International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 14, Issue 5, 2022

Original Article

FORMULATION AND OPTIMIZATION OF HYDROXYUREA LOADED NANOSTRUCTURED LIPID CARRIERS USING DESIGN OF EXPERIMENT FOR THE EFFECTIVE TREATMENT OF OVARIAN CANCER

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Received: 09 Jun 2022, Revised and Accepted: 15 Jul 2022

ABSTRACT

Objective: Ovarian cancer is the most deadly cancer in women, ranking fourth among all fatal diseases in women. Conventional chemotherapy has its own plethora of challenges, such as side effects and disease relapse. Hydroxyurea is a type of anticancer drug that is commonly used to treat malignancies. This study aims to develop and optimize hydroxyurea nanostructured lipid carriers (NLCs) to improve the therapeutic index and reduce its side effects in the effective treatment of OC.

Methods: NLCs were prepared by microemulsion technique. They were prepared and optimized using the design of experiment for particle size and drug entrapment efficiency. Particle size, polydispersity index, zeta potential, morphology, *in vitro* release, and stability were all examined in the optimized formulation.

Results: The results showed that the particle size of the NLCs was in the range of 224 nm to 634 nm. The drug entrapment efficiency of the NLCs was in the range of 46.33 % to 70.43 %. The optimized NLCs had a particle size of 237 nm, a polydispersity index of 26.9%, and a zeta potential of-29.7 mV. These NLCs were spherical, showed *in vitro* drug release of 92.21% up to 48 h, and were found to be stable from the stability studies.

Conclusion: This approach could be used as a better drug delivery platform to improve the drug's therapeutic index, reduce its side effects, and be feasible in the effective management of ovarian cancer.

Keywords: Ovarian cancer, Hydroxyurea, Nanostructured lipid carriers, Optimization

DOI: https://dx.doi.org/10.22159/ijap.2022v14i5.45464. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Ovarian cancer (OC) is the deadliest malignancy in women, ranking 4th among all fatal diseases in women. Patients with this lethal cancer have a 5-year survival rate of only 45.6 %. For the majority of patients, detection at the advanced stage of cancer results in a poor survival rate of 35 %. Debulking surgery, chemotherapy, and radiation therapy are the currently available treatment options. The most important aspect of OC treatment is chemotherapy. Depending on the stage of OC, chemotherapeutics drugs will be chosen for therapy. During the OC medication selection procedure, the sensitivity of the chemotherapeutic drug is critical. The use of high-dose chemotherapeutics will result in complications due to side effects, and the treatment plan may be terminated. Since OC cells go through molecular changes over time, they may become resistant to treatment [1-4]. Conventional treatment, on the other hand, has its own set of drawbacks, including toxicities and subsequent disease relapse due to multidrug resistance. Furthermore, because the chemo-drug is not specific for OC destruction, it causes dose cytotoxicity. Patients have a plethora of adverse effects, including excessive nausea, hair loss, and a decrease in plasma cell counts, as a result of taking chemotherapy for OC treatment. Several targeted drug delivery platforms have been developed to deliver antineoplastics to specific tumour sites, overcoming the treatment disadvantages of conventional antineoplastics [5-7].

Hydroxyurea (HU) is an anticancer drug that is commonly used to treat hematologic malignancies, sickle cell anaemia, breast cancer, ovarian cancer, and other conditions. HU prevents cells from leaving the G1/S phase of the cell cycle by inhibiting ribonucleoside diphosphate reductase, an enzyme necessary to convert ribonucleoside diphosphates to deoxyribonucleoside diphosphates. Long-term usage of high doses of this medicine has adverse effects on the blood and skin of the patients. Large amounts of drug release, deposits, and withdrawals of the drug via the reticuloendothelial system are some of the key issues with this type of drug delivery [8, 9]. To address these issues, nanostructured lipid carriers (NLCs) will be developed. NLCs are second-generation lipid nanoparticles developed using a blend of solid and liquid lipid. Nanoparticles have emerged as a promising strategy for the effective delivery of drugs [10, 11]. In cancer therapy, NLCs have recently emerged as a multifunctional platform for drug delivery. Many advantages of this delivery system have been reported in earlier research, including excellent entrapment efficiency, good stability, and sustained release of drugs at specific rhythmic intervals [12-15].

This study aims to develop and optimize HU NLCs to improve the therapeutic index and reduce its side effects in the effective treatment of OC. Altering the formulation aspects like stirring time and ingredients concentration and ratio can influence the essential parameters of NLCs, such as particle size and entrapment efficiency. Thus, an experimental design was used to predict the optimized NLCs.

MATERIALS AND METHODS

Materials

Hydroxyurea was purchased from Sigma Aldrich (Mumbai, India). Glycerol monostearate, oleic acid, poloxamer 407, tween 80, and mannitol were procured from Himedia Labs (Mumbai, India). The other chemicals and reagents used in the study were of analytical grade.

Preparation of NLCs

NLCs were prepared by the microemulsion method. The lipid mixture of glycerol monostearate and oleic acid (1.25 g/0.625 g) is weighed and melting them in a water bath at 75-80 °C. Hydroxyurea (150 mg) is added to the melted lipid phase. Under magnetic

stirring, the lipid melt was dispersed in a 20 ml hot aqueous surfactant solution of the same temperature containing 1 % Poloxamer 407 and 1 % tween 80 (w/v) to form an o/w emulsion. For 30 min, the o/w pre-emulsion was subjected to bath sonication. The formed o/w nanoemulsion was allowed to cool to room temperature. 5% Mannitol was added to the NLC solution and stirred for 15 min. Then, the NLC solution was subjected to lyophilization [16, 17].

Experimental design

There are several statistical models for designing nanoparticle optimization. Here, Box Behnken design (design-expert software version 13) was used to determine the appropriate parameters range that influence the preparation and optimization of NLCs. This allows for evaluating several independent variables on dependent variables using a set of experimental runs. The independent variables (or factors) for optimizing HU NLCs are stirring time (A), solid lipid: liquid lipid (B), and surfactant concentration (C). Particle size (Y1) and drug entrapment efficiency (DEE) (Y2) are the responses. The levels of these factors are shown in table 1. The design-expert software was used to create 13 tests in order to determine the relevant statistical aspects and optimize the preparation of HU NLCs. The major purpose of this approach was to reduce particle size while increasing DEE in HU NLCs. Based on the findings of the ANOVA (analysis of variance), the best model for each variable was selected [18, 19].

Table 1: Variables for the preparation and optimization of HU NLCs

Independent factors		Design level	
Uncoded	**Coded**	**Uncoded level**	**Coded level**
Stirring time (h)	A	3	-1
		6	0
		9	+1
Solid lipid: Liquid lipid (w/w)	B	1:0.5	-1
		1:1	0
		1:2	+1
Surfactant concentration (%)	C	1	-1
		1.5	0
		2	+1
Dependent factors		Constraints	
Y_1: Particle size		Minimize	
Y_2: DEE		Maximize	

Physicochemical characterization

- **Particle size, polydispersity index (PDI), and zeta potential**

After a sufficient dilution with distilled water previously filtered using a 0.45 membrane filter, the particle size, PDI, and zeta potential of the NLCs were evaluated using an Anton Paar Litesizer 500. A few drops of HU NLCs were taken and mixed with water to make a 10 ml solution, which was then put into the quartz cuvette [20].

- **Drug entrapment efficiency**

The DEE was estimated by calculating the free HU in the solution containing NLCs. The NLC solution was first centrifuged for 20 min at 14000 rpm. The free drug content was then determined by diluting the supernatant solution and using UV spectrophotometry at wavelength 214 nm (15). DEE was calculated using the following equation:

$$\text{DEE \%} = \frac{\text{Total HU taken} - \text{Free HU}}{\text{Total HU taken}} \times 100$$

- **Morphological studies**

Scanning electron microscopy (SEM) is used to examine the morphology (size and shape) of the optimized NLCs. The surface properties of the samples were examined using a Zeiss SEM with a 10-keV pulse and several resolutions.

- ***In vitro* release studies**

Under sink conditions, the release of HU from the NLCs was examined. Dialysis bags were filled with 5 ml of NLCs equivalent (MWCO 12000, HiMedia). The dialysis bags were immersed in 50 ml of dissolution medium and stirred at 37 °C with magnetic stirring. At each time interval, aliquots of the dissolving medium were taken and replaced with the same volume of fresh dissolution medium to maintain a constant volume. HU concentration was measured spectrophotometrically at 214 nm in samples extracted from phosphate buffer saline (pH 7.4) against a solvent blank [20-22].

- **Stability studies**

The optimized formulation packed in its primary pack 30 ml tubular vial with a 20 mm rubber stopper was subjected to accelerated stability studies using an environmental testing chamber (Remi instrument Ltd) for 30 d. Storage conditions of the testing chamber are maintained at 40°+2 °C and 75%+5% relative humidity (RH). In addition, the formulation was evaluated for appearance, drug entrapment efficiency, and *in vitro* drug release.

RESULTS AND DISCUSSION

Numerous researchers have developed NLCs using various techniques. Here, we have employed the microemulsion technique followed by bath sonication to prepare NLCs in an affordable, easy, and reproducible manner. The microemulsion must be formed at a temperature higher than the lipid's melting point in order to form with a lipid that is solid at room temperature [23].

Experimental design

- **ANOVA analysis**

Table 2 shows the independent variables with measured responses. The particle size of HU NLCs varies from 228 nm to 634 nm. DEE of HU NLCs varies from 46.33 % to 70.43 %. Tables 3 and 4 provide the ANOVA results and fit statistics for response 1. Tables 5 and 6 show the ANOVA results and fit statistics for response 2. To assess the influence of the parameters, mathematical equations were established for both responses.

The best-fitting equation for particle size was the linear model, as shown below:

Particle size = 433.77–118.88A+59.25B–63.63C

R^2 and adjusted R^2 scores can be used to assess model accuracy (table 4). For this model, these values were very similar and acceptable. This indicates that the model is capable of accurately predicting outcomes.

The best-fitting equation for DEE was the 2FI model, as shown below:

DEE = 56.25+5.53A+3.19B+7.47C+0.7475AB+2.62AC+1.35BC

R2 and adjusted R2 scores can be used to assess model accuracy (table 6). For this model, these values were very similar and acceptable. This indicates that the model is capable of accurately predicting outcomes.

Table 2: Experimental design with factors and their responses

Run	Factor 1 A: Stirring time (h)	Factor 2 B: Solid lipid: liquid lipid (% w/ w)	Factor 3 C: Surfactant concentration (%)	Response 1 Y_1: Particle size (nm)	Response 2 Y_2: DEE (%)
1.	9	1:1	1	398±0.86	51.22±1.06
2.	6	1:1	1.5	417±1.22	54.79±0.66
3.	6	1:2	2	429±0.74	69.52±1.72
4.	6	1:0.5	2	318±0.69	60.18±0.79
5.	3	1:1	1	634±0.38	46.33±1.28
6.	3	1:2	1.5	604±1.14	52.79±0.95
7.	6	1:0.5	1	445±0.57	46.96±1.46
8.	6	1:2	1	551±0.61	50.88±0.82
9.	3	1:1	2	496±1.41	55.05±0.68
10.	9	1:2	1.5	362±0.77	66.27±1.29
11.	3	1:0.5	1.5	481±0.59	48.15±0.98
12.	9	1:1	2	276±1.24	70.43±0.85
13.	9	1:0.5	1.5	228±1.44	58.64±1.42

Data is given in mean±SD, n=3.

Table 3: Response 1 ANOVA

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	1.735E+05	3	57839.92	179.97	<0.0001	significant
A-Stirring time	1.131E+05	1	1.131E+05	351.75	<0.0001	
B-Solid lipid: Liquid lipid	28084.50	1	28084.50	87.38	<0.0001	
C-Surfactant concentration	32385.13	1	32385.13	100.76	<0.0001	
Residual	2892.56	9	321.40			
Cor total	1.764E+05	12				

Table 4: Fit statistics for response 1

Std. Dev.	Mean	C. V. %	R^2	Adjusted R^2	Predicted R^2	Adeq precision
17.93	433.77	4.13	0.9836	0.9781	0.9655	36.7041

Table 5: Response 2 ANOVA

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	810.06	6	135.01	93.55	<0.0001	significant
A-Stirring time	244.65	1	244.65	169.51	<0.0001	
B-Solid lipid: Liquid lipid	81.47	1	81.47	56.45	0.0003	
C-Surfactant concentration	446.86	1	446.86	309.61	<0.0001	
AB	2.24	1	2.24	1.55	0.2597	
AC	27.51	1	27.51	19.06	0.0047	
BC	7.34	1	7.34	5.09	0.0649	
Residual	8.66	6	1.44			
Cor Total	818.72	12				

Table 6: Fit statistics for response 2

Std. Dev.	Mean	C. V. %	R^2	Adjusted R^2	Predicted R^2	Adeq precision
1.20	56.25	2.14	0.9894	0.9788	0.9524	29.5018

Adeq Precision is the model's signal-to-noise ratio, which must be more than 4 to be considered acceptable [24]. Adeq Precision for particle size and DEE are 36.7 and 29.5, respectively. This proves the model's sufficiency for both responses.

- **Variables interaction and optimization**

Response surface plots are used to understand the relationship between independent factors and responses. The perturbation and response surface plots in fig. 1 and 2 show that when stirring duration and surfactant concentration increased, particle size decreased. It increased when the ratio of liquid lipid to solid lipid increased. DEE increased as all three parameters were improved, as shown in fig. 3 and 4.

The numerical approach produced by the Design-Expert software was used to obtain the optimum formulation. The input parameters were restricted to a range, whereas the particle size and DEE desirability functions were based on the minimum and maximum levels, respectively. The optimal HU NLCs were made with a solid lipid to liquid lipid ratio of 1.04 %, a surfactant concentration of 2 %, and a stirring time of 9 h. For optimal NLCs, the anticipated particle size and DEE were 235 nm and 70.42 %, respectively. For the optimized formulation of NLCs, the experimental particle size and DEE values were 237 nm and 69.81 %, respectively, which were comparable to the predicted values. As a result, the models' reliability was confirmed.

Physicochemical characterization of optimized formulation

- **Particle size, polydispersity index (PDI), and zeta potential**

The average particle size of the optimized formulation was found to be 237 nm. The PDI of the optimized formulation was found to be 26.9%

(fig. 5), which was within the acceptable range, indicating homogenous particle size distribution. In addition, the average zeta potential of optimized nano-sponges was found to be-29.7 mV, which was sufficient to keep the nano-sponges away from aggregation.

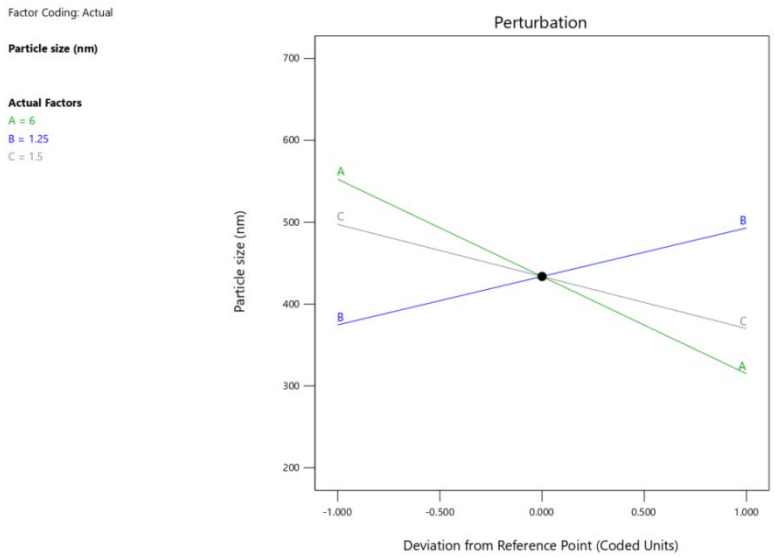
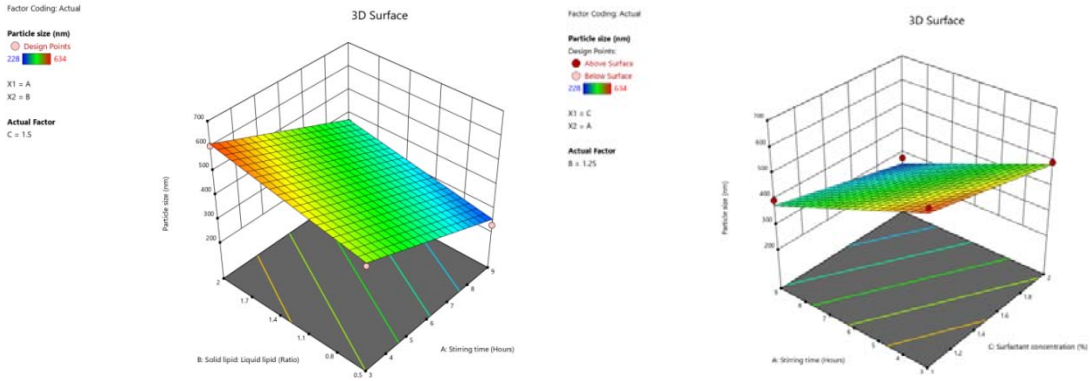


Fig. 1: Perturbation of factors on particle size



(a) **(b)**

Fig. 2: Response surface plots of particle size

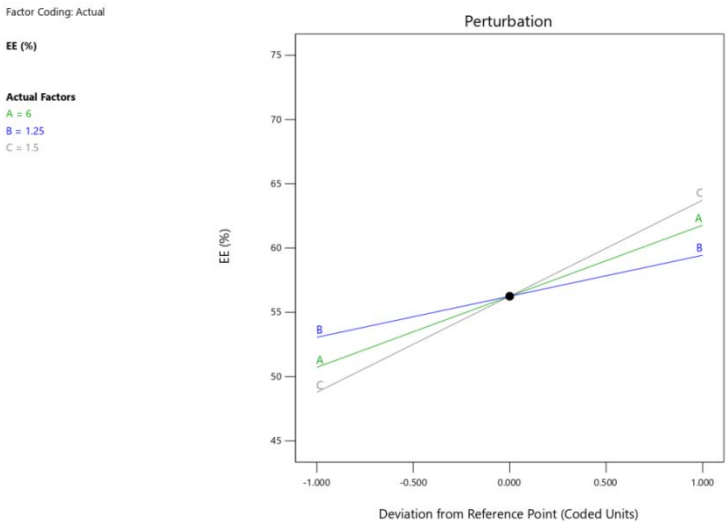
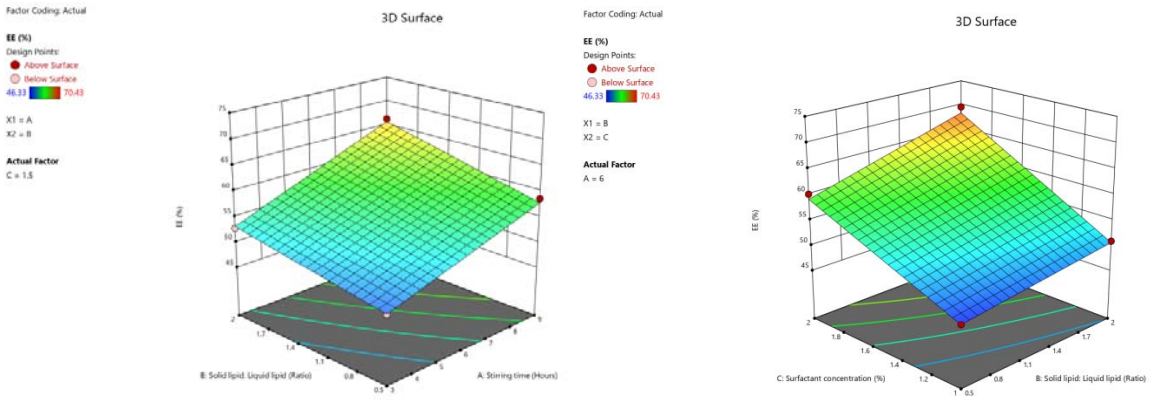


Fig. 3: Perturbation of factors on DEE



(a) **(b)**

Fig. 4: Response surface plots of DEE

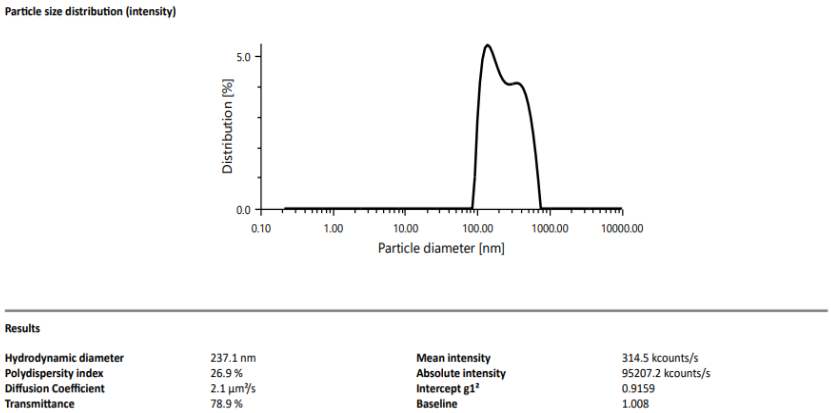


Fig. 5: Particle size and PDI of optimized HU NLCs

Investigations were done on how stirring time, surfactant concentration, and lipid ratio affected particle size. The optimum batch's particle size was reported to be 237 nm. With an increase in surfactant concentration and stirring time, the particle size gradually reduced. NLCs prepared without any charge modifiers have been shown to have negative zeta potential. The drug-loaded NLCs displayed a negative zeta potential. These NLCs had a surface charge that was nearly-30 mV, at-29.7 mV. The ideal zeta potential for effective nanoparticle stabilisation is considered to be-30 mV. It has been noted that serum proteins are less likely to bind to nanoparticles with negative surface charges, resulting in longer circulation half-lives and decreased accumulation in the liver and spleen [25-27].

- **Morphological studies**

The image of the optimized HU NLCs is shown in fig. 6. The SEM results revealed that the HU NLCs were spherical in shape. The nanoparticle size observed by SEM correlated well with the particle size measured by the particle size analyzer.

- ***In vitro* release studies**

Fig. 7 shows the drug release curve for the optimized HU NLCs (92.21% CDR up to 48 h). HU release from NLCs is biphasic, with early burst release followed by a controlled release in the latter hours. The initial burst release was followed by a sustained release in these nanoparticles. The drug is likely to be responsible for the initial *in vitro* burst release if it has been adsorbed on the surface of the nanoparticle or precipitated from the lipid matrix. The drug diffusing out of the lipid matrix is most probably the cause of the sustained release [28].

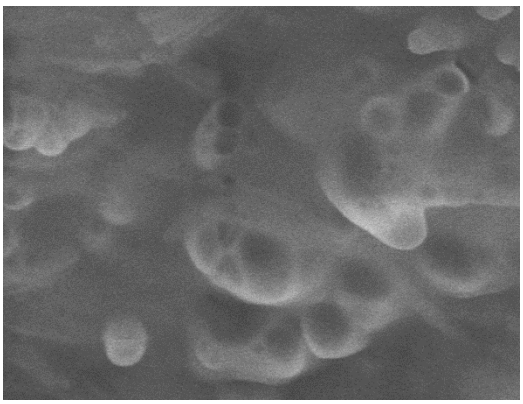


Fig. 6: SEM image of HU NLCs

- **Stability studies**

Accelerated stability studies showed that HU NLCs were stable, and the results are shown in table 7. Since the formulation remained stable during the stability studies at room temperature and there was no apparent change in appearance, DEE, or % CDR, the surfactant mixture proved suitable for long-term storage. Therefore, it can be concluded that the preparation method used here, along with the selected ingredients, resulted in the development of NLCs that were stable while stored. Furthermore, the high ZP values that cause strong inter-particular repulsive forces should be what gives the material its good stability and nanoaggregated pattern [29].

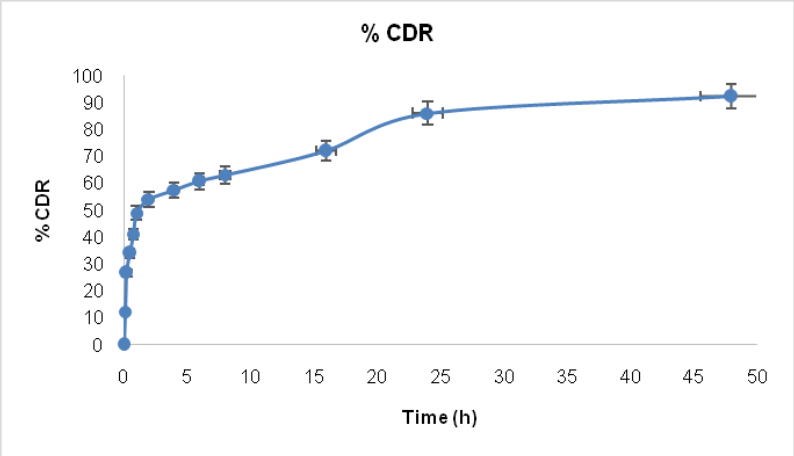


Fig. 7: *In vitro* release of HU NLCs, n=3

Table 7: Stability studies

	Storage conditions: 40 °C±2 °C/75%RH±5%RH			
Sample type	**Sampling interval**	**Appearance**	**% DEE**	**% CDR**
HU NLCs	0 d	White colour	69.81±27	92.21±73
	30 d	No change in colour	69.74±42	92.14±69

Data is given in mean±SD, n=3.

CONCLUSION

The application of nano-encapsulated HU in the form of NLCs for the treatment of ovarian cancer is the focus of this investigation. NLCs were prepared using the microemulsion technique and optimized for minimum particle size and maximum DEE by employing DoE. These HU NLCs confirmed the sustained release of the drug, which could be essential for patient compliance and reducing the dosage intervals. This approach could improve the drug's therapeutic index, reduce its side effects, and be feasible in the effective management of ovarian cancer. *In vivo* pharmacokinetic study is the future perspective of this study.

ACKNOWLEDGMENT

The authors would like to thank the Department of Science and Technology–Fund for Improvement of Science and Technology Infrastructure in Universities and Higher Educational Institutions (DST-FIST), New Delhi, for their infrastructure support to our department.

FUNDING

No funding was received for this study.

AUTHORS CONTRIBUTIONS

All the authors have equally contributed.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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