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ABSTRACT: This study aimed to examine the effects of various surfactants on the particle size distribution of albumin (HSA) nanoparticles and the binding efficiency of the active substance to albumin. Therefore, gefitinib, an EGFR tyrosine kinase inhibitor and albumin nanoparticles were manufactured using Nab[™] technology with various surfactants at different levels. Before producing gefitinib-albumin nanoparticles, the fluorescence spectroscopy method in the absence and presence of surfactants was used to demonstrate the binding of the gefitinib to albumin. Gefitinib binding to HSA in the presence and absence of surfactants, was demonstrated with Stern-Volmer plots. In order to optimize the nanoparticle production method, the effects of critical process parameters such as organic phase volume: total volume %, drug:HSA ratio, homogenization cycle number on particle size distribution were evaluated using the Box-Behnken design. After optimization of production method, the affects of surfactants on the particle size distribution and zeta potential were evaluated. Adding surfactants had no statistically significant effect on the Stern Volmer plots but they helped to produce uniform nanoparticles with PDI and particle size values of less than 0.2 and 130 nm respectively. It was observed that adding HSPC, DPPC, or oleic acid to the formulation enabled the production of uniform albumin nanoparticles.

KEYWORDS: Surfactant, HSPC, DPPC, oleic acid, gefitinib, human serum albumin, Nab™ technology

1. INTRODUCTION

Lung cancer is one of the most common types of cancer with the lowest survival rate. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers. The main treatment modality for NSCLC is chemotherapy. However, since the selectivity of traditional chemotherapeutic agents is low, they cause serious side effects (1). Gefitinib (Figure 1) is a epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor and effective in EGFR-mutated NSCLC (2). Although there is an FDA-approved gefitinib tablet (Iressa®), it has problems to be solved such as absorption variation, insolubility, adverse effects, drug resistance, and insufficient bioavailability (3-5). Therefore, there is a need to develop new formulation approaches to increase the anti-tumor effect and bioavailability of gefitinib and reduce its systemic adverse effects.



Figure 1. Chemical structure of gefitinib.

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As it is known, nanotechnological approaches can increase solubility, improve drug accumulation in tumor side, decrease side effects and also prevent drug resistance. For these reasons, nanoparticular systems have made great advances in cancer treatment.

Human serum albumin (HSA) (MW: 66.5 kDa) is a biocompatible, non-immunogenic, biodegradable, and natural biomaterial and can be used as a non-specific transport protein to assemble various insoluble organic molecules as it contains multiple hydrophobic binding pockets (6, 7). HSA nanoparticles can be actively targeted to the tumor side owing to the albumin-binding proteins: secreted protein, acidic and rich in cysteine (SPARC) and glycoprotein 60 (Gp60). Gp 60 is a widely distributed albumin receptor; however, it exists on the plasma membrane of the endothelium. Albumin binding of Gp60 occurs via a caveolin-dependent endocytotic process that results in transcytosis. Albumin nanoparticles passes through the plasma membrane, at the tumor site encounter SPARC which is highly expressed in malignant tumors. The therapeutic efficacy of paclitaxel-loaded albumin nanoparticles was shown to correlate with SPARC expression (8). Abraxane, which is containing paclitaxel and HSA, is the first commercial product of HSA nanoparticles, which has a high tolerated dose and decreases the hypersensitivity reactions such as anaphylaxis seen in formulations containing paclitaxel (e.g. Taxol) (9, 10). Therefore, in this study, nanoparticles containing gefitinib were prepared with HSA.

The most important goal in nanoparticle production is to produce stable, monodisperse, and reproducible particles. Passive targeting of nanoparticles to cancer cells is possible with enhanced permeability and retention (EPR) effect. The EPR effect is due to fast and disorganized growth of blood vessels of tumor, which increases porosity of the vascular endothelium that allow for nanoparticle transport to the tumor side. Passive targeting of nanoparticles by the EPR effect can be achieved with a particle size of 20-200 nm (11). For these purposes and to obtain nanoparticles smaller than 200 nm, the approach of adding surfactant to the formulation can be preferred. Although this approach can be preferred in the production of nanoparticles with Nab™ technology, the properties of the produced nanoparticles depend on the physicochemical properties of the active substance as well as the surfactants used. Surfactants that can be used for this purpose include HSPC, oleic acid, and DPPC (Figure 2).



Figure 2. Chemical structures of a) oleic acid, b) DPPC, and c) HSPC.

Various experimental designs are currently used to optimize formulation with less experimentation and provide estimations about the importance of different variables. The use of experimental design for formulation development is shown to be highly effective for understanding the relationship between independent and dependent variables. Response surface methodology allows simultaneous analysis of variables in cases of complex variable interactions. There are many studies that have applied response surface methodology to establish the optimal formulation in drug delivery systems (12-14). In this study, Box-Behnken design, which is a response surface methodology design, was used in order to avoid excessive formulation combinations and experimentation (15).

In this study, gefitinib loaded HSA nanoparticles was produced based on Nab[™] technology. Although Nab[™] technology has come to the forefront with the production of paclitaxel loaded HSA nanoparticles, it is a production method that allows drugs with low solubility to form nanoparticles with albumin, basically mixing and homogenizing the aqueous solution of HSA and the solution of the active substance in the organic phase, and nanoparticles are formed by evaporation (10).

This study aimed to evaluate the effect of surfactants on the albumin binding efficiency of the active substance and the particle size distribution of produced nanoparticles. For this purpose, different levels of HSPC, oleic acid, and DPPC were added to the formulations and the formulation was optimized. In this way, it is expected to overcome the difficulties of gefitinib such as low solubility and bioavailability, adverse effects, drug resistance, and variable absorption.

2. RESULTS AND DISCUSSION

2.1. Preformulation studies

2.1.1. Determination of organic solvent type

Generally, in Nab[™] technology, albumin is dissolved in water or an aqueous solution. Hydrophobic drugs should be dissolved in organic solvents such as ethanol, chloroform. The resulting emulsion is subjected to high pressure homogenization. Organic solvent is removed from the obtained dispersion under reduced pressure in a rotavapor (16). To determine the organic solvent type, 2 mg/mL gefitinib dissolved in organic phase, added to the 0.09% aqueous solution of HSA, and after homogenization and evaporation processes the particle size, PDI and zeta potential values were obtained (Table 1).

Table 1. The effect of the organic solvent type used in the preformulation studies of gefitinib-HSA nanoparticles on the particle size distribution (n=3) (mean±SD).

Organic solvent type	Particle s	size (nm)	PDI		
	Unfiltered	Filtered	Unfiltered	Filtered	
Ethanol	555.5±27.77	332.4±63.07	0.655 ± 0.048	0.427 ± 0.053	
Ethanol:chloroform (4:6)	716.7±21.58	161.4±18.48	0.458 ± 0.051	0.528±0.143	
Ethanol:chloroform (1:9)	639.0±17.64	205.6±99.97	0.316±0.061	0.407±0.034	

As seen in Table 1, the particle sizes obtained were far from being smaller than our target of 300 nm for the unfiltered samples. In the filtered samples, although this target was achieved for ethanol:chloroform mixture, it could not be achieved with just ethanol. PDI values were greater than 0.2, indicating that the obtained nanoparticles were polydisperse. In addition, it was observed that filtration through a 0.22 pore filter did not reduce the PDI value to the desired level. However, since the lowest particle size was obtained in the filtered samples, it was decided to continue the studies with ethanol:chloroform (4:6).

In a study by Lomis et al., albumin nanoparticles containing paclitaxel were produced by using different amounts of ethanol and chloroform mixture, and it was shown that the particle size and PDI value decreased with the increase in the amount of organic solvent ($170.2 \pm 1.4 \text{ nm}$, PDI: 0.14) (17).

2.1.2. Experimental design and optimization of the manufacturing process

The nanoparticle production parameters and the obtained responses were analyzed by Design-Expert software and are demonstrated in Table 2. The number of experiments included the midpoint of each edge and the repeated center points. Factors influencing the performance of nanoparticles were investigated using Box-Behnken Design, including organic phase volume:total volume %, gefitinib:human serum albumin ratio and homogenization cycle number.

					Unfiltered		Filtered (0.22 µm)		
Run	Factor 1: HSA:drug ratio	Factor 2: organic phase volume:total volume %	Factor 3: homogenization cycle number	Response 1: average particle size (nm)	Response 2: PDI	Response 3: zeta potential	Response 4: average particle size (nm)	Response 5: PDI	Response 6: zeta potential
1	7	10	10	195.4	0.302	36.1	148.3	0.180	35.1
2	10	2	20	217.1	0.447	34.6	106.9	0.330	23.4
3	7	6	20	211.0	0.264	25.8	146.4	0.260	35.7
4	4	10	20	218.1	0.252	31.8	154.5	0.182	37.6
5	4	2	20	348.8	0.460	33.0	163.4	0.459	32.8
6	7	2	10	297.3	0.626	30.8	115.8	0.539	29.9
7	10	6	30	242.7	0.359	27.0	140.0	0.220	26.2
8	7	2	30	261.4	0.472	33.8	153.3	0.487	22.9
9	7	10	30	218.5	0.405	42.4	191.5	0.354	35.6
10	7	6	20	181.0	0.352	34.5	120.5	0.334	38.1

Table 2. Experimental runs and observed responses for the Box-Behnken design.

11	10	6	10	184.9	0.227	39.7	136.3	0.196	36.9
12	4	6	30	244.1	0.403	35.7	117.6	0.228	25.7
13	7	6	20	204.9	0.416	30.0	135.5	0.389	42.0
14	4	6	10	189.7	0.346	33.6	123.9	0.199	37.3
15	10	10	20	252.1	0.361	37.2	169.0	0.250	27.8

The selected independent variables affected the observed responses for average particle size, PDI and zeta potential. The average particle size (unfiltered) ranged from 181.0 nm (formulation 10) to 348.8 nm (formulation 5) (Table 2) with the selected levels of variables. The independent factor affecting the average particle size (unfiltered) was organic phase volume:total volume %. The cross-interaction of organic phase volume:total volume % and HSA:drug ratio on average particle size (unfiltered) was significant (Table 3) (p < 0.05). The PDI (unfiltered) ranged from 0.227 (formulation 11) to 0.626 (formulation 6) (Table 2). The independent factor affecting the PDI (unfiltered) was organic phase volume:total volume % (Table 3) (p < 0.05). The PDI (filtered) ranged from 0.180 (formulation 1) to 0.539 (formulation 6) (Table 2). It was observed that the PDI (filtered) value decreased as the organic phase volume:total volume % increased (Table 3) (p < 0.05). B² (organic phase volume:total volume %)² was significant for both average particle size (unfiltered) and PDI (filtered) (Table 3) (p < 0.05). A² (HSA:drug ratio)² also was found significant for PDI (filtered) (Table 3) (p < 0.05). The negative regression coefficient of organic phase volume:total volume % for average particle size (unfiltered), PDI (unfiltered and filtered) suggests a decrease in these parameters with an increase in organic phase volume:total volume %.

	Intercept	Α	В	C	AB	AC	BC	A ²	B ²	C ²
	-									
average particle size	198.967	-12.9875	-30.0625	12.425	<u>41.425</u>	0.85	14.75	16.1292	<u>43.9292</u>	0.254167
(unfiltered)										
p-values		0.2404	<u>0.0274</u>	0.2586	<u>0.0300</u>	0.9532	0.3337	0.3122	<u>0.0281</u>	0.9866
PDI (unfiltered)	0.379467	-0.008375	<u>-0.085625</u>	0.01725						
p-values		0.7899	<u>0.0175</u>	0.5851						
zeta potential	33.7333									
(unfiltered)										
p-values										
average particle size	141.527	-0.9	15.4875	9.7625						
(filtered)										
p-values		0.9039	0.0570	0.2073						
PDI (filtered)	0.327667	-0.009	<u>-0.106125</u>	0.021875	0.04925	-0.00125	0.0565	<u>-0.100833</u>	<u>0.0784167</u>	-0.0160833
p-values		0.6193	<u>0.0015</u>	0.2546	0.0959	0.9606	0.0656	<u>0.0100</u>	0.0259	0.5488
zeta potential	32.4667	-2.3875	3.3875	-3.6						
(filtered)										
p-values		0.1965	0.0770	0.0624						

Table 3. Statistical analysis results of A: HSA:drug ratio, B: organic phase volume:total volume %, and C: homogenization number. (p-value shading: underlined: p < 0.05, bold: $0.05 \le p < 0.1$, normal: $p \ge 0.1$)

The plots show the region of maximum (region in red) and minimum (region in blue) for each response investigated (Figure 3).



Figure 3. Response surface plots (3D) showing the effects of variables on the responses.

The practical values of average particle size and PDI of unfiltered samples and average particle size of filtered samples were similar to predicted values, however, the measured value of filtered sample's PDI differed from the predicted value. Although the PDI of filtered sample could not predicted, the measured average particle size and PDI were small enough and met requirements (Table 4). These conditions were chosen as optimum formulation production parameters.

Table 4. Observed and predicted values of dependent variables of optimized nanoparticle using the Box-Behnken design.

				unfiltered		filtered (0.22 um)	
	hsa:drug ratio	organic phase volume:total	homogenization number	average particle size (nm)	PDI	average particle size	PDI
		volume %				(nm)	
predicted	9.835	6.461	10.000	188.054	0.344345	132.699	0.180
actual	9.835	6.461	10.000	215.4±2.89	0.360 ± 0.011	131.6±1.834	0.265 ± 0.008

2.2. Fluorescence spectroscopy

As shown in Figure 4, the fluorescence intensity of HSA decreased with increasing gefitinib concentration, indicating interaction between gefitinib and HSA (5). In the presence of HSPC, DPPC, oleic acid and gefitinib, the maximum emission wavelength of HSA shifted as seen in Figure 4 indicates that adsorption of surfactant changes the local environment (18).



Figure 4. Fluorescent intensity of HSA in the a) absence and presence (b) oleic acid, c) HSPC, d) DPPC) of surfactants.

Gefitinib binding to HSA in the presence and absence of surfactants, keeping the HSA and surfactants concentrations constant, was demonstrated with Stern-Volmer plots (Figure 5) (19). As shown in the Figure 5,

the fluorescence data showed good agreement with a Stern-Volmer model. The R² values were greater than 0.92 for all conditions except oleic acid. As can be seen from the graph, HSA binding was not changed significantly in the presence of surfactants. Therefore, the effects of surfactants on particle size distribution were evaluated in order to optimize the formulation and select the most suitable surfactant.



Figure 5. Stern–Volmer plots for the binding of gefitinib to HSA in the absence and presence of surfactants. (F0/F: fluorescence intensity value of the protein in the absence of the quencher / refer to the fluorescence intensity values of the protein in the presence of the quencher)

2.3. Nanoparticle production with surfactants

It is known that the particle size of nanoparticles can be reduced, more monodisperse particles can be obtained and physical stability can be increased by adding surfactant to nanosystems. Possible toxic effects of surfactants should also be considered during the formulation development phase (20). For this reason, it is aimed to use surfactants as low as possible. In this study, surfactant levels providing optimum particle size and PDI value were determined by using different levels of HSPC, oleic acid and DPPC (Table 5).

It was observed that the size of the nanoparticles decreased with the increase of DPPC concentration in the DPPC added samples, and the samples prepared with 1 mg/mL DPPC resultened in the most uniform particles (PDI= 0.236 ± 0.016). It was determined that the particle size increased with the increasing concentration of oleic acid, but more uniform particles were obtained (PDI decreased from 0.272 to 0.134). In the samples containing HSPC, the smallest and most monodisperse samples were obtained with 1 mg/mL HSPC (130.5 nm, PDI: 0.236). It was observed that particle sizes and PDI values of all samples filtered through a $0.22 \ \mu$ m pore diameter CA filter, except for the sample prepared with 1 mg/mL HSPC, decreased. Particle sizes of all filtered and unfiltered samples were less than 200 nm. No significant effect of filtration on the zeta potential was observed.

The first commercial product to apply HSA nanoparticles, the Abraxane formulation has a particle size of 130 nm. When the data obtained were analyzed accordingly, formulations containing 1 mg/mL and 2 mg/mL DPPC and 1 mg/mL HSPC were obtained below this size in unfiltered samples. In addition,

formulations containing 1 mg/mL DPPC or HSPC in unfiltered samples had a PDI value below 0.3. Therefore, nanoparticles containing 1 mg/mL DPPC or HSPC were determined as optimum formulations (21). **Table 5.** Effects of surfactants on the particle size distribution and surface charge (n=3, mean±SD).

Surfactant	Level	Particle s	size (nm)	Polydisper	rsity index	Zeta po	tential
	(mg/mL)			(PI	DI)		
		Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered
-	-	215.4±2.890	131.6±1.834	0.360±0.011	0.265±0.008	39.7±0.57	25.7±0.45
	0.2	144.0±1.908	113.5±3.179	0.396±0.013	0.319±0.067	45.2±1.32	46.3±2.29
DPPC	1	126.3±0.378	115.7±1.002	0.236±0.016	0.202±0.027	44.2±1.04	44.7±1.31
DITC	2	100.8 ± 3.749	94.04±1.160	0.351±0.009	0.302 ± 0.040	44.9±1.16	30.2±1.85
	0.2	162.9±1.159	120.0±1.007	0.272±0.003	0.202±0.027	42.6±2.15	46.5±2.19
Oleic acid	1	190.8±2.194	166.4±3.758	0.191±0.023	0.120±0.003	47.8±1.21	45.8±1.25
	2	192.3 ±1.361	176.9±2.577	0.134±0.020	0.080±0.024	47.8±2.40	49.8±1.79
	0.2	187.1±7.100	93.73±0.510	0.428±0.016	0.267±0.011	42.1±2.46	46.6±3.76
HSPC	1	130.5±2.721	161.9±3.647	0.275±0.015	0.275±0.003	44.2±1.91	43.4±0.15
	2	178.1±6.332	159.0±7.032	0.418 ± 0.027	0.277±0.009	42.5±1.78	44.3±0.74

In a study by Wan et al., lapatinib-loaded HSA nanoparticles were prepared by adding phosphatidylcholine, resulting in a particle size of less than 150 nm and a PDI value of less than 0.2 (22). Birnbaum et al., reported that PLGA nanoparticle production was affected depending on the solvent/surfactant combination. Particles of <100 nm were obtained with SDS, but these samples could not be redispersed after freeze-drying, however, samples containing PVA or HSA were able to redisperse (23).

3. CONCLUSION

This study was designed to evaluate the effects of surfactant on HSA nanoparticles using NabTM technology. Nanoparticle formulations were prepared with the various surfactants (HSPC, oleic acid, and DPPC) at three different levels. Adding surfactants had no visible changes on the Stern Volmer plots but nanoparticle properties were improved with surfactants by uniform nanoparticles with a PDI value of less than 0.2 and particle size of less than 200 nm. Considering the particle size (130 µm) and PDI value (<0.2) of the Abraxane formulation, the formulation containing 1 mg/mL DPPC was determined as the optimum formulation. In this way, it is aimed to obtain a treatment that is targeted to the tumor, whose effectiveness is increased with the EPR effect, and therefore, the systemic side effects are reduced while increasing the treatment efficiency.

4. MATERIALS AND METHODS

4.1. Materials

Gefitinib was kindly provided by Nobel (Istanbul, Turkey). Human Albumin Grifols® 20% (1 mL of the aqueous solution containing 0.2 g human albumin, 0.016 mmol sodium caprylate, 0.016 mmol sodium N-acetyltryptophanate) was used as HSA source. Hydrogenated-soy-phosphatidylcholine (HSPC) was purchased from Sigma (St. Louis, USA), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and oleic acid were purchased from Cayman Chemical (Michigan, USA). All other chemicals were of analytical grade.

4.2. Methods

4.2.1. Preformulation studies

Determination of organic solvent

Nanoparticles were prepared based on Nab[™] technology. In order to decide on the organic phase type, gefitinib:HSA ratio, organic phase volume:total volume %, and other process parameters were kept constant

and ethanol, ethanol-chloroform (4:6), ethanol-chloroform (1:9) were used (1%, v/v) to produce the nanoparticles. Gefitinib was dissolved in the mentioned organic solvent or organic solvent mixtures at a concentration of 2 mg/mL. The water phase containing 0.09% HSA was prepared by diluting from the commercially available solution containing 20% HSA. The organic phase was added drop wisely to the water phase at 11000 rpm for 5 minutes using ultraturax with stirring. The resulting coarse emulsion was subjected to 20 rounds of homogenization at 24000 psi in a high-pressure homogenizer. The organic solvent was evaporated at 25 °C at 23 mbar pressure for 2 min at a rotation speed of 100 rpm using a rotavapor (Vacuum pump v700). The resulting nanosuspension was filtered through a cellulose acetate (CA) membrane with a 0.22 μ m pore diameter. The mean particle size and PDI values of the nanoparticle suspensions obtained using different organic solvents were measured before and after filtration.

Optimization of the manufacturing process

Briefly, 1 mg gefitinib was dissolved in different volume of organic solvents (ethanol:chloroform, 4:6 v/v), and slowly injected into HSA solution (2%) using a high shear dispersion homogenizer (IKA, German) at 11,000 rpm for 5 min and the coarse emulsion was obtained. Then, obtained emulsion was homogenized using a high-pressure homogenizer at 24,000 psi with various cycles. The obtained nanoemulsion was rotary evaporated at 43 mbar and 35°C at a rotation speed of 100 rpm using a rotavapor (Vacuum pump v700) to remove the organic solvent. Thus, nanoparticles were obtained. Nanosuspension filtered using 0.22 μ m pore size filters, and the particle size distribution and zeta potential of obtained nanoparticles were measured before and after filtration.

4.2.2. Experimental design for nanoparticle production

For the optimization of the gefitinib-HSA nanoparticle formulation, Box-Behnken design is employed for three independent factors, organic phase volume:total volume % (x_1), drug:HSA ratio (x_2), homogenization cycle number (x_3) (Table 6). The dependent response variables measured are average particle size, pdi, and zeta potential of filtered and unfiltered nanoparticles. The formulations are listed in Table 7 in coded form. Each row in the matrix represents an experiment. The selected levels were chosen to have a significant effect on the responses. Experiments are performed in random order. Center points are repeated 3 times. The statistical experimental design was generated, evaluated for the quality of fit of the model using the Design-Expert® software (Version 12.0.3.0, Stat-Ease Inc.).

	Levels and ranges				
Independent variables	-1	0	+1		
<i>x</i> ₁ Organic/total volume % (v/v)	2	6	10		
x ₂ HSA/gefitinib (%)	4	7	10		
x ₃ Homogenization cycle number	10	20	30		
Dependent variables	Constraints				
<i>Y1 = average particle size (unfiltered)</i>		Minimize			
Y2 = pdi (unfiltered)		Minimize			
Y3 = zeta potential (unfiltered)		-			
<i>Y4 = average particle size (filtered)</i>	Minimize				
Y5 = pdi (filtered)	Minimize				
$Y6 = zeta \ potential \ (filtered)$		-			

Table 6. Variables and their levels in the Box-Behnken design	ı
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Formulation no.	x_1	x_2	x_3
1	0	+1	-1
2	+1	-1	0
3	0	0	0
4	-1	+1	0
5	-1	-1	0
6	0	-1	-1
7	+1	0	+1
8	0	-1	+1
9	0	+1	+1
10	0	0	0
11	+1	0	-1
12	-1	0	+1
13	0	0	0
14	-1	0	-1
15	+1	+1	0

	O 1 1	D D 1 1	1 .	C .1	<i>c</i> .
Table 7.	Coded	Box-Behnk	en design	for three	factors

The relationship between the dependent variables average particle size, PDI, zeta potential, and the independent variables organic phase volume:total volume % (x_1), drug:HSA ratio (x_2), homogenization cycle number (x_3) is demonstrated on the surface response plots in Figure 3.

4.2.3. Fluorescence spectroscopy

Before adding surfactants to the formulation, the albumin binding behavior of gefitinib in the presence of surfactants were examined. For this purpose, fluorescence spectroscopy method was used to investigate the affinity of gefitinib towards albumin.

Fluorescence spectroscopy is a widely used approach for evaluating drug binding to HSA (24). Drugs generally bind to the tryptophan residue of HSA, resulting in quenching of the intrinsic fluorescence of HSA (25). To assess the affinity of gefitinib for HSA, various amounts of gefitinib were added to a 1 μ M HSA solution in the presence or absence of HSPC, oleic acid, or DPPC. The surfactants were mixed with HSA at 1:50 molar ratio. The fluorescence spectra were measured by a fluorespectrophotometer, and excitation and emission wavelengths were adjusted as 280 nm and 290–455 nm, respectively.

To investigate the binding ability of gefitinib towards HSA with or without surfactants, the fluorescent quenching data was analyzed by the Stern-Volmer equation:

(Eq. 1)

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$F0/F = 1 + KSV[Q] = 1 + kq\tau 0[Q]$

F0 = fluorescence intensity value of the protein in the absence of the quencher

 \mathbf{F} = refer to the fluorescence intensity values of the protein in the presence of the quencher

[Q] = the concentration of the quencher

KSV = the Stern-Volmer quenching constant

4.2.4. Nanoparticle production using surfactants

The formulations were produced by optimized method and adding three different levels (0.2 mg/mL, 1 mg/mL, 2 mg/mL) of DPPC, HSPC, and oleic acid to the formulations. 1 mg gefitinib and a surfactant was dissolved in organic solvent mixture (ethanol:chloroform, 4:6 v/v) to obtain 6.461 ratio of organic phase/total volume (%, v/v) (Table 4), and slowly injected into HSA solution (0.21%) to obtain HSA:gefitinib ratio as 9.835

(Table 4), using a high shear dispersion homogenizer (IKA, German) at 11,000 rpm for 5 min and the coarse emulsion was obtained. Then, obtained emulsion was homogenized 10 times using a high-pressure homogenizer (Microfluidics LV1, USA) at 24,000 psi with 10 cycles. The obtained nanoemulsion was rotary evaporated at 43 mbar and 35°C to remove the organic solvent. Thus, nanoparticles were obtained. Nanosuspension filtered using 0.22 μ m pore size filters, and the particle size distribution and zeta potential of obtained nanoparticles were measured before and after filtration.

4.2.5. Particle size distribution and zeta potential measurements

To investigate the effects of chances in production method parameters on particle size distributions and zeta potential, produced nanoparticles were analyzed by laser diffraction method. The obtained suspensions were analyzed using a Malvern NanoZS (Zetasizer NanoSeries ZS, Malvern Instruments, UK), which derives the volume distribution from a laser diffraction intensity distribution. The size and zeta potential of each sample was measured in triplicate.

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