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Normal phase high performance liquid chromatography for determination of paclitaxel incorporated in a lipophilic polymer matrix☆

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Abstract

A normal phase (NP) high performance liquid chromatography (HPLC) method was developed for analysis of paclitaxel incorporated in poly(sebacic-co-ricinoleic acid), a lipophilic polymer matrix utilized for preparation of an injectable formulation for the localized delivery of paclitaxel. Thin layer chromatography experiments revealed that separation of paclitaxel from the polymer is dependent on the eluting strength (solvent strength) of the mobile phase. The HPLC system consists of a Purospher® STRAR Si analytical HPLC column (5 μm, 250 mm × 4 mm, Merck), and 1–2.5% (v/v) methanol in dichloromethane as the mobile phase. Detection was by UV absorbance at 240 and 254 nm. The effect of the mobile phase composition on paclitaxel retention, peak shape and column efficiency, and the influence of the sample loading on the shape of the paclitaxel peak were studied. The mobile phases used for the chromatography consisted of 1.5% (v/v) methanol in dichloromethane. Paclitaxel was determined in the formulation and in the samples from degradation studies using UV detection at a wavelength of 254 nm. UV detection at 240 nm has advantages for following polymer matrix degradation products due to higher detector response at this wavelength. The utility of the proposed NP HPLC approach was demonstrated by assessment of intra- and inter-batch content uniformity, and by the determination of paclitaxel content after 7 and 60 days exposure of the paclitaxel-loaded polymer matrix to in vitro and in vivo degradation.
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Keywords: Paclitaxel; Lipophilic polymer; Normal phase HPLC; Ricinoleic acid; Polymer formulation

1. Introduction

Paclitaxel is an antineoplastic agent with poor water solubility (Scheme 1A). The paclitaxel clinical formulation consists of a 1:1 (v/v) mixture of ethanol and Cremophor EL, which is diluted 5–20 folds in normal saline or dextrose isotonic solution prior to infusion. This formulation, however, presents a number of problems including stability, incompatibility with the components of infusion sets and the apparent side effects of Cremophor EL [1]. Therefore, a number of research groups have attempted to develop alternative paclitaxel formulations [1,2]. Biodegradable polymers have been used as carriers for the systemic [3] and localized delivery of paclitaxel [4] in the form of microspheres [5,6], pastes [7], implants [4,8] and micellar dispersions [3,9].

During the development of pharmaceutical formulations, it is necessary to specify their quality in terms of the drug content, formulation stability and content uniformity. This characterization is important for precise dosing of the therapeutic agent and assurance of the desired treatment efficiency.

Due to the low water solubility of paclitaxel, its separation from hydrophobic polymers usually required more sophisticated approaches than the simple liquid–liquid extraction procedures used for separation of water-soluble compounds from hydrophobic polymer matrices [8]. A number

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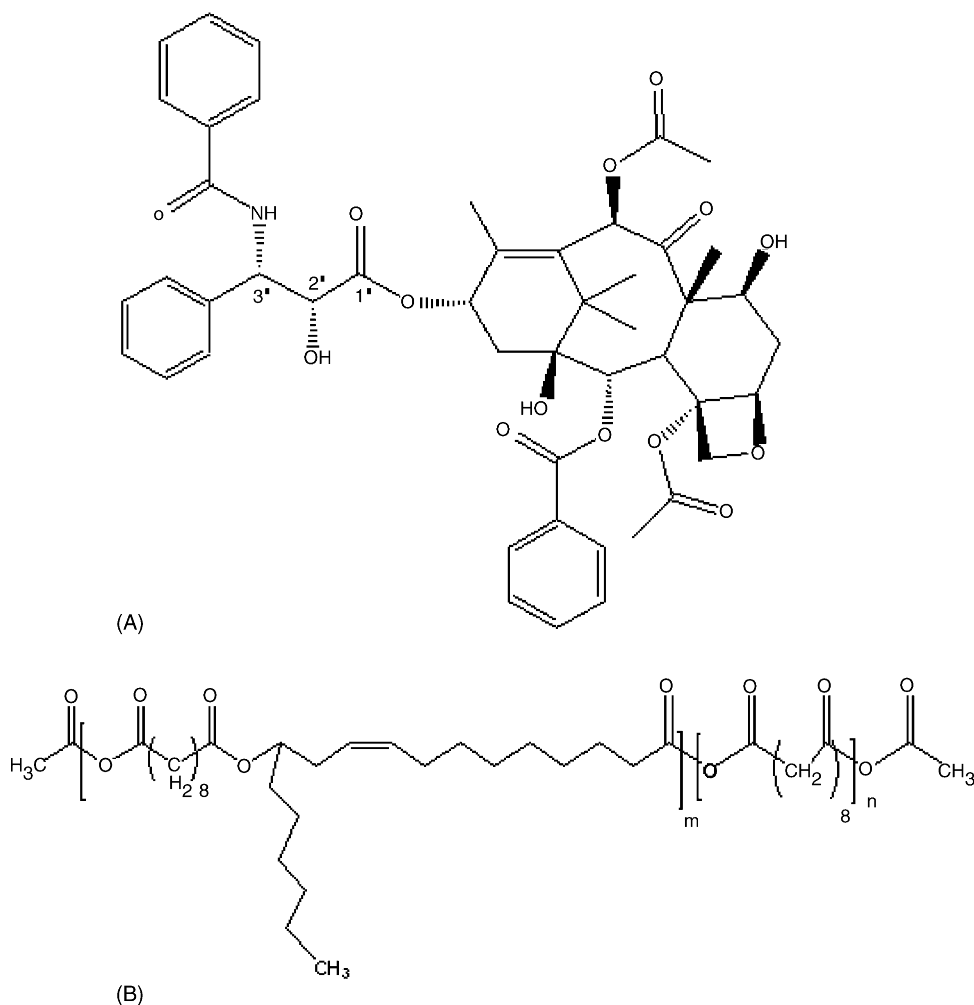

Scheme 1. Chemical structure of paclitaxel (A) and poly(sebacic–ricinoleic ester–anhydride) (p(SA–RA)) (B).

of practical techniques have been described for determination of paclitaxel without separating it from polymer carriers. These include indirect assessment of paclitaxel loading in microparticles [10], direct assessment of paclitaxel content in a polymeric device using radiolabeled paclitaxel [8] and UV absorbance measurements [11]. These methods are useful research tools, but they do not provide information about drug stability or drug carrier interactions that could occur during fabrication and storage. Chromatographic separation techniques using an RP HPLC have been successfully employed for the quantification [5,6], impurity determination [12], and characterization of paclitaxel metabolites [13,14].

Application of chromatographic analytical methods requires preparation of the homogenous samples. Physicochemical properties of polymers, including solubility, are dependent on their chemical composition, structure and molecular weight [15]. Biodegradable polymeric matrices, based on lactic acid and glycolic acid, were dissolved in mobile phases containing residual dichloromethane (DCM) [5,16,17]. This approach was utilized for analysis of paclitaxel incorporated in microspheres made from poly(lactic-co-glvcolic acid) (PLGA) and poly(L-lactic acid) (PLLA). However, it was reported that application of this approach might have resulted in inefficient extraction of paclitaxel by as much as 80% of the initially incorporated drug [5]. Incomplete recovery was also observed for other substances [18]. Liggins and coworkers described a sophisticated and successful method for separation of paclitaxel from a hydrophobic polymer carrier [6,19]. They used a phase separation technique based on precipitation of PLLA at the interface between organic and aqueous phases. The extraction efficiency of paclitaxel from microspheres using this approach was shown to be greater than 98%.

Recently, we described a new type of an injectable polymeric carrier—poly(sebacic acid–ricinoleic acid anhydride) 3:7 (p(SA–RA) 3:7) and its applicability for paclitaxel localized delivery [20]. This polymeric carrier is based on two naturally occurring fatty acids, ricinoleic acid and sebacic acid (Scheme 1B). The ester–anhydride copolymers of these fatty acids are highly hydrophobic with very low solubility in commonly applied solvents in RP HPLC, including acetonitrile,

methanol and ethanol. They are insoluble in non-polar solvents such as saturated hydrocarbons (e.g., hexane and heptane), but sufficiently soluble in dichloromethane and chloroform. Different approaches were considered for separation of paclitaxel from the polymer. Sample processing by selective polymer precipitation from the formulation's solution in dichloromethane according to the method of Liggins and coworkers revealed that p(SA–RA) 3:7 failed to precipitate at the interface between the aqueous and organic phases. This was in contrast to PLLA and PLGA. p(SA–RA) 3:7 formed in organic phase clouded dispersion system that composed of polymer particles. This different behavior of the polymers arises from differences in their physicochemical properties. It was found that application of this separation method using p(SA–RA) 3:7 resulted in irreproducible recovery ranging from 75 to 85% of the initially incorporated drug. Preparation of the sample, by dissolving the formulation in mixture of DCM/ACN with a high content of DCM for maintaining polymer solubility, resulted in peak distortion. The influence of DCM on the chromatographic behavior of paclitaxel was found to be a function of the amount of DCM entering the chromatographic system. The impact of the strong solvents on the peak characteristics is well outlined in the literature [21]. In contrast, in the phase separation approach [6,19] and the approach based on dissolving the polymeric device in the mobile phases containing dichloromethane residue [5,16,17], the amount of strong solvent entering the chromatographic system is low, and therefore, the analytical procedure is not comprised. Dissolving the formulation based on p(SA–RA) 3:7 in a mixture of DCM/ACN with a high content of DCM can also cause the polymer precipitation in the chromatographic system as samples diluted with mobile phase.

Polyanhydrides were found to be chemically incompatible with the reactive model drugs, *para* substituted anilines, when injection molded with the polymers at 120 °C [22]. However, no reaction was observed using compression molding at room temperature. As a result of this study, Leong and coworkers suggested that other groups such as hydroxyls and sulfhydryls, which are less reactive toward anhydrides, have the potential to react with polyanhydrides with the formation of ester and thioester bonds. It was also important that the drug should not react slowly with the matrix during storage. The nucleophilic properties of 2′-hydroxyl group of paclitaxel have been utilized for synthesis of its derivatives for targeted therapy of cancer [23,24]. Therefore, an analytical procedure that will allow reproducible and complete recovery of paclitaxel from the formulation, based on the carrier containing anhydrides bonds, is important for assessing the formulation's stability during preparation and storage.

In the literature, two approaches were considered as suitable for paclitaxel separation from the lipophilic polymer: solid phase extraction (SPE) and normal phase liquid chromatography (NPLC). SPE of paclitaxel based on a cyano or C_{18} modified stationary phase is described in the literature, especially for its separation from biological samples [14,17,25,26]. This approach necessitates multiple handling of the samples, requires recovery validation due to batch-to-batch retention variability of cartilage packing [27], and is a time-consuming and laborious process if not automated [25].

NPLC could be an alternative to reverse phase LC analytical protocols for determination of paclitaxel incorporated in lipophilic polymer matrices. In NPLC, the ability to operate with totally organic mobile phases and the flexibility in selection of the applied solvents [28] allows for adjusting the mobile phase composition to control the polymer solubility. A homogenous solution of polymeric matrices containing paclitaxel can be injected directly into the chromatographic system without extracting the drug from an organic solvent. NPLC was utilized for preparative isolation of paclitaxel from the yew tree crude extract [29–31]. This approach caused wide interest because RP HPLC was not sufficiently selective for closely eluting taxanes which need a gradient mode of elution. In addition, the low solubility of taxanes in aqueous solutions often results in increased column back-pressure, caused by precipitation of products in the crude sample whereas the higher solubility of taxanes in mobile phases of NPLC allows for purification of a larger quantity of paclitaxel when the separation procedure is performed under mass-overload conditions [30,31].

The objectives of the present study were to develop an NPLC analytical method for analysis of paclitaxel incorporated in a lipophilic polymer matrix and to assess the applicability of the NPLC method for paclitaxel determination during hydrolytic degradation of the polymeric device under in vitro and in vivo conditions.

2. Experimental

2.1. Chemicals and reagents

Paclitaxel (Lot. DF14, purity of 99.1%) was obtained from Bioxel Pharma (Sainte-Foy, Canada). HPLC grade acetonitrile, tetrahydrofuran, methanol, dichloromethane, and *n*-hexane were purchased from BioLab Ltd (Jerusalem, Israel). Poly(sebacic acid-co-ricinoleic acid) 3:7 (p(SA–RA) 3:7) was prepared as previously described [20,32]. The ricinoleic acid reference (Lot 77102, purity 99%) was purchased from ICN Biomedicals Inc. (Ohio, USA).

2.2. Thin layer chromatographic analysis (TLC)

TLC assessment of paclitaxel separation from the polymeric matrix was performed on silica gel 60 F_{254} (Merck, Darmastadt, Germany), 0.25 mm thick, 20 cm × 20 cm plastic sheets, utilizing a mobile phase consisting of mixtures of dichloromethane with methanol, acetonitrile or tetrahydrofuran at different ratios (see Table 1). Sample solutions were prepared in dichloromethane. Spots were detected by a UV lamp at 254 nm and confirmed by non-specific adsorption of iodine.

Table 1
Assessment by TLC of the influence of solvent B type on paclitaxel retention and separation from polymer

Solvent no.	Dichloromethane:MeOH			Dichloromethane:ACN			Dichloromethane:THF		
	Ratio (v/v)	Rf_{pacl}	Rf_{pol}	Ratio (v/v)	Rf_{pacl}	Rf_{pol}	Ratio (v/v)	Rf_{pacl}	Rf_{pol}
1	99:1	0.04	0.05	98:2	0.00	0.00	98:2	0.00	0.56
2	98:2	0.22	SF	96:4	0.00	SF	96:4	0.00	0.64
3	97:3	0.29	SF	80:20	0.14	SF	91:9	0.12	SF
4	96:4	0.35	SF	70:30	0.42	SF	80:20	0.52	SF
5	95:5	0.46	SF	50:50	0.82	SF	70:30	0.88	SF

Dichloromethane only: $Rf_{pacl} = 0$; $Rf_{pol} = 0.00$. Abbreviations: MeOH, methanol; ACN, acetonitrile; THF, tetrahydrofuran; Rf_{pacl}, retention factor of paclitaxel; Rf_{pol}, retention factor of the polymer; SF, solvent front.

2.3. Normal phase HPLC (NPLC)

2.3.1. Apparatus

NPLC analysis was carried out using an HPLC system consisting of an HP 1050 quaternary pump, an HP 1050 autosampler with a 200 μl loop, and an HP 1050 Photodiode Array Detector coupled with an HP ChemStation for LC 3D Systems intended for data processing and peak purity analysis (Agilent Technologies, Palo Alto, USA). A Purospher® STAR Si analytical HPLC column (250 mm × 4 mm; particle size, 5 μm) was used, which was protected with a Purospher® STAR Si guard column (4 mm × 4 mm; particle size, 5 μm) (Merck, Darmstadt, Germany). The column was at ambient temperature (25 ± 1 °C). The mobile phase consisted of DCM and MeOH at different ratios (1–2.5%, v/v). An isocratic mode of elution was utilized with a rate of 1 ml/min, and injection volumes varied as specified in the text. UV detection was at two wavelengths, 240 and 254 nm. Column maintenance was performed by flushing overnight with dry methanol once in 2 weeks as required. This maintained constant water content of the stationary phase and prevented wide variations in the retention time of the analyzed compound [33].

2.4. Polymeric device fabrication

Paclitaxel powder was incorporated in p(SA–RA) 3:7 by trituration [20].

2.5. In vitro degradation

In vitro degradation was performed as previously described [20]. Briefly, 20 mg of the polymeric formulation containing 10, 15 or 20% (w/w) of paclitaxel were incubated in 50 ml of 0.1 M phosphate buffer solution pH 7.4 at 37 °C with constant shaking (100 rpm). The phosphate buffer solution was replaced periodically After 60 days in phosphate buffer, the remnants of the polymer formulation were lyophilized, weighted and dissolved in a sufficient amount of dichloromethane so that the final concentration of paclitaxel did not exceed 0.15 mg/ml, assuming that the paclitaxel content in the device was equal to the initial loading. Degradation studies were performed in triplicates.

2.6. In vivo degradation

The in vivo degradation was performed by subcutaneous injection of the polymer formulation (200 μl) into the dorsal side of 8–9 weeks old Balb/c male mice (Harlan laboratories, Jerusalem, Israel). The injected formulation was p(SA–RA) 3:7 loaded with 5% (w/w) paclitaxel. After 1 week, the mice were sacrificed by cervical dislocation, the polymeric devices were removed from the injection site, lyophilized to dryness, and weighted. All samples were dissolved in a sufficient amount of dichloromethane so that the final concentration of paclitaxel did not exceed 0.15 mg/ml, assuming that paclitaxel content in the device is equal to the initial loading. Each group consisted of four mice.

All animals were kept under specific pathogen-free conditions and given free access to irradiated food and acidified water throughout the experiment. The ethics committee at the Hebrew University in Jerusalem (National Institutes of Health approval number: OPRR-A01-5011) reviewed our application for animal study and found it compatible with the standards for care and use of laboratory animals (ethics committee research number: MD-80.04-3, date: 05/01/2003).

2.7. Recovery study

Recovery studies included the determination of paclitaxel content in the dichloromethane solution containing both examined polymer and paclitaxel. The following two solutions were analyzed in triplicate for recovery studies: 0.2 mg/ml of paclitaxel and 3.8 mg/ml p(SA–RA) 3:7; and 0.2 mg/ml of paclitaxel and 1.8 mg/ml of p(SA–RA) 3:7. These solutions mimic the samples prepared from polymeric formulations containing 5 and 10% (w/w) paclitaxel. The injection volume was 15 μl to fit the concentration range of the validated calibration curve (0.04–0.18 mg/ml).

2.8. Validation

The validity of the analytical procedure was established through a study of specificity, precision, linearity, and accuracy according to the compliance criteria laid down in the ICH guidelines [34,35]. The ability to assess unequivocally the analyte in the presence of matrix components was val-

uated by analyzing the polymer matrix and paclitaxel separately. The selectivity of paclitaxel determination in the samples from the degradation studies was achieved by adjusting the separation and detection conditions (see explanation in the text). The linearity of the analytical procedure was evaluated by plotting the detector response (peak area) against analyte concentration. Linear regression analysis was applied to calculate the slope, intercept and linear correlation coefficient (R^2) [35]. The precision (RSD) of the analytical procedure was evaluated by determining the intra- and inter-day coefficients of variation [34,35]. The intra-day precision of the selected methods was estimated by analysis of six replicates of the quality control samples at four concentrations covering the specified range. The inter-day precision was assessed by analyzing quality control samples in the same mode as for the intra-day precision assay, and was repeated for three consecutive days [34,36]. The intermediate precision of linear response was also evaluated. The accuracy was established by quantitative determination of the paclitaxel amount in quality control samples and was expressed as percent recovery by the assay of a known amount of analyte in the samples [35]. For this determination, five different injected volumes of the quality control samples of known concentrations were repeatedly injected six times. The limit of detection (LOD) was calculated as signal-to-noise ratio of 3:1, and the limit of the quantification (LOQ) was determined as signal-to-noise ratio of 10:1 [35].

2.9. Calibration standards and quality control samples

Standard solutions and quality control samples for NPLC were prepared by dissolving a known amount of paclitaxel in dichloromethane. The number of points used in each curve was 8. Calibration curves were obtained by programmed injection of different aliquots (10–45 μl) of a standard solution with increments of 5 μl. The concentration of standard solutions was 0.1 mg/ml. For calculation of the concentration range, a fixed injection volume of 25 μl was assumed. Quality control samples were prepared separately at the same concentration and injected in the five different volumes that cover the specified range (Table 2). Each quality control sample and standard solution was divided into a number of vials and from each vial only one injection was taken to prevent concentration changes due to solvent evaporation after piercing of the vials' seal.

Table 2
Accuracy assessment of the NPLC analytical method for paclitaxel assay

Sample no.	Spiked concentration (mg/ml)	Recovery (%, $n=6$)	Deviation (%)
1	0.04	98.89	−1.11
2	0.07	98.77	−1.23
3	0.1	98.56	−1.44
4	0.14	97.95	−2.05
5	0.18	96.96	−3.04
Average absolute error (%)		1.77–0.80	

2.10. Calculations

The column's dead volume (t_0) was determined from the time of the negative peak of hexane [37]. Linear regression analysis and all calculations of peak characteristics' parameters were performed by HP ChemStation for LC 3D Systems (Revision: A.04.01) according to guidelines of the US Pharmacopoeia [38].

2.11. Peak purity assessment

Peak purity testing is based on evaluation of the degree of similarity of UV spectra across the peak. The peak purity was calculated using the HP ChemStation for LC 3D Systems software for peak purity evaluation. Noise contribution was calculated from the first 14 spectra in the run that consisted only of baseline and did not contain any peak in this region [14]. Linear interpolation of the two reference spectra (the integrated peak start and end) was subtracted from each spectrum in order to compensate for influence of the mobile phase spectral absorption. An appropriate threshold for each spectrum in the peak was calculated automatically by the software. Comparison of the degree of similarity of all spectra across the peak was performed against the apex spectrum within the peak [14]. Spectra were obtained during the entire separation run for each sample over a wavelength range of 240–450 nm. The peak was classified as pure if the purity factor was within the threshold value and at least 97% of the spectra were within the calculated threshold limit. Peak purity evaluation was performed with the purpose of obtaining additional supportive information during selection of the appropriate detection conditions that allowed specific determination of paclitaxel.

3. Results and discussion

TLC was applied for initial evaluation of the influence of mobile phase composition on the interaction of the solute with silica as adsorbent [28,29]. Three different polar modifiers, acetonitrile, methanol and tetrahydrofuran, were selected for the investigation of their influence on the paclitaxel separation from the polymeric matrices (Table 1). Paclitaxel and p(SA–RA) 3:7 eluted as single spots. All mobile phases showed a similar trend of faster elution of the polymer in comparison to paclitaxel.

According to results obtained in the TLC studies the localization capacity of paclitaxel and p(SA–RA) 3:7 are different, because paclitaxel and polymeric matrix retention exhibited significant differences in the sensitivity to the content of the polar modifiers in the mobile phase. This difference is originated from the diversity of their physicochemical properties.

p(SA–RA) 3:7 is a polyester-co-polyanhydride (Scheme 1B), made from ricinoleic and sebacic acid moieties connected by ester and anhydride bonds [32]. The high content of fatty side chains of ricinoleic acid along the polymer back-

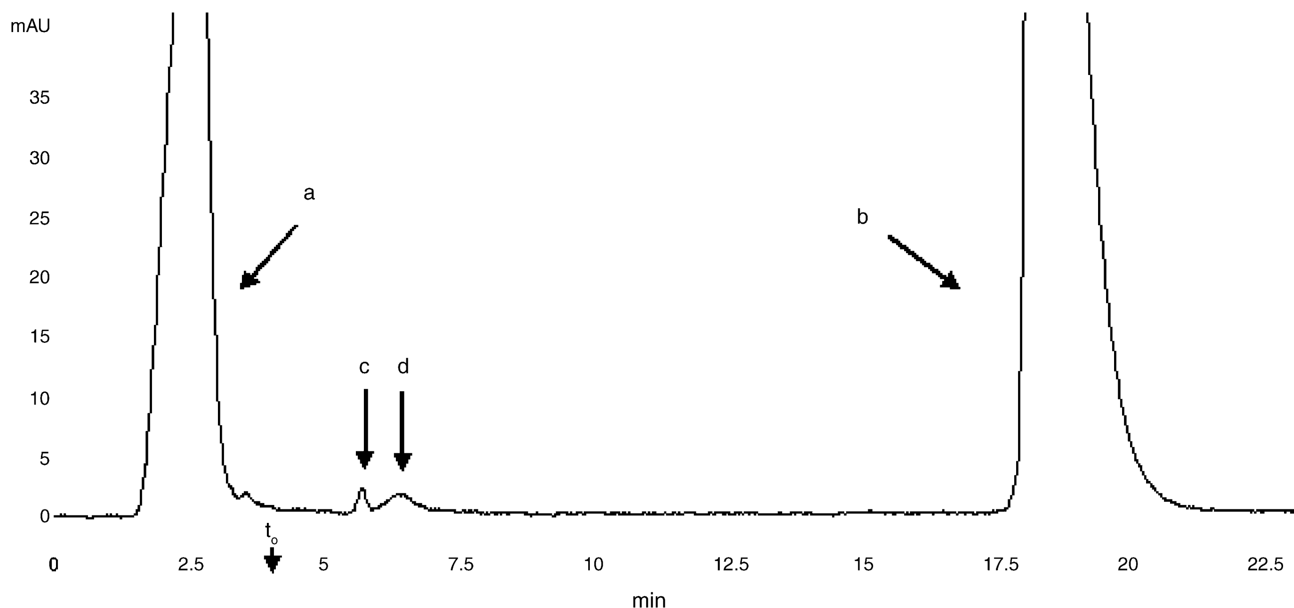

Fig. 1. Representative chromatograms obtained for the polymer carrier with paclitaxel. Elution conditions: mobile phase: dichloromethane:methanol, 99:1 (v/v); flow rate: 1 ml/min; UV detection was at 240 nm. Peak identification: (a) polymer carrier; (b) paclitaxel; (c) ricinoleic acid; (d) low molecular weight oligomers.

bone mask the majority of ester and anhydride bonds and thus determine the polymer lipophilicity in comparison with paclitaxel. Paclitaxel, on the other hand, is characterized by a more polar nature (Scheme 1). These TLC experiments indicate that regardless of the type of polar modifier, the polymer is easily separated from paclitaxel.

The HPLC experiments confirmed the TLC results, which showed that the polymer elution occurred very rapidly when paclitaxel was retained on the silica (Fig. 1). Moreover, HPLC experiments revealed that under the conditions studied, the main components of the p(SA–RA) 3:7 eluted mainly before t_0, indicating that the high molecular weight components of the polymer were excluded from the pores of the column packaging material.

Studies on the influence of the mobile phase composition on paclitaxel retention and peak shape were performed using mixtures of methanol–dichloromethane as the mobile phase. This simple binary mobile phase composition was selected, because methanol can control and maintain the activity of the silica surface [33]. The relationship between analyte retention and the eluting strength of mobile phase was investigated in the range of 1–2.5% (v/v) methanol in dichloromethane. Fig. 2 shows the paclitaxel retention factor (k') as a function of the methanol content in the mobile phase. This experimentally obtained dependence fits the power type equitation. Plotting the log retention factor against log methanol mole fraction in the mobile phase yields a linear dependence, which is described by the Soczewihski equation [39], and which was supported experimentally by a large number of reports of NPLC applications [28].

Changes in the retention time of the analyte with the increase of the eluting strength of the mobile phase were also followed by changes in peak symmetry. At a fixed analyte load the paclitaxel peak shape specified by the USP tailing factor (T) [33,40] also expressed power dependence as analyte retention: $T = 0.1158X_B^{-0.694}$, $R^2 = 0.9957$ ($n = 6$, RSDs were in the range of 0.5–1.0%). As peak asymmetry increases, integration and hence precision, becomes less reliable. Since peak tailing is also influenced by sample size [30,33] it is important to assess the influence of the sample loading on the paclitaxel peak shape at different contents of methanol in the mobile phase. Fig. 3 showed the influence of solute loading and mobile phase composition on the peak shape. The impact of band tailing on the alteration of column efficiency is well outlined in the literature [33,40]. Typical

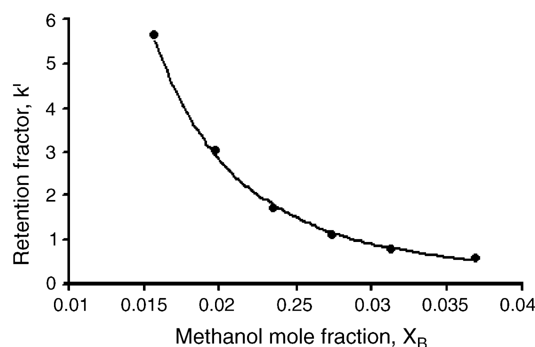

Fig. 2. Paclitaxel retention factor (k') as a function of methanol content in the mobile phase: $k' = 5E{-}05X_B^{-2.8025}$; $R^2 = 0.9971$. Analysis was performed using dichloromethane containing methanol (1; 1.25; 1.5; 1.75; 2 and 2.5%, all v/v) as mobile phase. Flow rate: 1 ml/min, sample concentration: 0.1 mg/ml, injection volume: 20 μl; and UV detection at 240 nm. Each point represents the average value of six measurements; RSDs were in the range 0.2–0.9%.

changes in theoretical plate numbers with increase of mobile phase strength were in the range of 4090–9050.

Use of protic organic modifiers instead of water for the control of silica surface activity is generally successful in NPLC [33]. However, misshaping or tailing some solutes' peaks could occur when the mobile phase containing low concentrations of protic solvent modifier is used [41]. Kirkland et al. [33] suggested that for the mobile phases based on dichloromethane the level of protic modifiers should exceed 0.2% (v/v) for most silicas. Park and coworkers studied the chromatographic behavior of paclitaxel in NPLC on bare silica with a fixed mobile phase composition and different sample loadings including mass-overload conditions [30]. In this study, the mobile phase was hexane:2-propanol:MeOH, 90:4:6% (v/v) and the isocratic mode of elution was utilized. They found that at a low paclitaxel amount, which is comparable with the maximum loading examined in the present work, the paclitaxel peak had a Gaussian elution profile. In the present work, results with the Purospher® STAR Si analytical column show that when the methanol concentration in the mobile phase exceeds 2% (v/v), the paclitaxel peak shape approaches the ideal Gaussian profile (Fig. 3). Park et al. as well as other researchers found that adjusting the separation conditions can be achieved by applying a mobile phase consisting of a major non-polar component such as hexane. They used a higher concentration of protic solvent modifiers than was used in the present work [29–31]. Thus, the future optimization approach could be based on the incorporation of a non-polar diluent into the mobile phase and increasing the content of the protic solvent modifiers.

Fig. 1 shows the chromatographic behavior of the polymeric matrix during elution with dichloromethane containing 1% (v/v) methanol. Only two additional peaks were observed together with the main peak of the polymer (peak a). One peak was ricinoleic acid as determined by an independent ran of standard ricinoleic acid (peak c) and the other was attributed to short oligomers (peak d). When the content of methanol was increased to 1.5% (v/v), two additional peaks (c and d)

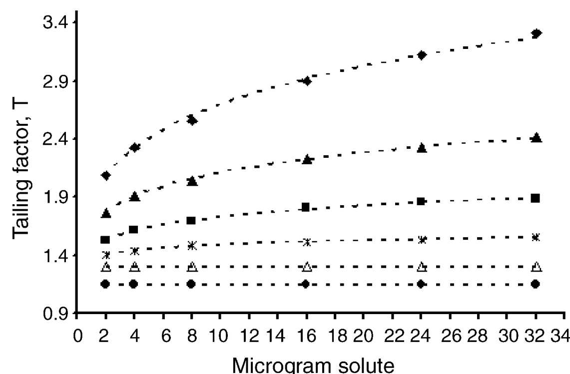

Fig. 3. Effect of sample loading and mobile phase composition on peak symmetry. Paclitaxel eluted by dichloromethane containing different amounts of methanol: 1% (v/v) (♦); 1.25% (v/v) (▲); 1.5% v/v (■); 1.75% v/v (*); 2% v/v (△); 2.5% v/v (●). RSDs were in the range of 0.5–1.2%.

merged to one that has the retention time of ricinoleic acid under these conditions (Fig. 5C).

The utilization of the proposed chromatographic method for paclitaxel determination during hydrolytic degradation of the polymeric device was assessed by analysis of the samples obtained from degradation studies. Representative chromatograms of polymeric formulations containing 10% (w/w) paclitaxel exposed to degradation in 0.1 M buffer phosphate pH 7.4 for 60 days at 37 °C are shown in Fig. 4. According to the retention times, the following peaks: a, b, c, and d were attributed to low molecular weight oligomers of the polymer carrier (Fig. 5A) and c′ is of ricinoleic acid.

In vivo degradation of the polymeric matrix with and without paclitaxel showed that incorporation of paclitaxel changed the overall profile of matrix degradation. Fig. 5 shows representative chromatograms of the polymeric formulation following in vivo degradation. After 1 week in mice both the blank polymer matrix and polymer containing 5% paclitaxel gave characteristic peaks of the partially degraded polymeric matrix (Fig. 5A and B, peak a). The profile of this peak is different form that of the non-degraded polymer (Fig. 5C). This difference could be explained by the rapid release of the sebacic acid component inserted among oligomeric esters chains of ricinoleic acid [32,42]. Additional degradation products of the blank polymeric matrix appeared in the chromatogram until 6.5 min. The height of the two peaks (peaks b, c; Fig. 5A) was found to be similar to the peaks of the partially degraded polymeric matrix, whereas in the paclitaxel-loaded formulation the relative response to peak (a) of the same peaks (b) and (c) was dramatically low (Fig. 5B). A comparison between the chromatographic profiles of the degradation products in vivo of the polymer with and without paclitaxel indicates that the paclitaxel-loaded formulation degrades at a slower rate than the polymer blank alone. This observation supports the in vitro degradation results [20], which showed that incorporation of paclitaxel in the polymeric matrix increases the overall hydrophobicity of the system and does not allow water to penetrate and degrade the polymer. Therefore, the subsequent hydrolysis of the partially degraded polymeric matrix (peak a, Fig. 5) to lower molecular weight oligomers (peaks b and c, Fig. 5) is retarded. Applying NPLC supported the interpretation of the hydrolytic degradation of the polymeric matrix and the influence of the drug carrier interactions on this process. Under conditions at which paclitaxel quantification was favored, the detailed separation of polymer degradation products was not achieved, because of the large difference in the physicochemical properties of the investigated drug, the polymer, and the products of its degradation.

The selection of the method for paclitaxel determination was based on peak performance characteristics and the specificity of the analyte assessment ensured by chromatographic separation and selective detection at a specified wavelength. Separation of paclitaxel from the non-degraded polymer can be achieved easily by the proposed approach (Fig. 5C). However, evaluation of purity of the paclitaxel peak obtained from

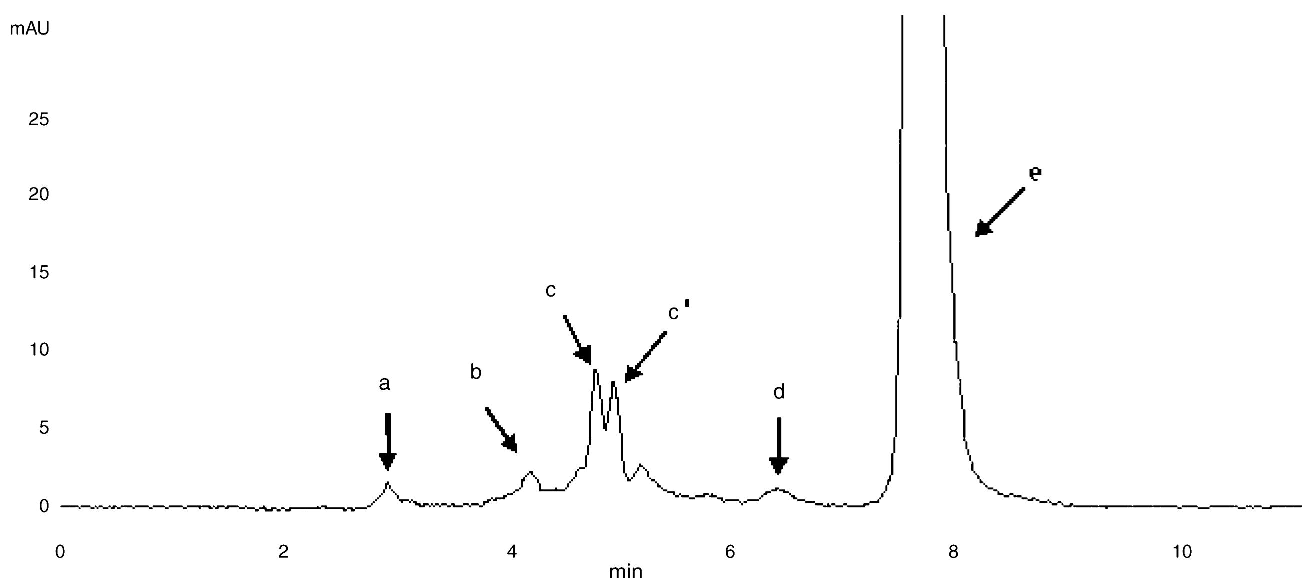

Fig. 4. Chromatographic separation of paclitaxel from polymer degradation products in vitro. Elution conditions: mobile phase dichloromethane:methanol, 98.5:1.5 (v/v); flow rate: 1 ml/min; injection volume: 20 μl; and UV detection at 240 nm. Peak identification: low molecular weight polymer degradation products: a, b, c and d, c′: ricinoleic acid and other low molecular weight oligomers (see Figs. 3, 5A and C); e: paclitaxel.

the samples from the in vitro and in vivo degradation when detection was performed at 240 nm revealed that they are not completely pure. Probably, this results from the simultaneous elution of the short oligomers and paclitaxel. These short oligomers are created during polymer degradation and their leaching from the polymer matrix bulk is delayed because of hydrophobic interactions with the drug. This is one of the causes of slower weight loss of the formulation in comparison to polymer blank [20]. In order to overcome the problem of the simultaneous elution of interfering compounds, chromatography using a mobile phase containing 1.5% (v/v) methanol and detection at a wavelength of 254 nm was selected. The choice of these conditions was made by testing the degree of similarity of UV spectra across the paclitaxel peak in the different ranges of wavelengths at maximum paclitaxel loading with acceptable peak characteristics (Fig. 3). At the range of 254–450 nm paclitaxel peaks for samples of in vitro and in vivo degradation studies was classified as pure. It should be noted that that UV detection at 240 nm has advantages for following polymer matrix degradation products due to the higher detector response at this wavelength.

The linearity of the selected method was established at a concentration range of 0.04–0.18 mg/ml. Linear regression analysis yields a slope of 10951 with an RSD of 0.06% for inter-day variation; the *Y*-intercept was 10 with RSD of 15% for inter-day variation and a linear correlation coefficient (R^2) of 0.99971. The limit of quantification (LOQ) and the limit of detection (LOD) were determined using six injections of independently prepared solutions at different concentrations. LOD was 1.40 μg/ml (14.3% RSD) and LOQ was determined as 3.42 μg/ml (5.3% RSD). Inter-day retention time repeatability was assessed during three consecutive days. These chromatographic conditions showed good intra- and inter-day retention time repeatability with 0.51–0.67% RSD The fluctuations of paclitaxel retention time between maintenance procedures were around 3%. The intra-assay precision was 1.25% and inter-day assay precision was 1.35%. The assay accuracy was within 2% (Table 2). Recovery studies showed that utilization of the NPLC analytical approach allows good recovery as high as 97.87 ± 1.22% for methods utilizing mobile phases with 1.5% (v/v) methanol.

The utility of the method was demonstrated by assessment of the intra- and inter-batch content uniformity and determination of the paclitaxel amount remaining after its release from the polymeric device exposed to in vivo and in vitro degradation. The content uniformity test was performed on three different batches of polymeric formulations containing 5 and 10% (w/w) of paclitaxel prepared by manual trituration. The obtained results are summarized in Table 3. The inter-batches and inter-formulations statistical comparison revealed that there is no significant difference between manually prepared batches containing 5% (w/w) paclitaxel, however, differences were found among batches containing 10% (w/w) paclitaxel. Despite the statistical difference, the obtained data indicate that the proposed method of paclitaxel incorporation in p(SA–RA) 3:7 is characterized by good inter-batch reproducibility (RSD < 6%, USP limitation for content uniformity variability) and enables an acceptable degree of intra-batch homogeneity that lies within the generally specified by USP tolerance limits of 85–115% of the labeled claim (RSD < 6%, $n = 10$ for first tier of the test) [43].

The estimated paclitaxel remnants in the polymeric formulation after 60 days of degradation in vitro indicated that the cumulative release of paclitaxel was influenced by the initial drug content in the formulation (Table 4). Moreover, recovered paclitaxel content was higher in comparison to the initial

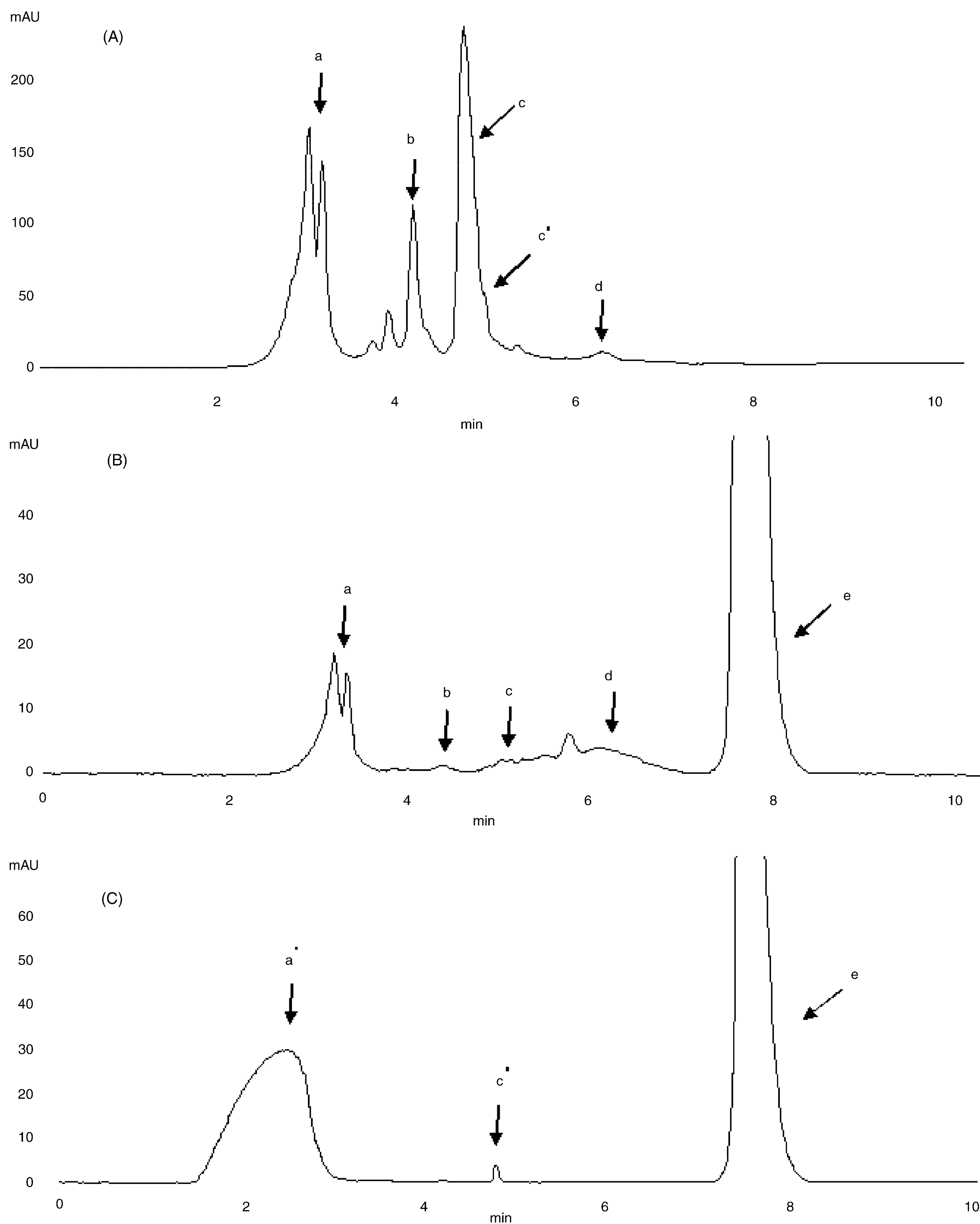

Fig. 5. Chromatographic separation of paclitaxel from polymer degradation products in vivo. Polymer carrier (A) and polymeric formulation containing 5% paclitaxel (B) after 1 week of degradation in vivo, non-degraded polymeric formulation with paclitaxel (C). Chromatographic separation was performed under conditions described in Fig. 4. Peak identification: (a) partially degraded polymer; (a′) non-degraded polymer matrix; (b–d) low molecular weight oligomers; (c′) ricinoleic acid; (e) paclitaxel.

Table 3
Content uniformity of polymer formulation based on p(SA–RA) 3:7 containing 5 and 10% (w/w) of paclitaxel[a]

Formulation	Batch	Maximum content	Minimum content	Mean content	SD	RSD (%)	Inter-batch comparison	Inter-formulation comparison
p(SA–RA) 3:7; paclitaxel 5% (w/w)	1	114.5	93.6	102.4	5.9	5.8	ns ($p > 0.05$) versus batch 2 and 3	vs ($p < 0.01$) p(SA–RA) 3:7 paclitaxel 5% w/w batch 2 versus p(SA–RA) 3:7 paclitaxel 10% batch 1
	2	113.3	98.1	105.8	4.9	4.6	ns ($p > 0.05$) versus batch 1 and 3	
	3	105.3	97.0	101.6	2.9	2.9	ns ($p > 0.05$) versus batch 1 and 2	
p(SA–RA) 3:7; paclitaxel 10% (w/w)	1	109.7	92.0	97.7	5.5	5.6	vs ($p < 0.01$) versus batch 2	
	2	109.9	97.2	103.6	4.1	4.0	ns ($p > 0.05$) versus batch 3	
	3	108.3	97.3	102.0	3.1	3.0	ns ($p > 0.05$) versus batch 1 and 2	

[a] Maximum, minimum and mean paclitaxel content expressed as percentage (w/w) of the labeled claim. Statistical comparison among batches was performed applying Kruskal–Wallis test (nonparametric ANOVA) followed by post-test Dunn's multiple comparisons test [44]. Number of the samples in each batch was 10. The samples ranged between 18 and 23 mg.

Table 4
Paclitaxel release from different polymer formulations exposed to degradation in vitro for 60 days (see details in text)

Formulation	Paclitaxel loading (%, w/w)	S.D. (%, w/w, $n = 4$)	Recovered paclitaxel amount (percentage of initial)	S.D. (%, $n = 3$)	Recovered paclitaxel content (%, w/w)	S.D. (%, w/w, $n = 3$)
p(SA–RA) 3:7; paclitaxel 10% (w/w)	10.3	0.59	45.6	3.4	18.4	1.4
p(SA–RA) 3:7; paclitaxel 15% (w/w)	14.65	0.81	51.7	4.0	24.8	2.0
p(SA–RA) 3:7; paclitaxel 20% (w/w)	21.0	1.0	94.4	1.1	43.8	5.3

content indicating that dissolution of the polymer degradation products is higher than that of paclitaxel.

Paclitaxel loading of the formulation utilized in the in vivo studies was 5.20% (w/w, ±0.29%, $n = 4$). After 1 week of degradation in vivo, the paclitaxel content in the polymeric formulation increased to 6.24% (w/w, ±0.92%, $n = 4$) and the average recovered paclitaxel amount was 71.5% (w/w, ±12%, $n = 4$) of the initial content. This data indicates that the formulation degrades in vivo much faster than in vitro conditions [20] thus the release of paclitaxel is enhanced.

In conclusion, a useful NPLC method for determination of paclitaxel incorporated in the lipophilic polymeric matrix was developed. This method allows complete chromatographic separation and recovery of paclitaxel from a polymeric matrix. It is characterized by good linearity, reproducibility and accuracy. The method applicability was demonstrated by assessment of the intra- and inter-batch content uniformity and determination of the paclitaxel remaining after its release from a polymeric formulation exposed to degradation in vitro and in vivo.

3.1. Prospective

Future investigations will include the optimization of the mobile phase composition that will allow better peak performance characteristics and guarantee of long-term retention time repeatability. This optimization will focus on development of stability indicated analytical method for more detailed characterization of the drug fate during formulation storage and at different stages of formulation production, such as fabrication, container filling and sterilization. They will also include utilization of the NPLC for the simulations of possible paclitaxel–polymer interactions.

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