Synthesis and biological evaluation of [^{99m}Tc]Lumbrokinase from earthworm *Lumbricus rubellus*

Sang Hyun Park* and Pradeep Kannampalli
Radiation Research Division for Biotechnology, Korea Atomic Energy Research Institute

Abstract

Lumbrokinase, a fibrinolytic enzyme isolated from the earthworm, *Lumbricus rubellus* is currently being investigated for its role in removing blood clots in conditions like thrombosis. The present investigation details a method for the synthesis of [^{99m}Tc]lumbrokinase and its distribution in a biological system. Radiolabelling of lumbrokinase with [^{99m}Tc] was accomplished by using tetraborohydrate exchange resin (BER) which afforded [^{99m}Tc]lumbrokinase with a radiochemical purity of > 90% as determined by radio-TLC and HPLC. Biodistribution studies in the blood, liver, heart, spleen, kidney and lung of Sprague-Dawley rats revealed that the maximum percentage of the injected dose (% ID) and the percentage of the injected dose per gram organ weight (% ID/g) of [^{99m}Tc]lumbrokinase were found in the blood when compared to the other organs at time periods of 0.5, 1, 2, 4, 8, 12 and 24 h after administration of a single dose of the radiotracer (3.7 MBq/0.1 mL). Results suggest that lumbrokinase is mainly concentrated in the target organ, namely the blood and hence can be effectively used in treating blood clotting diseases like thrombosis.

Key Words: [^{99m}Tc]lumbrokinase; *Lumbricus rubellus*; biodistribution

1. Introduction

Thrombosis, the formation of a clot or thrombus inside a blood vessel, thus obstructing the flow of blood through the circulatory system is a serious condition that can become fatal if untreated. Current therapies for thrombosis include bypass surgery, angioplasty, thrombectomy or administration of thrombolytic agents such as streptokinase, urokinase, tissue-type plasminogen activator and heparin (Wilson and Lampman, 1979; Yusuf et al., 1985). In spite of the various treatments available, there are severe limitations and side-effects associated with the present thrombolytic agents, hence a novel thrombolytic agent which not only has improved efficiency but also has the ability to directly degrade the fibrinogen and fibrin is being widely sought after (Park et al., 1998). Earthworms have been used in Chinese traditional medicine to treat clotting diseases for over 2000 years. In the early 19th century, it was found that certain components that were secreted from the alimentary tract of earthworm could dissolve fibrin (Fredericq, 1878). Mihara et al (1991) extracted a fibrinolytic enzyme from the earthworm, *Lumbricus rubellus* and named it lumbrokinase (LK), which was later found to be a group of six fibrinolytic isoenzyme proteins with molecular weights of 25 to 32 kDa. These fibrinolytic enzymes were found to hydrolyse not only plasminogen rich fibrin plates but also plasminogen free fibrin plates (Mihara et al., 1991) and also dissolve blood fibrin clots (Mihara et al., 1990), which makes them an excellent candidate for use in clinical application as a chemotherapeutic agent for the treatment of thrombosis (Mihara et al., 1989; Ryu et al., 1994; Ryu et al., 1995). This enzyme was also found to be heat stable and displayed a very broad optimal pH range (pH 2 – 11). It exhibited trypsin-like characteristics with high substrate specificity against fibrin, suitable as a fibrinolytic agent (Park et al., 1999). Studies using ^{125}I-radiolabelling technique have been reported which investigated the intestinal absorption of the full-sized protein (Shun et al., 1998). However, chemical modification of the protein during labelling and the release of ^{125}I during intestinal digestion is reported to interfere significantly with the result (Castell et al., 1997). Hence, in this study an attempt has been made to label the earthworm lumbrokinase with technetium-99m [^{99m}Tc] and investigate its biodistribution in Sprague-Dawley rats.

Technetium-99m [^{99m}Tc] is a widely used radioactive tracer in nuclear medicine. It has gamma ray energy of about 140 keV which is convenient for easy detection. The fact that both its physical half life and biological half life (6.01 h) are very short leads to

*** Corresponding author:**
Dr. Sang Hyun Park,
Radiation Research Division for Biotechnology,
Korea Atomic Energy Research Institute,
1266 Shinjeong-dong, Jeongeup,
Jeonbuk 580-185, Republic of Korea
Tel: +82-63-570-3370, Fax: +82-63-570-3371.
E-mail: parksh@kaeri.re.kr

its very fast clearance from the body after an imaging process. A further advantage is that the gamma emission from [^{99m}Tc] is a single energy, not accompanied by a beta emission and that permits a more precise alignment of imaging detectors. Different chemical forms of [^{99m}Tc] are used for imaging different organs like the brain, bone, liver, spleen and kidney and also for blood flow studies. [^{99m}Tc] is the most widely used radioactive isotope for diagnostic studies. The main objective of this investigation was to develop a method to label earthworm lumbrokinase with [^{99m}Tc] and to investigate the biodistribution of [^{99m}Tc]lumbrokinase in Sprague-Dawley rats for a period of 24 h following an i.v administration.

2. Materials and Methods

2.1 Chemicals

$Na^{99m}TcO_4$ was purchased from Sam Young Unitech Co., Ltd., Daejon, Korea. Lumbrokinase isolated from earthworm *Lumbricus rubellus* was obtained from Shin Poong Pharmaceutical Co., Ltd., Seoul, Korea. Unless otherwise stated, all the other chemicals and reagents used were of analytical grade and were used without any further purification.

2.2 Animals

Healthy male Sprague-Dawley rats (150 ± 5 g) obtained from Gyeryong Science Inc. (Daejon, Korea) were used for the biodistribution studies. Animals were randomly divided into seven groups with six animals in each group corresponding to each time period. The animals were acclimatized to the animal house conditions and all animal experiments were performed in accordance with guidelines prescribed by the institutional animal ethical committee.

2.3 Chemistry

The tetraborohydride exchange resin (BER) used for labeling lumbrokinase with [Tc^{99m}] acts as a reducing agent and was prepared by a previously reported method as described below. Chloride-form resin (Amberlite® ion exchange resin, 12.5 g) was slurry-packed with water into a 30 mL fritted glass funnel mounted on a filter flask. An aqueous solution of sodium borohydride (200 mL, 0.25 M) was slowly passed through the resin for over a period of 30 min. The resulting resins were washed thoroughly with distilled water until free of excess borohydride and finally with ethanol (10 mL × 3). The tetraborohydride anion exchange resin (BER) was then partially air-dried by removing the ethanol on the surface of BER. The resin was analyzed for its tetraborohydride content by hydrogen evolution upon acidification with 0.08 M HCl and the average capacity of BER was found to be 2.5 meq of tetrahydroborate ion per gram. The BER was stored under nitrogen at 48!. The hydride content was found to be constant for over a period of 5 weeks.

2.4 Radiolabelling of lumbrokinase

Radiolabelling of lumbrokinase was carried out as described below under three different conditions and by using two different types of reducing agents. The first condition involves the use of $SnCl_2$ as a reducing agent. A mixture of $SnCl_2$'''$2H_2O$ (0.1 mg), $Na^{99m}TcO_4$ (185 MBq/0.1mL) and lumbrokinase (1mg) were incubated at room temperature for 30 min in the presence of N_2 to yield [^{99m}Tc]lumbrokinase. In the second method, tetraborohydride exchange resin (BER) was used as a reducing agent. A mixture of BER (5 mg), $Na^{99m}TcO_4$ (185 MBq/0.1mL) and lumbrokinase (1 mg) were incubated at room temperature for 30 min in the presence of N_2 to yield [^{99m}Tc]lumbrokinase. The third condition also involved the use of tetraborohydride exchange resin (BER) as a reducing agent but at a higher concentration. A mixture of BER (10 mg), $Na^{99m}TcO_4$ (185 MBq/0.1mL) and lumbrokinase (1 mg) were incubated at room temperature for 30 min in the presence of N_2 to yield [^{99m}Tc]lumbrokinase. The labelling efficiency, stability and radiochemical purity of the [^{99m}Tc]lumbrokinase synthesized under the above three different conditions were then determined by HPLC and radio-TLC, respectively.

The labelling efficiency and stability were determined by radio thin layer chromatography (radio-TLC) system which consisted of a radio-TLC scanner (EC & G Berthold Linear Analyzer, Germany) and a one dimensional analysis of the Berthold chroma program. [^{99m}Tc]Lumbrokinase solution was spotted onto a silica gel coated fiber sheet (Gelman Sciences Inc., Ann Arbor, MI, USA). The sheet was eluted with three different solvent – methyl ethyl ketone, saline and phosphate buffered saline. The labelling efficiency of the [^{99m}Tc]lumbrokinase was calculated by comparing the radioactivity of [^{99m}Tc]lumbrokinase (at the origin) and the free [^{99m}Tc] peaks (at the solvent front). Labelling

yield of the [^{99m}Tc]lumbrokinase was checked by HPLC (Waters, USA), coupled with a ìBondapak C-18 column (3.9 × 300 mm, Waters, USA). A mixture of phosphate buffered saline: triethyl ammonium phosphate gradient system was used as a mobile phase, with a flow rate of 1 mL/min and an injection volume of 5 ìL.

2.5 Biodistribution studies

Biodistribution of [^{99m}Tc]lumbrokinase was investigated in healthy male Sprague-Dawley rats which were randomly divided into seven groups with six animals in each group corresponding to each time period. [^{99m}Tc]Lumbrokinase was injected into the tail vein under mild isofluorane anesthesia at a dose of 3.7 MBq/0.1 mL in saline. Animals were maintained on a normal diet and water ad libitum. Animals in all the groups were sacrificed by an excess anesthesia at different time intervals of 0.5, 1, 2, 4, 8, 12 and 24 h post injection with the radiotracer. Tissue samples (0.1 g) of the organs were removed, weighed and the radioactivity counts were measured in a ã-counter (Beckman model). The radioactivity in various organs were expressed as percent of the injected dose (% ID) and also as percent of the injected dose per gram of the organ (% ID/g organ) and presented as mean for six animals at each time period. In the case of the blood, this value was calculated by assuming that it constitutes 6% of the total weight.

3. Results and Discussion

Radiolabelling of lumbrokinase was carried out as described above under three different conditions by using two types of reducing agents. The results indicate that radiolabelling performed under Condition III (i.e., using 10 mg of BER) yielded [^{99m}Tc]lumbrokinase with a higher yield and purity. The labelling yield and radiochemical purity of the ^{99m}Tc-labelled lumbrokinase synthesized under the above three different conditions were then determined by HPLC and radio-TLC, respectively. The HPLC data (Fig. 1) showed a single peak confirming the presence of > 90% of the [^{99m}Tc]lumbrokinase with only a few traces of the unlabelled lumbrokinase and free ^{99m}Tc. Radio chromatograms from radio-TLC (Fig. 2) also confirmed the presence of > 90% of the [^{99m}Tc]lumbrokinase with only a few traces of the free technetium [^{99m}Tc].

The biodistribution of lumbrokinase was studied in rats by an administration of a single dose (3.7 MBq/0.1 mL) of [^{99m}Tc]lumbrokinase to Sprague-Dawley rats. The biodistribution pattern was obtained in rats at 0.5, 1, 2, 4, 8, 12 and 24 h post-injection of [^{99m}Tc]lumbrokinase from the uptake of the drug by the excised organs (blood, liver, kidney, spleen, heart, lung and stomach). The biodistribution data showed that the [^{99m}Tc]lumbrokinase was mainly accumulated in the blood as it showed a high level of radioactivity (%ID and %ID/g tissue) when compared to the other organs investigated (Fig. 3 and 4).

In conclusion the present investigation, demonstrates a method for the synthesis of [^{99m}Tc]lumbrokinase isolated from earthworm *Lumbricus rubellus*. We observed that there was > 90% yield of the labelled compound as determined by HPLC and radio-TLC in our study. Biodistribution studies carried on Sprague-Dawley rats revealed that [^{99m}Tc]lumbrokinase was mainly concentrated in the blood when compared to the other organs. Hence, we concluded that lumbrokinase isolated from *Lumbricus rubellus* can be effectively used in the treatment of thrombosis.

Fig. 1. HPLC chromatogram of [^{99m}Tc]lumbrokinase

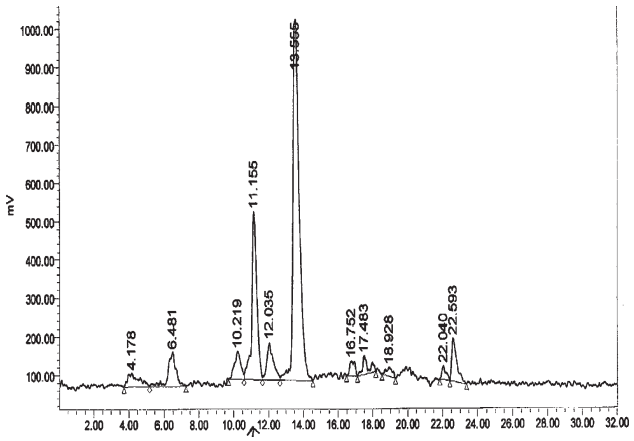


Fig. 2. Radio-TLC chromatogram of [^{99m}Tc]lumbrokinase

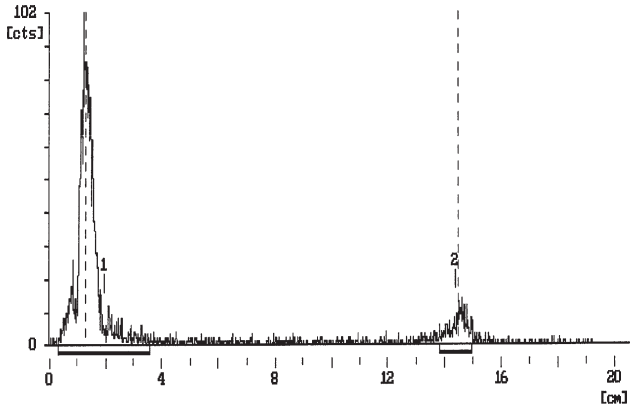


Fig. 4. Biodistribution of [^{99m}Tc]lumbrokinase in different organs of rats at different time intervals after an i.v administration expressed as % ID/g (n=6)

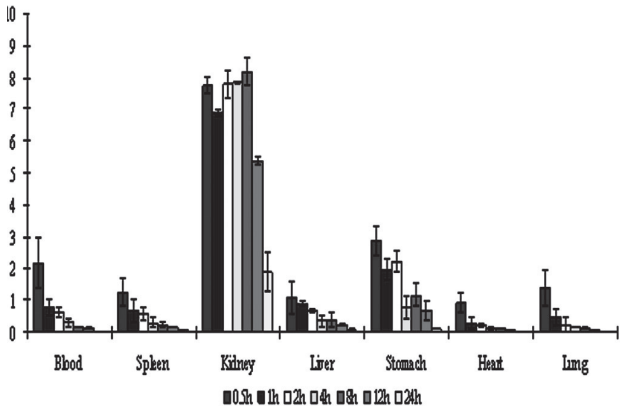
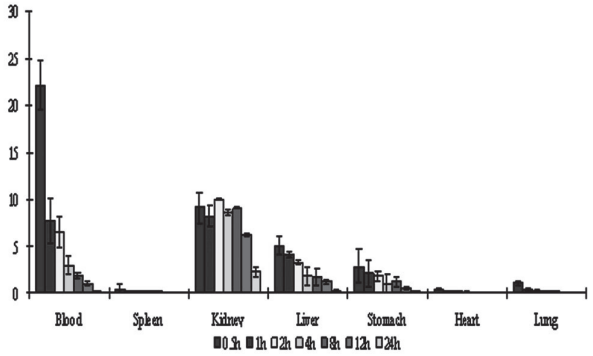


Fig. 3. Biodistribution of [^{99m}Tc]lumbrokinase in different organs of rats at different time intervals after an i.v administration expressed as % ID (n=6)



Acknowledgement

This work was supported by the nuclear research development project from Korea Ministry of Science and Technology.

References

Castell JV, Friedrich G, Kuhn CS and Poppe GE, Intestinal absorption of undegraded bromelain in humans. American Journal of Physiology, 273, 1997, 139 – 146.

Fredericq L, La digestion des matieris albuminiodes chez quelques invertebres. Arch Zool Expert, 7, 1878, 391.

Mihara H, Sumi H, Mizumoto H, Yoneta T, Ikeda R and Maruyama M,. Oral administration of earthworm powder as a possible thrombinlytic therapy. In: Tanaka, K., (Ed.,) Recent Advances in Thrombosis and Fibrinolysis. Academic Press, New York, 1990, 287-298.

Mihara H, Sumi H, Yoneta T, Mizumoto H, Ikeda R, Seiki M and Maruyama MA,. A novel fibrinolytic enzyme isolated from Lumbricus reubella. The Japanese Journal of Physiology, 41, 1991, 461 – 472.

Mihara H, Yineta T, Sumi H, Soeda M and Maruyama, M, A possibility of earthworm powder as therapeutic agent for thrombosis. Thromb Haemosta, 62, 1989, 545 – 549.

Park Y, Ryu E, Kim H, Jeong J, Kim J, Shim J, Jeon S, Jo Y, Kim W and Min B, Characterization of antithrombotic activity of lumbrokinase-immobilized polyurethane valves in the total artificial heart. Artificial Organs, 23, 1999, 210 – 214.

Park YD, Kim JW, Min BG, Seo JW and Jeong JM, Rapid purification and biochemical characteristics of lumbrokinase III from earthworm for use as a fibrinolytic agent. Biotechnology Letters, 20, 1998, 169 – 172.

Ryu GH, Han DK, Park SY, Kim M, Kim YH and Min BG, Surface characteristics and properties of lumbrokinase-immobilized polyurethane. Journal of Biomedical Material Research, 29, 1995, 403 – 409.

Ryu GH, Park S, Kim M, Han DK, Kim YH and Min BG, Antithrombogenicity of lumbrokinase mmobilized polyurethane. Journal of Biomedical Material Research, 28, 1994, 1069 – 1077.

Shun YP, Ging YY, Fan R and Wu C, Assay of the fibrinolytic activity of EFE-III-1 by intestinal gavage in vivo. Cap Med, 5, 199817 – 18.

Wilson JR and Lampman J, Heparin therapy: a randomized prospective study. American Heart Journal, 97, 1979, 155 – 158.

Yusuf S, Collins R, Peto R, Furberg C, Stampfer MJ, Goldhaber SZ and Hennekens CH, Intravenous and intracoronary fibrinolytic therapy in acute myocardial infarction: overview of results on mortality, reinfarction and side-effects from 33 randomized controlled trials. European Heart Journal, 6, 1985, 556 – 585.