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Development and Evaluation of ^{99m}Tecnetium-Ethionamide for Mycobacterial Infection Imaging

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A quick and reproducible method for radiolabeling of ethionamide, an antitubercular drug, with radioisotope of tecnetium, ^{99m}Tc was developed. The radiotracer was evaluated for radiochemical purity, stability, and tissue distribution in normal mice. The ^{99m}Tc labeled ethionamide, prepared by using reduced ^{99m}Tc, demonstrated good labeling efficiency (>85%) and reproducibility. The procedure offers minimum radiation exposure to the radiochemist as handling time is also less. Stannous chloride was used as reducing agent at a concentration of 15 μg/ml at pH 2.0. The radiocomplex was stable till 6 h at 37°. In vitro uptake study of ^{99m}Tc labeled ethionamide in *Mycobacteria tuberculosis* $H_{37}R_v$ showed higher uptake in live bacteria. The pharmacokinetic parameters of ^{99m}Tc labeled ethionamide, calculated by blood clearance studies in normal New Zealand white rabbits, were found to differ considerably from the native drug. ^{99m}Tc labeled ethionamide has low plasma protein binding of approximately 35%, and it showed no organ specific accumulation. The radiotracer was found to be a widely and rapidly distributed molecule in the body similar to native drug. Radiolabeled ethionamide penetrated all the organs and got cleared off due to short elimination half-life. The scintigraphic studies in infected rabbits confirmed that ^{99m}Tc-labeled ethionamide was preferentially taken up by the tubercular lesion.

Tuberculosis is a chronic granulomatous disease caused by *Mycobacterium tuberculosis*. It is a major health disorder killing about 3 million people all over the world every year[1]. Such a high prevalence of tuberculosis in developing and developed countries is attributed to poor hygiene, easy mode of transmission, unawareness about symptoms and delays in diagnosis. In the beginning tuberculosis (TB) is easier to cure but difficult to detect, but later it becomes easy to detect but difficult to cure[2]. A number of diagnostic tools like enzyme-linked immununo sorbent assay (ELISA), polymerase chain reaction (PCR), computed tomographic (CT) scan, magnetic resonance imaging (MRI), radiography and culture tests are available for the diagnosis of TB but they cannot detect the infection at an early stage and/or suffer from other limitations. Techniques like ELISA and PCR, though sensitive, but they cannot tell the exact site of infection. CT scan, MRI and radiography are not tubercular infection specific, diagnostic modalities. Therefore, there is a need for development of a specific and sensitive tool, which cannot only detect TB at an early stage but is also be able to tell the exact site of infection.

Nuclear medicine is a well-explored field in the diagnosis of infectious diseases. ^{99m}tecnetium-ciprofloxacin developed by Solanki et al in 1993 is the most commonly used infection specific imaging agent[3]. Britton *et al.* 2002 have reported 574 case studies in which ^{99m}tecnetium-ciprofloxacin has been used for the diagnosis of occult infections[4]. This radiopharmaceutical based on the affinity of ciprofloxacin for bacteria, cannot differentiate between a tubercular infection and any other bacterial infection. Antitubercular drugs like isoniazid, ethionamide and ethambu-

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tol are specifically used in the treatment of tuberculosis. Isoniazid and ethionamide act by inhibiting the synthesis of mycolic acid[5]. It is β–hydroxy α–alkyl fatty acid, present in the cell wall of the mycobacterium[6]. Our laboratory at INMAS is working on the development of tubercular specific, infection imaging agents. We have successfully labeled isoniazid and ethambutol with ^{99m}technetium (^{99m}Tc) and evaluated them for tubercular lesion detection in animal models[7]. Ethionamide (ETH) is a tuberculostatic drug of moderate efficacy introduced in 1956. It is a specific antitubercular drug interacting specifically with *Mycobacterium*. It acts by inhibiting the synthesis of mycolic acid[8] in the cell wall of *Mycobacterium*, which is an essential component for the *Mycobacterium* to be alive. So, this study was planned to exploit the therapeutic property of this drug for diagnosis of Mycobacterial lesions. In the present study, ETH was labeled successfully with ^{99m}Tc. All the essential in vitro and in vivo quality control parameters were studied to standardize the radiotracer. Scintigraphic studies of ^{99m}Tc-ETH in tubercular lesion bearing rabbits confirmed the utility of radiopharmaceutical as a tubercular infection-imaging agent.

MATERIALS AND METHODS

^{99m}Tc-pertechnetate was eluted from 99-Mo-^{99m}Tc generator by solvent extraction method. BRIT, Mumbai, India, supplied the molybedenum-99.

Radiolabeling:

Two milligrams of ethionamide (Panacea Biotec Laboratories, Delhi) was thoroughly dissolved in 1 ml of 0.1 N HCl, and the pH of the solution was 1.0. All the solutions used were nitrogen-purged. To this solution, 15μg of stannous chloride dihydrate (Sigma, St. Louis, MO, USA) in 15μl of 0.1 N HCl was added. The contents were passed through a 0.22 μM filter (Millipore Corporation, Bedford, MA, USA) into an evacuated sterile sealed vial. Sterile ^{99m}Tc-pertechnetate (1–2 ml; 70 MBq) was added drop wise to the vial with continuous mixing over a period of 30 s. The reaction mixture was incubated at room temperature for 15 min with continuous nitrogen gas purging.

Radiochemical purity:

The radiochemical purity of ^{99m}Tc-ETH was assessed by ascending instant thin layer chromatography (ITLC) using silica gel-coated fiber glass sheets (Gelman Sciences Inc., Ann Arbor, MI, USA) and solvent systems, containing acetone as mobile phase. The radioactive contaminants were identified as reduced/hydrolyzed (R/H) ^{99m}-technetium and free ^{99m}Tc-pertechnetate. Free pertechnetate and radiolabeled complex moves with the solvent front and R/H ^{99m}Tc remains at the point of application. Absence of free pertechnetate was confirmed by *in vivo* studies in mice and rabbits. The animal experimental protocols were approved by our Institutional Animal Ethics Committee.

In vitro Stability:

The radiolabel was tested for its *in vitro* stability by ascending ITLC. The preparation was incubated at 37° for 24 h. ITLC was carried out to assess the labeling efficiency at different time intervals.

In vitro uptake studies by *Mycobacteria tuberculosis:*

In vitro uptake of ^{99m}Tc-ETH was studied in *Mycobacteria tuberculosis* $H_{37}R_v$. ^{99m}Tc-ETH in the concentrations of 3, 6 and 12 μg/ml was incubated with $1x10^6$ cells of *M. tuberculosis*, $H_{37}R_v$, in 5 ml of Middlebrook 7H9 media (Difco) (each in triplicate). Number of cells was calculated by taking the absorbance of cell suspension at free pertechnetate was used as the negative control. Similar experiment was setup for the heat-killed bacteria. Four-hundered microlitres of the test samples were taken at the intervals of 2, 4 and 24 h and centrifuged at 10 000 rpm for 10 min. The pellets and supernatants were separated and the radioactivity was counted in well-type gamma ray spectrometer (ECIL, India). The percentage of the activity in pellet of the total (supernatant+ pellet) activity was calculated.

Blood clearance and plasma protein binding:

Blood clearance of ^{99m}Tc-ETH was studied in rabbits. Radiolabeled drug (70 MBq) was administered to each rabbit through the ear vein and blood samples were collected at different time intervals. The radioactivity in blood was calculated as percentage of the injected dose. From the blood samples, plasma was separated by centrifugation and the proteins were precipitated by adding equal volumes of 12.5 % trichloroacetic acid (TCA) and plasma. The radioactivity in the precipitate and supernatant was measured in a well-type gamma spectrometer. The plasma protein binding was expressed as a fraction of total activity in the sample.

Biodistribution:

In vivo distribution of ^{99m}Tc-ETH was studied in 2- to 3-month-old strain 'A' mice. ^{99m}Tc-ETH (50 μl, 40 KBq) was administered to each mice weighing 25-30 g through the tail vein. The animals were sacrificed at different time intervals, and different organs were removed, washed with normal saline, and dried in the paper folds. The radioactivity in

TABLE 1: EFFECT OF PH ON LABELING EFFICIENCY OF ^{99M}TC-ETH

pH	R/H ^{99m}Tc	^{99m}Tc-ETH
2	12.32	87.68
4	90.93	9.065
6	94.02	5.975

TABLE 2: EFFECT OF AMOUNT OF STANNOUS CHLORIDE DIHYDRATE ON THE LABELING EFFICIENCY OF ^{99M}TC-ETH.

Stannous chloride (μg)	^{99m}Tc-ETH	R/H ^{99m}Tc
10	80.98	7.85
15	87.38	10.2
25	77.96	22.44
35	70.5	28.44

each organ was counted using well-type gamma spectrometer and expressed as percent injected dose per organ.

Animal model and scintigraphy:

For the development of animal models of *M. tuberculosis*, healthy and infection free New Zealand white rabbits, each weighing approximately 2 kg were selected. The right thigh of each rabbit was shaved and disinfected with 70 % alcohol. 0.5 ml of $1x10^8$ live mycobacterial cells/ml in growing phase from clinical human isolates was injected intramuscularly. The animals were kept in isolation on normal feed and their weight, state of the site of injection and body temperature were monitored. The development of lesion in each animal was confirmed after four weeks by subjecting the aspirated samples to acid fast staining and culture test.

In vivo efficacy of the ^{99m}Tc-ETH was evaluated by scintigraphic studies under gamma camera (Electronics Corporation India Limited, ECIL). Seventy MBq of the ^{99m}Tc-ETH was administered to the infected New Zealand white rabbit via marginal ear vein. The rabbits were sedated with 0.75 ml/kg body weight of diazepam (Calmpose®, Ranbaxy Laboratories Limited) injected intravenously, and 1 mg/kg body weight of ketamine were administered intramuscularly. The rabbit was imaged under a Gamma camera at the time intervals of 2, 4 and 24 h. Scintigrams of rabbit showing the whole body distribution and accumulation of ^{99m}Tc-ETH in the infectious lesion were acquired.

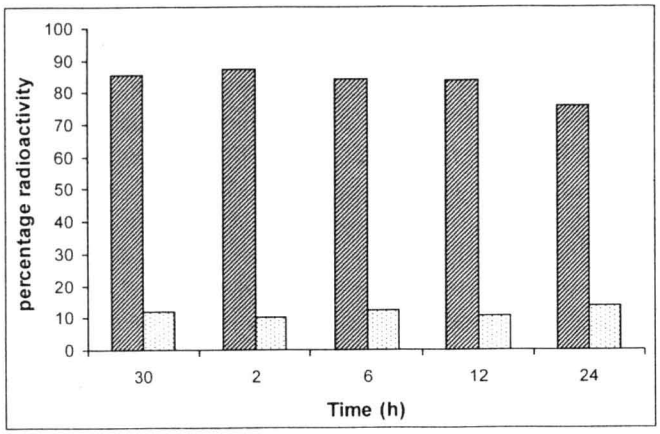

Fig. 1: *In vitro* stability of ^{99m}Tc-ETH

Percent radioactivity with time as (▨) ^{99m}Tc-ETH, (□) R/H ^{99m}Tc

RESULTS

ETH, a specific antitubercular drug, was labeled with ^{99m}Tc at pH 2.0 using 15 μg of stannous chloride dihydrate as a reductant. The radiochemical purity of the product was evaluated by ITLC, labeled preparations comprised of more than 85% of the ^{99m}Tc-ETH, with the rest being R/H ^{99m}Tc and free ^{99m}Tc. To achieve optimum labeling efficiency, the pH of the reaction mixture was varied from 1.0 to 6.0, while keeping rest of the factors constant. Similarly, the amount of stannous chloride dihydrate was also varied from 10–40 μg, while keeping the pH, amount of ETH, and volume of the reaction constant. Results of the effect of pH and amount of stannous chloride dihydrate on labeling efficiency are shown in Table 1 and 2, respectively. At pH 6.0, the R/H ^{99m}Tc fraction was very high, and at pH 4.0, it was found to be 90%. Similarly the effects of varying the concentration of stannous chloride dihydrate from 10 to 40 μg, keeping other conditions constant, is shown in Table 2. Fifteen μg of $SnCl_2.2H_2O$ and pH of 2.0 yielded the highest labeling efficiency of ^{99m}Tc-ETH. ^{99m}Tc-ETH complex was fairly stable *in vitro* up to 6 hours, and at 24 hours the percent of labeled product was only 75.9% as determined by ITLC (fig. 1).

In vitro uptake of radiolabeled ETH by *M. tuberculosis* was found to be 13.3 % in live bacteria at 24 h as compared to 8.2 % in heat-killed bacteria at 3 μl/ml (fig. 2). The uptake got increased at concentration 6 and 12 μg/ml but the ratio of percentage uptake in live to dead bacteria is maximum at 3 μg/ml i.e. 3.03 at 2 h. Blood clearance data in rabbits after intravenously administering 70 MBq of ^{99m}Tc-ETH exhibited fast and first order kinetics (fig. 3). Pharmacokinetic

TABLE 3: TISSUE DISTRIBUTION IN NORMAL MICE

Time	1 h	4 h	24 h
Blood	5.52±0.34	2.83±0.28	0.42±0.21
Liver	6.45±0.37	5.31±0.25	1.67±0.39
Kidneys	3.39±0.18	3.36±0.11	2.95±0.23
Spleen	1.89±0.15	1.48±0.14	0.52±0.13
Muscle	0.74±0.16	0.58±0.20	0.11±0.18
Bone	0.55±0.23	0.17±0.26	0.11±0.22
Heart	1.63±0.19	0.41±0.21	0.11±0.18
Lungs	2.28±0.12	1.37±0.12	0.65±0.13
Stomach	0.63±0.31	0.36±0.22	0.11±0. 28
Intestine	1.22±0.11	0.96±0.31	0.36±0.26

Tissue distribution in normal mice at 1, 4, and 24 h after intravenous administration of ^{99m} tc-ETH. Each value is expressed as percentage of the injected activity per g of an organ. Data represented as mean±SD (n=3)

parameters like elimination half life (0.004125 min^{-1}), plasma half-life, volume of distribution and blood clearance of ^{99m}Tc-ETH were calculated from the semi log plot of counts per gram of blood versus time (Table 4).

In vivo protein binding was 38 % at 30 min, and remained between 35-40 % till 6 h post administration of the complex as shown in fig. 4. Biodistribution data of ^{99m}Tc-ETH in 2-3 month old strain 'A' mice is summarized in Table 3. Based on the percent injected dose per whole organ, highest uptake of ^{99m}Tc-ETH was found in liver, blood, kidneys, lungs, intestines, and spleen averaging 6.45, 5.52, 3.39, 2.28, 1.22 and 1.89 %, respectively, at 1 h postadministration. Very little uptake of the radiotracer was seen in stomach and bone. Radioactivity in all the organs except kidneys got reduced considerably at 24 h. After administration of ^{99m}Tc-ETH, scintigraphy in lesion bearing rabbits showed a significant uptake at 4 hours. Maximum lesion-to-muscle ratio was 1.29 at 4 h postadministration (fig. 4).

DISCUSSION

Ethionamide, 2-ethyl-4-pyridine carbothioamide, is an antitubercular drug. The exact mechanism of action of ETH is not known. Rist *et al.* 1959 proposed that ETH acts by inhibiting the synthesis of mycolic acid[8]. It is almost insoluble in water[9] but because of being a basic drug, it was dissolved in 0.1 N HCl[13]. ETH was labeled successfully with ^{99m}Tc using stannous chloride as the reducing agent. Other solvents like 1 % acetic acid, ethanol and water with ethanol as a cosolvent, were also tried as solvents for ETH but the labeling efficiency of the drug went down remarkably. Therefore, 0.1 N HCl was found to be the best solvent to achieve maximum labeling of the drug. A simple and repro-

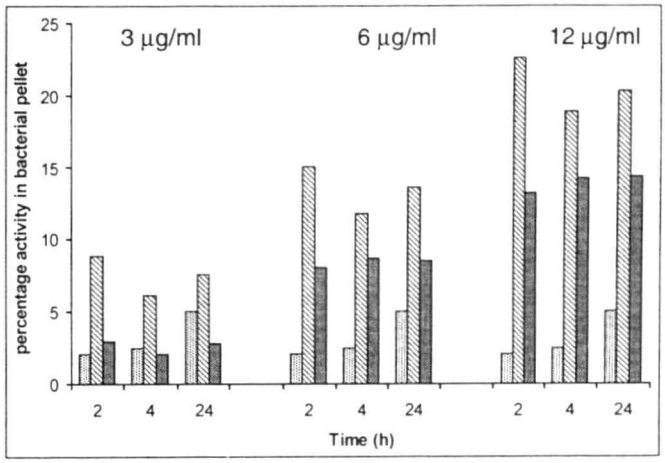

Fig. 2: *In vitro* uptake of the ^{99m}Tc-ETH by *M. tuberculosis* $H_{37}R_v$

***In vitro* uptake of the ^{99m}Tc-ETH by *M. tuberculosis* $H_{37}R_v$ at a concentration of 3, 6 and 12 µg/ml at 37°. ^{99m}Tc was taken as negative control and heat killed 1x10^6 *M. tuberculosis* cells also served as negative control. Each value is expressed as percentage activity of ^{99m}Tc-ETH or ^{99m}Tc bound to *M. tuberculosis* divided by total activity. Free pertecnetate in live bacteria (▥), ^{99m}Tc-ETH in live bacteria (▨) and ^{99m}Tc-ETH in heat-killed bacteria (■)**

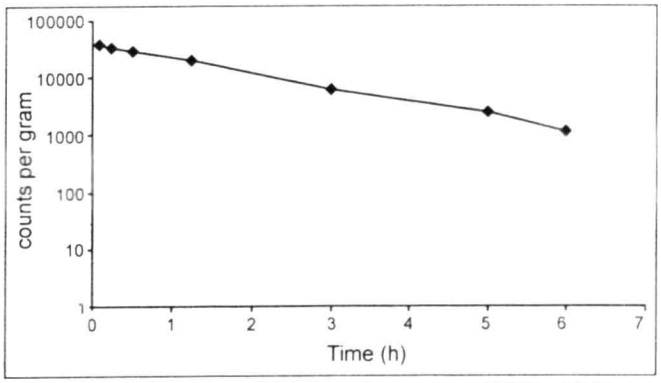

Fig. 3: Semi log plot of blood clearance of ^{99m}Tc-ETH.

Blood clearance of ^{99m}Tc-ETH in healthy New Zealand rabbit (average data of 3 rabbits)

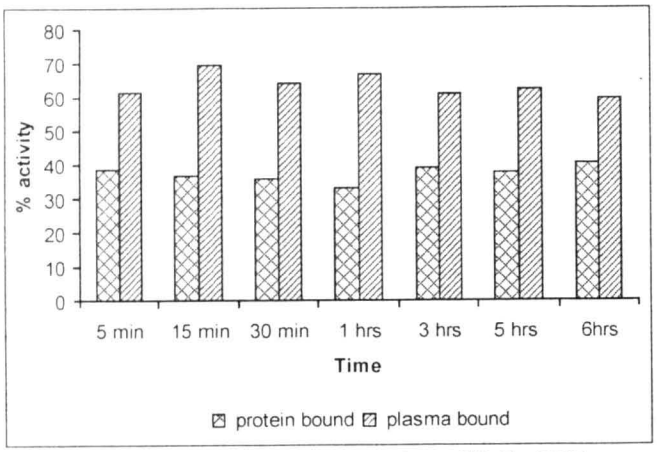

Fig. 4: *In vitro* plasma protein binding of ^{99m}Tc-ETH

Plasma protein binding expressed as a fraction of total radioactivity in the sample. Protein bound (▩) and plasma bound (▨)

Whole body scintigrams of tubercular lesion bearing New Zealand white rabbit obtained at 4 hours post-iv administration of 70 MBq of ^{99m}Tc-ETH. a) Scintigram of rabbit showing the lesion as hot spot b) whole body image of the rabbit depicts the distribution of the radiotracer in different organs.

ducible labeling procedure was developed at pH 2. At higher pH, the lower labeling efficiency was observed due to the precipitation of the drug. The radiochemical purity was studied by ITLC, and the radioactive contaminants were identified as R/H and free pertechnetate. Free ^{99m}Tc moved away with the solvent front whereas R/H ^{99m}Tc remained at the point of application when acetone was used as the mobile phase. Labeled ^{99m}Tc showed a Rf value of 0.8 in the above said system. The chromatogram when developed in iodine chamber confirmed the presence of labeled drug as a spot at Rf 0.8. Studies on the optimization of labeling parameters revealed that the optimal range of stannous ion concentration and pH were quite low. We have critically studied the stability, blood kinetics, biodistribution and tubercular infection imaging specificity of the radiopharmaceutical. For proceeding to the *in vivo* studies of ^{99m}Tc-ETH in animal models, in vitro uptake of the radiotracer by mycobacterium was studied.

^{99m}Tc-ETH was fairly stable till 24 h. The *in vivo* stability of the complex was evident from the negligible level of ^{99m}Tc-ETH for stomach, the target organ for free ^{99m}Tc[10]. Stability of the radiotracer was further confirmed by biodistribution studies indicating 0.63 and 0.11 % of the radiotracer in stomach at 1 and 24 h, respectively. *In vitro* uptake of ^{99m}Tc-ETH by mycobacterium showed active as well as passive diffusion. Considerable levels of the radiopharmaceutical were observed in heat-killed bacteria. However, higher uptake in live bacteria gave the lead to use this radiotracer as a tubercular infection-imaging agent.

Plasma protein binding of a drug depends upon the hydrophobicity and acidic or basic nature of the agent[11]. Buchanan *et al.* 1977 reported 30 % plasma protein binding of ETH, which decreased, to 24 % in kwashiorkor serum protein[12]. Plasma protein binding of ^{99m}Tc-ETH was found to be approximately 35-40 % over the period of 6 h. Low plasma protein binding of the radiotracer indicates that the drug would be able to cross the capillary walls and will have a wide volume of distribution. This observation is extended by the blood clearance studies. Pharmacokinetic parameters of ^{99m}Tc-ETH were calculated by plotting counts per gram of blood versus time on a semi logarithm paper[13]. Volume of distribution of the radiopharmaceutical was found to be 532.4 ml in New Zealand white rabbits. High volume of distribution signifies the accessibility of the radiotracer to different tissues. Biodistribution studies of ^{99m}Tc-ETH in 2-3 months old strain 'A' mice confirm this statement as higher levels of the radiopharmaceutical were seen in liver, kidneys, lungs, spleen, blood and intestine.

Greenberg *et al.* 1962 have carried out the pharmacokinetic studies of ETH in rabbits after its intravenous administration[14]. The drug distributed rapidly and widely in the

TABLE 4: PHARMACOKINETIC PARAMETERS OF ^{99M}TC-ETH AND ETH.

Pharmacokinetic parameters	ETH	^{99m}Tc-ETH
Plasma half life	35 min[a]	66 min
Volume of distribution	86% per Kg body weight[a]	12.6% per Kg body weight
Plasma protein binding	30%[b]	35-40%
Clearance	48 ml/min[a]	3.308 ml/min

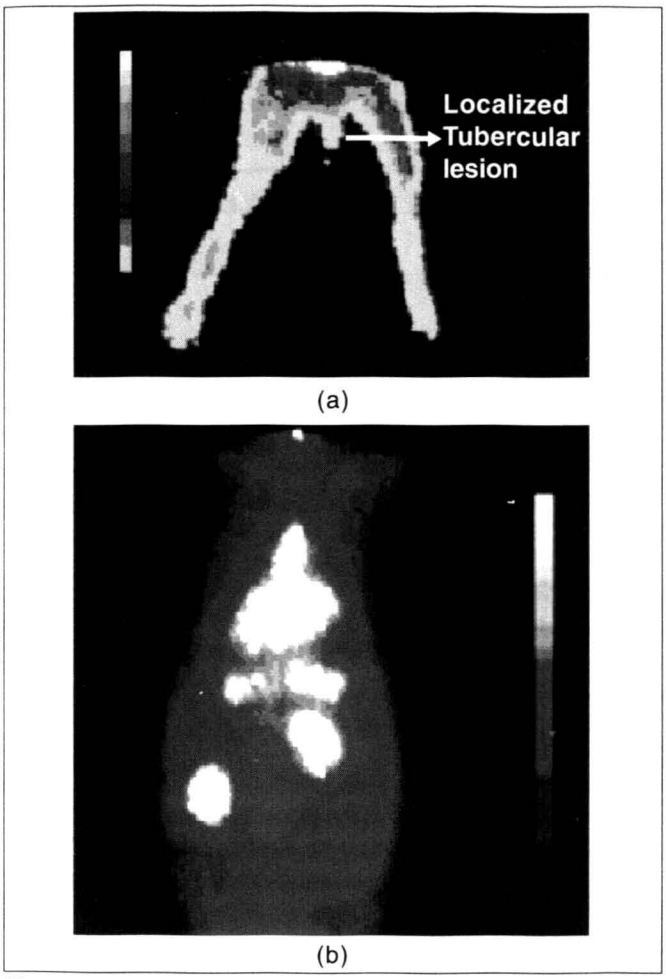

Fig. 5: Whole body scintigrams of tubercular lesion bearing rabbit

Whole body scintigrams of tubercular lesion bearing New Zealand white rabbit obtained at 4 hours post-iv administration of 70 MBq of ^{99m}Tc-ETH. a) Scintigram of rabbit showing the lesion as hot spot b) whole body image of the rabbit depicts the distribution of the radiotracer in different organs.

body. The scintigrams of the lesion bearing rabbits and biodistribution studies of ^{99m}Tc-ETH in mice showed that the radiotracer is a widely distributed agent. The pharmacokinetic parameters of ^{99m}Tc-ETH and ETH vary considerably (Table 4). These results suggest that ETH after labeling with ^{99m}Tc distributes less widely and less rapidly than the native drug. Increase in the half-life and decrease in clearance indicates longer residence of the radiotracer in body as compared to native drug. Greenberg *et al.* have also reported that there was no organ specific accumulation of ETH was seen. Similarly, the biodistribution of ^{99m}Tc-Eth in mice and whole body scintigrams of rabbits showed that the radiotracer distributed equally in all the organs. The radiopharmaceutical like the native drug washed out of the organs with the course of time as very low levels of it were observed at 24 h in mice.

The scintigraphic studies of the radiopharmaceutical in tubercular lesion bearing New Zealand rabbits showed significant uptake in lesion as compared to muscle. Lesion to muscle ratio observed in case of ^{99m}Tc labeled isoniazid is reported to be 2 after 2 h postadministration which increase to 3.5 at 24 h[7]. In case of ^{99m}Tc-ETH the ratio is less, i. e., 1.29 at 4 h but the radiopharmaceutical has pharmacokinetic advantages. Low plasma protein binding, faster elimination half-life and high volume of distribution of ^{99m}Tc-ETH in comparison to ^{99m}Tc labeled isoniazid makes almost all the organs accessible to the radiotracer. Moreover, no organ specific accumulation of the ^{99m}Tc-ETH was found.

The developed radiopharmaceutical, ^{99m}Tc-ETH, is shown to be stable and reproducible with good labeling efficiency. The radiotracer specifically accumulates in *M. tuberculosis* (both *in vitro* and *in vivo*). ^{99m}Tc-ETH has a limitation of being an acidic preparation. But low plasma protein binding and wide distribution of the radiotracer in the body makes the radiopharmaceutical accessible to all the tissues and thus it can be explored to detect deep-seated tubercular infection.

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