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ORIGINAL PAPER

A novel method for the simultaneous determination of 5-fluorouracil and oxaliplatin in new biodegradable PHBV/PLGA nanoparticles

Somayeh Handali[1] · Zahra Ramezani[1] · Eskandar Moghimipour[1,2] · Mohsen Rezaei[3] · Farid Abedin Dorkoosh[4,5]

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Abstract

The aim of the present study was to develop a novel HPLC method for the simultaneous determination of 5-fluorouracil (5-FU) and oxaliplatin (OXA) in the PHBV/PLGA nanoparticles (NPs). Chromatographic separation was performed using C18 column at a detection wavelength of 260 nm. The mobile phase consisted of 0.02 M phosphate buffer (pH 4) and methanol (90:10, V/V) and flow rate was 0.8 mL min^{-1}. Column temperature was also maintained at 30 °C. The calibration curves displayed linear concentration ranges of 0.05–40 μg mL^{-1} for 5-FU and 0.5–80 μg mL^{-1} for OXA with correlation coefficients of 0.998. The LOD and LOQ were, respectively, 0.01 and 0.03 μg mL^{-1} for 5-FU and 0.13 and 0.44 μg mL^{-1} for OXA. The values of intra- and inter-day precision and accuracy were within acceptable limits. According to the results, the method was found to be simple, reliable, rapid, and reproducible. In addition, the HPLC assay method presented was successfully applied to the simultaneous determination of 5-FU and OXA in PHBV/PLGA NPs.

Keywords 5-Fluorouracil · Oxaliplatin · HPLC · PHBV · PLGA · Nanoparticles

Introduction

Colorectal cancer is the second most common disease in the world which is causing about 6 millions death every year [1]. 5-Fluorouracil (5-FU), a pyrimidine analogue (Fig. 1a) is widely used in the treatment of colorectal cancer alone or in combination with other anti-cancer drugs [2]. 5-FU interferes with nucleoside metabolism and inhibition of thymidylate synthesis which leads to cytotoxicity and cell death [3, 4]. Despite its therapeutic efficacy, clinical application of 5-FU has been limited due to short half-life (5–20 min) and its severe toxicity on gastrointestinal tract, hematological system, and heart [3, 4].

Oxaliplatin (OXA) belongs to the third generation platinum-based anti-tumor drugs (Fig. 1b) which is also widely used in the treatment of colorectal cancer. OXA acts by damaging the structure of DNA; however, it has shown various side effects such as neurotoxicity, gastrointestinal, and hematological toxicity [1, 5, 6]. The combination of 5-FU and OXA is very common in the treatment of colorectal cancer; although, the adverse side effects are still quite severe [1].

Development of nano-drug delivery systems provides a possibility of co-delivering two or more drugs, which may enhance the anti-tumor activity, reducing side effects of drugs and overcoming drug resistance [5]. Polymeric nanoparticles (NPs) have been extensively studied as carriers for cancer chemotherapeutics. Polymeric NPs have great potential advantages such as protection of encapsulated drugs from degradation, sustained release of drugs, more chemical and physical stability and higher stability in biological fluids [7].

Poly (lactic-co-glycolic acid) (PLGA), a synthetic polymer is extensively employed in the preparation of drug delivery system for many drugs, including 5-FU and OXA due to its biodegradability, biocompatibility, low toxicity, sustainability and non-immunogenicity properties [8–12].

✉ Farid Abedin Dorkoosh
dorkoosh@tums.ac.ir

1 Nanotechnology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

2 Cellular and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

3 Department of Toxicology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

4 Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

5 Medical Biomaterial Research Centre (MBRC), Tehran University of Medical Sciences, Tehran, Iran

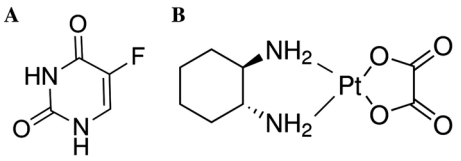

Fig. 1 Chemical structure of **a** 5-FU and **b** OXA

Poly (hydroxybutyric-co-hydroxyvaleric acid) (PHBV) is also a biocompatible and biodegradable polymer which is obtained by copolymerization of polyhydroxybutyrate (PHB) and hydroxyvalerate (HV) [13, 14]. In addition, PHBV can be acquired naturally by changing the growth conditions of bacteria [14]. It has been investigated as nanocarrier for drug delivery and also as a biomaterial for tissue engineering [13]. Unlike PLGA, PHBV is cheaper and does not produce acidic degradation products which may be hurtful for human tissues [15]. Nevertheless, entrapment of hydrophilic drugs in PHBV NPs is low; while hydrophilic drugs can be encapsulated in PLGA NPs with higher encapsulation efficiency [16]. Therefore, in the present due to good biocompatibility of PHBV, and its ability to improve the encapsulation efficiency, a biodegradable PHBV/PLGA NPs was introduced as a novel platform for co-delivery of 5-FU and OXA. To determine the drugs' encapsulation efficiency in NPs, a suitable and validated method is required for assessment. Although each of the drugs has been extensively studied, until now, no method for the simultaneous determination of both drugs has been reported. Therefore, the purpose of the present study was to develop and validate a new simple HPLC detection method for the simultaneous determination of 5-FU and OXA in PHBV/PLGA NPs.

Materials and methods

5-Fluorouracil (5-FU) and oxaliplatin (OXA) were obtained from Acros, USA and Afine Chemical, China, respectively. Poly (3-hydroxybutyrate-co-3-hydroxyvalerate acid) (PHBV) containing 2–3% polyhydroxyvalerate (PHV) by weight was acquired from Tianan Biologic Materials Ltd., Hangzhou, China. Poly (lactic-co-glycolic acid) (PLGA, 50:50) and polyvinyl alcohol (PVA) were purchased from Sigma-Aldrich, Germany. Other chemicals and solvents were of analytical grade and purchased from Merck, Germany.

Chromatographic conditions

5-FU and OXA were analyzed using high-performance liquid chromatography (HPLC, Waters, USA) and chromatographic separation was performed using C18 column (250 × 4 mm i.d., 5 µm, Capitalhplc, ODS-H, UK). The mobile phase was consisted of 0.02 M phosphate buffer (pH 4) and methanol at ratio of 90:10 (V/V). The detection was carried out at 260 nm and the column temperature was maintained at 30 °C. Eluent was pumped at a flow rate of 0.8 mL min^{-1} and injection volume was 50 µL.

Preparation of 5-FU and OXA stock and standard solutions

5-FU and OXA stock solutions (1 mg mL^{-1}) were prepared in deionized water. The standard solution of 5-FU and OXA was prepared by diluting stock solutions in the mobile phase at concentrations of 0.05–40 µg mL^{-1} for 5-FU and 0.5–80 µg mL^{-1} for OXA. The solutions were freshly prepared prior to injection into HPLC.

Method validation

Linearity

Calibration curves were achieved from different concentrations of 5-FU and OXA. Each solution was injected three times and calibration curve was analyzed by plotting the peak area ratio of 5-FU or OXA (y) versus the nominal concentration (x) of the standard solution.

Sensitivity

Sensitivity of the method was evaluated by means of the limit of detection (LOD) and limit of quantification (LOQ). LOD is the lower concentration of analyte which can be detected and LOQ is the lowest concentration of analyte which can be quantified with suitable precision and accuracy [17]. LOD and LOQ were calculated according to the Eqs. 1 and 2, respectively:

$$\mathrm{LOD} = 3 \times \sigma / S \quad (1)$$

$$\mathrm{LOQ} = 10 \times \sigma / S \quad (2)$$

where σ is the standard deviation of the response and S is the slope of the calibration curve [17].

Precision

To validate analytical method, the precision of the method was evaluated by repeatability (intra-day) and intermediate precision (inter-day). Precision was expressed as relative standard deviation (RSD%) of the analyte peaks. The precision of the method was evaluated by assaying a mixture of 5-FU and OXA at different concentrations (0.5, 10, and 40 µg mL^{-1} concentrations for 5-FU and 5, 25 and 80 µg mL^{-1} concentrations for OXA) in three injections

within 1 day for the intra-day precision and every day for 3 consecutive days for inter-day precision.

Accuracy

The accuracy of the method is the closeness of agreement between the value obtained and the true value [18]. It was determined at three concentration levels of 5-FU and OXA (0.5, 10, and 40 µg mL^{-1} concentrations for 5-FU and 5, 25 and 80 µg mL^{-1} concentrations for OXA) and analyzed on the same day and on 3 consecutive days. Then, accuracy (%) was calculated according to the Eq. (3):

$$\text{Accuracy (\%)} = (\text{observed concentration}/\text{nominal concentration}) \times 100 \quad (3)$$

Robustness

Robustness was performed by changing the mobile phase composition (the ratio of phosphate buffer to methanol; 85:15, 90:10, and 95:5), column-oven temperature (25, 30 and 35 °C) and flow rate (0.7, 0.8 and 0.9 mL min^{-1}) to evaluate the influence of these parameters on the efficacy of the method.

System suitability

The system suitability parameters such as resolution (Rs), selectively factor (a), tailing factor (T) and capacity factor (K') were studied.

Stability

Stability was evaluated by analyzing 5-FU and OXA at three concentrations (0.5, 10, and 40 µg mL^{-1} concentrations for 5-FU and 5, 25 and 80 µg mL^{-1} concentrations for OXA) at room temperature and at 2–8 °C for 24 h. The results obtained were compared with freshly prepared samples.

Method applicability

Preparation of 5-FU and OXA loaded PHBV/PLGA NPs

5-FU and OXA-loaded PHBV/PLGA NPs were prepared by double emulsion ($W_1/O/W_2$) method. Briefly, 5-FU (3 mg mL^{-1}) and OXA (2 mg mL^{-1}) were added dropwise into polymers solution dissolved in chloroform that contain PHBV and PLGA in a ratio of 2.75 (polymer concentration 2.5%) under homogenizer with 20,000 rpm (Heidolph, Germany) to form the primary emulsion (W_1/O). The mixture was then added dropwise into aqueous phase (W_2) containing PVA while homogenized. The resulting suspension was stirred to remove the organic phase and then centrifuged at 15,000 rpm for 30 min (MPW-350R, Poland). The schematics preparation of NPs is shown in Fig. 3a.

Characterization of NPs

Determination of entrapment efficiency (EE%) The amount of free 5-FU and OXA present in the aqueous phase were measured after centrifugation (15,000 rpm for 30 min). The supernatant was filtered by a 0.22 µm membrane and analyzed using HPLC (indirect measurement). Encapsulation efficiency (EE%) was calculated using the Eqs. 4:

$$\text{EE\%} = \left(W_t - W_f/W_t\right) \times 100 \quad (4)$$

where W_t is the initial added amount of the drugs and W_f is the amount of remained free drug in supernatant [18]. EE % of both drugs was also calculated based on direct measurement. Briefly, NPs obtained after lyophilization was digested with a mixture of 1:1 of deionized water (a solvent for 5-FU and OXA): chloroform (a solvent for PHBV and PLGA) at room temperature under sonication for 30 min. Then the mixture was centrifuged and the amount of drug was determined in aqueous phase using HPLC. EE % (direct measurement) was calculated using the Eqs. 5:

$$\text{EE\%} = \text{drugs encapsulated}/\text{total drugs} \times 100 \quad (5)$$

Morphology and particle size determination The morphology of the NPs was visualized by field emission scanning electron microscopy (FESEM, S4160, and Hitachi, Japan). The dimensions of the NPs were also measured by particle sizer (Qudix, ScatterOScope I, Korea) system at 25 °C.

Statistical analysis

Data were presented as mean ± SD of three samples for each determination. One-way ANOVA was performed to assess the statistical differences. The differences were considered statistically significant at $p < 0.05$.

Results and discussion

Linearity

Linearity was determined by calculating the regression equation and the correlation coefficient (R^2) of a calibration curve over the concentration range of 0.05–40 µg mL^{-1} for 5-FU and 0.5–80 µg mL^{-1} for OXA (Table 1). The coefficients of determination for the calibration curves of the both drugs were 0.998, confirming a good linearity over the studied concentration range.

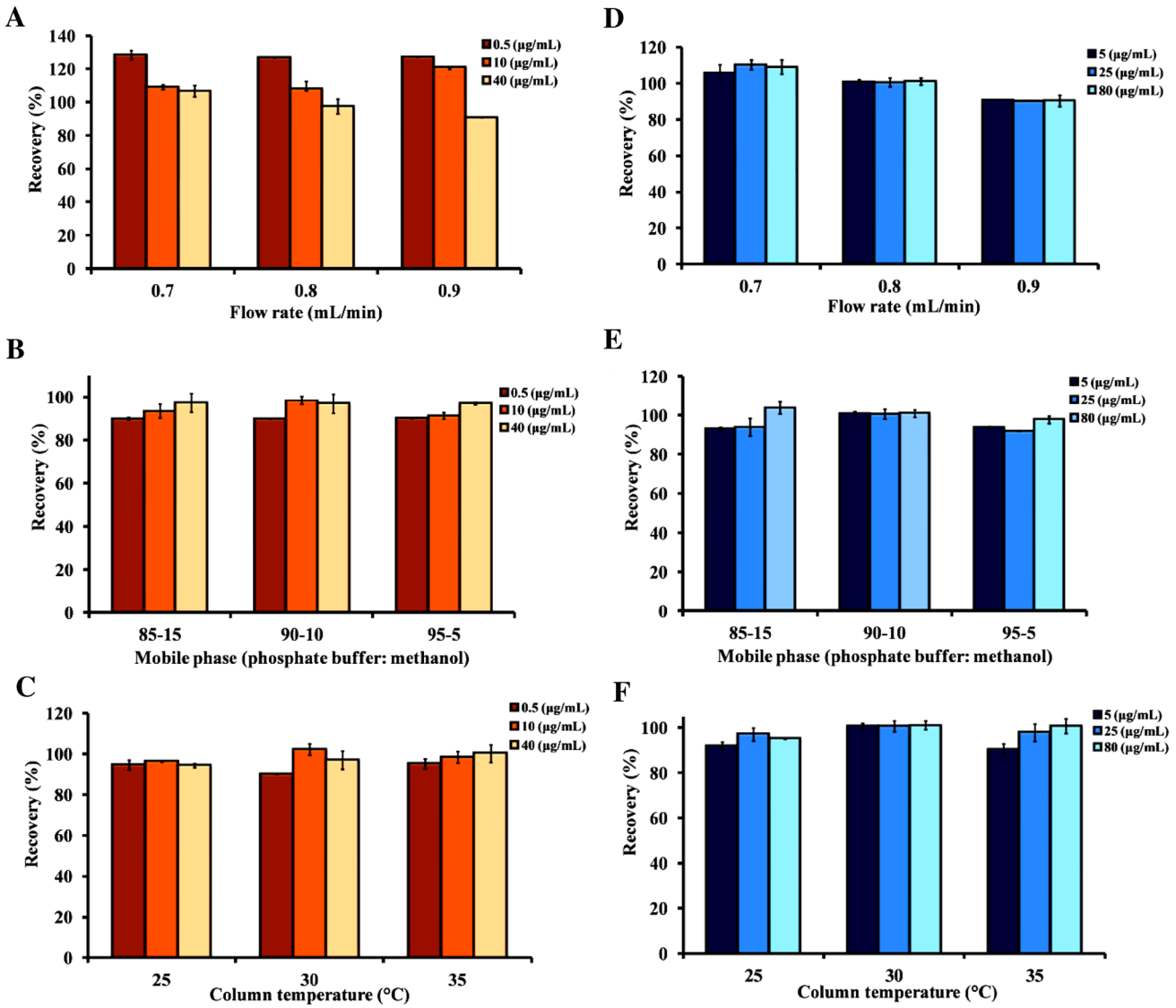

Fig. 3 **a** Schematic representation for preparation of 5-FU and OXA loaded PHBV/PLGA NPs and **b** chromatograms of simultaneously analyzed of 5-FU and OXA

Table 1 Calibration range, linearity of the method, LOD and LOQ

Drugs	Range (µg mL^{-1})	Regression equation	Correlation coefficient (R^2)	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	SE slope	SE intercept
5-FU	0.05–40	$y = 31{,}370x + 57{,}806$	0.9982	0.01	0.03	1805.78	5608.2
OXA	0.5–80	$y = 22{,}880x + 2982$	0.9989	0.13	0.44	263.27	445.51

Sensitivity

LOD and LOQ values of both drugs are presented in Table 1. According to the results, the LOD amounts for 5-FU and OXA were 0.01 and 0.13 µg mL^{-1}, respectively. The LOQ values for 5-FU and OXA were also found to be 0.03 and 0.44 µg mL^{-1}, respectively.

Precision

The inter-day and intra-day precision parameters were investigated for both drugs (Table 2). The intra- and inter-day RSD values did not exceed 5.62%. These results indicate that the developed method is accurate and reliable

Table 2 The validation of intra- and inter-day precision and accuracy

Concentration (μg mL^{-1})	Precision (RSD%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
5-FU				
0.5	3.75	5.62	90.26	90.41
10	1.82	3.37	101.53	100.35
40	1.26	1.88	101.96	99.51
OXA				
5	1.14	3.35	94.32	98.03
25	1.63	2.18	97.63	100.00
80	1.33	2.08	101.52	102.43

as RSD did not exceed 15%, which is in agreement with acceptance recommendations [19].

Accuracy

Accuracy values calculated for both drugs during the intra- and inter-day run are shown in Table 2. Regarding the results, there was no significant difference for the assay, which was tested within day and between days ($p > 0.05$). The accuracy values in intra- and inter-day variation studies at low, medium, and high concentrations for 5-FU and OXA were within the acceptable limits of 92% and 102%. These results indicated the good agreement between experimental and theoretical values; therefore, it can be deduced that this method was accurate and reliable.

Robustness

Robustness shows the ability of a method to tolerate small deliberate changes during analysis [20]. Robustness was evaluated by little changes in the flow rate, mobile phase, and column temperature. The recovery data of 5-FU and OXA under deliberate changes in the main factors of analysis are shown in Table 3 and Fig. 2. Based on the obtained results, there were no significant changes in the peak area under the modified conditions ($p > 0.05$) and all responses were within acceptable range. Consequently, the method proposed for the determination of 5-FU and OXA was reproducible.

System suitability

The results of system suitability are shown in Table 4 and proposed HPLC method was found to be a well-separated approach for 5-FU and OXA.

Stability

The stability of the 5-FU and OXA standard solution was examined at room temperature and at 2–8 °C after 24 h. The results of stability are summarized in Table 5. The results showed no significant change in peak areas and no degradation peak was found in the resulting chromatogram after this period of time. On the other hand, it was observed that the means of the recovery percentage was within the acceptance limit (90–110%) [18].

Method applicability

The proposed HPLC method was employed to determine 5-FU and OXA encapsulated in the PHBV/PLGA NPs. EE% of 5-FU and OXA based on indirect method were 56.77 ± 2.46% and 38.52 ± 1.76%, respectively. Moreover, the values of EE % for 5-FU and OXA based on direct determination were 55.75 ± 0.91% and 37.26 ± 1.76%, respectively. No interference was observed between both drugs

Table 3 Robustness of the method ($n = 3$, mean ± SD)

Parameter	Modification	Recovery (%)					
		5-FU			OXA		
		Concentration (μg mL^{-1})					
		0.5	10	40	5	25	80
Flow rate (mL min^{-1})	0.7	128.53 ± 2.76	108.86 ± 1.59	106.63 ± 3.45	105.04 ± 4.59	110.38 ± 2.68	109.15 ± 3.93
	0.8	126.81 ± 0.038	108.17 ± 4.30	97.46 ± 4.52	100.78 ± 1.18	100.66 ± 2.34	101.06 ± 1.91
	0.9	127.20 ± 0.07	121.06 ± 0.20	90.73 ± 0.13	90.81 ± 0.01	90.36 ± 0.01	90.49 ± 3.05
Mobile phase (phosphate buffer: methanol)	85:15	90.11 ± 0.58	93.54 ± 3.20	97.43 ± 4.28	93.02 ± 0.90	93.95 ± 4.56	103.79 ± 2.97
	90:10	90.001 ± 0.04	98.50 ± 1.85	97.007 ± 4.52	100.78 ± 1.18	100.66 ± 2.34	101.06 ± 1.91
	95:5	90.18 ± 0.05	91.40 ± 1.45	97.06 ± 0.46	93.78 ± 0.62	91.96 ± 0.24	97.78 ± 1.80
Column temperature (°C)	25	94.61 ± 2.38	96.51 ± 0.25	94.40 ± 0.94	91.91 ± 1.77	97.16 ± 2.88	95.19 ± 0.10
	30	90.001 ± 0.38	102.33 ± 2.74	97.004 ± 4.52	100.78 ± 1.18	100.66 ± 2.34	101.06 ± 1.91
	35	95.29 ± 2.34	98.39 ± 2.82	100.19 ± 4.18	90.56 ± 2.50	97.92 ± 3.90	100.64 ± 3.25

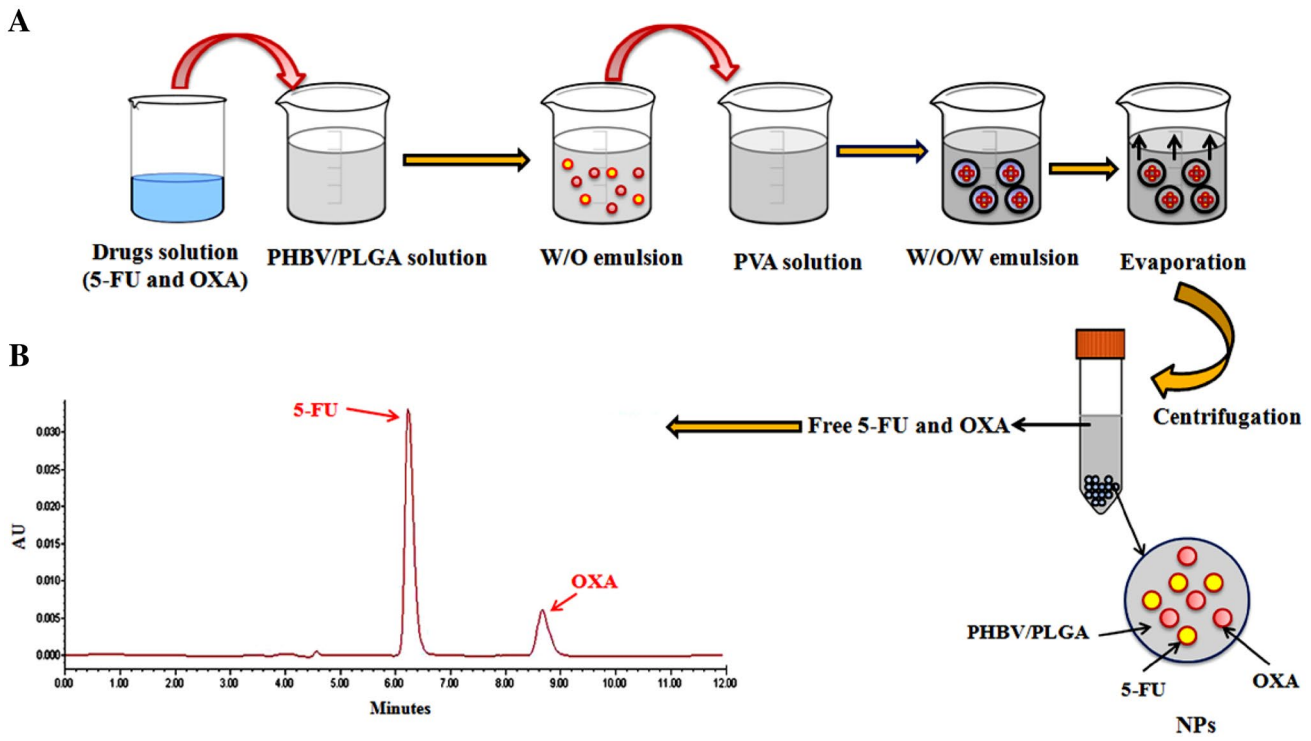

Fig. 2 Effect of change in chromatographic conditions: **a** flow rate, **b** mobile phase and **c** column temperature on recovery of 5-FU and effect of change in chromatographic conditions: **d** flow rate, **e** mobile phase and **f** column temperature on recovery of OXA

Table 4 System suitability parameters for 5-FU and OXA

Parameters	Obtained value		Reference values
	5-FU	OXA	
Resolution (Rs)	4.86		> 15
Selectivity factor (*a*)	1.58		> 1
Tailing factor (*T*)	1.12	1	~ 1
Capacity factor (*K'*)	1.83	2.93	1–10

Table 5 Results of the stability study of 5-FU and OXA at room temperature and at 2–8 °C for 24 h ($n = 3$, mean ± SD)

Concentration (μg mL^{-1})	Storage condition			
	2–8 °C		Room temperature	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
5-FU				
0.5	93.86 ± 5.07	5.40	93.88 ± 4.83	5.15
10	100.95 ± 1.91	1.89	99.51 ± 2.52	2.53
40	103.19 ± 0.67	0.65	98.05 ± 0.04	0.04
OXA				
5	97.52 ± 1.01	1.03	93.07 ± 1.68	1.80
25	104.10 ± 1.95	1.87	99.80 ± 1.00	1.00
80	106.70 ± 0.77	0.72	100.38 ± 0.95	0.94

at chromatographic conditions mentioned above (Fig. 3b). According to the results, it can be concluded that the developed method is suitable for the simultaneous analysis of 5-FU and OXA in the NPs.

Morphology and particle size determination

As it can be seen in Fig. 4a, the NPs were spherical in shape. Particle size analysis results showed that NPs were approximately 102 nm in size which is acceptable for drug delivery applications and NPs also exhibited a monodisperse distribution (Fig. 4B).

Conclusion

In the study, a new HPLC method for the determination of simultaneous 5-FU and OXA encapsulated in PHBV/PLGA NPs has been developed and validated. The proposed chromatographic method presented short chromatographic runs (< 10 min) and good separation was achieved indicating that the method was effective and rapid for determination of various amounts of 5-FU and OXA encapsulated in the NPs.

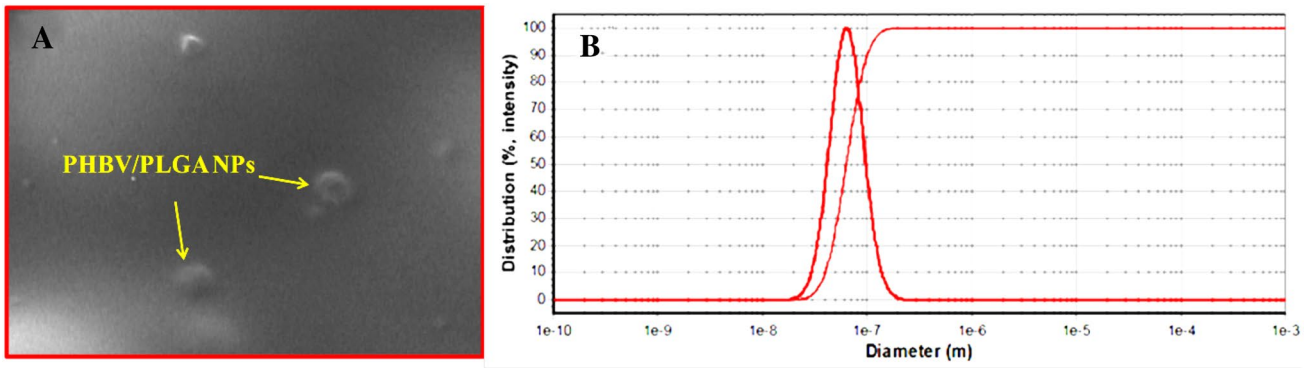

Fig. 4 **a** SEM images and **b** particle size distribution of 5-FU and OXA loaded PHBV/PLGA NPs

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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