Labeling of epirubicin with technetium-99m: Optimization, biodistribution and scintigraphic imaging in tumor bearing mice

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Abstract: Epirubicin is an antineoplastic agent of anthracycline antibiotic, used for treating a variety of tumor types such as lymphoma, cancer of the breast, lung, ovary and stomach. The objective of this work was to demonstrate direct radiolabeling of epirubicin with ^{99m}Tc, quality control, biological characterization and scientigraphic evaluation in tumor bearing mice. The ^{99m}Tc-epirubicin labeling was optimized by varying the amounts of ligand 100-350µg, stannous chloride dihydride 20-50µg and pH range 2-10 by using NaOH or HCl. The radiochemical purity of ^{99m}Tc-epirubicin was evaluated by chromatographic techniques (Whatman No. 3 paper and ITLC-SG). HPLC analyses were performed to check purity of epirubicin and radiochemical purity of labeled ^{99m}Tc- epirubicin. Biodistribution and scintigraphic imaging of ^{99m}Tc-epirubicin was performed in normal and tumor bearing mice at various time intervals. The optimum conditions ensuring ^{99m}Tc-epirubicin labeling yield as high as 99% by adding 35µg $SnCl_2.2H_2O$, 200µg of ligand at pH 6 for 30 min at room temperature (25°C±2°C). HPLC of ^{99m}Tc-epirubicin shows about 99% binding of the compound with technetium-99m. Electrophoresis study indicated the neutral nature of ^{99m}Tc-epirubicin. Biodistribution data and scintigraphic results showed that ^{99m}Tc-epirubicin accumulated in the liver as well as in tumor with significant uptake and excellent retention. ^{99m}Tc-epirubicin shows good stability in human serum. *In vitro* and *in vivo* studies revealed the significantly uptake of ^{99m}Tc-epirubicin in the tumor, and also indicating the efficiency of ^{99m}Tc-epirubicin as a tumor diagnostic agent.

Keywords: ^{99m}Tc-epirubicin, quality control, HPLC, tumor, biodistribution, scintigraphy.

INTRODUCTION

Cancer is a main cause of death throughout the world and needs accurate diagnosis for treatment. *In vivo* imaging can help in diagnosis, staging tumors, and improve drug scheduling, radiotherapy, and systemic treatment resulting advantage to both patient and oncologist (Hanahan and Weinberg, 2000; Munnink *et al*., 2009; Schottelius and Wester, 2009). Modern biomedical imaging technologies provide non-invasive imaging that helps in assessment of proliferation of cancer cells. SPECT and PET are two most important imaging procedures in nuclear medicine. Both of these can be used to estimate tumor growth potential, thus provide surgeons for estimation of treatment response. In present days research attention is focused on the designing of new radiopharmaceuticals which can concentrate in cancer cells selectively (Liu and Edwards, 1999). Technetium-99m based radio pharmaceuticals are used for diagnosis of cancerous and non-cancereous diseases involving different parts of body because Technetium-99m is the radionuclide having favorable nuclear properties such as half- life of 6 h, 140 keV, γ emitters and low cost (Jurisson and Lvdon, 1999; Jurisson *et al*., 1993). Technetium-99m is most widely eluted from ^{99}Mo/^{99m}Tc generator systems in all the radiopharmacy centers where radiopharmaceuticals are prepared for gamma imaging (Eckelman, 2009). Several pharmaceuticals are being designed to improve the diagnosis and treatment of cancers, such as ^{99m}Tc-spermine (Xu *et al*., 2013), ^{99m}Tc-doxorubicin (Fahem *et al*., 2012), ^{99m}Tc-5-fluorouracil (Dar *et al*., 2013), ^{99m}Tc-PyDA (Sakr *et al*., 2014), ^{99m}Tc-labeled Mitomycin C (Bokhari *et al*., 2015), ^{99m}Tc-methotrexate (Dar *et al*., 2012), ^{99m}Tc-BnAO-NI, ^{99m}Tc-DETA, ^{99m}TcN-MAG-AMCPP, ^{99m}Tc-TETA, ^{99m}Tc-TEPA, $^{99m}Tc(CO)_3$-labeled chlorambucil analog, ^{99m}Tc-nitride-pyrazolo[1,5-a] pyrimidine and $[^{99m}Tc(CO)_3(IDA\text{-}PEG3\text{-}CB)]_2$ (Mallia *et al*., 2010; Satpati *et al*., 2009; Ding *et al*., 2012), ^{99m}Tc-vincristine (Hina *et al*., 2015), ^{99m}Tc-glycopeptide (Wei *et al*., 2015), ^{99m}Tc-daunorubicin (Faheem *et al*., 2013), ^{99m}Tc-N-(2- Hydroxybenzyl)-2-amino-2-deoxy-D-glucose (Nadeem *et al*., 2013) and ^{99m}Tc-N_4-guanine (Tsao *et al*., 2013). Anthracyclines are are most effective among all chemotherapeutic drugs at early stages because of survival advantage as compared to non-anthracyclines having adjuvant treatments (Abe *et al*., 2005). Epirubicin is used to treat breast cancer, stomach cancer, lung cancer, ovarian cancer and myeloma (Robert, 1993). The mechanism of epirubicin cytotoxicity is by DNA

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intercalation, topoisomerase II inactivity, oxygen production and drug free radicals that end in interfering with DNA or protein synthesis and resulting cytocidal activity (Khasrawa *et al*., 2012; Bonadonna *et al*., 1993).

The main aim of present study was to check capability of ^{99m}Tc-epirubicin in detecting tumor sites in mice and visualize them by using a high-resolution gamma camera imaging. Labeling efficiency of epirubicin with ^{99m}Tc, quality control, stability, and biological distribution in tumor bearing Swiss Webster mice were evaluated.

MATERIALS AND METHODS

Reagents

All the chemical reagents of AR grade were used in experiment without further purification. Epirubicin hydrochloride for intravenous injection was the product of Pfizer, Australia was purchased from Lahore, Pakistan. Medical tracer Tc-99m was eluted as $^{99m}TcO_4^-$ from locally produced PAKGEN ^{99m}Mo/^{99m}Tc generator. A HPLC (C-18 column, waters, μ-Bondapak^{TM}C18, 3.9×300 mm) was used to examine the purity of the prepared ^{99m}Tc-epirubicin. The NaI(Tl) γ-ray scintillation counter (Ludlum model 261) was used for the measuring tissue and organ radioactivity.

Animals

Swiss Webster mice (25-28 g), with naturally developed tumors were obtained from the Animal House at School of Biological Sciences (SBS), University of the Punjab, Lahore and Swiss Albino mice weighing approximately 28 g were purchased from NIH, Islamabad, Pakistan. All the experiments with animals were performed according to the principles approved by the Animals Ethics committee of institute (Document No.IPDs-H-SOP-04-003). For SPECT imaging studies of mice Gamma scintillation camera was used.

Labeling methodology of ^{99m}Tc-epirubicin

The ^{99m}Tc-epirubicin labeling was optimized by varying the amounts of ligand 100–350 μg, stannous chloride dihydride 20–50 μg and pH range 2–10. The pH of solution under reaction was adjusted by using NaOH or HCl. The vial was incubated for different time intervals to determine stability and highest labeling efficiency of ^{99m}Tc-epirubicin at room temperature. Finally ^{99m}Tc-epirubicin was prepared by adding 200 μg of ligand and stannous chloride dihydride 35 μg at pH 6. After adding all reagents, ~ 296 MBq ^{99m}Tc in saline was added in the vial and incubated at room temperature for 30 min. In all experiments reaction mixture volume was 1.0 ± 0.2 ml.

Quality control of ^{99m}Tc-epirubicin

The quality control of ^{99m}Tc-epirubicin was done by ascending chromatographic methods. Free Pertehnetate ($^{99m}TcO_4^-$) in the reaction mixture was analyzed with Whatman No.3 paper (stationary phase) and acetone (mobile phase). The amount of reduced and hydrolyzed was checked by using ITLC-SG strips as the stationary phase and THF as a mobile phase. The stability of ^{99m}Tc-epirubicin was analyzed at room temperature for 5 h. The distribution of activity on strips was traced and quantitated using 2π Scanner (Berthold, Germany).

HPLC analysis of ^{99m}Tc-epirubicin

HPLC of ^{99m}Tc-epirubicin was performed *via* HPLC (D-200 Elite system). The C-18 column and mixture containing acetonitrile and Na_2HPO_4 buffer (pH was set at 5-6 by 0.5M NaOH) ratio 45:55 (v/v %) was employed as mobile phase. Sodium Iodide (NAI) crystal detector was used for measuring radioactivity. The flow rate was adjusted at 1 ml/min at wavelength of 254nm. UV detector was used for detection of pure epirubicin compound.

Stability studies of ^{99m}Tc- epirubicin

The stability of ^{99m}Tc-epirubicin was determined by ITLC-SG and paper chromatography at room temperature and human blood serum. The ^{99m}Tc-epirubicin complex was spotted on chromatographic strips after 30 min, 1h, 2 h, 4h, 5h and 24h, developed and scanned by virtue of which *in vitro* stability of the labeled preparation was ascertained. The stability of the labeled product was investigated in human serum sample by adding 0.2mL of ^{99m}Tc-epirubicin and 1.8ml of human serum at 35ºC. Then, the samples were analyzed at different time intervals up to 24h.

Electrophoresis procedure

Electrophoresis of ^{99m}Tc-epirubicin was done with Deluxe electrophoresis chamber of Gelman system. Whatman No. 1 strips were soaked with 0.05 M phosphate buffer, put a drop of ^{99m}Tc-epirubicin and introduced into the chamber. The strip was retained for 45-60 min under applied voltage (300 V), dried and scanned with the help of 2π scanner.

Biological distribution of ^{99m}Tc-epirubicin in normal and tumor bearing mice

Biological distribution studies of ^{99m}Tc-epirubicin were carried out in naturally developed mammary tumor bearing Swiss Webstar mice and normal Swiss Albino mice having weight ranges 25-28g. The mice were anestesized in a jar having cotton swab dipped in chloroform. The prepared ^{99m}Tc-epirubicin injection containing ~ 74 MBq (2 mCi) was injected intravenousl into the tail. The mice were weighed, sacrificed and biological distribution ^{99m}Tc-epirubicin in various organs was determined. Blood samples (0.2ml) were taken by heart rupture, weighed and blood activity was analyzed by supposing blood volume as 6.5% of body weight and activity was determined using a γ-counter. The results were expressed as the percentage of injected dose per gram of tissue (% ID/g).

Scintigraphic imaging of ^{99m}Tc- epirubicin

For evaluation of potential of prepared radiopharmaceutical as a feasible imaging agent, the scintigraphic study was carried out in mice bearing tumor. The animal was anesthetized and put on a hard flat board using surgical tape. After preparing the labeled kit under sterilized conditions, 200µL of ^{99m}Tc-epirubicin was intravenously injected in tail. The animal was placed under the head of gamma camera projecting the dorsal view of the animal. The energy window of 20% was set on 140 keV. Images were developed on 256×256 matrix size for five min each. Whole body static images were taken after ^{99m}Tc-epirubicin injection at various time intervals (30min 4h). Single headed Siemens Integrated Orbiter Gamma Camera System fitted with high-resolution parallel holes collimator connected to an on-line dedicated computer was used for study.

STATISTICAL ANALYSIS

The calculation of means and standard deviations were made on Microsoft Excel and t test was used to determine statistical significance. Differences at the 95% confidence level (pℜ0.05) were considered significant.

RESULTS

Structure and labeling of epirubicin with Tc-99m

The current study was done to assess the potential of chemotherapeutic drug epirubicin with Tc-99m for diagnostic purpose in normal and tumor-bearing animal models. The structure of epirubicin is given in fig. 1. The effect of amount of reducing agent is summarized in fig. 2. The highest labeling efficiency was achieved at 35 µg of $SnCl_2.2H_2O$. Radiolabeling was sufficiently increased with increase in amount of $SnCl_2.2H_2O$ from 10 to 35 µg and then decreased as the amount of reducing agent was further increased (fig. 1). The influence of pH on labeling yield of ^{99m}Tc- epirubicin is displayed in fig. 3. At low pH (2-5) the labeling was from 70-73%, while at pH 6 the labeling of ^{99m}Tc-epirubicin was increased to 99%. In basic environment the labeling efficiency was decreased to a small extent. Complexation of Tc-99m with epirubicin is quite rapid and maximum labeling is attained within min. The effect of amount of ligand on labeling efficiency of ^{99m}Tc-epirubicin is shown in fig. 4. As far as incubation time is concerned, 95% labeling took place in first 10 min which was maximum at 30min (fig. 5). So, by optimizing all the factors affecting the labeling yield of ^{99m}Tc-epirubicin beginning from amount of epirubicin, pH, amount of $SnCl_2.2H_2O$, and incubation time, it was found that the 99% labeling efficiency was acheived with 200µg epirubicin and 35µg $SnCl_2.2H_2O$ at pH 6 after 30 min retention time.

Quality control

Labeling efficiency was assessed by paper chromatography using acetone as the solvent, free technetium pertechnetate moved with solvent front, while ^{99m}Tc-epirubicin and reduced or hydrolyzed technetium-99m retained at origin. In ITLC-SG chromatography by using THF as solvent, reduced or hydrolyzed technetium-99m retained at the origin, whereas ^{99m}Tc-epirubicin and free technetium pertechnetate moved with solvent front. The electrophoresis of ^{99m}Tc-epirubicin showed the neutral nature of compound (fig. 6). High performance liquid chromatographic results of inactive ligands demonstrated that purity of ligand was 90%. The HPLC analysis of ^{99m}Tc-epirubicin revealed about 99% of the compound binds with technetium-99m as represented in fig. 7 and 8.

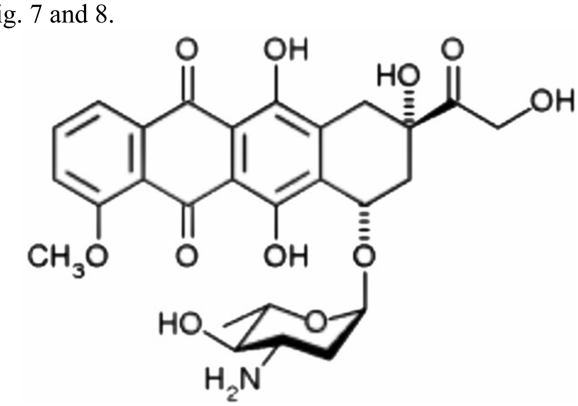

Fig. 1: Epirubicin chemical structure

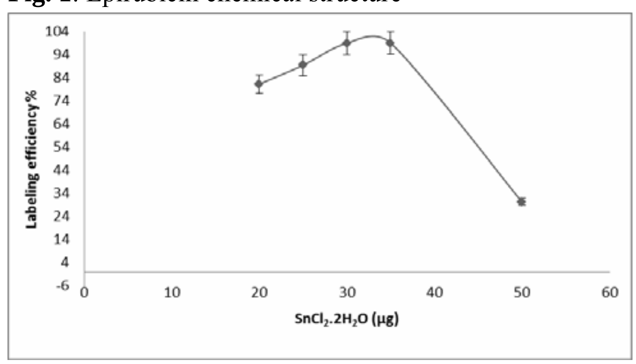

Fig. 2: Effect of amount of stannous chloride on percent labeling of ^{99m}Tc- epirubicin

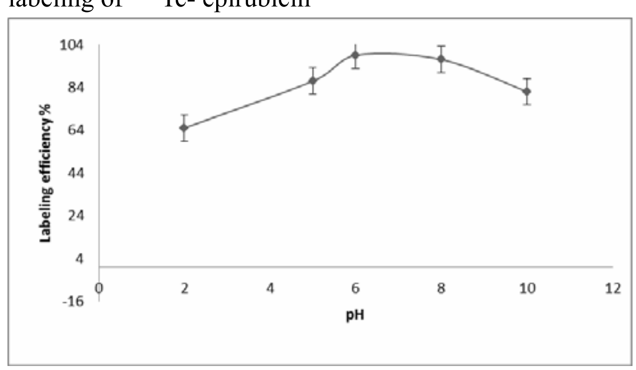

Fig. 3: Effect of pH of reaction medium on labeling yield of ^{99m}Tc-epirubicin

Stability of ^{99m}Tc-epirubicin at room temperature

In vitro stability of ^{99m}Tc-epirubicin at room temperature at 10min, 30min, 1h, 2h, 4h and 5h is represented in fig.

5. These results indicated that the labeled complex stayed stable for about 5h after labeling at room temperature.

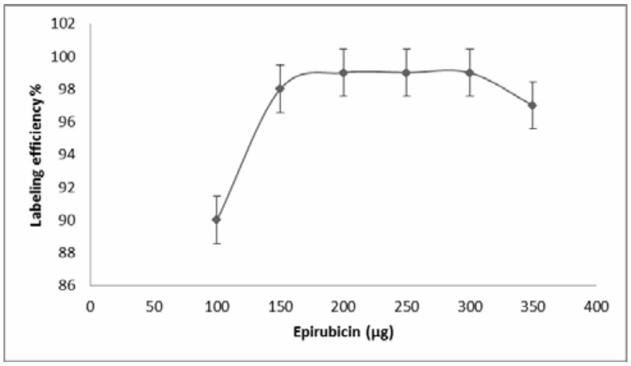

Fig. 4: Percent labeling yield of ^{99m}Tc-epirubicin as a function of amount of epirubicin

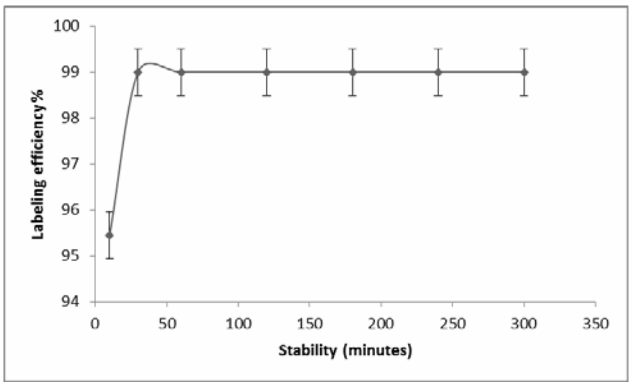

Fig. 5: ^{99m}Tc-epirubicin percent labeling yield according to reaction time at room temperature

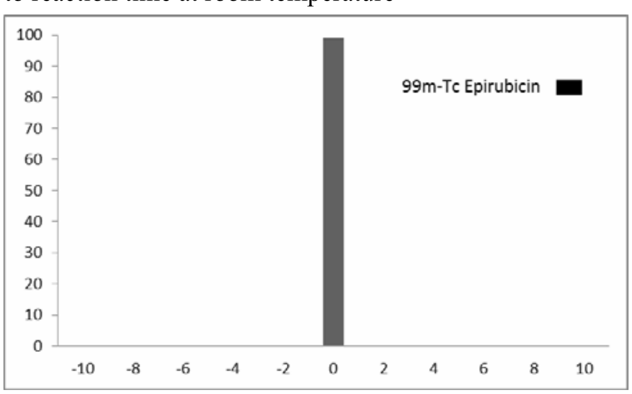

Fig. 6: Electrophoresis result of ^{99m}Tc-epirubicin

Stability of ^{99m}Tc-epirubicin in normal human serum

When ^{99m}Tc-epirubicin was incubated at 25ºC with normal human serum, it remained sufficiently stable upto 4 hours. After incubation of 4 hours, the total impurities were about 12% indicating that enzymes present in the blood serum were not able to degrade the ^{99m}Tc-epirubicin up to 4 hours of incubation period (fig. 9).

Biodistribution of ^{99m}Tc-epirubicin in normal mice

The *in vivo* study of ^{99m}Tc-epirubicin in normal mice illustrate that the large amount of drug accumulated in liver after injection. The amount of radioactivity observed in spleen, liver, lungs and stomach. The ^{99m}Tc-epirubicin revelead quick blood clearance, with only 0.96% ID/g at 30min, followed by more decrease at 4h. The activity found in spleen was 12.93±0.05, 3.57±0.05, 2.32±1.01 and 1.59±0.8 at 30 min, 1h, 4h and 24h respectively (table 1).

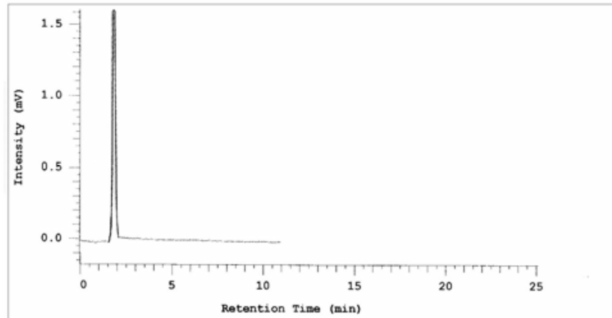

Fig. 7: HPLC analysis illustrate the percentage purity of epirubicin.

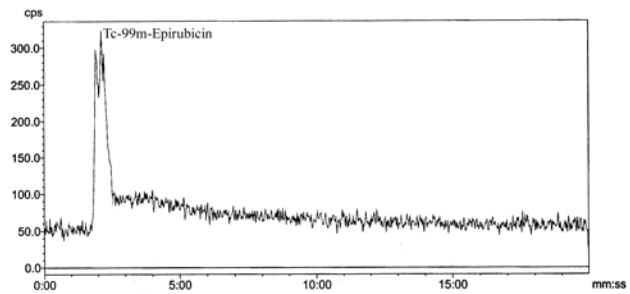

Fig. 8: HPLC study showing the percentage labeling of epirubicin with ^{99m}Tc

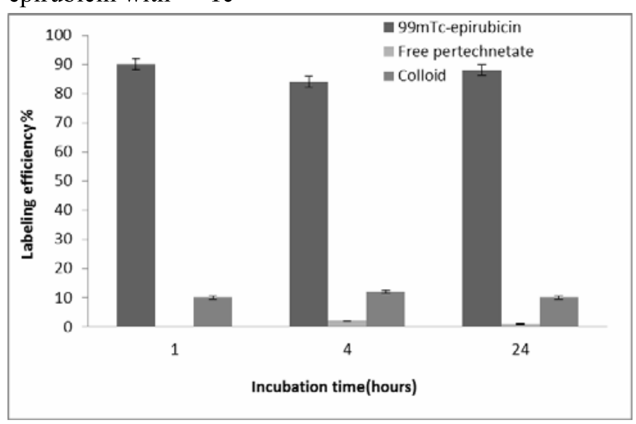

Fig. 9: Stability of ^{99m}Tc-epirubicin in normal human serum at different time intervals

Biodistribution of ^{99m}Tc-epirubicin in tumor bearing mice

The biodistribution analysis of ^{99m}Tc-epirubicin in mammary tumor bearing mice showed significant localization in liver, kidney and tumor sites. The activity observed at tumor sites was 19.03±0.7, 18.12±0.1, 18.99±0.2, 7.06±0.05 at 30min, 1h, 2h and 4h post administration which was higher than that in the brain, heart, spleen, stomach and lung (table 2).

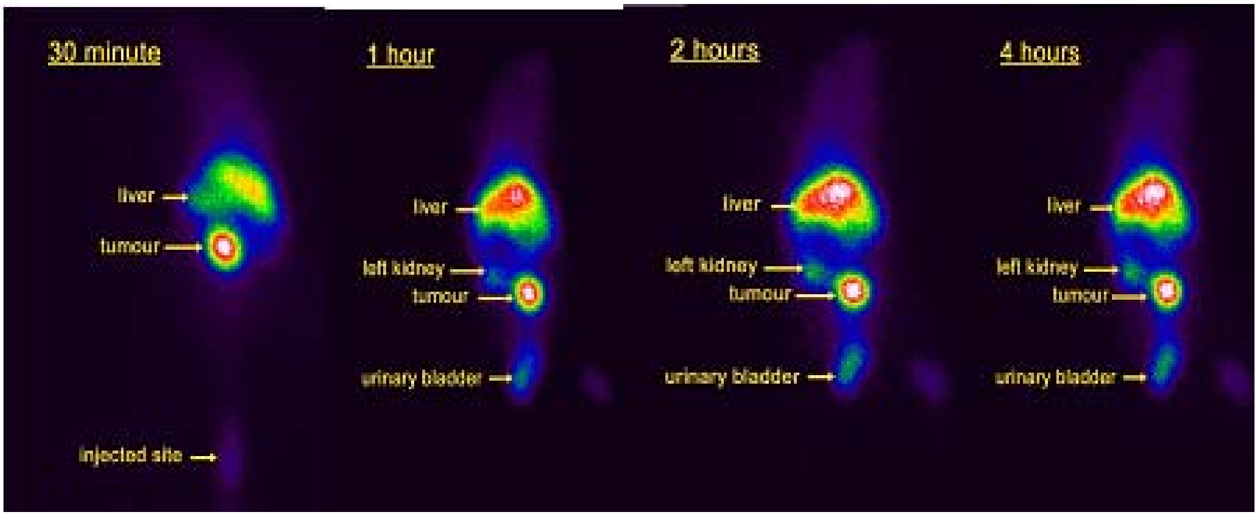

Fig. 10: Scintigraphic images of tumor bearing mice injected with ^{99m}Tc-epirubicin at 30min, 1h, 2h and 4h of intravenous post administration

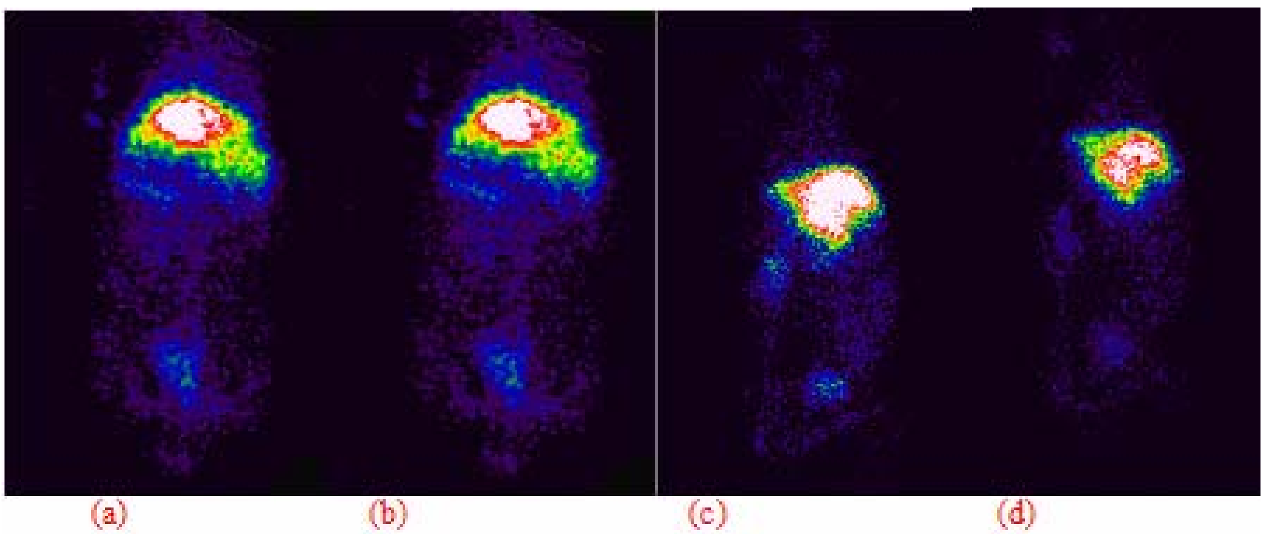

Fig. 11: Scintigraphic images of normal rabbits injected with ^{99m}Tc-epirubicin at 30min (a), 1h (b) 2h (c) and (d) 4 h of Post administration.

Imaging of ^{99m}Tc-epirubicin in normal rabbits and tumor bearing mice

Scintigraphic imaging have played an important role in cancer diagnosis and determining response to treatment. The results of imaging showed that ^{99m}Tc-epirubicin accumulated significantly in tumor sites of Swiss Webster mice bearing naturally developed tumors (fig. 10) which is in accordance to results obtained by biodistribution study. The imaging of ^{99m}Tc-epirubicin in normal rabbits showed significant localization in liver (fig. 11).

DISCUSSION

The design of radiopharmaceuticals to trace biochemical and physiological processes in small animal models are very important for preclinical radiobiological studies. The central emphasis of this work was to design and develop new ^{99m}Tc-labeled chemotherapeutic drug epirubicin which can specifically accumulate in cancer cells performing the function of imaging as well as chemotherapy. Radiochemical stability of radio pharmaceuticals is crucial for their effective and safe use in the diagnosis and therapy of disease. The final formulation for the radiopharmaceutical ^{99m}Tc-epirubicin hydrochloride was: Epirubicin hydrochloride 200µg; $SnCl_2.2H_2O$ 35µg; pH ~6; ^{99m}Tc 296 MBq and reaction mixture volume 1.5ml. As far stability data are concerned, epirubicin remained intact with the radio metal in complex. The ^{99m}Tc-epirubicin retained 99% labeling efficiency after 6h and hence could be considered quite stable and suitable for *in vivo* use for at least 6h following the preparation. In the electrophoresis process, the labeled compound moved neither toward the anode nor cathode, which suggests its neutral nature as shown in fig. 6 and all the activity of radiotracer stayed at the baseline. As shown in table 1, ^{99m}Tc-epirubicin was washed out from kidney

Table 1: Biodistribution of ^{99m}Tc-epirubicin in normal Swiss albino mice at 30min, 1h, 4h and 24h after intravenous post administration (%ID/g*).

Organ	Percent Injected Dose per gram organ			
	30 min	1 hours	4 hours	24 hours
Lungs	4.74±1.1	1.66±0.5	0.9±0.2	0.57±0.5
Kidney	2.41±1.25	0.97±0.2	0.66±1.05	0.35±0.01
Liver	23.85±0.05	24.24±0.09	27.45±0.09	15.16±1.05
Stomach	5.73±0.9	3.54±0.2	2.78±0.5	0.1±0.01
Intestine	0.54±0.5	0.53±0.2	0.5±0.3	0.57±0.05
Spleen	12.93±0.05	3.57±0.05	2.32±1.01	1.59±0.8
Heart	0.63±0.5	0.17±0.3	0.11±0.1	0.1±0.05
Blood	0.94±1.0	0.88±1.01	0.72±0.01	0.32±0.05
Brain	0.18±0.6	0.02±0.01	0.03±0.3	0.05±0.01
Carcass	9.38±0.2	6.07±0.2	2.7±1.0	2.55±0.1
Urine	0.05±0.9	0.01±0.02	6.41±1.09	0.08±0.05
Bladder	1.35±0.2	5.08±0.8	0.91±0.01	0.007±0.1

Table 2: Biodistribution of ^{99m}Tc-epirubicin at 30min, 1h, 4h and 24h after intravenous post administration in Swiss Webster mice with mammary carcinoma (%ID/g*).

Organ	Percent Injected Dose per gram organ			
	30 min	1 hours	4 hours	24 hours
Lungs	4.55±1.1	1.97±0.2	0.5±0.1	0.97±0.5
Kidney	2.21±0.15	0.5±0.5	0.42±0.1	0.13±0.05
Liver	21.45±0.05	21.24±0.09	25.45±0.09	12.16±1.05
Stomach	2.9±0.9	2.34±0.2	2.01±0.3	0.5±0.01
Intestine	0.44±0.1	0.48±0.6	0.15±0.9	0.57±0.5
Spleen	9.33±0.04	2.37±0.02	2.02±0.05	0.99±0.8
Heart	0.66±0.3	0.16±0.3	0.41±0.6	0.5±0.01
Blood	2.64±1.0	1.88±1.01	0.72±0.01	0.32±0.05
Brain	0.28±0.5	0.07±0.07	0.25±0.5	0.03±0.05
Carcass	7.38±0.2	4.07±0.2	3.7±1.0	2.25±0.1
Urine	0.15±0.5	0.01±0.02	7.05±1.09	0.9±0.05
Bladder	1.35±0.2	3.09±0.5	4.91±0.05	0.47±0.1
Tumor	19.03±0.7	18.12±0.1	18.99±0.2	7.06±0.05

*All data presented in mean percentage (n=3) of the injected dose of ^{99m}Tc-epirubicin per gram tissue±Standard deviation of the mean

after 1h but in liver significant amount remained even after 24h of post-administration in normal mice. ^{99m}Tc-epirubicin was distributed and retained in liver and that might be due to its metabolism in liver. The high amount of radioactivity observed in liver may also be contributed to its hepatobiliary route of excretion. Stomach and spleen showed a high uptake of 3.54±0.2 and 3.57±0.05 ID%/g at 4h of post administration respectively. Some organs showed significant decrease of ^{99m}Tc-epirubicin at 1h post injection like lung, kidneys, heart and stomach. The dissimilarities of the physiological processes between the tumor and normal cells allow understanding the differentiation of both types of tissues (Schottelius and Wester, 2009, Barros *et al*., 2010).

In tumor bearing mice, ^{99m}Tc-epirubicin showed very high uptake in tumor sites and and can be compared to ^{99m}Tc-labelled 5-fluorouracil (Dar *et al*., 2013) and T4CPP which selectively accumulated in rats having mammary tumours (Chatterjee *et al*., 1997) and T3,4BCPP in mice bearing abdominal sarcoma (Shetty *et al*., 1996). The chemotherapy dose of epirubicin resulting myocardial toxicity causing heart failure is typically 550 mg/m^2 and the risk increases quickly with increasing doses of epirubicin to 900mg/m^2 (Waters *et al*., 1999; Cunningham *et al*., 1999). The therapeutic efficacy of epirubicin resembles doxorubicin at equimolar doses. Due to hematologic and non-hematologic toxicity profile about cardiotoxicity, epirubicin is more favorable (Findlay *et al*., 2007). The amount of ^{99m}Tc-epirubicin administered for imaging purposes is 200μg and is very low as compared to chemotherapy doses. The amounts of ^{99m}Tc-epirubicin can be reduced further by the use of higher activities of ^{99m}Tc during labeling process. In tumor bearing mice the Scintigraphic imaging performed with ^{99m}Tc-epirubicin indicated good visualization of the tumors from 30min to 4h after administration showing good stability *in vivo*. Thus biodistribution and scintigraphy of ^{99m}Tc-epirubicin in mice bearing naturally developed tumors shows high efficacy of the prepared compound for diagnostic purpose.

In conclusion epirubicin (200µg) was labeled with ^{99m}Tc using 35µg of $SnCl_2{\cdot}2H_2O$ as a reducing agent at pH 6 in 30min reaction time at room temperature with a high radio labeling efficiency (99%) monitored by paper and ITLC-SG chromatography. HPLC represents single species of ^{99m}Tc-epirubicin and electrophoresis analysis shows neutral nature of resulting complex. ^{99m}Tc-epirubicin has shown good stability in human serum. ^{99m}Tc-epirubicin enters the circulation and reaches different body organs systems especially stomach, spleen and liver in normal mice. *In vitro* and *in vivo* evaluation of tumor bearing mice show the ability of ^{99m}Tc-epirubicin to accumulate significantly in the tumor. These results demonstrate that ^{99m}Tc-epirubicin may be used as a tumor specific diagnostic agent.

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