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Novel ^{99m}Tc labeled EDTA derivative with improved renal imaging efficiency

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Abstract

Renal imaging is a well-established nuclear medical investigation. Current renal imaging agents, dimercaptosuccinic acid, mercaptoacetyltriglycine, ethylenecysteine, diethylenetriamine pentaaceticacid and gluceptate have few drawbacks. Thus, there is a need for continuous research in designing and evaluating potentially superior renal imaging agents. Ethylenediamine-N,N-bis (disodiumacetato)-N^1-(2-hydroxyethylacetato) - N^1-(acetic acid) (EDHEAA), a derivative of a powerful chelating agent, EDTA was synthesized, characterized and radiolabeled with ^{99m}Tc by stannous chloride reduction method with a radiochemical purity of 94.08±4.82 % at a pH range of 6.0-7.0. The ratio of EDHEAA, ascorbic acid and $SnCl_2.2H_2O$ was optimized as 2.5 : 2.5: 0.5 respectively by ascending paper chromatographic analysis and was stable up to 6 h at room temperature and showed hydrophilic nature. Biodistribution of the labeled ^{99m}Tc-EDHEAA complex and the commercially available renal imaging agents (^{99m}Tc-DTPA and ^{99m}Tc-EC) in Wistar rats were studied and compared based on region of interest method. The labeled complex showed substantial retention in left and right kidneys at 20 min post injection and had rapid clearance in urinary bladder. Invitro stability in serum proteins was found to be stable up to 6 h.

Keywords: Technetium-99m (^{99m}Tc), Radiopharmaceuticals, Radiochemical purity, Radiolabeling, Biodistribution, Renal imaging.

1 Introduction

The primary goal in nuclear medicine is the development of target specific radiopharmaceuticals which helps in imaging the physiological function and metabolic activity of an organ and gives specific information about the organ function and dysfunction. Imaging the *invivo* distribution of radiopharmaceuticals provides functional morphology of organs in a non-invasive manner and helps in diagnosis of many common diseases associated with the improper functioning of the organs and in detection of certain type of cancers [1, 2]. This widespread utilization and growing demands of these techniques lead to the development and availability of a vast range of specific radiopharmaceuticals. ^{99m}Tc labeled diagnostic radiopharmaceuticals are common in use because of the superior imaging characteristics, convenient availability of the radioisotope via 99mMo/99mTc generators, reasonable cost, and ideal decay characteristics ($t1/2$ = 6.02 h, 140 keV, 89 % abundance) for Single Photon Emission Computed Tomography(SPECT) [1]. Designing radiopharmaceuticals by modifying the environment around the metal allows specific *in vivo* targeting and they must be stable sufficiently long to reach its destination [3, 4].

Radionuclide Nuclear imaging provides information on structure and function of various organs which reflect the biological process that take place at cellular and sub-cellular level [5]. ^{99m}Tc-dimercaptosuccinic acid (^{99m}Tc-DMSA), ^{99m}Tc-mercaptoacetytriglycine (^{99m}Tc-MAG3), ^{99m}Tc-Ethylenedicysteine (^{99m}Tc-EC), ^{99m}Tc-diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA) and ^{99m}Tc-gluceptate (^{99m}Tc-GH) are the commercially used renal imaging agents [6-8]. There are few drawbacks of these commercial renal agents such as ^{99m}Tc-DTPA is not advised for routine clinical applications and show low target to non-target ratio [9-11].^{99m}Tc-GH and ^{99m}Tc-DMSA produces delayed images i.e. only after 2 h of post injection and the radiation dose of ^{99m}Tc-DMSA to kidneys is high [12]. ^{99m}Tc-EC produces better images in 30 min but involves complicated synthetic procedure. ^{99m}Tc-MAG3 also produces better images in an hour but with high extraction fraction [13]. As the commercially available renal imaging agents have both advantages and disadvantages, a continuous search for a superior and improved renal imaging agent is in progress.

^{99m}Tc-EDTA was earlier used as renal imaging agents but it was replaced by ^{99m}Tc-DTPA due to high affinity of EDTA towards Ca^{2+} and Mg^{2+} ions in human body [9]. Many trials have been taken to modify EDTA molecule. ^{99m}Tc labeled EDTA-biotin monomer was developed as an imaging agent

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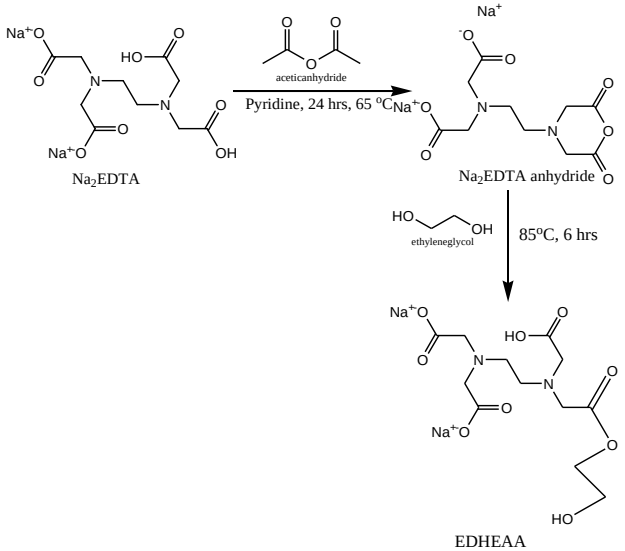
for soft tissue inflammatory lesions in horses [14]. A new technique for the development of ^{99m}Tc EDTA complex using copper as reducing agent was established which showed long term retention in kidneys [15]. A simple labeling of pertechnetate with versene(disodium calcium EDTA) was carried out which showed rapid transit time in the kidneys of the rabbit [16]. In the present work, a novel derivative of disodium EDTA, EDHEAA, was synthesized, characterized and radiolabeled with sodium pertechnetate to evaluate the optimum condition for percentage labeling efficiency and biodistribution studies were carried out in male Wistar rats based on region of interest method and compared with commercially available renal imaging agents (^{99m}Tc-EC and ^{99m}Tc-DTPA).

2 Experimental

All reagents (disodium EDTA, acetic anhydride, pyridine, ethylene glycol) were obtained from Merck chemicals. UV-visible spectrum was recorded in the region of 200-800 nm using Jasco UV-visible spectrophotometer. FTIR spectrum was recorded between 4000-400 cm^{-1} using Shimadzu FTIR spectrometer using KBr pellets. Mass spectrum was obtained using LC/MS agilent 1200 series with 6110-single quadrapole ESI detector. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Bruker DRX-500 spectrometer (Bruker, Germany) using TMS as reference. **^{99m}Tc** was eluted as $^{99m}TcO_4^-$ from ^{99m}Mo/^{99m}Tc generator (radionuclidic purity-99 %, activity-65-70 Ci/ml) supplied by Isorad Israel. TLC-SG aluminium plates (2.5 x 20 cm^2) and Whatmann No. 3 filter paper were used for ascending paper chromatography. Male Wistar rats each of 180-200 g were used for biodistribution studies and the images were obtained using GE health care Infinia Hawkeye SPECT-CT dual head gamma camera. Bio distribution of the amount of radioactivity present in organ was carried out by ROI (Region of Interest) using gamma camera applying advanced software capable of acquiring dynamic events with high resolution. In this method, a calculated quantity of the given pharmaceutical is injected into the animal through leg vein positioned under the gamma camera and the scanning produces several images of animal in dynamic mode for predefined time per image. ROI technique does not require elaborate arrangements and skilled man power to dissect the animal. In ROI method, animals injected with radiopharmaceuticals, can be used for several cycles after a particular interval of time before burial of the animal.

Synthesis of ethylenediamine-N, N-bis (disodiumacetato)-N^1-(2-hydroxyethylacetato)-N^1-(acetic acid) (EDHEAA)

A chelating ligand with active binding site was synthesized from disodium EDTA in a two-step process as shown in scheme1. Disodium EDTA (3.5 g) was dehydrated with pyridine and acetic anhydride (6 ml) by refluxing at 65 ^{o}C for 24 h to obtain the anhydride as white cream powder (Yield: 80 %; M.pt: 104-108^{o}C). The anhydride was converted to EDHEAA by refluxing with ethyleneglycol in 1:1 ratio at 85 ^{o}C for 6 h. The synthesized chelating ligand is soluble in water and insoluble in other solvents like ethanol, methanol, DMF, DMSO due to its ionic nature. (Yield: 68.2%; Color: White; M.pt.: 184-186^{o}C).



Scheme 1 Synthetic route of EDHEAA

Formulation and radiochemical purity of ^{99m}Tc-EDHEAA complex

Labeling of EDHEAA with **^{99m}Tc** was carried out by addition of the reagents given in Table 1 into an air tight vial. The percentage labeling efficiency was evaluated by ascending paper chromatography using Whatmann filter paper No: 3 in acetone and saline medium. The radioactivity was measured in the CRC^{15R} capintec radioisotope dose calibrator in **^{99m}Tc** window.

Table 1: The concentration of the reagents used in the labeled complex

Reagent	Concentration	Volume taken (ml)
Chelating ligand	20 mg/ml	0.5
$^{99m}TcO_4^-$	10.34 mCi	0.5
$SnCl_2.2H_2O$	10 mg/ml	0.2
Ascorbic acid	30 mg/ml	0.2

Optimization of percentage labeling efficiency

The factors affecting labeling efficiency such as reaction time, amount of chelating ligand, reducing agent, anti–oxidant and pH of the reaction were optimized by trial and error method [2]. The invitro stability of the labeled ^{99m}Tc-EDHEAA complex was measured at different time intervals from 15 min to 8 h using ascending paper chromatographic analysis.

Biodistribution studies

The biodistribution studies were carried out in male Wistar rats weighing 180-200g which were anesthetized

using diethyl ether and the study was carried out in dynamic mode in 256 x 256 matrix and 2.56 zoom factor keeping the rat in supine position under the GE Hawkeye dual head SPECT-CT gamma camera, by injecting 0.1 ml of 110-150 µCi (4070-5550 KBq) of the reconstituted solution intravenously into left leg. The dynamic mode study was carried out in two phases – first perfusion phase at the rate of 25 sec/framefor 2 min followed by second phase at the rate of 3 min/frame for 30 min.

The commercial renal imaging agents (^{99m}Tc-EC and ^{99m}Tc-DTPA) were supplied by BRITC, Board of Radioisotope technology, Government of India, which were reconstituted with 10 mCi of $Na^{99m}TcO_4$ in 1 ml of 0.1 N NaCl solution to obtain colourless clear solution with pH 6.5-7.0 and above 95 % of labeling efficiency. Serum proteins of human blood were used to analyze the pharmacokinetic invitro stability of the labeled complex. 10 mCi of the complex was added to blood which was incubated for 6 h and centrifuged at 3000 rpm for 10-15 min and a chromatogram was developed after separating the serum.

3 Results and Discussion

Spectroscopic techniques

The structure of the synthesized molecule (EDHEAA) was confirmed by UV-visible, FTIR, mass and ^{1}H-NMR analysis. UV-visible spectrum (Fig S1) in water at 10^{-6} M concentration exhibits a single broad absorption band from 240-300 nm which corresponds to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions occurring in the molecule. Absence of peaks in visible region indicates long shelf-life of the compound. The FTIR spectrum (Fig S2) showed peaks at 3528 cm^{-1} and 3379 cm^{-1} (O-H stretching), 3030 cm^{-1} (C-H stretching), 1624 cm^{-1} (C=O stretching), 1350-1200 cm^{-1} (C-O stretching), 1350-1000 cm^{-1} (C-N stretching) [17, 18]. The mass spectrum (Fig S4) shows the parent ion peak $[MH]^+$ at 333.2 corresponding to the molecular weight of the ion $[C_{12}H_{17}O_9N_2]^-$ and the base peak was observed at 315.1 which corresponds to anhydride molecule $[C_{12}H_{15}O_8N_2]$ [17, 18].

Radiochemical purity and optimization

Percentage of free pertechnetate (% F) and hydrolyzed ^{99m}Tc-colloid (% H) were obtained from acetone and saline chromatogram respectively and the radiochemical purity of the labeled ^{99m}Tc-EDHEAA complex was found to be 94.08±4.82 % and stable up to 6 h at room temperature.

Effect of reaction time:

The percentage binding of labeled complex (Fig 1a) was determined at different reaction time by keeping other parameters (amount of chelating ligand, reducing agent, anti-oxidant and pH) constant. Labeling efficiency significantly increased from 73% to 95% with increasing reaction time from 15 min to 30 min and remains constant from 30 min to 6 h after which it decreases drastically. The minimum optimum time was fixed as 30 min for optimizing other parameters.

Effect of anti-oxidant (ascorbic acid)

The dependence of percentage binding on concentration of anti-oxidant (Fig 1b) was determined by varying it from 2.5 mg to 15 mg and keeping other parameters constant. Trial was also made to check the labeling efficiency in the absence of ascorbic acid which showed no binding with ^{99m}Tc. The labeling efficiency increased significantly as the concentration of anti-oxidant increased from 2.5 mg to 10 mg at which maximum yield was obtained and remains same on further increasing the concentration.

Effect of reducing agent ($SnCl_2.2H_2O$)

The variation of percentage binding with reducing agent (Fig 1c) was determined by varying it from 0.5 mg to 2.5 mg and keeping other parameters constant. It was observed that the labeling efficiency was increased by increasing the amount of $SnCl_2.2H_2O$ from 0.5 mg to 2.0 mg at which maximum yield was obtained.

Effect of pH

The percentage binding of labeled complex on pH of the reaction medium from 3 to 7 was determined (Fig 1d) by varying the pH and keeping other parameters constant. It was observed that at pH 6.0-7.0, the ligand combined with maximum amount of reduced pertechnetate [19, 20]. The optimum pH range at which the maximum labeling efficiency was obtained was 6.0-7.0.

Effect of concentration of chelating ligand (EDHEAA)

The variation of percentage binding with concentration of ligand (Fig 1e) was determined by varying it from 2.5 mg to 20 mg and keeping other parameters constant. The increase in the concentration of ligand was accompanied by an increase in the labeling efficiency till 10 mg and no significant effect above 10 mg was observed. In general, increasing the concentration of the reactants increases the formation of the product and the maximum yield was found to be at 10 mg of ligand. Percentage of free pertechnetate and hydrolyzed ^{99m}Tc-colloid were high initially which gradually decreased on increasing the concentration of ligand.

Biodistribution studies

The percentage uptake of injected complex in different organs at different intervals of time up to 20 min were calculated based on region of interest (ROI) which showed slight uptake in all vascular structures such as heart, liver, lymph tissue etc., and high uptake of the complex is seen in left and right kidneys in 5 min to 20 min showing high organ specificity due to which this novel radiopharmaceutical may be used for renal imaging in time interval of 5 min to 20 min after post injection. As the images were obtained within 20 min, it reduces the radiation absorbed by the patients.

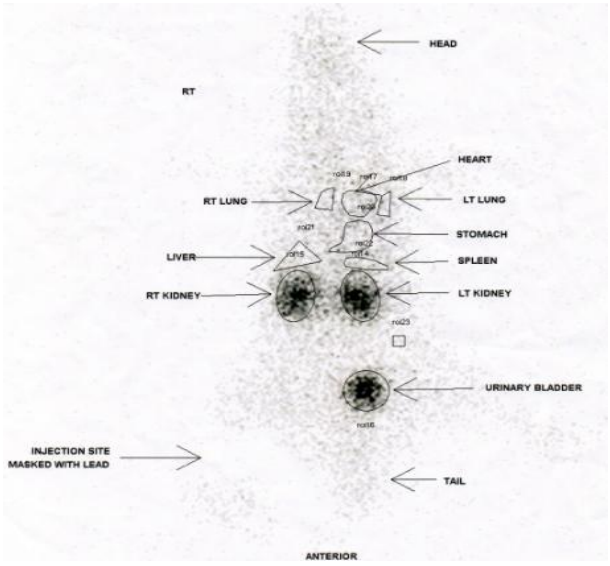


Fig 2 Anterior view of region of interest method in male Wistar rat using ^{99m}Tc-EDHEAA

Table 2 Biodistribution data of ^{99m}Tc-EDHEAA-Anterior view

S.No	ORGAN	^{99m}Tc-EDHEAA count at various time intervals					
		5 min	10 min	15 min	20 min	25 min	30 min
1	Heart	160±0.32	176±0.86	175±0.05	171±0.26	140±0.58	119±0.73
2	Left lung	49±1.26	43±1.07	41±1.28	42±0.58	34±1.49	21±0.76
3	Right lung	93±0.59	70±1.06	65±0.28	57±0.86	42±0.71	30±0.89
4	Liver	203±1.11	215±1.28	188±0.82	164±0.54	133±0.74	27±0.62
5	Stomach	152±0.15	165±0.37	144±0.84	124±0.56	110±0.41	98±0.70
6	Spleen	53±0.14	69±0.72	78±0.53	84±0.49	66±0.87	54±0.51

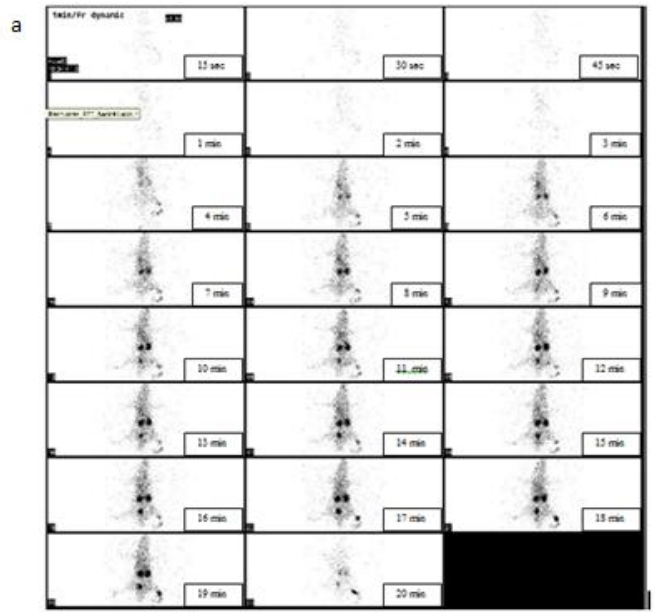


Fig 3: Gamma images of Wistar rat using ^{99m}Tc–EDHEAA, dynamic mode

The *invitro* analysis of the stability of the complex from the chromatogram of serum sample of blood was repeated several times which showed no radioactivity in RBC and was found to be stable up to 6 h.

The biodistribution studies of the labeled complex were compared with ^{99m}Tc-DTPA and ^{99m}Tc-EC (Fig 4), commercially available renal imaging agents. These were reconstituted with 10 mCi of $Na^{99m}TcO_4$ in 1 ml of 0.1N NaCl solution to obtain colourless clear solution with pH 6.5-7.0 and above 95% of labeling efficiency. The biodistribution studies were carried out by injecting 100-150 µCi of the commercial product in male Wistar rats. The images were acquired for 25 min at the rate of 5 min/frame for ^{99m}Tc-EC and for 30 min for ^{99m}Tc-DTPA in dynamic mode keeping the parameters same as for the labeled complex. The uptake of all the complexes were found to increase from 5 min to a particular time period in all organs and then decreased except in urinary bladder. The activity of labeled complex was in between the activity of ^{99m}Tc-EC and ^{99m}Tc-DTPA.The activity of ^{99m}Tc-EC was very high and that of ^{99m}Tc-DTPA was very low compared to the labelled complex. In commercial renal imaging

agents, the uptake in all organs is decreased after 35 min but the synthesized compound showed decrease in uptake in 25 min. This indicates early discharge of the complex from the organs without accumulating and the uptake of the complex was increased in urinary bladder which indicates that the clearance of the radiopharmaceutical is via glomerular filtration similar to commercial renal agents [6].

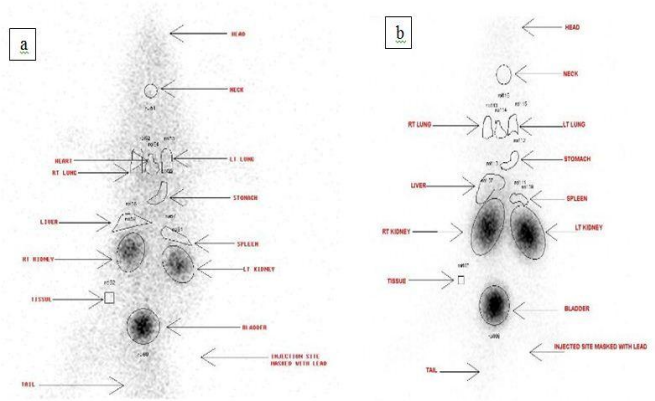


Fig 4: Anterior view of region of interest method of a) ^{99m}Tc-DTPA and b) ^{99m}Tc-EC in male Wistar rat

4 Conclusions

Ethylenediamine-N,N-bis(disodium acetato)-N^1-(2-hydroxyethylacetato)-N^1-(acetic acid) (EDHEAA) was synthesized from disodium EDTA in a two-step process and characterized by spectroscopic analysis and radiolabeled with sodiumpertechnetate and the optimum conditions for maximum percentage labeling efficiency were evaluated after 30 min as the ratio of ligand, ascorbic acid and stannous chloride dihydrate as 2.5:2.5:0.5 at pH range of 6.0-7.0. Radiochemical purity and *invitro* stability were evaluated using ascending paper chromatography and was found to be 94.08±4.82 % and stable up to 6 h. Biodistribution studies on Wistar rats showed clear spots at kidney region and shows high target to non-target ratio and was compared with the commercially available renal imaging agents. The labeled^{99m}Tc-EDHEAA complex showed almost same pattern of perfusion, cortical uptake and clearance phase as such as the commercially available renal imaging agents. The synthesized chelating ligand was colourless, water soluble and shows no absorption in visible region which supports the properties of a pharmaceutical drug with long shelf-life.

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Supplementary data

The supporting spectrums for the manuscript are given below.

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2. Figure S2 – FTIR spectrum of EDHEAA
3. Figure S3 – Mass spectrum of EDHEAA
4. Table S1 - Biodistribution data of ^{99m}Tc –EC and ^{99m}Tc-DTPA– Anterior view

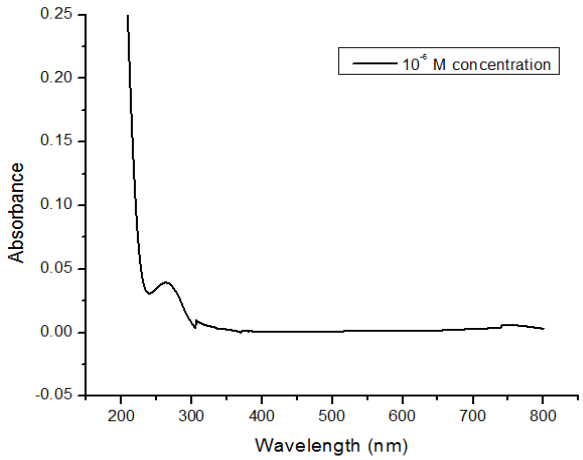

Fig S1 UV-visible spectrum of EDHEAA

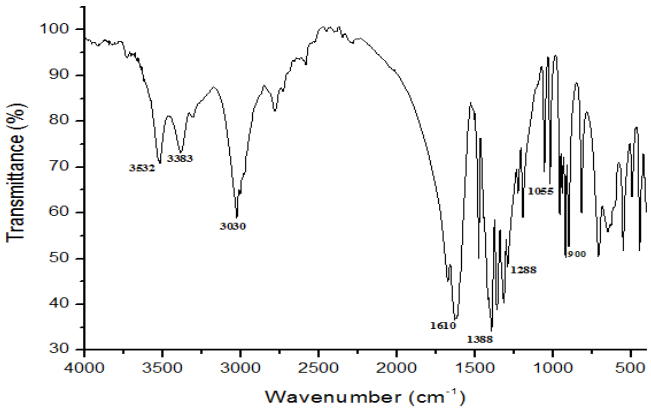

Fig S2 FTIR spectrum of EDHEAA

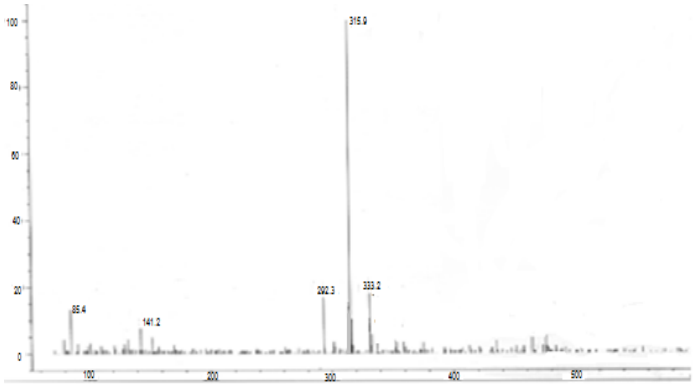

Fig S3 Mass spectrum of EDHEAA

Table S1: Biodistribution of ^{99m}Tc –EC and ^{99m}Tc-DTPA– Anterior view

		^{99m}Tc –EC					^{99m}Tc-DTPA					
S.No	**Organ**	**5 min**	**10 min**	**15 min**	**20 min**	**25 min**	**5 min**	**10 min**	**15 min**	**20 min**	**25 min**	**30 min**
1	R. lung	504 ±0.58	584 ±1.02	513 ±0.26	457 ±0.32	330 ±0.52	269 ±1.21	309 ±0.65	410 ±0.69	408 ±0.35	343 ±0.82	230 ±0.75
2	L. lung	555 ±1.08	584 ±0.78	477 ±0.71	420 ±0.53	326 ±1.29	368 ±0.51	385 ±0.76	383 ±0.92	370 ±1.04	368 ±0.64	202 ±0.36
3	Heart	644 ±0.48	387 ±0.28	535 ±0.31	394 ±0.25	307 ±0.85	346 ±0.76	405 ±0.28	415 ±0.39	385 ±0.48	373 ±0.75	226 ±1.15
4	Stomach	1027 ±0.75	987 ±0.18	805 ±0.92	684 ±0.76	531 ±0.48	275 ±0.76	329 ±0.34	397 ±0.67	356 ±1.36	341 ±0.78	188 ±0.85
5	Liver	920 ±0.45	1331 ±0.86	1188 ±0.71	1013 ±0.28	927 ±0.49	335 ±0.58	436 ±0.27	495 ±0.93	474 ±0.34	443 ±0.64	291 ±0.98
6	Spleen	416 ±1.08	387 ±0.79	398 ±0.28	376 ±0.78	357 ±0.39	147 ±0.48	291 ±0.76	315 ±0.22	264 ±0.18	299 ±0.75	203 ±0.19
7	**R. kidney**	**6507** ±1.05	**15176** ±1.35	**20817** ±0.98	**25224** ±0.76	**24859** ±1.39	**731** ±1.28	**923** ±0.99	**1420** ±0.48	**2120** ±0.28	**2477** ±0.79	**1072** ±1.68
8	**L. kidney**	**6887** ±0.58	**14497** ±0.49	**20387** ±0.39	**22025** ±0.62	**24035** ±1.36	**960** ±0.87	**1188** ±0.69	**1933** ±1.27	**2616** ±0.77	**2796** ±0.66	**965** ±0.98
9	U. Bladder	1237 ±0.65	3950 ±1.45	10749 ±0.59	15377 ±0.76	21117 ±0.13	258 ±0.95	336 ±0.76	421 ±0.58	807 ±0.76	3715 ±0.83	4142 ±0.74
10	Tissue	147 ±0.96	198 ±0.76	192 ±0.48	209 ±0.39	147 ±0.92	35 ±0.75	57 ±0.36	59 ±0.74	65 ±0.59	61 ±0.74	41 ±0.59