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Preparation, Characterization and *In Vitro* Evaluation of Etoposide Loaded PCL Nanoparicles

Riddhi M. Dave*[1], Rakesh K. Patel[2]
1 S.K.Patel Colleges of Pharmaceutical Education and Research, Ganpat University, Kherva-. Gujarat, INDIA

ABSTRACT

The present investigation involves the Preparation and Characterization of etoposide loaded PCL Composite micropartices on account to control initial burst release. The prepared composite particles were characterized physicochemically for Encapsulation efficiency, Mean particle size, Release kinetic and compared with nanoparticles and simple microparticles prepared by the same double emulsion method. The major objective of the present study is to incorporate a hydrophilic drug etoposide within hydrophobic polymer poly (ε-caprolactone) for the preparation of composite micro particles to minimize initial burst release of the drug which is generally associated with micro and nanoparticles. Micro particles and nanoparticles were prepared by W/O/W emulsion solvent extraction and W/O/W solvent evaporation method respectively using different ratios of drug to polymer (0.1:1, 0.2:1 and 0.4:1). These prepared nanoparticles were further fabricated in micro particles using double emulsion method in ratio of (0.05:1, 0.1:1, 0.2:1).When PCL nanoparticles were encapsulated into the microparticles, there was a large decrease in the burst release again; this decrease is much more marked when $p < 0.05$. When nanoparticles formulated in to composite micropartices the burst released is suppressed only 50% of the drug was released in 8 hrs. Therefore, the advantage of encapsulating nanoparticles in microparticles (composite microparticles) has been definitely demonstrated for a hydrophilic drug.

Keywords: Ex-Vitro, Microparticles, Nanoparticles

*Corresponding Author Email: riddhi_dave2002@yahoo.com
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INTRODUCTION

Several techniques can be used to prepare polymeric micro particles, providing controlled drug delivery[1]. Most commonly, organic solvent evaporation and/or extraction methods are applied. Depending on the solubility of the drug, simple or multiple emulsion techniques, e.g. oil-in-water (O/W) and water-in-oil in-water (W/O/W) methods, are used[2]. The popular method for the encapsulation of water-soluble drugs within water insoluble polymers is the double-emulsion solvent diffusion method[3]. In these works, the water soluble drug etoposide is dissolved in water, and this solution is emulsified in an organic solution of the polymer to be used for the wall material. This primary emulsion is then emulsified in an aqueous phase to form a W/O/W emulsion. The organic solvent diffuses into the external water phase and evaporates at its surface. The main disadvantage of this method is its limited ability to encapsulate hydrophilic drugs, as partitioning into the aqueous phase of the emulsion readily occurs[3, 4]. A further effect of partitioning is the accumulation of drug crystals on the surface of micro particles, which produce a burst release of the drug upon administration[5,6]. Burst release is often observed with microparticulate systems; it is unpredictable and generally difficult to control, but may be prevented by changing the drug distribution within the polymer matrix [7] or by developing more sophisticated drug delivery systems. Examples of the latter are liposomes encapsulated inside dextrin[8], and alginate microcapsules, allowing the release of the drug in a controlled way and eliminating the burst effect[9]. Double-walled micro particles[10], double-layered minipellets[11], and coated micro particles [12,13] have all been developed to reduce the initial burst and provide sustained release profiles of the drug. Indeed, the release of ibuprofen from microparticles prepared with a blend of ethylcellulose and polystyrene was prolonged over 24 h with a reduced burst, compared with microparticles prepared with ethylcellulose alone[14]. Recently, solvent evaporation methods were developed to incorporate a hydrophilic drug within biodegradable, poly (-ε-caprolactone)-based microparticles[1]. The aliphatic semi-crystalline polyester, poly-ε-caprolactone (PCL), has been used in the field of controlled drug release. When used alone, PCL produces controlled release over extended periods of up to 1 month[15]. However, due to its hydrophobic and semi-crystalline nature, the degradation of PCL is much slower than established polymers based on poly (lactic acid) (PLA) derivatives. PCL polymer tends to produce drug-loaded microparticles with an initial burst drug release. The degradation of these polyesters involves a bulk erosion process[5, 16 ,17]. The size and release properties of microparticles are key considerations when designing microsphere delivery systems[5], since the release kinetics of the

drug dominantly depend on the polymer nature. The physical states of the polymer and drug (e.g. crystalline, amorphous, glassy, rubbery, and molecularly dispersed) are of major importance for the underlying drug release mechanism[1,2]. The morphology and drug distribution within microparticles and a fundamental understanding of the relationship between these key characteristics and release mechanisms is essential to yield useful products[5]. For example, within an amorphous polymer, the diffusion coefficient of a drug is much higher, compared to that within a crystalline polymer[1,2].

MATERIAL AND METHODS

Materials

Etoposide was obtained as a gift samples from Astron Research Ltd. Ahmedabad. Poly (ε-caprolactone) (MW 40,000 Da), an acrylic polycationic non biodegradable polymer (copolymers of acrylic acid esters with a low content of quaternary ammonium groups 0.5-0.8%) (4.48-6.77% ammonium methacrylate units by dry weight), Ethylcellulose powder (viscosity 7 Cp) and Polyvinyl alcohol (PVA) (Mw 95000-110000 Da), were supplied by Aldrich, USA. Ethyl acetate, Methylene chloride, Buffer phosphate and Buffer phosphate saline (pH 7.4) were obtained from Merck (Darmstadt, Germany). All solvents and reagents were of analytical grade.

Preparation of Simple Microparticles

Etoposide-loaded ethyl cellulose microparticles were prepared by the $W_1/O/W_2$ emulsion solvent extraction method using different ratios of drug to polymer (0.1:1, 0.2:1 and 0.4:1). [7, 18] In the first step (W1/O emulsion), an aqueous solution (1 ml) of the drug used as the internal aqueous phase was emulsified into an organic solution of the polymer (ethylcellulose 250 mg) in ethyl acetate (5 ml) by a homogenizer with 22000 rpm. After 2 minutes, the primary emulsion was poured into 20 ml of 0.1% PVA aqueous solution in order to obtain a $W_1/O/W_2$ pre-emulsion. After magnetically stirring for 1 min (1000 rpm) at room temperature, this pre-emulsion was added to 400 ml of a 0.1% PVA aqueous solution and stirred mechanically (three blended propeller, 1600 rpm) for 10 min to form the final $W_1/O/W_2$ emulsion and allow microparticles hardening. Blank microparticles (without the drug) were prepared under the same conditions as without the drug. Microparticles were collected by vacuum filtration (Heidolph, USA) and freeze-dried.[18]

Preparation of Nanoparticles

Etoposide-loaded PCL were prepared by the $W_1/O/W_2$ solvent evaporation method using different ratios of drug to polymer (0.1:1, 0.2:1 and 0.4:1). Briefly, 1 ml of aqueous internal

phase was emulsified for 15 minutes in 5 ml of methylene chloride (containing 125 mg of PCL and 125 mg ethylcellulose) using a homogenizer with 22000 rpm. This primary emulsion was poured into 40 ml of 0.1% PVA aqueous solution while stirring using a homogenizer for 1 min, under the same conditions, in order to create the water in the oil-in-water emulsion. Three to four ml of NP suspension was obtained after solvent evaporation under reduced pressure (Evaporator, Heidolph, USA). Nanoparticles were separated from the bulk suspension by centrifugation (Hettich universal 320R, USA) at 42,000 x g for 20 min. The supernatant was kept for drug assay, as described later, and the sediment nanoparticles were re-dispersed in 3ml of purified water before freeze-drying. After lyophilization, the dried nanoparticles were re-suspended in 2ml of purified water shortly before preparing the composite microparticles. Blank nanoparticles (without the drug) were prepared under the same conditions without the drug.[18, 19]

Preparation of Composite Microparticles

For the preparation of composite microparticles in the primary emulsion (W_1/O), PCL and ethylcellulose nanoparticle suspension (2 ml), used as the internal aqueous phase (instead of 2 ml of drug aqueous solution), was emulsified in an organic solution of polymer in ethyl acetate. Blank composite microparticles (without drug) were prepared under the same conditions without drug.[18]

Determination of Loading Efficiency and Production Yield (%)

The encapsulation efficiency of the PCL nanoparticles was defined as the percentage of etoposide encapsulated in respect to the total amount of etoposide used to prepare the PCL nanoparticles. The amount of etoposide entrapped within the PCL nanoparticles was determined by measuring the amount of non-entrapped etoposide by RP-Phased HPLC quantification that was recovered in the supernatant after ultracentrifugation and wash the etoposide loaded PCL nanoparticles for three times at 15000 rpm for 30 min. Each sample was assayed in triplicate and the results were reported as average ± S.D. The loading efficiency (%) was calculated according to the following equation:

Loading efficiency (%) = (actual drug content in microparticles/theoretical drug content) x 100

The production yield of the microparticles was determined by calculating accurately the initial weight of the raw materials and the final weight of the polymeric particles obtained. All experiments were performed in triplicate.

Particle Size Distribution Characterizations

The particle size and particle size distribution of the etoposide-loaded PCL nanoparticles were determined using a laser diffraction particle size analyzer (The Shimadzu SALD-3101, Japan).

Surface Morphology

The surface morphological examinations of the etoposide-loaded PCL nanoparticles were performed using a scanning electron microscope (JSM 5610 LV SEM, JEOL, Datum Ltd, Tokyo, Japan).

Differential Scanning Calorimetry (DSC)

Typically, about 5mg of the sample were weighed into an aluminum pan, which was crimped non-hermetically, and heated in the differential scanning calorimeter (DSC 60, Shimadzu, Japan) from 30 to 300 ^{o}C at a rate of 10 ^{o}C per min.

In Vitro Release Study

The in vitro release profiles were studied by dialysis tube diffusion method using phosphate buffer saline at pH: 7.4, as a release medium. The in vitro release experiment was carried out as follows: 15 mg of etoposide loaded PCL nanoparticles and 5 ml Phosphate buffer saline (PBS) (pH: 7.4) was placed into dialysis diffusion bag that immersed into 100 ml PBS solution and the system was placed in a orbital shaker bath, which was maintained at 37±0.1^{o}C and shaken horizontically at 100 rpm/min. At predetermined intervals, aliquots of the release medium (5 ml) was taken out and assayed for drug release and replaced by 5 ml of fresh buffer at each sampling point and agitation was continued.[20] Etoposide release was quantified at 254 nm by reversed phase HPLC method. While at the same wavelength there was no interference of PCL observed. All release experiments were performed in triplicate.

RESULTS AND DISCUSSIONS

Determination of Loading Efficiency

An important prerequisite for high encapsulation efficiency by the W/O/W method are:

1. The insolubility of the drug in the external phase from the internal aqueous phase,

2. The fine dispersion of the aqueous drug solution into the organic polymer solution to form a W/O emulsion [21], In order to obtain a fine dispersion, the aqueous etoposide solution was added to the organic phase.

To ensure the high entrapment efficiency of water-soluble drug a hydrophobic processing medium is used, into which the hydrophobic macromolecules are unlikely to migrate. It is assumed that etoposide is concentrated at the interfaces (either internal water in oil or external oil in water) and hence a significant amount of the drug is assumed to be adsorbed at the outer surface. With increasing drug concentration, saturation of the outer surface is obtained. The encapsulation efficiency of the drug depends on the solubility of the drug in the solvent and

continuous phase. In addition, the removal of the organic solvent under reduced pressure favors its fast evaporation, followed by the polymer precipitation, thus reducing the migration of the drug to the external phase. Indeed, the faster the solvent is evaporated, higher the encapsulation efficiency will be.

In all formulations, the mean amount of drug entrapped in prepared nanoparticles and composite microparticles was near to the theoretical value, since the drug loading efficiency is almost 100% (Table 1). The encapsulation efficiency of the drug depended on the solubility of the drug in the solvent and continuous phase Youan et al. reported similar observation[22]. The entrapment efficiency of polypeptides was increased by enhancing the viscosity builders[23]. In simple microparticles prepared by the extraction method, the amount of drug entrapped in microparticles was lower than the theoretical value which indicates the loss of some free drug crystals during encapsulation. As the ratio of drug to polymer increased, the amount of free drug loss decreased (Table 1), so that at the ratio of drug to polymer, 0.4:1, the amount of drug entrapment was 32.09%. Using higher amounts of the drug caused a slight increase in the viscosity of the dispersed phase. The entrapment efficiency of polypeptides was increased by enhancing the viscosity builders[21].

Table 1: Effect of Drug/Polymer Ratio on Drug Loading Efficiency, Production Yield And Particle Size Of Etoposide Microparticles, Nanoparticles And Composite Microparticles

Process Variable	Formulation code	Drug: Polymer ratio	Production Yield (%±SD)	Loading Efficiency (%±SD)	Mean particle size (µm±SD)
Microparticle	M1	0.1:1	51.16 ±3.67	67.25± 6.73	35.68 ±0.51
	M2	0.2:1	45.12 ±3.87	76.88± 9.08	19.22 ±0.48
	M3	0.4:1	32.09± 3.08	95.22± 7.89	14.13 ± 0.51
Nanoparticle	N1	0.1:1	60.18 ±7.21	98.12 ±8.98	4.64± 0.56
	N2	0.2:1	57.01 ±3.46	98.21± 6.74	1.56 ± 0.53
	N3	0.4:1	70.03± 5.32	98.24± 6.32	1.06 ± 0.19
Composite microparticle	C1	0.05:1	78.98 ± 1.32	99.17 ±4.23	10.25 ± 0.39
	C2	0.1:1	81.91 ± 3.56	98.28± 6.43	15.22± 0.71
	C3	0.2:1	87.93 ± 6.78	98.24± 7.12	14.05± 0.69

Production yield

The prerequisite to obtain microspheres is that the selected solvent system for the polymer should be immiscible with a non-aqueous processing medium [18] here ethylacetate was an organic solvent which was polar, water miscible and oil immiscible. Poly-ε-caprolactone was dissolved in methylene chloride was used to formulate nanoparticles, whereas ethylcellulose dissolved in ethylacetate, a non-solvent of PCL, was used for the preparation of microparticles. Generally,

increasing the drug-polymer ratio increased the production yield (in the nanoparticles and composite microparticles, Table 1). When the ratio of drug/polymer increased from 0.1:1 to 0.4:1, the production yield was decreased (p < 0:05). The reason for decreased microparticles) could be due to the decreased diffusion rate of solvents (ethyl acetate) from concentrated solutions into initial emulsion.

Particle Size

The size of microparticles was found to be increase with an increase in the concentration of the drug (Table 1). It can be attributed to the fact that with the higher diffusion rate of non-solvent to the polymer solution, a smaller size of microcapsules are easily obtained [24,25]. As can be seen, the sizes of particles were decreased with an increase in the amount of the drug. These results are correlated with those observed by Kumar et al. [18, 26] indeed, they stated that the nanoparticle size decreased with increasing drug concentration in the internal aqueous phase. Comparison of the size of macroparticles, nanoparticles and composite microparticles prepared by W1/O/W2 emulsification with etoposide as a surfactant by stabilizing the first emulsion, and consequently, hampering the fast coalescence of the droplets. The chemical structure of etoposide, and a hydrophilic molecule circled with a hydrophobic acidic group may confer a relative amphiphilicity to the drug, which could behave as a surfactant.

Surface morphology

As shown in the figure 1, the etoposide loaded nanoparticles presented smooth surface without apparent porosity. Moreover, nanoparticles were of good surface morphological characteristics, spherical with smooth surface, no crystals of drug were observed on the surface and without any aggregation homogeneously distributed.

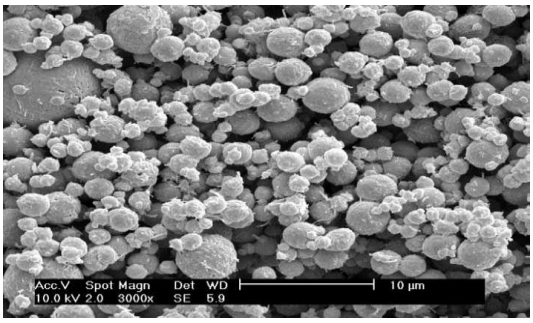

Figure 1: Scanning electron micrograph of etoposide nanoparticles (Batch N3, Etoposide: poly (ε-caprolactone, 0.4:1) Original magnification of electron micrograph was 1000x and scale bar represents a distance of 10 µm.

Drug Polymer Compatibility

Drug-loaded nanoparticles indicated a positive charge, because poly (ε-caprolactone) was a

polycationic polymer and changed the charge of nanoparticles. The endothermic peak of the pure drug was observed at about 280 °C (Figure 2) and ethylcellulose showed an amorphous state. Indeed, in the thermogram of the nanoparticles containing poly (ε-caprolactone), there was endothermic peak at 59 °C. However, in the thermogram of nanoparticles there was endothermic peak of the drug melting, but in the thermogram of the composite microparticles, there was no endothermic peak of the poly (ε-caprolactone). In the thermogram of nanoparticles, there was no endometric peak of etoposide.

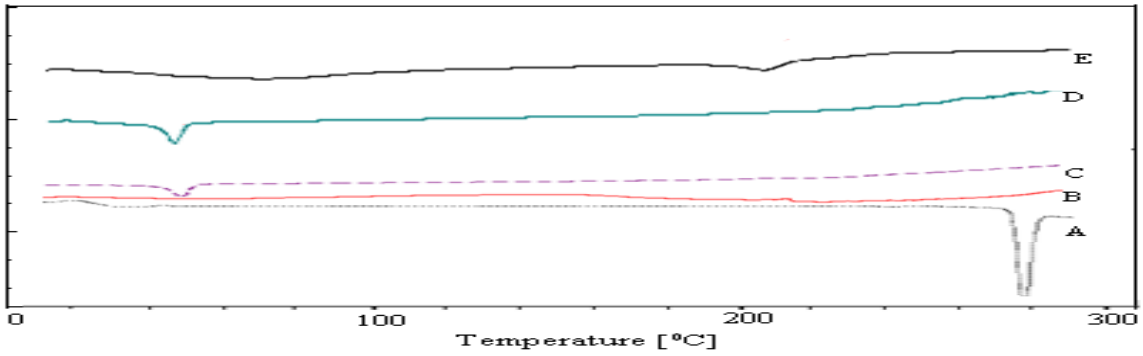

Figure 2: DSC Thermograms of A) Etoposide; B) Ethylcellulose; C) Poly (ε-caprolactone) D) Nanoparticles; E) Composite microparticles

In Vitro Release Profile

The in vitro release profiles of etoposide from microparticles and nanoparticles exhibited in Table 2 also showed an initial burst effect, which may be due to the presence of some drug particles on the surface of the micro and nanoparticles. For microparticles, the dissolution of etoposide at pH 7.4 strongly showed an initial burst effect with a biphasic dissolution profile which ended very early, and for the remaining time, nearly linear behavior was observed. After such a phase, two phenomena can combine in enhancing the diffusion of the remaining dispersed drug into the bulk phase, as well as the formation of pores within the matrix due to the initial drug dissolution; particle wetting and swelling enhances the permeability of the polymer to the drug[27]. The results indicated that some factors, such as drug/polymer ratio, governed the drug release from these microparticles. In order to keep the total surface area of the microparticles constant and, thus get comparable results, the release studies were carried out using the same size fractions of microparticles containing equivalent amounts of etoposide from different batches. Drug release rates were decreased with increasing amounts of etoposide in the formulation. Higher levels of etoposide corresponding to lower levels of the polymer in the formulation resulted in an increase in the drug release rate (M1). As more drugs are released from the microparticles, more channels are probably produced, contributes the faster drug release rates.

However, data shows that the burst effect is lower when the drug/polymer ratio is 0.1:1 (M1) compared with 0.4:1 (M3). In the formulation M3, an increase of the internal phase viscosity, due to the different etoposide concentrations, could reduce the leakage of the drug towards the external aqueous phase and decrease the burst effect (to compare with M1 and M2). PCL-loaded nanoparticles of each formulation displayed an immediate and important initial drug release in the first 15 min, followed by a 40-90% release obtained over 24 h. This immediate high release may be due to the small diameter of nanoparticles leading to a large exchange surface and probably to a more porous structure, owing to the solvent evaporation method, favoring the release of the encapsulated drug[28]. Indeed, it has been already demonstrated that the slow precipitation of microparticles after solvent evaporation leads to more porous particles, compared to the fast polymer precipitation obtained after solvent extraction.[1] Although not all the encapsulated drug was released in 24 h, the dissolution test was limited to this time, because the aim of this research was to demonstrate the influence of the encapsulation of nanoparticles within microparticles on the initial burst release[18].

The initial percent of drug release and dissolution profiles were very different with all types of microparticles, compared with nanoparticles, as shown in table no 2. However, composite microparticles tend to reduce the initial burst effect, especially for microparticles prepared from ethylcellulose used alone (simple microparticles). Reduction in the initial burst effect can be described not only by the rather hydrophilic properties of etoposide, which prefers to diffuse towards the surrounding dissolution in aqueous media, but also to the high encapsulation ratio of PCL nanoparticles[5,18]. The encapsulation of nanoparticles into microparticles also had a strong effect on the dissolution profiles. The presence of ethylcellulose in the matrix of microparticles conferred a slower and more progressive release of etoposide during the time of the experiment [2]. Therefore, any mechanism which is able to restrict this diffusion of etoposide towards water would be easily observed. This is, indeed, due to the slow diffusion of water into the lipophilic ethylcellulose matrix[18, 28].

When PCL nanoparticles were encapsulated into the microparticles, there was a large decrease in the burst release again; this decrease is much more marked when $p < 0.05$, value of P shows that burst release is decreased. Therefore, the advantage of encapsulating nanoparticles in microparticles (composite microparticles) has been definitely demonstrated for a hydrophilic drug[3, 18]. For nanoparticles, the burst was higher than composite microparticles prepared with poly (ε-caprolactone). However, composite microparticles tend to reduce the initial burst effect. Indeed, for the composite microparticles, the total etoposide released was much lower after 24 h.

The presence of ethylcellulose in the matrix of microparticles conferred a slower and more progressive release of drug during the time of the experiment. On the other hand, the effect of burst is much more marked with nanoparticles. Indeed, due to the high hydrophilicity of the drug, this compound has a natural tendency to diffuse very rapidly towards an aqueous phase. The burst effect of simple microparticles was intermediate between nanoparticles and composite microparticles when taking into account the whole 24 h of the experiment. This is probably due to the slow diffusion of water into the lipophilic ethylcellulose matrix.

Table 2: % Drug release from different formulations

Time(hr)	M1	M2	M3	N1	N2	N3	C1	C2	C3
0	0	0	0	0	0	0	0	0	0
1	17	15	12	14	12	10	8	7	5
2	32	30	25	23	21	20	14	12	10
3	45	43	38	35	33	31	24	23	21
4	62	60	50	46	43	41	34	30	25
5	67	65	56	55	51	49	45	42	40
6	72	70	60	63	59	56	50	46	42
8	83	80	75	72	70	68	58	54	50
10	94	90	85	80	79	77	65	62	57
12	99	99	95	90	92	85	70	68	63
16	99	99	99	98	99	91	85	81	75
20	100	99	99	99	99	95	95	90	88
24	100	100	99	99	100	99	99	96	93

CONCLUSION

The advantage of encapsulating nanoparticles in microparticles (composite microparticles) has been definitely demonstrated for a hydrophilic drug etoposite.

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