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Preclinical assessment of 99mTc labeled liposome agents as an effective tracer in nuclear medicine

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Abstract

This study investigates the use of [99mTc] liposome agents for the assessment of nuclear medicine purposes. A variety of [99mTc] liposome formulations were compared with common lymphoscintigraphic agents including [99mTc] regular-sulfur colloid (SC), and [99mTc] human serum albumin (HSA) besides assisting the use of positive charged liposomes in rabbits. 10 male rabbits (2–2.5 kg) were anesthetized with ketamine:xylazineintramuscularly. Then they were injected with different [99mTc] liposome agents subcutaneously in the dorsum of each hind foot over the region of the metatarsals at the midline as well as intravenously. Dynamic (1 minute) scintigraphic images were acquired in a 256x1024 image matrix using a symbia gamma camera as early images andfor the first 60 minutes post injection. This was followed by testing the tissue biodistribution by calculating the percentage of the injected dose (% ID) per organ for each (99mTc) agent and studying the heart/liver, heart/bone marrow, and heart/kidneys ratio. All agents demonstrated good migration from the injection site to blood pool but not to lymphatic drainage. Agents were starting to clear out rapidly after 60 minutes. [99mTc] liposome imaging can be used to develop novel liposome compositions with improved cardiac diagnostic and drug delivery characteristics.

Key words: Liposomes, nuclear medicine, nanotechnology,nano colloid, human serum albumin, tracer

1. Introduction

Liposomes have useful properties that promote them for the use as a drug delivery system, particularly in the targeted administration in order to enhance efficiency and reduce related toxicity of the drugs as well as other therapeutic purposes*(1)*. However, few studies examined the lymphoscintigraphic agents added to liposomes as in an animal stage researches, it has supported the use of liposomes for the administration of lymphoscintigraphic agents. The reason behind was not only to detect the lymph nodes but also to inquire other findings that could be illustrated by using different lymphoscintigraphic agents*(2)*.Liposomes are an ideal vehicle of drug administration to allow certain agents to concentrate into specific targeted cells as many researches approved that several drugs have been linked to less toxicity when delivered in a liposome encapsulated formulation*(3)*.A liposome consists of a region of aqueous solution inside a hydrophobic membrane. Hydrophobic chemicals can be easily dissolved into the lipid membranes; in this way liposomes are able to carry both hydrophilic and hydrophobic molecules. While the extent of location of the drug will depend upon its physiochemical characteristics and composition of lipid. For the deliverance of necessary drug molecules to the site of action, the lipid bilayers fuse with other bilayers of the cell (cell membrane) to release the liposomal content. Liposomes are ideal structures for delivering therapeutic agents to the lymph nodes. Their ideal features are based on their size, which prevents their direct absorption into the blood, the large amount of drugs and other therapeutic agents that liposomes can carry, and their biocompatibility.For medications with a diagnostic or therapeutic index, a drug carrier is used to help in the drug delivery to increase the safety and efficacy of administration. William T. Phillips accomplished another research about the delivery of gamma-imaging agents by liposomes in 1999. It described the liposomes structure in order to deliver gamma imaging agents and how does the ability to modify the surface of the liposomes permits the customization of liposome formulations for each particular diagnostic use *(4)*.This research aimed to study the lymph node delivery system by adding liposomes to different lymphoscintigraphic tracers such as 99mTc human serum albumin (HSA), 99mTc sulfur colloid. Also study the effect of positive charged liposomes on the lymphatic imaging. Furthermore, in the present study, subcutaneous and intravenous injections were used to investigate other biodistribution of liposomes and lymphoscintigraphic combination.

2. Materials and Methods

2.1 Liposome preparation

Neutral multilamellar vesicles (MLVs) of liposomes were prepared by the thin film hydration method as described previously *(5, 6)*. Briefly, L-α-Dipalmitoyl phosphatidylcholine (DPPC) was dissolved in ethanol in a round bottom flask. The solution was shaken well for a few minutes then vigorous vortexing took place to assure complete solvation. The organic solution was removed gradually using a rotary evaporator (Re- 2010, Lanphan Zhengzhou, Henan, China) under vacuum produced by a circulating water aspiration vacuum pump (SHB-III, USA Lab Equipment, USA) in a warm water bath at a temperature above the phase transition temperature of the suspended lipid (50°C) at 60 rpm to produce a uniform thin film of lipid on the inner wall of the flask.The flask was then left under vacuum for 12 hours to ensure the evaporation of all traces of ethanol. The lipid film was hydrated with 10 ml tris buffer (pH 7.4 in 37°C) in a water bath at 50°C for 15 min at 60 rpm to form multilamellar vesicles (MLVs) serving as control (empty) liposomes.The flask was mechanically shaken for 1 h at 50 °C. Then the flask was flashed through with nitrogen stream and immediately closed. Parallel to the control DPPC, HSA-loaded liposomes were prepared following the same method as described using only aliquots of mass HSA at molar ratios to lipid 2:7. Cationic HSA liposomes were prepared by an addition of 1 mg of stearyl amine to the lipid composition to introduce a net positive charge at molar ratio = 1:7.

2.2.1. (99mTc) labeling of liposomes

All kits were checked for contamination using the paper chromatography test to ensure the absence of free technetium pertechnetate impurities, and the labeling efficiency was > 85 *(7)*. Sterile apyrogenic vacuumed vials were used. All filled vials were included stannous chloride and have been lyophilized. The solution was purged with sterile nitrogen gas for 20 to 30 minutes and then vials were stoppered and kept in refrigerator until use. All liposome agents were labeled with 99mTc. After generator elution, 814 MBq of 99mTc pertechnetate was diluted with 3 ml of saline. Aliquots of each (99mTc) liposome formulation (0.3 ml, 81.4 MBq) were prepared for the injection of each rabbit.

2.2.2. (99mTc) labeling of positive charged liposomes

After preparing positive charged liposomes, 99mTc pertechnetate was added. The kit volume after adding saline was 1.5 ml with activity equal 444 MBq. Aliquots of (99mTc) positive charged liposomes for each injection was (0.15ml, 44.4 MBq).

2.2.3. (99mTc) labeling of liposomes and sulfur colloid (SC)

A commercial kit of sulfur colloid (SC) and liposomes labeled with regular (99mTc) were prepared for injection one group of rabbits. It contained 777 MBq in 2 ml saline. Aliquot of each injection prepared was (0.2ml, 77.7 MBq).

2.2.4. (99mTc) labeling of liposomes and human serum albumin (HSA)

The (99mTc) was added to human serum albumin (HSA) and liposomes kit (555 MBq, 1.5ml saline). Aliquots of (99mTc) HSA and liposome (0.15ml, 55.5 MBq) were prepared for injection *(8)*.

2.3. Imaging studies

All proposed animal work has been reviewed and approved by the Institutional Animal Care and Use Committee**.** Animal experiments were conducted on ten male New Zealand white rabbits (2–2.5 kg) which were anesthetized with ketamine:xylazine cocktail (10 mg/kg:10 mg/kg) in the brachial muscle. An aliquot (0.3-1.5 ml) of each (99mTc) agent was injected subcutaneously in the dorsum of each foot over the region of the metatarsals at the midline. Dynamic (1 minute) scintigraphic images were acquired in a 256x1024 image matrix using a symbia gamma camera. The subcutaneous bleb was massaged gently for 5 minutes. At 60 minutes post injection, dynamic acquisition was halted.

2.4. Biodistribution

A biodistribution study was performed for all rabbits in the imaging study thus one-hour post injection imaging was acquired. Another imaging was involved for two rabbits injected intravenously in the marginal ear vein. After imaging the percentage of the injected dose (% ID) per organ for each (99mTc) agent was calculated by comparison with a standard aliquot of the respective (99mTc) agent.

2.5. Image analysis

For every rabbit, images were acquired at 1- and 60- minutes post injection. All images were corrected for background activity and analyzed. The baseline images were specified as the image was acquired at 1-minute post injection which was the first image acquired in the dynamic acquisition and represented the distribution from 0–1-minute post injection and also compared to the image that was acquired with 99mTc and manufactured kit of albumin nano colloid for each particular rabbit. For all images, the regions of interest weredrawn, after being corrected from decay, to calculate the percentage of injected dose per gram of tissue of organ %ID/g. The %ID in

the injection site of each rabbit foot was considered to be 100%. Also, the %ID in region of interest of the injection site at 60 minutes was calculated.

2.6. Statistical analysis

Values are reported as mean ± s.e.m. Other calculations were used to compare the heart-to-kidneys ratios, heart-to-liver ratios and heart-bone marrow ratios. Also, bladder activity was examined for each agent at a given time. Blood clearance and injection site curves were fitted to an exponential model with a consideration of radiopharmaceutical half-life. A p value <0.05 was considered to be statistically significant.

2. Results

All studies were performed on 10 male rabbits, images were acquired immediately after injecting them and 60 minutes post injection as whole-body images as shown in Figure(1).

1. ***Clearance*:** [99mTc] liposome agents have a significant amount of clearance in the bladder, as seen in Figure(2), and the site of the injection, in Figure (3), after 60 minutes. Also [99mTc] liposome agents were only faintly visualized and had poor retention in the popliteal node thus there was low uptake in [99mTc] liposome by the popliteal node at early images and 60 minutes images. Images were acquired for the injection site and bladder as an early image and after 60 minutes for all [99mTc] agents and ID% was analyzed. Overall at 60 minutes for all [99mTc] liposome formations the activity in the injection site was reduced compared to the earlyimages. Obviously, the clearance of [99mTc] positive liposome HSA was the greatest. It was observed that the clearance in the injection site of [99mTc] liposome was significantly different in early image compared to 60 minutes image. The (ID %) in 60 minutes images was (10.8±0.17%) leads to clearance(89%) in the injection site.

It was interesting to find the significant difference in the injection site clearance between early and 60 minutes images in [99mTc] labeled positive charged liposome HSA. The (ID% =13.5±0.2%) after 60 minutes resulting in clearance of (86%). Additionally, the bladder clearance in [99mTc] labeled positive charged liposome HSA early images were significantly different than 60 minutes images. The clearance was (65%) in 60 minutes images.

2. ***Cardiac activity retention*:**In order to assist the cardiac ejection fraction efficiency, a relationship was applied comparing the heart count rate to other organs such as liver, kidneys, and bone marrow to determine the cardiac function using different agents. All images using different agents combined with liposomes did not shown any lung

activity or lymph nodes. It was observed that the heart ratio compared to the other blood pool organs using different types of liposome formations was ≥ 1.

Table (1): Heart to liver ratio

The ratio of [99mTc] labeled positive charged liposome HSA early images were greater and significantly different than [99mTc] labeled liposome HSA. Generally, it was found that heart to liver ratio was the highest in early images of [99mTc] labeled positive charged liposome HSA compared to all other [99mTc] liposome agents Table(1).

Table (2): Heart to kidney ratio

Early images of heart to kidney ratio were uppermost in [99mTc] labeled liposome. In early images of [99mTc] labeled positive charged liposome HSA there was significant difference compared to [99mTc] labeled liposome HSA and [99mTc] labeled liposome. Delayed images of [99mTc] labeled liposome HSA and early images of [99mTc] labeled liposome nano colloid showed no significant difference ($P<0.05$) Table(2).

Table (3): Heart to Bone Marrow ratio

Heart to bone marrow ratio showed no significant difference between delayed images of [99mTc] labeled liposome HSA and early images of [99mTc] labeled positive charged liposome HSA. However, there was major significant difference between early and delayed images of all [99mTc] liposome agents. Early images of [99mTc] labeled positive charged liposome HSA was the highest in ratio whereas [99mTc] labeled liposome HSA was the lowest but > 1 Table(3).

Discussion

In this study, [99mTc] liposome, [99mTc] liposome nano colloid, [99mTc] positive charged liposome HSA, and [99mTc] liposome HSA was investigated. First of all, the labeling efficiency was determined by instant thin layer chromatography (ITLC) test of each [99mTc] liposome agent. A particularly interesting finding in this research was that there were remarkable results in the clearance of the activity in the bloodstream. Furthermore, there were significant differences between the injection site and between bladder activity in early images and 60 minutes post injection. Although [99mTc] liposome nanocolloid was the lowest in bladder activity clearance, it showed

significant difference an hour after the injection. It was noticeable that [99mTc] positive charged liposome HSA was the highest in bladder activity clearance compared to all other [99mTc] liposome agents. When comparing bladder clearance between [99mTc] positive charged liposome HSA with [99mTc] liposome HSA, clearly the positive charged liposome was the reason of the high blood pool clearance. Consequent blood retention activity is increased as the bladder clearance is increased too. This result will lead to better safety for patient and for the people exposed to this patient, because as fast as the radiopharmaceutical is cleared out from the patient's body, the remaining dose will be less. A further advantage is that preparing [99mTc] liposome was not difficult. It is widely available and affordable as well; it can also be prepared before the patient attends. Long term storage of the liposome by lyophilization is another positive feature. Using HSA and nanocolloid is easy and safe too, because they come in well packed, sterile, ready to use kits. It is believed that [99mTc] positive charged liposome HSA had higher heart to liver ratio when compared to [99mTc] liposome HSA, due to the presence of positive charged liposome. Obviously the highest in range between all agents was [99mTc] positive charged liposome HSA as heart to liver and heart to bone marrow ratio. According to a research project which was studying the liposomes distribution by comparing 99mTc HMPAO-labeled PEG-coated liposomes with 99mTchydrazinonicotinyl -HYNIC- labeled PEG-coated liposomes. The researchers conclude that abscess uptake was higher in 99mTc-HYNIC-liposomes, however it was less in kidneys. They also clarify that the reason behind low liposomes tissue retention was because of the liposome surface charge *(9)*.However, heart to liver ratio was not showing significant difference ($P<0.05$) as well as the kidneys. Thus, the clearance was slower comparing to [99mTc] labeled positive charged liposome HSA. In this trial, the same tracer (HSA) was used; accordingly, that is another proof of the effectiveness of positive charged liposome in cardiac studies. It must be noted that in this study two of the rabbits were injected with [99mTc] positive charged liposome HSA intravenously. A promising result was showing significant activity retention in the cardiac muscle, accordingly, it could be indicated for quantitative gated blood pool imaging. Overall in this study it was noticeable that the heart ratios in the early images are higher than 60 minutes post injection images; also, in all [99mTc] liposome agents the ratios were greater than one. These results indicate the rapid clearance in the blood pool when using [99mTc] liposome agents, for this reason [99mTc] liposome can play an important role in myocardial perfusion and function qualitative and quantitative assessment. Another benefit is that liposomes are not interacting with patient's other medications. This research found that [99mTc] liposome agents are in the blood circulation even with subcutaneous injection as with intravenous injection. As a result, it could be recommended for patients with

long standing hospital stay and multiple IV injections resulting in damaged vessels or vascular trauma. It was noted that all [99mTc] liposome formations tested were poorly retained by the lymph node and for this reason would not be useful for sentinel node detection in lymphoscintigraphy imaging. However, this feature could be ideal to detect lymph node tumors by retention of subcutaneously administered liposomes with specific tumor receptors attached to their surfaces in a particular lymph node, which was obscured by using the frequent [99mTc] tracers such as sulfur colloid because of the lymph nodes activity retention. Moreover, the liposome is a phospholipid just like the myoview tracer, sestamibi, thus it passes the cardiac cell membrane and causes activity retention *(10)*. The early washout of the liposomes is another advantage over other tracers. Accordingly, one day myoview protocol will be appropriate and more reliable. In addition, liposomes are costing less comparing to sestamibi and pyrophosphate PYP. Furthermore, liposomes agents could be useful for detecting GI bleeding because of its rapid clearance of the bowel activity. Due to the current circumstances and the surrounding of corona virus outbreak (Covid-19), future studies could fruitfully explore this issue further by using monoclonal antibodies in addition to positive charged liposomes to develop specific anti-viral against this newly emerging pathogen.

Further future long-term tests required for the use of positive charged liposomes 99mTc geared towards the development of more effective tracers, evaluate the efficiency, safety, and toxicity in different animal testing before starting with clinical trials, and comply with good manufacturing practices.

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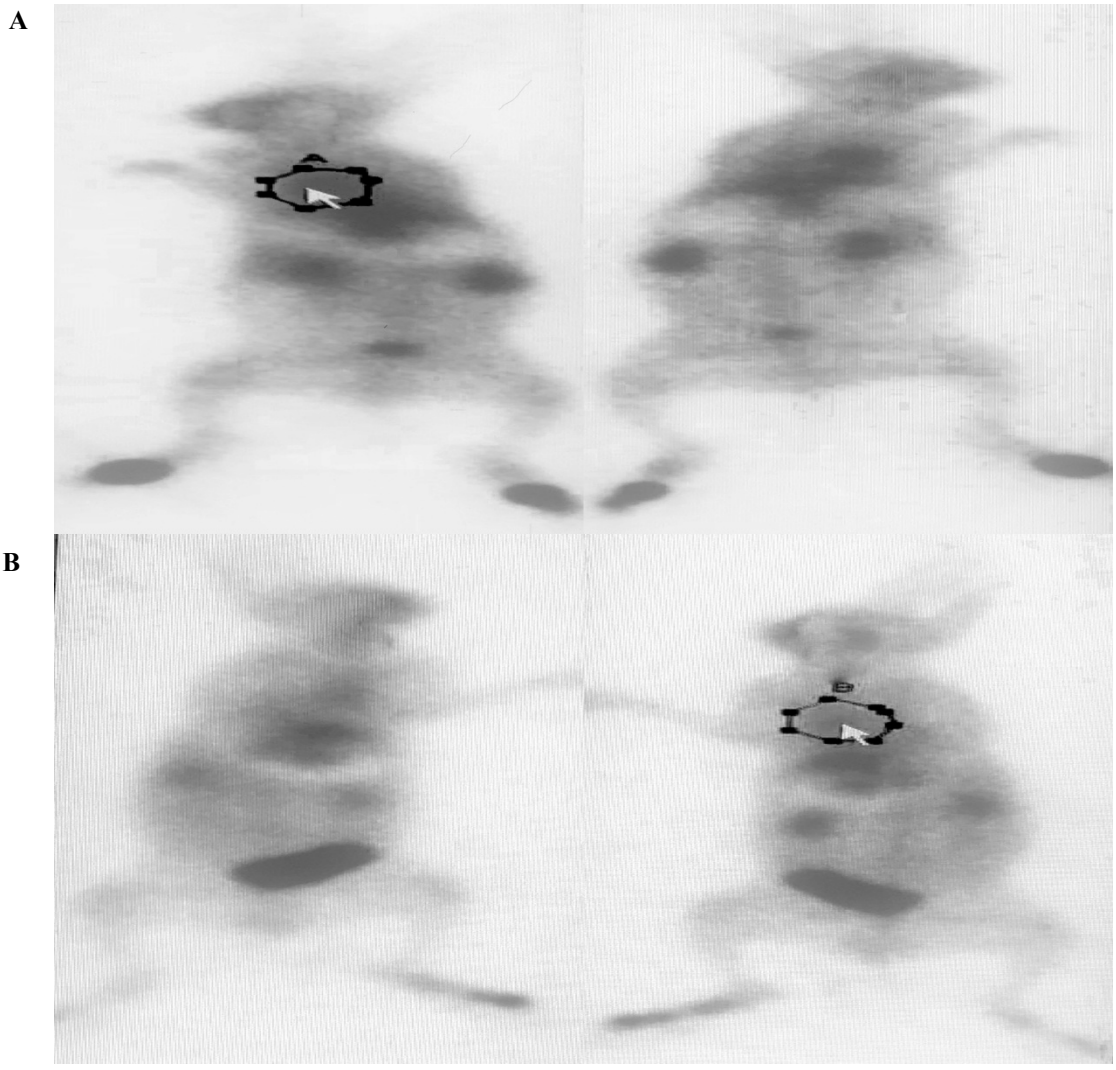

Figure(1) A. Anterior posterior early images of a rabbit injected with [99mTc] labeled positive charged liposome HSA. B. Anterior posterior 60 minutes post injection images of a rabbit injected with [99mTc] labeled positive charged liposome HSA.

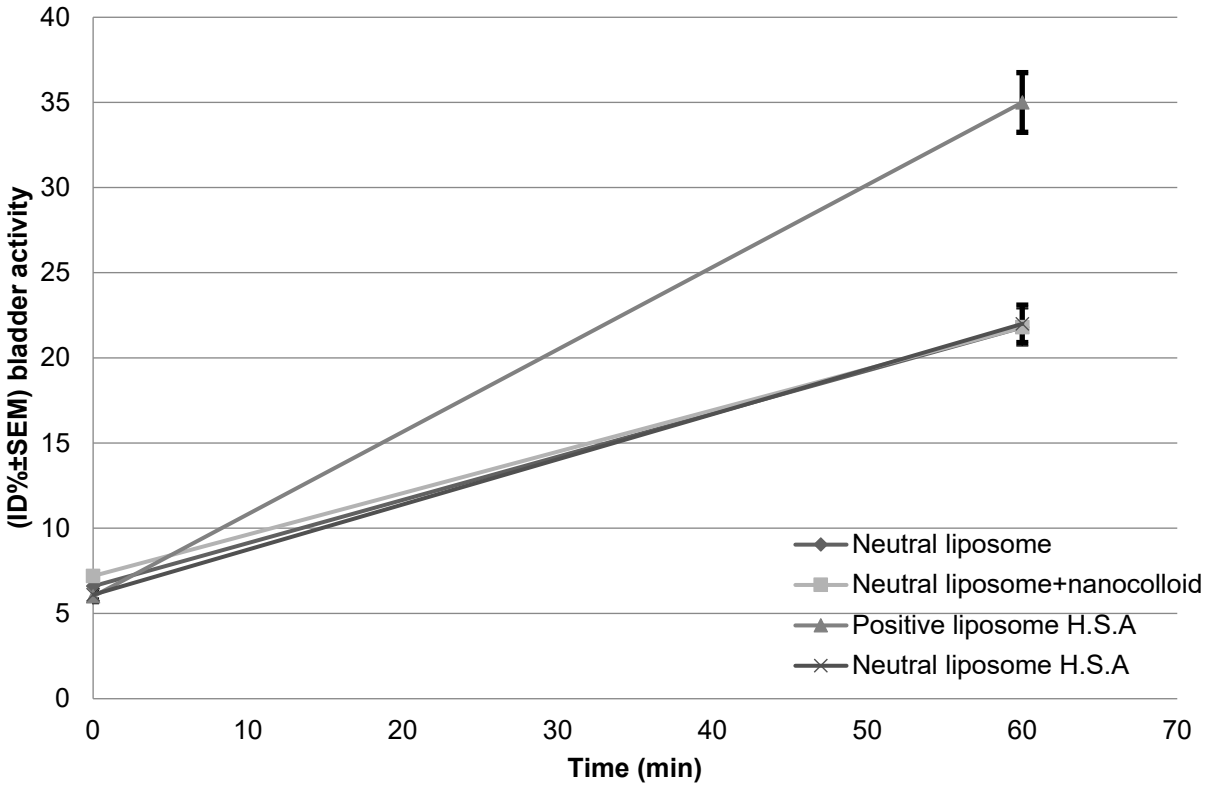

Figure (2): Bladder clearance according to injection time

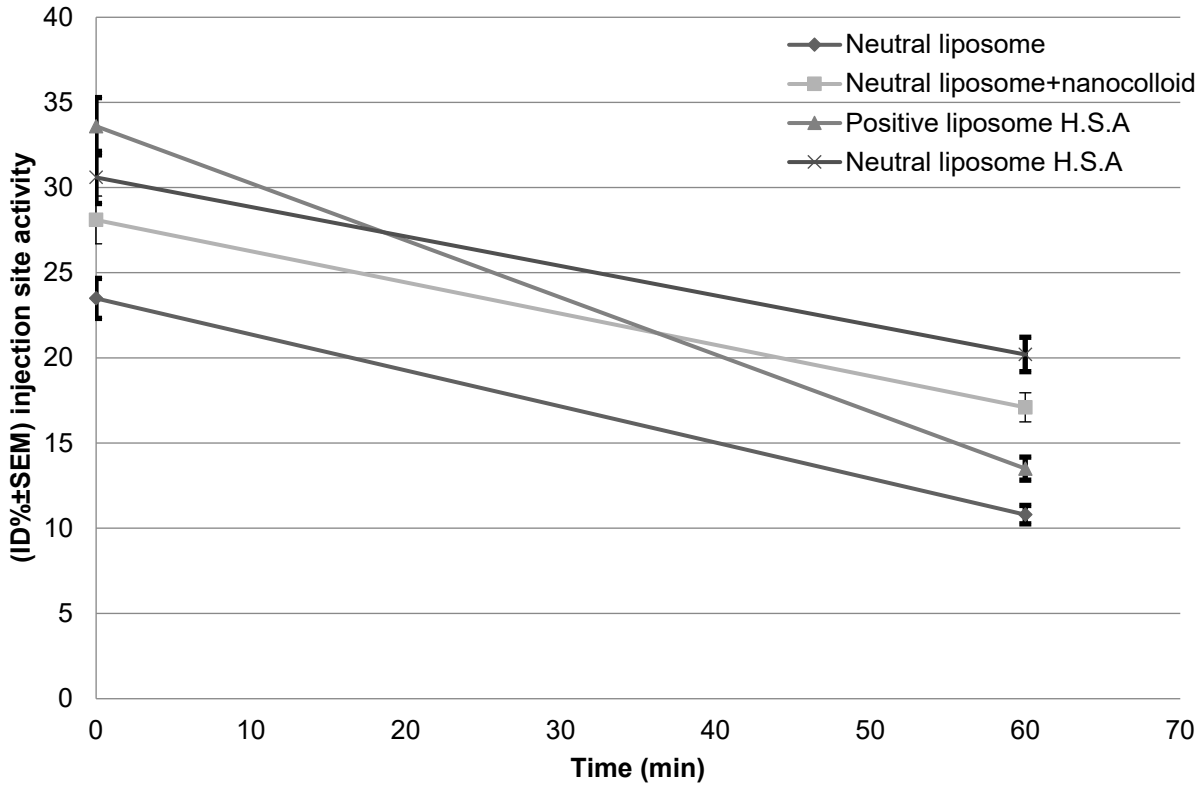

Figure (3): Injection site clearance according to injection time

Table (1): Heart to liver ratio

Time (min)	Liposome	Liposome,Nano colloid	+ve liposome, HSA	Liposome, HSA
early	1.15+-0.3$^{\infty}$	1.08+-0.3	1.4+-0.2	1.01+-0.2$^{\updownarrow}$
60	0.72+-0.1*	0.78+-0.1$^{\infty}$	0.7+-0.2$^{\infty}$	0.76+-0.2*$^{\updownarrow}$

*P < 0.01 versus liposome HSA

$^{\updownarrow}$P < 0.05 versus lipososme HSA

$^{\infty}$P < 0.05 versus liposome

Table (2): Heart to kidney ratio

Time (min)	Liposome	Liposome,Nano colloid	+ve liposome, HSA	Liposome, HSA
early	1.54+-0.5	0.77+-0.2*	1.2+-0.1	0.74+-0.1[↕]
60	1.08+-0.3	0.87+-0.3	0.87+-0.4*	0.58+-0.09*[↕]

*P < 0.01 versus liposome HSA

[↕]P < 0.05 versus lipososme HAS

Table (3): Heart to Bone Marrow ratio

Time (min)	Liposome	Liposome,Nano colloid	+ve liposome, HSA	Liposome, HSA
early	2.6+-0.8	2.07+-0.6	3.1+-0.5*	1.85+-0.3
60	1.92+-0.6	1.8+-0.6	1.4+-0.4	1.56+-0.3*

*P < 0.01 versus liposome HAS