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Research Article

Preparation of Intravenous Stealthy Acyclovir Nanoparticles with Increased Mean Residence Time

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Abstract. A major cause of thromboplebitis, during acyclovir (ACV) parenteral administration is the high pH of its reconstituted solution (pH 11). Its plasma half life is 2.5 h, requiring repeated administration which may result in excess of drug solubility leading to possible renal damage and acute renal failure. The present study reports the efficiency of stealthy ACV nanoparticles (NPs) to increase the mean residence time of the drug 29 times. It caused a marked decrease in thrombophlebitis when injected into rabbit's ear vein. The polymers used were (Poly lactic acid, polylactic-co-glycolic (PLGA) 85/15, PLGA 75/25, PLGA 50/50). Particles were evaluated for their encapsulation efficiency, morphology, particle size and size distribution, zeta potential, and *in vitro* drug release. Small NPs (280–300 nm) with 60% drug release after 48 h were obtained. Among the block copolymer used, poloxamer 407 was of superior coating properties with a coat thickness in the range of 1.5–8.3 nm and a decreased surface charge.

KEY WORDS: acyclovir; nanoparticles; parenteral; pharmacokinetics, thrombophlebitis; poly (lactide-co-glycolide).

INTRODUCTION

Homo- and copolymers of lactic acid and glycolic acid are being extensively used as controlled release carriers. Being biodegradable, these polyesters do not accumulate in the body even in case of repeated injections, which makes them very attractive materials for preparing nanocarriers. Polylactic acid (PLA) has an excellent loading capacity, while polylactic co-glycolic (PLGA) has higher degradation kinetics than PLA.

Once injected, drug delivery nanocarriers are rapidly removed from the bloodstream as a result of interaction with the mononuclear phagocyte system or with the complement system (1,2). The opsonization of nanoparticulate drug carriers from the body by the reticuloendothelial system (RES) is a major obstacle that affects the efficiency of the nanoparticulate drug delivery systems. In this respect, the properties of the nanocarriers are important. Charged polymers are recognized by the plasmatic proteins and oriented towards the macrophages of the RES (3). Whenever the plasma proteins are not deposited on their surface, the nanocarriers are not recognized by the RES, opsonization is delayed and they belong to the sterically stabilized nanocarriers. Their lifetime in the bloodstream may be longer. Poly(ethylene oxide) (PEO), and block copolymer are commonly used to modify the surface of carriers for making them "stealthy". PEG derivatives (4) and block copolymers are used in many drug delivery systems, because their physico-chemical properties, such an amphiphilicity and degradation rate, can be changed by the choice, content, and molecular weight of the constitutive blocks.

Acyclovir (ACV) is available for parenteral administration as acyclovir sodium for intravenous (IV) infusion only, with a pH 11 for its reconstituted solution. Its plasma half life is 2.5 h (5). The most frequent adverse reactions reported during administration of acyclovir sodium were phlebitis at the injection site and transient elevations of serum creatinine with higher incidence following rapid intravenous infusion (less than 10 min). Precipitation of ACV crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/mL at 37°C in water) is exceeded or if the drug is administered by bolus injection. Ensuing renal tubular damage can produce acute renal failure.

Aiming at extending its plasma half- life, hence reducing the daily dose and the accompanied side effects (including thrombophlebitis), ACV NPS were fabricated. Meanwhile the influence of the polymer type, the lactide to glycolide (L/G) ratio of PLGA on the encapsulation efficiency (EE) and particle size were investigated. To prevent opsonization, block copolymer with hydrophobe molecular weight 4000 and 70% ethylene oxide, Poloxamer 407 was used during NPs preparation. NPs were also prepared using Tween 80 which were then protected from opsonization by external surface adsorption with different block copolymers with different ethylene oxide/propylene oxide chain length. The blood circulation time and pharmacokinetics parameters, after IV administration, of ACV NPs prepared by incorporating the block copolymers during fabrication or their

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surface adsorption after fabrication were studied in rabbits. The results were compared with those of equivalent doses of commercial ACV sodium injections. As far as we know, stealthy ACV PLGA NPs for IV administration have not yet been reported. However, self assembled lipid conjugate ACV NPs were prepared (6–8), but were cleared from the blood circulation very rapidly (90% within 5 min) after IV administration to rabbits. ACV loaded PLA nanospheres, with very low EE, for ophthalmic drug delivery were also prepared (9). Valaciclovir NPs bovine serum albumin were used for liver targeting (10). PLGA-ACV-loaded microparticles were proven to be promising carriers for treatment of HSV-1 infections (11). Furthermore they have been broadly used in ocular (12–17) and topical (18) delivery.

MATERIALS AND METHODS

Materials

Acyclovir was a gift from Glaxo–smithkline (Cairo, Egypt). Poly (D, L-lactide) (DL-PLA) inherent visc.: 0.67 dL/g molecular weight (MW) 93,200 Da, 85/15 poly DL-lactide-co-glycolide (85/15 DL-PLGA) inherent viscosity:0.64 dL/g, MW 74,000–94,000 Da, 75/25 poly DL-lactide-co- glycolide (75/25 DL-PLGA) inherent viscosity: 0.67 dL/g MW 97,000–105,000 Da, 50/50 poly DL-lactide-co- glycolide (50/50 DL-PLGA) inherent viscosity: 0.58 dL/g MW 75,000–80,000 Da were purchased from Birmingham polymers, Inc. USA. Poly vinylalchol (PVA) MW 14000, Elgomhoria company (Cairo, Egypt). Tween 80: Atlas chemical Industries, USA. Poloxamer 188 (F-68), Poloxamer 338 (F-108), Poloxamer 407 (F-127) were purchased from BASF Corporation, USA.

Preparation of Nanoparticles

Aqueous PLA NP dispersions were prepared according to the SESD method developed by Niwa *et al*. (19) with slight modification. Briefly 200 mg of PLA and 100 mg of ACV were dissolved and suspended respectively in 25 ml acetone. This organic phase was then poured slowly into 50 ml of aqueous surfactant solution under stirring at 15,000 rpm using a homogenizer (Heidolph Diax 900, Germany) for a few minutes at room temperature. The prepared system was then stirred using a magnetic stirrer. NPs were immediately formed, and acetone was then removed from the colloidal suspension by roto evaporation (Bibby sterilin LTD, RE200B, U.K) under reduced pressure. The resulting particle suspension was filtered through a 1.2 μm membrane filter (Micro filtration systems, California, USA) to ensure the absence of microparticles. The raw suspension was centrifuged at 15,000 rpm at 4°C for 30 min. (Cooling centrifuge, Heraeus, Germany). The supernatant was discarded and the NP precipitate was then washed with distilled water and again centrifuged three times. The washed NPs were then freeze dried. A full factorial design was built to study the effect of changing in the polymer, surfactant types and surfactant concentration. Polymer type was studied at four levels namely (DL-PLA, 85/15 DL-PLGA, 75/25 DL-PLGA and 50/50 DL-PLGA), surfactant type at three levels (PVA, Tween 80 and Poloxamer 407) and surfactant concentration at three levels viz (0.5, 1, and 2% *w*/*v*). A typical design of the full factorial experiment with the composition of different formulas is shown in Table I. Triplicates were prepared for each formulation.

Evaluation of the Prepared Nanoparticles

Particle Morphology

The morphological examination of nanospheres was performed using transmission electron microscopy (Jeol Jem 1230, Tokyo, Japan with Semafore program ver. 4.01 for particle size detection).The dried NP samples were suspended in distilled water before examination.

Particle Size

Particle diameter was determined using the particle size analyzer (Malvern Mastersizer, Malvern Instruments Ltd., Malvern, UK). Accordingly, the dried NP samples were suspended in distilled water. The obtained homogenous suspensions were examined to determine the mean diameter and polydispersity index.

Determination of Drug Encapsulation Efficiency

The amount of drug incorporated in the NPs was determined using a UV spectrophotometer (Unicam, Helios Alpha, England). Freeze dried NPs were dissolved in 5 ml methylene chloride. ACV was then extracted from the methylene chloride by three portions of 20 ml 1 M sodium hydroxide solution, and measured at 254 nm. The individual values are reported according to the following equation and the results expressed are the mean of three determinations:

$$\text{Encapsulation efficiency \% (EE)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of drug used in formulation}} \quad (1)$$

Zeta Potential

The zeta potential of the NPs was measured using the zetasizer 2000 (Malvern instruments Ltd. Malvern UK). The freeze dried NPs were suspended in bidistilled water. Measurements were carried out in triplicate at 25°C in 10^{-3} M NaCl solution to keep the ionic strength constant.

In Vitro Release Study

The *in vitro* drug release studies were performed using a dialysis membrane with 12000 MWCO (Spectra Por, Sigma, USA) retaining NPs and allowing free drug into the release media. Briefly, 2 mg of ACV and amount equivalent to 2 mg ACV in drug-loaded NPs were transferred to a glass cylinder having the length of 10 cm and diameter of 2.5 cm fitted with a membrane presoaked in distilled water. The cylinder was placed in a receiving compartment containing 50 ml phosphate buffer saline (pH 7.4). This volume provided complete sink conditions for the drug. The entire system was kept at 37±0.5°C with continuous magnetic stirring at 100 rpm. At predetermined time intervals (1, 2, 3, 4, 5, 6, 8, 10, 12, 24, and

Table I. Composition of Different ACV-NPs Prepared by SESD According to the Factorial Design

Surfactant type	Surfactant concentration (%)	Polymer type			
		DL-PLA	85/15 DL-PLGA	75/25 DL-PLGA	50/50 DL-PLGA
Tween 80	0.5	1	10	19	28
	1	2	11	20	29
	2	3	12	21	30
PVA	0.5	4	13	22	31
	1	5	14	23	32
	2	6	15	24	33
F-127	0.5	7	16	25	34
	1	8	17	26	35
	2	9	18	27	36

48 h), aliquots of the release media were withdrawn and assayed spectrophotometrically at 254 nm. Withdrawn samples were replaced by fresh buffer.

Coating of ACV NPs

The dispersion of the prepared NPs of formulas 4, 14, 20, 22, and 23 was prepared, divided into three portions and then coated with different copolymers. The first portion was mixed with appropriate amount of poloxamer 188, the second with poloxamer 338, and the third with poloxamer 407 to produce a final surfactant concentration of 1% *w/v*. The samples were incubated over night at room temperature and then freeze dried. For each poloxamer, the thickness of the coating layer with each poloxamer was determined by subtracting the diameter of the uncoated particles from that of the coated ones and then dividing the obtained figure by two (20). Due to batch-to-batch variation, all comparative studies were done on the same NPs batch. The *in vitro* drug release of the most efficiently coated NPs was studied as well as their zeta potential.

Sterilization by Gamma Radiation

Different batches of the selected formulas were sterilized using gamma radiation. They were first packed in dry ice inside polyurethane container and then sterilized. The irradiation facility used was cobalt-60 Gamma Chamber 4000-A. According to European pharmacopoeia, the received dose was 25 KGy. This was considered as adequate for sterilizing pharmaceutical products when the bioburden is not known. After sterilization, the NPs were evaluated for their EE, particle size, and ACV release.

Differential Scanning Calorimetry

The thermal properties of ACV, selected polymer, blank NPs and selected ACV-loaded NPs were investigated using (differential scanning calorimetry (DSC)60 Shimadzu, Japan). Samples (3–5 mg) were put in hermetically sealed aluminum pans, heated, and scanned, first, from room temperature to 80°C at a heating rate of 10°C /min. After cooling the sample below zero, using liquid nitrogen, a second scan was performed from 0° to 300°C at a heating rate of 4°C/min.

Pharmacokinetic Study

Intravenous Administration of ACV to Rabbits

Twelve white male albino rabbits were divided into four groups (three for each group) and used for the pharmacokinetic study. The study protocol was reviewed and approved by the Experiments and Advanced Pharmaceutical Research Unit (EAPRU), Faculty of Pharmacy; Ain shams University on the use of the animals. The treatment was as follows:

Group 1: received commercial ACV sodium injection.
Group 2: received uncoated ACV NPs prepared with 75/25 PLGA and 1% Tween 80 (formula 20).
Group 3: received formula 20 coated with poloxamer 407 by surface adsorption (formula 20C).
Group 4: received the formula prepared with 75/25 PLGA using 1% poloxamer 407 by incorporation during fabrication (formula 26).

The rabbits were anesthetized using an intramuscular injection of 8.9 mg/kg of xylazine hydrochloride. A dose of 10 mg/kg (21) of ACV solution and an equivalent amount of ACV in NPs were given through the marginal vein of the rabbit's ear as a bolus injection. Samples of 2 ml blood were then withdrawn from the other ear at different time intervals of 0.083, 0.25, 0.5, 1, 2, 3, 4, and 6 h for commercial product and 0.083, 0.25, 0.5, 1, 2, 3, 6, 12, 24, and 48 h for ACV NPs. Samples were collected in heparinized tubes and centrifuged at 3,000 rpm for 15 min. The separated plasma was transferred into tubes, sealed, and stored at −20°C until assayed.

Sample Preparations and Analysis

At time of analysis 0.5 ml of ethyl paraben solution in methanol (1 μg/ml) and 0.5 ml acetonitrile were added to 0.5 ml of each plasma sample. The samples were vortexed, centrifuged at 3,000 rpm for 10 min, then filtered through a 0.45 μm membrane filter. The filtrate was evaporated to dryness using vacuum concentrator. The obtained residue was reconstituted in 100 μl of mobile phase. Samples of 20 μl were injected into the high-performance liquid chromatography (HPLC) apparatus following experimental conditions: HPLC apparatus (model LC–10 AS, Shimadzu, Japan) connected to an ultra-violet variable wavelength detector (Model SPD-10 A, Shimadzu,

Japan). A C_{18} reversed-phase column (Nova-Pak®, 250× 4.6 mm), Water, MA, USA. An isocratic pump (model LC-10 AS, Shimadzu, Japan). The mobile phase consisted of 0.03 M phospate buffer/methanol/acetonitrile (35:40:25) adjusted to pH 4 with phosphoric acid. The system was operated at a flow rate of 1.5 ml/min and the detection wavelength was 230 nm.

Data Analysis

Plasma concentrations *versus* time data for commercial ACV sodium and ACV NPs in individual rabbits were analyzed by non-compartmental estimations using Kinetica software (version 4.4.1). The area under the plasma concentration-time curve $AUC_{(0-\infty)}$, area under the moment concentration-time curve $(AUMC_{(0-\infty)})$ and mean plasma residence time $MRT_{(0-\infty)}$, expressed by the following equations were calculated by the trapezoidal rule plus monoexponential extrapolation to infinity using the same pharmacokinetic program.

$$AUC_{(0-\infty)} = \int_0^\infty Cpdt \quad (2)$$

$$AUMC_{(0-\infty)} = \int_0^\infty (Cp \times t)dt \quad (3)$$

$$MRT_{(0-\infty)} = AUMC_{(0-\infty)}/AUC_{(0-\infty)} \quad (4)$$

Statistical Analysis

All statistical analysis was undertaken using ANOVA test followed by Fisher's PLSD (pairwise least significant difference) for multiple comparisons at $p \leq 0.0001$ with Stat view statistical software program.

RESULTS AND DISCUSSION

All NPs prepared showed discrete spherical appearance (Fig. 1) with mean diameters in the range of 190 to 765 nm (Table II). The polydispersity index (PI) varied from 0.1–0.13, indicating a narrow particle size distribution.

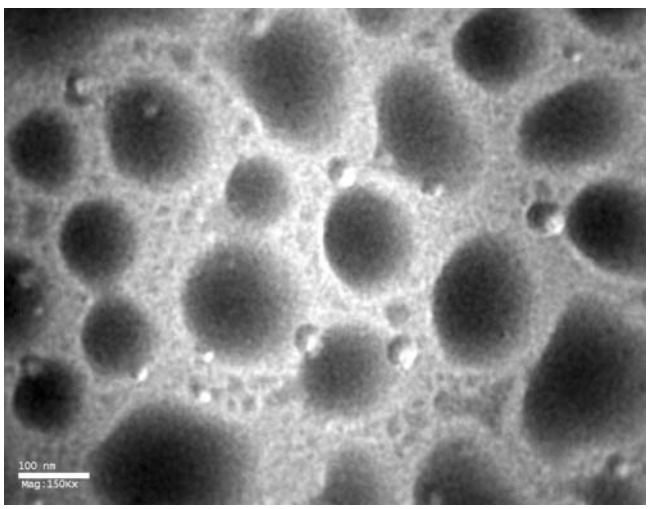

Fig. 1. TEM photomicrographs of ACV-loaded NPs prepared by SESD (×150,000)

Table II. EE, Particle Diameter, and Zeta Potential of the Different Formulas Prepared by SESD

Formulation	EE%±SD	Particle diameter (nm)±SD	Zeta potential±SD
1	15.71±1.66	290±14.1	−16.6±2.4
2	20.36±1.05	350±28.2	−16.6±5.4
3	26.14±2.86	270±42.4	−16.5±0.9
4	40.32±2.52	620±14.2	−14.8±1.5
5	33.71±2.34	655±49.5	−14.7±2.4
6	34.95±0.43	765±12.0	−14.7±1.4
7	32.92±1.45	285±21.2	−9.3±1.8
8	28.97±6.76	220±28.3	−9.2±2.5
9	36.77±1.05	190±14.13	−9.1±2.7
10	18.36±1.90	265±35.4	−18.4±1.1
11	21.64±0.12	230±84.9	−18.2±0.9
12	23.57±3.43	250±70.7	−17.8±0.5
13	40.39±0.60	715±17.7	−15.5±1.4
14	43.61±1.87	655±21.2	−15.5±2.2
15	34.86±2.33	625±35.4	−15.4±1.2
16	19.33±0.03	310±14	−12.2±2.9
17	26.60±1.88	190±14.4	−11.9±1.4
18	27.98±2.42	190±14.2	−11±3.2
19	16.96±0.23	260±28.3	−17.6±0.5
20	32.83±6.44	305±35.4	−17.1±0.9
21	24.27±5.85	220±28	−16.8±0.6
22	39.56±3.13	560±56.6	−15.4±3.3
23	35.91±0.71	290±14.1	−15.3±0.5
24	25.12±0.67	455±30.4	−14.9±5.1
25	14.72±1.11	270±42.4	−10.1±0.2
26	14.02±1.19	285±12	−10.1±1.3
27	14.14±2.90	265±35.3	−9.7±1.2
28	30.27±2.49	290±14.13	−22.1±3.5
29	33.98±1.84	305±35.3	−21.4±7.5
30	24.00±1.89	270±42.4	−19.2±2.5
31	48.38±4.37	430±14	−16.1±1.9
32	36.13±3.60	325±35	−16.1±1.8
33	32.68±2.18	245±63.6	−15.5±4
34	32.17±3.57	265±21.2	−13.3±1.6
35	36.52±1.82	190±14.5	−12.7±1.7
36	30.67±1.46	270±42.4	−12.5±2.4

ANOVA showed that for ACV NPs particle size, the main effects of polymer and surfactant types were significant ($p \leq 0.0001$) while that of surfactant concentration was not.

Figure 2a shows that the particle size of the nanospheres prepared with different polymers can be arranged in ascending manner: 288<323<381<405 for the respective polymers 50/50 PLGA<75/25 PLGA<85/15 PLGA<DL-PLA. The smallest particle size given with the lowest MW polymer (50/50PLGA) can be due to the influence of the copolymer MW on the viscosity of the organic polymer solution.

Figure 2b shows that PVA gave higher particle size (528 nm) compared to with Tween 80 (275 nm) and poloxamer 407 (244 nm). The relatively lower value obtained with poloxamer 407 can be attributed to its relatively higher HLB value (HLB=18). The surfactants with high HLB are better o/w emulsifier thus stabilizing the aqueous phase and reducing the size of the particles (22).

The EEs of various formulas shown in Table II ranged from 14.02% to 48.38%. ANOVA of the EE values of all the tested formulas showed that the polymer and surfactant type and surfactant concentration were significant ($p \leq 0.0001$), but

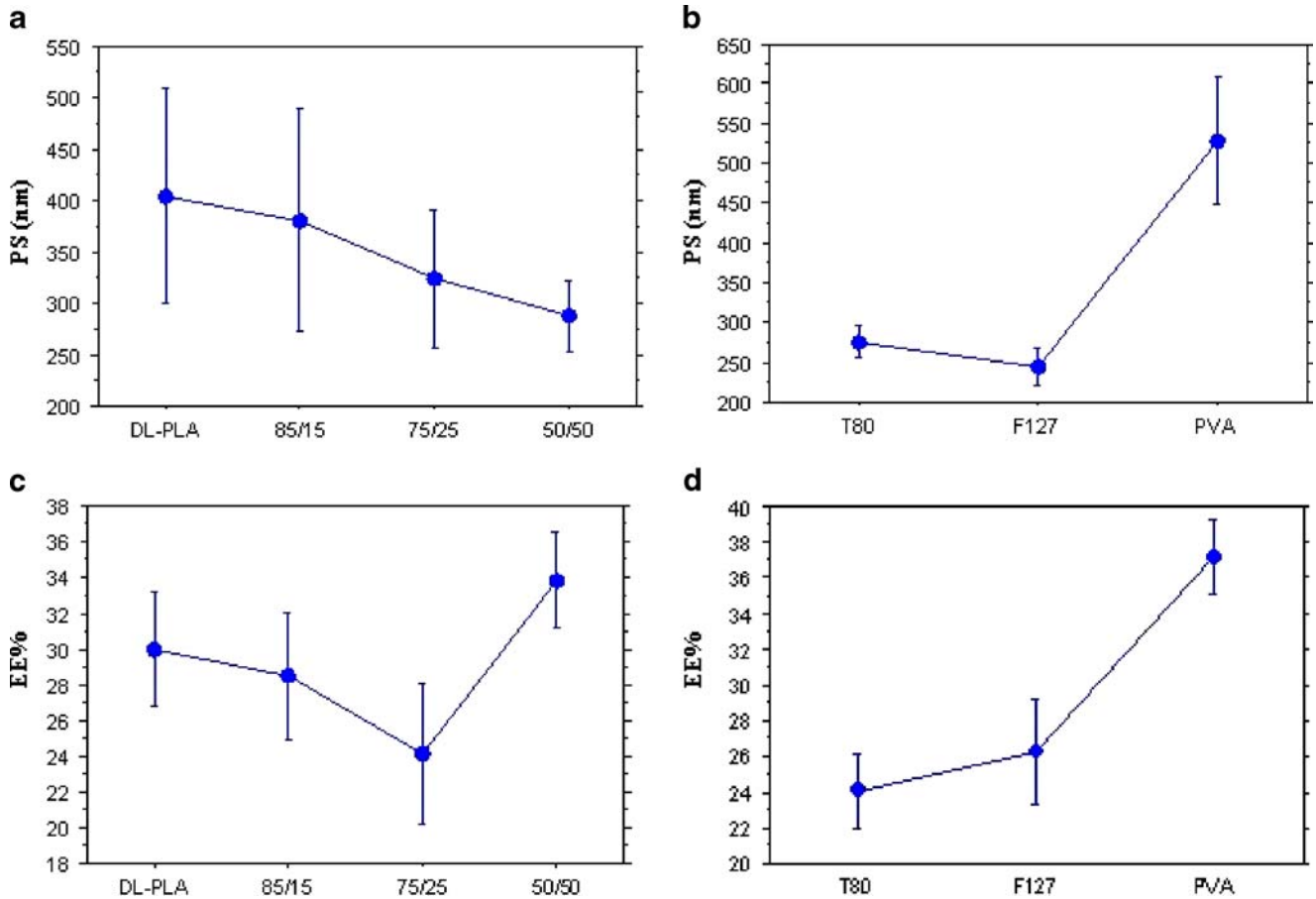

Fig. 2. Plots for the main effect of the different factors: **a** Polymer type on particle size. **b** Surfactant type on particle size. **c** Polymer type on EE%. **d** Surfactant type on EE%

not the surfactant concentration. For the polymer type (Fig. 2c) the EE increased significantly from 24.169% for 75/25DL-PLGA to 30.019% for DL-PLA. It is claimed that the EE depends mainly on the lactide/glycolide ratio of polymer; the higher the lactide ratio, the higher the EE (23). Unexpectedly, 50/50 showed the highest EE (33.866%) although it has the lowest lactide/glycolide ratio. This might be due to the increased polymer hydrophilicity enhancing its affinity towards the hydrophilic ACV.

As for the surfactant type, it is obvious from Fig. 2d that PVA shows the highest significant effect on EE (37.162%) followed by poloxamer 407 (26.232%) and finally Tween 80 (24.036%). Poloxamer 407 with HLB 18 and Tween 80 with HLB 15, have the tendency to solubilize the drug in the aqueous phase hence reducing the EE.

Zeta potential results (Table II) show that all formulations exhibited a negative charge with values ranging from −22.1 to −9.1 mv. This negative value was due to the presence of terminal carboxylic groups in the polymers. Compared with plain PLA or PLGA nanoparticles with zeta potential around −49.2 mv (24), a decrease in zeta potential was likely to occur due to a possible ionic interaction between carboxylic terminal group in the polymer and amino group in ACV.

Figure 3 shows that a marked decrease in zeta potential was observed with NPs prepared with poloxamer 407. This might be due to poloxamer adsorption on the NPs surface forming a coating layer that shield the surface charge and move the plane of shear outwards from the particle surface (20).

In Vitro Drug Release

Figure 4 shows the release behavior of ACV from NPs of the four tested copolymers using 1% Tween 80 as a representative for the release of all the prepared NPs. The release is characterized by an initial burst effect of approximately 12.91% to 37.26% in the first hour followed by a slower exponential release of the remaining drug over the next 48 h. The rapid initial release may be either due to surface drug or to a part of it lying beneath the surface of the NPs dissolving faster than drug embedded in the

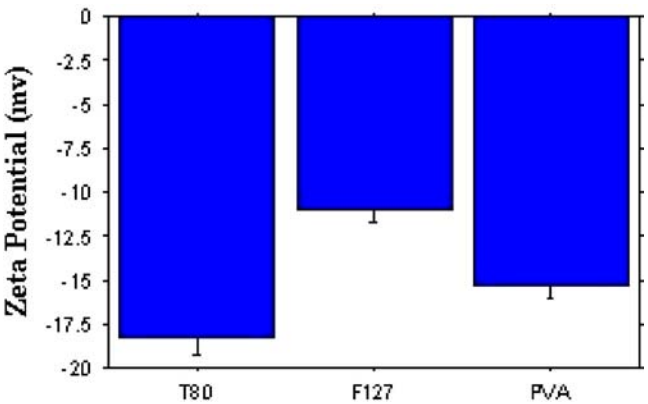

Fig. 3. Effect of surfactant type on zeta potential

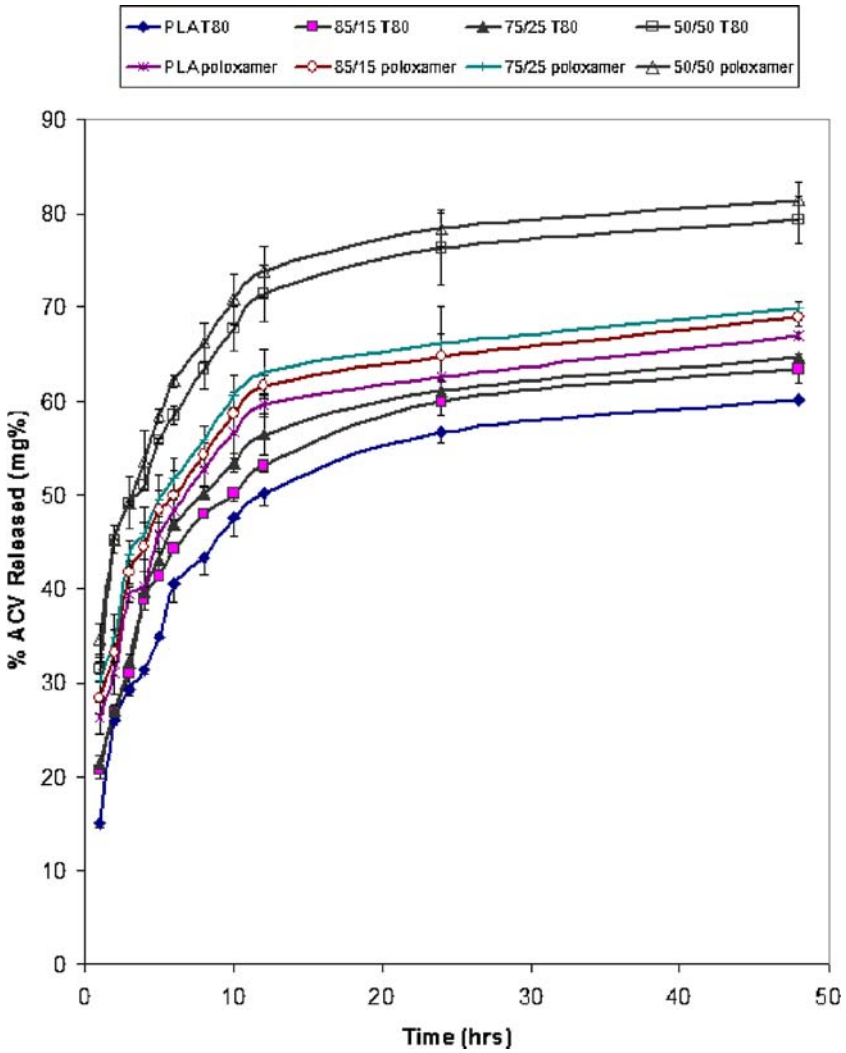

Fig. 4. Release profile of ACV-NPs prepared using 1% Tween 80 and 1% poloxamer 407

center of the spheres. The dissolution medium enters the sphere through pores, dissolving the drug. A saturated drug solution will be formed from which drug diffuses slowly into the surrounding medium. Further drug will diffuse through the polymer into the water-filled channels until drug depletion from the NPs. The small size of the NPs is also a major factor which influences the burst release effect.

Figure 4 shows also that the presence of poloxamer 407 in the NPs enhanced the release of ACV compared to those prepared using Tween 80 and PVA, where a higher burst effect was always noticed in all tested polymers. As an example, 75/25 PLGA released 30.30% in the first hour using 1% poloxamer 407 compared to 21.23% and 21.41% in case PVA and Tween 80, respectively, with the same concentration.

The polymer composition (ratio of lactic to glycolic acid moiety) and its molecular weight influenced the pattern of ACV release from nanospheres. ACV was released faster from nanospheres prepared using 50/50 PLGA polymer containing higher fraction of glycolic acid moiety and is of lower molecular weight compared with other polymers. Rate of hydration of polymeric materials depend on water uptake of the polymer. Lactic acid polymer because of the methyl group is more hydrophobic than glycolide polymer leading to lower water uptake. The water uptake increases as the glycolide ratio in copolymer increases (25) and hence PLGA 50/50 showed faster release compared with PLA, PLGA 85/15, and PLGA 75/25.

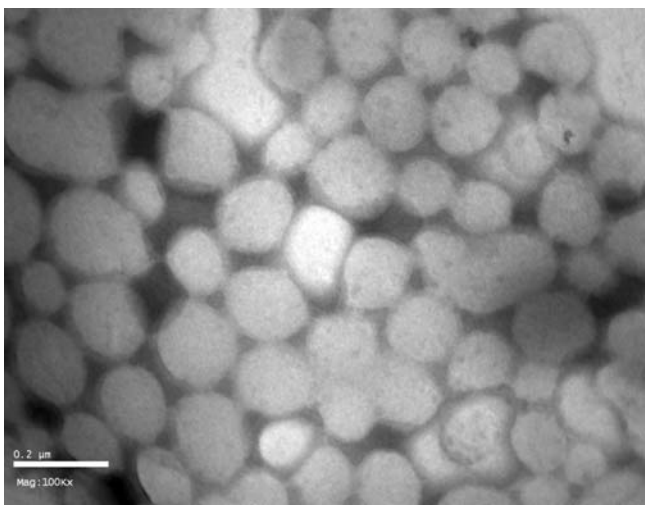

Fig. 5. TEM photomicrographs of 75/25 PLGA ACV NPs using 1% tween 80 and coated with poloxamer 407

Table III. Effect of Different Poloxamers on the Coating Layer Thickness of ACV NPs

Formula	Diameter of uncoated NPs (nm)±SD (polydispersity index)	Adsorbed layer thickness (nm) Coating substance(Poloxamer) 188	338	407
4	625±65 (0.8)	ND	ND	ND
14	640±60 (0.3)	ND	ND	ND
20	300±10 (0.6)	1.65	5	8.3
22	570±15 (0.3)	ND	ND	ND
23	280±10 (0.5)	1.5	6.1	6.65

ND not detected

Effect of Coating on ACV NPs

Figure 5 shows the TEM photomicrographs of formula 20C (75/25 PLGA with 1% Tween 80 and coated with poloxamer 407) as a representative of the coated NPs. NPs appeared brighter than the uncoated ones (26).

Coating with the different block copolymers *viz* poloxamer 188, 338, and 407 caused nonsignificant change in the EE of all tried formulas at $p \leq 0.001$ (results not shown).

Table III shows the coating layer thickness of the different poloxamers used. All NPs with large particle diameter showed undetectable coat layers. However, the thickness of the poloxamers layers in smaller particles (280 and 300 nm) was in the range 1.5–8.3 nm. These values were somewhat lower than those reported for the same block copolymers adsorbed to polystyrene particles (10–15 nm) (27). The difference can be attributed to the different nature of the NP surfaces. In general, during coating, the block (PPO) of the polymer will be attached to the polymer surface while (PEO) blocks will extend into the dispersion medium. A block copolymer such as poloxamer adsorbed to a NP surface will exist in different conformations that will depend on the packing of the polymer at the surface. A close packing of the polymer molecules at the surface will result in an extended polymer "brush" arrangement which will provide a greater adsorbed hydrodynamic thickness than less closely packed polymer chains. Accordingly, the more hydrophobic the NP polymer is, the more it attracts PPO and is better coated than less hydrophobic one. Hence polystyrene particles being hydrophobic in nature had a thicker coat than PLGA particles (more polar) (27).

Table IV. Effect of Coating with Different Poloxamers on Zeta Potential of NPs

Formula	Zeta potential (mv)±SD Uncoated	Coating substance (Poloxamer) 133	338	407
4	−14.6±3	−14.5±1.6	−14.4±1.6	−14.4±1.6
14	−15.2±1.6	−15.6±1.6	−15.7±1.4	−15.9±1.6
20	−17.6±1.8	−7.6±0.8	−6.6±1.4	−6.5±1.6
22	−14.2±1.7	−14.2±4	−14.3±1.6	−14.6±1.2
23	−15.1±3.5	−7.7±0.6	−6.9±4.8	−6.8±0.4

Both copolymers poloxamer 338 and 407 gave thicker layers compared with 188, this might be due to the higher molecular weights hence higher content of their poly(oxyethylene) chain compared with poloxamer 188 providing

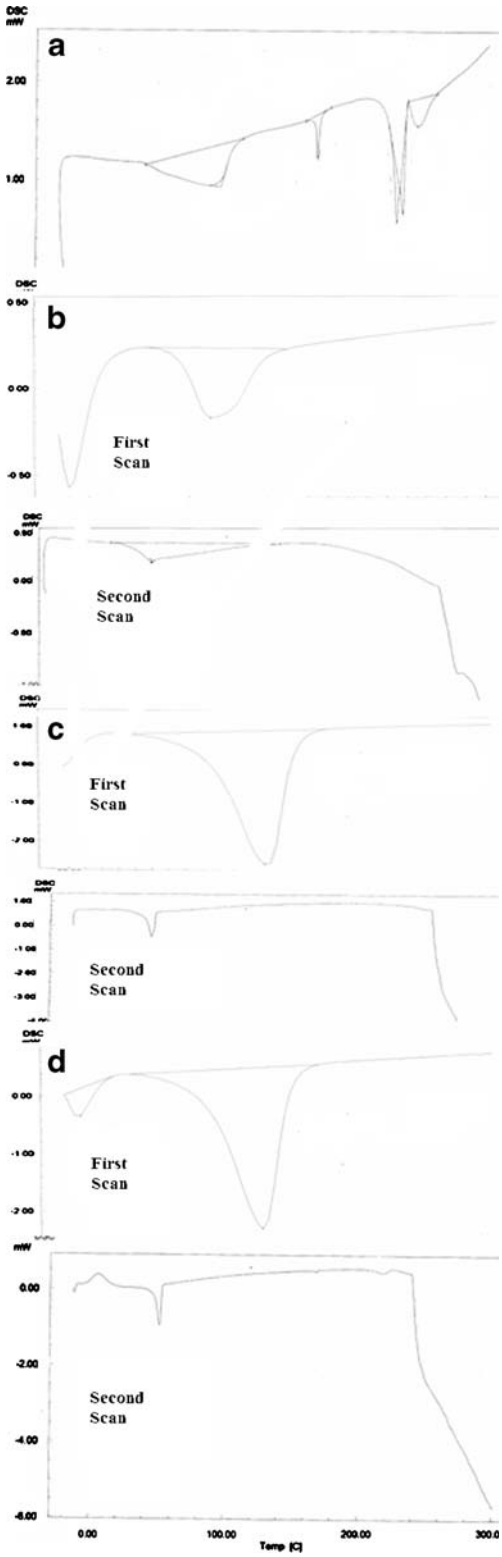

Fig. 6. DSC thermograms during heating from room temperature to 80°C in the first run and during heating from 0°C to 300°C in the second run. **a** ACV. **b** Polymer. **c** Unloaded NPs. **d** Loaded NPs

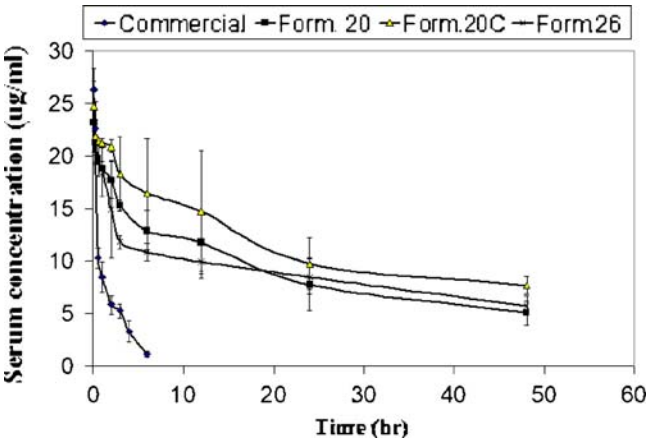

Fig. 7. Mean plasma concentrations—time profiles of ACV from different formulas after i.v. administration

greater adsorbed hydrodynamic thickness and expected reduction in the opsonization effect. Gref *et al.* (28) optimized the thickness and density of a PEO coating at the surface of biodegradable PLA nanoparticles, in order to reduce simultaneously surface charge, plasma protein adsorption, and interaction with phagocytic cells. They observed a sharp decrease in the protein adsorption upon increasing the molecular weight of the polyether chains.

The presence of adsorbed coating layers of poloxamer 188, 338 and 407 on both formulas 20 and 23 was reflected in their zeta potential results (Table IV) where a reduction was seen in the zeta potentials after coating. This might be because of a higher concentration of surfactant adsorbed on the particle surfaces with the formation of a denser surfactant film on the NP surface, thus, eliciting a reduced electrophoretic mobility, and a higher absolute potential value ensures a high-energy barrier that stabilizes the nanosuspension (29). The unchanged zeta potential of the large particles (formulas 4, 14, 22) confirmed the undetectable coat thickness of these formulas.

Coating with any of the three block copolymers enhanced the drug release especially after the first hour (results not shown). This might be due to the greater hydrophilic character of the coated surface leading to an easier penetration of the release medium to the particle and hence faster drug release.

From the previous results it is obvious that the uncoated Formula 20 made from 75/25 PLGA with the use of 1% Tween 80 and formula 20C (75/25 PLGA with 1% Tween 80 and coated with poloxamer 407) had suitable diameters (300 and 316.6 nm, respectively), and optimum drug release (64.26 and 68.56 after 48 h, respectively). Besides the block copolymer, poloxamer 407 gave thicker coat layer (8.3 nm) compared to other block copolymers. Hence they were chosen for further studies. For comparison purpose, formula 26 which was prepared from the same polymer (75/25 PLGA) and 1% poloxamer 407 internally incorporated during fabrication was also chosen in order to compare between the effect of coating by adsorption and by incorporation methods.

DSC Studies

Figure 6 shows the DSC thermograms of 75/25 PLGA, unloaded NPs and ACV-loaded NPs (formula 20) during the first and the second heating runs. The DSC thermogram of ACV showed a small endothermic peak at 171.77°C, a broad endotherm at 98.44°C and a sharp narrow melting endotherm at 236.78°C. The last two endotherms corresponds to loss of residual water and melting temperature of the drug, respectively. So according to Sohn and Kim, the used drug might be the hydrate form (30). In the thermogram of ACV NPs, the endothermic peak of ACV disappeared indicating the absence of any crystalline drug materials in the NPs. This might be due to the polymer inhibiting crystallization of ACV during NP formation. So, it can be concluded that ACV in the NPs was in an amorphous or a solid solution state in the polymer matrix after the fabrication. The Tg of the polymer was shifted from 49.15°C in case of raw polymer to 51.28°C after fabrication of unloaded NPs. This might be due to the reduction in molecular mobility of the polymer. One possible explanation for this reduction may be the restriction of PLGA molecular mobility at the surface of particles magnified by the higher surface to volume ratio of the nanometer scale particles (31). The Tg of the polymer was further shifted

Table V. Pharmacokinetic Parameters of ACV After IV Administration of Different Formulas

Parameter	Mean value±SD			
	Commercial	20	20C	26
C_{max} (μg/ml)[a]	26.37±1.96	23.22±2.94	24.73±2.41	23.15±1.91
$AUC_{(0-6)}$ (μg/ml.h)	36.24±5.36	–	–	–
$AUC_{(0-48)}$ (μg/ml.h)	–	442.64±76.23	564.41±110.92	428.97±58.98
$AUC_{(0-\infty)}$ (μg/ml.h)[b]	38.52±6.11	612.56±51.65	821.69±83.88	803.34±126.79
$AUMC_{(0-\infty)}$ (μg/ml.hr^2)	82.05±18.55	22695.63±2949.7	33562.9±3233.01	50996.61±11333.62
$t_{1/2}$ (h)[c]	1.34±0.09	25.94±5.14	27.55±4.02	45.10±5.10
Clearance (L/h)[d]	0.5284±0.08	0.0328±0.003	0.02451±0.003	0.0252±0.01
MRT (h)[e]	2.11±0.16	37.35±6.66	41.31±7.28	63.22±6.22

All formulas between the same parentheses are not significantly different from each other but differ significantly from those included in other
[a] (Commercial, 20, 20C, 26)
[b] (Commercial), (20, 20C, 26)
[c] (Commercial), (20, 20C), (26)
[d] (Commercial), (20, 20C, 26)
[e] (Commercial), (20, 20C), (26)

to 52.38°C after ACV encapsulation. This might be due to an antiplasticizing drug effect (32).

Effect of Sterilization by Gamma Irradiation on NPs

Sterilization did not affect the EE, where no significant difference between unsterilized and sterilized formulas at $p \leq 0.001$ was seen (results not shown). The mean diameters of sterilized NPs irradiated with the use of dry ice and non sterilized ones were not significantly different at $p \leq 0.01$.

There was no significant difference ($p \leq 0.001$) in the burst effect between sterilized and unsterilized particles. Furthermore, their release profiles were similar with f_2 values 85.6, 80.34, and 89.49 and f_1 values 3.39, 4.26, and 2.27 for uncoated formula 20, coated one (20C) and formula 26, respectively.

In Vivo study

Commercial ACV sodium caused severe thrombophelebitis in the rabbit's ear, while ACV NPs showed no inflammation or phelebitis but only slight redness at the site of injection.

The mean plasma concentrations *versus* time profiles of all tested formulas are illustrated in Fig. 7. ACV commercial solution after administration gave plasma concentration of 26.37 and 22.61 μg/ml at 0.083 and 0.25 h, respectively. These values were insignificantly higher than their corresponding values achieved after administration of ACV NPs ($p<0.001$). However, starting from 0.5 h, the ACV plasma concentration produced by NPs were significantly higher than those produced by the commercial solution.

Figure 7 shows that in case of IV administration of ACV commercial product, the drug disappeared from the circulating blood in less than 6 h. In contrast, ACV entrapped in NPs showed a long circulating time which extended up to 48 h. The drug levels of the NPs of formula 20C were higher than those of formulas 20 and 26. However, these later showed extended circulation time. This might be due to the influence of the surfactants used internally during their preparation. According to Araujo *et al.* (33), poloxamers were considered very effective in increasing blood circulation time and reducing liver uptake, whereas Tween 80 was a potent substance to target particles to organs that do not belong to the reticuloendothelial system.

The comparative pharmacokinetic parameters after IV administration of ACV formulas are reported in Table V. The plasma pharmacokinetic parameters of the three tested ACV NPs were significantly enhanced ($p<0.001$) compared with the commercial ACV solution, except C_{max}. The $AUC_{(0-\infty)}$ of formulas 20, 20C, and 26 were 612.56, 821.69, and 803.34 μg/ml.h, respectively compared to 38.52 μg/ml.h for the commercial ACV. The $t_{1/2}$ of formulas 20, 20C, and 26 were 25.94, 27.55, and 45.10 h, respectively, while for commercial solution was 1.34 h. The clearance of ACV was not significantly different whether it was delivered from formulas 20, 20C, or 26; however, the commercial solution gave significantly higher clearance than the three NP formulas. A significant increase in the MRT values of the three tested NPs compared to the commercial solution was also observed, where the respective MRT of formulas 20, 20C, and 26 increased with about 18-, 20-, and 29-fold in plasma respectively compared to the same dose of commercial solution. Possible explanation for the higher AUC, elimination $t_{1/2}$ and MRT of ACV-NPs was the slower release of ACV from the biodegradable NPs which led to lower clearance.

The MRT of formula 26 was significantly higher ($p<0.001$) than those of formulas 20 and 20C. The significantly higher MRT noticed with formula 26 in comparison to formula 20C showed the superiority of the quality of NP when poloxamer was incorporated during the preparation than when it was used as a coat only. This might be due to possible adsorption of the surfactant on the NP surface during processing in a manner similar to coating. Moreover, the presence of poloxamer in the core of the particle offered further protection against adhesion of opsonins after the surface polymer degradation.

The relatively higher MRT observed with formula 26 in comparison to formula 20 with respective values 63.22 and 37.35 hrs proved the superiority of poloxamer compared to tween 80 for extending blood circulation of ACV NPs.

The nanometric polymeric particles covered by a layer of PEO chains can prevent the physiological defense processes stimulated by intravenous injections from being triggered, which accounts for a longer residence time observed in the systemic circulation. In this respect, the lifetime in the blood stream was increased.

CONCLUSIONS

In vivo pharmacokinetic studies proved ACV biodegradable polymeric NPs to be a safe parenteral sustained release drug delivery system for IV administration.

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