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PREPARATION AND CHARACTERIZATION OF BIODEGRADABLE PACLITAXEL LOADED CHITOSAN MICROPARTICLES

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At the present study, paclitaxel loaded chitosan microspheres were prepared by an emulsification technique and crosslinked with tripolyphosphate. The effect of chitosan molecular weight and the pH of the crosslinking medium on physicochemical characteristics of the prepared microparticles were investigated. Particle size, size distribution, zeta potential, entrapment efficiency, powder X-ray diffraction, thermal analysis and *in vitro* release profile were determined for each formula. The particle sizes showed a wide range of variability ranging from 1.206 to 8.727 µm depending on the chitosan composition and the pH of the formulations. All formulae showed narrow particle size dispersion except TM7, TH3 and TH7. Zeta potential values ranged between +7.81 to +30.72 mV. Formulae TL5, TM3 and TM5 exhibited the highest three entrapment efficiencies among all the prepared formulae with values equal to 83%, 83.7% and 73.7%, respectively. Both X-ray diffractomy and DSC confirmed the amorphous state of the encapsulated paclitaxel. The release profile showed tremendous enhancement in the cumulative % released for formulae TM3 and TM5 reaching a magnitude around 90%.

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Keywords: Paclitaxel, Chitosan, Microparticles, Emulsification, Characterization

1. Introduction

Paclitaxel (PTX), a naturally hydrophobic diterpeneoid product extracted from the bark of the Pacific yew tree (*Taxus brevifolia*) [1]. PTX is a powerful anticancer drug with special effects against a wide spectrum of cancers, including breast and ovarian cancers, small cell and non-small cell lung cancer, colon cancer, head and neck cancer, multiple myeloma, melanoma, and Kaposi's sarcoma [1-7]. In a unique mechanism of action, PTX enhances microtubule assembly and prevents microtubule deploymerization. This action leads to cell synchronization in G_2/M, and subsequently to cell death by apoptosis [8]. In addition, PTX is a potent anti-cell proliferation agent at low concentrations [5]. However, clinical applications of PTX have been hampered by its extremely poor water solubility (less than 1 µg/mL) [9]. Although PTX is currently used by

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dissolving it in a 50/50 (V/V) mixture of Cremophor EL (polyoxyethylated castor oil)/absolute ethanol, this formulation is known to induce severe side effects such as neurotoxicity, nephrotoxicity and hypersensitivity, in up to 30% of the patients [10–12].

Abraxane, an injectable suspension of albumin-bound paclitaxel nanoparticles, has been marketed lately and shown effective [13-14]. However, the reported bone marrow suppression and neuropathy toxicities were higher compared with the conventional PTX injection [15].

Since PTX is poorly soluble in water and peroral delivery is not effective, it is mainly given by intravenous administration. Many researchers are attempting to formulate PTX in delivery systems using ulternatives to Cremophor EL for better solubility.

These systems include water soluble PTX pro-drugs [16,17], liposome [18-20], microspheres [21], emulsion [22, 23], cyclodextrin inclusion complex [24-26], polymeric micelles [27-29] and polymeric nanoparticles.

The use of microspheres for the delivery of anticancer agents has generated considerable interest. Biodegradable polymers such as poly lactic acid (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) has been extensively studied as a drug transport vehicle in controlled release delivery systems [30]. Kang et al., [30] successfully prepared PTX loaded PLA microparticles using supercritical CO_2. *In vitro* cytotoxicity evaluation indicated that PTX-PLA microparticles had superior antiproliferation activity against the A549 and SKOV3 cell lines, compared with free PTX formulations [30]. PTX loaded poly(lactide)-tocopheryl polyethylene glycol succinate nanoparticles were introduced by Zhang and Feng, [31].

Mita et al. [32] introduced porous hydrophobic PTX microspheres as novel drug delivery system free of Cremophore EL. The system was manufactured by spray-drying of a PTX solution containing volatile salt and water soluble excipients such as mannitol, polysorbate 80, and povidone [32].

Chitosan, the N-deacetylation form of chitin, mostly found in the exoskeleton of crustacean, insects, and fungi, is a natural polysaccharide. It has been recogonized as a promising polymer for drug delivery, more specifically, for the delivery of macromolecules [33]. Chitosan is not only non-toxic and biodegradable with low immunogenicity, but also possesses a high density of positive charge in an acid solution attributed to the glucosamine group on its backbone. In addition, chitosan has a strong mucoadhesive character due to interactions with the mucous membranes associated with epithelial barriers and tumors, a fact that makes it a useful polymer for mucosal drug delivery [31,33]. Because of these beneficial characteristics, increasing attention has been drawn to the applications of chitosan based micro- and nanoparticles in the pharmaceutical and nutraceutical field [35-36]. It has the ability to entrap macromolecules into colloidal systems through different mechanisms, including ionic crosslinking, desolvation, or ionic complexation [33].

This study aims to optimize the loading of PTX in chitosan microparticles as a new formulation that are devoid of Cremophor® EL. The effect of chitosan molecular weight and the pH of the crosslinking medium on the particle size, surface charge, entrapment efficiency, and *in vitro* release profile were investigated.

2. Methods

Materials

PTX was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Low, medium and high molecular weight (M wt.) chitosan were purchased from Fluka BioChemika (Buchs, Switzerland). Tween 80 and Tripolyphosphate (TPP) were obtained from BDH Laboratories (Poole, England). All other reagents and chemicals were of analytical grade and used as received.

Preparation of Paclitaxel loaded chitosan microparticles

Chitosan solution (2.5mg/ml, 25ml) was prepared by dissolving chitosan (low, medium, or high molecular weight) in dilute acetic acid (1% v/v) and Tween-80 was added as a surfactant to

give final concentration of 2% V/V. PTX was dissolved in 2.5 ml of dichloromethane and mixed with the aqueous phase by vigorous stirring for 20 min to form an O/W emulsion. Then, 10 ml of TPP solution (with different pH 3, 5, and 7) were added to the formed emulsion under magnetic stirring. After 1 h of cross-linking, microparticles were isolated by centrifugation at 9000 rpm for 30 min. Table.1 represents the exact composition of each of the prepared formula. The effects of the chitosan molecular weight and the pH of the hardening medium were investigated.

Table 1: Formulation of Paclitaxel Loaded Chitosan Microspheres with different chitosan molecular weight and different pH.

Formulas	Chitosan Molecular weight	pH
TL3	Low	3
TL5		5
TL7		7
TM3	Medium	3
TM5		5
TM7		7
TH3	High	3
TH5		5
TH7		7

* T: Taxol (Paclitaxel), L: Low MW Chitosan, M: Medium MW Chitosan, H: High MW Chitosan, pHs: 3, 5 and 7.

Measurement of particle size

The mean particle size, polydispersity index and zeta potential of the size distribution for each formula were determined by photon correlation spectroscopy using 90 Plus particle size analyzer, Brookhaven Instruments Corporation (BTC), (Holtsville, New York, USA). Analysis was performed at 25 °C with an angle of detection of 90°. Each value reported is the average of three measurements. The polydispersity index measures the size distribution of the microparticles population.

Differential scanning calorimetry

Thermograms of the microparticle samples were obtained by a differential scanning calorimeter DSC-60 (Shimatzu, Japan). Samples of 5 mg were accurately weighed into aluminum pans and then hermetically sealed with aluminum lids. The thermograms of samples were obtained at a scanning rate of 10° C/min over a temperature range of 50 to 400° C. All tests were performed twice.

X- ray Powder Diffractometer of paclitaxel loaded microparticles

The X- ray Powder Diffractometer of PTX loaded microspheres were measured using P analytical X-ray diffractometer (PW3719). Samples of 30 mg were loaded into the sample holder for study.

Determination of % entrapment efficiency

The content of PTX was determined in the supernatant applying a simple and sensitive high performance liquid chromatography (HPLC) with minor modification [37]. Separations were carried out using mobile phase consisting of acetonitrile and 20 mM potassium dihydrogen phosphate (50:50, V/V) on a µ Bondapack C18 column (150 mm 3.9 mm) at a flow rate of 1 ml/min and detection wavelength of 227 nm. The method exhibited linearity over an analytical range of 50-2000 ng/ml (R2 =0.9999).

The drug entrapment efficiency (EE) was calculated from the following formula:

$$EE = \frac{(w - w_0)}{w} \times 100\ \%$$

Where EE, W, and Wo are the entrapment efficiency, the weight of the drug added in the system, and the weight of the drug in the supernatant after centrifugation, respectively.

***In vitro* release study**

Certain weights from each of formula equivalent to 10 mg PTX were suspended in 50 ml of 1M sodium salicylate in screw capped tubes. The tubes were incubated in shaker water bath at 37± 0.5°C and 100 rpm speed. Aliquots of 1 ml were withdrawn at specified time intervals (0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, 7 and 24 hours) and replaced with fresh preheated at 37°C medium. Samples were centrifuged at 1000 rpm for 10 minutes; then supernatant was membrane filtered (0.45 µm) and analyzed in triplicate for drug release by the above described HPLC method.

3. Results and discussion

Particles characteristics

Table 2 presents the mean particle size, polydispersity, zeta potential and entrapment efficiency for all the prepared formulae. Figure 1 showed the particle sizes and polydispersity indices for all the formulae. The polydispersity index is a measure of the width of the dispersion of particles. The used dynamic light scattering system reports a polydispersity index with a value between 0 and 1. A polydispersity index of 1 indicates large variations in particle size while a polydispersity value less than 0.1 is regarded as monodisperse. Narrow dispersions comprise polydispersity index values between 0.1 and 0.2. Hence, according to Figure 1 and Table 2, most of the dispersions can be labeled as narrow disperse except TM7, TH3 and TH7 polydispersity index is slight higher. Generally, the particle size showed a wide range of variability ranging from 1.206 to 8.727 µm depending on the chitosan composition and the pH of the formulations. One can notice a correlation between the particle size and chitosan M wt. Particles with sizes below 2 µm were obtained with two low M wt chitosan particle formulae (TL5 and TL7) and one medium M wt formula (TM3). No correlation was detected with the pH of the cross linking media and the particles' sizes.

The zeta potential of microspheres is commonly used to characterize the surface charges property of microspheres. It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. Higher values of zeta potential imply more stable microparticles, and lower values indicate colloid instability. Microspheres with a Zeta potential above (+/-) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles [38]. As shown in Table 2, Zeta potential values ranged between +7.81 to +30.72 mV. The determined Zeta potential of TM3 microspheres were 30.7 mV (above + 30 mV) indicating the stability of this formula. Generally, higher zeta values were found at lowest pH used (pH 3) in all the chitosan M wts used. This was expected as the glucosamine group on chitosan backbone may take more protons as the medium gets more acidic.

The molecular weight variation of chitosan and pH of composition were found to affect the extent of PTX entrapment in microparticles. Entrapment efficiencies of PTX ranging from 21-83.7% were also observed at different molecular weight of chitosan and pH of TPP solutions. As shown in Table 2, formulae TL5, TM3 and TM5 exhibited the highest three entrapment efficiencies among all the prepared formulae with values equal to 83%, 83.7% and 73.7%, respectively.

It is clear that the high entrapment efficiencies were achieved in moderate acidic medium (pH 3 and 5). Microparticles were prepared by the ionic interaction between a positively charged amino group of chitosan and a negatively charged gelling counterion TPP. The ionization of TPP is dependent on the pH value of solution. In original TPP solution (pH 7), TPP is dissociated into OH- and phosphate ions. However, at Lower pH 3 and 5 only phosphate anion of TPP are formed. At lower pH the ionization of amine group of chitosan is increased due to its basic nature. Therefore, chitosan microparticles prepared in the original TPP solution are dominated by deprotonation and slightly ionic-crosslinking, but chitosan microparticles prepared in acidic TPP solution are completely ionic-crosslinking dominated [39, 40].

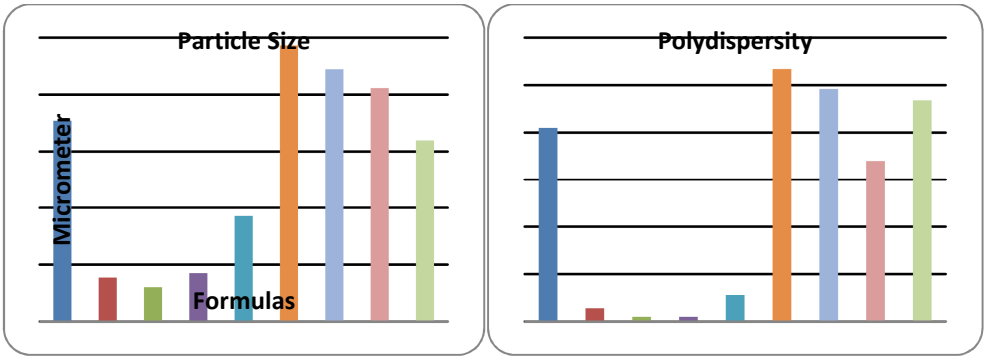

Fig. 1. The mean particle size and polydispersity index of all the prepared formula.

Table 2: Particle size, Polydispersity, Zeta Potential and Encapsulation Efficiency of Paclitaxel Loaded Chitosan Microspheres.

Formulas*	Particle size (µm, n=3)	Polydispersity	Zeta Potential (mV)	% EE
TL3	7.082 ± 0.325	0.205	27.16 ± 1.09	33.3 ± 1.34
TL5	1.545 ± 0.136	0.010	24.74 ± 0.91	83 ± 1.89
TL7	1.206 ± 0.064	0.005	7.81 ± 0.79	51 ± 1.23
TM3	1.702 ± 0.092	0.005	30.72 ± 0.94	83.7 ± 1.55
TM5	3.710 ± 0.150	0.028	17.55 ± 0.96	73.7 ± 1.80
TM7	8.727 ± 0.086	0.267	12.11 ± 1.01	29.1 ± 1.55
TH3	7.892 ± 0.120	0.246	11.86 ± 0.75	61 ± 0.80
TH5	7.230 ± 0.033	0.170	12.15 ± 1.10	38.4 ± 2.12
TH7	5.390 ± 0.140	0.234	10.71 ± 1.18	21 ± 1.99

* T: Taxol (Paclitaxel), L: Low MW Chitosan, M: Medium MW Chitosan, H: High MW Chitosan, pHs: 3, 5 and 7.

X- ray Powder Diffractometer of paclitaxel loaded chitosan microparticles

X- ray powder diffraction pattern of different formulae of PTX loaded chitosan microparticles were obtained and compared with PTX and blank particles made with medium M wt chitosan (Figure 2). The X-ray powder diffraction patterns of PTX showed various intense peaks between 0-20° 2θ revealing crystallinity. Amorphous patterns, however, were observed for blank containing medium molecular chitosan. The diffractograms of the TM3, TH5 and TH7 microparticles were mostly dominated by the amorphous character hence it was confirmed that drug is loaded successfully. While in other formulations crystalline characteristics were noticed.

Differential Scanning Calorimetry (DSC)

Thermogram of paclitaxel (Figure 3 b) showed two close melting endotherms at 219.6°C and 224°C just prior to an exotherm of degradation peak. Pure chitosan (medium M wt) exhibited an exothermic peak at approximately 300 ºC (curve c Figure 3). The main event in the blank microparticles thermogram is the presence of an exthoermic peak at 240 ºC, as shown in curve a in Figure 3. This peak is attributed to the polymer crosslinking. The same chitosan exothermic degradation peak around 300 ºC also appeared in the plain microparticle thermogram. Except formula TH3, all the tested PTX loaded microparticles showed the cross linking exthothermic event with variable intensities. The absence of the PTX melting endotherm was also witnessed in all the tested microparticles suggesting PTX formulated in the chitosan microparticles existed in amorphous form of molecular dispersion or solid state in chitosan polymer matrix.

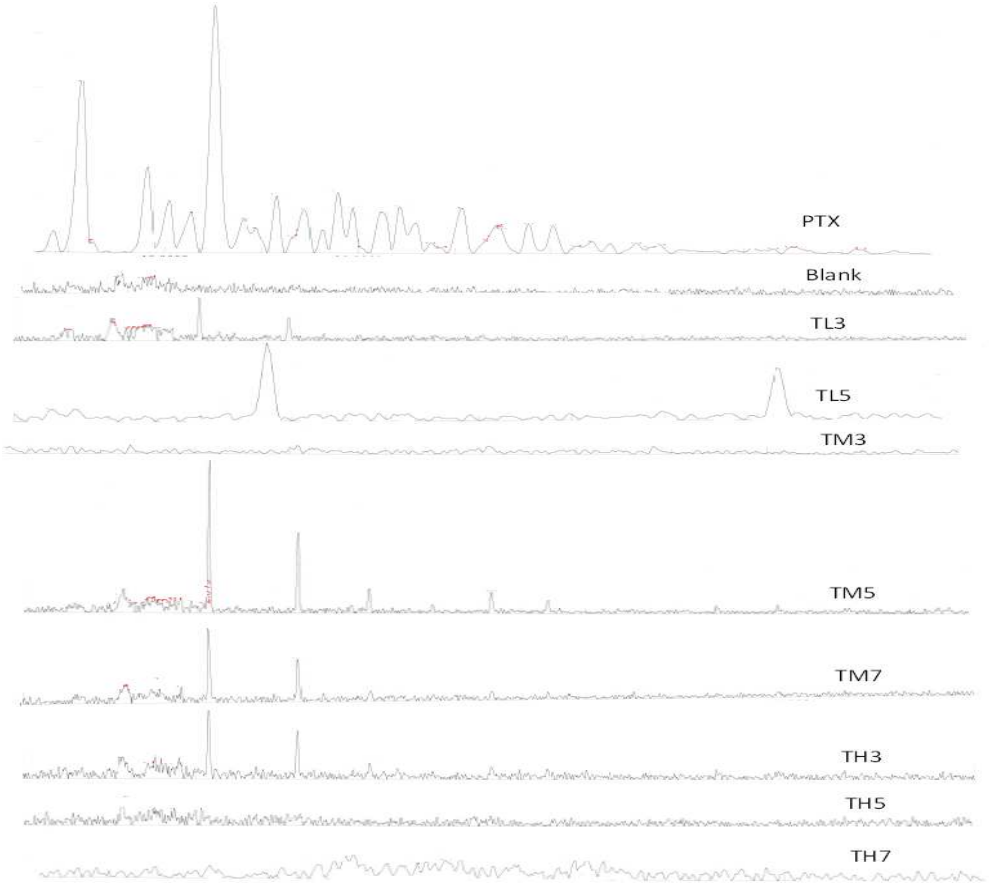

Figure 2: X- ray Diffraction patterns of paclitaxel loaded microparticles

***In vitro* release study**

The *in vitro* drug release profiles of entrapped PTX from the chitosan loaded microparticles are shown in the Figure 3. The release was monitored in sodium salicylate 1M medium as hydrotropic agent to maintain sink conditions. The use of sodium salicylate as medium for studying the release of PTX was first presented by Cho et al [41]. They found that sodium salicylate increased the aqueous solubility of PTX by 100 times without destroying the micellar structure of a polymeric micelle drug delivery. Generally, all the formulae exhibited a rapid release profile with most of the amount released within the first hour. Slight increase in the cumulative amount released was noticed, in few formulae, after 24 hours compared with the 7 hours amounts. The cumulative amount released after 24 hours tremendously varied between the different microspheres formulae. TM3 and TM5 showed relatively high cumulative % drug released with values around 91.3% and 88.5% after 24 hours. On the other hand, TH3, TH5, and TM7 showed low magnitude of the % drug released with values equal to 2.6%, 12.8% and 23.7% respectively. The existence of PTX in the amorphous form and the low particle size seemed critical for the enhancement of PTX dissolution as the top four formulae with the highest amount released exhibited particle sizes < 4µm. The highest magnitude of PTX released from TM3 and TM5 may be attributed to their high relative zetapotential values, allowing them to maintain their low mean particle sizes prohibiting particles' aggregation. The medium M wt chitosan appeared

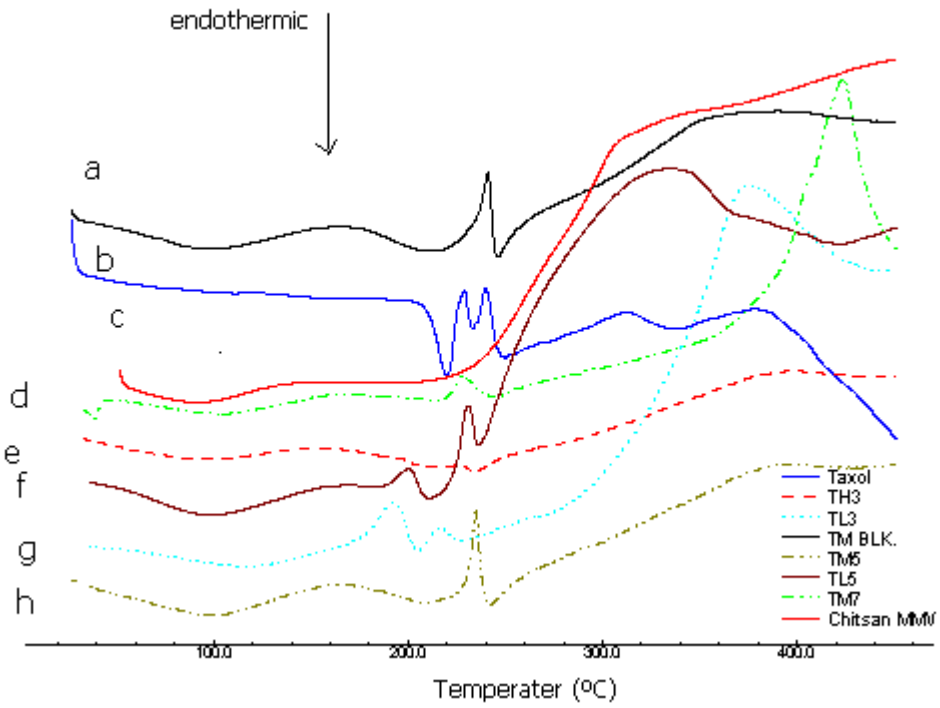

Fig. 3. DSC thermograms of blank microparticles (a), pure paclitaxel (b), pure chitosan medium M wt (c), TM7 (d), TH3 (e), TL5 (f), TL3 (g), and TM5 (h).

superior with regard to forming high aqueous perfusable microparticles that provide high PTX dissolution rate. This significant enhancement of PTX dissolution is comparable to that reported by Wang et al [42]. They showed more than 90% cumulative % released from nanosuspention prepared using high pressure homogenization. Another study showed only 30% cumulative amount released from the best in situ gel formula [43].

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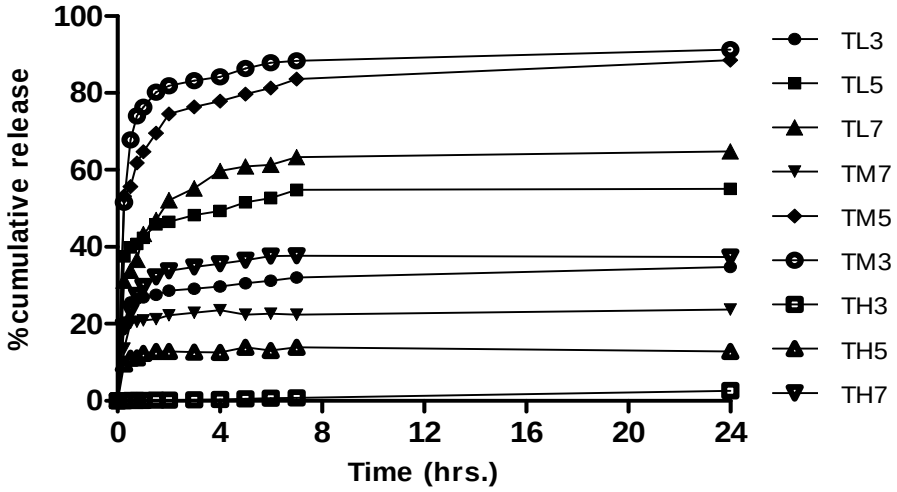

Fig 4: Release profile of Paclitaxel from Paclitaxel loaded microparticles.

4. Conclusions

The present study demonstrated that chitosan-loaded PTX microparticles can be successfully prepared by simple emulsification method using optimum conditions. Physicochemical properties, such as particle size, zeta potential surface charge, encapsulation efficiency, and release can be modulated by parameters including the molecular weight of chitosan and pH of the formulation. The formula TM3 combined all the beneficial attributes of low particle size (1.702 µm), low polydispersity (0.005), high surface charge density (+30.72 mV), high entrapment efficiency (83.7 %) and high magnitude of cumulative % amount released (around 90%). After the entrapment of PTX into low molecular weight chitosan at pH 3, the encapsulation efficiency and release profile greatly increased, indicating that chitosan loaded PTX microparticles possess high potential to be developed as an alternative to formulation available in the market.

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