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ORIGINAL ARTICLE

Validated HPLC method for paclitaxel determination in PLGA submicron particles conjugated with α-fetoprotein third domain: Sample preparation case study

Méthode HPLC validée pour la détermination du paclitaxel dans des submicroniques PLGA conjuguées au troisième domaine de l'α-foetoprotéine : étude de cas sur la préparation des échantillons

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HIGHLIGHTS

- We designed paclitaxel loaded PLGA submicron particles conjugated with a recombinant alpha fetoprotein third domain (rAFP3d-SP).
- Simple, fast and efficient sample preparation method for HPLC determination of paclitaxel in rAFP3d-SP formulation was developed.
- HPLC method with UV detection was successfully validated as per as ICH Q2 (R1) guideline.
- The method can be used for paclitaxel determination in such formulation like PLGA particles conjugated with protein vector molecule.

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KEYWORDS
HPLC;
Paclitaxel;
Alpha-fetoprotein third domain;
PLGA;
Sample preparation

Summary
Objectives. — The goal of this study was to develop sample preparation method and validate the HPLC method for precise determination of paclitaxel (Ptx) in PLGA submicron particles conjugated with protein vector molecule.
Methods. — Ptx loaded PLGA submicron particles were formulated by a single emulsification method. PLGA submicron particles were conjugated with alpha fetoprotein third domain (rAFP3d) via standard carbodiimide technique. The obtained conjugate was analyzed using 1525 binary pump and 2487 UV—VIS detector system (Waters, USA) and Reprosil ODS C-18 analytical column with the dimensions of 150 mm × 4.6 mm ID × 5 μm (Dr. Maisch GmbH, Germany). Sample preparation method was developed utilizing guard cartridge with C18 stationary phase (Phenomenex, USA). HPLC method was validated according to the international conference on harmonization guidelines.
Results. — Efficient sample preparation was achieved using 4% of DMSO pre-dissolution, following by 10 min of centrifugation at 4500 g. Ptx determination was performed using acetonitrile/0.1% phosphoric acid (50:50 v/v) mobile phase at a flow rate of 1.0 mL/min, injection volume of 10 μL, and at 227 nm. The developed method showed linearity, accuracy and precision in the range from 0.03 to 360 μg/mL, with LOD and LOQ values of 0.005 and 0.03 μg/mL, respectively. The intra- and inter-day precisions presented RSD values of lower than 2%.
Conclusion. — The validated method was successfully applied to calculate Ptx encapsulation efficacy and drug loading in the developed formulation.

MOTS CLÉS
HPLC ;
Paclitaxel ;
Troisième domaine alpha-foetoprotéine ;
PLGA ;
Préparation d'échantillons

Résumé
Objectifs. — Le but de cette étude était de développer une méthode de préparation d'échantillons et de valider la méthode HPLC pour la détermination précise du paclitaxel (Ptx) dans des particules submicroniques PLGA conjuguées à une molécule de vecteur protéique.
Méthodes. — Des particules submicroniques PLGA chargées de Ptx ont été formulées par une seule méthode d'émulsification. Les particules submicroniques PLGA ont été conjuguées avec le troisième domaine de l'alpha foetoprotéine (rAFP3d) via la technique standard de carbodiimide. Le conjugué obtenu a été analysé en utilisant une pompe binaire 1525 et un système de détection UV-VIS 2487 (Waters, USA) et une colonne analytique Reprosil ODS C-18 avec des dimensions de 150 mm × 4,6 mm DI × 5 μm (Dr. Maisch GmbH, Allemagne). La méthode de préparation des échantillons a été développée en utilisant une cartouche de garde avec une phase stationnaire C18 (Phenomenex, USA). La méthode HPLC a été validée conformément à la conférence internationale sur les directives d'harmonisation.
Résultats. — Une préparation efficace des échantillons a été obtenue en utilisant 4 % de prédissolution de DMSO, après 10 min de centrifugation à 4500 g. La détermination du Ptx a été réalisée en utilisant une phase mobile acétonitrile/0,1 % d'acide phosphorique (50 :50 v/v) à un débit de 1,0 mL/min, un volume d'injection de 10 μl et à 227 nm. La méthode développée a montré une linéarité, une précision et une précision comprises entre 0,03 et 360 μg/mL, avec des valeurs LOD et LOQ de 0,005 et 0,03 μg/mL, respectivement. Les précisions intra- et inter-journalières présentaient des valeurs RSD inférieures à 2 %.
Conclusion. — La méthode validée a été appliquée avec succès pour calculer l'efficacité d'encapsulation de Ptx et la charge de médicament dans la formulation développée.

Introduction

Paclitaxel is a mitotic inhibitor of plant origin used in chemotherapy of patients with malignant tumors. The drug stimulates the microtubules assembly of tubulin dimers and inhibits it's depolymerization. This leads to disruption of the normal process of dynamic reorganization of the microtubule network, which is important for cellular functions at the stage of mitosis and cell cycle interphase [1]. Paclitaxel belongs to the class of taxanes, being a diterpenoid pseudo-alkaloid with the empirical formula C47H51NO14 and a molecular weight of 853.9 g/mol (Fig. 1) [2].

One of the main disadvantages of paclitaxel is low solubility in water. Cremophor EL is used to enhance paclitaxel solubility in market-available drugs and may cause serious allergic reactions [3]. Moreover, biodistribution of paclitaxel in the body is nonspecific, which leads to the nonspecific toxicity of healthy tissues and side effects [3]. Drug encapsulation into PLGA submicron particles increase its solubility, thus avoiding the use of harmful detergents. In addition, the preparation of conjugates of polymeric particles with a vector molecule promotes their selective accumulation in target organs and tissues and increases the specificity of the action. Thus, we synthesized a conjugate of Ptx loaded PLGA submicron particles with a recombinant alpha fetoprotein third domain (rAFP3d-SP) to enhance Ptx selective tumor accumulation. Alpha-fetoprotein (AFP) and its recombinant 26-amino acid peptide receptor-binding fragment (rAFP3d) are well-known tumor-associated markers [4].

RP-HPLC is one of the most abundant and informative methods in pharmaceutical analysis. Several works on validated HPLC determination of paclitaxel both as free form and as encapsulated molecule are easy to find in the literature [5—8]. However, to our knowledge, no RP-HPLC validation research on PLGA submicron particles conjugated with AFP is published. Such complex systems are difficult to analyze mainly because of highly variable physicochemical properties of the components. Thus, polymer molecules tend to be adsorbed on the stationary phase and incompletely eluted by the Ptx mobile phase, which can adversely affect the column performance. Properly done sample preparation is the cornerstone of the robust and precise HPLC method. The aim of this work was to develop an efficient sample preparation and validate HPLC method for Ptx determination in such multicomponent system, as targeted delivery system based on PLGA submicron particles with a protein vector molecule.

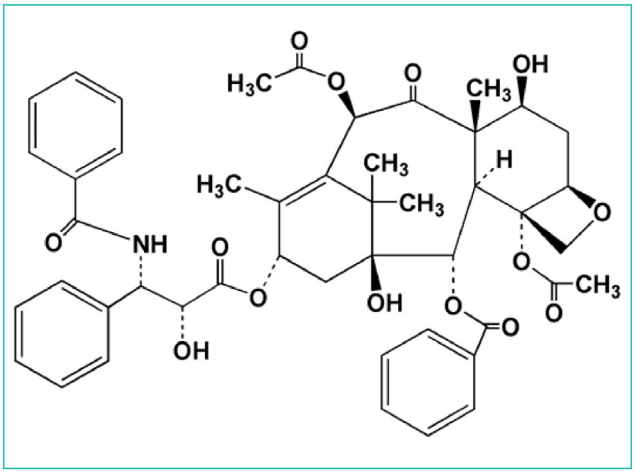

Fig. 1. Chemical structure of paclitaxel [2].
Structure chimique du paclitaxel [2].

Materials and methods

Materials

Poly(lactic-co-glycolic acid) acid terminated with a monomer ratio of 50:50 and inherent viscosity 0.2 dL/g in HFIP was obtained from PURASORB PURAC, the Netherlands. Poly(vinyl alcohol) (PVA) with average Mw 25000, powder, 88% hydrolyzed and D-Mannitol, as well as the reference standard for paclitaxel (99%) was purchased from Sigma Aldrich, USA. The solvents acetonitrile, dichloromethane, dimethyl sulfoxide (DMSO) were all obtained from Fisher Scientific, UK. For the hydrolysis and pH adjustment, sodium hydroxide (NaOH) and aqueous hydrochloric acid (HCl) were purchased from Khimmed Syntes, Russia. 85% phosphoric acid, hydrochloric acid and 30% hydrogen peroxide were purchased from Merck, USA. Water was purified using Milli-Q Plus system (Millipore®). All other solvents and chemicals were of analytical or HPLC grade.

rAFP3d-SP synthesis

Ptx loaded PLGA particles were formulated by a single emulsification method as was described previously [9,10]. Briefly, a mixture of PLGA and Ptx dissolved in dichloromethane was stirred for 30 min followed by adding dropwise to 1% PVA solution. The resulting solution was emulsified with ultrasound homogenization (Labsonic U.B. Braun, Germany) and the organic solvent was removed with rotary evaporator (IKA, Germany).

PLGA particles were conjugated with rAFP3d via standard carbodiimide technique [11,12]. Briefly, preliminary washed submicron particles were incubated with N-hydroxysuccinimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride. Then β-mercaptoethanol was added, followed by incubation with rAFP3d, pH being adjusted to 8.5. Ethanolamine was added to the solution to stop the reaction. Gel-filtration was carried out on Superose 12 (GE Healthcare, USA) to purify the resulted conjugate. Pure fraction was lyophilized with D-mannitol and stored at -20°C.

rAFP3d-SP characterization

To determine particle size, zeta-potential and PDI of particles, rAFP3d-SP was weighted and suspended in distilled water at a concentration of 1 mg/mL. Measurements were performed on a Zetasizer Nano ZS ZEN 3600 analyzer (Malvern Instruments, Great Britain) using a standardized protocol.

Particle shape was investigated by transmission electron microscopy (TEM). 5 μl of rAFP3d-SP water suspension were applied to freshly ionized coal-formed films, and the sample was contrasted with 1% uranium acetate aqueous solution. TEM was performed using JEOL 100CX(JEOL, Japan) at accelerating voltage of 80 kV.

The crystallinity of Ptx encapsulated in rAFP3d-SP was investigated by *X-ray diffraction* (XRD) (D/MAX-2500 V/PC, Japan). The XRD was performed with a Cu Kα radiation and a diffraction 2θ range of 2—80°.

Instrumentation

The analysis was performed using a chromatographic system consisting from 1525 binary pump (Waters, USA) and 2487 UV—VIS detector (Waters, USA). Chromatographic data was

Table 1 Gravimetric experiment layout.
Disposition de l'expérience gravimétrique.

	#	DMSO, %	Mobile phase, μL	Centrifugation time, min
DMSO concentration test	1	0	1000	5
	2	2	980	
	3	4	960	
	4	8	920	
Centrifugation time test	5	4	960	2
	6			5
	7			10

acquired and processed using the Breeze 3.0 data processing system (Waters, USA).

Chromatographic conditions

The chromatographic separation was performed using a Reprosil ODS C-18 analytical column with the dimensions of 150 mm × 4.6 mm ID × 5 μm (Dr. Maisch GmbH, Germany). The flow rate was set at 1 mL/min with UV detection at 227 nm. Mobile phase consisted of acetonitrile: 0.1% phosphoric acid solution (50:50 v/v). The mobile phase was filtered through a 0.45 μm nylon membrane filter (Phenomenex, USA). The sample injection volume was 10 μL and the analysis was carried out at ambient temperature.

Sample preparation method development

Sorption of model samples on guard cartridge

Guard cartridge with C18 stationary phase (Phenomenex, USA) was used as column model to reveal undesirable protein and PLGA trapping on stationary phase. To evaluate sorption level a model mixture was used. To prepare the model mixture 5 mg of rAFP3d were dissolved in 200 μL of 0.1% orthophosphoric acid and 35 mg of PLGA were dissolved in 300 μL of acetonitrile. The solutions were mixed together thoroughly with V-1 vortex (Biosan, Latvia), yielded PLGA: rAFP3d concentration ration as 7:1. The injection volume was 10 μL and detection wavelengths were 214 nm (carboxylate ester functional groups of PLGA) and 280 nm (tyrosine and tryptophan side chains of rAFP3d). Peak area of direct injection (no column or cartridge) was used as 100% recovery reference.

Gravimetric assay of residual excipients in HPLC sample

To evaluate the efficacy of exipients' precipitation the gravimetric assay was used. 5 mg of lyophilized rAFP3d-SP formulation were weighted with 0.01 mg precision with analytical balance EX225D (Ohaus, USA) in 1.5 mL polypropylene tubes (SSI, USA) and dissolved in DMSO portions. Tubes with DMSO solutions were treated accordingly experiment layout (Table 1).

Treated samples were centrifuged at 12 100 g (MiniSpin, Eppendorf) and supernatant was transferred into dried borosilicate glass vials. Supernatant was frozen and freeze dried with final drying mode (pressure < 0.01 mbar at 30 °C) to constant mass with the freeze-drier Alpha 3-4 LSC basic dryer (Martin Christ, Germany). Vials were weighted and dry residue weight was calculated.

Glass vials preparation

Borosilicate glass vials were enumerated with permanent marker and weighted with 0.01 mg precision. Vials were dried in vacuum < 0.01 mbar at 30 °C to constant weight.

Paclitaxel recovery

Model mixtures were used to evaluate paclitaxel recovery. Paclitaxel peaks area were measured and standard paclitaxel solution was used as 100% recovery reference.

Model mixtures were prepared by mixing stock solutions (Table 2): 10 μL PLGA, 5 μL rAFP3d and 5 μL paclitaxel, followed by DMSO and mobile phase addition according to experiment layout in Table 1. The model mixtures were centrifugated and supernatant was collected and analyzed as indicated in section 2.4.

Preparation of standard and sample solutions

Paclitaxel was accurately weighed, and the standard solution was prepared by dissolving approximately 6 mg of the drug in 5 mL DMSO resulting in a concentration of 1.20 mg/mL. 1 mL of the resulting solution was transferred into a 5 mL volumetric flask with mobile phase added to 5 mL to achieve a concentration of 0.24 mg/mL. To prepare a sample solution, 24 mg of rAFP3d-SP were accurately weighed and pre-dissolved with 200 μL DMSO followed by transferring into a 5 mL volumetric flask and diluted to volume with the mobile phase to achieve a concentration equivalent to that one in standard solution.

Method validation

Linearity

The method linearity was evaluated from the standard curve of detector response (peak area) against analyte concentration. The concentration range was selected on the basis of anticipated drug concentration in the drug loading study. 6-point calibration curve was generated at a concentration

Table 2 Preparation of the stock solutions.
Préparation des solutions mères.

Model mixture 1			
Mixture component	Weighted, mg	Solvent volume, μL	Stock concentration, mg/mL
PLGA	17.50	50 (acetonitrile)	350.00
paclitaxel	5.00	17.7 (acetonitrile)	280.00
rAFP3d	14.00	140 (0.1% orthophosphoric acid)	100.00
Model mixtures 2—7			
Mixture component	Weighted, mg	Solvent volume, μL	Stock concentration, mg/mL
PLGA	17.50	50 (DMSO)	350.00
paclitaxel	5.00	17.7 (DMSO)	280.00
rAFP3d	14.00	140 (DMSO)	100.00

range 0.03—360 μg/mL. Three calibration curves were prepared and each solution was injected three times.

Accuracy and precision

The accuracy of the method was carried out using one set of different standard addition methods at different concentration levels, 12.5%, 100% and 150% around the paclitaxel test concentration 0.24 mg/mL, and then comparing the difference between the spiked value (theoretical value) and actual found value. The percentage recovery and RSD were calculated.

The precision of this method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability was assessed by analyzing six replicates of a fixed amount of the drug (0.24 mg/mL) on the same day. Intermediate precision was evaluated through the analysis of the standard solution by another analyst on a different day. Results were expressed as % RSD.

Limit of detection (LOD), limit of quantification (LOQ)

LOD and LOQ of paclitaxel were determined by analyzing different paclitaxel standard diluted solutions and measuring signal-to-noise ratio. LOD is the concentration that gives a signal-to-noise ratio of approximately 3:1, while LOQ is the concentration that gives a signal-to-noise ratio of approximately 10:1 with % RSD of less than 10%.

Robustness

To assess the results' reliability, the robustness of the method was examined by small variations of critical parameters. For this purpose, the flow rate (0.9 and 1.1 mL/min), the ratio of acetonitrile in the mobile phase (48 and 52%), and the concentration of phosphoric acid (0.09 and 0.11%) were varied. Paclitaxel concentration of 0.24 mg/mL was selected to perform the robustness study. Assessment of change in these parameters was based on the percent recovery and RSD.

Specificity

Specificity was determined by comparing the representative chromatograms of samples obtained from the supernatant of rAFP3d-SP (without Ptx) and samples containing Ptx.

Stability

Stability was evaluated by analyzing Ptx standard samples in DMSO at a concentration of 1.2 mg/mL at room temperature for 24 h and after storage at +4 °C for 24 h and 14 days, respectively. The stability of both lyophilized and dissolved rAFP3d-SP was verified at the abovementioned conditions. The stability of dissolved rAFP3d-SP was determined at a concentration of 1.2 mg/mL, which corresponds to Ptx concentration of 0.24 mg/mL. The results obtained were compared to those corresponding to freshly prepared samples. The analyte was considered stable under test conditions if changes obtained were within ±2%.

Method applicability

To determine the drug loading of rAFP3d-SP 24 mg was accurately weighed and transferred into a 5 mL volumetric flask with mobile phase added to 5 mL. 1 mL of the resulting solution was centrifuged for 10 min at 4500 g at room temperature. The supernatant was transferred to a chromatographic vial and injected into the HPLC system.

To determine the entrapment efficacy of paclitaxel, 24 mg of rAFP3d-SP were accurately weighed and resuspended in distilled water (4 mL) and centrifuged for 30 min at 4 °C and 15,000 g. The supernatant was removed and the procedure was repeated. Then the precipitate was transferred into a 5 mL volumetric flask with mobile phase added to 5 ml. The resulting solution was injected into the HPLC system.

The drug concentration in the precipitate was obtained by comparing the concentration to a previously constructed analytical curve. Drug loading and encapsulation efficiency were calculated as follows:

Drug loading % = (weight of remained drug in the submicron particles/weight of polymeric submicron particles) × 100 (1)

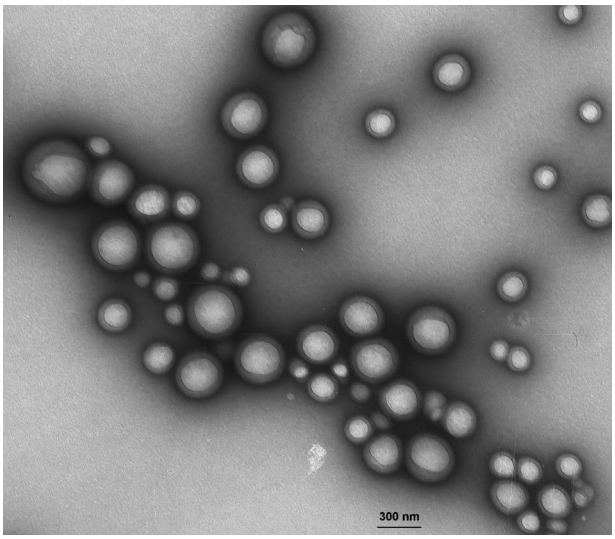

Fig. 2. TEM image of rAFP3d-SP.
Image TEM de rAFP3d-SP.

Encapsulation efficiency % = 100 — ((total amount of drug—actual amount of drug in precipitate)/total amount of drug)) × 100 (2)

Results and discussion

rAFP3d-SP synthesis and characterization

rAFP3d-SP was successfully synthesized via standard carbodiimide technique. Based on the analysis of three batches, the average diameter of rAFP3d-SP was 280 ± 12 nm and the values of the zeta potential were −25 ± 4 mV. Spherical shape of rAFP3d-SP particles was confirmed by TEM (Fig. 2).

The XRD patterns for free Ptx, PLGA, SPs, and rAFP3d-SP are shown in Fig. 3. Ptx powders displayed a few typical diffraction peaks at 5.3°, 9.6°, 12.6° and 16.5°, confirming its crystalline state. There were no peaks observed on the PLGA XRD pattern, indicating its amorphous state. These findings are in agreement with previous results [13]. SPs and rAFP3d-SP formulations exhibited a number of different peaks from Ptx pattern, both of drug formulation patterns being similar to each other. The absence of peaks corresponding to the pure Ptx on the rAFP3d-SP pattern is likely due to Ptx distribution in amorphous state. The last is readily bioavailable state, which allowed fast drug release from conjugate following administration.

Chromatographic conditions

Ptx quantification was successfully achieved with the isocratic elution method (Fig. 4). The best resolution was obtained using the mobile phase consisting from acetonitrile: 0.1% phosphoric acid solution (50:50 v/v) and a flow rate of 1 mL/min. Ptx retention time was 10.12 min. These chromatographic conditions provided satisfactory suitability parameters: the number of theoretical plates (10212) and the asymmetry factor (1.2). The peak with retention time of about 2.5 min refers to DMSO presented in the solvent.

Sample preparation method development

Test samples consist of several components: the drug, the polymer matrix, and the protein covalently linked to the matrix. To achieve reproducibility of the results and maintain desirable performance, it is important for all components to be completely eluted with the selected mobile phase.

To confirm the need of selection of specific conditions for sample preparation, a model mixture consisting of PLGA and rAFP3d was analyzed, and the percentage of sorption on the

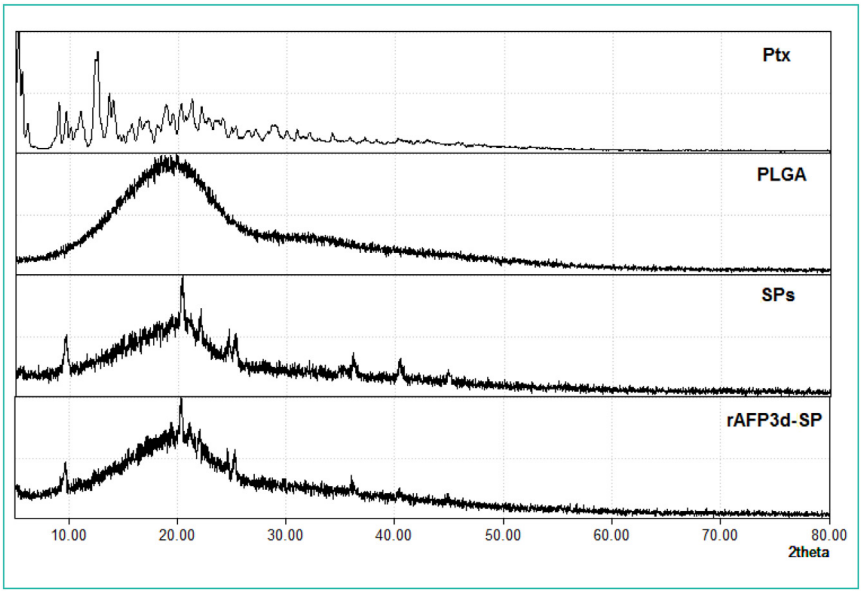

Fig. 3. XRD spectra of Ptx, PLGA, SPs and rAFP3d-SP.
Spectres XRD de Ptx, PLGA, SPs et rAFP3d-SP.

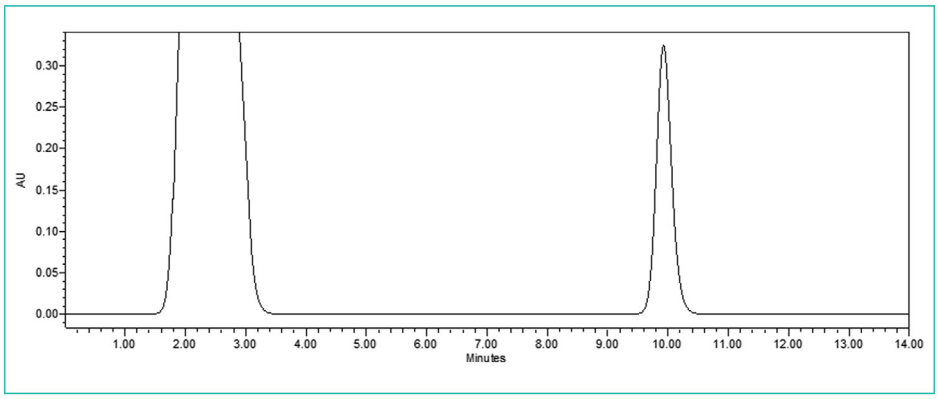

Fig. 4. Chromatogram of Ptx standard solution (0.24 mg/mL).
Chromatogramme de la solution étalon Ptx (0,24 mg/mL).

Table 3 Model mixture for sorption assay on the C18 guard cartridge.
Mélange modèle pour le test de sorption sur la cartouche de garde C18.

	Peak area, no column	Peak area, C18 cartridge
PLGA	Mean = 86322038 RSD = 1.7%	Mean = 77859823 RSD = 0.7%
Difference in peak areas (sorption on the cartridge)	8462215	10%
rAFP3d	Mean = 5180648 RSD = 1.5%	Mean = 3666687 RSD = 1.8%
Difference in peak areas (sorption on the cartridge)	1513961 29%	

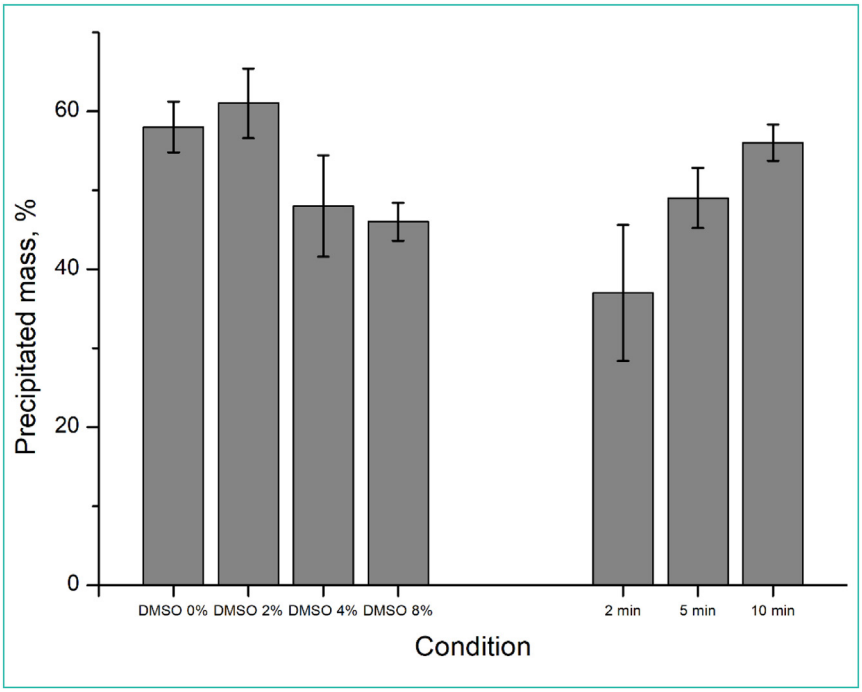

Fig. 5. Selection of DMSO concentration and centrifugation time for efficient precipitation of rAFP3d-SP excipients ($n = 3$, mean ± SD).
Sélection de la concentration de DMSO et du temps de centrifugation pour une précipitation efficace des excipients rAFP3d-SP ($n = 3$, moyenne ± ET).

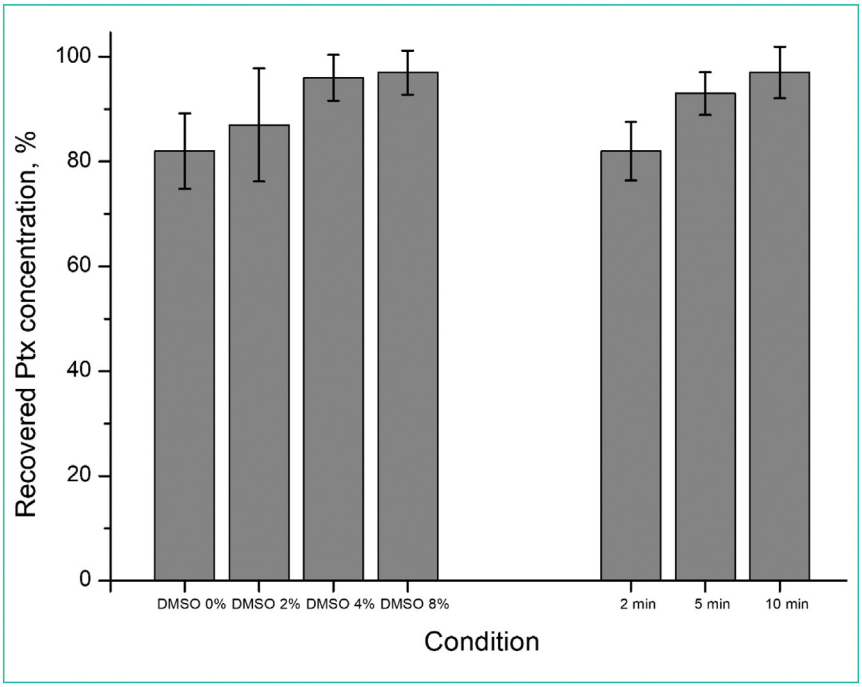

Fig. 6. Selection of DMSO concentration and centrifugation time for efficient paclitaxel recovery ($n = 3$, mean ± SD).
Sélection de la concentration de DMSO et du temps de centrifugation pour une récupération efficace du paclitaxel ($n = 3$, moyenne ± ET).

guard cartridge with C18 stationary phase was determined. Protein recovery from C18 column usually is significantly less than 100%. So regular analysis of protein-containing samples without proper elution method can change column performance. The net interaction caused by protein hydrophobic adsorption is very strong resulting in the protein remaining adsorbed to the surface until a specific concentration of organic solvent is reached, at which time the protein desorbs from the surface and elutes from the column [14]. So, it is applicable to use much shorter and cheaper guard cartridge as model column to evaluate polymers trapping on stationary phase.

Detection was carried out simultaneously at 214 nm (carboxylate ester functional groups of PLGA) and 280 nm (tyrosine and tryptophan side chains of rAFP3d). Peak area of direct injection (no column or cartridge) was used as 100% recovery reference. 10% of PLGA and 30% of rAFP3d were adsorbed on the stationary phase of the cartridge (Table 3.). During subsequent elution in a gradient mode (acetonitrile 60—90% in 10 min), 4% of adsorbed PLGA were eluted (data not shown). The results obtained confirm the need for careful selection of sample preparation conditions to prevent matrix sorption on the stationary phase of the column.

We assumed that centrifugation with the precipitation of matrix components would be a simple and efficient way of rAFP3d-SP sample preparation. PLGA readily dissolves in DMSO, but rapidly precipitates, when aqueous solutions are added [15].

DMSO protein solution precipitated after the addition of acetonitrile. Thus, to precipitate the matrix we predissolved test sample in DMSO, added mobile phase and centrifugated, selecting optimal centrifugation mode and DMSO concentration.

The most efficient sedimentation was after 10 min of centrifugation, while 56% of the initial weight of the test sample was precipitated (Fig. 5). When centrifuged for two minutes, precipitation occurred incompletely, resulting in inappropriate sampling, as evidenced by the high value of RSD (8.6%). The higher DMSO concentration, the higher solubility of the compounds in the model mixture and the less mass of the precipitate after centrifugation.

The goal of this study was to achieve Ptx recovery level more than 90%. The highest paclitaxel recovery was with 4% or 8% DMSO (96 and 97%, respectively) and centrifugation for 10 min (97%), as shown in Fig. 6. Low paclitaxel recovery after centrifugation for 2 min is apparently due to insufficient centrifugation and inappropriate sampling.

Mass sedimentation data and paclitaxel recovery level correlate well with each other. Thus, the higher DMSO concentration is, the less total sediment weight occurs, and the higher paclitaxel amount in the supernatant presents. We used 4% DMSO in further experiments since the results with 4% and 8% DMSO differed insignificantly ($P > 0.05$) and the smaller DMSO concentration is, the smaller dead volume DMSO peak and the lower on-injection elution of analyte by DMSO occurs. Since the results with 4% and 8% DMSO differed insignificantly, we used 4% DMSO and centrifugation for 10 min for further experiments.

Method validation

Linearity

Method linearity was observed in the concentration range of 0.03—360 μ/mL, demonstrating its suitability for analysis. The regression evaluation was carried out by ordinary least squares method. The response of the drug was found to be

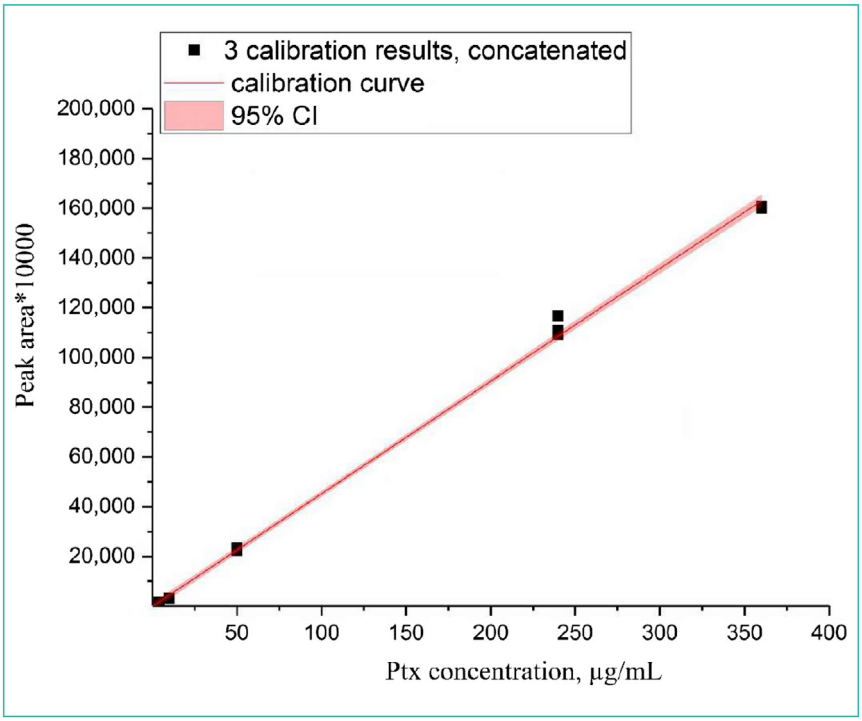

Fig. 7. Plot of a calibration curve (OLS fitting). The band indicates the 95% confidence interval.
Tracé d'une courbe d'étalonnage (ajustement OLS). La bande indique l'intervalle de confiance à 95 %.

Table 4 Method accuracy data.
Données de précision de la méthode.

Spiked level, %	Recovery, %	% RSD
12.5	99.27	1.56
100	98.17	1.80
150	100.62	0.89

Table 5 Precision study data.
Données d'étude de précision.

Set	Intra-day recovery, % ($n = 6$)	Inter-day recovery, % ($n = 6$)
1	99.6	100.0
2	99.4	100.8
3	100.6	99.6
4	101.8	99.2
5	101.0	101.0
6	99.8	99.8
Mean	100.4	100.1
% RSD	0.93	0.70

linear in the investigation concentration range and the linear regression equation was $y = 4531300\ (\pm 42166) \times -1518690\ (\pm 7496810)$ with Pearson's correlation coefficient R 0.9993 ($P = 2.6 \times 10^{-24}$). The resulting calibration curve including a 95% confidence interval is shown in Fig. 7.

Accuracy and Precision

The results of accuracy testing showed that the method is accurate within the acceptable limits. The percentage recovery and RSD were calculated. Acceptable accuracy was within the range of 98.0% to 102.0% recovery and not more than 2.0% RSD, as demonstrated in Table 4.

The results for intra-day and inter-day precision are shown in Table 5. The RSD values for intra-day precision study and inter-day precision study were < 2.0%. It was confirmed that the developed method was precise for analysis.

LOD and LOQ

The method showed a LOD of 0.005 µg/mL (s/n > 3) and a LOQ of 0.03 µg/mL (s/n > 10). In a previously published method [16] for the determination of Ptx, the LOD and LOQ of the method were found to be 2 and 10 µg/mL, respectively. Other HPLC methods [17,18], developed for the determination of Ptx in different formulations, have also been published, and LOD and LOQ were reported from 0.04 to 1 and from 0.15 to 5 µg/mL.

Robustness

A study of the robustness was carried out in order to determine its ability not to be affected by small changes in the conditions of the analysis. The results of robustness study of the developed method are established in Table 6. The values of the system suitability parameter for all variations of the chromatographic conditions were satisfactory and differed only slightly from the standard ones (RSD less than 2%). Retention time was also changed slightly.

Table 6 Robustness study data.
Données de l'étude de robustesse.

Parameter	Condition variances	System suitability	
		Retention time, min	Percent recovery	% RSD
Flow rate	0.9 mL/min	10.13	99.2	0.5
	1.1 mL/min	8.35	98.4	0.6
Organic percentage in m.ph.	48%	10.52	100.1	0.4
	52%	8.75	98.5	0.2
Phosphoric acid percentage in m.ph.	0.09%	12.90	97.6	0.6
	0.11%	9.13	98.3	0.6

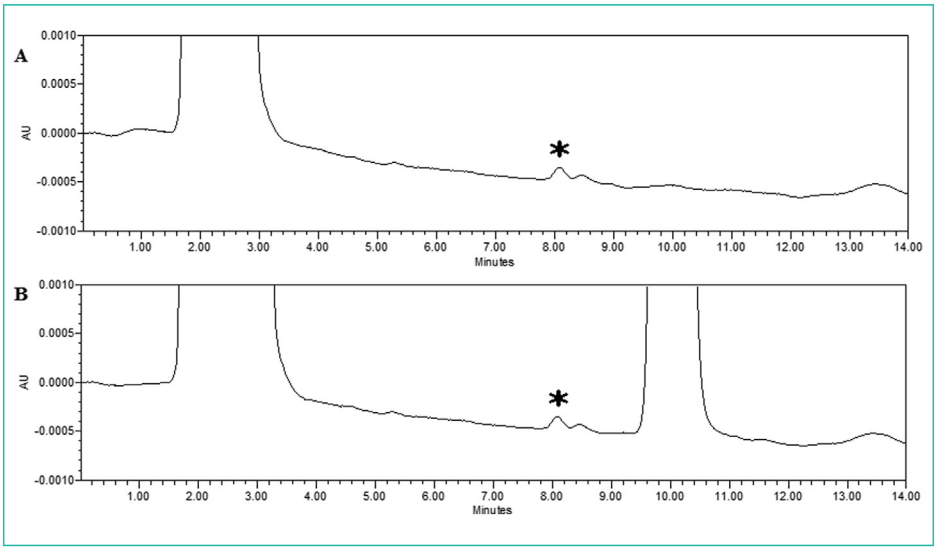

Fig. 8. Chromatogram of (A) rAFP3d-SP without Ptx and (B) rAFP3d-SP (Ptx sample).
Chromatogramme de (A) rAFP3d-SP sans Ptx et (B) rAFP3d-SP (échantillon Ptx).

Specificity

To assess the specificity of the method, the effect of all components in the rAFP3d-SP formulation on the type of chromatogram was determined. For this purpose, rAFP3d-SP solution without Ptx (blank solution) was subjected to HPLC analysis. When comparing the obtained chromatograms of the blank solution and the rAFP3d-SP formulation with Ptx, it was concluded that rAFP3d-SP excipients do not interfere the Ptx peak (Fig. 8). The small peak associated with the blank solution is indicated by *.

Stability

We compared the stability of Ptx in a stock solution with a freshly prepared standard under various conditions (Table 7). In the case of stability of Ptx in rAFP3d-SP, we compared the test sample with the freshly prepared one. All the conditions tested changes found were lower than 2%, which confirms the stability of both stock and rAFP3d-SP solutions.

Method applicability

Validated HPLC method was utilized to determine DL and EE of Ptx in rAFP3d-SP. To determine EE of Ptx we used a direct method, which effectiveness was proven for polymer carriers with paclitaxel [8]. The direct method consists in the analysis of the precipitate obtained after centrifugation of rAFP3d-SP aqueous suspension. Drug loading and encapsulation efficacy were found to be $5.1 \pm 0.2\%$ and $87.3 \pm 0.6\%$, respectively, which corresponds Ptx concentration of 0.24 ± 0.01 mg/mL and 0.21 ± 0.01 mg/mL, respectively, and lies within the linear range. Such DL and EE values indicate the efficiency of paclitaxel encapsulation, which makes the developed conjugate a promising agent for the therapy of oncological diseases.

The object of this study can be considered as a model targeted delivery system consisting of PLGA particles, encapsulated hydrophobic drug and a protein vector molecule conjugated on the surface of the particles. The issue of analyzing such systems is relevant, since drug delivery systems with vector molecules are currently one of the most promising and widely demanded methods of targeted therapy for tumor diseases. Analyzing delivery systems with multiple components can be complex and time consuming. The approaches to optimizing sample preparation and validation of the method for quantitative determination of an encapsulated hydrophobic molecule described in detail in this work can be useful basis in the analysis of multicomponent drug delivery systems.

Table 7 Stability study data of stock and analyzed solutions ($n = 3$).
Données de l'étude de stabilité du stock et des solutions analysées ($n = 3$).

Condition	Ptx in rAFP3d-SP (nominal concentration 0.24 mg/mL)		Ptx in stock solution (nominal concentration 1.2 mg/mL)	
	Recovery, %	RSD, %	Recovery, %	RSD, %
RT for 24 h	98.1	5.2	99.1	5.8
+4 °C for 24 h	97.6	6.1	101.1	4.9
+4 °C for 14 d	102.4	4.8	101.5	5.1

Conclusion

We developed effective and simple sample preparation method to provide high Ptx recovery from polymer matrix and to maintain column performance. HPLC method was validated by the following parameters: specificity, accuracy, and linearity. The method linearity was proven in the range of 0.03—360 μg/mL, with a correlation coefficient above 0.999. The LOD and LOQ were 0.005 and 0.03 μg/mL, respectively, which is much less than the expected analyte concentration and allows quantitative Ptx determination in a wide concentration range. The method is reliable enough to reproduce accurate results under various chromatographic conditions. Described approaches may be considered as useful basis for analysis of multicomponent drug delivery systems.

Authors' contribution

M. Sokol, V. Zenin and E. Nikolskaya validated the analytical method. The rest of the authors synthesized and characterized the conjugate. All the authors participated in the preparation of the manuscript.

Disclosure of interest

The authors declare that they have no competing interest.

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