Original Article

Freeze-Dried Cold Kit for Preparation of ^{99m}Tc-Ciprofloxacin As an Infection Imaging Agent

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ABSTRACT

Introduction: Radiolabeled antibiotics are being used for the specific diagnosis of infection by exploiting their specific binding properties to the bacterial components, thereby making it possible to differentiate infection from sterile lesions. The aim of this work was to prepare and evaluate a freeze-dried kit of ciprofloxacin designed for the labeling with ^{99m}Tc.
Methods: Factors like amount of reducing agent and optimum pH were investigated to make the ciprofloxacin kit. The kit was reconstituted with ^{99m}Tc at room temperature and the radiochemical purity was evaluated by ITLC method. Stability and protein binding in human serum followed by *in vitro* binding to bacteria were assessed. Biodistribution of labeled kit in *staphylococcus aureus* infected rats muscles were studied using *ex vivo* counting and scintigraphy.
Results: Labeling yield of >90% was obtained corresponding to a specific activity of 178 GBq/mmol. The stability of radiolabeled kit in human serum was 84.2% after 1 hour post incubation. *In-vitro* studies showed 75 % of radioactivity was bound to bacteria. After injection into mice clearance from the circulation occurred mainly by biliary-renal clearance and site of infection was rapidly detected within 30 min. Target to non-target muscle ratio was 3.23 ± 0.05 % at 30 min post injection.
Conclusion: ^{99m}Tc-ciprofloxacin showed favorable radiochemical and biological characteristics which permitted detection of the infection with optimal visualization.

Keywords: ^{99m}Tc- ciprofloxacin, Infection imaging, Kit preparation.

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INTRODUCTION

A wide range of radiopharmaceuticals have been proposed to visualize infection and inflammation scintigraphically. Radiolabeled leukocytes and ^{67}Ga-citrate are the most commonly applied radiopharmaceuticals (1, 2). Various ^{99m}Tc labeled agents such as polyclonal and monoclonal antibodies (3), cytokines (4), chemo tactic peptides (5, 6), HMPAO leukocytes (7) and human defensins (8-11) have been introduced or proposed to visualize bacterial infections and sterile inflammatory processes. Also radiolabeled leucocytes can be considered as "gold standard" that can visualize a majority of infectious and inflammatory lesions but it is labor-intensive and the in vitro labeling carries risks of handling potentially contaminated blood and also requires specialized equipment, taking approximately three hours (12).

The use of radiolabeled antibiotics presents a promising approach for the precise diagnosis and detection of infectious lesions, because they specifically bind to the bacterial components, making it possible to differentiate between infectious and sterile lesions (13). Antibiotics localize in the infectious focus, where they are frequently taken up and metabolized by microorganisms. Ciprofloxacin is a first generation fluroquinolone antibiotic which is active against both gram positive and gram negative bacteria. It binds to DNA-gyrase and topoisomerase IV enzymes and thus interferes with the strand cutting and resealing function during DNA replication in bacteria (14). Solanki et al. labeled ciprofloxacin with ^{99m}Tc in 1993, supplied under the name of Infecton (15). They used formamidine sulphonic acid as the reducing agent that had to be boiled before use. Later the preparation was modified by using stannous tartarate reduction method that did not require boiling, but the pH of injectable radiopharmaceutical was quite low at 4 (13). Both these methods used two-vial kits for final preparation, whereas most of the clinically used radiopharmaceuticals in nuclear medicine imaging are single-vial kits. Besides, significant amount of colloid formation upon reconstitution with $^{99m}TcO_4^-$ has also been reported with this kit (16). Bhardwaj et al. have recently reported formulating a single vial kit for the preparation of ^{99m}Tc-ciprofloxacin which appears to have better shelf life and stability as compared to the available alternatives (17). This kit contains stannous tartarate as a reducing agent and NaCl or KCl as excipients.

Here we present data on the development of a biologically active single-vial ciprofloxacin kit and describe optimum condition for radiolabeling with ^{99m}Tc using $SnCl_2.2H_2O$ as a reducing agent and without use of excipients. Stability and protein binding in human serum followed by *in vitro* binding to bacteria and biodistribution in infected mice were assessed.

METHODS

Ciprofloxacin hydrochloride was obtained from Temad Pharmaceutical Company, Iran. Other chemicals were obtained from Merck or Fluka. Chemicals and solvents were of highest purity and analytical grade and used without further purification. ^{99m}Tc-pertechnetate was supplied by AEOI, as ^{99}Mo/^{99m}Tc generator. All radioactivity measurements were carried out using Na (Tl) scintillation counter (ORTEC Model 4001M Minibin & Power Supply). For sterility filtration, 20 µm Millex-GS filters from Millipore were used.

Preparation of kit

100 mg (15 mmol) of ciprofloxacin hydrochloride (2 mg/ml) was dissolved in 10 ml double distilled water in a vial. Then 1.25 ml (1 mg/ml) of freshly prepared $SnCl_2.2H_2O$ in nitrogen purged HCl 0.1 M

was added into the vial. The solution was filtered through a cellulose ester filter (0.22 µm). Aliquots of 1 ml were transferred to glass vials and lyophilized for 18 hours. After that, the vials were sealed under vacuum. Effect of different amounts of stannous chloride and also change of pH factor in formulation were investigated.

Labeling and radiochemical purity

Radiolabeling of the kit involves initial warming up of the vial to the room temperature followed by the addition of 370-740 MBq (10-20mCi) of freshly eluted $^{99m}TcO4^-$ in maximum 1 ml of normal saline and incubation of the vial for 20 min at room temperature. Radiochemical purity was determined by thin layer chromatography. The reaction product was spotted on silica gel ITLC-SG strips (Sigma Chemical Company, USA) (10×1.5 cm^2 sheets) and developed in acetone and a triple solvent including ethanol/ammonia/water: 2/1/5 as the mobile phases. After developing, they were cut into 1 cm pieces and counted with a NaI (Tl) detector equipped with a single channel analyzer. By using Acetone as the mobile phase, reduced ^{99m}Tc and ^{99m}Tc-ciprofloxacin remained at the point of spotting, while free $^{99m}TcO_4^-$ moved towards the solvent front. In using Ammonia/Acetone/H_2O: 2/1/5 solution as another mobile phase, ^{99m}Tc-ciprofloxacin and $^{99m}TcO_4^-$ moved to the front, where as reduced ^{99m}Tc remained at the point of spotting. For the labeled kit radiochemical purity was evaluated at two, four and six hours post labeling.

Affinity to human serum and stability

The affinity of the labeled antibiotic to human serum proteins was examined by mixing 1 ml of labeled kit with activity between 5-20 mCi and 1 ml of human serum. The mixture was incubated in 37 ºC for 24 hours and 100 µl of reaction mixture was placed on a sephadex G25-column to evaluate the complex affinity to plasma protein. After washing the column with PBS or normal saline, activity bound to serum protein was measured with a well-type gamma counter.

To test the serum stability of ^{99m}Tc-Ciprofloxacin complex, we added 1 ml of freshly prepared human serum to 100 µl of labeled antibiotic. The mixture was incubated in 37 ºC for 24 h. then the serum protein was denatured by mixing 100 µl of the solution with 100 µl absolute ethanol. After that, the mixture was centrifuged at 2000 g in 4 ºC for 10 min. Radiochemical stability was determined by taking samples of 10 µl of supernatant at different times up to 24 h of incubation that were analyzed by ITLC.

In vitro binding

Binding of ^{99m}Tc-ciprofloxacin to bacteria was assessed by the method described previously (10). Briefly, 0.1 ml ^{99m}Tc-ciprofloxacin (37MBq) was transferred to a test tube. Then, 0.9ml of 50% (v/v) 0.01M acetic acid in phosphate buffer (Na-PB, pH 7.5) containing approximately 1×10^8 colony forming units (CFU) per ml viable *S. aureus* were added. The mixture was incubated for 1h at 4ºC and thereafter the vials were centrifuged in a pre-cooled centrifuge for 5 min at 2000 g at 4 ºC. The supernatant was removed, and the radioactivity in the bacterial pellet was gently re-suspended in 1ml of Na-PB and re-centrifuged as above. The supernatant was removed and the radioactivity in the bacterial pellet was determined by gamma counter. The radioactivity related to bacteria was expressed in percent of the added ^{99m}Tc activity bounded to viable bacteria in regard to total ^{99m}Tc.

Biodistribution

Male Swiss mice, weighing 25-30 g were infected by injecting 0.1 ml of saline containing 1×10^8 CFU bacteria into right

thigh muscle. 24 h later, they were injected under ether anesthesia with 0.1 ml (74 MBq) of ^{99m}Tc-ciprofloxacin in saline via the tail vein. At 1 h after injection, accumulation of the tracer in infected area was assessed by planar scintigraphy under ether anesthesia. For ex vivo counting, mice were sacrificed after 1 h, 2 h and 24 h and organs of interest were collected, weighed and radioactivity was measured in a γ-counter.

Statistical analysis

The calculations of means and standard deviations were made on Microsoft Excel. Student's t-test was used to determine statistical significance. Differences at the 95% confidence level ($p<0.05$) were considered significant.

RESULTS

The ciprofloxacin (Figure 1) was labeled with ^{99m}Tc in high labeling yield.

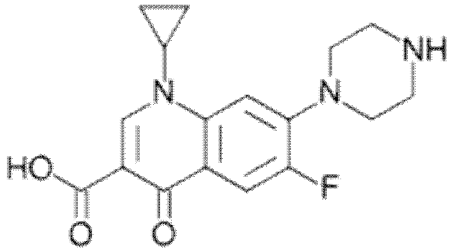

Figure 1. Chemical structure of ciprofloxacin.

The quality control with ITLC indicated that the complex purity was >90±4 % (N=12) at a specific activity of 178 GBq/mmol (Figure 2). As showed in Figure 3 the highest labeling yield was obtained by using 100 µg stannous chloride. The effect of pH on the radiolabeling yield was evaluated on pH 2.5 and 4 while for more than that the solubility decreased. The best pH to get the maximum labeling yield was 2.5 (Figure 4). The radiochemical purity of labeled kit retained more than 90% of the initial activity 6 h after preparation. The amount of ^{99m}Tc that was displaced from the labeled kit to human serum after 24 hour was 75%. The radiochemical yield of labeled kit in human serum plasma was more than 84.2 % and 79.6 % at 1 and 2 hours post incubation. Results for *in vitro* testing showed 75% binding of the added activity to bacteria.

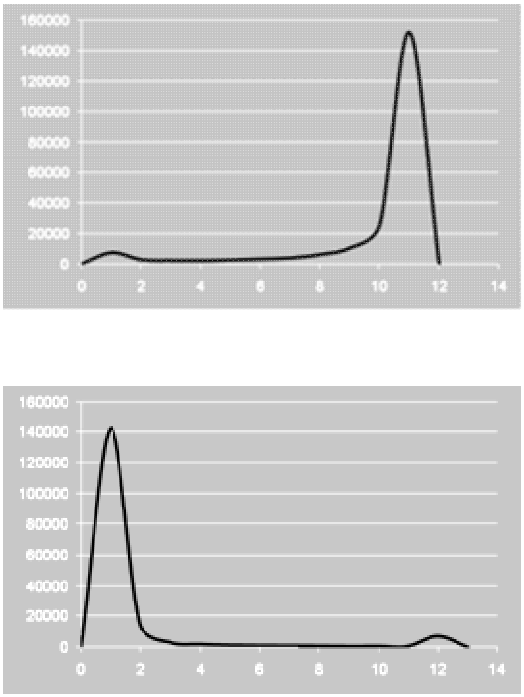

Figure 2. Radiochromatogram of ^{99m}Tc-ciprofloxacin by ITLC method in ammonia/acetone/H_2O: 2/1/5 solution (above) and acetone (below).

Table 1 shows the organ distribution of ^{99m}Tc-ciprofloxacin at 2 and 4 hours post injection. The results were expressed as the percent uptake of injected dose per gram of tissue (%ID/g) and at least in 3 mice. High uptakes were found in liver, kidneys, and blood averaging 16.1, 14.1 and 5.9% at 1 hour post injection respectively. Specific accumulation in infected thigh muscles, as indicated by T/NT ratios, was 3.2 and 1.8 at 1 and 4 h after injection.

Table 1. Biodistribution in mice (% injected dose per gram organ ± SD, n = 3)

Organ and body fluid	1 hour post injection	4 hour post injection
Blood	5.95±0.28	6.66±0.12
Heart	2.14±0.14	1.18±0.09
Lung	4.26±0.09	2.8±0.07
Stomach	1.17±0.11	1.6±0.26
Intestine	2.61±0.22	4.4±0.18
Liver	16.1±0.34	19.5±0.27
Spleen	3.39±0.19	5.25±0.09
Kidney	14.08±0.09	6.59±0.17
Non-infected muscle	1.14±0.03	1.32±0.7
Infected muscle	3.63±0.08	2.37±0.10

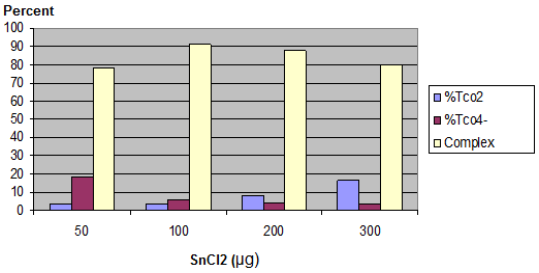

Figure 3. Effect of different amounts of reducing agent on radiochemical purity of labeled compound.

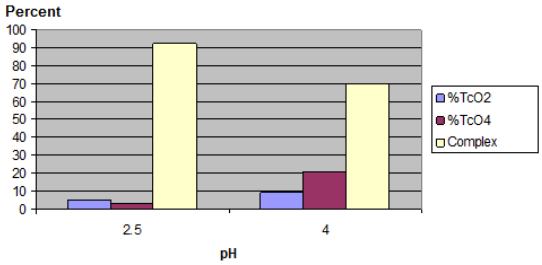

Figure 4. Effect of pH on ^{99m}Tc-ciprofloxacin radiolabeling yield.

Scintigraphic study in mice bearing the infectious lesion over tight muscle at 1 hour post injection showed high uptake of activity in infection site compared to normal site (Figure 5).

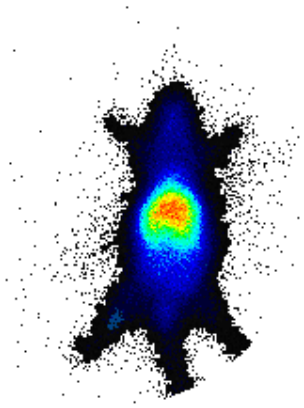

Figure 5. Typical scintigram of mice with right thigh muscle infection 60 min post injection.

DISCUSSION

Ciprofloxacin is a broad spectrum antibiotic active against most of the gram positive and gram negative bacteria. ^{99m}Tc-ciprofloxacin is a specific infection-imaging agent by virtue of its specific binding to bacterial DNA gyrase (18). ^{99m}Tc-ciprofloxacin has been in use for the past several years and it is now a well-established radiotracer, used for infection imaging. A two-vial kit,

Infecton, developed at St. Bartholomew's Hospital, London in early 1990s (15), has been used for the localization of bacterial infections. The preparation of ^{99m}Tc-ciprofloxacin using Infecton involved boiling at 100°C for 10 min and the radiopharmaceutical was found to be stable only for 8 h (19). Later Singh et al. developed a single-vial kit for the preparation of ^{99m}Tc-ciprofloxacin having high labeling efficiency, and with minimal colloid content (20). Their kit contained lyophilized stannous tartarate and ciprofloxacin but it was not stable enough to produce acceptable labeling efficiency, as the percentage of the free sodium pertechnetate started to rise within one week of preparation. The suggested cause of low shelf life was thought to be inefficiency of the reducing agent. Recently they reported development of a single vial cold kit which in order to obviate the interaction of ciprofloxacin with stannous tartarate, pellets of ciprofloxacin with NaCl and KCl were used in the preparation of kit (17). It may be noted that stannous tartarate is a reducing agent that should be freshly prepared before use. Also solubility of ciprofloxacin decrease when normal saline is used instead of distilled water.

Based on these facts, in the present study we evaluated a single vial kit for the labeling with ^{99m}Tc while stannous chloride used as a reducing agent and no excipient was used. The preparation of the ciprofloxacin kit utilized a mixture of solutions containing antibiotic ciprofloxacin (2 mg/ml) and stannous chloride as a reducing agent (100µg/ml) that was lyophilized for a period of 18 hours. The products were maintained under vacuum. We evaluated the stability of the radiopharmaceutical, its biodistribution and its infection imaging specificity. The *in vitro* stability of ^{99m}Tc-ciprofloxacin was assessed by ITLC and the radioactive contaminates were identified as R/H and free pertechnetate. The use of two solvent systems as described before was found to be an accurate method to clearly distinguish and quantify the relative amounts of free ^{99m}Tc, R/H ^{99m}Tc and ^{99m}Tc-ciprofloxacin.

The biodistribution of ^{99m}Tc-ciprofloxacin in mice shows radioactivity in the liver and kidney, thereby indicating that the major route of excretion is through hepato-biliary system and kidney. The scintigraphic studies of the ^{99m}Tc-ciprofloxacin in animal models confirmed the specificity of the radiopharmaceutical for the infectious sites. Based on the findings of this study the complex formed by reconstitution of this kit has the features of a specific infection imaging agent.

CONCLUSION

In this work ciprofloxacin cold kit was developed and evaluated. Based on the data obtained from this study, the product was stable, reproducible with high labeling efficiency with desirable characteristics making it a promising agent for imaging of infectious lesions.

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