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Research Article

Synthesis of $^{99m}Tc(CO)_3$-mebrofenin via $[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor and comparative pharmacokinetics studies with ^{99m}Tc-mebrofenin

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Summary

The organometallic precursor fac-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ was reacted with trimethyl-bromoacetanilido-iminodiacetic acid (mebrofenin) in phosphate buffered saline (pH 7) at 70°C for 1 h to produce the complex, $^{99m}Tc(CO)_3$-mebrofenin, in >95% yields. High performance liquid chromatography analysis indicated the formation of a single species. *In vitro* studies of $^{99m}Tc(CO)_3$-mebrofenin showed that the complex is stable for 24 h. No decomposition or alteration of the complex was observed in the presence of excess amount of cysteine and histidine. ^{99m}Tc-mebrofenin was also prepared for comparative evaluation by the conventional method using a ready to use kit HPLC analysis of this complex showed presence of two species. Biodistribution studies in normal Swiss mice with $^{99m}Tc(CO)_3$-mebrofenin showed hepatobiliary clearance. However, the retention in liver was higher (13% at 1 h p.i.) as compared to that of ^{99m}Tc-mebrofenin (3.4% at 1 h p.i.). Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: technetium-99m; ^{99m}Tc-carbonyl; mebrofenin; hepatobiliary agents; ^{99m}Tc-iminodiacetic acid

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Introduction

Potential usefulness of organometallic carbonyl compounds of technetium and rhenium for nuclear medicine applications was proposed in the literaure.[1,2] A convenient method for the synthesis of the hydrophilic precursor fac-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ in aqueous solution directly from $^{99m}TcO_4^-$ in saline and carbon monoxide at normal pressure has been developed by Alberto *et al*.[3] The precursor reacts with a variety of ligands under moderate conditions due to substitution lability of the three water molecules.[4–7] The major advantage of using the carbonyl precursor is that high specific activity labeling of biomolecules can be obtained with minimum amount of ligands.[8,9] Also the carbonyl precursor does not need multidentate chelator to make stable complexes. A large number of ligands have been studied for choosing ideal chelating systems for the carbonyl precursor.[10–13] Previous *in vitro* studies have suggested that the ideal chelating system for the precursor fac-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ in respect of a potential radiopharmaceutical application should contain one or more amine functional groups (preferably aromatic nitrogen heterocycles), in combination with a carboxylic acid functional group.[3] A variety of bidentate and tridentate model ligand systems containing amines and carboxylic acid functional groups have been evaluated by Schibli *et al*. for the formation of fac-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ complexes which are stable *in vitro* and *in vivo*.[6] The complexes with tridentate ligands were preferable for the functionalization of biomolecules since they formed organometallic compounds with more favorable pharmacokinetics.[6] Among the molecules, histidine and iminodiacetic acid (IDA), have been extensively used for functionlisation of biomolecules.[14–17]

It is of interest to have a comparative evaluation of the existing ^{99m}Tc radiopharmaceutcals prepared by conventional methods with the one prepared through the carbonyl precursor, $[^{99m}Tc(OH_2)_3(CO)_3]^+$. The complexes with ^{99m}Tc carbonyl precursor may be formed with better pharmacokinetics or may result in the formation of novel radiopharmaceuticals, due to entirely different radiochemical characteristics of the complexes with the carbonyl core. Such comparative studies are reported with methoxy isobutyl isonitrile (MIBI) ligand used for the preparation of $[^{99m}Tc(MIBI)_6]^+$, an extensively used myocardial perfusion imaging agent.[18]

Hepatobiliary function imaging using radiotracers offer many advantages. Derivatised acetanilido iminodiacetic acid (IDA) ligands

have been extensively investigated in the eighties in search of the ideal hepatobiliary imaging agent and mebrofenin was found to be the most used.[19–22] Hence our attention was drawn to study $^{99m}Tc(CO)_3$-mebrofenin complex and its comparison with ^{99m}Tc-mebrofenin (prepared by the conventional method). ^{99m}Tc-HIDAs, first extensively investigated series for structure distribution relationships (SDKs), are reported to be bis complexes of the ligand with $^{99m}Tc(+3)$ core and carrying an overall negative charge.[23] A mono complex with a single ligand is expected to be formed in the case $^{99m}Tc(CO)_3$-mebrofenin. It is possible that this will lead to a product of distinct features of its own which may not necessarily match with that of ^{99m}Tc-mebrofenin. Hence, we chose to study this aspect in our present work.

Results and Discussion

Preparation of $^{99m}Tc(CO)_3$–mebrofenin

Formation of $^{99m}Tc(OH_2)_3(CO)_3$ was revealed by HPLC. The carbonyl precursor was formed in yields of 95–98% with an HPLC retention time of 13.7 min. Mebrofenin was labeled with $^{99m}Tc(OH_2)_3(CO)_3$ in 95–98% yields under optimized conditions. The labeled compound was eluted out as single species with retention time of 17.4 min (Figure 1(a)). No activity was retained on the column.

The radiolabeling yield was dependant on the reaction pH as well as concentration of the ligand. The results of complexaton studies with the ligand at various pH are given in Table 1. Maximum complexation yield was obtained at pH 7. In alkaline medium the yield was lower with pertechnatate as the major radiochemical impurity and the rest being ^{99m}Tc carbonyl, as detected by HPLC. Overall yield was better in acidic medium with mainly ^{99m}Tc carbonyl as the radiochemical impurity. At pH 7, complexation of the ligand with ^{99m}Tc carbonyl was studied at different ligand concentration. The ligand reacted with ^{99m}Tc carbonyl at as low as 25 μg in 1 ml reaction volume (0.7×10^{-5} M) (Figure 2). Studies on the effect of temperature on reaction kinetics showed that the reaction rate was slow at room temperature, the yield being 91% for a reaction time of 24 h at pH 7 with optimized amount of ligand concentration. Complexation yield of 95–97% could be obtained when the reaction was carried out at $\sim 70^{\circ}$C for 1 h.

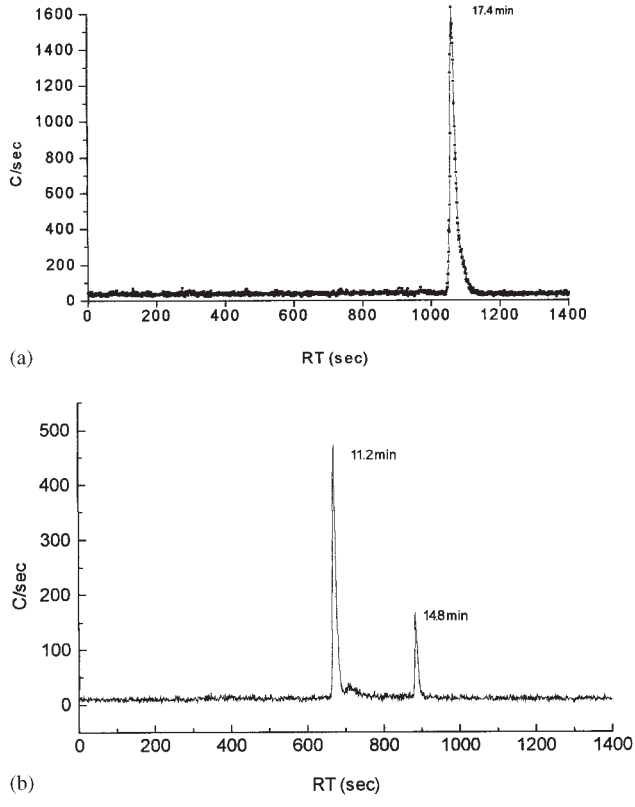

Figure 1. HPLC pattern of (a) $^{99m}Tc(CO)_3$-mebrofenin (b) ^{99m}Tc-mebrofenin

Table 1. Effect of pH on labeling yield of $^{99m}Tc(CO)_3$-mebrofenin (The reaction was carried out with 1.4×10^{-4} M mebrofenin at 70°C for 1 h)

Species	% Activity		
	pH 5	pH 7	pH 9
$^{99m}TcCO_4^-$	4	4	28
$[^{99m}Tc(OH_2)_3(CO)_3]^+$	5	1.8	14
$^{99m}Tc(CO)_3$-mebrofenin	91	95	51

Characterization of $^{99m}Tc\ (CO)_3$–mebrofenin

Multiple quality control techniques such as partition chromatography, paper chromatography and paper electrophoresis were used along with HPLC to study the charge and the lipophilicity of the product. The distribution ratio of the complex determined from three consecutive

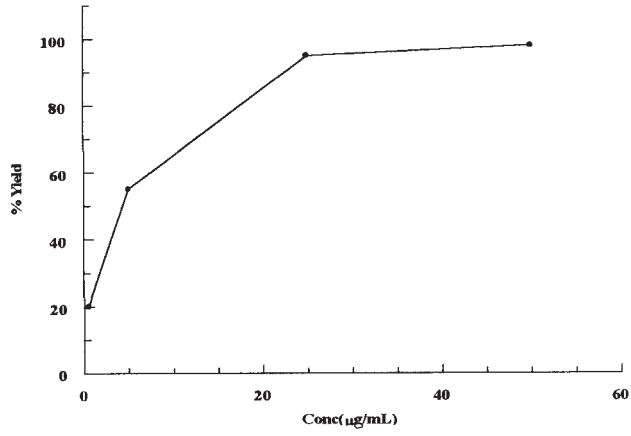

Figure 2. Effect of mebrofenin concentration on labeling yield of $^{99m}Tc(CO)_3$-mebrofenin

back extractions in octanol/saline was found to be 3 indicating that the product is lipophilic. The complex migrated with R_f of 0.9 in paper chromatography using methanol as solvent. Paper electrophoresis pattern revealed that the complex is negatively charged with a migration rate of 0.5 cm/ volt/ h towards anode.

Stability and challenge studies with histidine and cysteine

In vitro stability of $^{99m}Tc(CO)_3$-mebrofenin in saline was excellent. When HPLC was carried out with the preparation stored at room temperature for 24 h, the major peak was that of the complex (R_t 17.4 min) with negligible amount of $^{99m}TcO_4^-$ ($\sim$4%).

The complex was challenged with large excess of histidine and cysteine for 1 h at 37°C to determine the resistance to ligand exchange. No release of the radiolabel from the complex was observed after 1 h incubation at 37°C.

^{99m}Tc-mebrofenin

HPLC pattern of ^{99m}Tc-mebrofenin is depicted in Figure 1b. The complex prepared by conventional method showed two species with retention time 11.2 and 14.8 min (Figure 1(b)). Both the species formed were more hydrophilic than the one prepared by using ^{99m}Tc carbonyl precursor as revealed by the longer retention time of [$^{99m}Tc(CO)_3$]-

mebrofenin (17.4 min). However, no difference in paper chromatogrphy pattern and paper electrophoresis pattern was noticed between the two products.

Biodistribution

Biodistribution patterns of $^{99m}Tc(CO)_3$-mebrofenin and ^{99m}Tc-mebrofenin is given in Table 2 and the activity retained in liver, intestine, stomach, kidney and blood at 30 min p.i is depicted in Figure 3. The complex showed rapid clearance from the blood and excretion through

Table 2. Biodstribution (%ID/organ) of $^{99m}Tc(CO)_3$-mebrofenin and ^{99m}Tc-mebrofenin in Swiss mice as a function of time after intravenous administration

Organ/tissue	[$^{99m}Tc(CO)_3$]-mebrofenin			^{99m}Tc–mebrofenin		
	15 min	30 min	1 h	15 min	30 min	1 h
Blood	0.46 (0.23)	0.44 (0.03)	0.38 (0.17)	0.61 (0.26)	0.30 (0.17)	0.47 (0.1)
Liver	24.6 (3.59)	18.2 (1.5)	13.1 (1.8)	5.8 (0.5)	3.2 (0.9)	3.4 (0.9)
Intestine	53.8 (2.4)	77.9 (1.5)	71.7 (0.7)	77.6 (7.9)	77.4 (1.5)	88.4 (5.6)
Kidney	1.2 (0.10)	0.8 (0.1)	0.6 (0.1)	1.2 (0.2)	0.5 (0.1)	0.4 (0.1)
Stomach	0.8 (0.5)	1.0 (0.4)	0.8 (0.2)	1.3 (0.2)	1.2 (1.7)	1.0 (0.5)
Heart	0.2 (0.04)	0.14 (0.04)	0.1 (0.09)	—	—	—
Thyroid	0.1 (0.1)	0.11 (0.02)	0.03 (0.01)	0.09 (0.04)	0.03 (0.01)	0.06 (0.03)
Lungs	0.7 (0.2)	0.3 (0.06)	0.3 (0.08)	0.15 (0.03)	0.08 (0.03)	0.08 (0.02)
Spleen	0.19 (0.02	0.15 (0.07)	0.11 (0.07)	0.06 (0.03)	0.07 (0.04)	0.07 (0.02)

Values in the bracket represent standard deviation ($n = 3$).

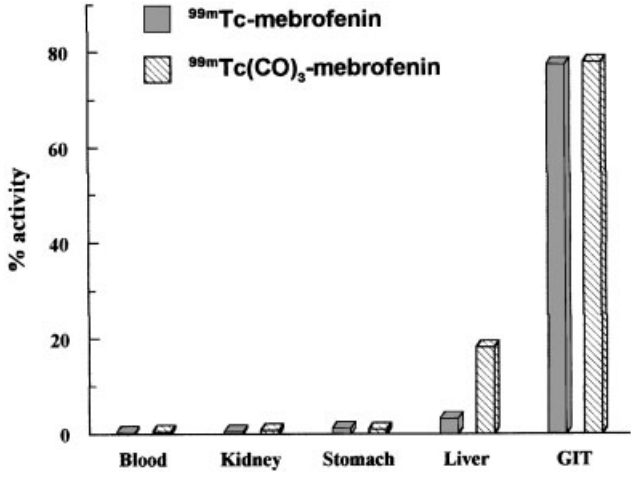

Figure 3. Biodistribution pattern (% activity/organ) of ^{99m}Tc-mebrofenin and $^{99m}Tc(CO)_3$-mebrofenin at 30 min p.i.

hepatobiliary system similar to ^{99m}Tc-mebrofenin. Uptake of the product in intestine after 15 min was 55.8 ($\pm$ 2.4)% which increased to 77.9 ($\pm$ 1.54)% in 30 min and marginally decreased to 72 ($\pm$ 0.7)%, at 1 h with respective liver uptake being 24.6 ($\pm$ 3.5)%, 18.2 ($\pm$ 1.53)% and 13.14 ($\pm$ 1.8)%. Retention of activity in liver was higher as compared to that with ^{99m}Tc-mebrofenin [5.8 ($\pm$ 0.46)%, 3.23 ($\pm$ 0.9)% and 3.4 ($\pm$ 0.9)% at 15 min, 30 min and 1 h post injection, respectively]. The higher retention in the liver may be due to the higher lipophilicity of the complex as revealed by HPLC. The amount of radioactivity found in the stomach, an indication of *in vivo* decomposition and reformation of $^{99m}TcO_4^-$, was negligible [0.75 ($\pm$ 0.19)%]. The rate of renal clearance for both the complexes was similar.

^{99m}Tc labeled *N*-(2,6-dimethylphenylcarbmoylmethyl)-iminodiacetic acid (^{99m}Tc-HIDA) is a representative of the class of radiopharmaceuticals, all of which contain N substituted iminodiacetic acid. Loberg *et al*. have reported that ^{99m}Tc-HIDA exists in solution as sodium bis [*N*-(2,6-dimethylphenylcarbmoylmethyl) iminodiaceto] technatate (III).[23] Since ^{99m}Tc- mebrofenin used in regular clinical use belongs to the same class of radiopharmaceuticals, it is also expected to exist as bis complex of the ligand with ^{99m}Tc(III) and carrying an overall anionic charge.

Formulations of ^{99m}Tc containing well characterized single radiochemical species are being sought to develop new radiopharmaceuticals for clinical use and for medical research. $[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor capable of forming complexes with suitable ligands offer an attractive pathway for developing relatively small complexes with biologically useful molecules. IDA and histidine molecules have been shown to be useful for displacing the H_2O molecules from the precursor leading to the formation of $[^{99m}Tc(CO)_3L]$ type of mono complexes.[6] Depending on the charge of the tridentate ligand in the complex, one can aim to prepare neutral, cationic or anionic complexes. IDA being a ligand with two carboxylic acid groups would lead to anionic complex (Figure 4(b)) In view of the distinctly different nature of the mono complex (probable structure in Figure 4(c)), difference in *in vivo* behavior in test animals can be expected. Mice studies have accordingly shown greater and prolonged retention of the $^{99m}Tc(CO)_3$-mebrofenin in liver. On the other hand an ideal hepatobiliary tracer such as ^{99m}Tc-mebrofenin is hardly retained in normal liver 30 min p.i. with most of the tracer being cleared into the intestines. It should, however, be pointed out that the aim of the present study was to assess the extent of

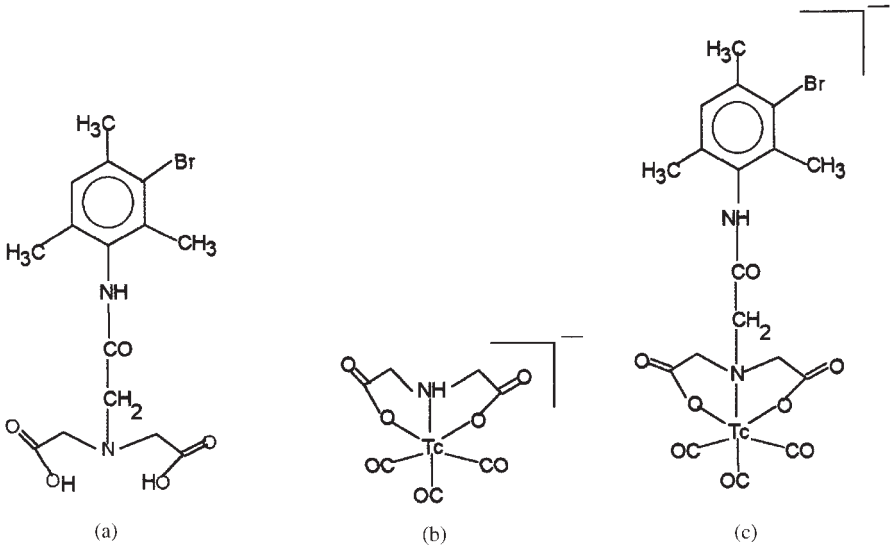

Figure 4. (a) Trimethyl-bromo iminodiacetic acid mebrofenin, (b) $^{99m}Tc(CO)_3$-IDA (c) probable structure of $^{99m}Tc(CO)_3$-mebrofenin

the differences in biological behavior of the two formulations. Such comparative studies could help in understanding the nature of the new products as well as to evolve strategies for further exploitation of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor route for the development of new radiopharmaceuticals.

The biodistribution studies with $^{99m}Tc(CO)_3$-IDA revealed negligible retention of the activity in soft tissues.[6] The ability of *N* substitution in IDA with molecules of interest e.g. sugar, peptides, drugs etc. to prepare $^{99m}Tc(CO)_3$ conjugated ligand complexes could open up new avenues for functional imaging with ^{99m}Tc. Molecules with an additional functional group other than the moiety needed for biological activity/specificity could be deployed in such strategies. The merit of ^{99m}Tc as an ideal radionuclide for imaging and the biological specificity of the biochemical/drug could be then availed leading to more efficacious diagnostic imaging capabilities. Work on such lines is in progress in our group and the present study is a forerunner in the series.

Experimental

All chemicals and solvents were reagent grade used without further purification. Sodium borohydride, Na/K tartrate and Carbon monoxide

in 0.5 L refillable canisters were obtained from Aldrich Chemicals. $^{99m}TcO_4^-$ was eluted from a $^{99}Mo/^{99m}Tc$ column generator using 0.9% saline.[24] Mebrofenin (Figure 4(a)) synthesized by a 3-step route and characterized by MS, IR and ^1H NMR was available in house ex-stock. Lyophilized kit for ^{99m}Tc-mebrofenin (mebrofenin 25 mg, stannous chloride dihydrate 0.2 mg, pH 5.5) was also available in house ex-stock and used. HPLC analysis was performed on a Jasco PU 1580 system with a Jasco 1575 tunable absorption detector. A radiometric detector system along with the software developed locally was used for radiochromatography. For radiochemical purity (RCP) analysis a C_{18} reversed phase HiQ Sil (5 μm, 4 × 250 mm) column was used.

$[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor preparation

The precursor was prepared by a modified procedure reported by Alberto and coworkers.[3] Briefly $NaBH_4$ (5.6 mg), Na_2CO_3 (4 mg) and Na/K tartrate (15 mg), were dissolved in 0.5 ml double distilled water in a glass serum vial. The vial was sealed and a needle was introduced through the rubber stopper to equilibrate with the atmospheric pressure. CO gas was purged through the solution for 5 min followed by the addition of 1 ml of $^{99m}TcO_4^-$ containing 37–74 MBq. The needle was removed and the vial was heated at 80°C for 15 min. After cooling the vial for 10 min and re-equilibration to atmospheric pressure, the reaction pH was adjusted to 7 with 300 μl of 1:3 mixture of 0.5 M phosphate buffer pH 7.5: 1 M HCl. The precursor was characterized by HPLC.

Preparation of $^{99m}Tc(CO)_3$–mebrofenin

A 100 μl of aliquot of an aqueous solution of mebrofenin was added to 500 μl of the $[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor solution and incubated at 70°C for 15 min.

Characterization of $^{99m}Tc(CO)_3$-mebrofenin. HPLC: Formation of the complex and nature of the species formed was determined by C_{18} reverse phase HPLC. HPLC eluting solvents consisted of H_2O containing 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). The HPLC gradient system for analysis of the product started with 90%A/10%B with a linear gradient to 10%A/90%B from 0–28 min with no change in the eluant composition from 28–30 min. The flow rate was

1 ml/min. 25 µl of the sample was used for analysis. Recovery of the activity was determined by summing the total counts in all fractions and comparing it to the total injected activity.

Solvent extraction: Solvent extraction was performed by vortexing 1 ml of the reaction mixture with 1 ml of octanol for 1 min. Equal aliquots of the organic and aqueous layers were withdrawn and measured for radioactivity. The organic extract was back extracted repeatedly with saline to estimate the distribution ratio.

Paper chromatography: Paper chromatography was carried out with ^{99m}Tc-mebrofenin and ^{99m}Tc[$(CO)_3$]-mebrofenin. The samples were spotted on whatman 3 chromatography paper at one end. The strips were developed up to 6.5 cm using 70% methanol as solvent, dried and cut into equal segments and measured for radioactivity in a gamma counter with a Nal (Tl) detector.

Paper electrophoresis: Paper electrophoresis was carried out with $^{99m}TcO_4^-$, ^{99m}Tc-carbonyl, ^{99m}Tc$(CO)_3$-mebrofenin and ^{99m}Tc-mebrofenin. The samples were spotted on Whatman 3 chromatography paper (40 cm) at 10–12 cm from the cathode and electrophoresis was carried out at 8 V/cm for 1 h in 0.02 M phosphate buffer (pH 7.5). The strips were dried cut into 1 cm segments and counted.

Stability studies and challenge with histidine and cysteine. The stability of the complex was studied for a period of 24 h by HPLC analysis. Challenge experiment with histidine and cysteine were performed with [^{99m}Tc$(CO)_3$]-mebrofenin. Solution of histidine and cysteine (100 µl, 0.1 M) in distilled water was reacted with 500 µl of the complex at 37°C for 1 h and the resultant product was analyzed by HPLC.

Preparation of ^{99m}Tc-mebrofenin. ^{99m}Tc-mebrofenin was prepared by using a ready to use kit and by following the kit protocol. 3 ml of $^{99m}TcO_4^-$ (37–74 MBq/ml) in saline was added to the kit vial (containing 25 mg of mebrofenin and 0.2 mg of stannous chloride) and mixed well. The vial was heated in a boiling water bath for 5 min and allowed to cool for 10 min.The product formed was characterized as described earlier.

Biodistribution. Redistribution studies were carried out with ^{99m}Tc$(CO)_3$-mebrofenin as well as ^{99m}Tc-mebrofenin. Swiss mice (25–30 g) were injected in tail vein with 0.1 ml of the products each containing 3–7 MBq of activity. The mice were sacrificed at 15 min,

30 min and 1 h post injection. Tissues and organs were excised, rinsed, weighed and counted in a NaI (Tl) flat geometry detector. The percent injected dose in each tissue was calculated from the above data. The percent activity in the blood was calculated by measuring the activity in 0.5–1 g of the blood withdrawn by cardiac puncture immediately after sacrifice and assuming the whole blood volume as 6.5% of the body weight. All the animal experiments were carried out in compliance with the relevant national laws relating to the conduct of animal experimentation.

Conclusion

Labeling of mebrofenin via the ^{99m}Tc-carbonyl precursor could be achieved in high yields with the formation of a single species. The species formed was radiochemically different than ^{99m}Tc-mebrofenin prepared by the conventional method. In the present case, the product did not reveal any superior biological features as a hepatobiliary radiopharmaceutical. However, it may be of interest to probe the ^{99m}Tc carbonyl chemistry with other ligands used for the preparation of existing radiopharmaceuticals in order to develop novel complexes that may yield better diagnostic agents.

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