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Research Article

TRIMETHYLCHITOSAN COATED INSULIN LIPOSOMES FOR ORAL DELIVERY, PREPARATION AND CHARACTERIZATION

[1,2,*]ESKANDAR MOGHIMI POUR AND [2]ALI YADEGARI

[1]Nanotechnology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

*Corresponding Author: moghimipour@yahoo.com/aliyadegari90@gmail.com

Abstract

Insulin can use for treatment of diabetes. The effectiveness of this compound is dependent on its absorbance and hence, oral drug delivery is preferred than parenteral drug delivery. There have been extensive researches to formulate non-injectable preparations of insulin. The goal of the present study was to formulate a relatively stable form of insulin using different vehicles such as liposome, alone and in combination with trimethylchitosan. Different (6:2, 7:2 and 8:2) ratios of phosphatidylcholine and cholesterol were utilizes to formulate liposomes by dehydration-rehydration method. DRVs were then placed in trimethylchitosan solution (pH=7) in 1:1 ratio for one hour to form the complexes. Different formulations were characterized and their physico-chemical properties, such as particle size and surface charge were evaluated. They were also examined for their release behavior utilizing a specially designed diffusion cell, respectively. Encapsulation values of the formulations were also determined. The drug was assayed by HPLC method using a C8 column and UV detector at 214 nm. The results showed that there was significant difference between the formulations regarding their size and encapsulation efficacy significantly differed. The loading of liposomes and their size were increased when incorporated with trimethylchitosan and increased amount of insulin. The results indicated that comparing the formulations, the surface charge increased significantly by increasing the amount of trimethylchitosan, and decreasing the lipid ratio. The drug release profile included two steps, rapid release and sustained. Drug release was affected by presence of trimethylchitosan, and release profile was affected by amount of insulin, too.

Keywords: Insulin – Trimethylchitosan – Liposome – Chitosome.

1. Introduction

Type 1 diabetes is generally destruction of insulin-producing pancreatic β cells. Polydipsia, polyphagia, and polyuria are the classic trio ofsymptoms associated with disease onset. An immediate need for exogenous insulin replacement is also a hallmark of type 1 diabetes, for which lifetime treatment is needed.(1), (2), (3), (4).

Insulin, a major protein hormone, is secreted by the β-cells of the pancreas and plays a critical role in controlling diabetes. Daily subcutaneous injection is the most common form of insulin therapy. Oral drug delivery is the preferred route of long-term administration because it offers several advantages over other routes such as convenience, less invasive and expensive. Proteins are generally water-soluble compounds with abundant hydrophilic polar groups on their surface, which lead to an inefficient absorption of oral insulin and low permeability. (5), (6), (7).

New delivery approaches depend on protecting insulin against enzymatic degradation and enhancing their transport across the intestinal mucosa into the systemic circulation. Various approaches have been proposed to overcome barriers and to attain better oral bioavailability, including the use of surfactants, permeation enhancers, protease inhibitors, enteric coatings, carrier systemsand chemical modifications of insulin.(8), (7)

Amongst these, the use of colloidal polymeric particulate delivery systems, particularly muco adhesive nanoparticles represents a promising concept. Then, it can enhance the permeability as well as reduce degradation of drugs.(9), (10) Chitosan is biocompatible, non-immunogenic, nontoxic and biodegradable, making it an excellent choice as a component of oral drug delivery systems and making it highly attractive for biomedical and drug delivery applications.(11), (12)

Trimethyl chitosan (TMC) is a partially quarternised derivative of chitosan which is well soluble in a wide pH

range (pH1–9). TMC has been proven to be a potent intestinal absorption enhancer of peptide and protein drugs, especially in neutral environments where chitosan is ineffective as an absorption enhancer.(8), (13), (14)

Liposomes have been shown to improve the enteral absorption of peptide and protein drugs.

Unfortunately, most liposome formulations cannot be used for oral delivery because liposomes dissolve in intestinal detergents such as bile salts, and they are readily degraded by intestinal phospholipases. Tominimise these disruptive influences, accordingly, polyelectrolyte coatings obtained by alternating the deposition of polyanions and polycations emerged as a novel way to functionalise surfaces. This method has been quickly applied to the drug delivery field by combining trimethylchitosan. The mucoadhesive property of CS-coated liposomes has been demonstrated to help delay the intestinal transit time to increase the absorption of insulin.(15), (16), (17)

In this study, liposomal and chitosomal insulin formulations were prepared by thin film-hydration method using cholesterol, lecithin, and trimethylchitosan. In this paper, thin film-hydrated liposomes and chitosomes were prepared and evaluated in terms of potential new insulin carriers for effective oral delivery and their ability to increasing of absorbance insulin.

2. Materials and Methods

2.1. Chemicals

Cholesterol, lecithin, actone, triflouroaceic acid(TFA) and trichloroacetic acid(TCA) were purchased from Merck (Germany) and chitosan, sodium hydroxide, sodium iodide and methyl pyrollidone from Sigma (Germany).Chloroform, methanol, acetonitrile and methyl were purchased from sachmun (southkorea). Insulin was provided by Novonordisk (switzerland). All other materials and solvents used in this study were of analytical grade.

2.2. Insulin assay

The drug was assayed by HPLC (Agilent, 1260Iinfinity) method using a C8 column and UV detector at 214 nm by mobile phase containing of water: Acetonirille: TFA (1: 34: 65).

2.3. Vesicle preparation

Thin film-hydration method. Cholesterol and lecithinwere dissolved in 6mL of solvent system consists of chloroform: methanol (3:1) in a round-bottom flask. The organic solvents were removed under vacuum in a rotary evaporator at 50^{o}C for 10 min to form a thin film on the wall of the flask. After removal of the last trace of organic solvents, hydration of the lipid film was carried out using 50 mL of buffer phosphate (pH=7) which is containing variable amount of insulin. Then, the vesicle suspension was sonicated in 3 cycles of 5min “on”2min “off” to the formation of liposomes.

For preparation of chitosome formulations, TMC solution 10% w / v is made at buffer phosphate pH = 7. Then, the variable ratio of chitosan: lipid added to liposome formulations, and stirred in 1 hours at 4°C.(18), (19)

2.4. Characterization of insulin liposomes and chitosmes

2.4.1. Particle size distribution and zeta potential

Particle size and zeta potential of vesicles were measured by particle size analyzer and zeta analyzer. The mean droplet size of samples was determined at 25 ºC by scatter scope 1 quidix (South Korea) and zeta potential was determined by Malvern ZEN3600 (England). Each sample was measured three times.

2.4.2. Content of drug in vesicles

In order to increase the stability of the prepared vesicles placed at 0 ° C, then 10 ml of vesicles formulations containing insulin centrifuged at 25,000 rpm and 0 ° C for 40 minutes, and then the supernatant phase filtered and the diffused drug was determined by HPLC method using a C8 column and UV detector at 214 nm by mobile phase containing of water: Acetonirille: TFA (1: 34: 65). By subtraction of unloaded drug content from total drug amount, the major amount of loaded drug achieves. Drug entrapment efficiency is illustrated as percent of initial content of dug that was entrapped within liposome by the following equation:

$$Loading\ Efficiency\% = \frac{total\ drug - diffused\ drug}{total\ drug} \times 100$$

2.4.3. Physical stability of insulin liposomes and chitosomes

To investigate the stability, vesicles stored at 30 º C for 3 months and the change in particle size and loading amount of drug will be reviewed.

2.4.4. Investigation of drug release from vesicles

Franz diffusion cells with a cellulose membrane were used to determine the release rate of insulin from different formulations. The membrane was then clamped between the donor and receptor chambers. Diffusion cell was filled with 35 ml of buffer phosphate.

The receptor medium was constantly stirred by externally driven magnetic beads at 300 rpm throughout the experiment. 2 ml of solution containing insulin loaded liposome and chitosomes was placed in donor compartment. At predetermined time intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 8h), a 2ml sample was removed from receptor for spectrophotometric determination and replaced immediately with an equal volume of fresh receptor medium. Samples were analyzed by by HPLC method using a C8 column and UV detector at 214 nm by mobile phase containing of water: Acetonirille: TFA (1: 34: 65). The results were plotted as cumulative released drug percentage versus time.

2.4.5. In vitro stability of insulin

The stabilities of free insulin, insulin entrapped in the liposomes and insulin entrapped in the chitosomes in the mimicked conditions of the GI tract were studied as follows. The samples were incubated at 37 °C for 5, 15, 30, 45, and 60 min in equal volumes of simulated intestinal fluid at pH= 7.5. At a certain time, the reaction was stopped by using TCA precipitation technique. Proteins and non-proteins were recovered by centrifugation at 10,000 rpm and 4 _C for 5 min, and then the collected pellets were washed with cold acetone and methanol. The precipitated proteins were resuspended with a buffer solution at pH 7.0 and analysed by HPLC using the buffer as the eluent. Degradation of insulin was determined by HPLC by HPLC method using a C8 column and UV detector at 214 nm.(15)

2.4.6. Thermal analysis

Differential scanning Calorimetery (DSC) analysis was performed using DSC-1 Series Differential Scanning Calorimeter (Mettler -Toledo DSC, Schweiz). Approximately 10 – 20 mg of different liposome and chitosome formulations were accurately weighed in an aluminum pan and sealed for analysis. A heating rate of 5°C/min was employed in the temperature range of (+20) – (+200) centigrade.

2.4.7. Experimental design for preparation of liposomes and permeation

Full-factorial design was used concerning with 3 variables at 3 levels. Lipid weight ratio of lecithin: cholesterol, lipid/ polymer ratio (L/P) and the amount of insulin were chosen as independent variables (Table 1). Dependent variables were including drug loading, particle size and drug release. Components of liposomal and chitosomal formulations are presented in Table 2.

Table 1. Independent variables and levels

Variable	Low level	Medium level	Medium level
Lipid weight ratio of lecithin: cholesterol	6: 2	7: 2	8: 2
Lipid / polymer ratio	1:0	1:0.5	1:1
Amount of insulin	5	7.5	10

Table 2. Components of liposomal formulations

Formulation NO.	State in full-factorial design	cholesterol (g)	lecithin (g)	TMC (g)	Insulin (g)
1	+++	0/2	0/8	1	10
2	++x	0/2	0/8	1	7/5
3	++-	0/2	0/8	1	5
4	+x+	0/2	0/8	0/5	10
5	+xx	0/2	0/8	0/5	7/5
6	+x-	0/2	0/8	0/5	5
7	+-+	0/2	0/8	-	10
8	+-x	0/2	0/8	-	7/5
9	+--	0/2	0/8	-	5
10	x++	0/22	0/78	1	10

11	x+x	0/22	0/78	1	7/5
12	x+-	0/22	0/78	1	5
13	xx+	0/22	0/78	0/5	10
14	xxx	0/22	0/78	0/5	7/5
15	xx-	0/22	0/78	0/5	5
16	x-+	0/22	0/78	-	10
17	x-x	0/22	0/78	-	7/5
18	x--	0/22	0/78	-	5
19	-++	0/25	0/75	1	10
20	-+x	0/25	0/75	1	7/5
21	-+-	0/25	0/75	1	5
22	-x+	0/25	0/75	0/5	10
23	-xx	0/25	0/75	0/5	7/5
24	-x-	0/25	0/75	0/5	5
25	--+	0/25	0/75	-	10
26	--x	0/25	0/75	-	7/5
27	---	0/25	0/75	-	5

Results and Discussion

3.1. *Particle size distribution and zeta ptential*

The mean particle size, polydispersity index and zeta potential describe in table 3.

Table 3. Liposomes mean particle size and polydispersity index and zeta potential

Formulation NO.	State in full-factorial design	The mean particle size (nm)	PDI	Zeta potential
1	+++	606	0/44	5.38
2	++x	587	0/47	5.31
3	++-	563	0/35	5.36
4	+x+	486	0/36	-1.15
5	+xx	463	0/29	-1.11
6	+x-	458	0/38	-1.11
7	+-+	391	0/19	-25.33
8	+-x	386	0/41	-25.31
9	+--	369	0.36	-25.27
10	x++	593	0.42	4.63

11	x+x	589	0.28	4.66
12	x+-	567	0.39	4.61
13	xx+	521	0.47	0.76
14	Xxx	517	0.31	0.68
15	xx-	503	0.44	0.69
16	x-+	304	0.29	-21.05
17	x-x	303	0.37	-21.07
18	x--	286	0.33	-21.01
19	-++	595	0.46	5.89
20	-+x	591	0.44	5.67
21	-+-	581	0.28	5.86
22	-x+	507	0.36	1.36
23	-xx	516	0.33	1.24
24	-x-	515	0.41	1.16
25	--+	362	0.29	-14.22
26	--x	382	0.32	-14.25
27	---	377	0.47	-14.21

Particle size ranges was between 303 to 606 nm and poly dispersity index below than 0.5. It seems that method preparation was appropriate for liposome and chitosome preparation.

The results showed that there was significant difference between the formulations regarding their size. The particle size of formulations were increased when incorporated with trimethylchitosan (TMC) and increased amount of insulin. The results indicated that comparing the formulations, the surface charge increased significantly by increasing the amount of TMC, and decreasing the lipid ratio. In other words, increase in TMC content and decrease in lipid ratio leads to increase of the surface charge.

3.2. Loading capacity of liposomes and chitosomes

The loading efficiency percentage is important for evaluating therapeutic, pharmaceutical reasons and cost. The loading capacity describes in table 4**.**

Table 4.Loading capacity of liposomes and chitosomes.

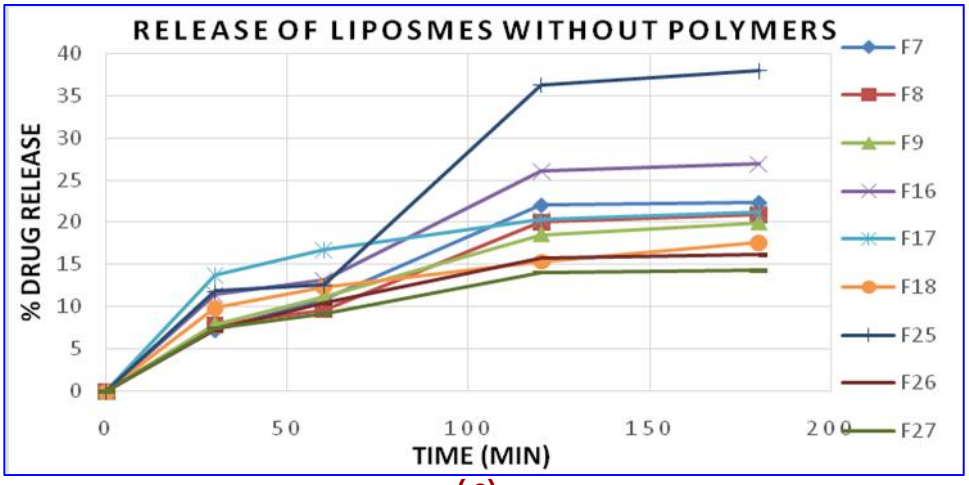
Formulation NO.	State in full-factorial design	Average percentage of loading efficiency
1	+++	65.71
2	++x	61.32
3	++-	52.69
4	+x+	51.16
5	+xx	47.26
6	+x-	44.36
7	+-+	42.35
8	+-x	37.32

9	+--	35.26
10	x++	60.16
11	x+x	59.87
12	x+-	54.49
13	xx+	58.13
14	Xxx	48.23
15	xx-	45.23
16	x-+	40.12
17	x-x	39.25
18	x--	35.23
19	-++	61.23
20	-+x	59.91
21	-+-	51.23
22	-x+	58.46
23	-xx	53.27
24	-x-	46.78
25	--+	51.62
26	--x	36.25
27	---	34.62

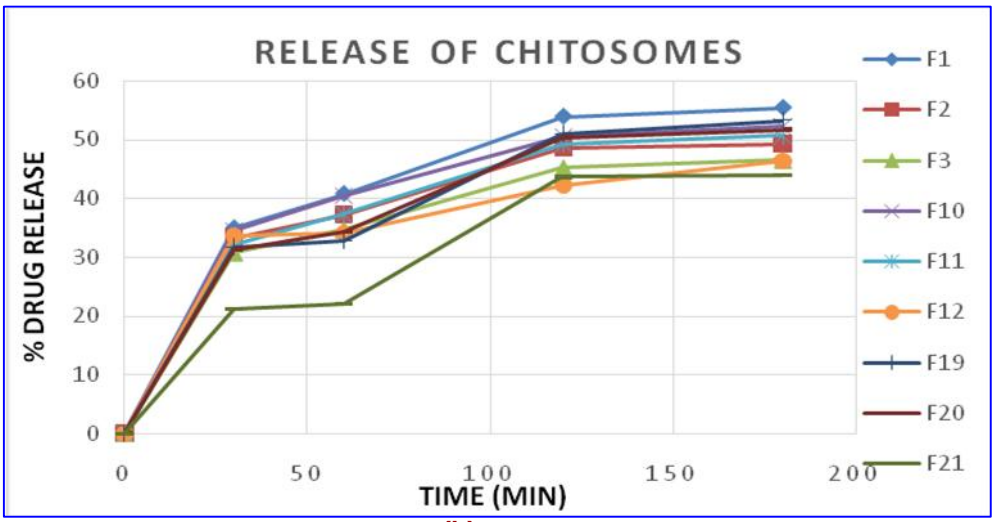
Insulin liposomes and their chitosomes loaded with a range of 34 percent to 65 percent. The maximum loading efficiency was shown by chitosome formulations. Results describe presence of TMC has a significant impact on loading efficiency and increase in amount of insulin leads to increase of drug loading. In addition, insulin is peptide macromolecule that indicates the main location for drug loading is the core of liposomes and chitosomes.

3.3. Drug release from liposomes and Chitosomes

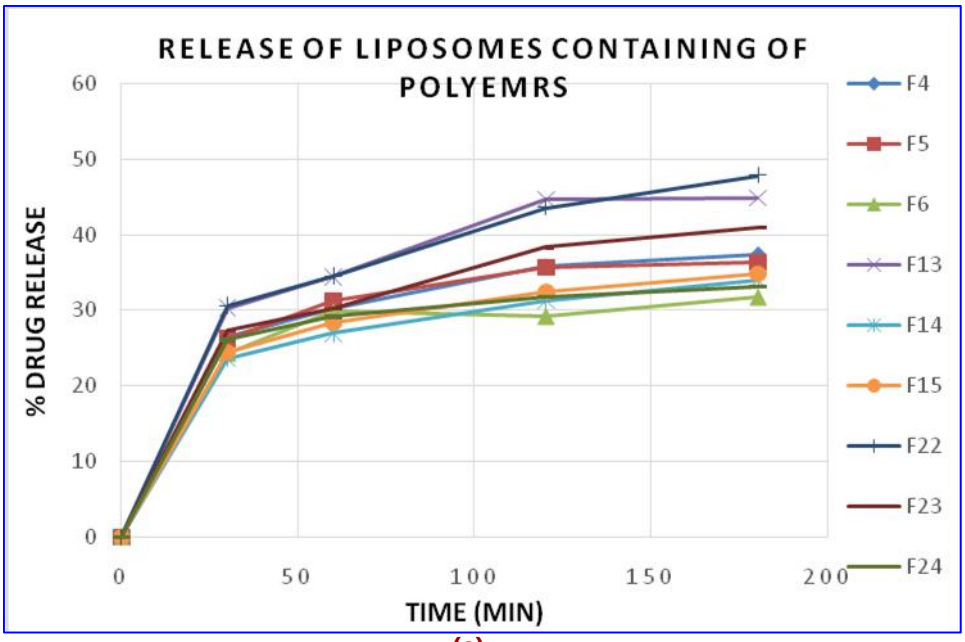
The recent study was carried out in phosphate buffer pH=7 and drug release was followed during 3 hours. In order to determine the effect of independent variables on drug release profile, drug release profile indicated in figure 1.



(a)



(b)



(c)

Fig 1.The Drug Release profile for (a) liposomes without polymers, (b)liposomes with polymers and (c) chitosomes.

The drug release profile of liposomes and chitosomes were good features. The drug release profile included two steps, rapid release and sustained. Drug release was affected by presence of TMC, and release profile was affected by amount of insulin, too.

Based on above figures, TMC has a significant direct impact on two step drug release. In other words, increase the loading efficiency leads to increase drug release from liposome and chitosomes.

In finally, results indicated drug release profile of chitosomes were prefer than the other formulations because drug release after 30 minutes is more than 30% and these could release to reach 60% in 3 hours with continuous speed.

3.4. In vitro stability of insulin

The stabilities of free insulin, insulin entrapped in the liposomes and insulin entrapped in the chitosomes were examined at 37°C in in simulated intestinal fluid (SIF) at pH 7.5 and it indicate in figure 2. The results show that insulin degradation essentially occurred during the first 10 min of incubation and little further degradation occurred up to 60 min. The degradation of free insulin was significantly higher than that of insulin entrapped in the liposomes and insulin entrapped in the chitosomes. However, the degradation of insulin when entrapped in the chitosmes was lower than when insulin was with the liposomes. These finding suggest that the chitosomes could potentially protect proteins from degradation by enzymes.

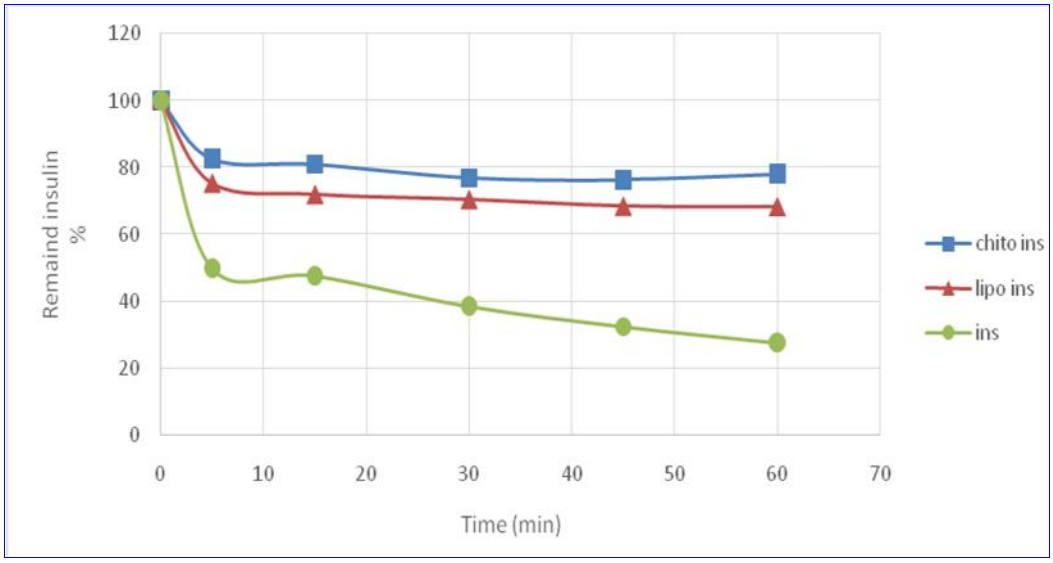


Fig 2. Degradation of insulin in simulated intestinal fluid at 37°C (pH 7.5) in presence of free insulin, insulin entrapped in the liposomes and insulin entrapped in the chitosomes.

3.5. Thermal analysis:

The thermogram of thermal analysis of liposomes and chitosomes containing of insulin in the heating program in range of 20 °C to 200 °C indicated in figure 3.

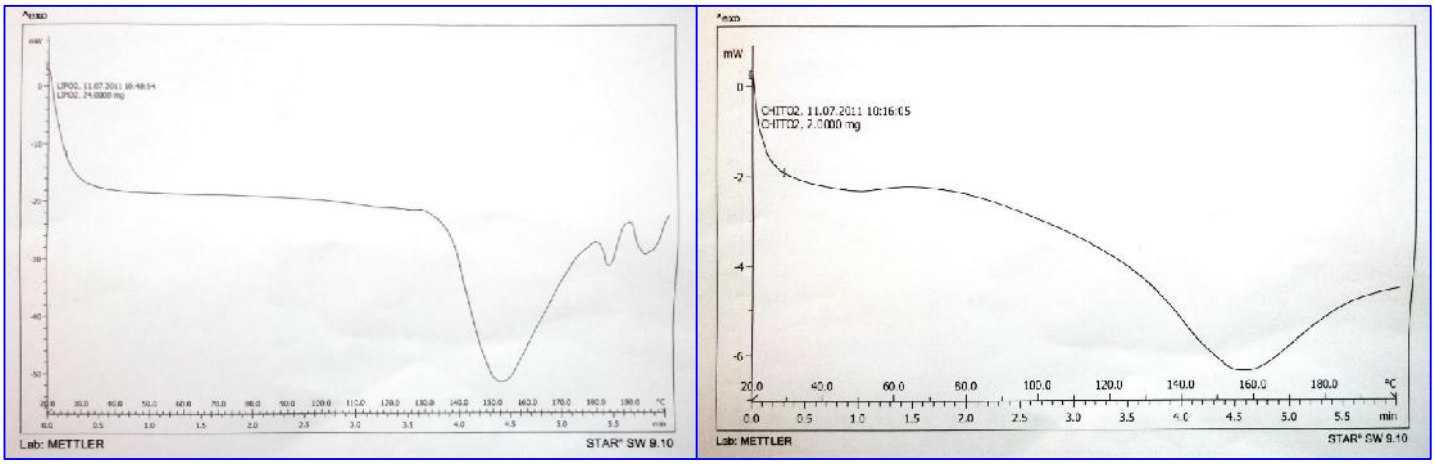


Fig 3. Thermogram of liposomes (left) and chitosome (right) containing of insulin

It is observed that all of formulation exhibits a peak endotherm in the range 140 °C to 160 °C, that this peak resemble in cholesterol thermogram. The broad peak in the chitosomes formulation, indicates hydrophilic and cationic nature of TMC. The peak of liposome was usually lower by 5–10 °C than this peak in chiotosome thermogram, that it due to difference between hydrocarbon chain of lipids and polar groups of TMC, that occur because of the molecular interaction lead phospholipid bilayer fluidity increased.(20), (21)

Conclusion

In conclusion, physicochemical properties of chitosome such as particle size, loading efficiency, and release profiles demonstrates chitosomes as a good carrier for insulin that can improve absorbance and efficacy of insulin.

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