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Review

In vivo behaviour of vesicular urokinase

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Abstract

Thromboembolic diseases including deep vein thrombus (DVT) are major causes of morbidity and mortality. Detection of DVT in low extremities is difficult. There are some accepted imaging techniques in clinic but most of them have several disadvantages limiting their effective use. Because of this, researchers are still performed to develop a rapid, specific means of detecting and/or imaging venous thrombi-based on the changing composition of the thrombus.

Urokinase, fibrinolytic enzyme isolated form human urine, is a direct activator of plasminogen. In thrombus formation, plasminogen seems to be trapped in or absorbed onto fibrin matrix thus leading to a localised concentration of plasminogen. This suggests that radiolabelled urokinase would be a suitable compound for the detection of thrombi. The most important disadvantage of this enzyme is short plasma half life. To overcome this problem, it was decided to encapsulate the enzyme in drug delivery systems such as liposomes, niosomes or sphingosomes.

In this study, we prepared, characterized and monitored the biodistribution of three types of vesicular systems containing urokinase. All types of prepared vesicles show in vitro an acceptable encapsulation, stability and release profile. Thrombus uptake was increased by encapsulation of urokinase into vesicles.

Keywords: Thrombosis; Urokinase; Liposome; Niosome; Sphingosome; Biodistribution studies

Contents

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1. Introduction

Thromboembolic diseases including deep vein thrombus (DVT), pulmonary embolism (PE), myocardial infarction (MI), stroke and arterial thrombosis are major causes of morbidity and mortality. Coronary artery diseases and PE are among the leading causes of death in developed countries. Other thromboembolic disorders are congestive heart failure, recurrent DVT and PE, postphlebitic syndrome and lost of limb. With the increase in the life expectancy of the population, it can be anticipated that these condition will become more common.

Detection of DVT in the lower extremities is difficult. Since a reliable diagnosis is often most important, a number of diagnostic techniques have been developed. One of the techniques of diagnosis of DVT is a radionuclidic technique. Nuclear medicine has been instrumental in establishing and monitoring safe and effective therapeutic regimens. There are some accepted imaging techniques in the clinic but most have several disadvantages limiting their effective use. Because of this, researchers are still developing a rapid, specific means of detecting and/or imaging venous thrombi-based on the changing composition of the thrombus.

Urokinase, fibrinolytic enzyme isolated form human urine, is a direct activator of plasminogen. During the formation of thrombi, plasminogen seems to be trapped in or absorbed onto fibrin matrix thus leading to a localised concentration of plasminogen. This suggests that radioactively labelled urokinase would be a suitable compound for the detection of thrombi (Millar, 1974). The most important disadvantage of this enzyme is short plasma half life (10–20 min) (Som et al., 1975). To overcome this problem, it was decided to encapsulate the enzyme in drug delivery systems such as liposomes, niosomes or sphingosomes.

2. Materials and methods

2.1. Chemicals

The lipids used to prepare vesicles: phospholipids (DMPC, dimyristoil phosphatidyl choline) were 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) was purchased from Sigma Chemicals (USA). The stability of the resulting liposomes, niosomes and sphingosomes was increased by addition of dicetylphosphate (DCP) as a negative charge inducing agent. To avoid leakage of the encapsulated urokinase, cholesterol (CHOL) was added. Ukidan was kindly supplied from Serono Company. Urokinase, plasminogen, D-Val-Leu-Lys-*p*-nitroanilide and bovine serum albumine were purchased from Sigma Chemicals (USA). All other chemicals were of analytical grade.

2.2. Preparation method

In this study, liposome, niosome and sphingosome dispersions containing urokinase were prepared by the film method described by Bangham et al. (1965). Briefly, mixtures of the appropriate amounts of lipids in ethanol were dried by using a rotavapor until a homogenous film was formed. Nitrogen stream was passed over the lipid film to remove the ethanol residue. This film was dispersed using a urokinase solution (30,000 units ml^{-1}) 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 μm (once) and 0.2 μm (10 times). Then, the dispersions were subsequently freeze–thawed 10 times to increase the encapsulation capacity of vesicles (Herremans,

1995). Non-entrapped enzyme was removed by ultracentrifugation (Beckman Instruments Inc., CA, USA) for 30 min at 55,000 rpm and 4 °C. The pellet was subsequently redispersed in buffer.

2.3. Particle size

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

2.4. Phospholipid content

Phospholipid concentrations were assessed by a phosphate assay according to Rouser method (Rouser et al., 1970).

2.5. Urokinase activity determination

Urokinase activity assay was also carried out according to the previously described method (Barlow, 1976), which was modified to allow quantification of liposomal urokinase. Triton X-100 was used to distrup the bilayer.

2.6. Release of urokinase from liposome/niosome/sphingosome dispersions

The release studies were carried out in 10 mM HEPES (pH 7.5, 0.8% NaCl) with and without albumin. It has been reported that 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.

2.7. Radiolabelling of urokinase with Tc-99m

Urokinase was labelled with Tc-99m by tin reduction method, 0.2 ml of $SnCl_2$ (1 mg ml^{-1}) solution was added to urokinase solution (30,000 IU ml^{-1}) and 70–80 mCi of $^{99m}TcO_4^-$ (pertecnetate) was added to the mixture. After 1 h incubation, free Tc was removed by passing through a Sephadex G5 column. The liposome/niosome/sphingosome dispersions containing radiolabelled urokinase were then prepared by film method.

Quality control of the binding was checked by miniaturised ITLC-SG plates in saline and 85% methanol as a running solvent.

2.8. Animal thrombus model

Before thrombus formation, rabbits were anaesthetised with xylazine (12 mg kg^{-1} Rompun®) and ketamine (50 mg kg^{-1} Ketalar®) i.m. The right jugular vein was blocked by clamps to occlude venous flow. Firstly, a clamp was placed around the proximal end of the jugular vein and then placed around the 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).

2.9. Biodistribution studies of urokinase dispersions

The liver was saturated by injection of empty liposome/niosome/sphingosome dispersions. After pre-saturation, dispersions containing radiolabelled urokinase were injected into the ear vein of female rabbits which have 2.5–3 kg weight. Following administration, rabbits were sacrificed by injection of 2 ml of Lysthenon® forte (succinyl-bis-cholin chloride 100 mg/5 ml) intracardiacally at first and forth hours. The liver, lung, kidneys, spleen, heart, vein without thrombi and vein with thrombi were removed and all organs were washed with saline to remove the blood and weighed. In addition, blood samples (1 ml) were taken to scan the activity remaining in blood, then, the radioactivity of organs was measured by well-type gamma counter. Percentage uptake per gram organs was calculated. For in vivo evaluation, three parallel studies were carried out ($n = 3$).

3. Results and discussion

The molar composition and codes of liposome/niosome/sphingosome dispersions containing urokinase used in this study are given in Table 1. The physicochemical properties of the vesicles are shown in Table 2. The entrapment efficiency for urokianse was between 9 and 12%. At a 30 μmol ml^{-1} lipid

Table 1
Molar composition of dispersions containing urokinase

Formulation	Molar composition	Codes
DMPC:DCP:CHOL + urokinase	10:1:4	LUK (liposome)
SUR I:DCP:CHOL + urokinase	10:1:4	NUK (niosome)
SPH:DCP:CHOL + urokinase	10:1:4	SUK (sphingosome)

concentration, an entrapment efficiency of 11.6% for the liposomes and 9.7% for the sphingosomes was obtained. Activity of urokinase was checked after the preparation of vesicles. It was found that 70–84% of the initial activity of the enzymes was recovered in the preparations.

Release of urokinase in buffer with and without albumin at 37 °C was investigated (Fig. 1a and b). For all dispersions, a burst type release was found in the first hour possibly caused by the charge inducer component incorporated in the vesicles.

In both media, leakage of urokinase from niosomes was found to be higher in case of liposomes and sphingosomes. In HEPES buffer 78% of entrapped urokinase released from niosomes while 60 and 69% of entrapped urokinase were released from liposome and sphingosome dispersions, respectively, at the end of the 24-h observation period ($p < 0.05$). Release of urokinase from vesicles was increased by addition of albumin to release medium, with all types of vesicles showing a final degree of release up to 80–95% after 6–7 h ($p > 0.05$) (Fig. 1a and b).

The labelling efficiency was found 90% for urokinase. Results are in agreement with the literature (Dugan et al., 1973). The stability of labelling was checked at 2 h after labelling. It was found that the change in labelling was not important ($p > 0.05$). In order to understand, whether there is any complex or not between buffer and Tc-99m, HEPES was also labelled with Tc-99m and binding efficiency was found as 4% which is negligible.

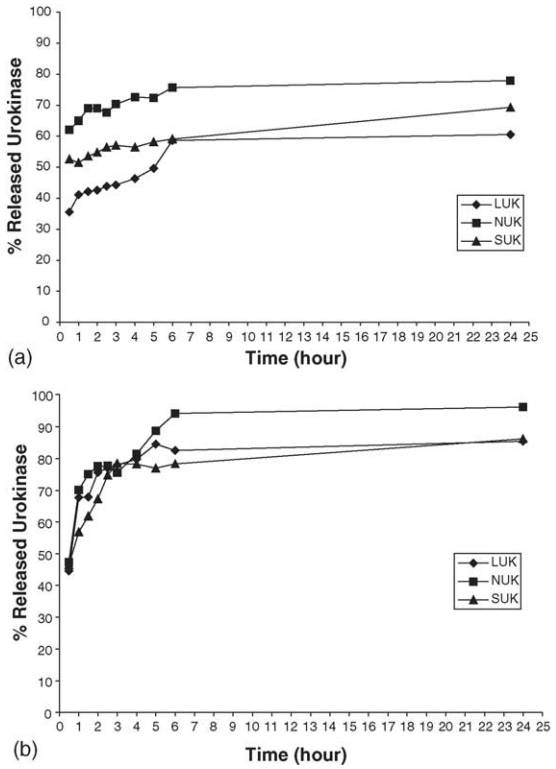

Fig. 1. Release of urokinase from liposome/niosome/sphingosome dispersions incubated in 10 mM HEPES buffer (a) without albumin and (b) with albumin at 37 °C.

For biodistribution studies, liposome/niosome/sphingosome dispersions containing radiolabelled urokinase were injected to the rabbits via ear vein. Tc-99m labelled urokinase solutions were applied to animals as control groups.

When liposomes are injected to the body, they can be taken-up by RES and cleared from the organism. There are many parameters which are vital in the interaction of microparticulates with macrophages. Vesicle size is one of them. Another parameter is charge. Negatively charged liposomes can be taken up and phagocytosed

Table 2
Physicochemical properties of urokinase vesicles

Formulation	Molar composition	Mean particle size (nm) (PI)	Phospholipid Concentration (μmol ml^{-1})	Entrapment efficiency (%)
LUK	10:1:4	183 (0.1)	28.9 ± 1.06	11.6 ± 0.74
NUK	10:1:4	172 (0.2)		12.4 ± 1.25
SUK	10:1:4	193 (0.1)	24.9 ± 0.65	9.7 ± 0.87

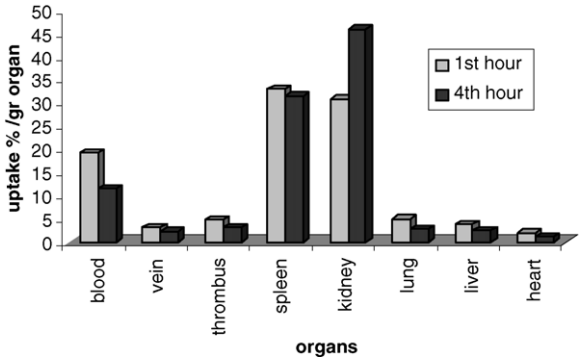

Fig. 2. Biodistribution results of urokinase liposomes (LUK) ($n = 3$).

more readily by macrophages. Because of this, spleen was found as organ showed highest activity (Betageri et al., 1993).

When compared to first and forth hour uptake of urokinase liposomes, the uptake of all organs decreased with time except for kidneys. Only, renal activity increased with time. Thrombus uptake decreased with time. Fig. 2 shows the biodistribution results of urokinase liposomes.

Nearly the same results were obtained from administration of urokinase niosomes. Uptake in all organs decreased only renal uptake increased when compared to first and forth hour results. The lowest blood activity was obtained with urokinase niosomes, suggesting that niosome dispersions were rapidly cleared from blood circulation (Fig. 3).

When biodistribution results of urokinase sphingosomes were investigated, it was clearly observed that spleen and kidneys had the highest drug concentration

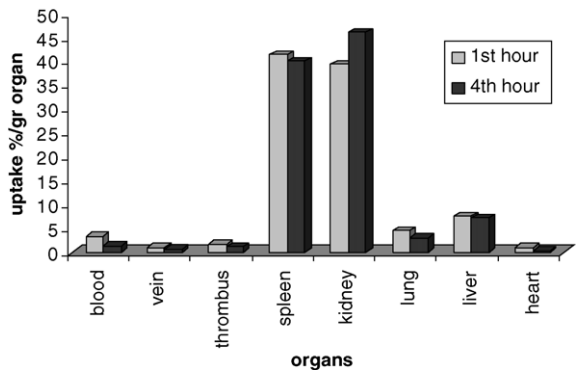

Fig. 3. Biodistribution results of urokinase niosomes (NUK) ($n = 3$).

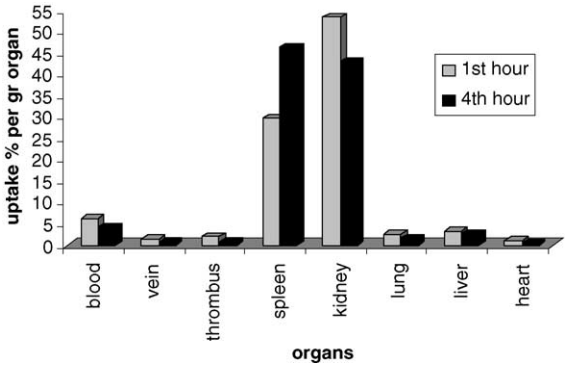

Fig. 4. Biodistribution results of urokinase sphingosomes (SUK) ($n = 3$).

like the other dispersion results. The activity in all organs decreased with time except for in spleen. Thrombus uptake of urokinase sphingosomes decreased with time (Fig. 4).

Radiolabelled free urokinase was applied as a control. The highest drug concentration was measured in spleen but on the contrary activity in the kidney was found to be very low. Liver uptake was higher than vesicular urokinase, proving that saturation of liver with the empty vesicles prevented liver uptake. Low blood activity even at 1 h showed that free urokinase was rapidly cleared from the circulation. The most important point is that thrombus uptake of free urokinase is lower than that caused by vesicles containing urokinase. Biodistribution results are given in Fig. 5.

The thrombus/vein ratio also calculated for urokinase dispersions and free urokinase (Table 3). According to these ratio, it is clear that thrombus uptake

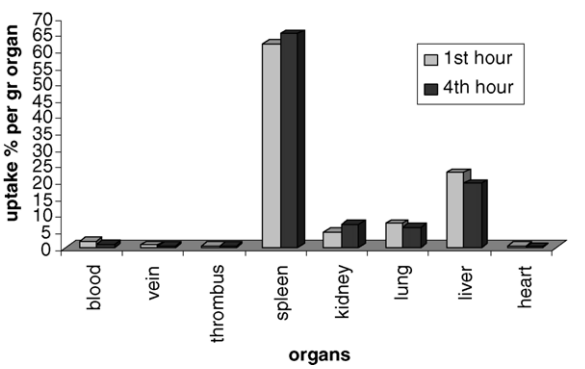

Fig. 5. Biodistribution results of free urokinase (UK) ($n = 3$).

Table 3
Thrombus/vein ratio obtained from biodistribution studies of dispersions containing urokinase and free urokinase

Formulations	Thrombus/vein	
	First hour	Forth hour
LUK	1.568	1.703
NUK	1.542	1.333
SUK	1.452	1.032
Free urokinase	0.863	0.982

increased by encapsulation of urokinase into vesicles such as liposome/niosome/sphingosome ($p < 0.05$).

4. Conclusion

All three types of urokinase vesicles show in vitro an acceptable encapsulation, stability and release profile. Although thrombus uptake increased by encapsulation of urokinase into vesicles, imaging of thrombus could not succeed. Further experiments are necessary to prove whether these vesicles can be used for scintigraphic imaging of deep vein thrombus.

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