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Dynamic dialysis for the drug release evaluation from doxorubicin–gelatin nanoparticle conjugates

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Abstract

The drug release from doxorubicin (DXR)–gelatin nanoparticle conjugates was evaluated by means of a dynamic dialysis technique. The study was carried out in absence and in presence of a proteolytic enzyme (trypsin) able to degrade the carrier. In a preliminary study the apparent permeability constant (K_{cv}) of the drug through the dialysis bag was evaluated in several media. On the basis of this screening, a saline solution (NaCl 0.9%, w/v) resulted appropriate to carry out the dialysis study since, in this medium, the K_{cv} did not depend on the drug concentration in the donor solution. In absence of the enzyme only a little fraction (from 9 to 13%, w/w of the drug content) was released from nanoparticles. This fraction was considered as the evidence of the free drug fraction. After the addition of trypsin, the diffusion of a further drug fraction was observed. This fraction is probably due to a fraction of the DXR–peptide conjugates characterised by a molecular weight lower than membrane cut-off (3500 Da).

Keywords: Gelatin nanoparticles; Dynamic dialysis; Drug release; Doxorubicin hydrochloride

1. Introduction

Doxorubicin (DXR) is an anthracycline antineoplastic agent that is commonly used in the treatment of neoplasms, including leukemias and lymphomas. However, its therapeutic use is limited by a cumulative dose-dependent irreversible cardiotoxicity (Young et al., 1981). To overcome this drawback, an appropriate drug targeting device can be considered as a reasonable approach since it allows the drug concentration to be increased at the desired site reducing the administered dose. Many studies showed that nanoparticles are a promising system for the targeting of anticancer drugs (Douglas et al., 1987; Couvreur et al., 1990; Narayami and Panduranga Rao, 1994). The characterisation of the

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in vitro drug release from nanoparticles is technically difficult to achieve. The problems can be ascribed to the inability to separate in an effective and rapid way the nanoparticles from the release medium owing to the their extremely small size. In order to minimise these problems, the dynamic dialysis technique may be a useful approach (Gupta et al., 1987).

In a previous paper (Leo et al., 1997) it was demonstrated that a fraction of DXR remained linked to the protein matrix as glutaraldehyde was used to cross-link gelatin nanoparticles. To our knowledge, no studies have been focused on the application of the dynamic dialysis technique in the determination of drug release from a drug-carrier conjugate. Therefore, this work aimed to evaluate, by a dynamic dialysis technique, the in vitro release of the drug from glutaraldehyde cross-linked nanoparticles in the absence and in the presence of a proteolytic enzyme. This procedure should allow the release of the free drug fraction to be distinguished from that of the drug covalently bound to the gelatin nanoparticles. However, in the application of this technique it is essential that the drug diffusion through the membrane results constant and independent from the drug concentration inside the dialysis bag (Washington, 1990). Hence, before carrying out the dialysis of the DXR-loaded nanoparticles, different media were checked in order to found the medium in which the apparent permeability constant (K_{cv}) was not affected by the drug concentration.

2. Materials and methods

2.1. Materials

Gelatin type A from porcine skin (250 Bloom, pH of 1%, w/v water solution: 3.5–5) and glutaraldehyde (25%, w/w water solution), used in the preparation of the nanoparticles, were purchased from Fluka (Buchs, Switzerland). DXR hydrochloride (Adriblastina®) was supplied from Pharmacia (Milan, Italy). Sephadex G-50m (Sigma, St. Louis, MO) was used as column packing in the gel-filtration purification procedure. All solvents were of analytical grade (Carlo Erba, Milan, Italy). All the products and chemicals were used as received from the manufacturers.

2.2. Preparation of gelatin nanoparticles

The preparation of gelatin nanoparticles was performed using the coacervation-phase separation technique (Oppenheim and Stewart, 1979) modified as previously described (Leo et al., 1997).

Briefly, a sodium sulphate water solution (20%, w/v; 7 ml) was added at 37°C under magnetic stirring to a water gelatin solution (1% w/v; 10 ml) containing Polysorbate 20 as surfactant (0.5%, w/v) and the drug (DXR) (5 or 10 mg for the batches A or B, respectively). Thus, a permanent faint turbidity due to gelatin coacervate was obtained. Then, isopropanol (1.2 ml) was added until the coacervate desolvated and the turbidity disappeared. The magnetic stirring was replaced with an Ultra Turrax® (Janke & Kunkel-Ika-Laboratortechnick, Staufen, Germany) and 0.4 ml of the 25% glutaraldehyde water solution were added. The cross-linking process was completed after 11 min by the addition of 5 ml of a water solution of sodium metabisulphite (12%, w/v). After 1 h, the nanoparticles suspension was purified from the excess of salts and unloaded drug by gel-chromatography using a column (diameter 25 mm; height 50 cm) filled by Sephadex G-50 m.

The purified nanoparticles were lyophilised and stored in the dark as freeze-dried powders under vacuum (2 mmHg) and at 25°C.

2.3. Determination of the drug content

The drug content in the DXR-loaded nanoparticles was determined upon enzymatic degradation of nanoparticles by a proteolytic enzyme (trypsin, from bovine pancreas, Sigma). An amount exactly weight of DXR-loaded nanoparticles (batch A or B) or unloaded nanoparticles (10 mg) was dispersed in a solution of NaCl (25 ml; 0.9%, w/v) containing trypsin (80 μg/ml) to have a ratio trypsin/nanoparticles of 1:5, w:w. The dispersion was kept for 5 h at 37°C in the dark under

magnetic agitation. After this period a clear solution was obtained. The digest solution prepared from the loaded nanoparticles resulted in faint red colour since contained free and coupled DXR (Leo et al., 1997). As DXR covalent coupling can be quantitated as DXR equivalents using absorption at 480 nm (Cummings et al., 1991), the drug content after enzymatic degradation was determined spectrophotometrically at 480 nm (Lambda 3B, Perkin-Elmer, Norwalk, USA) against a blank formed by the solution obtained from the unloaded nanoparticles in the same experimental conditions.

2.4. Size analysis

The nanoparticle size was evaluated by means of a light scattering method (Nanosizer, Coulter Electronics, Harpenden, England).

2.5. Dynamic dialysis application

The determination of the drug release from DXR–gelatin nanoparticle conjugates was performed by a dynamic dialysis technique monitoring the drug concentration in the receiver solution. According to the method, the drug is firstly released from the nanoparticles into the donor solution contained in the dialysis bag. Subsequently, the drug can diffuse through the dialysis bag in the receiver solution where the drug concentration was determined.

To calculate the apparent permeability constant (K_{cv}) of the drug diffusion through the dialysis membrane, the mathematical model proposed by Gupta et al. (1987) was applied. According the model:

$$\ln[Q_s^0 - C_2(V_1 + V_2)] = \ln Q_s^0 - K_{cv}t \quad (1)$$

where Q_s^0 is the total amount of the drug in the system; C_2 is the amount of the drug in the receiver solution at time t; V_1 and V_2 are the volumes of the donor and receiver solution, respectively.

A plot of $\ln[Q_s^0 - C_2(V_1 + V_2)]$ versus time would give a straight line with a slope equal to $-K_{cv}$ and an intercept of $\ln Q_s^0$.

As the drug diffusion can be affected by the characteristics of the diffusing medium (Washington, 1990), the apparent permeability constant (K_{cv}) was evaluated in the following aqueous media: deionised water and water solutions containing different sodium chloride concentrations (0.1, 0.4, 0.9 and 2%, w/v).

Practically, a solution of DXR (2 ml; donor solution, V_1) in one of the media above indicated, was prepared at a concentration range approximately between 25 and 250 μg/ml and introduced in a dialysis bag (length 8 cm) formed by a Spectra/Por® 3 membrane (MWCO 3500; diameter 2.5 cm, Spectrum, Los Angeles, USA). Before the use, the dialysis bag was soaked with deionised water for 3–4 h to remove all the glycerol on the membrane surface. The dialysis bag filled with the donor solution was immersed in a jacked-beaker thermostated at 37°C ($\pm$ 1°C) contained 80 ml of the receiver solution (V_2) and protected from light in order to avoid DXR photodegradation. Obviously, the same dialysis medium was used as donor and receiver solution. At fixed time intervals an aliquot (15 ml) of the receiver solution was withdrawn and the amount of DXR was determined spectrophotometrically at 480 nm. After each determination, the sample was reintroduced in the receiver solution.

2.6. Drug release study from nanoparticles

Unloaded or loaded nanoparticles (batches A or B) (40 mg) were dispersed in NaCl (2 ml; 0.9%, w/v) and introduced in a dialysis bag (donor solution, V_1). This dispersion was dialysed against 80 ml of NaCl (0.9%, w/v) (receiver solution, V_2) for 72 h. Throughout the experimental procedure, both the donor and the receiver solutions were agitated by magnetic stirring. To determine the DXR amount diffused through the dialysis bag, samples (15 ml) were withdrawn from the receiver solution at prefixed times and the drug concentration was measured spectrophotometrically at 480 nm; after each determination, the sample was reintroduced in the receiver solution.

To study the drug release in the presence of a proteolytic enzyme the dialysis test was performed for 24 h without enzyme as above described. After

Table 1
Mean diameter, doxorubicin (DXR) content and encapsulation efficiency of the gelatin nanoparticles (± S.E.)

Batches	Initial DXR/gelatin ratio	Mean diameter (nm)	DXR content (%, w/w)	Encapsulation efficiency (%)
Unloaded	–	194 ± 13	–	–
A	5:100	213 ± 12	2.0 ± 0.12	40 ± 2.4
B	10:100	205 ± 10	3.1 ± 0.15	31 ± 1.5

this time period, 0.5 ml of a trypsin solution (16 mg/ml in NaCl 0.9%, w/v) was added to the donor solution. Thus, the dialysis experiment was performed for a further 48 h as described above.

A V_2 sample obtained from a dialysis test carried out using unloaded nanoparticles was placed in the reference cell.

3. Results and discussion

The dimension, the drug content and the encapsulation efficiency of nanoparticles are reported in Table 1. The dimensions of the unloaded and loaded nanoparticles were practically the same (about 200 nm). As expected, the highest was the amount of DXR used in the preparation, the highest was the drug loaded. However, the highest was the initial amount of DXR, the lowest was the encapsulation efficiency probably owing to the easy washing out of the drug before the cross-linking procedure of nanoparticles.

The dynamic dialysis was carried out using a membrane cut-off of 3500 Da in order to assure both the diffusion of the drug (MW = 580 Da) and the complete separation from the receiver solution of the nanoparticles.

In a preliminary study, deionised water and NaCl solutions at different concentrations (0.1, 0.4, 0.9 and 2%, w/v) were tested as possible dialysis media for the drug release studies from DXR-loaded nanoparticles. For this aim, the dialysis behaviour of solubilised DXR was analysed. In deionised water, the equilibrium between the donor and the receiver solution was accomplished after 3 h without reaching the complete recovery of the drug in the donor solution (Fig. 1). At the optical inspection, the tube appeared coloured in red probably owing to the partial absorption of the drug on the membrane material. Since plotting $\ln[Q_s^0 - C_2(V_1 + V_2)]$ versus time no straight lines were observed (Fig. 2), the K_{cv} value was calculated from the initial portion of the curve. However the K_{cv} values were related to the initial drug concentration and a remarkable amount of DXR could be considered adsorbed on the membrane. Therefore, it cannot be considered as an appropriate dialysis medium for the study of DXR release.

In the presence of NaCl no drug absorption was observed on the bag material. The largest part of the drug dissolved in the donor solution (90–100%) was recovered at equilibrium (5–6 h) in the receiver solution. Using solutions of NaCl 0.1 and 0.4% w/v, the K_{cv} values resulted the same for DXR concentrations ranged between 50 and 80 μg/ml. Increasing the drug concentration in the donor solution, the K_{cv} values did not remain constant (Figs. 3 and 4). Plotting $\ln[Q_s^0 - C_2(V_1 +$

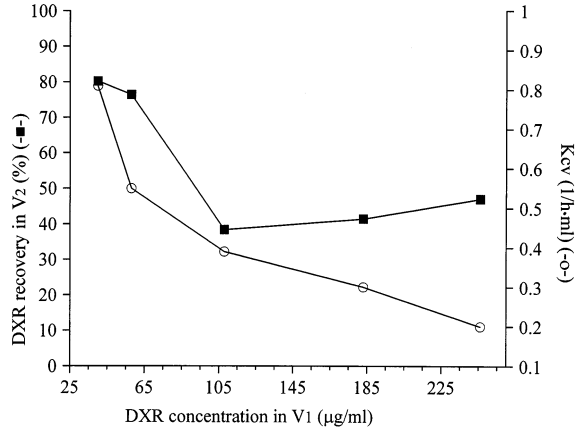

Fig. 1. Doxorubicin (DXR) recovery percentage (-■-) and the apparent permeability constant (K_{cv}) (-○-) values obtained from dialysis of different DXR concentrations in deionised water (V_1: donor solution; V_2: receiver solution).

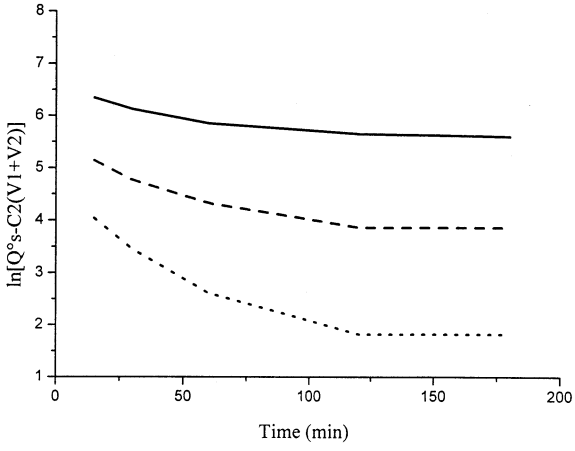

Fig. 2. Plot of $\ln[Q_s^0 - C_2(V_1 + V_2)]$ versus dialysis time of three different concentrations of DXR in deionised water. Concentration of DXR in the donor solution (μg/ml): (—) 246; (-----) 108; (· · · · ·) 40.

V_2)] versus time, straight lines ($r^2 = 0.99$) were pointed out only for the concentrations lower than 80 μg/ml (data not shown) On the contrary, using DXR solutions in NaCl 0.9% w/v, the K_{cv} value was easily determined from the slope of the straight lines obtained applying Eq. (1) (Fig. 5). The calculated value (0.46 ± 0.02/h/ml) was not affected by the initial drug concentration (Fig. 6). Finally, using as dialysis medium an higher NaCl concentration (2% w/v), the apparent permeability

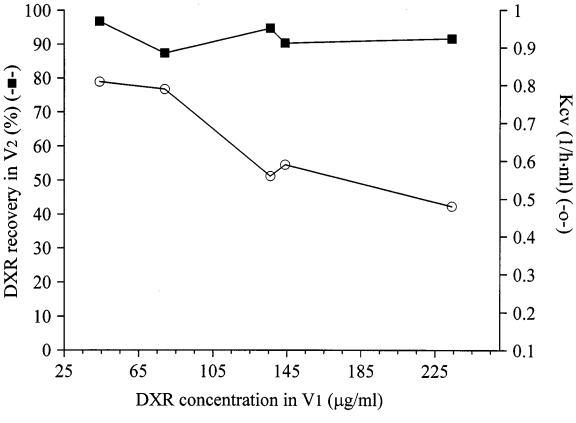

Fig. 3. Doxorubicin (DXR) recovery percentage (-■-) and the apparent permeability constant (K_{cv}) (-○-) values obtained from dialysis of different DXR concentrations in NaCl 0.1% w/v (V_1: donor solution; V_2: receiver solution).

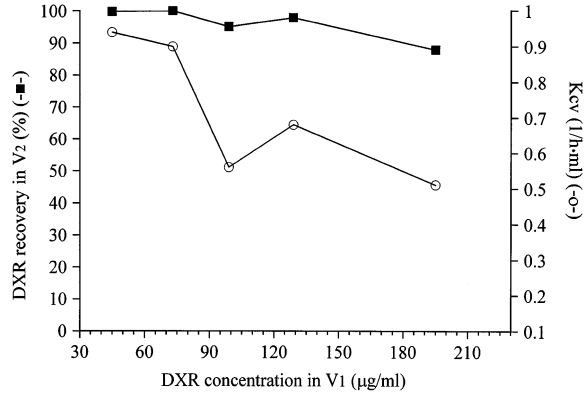

Fig. 4. Doxorubicin (DXR) recovery percentage (-■-) and the apparent permeability constant (K_{cv}) (-○-) values obtained from dialysis of different DXR concentrations in NaCl 0.4% w/v (V_1: donor solution; V_2: receiver solution).

constant was affected by the DXR (Fig. 7). These findings allow a conclusion to be drawn that the ionic strength of the solution medium plays a key role on the diffusion rate of the drug through the membrane. Based on the results of this screening, only the solution of NaCl 0.9%, w/v can be considered as a suitable medium to perform the dialysis study of loaded nanoparticles.

Therefore, the dialysis of the two batches of DXR-nanoparticles conjugates was carried out in NaCl 0.9%, w/v for 72 h. After 6 h only a little

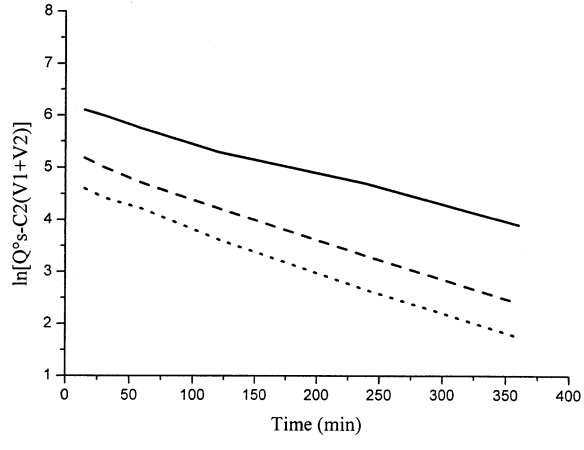

Fig. 5. Plot of $\ln[Q_s^0 - C_2(V_1 + V_2)]$ vs dialysis time of three different concentrations of DXR in NaCl 0.9%, w/v. Concentration of DXR in the donor solution (μg/ml): (—) 251; (-----) 95; (· · · ·) 55.

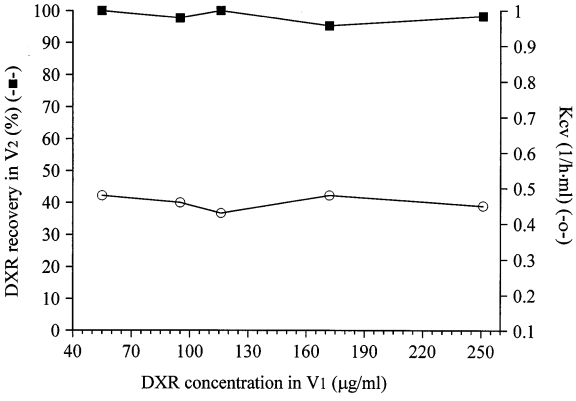

Fig. 6. Doxorubicin (DXR) recovery percentage (-■-) and the apparent permeability constant (K_{cv}) (-○-) values obtained from dialysis of different DXR concentrations in NaCl 0.9% w/v (V_1: donor solution; V_2: receiver solution).

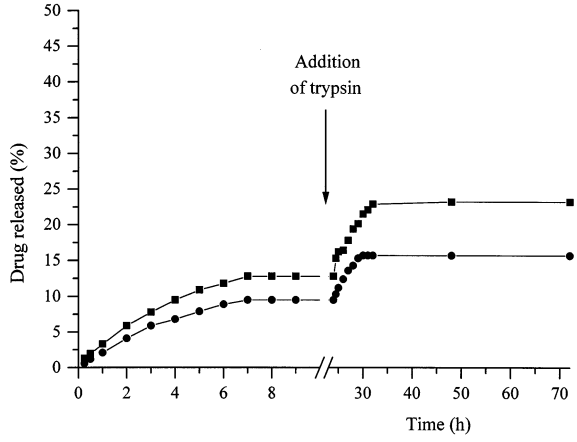

Fig. 8. DXR released in the receiver solution from DXR–gelatin nanoparticles conjugates: Batch A (-●-); Batch B (-■-).

percentage of the drug loaded in the nanoparticles diffused through the membrane (9 and 13% for the batches A and B, respectively) (Fig. 8). Subsequently, no additional diffusion was observed for a further 66 h. In order to establish if the diffusion process from the matrix can be the limiting factor in the drug release from DXR-nanoparticles, the K_{cv} value of the free drug was compared to that calculated from the drug release from the colloidal carrier. For this aim, Q_s^0 was considered as the total amount released upon 72 h. The K_{cv} value obtained was 0.44 ± 0.01/h/ml for the batch A and 0.45 ± 0.01/h/ml for the batch B. These values agreed with the K_{cv} value of the free DXR (0.46 ± 0.02/h/ml) in the same dialysis medium. Therefore, the fraction released during the first 8 h could correspond to the fraction of the free drug that is released without any control from the carrier. Therefore, it is possible to hypothesise that a part of the drug can escape the separation by gel-filtration remaining adsorbed on the nanoparticle surface. According to the expectations, the batch B prepared with the highest amount of the drug and showing the highest drug content, presented the highest percentage of a free drug (13%) with respect to batch A (8%).

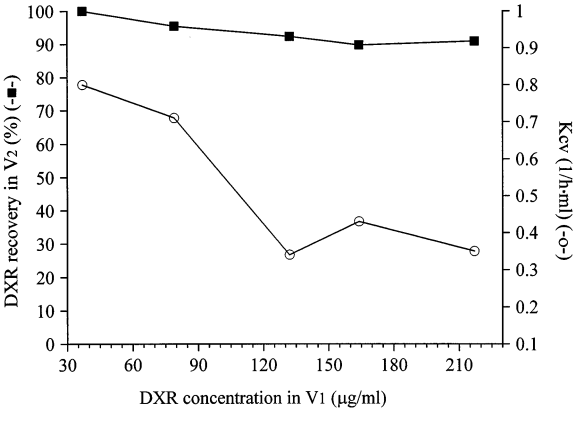

Fig. 7. Doxorubicin (DXR) recovery percentage (-■-) and the apparent permeability constant (K_{cv}) (-○-) values obtained from dialysis of different DXR concentrations in NaCl 2% w/v (V_1: donor solution; V_2: receiver solution).

In order to evaluate the release of the fraction linked to the protein matrix, the dialysis of nanoparticles was carried out in presence of a proteolytic enzyme (trypsin) able to degrade rapidly the gelatin nanoparticles (Leo et al., 1997). The dialysis of the two batches of nanoparticles was performed adding the enzyme to the donor solution after the complete diffusion of the free drug (24 h). Following the addition of trypsin, only a little fraction of DXR diffused in the receiver solution during the further 8 h (12.6 and 23.5% for the batches A and B, respectively) (Fig. 9). Although the dialysis experiments were carried out for 48 h, no additional drug was released from the nanoparticles.

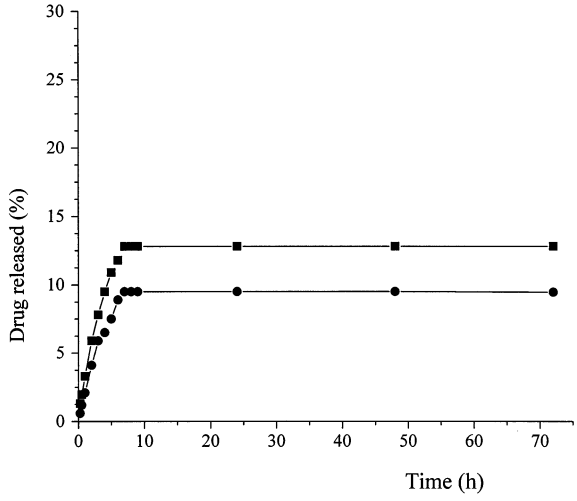

Fig. 9. DXR released in the receiver solution from DXR–gelatin nanoparticles conjugates before and after addition of trypsin solution (0.5 ml; 16 mg/ml in NaCl 0.9%, w/v): Batch A (-●-); Batch B (-■-).

The incomplete diffusion of the drug covalently bound may be explained by two working hypotheses. According to the first ones, it can be considered that trypsin prevents the drug diffusion hindering the membrane pores or interacting with the drug. According to the second hypothesis, the incomplete drug diffusion can be attributable to the enzymatic degradation of nanoparticles. Upon enzymatic degradation, in fact, free or drug-coupled peptides with a molecular weight higher than 3500 Da could take place. As 3500 Da is the value of the membrane cut-off, these peptides should be unable to pass through the membrane. As a consequence, the DXR fraction bound to these peptides cannot be determined in the receiver solution. According to this hypothesis, the incomplete recovery of the drug can be due to the DXR–peptide conjugates having molecular weight higher than the membrane cut-off.

To confirm these hypotheses an additional dialysis experiment was carried out. Free DXR, in the presence of the amount of trypsin (0.5 ml; 16 mg/ml in NaCl 0.9%, w/v) used for the nanoparticle degradation, was dissolved in the donor solution (NaCl 0.9% w/v; 2 ml) and the dialysis procedure was carried out as described. The results showed that the K_{cv} value (0.45 ± 0.02/h/ml) agreed to that observed in the absence of trypsin (0.46 ± 0.02/h/ml). On the basis of this experiment, it is possible to conclude that the incomplete release of the total loaded drug is probably due to the molecular weight (> 3500 Da) of the DXR–peptide conjugates derived from nanoparticle degradation.

4. Conclusions

According to experimental data, although trypsin degraded the carriers, it was unable to cleave DXR bound to the protein matrix. As a consequence, the drug linked to peptides having a molecular weight higher than membrane cut-off cannot diffuse freely through the membrane. Therefore, the little fraction diffused in the receiver solution, after the enzyme addition, could be attributable to a fraction of DXR–peptide conjugates characterised by a molecular weight lower than 3500 Da.

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