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Evaluation of Thrombolytic and Antithrombin Activity of Rivaroxaban Loaded Liposomes

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Abstract:

The active drug loading approach, also called remote drug loading, involves loading the drug agent after empty liposomes are produced. The transmembrane gradient of pH or ion concentration is the driving force to promote the drug diffuse across the membrane into the inner core of liposomes. Based on the rank order performed for all conventional RIV liposomes formulae depending on their characterization and evaluation tests, one optimized liposome formulation was selected. From the *in* vitro drug release data, drug loading efficiency and particle size analysis formulae F4 were selected as optimized formulation. An *in vitro* study of the thrombolytic activity of liposomes was carried out using a similar procedure to that used for RIV. The results indicate that the formulation of RIV into liposomes doesn't result in a significant loss of activity *in vitro*. This might be due to the effect of polymers used in the formulation and the properties of liposomes like porous surfaces.

Key words: Rivaroxaban; Chromogenic Assay; Antithrombin Activity; Prothrombin time; Anticoagulant

1. INTRODUCTION

An anticoagulant, commonly known as a blood thinner, is a chemical substance that prevents or reduces the coagulation of blood, prolonging the clotting time. Some occur naturally in blood-eating animals, such as leeches and mosquitoes, which help keep the bite area unclotted long enough for the animal to obtain blood [1]. As a class of medications, anticoagulants are used in therapy for thrombotic disorders. Oral anticoagulants (OACs) are taken by many people in pill or tablet form, and various intravenous anticoagulant dosage forms are used in hospitals [2]. Some anticoagulants are used in medical equipment, such as sample tubes, blood transfusion bags, heart–lung machines, and dialysis equipment. One of the first anticoagulants, warfarin, was initially approved as a rodenticide. Anticoagulants are closely related to antiplatelet drugs and thrombolytic drugs by manipulating the various pathways of blood coagulation [3]**.** Specifically, antiplatelet drugs inhibit platelet aggregation (clumping together), whereas anticoagulants inhibit specific pathways of the coagulation cascade, which happens after the initial platelet aggregation but before the formation of fibrin and stable aggregated platelet products. Common anticoagulants include

warfarin and [4] The use of anticoagulants is a decision based on the risks and benefits of anticoagulation. The biggest risk of anticoagulation therapy is the increased risk of bleeding. In otherwise healthy people, the increased risk of bleeding is minimal, but those who have had recent surgery, cerebral aneurysms, and other conditions may have too great a risk of bleeding. Liposomes are most often composed of phospholipids especially phosphatidylcholine, and cholesterol but may also include other lipids [5] such as those found in egg and phosphatidylethanolamine, as long as they are compatible with lipid bilayer structure. A liposome design may employ surface ligands for attaching to desired cells or tissues. To deliver the molecules to a site of action, the lipid bilayer [6] can fuse with other bilayers such as the cell membrane, thus delivering the liposome contents; this is a complex and non-spontaneous event, however that does not apply to nutrients and drug delivery. We searched the approved drug database published on the website of the FDA and EMA, and found that 14 types of liposomal products have been authorized. It should be noted that this list excludes generics, lipid complexes (e.g., Abelcet, Amphotec, and Onpattro), and nationally authorized liposomal products in Europe. Doxil (Doxorubicin HCl liposome injection) was the first liposomal product approved by the FDA in 1995. Among these marketed products, 43% of products were approved before the year 2000, and 57% of products were approved before the year 2010 [7].

2. MATERIALS AND METHODS

Chromogenic thrombin substrate received from Biophen, Tris-HCl buffer, Heparin (5000IU) received from Samarth life sciences etc.

2.1 Handling and storage of blood sample

In order to avoid decomposition of blood sample or other potential chemical changes in the drugs to be analyzed. Blood samples were being frozen immediately in deep freezer at (-70^{O}C) temperature upon collection and thawed before analysis.

The sample stored in portable deep freezer with temperature probe, when move from one facility to another facility

2.2 *In vitro* and *in vivo* studies

2.2.1 Selection of Rabbits

In vitro and *in vivo* studies were carried out on healthy white rabbits with an average body weight of 2400 ± 50 g. Rabbits were chosen due to the larger volume of blood required to perform the blood coagulation test and other parameters of the study. Rabbits were kept at a prerequisite temperature of 23 °C ± 2 °C with a humidity of 50–60%. Both control group and test group rabbits were kept in the same environment and provided with standard food. Steel-bottom cages were used to keep each rabbit separately, with free access to food and water [8].

2.2.2 Animal experimentation

Species: Rabbit (*Angora* rabbit breeds), Sex: Males and Females (Females will be nulliparous and non-pregnant), Body Weight Range: 2-3 kg at the start of treatment Number of Animals: 24* at receipt, No. of Animals/group: 06 animal in each groups Total No. of Groups: 04 Source: B. N University, Department of pharmacy, Udiapur. (CPCSEA Registration No 870/00/Re/S/05/CPCSEA)

Group I- Receiving control, Group II- vehicle placebo administration, Group III- Treated with trial optimized formulation-1, Group IV- Treated with trial optimized formulation-2

2.2.3 Blood Withdrawal Method

Healthy rabbits in the range of 2–3 kg were used for the collection of blood samples. Blood was drawn from the auricular marginal veins of both ears using a 22G to 25G needle [9]. Before starting the sampling, the fur on the ear was shaved and

the skin cleaned with alcohol. As the skin on the ear is very sensitive, it was anesthetized locally with a lidocaine-containing cream. The cream-colored spot was wrapped with a plastic sheet and a protective adhesive bandage. After 45 minutes, the full thickness of the skin was numb. Dilation of the vessel had been obtained by massaging the ear [10]. The needle was carefully inserted, and blood was collected in tubes. After the removal of the needle, cotton gauze was firmly applied to the site of venipuncture until the bleeding stopped. The rabbits were kept under observation for the next few hours to ensure that homeostasis had been completed [11].

2.2.4 Bleeding Time Estimation

Bleeding time in studied animals was estimated according to the modified method [12] briefly, a minor incision (5 mm long and 1 mm deep) was made in the central artery of ear after removal of hairs. The slit opening was dried using blotting paper after every 30 s till the bleeding was stopped.

2.2.5 Preparation of Plasma

The plasma was obtained by an optimized centrifugation method. The whole blood was collected into a commercially available anticoagulant treated tube (lavender top), and the tube was inverted 8–10 times for mixing of blood and anticoagulant. Then it was centrifuged at 3000 rpm for 15 minutes [13]. The supernatant liquid was transferred immediately into a clean polypropylene tube using a Pasteur pipette.

2.3 Antithrombin Activity

This study was carried out by screening method, chromogenic assay, and clotting time (PT and aPTT).

2.3.1 Initial Screening Assay

This assay was performed by adding 50µl of drug loaded liposomes to an equal volume of thrombin, incubating the mixture at 37^0c for 5 minutes, and then adding 50µl normal human plasma obtained from a normal volunteer in the laboratory. The clot time was determined by taking a reading of the optical density in the coagulation analyzer. The sample with the procedure was sent to Lalpath Laboratory, udaipur, for initial screening [14].

2.3.2 Chromogenic Assay

For specific elucidation of antithrombin activity, the ability of an drug loaded liposomes to specifically inhibit thrombin was determined using a chromogenic assay. The chromogenic assay produces a yellow color that absorbs light at 405 nm. It was performed by placing 50 µl of various concentrations (20, 40, 60, 80, and 100 mg/mL) of RIV-liposomes. Each concentrate was further diluted with 50 µl of Tris-HCl buffer (50mM, pH 8.0), blood plasma was added, and it was incubated at 37^0c for 10 minutes [15]. Then 50 µl chromogenic substrate was added, and the change in absorbance at 405 nm was monitored for 10 minutes. The comparison was carried out by using heparin, at 5000 units/mL (1 unit of heparin = 0.002 mg of pure heparin). The dose of heparin was calculated by using the dose conversion method HED to AED [16]. Animal dose (mg/kg) = HED (mg/kg) × Km ratio (H/A). The maximum rate of absorbance change was calculated and recorded. Antithrombin level (B) was computed as; B (%) = {(Ao – At)/Ao}100, Where B= Antithrombin activity, Ao and At= the absorbance of negative control and the test drug, respectively [16]. As a control, the solvent used for extraction was diluted in Tris-HCl buffer to an equivalent degree as the sample. This was used as a blank. Reaction rates less than the solvent blank were interpreted as indicating the presence of antithrombin activity [17].

2.3.3 Clotting Time Study

A clotting time study was carried out to examine the hemostatic efficacy of the Drug loaded liposomes. Anticoagulant activity at different concentrations (20, 40, 60, 80, and

100 mg/mL) was determined by measuring the clotting time (in seconds) of unfrozen blood plasma using an ACL Elite coagulometer, at the Lal path Laboratory. All measurements were carried out in triplicate [18, 19].

2.3.4 Prothrombin Time (PT) Assay

The 50µL normal citrated plasma was incubated with different concentrations of RIV for 5 min at 37 °C and clotting time was immediately recorded after the addition of 100 µL PT reagent. The same procedure was carried out with standard heparin [20].

2.3.5 Activated Partial Thromboplastin Time (aPTT) Assay

The 50 µL normal citrated plasma was incubated with different concentrations of RIV for 2 min at 37 °C. Then 50µL aPTT reagent was added and incubated for a further 3min at 37 °C. The aPTT clotting time was immediately recorded after the addition of 100 µL aPTT reagent, and the same procedure was carried out with standard [21].

3. RESULT AND DISCUSSION

3.1 Antithrombin Activity (*In Vitro*):

The Liposomes of RIV evidenced a maximum clotting time after 120 minutes of 70 sec and 290 sec at dilutions of 1/500 and 1/100, respectively, whereas the extract showed a maximum of 200 sec and prevented clotting at the same dilution.

Table 1: Clot Time by initial screening method

Sr. No.	Dose form	Dilution in 1L	Clot time (Sec.)				
		Reading after min.	5 min	30 min	60 min	90 min	120 min
1	Sterile water	Control	25	25	24	26	25
2	Liposomes	1/100	45	87	135	210	290
		1/500	29	35	44	58	75
3	Rivaroxaban	1/100	Prevented	Prevented	Prevented	Prevented	Prevented
		1/500	200	-	-	-	-
4	Heparin	547.2 IU	290	-	-	-	-

The result revealed that the Liposomes enhanced the effectivity of rivaroxaban and prolonged the clotting time. Liposomes after 120 min, at dilution 1/100 shows an approximate equal enhanced effect to that of standard heparin at dose 547.2 IU (after 5min).

3.2 Chromogenic Assay Method

Antithrombin activity of Liposomes of rivaroxaban was carried out and compared with that of the rivaroxaban and standard heparin. The output is given in mOD units/ minute, as shown in table 2.

Table 2: Comparison of antithrombin activity of rivaroxaban, Liposomes and Heparin

Concentration(mg/mL)		OD units/minute		% Antithrombin activity	
RIV	Weight of Liposomes equivalent to RIV	RIV	Liposomes	RIV	Liposomes
Control		0.37			
20	20	0.190	0.185	48.08	55.18
40	40	0.150	0.150	58.21	61.46
60	60	0.142	0.139	60.30	63.97
80	80	0.118	0.117	66.46	66.78
100	100	0.107	0.93	69.30	83.52
Heparin (547.2unit)		0.005		96.11	

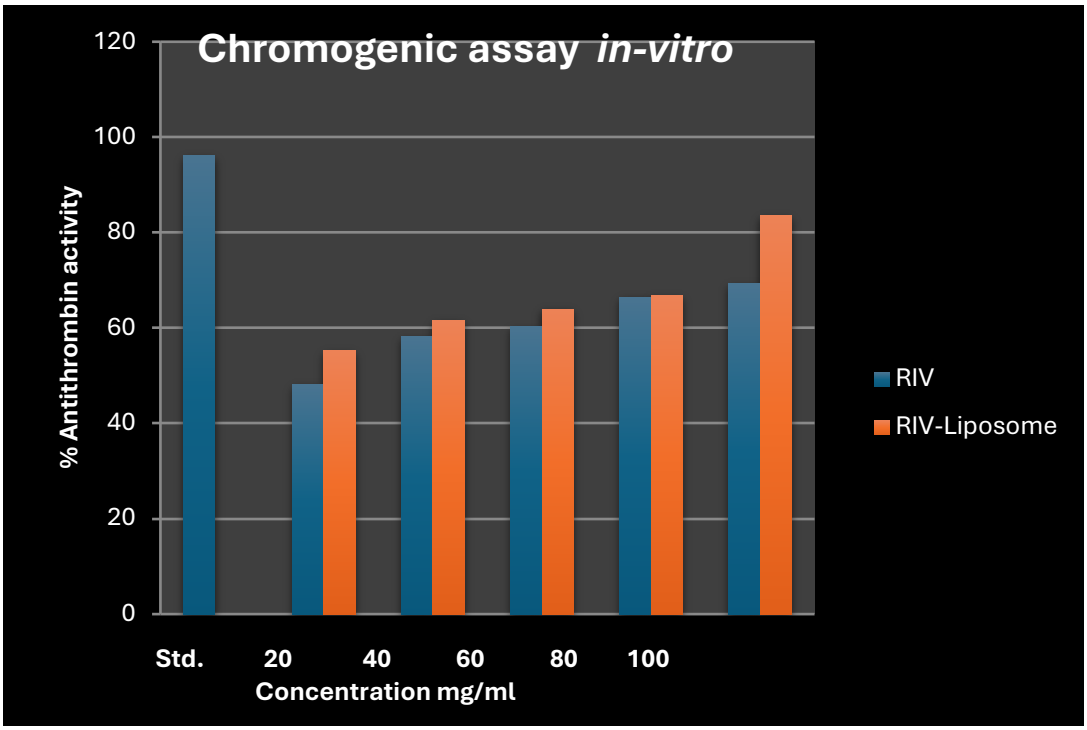

Figure 1: Comparison of antithrombin activity of rivaroxaban, Liposomes (F-4) and Heparin

By chromogenic assay, Liposomes of Rivaroxaban have shown 55.18%, 61.46%, 63.97%, 66.78%, and 83.52% antithrombin activity at 20, 40, 60, 80, and 100 mg/mL concentrations, respectively. Heparin has shown 96.11% antithrombin activity at 547.2 IU.

Liposomes of rivaroxaban at various concentrations showed approximately equal antithrombin activity to that of the rivaroxaban these results demonstrate that prepared Liposomes of rivaroxaban have significant antithrombin activity.

3.3 Clotting Time of Liposomes

The Liposomes of RIV and standard drug were tested for Prothrombin time (PT) and activated partial thromboplastin time (aPTT) activity.

Table 3: Prothrombin time (PT) and Activated partial thromboplastin time (aPTT) values of the RIV & Liposomes

Concentration (mg/mL)	RIV		Liposomes (Weight of Liposomes equivalent to RIV)	
	PT(Sec)	aPTT(Sec)	PT(Sec)	aPTT(Sec)
Control (0.0)	11.6±0.0	12.7±0.0	11.6±0.0	12.7±0.0
20	21.0±0.1	12.01±0.3	25.8±0.5	13.25±0.7
40	29.8±0.4	14.00±0.3	34.3±0.7	14.07±0.1
60	37.2±0.3	14.1±0.4	45.7±0.6	15.4±0.2
80	44.6±0.0	15.3±0.7	50.0±0.0	16.3±0.1
100	50.8±0.1	16.8±0.8	61.2±0.1	17.3±0.5
Std. Heparin (547.2 unit)	64.0±1.4	31.0±0.4	64.0±1.5	31.0±0.4

Clotting time (n=3) are expressed (in seconds) as the mean ± SD.

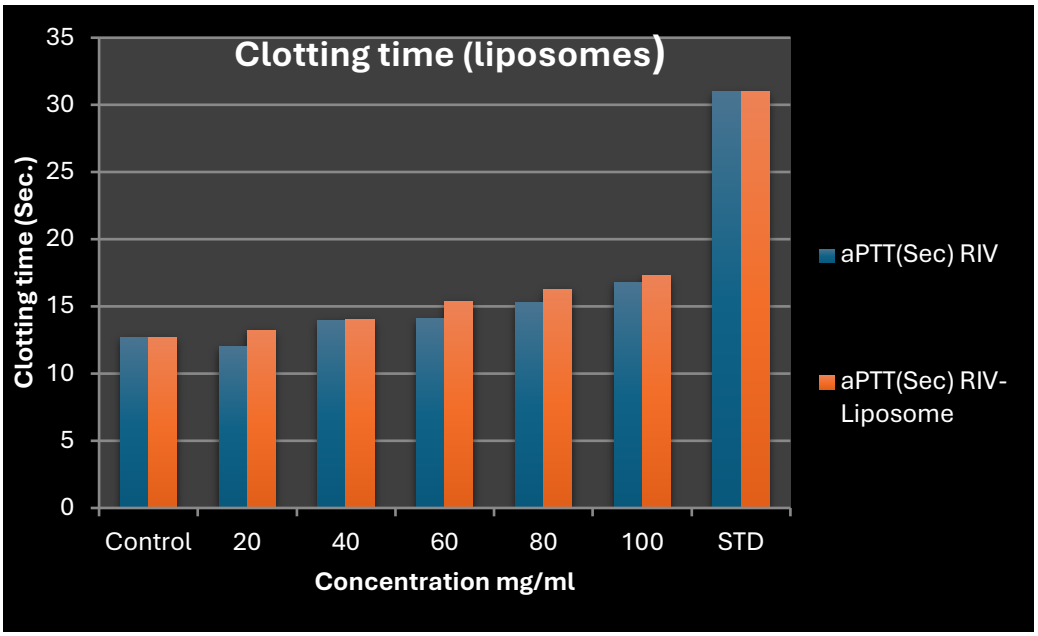

Figure 2: Clotting time of Liposomes (F-4), RIV and standards

The clotting time of Liposomes was determined by evaluating the PT and aPTT times of rabbit blood. The PT and aPTT times of Liposomes were 61.2% and 17.3% of that RIVand 64.0% and 31.0% of standard drug heparin, respectively, at the concentration of 100 mg/ml. Thus, it reveals that the Drug RIV slowly release from Liposomes, showing the extended activity of the drug.

3.4 *In Vivo* Study

This study was carried out by determining the bleeding time and clotting time of rabbit blood. The same procedure was used as mentioned in the Materials and method chapter.

3.4.1 Bleeding Time

The bleeding time of Liposomes is expressed in seconds. The result of bleeding time after ingestion of Liposomes is shown in table no. 4.

Table 4: *In vivo* study of bleeding time of the Liposomes

Days	Liposomes concentration (mg) to the equivalent weight of RIV	Bleeding time (sec)		
		Control	Test	Std. Heparin (547.2 unit)
Day 0	10	168.6 ± 17.36	252.0 ± 15.50	258.18± 08.09
Day 1	10	169.4 ± 10.36	288.0 ± 14.21	292.00± 7.00
Day 2	10	168.6 ± 12.36	298.0 ± 14.31	297.08± 05.09
Day 3	10	178.4 ± 11.36	329.1 ± 12.36	343.09± 10.28
Day 4	10	178.6 ± 12.10	353.0 ± 22.58	366.09± 09.08
Day 5	10	174.3 ± 09.23	356.4 ± 24.31	367.04± 08.04
Day 6	10	169.2 ± 20.80	357.0 ± 10.36	369.00± 08.00
Day 7	10	178.0 ± 08.20	357.2 ± 12.58	372.13± 5.28

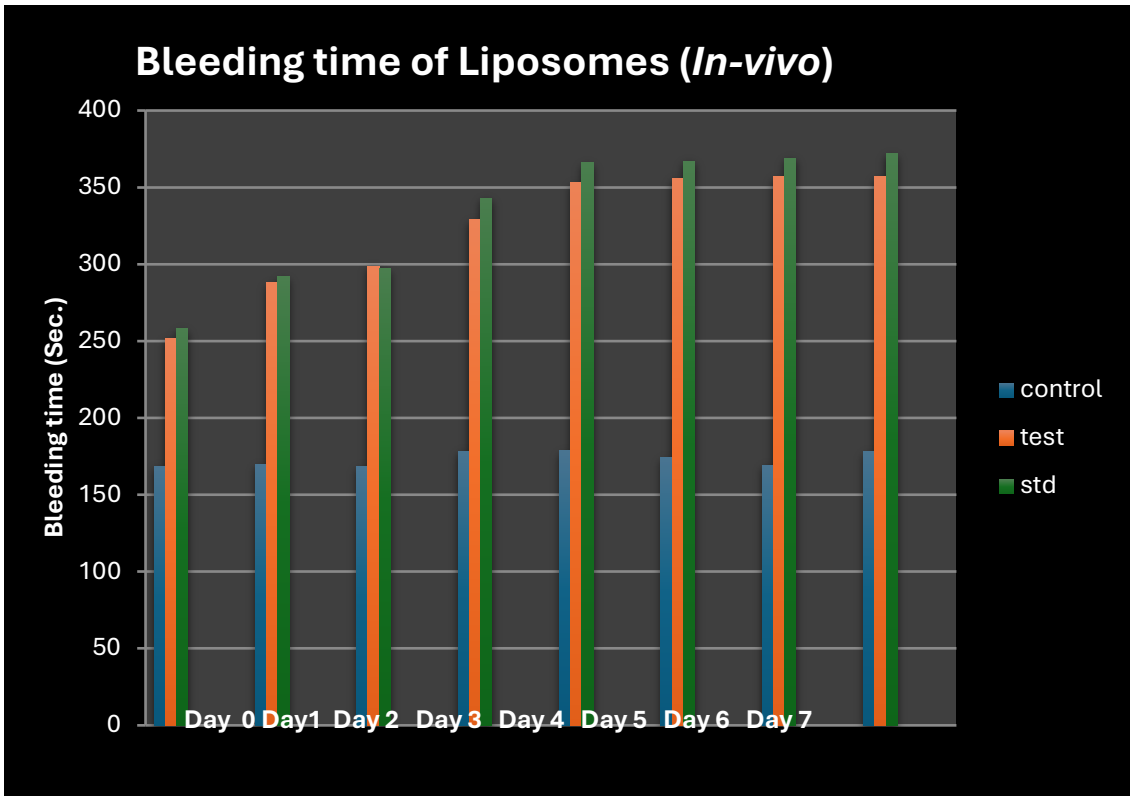

Figure 3: *In vivo* study of bleeding time of RIV & Liposomes (F-4)

The Liposomes of RIV prolonged bleeding time, indicating potential antithrombin activity. At day zero, the bleeding time was less than days 1 to 7. This study has revealed that the cumulative effect of RIV is dose-dependent. At day 7, the bleeding time of Liposomes was 96.05% of standard heparin. Hence, the study revealed that the Liposomes enhanced the effectivity of the RIV and had a cumulative effect.

3.5 Clotting Time

The clotting time of Liposomes is expressed in seconds. The clotting time of rabbit blood after ingestion of Liposomes is depicted in table no. 5.

Table 5: *In vivo* study of clotting time of the Liposomes

Days	Liposomes Concentration (mg) to the equivalent weight of RIV	PT(sec)		aPTT(sec)		Heparin(547.2 IU) daily upto 7 days	
		Control	Test	Control	Test	PT(sec)	aPTT(sec)
Day 0	10	10.6 ± 0.84	24.7 ± 0.3	13.5± 1.4	13.00± 1.4	44.00± 0.4	18± 0.4
Day1	10	10.0 ± 0.04	29.04± 0.7	13.1± 1.4	14.55± 1.4	50.10± 1.4	254± 0.2
Day 2	10	11.3 ± 0.31	30.3± 0.6	12.8± 1.4	15.07± 1.4	55.00± 1.0	27± 0.5
Day 3	10	11.21±0.53	34.7 ± 0.3	10.5± 1.4	15.4± 1.4	61.20± 0.7	31± 0.7
Day 4	10	12.3 ± 0.20	37.0 ± 1.4	12.5± 0.4	16.3± 1.4	67.19± 1.0	34± 0.6
Day 5	10	10.1 ± 1.18	38.09± 0.4	13.5± 1.4	18.00± 1.4	73.21± 0.6	37± 0.6
Day 6	10	11.21± 0.53	38.52± 8.4	12.2± 1.4	18.8± 1.4	78.09± 0.5	40± 1.0
Day 7	10	11.31± 0.33	39.04± 0.7	11.7± 1.3	20.2± 1.4	83.00± 1.4	41± 1.0

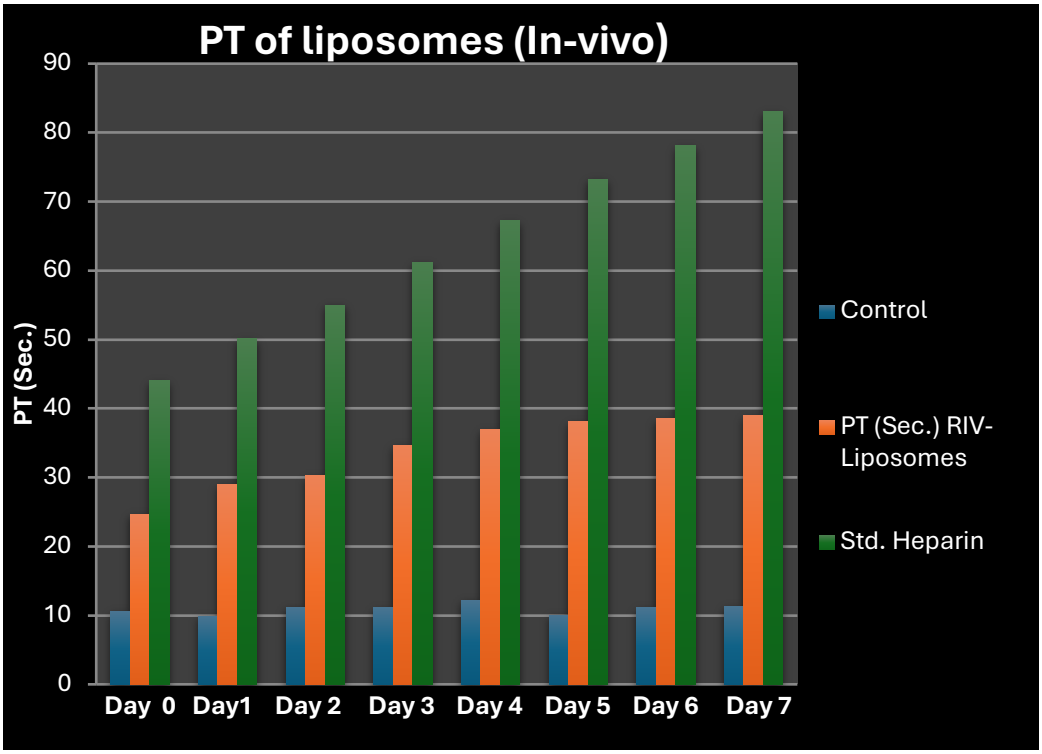

Figure 4: *In vitro* study of PT time of the Liposomes (F-4)

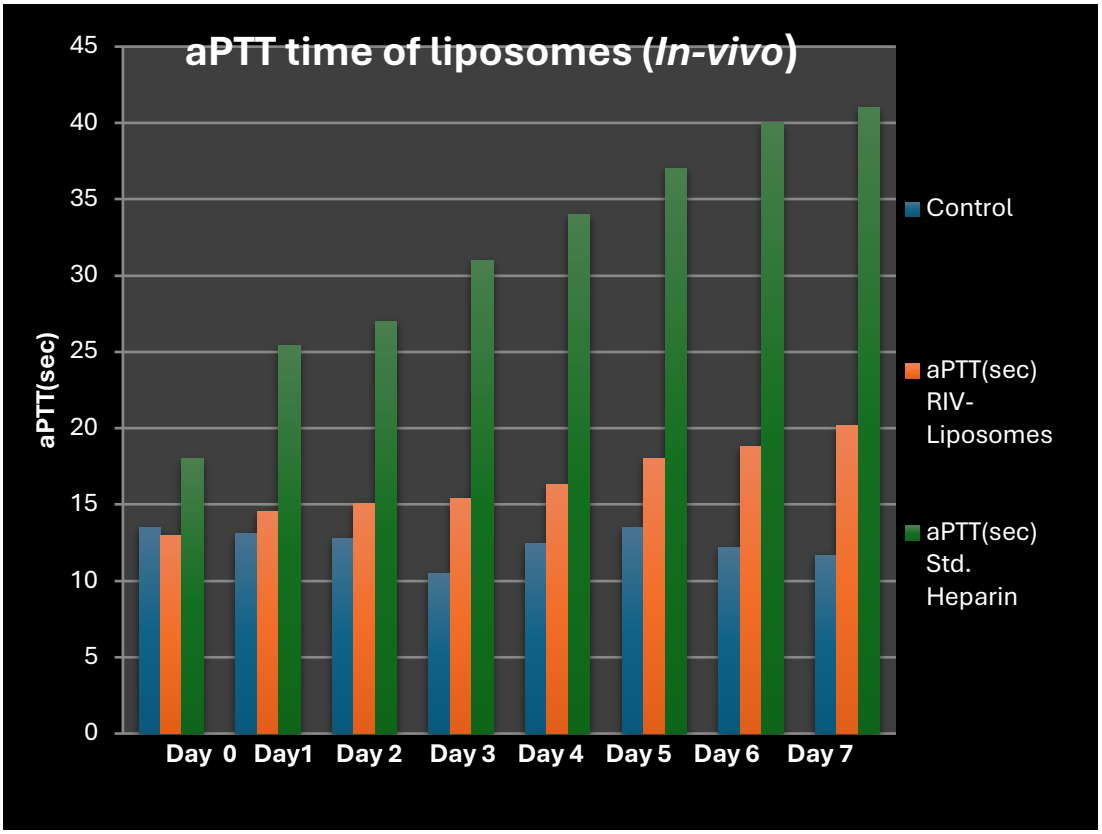

Figure 5: *In vitro* study of aPTT time of the Liposomes (F-4)

The Liposomes of *RIV* had an effect on the coagulation cascade by increasing PT and aPTT time. The liposomes cumulatively prolonged PT and aPTT values significantly at a concentration of 10 mg/ml once a day for 7 days. The result was compared with standard heparin (547.2 IU). The result has revealed that the clotting time of liposomes at 10 mg/ml was approximately 50% of that of standard heparin. Therefore, the concentration of liposomes could be increased to achieve the approximate clotting time of heparin.

3.6 Thrombolytic Activity

The thrombolytic activity carried out by measuring equivalent weight of liposomes to the RIV. The liposomes of RIV at concentration 10mg/ml shown 35.5± 1.20 % clot lysis after 24 hrs.

Table 6: Thrombolytic activity of the Liposomes

Equivalent Wt. of liposomes	Incubation Time	Clot Lysis % of RIV (Mean±SD)	Clot Lysis % of liposomes (Mean±SD)
10 mg/ml	6hrs	13.2±1.03	10.3 ±1.01
	12hrs	15.0 ±1.00	18.6±1.10
	24hrs	30.6±1.20	35.0±1.24

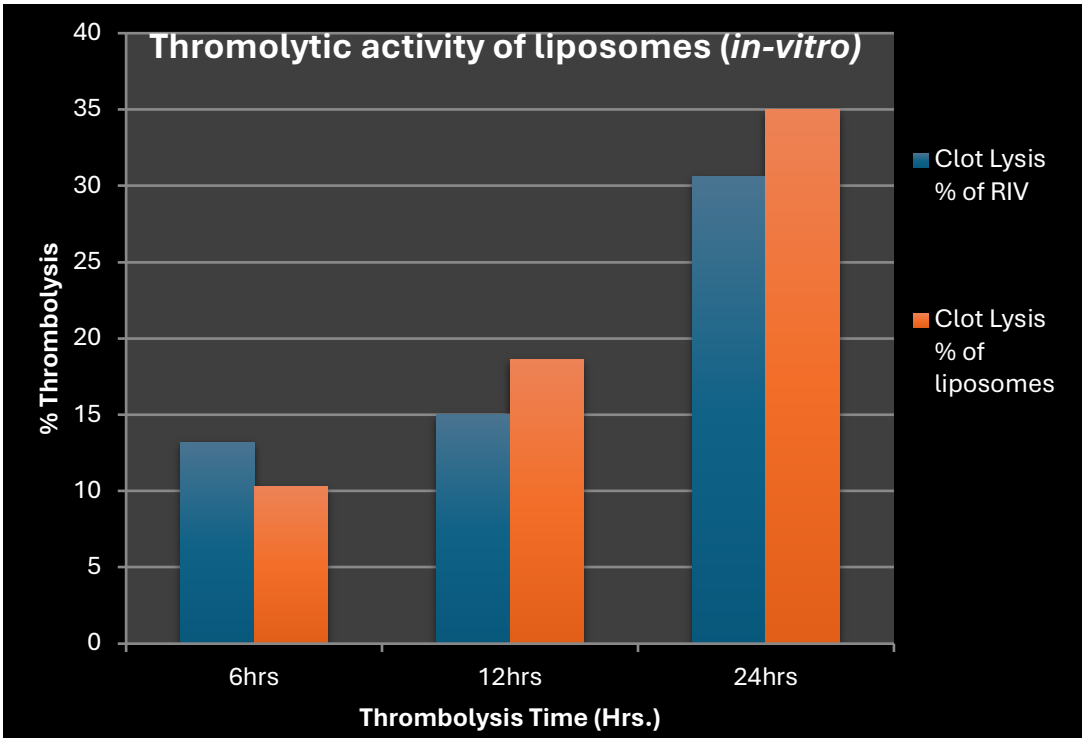

Figure 6: Thrombolytic activity of the RIV-Liposomes (F-4)

The percentage clot lysis of liposomes was compared with standard streptokinase (27353 IU), and *RIV* showed a significant effect. Liposomes found 30.6% thrombolytic activity of streptokinase. The results revealed that liposomes showed the maximum thrombolytic effect at the initial stage and extended slowly with time.

4. CONCLUSION

The study of antithrombin activity has revealed that the liposomes of RIV prolonged the bleeding time as well as both PT and aPTT values instantly after administration of dose and maintained the values for subsequent administration of liposomes, so they could be considered to have antithrombin activity with a significant effect.

REFERENCES

1. J. Hauptmann, T. Steinmetzer, H. Vieweg, P. Wikstrçm, J. Stìrzebecher, Pharm. Res. 2002, 19, 1027–1033.
2. T. Steinmetzer, A. Schweinitz, S. Kìnzel, P. Wikstrçm, J. Hauptmann, J. Stìrzebecher, J. Enzyme Inhib. 2001, 16, 241–249.
3. J. Stìrzebecher, D. Prasa, J. Hauptmann, H. Vieweg, P. Wikstrçm, J. Med. Chem. 1997, 40, 3091–3099.
4. T. Steinmetzer, B. Baum, A. Biela, G. Klebe, G. Nowak, E. Bucha, Chem-MedChem 2012, 7, 1965–1973.
5. A. Straub, S. Roehrig, A. Hillisch, Angew. Chem. Int. Ed. 2011, 50, 4574 – 4590; Angew. Chem. 2011, 123, 4670–4686.
6. B. Baum, M. Mohamed, M. Zayed, C. Gerlach, A. Heine, D. Hangauer, G. Klebe, J. Mol. Biol. 2009, 390, 56–69.
7. B. Baum, L. Muley, M. Smolinski, A. Heine, D. Hangauer, G. Klebe, J. Mol. Biol. 2010, 397, 1042–1054.
8. A. Biela, F. Sielaff, F. Terwesten, A. Heine, T. Steinmetzer, G. Klebe, J. Med. Chem. 2012, 55, 6094–6110.
9. S. Anabousi, M. Laue, C. M. Lehr, U. Bakowsky, C. Ehrhardt, Eur. J. Pharm. Biopharm. 2005, 60, 295–303.

10. H. Bakowsky, T. Richter, C. Kneuer, D. Hoekstra, U. Rothe, G. Bendas, Ehrhardt, U. Bakowsky, Biochim. Biophys. Acta, Gen. Subj. 2008, 1778, 242–249.
11. M. Petitou, V. Nancy-Portebois, G. Dubreucq, V. Motte, D. Meuleman, M.de Kort, C. A. A. van Boeckel, G. M. T. Vogel, J. A. J. Wisse, Thromb. Haemostasi 2009, 102, 804–810.
12. M. Reers, R. Koschinsky, G. Dickneite, D. Hoffmann, J. Czech, W. Stìber, J. Enzyme Inhib. 1995, 9, 61–72.
13. M. Petitou, C. A. van Boeckel, Angew. Chem. Int. Ed. 2004, 43, 3118–3133; Angew. Chem. 2004, 116, 3180–3196.
14. S. T. Olson, R. Swanson, M. Petitou, Blood 2012, 119, 2187–2195.
15. S. Shrivastava, T. Bera, S. K. Singh, G. Singh, P. Ramachandrarao, D. Dash, ACS Nano 2009, 3, 1357–1364.
16. D. Peters, M. Kastantin, V. R. Kotamraju, P. P. Karmali, K. Gujraty, M. Tirrell, E. Ruoslahti, Proc. Natl. Acad. Sci. USA 2009, 106, 9815–9819.
17. J. Myerson, L. He, G. Lanza, D. Tollefsen, S. Wickline, J. Thromb. Haemostasis 2011, 9, 1292–1300.
18. N. Vyavahare, N. A. Scott, S. R. Hanson, J. Kohn, J. Controlled Release 1993, 27, 165–173.
19. S. R. Hanson, L. A. Harker, Proc. Natl. Acad. Sci. USA 1988, 85, 3184 –3188.
20. J. W. Myerson, L. He, J. S. Allen, T. Williams, G. Lanza, D. Tollefsen, S. Caruthers, S. Wickline, Nanotechnology 2014, 25, 395101.
21. R. U. Palekar, J. W. Myerson, P. H. Schlesinger, J. E. Sadler, H. Pan, S. A. Wickline, Mol. Pharm. 2013, 10, 4168–4175.