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Our research focused on the preparation of vesicular drug delivery systems, such as liposomes, noisomes, and sphingosomes, for achieving slow release of entrapped proteins in the circulation to increase half-life, to mask immunogenic properties, and to protect against loss of enzymatic activity. We prepared, characterized, and monitored the biodistribution of three types of vesicular systems (liposomes, niosomes, and sphingosomes) containing streptokinase. For biodistribution stuides, radiolabelled streptokinase dispersions were injected into the ear vein of female rabbits in the weight of 2.5–3 kg weight. Following the application, rabbits were sacrificed, then organs of these animals were removed and radioactivity of organs was measured by well-type gamma counter. The comparison of the biodistribution results of the free streptokinase with the streptokinase vesicles showed that incorporation of the enzyme into the vesicles changed the biodistribution of the drug and by the entrapment of the streptokinase in the vesicles, thrombus uptake and imaging quality were improved.

The fibrinolytic system involving enzymes like streptokinase offers opportunities for the development of new radiopharmaceuticals for thrombus detection. The thrombolytic activity of these enzymes results from indirect activation of the fibrinolytic system following combination of the thrombolytic agent with plasminogen to form a plasminogen activator complex. In the presence of fibrin, this complex is converted rapidly to a streptokinase-plasmin complex that is responsible for the conversion of plasminogen to plasmin. Insoluble fibrin within the thrombus is lysed by plasmin to form fibrin degradation products.

Streptokinase is an antigenic bacterial protein produced by Lancefield group C β-hemolytic streptococcus having fibrinolytic properties. Streptokinase has been used as a fibrinolytic agent for several decades in the treatment of acute myocardial infarction, deep vein thrombosis, and other thrombosis-related pathogenis states with favorable results (Jackson and Tang 1982).

There are some limitations associated with the use of these enzymes for diagnostic or therapeutic purposes. The most important limitation is that they generally exhibit a short half-life. The pharmacokinetics of streptokinase was shown to be biphasic: a first phase with a half-life (t1/2) of 11 to 17 min that thought to be result of clearance by specific antibodies and the second phase with a t1/2 of ∼83 min thought to reflect nonantibody mediated clearance mechanism (Mentzer, Budzynski, and Sherry 1986).

Coates, DeNardo, and DeNardo (1975) studied the pharmacokinetics of I125-streptokinase and reported that this enzyme has restricted usefulness as a radiopharmaceutical for detecting thrombi because of its very rapid plasma clearance. They suggested to covalently modify the enzyme such that its kinase activity is retained but its immunological properties yielding rapid plasma clearance are eliminated.

Our research is focused on the preparation of vesicular drug delivery systems, such as liposomes, niosomes, and sphingosomes, for achieving slow release of entrapped proteins in the circulation to increase half-life, to mask immunogenic properties, and to protect against loss of enzymatic activity. In this study, we prepared, characterized, and monitored the biodistribution

of three types of vesicular systems (liposomes, niosomes, and sphingosomes) containing streptokinase.

MATERIALS AND METHODS

Different types of lipids were used to prepare vesicles: phospholipids (DMPC = dimyristoil phosphatidyl choline) obtained from Natterman GmbH (Germany), synthetic surfactants (SUR I = hexadecyl poly (3) glycerol) was obtained from L'Oreal (France) as a generous gift, and sphingomyeline (SPH) purchased from Sigma Chemicals (USA). The stability of the resulting liposomes, niosomes, and sphingosomes was increased by addition of stearylamine (SA) as a positive charge-inducing agent. To avoid leakage of the encapsulated streptokinase, cholesterol (CHOL) was added. Kabikinase and streptase were kindly supplied by Pharmacia & Upjohn, Hoechst-Marion Roussel, respectively. Streptokinase was purchased from Sigma Chemicals (USA). All other chemicals were of analytical grade.

Preparation Method

In this study, we prepared liposome, niosome, and sphingosome dispersions containing streptokinase by the film method described by Bangham, Standish, and Watkins (1965). Briefly, mixtures of the appropriate amounts of lipids in ethanol were dried by using rotavapor until a homogenous film was formed. Nitrogen stream was passed over the lipid film to remove the ethanol residue. This film was dispersed by streptokinase solution (120,000 units/ml) in 10 mM HEPES (pH 7.5) buffer containing % 0.8 NaCl. All dispersions were extruded through polycarbonate membrane filters with pores of $0.6\,\mu$m (once) and $0.2\,\mu$m (10 times). Then, the dispersions were subsequently freeze-thawed 10 times to increase the encapsulation capacity of vesicles. Nonentrapped enzyme was removed by ultracentrifugation (Beckman Instruments, California, USA) for 30 min at 55,000 rpm and 4°C. The pellet was subsequently redispersed in buffer.

Particle Size and Phospholipid Content

Mean particle size was determined by dynamic light scattering with a Malvern 4700 system. As a measure of the particle size distribution, the polydispersity index (PI) was used. PI can range from 0 (monodisperse) to 1.0 (polydisperse).

Phospholipid concentrations were assessed by a phosphate assay according to the Rouser method (Rouser, Fleisner, and Yamamoto, 1970).

Protein and Streptokinase Activity Determination

Protein determinations were carried out with a modified Lowry et al. method (1951). Briefly, lipid bilayer was destroyed by Triton X-100 and sodium dodecylsulphate (SDS) and final protein concentration represents total protein amount associated with vesicles after the removal nonencapsulated protein.

Streptokinase activity assay was carried out according to the previously described method (Jackson, Esmon, and Tang, 1980)

that was modified to allow quantification of liposomal streptokinase. Triton X-100 was used to distrup the bilayer. This detergent does not affect the enzyme activity (Corvo 1998).

Release of Streptokinase

The release studies were carried out in 10 mM HEPES (pH 7.5,%0.8 NaCl) with albumin. There is an interaction between liposomes and serum protein or plasma, and in vitro studies have been shown to enhance the leakage of solutes from vesicles (Landbrooke, Williams, and Chapman 1968).

Radiolabelling of Streptokinase with Tc-99m

For in vivo studies, first streptokinase was labelled with Tc-99m by tin reduction method (Dugan et al. 1972). Then, liposome/niosome/sphingosome dispersions containing radio-labelled streptokinase were prepared by film method. For labelling, 0.2 ml of $SnCl_2$ (1 mg·ml^{-1}) solution was added to streptokinase solution (120,000 IU·ml^{-1}) and 70–80 mCi of $^{99m}TcO_4^-$ (pertecnetate) was added to the mixture. After 1 hr incubation, free Tc was removed by passing through a Sephadex G5 column (Sigma Chemicals, USA).

After labelling, quality control of the binding was checked by miniaturized ITLC-SG plates (Gelman Sci, Germany) in saline and 85% methanol as a running solvent. At the end of the development procedure, the cut strips were measured in a well-type gamma counter (CDPC GamBit CR Gamma Counter, USA) for radioactivity .

Animal Thrombus Model

Before forming thrombus, rabbits were anesthetized with xylazine (12 mg·kg^{-1} Rompun®) and ketamine (50 mg·kg^{-1} Ketalar®) i.m. Right jugular vein was blocked by clamps to occlude venous flow. First, clamp was placed around the proximal end of the jugular vein and then placed around distal of the vein. To enhance clot formation, 100 μl thrombin (10 IU/100 μl) was injected into the middle portion of the vein. After 5 min, clamps were removed (Perkins et al. 1997).

Biodistribution Studies of Streptokinase Dispersions

For biodistribution studies, streptokinase solution was labelled with Tc-99 m and then liposome/niosome/sphingosome dispersions containing radiolabelled streptokinase were prepared. Particulate drug delivery systems such as liposomes were phagocyted by liver and removed from blood circulation in a short time after injection. Depending on preliminary scintigraphic studies, the highest organ uptake was observed in liver. To prevent this, in biodistribution studies liver was saturated with empty liposome/niosome/sphingosome dispersions.

For this purpose, 20 min after the application of 4 ml of empty liposome/niosome/sphingosome dispersions, radiolabelled streptokinase dispersions were injected into the ear vein of female rabbits at 2.5–3 kg weight. Following the application,

rabbits were sacrificed by injection of 2 ml of Lysthenon®forte (süksinil-bis-kolin klorid 100 mg/5 ml) intracardiacally at 1 and 4 hr. Then, organs of these animals (liver, lung, kidneys, spleen, heart, vein in natural forms [i.e., without thrombus]) and thrombus (vein having thrombus) were removed, and all organs were washed with saline to remove the blood and were weighted. In addition, 1 ml blood samples were taken to scan the activity remained in blood, then radioactivity of organs was measured by well-type gamma counter. Percent uptake per gram organs was calculated. The ratio between the activity in the jugular vein with thrombosis and the other jugular vein was calculated (called thrombus/vein ratio).

Scintigraphy Studies

First, liver was saturated with empty dispersions as described in biodistribution studies. Rabbits were anesthetized with xylazine (12 mg·kg^{-1} Rompun$^{®}$) and ketamine (50 mg·kg^{-1} Ketalar$^{®}$) intramuscularly to carry out the scintigraphy studies. Animals were placed under the gamma camera; 20 min after the application of empty dispersions, liposome/niosome/sphingosome dispersions containing radiolabeled streptokinase were injected into the ear vein of female rabbits having 2.5–3 kg weight and at predetermined intervals of 5, 10, 15, 30, 45, and 60 min following the injection, scintigraphic images were taken.

RESULTS AND DISCUSSION

Molar composition and codes of liposome/niosome/sphingosome dispersions containing streptokinase used in this study are given in Table 1.

Physicochemical properties of the vesicles are shown in Table 2. A slight difference in mean particle size was observed between the liposomes (0.15 μm) compared with the niosomes/shingosomes (0.19–0.20 μm). The entrapment efficiency for streptokinase ranged from 10–13%. We found that 70–84% of the initial activity of the enzyme was recovered in the preparations.

One of the important physical characteristics of particulate drug carrier systems is the drug release profile, i.e., the fraction of drug released from the dispersion as a function of time after administration of the system. In Figure 1, the release of streptokinase in 10 mM Hepes buffer with albumin at 37°C is shown. A burst-type release was found in the first hour for all dispersions. This burst type release might be caused by the charge inducer

TABLE 2
Physicochemical properties of streptokinase vesicles

Formulation	Size (nm)	Phospholipid concentration (μmol/ml)	Entrapment efficiency (%)
Liposomes	150 (0.1)	28 ± 2	11 ± 0.4
Niosomes	190 (0.2)	–	13 ± 0.1
Sphingosomes	200 (0.1)	23 ± 2	10 ± 0.1

Initial streptokinase concentration was 120.000 units·mL^{-1}. The size values represent mean ± SD of at least 6 determinations.

(SA) component incorporated in the vesicles. A phase of rapid release previously has been described for hydrophilic drug from charged liposomes. Hence, this effect may be the result of an inherent property of the bilayer or liposome structure or reflect loss of surface associated material (Taylor et al. 1990; Alpar, Bamford, and Walters 1981). In medium containing albumin, all three vesicle types showed a release up to 70–80% at the end of the 24-hr observation period.

For biodistribution studies, streptokinase was labelled with Tc-99m by tin reduction method. The labelling efficiency was found to be 86% for streptokinase. Our results are in agreement with Dugan et al. (1972). Liposome/niosome/sphingosome dispersions were prepared after the labelling of streptokinase. For this reason, stability of labelling was checked after preparation. Triton X-100 was added to the dispersions to lyse the bilayer, and labelling stability was checked again and found satisfactory ($p > 0.05$).

Liposome/niosome/sphingosome dispersions containing radiolabelled streptokinase were injected into rabbits via the ear vein. For comparative reasons, the biodistribution of iv injected free streptokinase was studied as well. Generally, the highest label concentration of liposomes and sphingosomes was observed in spleen and kidneys (Figures 2–4). The highest label concentration of niosomes was also observed in spleen. Kidney uptake, however, was found to be substantially lower (Figure 3).

Detectable amounts of label were recovered from the thrombi in all three vesicle types, with higher amounts at 4 hr than 1 hr. While the activity in vein, lung, heart, and blood decreased with time, it increased in liver and thrombus.

There are two reasons for the high label accumulation in the spleen. First, spleen has high phagocytic activity. Second, the

TABLE 1
Composition of vesicular systems containing streptokinase

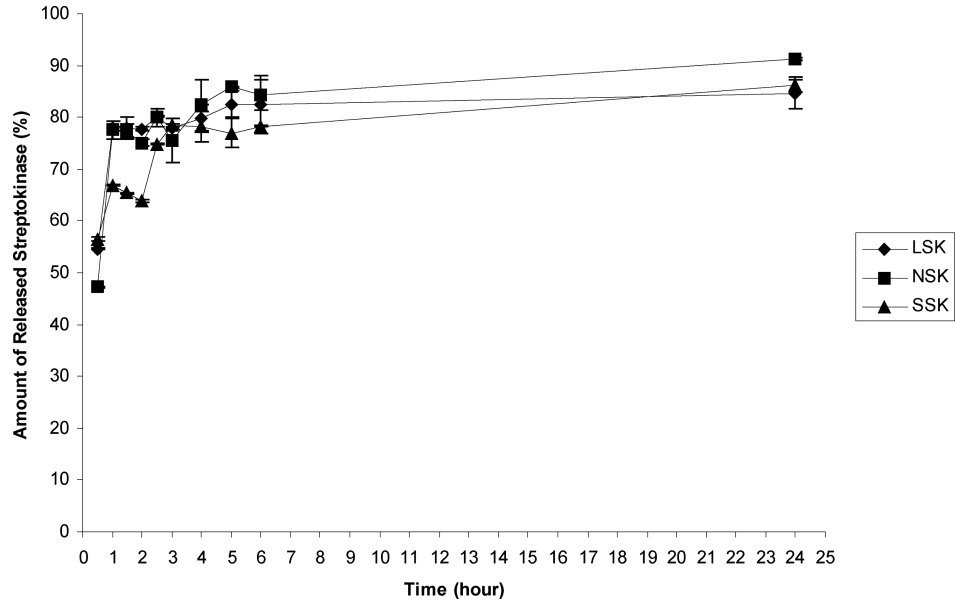| Formulation	Molar composition	Codes
DMPC:SA:CHOL + streptokinase	10:1:4	LSK (liposomes)
SUR I:SA:CHOL + streptokinase	10:1:4	NSK (niosomes)
SPH:SA:CHOL + streptokinase	10:1:4	SSK (sphingosomes)

FIG. 1.   Release of streptokinase from liposome/niosome/sphingosome dispersions incubated in 10 mM HEPES buffer with 0.02% albumin at 37°C.
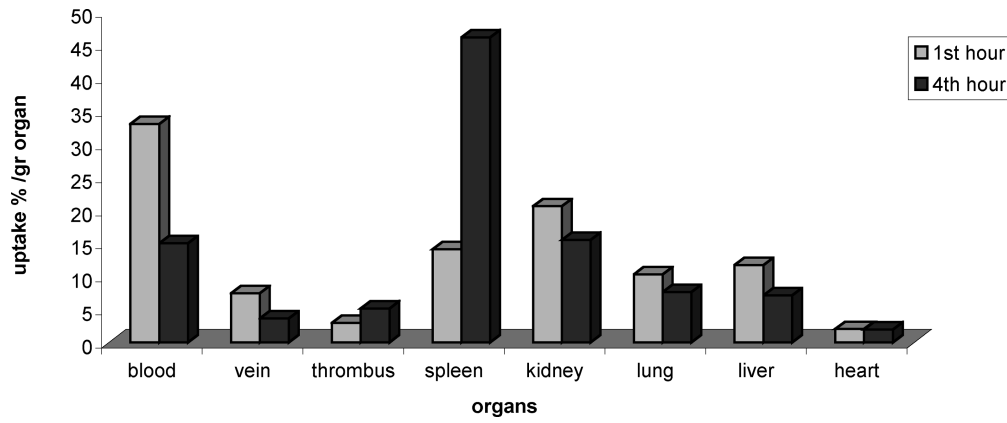

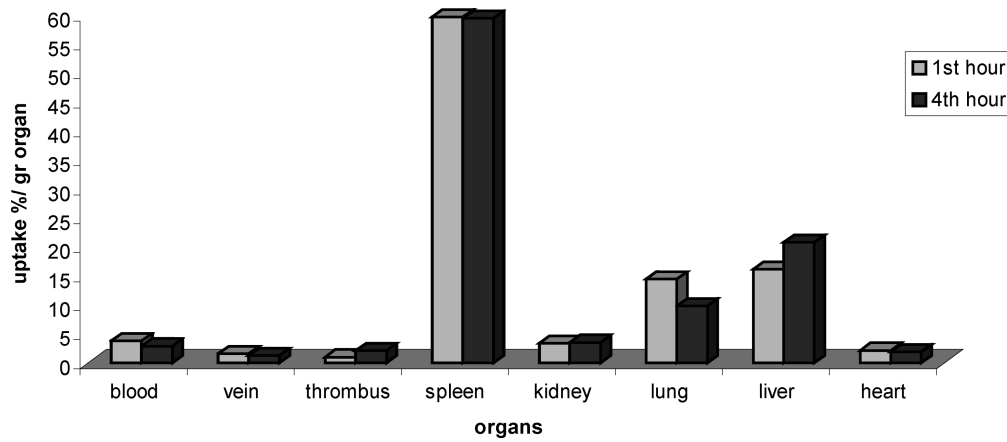FIG. 2.   Biodistribution results of streoptokinase liposomes ($n = 3$).

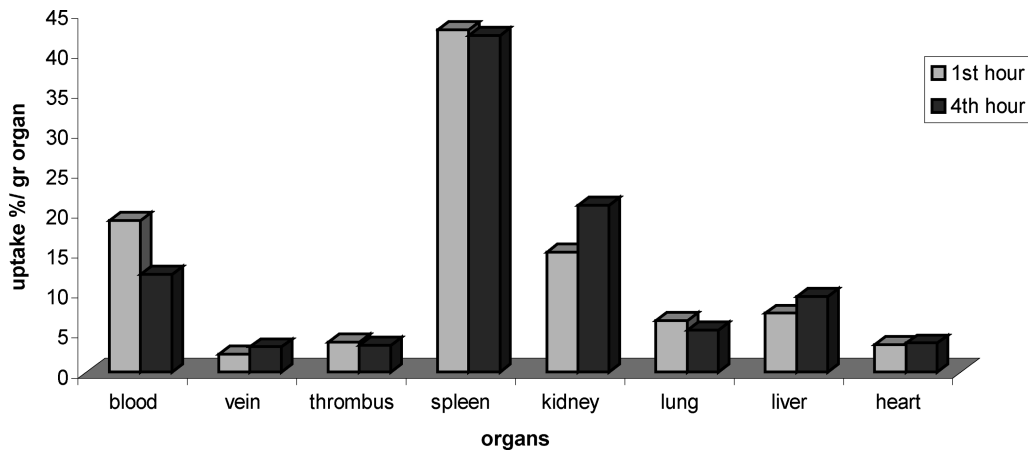FIG. 3.   Biodistribution results of streoptokinase niosomes ($n = 3$).

FIG. 4.   Biodistribution results of streptokinase sphingosomes ($n = 3$).

saturation of the liver may cause higher uptake by the spleen. Studies show that saturation of (RES) will not cause higher uptake of liposomes, it will only change the distribution of liposomes among the RES organs. The circulation time of liposomes in the blood can be increased by the saturation of RES (Betageri, Jenkins, and Parsons 1993). The high activity level in the kidney may be explained by the elimination of enzymes through kidney. When streptokinase is released from the vesicles, it will be metabolised in liver, then metabolite will be eliminated from the kidney. Therefore, higher activity can be found in the kidney. The increasing activity in the kidney in the course of time confirms this hypothesis.

Uptake of Tc-99m-labelled streptokinase by the thrombus can be explained by the mechanism of thrombolysis produced by streptokinase. This mechanism involves a series of reactions where streptokinase adsorbs to and penetrates in and around the thrombus; it activates plasminogen located within the thrombus, and yields sufficient plasmin for fibrin dissolution and thrombus lysis (Dugan et al. 1972). Because of adsorption and penetration properties of streptokinase in thrombi, 99mTc-labelled streptok-

inase was accumulated in thrombus, and by this way thrombus can be imaged by scintigraphy.

Liposomal encapsulation would provide protection to streptokinase from inactivation by plasma components and prolong the plasma circulation time. In addition liposome accumulation in the areas of myocardial infection has been reported which suggesting that liposomal encapsulation also would improve clot selectivity (Perkins et al. 1997).

Free radiolabelled streptokinase also was administered to the rabbits. The highest label concentration was detected in the kidneys. While the activity in blood, vein, and heart decreased, the activity in liver, kidneys, and spleen increased in time. The highest label concentration in thrombus was measured at 1 hr and then, decreased at 4 hr. Activity in control jugular vein decreased in parallel to thrombus uptake (Figures 2–5).

The comparison of the biodistribution results of the free streptokinase with the streptokinase vesicles showed that incorporation of the enzyme into the vesicles changed the biodistribution of the drug. Kidney was the second organ from uptake of the drug point of view. There is an entrapment of the drug in the outer
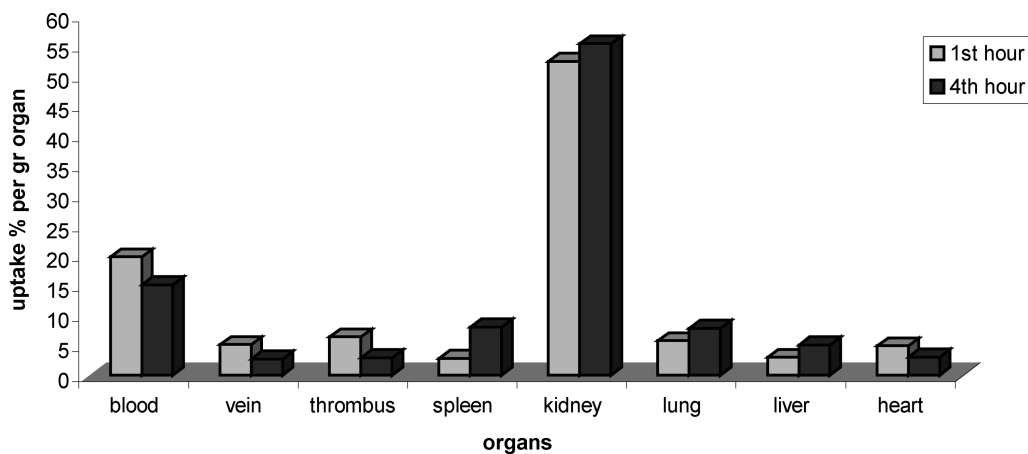

FIG. 5.   Biodistribution results of free streptokinase ($n = 3$).

TABLE 3
Thrombus/vein ratio obtained from biodistribution studies of
dispersions containing streptokinase and free streptokinase

Formulations	Thrombus/Vein	
	1st hr	4th hr
Liposomes	0.394	1.664
Niosomes	0.639	1.420
Sphingosomes	1.674	1.060
Free streptokinase	0.973	0.862

layer of vesicles, and the primarily released drug goes to the kidneys. Surprisingly, the localization in the kidney was low for niosomes. The free enzyme targets the thrombus as good as the vesicle-bound enzyme. However, thrombus/vein ratio is lower.

Another important parameter for the biodistribution results was the comparison of the thrombus/vein ratio (Table 3). With entrapment of streptokinase in the vesicles, thrombus uptake and imaging quality were improved and at 4 hr after administration, a higher thrombus/vein ratio was obtained when compared with free streptokinase ($p < 0.05$).

After the injection of dispersions containing radiolabelled enzyme, thrombus/vein ratios were calculated, according to biodistribution results. These values were obtained in the range 0.8–0.9

for free streptokinase. Thrombus/vein ratios increased to 1.7 by the encapsulation of enzymes into vesicles.

Scintigraphic imaging studies of Tc-99m-labelled dispersions containing streptokinase were carried out. At 20 min after injection of empty dispersions, Tc-99m labelled drug loaded dispersions were injected and scintigraphic images were taken at predetermined intervals.

Although the thrombus/vein ratio was found to be almost the same for all types of vesicles, positive scintigraphic images of thrombi could only be obtained in the liposomes. Thrombus started to come into view in a few minutes after injection and quality of images increased in time. Of 3 rabbits, with thrombus, 2 showed a positive result (Figure 6). Our results are in agreement with Sam, Rhodes and Bell (1975) reported that biodistribution studies of streptokinase were changeable and reproducible results could not be obtained. They indicated that stabilizing agents and streptococcal antibodies caused these results (San et al. 1975). Thrombus cannot be imaged by free streptokinase. Although the enhanced uptake was observed in the jugular vein formed by experimental thrombus in biodistribution studies, it was insufficient to be detected with naked eye when compared with the other jugular vein (control area) on scintigraphic images.

The aim of vesicle incorporation of streptokinase is to use the potential microreservoir function for entrapment enzymes
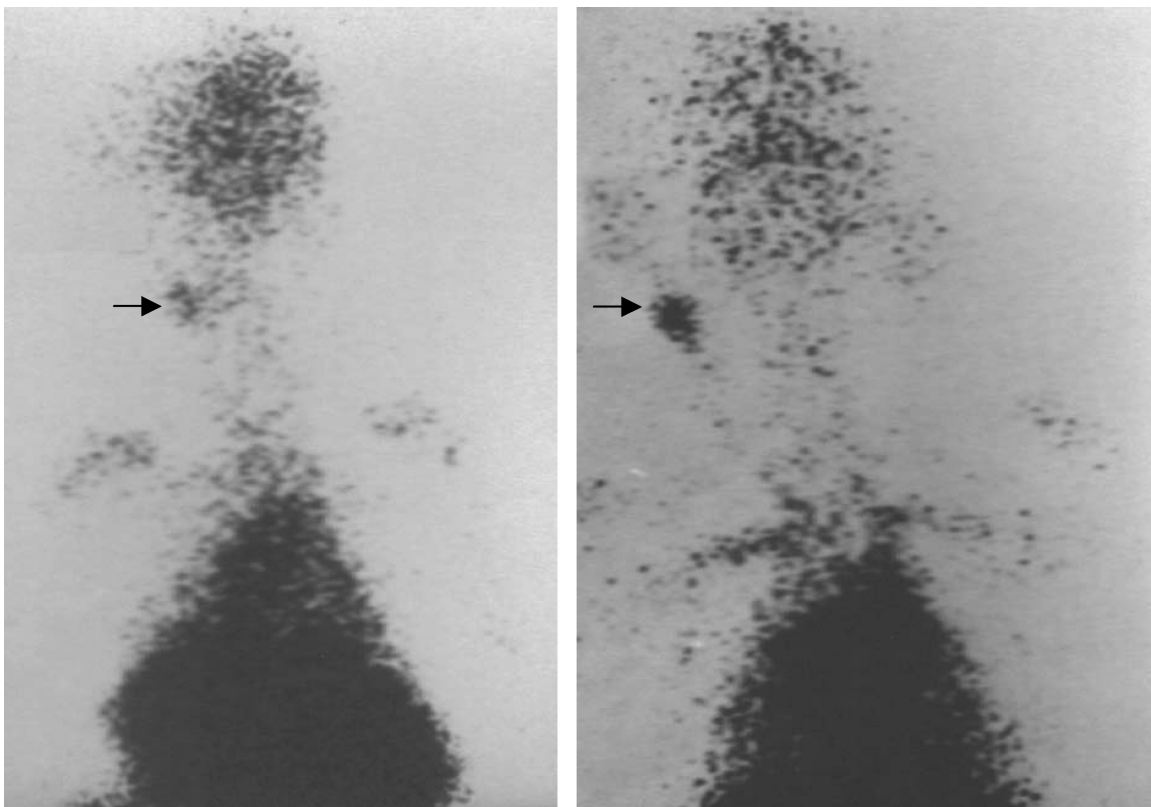

FIG. 6. Scintigraphic images of thrombus after injection of streptokinase liposomes.

until they reach the target area. By the protection of enzymes from antibodies in the circulation, better scintigraphic images may be obtained. But, thrombus/vein ratio should be more than 2 for good images (Korkmaz, Özer, and Hıncal 2000). Even though the better accumulation in the thrombus was obtained ($p < 0.05$), this ratio should be increased further for sufficient scintigraphic images.

CONCLUSION

We were able to encapsulate efficiency streptokinase in vesicles such as liposomes, niosomes, and sphingosomes with a little loss of fibrinolytic activity and prove that encapsulation of enzymes enhanced the accumulation of streptokinase into thrombi. Further studies are required to improve further the quality of the scintigraphic images.

REFERENCES

Alpar, H. O., Bamford, J. B., and Walters, V. 1981. The in vitro incorparation and release of hydroxocobalamin by liposomes. *Int. J. Pharm.* 7:349–351.

Bangham, A. D., Standish, M. M., and Watkins, J. C. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* 13:238–252.

Betageri, G. V., Jenkins, S. A., and Parsons, D. L. 1993. Pharmacokinetics of liposome products, In: *Liposome Drug Delivery Systems*, eds. G. V. Betageri, S. A. Jenkins, and D.L. Parsons, Basel: Technomic Publishing, 47–64.

Coates, G., De Nardo, S. J., and DeNardo, G. L. 1975. Pharmacokinetics of radioiodinated streptokinase. *J. Nucl. Med.* 16:136–142.

Corvo, M. L. 1998. Liposomes as delivery system for superoxide dismutase in experimental arthritis, Ph.D. Thesis, Utrecht University, The Netherlands.

Dugan, M. A., Kazar, J. L., Ganse, G., and Chakes, N. D. 1972. Localization of deep vein thrombosis: radioactive streptokinase. *J. Nucl. Med. Concise Commun.* 14:233–234.

Jackson, K. W., and Tang, J. 1982. Complete amino acid squence of streptokinase and its homology with serine proteases. *Biochemistry* 21:6620–6625.

Jackson, K. W., Esmon, N., and Tang, J. 1980. Streptokinase and staphylokinase, In *Methods in Enzymology,* vol. 80, eds. Perlman, G. E., and Lorand, L. New York: Academic Press, 387–394.

Korkmaz, M., Özer, A. Y., and Hıncal, A. A. 2000. DTPA niosomes in diagnostic imaging, In: *Synthetic Surfactant Vesicles and Other Non-Phospholipid Vesicular Systems*, eds. I.F. Uchegbu, Singapore: Harwood Academic Publishing, 227–242.

Landbrooke, B. D., Williams, R. M., and Chapman, D. 1968. Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. *Biochim. Biophys. Acta* 150:333–340.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265–275.

Mentzer, R. L., Budzynski, A. Z., and Sherry, S. 1986. High dose, brief duration intravenous infusion of streptokinase in acute myocardial infarction: description of effects in the circulation. *Am. J. Cardiol.* 57:1220–1226.

Perkins, W. R., Vaughan, D. E., Plavin, S. R., Daley, W. L., Rauch, J., Lee, L., and Janoff, A. S. 1997. Streptokinase entrapment in interdigitation-fusion liposomes improves thrombolysis in an experimental rabbit model. *Thromb. Haemost.* 77:1174–1178.

Rouser, G., Fleisner, S., and Yamamoto, Y. 1970. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5:494–496.

Sam, P., Rhodes, B. A., and Bell. W. R. 1975. Radiolbelled steptokinase and urokinase and their comparative biodistribution. *Thromb, Res.* 6:247–253.

Taylor, K. M. G., Taylor, G., Kelaway, I. W., and Stevens, J. 1990. Drug entrapment and release from multilamellar and reverse-phase evaporation liposomes. *Int. J. Pharm.* 58:49–55.