IJPSR (2015), Vol. 6, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 22 November, 2014; received in revised form, 20 January, 2015; accepted, 27 March, 2015; published 01 July, 2015

DEGRADATION STUDY OF LIPOSOME DRUG DELIVERY PREPARED WITH PALM OIL; USING ^{1}H NMR

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Keywords:

Liposome, Drug delivery systems, Palm oil, Nuclear magnetic resonance (NMR), Hydrolysis.

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ABSTRACT: This study aims to utilize natural palm oil in the formulation of liposome and evaluate its stability and degradation via the ^{1}H NMR technique. In this study, the conventional method was used to prepare liposome consisting 0, 5, 10, 15, 20 and 25% of palm oil in their structure, and the liposome were characterized *in vitro* afterwards. The chemical degradation of liposome in the presences of blood component was detected using ^{1}H NMR after incubation period of 2 hours, one day and one week. According to ^{1}H NMR spectrums, samples that lack palm oil or those exceeding 15% palm oil concentration underwent decomposition in the first 2 hours, while samples that were loaded with 5,10 and 15% or palm oil showed no such degradation. However, after being incubated for a day with serum component, the samples loaded with 10 and 15% palm oil remained stable and did not succumb to degradation. It can be surmised that this approach is a viable analytical concept based on the chemical characteristics and the stability of liposomal drug carriers, which complements that of conventional analytical techniques.

INTRODUCTION: Liposomes are utilized as a drug delivery component, and are used to treat various illnesses, infections, cancers, and genetic pathologies [1-3]. Liposomes are made up of two heads, one hydrophilic while the other hydrophobic; which makes them capable of delivering drugs with various physicochemical properties [4-5]. They can also be developed to transport and transfer toxic substances and drugs that have poor solubility, biologically active materials, and vaccines to enhance their pharmacological functions [6-8].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(7).2741-47

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(7).2741-47

Liposomes are also a potential carrier for controlled drug release of tumors' therapeutic agents and antibiotic, for gene and antisense therapy through nucleic acid sequence delivery, immunization through antigen delivery, and for anti-Parkinson medications [9-12]. Palm oil is made up of saturated fatty acid possessing antioxidant properties due to presence of certain components, such as carotenes, tocopherol, tocotrienols, terpenoids and flavonoids; it has the capacity to be used in pharmaceutical products on top of its nutritional advantages. The antioxidants in palm oil are thought to resist decomposition of lipids, which naturally protect vesicles from deterioration in the presence of both oxygen and heat and stabilize liposomes structure respectively [13-17].

This study aims to utilize palm oil as a stabilizing agent for liposome, which will in turn help enhance

its physical and chemical stability in its function as a drug delivery device.

In the previous decade, pharmaceutical researchers utilized biophysics to evaluate liposomal dosage forms. Liposome consisting of various percentage of palm oil were prepared and characterized, while oxygen and heat accelerate the interface interactions between liposome and serum components. The stability of vesicles was evaluated after 2h, one day, and one week of incubation periods using the ^{1}H NMR technique [18].

MATERIALS AND METHODS:

Materials: Palm oil (PO), cholesterol (CH), L-alpha-phosphatidylcholine (PC), diethyl ether, methanol, chloroform, and RPMI 1640, Phosphate Buffered Saline tablets were provided from Sigma-Aldrich (Germany). Sodium carbonate was purchased from MERCK (Germany). Fetal bovine serum (FBS) and Penicillin G plus Streptomycin were obtained from GIBCO (US).

Method: as it is shown in **Table 1**, six formulations of liposome with the different ratios of PC and palm oil were prepared using the conventional method [19-20]. Each formulation was made up of CH with varying amounts of PC and palm oil. The lipid components were dissolved in chloroform:methanol mixture of (2:1, v/v) in a round-bottom flask. The solvent was removed under vacuum conditions using a rotary evaporator (Rotavapour R-124, BÜCHI) at 50°C and 50 rpm. After a thin lipid film begins to form in the interior of the flask, the system was purged with nitrogen to completely remove any remaining organic solvents. The lipid film was hydrated in 10 ml phosphate-buffered saline (PBS, pH 7.4) and sonicated for 15 min in a bath-type sonicator (Sonicor). The liposomes were allowed to form at room temperature and refrigerated overnight after the fact. The final mixture was then filtered through a syringe filter (0.45 μm).

TABLE 1: COMPOSITION OF LIPOSOME FORMULATIONS

Formulation	Content of lipids (%w/w)			
	Phosphatidylcolin	Cholestrol	PEG	Palm oil
FI	50	45	5	-
FII	45	45	5	5
FIII	40	45	5	10
FIV	35	45	5	15
FV	30	45	5	20
FVI	25	45	5	25

Formation and morphology:

The formation of liposomes was observed with a transmission electron microscopy (TEM). Samples were prepared by applying a drop of mixture to a carbon-coated copper grid and left for a minute to allow some of the particles to stick onto the carbon substrate. After removing the excess dispersion using a piece of filter paper, a drop of 1% phosphotungstic acid solution was applied for a minute and air-dried. The samples were then viewed using a TEM (ABFETEM Leo 9112) [20-21].

Particle size distribution and zeta potential measurement:

The value of Zeta Potential (ZP) defines the stability of particulate systems, due to the fact that it measures the repulsive forces between particles. Particles having ZPs of less than -30mV or more than +30mV are usually regarded as stable [22]. The ZP values were obtained by inserting each sample into the cell attached to a zetasizer.

Degradation study of liposome by ^{1}H-NMR:

100mg liposome per formulation was weighted separately and added to 10ml RPMI 1640 with 10% FBS plus 1ml Penicillin G. Then, the mixture was incubated at 37^{0}C in the presence of atmospheric oxygen for 2hrs, one day, and one week, respectively. After incubation, acid-base treatment was conducted in order to improve the separation of basic extracts. First, NaOH 0.1N was added until the mixture arrives at a basic pH, then the reaction products were extracted with Ethyl acetate and Diethyl ether (60:40, v/v). The aqueous and organic phases were then separated. The organic phase, which has a basic and non-ionizable component, was washed with 10ml HCL 0.1N, then the aqueous and organic phases were re-separated, with

the organic phase being dried over Na_2SO_4 and then concentrated. Consequently, the serum component and lipoprotein extraction remained in the interphase. The aqueous phase that contains acid ionizable substances was washed with 10 ml HCL 0.1 N, then ethyl acetate and diethyl ether (60:40,v/v) was added as well. The organic phases were separated, dried over Na_2SO_4, and concentrated. ^{1}H-NMR measurements were performed at room temperatures using CHLOROFORM-D ($CDCL_3$) as a solvent [18].

RESULTS AND DISCUSSION:

Liposome formation and morphology: According to the TEM images in **Fig.1**, Large unilamellar vesicles (LUVs) were formed in all of the formulations. FI and FII were morphologically spherical although FIII and FIV showed ellipsoidal formation. While the morphology of liposomes changed significantly at the highest ratio of palm oil in FV and FVI. TEM images in **Fig. 1** shows the fine formation vesicle of FI and FIII, while in FIV, as the ratio of palm oil was increased to 20%, deformed vesicles were discovered[19-21].

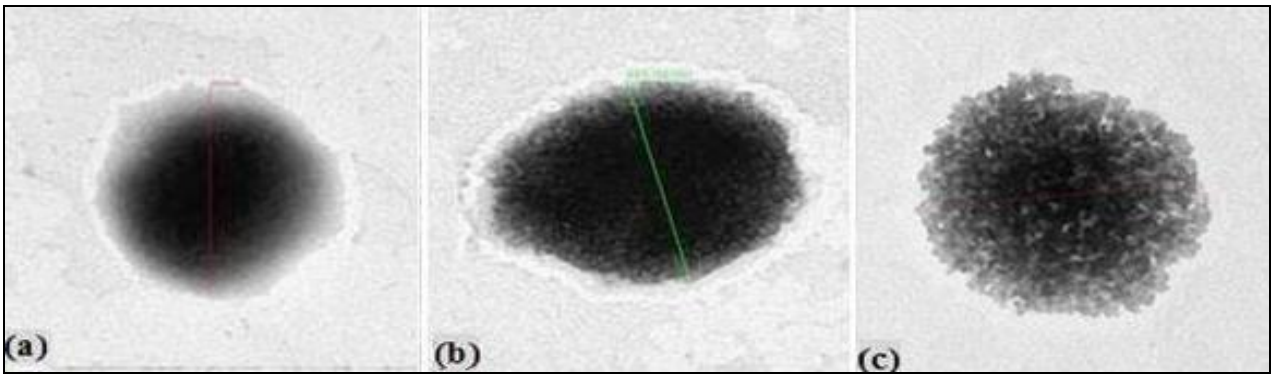

FIG.1: TEM IMAGES OF LUV LIPOSOME. KEY: (A) FI VESICLE WITH MAGNIFICATION16000x, (B) FIII VESICLE WITH MAGNIFICATION16000x, (c) FIV VESICLE WITH MAGNIFICATION 20000x

Particle size distribution and zeta potential measurement:

As can be seen in **Table 2**, all liposomes have a mean particle size between 340-450nm, which confirms the figuration of LUVs. FV & FVI demonstrated the smallest size, whereas FIII & FIV were among the largest. Considering the ZP value of liposomes, and since particles containing ZP $\leq$-30mV or $\geq$+30mV are typically more stable [22], only FIII & FIV liposome showed satisfactory ZP values in both fresh mixture and after 30 and 60 days incubation at -4 ^{0}C.

TABLE 2: PARTICLE SIZE (nm ± SD) AND ZETA POTENTIAL (mV) OF LIPOSOME

Formulations	Mean particle size	ZP fresh mixture	ZP after 30 days	ZP after 60 days
FI	378.84±2.1	-26.6±1.8	-24.7±3.3	-22.2±4.1
FII	407.45±1.3	-27.5±2.3	-26.3±0.9	-25.1±2.6
FIII	447.21±2.7	-32.2±1.1	-31.8±2.1	-30.6±1.9
FIV	438.74±1.9	-31.7±4.2	-31.0±2.8	- 30.3±4.3
FV	356.67±0.9	-29.3±2.3	-27.1±1.4	- 26.5±4.1
FVI	341.45±0.8	- 28.9±2.1	-27.6±2.2	-25.7±3.8

Degradation study using ^{1}H-NMR:

Phosphatidylcholin, with its lipid bilayer structure, is the main compnent of any type of liposome. However, its poor stability and high tendency to oxidize causes the degradation and depreciation of vesicles [23]. Since oxygen and heat accelerate degradation, signals from this lipid (**Fig. 2**) was used as a reference to compare the degradation of formulations and intraction of vesicles with the serum after 2hrs, one day, and one week of incubation with serum component.

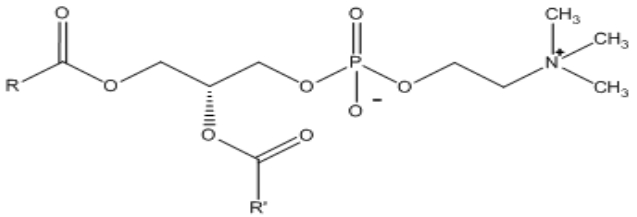

FIG.2: PHOSPHATIDYLCHOLIN STRUCTURE

^{1}H NMR spectrums of acidic and alkaline extracts from each formulation was measured. **Fig.3 & 4** demonstrate the ^{1}H NMR spectra of FI liposome after 2 hours and one day of incubation with serum component. Considering **Fig. 3**, signals observed at 2.0103, 2.2757, 2.8108 and 5.3568 ppm which are corresponding to **-CH_2-C=C-**, **-CH_2-C=O** and **=CH-CH_2-CH=**, **–CH=CH-.** Further in **Fig. 4**, signals presented at 2.0761, 2.3190, 2.8084, 3.8228, 4.2912 and 5.3917ppm which are result of **-CH_2-C=C**, **-CH_2-C=O, =CH- CH_2-CH=**, **CH_2-N, CH_2-O-P** and **-CH=CH-** respectively [10, 24]. No signals observed after 2h incubation of FII, FIII and FIV vesicles with serum component whilst passing one day signal of PC confirm the degradation of liposome during 24h incubation[18,24].

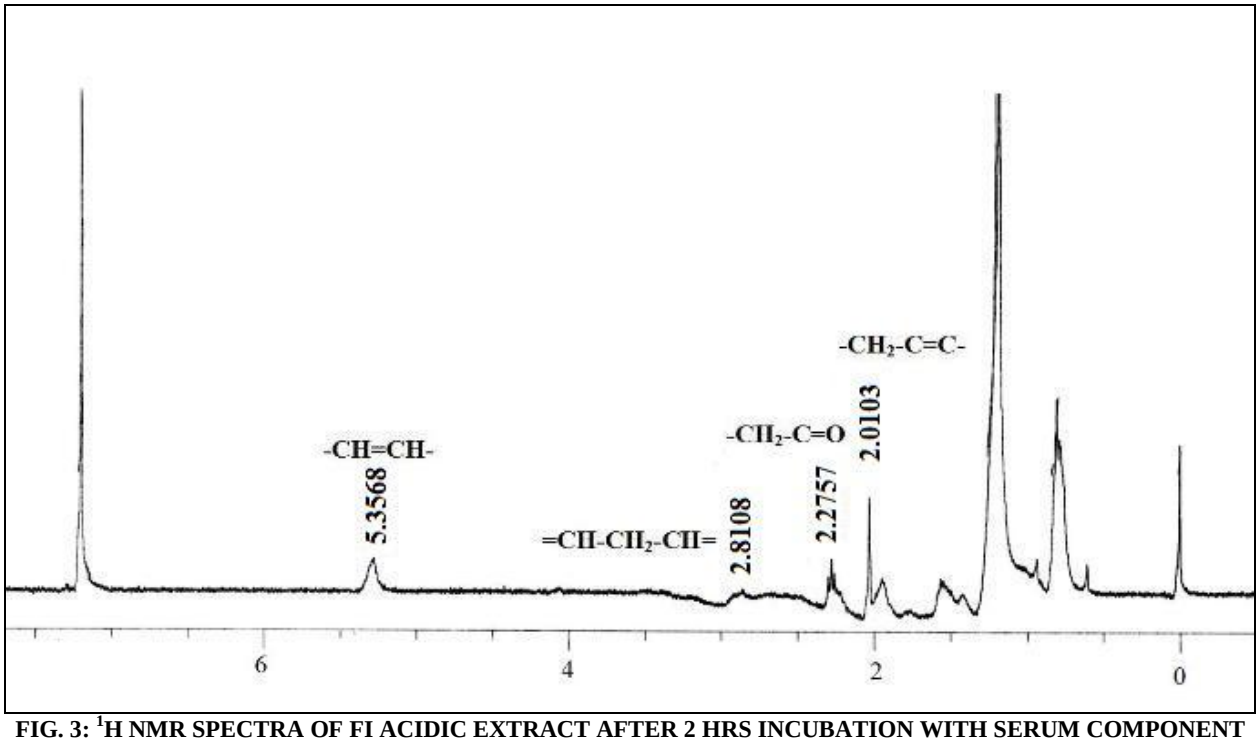

FIG. 3: ^{1}H NMR SPECTRA OF FI ACIDIC EXTRACT AFTER 2 HRS INCUBATION WITH SERUM COMPONENT

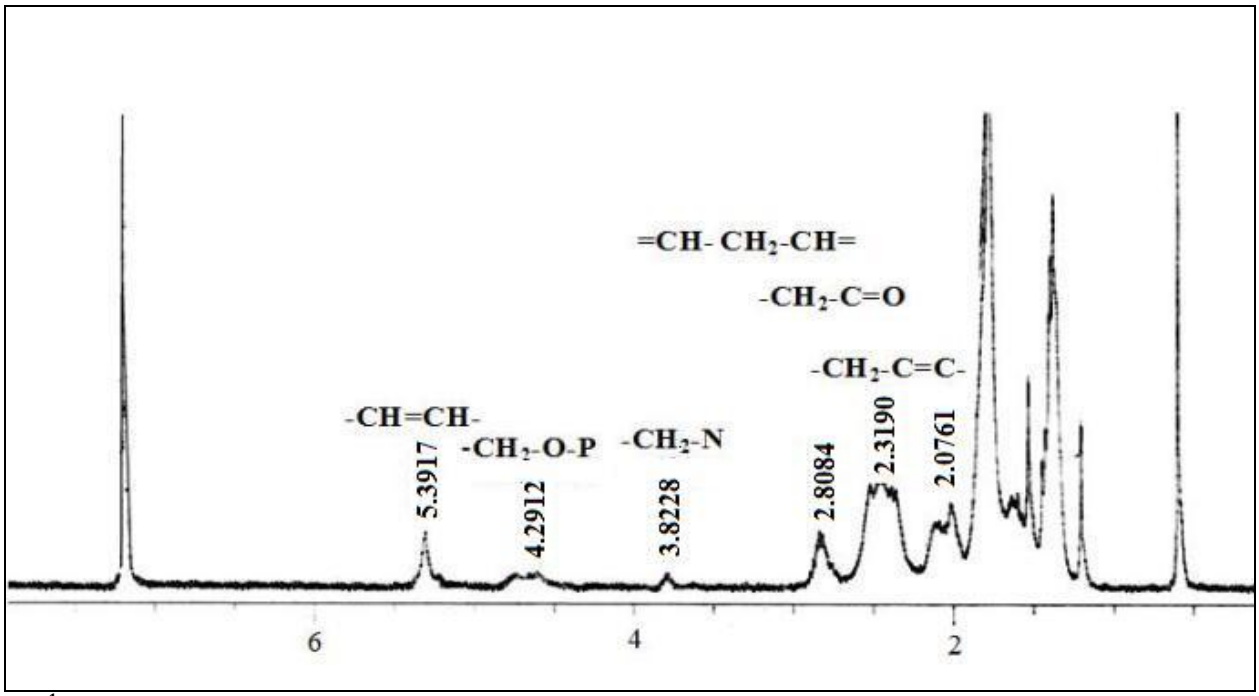

FIG.4: ^{1}H NMR SPECTRA OF FI ALKALINE EXTRACT AFTER ONE DAY INCUBATION WITH SERUM COMPONENT.

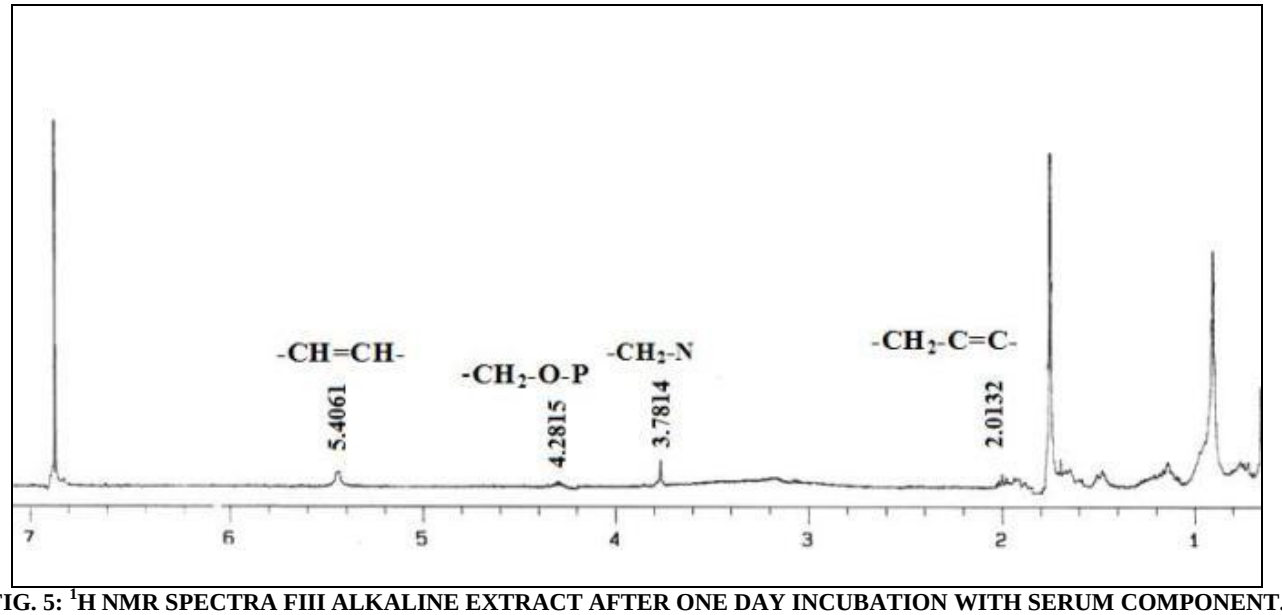

FIG. 5: ^{1}H NMR SPECTRA FIII ALKALINE EXTRACT AFTER ONE DAY INCUBATION WITH SERUM COMPONENT.

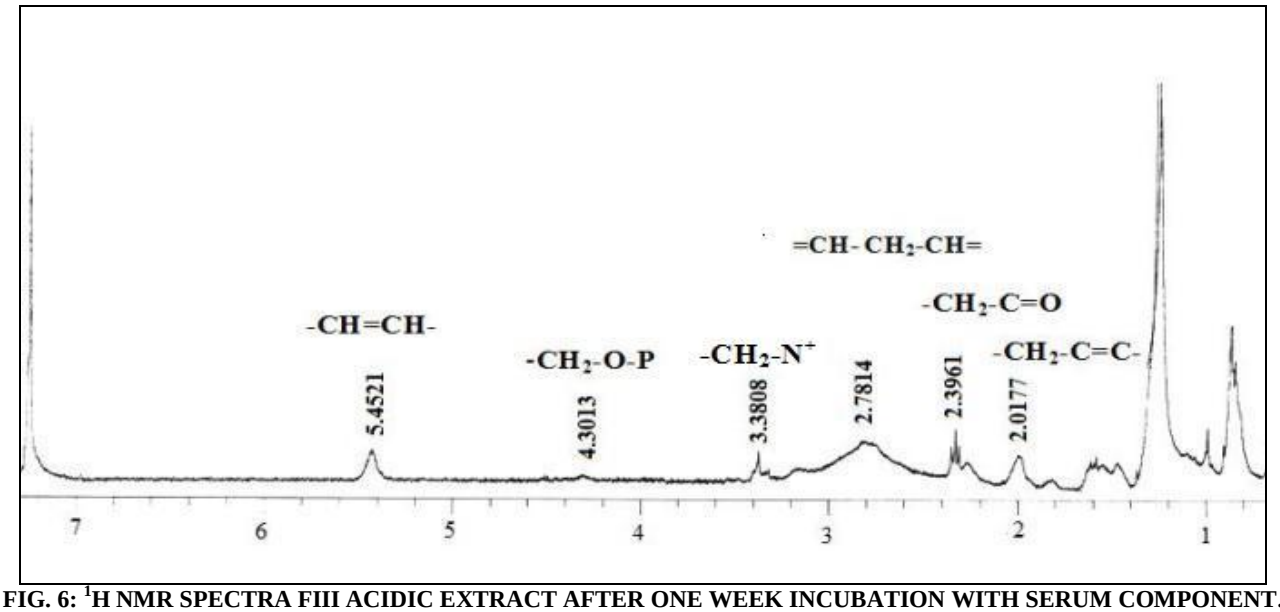

FIG. 6: ^{1}H NMR SPECTRA FIII ACIDIC EXTRACT AFTER ONE WEEK INCUBATION WITH SERUM COMPONENT.

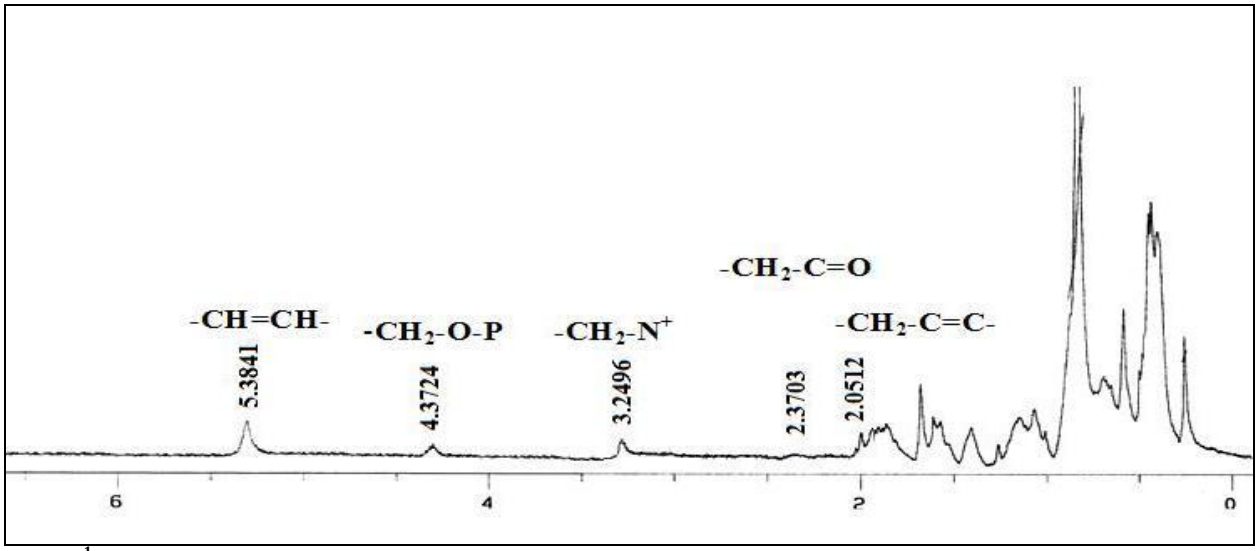

FIG. 7: ^{1}H NMR SPECTRA OF FVI ACIDIC EXTRACT AFTER 2 HRS INCUBATION WITH SERUM COMPONENT.

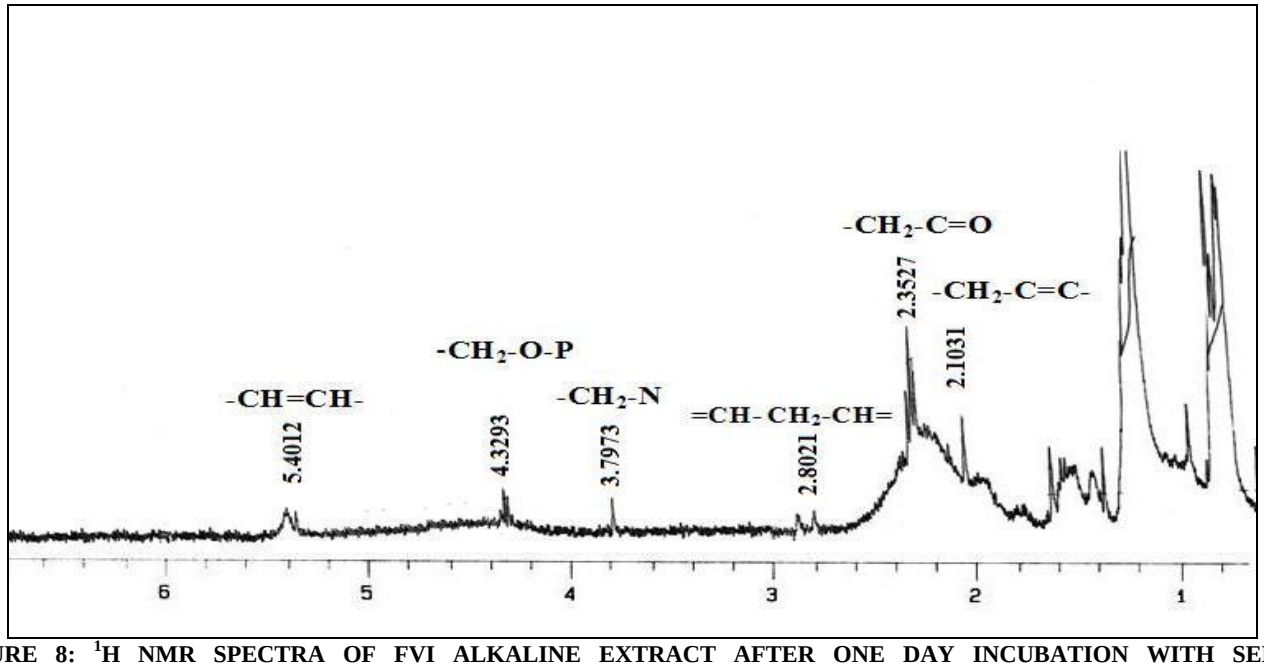

FIGURE 8: ^{1}H NMR SPECTRA OF FVI ALKALINE EXTRACT AFTER ONE DAY INCUBATION WITH SERUM COMPONENT.

Fig.5 & 6 demonstrate ^{1}H NMR spectra of FIII liposome after one day and one week of incubation with serum component. **Fig. 5** shows four signals at 2.012, 3.7814, 4.2815, and 5.4061 ppm to that are matching to **-CH_2-C=C**, **CH_2-N^+**, **-CH_2-O-P** and **-CH=CH-**. Further in **Fig. 6** five signals were identified in ^{1}H NMR spectra of FIII acidic extract after one week incubation with serum component. Signals at 2.0177, 2.3961, 2.7814, 3.3808, 4.3013 and 5.4521 ppm which are corresponding to **-CH_2-C=C-, -CH_2-C=O, =CH-CH_2-CH=**, **CH_2-N^+** and **-CH=CH-** [18, 24].

Both FIV and FVI liposome showed early degradation after 2 h of incubation. **Fig. 7 & 8** demonstrate ^{1}H NMR spectra of FVI liposome after 2 h and one day of incubation with serum component. As **Fig. 7** shows, after 2 hours incubation five signals observed at 2.0512, 2.3703, 3.2496, 4.3724 and 5.3841 that are corresponding to **CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N^+**, **-CH_2-O-P** and **-CH=CH**. **Figure 8** the alkaline extract of FVI vesicles after one day incubation showed six signals at 2.1031, 2.3527, 2.8021, 3.7973, 4.3293 and 5.4012 ppm that are corresponding to **-CH_2-C=C-**, **-CH_2-C=O**, **=CH- CH_2-CH=**, **CH_2-N**, **-CH_2-O-P** and **-CH=CH-** [18,24].

Overall, replacing various percentage of palm oil showed a variety of changes in the characteristics of the vesicles. In FI liposome possessed fine formations, although they show poor ZP values and fast degradation, due to early hydrolysis of PC during incubation with the serum component. In FII liposome, fine shape observed plus improved the steadiness of vesicles during the 2 hours incubation but FII vesicles still have poor ZP and high degradation after one day incubation with serum component. Impressive results were obtained from FIII and FIV liposome, showing superior formation and satisfactory ZP values with advanced stability toward oxidation and hydrolysis during 2 h incubation and also the least degradation after one day and one week incubation compare with other formulations. FV & FVI formulations were enabled of preparing qualified liposome due to the lack of PCs. Although FV & FVI showed higher ZP value compare to FI and FII, they didn't have good shape and formation which lead to fast hydrolysis of them during incubation with serum component.

CONCLUSION: This study replaces percentages of PCs with palm oil in the preparation of liposome, and this change was carefully vetted. Although the use of PC for making liposome seems to be necessary, palm oil seems to be a viable replacement. The addition of controlled amounts of palm oil would result in the optimization and stabilization of liposome, along with helping it avoid both oxidation and hydrolysis. TEM images

confirm the formation of LUV under loading with palm oil, and liposomes loaded with 5, 10 and 15% palm oil showed well-defined morphologies compared with vesicles consisting of 20 and 25% palm oil. Zeta potential values showed that 10 and 15% loading of palm oil into liposome would increase the ZP value in fresh and incubated vesicles additionally. Degradation of LUV was studied using ^{1}H-NMR to check for oxidation and hydrolysis of PC during incubation with serum component. ^{1}H-NMR is a qualified analytical technique to accomplish the stability and degradation study of liposome in serum component.

Looking at the ^{1}H-NMR spectrums, vesicles with 5, 10 & 15% palm oil did not decompose in the first 2 hours, although after a day; only the liposomes loaded with 10 and 15% of palm oil remain less affected by oxidation and hydrolysis. However, after a week of being incubated, all of the samples consistently degraded.

ACKNOWLEDGMENTS: This research work was funded by ERGS grant ERGS/1/2011/SKK/ CUCMS/03/3, and supported by Cyberjaya University Collage of Medical University (CUCMS) and University of Malaya (UM).

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How to cite this article:
Sabeti B, Md. Noordin IB, Mohd S, Hashim R, Dahlan A and Javar HA: Degradation Study of Liposome Drug Delivery Prepared With Palm Oil; Using ^{1}H NMR. Int J Pharm Sci Res 2015; 6(7): 2741-47.doi: 10.13040/IJPSR.0975-8232.6(7).2741-47.