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LIPOSOMAL ASPIRIN PREPARATION AND CHARACTERIZATION

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ABSTRACT
Aspirin (acetylsalicylic acid, ASA) has been used as one of the most well-known, inexpensive, easily offered and commonly used non-steroidal Anti Inflammatory Drug (NSAID) causing many side effect one of them upper gastrointestinal (GI) bleeding and ulceration. Liposome is considered most successful in a nanotechnology applying drug delivery systems to improve therapeutic effectiveness, decrease drugs side effects and toxicities to conduct this aim the liposomal aspirin was prepared from lecithin and cholesterol 1:1 by bangham ordinary method these lipid film hydrated by aqueous phosphate buffer containing aspirin. The liposome entrapment efficiency was 85.5% multilamellar and multi-vehicles shape, with size range (165 ±5.82 nm). The new formula of multilamellar liposome carrying aspirin was prepared with efficient and positive tolerance to GIT media and pH stability and osmotic tolerance.

KEYWORDS: ASA, NSAID, liposome, GIT media, ulceration.

INTRODUCTION

Aspirin (acetylsalicylic acid, ASA) has been used as one of the most well-known, inexpensive, easily offered and commonly used non steroidal Anti Inflammatory Drug (NSAID). Aspirin's ability to inhibit the production of prostaglandins and thromboxanes is due to its irreversible inactivation of the cyclooxygenase. Aspirin irreversibly inhibits COX-1 and modifies the enzymatic activity of COX-2[1]. COX-2 normally produces prostanoids, most of which are proinflammatory, aspirin, like other medications affecting prostaglandin synthesis, has profound effects on the pituitary gland, it uncouples oxidative phosphorylation in cartilaginous (and hepatic) mitochondria**.** Salicylic acid was found to activate AMP-activated protein kinase[2]**.** Ulcers are reported as the most common cause of hospitalization for upper gastrointestinal (GI) bleeding and are often a clinical concern due to the widespread use of aspirin[3]**.**

A liposome is a vesicular form, as the artificial primer cell; formed by dispersing the phospholipids in aqueous solution. These lipid vesicles have cell bilayer structure [4]. Liposome is considered most successful in a nanotechnology applying drug delivery systems to improve therapeutic effectiveness, decrease drugs side effects and toxicities [5,6]. Liposome is used as an oral dosage in order to protect the encapsulated drug particles from degradation in digestive system[4,7] and selectively targeted tissues entire active site and prevent drug enter into blood-tissue barriers[8]. Attendances to improve the undesired effect of aspirin with minimized its unwanted effect by using liposomal aspirin.

MATERIALS & METHODS

Liposome Preparation Technique

The liposome was created and formulated according to the Bangham technique[9] in which settling steps was as follows:

A. The mixture of both cholesterol (0.25 gram) and (0.25 gram) phosphatidylcholine (lecithin); 1:1 w/w were dissolved in a combination of both chloroform and methanol 15ml 2:1v/v. The prepared phosphatidyl choline-cholesterol in the solvent was vortex continuously for 30 minutes in the tube (25 ml).

B. The solvent was evaporated by conducted with rotary evaporator; vacuum pump joined incubated in 40°C yield dry thin film foam like appearance deposited on the tube wall. Then a phosphate buffer of pH 7.2 [10] (2 ml) with and without Aspirin mix with the thin film of lipid to prepare liposomal aspirin and empty liposome, respectively

C. Aspirin entrapment by sequence steps:

1. Aspirin stock solution was mixed with athin film of liposome and vortexes at temperature 40°C for 15 minutes.
2. The formed aspirin liposome suspension was centrifuged at 5000 rpm for 15 min; 4°C separate the free drug entrapped aspirin solution.
3. The supernatant was collected and filtered through 0.2 µm membrane filters

The aspirin was measured by UV-Absorbance spectrophotometer of supernatants and liposome

$$\text{Aspirin Entrapment \%} = \frac{\text{amount of aspirin entrapped}}{\text{Total amount of aspirin}} \times 100$$

$$\text{Aspirin entrapment efficiency \%} = \frac{\text{Totalasprin} - \text{freeaspirin(supernantant)}}{\text{Totalamount of aspirin}} \times 100$$

Standardization of liposome

Liposome type, lamellar and size was examined by light and electron micros

Light Microscopic identity of liposome

Examination of the prepared liposome by light microscope in order to characterize the following parameters particle size, type, Lamellar (layers numbers).

Liposome smear was prepared 50µl of 0.1% liposome suspension. The drop was separated on a slide with a cover slip and examined under oil immersion. The examination was done on empty liposome and liposomal aspirin for the general outline and unity of size[11].

The technique was achieved for estimation the heterogeneity and particles size of liposome with a light microscope linked with image J software. Measurements include 100 liposomes. Morphology and particle size were calculated[12].

Electron microscope (scan and transmission)

The samples of liposome were preserved in deep freeze −20°C after packing sealed in Eppendorf tubes with1ml suspension of both empty liposome and liposomal aspirin. Scanning and transmitted micrograph were done in Lose Alamos National Laboratory, USA, for E.M. imaging scan type and transmission for laminar and sizing of liposome with general surface appearances laser beam scatting technique[13,14].

Aspirin and liposomal aspirin absorbance curve

Prepare empty liposome and liposome carrying Aspirin by mixing 0.5g of liposome with 2ml phosphate buffer pH 7.2 only and with phosphate buffer containing aspirin, respectively. The spectrophotometric analysis, a dilution of the prepared liposome to reach a concentration 0.25% of liposome for all solutions at room temperature. The control blank was used phosphate buffer pH 7.2 measured the absorbance at multiple wave length scanning from 300 to 900 nm according to Beer-Lambert law [15,16] use software to determine the λ max was 418 nm [17].

Liposome Counting

The liposome stock solution was suspending a 100 mg of the liposome in 1ml of normal saline 0.85%. The serial dilutions used in absorbance curve estimation from 0.1 ml of stock solution. The stock solution and its dilution were counted by hemocytometer by the following steps:

Liposome suspension placed on the Neubauer chamber and covers with slip and allocated the slide on the hot plate 37°C for 5 minutes for uniform distribution, count total liposome number on large grade square and calculated by the equation (18).

$$\text{Total liposome per ml} = \frac{\text{liposome number} \times \text{dilution factor}^{**}}{\text{squares number} \times \text{volume of square} *}$$

*Volume of square=1mmx1mmx0.1mm

**Dilution factor 1:10

Liposome pH stability

The assessment of liposome carrying aspirin stability in pH changes was represented tolerance oral passage, the test was prepared and planned according to the method described by Semalty *et al.* (2010) [19]. The concept plan determine the number of liposome survival during the serial increment acidity values, the liposome formula stock suspension 0.25% was added to acidified tube labeled as 2, 3, 4, 5, 6, 7 and 8 pH and stand one hour the liposome concentration was measured in zero and 1hour by spectrophotometer at 418ʎ max.

Osmo-tolerance

Spectrophotometric assessment of osmoletonicity tolerance of liposomal aspirin via estimation the liposome survival numbers with the challenge the dilution assay (20). Liposome formulated aspirin 0.25% 1ml added to normal saline NaCl and descending hypotonic dilutions (8.5, 7.5, 6.5, 5.5, 4.5, 3.5, 2.5, 1.5 and 0 %) set recording in zero and incubated at 37 °C for one hour. Measured liposome fragility at 418 ʎ max

Sterilization by filtration

The aspirin solution, empty liposome and liposomal aspirin were sterilized by membrane filtration via 0.2 µm zeta filter. The fertilized liposome suspension and Aspirin were transferred to the sealed amber container under a flow of nitrogen gas for storage [21].

RESULTS

Entrapping efficiency and entrapment percentage

The entrapment percentage and efficiency of liposome carrying aspirin formula as given in table (1) for 10 patches, each one 500 mg liposome of aspirin stock 4%. Entrapment efficiency for formulation 85.5% was created entrapment % 64%. These data calculated according to aspirin standard curve.

TABLE 1: The entrapment of liposome carrying aspirin formula

Entrapping amount ml	Non-Entrapping amount ml	Entrapping efficiency %	Entrapping %
0.8	1.2	85.50±5.67	64.00±4.12

Number of patches 10

Liposome characterization

The prepared dispersions liposome characterized as the following:-

Liposome size The size of liposome carrying Aspirin was 165 ±5.82 nm and the size of empty liposome was 143 ±6.66 nm as shown in table 2, Liposome carrying aspirin covered the size range of 63.18-178.04 nm and empty

liposome 59.66-122.11nm but it was limited to the detection of particles less than approximately 30 nm were undetectable, the liposome size entrapped aspirin larger than empty significantly $p \leq 0.05$.

TABLE 2: Size of liposome carrying Aspirin and Empty liposome

Liposome formula	Size of liposome		
	Range	Mean ± SE	
Liposome carrying Aspirin	63.18 -178.04	165±5.82	a
Empty liposome	59.66 -122.11	143±6.66	b

The data presented as Mean ± SE
Small letters denoted to differences between types liposomal types
Number of patches 10

Lamellar of liposome

The lamellae of liposome carrying Aspirin and empty liposome was shown in table 3 there were no significant $p<0.05$ between empty and entrapped liposome. Rang values of liposome lamellar were 2-5 of both empty liposome and liposome carrying Aspirin, and mean of lamellae of the empty liposome and Liposome carrying Aspirin were 3.79 ±0.42 and 3.88 ±0.69 respectively

TABLE 3: Lamellarnumber of liposome carrying aspirin and empty liposome

Liposome formula	Lamellar number of liposome	
	Range	Mean ± SE
Liposome carrying Aspirin	2-5	3.79±0.42
Empty liposome	2-5	3.88±0.69

The data presented as Mean ± SE
Number of patches 10

Absorbance curve of liposome carrying Aspirin and empty liposome

The absorbance of aspirin, liposomal aspirin, empty liposome and phosphate buffer are shown in figure 2. All reading was done at room temperature, 2 nm intervals. The result in the plotted graph displays the λ max peak absorbance at 418 ±2 nm for aspirin, 422 nm for liposomal aspirin and empty liposome 420nm.

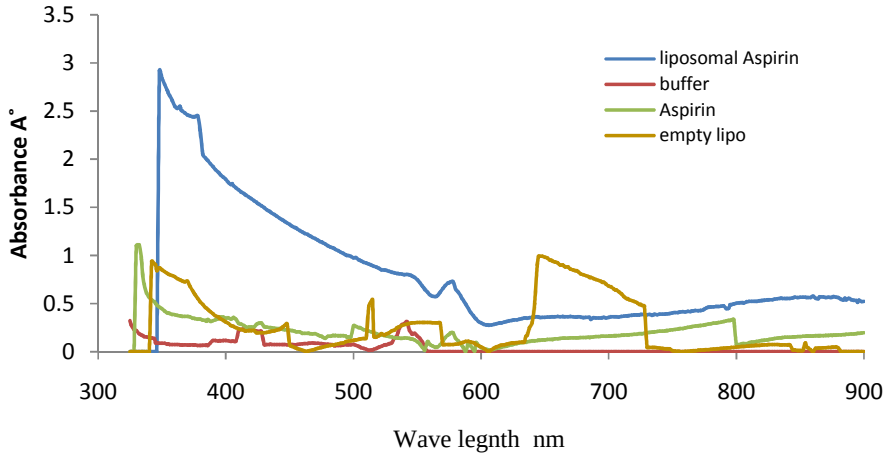

FIGURE 2: Absorbance spectra of the liposome in phosphate buffer (3%) and overlay of the absorbance spectra of the empty liposome, liposomal Aspirin, Aspirin and phosphate buffer,the selective ʎ spectra interactive peak 420 nm.

Counting of liposome and calibration curve

Liner curve liposomal aspirin in normal saline 0.85% at 37°C aliquots standardization stock solutions for liposomal aspirin and empty liposome were prepared to get the final concentration (0.25 mg/ml) and five serial dilutions from the stock solutions which were counted as a number of Liposome per mm^3. The data were plotted with concentrate showed r^2= 0.975 the absorbance data was plotted against dilution factor figure 3. While plotted calibration curve of the absorbance at λ max 420 nm against liposome number figure 4 showed correlation r^2= 0.967 and the number of liposomes plotted against dilution factor showed correlation liner regression r^2=0.983 figure 5.

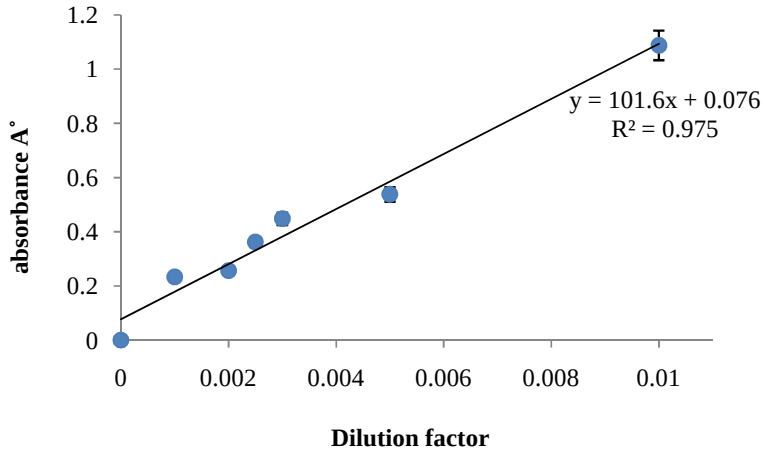

FIGURE 3: Spectroscopic estimation of calibration curve of liposome carrying Aspirin concentrations versus absorbance at 420 nm at 37

The data presented as Mean ± SE
Number of patches 10

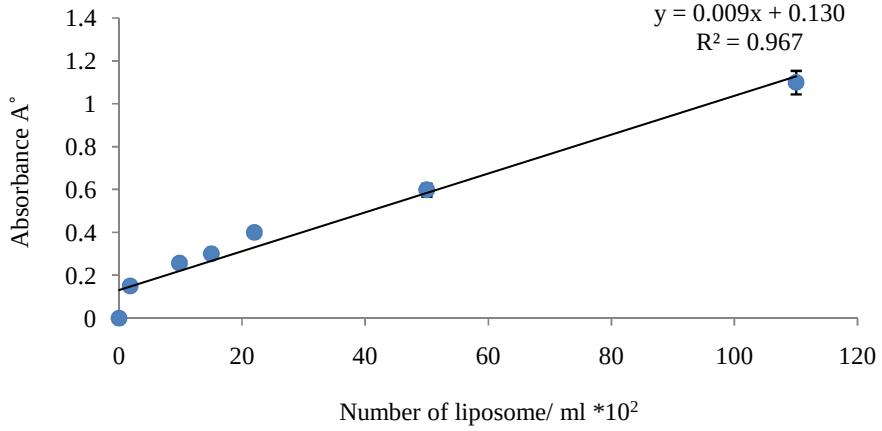

FIGURE 4: Spectroscopic estimation of calibration curve of liposome carrying aspirin number versus absorbance at 420 nm at 37°C

The data presented as Mean ± SE
Number of patches 10

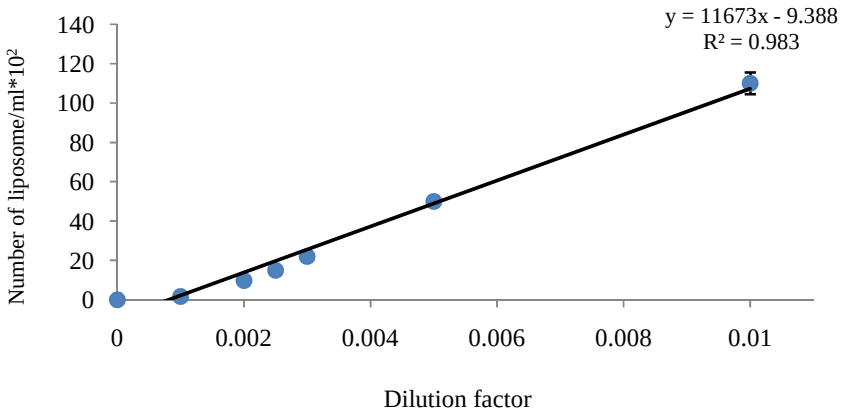

FIGURE 5: Calibration curve of liposome carrying aspirin concentration versus liposome number /ml
The data presented as Mean ± SE
Number of patches 10

MORPHOLOGY OF LIPOSOME

Light microscope

Liposomal aspirin and empty liposome were examined by thelight microscope with magnifications X400and X1000forestimation liposome morphology with and without aspirin. The empty liposome showed regular rounded vesicle shape figure 6A.while the liposomal aspirin showed heterogeneity in size and shape of theliposome as multivesicular like grab unit Figure 6B.

Electron microscope morphology

The characteristics of liposomal aspirin and empty liposome showed the multilamellar,and size and diameter

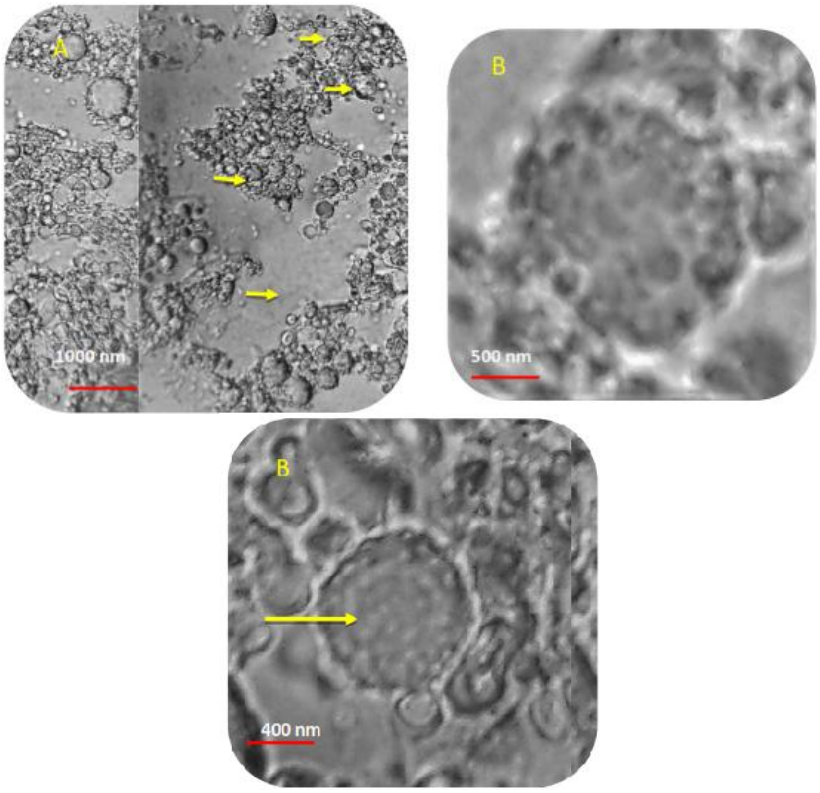

FIGURE 6: Micrograph of formulated liposome carrying Aspirin by thin film "Bangham method" light microscope-phase contrast

A. Multilamillar, multivesicle and different size of liposome denoted by arrow
B. Large scale of multivehicle
C. Multilaminar layer liposome composed arrow denoted to the liposome layers

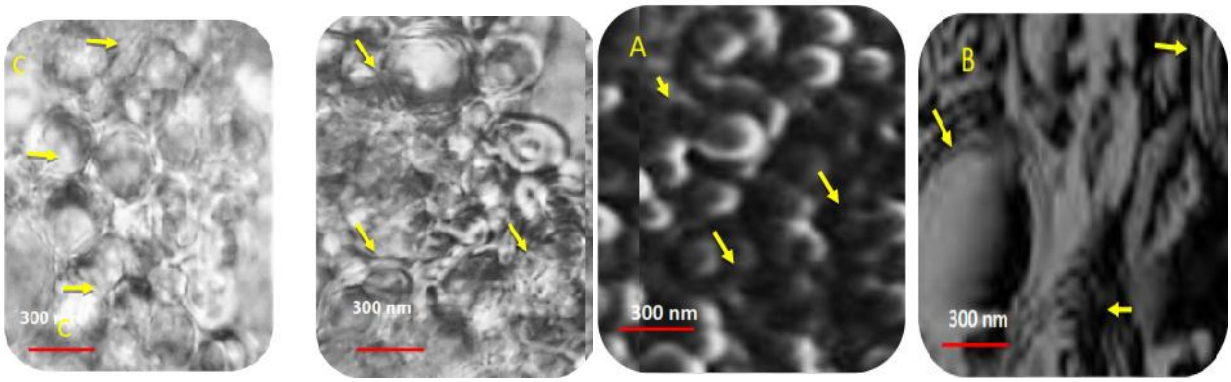

FIGURE 7: Micrograph of formulated liposome carrying aspirin by thin film "Bangham method" Electron microscope

A. Scanning mode picture of different size liposome denoted by arrow
B. Multilaminar layer liposome arrow denoted to the liposome layers

Effect of pH on the liposomal Aspirin

The liposome pH tolerance curve showed adecline with decreased pH value. Liposome was stable at pH 7 and less stable at pH 2 and 3 after incubation for one hour in different pH media at room temperature figure 9.

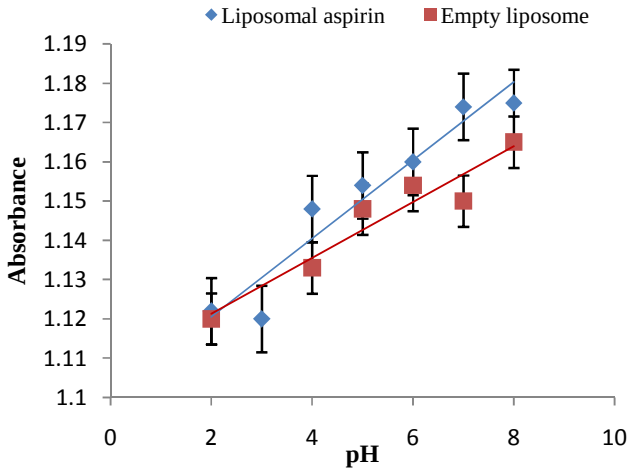

FIGURE 8: pH challenge of Liposomal aspirin and empty liposome at zero time

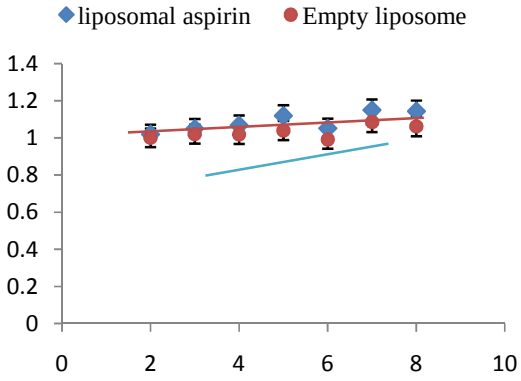

FIGURE 9: pH challenge of Liposomal aspirin and empty liposome after one hour

Osmotonicity

The effect of different NaCl concentrationon liposomal aspirin and empty liposome showed increased in the absorbance curve after introducing empty liposome and liposomal aspirin to gradual decreased in solution tonicity starting from isotonic to hypotonic solutions after one hour figure 10 and 11.

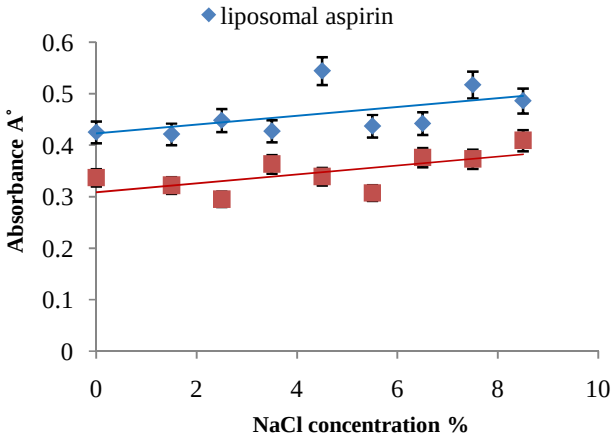

FIGURE 10: Effect of different osmolarity on Liposomal aspirin and empty liposome at zero time

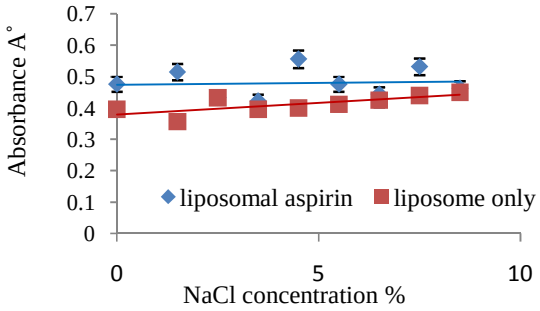

FIGURE 11: Effect of different osmolarity on Liposomal aspirin and empty liposome after one hour

DISCUSSION

Liposomal aspirin entrapping efficiency and characterization

The efficacy of therapeutic molecules is often limited by the insufficient delivery, accumulation in target tissues or undesirable side effects even moderate to severe toxicities in non-aimed organs[20]. Liposome is the delivery systems, because of their biocompatible composition as well as superior efficacy, especially the significant improvement in drug circulation[6]. The liposome formulary aspirin has an impact improved aspirin kinetically and dynamically. The instance dramatic evolution the both: i. Physical appearance: size, shape and lamella ii. Chemical: pH and osmotolerance.

The vesicle size is an acute parameter in determining the circulation half-life of liposome and both size and number of bilayers affect the amount of drug encapsulation in the liposome[21]. The current study has shown that the liposome prepared by Bangham ordinary methods size mean was 165 ±5.82 nm agreed with yaha, 2017[22] table (2), which was large multilamellar vesicle[23]. Woodle *et al.* (1992) [24] recorded that the mean particle diameter was increased from 100 to 200 nm leads to shorter elimination half-life of liposome and decreases the plasma protein binding which increases the liposome clearance. The size of liposome permitted stable for lamaller in GIT canal, that liposome structure MLVs and MVs display the high entrapment according to the using of miscible solvent (methanol-chloroform)[25]. The polarity of the solvent can influence the size and lamellar type of liposome and also could modify the lamellarity property and entrap liposome [26].

Another fact was the preparation of liposome rich with cholesterol derived MLVs and maintained liposome stability in internal body environment that liposome had charged lipid into liposome surface maintain certain drug through electrostatic bounds [27]. The impact of cholesterol on liposomal clearance was assessed in the present study that cholesterol was required to maintain the stability of liposome in the plasma [28]. Additionally, it may also inhibit protein binding by shielding defects on the surface of liposomes[26] thereby presumably preventing them from recognition by opsonizing plasma proteins [23]. Aspirin is a water-soluble drug and dissolved in the aqueous buffer encapsulated inside the liposomes, leading to increasing vesicles size as compared to empty liposome (145 nm) the greater encapsulated fluid causing greater encapsulation efficiency [29]. The prepared liposomal aspirin had 85.5% entrapment efficiency table 1, which was considered good entrapping efficacy due to the solubility of aspirin in aqueous compartment of the liposome. Previously records of Ekanayake *et al.* (2012)[30] showed that aspirin encapsulation efficiency in phospholipids was 72%. The increase in the encapsulation efficiency may due to Incorporation of cholesterol[31] which, tighten fluid bilayers and reduce the leakage of contents from the liposome. Switching from a fluid phase phospholipid bilayer to a solid phase bilayer also reduced leakage [26,31]. It was found that used lecithin and cholesterol at 1:1 had agood entrapping efficacy agreed with Marei (2012) [9] that found reducing the amount of cholesterol lead to reduction in encapsulation efficiency. This is probably because cholesterol imparts rigidity and stability to the liposome wall and reducing its amount causes lyses and fusion of the liposome which resulted in low encapsulation efficiency [29]. The selection of the Bangham ordinary liposome preparation methods to enhance aspirin bioavailability by decreasing the metabolism of aspirin through first pass effect in liver or within intestinal cells by the action of esterase[32] and decrease its side effect on stomach via entrapping to MLVs and MVs which act as shield to prevent unwanted effect of aspirin[33]. MLVs are formed more easily at larger hydrodynamic diameters, and thus have greater entrapped volume exhibit a moderate release rate[6]. These due overall to the number of a phospholipid bilayer that it has to cross before being released. The morphology of liposome vesicles was shown in figure 5A and B, using an optical microscope. The images revealed that the suspension contains multilamellar vesicles (MLVs) and multivesicular vesicles (MVs), the confirmation of the liposome formation by scan EM was also used. The image in figure 6 confirms the presence of typical multilamellar liposome enclosing internal aqueous phase in which the drug is soluble.

Absorbance curve and liposome concentration

The λ max was determined through scan by spectrophotometer, different particles have unique absorbance properties the λ max range from 418-420 nm figure 2 has proven that maximal absorbance peak was 420nm consider the best wave length to record measurement of real sense it showed highest absorbance values and the highest sensitivity[34]. For comparison of absorbance curve result with liposomal aspirin, aspirin, and empty liposome they have to vary the absorptive and number of entrapped molecules. The spectrum of liposome recorded at 420 nm.

The vesicle size facilitated to calculate recording many peaks nanometer[35]. The turbidity originated from empty

liposome and liposomal aspirin was 418 nm absorbance was not only depended on concentration but also on partials size [36]. Water solubility of aspirin was increase liposome size because aspirin incorporated in the liposome core [9]. For recovering determination of absorbance by spectrophotometer distinct concentration of liposome and measured aslope of the plot of absorbance 418 nm versus concentration. The number of liposomes calculated per ml using hemocytometer was depicted versus concentration. The number of liposomes depicted visible spectroscopy absorbance nm to at the calibration curve quantifying the dose administered of aspirin entrapped.

The effect of pH on liposomal aspirin

Liposome is drug carrier vehicle that can be used to keep advantageous interaction with gastric secretion through oral drug delivery to achieve oral form [37]. For this result showed tolerance in different pH value with less reduction in the absorbance of liposome at pH2 and 3, figure (9) The tolerated acidic effect on the liposome stability in oral route after one hour indicated that liposomal aspirin can pass GIT and exert aspirin dynamic effect [38,39].

Osmotnicity and liposome

Osmotic effect test independently on electrostatic challenge interaction, the osmotic pressure are measured by addition of series concentration of NaCl which was of molecular weight 58.4428g/mol and has the capability to penetrate phospholipid bilayers [40]. The hypoosmolarity increased the particle size after one hour and increase absorbance curve figure (11) which may be due to the large particle which undergoes swelling to more than 100 nm due to that hypotonic environment causes a net movement of water into the liposome, causing it to swell which gave impression that lecithin and cholesterol had fluidity and elasticity give compliance the increase in their size[41].

CONCLUSION

The new formula of multilamellar liposome carrying aspirin was prepared with efficient and positive tolerance to GIT media and pH stability and osmotic tolerance.

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