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Liposomal Encapsulation of Amikacin Sulphate for Optimizing Its Efficacy and Safety

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Authors' contributions

This work was carried out in collaboration between all authors. Author MSE designed the study, wrote the protocol, managed the analyses of the study and reviewed the manuscript. Author ABD carried out the experimental work, performed the statistical analysis and wrote the first draft of the manuscript. Authors AAE, GAA and AMM checked and revised the manuscript thoroughly and confirmed all the data given in manuscript and managed the literature. Author AE performed sterilization section. Authors AR and RFA performed the microbiological experiments. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The abstract of the current study was to formulate amikacin sulfate in a liposomal formulatiom for enhancing its efficacy and safety.
Place and Duration of Study: Pharmaceutical Technology Department, Pharmaceutical and Drug Industries Research Division, National Research Centre (NRC), Dokki, Cairo, Egypt, between 2010-2013.

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Methodology: Amikacin sulfate liposomes were prepared by the vortex dispersion method using dipalmitoyl phosphatidyl choline (DPPC), cholesterol (CHOL) and charge inducing agent (CIA). Dicetyl phosphate (DCP) and Stearyl amine (SA) were added as the negative and positive charge inducing agents respectively. Characterization of the prepared amikacin sulphate liposomes was performed. *In-vitro* release of selected formulations was estimated. A stability study for 45 days was performed. Investigation of the optimum dose for sterilization of amikacin sulfate liposomes was carried out. Selected amikacin sulfate liposomal formulations activities were evaluated against *Escherichia coli* infection in mice and compared to the free drug.
Results: The entrapment efficiencies ranged from 43.6±1.81 to 62.5±2.57%, the vesicles are well identified and present in a nearly perfect sphere like shape ranging in size from 54.3±11 to 362.1±56 nm and the polydospersity index values of all liposomal formulations were < 0.3. DSC of different liposomal formulations shows a change in transition temperature of the main phospholipids. *In-vitro* release profiles revealed biphasic release of the drug from liposomes. Physical stability performed at 2-8ºC for 45 days revealed low leakage of drug from all liposomal formulations investigated. Sterilization using gamma radiations revealed that a dose of 25 KGy was the optimum sterilization dose. The results also revealed less number of colonies forming units (cfu/ml) in the case of amikacin sulfate liposomes than the unentrapped drug.
Conclusion: It can be fulfilled from this work that amikacin sulfate liposomes represent promising carrier for delivery of amikacin offering good physical stability, high entrapment effeciencies and controlled drug release.

Keywords: Amikacin sulfate; Liposomes; Antimicrobial activity; E. coli.

1. INTRODUCTION

Amikacin sulfate is a semi-synthetic aminoglycoside antibiotic derived from kanamycin [1]. It is commonly administered parenterally for the treatment of Gram-negative infections which are resistant to gentamicin, kanamycin or tobramycin because the amikacin molecule has fewer points susceptible to enzymatic attack than most other aminoglycosides [2,3]. It is useful therapeutically and prophylactically in the treatment of serious, often life-threatening, bacterial infections. It is most often used for treating severe hospital-acquired infections with multidrug resistant gram negative bacteria such as, *Pseudomonas aeruginosa, Acinetobacter and Enterobacter* [4].

Clinical studies have shown that amikacin sulfate injection is effective in treatment of bacterial septicemia (including neonatal sepsis); serious infections of the respiratory tract, bones and joints, central nervous system infections (including meningitis), skin and soft tissue infections, intra-abdominal infections (including peritonitis); and burns and post operative infections (including post vascular surgery). Amikacin sulfate works bactericidally by binding to the bacterial 30S ribosomal subunit, causing misreading of mRNA and leaving the bacterium unable to synthesize proteins vital to its growth [4]. But as all amino glycosides, it has some side effects like hearing loss (Ototoxicity), kidney damage (Nephrotoxicity) and neuromuscular blockade (Neurotoxicity) [5,6].

Liposomes are spherical lipid bilayer which varies in size from 250 Å to >2 µm in diameter that serve as a convenient delivery vehicle for biologically active compounds [7]. They are formed by the interaction of amphiphilic lipids suspended in an aqueous phase, the bilayers are usually composed of natural or synthetic phospholipids and cholesterol, but the incorporation of other lipids or their derivatives, as well as proteins, is also possible [8].

Encapsulation of drug into liposomes alleviates many of these obstacles as liposome-encapsulated drugs often exhibit reduced toxicity, allowing for parenteral administration of much higher dose of drug which could not be tolerated with the free form. Liposome encapsulation has also been shown to enhance retention of drugs in the tissues resulting in sustained drug release. Liposomes can be used to improve penetration and diffusion of active ingredients, achieve selective transport of active ingredients, increase biocompatibility, reach greater stability, minimize dose of drug and reduce unwanted side effects. Thus, encapsulation of drugs in liposomes has often resulted in an improved overall therapeutic efficacy [9].

The objective of the present study is to develop amikacin sulfate liposomal preparation with enhanced activity and limited side effects.

2. MATERIALS AND METHODS

2.1 Materials

Amikacin sulfate was kindly provided by EIPICO Pharmaceutical Company, Egypt. Dipalmitoyl Phosphatidyl Choline (DPPC) and Stearylamine (SA) minimum 97% (GC) were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Cholesterol and Dicetyl phosphate (DCP) were purchased from Sigma Chemical Company; Germany.

2.1.1 Biological materials

Mature albino mice of 20-25 gm utilized in this study were purchased from the Animal House Colony at the National Research Centre (NRC), Cairo, Egypt. All animals were housed under standard conditions of natural 12 h light and dark cycle with free access to food and water. Animals were allowed to adapt to the laboratory environment for one week before experimentation. All animal procedures were performed after approval from the Ethics Committee of NRC - Egypt and in accordance with the recommendations of the proper care and use of laboratory animals. *E. coli* serotype (O-111) was procured from Microbiology department, Faculty of Veterinary Medicine, Cairo University. All other chemicals were of analytical grade.

2.2 Methods

2.2.1 Preparation of liposomes

Liposomes were prepared by the thin-film hydration method (vortex dispersion method) [10,11]. Accurately weighed 10 mg of amikacin sulfate was solubilized in 10 ml borax buffer till obtaining a clear solution. In a 100 ml pear-shaped flask of the (Buchi–M/HB-140, Switzerland) Rotary evaporator, 100 mg of DPPC and cholesterol with or without charge inducing agent (CIA) were dissolved in 10 ml chloroform and rotated at 56°C for 10 minutes. The chloroform was then evaporated under reduced pressure in order to form a thin film of phospholipids on the wall of the flask. The thin phospholipids film was hydrated with 10 ml of Borax buffer containing 10 mg of amikacin sulfate under rotation at 56°C. Different formulations are presented in Table 1.

2.2.2 Determination of amikacin sulfate entrapment in liposomes

To determine the amount of drug entrapped in liposomes, the unentrapped amikacin sulfate was separated from the liposomal entrapped amikacin sulfate by cooling centrifugation at 5200 x g at - 4ºC for 30 minutes using the refrigerated centrifuge (Union 32R Hanil Science industrial Co., LTD., Korea) [11-13]. The liposomal pellets were then washed with 10 ml borax buffer. The pellets were resuspended in 10 ml borax buffer and the free amount of amikacin sulfate was assayed spectrophotometrically using Chloranil reagent at λ_{max} 348.6 nm [14]. The amount of amikacin sulfate entrapped was then calculated by subtracting the free amount of amikacin sulfate from the amount added at the start of the preparation.

Table 1. Composition of the prepared liposomal formulations

Formula	Liposomes type	Molar ratio			
		Phospholipid		Charge inducing agent	
		DPPC[a]	CHOL[b]	DCP[c]	SA[d]
F_1	Neutral liposomes	7	2	-	-
F_2	Negative charged liposomes	7	2	1	-
F_3	Positive charged liposomes	7	2	-	1
F_4	Neutral liposomes	7	4	-	-
F_5	Negative charged liposomes	7	4	1	-
F_6	Positive charged liposomes	7	4	-	1
F_7	Neutral liposomes	7	7	-	-
F_8	Negative charged liposomes	7	7	1	-
F_9	Positive charged liposomes	7	7	-	1

a: Dipalmitoylphosphatidylcholine, b: Cholesterol, c: Dicetyl phosphate, d: Steraylamine

Entrapment efficiency (EE %) is expressed as (amount of drug entrapped/ total amount of drug added × 100) [15,16].

2.2.3 Characterization of liposomes

2.2.3.1 Transmission electron microscopy

The samples were then examined by the transmission electron microscopy (JEOL, JEM-1230, Tokyo, Japan), with an accelerating voltage of 80 KV. Photographs were taken at suitable magnifications (20Kx).

2.2.3.2 Differential Scanning Calorimetry

The thermal properties were analyzed using differential scanning calorimetry (DSC; Shimadzu, DSC-60 with TA-60 WS thermal analyzer, Tokyo, Japan) calibrated with indium. Thermograms were analyzed using Shimadzu TA-60 software (Shimadzu, Tokyo, Japan).

2.2.3.3 Determination of vesicle size and Zeta Potential

Liposomal vesicle size as well as estimation of their zeta potential were determined using dynamic light scattering based on laser diffraction in a multimodal mode using the Nicomp particle sizing system [17,18] (Zeta Potential / Particle Sizer, NICOMP TM 380 ZLS, Santa Barbara, CA) with He-Ne laser at 632.8 nm at a scattering angle of 90.0° which is capable of measuring vesicles in the 1nm to 5 µm size range. For vesicle size and zeta potential determination, the liposomal preparation was diluted with double distilled water (1:100 vol/ vol). After dilution, the sample was transferred to a quartz cuvette and measured at room temperature. Size distributions were displayed in term of number versus vesicle size. The polydispersity index (P.I.) was determined as a measure of homogeneity [18]. Small values of P.I. (< 0.3) indicate a homogenous population, whereas high P.I. values (> 0.3) indicate heterogeneity [19,20].

2.2.4 Effect of swelling time on the percentage of amikacin sulfate entrapped

The swelling of amikacin sulfate liposomes was conducted by incubating the liposomal suspension at 56°C, viz., above the transition temperature of the investigated material in the incubator for the required time. A 1 ml sample from each preparation was taken at different time intervals, viz., zero, 3, 6 and 24 hours after the start of the experiment. The samples were separated and washed twice and the percentage of amikacin sulfate entrapped was investigated at each of the selected swelling times.

The percentage of increase in entrapment was calculated by the equation:

$$\frac{\text{Mean drug entrapment at specific time} - \text{Mean drug entrapment at zero time}}{\text{Mean drug entrapment at specific time}} \text{ x } 100$$

2.2.5 *In-vitro* release profile of amikacin sulfate liposomes

The method adopted for this study was previously discussed by El-Ridy et al. [13] and Mokhtar et al. [21]. Each liposomal formulation was separated, washed and the amount of drug entrapped at zero time was considered as the total amount of the drug and was determined spectrophotometrically at 348.6 nm. The pellet of each preparation was then suspended using normal saline to exactly 10 ml. The (Hilab, GLF 3202, Germany) rotary shaker was adjusted to a rate of 150 strokes/min and the temperature was adjusted to 37±0.2°C. A 1 ml sample from each of the liposomal suspensions was taken at different time intervals, namely at 3, 6, 24, 48 and 72 hours after the start of the experiment. The samples were separated and washed, the amount of amikacin sulfate released was determined at each time interval by direct spectro-photometric assay at 348.6 nm.

The mean amount of amikacin sulfate released was then calculated at each time interval for each of the formulations investigated. The mechanisms of amikacin sulfate release from liposomal formulations was determined using the following mathematical models: zero-order kinetics (cumulative % drug released vs. time), first-order kinetics (log % drug retained vs. time), second-order kinetics (inverse % drug retained vs. time), Higuchi model (cumulative % drug retained vs. square root of time), Hixson-Crowell model (cube root of drug % remaining in matrix vs. time), Korsmeyer-Peppas model (log cumulative % drug release vs. log time) and Baker-Lonsdale model. The R^2 and K values were calculated for the linear curve obtained by regression analysis of the above plots.

2.2.6 Determination of the physical stability of liposomes

The selected liposomal batches were prepared, separated, washed, and resuspended in borax

buffer. Liposomal suspensions were sealed in 20 ml glass vials and stored at (2-8ºC) [22]. Samples from each batch were withdrawn at specified time intervals to determine the amount of drug retained in the vesicles.

2.2.7 Sterilization of amikacin sulfate Liposomes

The aim of this study to investigate the optimum sterility conditions required to provide a sterile parenteral amikacin sulfate liposomal formulation which could be used safely as an injectable suspension. The irradiation of the drug liposomal suspensions was conducted using a Cobalt-60 source at ambient temperature by Canadian Gamma Cell at the National Centre for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. Two γ-irradiation doses were attempted for irradiation of amikacin sulfate liposomes, viz., 15 and 25 kGy; eighteen vials (9b & 9c) were exposed to each irradiation dose while nine vials (9a) were left unirradiated as control.

2.2.8 *In-vivo* study

The antibacterial activity of amikacin sulfate liposomes was monitored and evaluated against infection with *E. coli*. Two liposomal formulations, F_2 and F_8 amikacin sulfate liposomes were used to compare their efficacy with free amikacin sulfate, in addition to F_2 and F_8- drug free liposomes. A group which did not receive drug or infection was considered as a control (-*ve*), while a group which receive infection was considered as positive control.

2.2.8.1 Experimental design

Seventy mature albino mice weighing 25-30g were allocated into 7 groups, each of ten animals. The first group was the (-*ve)* control group (normal group). The second group was the (+*ve*) control group (infected but not treated). The third group received free amikacin sulfate (40 mg/ kg) [23,24]. The fourth group received F_2 amikacin sulfate liposomes (40 mg/ kg). The fifth group received F_8 amikacin sulfate liposomes (40 mg/ kg). The sixth group received F_2-drug free liposomes (40 mg/ kg). The seventh group received F_8-drug free liposomes (40 mg /kg). All mice groups except the normal control group (untreated) were inoculated with (1.5×10^8/ mice) of *E. coli* serotype (O-111) in MacConkey broth through single intra-muscular (i.m.) injection.

Twenty four hours post-infection, mice were randomized to receive different formulations intra-muscularly daily for 6 successive days. After 3 days of treatment half of animals were sacrificed. Blood and infected organs (spleen, liver and kidney) were collected for analysis. After 6 days of treatment the rest of animals were sacrificed, blood and infected organs (spleen, liver and kidney) were collected for analysis. Blood samples were collected in sterile heparinized tubes for further culture on MacConkey agar, incubation at 37ºC for 18 hrs. The infected organs were dissected and homogenized in saline (1 g tissue/2 ml saline) for 30 s and were centrifuged at 3000 rpm for 15 min. Tissue homogenate suspensions and blood were cultured on MacConkey agar incubated at 37ºC for 18 hrs.

2.2.8.2 Blood and tissues cultures

The colony forming unit (CFU) counts were determined in blood and tissue samples (spleen, liver and kidneys), following homogenization and plating on MacConkey agar. The numbers of viable cells of *E. coli* serotype (O-111) in blood and tissue homogenates were expressed as $\log_{10}$. The free and liposomal amikacin sulfate in the collected samples after 3 and 6 days against E-coli serotype (O-111) in mice were estimated by microbiological assay technique [25,26].

2.2.9 Data analysis and statistics

Results were expressed as mean values ± standard error. Statistical analysis of results, was done using analytical software named SPSS statistics (version 17.0), released at (Aug. 23, 2008), Chicago, USA. Analysis of variance (ANOVA, single factor) followed by LSD, was employed in the statistical analysis of the determined parameters. Significance was defined at P values = 0.05.

3. RESULTS AND DISCUSSION

3.1 Entrapment Efficiency of Amikacin Sulfate Inside Liposomes

The entrapment efficiency % of amikacin sulfate inside liposomal formulations was tabulated in (Table 2). Amikacin sulfate was successfully entrapped in all the prepared formulations. The entrapment of amikacin sulfate was influenced by

the physicochemical characteristics of the liposome, like types of lipids, the composition and proportions of lipids in the liposomal formulation ,particle size, surface charge: positive, negative or neutral, sensitivity to pH changes temperature sensitivity, the fluidity of the liposomal membrane and bilayer rigidity [27,28].

The results tabulated in Table 2 and shown in Fig. 1 reveal a comparison between neutral, negatively and positively charged amikacin sulfate liposomes in three different molar ratios. The results revealed that the effect of cholesterol content increased the drug EE%, depending on the type of liposomes charge. Accordingly, neutral amikacin sulfate liposomes of the molar ratios DPPC/Cholesterol (7:2 and 7:4), F_1 and F_4 exhibited nearly similar EE% (48.25% and 48.59%) respectively, with statistical nonsignificant difference ($P > 0.05$). However the molar ratio DPPC/Cholesterol (7:7), F_7, exhibited the lowest EE% of (43.64%) with statistical significant difference ($P = .05$) from both F_1 and F_4. On the other hand, for negative liposomes the highest EE% was noticed with F_2 of the molar ratio DPPC/CHOL/DCP (7:2:1) with a mean of (54.5%) which was only significantly higher at ($P = .05$) than F_5 of the molar ratio DPPC/CHOL/DCP (7:4:1) but not from F_8 of the molar ratio DPPC/CHOL/DCP (7:7:1) with respective EE% values of 51.1 and 52.9%.

Finally, for positively charged liposomes the highest EE% was seen with F_9 of the molar ratio DPPC/CHOL/SA (7:7:1) with a mean of (62.43%). This was followed by F_3 with the molar ratio (7:2:1) and a mean of (56.69%). The least entrapment was exhibited by F_6 of the molar ratio (7:4:1) with a mean of (53.16%) with statistical significant difference ($P < 0.01$) when compared to the molar ratios (7:2:1 and 7:7:1). Increasing the cholesterol content, e.g., in case of the molar ratio DPPC/CHOL/SA (7:7:1) led to a significant increase in the percentage of amikacin sulfate entrapped in positive liposomes.

In general, it was obvious that at the same cholesterol content, positively charged liposomes exhibited the highest drug EE%. Furthermore, the effect of variable changes was the most prominent when phospholipid ratio to cholesterol was 7:7 and the least at the ratio 7:4. The inclusion of dicetyl phosphate (-ve CIA) or stearyl amine (+ve CIA) tend to increase the interlamellar repeat distances between successive bilayers in the MLV, swelling the structure with the greatest proportion of the aqueous phase. These effects lead to a greater overall entrapped volume especially for hydrophilic drugs like amikacin sulfate [29].

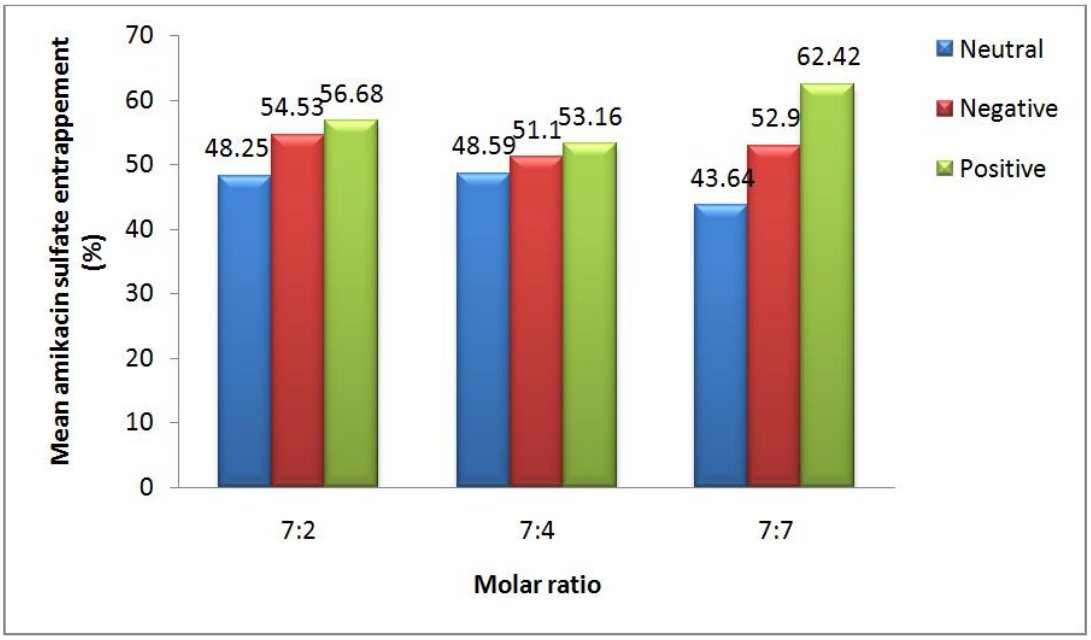


Fig. 1. Effect of different phospholipids surface charges and molar ratios on the percentage of amikacin sulfate entrapped

Table 2. Encapsulation efficiencies of entrapped amikacin sulfate in different liposomes

Formula code	Surface charge	Molar ratio DPPC:Chol:CIA	Mean Drug* entrapped (%)	Standard deviation
F_1	Neutral	7:2	48.3	1.32
F_2	Negative**	7:2:1	54.5	0.78
F_3	Positive***	7:2:1	56.7	0.55
F_4	Neutral	7:4:1	48.6	1.35
F_5	Negative**	7:4:1	51.1	1.39
F_6	Positive***	7:4:1	53.2	1.33
F_7	Neutral	7:7	43.6	1.81
F_8	Negative**	7:7:1	52.9	2.54
F_9	Positive***	7:7:1	62.5	2.57

*Mean of six batches, **Negatively charged by dicetyl phosphate, ***Positively charged by stearylamine.

3.2 Characterization of Liposomes

3.2.1 Transmission electron microscopy

Fig. 2 shows the different vesicle shapes of some of the selected liposomal formulations. All fields were negatively stained with 1% pohosphotungestic acid at room temperature. It demonstrated that vesicles were well identified and present in a nearly perfect sphere like shape, having a large internal aqueous space and a smooth vesicle surface. Fig. 5 reveals also, well-stained liposomal vesicles, where the outer lipophilic domain was black stained and the inner hydrophilic domain was light stained. The figure also shows aggregation and accumulation of liposomal vesicles due to positive charge added, this was in accordance with previously reported publications [30,31], confirming visually seen aggregations.

3.2.2 Differential scanning calorimetry

Tables 3 and 4 and Figs. 3, 4, and 5 reveal the themograms as well as the phase transition temperatures (T_c) of individual components of liposomes like DPPC, cholesterol, DCP, amikacin sulfate and saccharose. In addition, thermodynamic parameters of twelve liposomal formulations which were F_1, F_2, F_4, F_5, F_7 and F_8 lyophilized with and without a lyoprotectant were investigated. The thermodynamic behavior of drug-free liposomes of the same molar ratios was also investigated. All the thermograms were done for one scanning cycle as it was previously reported that repeated cycling through the main transition temperature could convert lipid-containing liposomes to disc-like micelles [32].

Table 3. Thermodynamic parameters of different lyophilized amikacin sulfate liposomal suspensions with and without a lyoprotectant

Formulations	Formulae code	Surface charge	Molar ratio DPPC /Chol /CIA	Transition Temperature* (T_c°) (°C)	Enthalpy of Transition [ΔH]* (Kcal/mol)	Transition Temperature** (T_c°) (°C)		Enthalpy of Transition [ΔH]** (Kcal/mol)	
	F_1	Neutral	7:2	65.96	26.02	38.29	104.29	10.2	40.7
	F_2	Negative	7:2:1	68.27	38.50	-	105.15	-	95.7
	F_4	Neutral	7:4	77.12	4.742	53.58	129.59	27.3	4.81
	F_5	Negative	7:4:1	72.54	8.442	-	102.15	-	118.15
	F_7	Neutral	7:7	67.09	5.079	47.01	89.73	3.3	3.32
	F_8	Negative	7:7:1	74.44	5.711	29.88		- 0.64	

* Transition temperature and Enthalpy of Transition of lyophilized liposomes without a lyoprotectant.
** Transition temperature and Enthalpy of Transition of lyophilized liposomes with a lyoprotectant

Table 4. Thermodynamic parameters of individual components of liposomes

	Component	Transition temperature (Tc^o) (^oC)	Enthalpy of transition [ΔH] (Kcal/mol)
Individual components	Dipalmitoyl phosphatidyl choline	62.86, 67.11	-5.439, -2.275
	Amikacin sulfate	145.83	2.180
	Cholesterol	145.4	145.4
	Dicetyl phosphate	74.75	30.786
	Saccharose	93.86, 192.80	88.68, 17.217

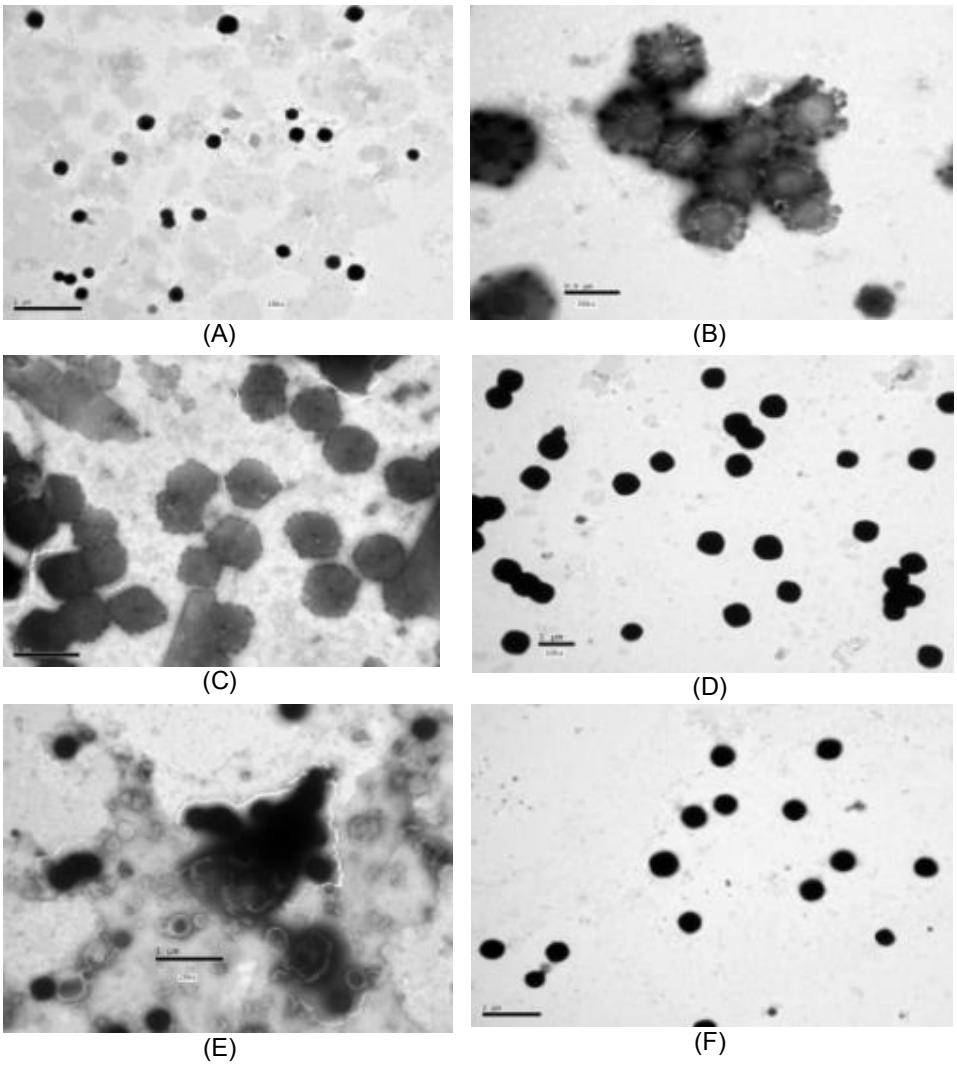


Fig. 2. TEM micrographs of selected liposomal formulations

(A) F_1-drug free liposomes (7:2). (B) F_1–drug liposomes (7:2. (C) F_2-drug free liposomes (7:2:1). (D) F_2-drug liposomes (7:2:1). (E) F_3-drug liposomes (7:2:1). (F) F_8-drug liposomes (7:7:1)

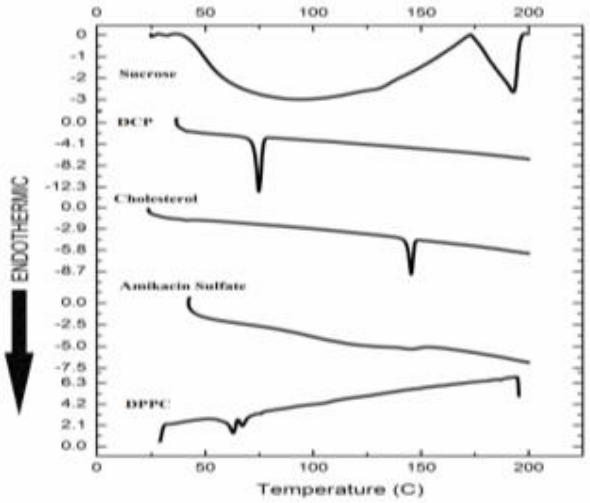
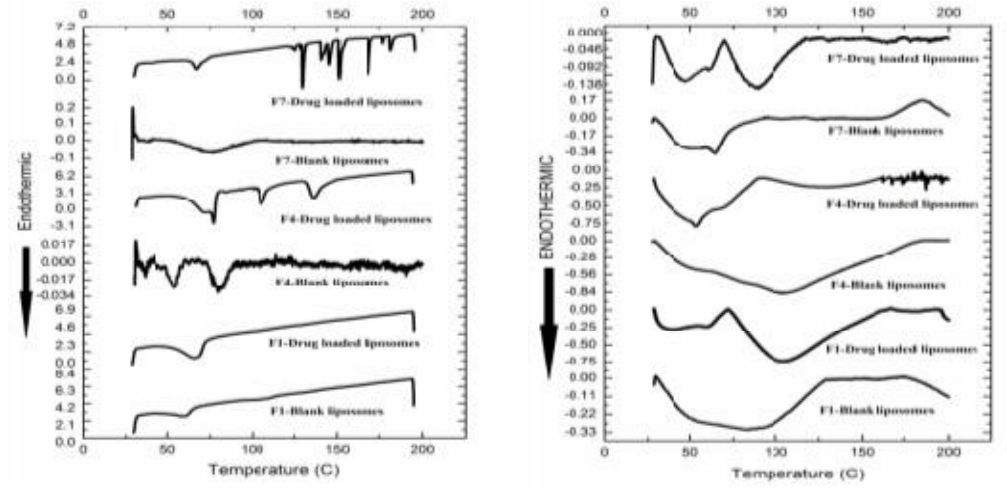


Fig. 3. DSC of DPPC, amikacin sulfate, cholesterol, DCP and sucrose



(a) (b)

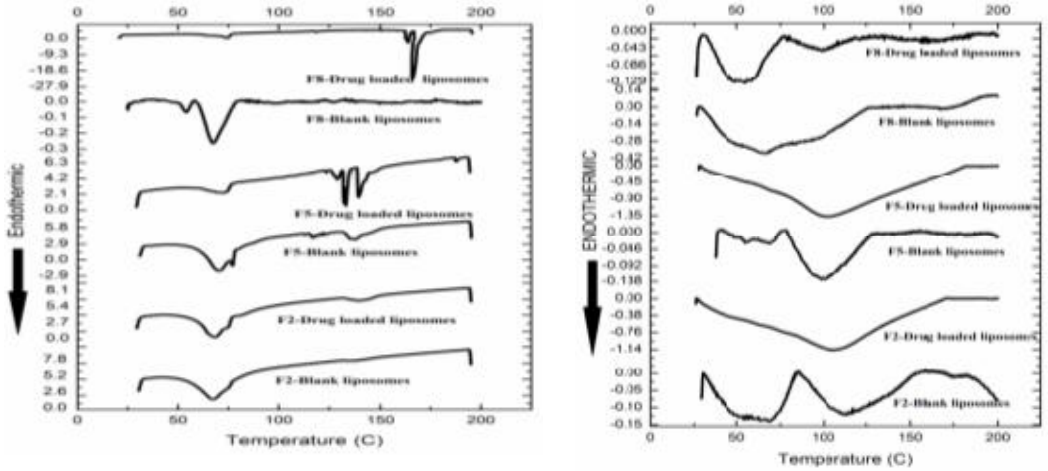
Fig. 4. DSC thermograms of : (a) Neutral liposomes without lyoprotectant. (B) Neutral liposomes with a lyoprotectant

3.2.3 Determination of vesicle size

The vesicle sizes of F_1, F_2, F_4, F_5, F_7 and F_8 amikacin sulfate liposomes are presented in Table 5. It was found that neutral amikacin sulfate liposomes of the molar ratio DPPC/ Cholesterol (7:2), F_1, was the largest vesicle size, followed by neutral amikacin sulfate liposomes of the molar ratio DPPC/ Cholesterol (7:4), F_4, and the smallest vesicle size was exhibited by neutral amikacin sulfate liposomes of the molar ratio DPPC/ Cholesterol (7:7), F_7, So it could be expected that by increasing the cholesterol content in neutral liposomes, the vesicle size decreased.

3.2.3.1 Polydisperisity Index (P.I.)

To investigate the width and homogeneity of the vesicle size distribution, P.I. was calculated. Table 5 reveals that the P.I. values of all liposomal formulations were < 0.3, thus indicate a homogenous vesicular population [18]. A polydispersity index of 1 indicated large variations in particle size; a reported value of 0 means that size variation is absent [33]. The obtained low values of P.I. of the prepared liposomes indicate a limited variation in vesicle size.



(a) (b)

Fig. 5. DSC thermograms of : (a) Negative liposomes lyophilized without a lyoprotectant. (b) Negative liposomes, lyophilized with a lyoprotectant

Table 5. Vesicle size and polydisperisity index (P.I.) of the prepared liposomes

Formulae code	Surface charge	Molar ratio DPPC/Chol/CIA	Vesicle Size (nm ± SD)	Variance (P.I.)*
F_1	Neutral	7:2	313.1±53.7	0.172
F_2	Negative	7:2:1	362.1±56	0.155
F_4	Neutral	7:4	165.8±29.5	0.178
F_5	Negative	7:4:1	54.3±11	0.203
F_7	Neutral	7:7	126.3±26.4	0.209
F_8	Negative	7:7:1	79.5±18.1	0.227

**P.I.: Polydisperisity Index obtained as P.I. = (SD/ mean vesicle size).*

3.2.4 Determination of zeta potential

The results shown in Table 6 revealed that the surface zeta potential charge of all liposome vesicles displayed negative values. It was also observed that the charge containing substances used in the formulation affected the surface charge of liposomes compared to the uncharged formulation. The highest negative values obtained were exhibited by the formulations containing DCP (−6.86 mV and −14.49 mV for negative amikacin sulfate liposomes of the molar ratios DPPC/Cholesterol/DCP 7:2:1 and 7:7:1 respectively), whereas the lowest negative values were exhibited by uncharged liposomal formulations (−3.87mV and −4.08 mV for neutral amikacin sulfate liposomes of the molar ratios DPPC/ Cholesterol 7:2 and 7:7 respectively.

3.3 Effect of Swelling Time on the Percentage of Amikacin Sulfate Entrapped

The swelling time study was conducted to prove that when we made changes in temperature with time, an increase in the percentage of drug entrapped might occur. The chosen formulations for this study were F_1, F_2 and F_5 with respective molar ratios of DPPC/ Cholesterol (7:2), DPPC/ Cholesterol /DCP (7:2:1 and 7:4:1). In general, the results tabulated in Table 7 and shown in Fig. 6, revealed that swelling lead to an increase in the percentage of amikacin sulfate entrapped for both neutral and negatively charged liposomes. The increase in the percentage of amikacin sulfate entrapped increased by increasing the swelling time from 3 hours to 6 hours and from 6 hours to 24 hours. The results were in accordance with that reported by Mayer et al. [34].

3.4 *In-vitro* Release Profile of Amikacin Sulfate Liposomes

Considering the release pattern for amikacin sulfate from liposomes (Table 8 and Fig. 7), it was observed that amikacin sulfate release occurred in two phases, an initial burst release that extended for 3 hours, followed by a

sustained and reduced release maintained at least for 72 hours. This biphasic release pattern of the investigated water soluble drug, amikacin sulfate, seems to be the characteristic of bilayered vesicles [35,36]. The first fast release phase can be explained, as previously reported , for *in-vitro* dissolution medium, drug particles initially pass into solution from the surface (immediate release) [10]. This fast release is followed by a slow sustained release of the drug as the solid matrix also begins to swell as soon as hydration with solvent molecules occurs. This causes diffusion of the dissolved drug and erosion of vesicle layer into aggregates or granules and this, in turn, deaggregate into fine particles that also release their drug content by dissolution [37]. Higuchi model describes the release of the drug from the matrix system through diffusion followed by pore formation as described by Yesmine et al. [38]. The same was observed for the release of amikacin sulfate from liposomes in our experiment (Table 9).

Table 6. Average zeta potential of the liposomal formulations

Formulae code	Surface charge	Molar ratio DPPC/Chol/CIA	Avg. Zeta potential (mV)
F_1	Neutral	7:2	-3.87
F_2	Negative	7:2:1	-6.86
F_7	Neutral	7:7	-4.08
F_8	Negative	7:7:1	-14.49

Table 7. The effect of swelling time on the percentage of amikacin sulfate entrapped

Swelling time (Hour)	Mean amikacin sulfate entrapped (%) ±S.D					
	Neutral (7:2)	Entrapment increase (%)	Negative (7:2:1)	Entrapment increase (%)	Negative (7:4:1)	Entrapment increase (%)
Zero	47.81±1.03	-	54.27±0.29	-	49.08±0.69	-
3	49.56±0.12	3.53	55.04±0.19	1.39	51.71±1.03	5.08
6	51.02±0.57	6.29	57.10±0.37	4.95	52.86±1.03	7.15
24	54.69±0.12	12.58	60.39±0.96	10.13	54.58±0.46	10.07

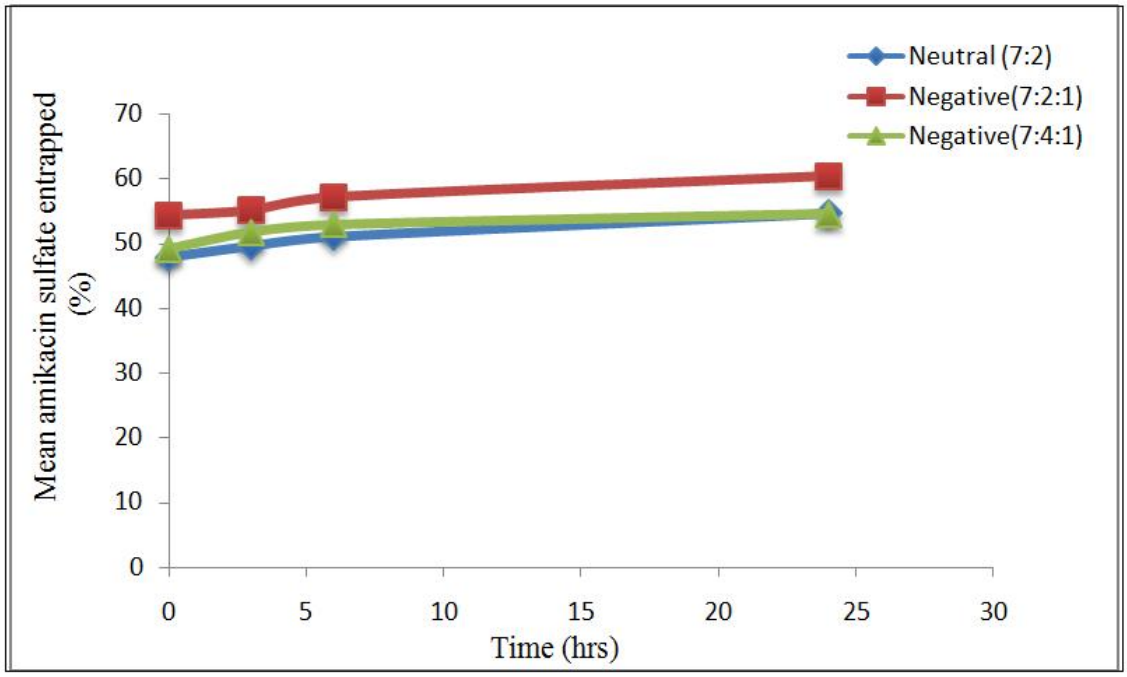


Fig. 6. The effect of swelling time on the percentage of amikacin sulfate entrapped in liposomes

Table 8. *In-vitro* release profiles of amikacin sulfate liposomes of different charges and molar ratios

Time (Hour)	Mean amikacin sulfate released (%)±S.D				
	Neutral 7:2	Neutral 7:4	Negative 7:2:1	Negative 7:4:1	Negative 7:7:1
Zero	0±(0)	0±(0)	0±(0)	0±(0)	0±(0)
3	7.12±0.53	10.16±0.33	5.61±0.32	12.88±0.33	13.17±0.76
6	8.60±0.45	12.85±0.33	6.37±0.41	17.07±0.51	15.85±0.96
24	11.37±0.36	16.33±0.39	10.19±0.48	18.07±0.43	19.02±0.69
48	14.09±0.48	19.22±0.39	13.72±0.42	21.77±0.44	21.83±0.54
72	17.20±1.32	21.62±0.39	15.89±0.58	25.14±0.60	25.72±0.48

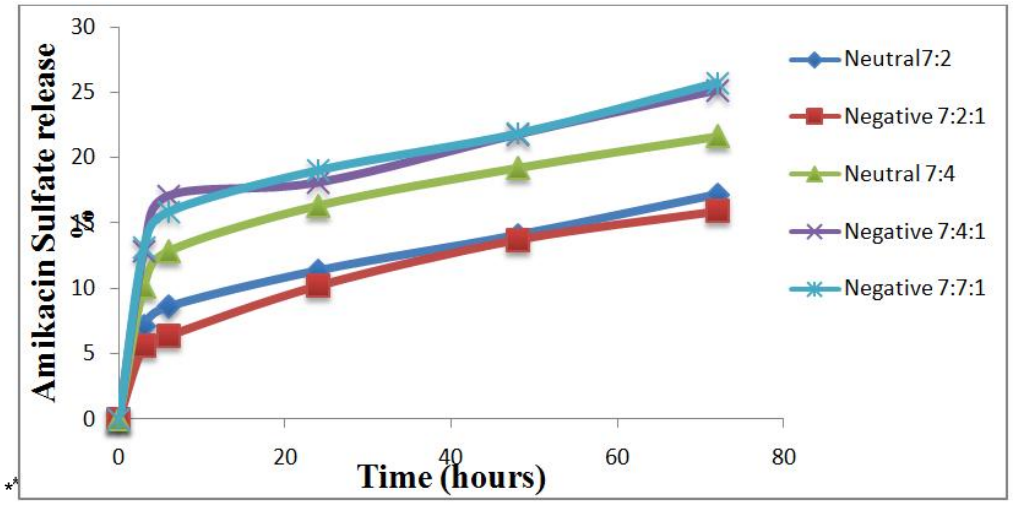


Fig. 7. *In-vitro* release profiles of amikacin sulfate liposomes of different charges and molar ratios

3.4.1 Kinetics studies of amikacin sulfate release data

Amikacin sulfate liposomes release data were kinetically treated and the results are presented in Table 9. Swelling and erosion of vesicles may have occurred during dissolution so; the kinetics of drug release was analyzed by applying Korsmeyer-Peppas equation which often used for identifying the release mechanism. Data shown in (Table 10) revealed that the drug release from liposomes follow Fickian diffusion release. The table reveals that amikacin sulfate release pattern from different liposomal formulations was most fitted to diffusion-controlled mechanism (Higuchi release kinetics model). Comparing the release efficiencies of amikacin sulphate from the liposomal formulations investigated (F_1, F_2, F_4, F_5 and F_7), it could be concluded that the release efficiency of the drug from F_7 liposomal formulation with a release rate constant of (1.697 mg $h^{-1/2}$), was higher than that calculated for the other liposomes investigated. These results pointed to sustained release characteristics with a Higuchi pattern of drug release, where liposomes act as a reservoir system for continuous delivery of drug. The ability to control drug release rates, combined with the ability to protect associated drugs from degradation, allows properly formulated liposomes to function as sustained release systems, continually releasing their store of drugs over several hours to several days [39,40].

3.5 Determination of the Physical Stability of Liposomes

Negatively charged amikacin sulfate liposomes of the molar ratios DPPC/ cholesterol/ DCP (7:2:1 and 7:7:1) were considered in this study as they represented formulations of the highest entrapment efficiency. Furthermore, the corresponding neutral liposomes were included in this study for comparison to investigate effect of DCP on liposomes stability. Physical stability studies on the selected liposomal formulations stored at refrigerator temperature (2-8ºC) for a period of 45 days were conducted by monitoring the leakage of encapsulated amikacin sulfate from liposomes.

3.5.1 Effect of storage on drug leakage from liposomes

Drug leakage from the liposomes was evaluated after 7, 15, 30 and 45 days and the results of the stability study are demonstrated in Table 11 and graphically illustrated in Fig. 8 in terms of percentage drug retained in the liposomes versus time. In general, the four liposomal formulations under study did not show a decrease in EE% after 45 days storage indicating their stability. Incorporation of cholesterol generally enhanced bilayer stability against leakage. Furthermore, it appeared that inclusion of DCP in the liposomes was beneficial as it gave the particles negative charge, which will help in decrease particles aggregations without affecting the bilayers elasticity. Hence, these two formulations with the highest EE% and stability will be subjected to microbiological studies.

Table 9. The calculated correlation coefficients of amikacin sulfate release profile from different liposomal formulations

Formulae	Zero order		First order		Second order		Higuchi model		Hixson-Crowell		Baker-Lonsdale model	
	R^2	K	R^2	K	R^2	K	R^2	K	R^2	K	R^2	K
F_1	0.983	0.138	0.941	0.005	0.872	-0.00	0.990	1.416	0.792	0.003	0.962	0.832
F_2	0.973	0.150	0.923	0.006	0.856	-0.00	0.998	1.558	0.857	0.003	0.949	0.816
F_4	0.934	0.153	0.874	0.004	0.798	-0.00	0.984	1.602	0.715	0.003	0.876	0.865
F_5	0.919	0.153	0.862	0.003	0.785	-0.00	0.927	1.568	0.658	0.004	0.837	0.886
F_7	0.962	0.165	0.921	0.003	0.862	-0.00	0.980	1.697	0.679	0.004	0.898	0.885

Table 10. Fitting of release data of amikacin sulphate from different liposomal formulations according to Korsmeyer-Peppas model

Formula	Korsmeyer-Peppas parameters			
	R^2	K	n	Mechanism of elease
F_1	0.981	5.28	0.261	Fickian diffusion
F_2	0.989	3.67	0.335	Fickian diffusion
F_4	0.988	8.18	0.223	Fickian diffusion
F_5	0.907	11.11	0.178	Fickian diffusion
F_7	0.973	10.81	0.190	Fickian diffusion

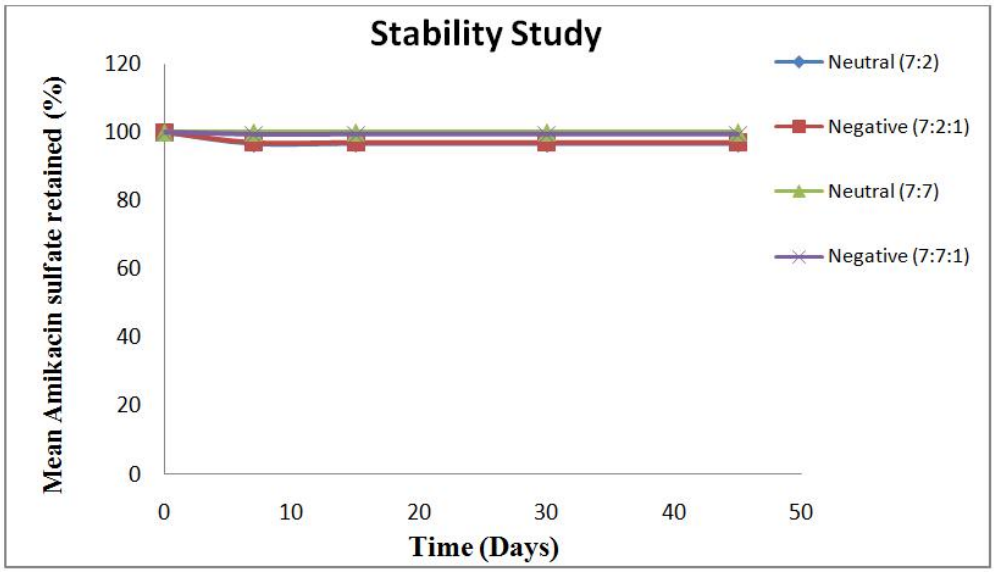


Fig. 8. Percentage of amikacin sulfate retained in liposomes after storage for 45 days at 2-8º

Table 11. Physical stability of amikacin sulfate liposomes stored for 45 days at 2-8ºC

Time	Mean amikacin sufate retained %			
	Neutral (7:2)	Neutral (7:7)	Negative (7:2:1)	Negative (7:7:1)
0	100	100	100	100
7	96.71	100	96.99	99.41
15	96.71	100	96.99	99.41
30	96.71	100	96.99	99.41
45	96.71	100	96.99	99.41

3.6 Sterilization of Amikacin Sulfate Liposomes

The aim of this study to investigate the optimum sterility conditions required to provide a sterile parenteral amikacin sulfate liposomal formulation which could be used safely as an injectable suspension. Twelve vials of amikacin sulfate loaded liposomes were exposed to two doses of Gamma irradiation, viz , 15 and 25 KGy , such that six vials were irradiated for each irradiation dose (three vials for DPPC/ CHOL/ DCP (7:2:1), and three vials for DPPC/ CHOL/ DCP (7:7:1)), and six vials were left unirradiated, as control, representing a radiation dose of zero KGy.

Table 12 presents the results of the sterility testing for irradiated amikacin sulfate liposomes, F_2 and F_8, of the molar ratios DPPC/ CHOL/ DCP (7:2:1 and 7:7:1). The data presented, revealed that there was no growth in negative control group indicating that the tubes containing the medium were well sterilized, while growth took place in the two positive control groups representing faculatative anaerobic bacteria (*Escherichia coli*) and anaerobic bacteria (*Clostridium sporogenes*) thus, proving the suitability and ability of the medium to support the growth of both aerobic and anaerobic types of bacteria. This is important as if any microorganism was contaminating the irradiated vials; the medium used will enable their growth and subsequent detection whether aerobic or anaerobic.

In tubes inoculated with the biological indicator, *Bacillus pumilus* E601, growth was detected in the tubes having radiation doses of zero and 15 kGy while no growth was observed at the radiation dose of 25 kGy, which was the radiation dose known to stop the viability of this microorganism. Considering tubes inoculated with amikacin sulfate liposomes suspension, growth was observed at the doses zero and 15 kGy while no growth was observed at the radiation dose of 25 kGy in the three amikacin sulfate liposomes formulations investigated indicating that the irradiation dose of 25 kGy was sufficient for accomplishing radiation sterilization of the product.

These results indicated that the irradiation dose of 25 kGy was the optimum sterilization dose for the investigated amikacin sulfate liposomal formulations. It should be mentioned that this study was performed for elucidating the optimum gamma radiation dose that could be used for sterilization of amikacin sulfate liposomes expected to be used for future studies or future injectable liposomal formulations.

Table 12. Sterility testing results for F_2 and F_8 amikacin sulfate liposomes

Radiation dose (KGy)	Amikacin sulfate liposomes on thioglycolate		Biological indicator *Bacillus pumilus* E601 on thioglycollate	Control tests		
				Positive control		Negative control
	F_2 – Amikacin sulfate liposomes	F_8– Amikacin sulfate liposomes		*E. coli*	*Cl. sporogenes*	
0	+	+	+	+	+	-
15	+	+	+			
25	-	-	-			

3.7 Antibacterial Efficacy in Blood Samples

Results tabulated in (Tables 13 and 14) and Figs. (9 and 10) revealed that the antibacterial efficacy of amikacin sulfate liposomes of the molar ratios DPPC/CHOL/DCP (7:2:1 and 7:7:1), F_2 and F_8 in elimination of *E. coli* infection from blood was significantly different from free amikacin sulfate, drug-free liposomes of the same molar ratios and the control group with statistical significant difference ($P < 0.05$). Also results showed that amikacin sulfate liposomes of the molar ratio DPPC/CHOL/DCP (7:7:1), F_8 was more effective than amikacin sulfate liposomes of the molar ratio DPPC/CHOL/DCP (7:2:1), F_2 with statistical significant difference ($P = 0.05$).

3.8 Antibacterial Efficacy in Organs Samples

3.8.1 Antibacterial efficacy in spleen samples

Results tabulated in (13 and 14) and Figs. (9 and 10) proved that the antibacterial efficacy of amikacin sulfate liposomes of the molar ratios DPPC/CHOL/DCP (7:2:1 and 7:7:1), F_2 and F_8, in elimination of *E.coli* infection from spleen tissues was significantly different from free amikacin sulfate, drug-free liposomes of the same molar ratios and the control group with statistical significant difference ($P < 0.05$). Also the results proved that amikacin sulfate liposomes of the molar ratio DPPC/CHOL/DCP (7:7:1), F_8 is more effective than amikacin sulfate liposomes of the molar ratio DPPC/CHOL/DCP (7:2:1), F_2 with statistical significant difference ($P = 0.05$).

3.8.2 Antibacterial efficacy in liver samples

Results presented in (Tables 13 and 14) and Figs. (9 and 10) revealed that the antibacterial efficacy of amikacin sulfate liposomes of the molar ratios DPPC/CHOL/DCP (7:2:1 and 7:7:1), F_2 and F_8 in elimination of *E. coli* infection from liver tissues was significantly different from free amikacin sulfate, drug-free liposomes of the same molar ratios and the control group with statistical significant difference (P =0.05). Liposome encapsulation increased the delivery of amikacin sulfate to the liver and spleen, and in each of our experiments, liposomal amikacin was more active than free amikacin in reducing the number of hepatic and splenic organisms, which indicated that liposomal formulation was expected to target the drug to a particular organ for improving its efficacy [23].

In our study, we found very high levels of amikacin activity in the spleen and liver as well as sustained levels in the serum, suggesting that the reticuloendothelial system can be saturated to some extent with the lipid doses investigated in this thesis. We also found that the treatment with empty liposomes resulted in decreased numbers of organisms in the spleens and livers of mice. Although, in neither study was the infection completely eliminated, it was apparent that in both cases, liposome encapsulation provided a therapeutic benefit more than the free drug. It is likely that further work to optimize liposome formulation as well as treatment regimens (i.e., dosage, dosage interval, length of therapy, and combination with other agents) will

Table 13. Log_{10} CFU/ml or gm, blood, spleen, liver and kidneys (Mean ± S.E.) For free and liposomal amikacin sulfate after 3 days against *E. coli* serotype (O-111) in mice (n=5)

Groups	Log $_{10}$ CFU/g tissue samples (Mean ± S.E.)*			
	After 3 days			
	Blood	Spleen	Liver	Kidneys
Control (–ve)	------	----	----	----
Control (+ve)	$7.88±0.006^{b,c,d}$	$4.15±0.016^{b,c,d}$	$3.74±0.060^{b,c,d}$	$3.98±0.023^{b,c,d}$
F_2 drug-free liposomes	$6.15±0.016^{a,b,c,}$	$3.83±0.065^{a,b,c,d}$	$3.38±0.109^{a,b,c,d}$	$3.73±0.182^{b,c,d}$
F_8 drug-free liposomes	$5.10±0.009^{a,b,c,d}$	$4.03±0.026^{b,c,d}$	$3.58±0.032^{b,c,d}$	$3.94±0.010^{b,c,d}$
Free amikacin	$2.92±0.004^{a,c,d}$	$3.36±0.070^{a,c,d}$	$3.06±0.220^{a,c,d}$	$2.20±0.081^{a,c}$
F_2-drug liposomes	$2.50±0.015^{a,b,d}$	$3.18±0.095^{a,b,d}$	$2.79±0.112^{a,b}$	$2.89±0.057^{a,b,d}$
F_8-drug liposomes	$2.36±0.008^{a,b,c}$	$2.92±0.120^{a,b,c}$	$2.67±0.150^{a,b}$	$2.37±0.060^{a,c}$

** Values represented the mean ± S.E. of five mice for each group.*

[a]: Values exhibit statistical significant difference from control positive at P = 0.05.

[b]: Values exhibit statistical significant difference from free amikacin sulfate at P = 0.05 .

[c]: Values exhibit statistical significant difference from amikacin sulfate liposomes (7:2:1) at P = 0.05.

[d]: Values exhibit statistical significant difference from amikacin sulfate liposomes (7:7:1) at P = 0.05.

be necessary to provide further therapeutic advantages according to USB directions and FDA regulations.

3.8.3 Antibacterial efficacy in kidney samples

Results presented in (Tables 13 and 14) and Figs. (9 and 10) proved that the antibacterial efficacy of amikacin sulfate liposomes of the molar ratios DPPC/CHOL/DCP (7:2:1 and 7:7:1), F_2 and F_8 in elimination of *E. coli* infection from kidneys tissues, was significantly different ($P < 0.05$) from drug-free liposomes of the same molar ratios and the control group with statistical significant difference. Also, results showed that amikacin sulfate liposomes of the molar ratio

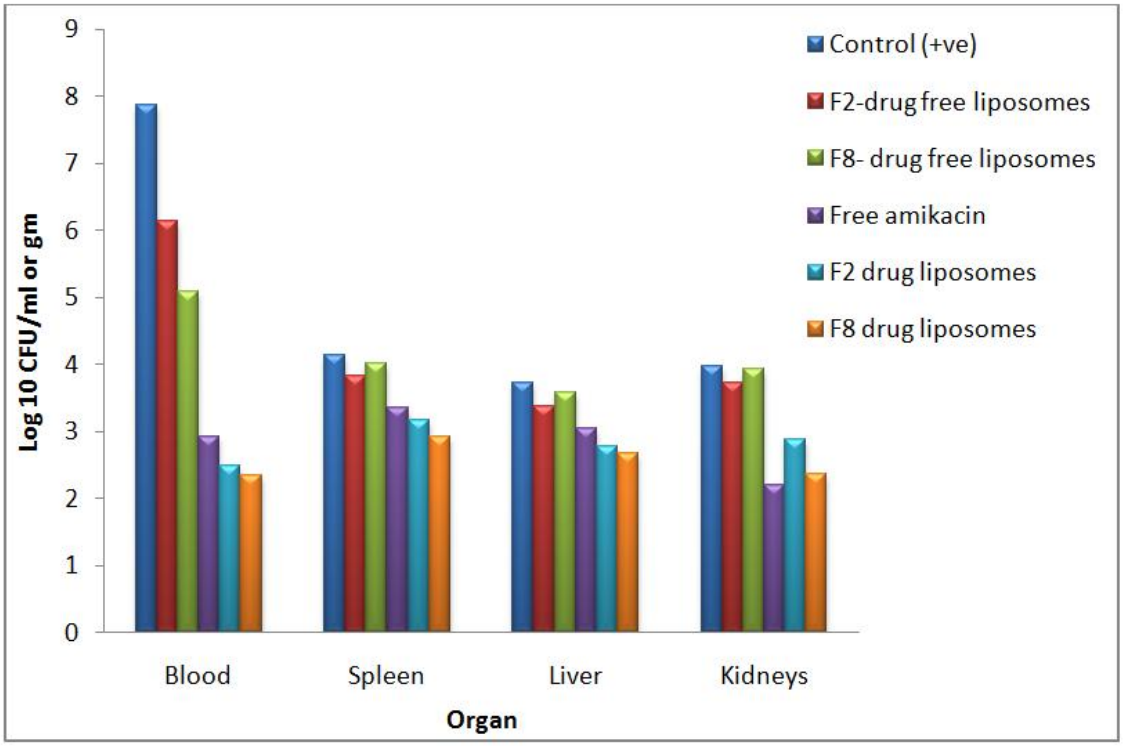


Fig. 9. Comparative efficacy of different treatments against *E. coli* infection in mice after 3 days of infection. Data presented as mean log_{10} CFU/ml or gm.

Table 14. Log_{10} CFU/ml or gm, blood, spleen, liver and kidney (Mean ± S.E.) for free and liposomal amikacin sulfate after 6 days against *E. coli* serotype (O-111) in mice (n=5)

Groups	Log $_{10}$ CFU/ml samples (Mean ± S.E.)*			
	After 6 days			
	Blood	Spleen	Liver	Kidneys
Control (–ve)	------	----	----	----
Control (+ve)	$7.92\pm0.005^{b,c,d}$	$4.20\pm0.009^{b,c,d}$	$3.76\pm0.050^{b,d}$	$4.08\pm0.026^{b,c,d}$
F_2 drug-free liposomes	$6.09\pm0.016^{a,b,c,d}$	$4.02\pm0.021^{b,c,d}$	$3.69\pm0.028^{b,c,d}$	$3.50\pm0.007^{a,b,c,d}$
F_8 drug-free liposomes	$4.05\pm0.013^{a,b,c,d}$	$4.12\pm0.008^{b,c,d}$	$3.66\pm0.030^{b,c,d}$	$3.88\pm0.024^{a,b,c,d}$
Free amikacin	$2.85\pm0.016^{a,c,d}$	$3.69\pm0.073^{a,c,d}$	$3.15\pm0.271^{a,c,d}$	$2.29\pm0.092^{a,c,d}$
F_2-drug liposomes	$2.43\pm0.014^{a,b,d}$	$3.14\pm0.146^{a,b}$	$2.69\pm0.063^{a,b}$	$2.86\pm0.042^{a,b,d}$
F_8-drug liposomes	$2.30\pm0.014^{a,b,c}$	$2.79\pm0.420^{a,b}$	$2.66\pm0.091^{a,b}$	$2.52\pm0.067^{a,b,c}$

** Values represented the mean ± S.E. of five mice for each group.*
[a]: Values exhibit statistical significant difference from control positive at P < 0.05.
[b]: Values exhibit statistical significant difference from free amikacin sulfate at P < 0.05 .
[c]: Values exhibit statistical significant difference from amikacin sulfate liposomes (7:2:1) at P < 0.05.
[d]: Values exhibit statistical significant difference from amikacin sulfate liposomes (7:7:1) at P < 0.05.

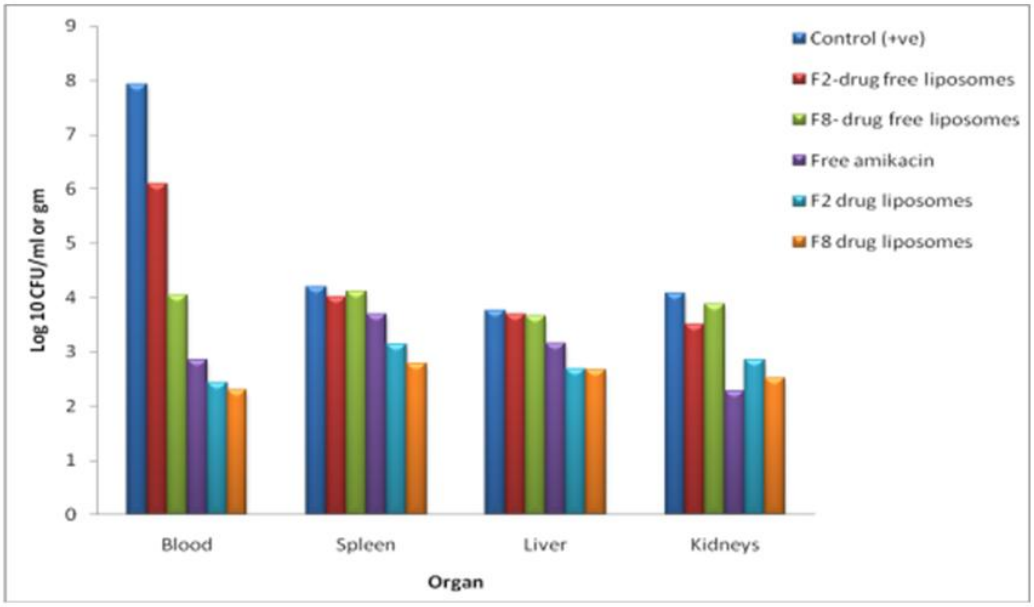


Fig. 10. Comparative efficacy of different treatments against *E. coli* infection in mice after 6 days of infection. Data presented as mean log_{10} CFU/ml or gm.

DPPC/CHOL/DCP (7:7:1), F_8 is more effective than amikacin sulfate liposomes of the molar ratio DPPC/CHOL/DCP (7:2:1), F_2 with statistical significant difference (*P* =0.05). The results showed that the kidney was the site of amikacin sulfate deposition in mice as free amikacin sulfate is more effective in treatment than amikacin sulfate liposomes of the molar ratio DPPC/CHOL/DCP (7:2:1 and 7:7:1), F_2 and F_8, r espectively. The mode of deposition of aminoglycoside antibiotics is of particular interest because of their nephrotoxic potential [41] and a drug delivery system that helps to increase the therapeutic index of amikacin sulfate by increasing the concentration of the drug at the site of infection and/or reducing the nephrotoxicity would attract considerable interest. The liposomal encapsulation of amikacin sulfate provided the previous postulation [42,43].

4. CONCLUSION

Amikacin sulfate was succsefully encapsulated in all liposomal formulations investigated. Characterization revealed that vesicles were well identified and present in a nearly perfect sphere like shape in the nano range with a negative surface charge swelling led to an increase in the percentage of amikacin sulfate entrapped. *In-vitro* release profiles revealed that amikacin sulfate release occurred in a biphasic pattern. Physical stability performed at 2-8ºC for 45 days, revealed low leakage of drug from all liposomal formulations investigated. Sterilization revealed that a dose of 25 KGy was the optimum sterilization dose for the amikacin sulfate liposomal formulations investigated. The results revealed very high levels of amikacin activity in the spleen and liver as well as sustained levels in the serum. Encapsulation of drug in liposomes decreased its concentration in kidney, with expected potential to decrease its nephrotoxicity. Liposomal encapsulation provides a therapeutic benefit compared to free drug. The mode of deposition of aminoglycoside antibiotics is of particular interest because of their nephrotoxic potential and a drug delivery system, e.g. liposomes that helps to increase the therapeutic index of amikacin sulfate by reducing the nephrotoxicity was our goal.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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