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## Experimental design and desirability function approach for development of novel anticancer nanocarrier delivery systems

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The therapeutic effects of anticancer drugs would highly improve if problems with low water solubility and toxic adverse reactions could be solved. In this work, a full factorial experimental design was used to develop a polymeric nanoparticulate delivery system as an alternative technique for anticancer drug delivery. Nanoparticles containing tamoxifen citrate were prepared and characterized using an O/W emulsification-solvent evaporation technique and different analytical methods. Scanning Electron Microscopy (SEM), particle size analysis and High Pressure Liquid Chromatography (HPLC) were used for characterization of nanoparticles. Nanoparticles' characteristics including size, size distribution, drug loading and the efficiency of encapsulation were optimized by means of a full factorial experimental design over the influence of four different independent variables and desirability function using Design-Expert software. The resulting tamoxifen loaded nanoparticles showed the best response with particle sizes less than 200 nm, improved encapsulation efficiency of more than 80% and the optimum loading of above 30%. The overall results demonstrate the implication of desirability function in experimental design as a beneficial approach in nanoparticle drug delivery design.

### 1. Introduction

General characteristics of novel anticancer lead compounds, including low solubility in biological systems, short *in vivo* half-life and side effects have made new resilient types of delivery systems crucial for future developments (Freitas 2005). Among new delivery systems, nanoparticles which can be designed to control drug release and reach the target tissues seem to be promising to reduce the dosage frequency and also the side effects (Moses et al. 2003). Tamoxifen citrate, as an anticancer drug, has been approved and registered for breast cancer therapy for more than 25 years (Berzas et al. 2003). It is commonly used as a primary therapy for breast cancer in elderly women, despite many adverse effects like carcinogenicity, weight gain, blood clotting and ocular side effects (Curtis et al. 1996; Corrada et al. 2004).

Biodegradable nanoparticles in which the therapeutic agent is entrapped in, adsorbed or chemically coupled onto the polymer matrix, can improve the bioavailability of poorly absorbable drugs for oral delivery (Brannon-Peppas 1995; Govender et al. 1999). Moreover, they are able to permeate cells for cellular internalization and connective tissue permeation, and so deliver the drug efficiently to the targeted tissues without clogging capillaries (Astier et al. 1988). The ability of nanoparticles to improve drug diffusion through biological barriers is a typical advantage for the delivery of anticancer agents. The enhanced endocytic activity and leaky vasculature in the tumor could result in accumulation of intravenously administered nanoparticles (Couvreux et al.

1977). As a result, the application of biodegradable polymeric nanoparticles for controlled delivery of anticancer drugs inspired scientists. Recent studies on nanoparticulate anticancers demonstrated drug retention in tumor tissue, tumor growth reduction and prolongation of the animal life (Mu and Feng 2003).

The polymeric matrix of nanoparticles should contain several requirements such as biocompatibility, biodegradability, mechanical strength, and ease of processing. Homopolymers of lactic acid (PLA) and copolymers of lactic and glycolic acid (PLGA) have been widely studied, due to their desirable biocompatibility and biodegradability properties and also FDA approval (Hans and Lowman 2002; Vandervoort and Ludwig 2002; Sahoo et al. 2002). Decreased tumor tissue pH compared to the normal tissue can be considered as a target for site specific drug delivery (Lee et al. 2003). The pH-sensitive particles are expected to have application for solid tumor treatment, exploiting the fact that most solid tumors have an acidic extracellular pH (Dai et al. 2004; Garnett 2001).

In a series of ongoing work to develop biodegradable polymeric nanoparticles, preparation and characterization of tamoxifen-loaded PLGA nanoparticles was already reported (Mirzajani et al. 2010). Since it is difficult to assess the effect of the variables individually or in combination on the responses (nanoparticle characteristics), a mathematical model suitable for establishing a quantitative relationship between the variables and responses is used (Duan et al. 2006). Also, the influence of different factors on the nanoparticle's properties have been optimized using desirability function.

**Table 1: Selected variables and their levels**

Variables	Coded units	Levels	
		-1	+1
PVA (%)	A	7	10
Speed (rpm)	B	13500	24000
PLGA (mg ml <sup>-1</sup> )	C	3.00	5.00
Organic phase (ml)	D	20	40

## 2. Investigations, results and discussion

Rapid opsonization and uptake of the injected particulate carriers by mononuclear phagocytes in liver and spleen (also known as reticuloendothelial system or RES) is the main obstacle in the targeted drug delivery. Intravenous distribution of nanoparticles is significantly influenced by the particles' size and surface hydrophobicity, where the particles with diameter larger than 300 nm are eliminated rapidly from blood circulation. Previous studies demonstrated that drug bioavailability can be improved using nanoparticulate delivery systems in the size range of 100–300 nm (Kumar 2006; Owens and Peppas 2006).

In the present work, nanoparticles were prepared by an O/W emulsification-solvent evaporation method, where the organic phase containing drug and polymer was added to an aqueous phase containing stabilizer (Sahana et al. 2008). This simple preparation technique may involve complex interfacial hydrodynamic phenomena, where the concentration gradient near the interface, solute transfer out of the dispersed phase and the interfacial tension sensitive to the solute concentration are the most important factors. The presence of a surfactant may markedly complicate the situation since it acts to suppress the interfacial flow and the diffusion of dichloromethane to the aqueous phase. The main advantage of using surfactants in the process is the instantaneous and reproducible formation of nanometric, monodispersed nanospheres exhibiting a high drug loading capacity (Rafati et al. 1997).

Different factors affecting the nanoparticle formation and drug loading using O/W emulsion-solvent evaporation method were selected (Table 1): PVA concentration (A), homogenization speed (B), PLGA concentration (C) and the volume of organic phase (D). In order to adjust the suitable levels of each fac-

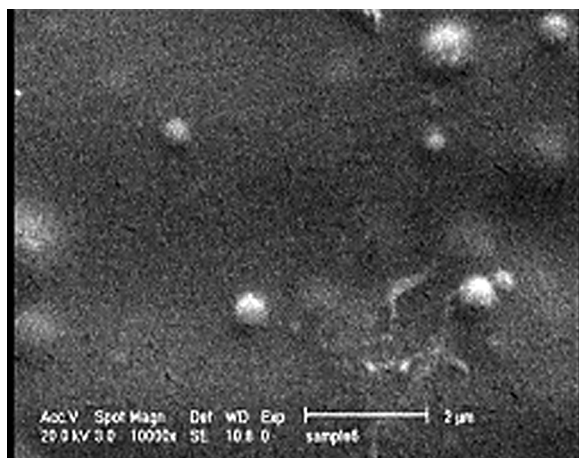


Fig. 1: Scanning electron microscopy image of tamoxifen nanoparticles

tor, some preliminary studies were performed, which clearly showed that higher PVA aqueous concentration and homogenization speed evolved particles with smaller particle size and narrower size distribution. At the same time, it was evident that using 2 and 3% w/v PVA concentrations led to polydispersed and micron size particles, which was attributed to the insufficient concentration of stabilizer to break down organic droplets and cover the nanoparticles surface (Rafati et al. 1997). Therefore, PVA as a common surfactant in the preparation of PLGA nanoparticles that strongly influences the particles' characteristics was used in concentrations of 7.5 to 10% (w/v) (Pasandideh and Niaki 2006). Also, in view of previous reports on the PLGA nanoparticle formulation, 3 and 5 mg ml<sup>-1</sup> polymer concentrations were selected. Finally, due to the instrument limitations, the levels of homogenization process were fixed to 13500 and 24000 rpm.

For visual evaluation of the manufactured nanoparticles, the SEM image of the resulted particles and their surface morphology were studied. The resulting tamoxifen nanoparticles showed slick and spherical particles, as shown in Fig. 1.

Based on the screening results, a technique of two-level factorial design offered the possibility of investigating four independent variables and the possible interactions after performing only sixteen experiments. In order to minimize the effect of

**Table 2: Full factorial design experiments and desirability function.**

Batch No.	Rand.	Block	A	B	C	D	L	E	Z-A	PdI	ODF
1	15	2	-1	-1	-1	-1	18.12	17.11	171	0.138	0.09
2	6	1	1	-1	-1	-1	1.79	11.44	159	0.238	0.24
3	4	1	-1	1	-1	-1	33.81	18.98	122	0.273	0.22
4	14	2	1	1	-1	-1	17.44	16.01	91	0.349	0.25
5	8	1	-1	-1	1	-1	25.02	7.84	210	0.185	0.16
6	13	2	1	-1	1	-1	10.91	11.93	161	0.222	0.01
7	12	2	-1	1	1	-1	14.31	10.79	123	0.165	0.22
8	2	1	1	1	1	-1	29.45	7.88	108	0.188	0.07
9	7	1	-1	-1	-1	1	54.46	28.61	157	0.227	0.33
10	16	2	1	-1	-1	1	12.22	26.12	192	0.986	0.52
11	10	2	-1	1	-1	1	27.99	33.18	69	0.083	0.15
12	3	1	1	1	-1	1	33.56	16.33	124	0.233	0.15
13	11	2	-1	-1	1	1	14.62	25.21	171	0.168	0.10
14	1	1	1	-1	1	1	13.99	7.69	177	0.395	0.26
15	5	1	-1	1	1	1	13.48	1.00	119	0.218	0.12
16	9	2	1	1	1	1	19.89	17.25	106	0.189	0.10

Rand: randomization, L: loading, E: efficiency, ZA: Z-average, PdI: poly disparity index; ODF: overall desirability functions.

**Table 3: Analysis of variances for a 16 factorial design experiment**

Source		SS <sup>a</sup>	D <sub>f</sub> <sup>b</sup>	MS <sup>c</sup>	Fvalue	Prob>F	
Block		0.020	1	0.020			
Model		0.22	6	0.037	6.00	0.012	Sig*
	A	0.039	1	0.039	6.25	0.037	
	B	0.028	1	0.028	4.47	0.067	
	AC	0.065	1	0.065	10.42	0.012	
	AD	7.12E-003	1	7.12E-003	1.15	0.315	
	BD	0.013	1	0.013	2.05	0.190	
	CD	0.073	1	0.073	11.67	0.009	
Residual	0.050	8	6.21E-003				
Cor Total	0.29	15					
Std. Dev.	0.079						
R-Squared	0.8182						
Pred R-Squared	0.6819						

<sup>a</sup> Sum of Square,<sup>b</sup> Degree of freedom,<sup>c</sup> Mean Square,

\* Significant factor.

uncontrolled variables on the response, the experiments were performed based on the randomization law during two consecutive days (2 blocks). Corresponding design matrix and the responses are shown in Table 2.

As described before, to find the best compromise between responses, the desirability function was used. From Eq. (1) it can be seen that the maximum overall desirability will be at the level of the independent variables that simultaneously produce maximum desirability for each response (Table 2). Depending on whether a particular response is to be maximized, minimized or assigned a target, there are three forms of the desirability functions. First of all, the larger-the-best (LTB-type) - for an objective function to be maximized; the second one - for an objective to be minimized; and the third one the nominal-the-best (NTB-type) for an objective function required to achieve a particular target. In our study, two responses (loading and efficiency) should be maximized and two others (particle size and size distribution) should be minimized.

If a response is to be maximized, its individual desirability function is defined as

$$d_i(\hat{Y}_i(x)) = \begin{cases} 0, & \text{if } \hat{Y}_i(x) < Y_i^- \\ \left( \frac{\hat{Y}_i(x) - Y_i^-}{T_i - Y_i^-} \right)^g, & \text{if } Y_i^- \leq \hat{Y}_i(x) \leq T_i \\ 1, & \text{if } \hat{Y}_i(x) > T_i \end{cases} \quad (4)$$

and if a response is to be minimized instead, then its individual desirability function is defined as

$$d_i(\hat{Y}_i(x)) = \begin{cases} 1, & \text{if } \hat{Y}_i(x) < T_i \\ \left( \frac{\hat{Y}_i(x) - Y_i^+}{T_i - Y_i^+} \right)^g, & \text{if } T_i \leq \hat{Y}_i(x) \leq Y_i^+ \\ 0, & \text{if } \hat{Y}_i(x) > Y_i^+ \end{cases} \quad (5)$$

where  $Y_i^-$  and  $Y_i^+$  present the lower and upper tolerance limits of the response, respectively. The  $T_i$  in Eq. (4) interpreted as a large enough value of the response, wherever it denoting a small enough value of the response in Eq. (5). The super index  $g$  represents the weight factor. As a result that all responses have the same importance like this case, the  $g$  is 1.

According to the introduced equations, desirable responses are calculated, which can be seen in Table 2, the software offered the optimum setting of formulation.

The mathematical modeling offered by the software for the preparation of tamoxifen nanoparticles was developed in Eq. (6), where  $Y$  is the dependent variable of overall desirability function (ODF).

$$Y^{0.4} = +0.48 - 0.049 \times A - 0.042 \times B - 0.064 \times AC - 0.021 \times AD - 0.028 \times BD + 0.067 \times CD \quad (6)$$

The mathematical modeling of the best response was carried out by Eq. (6) according to the ANOVA calculation (Table 3). The F value in this table was the ratio of mean-squared error (MS) of each treatment to the one of residuals. The implication of the F value depends on the degree of freedom of the model and the effect, where  $F < 0.012$  in this column was statistically significant. The R-squared and the predicted R-squared were 81.82% and 68.19% for desirability function, respectively. The standard error of the estimate showed that the standard deviation of the residual was 0.079.

Based on the results, the percentage of PVA (A) has the main influence on the formulation process as was evident by the screening studies, however, the rest of the factors including homogenization speed (B), the concentration of PLGA polymer and the organic phase volume (D) have also some influence on the particle formation. Having selected the PVA concentration within the appropriate concentration range (i.e. 7.5–10%W/V), this factor has shown no direct effect on the responses by providing sufficient shear stress to break the droplets and cover the nanoparticle surface. However, the interactions between the concentration of PVA and the PLGA concentration (AC) and homogenization speed (BC), and also the organic phase volume (CD) have shown important contribution to the responses. Fig. 2 shows the response surface function developed by the model considering the effective parameters on the overall desirability function (ODF). Fig. 2(A) shows that as PVA concentration increases from 7.5 to 10%W/V the desirability response slightly increases. Whereas, reducing the PLGA concentration from 5 to 3%w/v, nearly doubles the ODF. Also, by increasing the organic phase ratio, the overall desirability slightly increases from 0.38 to 0.48 (Fig. 2(B)). On the other hand, the reduction of PLGA concentration and organic phase volume, (Fig. 2(C)) improved the overall desirability (ODF) and also produced smaller par-

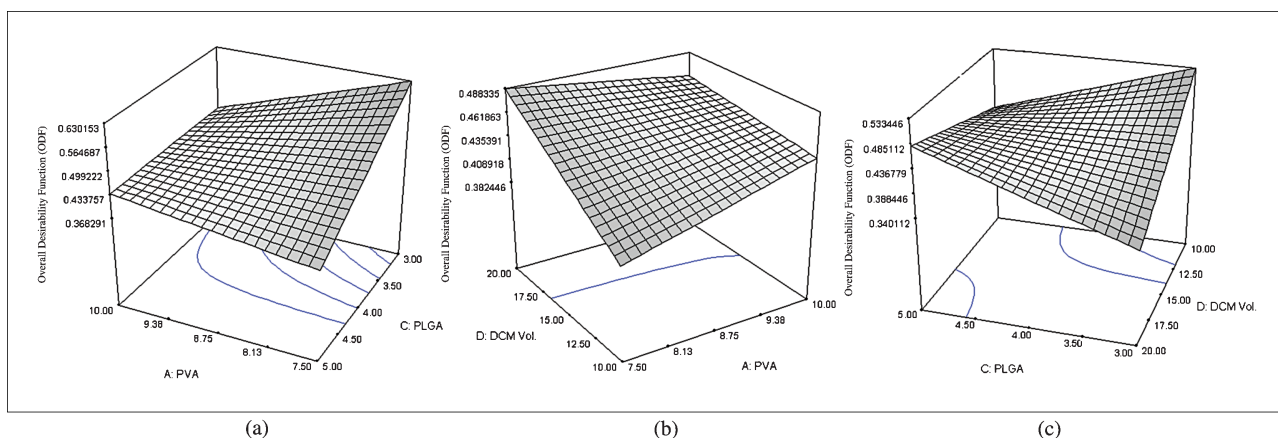


Fig. 2: Response surfaces estimated from the full factorial design, (A) amount of PVA vs. PLGA, (B) PVA percentage vs. organic phase volume, and (C) PLGA vs. organic phase volume

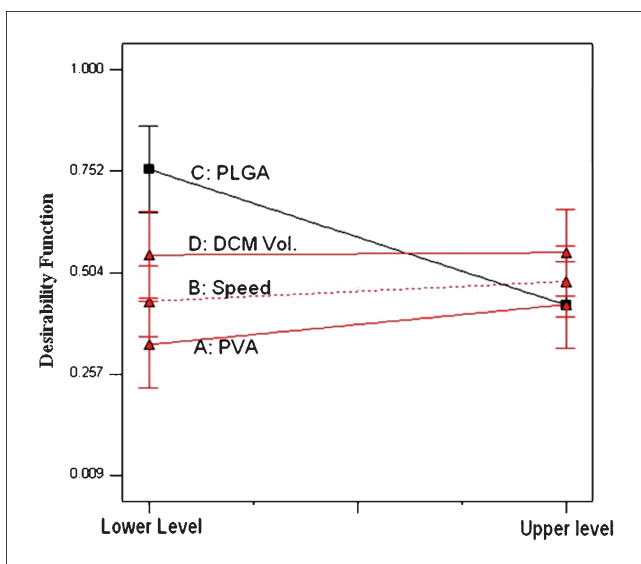


Fig. 3: Interaction graph between inflectional parameters

ticles. Due to the fact that the homogenization speed (B) is a qualitative parameter and does not have three dimensional response surface, its interaction with the PLGA concentration (C), can be graphically demonstrated by Fig. 3. This figure represents the above mentioned results and also demonstrate that the reduction of homogenization speed leads to decrease in the ODF. In other words, producing smaller particles with the high drug loading and efficiency requires a compromise between the shear stresses/rate produced by homogenization and viscosity of the medium (PVA Conc.) in one hand, and the volume and the solid content of organic phase droplets in another hand (Rafati et al. 1997). Based on these results, the software offered an optimum setting of conditions (Table 4).

As shown in Table 4, the optimum formulation with highest desirability factor was achieved by 8.75% of the emulsifier,

**Table 4: Factorial design optimized formulations**

Variables	Optimum condition
PVA (%)	8.75
Speed (rpm)	24000
PLGA (mg mL <sup>-1</sup> )	4.00
Organic phase (mL)	15

4.00 mg mL<sup>-1</sup> of the polymer, 15 ml of DCM and 24000 rpm homogenization speed. The resulting tamoxifen nanoparticles showed the best response in size with average of 192 nm, best encapsulation efficiency of 82% and optimum loading of 33%.

### 3. Experimental

#### 3.1. Materials

Tamoxifen citrate obtained from Chemische Fabrik Berg Co., (Bitterfeld, Germany). PLGA copolymer (50/50; MW 40000–75000) Boehringer Ingelheim Co., (Ridgefield, USA) and PVA copolymer (poly vinyl alcohol copolymer, 88% hydrolyzed, MW 22000) was purchased from Acros (New Jersey, USA). HPLC grade methanol was obtained from Caledon Co. (Georgetown, Canada), and water was purified by reverse osmosis (Milli-Q Millipore). Dichloromethane (DCM) was purchased from Qualigenes Co. (Bombay, India). Sodium 1-octanesulfonate and glacial acetic acid were supplied by Merck (Darmstadt, Germany).

#### 3.2. Preparation of nanoparticles

Emulsification-solvent evaporation technique is a well known technique for preparation of PLGA nanoparticles (Esmaili et al. 2008). The preparation procedure used in this study was as follows:

A. Exact quantities of PLGA polymer and tamoxifen citrate (1 mg) were accurately weighed and dissolved in dichloromethane.

B. The organic phase was added into a PVA aqueous solution and immediately homogenized using a high speed homogenizer at 24000 rpm, unless otherwise stated, using an IKA, Ultra-Turax® T25 Basic (S25N-18G head) at room temperature for 20 min.

C. The emulsion was stirred magnetically at room temperature overnight until the organic solvent was completely evaporated. Subsequently, nanoparticles were separated by centrifugation (Hettich, EBA-20). The separated nanoparticles were redispersed and centrifuged three times in distilled water to completely remove free drug and excess surfactants.

#### 3.3. Experimental design

Screening studies showed that four independent factors including; PVA and PLGA concentrations, homogenization speed and the volume of organic solvent were the influencing parameters on the nanoparticle characteristics. Design expert 6.0.10 trial software was applied for designing the experiments. Due to the factors contribution and laboratory equipments, four variables were taken at two levels: low and high, which were represented by transform values of -1 (for the lowest limit) and +1 (for the highest limit), respectively. Values of these selected variables and their levels are shown in Table 1.

Batches of nanoparticles were prepared according to the full factorial design process and four responses including Z-average mean particle size, size distribution, drug loading and the efficiency of the encapsulation were monitored. The main objective in optimization studies is to determine the experimental conditions which yield the best responses. However, sometimes this is not so easy because experimental responses may be contradictory and require optimal compromises to be identified between them. To overcome this problem, known as multi-objective optimization, desirability functions are used (Bourguignon and Massart 1991; Jimidar et al. 1996). These functions require the definition of acceptable results for each

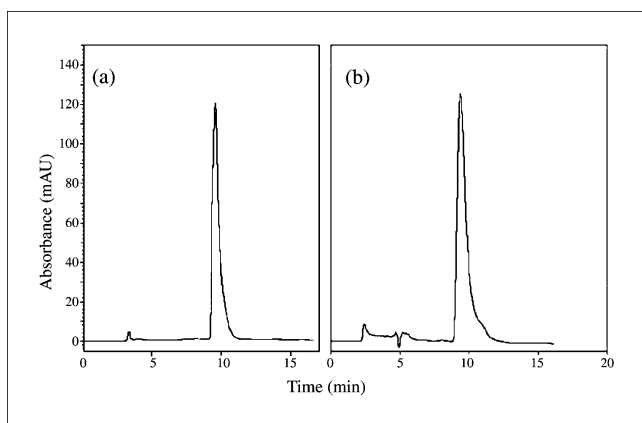


Fig. 4: Tamoxifen citrate HPLC chromatograms for a standard solution (a) and a sample solution (b).

individual response and results which are not acceptable at all. Additionally, different degrees of importance are attributed to each experimental response, based on its contribution to the required characteristics (Sáiz-Abajo et al. 2005). The selection of factors and levels in the design, which mostly affects the nanoparticles characterization, would be based on the results of preliminary investigations. Having generated the polynomial equation relating to the four important responses (mentioned above), and in order to find the best compromise between them, the desirability function was used (Myers and Montgomery 1995; Bempong 2005).

According to this approach, each response  $Y_i$  and predictor of the response  $\hat{Y}_i(x)$  is first converted to an individual desirability function ( $d_i$ ) that varies from 0 to 1, where  $d_i=0$  represents completely undesirable response and  $d_i=1$  represents completely desirable or ideal response. The individual desirabilities of each  $d_i$  are then combined using geometrical mean, which gives the overall (global) desirability  $D$ , with  $n$  denoting the number of responses.

$$D = \left( \prod_{i=1}^n d_i \right)^{1/n} \quad 0 \leq D \leq 1 \quad (1)$$

### 3.4. Particle size and size distribution

Nanoparticles Z average diameter and polydispersity index (PDI) were determined using dynamic light scattering technique (DLS) (Malvern NIBS nanosizer, Worcestershire, United Kingdom). The analysis was performed at a temperature of 25 °C using samples appropriately diluted with ultra purified water in the range of 0.6 nm-6 μm.

### 3.5. Scanning electron microscopy

The morphology of the nanoparticles was determined using scanning electron microscopy (SEM) (Phillips, the Netherlands), the sample were prepared on aluminum stubs and coated with gold prior to examination by SEM.

### 3.6. Drug entrapment study

A sample of nanoparticles prepared in each run was accurately weighed and dissolved in 2 ml dichloromethane, which then was evaporated under a gentle stream of nitrogen gas and dissolved in 1 ml HPLC grade methanol for HPLC assay. The concentration of tamoxifen citrate was analyzed by the reported USP HPLC method. In brief, the HPLC system consisted of a model K-1001 solvent delivery equipped with a model PDA K-2700 ultraviolet detector at 254 nm (all from Knauer, Germany). The analytical column was (4.6 × 150 mm, 5 μm) CLC-Phenyl L11, (Shimadzu-Japan). The mobile phase was a methanol solution containing, 320 ml of water, 2 ml of glacial acetic acid, and 1.08 g of sodium 1-octanesulfonate in a liter. The retention time of tamoxifen was about 6.68 min (Fig. 4).

The amount of tamoxifen loading in nanoparticles (Eq. 2) and the encapsulation efficiency (Eq. 3), also known as nanoparticle yield, was calculated using the following equations; where TNW is the total nanoparticle weight:

$$\begin{aligned} & \% \text{ Tamoxifen loading} \\ & = \left( \frac{\text{Weight of loaded tamoxifen}}{\text{Weight of tamoxifen loaded sample}} \right) \times 100 \quad (2) \end{aligned}$$

% Encapsulation efficiency

$$= \left( \frac{\text{Loading} \times \text{TNW (mg)}}{\text{Added tamoxifen (mg)}} \right) \times 100 \quad (3)$$

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