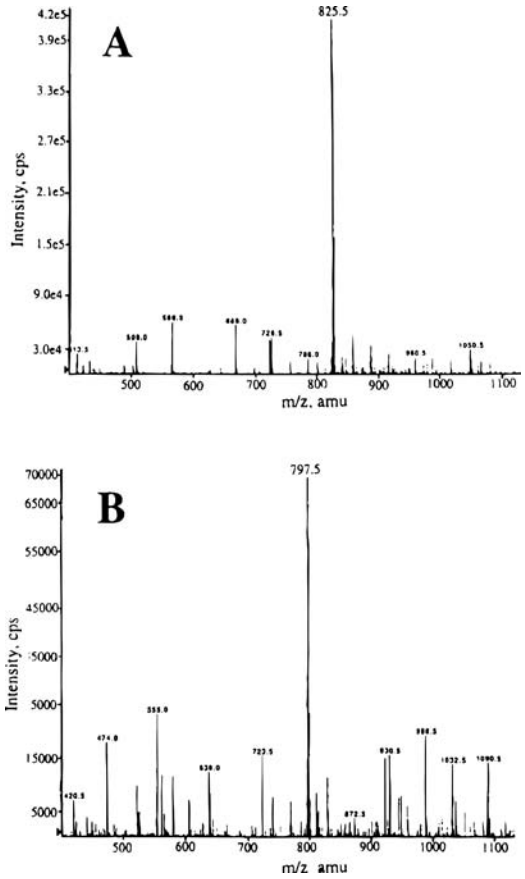

Fig. 5. Mass spectra of vincristine (A) and main acidic degradation product in microspheres (B).

base/wt. polymer) loading. The addition of base did not change the spherical appearance of microspheres, although the particle surface at high base content became less smooth due to protruding base particles (Fig. 2C). A microsphere particle size of ~50 μm, a loading of ~0.2%, and an encapsulated efficiency in a range of 76 to 98% were obtained (Table 1, Protocols C-E).

The acidic degradation of VCR was fully inhibited by addition of 3 and 10% of $Mg(OH)_2$ (Fig. 3B). However, in the microspheres containing only 0.5% $Mg(OH)_2$ the deformyl degradation product appeared after 2 weeks of incubation. This can be attributed to non-homogeneity of microclimate neutralization by $Mg(OH)_2$ (19) and/or an insufficient supply of base for the neutralization of acidic groups formed as PLGA hydrolysis proceeded.

Despite VCR stabilization during release, the addition of $Mg(OH)_2$ induced the appearance of a second degradation product formed during microsphere preparation (peak III in Fig. 4B). The degradation product was more hydrophilic with a retention time of 2.6 min compared to 5.5 min for VCR. The retention time of peak III is consistent with that of the VCR degradation product formed in solution at pH ~7.3 in the study by Vendrig *et al.* (15). Roughly 12% of the drug was degraded during the preparation of the microspheres containing 3 and 10% $Mg(OH)_2$. No further formation of the basic degradation product was observed during microsphere incubation (Fig. 3B). It is probable that VCR is either exposed to a higher pH or is more reactive in the polymer-base solutions during microsphere preparation than in the polymer microclimate during incubation.

Substitution of $Mg(OH)_2$ with $ZnCO_3$ Inhibits Alkaline Degradation

To inhibit formation of the basic degradation product a weaker base, $ZnCO_3$, was used for microclimate pH neutralization instead of $Mg(OH)_2$. The pH of a saturated aqueous solution of base is 7.3 for $ZnCO_3$ and 9.8 for $Mg(OH)_2$ (23). The hydronium ion activities in non-aqueous solvents (pa_H^*) of the polymer solutions with and without bases were measured to evaluate the conditions affecting VCR stability during microsphere preparation (Table 2). The pa_H^* of PLGA solution containing no additives was low at 3.9. This value increased with addition of 0.5, 3, and 10% of $Mg(OH)_2$ to 4.8, 6.1 and 7.3, respectively. The addition of $ZnCO_3$ also increased pa_H^* but to a lesser extent than the addition of $Mg(OH)_2$ on a weight basis.

The substitution of $Mg(OH)_2$ with $ZnCO_3$ did not change the physical characteristics of the microspheres. Spherical microspheres with ~0.17% drug loading, 85% encapsulation efficiency, and the ~48 μm particle size were obtained (Table 1, Protocols F-G, Fig. 2D). However, only 3% of VCR converted to the basic product during microsphere preparation with $ZnCO_3$ compared to 12% with $Mg(OH)_2$. The acid-catalyzed VCR degradation was inhibited resulting in 97% of the drug remaining intact after 3 weeks and 92% intact after 4 weeks (Fig. 3C). Hence, the substitution of $Mg(OH)_2$ with $ZnCO_3$ further improved the stability of encapsulated VCR in PLGA microspheres.

Drug Release Kinetics

Drugs were released in sustained manner from all the formulations (Fig. 6). VBL microspheres released drug nearly linearly for 4 weeks (66% of encapsulated drug released). The VCR formulations contained 3 and 10% $ZnCO_3$ and released 56 and 31% of the stable drug, respectively, at the end of incubation period. VCR was released faster from the formulation containing more $ZnCO_3$, probably because the co-encapsulation of this base increased polymer water content leading to a faster drug transport. The water content of PLGA usually increases with co-encapsulation of basic additives and microclimate neutralization (23). For example, the water content was reported to increase 2.5 and 4 times by co-encapsulation of 3% $Mg(OH)_2$ and 3% $ZnCO_3$, respectively, for PLGA millicylinders containing 15% protein (23). Hence, we expected to observe an increase in VCR release rate from the

Table 2. Neutralization of PLGA Solutions with Basic Salts

Base added	Base loading, % (w/w)	pa_H^{*a}
—	—	3.9 ± 0.1
$Mg(OH)_2$	0.5	4.8 ± 0.3
$Mg(OH)_2$	3	6.1 ± 0.4
$Mg(OH)_2$	10	7.3 ± 0.2
$ZnCO_3$	3	5.0 ± 0.1
$ZnCO_3$	10	6.4 ± 0.3

[a] N = 5 ± SD.

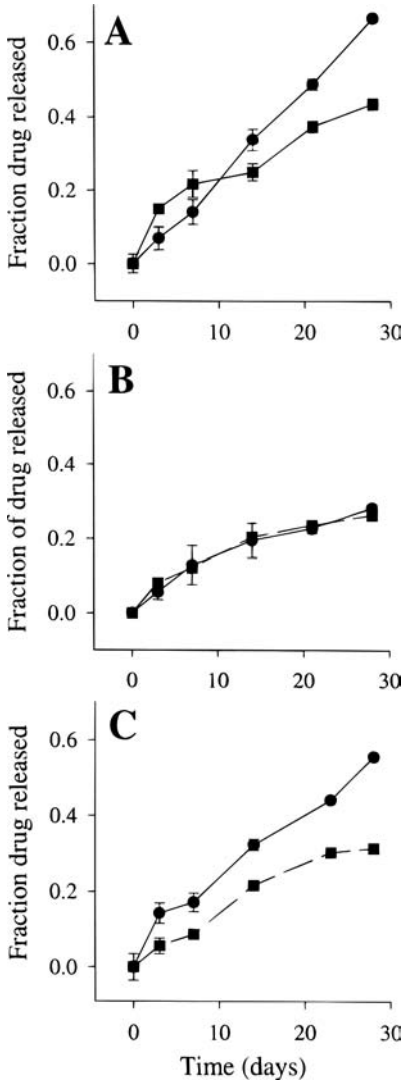

Fig. 6. Drug release from the microspheres containing: (A) VCR (■) and VBL (●); (B) VCR with 3% (■) and 10% (●) $Mg(OH)_2$, (C) VCR with 3% (■) and 10% (●) $ZnCO_3$. The release kinetics was derived from decrease in total peak area of encapsulated drug in microspheres (Mean ± S. D., n = 2).

formulations containing bases compared to the formulations without base. However, just the opposite was observed as less dug was released after 28 days from the microspheres containing either 3–10% $Mg(OH)_2$ or 3% $ZnCO_3$ compared to microspheres without additives. A possible explanation is that the solubility of weakly basic drug decreased in the neutralized microclimate (VCR has pK_as of 5 and 7.4 (24)). In addition, the positively charged drug may have interacted with the negatively charged polymer end-groups, which become ionized in the neutralized microenvironment.

Potential Clinical Applications of PLGA Microspheres Containing Vinca Alkaloids

Malignancies which present discreet and visible tumor foci and are accessible by local intervention, such as stage I Hodgkin's disease, mycosis fungoides, and mucocutaneous AIDS-related Kaposi's sarcoma, are amenable to local treatment by controlled delivery of vinca alkaloids. AIDS-KS is the most prevalent HIV associated malignancy, with mucocutaneous lesions being the most common presentation (8,25). VCR and VBL are commonly used as single agents or in combination for systemic treatment of AIDS-KS (9). However, the HIV^+ population is less tolerant to systemic delivery of vinca alkaloids, since hematological toxicity of VBL is dangerous in conjunction with the immune suppression and many anti-HIV agents are neurotoxic as is VCR (2,10). Recently, intralesional bolus injection of VBL was found to be successful in AIDS-KS management (26). Controlled release is known to reduce injection frequency and to prolong drug exposure relative to local bolus injections (2,3). Therefore, local administration of PLGA microspheres containing vinca alkaloids represents a promising alternative therapy for AIDS-KS.

CONCLUSIONS

VCR and VBL were successfully stabilized in PLGA microspheres and continuously released for more than 4 weeks. The microspheres prepared here may have applications for site-specific chemotherapy for a variety of malignancies, particularly AIDS-KS. The acid-catalyzed deformylation of VCR in PLGA microspheres was strongly inhibited by the addition of poorly soluble basic additives. The central role of microclimate pH in PLGA for controlling the stability of encapsulated substances has now been shown for numerous molecules including: VCR, the camptothecins (7,19), bovine serum albumin (BSA) (20, 23), and basic fibroblast growth factor (20). Hence, development of ways to measure and control microclimate pH continues to be an important objective to help realize the full potential of PLGA to stabilize and deliver chemically labile drugs.

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