Development of Stable Freeze Dried Kits of ^{99m}Tc-Ciprofloxacin for Infection Imaging

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Summary: Radio-labeled antibiotics are being employed for the particular diagnosis of infections utilizing their specific binding properties to the bacterial components. Stable freeze dried kits of ciprofloxacin is developed through simple stirring at room temperature and labeled with ^{99m}Tc after incubation at 35°C.Kits produced through this method showed high stability both at room temperature and in serum at 37°C. The radiochemical purity of the complex was 99.6% when 1.0 mg ciprofloxacin in the presence of 100.0 µg $SnCl_2$, 2.0 mg gentistic acid, 3.0 mg penicillamine and 20.0 mg d-mannitol was labeled by 10-20 mCi sodium pertechnetate. Serum protein binding, lipophilicity, in-vitro binding with *Pseudomonas aeruginosa, Salmonella typhi* and *Escherichia coli* as well as in-vivo biodistribution in infected rabbits were also investigated. Biodistribution results exhibited that radio ligand had good affinity in the infected site in rabbit. The uptake for *Pseudomonas aeruginosa, Salmonella typhi* and *Escherichia coli* induced infections as target to non-target ratio (T/NT) 5.425 ± 0.17, 5.397±0.15 and 4.890 ± 0.13 at 1 hr post injection respectively.

Keywords: Antibiotic; Scintigraphy; Bacterial strains; Radio-labelling.

Introduction

The initial diagnosis of the infection is one of most universal challenge in nuclear medicines and for this purpose numerous radiopharmaceuticals have been developed to resolve this problem. Leukocytes category is difficult and time taking with a potential risk of contamination and spreading of blood borne pathogens to the persons involved [1]. ^{67}Ga-citrate is one of the early radionuclides for this purpose, but it has a drawback like long physical half-life, extreme and various energy gamma rays generating tremendous radiation absorb doses, non- specific in infectious or non-infectious inflammations and at the last not being available as a generator [2]. Antimicrobial peptides, produced through epithelial cells, phagocytes, endothelial or several other cell types demonstrate antiviral, antibacterial and antifungal activities in vitro [3]. ^{99m}Tc or ^{111}In labelled leukocytes are understood as a standard in nuclear medicine for scintigraphy of infection [4]. Three phase bone scanning is remarkably sensitive tool for the difference between bone infection against tissues primarily when positive uptake is observed on all three phases but it is unfortunately not specific [5]. In numerous former studies, cationic antimicrobial peptides labeled with ^{99m}Tc to investigate bacterial and fungal infections [6-9]. Ciprofloxacin is a first generation antibiotic from fluoroquinolone family which is active against both gram negative and gram positive bacteria. It combines to DNA gyrase, topoisomerase IV enzymes, intervenes with strand opening and resealing function through DNA replication in the bacteria [10]. Labeled ciprofloxacin by ^{99m}Tc is being sold under the trade name of Infecton. This processes used two-vial kits for concluding preparation, whereas the majority of radio-pharmaceuticals utilized clinically for imaging are single-vial kits. Besides, considerable amount of colloid formation on reconstitution has also been recorded with this kit [11]. A single vial kit also formulated to prepare ^{99m}Tc-ciprofloxacin which shows better shelf life and stability in contrast to available alternatives [12]. Ciprofloxacin (Fig. 1) structure shows electron donor atoms, reduced sodium pertechnetate and can easily react with this ligand. Various complexes of ^{99m}Tc are feasible due to sulphur, nitrogen, oxygen and reduced ^{99m}Tc interactions [13]. In the present work, keeping in view the potential of ciprofloxacin against gram positive and gram negative bacteria we present the development of a biologically active single-vial ciprofloxacin kit at room temperature without HCl and also described optimum condition for its radiolabeling with ^{99m}Tc using $SnCl_2.2H_2O$ as a reducing agent and D-penicillamine as co-ligand and successfully used for localization in infection created by *Pseudomonas aeruginosa, Salmonella typhi* and *Escherichia coli*.

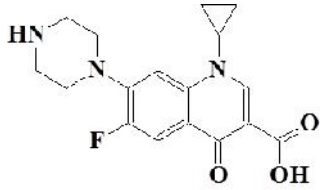

Fig. 1: Chemical structure of the ciprofloxacin.

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Experimental

Reagents

Ciprofloxacin (Java pharmaceutical, Pakistan), D-mannitol, D-penicillamine, Octanol, Stannous chloride, Gentistic acid (Sigma Aldrich, USA), Sodium pyrophosphate, Ascorbic acid, Acetone (Riedel, Germany), Saline (Otsuka, Pakistan), Tricholoacetic acid (Fisher Scientific, UK). All the reagents of analytical grade used as such. Technetium-99m as sodium pertechnetate (^{99m}NaTcO4) was prepared in house from ^{99}Mo/^{99m}Tc generator using 0.9% saline.

Instrumentation

Monitoring of all complexes was made by paper chromatography (PC) (Agilent, USA), instant thin layer chromatography (ITLC) (Agilent, USA) and RP-HPLC (Shimadzu, Japan). Radioactivity measurements were performed using Na(Tl) scintillation counter (ORTEC Model 4001 M Minibin, USA), Dose Calibrator, (CAPINTEC-INC by USA used to measure the amount of radioactivity of a particular radionuclide, pH meter (UTECH, Germany), SPECT dual head gamma camera (Infinia GE, USA) employed to take images of rabbits. Laminar flow hood (Technico Scientific, Lahore) employed to prepare the kits in sterile conditions. Autoclave (Astell, UK): was employed to make apparatus sterile. Magnetic Stirrer (J.P Selecta, Spain) employed to stir the solution. Water bath (Vision Scientific, Pakistan): was employed to sustain temperature of different samples at diverse temperatures. UV/Visible spectrophotometer (Shimadzu UV-2450) employed to measure bacterial strains optical density.

Ciprofloxacin Kit Formulation

Each of ciprofloxacin kit contained following constituents: 1.0 mg ciprofloxacin, 2.0 mg gentistic acid, 3.0 mg L-Penicillamine, 20.0 mg D-mannitol dissolved in 1.0 ml distilled water with100 µg stannous chloride at pH 4. The solution was then filtered via 0.22 µm filter. After the lyophilization, freeze dried kits were stored at 4°C. The whole process was conducted under sterile conditions of class A.

Radiolabeling

For radiolabeling of kits, sodium pertechnetate (10-20 mCi) was drawn from Tc - generator, freshly eluted was added to the vials containing cold kit. After the incubation period of 15-20 min, saline (0.9% NaCl) was added and QC was performed to check the labelling efficiency.

Radiochemical Analysis

Radiochemical purity of the ^{99m}Tc-ciprofloxacin was analyzed by ITLC, PC and RP-HPLC. To determine the amount of free pertechnetate in the kit, acetone was used as the mobile phase on a Whatmann No.3 paper. To calculate the reduced activity, ITLC as stationary phase and saline was used as mobile phase. For radiochemical analysis of ^{99m}Tc-ciprofloxacin by HPLC a volume of 10 µL of the test solutions was injected. Analytical separations were performed on Nucleosil C-18 column (100 Aº, 5µm, 250×4 mm) which was eluted gradient with a flow rate of 0.5 mL/min using 0.1% TFA in H_2O (solvent A) and acetonitrile (solvent B).The gradient program is summarized in Table-1. The ^{99m}Tc-complexes were detected by radio-HPLC. The radioactive detector with a NaI (Tl) scintillation detector separated by a Teflon tube from UV detector set about a 0.4-0.7 min delay. The eluent gradients and columns were kept same as described above for RP-HPLC.

Table–1:Gradient Program.

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
5	95	5
25	0	100
30	0	100

Stability at Room Temperature and in Serum at 37ºC

In-vitro degradation of ^{99m}Tc-ciprofloxacin complex was determined both at room temperature and in human blood serum. In both radio-labeled compounds was added and incubated for 6 hrs at room temperature /freshly prepared serum from a healthy donor blood at 37ºC respectively. Samples were analyzed after every hour with PC and ITLC.

Partition Coefficient

The partition coefficient of the complex was analyzed after 15-20 min by vigorous vortex mixing of 2.0 ml of octanol, 2.0 mL saline and 1.0 mL of the radiolabelled. The counts in 100 µL of both organic and inorganic layers were calculated. The reported octanol/saline partition coefficient (Log P) represents the mean ± standard deviation (SD) of the three measurements.

Protein Binding

In fresh human blood (5mL) radiolabeled ciprofloxacin kit was added and incubated for 1 hr at

25˚C. This blood was centrifuged at 3000 rpm after 10 min incubation at 37˚C. Equal volume of 10 % trichloro acetic acid (TCA) was added in serum with strong shaking. Serum was centrifuged at 3000 rpm (10 min). Both supernatant and residue were counted for radioactivity.

Binding of ^{99m}Tc-ciprofloxacin to different strains of bacteria

^{99m}Tc-ciprofloxacin binding was studied against *Pseudomonas aeruginosa*, *Salmonella typhi and Escherichia coli* using the cylinder plate method of the microbiological assay (utilized for potency determination of the antibiotics) [14].

Bio distribution study of ^{99m}Tc-ciprofloxacin normal and infected rabbits

Biodistribution in normal rabbit was studied after anesthesia of diazepam (2.0 mg/mL). ^{99m}Tc-ciprofloxacin (1 mL) was injected in iliac vein of the rabbit. Scintigraphy study was performed after 15 min, 30 min and 1 – 4 hrs post-injection. For the bio-distribution study of complex, 1mL (3×10^8 cfu/mL) suspension of *Pseudomonas aeruginosa, Salmonella typhi* and *Escherichia* coli were injected in thigh muscle of the rabbits. After the start of infection and the swelling at infected site, ^{99m}Tc-ciprofloxacin (1mL =2.5mCi) was injected in the iliac vein of all the rabbits separately after diazepam anesthesia (1mL). Scintigraphy images were taken at (1-4 hrs) and 24 hrs post injection with Gamma Camera.

Results and Discussion

Labelling of ciprofloxacin with ^{99m}Tc was optimized at pH 4 by using 1.0 mg ciprofloxacin as a bidentate ligand, 100 µg stannous chloride dihydrate as reducing agent with 10-20 mCi sodium pertechnetate as a radio-metal. The effect of reducing agent on labelling efficiency is presented in Fig. 2. Labelling efficiency was lower at 25 µg and reaches at its maximum when amount of reducing agent was 100 µg and labeling efficiency again decreased above this concentration. Maximum labelling efficiency is obtained when 1.0 mg ciprofloxacin at pH 4 was used. The results are summarized in Table-2. In radiochemical purity assessment of ^{99m}Tc-ciprofloxacin by ITLC, only insignificant activity remained in origin which corresponds to the reduced ^{99m}Tc. In PC main part of the activity stayed at the origin and less than 2-3% of total activity was travelled which is belonged to free ^{99m}TcO4. ^{99m}Tc-ligand HPLC studies demonstrated that the reaction lead to a single complex and its retention time was found to be 10.95 min with the radioactive detector and has a yield of > 95%. The chromatogram is presented in Fig. 3. No major degradation in ^{99m}Tc-ciprofloxacin was seen and complex stability was > 99% (Table-3) over the observed time period of 6 hrs both at room temperature and in blood serum at 37ºC. The serum protein binding of labelled complex was 55% while 45% remained unbound. So far the major drawback in labelling of ciprofloxacin is the colloid impurity and the instability which were the subject of discussion in earlier studies by the different groups [15, 16] but in present study with the optimization it is controlled. The partition coefficient (log P = -1.69) of the ^{99m}Tc-ciprofloxacin signifying its low lipophilicity which could explain that there is no noteworthy accumulation of the radio-ligand in the liver and its rapid wash out from it. In vitro binding efficiency of ^{99m}Tc-ciprofloxacinto *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli* was good. Scintigrams in Fig. 4 and biodistribution of the complex showed that maximum uptake is at 1 hr post injection and clearance from blood circulation was pretty swift after it and suggests that image taken at 1 hr will be the best for visualization in the case of all bacterial strains. Because of large activity in kidney and bladder suggest the urinary systems being the main route of clearance of administered dose. From scintigrams it is clear that no uptake in liver is seen which suggests its stability and low colloidal formation. The percentage injection dose (ID) showed three fold decreases after 4 hrs and it is almost same for *Pseudomonas aeruginosa and Salmonella typhi* although its value is low in *Escherichia coli.* The radioactivity concentration at infected muscle at 1 - 4 hrs post injection is summarized in Table-4. The ratio of activity in an infected muscle to non-infected muscle was more than two-fold (T/NT) 4.890 ± 0.17, 5.397 ± 0.15 and 5.424 ± 0.22 at 1hr post injection and1.930 ± 0.21, 2.064 ±0.22, 2.064±0.23 at 4 hrs post injection for *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* respectively. High retention at infection site indicates ^{99m}Tc-ciprofloxacin has specific affinity to all above mentioned bacterial strains even though it is higher for *Salmonella typhi* and *Pseudomonas aeruginosa* than *Escherichia coli*. The uptake in all organs was reduced drastically after 1 hr demonstrating that removal is time dependent and early images obtained up to 1 hr is best for detection of infection.

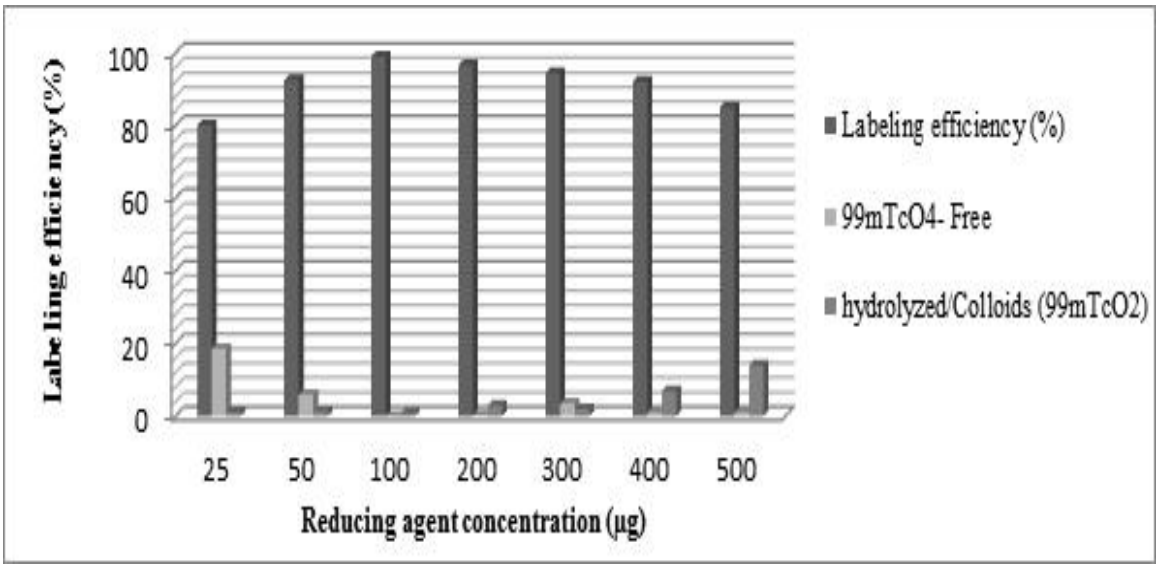

Fig. 2: Effect of concentration of stannous chloride on labelling efficiency of ^{99m}Tc-ciprofloxacin.

Table-2: Effect of ciprofloxacin concentration and pH on labelling efficiency.

	Concentration of ciprofloxacin (mg)					pH				
	0.5	1.0	2.0	2.5	3.0	2	3	4	5	6
Labelling efficiency (%)	83.7	99.5	99.6	97.5	97.4	89.5	95.2	99.7	97.5	97.4
Free $^{99m}TcO_4^-$	14.3	0.2	0.2	1.0	1.4	9.5	4.2	0.2	1.0	1.4
Colloids/hydrolyzed ($^{99m}TcO_2$)	2.0	0.3	0.2	1.5	1.2	1.0	0.6	0.1	1.5	1.2

Table – 3: In vitro stability of ^{99m}Tc-ciprofloxacin complex at room temperature and 37˚C

	Room Temperature							37˚C						
	Time (hrs)							Time (hrs)						
	1	2	3	4	5	6	24	1	2	3	4	5	6	24
Labelling efficiency (%)	99.73	99.66	99.61	99.58	99.53	99.50	97.29	99.61	99.56	99.55	99.49	99.31	99.23	97.13
Free $^{99m}TcO_4^-$	0.20	0.17	0.20	0.20	0.27	0.30	1.71	0.20	0.22	0.25	0.26	0.39	0.40	1.77
Colloids/hydrolyzed ($^{99m}TcO_2$)	0.07	0.17	0.19	0.22	0.20	0.20	1.00	0.19	0.20	0.20	0.25	0.30	0.37	1.10

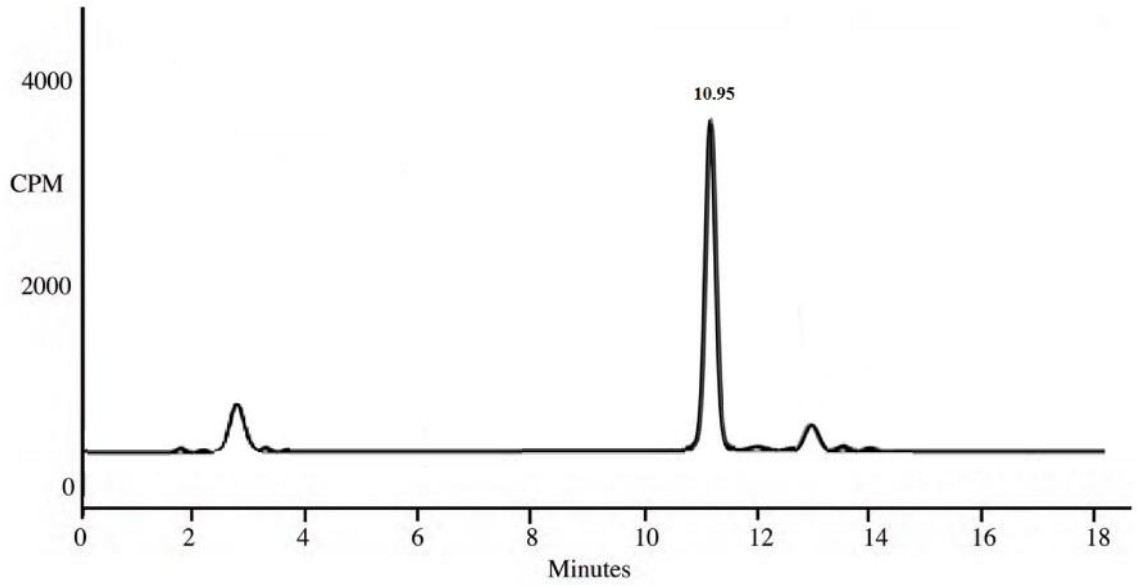

Fig. 3: Chromatogram.

Table–4:Biodistribution of ^{99m}Tc-ciprofloxacin in E. coli, *Ps. aeruginosa and Salmonella typhi* (% ID/g ± SD, n=3)

Bacterial strain	Time (hrs)	Organ					
		Thigh (T)	Thigh (NT)	Kidneys (RT)	Kidneys (LT)	Liver	Urinary bladder
Escherichia coli	1	0.93±0.21	0.19±0.22	1.80±2.49	1.08±2.20	1.39±1.90	37.22±4.29
	2	0.81±0.19	0.32±0.21	1.17±2.59	0.92±2.00	0.50±1.80	80.31±4.10
	3	0.43±0.20	0.20±0.19	0.50±2.39	0.59±1.87	0.27±1.70	85.79±3.20
	4	0.36±0.17	0.18±0.17	0.55±2.48	0.64±1.59	0.14±1.56	97.63±2.90
Pseudomonas aeruginosa	1	1.55±0.21	0.52±0.18	0.82±0.45	0.79±0.43	0.91±1.1	51.89±1.2
	2	1.12±0.17	0.77±0.20	2.00±0.46	0.61±0.24	7.78±1.01	72.40±1.3
	3	0.76±0.14	0.54±0.15	0.96±0.44	0.74±0.34	5.07±1.2	81.39±1.1
	4	0.56±0.09	0.41±0.17	1.03±0.49	0.76±0.33	1.98±1.09	73.00±1.08
Salmonella typhi	1	1.55±0.01	0.28±0.03	0.82±0.32	0.79±0.31	0.82±0.22	51.89±1.2
	2	1.12±0.02	0.47±0.08	2.00±0.23	0.61±0.22	7.78±0.21	72.40±1.1
	3	0.76±0.05	0.36±0.03	0.96±0.22	0.74±0.23	5.07±0.19	81.39±1.09
	4	0.56±0.04	0.27±0.08	1.03±0.24	0.76±0.25	1.98±0.11	73.00±1.08

*T = target, NT = non-target, RT = right, LT = left

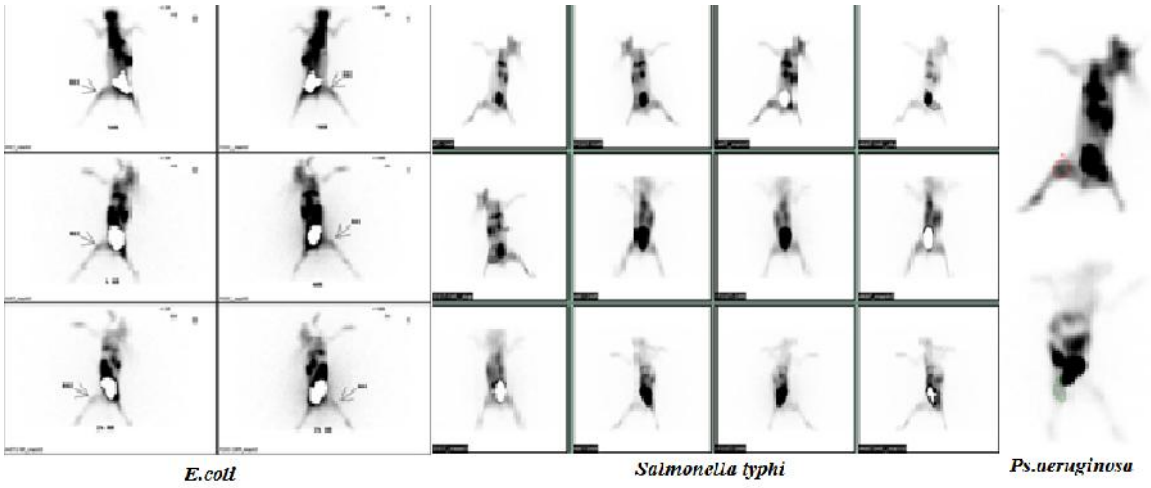

Fig. 4: Scintigrams of biodistribution of ^{99m}Tc-ciprofloxacin

Conclusion

A new method for the preparation of an infection imaging agent ^{99m}Tc-ciprofloxacin is developed with highest labelling efficiency, stability, long shelf life and attractive characteristics making it a guaranteed agent for imaging of infectious lesions. Biodistribution results showed high uptake at infection site with small amount of colloidal formation which was a great issue in previous studies with the labelling of ciprofloxacin kits.This complex may guide to further encouragement of a radiotracer for the imaging of infections induced through gram positive and gram-negative bacteria.

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