Formulation of lyophilized single vial kit of N-N-Ethylene-L-Dicysteine (EC) for labeling with ^{99m}Tc. II: Radiochemical and clinical evaluation as a renal tubular agent in comparison with ^{99m}Tc-MAG$_3$

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Abstract: A Technetium ^{99m}Tc labeled lyophilized single component kit of N-N-ethylene-I-dicysteine (EC) is developed to replace multiple step kit developed by others. The aim of study is to formulate a radionuclide that is easy to prepare, has rapid plasma clearance, produce high quality images and is an affective alternative to radioiodine labeled orthoiodohippurate, which has been remained the physiological 'gold standard' since long time. To achieve this goal, the systematically varied key parameters such as pH, the use of reducing agents, stabilizers and additives are optimized to obtain maximum radiochemical purity and optimum bio distribution in non human and human primates. Various pH levels of EC showed equally good results in animal experiments but only pH 10 was suitable for human use. Dynamic and renal Scintigraphic studies are carried out with ^{99m}Tc-EC at pH 8 in 12 volunteers and at pH 10 in 18 volunteers and compared with ^{99m}Tc-MAG$_3$, Background ratios, renograms, relative renal function and semi quantitative parameters are available in all studies. The background ratios (mean ± SD) at 30th minute are 0229±0.024 and 0.236±0.018 for ^{99m}Tc-EC at pH 10 and ^{99m}Tc-MAG$_3$ respectively. The mean ± standard error of mean (SEM) values of T_{MAX} and time to half activity (T_{12}) for ^{99m}Tc-EC (pH10) are 3.7±0.6 and 7.3±1.0 respectively while for ^{99m}Tc-MAG$_3$, they are 4.0±0.8 and 7.9±1.4 with p values 0.001 and 0.049 respectively. The values of relative renal function (RRF) for ^{99m}Tc-EC and ^{99m}Tc-MAG$_3$ are 50.8±3.11 and 51.2±3.4 respectively with p value of 0.822. The residual activity at 25th minute (A_{25} / A_{MAX}) and renal uptake are 0. 209±12.67±2.80 for ^{99m}Tc-EC and 0.218±0.035 and 1053±2.98 for ^{99m}Tc-MAG$_3$ (p=0.031 an 0.0003) respectively. The correlation coefficient (R^2) for T_{MAX}, $T_{1/2}$, A_{25}/A_{MAX} and renal uptake are 0.96, 0.69, 0.93 and 0.85 respectively.

Keywords: Renal tubular agent, ^{99m}Tc-EC, formulation, chemical and clinical evaluation.

INTRODUCTION

Many efforts have been made over the past two decades to formulate a ^{99m}Tc-labelled renal tubular agent as an alternative to radioiodinated-2-iodo-hippurate-OIH. In order to obtain a ^{99m}Tc-agent, an anionic chelate complex containing a TcO_4-CO-NH-$(CH2)_n$-COOH moiety has been used. Technetium labeled tetradentate bisamide bisthiol derivatives (^{99m}Tc-DADS) and its carboxylate derivatives (^{99m}Tc-CO_2-DADS) has also shown favorable renal characteristics (Davison, et al., 1979; Frtizberg, et al. 1981 and 1982). By changing the core donor atoms from N_2S_2 to N_3S and placement of carboxylate on the third amide nitrogen, mercapto-acetyl-triglycine (MAG_3) was developed. MAG_3 shares many characteristics with OIH and has evaluated as a clinically useful agent, although the plasma clearance of MAG_3 is 49%-67% than that of OIH (Taylor Jr, *et al.,* 1986 and 1987; Bubeck *et al.,* 1990; Kengen *et al.,* 1991). However MAG_3 is still not an ideal agent. Amongst its disadvantages are, that after its reconstitution with Technetium-99m needs to be boiled for 15 minutes before its clinical use and then its slow hepatic clearance. A new agent N-N ethylene-1-dicysteine (EC) when labeled with ^{99m}Tc has been found to have renal pharmacodynamics comparable to MAG_3 and OIH. The formulation of EC has been achieved as a two (AM Verbruggen, et al. 1992) or a three (S Mansur, et al. 1993) component kit. But these formulations have been found time consuming and rather cumbersome to reconstitute with Technetium-99m before clinical use. In current study It is set out to formulate a single vial kit, with its inherent advantage of ease of preparation, which is cheap to prepare whilst it maintaining the physical and clinical characteristics of a two or three component kits.

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MATERIALS AND METHODS

Radiopharmaceuticals

The ^{99m}Tc-MAG_3 used for the comparative studies was prepared in our laboratory using the method formulated by (Fritzberg *et al.*, 1986). The cold ligand of EC was synthesized by the method described by (Blondeau *et al.*, 1967). Its characterization was carried out using melting point determination, IR, NMR, and elemental analysis.

Study on Effect of pH and Excipients on the Radiochemical Purity of ^{99m}Tc-EC Complex.

To evaluate the effect of pH, freeze dried kits were prepared at pH values ranging from 6 to 11 keeping all other parameters constant. Similarly the amount of other excipients in the final formulation was selected by

optimization process i.e., the quantity of selected substance was altered and analyzed for the labeling yield of EC with ^{99m}Tc, keeping other parameters constant.

Formulation of ^{99m}Tc-EC Kit

The phosphate buffer was prepared with, Na_2HPO_4 (13mg), KH_2PO_4 (18mg), was dissolved in sufficient deoxygenated water, then few drops of N/20 NaOH and make the volume to 100 ml with deoxygenated water.

Each vial of the formulation contained EC (2mg), mannitol (30mg), disodium ethylene-diamine tetraacetic acid (EDTA) (0.5mg), ascorbic acid (24mg), and $SnCl_2$ $2H_2O$(200µg).

The preparation of bulk solution in deoxygenated water was carried out in a fume hood with laminar flow and under N_2 gas atmosphere at a room temperature of 20^0-25^0C, under sterile conditions. Finally phosphate buffer solution was added dropwise to bring the pH of the bulk solution close to 9. Then N_2 gas was purged into the prepared stock solution for 15 minutes and at the end $SnCl_2$, $2H_2O$ (200µg/100µL/vial) was added. The final pH was again adjusted to pH 9. N_2 gas was purged once again for 5 minutes and then the stock solution was dispensed into the vials which were freeze dried for 48 hours

Labeling of EC with technetium-99m

The kit was kept in a lead container at room temperature. Then 2 milliliters of freshly eluted $^{99m}TcO_4$ solution (1850MBq/ml) from an Ameritech-II generator was added to the kit. The reconstituted kit was incubated for 15 minutes at room temperature and then radio-pharmaceutical purity was checked.

Stability of ^{99m}Tc-EC Complex

Six kits of EC were labeled in the morning and incubated for 15 minutes. Paper chromatography was carried out at various intervals over a period of 24 hours. Stability of six MAG_3 kits was studied simultaneously for comparison.

Radiochemical analysis of ^{99m}Tc-L, L-EC Complex

Paper Chromatography, Instant Thin Layer Chromatography (ITLC) and HPLC were used to assess radiochemical purity of the ^{99m}Tc-EC complex in terms of free $^{99m}TcO_4$ and reduced hydrolyzed technetium (Verbruggen *et al.,* 1992; IAEA-TEC.DOC. 1995). A stationary phase (Whatman no.1) and mobile phase of 99.9% acetone (system 1) and 50% acetonitrile solution (system II) were used for ascending paper chromatographic analysis. Whilst Stationary phase of ITLC-SG, Gelman and mobile phase of acetone 99.9% (system I) and 0.5.M acetic acid (system-III) were used for instant thin layer chromatography The R_f values found were similar to paper chromatography.

HPLC was done in reverse phase column RP-I8 using gradient system 0.0125M phosphate buffer pH 2.5 (solution A) and ethanol solution 99.99% (solution B) as solvent system. Elution was obtained by using gradient 0 min: 100% (A). At 10min: 91% (A): 9% (B). At 20 min 91% (A) 9% (B).

Biodistribution studies in rats

The time course of organ distribution was evaluated in rats with average body weight 300g in a group of 5 for each kit. All animal studies were conducted in accordance with United Kingdom Biological Council's Guide lines on the Use of Living Animals in Scientific Investigations, 2nd edition. Each animal was injected with 0.222 MBq in 0.2ml preparation of each ^{99m}Tc-EC complex and ^{99m}Tc-MAG_3.The rats were sacrificed with lethal dose of ether at 2 and 10 minutes post injections. The organs were excised, weighed and counted in well counter.

Scintigraphic imaging in monkeys

A 10 year old Rhesus monkey (body weight 12kg) was selected for study. ^{99m}Tc-EC complex was used for renal studies in the monkey, one week later the same monkey was used to carry out renal studies with ^{99m}Tc-MAG_3. Anesthesia was induced by I/M injection of 100mg ketamine hydrochloride and sustained by a 10mg dose after every 25 minutes. An intravenous line was maintained throughout the procedure. A single headed Siemen's integrated ORBITOR gamma camera system interfaced with high resolution parallel holes collimator, connected with an online dedicated computer was used.

A 1-minute full syringe count and empty syringe counts were taken under the camera keeping the syringe at a 30 cm distance from the head before and after the injection and completion of dynamic studies. The injection 100MBq of radiopharmaceuticals under study was given to a monkey in a posterior position under the camera. A 3 phase dynamic study was acquired using 64 x 64-matrix size. In the posterior projection, 60 images of 1 sec/frames. 24 of 5 sec/frame and 54 of 30 sec/frame were obtained.

Regions of interest (ROI) were drawn over the kidneys with appropriate background subtraction and time to peak activity (T_{MAX}) and the percentage of activity at T_{MAX} to the injected dose (R_{MAX}) were calculated.

Human study

Nine healthy volunteers (2 females and 7 males) with an age range of 23-70 years and weight 45-100 kg were studied. The local ethical committee approved the study and an informed written consent was obtained from all volunteers. Dynamic Scintigraphic study was carried out with ^{99m}Tc-EC complex prepared at pH 8 in 6 volunteers (6 kidneys); and at pH 9 and 10 in 12 volunteers (13 kidneys) the study was repeated a week later with ^{99m}Tc-MAG_3) in each case. To ensure an adequate hydration the subjects were encouraged to drink 500ml of water prior to the procedure. All volunteers were scanned in the supine

position, with camera heads placed underneath the table keeping the kidneys, ureters and the bladder within the fields of view. Using the same imaging protocol as it was used in the rhesus monkey. 95.5-148.0 MBq of ^{99m}Tc-EC or ^{99m}Tc-MAG_3 was injected as a bolus in the antecubital vein, followed by the flush of 10-ml of saline. The dynamic images were acquired immediately.

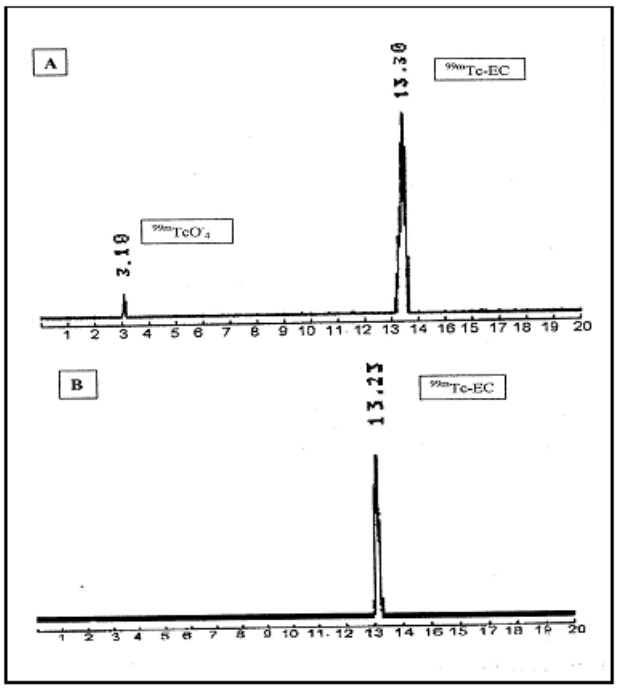

Fig. 1: HPLC of ^{99m}Tc-EC-complex prepared at pH 8(A) and pH 9(B)

The parameters computed were T_{MAX}, time to half of peak activity ($T_{1/2}$) and relative renal function (RRF). Two different ratios i.e., background to renal ratio at 30th minute (B/K) and the ratio of residual activity at 25th minute to the activity at T_{MAX} ($A/_{25}$ / A_{MAX}) were calculated manually. The later, was calculated by dividing the decay corrected of the kidney at the 25th minute by the counts at $T_{MAX.}$

The percentage of renal uptake was calculated in each case study with each renal imaging agent as a parameter of renal clearance by the following relationship (K Itoh, et al. 1996).

$$\%RU = \frac{C}{De^{-0.15d}} \times 100 \quad (1)$$

Whereas RU is renal uptake, C is 1-2 minute renal counts (corrected for background), D is total injected dose (corrected for empty syringe counts) and d is renal depth. The renal depth was measured by following regression equation for right (d_R) and left (d_L) kidneys respectively.

$$d_R = 13.63\ (W/H)^{0.6996} \quad (2)$$

$$d_L = 14.0285\ (W/H)^{0.7557} \quad (3)$$

W and H are the weight and height of the patient.

For parameters like T_{MAX}, $T_{1/2}$ and residual activity, standard error of mean (SEM) was calculated because their standard deviation had high values. Student paired T test was applied to clinical data using and p value was determined to find out the significance of the results.

RESULTS

Chemicals

The analytical data obtained by paper chromatography and ITLC showed that the radiochemical purity of ^{99m}Tc-Ec complex was >98.5% at pH values 8 to 11. Only traces of $^{99m}TcO_4$ and $^{99m}TcO_2$ were found), The R_f values of ^{99m}Tc-EC complex, $^{99m}TcO_2$ and $^{99m}TcO_4$ for system 1 were 0, 0 and 1, while those for system II were 1, 0 and 1. HPLC analysis (fig. 1) showed that the percentage yield of ^{99m}Tc-EC complex prepared at pH 10 was 99.95%± 0.16 with retention time (t_R) 13.42±0.50 minutes for both the complexes. t_R for free $^{99m}TcO_4$ was 3.00±0.15 minutes.

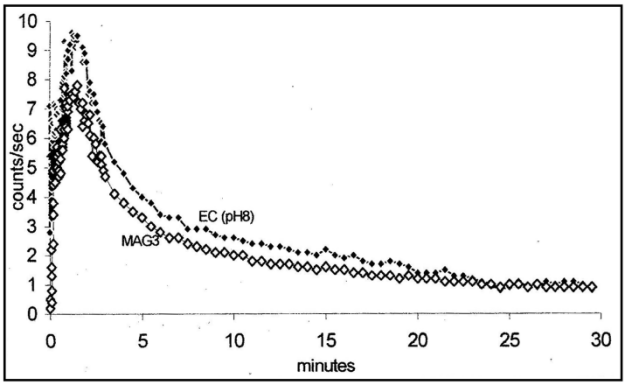

Fig. 2: Computer-generated 0-30 minutes renograms of Lt. Kidney of Rhesus Monkey using ^{99m}Tc-MAG_3 and ^{99m}Tc-EC at pH 8.

The ^{99m}Tc-EC complex was found to be stable even after 24 hours. The percentage yield of the labeled complex at 15 minutes was 99.67±0.53% which after 8 hours was still 99.59±0.60. After 24 hours the labeling yield was found to be 98.40±0.55%. ^{99m}Tc-MAG_3 complex was however less stable which after reconstitution was dropped down from 96.60±0.32 to 94.60±0.40% after 24 hours.

Biological

Biodistribution studies in rats of ^{99m}Tc-EC complex prepared at pH 8 showed uptake of the activity in blood, liver, and GIT at 10th minute almost similar to ^{99m}Tc-MAG_3 (table 1). The renal uptake of ^{99m}Tc-$MAG_{3,}$

Whilst the results ^{99m}Tc-EC complex at higher pH (9.5-10) values were even better than ^{99m}Tc-$MAG_{3.}$ No adverse effects occurred in the monkey with ^{99m}Tc-EC (pH8). Good quality images were obtained, identical to ^{99m}Tc-MAG_3 images acquired a week later. The Reno-gram

parameters of the two agents are compared in fig. 2 and table 2, respectively.

During human evaluation, the background activity at the 30th minute was found to be higher in case of ^{99m}Tc-EC (pH 8) than ^{99m}Tc-MAG$_3$ and ^{99m}Tc-EC (pH 10). Fig 3 shows that the renal cortex is visible in case of ^{99m}Tc-EC (pH 9/10) even at the end of study. More activity was seen in the liver in case of ^{99m}Tc-MAG$_3$ as compared to ^{99m}Tc-EC (pH 9/10). The B/K (Background/Kidney) ratio was considerably higher in ^{99m}Tc-EC (pH 8) as compared to ^{99m}Tc-EC (pH 9/10) and ^{99m}Tc-MAG$_3$, whereas for ^{99m}Tc-EC (pH 9/10) the B/K ratio was even better than MAG$_3$ (table 3).

The values (mean ± SEM) of T_{MAX}, $T_{1/2}$, A_{25}/A_{MAX} and uptake of ^{99m}Tc-EC at pH 8 and 10 were compared with ^{99m}Tc-MAG$_3$ in table 4, it was seen that T_{MAX}, were comparable, with p values 0.549 and 0.514 respectively. The values of PRF of the two agents were also almost similar i.e, 52.0±2.0 and 52.7±1.8 (n=3) respectively with p values 0.529. However, A_{25} /A_{MAX} value of ^{99m}Tc-EC (pH 8) was quite higher as compared to ^{99m}Tc-MAG$_3$ in the same subjects (p= 0.0012). Similarly the renal uptake of ^{99m}Tc-MAG$_3$ in the uptake of ^{99m}Tc-MAG$_3$ was more than ^{99m}Tc-EC (pH 8) (11.00±2.70 and 8.96±3.96, respectively) having p value 0.0691.whereas ^{99m}Tc-EC at pH 10 showed better values of T_{MAX} and $T_{1/2}$ as compared to ^{99m}TC-MAG$_3$, with p values 0.001 and 0.049.

RRF also gave similar results i.e., 50.8±3.11 and 51.2±3.4 respectively, and p value of 0.822. Similarly, in contrast to ^{99m}Tc-EC (pH 8), ^{99m}Tc-EC (pH 9/10) gave better results of residual activity (A_{25} / A_{MAX}) and renal uptake. A_{25} / A_{MAX} of ^{99m}Tc-EC (pH 9/10) was almost similar to ^{99m}Tc-MAG$_3$ in same volunteers (p=0.031), ^{99m}Tc-EC (pH 10) on the other hand showed better renal uptake then ^{99m} Tc-MAG$_3$ (p=0.0003). fig. 4 shows the correlation and regression equation between ^{99m}Tc-EC (pH 9/10) and ^{99m}Tc-MAG$_3$.

DISCUSSION

Tubular agents can be used to evaluate renal function in the same way as the glomerular filtration rate (GFR) agents like ^{99m}Tc-DTPA. For quantitative analysis, both MAG$_3$ and EC can be used to measure effective renal plasma flow (ERPF). From the clinical point of view, ERPF can be used in the same way as the GFR (CD Russell, et al. 1991). However from the standpoint of nuclear medicine laboratory; the compelling advantage of ERPF is that it can be accurately measured within one hour in contrast to GFR which needs 3 hours for the same accuracy.

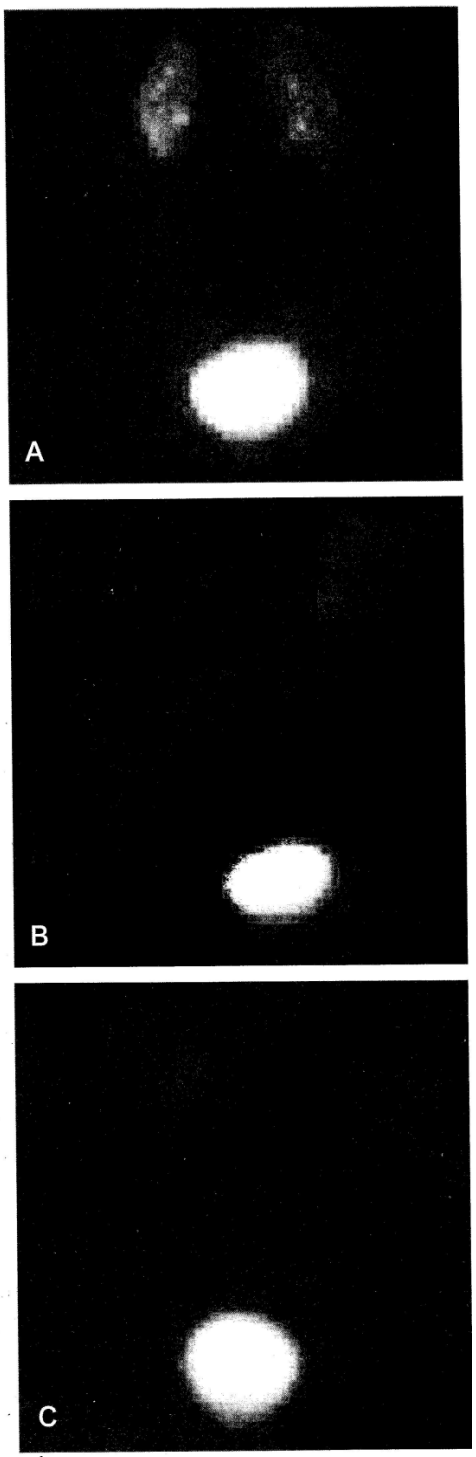

Fig. 3: 30th minute images of lower abdomen of human volunteers obtained for ^{99m}Tc-EC(pH 8), ^{99m}Tc-MAG$_3$ (B) and ^{99m}Tc-EC(pH 9) (C).

OIH, although regarded as a gold standard for the measurement of ERPF, carries the disadvantages of radioiodine labeled agent which has elimination half-life more than 8 days. On the other hand ^{99m}Tc- MAG$_3$ has may excellent characteristics and is widely used in the clinical setting. It however, differs significantly from OIH

Table 1: Biodistribution in rats (% Dose/organ) of ^{99m}Tc-EC at various pH values compared with ^{99m}Tc-MAG_3 (n=5)

Organs	^{99m}Tc-EC(pH8) (mean±SD)	^{99m}Tc-EC(pH9) mean±SD)	^{99m}Tc- MAG_3 (mean±SD)
Blood	2.87 ± 0.93	1.12 ± 0.42	2.99±0.75
Kidneys (1-2min)	9.09 ± 185	8.61 ± 2.1	9.05 ±1.11
Kidneys (10min)	4.10 ± 0.75	3.55 ± 0.75	6.78±1.13
Liver	2.46 ± 0.53	1.71 ± 0.25	2.39±0.47
Intestine	3.31 ± 0.67	2.80 0.79	3.97±1.32

Table 2: Renogram Parameters Of Rhesus Monkey

Parameters	^{99m}Tc-EC (pH8)		^{99m} Tc-MAG_3	
Kidneys	Rt	Lt	Rt	Lt
T_{MAX} (min)	1.25	1.25	1.33	1.5
$T_{1/2}$ (min)	4.05	4.13	3.90	3.93
R_{max} (% of Counts at T_{max} to ID	8.87	8.74	6.67	7.60

Table 3: Background to kidney ratio at 30th minute of various renal agents used in the study

Agents	Background/Kidney Ratio means+- SD
^{99m}Tc-EC (pH8) n=6)	0.299 ± 0.017
^{99m}Tc- MAG_3 (n=81)	0.236 ± 0.018
^{99m}Tc-EC (pH9) (n=12)	0.229 ± 0.024

Table 4: Comparison of ^{99m}Tc-EC (pH8), ^{99m}Tc-MAG_3 and ^{99m}Tc-EC (pH9) for Scintigraphic studies of various parameters in human.

Parameters	^{99m} Tc-EC (pH8) (n=6, mean±SEM	^{99m} Tc-MAG_3 (n=6, mean±SEM	^{99m}Tc-EC (pH9) (n=12,mean±SEM	^{99m}Tc-MAG_3 (n=12, mean±SEM
T_{MAX}	3.4 ±0.7	4.1 ± 1.6	3.7 ± 0.6	4.0 ±0.8
$T_{1/2}$	8.8 ± 2.1	7.5 ±3.4	7.3 ± 1.0	7.9 ±14
A_{25}/A_{MAX}	0.368 ± 0.032	0.201 ±0.033	0.029 ± 0.033	0.218 ±0.035
Renal Uptake	8.96 ± 3.96	11.00 ± 2.70	12.67 ±3.80	10.53 ± 2.98

in its pharmacological behavior. Its plasma clearance is 67% of OIH (Davison *et al.*, 1979; Frtizberg *et al.*, 1981; Frtizberg *et al.*, 1982). (Verbruggen *et al.*, 1990) introduced ^{99m}Tc-EC during a mice biodistribution study of the metabolites of ^{99m}Tc-L,L ethylenedicysteine diethylester (^{99m}Tc-ECD), a brain imaging agent. In animal experiments EC has shown better renal excretion characteristics than ^{99m}Tc-MAG_3 with significantly lower retention in the kidneys, liver, bowel and the blood pool (Verbruggen *et al.*, 1992), similar characteristics have been sown in human volunteers (Surma *et al.*, 1994; Gupta *et al.*, 1995). However, the commercially available EC kits have 2 or 3 components and have disadvantage of cumbersome radiolabeling procedure which is less than ideal.

In the current study we have formulated a single vial EC kit. The kit is easy to prepare and no boiling is required. Furthermore, it is remained stable throughout the day. Initially it is formulated at pH ranging from 8 to 11. Paper chromatography and HPLC showed adequate labeling in all the formulation. During the biodistribution studies in rats, ^{99m}Tc-EC at all pH levels showed results comparable to ^{99m}Tc-MAG_3 (table 1). The uptake in the kidneys at 1-2 minutes is almost similar to that of MAG_3, but at 10 minutes the remaining activity is less for EC (pH 8). Showing its better clearance at higher pH the results are even better than pH 8. (Verbruggen, *et al.*, 1992) studied comparative biodistribution of two vial kit of ^{99m}Tc-EC and ^{99m}Tc-MAG_3 in mice (n=10). The uptake in the kidneys at 10 minutes was reported as 4.87±2.48 (^{99m}Tc-MAG_3) and 6.76±2.28 (^{99m}Tc-EC) for the two agents respectively. Our results are similar to those of the Verbruggen study.

The parameters obtained from the renograms curves of monkey showed good agreement between ^{99m}Tc-EC (pH 9) and ^{99m}Tc-MAG_3 (table 2). R_{MAX} in case of EC is higher as compared to MAG_3 showing its better uptake by the kidneys. These results are also in agreement with verbruggen's findings (AM Verbruggen, et al. 1992) in the case of a baboon (R_{MAX} = 8.20 for left kidney).

The promising results of ^{99m}Tc-EC (pH 9) in animal experiments prompted its evaluation in human volunteers and it is found however, in contrast with the animal studies. The high background at 30th minute (fig. 3), high residual activity and low renal uptake (table 4) as

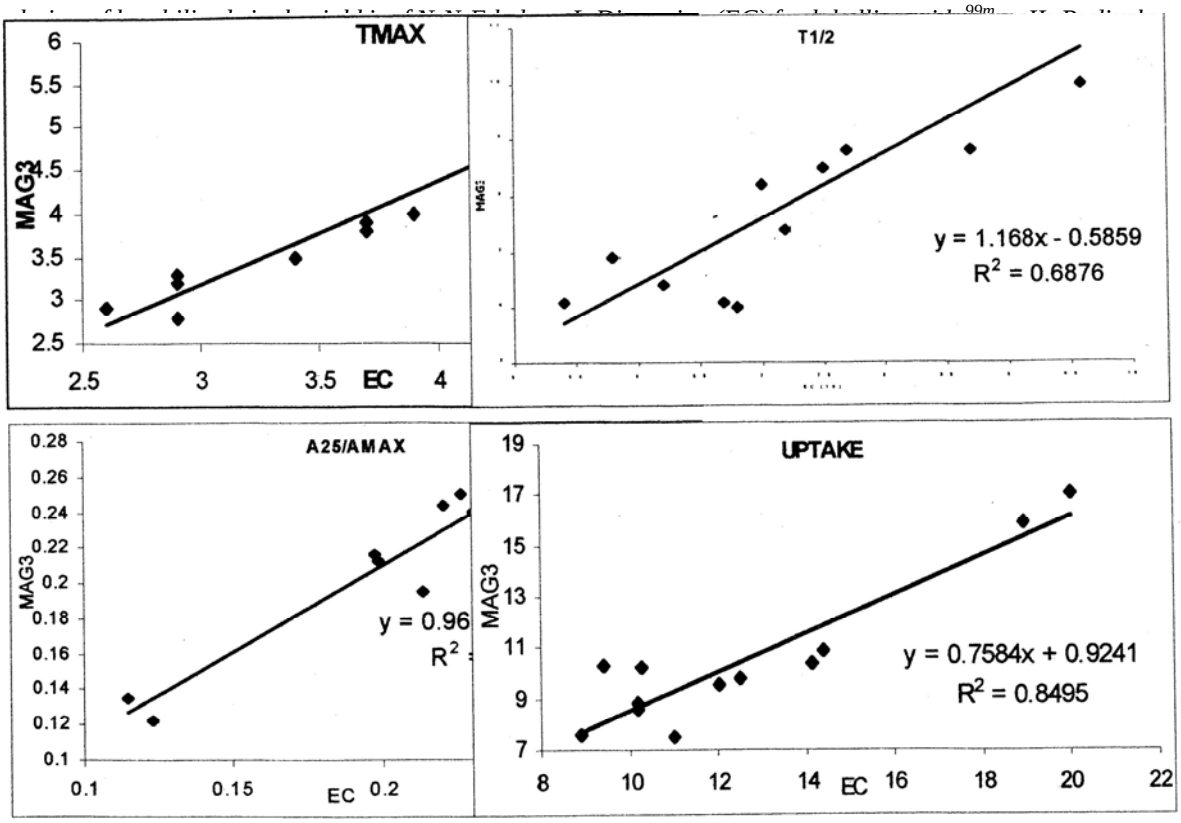

Fig. 4: Correlation of T_{MAX}, $T_{1/2}$, $A_{25/}$ A_{MAX} and uptake between ^{99m}Tc-EC(pH) and ^{99m}Tc-MAG_3 in normal volunteers

compared to ^{99m}Tc-MAG_3 are seen. This could be due to the formation of specie at this pH, which has low plasma clearance and/or tendency to bind with renal parenchyma. However no such specie is isolated by HPLC gradient system used. Hence there is need of further evaluation of ^{99m}Tc-EC at lower pH by HPLC using various solvent systems.

The single vial EC kit formulated at higher pH i.e. pH 9-10 is then tested for clinical evaluation. The background observed at these cases is less than ^{99m}Tc-MAG_3 (fig 3) and B/K ratio being almost similar (table 3). The residual activity at pH 9 and10 was better than ^{99m}Tc-MAG_3 (table 4) and two vials EC kit (0.223) reported by (Gupta, *et al 1995)*. Its renal uptake was superior to MAG_3. This suggests that the clearance of ^{99m}Tc-EC (pH 9 and 10) is higher than ^{99m}Tc-MAG_3.

CONCLUSION

The single vial EC kit at pH 8, gave a good labeling yield with ^{99m}Tc associated with longer stability.

Whilst in the animal study the best results are obtained with pH 8, whilst the results in rhesus monkey and human volunteers are better at pH 9- 10.

Unlike ^{99m}Tc-MAG_3, ^{99m}Tc-EC complex does not need boiling prior to clinical use.

EC can be used both for static as well as for dynamic renal scintigraphy studies.

Renal excretion is the primary route of EC elimination with no other excretory pathways.

Clinical evaluation in a large series is needed to reach better understanding of renal handling of ^{99m}Tc-EC before routine clinical usage can be recommended.

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