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Research Article

On the ^{99m}Tc-labeling of isoniazid with different ^{99m}Tc cores

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Summary

Recent advances in the chemistry of radiolabeling with ^{99m}Tc such as use of the ^{99m}Tc tricarbonyl and ^{99m}Tc–HYNIC cores have revived interest in ^{99m}Tc-labeling of small biomolecules and further investigation as potential radiopharmaceuticals. Isoniazid, a drug commonly used for treatment of tuberculosis, has been chosen for the present study. Three distinct strategies were utilized to radiolabel isoniazid with ^{99m}Tc. In the direct labeling protocol, the hydrazino amide functional group of isoniazid was used for ^{99m}Tc-labeling in the HYNIC sense using tricine and EDDA as co-ligands. The other strategies of ^{99m}Tc-labeling involved the derivatization of isoniazid to its *N*, *N*-diacetic acid derivative, which in turn was either used as a tridentate ligand for labeling with the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ synthon or directly labeled by the conventional route wherein ^{99m}Tc is in the $+3$ oxidation state. The complexes prepared in >95% yields were characterized by paper chromatography, thin layer chromatography and HPLC. Comparison of the three approaches showed that maximum specific activity and stability was obtained in the ^{99m}Tc–isoniazid derivative synthesized via the tricarbonyl method. However, *in vitro* cell-binding studies indicated that none of the ^{99m}Tc–isoniazid complexes prepared had any appreciable uptake in *Mycobacterium tuberculosis*.

Key Words: isoniazid; technetium-99m; ^{99m}Tc–isoniazid; tuberculosis

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Introduction

Tuberculosis caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is one of the biggest health hazards to the population of tropical countries and the world's leading cause of death from a single infectious agent.[1] WHO has estimated about 2 million cases of tuberculosis annually in India alone of which over 450 000 are fatal.[2] In up to 20% of cases the active disease may develop in extra pulmonary regions like the lymph nodes, brain, bone, etc.,[3] which cannot be detected by the conventional technique of sputum analysis. This percentage may be even higher in HIV-infected patients. While the development of newer PCR-based methods[4,5] alleviates the need to obtain a significant number of organisms from the site of infection, detection of non-pulmonary tuberculosis still depends on obtaining samples from accurately localized foci of infection based on non-specific clinical symptoms. It would be thus advantageous to have a direct infection imaging technique for detection and localization of the disease caused by *M. tuberculosis*. Conventional agents like Ga-67 citrate, ^{99m}Tc nanocolloid or ^{99m}Tc immunoglobulin are non-specific and may not be suitable for differentiating infective conditions from inflammatory ones.[6] ^{99m}Tc–tetrofosmin and ^{99m}Tc MIBI have also been used in the diagnosis of pulmonary tuberculosis, but these are myocardial imaging agents and lack specificity.[7,8]

Isonicotinic acid hydrazide (isoniazid) is one of the most effective agents in tuberculosis therapy.[9] This agent rapidly permeates the bacterial cell membrane via passive diffusion.[10] It has been shown that isonicotinic acid is one of the metabolites of isoniazid which is trapped in the bacterial cell.[11] Studies on introduction of radiolabels such as ^{11}C and ^{123}I on a substituent of the pyridine ring of isoniazid for the development of potential radiopharmaceuticals have been reported earlier.[12,13] Isoniazid derivatized with 2 iminothiolane has been labeled with ^{99m}Tc by conventional method and has shown promising results as infection imaging agent.[14] The conventional method of labeling[15–18] biomolecules possesses inherent limitations of low specific activity of the radiolabeled complexes emerging from the use of excess amount of ligand. To circumvent this drawback, a considerable amount of attention is focussed over the years on the development of ^{99m}Tc radiopharmaceuticals with novel cores like hydrazinonicotinic acid (HYNIC) core and carbonyl core,[19–25] yielding ^{99m}Tc complexes with high specific activity, good stability and retention of biological activity. Here we report work carried out on radiolabeling of isoniazid with ^{99m}Tc using different approaches to explore its potential as a tuberculosis-imaging agent.

Results and discussion

^{99m}Tc–isoniazid (HYNIC core)

Hydrazinonicotinic acid amide is the most commonly used precursor ligand for ^{99m}Tc-labeling of molecules along with co-ligands ethylene diaminediacetic acid (EDDA) and tricine for the preparation of ^{99m}Tc–HYNIC core. In this respect, isoniazid, which contains the isonicotinic ring and an amidohydrazide (-CONH-NH_2) substituent, can be envisaged to furnish identical donors for building up the HYNIC core in presence of the usual co-ligands, EDDA and tricine. Utilizing this protocol, ^{99m}Tc-labeling of isoniazid could be accomplished in >90% yields. It was observed that in presence of only tricine, the yield was less than 70% whereas in presence of EDDA and tricine the yield increased to >90%. The yield was maximum within 15 min both at room temperature (RT) as well as at 70°C as estimated from paper chromatography (PC) using acetone and saline as eluants (Figure 1). In PC with acetone, ^{99}Tc–tricine and ^{99m}Tc–EDDA moved to solvent front whereas the ^{99m}Tc–isoniazid complex remained at the point of spotting (Figure 1). In PC/saline, it was observed that the complex moved to the solvent front thereby ruling out the presence of reduced technetium. The HPLC chromatograms of ^{99m}Tc–tricine, ^{99m}Tc–EDDA and the ^{99m}Tc–isoniazid complex showed that the radiolabeled species were eluted at different retention times, being 7.9 min for the complex and 2.5 and 2.9 min for ^{99m}Tc–EDDA and ^{99m}Tc–tricine, respectively (Figure 2). Paper electrophoresis revealed the complex with negative charge. The migration rate was 5 cm/h/10 V toward anode.

A typical complexation protocol required 0.5 mg of isoniazid as an optimum amount. The complex prepared at room temperature retained the radio-

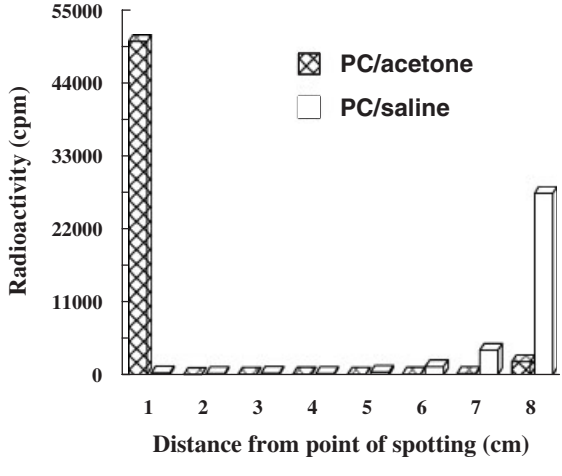

Figure 1. Paper chromatography pattern of ^{99m}Tc–isoniazid (HYNIC core) in saline and acetone

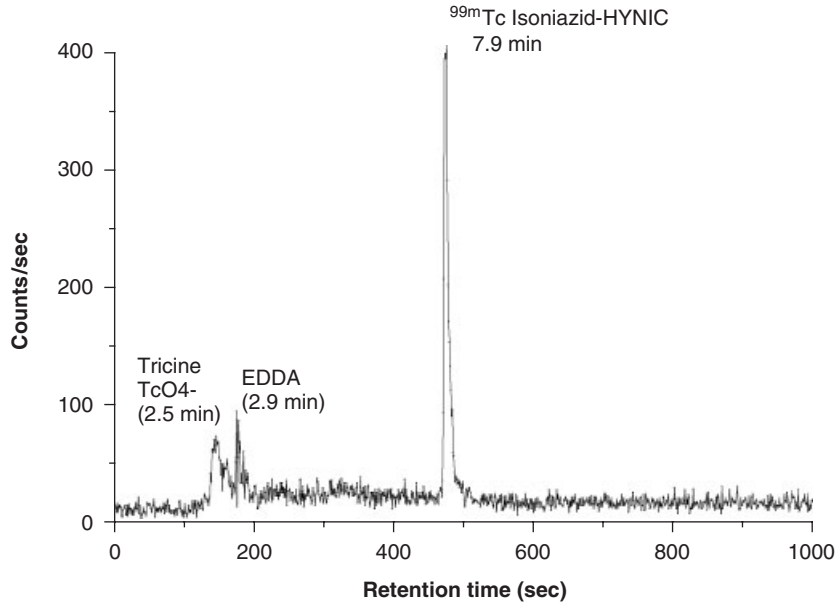

Figure 2. HPLC pattern of ^{99m}Tc–isoniazid (HYNIC core)

chemical purity of >90% for 5 h whereas the complex prepared by heating showed a decrease in RC purity (~85%) when stored at room temperature.

Isoniazid–diacetic acid

The *N*, *N*–diacetic derivative of isoniazid was synthesized to facilitate labeling with ^{99m}Tc by conventional approach and carbonyl approach. ^{1}H-NMR signal of the derivative at 3.515δ ppm, which corresponds to 4 number of protons, clearly indicates the introduction of two $-CH_2COOH$ groups in the isoniazid moiety and this gives the conclusive evidence in favor of the formation of isoniazid–*N*, *N*-diacetic acid.

With conventional approach monomeric octahedral anionic complex $[Tc(III)(R\text{-}IDA)_2]^-$ is formed while with carbonyl approach the complex $[Tc(I)(R\text{-}IDA)]^-$ formed is monomeric octahedral with −1 charge.[26,27]

^{99m}Tc–isoniazid (conventional core)

The complexation yield of the diacetic acid derivative of isoniazid with ^{99m}Tc at a ligand concentration of 15 mg/ml was >95% and the complex was characterized by PC. It was observed that in PC/acetone, >95% of the activity remained at the point of spotting ($R_f = 0$). This indicated that the reaction mixture did not contain any $^{99m}TcO_4^-$. On the other hand, when the PC was carried out with saline, most of the activity was observed to move toward the

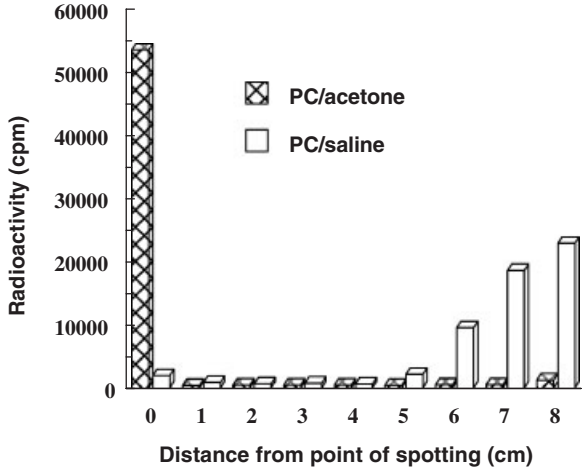

Figure 3. Paper chromatography pattern of ^{99m}Tc–isoniazid (conventional core) in acetone and saline

solvent front ($R_f = 0.8$–1). This ruled out the possibility of the presence of reduced technetium as it remains at the point of spotting under identical conditions. Characterization as well as determination of the extent of complexation of isoniazid diacetic acid with ^{99m}Tc could be obtained by a combination of the above PC methods. The paper chromatography patterns of ^{99m}Tc–isoniazid (conventional core) using acetone and saline as solvents are shown in Figure 3. HPLC analysis revealed formation of single species with a retention time of 11 min (Figure 4). Paper electrophoresis revealed the complex with negative charge. The migration rate was 5 cm/h/10 V toward anode.

Various parameters, such as, ligand concentration, stannous ion concentration, reaction time and temperature were optimized to obtain maximum complexation yield. Variation of the ligand concentration from 1 to 30 mg/ml in order to maximize the complexation yield was carried out and it was observed that an optimum of 15 mg/ml ligand is required in order to obtain a complexation yield of >95%. Hence all the subsequent optimization studies were carried out with similar ligand concentration. Saturated stannous tartrate solution in saline was used as the reducing agent and varying amount of this reducing agent was added in the reaction mixture in order to optimize the complexation yield. While 64.5% yield was obtained when 0.05 ml of saturated stannous tartrate solution was used, it increased to 94.5% when 0.2 ml of it was added in the reaction mixture. Hence, 0.2 ml saturated stannous tartrate was considered as the optimum reducing agent concentration. The reaction mixture was incubated at room temperature and extent of complexation was studied at different time intervals. Seventy-seven percent complexation was

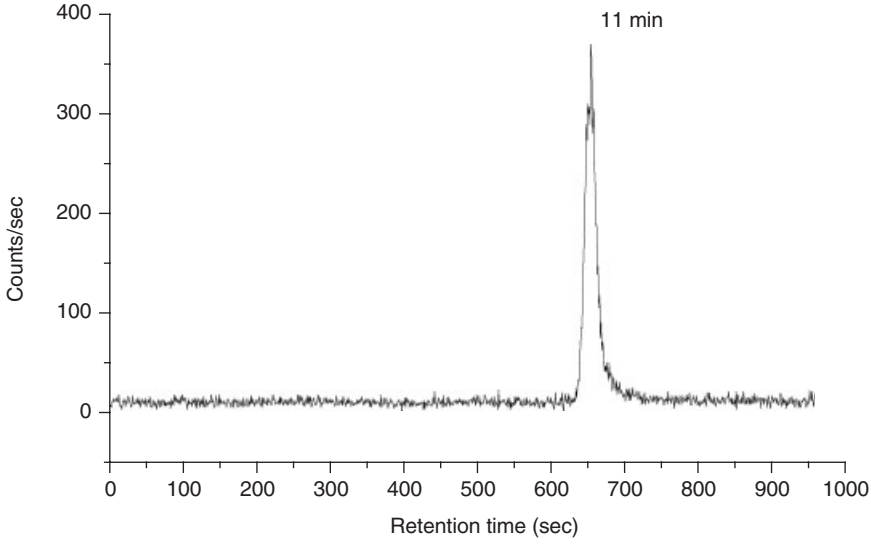

Figure 4. HPLC pattern of ^{99m}Tc–isoniazid (conventional core)

achieved within 10 min of incubation, which gradually increased to ~95% after 30 min. Incubation beyond 30 min did not show any further increase in extent of complexation.

^{99m}Tc–isoniazid (carbonyl core)

$[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor was synthesized in 95–98% yields. In HPLC this precursor had a retention time of 13.7 min. Small traces of $^{99m}TcO_4^-$ could be detected as radiochemical impurity. The precursor reacted with isoniazid diacetic acid to form ^{99m}Tc–isoniazid carbonyl core in high yields (95–98%). HPLC revealed two species with retention time of 8 min (25%) and 9 min (75%) (Figure 5).

The reaction conditions for the synthesis of ^{99m}Tc–isoniazid (carbonyl core) were optimized to get high labeling yields. Optimum concentration of isoniazid diacetic acid required for complexation was 60 μg/ml. At lower concentration, the yield decreased with unreacted carbonyl precursor as radiochemical impurity. It was also observed that it was essential to heat the reaction mixture at 70°C for 5 min to achieve complexation. The reaction progressed at RT with slower kinetics.

Formation of multiple species in case of ^{99m}Tc–isoniazid (carbonyl core) due to partial substitution of water molecules from the precursor has been reported earlier.[28] Formation of multiple species is attributed to the denticity as well as stereochemistry of the ligand used for complexation. The species can be identified with different retention time on reverse phase HPLC due to different lipophilicities. Many times non-substituted water molecule is replaced by

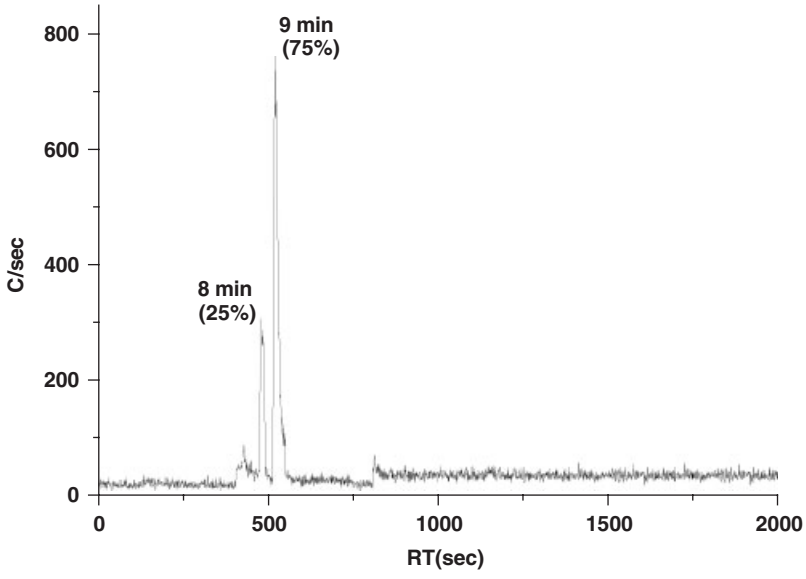

Figure 5. HPLC pattern of ^{99m}Tc–isoniazid (carbonyl core)

chloro ion depending on chloro ion concentration in the solution. This can be confirmed by carrying out stability studies of the complexes with time and studying effect of dilution as well as excess of chloride ion concentration on the multiple species formed and their interconversion.[29] In case of ^{99m}Tc–isoniazid (carbonyl core), the two species formed did not show any inter conversion either due to dilution or addition of excess chloride ion concentration, indicating formation of two species cannot be attributed to the presence of chloro and aqua species. The formation of two species could be due to the possibility of coordination of isoniazid with precursor via the pyridine nitrogen.

Optimized conditions required for the synthesis of ^{99m}Tc–isoniazid with three different cores (conventional, HYNIC and carbonyl) are given in Table 1. It was observed that ^{99m}Tc–isoniazid complexes with three different cores could be prepared in high yields. It was essential to heat the reaction mixture to 70°C for the synthesis of ^{99m}Tc–isoniazid (carbonyl core) while formation of ^{99m}Tc–isoniazid (HYNIC-like core) and ^{99m}Tc–isoniazid (conventional core) is favored at RT. Optimum reaction pH for the synthesis of the complexes with different cores also varied (acidic for carbonyl core, neutral for HYNIC and alkaline for conventional core). ^{99m}Tc–isoniazid (HYNIC-like core) and ^{99m}Tc–Isoniazid (conventional core) could be formed as single species, while ^{99m}Tc–isoniazid (carbonyl core) was formed with two species. ^{99m}Tc–Isoniazid complexes with different cores were formed with negative charge. Charge with HYNIC and conventional

Table 1. Optimized parameters for the synthesis of ^{99m}Tc–isoniazid by different chemical approaches and their stability

	Conventional approach	HYNIC approach	Carbonyl approach
Ligand Conc.	15 mg/ml	0.5 mg/ml	0.06 mg/ml
pH	~9	~7	5
Temperature	RT	RT	70°C
Time	30 min	15 min	15 min
Stability	1 h	5 h	24 h

core was determined by paper electrophoresis technique. Though paper electrophoresis was not done for ^{99m}Tc–isoniazid (carbonyl core), structure of ^{99m}Tc–IDA complexes with negative charge is reported.[27] Probable structures of ^{99m}Tc–isoniazid with three different cores are depicted in Figure 6. Specific activity and stability of the complex with carbonyl core was highest followed by that with HYNIC-like core and ^{99m}Tc (III)core. Preparation of ^{99m}Tc complexes by conventional method is carried out by reduction of pertechnetate with $SnCl_2$ and such complexes are known to be formed with poor stability. Stability of complexes prepared with HYNIC approach depends on type of the co-ligand used for complexation. In our studies the molecule selected for labeling with HYNIC approach is only a HYNIC mimic and it may be an additional factor responsible for the poor stability of the complex.

Earlier studies on derivatization of isoniazid with 2 iminothiolane (at terminal amino group of hydrazine) and complexation of derivatized isoniazid with ^{99m}Tc have reported uptake of the labeled isoniazid derivative by *M. tuberculosis*.[14] However, in our studies, complexes with isoniazid directly as well as with derivatized isoniazid (at terminal amino group of hydrazine) have not shown any retention by *M. tuberculosis*. The *M. tuberculosis* strain employed is known for its sensitivity to isoniazid and a sufficient quantity of *M. tuberculosis* cells was used for the uptake studies. The lack of observable uptake of the labeled product may be due to the nature of the labeled products used in these studies and the way they differ from normal isoniazid. Since the entry of isoniazid into *M. tuberculosis* cells is via passive diffusion, whose rate is a function of concentration difference inside and outside the cell, one would normally expect a significant proportion of the labeled product to be taken up in the cells on direct exposure. Since all the labeled products in our study have charge it may be that charged nature of the products prevents uptake by simple diffusion. Secondly there is the aspect of retention of the labeled product within the mycobacterial cell. Under normal circumstances, by action of catalase enzyme, isoniazid is converted by removal of the hydrazine moiety to isonicotinic acid, which is retained. It may be that specific modification

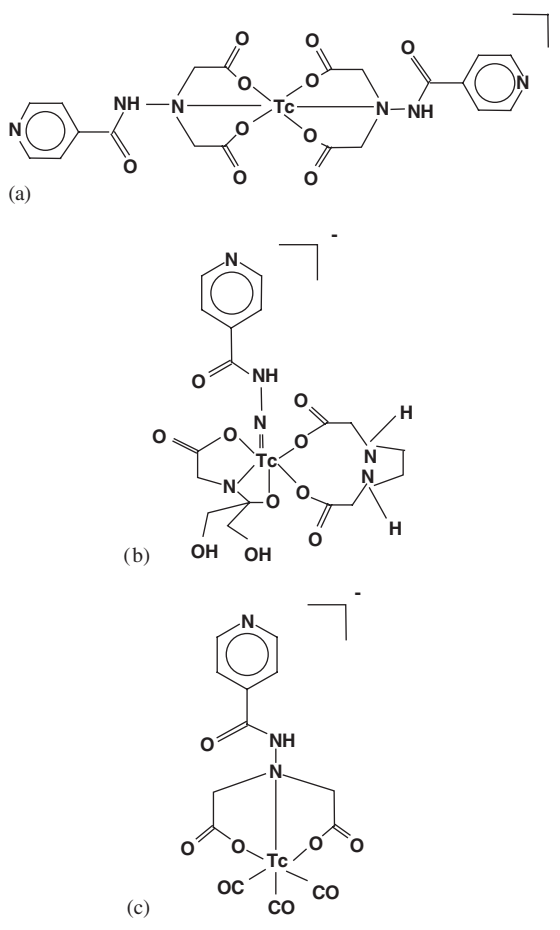

Figure 6. Probable structure of ^{99m}Tc–isoniazid with (a) conventional core, (b) HYNIC core and (c) carbonyl core

made at the hydrazine region and involvement of hydrazine group in complexation with ^{99m}Tc hinders the action of the enzyme and thus the retention of the labeled product; the product would then be leached out in subsequent washings. The other possibility is that since the cleavage would separate the radiolabeled component from isonicotinic acid, this group may be effluxed by active transport mechanisms in the cell. Studies using inhibitors of active and facilitated transport could give further illumination on this aspect. In either case, the modification performed at the hydrazino end and the nature of complex formed need to be revised.

Our observation indicates that the nature of the chelating agent used as well as the site of functionalization of isoniazid plays a critical role in obtaining mycobacterial uptake. Efforts are underway to utilize the above labeling

protocols involving the $^{99m}Tc(CO)_3$ core and the HYNIC approaches by careful alteration of co-ligands to yield neutral complexes, which ensure that the radiolabel is retained with isonicotinic acid. These could possibly form suitable diagnostic agents for tuberculosis detection.

Experimental

Isoniazid, ethylenediamine diacetic acid (EDDA), tricine, bromoacetic acid, sodium borohydride and Na/K tartrate were obtained from Aldrich Chemicals Co., USA. All chemicals and solvents were of reagent grade, which were used without further purification. Carbon monoxide in 0.5 l refillable canisters was obtained from M/s Alchemie Gases & Chemicals Pvt. Ltd., Mumbai, India. $^{99m}TcO_4^-$ was eluted from a $^{99}Mo/^{99m}Tc$ column generator using normal saline.[30]

HPLC analysis was performed on a Jasco PU 1580 system and a Jasco 1575 tunable absorption detector and a radiometric detector system. For radiochemical purity (RCP) analysis a C-18 reversed phase HiQ Sil (5 μm, 4×250 mm) column was used. FT-IR spectrum was recorded in a JASCO-FT/IR-420 spectrometer. ^{1}H-NMR spectrum was recorded in a 300 MHz Varian VXR 300S FT-NMR spectrometer.

For the biological evaluation, *M. tuberculosis* ($H37R_v$ strain, isoniazid-sensitive) was obtained from American Type Culture Collection – ATCC (Virginia, USA). Dubois medium was obtained from HiMedia laboratories (Mumbai, India), fetal bovine serum from Gibco BRL (Maryland, USA) and Tween-80 from Sigma (USA). Spectrophotometric measurements were taken on a Shimadzu UV-240 spectrophotometer (Kyoto, Japan).

Direct labeling of isoniazid with ^{99m}Tc using HYNIC approach

Synthesis of ^{99m}Tc–isoniazid (HYNIC core) was carried out by using co-ligands tricine and EDDA and reducing agent stannous chloride at concentrations reported earlier.[20] Five hundred microlitres of ethylenediamine diacetic acid (EDDA) (20 mg/ml in 0.1 N NaOH) and 500 μl of tricine (40 mg/ml in 0.2 N phosphate buffer, pH 7.2) were added to 1 mg of isoniazid. To this was added 1 ml of TcO_4^- ($\sim$3.7–37 MBq) and 15 μl of stannous chloride (2 mg/ml in 0.1 N HCl). The reaction mixture was incubated for 15 min at RT. Characterization of ^{99m}Tc–isoniazid (HYNIC core) was carried out by PC using acetone and saline as solvent systems. Characterization was also carried out by HPLC employing gradient elution technique using acetonitrile (A)/water (B) with 0.1% trifluoroacetic acid [0–1 min 80% A, 1–10 min 80–20% A, 10–15 min 20–80% A, 15–20 min 80% A]. Paper electrophoresis was carried out in 0.025 M phosphate buffer pH 7.5 to determine the charge on the complex.

Synthesis of isoniazid diacetic acid

Isoniazid was derivatized to its diacetate derivative on reaction with bromoacetic acid in a strongly alkaline medium. In a typical reaction, 1.75 g (1.3 mmol) of isoniazid and 3.90 g of bromo acetic acid (2.8 mmol) were dissolved in 10 ml of double distilled water and the reaction mixture was heated at 60–70°C after adjusting its pH to ~10 using 5 N aqueous NaOH solution. The progress of the reaction was marked with a decrease in the pH which was required to be maintained at ~10 by addition of 5 N aqueous NaOH solution. The reaction was continued for 3 h. The progress and completion of the reaction was monitored by thin-layer chromatography using 30% methanol in ethyl acetate as the solvent. After the completion of the reaction, the solvent was removed under vacuum whereby an orange–yellow product was obtained. This crude product was purified by silica gel column chromatography using 10% methanol in ethyl acetate as the eluting solvent. The scheme for the synthesis of isoniazid diacetic acid is shown in Figure 7. The derivative of isoniazid was characterized using usual spectroscopic techniques such as Fourier transform infra red (FT-IR) spectroscopy and ^{1}H-NMR spectroscopy. IR (KBr,ν/cm): 3463 (–NH), 1635 (>C=O). ^{1}H-NMR (CD_3OD, δ ppm): 3.515 (4H s, $-CH_2COOH$), 8.335–8.346 (2H d, Ar-H), 8.991–8.997 (2H d, Ar-H).

Labeling of isoniazid diacetic acid with ^{99m}Tc using conventional approach [^{99m}Tc–isoniazid (conventional core)]

Fifteen milligrams of isoniazid diacetic acid was dissolved in 0.2 ml of 0.5 M sodium bicarbonate buffer (pH 9). To the resulting solution, 0.1 ml (~3.7–37 MBq) of freshly eluted TcO_4^- and 0.2 ml of saturated stannous tartrate solution (in saline) were added. The volume of the reaction mixture

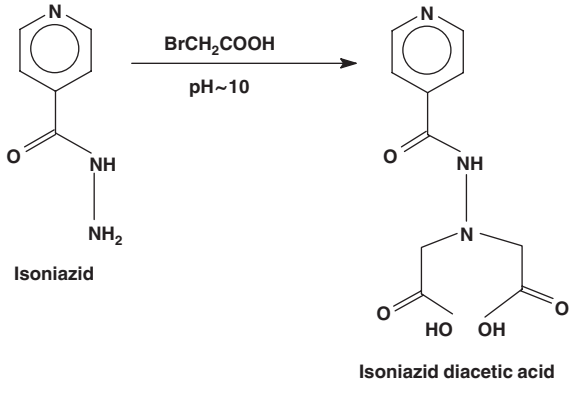

Figure 7. Scheme for the synthesis of isoniazid *N*, *N*′ diacetic acid

was made up to 1 ml by the addition of normal saline. Nitrogen gas was purged through the reaction mixture for 2 min and it was incubated at room temperature for 30 min. ^{99m}Tc–Isoniazid (conventional core) was characterized by PC using acetone and saline as the eluting solvents. The complex was further characterized by HPLC, employing gradient elution using acetonitrile (B)/water (A) with 0.1% trifluoroacetic acid [0–1 min 80% A, 1–10 min 80–20% A, 10–15 min 20–80% A, 15–20 min 80% A].

Labeling of isoniazid diacetic acid with ^{99m}Tc using carbonyl approach [^{99m}Tc–isoniazid (carbonyl core)]

The $[^{99m}Tc(OH_2)_3(CO)_3]^+$ synthon was prepared by a modified procedure reported by Alberto and co-workers.[16] Briefly $NaBH_4$ (95.5 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 introduced through the rubber stopper to equilibrate the atmospheric pressure. Carbon monoxide was purged through the solution for 5 min. After the addition of 1 ml of the generator eluate containing 37–74 MBq of $^{99m}TcO_4^-$, the needle was removed and the vial heated at 80°C for 15 min. After cooling the vial for 10 min and re-equilibration to atmospheric pressure, the reactants pH was adjusted to 5 with 1:3 mixture of 0.5 M phosphate buffer (pH 7.5): 1 M HCl. One hundred microlitres of aqueous solution of isoniazid derivative (100 μg, ~4 mmol) was added to 400 μl (12–37 MBq) of the $[^{99m}Tc(OH_2)_3(CO)_3]^+$ precursor at pH 5 and the reaction mixture was heated at 70°C for 5 min.

Formation of the precursor as well as the complex was determined by HPLC employing gradient elution using water (A) / acetonitrile (B) with 0.1% trifluoroacetic acid [0–28 min 90–10% A, 28–30 min 10% A, 30–32 min 90% A]. Recovery was determined by summing up the counts in all fractions and comparing them to the total injected activity. Paper electrophoresis was carried out in 0.025 M phosphate buffer pH 7.5 to determine the charge on the complex.

Stability

The stability of ^{99m}Tc–isoniazid with conventional core, ^{99m}Tc HYNIC core and ^{99m}Tc carbonyl core was studied for a period of 24 h. HPLC analysis was used to estimate the stability of the complex.

Cell culture and processing

M. tuberculosis ($H37R_v$) was cultured in liquid Dubois medium with 0.02% Tween-80 for surfactant action and 2% fetal bovine serum as a growth supplement. The cultures were maintained at 37°C. After sufficient growth of the cells was obtained, they were harvested by centrifugation to separate out the bulk of the media and then subjected to gentle dispersion using glass beads.

The dispersed cells were then washed twice with 0.02 M phosphate buffered saline (pH 7.5) and diluted as required in the same buffer. One hundred microlitres of cells corresponding to a spectrophotometric absorbance of 0.1 was taken in each tube for the binding studies.

Cell binding

The cells were dispensed into screw-capped reaction tubes. The respective labeled compounds were added at various concentrations (400, 200 and 100 ng/tube) in a 100 µl volume. Total reaction volume was adjusted using phosphate buffered saline to 300 µl/tube. The tubes were incubated at room temperature for various time periods (15, 30, 60, 120 and 180 min). At the end of the incubation the cells were washed twice with 1 ml phosphate buffered saline and the activity associated with the cell pellet was estimated. Counting was done in a single-channel Na(Tl)I scintillation counter with the window adjusted for ^{99m}Tc.

Conclusion

^{99m}Tc–isoniazid could be synthesized in high yields with different ^{99m}Tc cores. Specific activity and stability of ^{99m}Tc–isoniazid synthesized with carbonyl approach was the highest. A study reported earlier wherein isoniazid derivatized at the hydrazino end and labeled with ^{99m}Tc has been successfully proposed as agent for diagnosis of tuberculosis, provided impetus for the present study. However, in the present case there was no mycobacterial uptake seen with any of the complexes indicating nature of chelating agent used as well as that site of functionalization of isoniazid plays a critical role in getting mycobacterial uptake with ^{99m}Tc–isoniazid.

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