

# Technetium-99m-Labeled Diethylcarbamazine Citrate ( $^{99m}\text{Tc}$ -DEC) as a New Diagnostic Agent for Lymphatic Filariasis Detection

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## ABSTRACT

Lymphatic filariasis or more commonly known as elephantiasis disease has infected more than 120 million people in 80 countries; and more than 40 million of them are unable to work, in addition to disruption of aesthetic values. The problem faced by almost all people in combating this infectious disease is delayed diagnosis. The sufferers do not realize that they have been infected with this disease. A specific and accurate method of early detection is therefore needed.  $^{99m}\text{Tc}$ -labeled Diethylcarbamazine-citrate ( $^{99m}\text{Tc}$ -DEC) has been successfully prepared. However, as part of the discovery and development of new drugs, the fulfillment of pharmaceutical and safety requirements have to be evaluated. Physico-chemical aspects such as stability, purity levels, and other pharmaceutical requirements, as well as pharmacokinetic studies and route of administration, are important parameters to be studied. The stability test showed that after seven months of storage, a preparation in a dry kit retains an efficiency of labeling and a purity level of more than 90 % and its physico-chemical and biological characteristics remained steady. Biodistribution test in Wistar rat showed that the greatest accumulation occurred in the lymphatic system, especially in the popliteal glands and in lumbar and mesenteric lymph nodes. Imaging with a gamma camera after intradermal and intravenous injections to the experimental animals resulted in a positive image that showed  $^{99m}\text{Tc}$ -DEC accumulation in the target organ. The results of this innovation are expected to contribute significantly to improving public health, particularly in early detection of filarial infections. In addition, this result is expected to be a concrete contribution to the program of "The Global Goal of Elimination of Lymphatic Filariasis as a Public Health Problem by the Year 2020".

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## INTRODUCTION

Filariasis or commonly known as elephantiasis disease is one of the diseases that attract particular interest from stakeholders in the health sector all over the world. Although it rarely causes death, but at its late stages, the disease can make a person suffer a permanent physical disability; thus, it has resulted in a significant impact especially in the community of developing countries, either in the tropics or subtropics, that are suffering social-economic problems [1].

Lymphatic filariasis (LF) is one of the major causes of acute and chronic morbidity that affect the population of tropical and subtropical areas of Asia, Africa, Western Pacific, and several regions of the Americas. It is estimated that over 1.2 billion people live in areas at risk of the disease, and among the 120 million actual cases, 91 % are caused by the worm *Wuchereria bancrofti* [2,3]. It was recently reported that more than one billion people in the world are at risk of suffering of filariasis. More than 120 million people from 80 countries have been infected by microfilaria; the disease was even declared endemic in thousands of villages in 26 provinces in Indonesia. These are the reasons the World Health Organisation (WHO) declared a global agreement to combat this disease with the

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theme of the Global Goal of Elimination of Lymphatic Filariasis as a Public Health Problem by the Year 2020 [4].

Detection of filarial worms depends on the presence of microfilariae in the peripheral blood stage, or is known as periodicity stage. Interestingly, filarial periodicity is found in between 10 pm and 2 am (nocturnal). Therefore, blood sampling should be done at night. In addition, the active larvae can only be discovered 6-12 months after a person has been infected with filaria, and the manifestations of filariasis only become visible after around four years. Therefore, early detection for this case is quite difficult to attain. Laboratory tests for identification of filarial antigens by enzyme-linked immunosorbent assay (ELISA) technique or rapid immunochromatographic card can actually be done, but those techniques are complicated and often give false positive results [5-12]. Diagnosis techniques with higher sensitivity and specificity are still needed.

Nuclear medical techniques using radiopharmaceuticals provide an alternative approach to solve this problem. Diethylcarbamazine citrate (DEC) is a drug for the treatment of filariasis in humans and animals (Fig. 1). DEC has had a long history of safe use in mass drug administration (MDA) lymphatic filariasis eradication program. Furthermore, *W. bancrofti*, the human parasitic roundworm that causes lymphatic filariasis, do not appear to have developed resistance to DEC [13-15]. We hypothesized that DEC could be labeled with radionuclide technetium-99m ( $^{99m}\text{Tc}$ ) to form the organometal complexes of  $^{99m}\text{Tc}$ -DEC. The  $^{99m}\text{Tc}$ -DEC radiopharmaceutical is expected to be uptaken by microfilariae in the body of an infected person due to binding between  $^{99m}\text{Tc}$ -DEC and the filariae's DEC receptor. Thus, the presence of microfilariae in the body can be detected externally by gamma camera; therefore, the early detection of filariasis can be established.

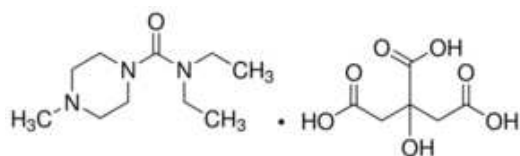


Fig. 1. Chemical structure of DEC.

The working targets of DEC are arachidonic acid (arachidonate 5-lipoxygenase) and cyclooxygenase pathway (cytochrome-C-oxydase subunit-1) located on microfilaria sheaths. DEC mechanism as an antifilarial parasite is estimated to be reduction of muscle activity causing the filaria to be paralyzed and easily moved out from the host [13,16]. Another suggestion is that

DEC may cause changes in microfilaria membrane surface, so it is more easily destroyed by the host defense system. This mechanism of action is hypothesized to be identical when  $^{99m}\text{Tc}$ -labeled DEC is used as a diagnostic agent. The imaging process of  $^{99m}\text{Tc}$ -DEC bound to microfilariae will easily be done from outside the body as a result of gamma radiation emitted from the technetium-99m that is attached to the DEC. Therefore, the presence of microfilariae in the body can be detected precisely and accurately anytime and anywhere.

The formulation and preparation of  $^{99m}\text{Tc}$ -DEC radiopharmaceutical dry kit had been conducted in the previous research [17]. The kit's labeling efficiency and radiochemical purity was higher than 95 %, and its physico-chemical characteristics were good. However, the results of the aforementioned research could not be applied clinically when several pharmaceutical and safety testing requirements had not been carried out. Therefore, various other parameters, such as biological, pharmacological, and toxicological aspects, as well as dose conformity and route of administration, have also to be studied. It is important to verify the uptake of  $^{99m}\text{Tc}$ -DEC by filaria. The verification could not be done only through *in-vitro* studies, but must also be accompanied by *in-vivo* testing in animals infected with filaria.

Therefore, the objectives of this research were to obtain data which indicate that  $^{99m}\text{Tc}$ -DEC meets pharmaceutical requirements, and to convince potential users (end user) that the resulting formula can be used as a diagnostic agent for early detection of filariasis. The use of this new radiopharmaceutical is expected to give a real contribution in solving the present problem of filariasis infection of the Indonesian community. This in turn will support the Global Goal of Elimination of Lymphatic Filariasis as a Public Health Problem by the Year 2020 program in particular and the Indonesian health program in general.

## EXPERIMENTAL METHODS

### Radiolabeling and stability study of $^{99m}\text{Tc}$ -DEC

The labeling of DEC with  $^{99m}\text{Tc}$  was conducted using a direct method. Two forms consisted of 4 mg DEC-citrate and 100 mg  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ , pH 4 were prepared. The first formula was freshly prepared and the second one was freeze dried and re-constituted just prior addition of  $^{99m}\text{Tc}$ . Labeling of DEC with  $^{99m}\text{Tc}$  was performed by

adding an aliquot of  $^{99m}\text{Tc}$ -pertechnetate into the aforementioned forms followed by incubation at room temperature for 10-20 minutes. The results of radiolabelling, which is stated as radiochemical purity, was determined using two-system chromatography. The first system was chromatography using Whatman 3MM paper (1×10 cm) as stationary phase and dry acetone as mobile phase. This system was for separating  $^{99m}\text{Tc}$ -DEC from free  $^{99m}\text{Tc}$  in form of  $^{99m}\text{Tc}$ -pertechnetate ( $^{99m}\text{TcO}_4^-$ ). The second system was chromatography using ITLC-SA (1×10 cm) as stationary phase and water as mobile phase. This system was created for separating  $^{99m}\text{Tc}$ -DEC from free  $^{99m}\text{Tc}$  in its reduced form ( $\text{TcO}_2$ ). The labeling radiochemical purity of  $^{99m}\text{Tc}$ -DEC can be calculated with eq. (1):

$$\text{Labeling Efficiency} = 100\% - (\% \text{ } ^{99m}\text{TcO}_4 + \% \text{ } ^{99m}\text{Tc-red}) \quad (1)$$

The stability of  $^{99m}\text{Tc}$ -DEC (indicated as radiochemical purity) after storage at certain periods of time was tested with the aforementioned chromatograph. Stability test of freeze dried DEC/DEC kit over certain period of time during seven months of storage was also performed. The stability is considered good when labeling efficiency and purity level are both higher than 90 %, and there are no changes in physico-chemical and biological performance during storage. The test was performed in a similar manner to the radiolabeling procedure as previously mentioned, followed by radiochemical purity determination.

### Biological evaluation

Biological tests on animal were conducted after obtaining approval letter No. 001/KEPPHP/BATAN/III/2012 dated March 20, 2012 from the Animal Ethics Committee (KEPPHP-BATAN).

### Sterility test of DEC kits

The sterility test was performed according to the procedure in Indonesian Pharmacopoeia and published journal papers using two types of media, namely the Fluid Thioglycolate Medium (FTM) for aerobic and anaerobic bacteria and the Soybean Casein Digest Medium (SCDM) for fungus/mold [18]. Two vial DEC kits (one batch consists of 25 DEC kits) were reconstituted with one mL of saline solution. Each solution was then added into glass tubes containing 5 mL of FTM or 5 mL of SCDM respectively. FTM tubes were incubated in

the incubator (37 °C), and SCDM tubes were placed at room temperature (25-27 °C). Media tubes were observed for up to 14 days; if there was no growth of bacteria or mold colonies, then the DEC kit was sterile. However, if there was a growth of bacteria or mold, as indicated by the formation of cloudy areas or colonies in the media, experiments should be repeated with twice the number of samples (four vials of DEC kits), and if after 14 days of observation growth of colonies of bacteria or mold was still found, the DEC kits were not sterile.

### Pyrogenity test of DEC kits

The pyrogenity test of  $^{99m}\text{Tc}$ -DEC was conducted in accordance with the methods and procedures in Indonesian Pharmacopoeia [18] using three white rabbits (2-3 Kg). The rabbits' body temperatures were recorded at 30 minutes before the injection of radiopharmaceuticals as the initial temperature. Afterward, 500  $\mu\text{L}$  of  $^{99m}\text{Tc}$ -DEC was injected intravenously through the auricular vein in each rabbit's ear at minute 0. The body temperatures of the rabbits were measured again after one, two, and three hours after injection. The highest temperature obtained from the 1 hour until the 3 hour after injection is called the maximum temperature. The  $^{99m}\text{Tc}$ -DEC was declared pyrogen-free if the rise in body temperature of the rabbit after injection was not greater than 0.5 °C.

### Acute toxicity determination

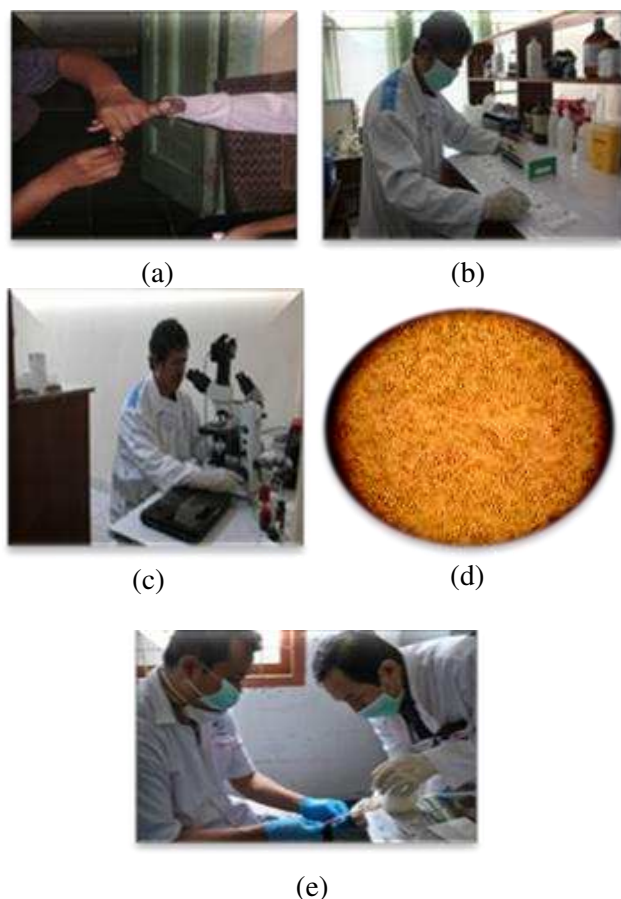
The acute toxicity determination of  $^{99m}\text{Tc}$ -DEC was conducted in accordance with the methods and procedures in Indonesian Pharmacopoeia with a slight modification [19] by multiplying certain number of dose given to the test animals (Swiss mice) after converted into common dose received by humans. Each treatment was carried out on five mice.  $^{99m}\text{Tc}$ -DEC was injected intradermally and intravenously at a dose which was increased gradually. Test animals were observed daily for up to 14 days, and if there were physical abnormalities or abnormal movements, the  $^{99m}\text{Tc}$ -DEC was declared toxic.

### Biodistribution of $^{99m}\text{Tc}$ -DEC

#### Induction of microfilariae into experimental rats

Figures 2(a) to 2(e) show the sequence of events of induction activities through the injection of the blood from patients infected by microfilariae

to the test animals in the middle of the night (after 22:00 pm). Blood samples from five filarial volunteers (Fig. 2(b)) were collected and 1 mL was injected to 10 experimental Wistar rats (250-300 g) on the same day (Fig. 2(e)). The remain blood samples were analyzed in the laboratory with a Nikon microscope (Fig. 2(c)) and Fig. 1(d)).



**Fig. 2.** Sampling of filarial perifer blood (a); blood analysis (b & c); microscopic image of microfilaria in the blood (d); injection of infectious blood into rats Wistar (e).

All injected experimental rats were maintained for several months with a special treatment. The conditions of those animals were observed every day and blood samples were taken and analyzed at certain times by using microscope to see if there has been a growth of microfilariae.

#### Biodistribution study of $^{99m}\text{Tc-DEC}$

Three months post infection, three experimental rats were injected intravenously with 1 mL of 1-mCi/mL  $^{99m}\text{Tc-DEC}$ , and three others were injected intradermally with 0.1 mL of the same activity concentration of  $^{99m}\text{Tc-DEC}$ . After 15 minutes post injection, the rats were anesthetized by ketamine solution injection to death, followed by dissection. Several critical organs were taken, and

the radioactivity accumulation of  $^{99m}\text{Tc-DEC}$  was determined by using a single channel analyzer.

#### Determination of the kinetic profile $^{99m}\text{Tc-DEC}$

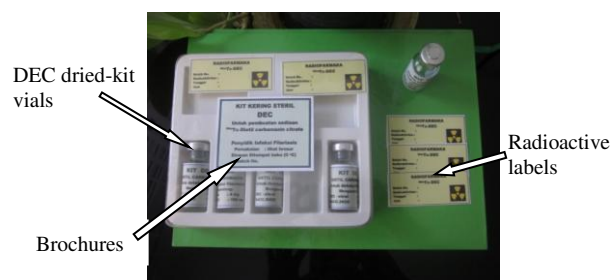
The determination of the kinetic profile in test animals was done to three normal and three infected Wistar rats. The injection dose was similar to the dose used in the biodistribution study. After intradermal and intravenous administration of  $^{99m}\text{Tc-DEC}$ , blood samples were taken from tail vein of each rat at the times of 5, 15, 30, 45, 60, 120, and 180 minutes post injection. Blood samples were weighed and their radioactivities measured with a single channel analyzer. The biological half-life was then calculated from the kinetic profile of  $^{99m}\text{Tc-DEC}$  by using computerized Multivit program.

#### Gamma camera imaging

Imaging was performed with a gamma camera (GE Infinia-Hawkeye SPECT/CT) on normal and filarial-infected Wistar rats after injection of 0.1 mCi (0.1 mL) of  $^{99m}\text{Tc-DEC}$  intradermally and intravenously.

## RESULTS AND DISCUSSION

Figure 3 shows DEC kits that had been prepared and was used throughout this research. These kits are easily distributed to nuclear medicine departments in hospitals for preparation of the  $^{99m}\text{Tc-DEC}$  radiopharmaceutical.



**Fig. 3.** DEC kits.

The stability of the DEC kits is indicated by the absence of physical changes during storage, as well as by the labeling efficiency and radiochemical purity level of no less than 90 %. The labeling efficiency and radiochemical purity level of the kit used, during the time period of storage in the refrigerator, is shown in Table 1.

**Table 1.** Labeling efficiency and radiochemical purity levels during the storage of the DEC kits labelled with <sup>99m</sup>Tc

No.	Storage Time (months)	Labeling Efficiency and Radiochemical Purity (%)
1.	0	95.4 ± 3.3
2.	1	97.2 ± 0.8
3.	2	98.3 ± 0.2
4.	3	98.3 ± 0.4
5.	4	96.4 ± 1.3
6.	5	97.3 ± 0.5
7.	6	98.0 ± 0.0
8.	7	98.2 ± 0.8

Table 1 shows that labeling efficiency and radiochemical purity of DEC kits (stored for up to 7 months) labeled with <sup>99m</sup>Tc were higher than 90 %. This indicates that the DEC kits were stable during this storage period. When compared with ciprofloxacin kits that are stable in storage for up to 17 weeks [20], the DEC kits were more stable.

The stability of DEC kit labeled with <sup>99m</sup>Tc (<sup>99m</sup>Tc-DEC) under up to four hours of storage is shown in Table 2. This result shows that for up to two hours of storage, the <sup>99m</sup>Tc-DEC still had an acceptable radiochemical purity (> 90 %). The decrease of radiopharmaceutical purity levels of <sup>99m</sup>Tc-DEC during storage might be caused by radiolysis or by conformation of the <sup>99m</sup>Tc-DEC complex itself. However, compared with <sup>99m</sup>Tc-ciprofloxacin radiopharmaceuticals, this <sup>99m</sup>Tc-DEC complex was more stable. The radiochemical purity of <sup>99m</sup>Tc-ciprofloxacin for just 45 minutes of storage is not acceptable (< 90 %) [20].

**Table 2.** Effect of solution storage after addition of <sup>99m</sup>TcO<sub>4</sub><sup>-</sup>

Stability after reconstitution with Sodium <sup>99m</sup> Tc-pertechnetate solution				
Storage period at room temperature (h)	1	2	3	4
Yield or radiochemical purity (%)	96.31	90.48	88.90	87.16

Considering the data in Table 2, the user should not keep <sup>99m</sup>Tc-DEC for too long or use it for more than two hours when it is reconstituted with a solution of sodium pertechnetate-<sup>99m</sup>Tc.

The pyrogenicity and sterility test of <sup>99m</sup>Tc-DEC made afresh (*recentus paratus*) or from a kit from that had been stored up to eight months found it sterile and pyrogen-free.

On the other hand, because the injection volume should be as small as possible, the amount of sodium pertechnetate added must be as concentrated as possible. There has been a concern that radiolabeling of DEC with a large volume of <sup>99m</sup>Tc-pertechnetate might hinder the radiolabeling process. Therefore, radiolabeling of DEC with

<sup>99m</sup>Tc-pertechnetate with volume range of 1-4 mL was carried out. Table 3 shows that the volume of <sup>99m</sup>Tc-pertechnetate slightly influenced the radiolabeling efficiency of DEC. It can be seen that radiolabeling with <sup>99m</sup>Tc-pertechnetate volumes larger than 1 mL decreased the radiolabeling efficiency to 92.23-94.58 %. However, these results still met the requirement through its average value of 94.65 ± 2.38 %.

**Table 3.** Effect of volume <sup>99m</sup>Tc-pertechnetate on DEC labeling

Volume of <sup>99m</sup> Tc-pertechnetate (ml)	Labeling Efficiency (%)
1.0	97.90
2.0	92.23
3.0	93.89
4.0	94.58

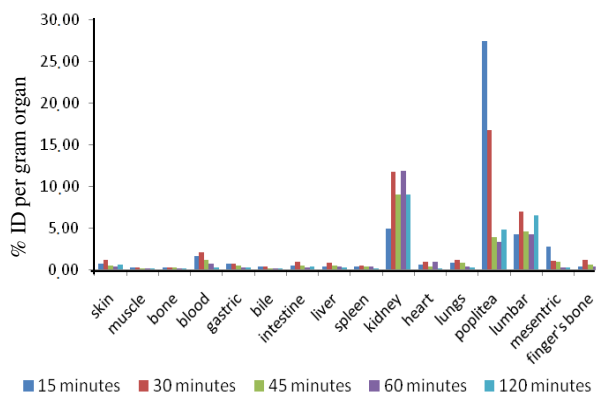
The acute toxicity test was conducted by the administration of <sup>99m</sup>Tc-DEC through two routes of injection, namely intradermal and intravenous injections. Observations were carried out for seven days and can be continued to up to 14 days in order to notice if there were abnormalities that may occur. The results of the toxicity tests are shown in Table 4. The data show that there was no abnormalities or death on test mice despite being given doses much higher than that expected to be used for humans. According to the test data on acute toxicity, it can be stated that the <sup>99m</sup>Tc-DEC is safe to be used.

**Table 4.** Observations acute toxicity tests of <sup>99m</sup>Tc-DEC on test animals

Injection routes	Weight of mice (gram)	Dose of <sup>99m</sup> Tc-DEC	Human Conversion dose	Abnormalities during 7-14 days of observation
intradermal	30.4 ± 2.70	462 µCi (15.2mCi/kg)	912 x	-
intravenous	38.0 ± 1.97	430 µCi (11.3mCi/kg)	135 x	-

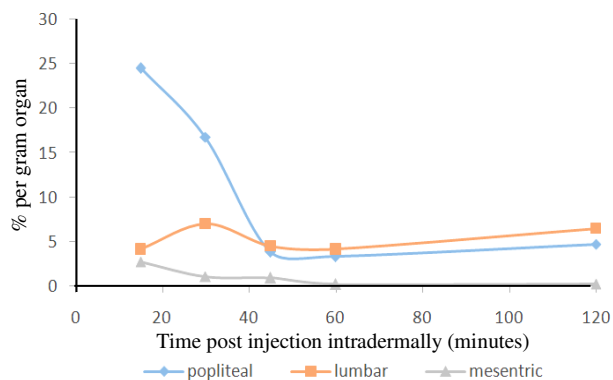
The biodistribution data displayed in Fig. 4 showed that at 15 minutes after intradermal injection the highest accumulation of radioactivity was found in the popliteal lymph nodes (27.5 %); it decreased to 4.76 % at 120 minutes after injection.

Accumulation was also found in several other lymphatic organs such as lumbar and mesenteric lymph nodes. Significant radioactivity was also found accumulating in the kidneys. This can be understood as the kidney is a main excretory organ and <sup>99m</sup>Tc-DEC is a polar compound. This biodistribution data indicates that <sup>99m</sup>Tc-DEC can reach the target organ.



**Fig. 4.** Biodistribution of  $^{99m}\text{Tc}$ -DEC in rat after intradermal injection.

Although the highest uptake of radioactivity was found in the popliteal lymph nodes at the beginning of injection as shown in Fig. 5, but the sharp decline occurred at minute 45 when compared with the lumbar and mesenteric nodes. This was possibly because the popliteal nodes are closer to the area of injection compared with lumbar and mesenteric nodes. Then, after decreasing, the accumulation of radioactivity rose in similar pattern for all of three lymphatic glands.

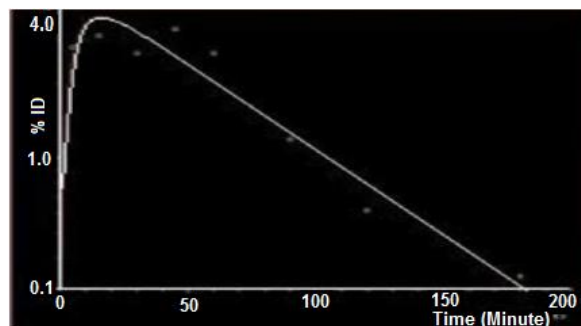


**Fig. 5.** Lymphatically uptake of  $^{99m}\text{Tc}$ -DEC.

A pharmacokinetic study was performed by injection of  $^{99m}\text{Tc}$ -DEC through intradermal and intravenous routes in normal mice and infected mice. Blood samples were collected from the mice tails at 5, 15, 30, 45, 60, 90, 120, and 180 minutes after injection. The blood samples were weighed and measured using a single channel analyzer (ORTEC 4890), and the calculation of radioactivities were corrected to the half-life ( $t_{1/2}$ ) of  $^{99m}\text{Tc}$ . Activity concentration in blood from the intradermal route of injection as a function of time is shown in Fig. 6.

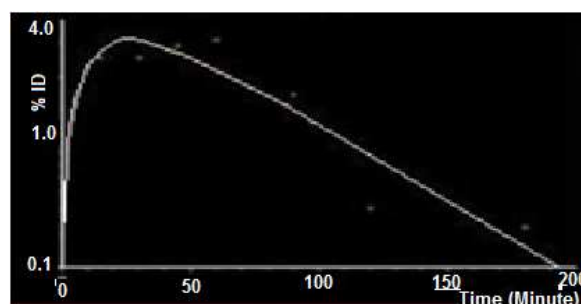
Assuming that  $^{99m}\text{Tc}$ -DEC was in one biocompartment, the value of biological half-life after intradermal injection in normal mice obtained at 44.32 minutes (Fig. 6(a)), whereas in infected

mice by 37.83 minutes (Fig. 6(b)). By considering the individual characteristics of living creatures, these values did not show a significant difference. Therefore, the biological half-life can be expressed as approximately 40 minutes. The  $^{99m}\text{Tc}$ -DEC that was administered via intradermal injection seemed to have a relatively longer biological half-life.



Kinetic profile of  $^{99m}\text{Tc}$ -DEC through intradermal administration in normal mice  $t_{1/2}$  distribution = 4 minutes, and  $t_{1/2}$  elimination = 44.32 minutes

(a)



Kinetic profile of  $^{99m}\text{Tc}$ -DEC through intradermal administration in infected mice  $t_{1/2}$  distribution = 10.49 minutes, and  $t_{1/2}$  elimination = 37.83 minutes

(b)

**Fig. 6.** Pharmacokinetic profile (intradermal route injection) of  $^{99m}\text{Tc}$ -DEC in normal mice (a) and infected mice (b).

This indicated that  $^{99m}\text{Tc}$ -DEC was retained in the body for a significant duration. On one hand, this provides a distinct advantage because the lymphoscintigraphy technique for detecting filariasis also takes a long time, so there is flexibility for physicians to perform diagnostic management. On the other hand, administration via intradermal injection would cause difficulties in distinguishing between the physical blockage or obstruction of infections caused by microfilariae through lymphoscintigraphy techniques; consequently, administration through intravenous injection becomes the preferred technique.

Figure 7 indicates that the biological half-life in one compartment model with intravenous injection give the value of 29.7 minutes. This value was sufficient to discover the uptake of  $^{99m}\text{Tc}$ -DEC in the body when an imaging was performed using a gamma camera.



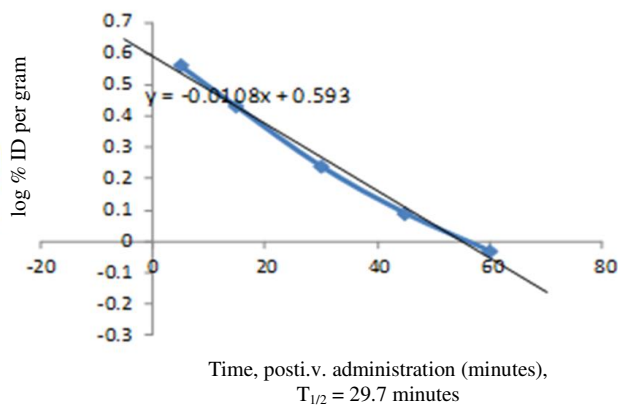
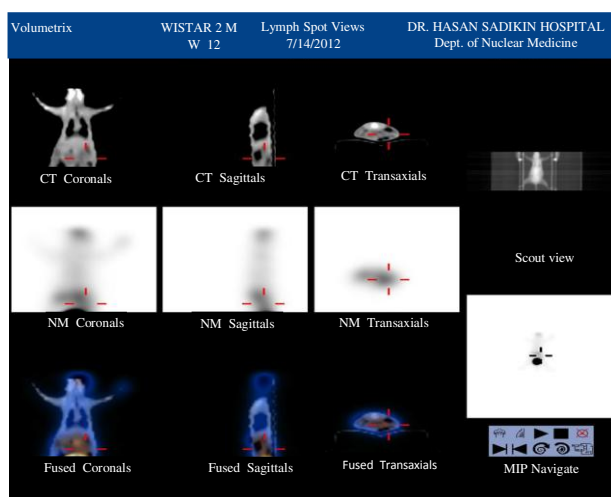
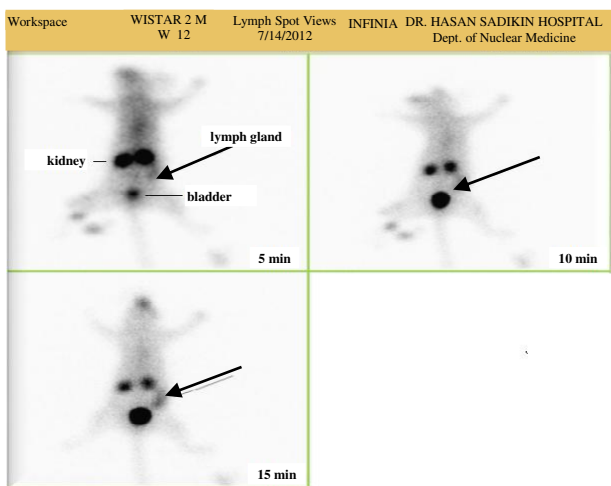


Fig. 7. Kinetic profile of <sup>99m</sup>Tc-DEC after intravenous injection.

Imaging with a gamma camera can be used as key evidence to indicate that this agent is acceptable for use. Imaging is intended to ensure that this agent accumulates in the target organ. Figure 8 (a and b) shows the result of gamma camera imaging after intravenous and intradermal administration of <sup>99m</sup>Tc-DEC in rat infected with filariae.



(a)



(b)

Fig. 8. Imaging in rat infected with filarial with <sup>99m</sup>Tc-DEC intradermally (a) and intravenously (b).

While gamma camera imaging results as displayed in Fig. 8(a) and 8(b) show <sup>99m</sup>Tc-DEC distributed in several organs of the rat, the evaluation is still difficult. The presence of blackening in the intestine after intravenous injection also gives doubt to determination if the abnormalities are caused by filariae. This is possible because the rodent's body is too small. Therefore, the final verification as an evidence for the solution should be performed on voluntary patients with filariae.

### Potential development for the future

Given the large number of filariasis cases found, it is hoped that this diagnostic agent can be immediately utilized. The preparation and analysis technology has been owned and mastered. Potential development is possible to follow up. Clinical evaluation of <sup>99m</sup>Tc-DEC to filarial patients is very important and absolutely must be done. The diffusion of technology and the development of science and technology capacity of the production system is expected to accelerate.

### CONCLUSION

Diagnostic kits of DEC in a freeze dried and sterile form have been successfully formulated and ready to be labeled with <sup>99m</sup>Tc to produce <sup>99m</sup>Tc-DEC for diagnosis filariasis infection. *In-vitro* and *in-vivo* evaluations such as testing stability of <sup>99m</sup>Tc-DEC, biodistribution, pharmacokinetic, and toxicological studies which have been conducted and showed very promising results.

In a new drug development, there are many requirements that must still be met prior to use by the public, especially the completion of the preclinical and clinical data, particularly in voluntary patients with filariae.

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