

# Suppression of Agglomeration of Ciprofloxacin-Loaded Human Serum Albumin Nanoparticles

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## ABSTRACT

The present study is aimed at developing and exploring the use of pectin in suppression of agglomeration of ciprofloxacin-loaded human serum albumin (HSA) nanoparticles. The HSA-pectin nanoparticles loaded with ciprofloxacin were prepared by the pH-coacervation method, and various physicochemical parameters such as particle size, morphology,  $\zeta$ -potential, electrolyte-induced flocculation, pH-dependent  $\zeta$ -potential, drug loading, in vitro drug release, and stability of nanoparticles, were evaluated. The size of the HSA-pectin nanoparticles (F3) was found to be 180 to 290 nm. The HSA nanoparticles were modified with pectin when the critical flocculation concentration of nanoparticles in Na<sub>2</sub>SO<sub>4</sub> solution was increased from 0.3 M to 0.9 M. The isoelectric points of the formed nanoparticles were found to be relatively lower between pH values 3 and 6. Pectin may be used as a pharmaceutical additive for the suppression of particle agglomeration in HSA nanoparticles, and the effect may be attributed to the pectin segments present on the surface of nanoparticles.

**KEYWORDS:** Ciprofloxacin, human serum albumin, pectin, nanoparticles, agglomeration.

## INTRODUCTION

Over the past few decades, biodegradable nanoparticles have created considerable interest as effective drug carrier devices. The biodistribution of colloidal drug delivery systems after intravenous administration is mainly determined by their physicochemical properties, such as size and surface characteristics, which is achieved through recognition or non-recognition of the colloidal system by the body's defense system.<sup>1,2</sup> Particles that are small enough to escape the capillary beds of the lungs are normally sequestered rapidly by the cells of the reticuloendothelial system, particularly the Kupffer cells of the liver.<sup>3</sup> This sequestration has been identified as a major obstacle to targeting cells or tissues elsewhere in the body, such as the bone marrow and solid

tumors.<sup>4,5</sup> However, the protein absorption in biological fluids may modify particle interaction with specific plasma membrane receptors, leading to elimination of the particles from the systemic circulation. The mechanism of protein absorption on the particle surface in conjunction with the recognition of such coated particles by monocytes and macrophages is called opsonization. Opsonization seems to be influenced by the surface curvature of the carrier systems, with smaller carriers having reduced adsorption of proteins and opsonins and, in turn, reduced uptake of such systems by phagocytic cells.<sup>6</sup> It was observed that in the case of liposomes the extent of opsonization decreased with a decrease in particle size from 800 to 200 nm, and no enhancement of phagocytic uptake due to opsonization was recorded at particle sizes below 200 nm.<sup>7</sup>

Among the available potential colloidal drug carrier systems covering the size range described, protein-based nanoparticles play an important role. Basically, 3 different methods for their preparation have been described, based on the emulsion formation, desolvation, and coacervation. Most often, serum albumin obtained from human, bovine, etc., as well as gelatin was used as the starting material for the preparations. With respect to emulsion techniques applying human serum albumin (HSA), a complete and systematic study concerning the influence of protein concentration, emulsification time and power, stirring rate, heat stabilization temperature, and type of nonaqueous phase was performed by Gallo et al.<sup>8</sup> A method for the preparation of bovine serum albumin nanoparticles in the sub-200 nm range was described by Müller et al.<sup>9</sup> The disadvantage of the emulsion methods for particle preparation is the need to apply organic solvents to remove both the oily residues of the preparation process and the surfactants required for emulsion stabilization. Therefore, as an alternative method for the preparation of nanoparticles a desolvation process derived from the coacervation method of microencapsulation was developed. In 1993, Lin et al described the preparation of HSA nanoparticles of a diameter of around 100 nm using a surfactant-free pH-coacervation method.<sup>10</sup> The particles were prepared by the dropwise addition of acetone to an aqueous HSA solution at pH values between 7 and 9, followed by glutaraldehyde cross-linking and purification by gel permeation chromatography. It was found that with increasing pH of the HSA

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solution, particle size was reduced, apparently because of increased ionization of the HSA (isoelectric point = 5.3), which leads to repulsion of the HSA molecules and aggregates during particle formation. HSA nanoparticles were obtained in a size range between 90 and 250 nm, by adjusting the pH and by controlling the amount of added acetone. The prepared nanoparticles were spherical, but scanning electron microscopy (SEM) revealed a broad size distribution. No further data concerning the polydispersity of the nanoparticles prepared under different conditions were given.<sup>11</sup> The obvious drawbacks of such systems are (1) the formation of large aggregates whose size falls outside the apparently preferred size range for drug delivery using nanoscale particles and (2) lack of stability in aqueous dispersion leading to phase separation. However, the HSA nanoparticles prepared in this way had low stability and eventually reverted to the phase-separated state.

The object of the present study was to overcome these limitations by incorporating pectin into the preparation of HSA nanoparticles with particle diameters between 180 and 290 nm and suppressing particle agglomeration more efficiently. The antibiotic ciprofloxacin hydrochloride was selected for incorporation into nanoparticles based on its short biological half-life and solubility characteristics. More recently, Bozdag et al<sup>12</sup> attempted to suppress nanoparticle agglomeration in ciprofloxacin-HCl-loaded poly(D,L-lactide-glycolide) nanoparticles using different cryoprotective agents. The present study is intended to establish a rational basis for the protection and promotion of protein-based nanoparticles as drug carrier systems.

## MATERIALS AND METHODS

### Materials

Ciprofloxacin hydrochloride was a generous gift from Torrent Laboratories (Aurangabad, India). HSA was from Fluka (Mannheim, Germany), glutaraldehyde was from Loba Chemics Pvt Ltd (Mumbai, India), pectin was from SD Fine Chemicals (Mumbai, India), and acetone was from CDH (New Delhi, India). Dialysis membrane bag pore size 2.4 nm was from Himedia (Mumbai, India). All other chemicals were of reagent-grade and used without further modification.

### Methods

#### Preparation of HSA-Pectin Nanoparticles

HSA-pectin nanoparticles were prepared by the pH-coacervation method<sup>10,11,13</sup> with slight modification, and the drug was encapsulated by the incorporation process. Briefly, 5 mL of 4% solution of HSA in a pH 8.0 phosphate buffer, 1.125 mL of phosphate-buffered saline (PBS, 0.01 mol, pH = 7.4), 20 mg of ciprofloxacin hydrochloride, and pectin were

added to a beaker and sonicated in a bath-type sonicator for 30 minutes at 60°C. After sonication, the solution was stirred with a mechanical stirrer and a suitable amount of acetone was added dropwise (1 mL/min) from a syringe until the solution become turbid. The nanoparticles formed were cross-linked by adding 8% vol/vol glutaraldehyde in water (1.5 µL/mg HSA) and stirred continuously for 24 hours at room temperature. The resulting nanoparticles were purified by 4 cycles of different centrifugations in 30 000 × g for 10 minutes (Beckman model L 7-55 ultracentrifuge, Gagny, France), and the sediment was redispersed in an equal volume of distilled water. Each redispersion step was performed in an ultrasonication bath for 5 minutes. For lyophilization, the cleaned nanoparticles were dispensed into 10-mL vials and transferred to a freeze-dryer (Heto Drywinner, Heto Holtan, Germany). Drying lasted for 48 hours at -20°C with a secondary cycle of 3 hours with the temperature adjusted to 20°C. The pressure during drying was 1030 mbar. Lyophilized powder was stored at 4°C and was easily re-dispersible in aqueous buffer solution by hand-shaking for 30 seconds.

#### Particle Size, Morphology, and ζ-Potential of Nanoparticles

The lyophilized formulations F1 to F5 were redispersed separately in phosphate buffer (pH 7.0, ionic strength 0.005 M) and ζ-potential was measured with the help of electrophoretic laser Doppler anemometry in a Malvern Zetasizer 4 (Malvern Instruments, Worcestershire, UK). The size of the nanoparticles was determined by photon correlation spectroscopy with a Malvern 4700 submicron particle analyzer system (Malvern Instruments). SEM (LEO 435 VP, Cambridge, UK) was also used to investigate nanoparticle morphology.

#### Electrolyte-Induced Flocculation of HSA Nanoparticles

Formulations F5 and F3 (1 mg) were incubated in 1.0 mL of sodium sulfate solution of different concentrations (0.0-1.0 M) to assess their electrolyte-induced flocculation. The optical turbidity of the resultant suspension was measured at 600 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

#### Determination of pH-Dependent ζ-Potential of Nanoparticles

For the analysis, the 1-mg formulations F5 and F3 were dispersed in 1.0 mL of a constant-ionic-strength (0.01 M) acetate buffer of different pH values between 3 and 6, and the ζ-potential of the nanoparticles was measured.

**Table 1.** Influence of Pectin on Particle Size and  $\zeta$ -Potential of Ciprofloxacin-Loaded HSA Nanoparticles\*

Formulation Code	Amount of Pectin in Ciprofloxacin-HSA Nanoparticles (%)	Size (nm)	$\zeta$ -Potential <sup>†</sup>	Drug Entrapment (%)
F1	0.5	190.8 ± 40.7	-12.6 ± 0.7	40 ± 2.36
F2	1.0	230.5 ± 32.8	-5.3 ± 0.9	39 ± 1.77
F3	1.5	246.6 ± 28.9	-3.5 ± 1.1	37 ± 1.83
F4	2.0	290.7 ± 29.9	-3.4 ± 1.4	34 ± 2.61
F5	Ciprofloxacin-HSA nanoparticles	832.0 ± 24.8	-26.7 ± 3.6	42 ± 0.84
F6	Ciprofloxacin-HSA nanoparticles (before lyophilization)	142.0 ± 28.8	-20.5 ± 1.3	42 ± 1.33

\*Mean ± SD, n = 3. HSA indicates human serum albumin.

<sup>†</sup>pH 7.0, ionic strength 0.005.

#### Determination of Concentration of Ciprofloxacin Loaded in Nanoparticles

Accurately weighed nanoparticles were hydrolyzed by incubating with 100 mL of 6 mol hydrochloric acid at 100°C for 24 hours and filtered through a membrane filter to remove the filtrate.<sup>14</sup> The amount of ciprofloxacin present in the filtrate was determined in a UV-visible spectrophotometer at 271 nm.<sup>15</sup>

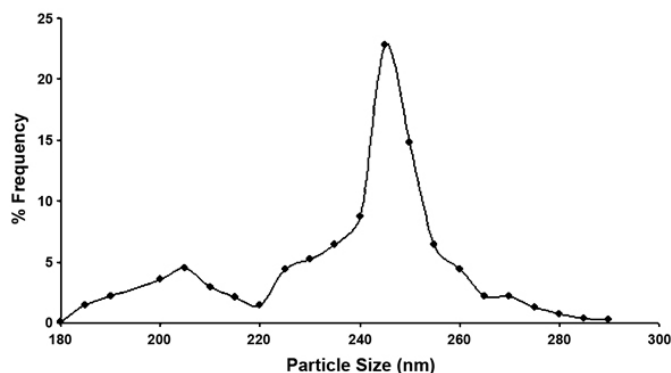
#### In Vitro Drug Release

Drug release from known amounts of nanoparticles (F3) was determined by using a modified dissolution method. The dialysis bags (2.4- $\mu$ m pore size) were filled with a known amount of F3 and placed in 50 mL of PBS (pH 7.4). The device was thermostated at 37 ± 0.5°C and shaken at 70 ± 2 rpm. Aliquots (1 mL) were withdrawn from the release medium and replaced by an equal volume of PBS (pH 7.4) at each sampling time. The content of each sample and the mean cumulative release rate were calculated. The procedures were applied to the 3 batches of F3, before and after storage.

#### Stability of Ciprofloxacin-HSA-Pectin Nanoparticles

##### Physical Stability

Ciprofloxacin-HSA-pectin nanoparticles (F3) were stored for 3 months at 37°C in 75% relative humidity (RH) in the dark.



**Figure 1.** Particle size distribution graph of human serum albumin-pectin nanoparticles (F3).

The appearance of the nanoparticles was monitored by SEM. These storage conditions instead of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines were used since our product is in the process of development.

#### Chemical Stability

After storage for 3 months at 37°C in 75% RH, the drug content and release rate of ciprofloxacin-HSA-pectin nanoparticles (F3) were determined.

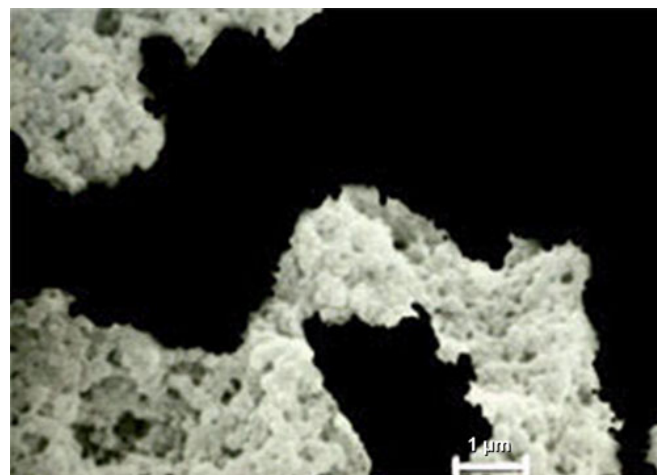
#### Statistical Analysis

The results were expressed as mean ± SD (n = 3), and statistical analysis was performed with SPSS 10.1 for Windows (SPSS, Chicago, IL).

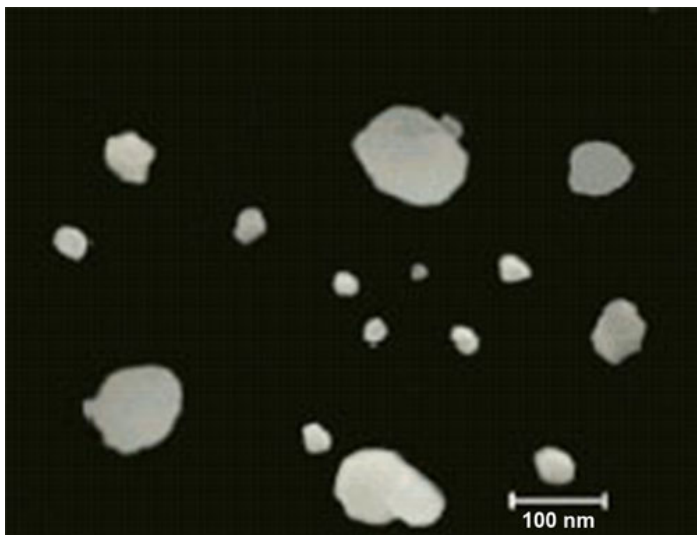
## RESULTS AND DISCUSSION

#### Preparation, Morphology, and $\zeta$ -Potential of Ciprofloxacin-HSA-Pectin Nanoparticles

HSA is characterized as having a high content of charged amino acids and is insoluble in organic solvents. Ciprofloxacin-HSA-pectin nanoparticles (F3) within the size range



**Figure 2.** Scanning electron microscope image of freeze-dried agglomerated human serum albumin nanoparticles (at 10 000 $\times$ ).



**Figure 3.** Scanning electron microscope image of freeze-dried ciprofloxacin hydrochloride-loaded human serum albumin-pectin nanoparticles prepared by the pH-coacervation method (at 100 000 $\times$ ).

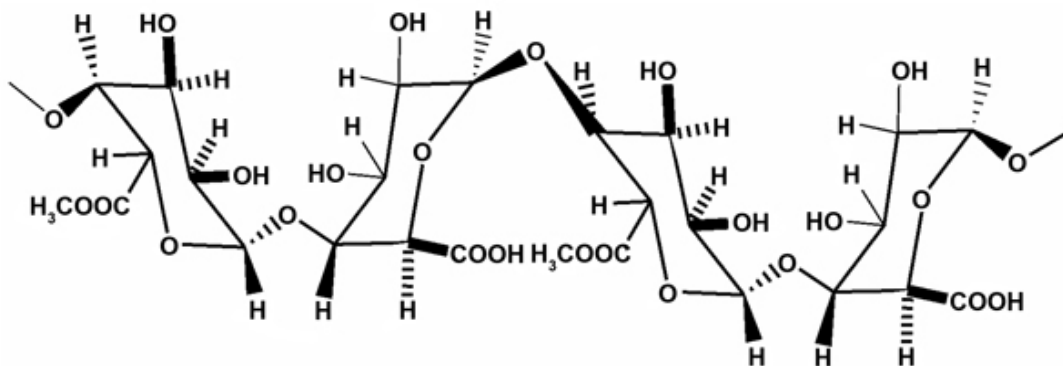
of ~180 to 290 nm were prepared using the modified pH-coacervation method (Table 1 and Figure 1). The SEM micrograph in Figure 2 shows the agglomeration of HSA nanoparticles (F5) after lyophilization, while Figure 3 shows that more or less spherical ciprofloxacin-HSA-pectin nanoparticles (F3) were obtained.

A change in  $\zeta$ -potential with the addition of pectin was observed in HSA nanoparticles. F3 had a  $\zeta$ -potential value that was significantly lower than that of the HSA nanoparticles at pH 7, as shown in Table 1, which may be attributed to the shift in the shear plane due to the presence of pectin molecules on the surface. Interestingly, no significant  $\zeta$ -potential difference was observed between the nanoparticles prepared from 1.5% and 2% pectin. Pectin is one of the structural polysaccharide components of primary cell walls of plants and is used as a gel-forming, thickening, fat-replacing, and stabilizing agent.<sup>16,17</sup> Sugar beet pectin differs from citrus pectin in that sugar beet pectin is partially

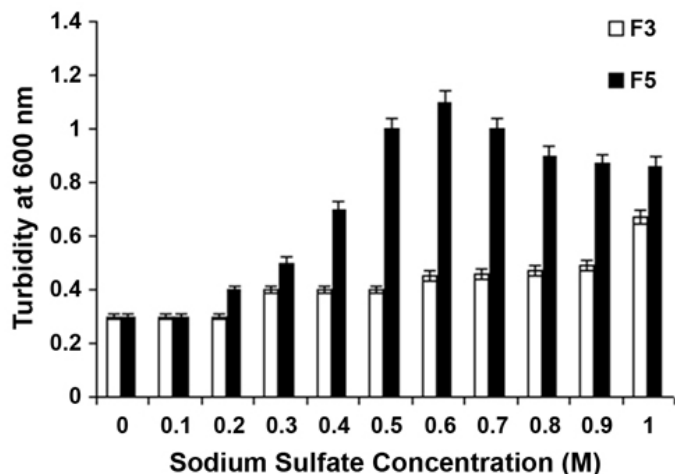
acetylated. O-acetyl pectin is not immunogenic in mice, probably because of its low molecular weight.<sup>18</sup> According to Plaschina et al,<sup>19</sup> the  $p^{K_a}$  of pectin is in the range of 3.55 to 4.10. The fact that 2% pectin in ciprofloxacin-loaded nanoparticles has a less negative charge ( $-3.4 \pm 1.4$ ) than 0.5% pectin in the nanoparticles ( $-12.6 \pm 0.7$ ) indicates that the concentration of pectin exerts a pronounced influence on the  $\zeta$ -potential of the nanoparticles. This may be due to the polyanionic nature of galacturonic acids in pectin, as shown in Figure 4, that produces electric charge in colloid stabilization.

#### ***Turbidity Ratio Test (Electrolyte-Induced Flocculation of Nanoparticles)***

In the present study, the existence of a pectin barrier was investigated by an electrolyte-induced flocculation of the nanoparticles, which is based on the fact that the physical stability of a colloidal system is mainly dependent upon the competitive processes of attraction (van der Waals forces) and repulsion (either electrostatic repulsive force or steric stabilizing barrier or both).<sup>20</sup> If particles are mainly stabilized electrostatically, the destruction of the electrostatic double layer surrounding the particles will result in the aggregation of particles into clusters, with a corresponding increase in optical turbidity. However, if the particles are mainly stabilized by a pectin barrier, the colloidal system is stable even if the electrostatic double layers have been destroyed. The stabilization of HSA nanoparticles is due to steric hindrance resulting from the adsorbed pectin molecules on the particle surface. The influence of the  $\text{Na}_2\text{SO}_4$ -induced flocculation of F3 is shown in Figure 5. When HSA nanoparticles were modified with pectin, the critical flocculation concentration of the nanoparticles in  $\text{Na}_2\text{SO}_4$  solution increased from less than 0.3 M to ~0.9 M. It was reported that when HSA nanoparticles were modified by HSA-methoxy poly ethylene glycol (mPEG), the stability of the resulting colloidal systems in  $\text{Na}_2\text{SO}_4$  solution was increased up to 0.5 M,<sup>5</sup> while when HSA nanoparticles



**Figure 4.** Structure of pectin shows that pectin molecules have a linear backbone composed of units of (1,4)-linked  $\alpha$ -D-galacturonic acid and its methyl ester.

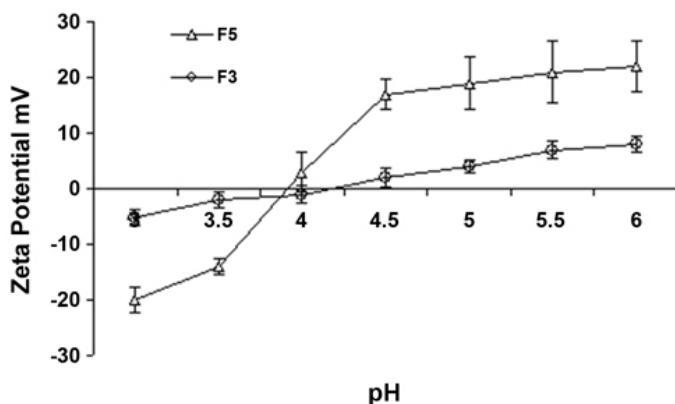


**Figure 5.** Influence of sodium sulfate concentration on turbidity of the nanoparticle solution. Mean  $\pm$  SD, n = 3.

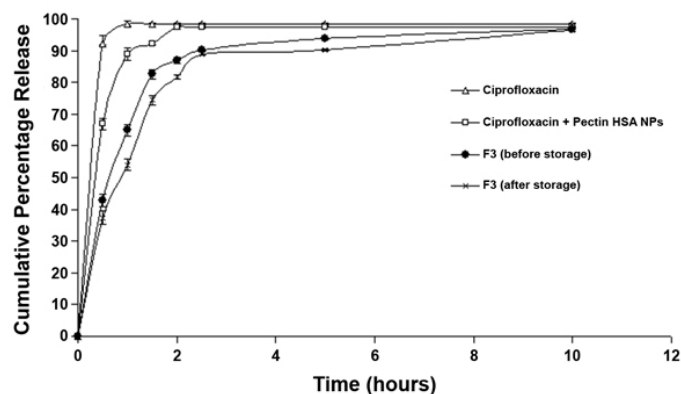
were modified by dextran-mPEG, the particles were stable in Na<sub>2</sub>SO<sub>4</sub> solution until the concentration was higher than 1.2 M.<sup>21</sup> Stolnik et al<sup>22</sup> reported a similar result and found that when poly lactic glutamic acid (PLGA) and polystyrene nanoparticles were coated with poloxamine 908 or PLA-mPEG copolymer, which creates a steric poly ethylene oxide (PEO) barrier on the particle surface, the stability of the resulting colloidal systems in Na<sub>2</sub>SO<sub>4</sub> solution was increased but still flocculated at a critical concentration around 0.5 M.

#### *ζ-Potential of Nanoparticles at Different pHs*

The ζ-potential of F3 at different pHs was relatively lower as compared with that of F5, as shown in Figure 6, which may be ascribed to the chemical absorption of the pectin on the particle surface, which results in a relatively lower isoelectric point of the formed nanoparticles. The pectin layer, which exists on the surface of ciprofloxacin-HSA-pectin nanoparticles (F3), plays an important role in the stability



**Figure 6.** Influence of pectin on the ζ-potential of human serum albumin nanoparticles at different pHs. Mean  $\pm$  SD, n = 3.



**Figure 7.** In vitro cumulative percentage release of ciprofloxacin from ciprofloxacin-loaded HSA nanoparticles incubated at 37°C with phosphate buffer solution (pH 7.4). Mean  $\pm$  SD, n = 3. HSA indicates human serum albumin; NPs, nanoparticles.

of nanoparticles, by suppressing the agglomeration of HSA nanoparticles.

#### *Drug Release In Vitro*

Figure 7 shows the in vitro cumulative percentage release profiles for the pure ciprofloxacin, ciprofloxacin-HSA-pectin nanoparticles, and ciprofloxacin-HSA-pectin nanoparticles (F3) before and after storage. The in vitro release rate of ciprofloxacin-HSA-pectin nanoparticles, ciprofloxacin-HSA-pectin nanoparticles (F3), and pure ciprofloxacin hydrochloride were compared. In this comparison, the in vitro release rate of ciprofloxacin-HSA-pectin was faster than the release rate of F3 but, unexpectedly, it was slower than that of pure ciprofloxacin hydrochloride. The release behavior of ciprofloxacin-HSA-pectin nanoparticles can be explained partially by the absorption of part of the drug by the HSA-pectin nanoparticles and partially by the blank nanoparticles' suppressing the drug from diffusion in the dialysis tube.

#### *Test for Stability*

After the nanoparticles (F3) were stored, no detectable change in appearance or redispersing ability could be detected, and the drug content was found to be 24.09  $\pm$  1.86%. A total amount of 37% drug was released in vitro from F3 in the first half-hour, while 94% was released in 10 hours, as shown in Figure 7.

#### **CONCLUSION**

The addition of pectin to the ciprofloxacin-loaded HSA nanoparticles did not affect the drug-loading efficiency or the release rate of the drug in the nanoparticles. A small quantity of pectin could suppress the agglomeration of

ciprofloxacin-loaded nanoparticles. This should have practical implications for nanoparticle preparation technology.

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