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FORMULATION AND EVALUATION OF DOXORUBICIN HYDROCHLORIDE LOADED BOVINE SERUM ALBUMIN NANOPARTICLES

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ABSTRACT
Nanoparticles are small colloidal particles measuring 100 nanometers which were simple, stable and easy to scale-up. The nature of many conventional drugs changes when converted to nanoparticles. Nanoparticles have more surface area than larger particles which makes them more reactive (protecting against chemical and enzymatic degradation, ability to reduce side effects). Doxorubicin Hydrochloride is one of the commonly used drugs for the treatment of the metastatic breast cancer or ovarian cancer. In this study, Albumin was used to prepare Nanoparticle, in different compositions and particles were numerous and Nano metric in size. To formulate Doxorubicin Hydrochloride encapsulated nanoparticles. To assess the impact of polymer ratio, surfactant and homogenization speed on particle size, entrapment efficiency and in vitro release of Doxorubicin Hydrochloride. The linearity was demonstrated by 50, 75, 100, 125 and 150% of the normal concentration of 0.5mg/mL using 5 concentrations between these ranges. These concentrations were prepared by coacervation method. Based on the entrapment efficiency and particle size a set of formulations (F11, F19 and F21) were considered as lead formulations which are evaluated for the diffusion studies. By observing the results it can be concluded that the formulation F19 has the ability to prolong the release and reduce the side effects by targeting the drug to the desired site and reduce the dosing frequency of the drug for better patient compliance.

KEYWORDS: Doxorubicin Hydrochloride, Serum albumin, Nanoparticle, Entrapment efficiency, Linearity.

INTRODUCTION
The aim of any drug delivery system is to provide therapeutic concentrations of the drug at the site of action and then maintain concentrations within the therapeutic range. Administering drugs orally, intravenously or rectally doesn't maintain drug concentration within the therapeutic range for longer duration of time. The shorter duration of action of conventional dosage forms lead to formulation of control temporal delivery. An appropriately designed controlled release dosage form can be a major step in solving this problem. It is the reason that the science and technology responsible for development of controlled-release pharmaceuticals has been, and continues to be the focus of a great deal of attention. One of the most interesting concepts is target-organ drug delivery system. Injecting drugs into whole body is not only wasteful but also cause harmful effects which can be eliminated by target-organ drug delivery. Targeted delivery has no restrictions to any one route of administration. Oral formulations, parenterals, transdermal and pulmonary route and many other routes can be used for effective drug targeting. Targeted drug delivery can also be done by changing the formulation in such a way that alters its distribution profile in the body, thereby minimizing contact with healthy tissues e.g., encapsulation in a liposome formulation. Nanoparticles are small colloidal particles which are made up of non-biodegradable and biodegradable polymers. Their diameter is from 1-1000nm. Two types of nanoparticles werenanospheres, which are matrix systems and nanocapsules, which are reservoir systems composed of a polymer membrane surrounding an oily or aqueous core. These systems were developed in early 1970s. This approach was attractive because the methods of preparation of particles were simple and easy to scale-up. The particles formed were stable and easily freeze dried. Due to these reasons, nanoparticles made of biodegradable polymers were developed for drug delivery. Nanoparticles were able to protect drugs against chemical and enzymatic degradation and were able to reduce side effects of some active drugs. Due to small size of these systems limits their use, as only small quantities of material can be encapsulated. Other types of nanoparticles systems include colloidal sulfur and colloidal gold. Colloidal sulfur (^{99m}Tc) is used as a diagnostic agent. It is usually protected from aggregation by the addition of gelatin as a polymeric stabilizer.

Colloidal gold is also used as a diagnostic (^{198}Au) and as a therapeutic agent.

MATERIALS

The gift sample obtained by Doxorubicin Hydrochloride (from RPG life sciences), bovine serum albumin(BSA) (Sisco Research lab pvt.ltd, Mumbai), tris buffer (Merck limited, Mumbai), TocopherolPolyethyelene glycol-1000 succinate (TPGS) (Peboc division of Eastman company UK ltd), hydrochloric acid (Merck limited, Mumbai), 25% glutaraldehyde (Nice, Mumbai), ethanol (Shymlakhs international), ethanolamine (Merck limited, Mumbai), sodium hydroxide (Merck limited, Mumbai).

EQUIPMENTS USED

Homogenizer (Kinematica AG), hot plate magnetic stirrer (Remi), analytical balance (ShincoDeshi., Ltd, Japan), pH meter (Polmon, LP-139S), microscope (Olympus, CH20), HPLC (Shimadzu), centrifuge (REMI), sonicator (Enertech electronic Pvt.Ltd).

METHODS

Demonstration of linearity: The linearity of the method was demonstrated by 50, 75, 100, 125 and 150% of the normal concentration of 0.5mg/mL using 5 concentrations between these ranges.

Preparation of standard solution: 2.5mg of Doxorubicin Hydrochloride working standard taken into 25mL volumetric flask dissolve and made up to the mark with diluents.

System suitability for linearity: Injected 20µL of standard solution into the chromatograph for 6 times and calculated the relative standard deviation (RT-1.0%, peak area-2.0%) for Doxorubicin peak.

Table 1.

S.No	Injection number	Retention time	Peak area
1	Injection-1	6.523	3253245
2	Injection-2	6.503	3281639
3	Injection-3	6.556	3252490
4	Injection-4	6.554	3249954
5	Injection-5	6.537	3243899
6	Injection-6	6.525	3252802
Mean		6.533	3255671
Acceptance		NMT-1.0%	NMT-2.0%
Result		Pass(RSD 0.31)	Pass(RSD 0.4)

Linearity of test solutions: Prepared the series of linearity test solutions in 6 concentrations of 50%, 75%, 100%, 125%, 150% and 200% using the stock solution. Transferred accurately the volume of stock solution mentioned below into the volumetric flask of specified capacity and brought to volume with diluent, prepared the series of solutions each in triplicate.

Preparation of stock solution: 12.58mg of Doxorubicin Hydrochloride taken into 25mL volumetric flask dissolve and made upto the mark with diluent.

Linear solution 50%: Transferred 1.0mL of stock solution into the 10mL volumetric flask and brought to volume with diluent (concentration 0.05032mg/mL).

Linear solution 75%: Transferred 1.5mL of stock solution into the 10mL volumetric flask and brought to volume with diluent (concentration 0.07548mg/mL).

Linear solution 100%: Transferred 2.0mL of stock solution into the 10mL volumetric flask and brought to volume with diluent (concentration 0.10064mg/mL).

Linear solution 125%: Transferred 2.5mL of stock solution into the 10mL volumetric flask and brought to volume with diluent (concentration 0.1258mg/mL).

Linear solution 150%: Transferred 3.0mL of stock solution into the 10mL volumetric flask and brought to volume with diluent (concentration 0.15096mg/mL).

Linear solution 200%: Transferred 4.0mL of stock solution into the 10mL volumetric flask and brought to volume with diluent (concentration 0.20128mg/mL).

Procedure: injected 20µL of each solution into the chromatograph (in duplicate) and measured the area of the peak due to doxorubicin and then calculated the average area in each case.

Statistical evaluation: A graph between the concentration and average area was plotted. Points for linearity were observed. Using method of least squares a line of best fit was taken and calculated the correlation coefficient, slope, and y-intercept.

Table 2: Demonstration of linearity between different Doxorubicin concentrations.

S.No	Linear solution range	Concentration (mg/mL)	Peak area	Mean area
1	50%	0.05032	1600927 1621121	16110324
2	75%	0.07548	2462217 2435272	2448745
3	100%	0.10064	3265086 3250324	3257705
4	125%	0.1258	4063870 4064792	4064331
5	150%	0.15096	4910948 4912697	4911823
6	200%	0.20128	6517611 6514749	6516180

METHOD OF PREPARATION OF DOXORUBICIN Hydrochloride LOADED NANOPARTICLES

Coacervation method

Bovine Serum Albumin and drug were dissolved in 10mL of purified water. After a short time, 6mg of Tris-Buffer was added to the aqueous solution and TPGS was added, and thensonicated for 2mins. At the same time pH of the solution was adjusted to 6.5 by 0.1M Hydrochloride using digital pH meter. BSA Nanoparticles were prepared by coacervation method. In this method nanoparticles were obtained by the addition of 15mL of ethanol drop wise to 10mL of aqueous polymer and drug solution under continuous stirring until the solution become turbid at 4ºc. Coacervates thus obtained were hardened for 1hr with glutaraldehyde. Then ethanolamine was added which block the free aldehyde groups. Afterwards, nanoparticles purified by centrifugation (12,000 rpm/4ºc/45min) to eliminate free albumin and the excess of glutaraldehyde. Then the bottom part re-suspended in same volume of distilled water.

FORMULATION DEVELOPMENT

Table 3: Formulation of Doxorubicin Hydrochloride loaded nanoparticles.

Ingredients	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Bovine Serum Albumin(mg)	50	50	50	50	100	100	100	50	50
Doxorubicin Hydrochloride(mg)	10	10	10	10	10	10	10	10	10
Tris buffer(mg)	6	6	6	6	6	6	6	6	6
TPGS(%)	1.0	0.5	0.25	0.1	0.1	0.25	0.5	0.1	0.25
Ethanol(mL)	15	15	15	15	15	15	15	15	15
pH	6.5	6.5	7.5	6.5	6.5	6.5	6.5	6.5	6.5
Speed(rpm)	11,000	11,000	11,000	11,000	11,000	11,000	11,000	15,000	15,000
25% glutaraldehyde	60	60	60	60	150	150	150	60	60
Ethanolamine(µL)	25	25	25	25	25	25	25	25	25

Table 4: Formulation of Doxorubicin Hydrochloride loaded nanoparticles

Ingredients	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**	**F16**	**F17**
Bovine Serum Albumin(mg)	50	100	100	100	150	150	50	50
Doxorubicin Hydrochloride(mg)	10	10	10	10	10	10	10	10
Tris buffer(mg)	6	6	6	6	6	6	6	6
TPGS(%)	0.5	0.1	0.25	0.5	0.1	0.25	0.1	0.25
Ethanol(mL)	15	15	15	15	15	15	15	15
pH	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Speed(rpm)	15,000	15,000	15,000	15,000	15,000	15,000	20,000	20,000
Glutaraldehyde(µL)	60	150	150	150	150	150	60	60
Ethanolamine(µL)	25	25	25	25	25	25	25	25

Table 5: Formulation of Doxorubicin Hydrochloride loaded nanoparticles.

Ingredients	F18	F19	F20	F21	F22	F23	F24	F25
Bovine Serum Albumin(mg)	50	100	100	100	50	50	100	100
Doxorubicin Hydrochloride(mg)	10	10	10	10	10	10	10	10
Tris buffer(mg)	6	6	6	6	6	6	6	6
TPGS(%)	0.5	0.1	0.25	0.5	0.1	0.25	0.1	0.25
Ethanol(mL)	15	15	15	15	15	15	15	15
pH	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Speed(rpm)	20,000	20,000	20,000	20,000	24,000	24,000	24,000	24,000
Glutaraldehyde(µL)	60	150	150	150	60	60	150	150
Ethanolamine(µL)	25	25	25	25	25	25	25	25

EVALUATION OF DOXORUBICIN Hydrochloride LOADED NANOPARTICLES.

Particle size: Particles size was determined by using MALVERN instrument.

Zeta potential: Zeta potential was determined by using MALVERN instrument UK.

Lyophilization: The obtained centrifuged samples were lyophilized and stored at 2-8ºC. The samples are lyophilized to attain stability. The obtained lyophilized powder is utilized for in-vitro drug release parameters.

Drug Encapsulation Efficiency: After centrifugation the supernatant liquid was taken, the amount of free drug was present in supernatant was estimated by HPLC method. The amount of free drug in supernatant was then substracted from the total amount of drug added during the coacervation process given amount if drug entrapped. Then entrapment efficiency calculated by given formula.

$$\text{Entrapment Efficiency} = \frac{\text{Amount entrapped}}{\text{Total drug loaded}} \times 100$$

RESULTS

Table 6: Calibration curve for the estimation of Doxorubicin Hydrochloride.

Concentration (mg/mL)	Peak Area
0.05032	1611024
0.07578	244845
0.10064	3257705
0.1258	4064331
0.15096	4911823
0.20128	6516180

Table 7: Microscopic observations of prepared nanoparticle formulations.

Formulation code	Observation	Remarks on the overall preparation
F1	Hazy solution observed	Large size particles observed
F2	Hazy solution observed	Lumps formed
F3	Hazy solution observed	Coagulation formed
F4	Hazy solution observed	Medium and more large size particles observed
F5	Hazy solution observed	Medium and large size particles observed
F6	Hazy solution observed	Medium and large size particles observed
F7	Hazy solution observed	Medium and large size particles observed
F8	Hazy solution observed	Medium and large size particles observed
F9	Hazy solution observed	Coagulation formed
F10	Hazy solution observed	More medium size particles observed
F11	Hazy solution observed	Less medium and more small size particles observed
F12	Hazy solution observed	Less medium and more small size particles observed
F13	Hazy solution observed	Less no.of small and more no.of medium size particles observed
F14	Hazy solution observed	Large size particles observed
F15	Hazy solution observed	Coagulation formed
F16	Hazy solution observed	Small and medium size particles observed
F17	Hazy solution observed	More medium size particles observed

F18	Hazy solution observed	Large size particles observed
F19	Hazy solution observed	Small size particles observed
F20	Hazy solution observed	Small size particles observed
F21	Hazy solution observed	Small size particles observed
F22	Hazy solution observed	More medium size particles observed less large size particles observed
F23	Hazy solution observed	More medium size particles observed
F24	Hazy solution observed	Medium and small size particles observed
F25	Hazy solution observed	Coagulation observed

Table 8: Entrapment Efficiency, Particle size and Drug loading.

Formulation code	Particle size(nm)	Zeta potential(mv)	Polydispersy index	Entrapment Efficiency	Drug loaded(mg)
F1	---	---	---	24.98	0.24
F2	---	---	---	31.24	0.31
F3	2037	-3.16	0.165	38.43	0.38
F4	---	---	---	29.76	0.29
F5	---	---	---	63.2	6.32
F6	---	---	---	70.9	7.09
F7	---	---	---	76.1	7.61
F8	---	---	---	49.6	4.96
F9	---	---	---	44.6	4.46
F10	---	---	---	49.1	4.91
F11*	205.1	-36.9	0.011	88.9	8.89
F12	341.5	16.5	1.000	74.1	7.41
F13	366.6	27.9	0.240	72.2	7.22
F14	1056	17.8	0.438	96.2	9.62
F15	---	---	---	95.8	9.58
F16	320.2	7.43	0.473	55.4	5.54
F17	497.8	23.6	0.264	51.2	5.12
F18	798.0	15.2	0.330	53.6	5.36
F19*	90.44	-16.1	0.692	94.5	9.45
F20*	179.7	-25.6	0.034	92.6	9.62
F21	291.6	-19.9	0.098	91.3	9.13
F22	---	---	---	61.8	6.18
F23	---	---	---	68.7	6.87
F24	---	---	---	86.2	8.62
F25	---	---	---	80.3	8.03

LEAD FORMULATIONS:

Based on the entrapment efficiency and particle size a set of formulations (F11, F19 and F21) were considered as Lead formulations which can be taken up further studies and evaluated for the diffusion studies.

Table 9: Lead formulations of Doxorubicin Hydrochloride loaded nanoparticles,

Ingredients	F11	F19	F20
Bovine Serum Albumin(mg)	100	100	100
Doxorubicin Hydrochloride(mg)	10	10	10
Tris buffer(mg)	6	6	6
TPGS(%)	0.1	0.1	0.25
Ethanol(mL)	15	15	15
Speed(rpm)	15,000	20,000	20,000
Glutaraldehyde(µL)	150	150	150
Ethanolamine(µL)	25	25	25

Table 10: Diffusion profiles for F11, F19 & F20.

S.No	Time (hrs)	Percentage drug diffused		
		F11	F19	F20
1.	0	0	0	0
2.	1	0.1±0.09	0.3±0.04	0.1±0.56
3.	6	3.1±1.23	4.5±0.67	4.3±0.34
4.	12	7.5±0.67	7.2±0.56	5.6±1.55
5.	24	12.6±0.54	16.8±0.15	17.2±1.22
6.	36	24.5±0.29	25.5±0.51	20.8±1.72
7.	48	32.6±0.56	34.6±0.67	27.2±0.98
8.	60	40.8±1.25	45.2±0.85	43.1±0.45
9.	72	45.6±0.27	50.1±0.44	51.7±0.34
10.	84	52.2±0.112	62.3±0.69	59.8±0.88
11.	96	65.3±0.62	73.5±1.22	68.9±0.67
12.	108	73.5±0.44	79.9±0.36	72.5±0.46
13.	120	80.1±0.78	91.9±0.45	86.5±0.99

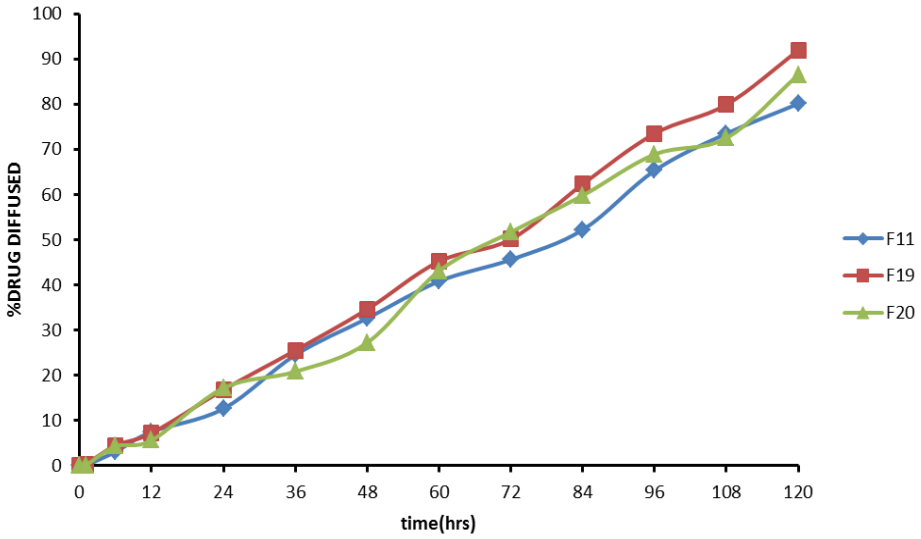

Fig 1: Diffusion profile plot for F11, F19, F20.

DISCUSSION OF RESULTS

Doxorubicin Hydrochloride is one of the commonly used drugs for the treatment of the metastatic breast cancer or ovarian cancer. In the present study, an attempt was done to entrap a new drug i.e. Doxorubicin Hydrochloride in the preparation of the Nanoparticles.

In these studies, Nanoparticles were prepared using the Albumin, in different compositions and particles are numerous and nano metric in size. Based on the requirement it has been postulated to prepare formulation with nano size and a good balanced Zeta potential.

From the prepared Nanoparticles formulations the following results are observed.

Microscopic observation: The prepared Nanoparticles formulations were observed under microscope given in table-12, the following results are obtained. Among from these F1, F2, F3, F4, F9, F14, F15, F18, F25 are appeared as large particles F5, F6, F7, F8, F10, F22, F23, F24 are appeared as medium size particles and F11, F12, F13, F16, F17, F19, F20, F21 appeared as small size particles even though F11, F19, F20, F21 are having good particle size when compared to other formulations. By using the increased ratios of emulsifier, polymer concentrations and homogenization speeds.

Entrapment Efficiency: The formulations F1 to F25 subjected to entrapment efficiency are given in table-13. From that result F11, F14, F15, F19, F20, F21 were getting good entrapment efficiency when compared to other formulations. But F14, F15 not getting good particle size. So that we are finally concluded the F11, F19, F20 formulations were subjected to further evaluation test i.e. particle size and SEM.

Particle size: From the selected formulations F11, F12, F13, F16, F17, F19, F20 and F21 (from table-13), shows good particle size, when compared to other selected formulas. And formulations F11, F19, F20 were considered as lead formulations. Three polymer concentrations used (0.5%, 1.0%, 1.5%), in that 1.0% solution (from fig-16) gave good particle size compared

to other two concentrations. I used different homogenized speeds 11,000, 15,000, 20,000 & 24,000rpm in that 20,000rpm gave good particle size F19 (from fig-18) and different stabilizer concentrations like 0.1%, 0.25%, 0.5% & 1.0% solutions used. The 1.0% solution have given very large particle size then optimized remaining three concentrations, in that 0.1% solution (from fig-17) gave particle size.

Zeta potential: Higher zeta potential means 15-30mV shows high stability of colloidal system. The obtained zeta potential result range from 3.16 to 36.9(from table-13) even though most of the formulations are within the range. Except F3 & F16 remaining formulations have zeta potential between 15-36mV. The prepared formulations have good stability. F3 & F16 formulations are not in the range.

Poly dispersity index: Polydispersity indicates the degree of non-uniformity of the particle size. Below 0.7indicates the good dispersity, the prepared formulations have 0.01-1.00 (from table-13), most of the prepared formulations have poly dispersity index value below 0.7. So the formulations have uniform particle size distribution.

Diffusion Studies: The above Lead formulations are subjected to diffusion studies (actual dose of the drug 10mg was incorporated) were given in table-13. From the obtained the graphs were plotted like Zero order, First order, Higuchi, KorsmeyerPeppes for determination of order of drug release and mechanism of drug release (table-15 to 21 & from fig 11-15). From Zero & First order plots, the lead formulation F19 indicates it follows zero order release. Korsmeyerpeppas equation shows "n" value 1.071 means it follows case-II transport. From the obtained results we suggested the F19 is better when compared to the other formulations F19 satisfy the controlled and nanoparticles drug delivery system limitation under conducting of various evaluation tests.

The optimized F19 specifications.
Particle size: 90.44 nm
Entrapment efficiency: 94.5%
Diffusion studies:
Drug release-91.9%
Order of drug release
a) Zero order-R^2-0.997
b) First order-R^2-0.878
Mechanism of drug release
a) Higuchi-R^2-0.940
b) Korsmeyerpeppas: n=1.070

CONCLUSION

By observing the above results & discussion it can be concluded that the formulation F19 has the ability to prolong the release and reduce the side effects by targeting the drug to the desired site and reduce the dosing frequency of the drug and to obtain a better patient compliance.

A good controlled release dosage form should deliver therapeutic agents at a precisely controlled rate for the sake of safety and therapeutic efficacy. The BSA nanoparticles satisfy the characters of controlled release formulations because it releases the Doxorubicin Hydrochloride in controlled manner. In this present study the particle size optimized by different polymer concentrations, stabilizer concentrations and homogenization speed. Good particle sizes are obtained at 1.0% polymer solution (from fig-16), 0.1% stabilizer concentration (from fig-17) and 20,000rpm of homogenization speed (from fig-18).

SCOPE OF THE FURTHER WORK

The clinical application of the Doxorubicin Hydrochloride in nanoparticle formulations in intravenous dosage forms can be studied, by preparing the formulations and administering to different animal species. The author has limited time for the continuation of the studies. However the stipulated time is short, an attempt was made to conduct studies in rat species, but the Ethical Committee has been turned down the request citing different reasons. A further refinement in the formulations and drug release studies can be done for subsequent studies.

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