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In-vitro binding assay study of ^{99m}Tc-flouroquinolones with *E. coli*, *Salmonella* and *Ps. aeruginosa*

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Abstract A simple methodology was developed to evaluate binding efficiency of antibiotic members of fluoroquinolones, namely ciprofloxacin, ofloxacin and enorfloxacin, complexed with ^{99m}Tc, against *Escherichia coli*, *Salmonella* and *Pseudomonas aeruginosa* bacterial strains. Radioactivity in the pellet, tips supernatant and micro-centrifugation tubes was counted separately for 5 s in a sodium iodide well-counter with dedicated nuclear medicine software. The overall percentage activity of the live and killed bacteria was found in the range of 5–46% with different types of labelled quinolones and bacteria. Activity of the labelled enorfloxacin and ciprofloxacin indicated acceptable results for both live and killed *E. coli* and *Ps. aeruginosa*. However, ofloxacin was found to be moderate for all the live bacterial stains. This developed methodology has achieved more than 95% labelling efficiency of ^{99m}Tc with derivatized quinolones and also the observed results indicated that these complexes may be used as an infection specific imaging agents.

1. Introduction

Scintigraphic imaging of infection and inflammation is a powerful diagnostic tool in the management of patient with infectious diseases. Most infections and inflammatory foci may be seen accurately with radio labelled autologous leukocytes. The preparation of these radiopharmaceuticals is laborious and requires the handling of contaminated blood.

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Few radiopharmaceuticals are available that could be used instead of radio-labelled leukocytes to scintigraphically visualize infections and inflammation foci, such as ^{99m}Tc labelled antigranulocyte antibody and ^{67}Ga-citrate.[1] Various agents labelled with ^{99m}Tc have been developed for this application. Most of these newly developed agents are those that bind receptors on white blood cells subpopulations, i.e. monoclonal antibodies, chemotactic peptides and cytolines.[1] The ideal radiopharmaceutical for imaging infection/inflammation should be efficiently accumulated and has good retention in inflammation foci. Moreover, it should have rapid clearance from the background with no accumulation in non-inflamed tissues, no side effects, low cost (^{99m}Tc), easy preparation (kit formation), and should have the ability to discriminate infection from non-microbial inflammation cells.

^{67}Ga-citrate has been widely used for imaging infection and inflammation ever since its discovery in 1971. On intravenous injection, ^{67}Ga-citrate binds to transferring and this complex extravagates at the site of inflammation because of the locally enhanced vascular permeability.[2–4] This use of radio-labelled autologous leukocytes for imaging infection and inflammation has been a major breakthrough in radio-labelled imaging because they rapidly clear from the blood and migrate actively from the circulation into the infected tissues.[5–7]Also the use of radio-labelled monoclonal antibodies against surface antigens of granulocytes was one of the first attempts to accomplish *in-vivo* labelling of leukocytes.

Now several monoclonal antibodies reactive with antigens expressed on granulocytes have been developed,[1] and each of these anti-granulocyte antibodies with ^{99m}Tc allowed the accurate delineation of infection and inflammation. Rubin et al.[8] used radio-iodinated IgG as a control in an experiment to image *Pseudomonas aeruginosa* infection with a radio-labelled monoclonal antibody against a surface epitope of the *Pseudomonas* bacterium and observed that the control antibody visualized the good infectious focus as the anti-Pseudomonas antibody did. They proposed the use of non-specific IgG as an infection and inflammation imaging agent. The literature[1,5–9] indicated that different radio-labelled compounds like ^{67}Ga-Citrate, radio-labelled leukocytes (white blood cells-WBCs), labelled antigranulocyte antibodies, radio-labelled non-specific human IgG, anti-e-selection antibodies, radio-labelled liposomes, bacterial chemotactic peptides, Interleukin-1, 2 and 8, Tuftsin, platelet factor-4, and antimicrobial peptides have been investigated for imaging infections and inflammations.

None of the agents discussed above can discriminate between infection and inflammation, because of their binding to the DNA gyrase enzyme present in all dividing bacteria. Fluoroquinolones does not accumulate in dead bacteria and non-inflammatory sites. Ciprofloxacin is a fluoroquinolone antimicrobial agent that binds with the gyrase enzyme, even to those resistant to ciprofloxacin. It is stated[9] that ^{99m}Tc-labelled ciprofloxacin can distinguish between infection and sterile inflammation. Another study[10] indicated that it also shows high accuracy in the detection of bacterial infection. Preliminary studies with sparfloxacin, norfloxacin, pefloxacin, and lomefloxacin derivatives have shown significant *in-vitro* uptake in bacteria as well as accumulation in sterile inflammatory sites. Critical evaluation of these investigated compounds[11–15] was warranted to ultimately prove their value for specific imaging of infections.

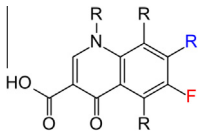

Figure 1 Essential structure of all quinolone antibiotics: the blue drawn remainder of R is usually piperazine; if the connection contains fluorine (red), it is a fluoroquinolone.

Quinolones are a family of synthetic broad spectrum antibacterial drugs. The essential structure of all quinolone antibiotics is given in Fig. 1. Fluoroquinolones are antibiotics that are commonly used to treat a variety of illnesses such as respiratory and urinary tract infections. These medicines include ciprofloxacin (Cipro), gemifloxacin (Factive), levofloxacin (Levaquin), moxifloxacin (Avelox), norfloxacin (Noroxin), and ofloxacin (Floxin). In the present work, ^{99m}Tc labelling has been carried out with derivatized ciprofloxacin, ofloxacin and enorfloxacin to investigate their accumulation in both live and dead *Escherichia coli, Salmonella* and *Ps. aeruginosa* bacterial strains.

2. Material and methods

All reagents and solvents used in this study were of reagent grade purity and were used without further purification. All apparatus used were freshly autoclaved and were free from any micro-organisms. Experiments were performed in triplicate and the results reported were the average of triplicates. The isolated bacterial stains, obtained from the department of zoology, University of the Punjab, were identified as *E. coli*, *Salmonella* and *Ps. aeruginosa* bacterial strains, and were incubated in different flask mediums by using the standard conditions of laminar flow chamber.

The bacterial cultures were poured into falcon tubes and centrifuged at 16,000 rpm in freezing conditions for 10 min to convert them into supernatant and pellets. The pellets were dissolved in autoclaved solvent solution. The suspensions were checked for their optical density and diluted enough to give the solvents of 0.14 optical densities which referred to approximately 1.00×10^8 cfu/mL of each stain. The bacteria were not multiplying during the study. Half of each aliquot was taken into a separate sterile tube and was heated at 70 °C for 40 min in a water bath. 1.00 mL sample from all killed suspensions of bacterial stains was placed in sterile glass tubes to confirm their sterility and cell morphology. No liability was found in any of these controls and the morphology was similar to that of the original stains from fresh culture.

Each sample was evaluated after 30 min, 1.0 h and 4.0 h of incubation interval with ^{99m}Tc-fluoroquinolones or ^{99m}Tc$^-$ (blank) at room temperature. Experiments for each labelled fluoroquinolones were conducted on three separate days, so that 12 samples from each group were evaluated. The ^{99m}Tc-fluoroquinolone was constituted on experimental days.[16]

2.1. Pertechnetate kit formation

Each of ciprofloxacin, ofloxacin and enorfloxacin kit contained the following constituents: 3.00 mg of fluoroquinolones, 3.00 mg of cysteine monohydrochloride, 2.00 mg of ascorbic

acid, 20.00 mg of NaCl dissolved in 800 µL distilled water, mixed on stirring and followed by addition of 200.00 µg of $SnCl_2{\cdot}2H_2O$ from a solution containing 100.00 mg stannous tartrate in 0.4 mL conc. HCl by gentle heating and diluted up to 10 mL with distilled water. pH was adjusted to 4.5 by using 0.1 N HCl/0.1 N NaOH solutions. Stability of pH value was monitored for 15 min during the stirring process. After lyophilization, the freeze dried kits were stored at 4 °C.

2.2. Radio-labelling of kit

For radio-labelling of the freeze dried kits, sodium per technetate (300 MBQ) was drawn up from a freshly eluted in-house developed 'Sterile PAKGEN $^{99}Mo/^{99m}Tc$ generator'[16] (PINSTECH, Islamabad-Pakistan), and put into the vials containing ciprofloxacin, ofloxacin and enorfloxacin kits by using insulin syringes. The vials were shaken for 30 s, incubated at room temperature for 10–15 min, and volume was brought to 1.00 mL by using saline solution (0.9% NaCl). Quality control of the radio-labelled kits was performed with the help of paper chromatography in acetone and ITLC (instant thin layer chromatography). Reaction mechanisms of ^{99m}Tc with studied antibiotics are reported in Figs. 2–4. The labelling efficiency of fluoroquinolones with ^{99m}Tc is reported in Table 1.

2.3. Radiopharmaceutical incubation and measurement

Culture reviving and heat killing of bacterial stains are repeated as follows; 1.00 mL of each sample was withdrawn in a sterile glass pipette and placed in a polypropylene micro-centrifugation tube (VWR international). 50 µL ^{99m}Tc-fluoroquinolones along with 50 µL ^{99m}Tc (blank) were placed in each sample of live or killed bacterial stains. At the spent of time, each sample tube was spun on 14,000 rpm (Eppendorf Minispin Plus Microcentrifuge) at 4 °C for 5 min. The supernatant was transferred via micro pipette (Redi-IPS Fisher brand) to a new sterile glass tube. The pellet was re-suspended with 1.00 mL sterile saline solution and centrifuged. The re-suspended supernatant was added to initial supernatant. This pellet was then transferred via pipette into a new sterile glass test tube. This pipette tip was saved, as the bacteria within it could not be completely evacuated. Radioactivity in the pipette

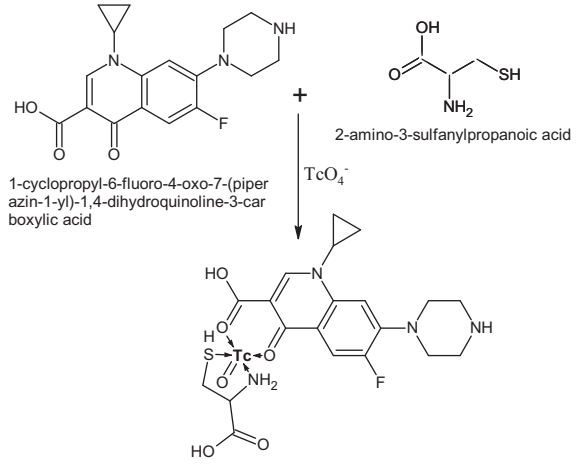

Figure 2 Complex formation of ^{99m}Tc-ciprofloxacin.

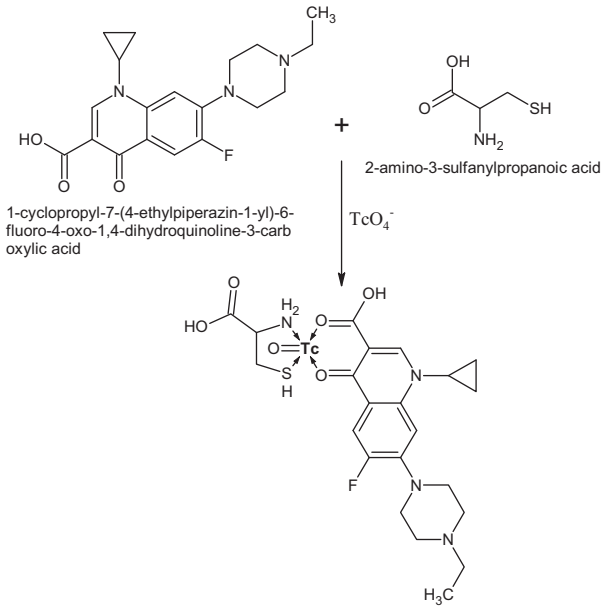

Figure 3 Complex formation of ^{99m}Tc-enorfloxacin.

tip was considered to be the part of bacterial pellet. Transfer of the pellet to a new glass test tube was necessary due to some binding of ^{99m}Tc-quinolones to the micro-centrifugation tube, which was then preserved and considered the part of supernatant after being washed with 1.00 mL of sterile saline solution.

The radioactivity in the pellet, tips supernatant and micro-centrifugation tubes was counted separately for 5 s in a sodium iodide well counter equipped with dedicated nuclear medicine software. The percentage of total activity in the pellet was calculated as follows:

$$\% \text{ total activity} = \frac{\text{Pellet activity}}{\text{Pellet activity} + \text{Supernatantactivity}} \times 100 \quad (1)$$

The results thus obtained for each labelled antibiotics for live and killed microbes and for blank solution after 30 min, 1 h and 4 h are reported in Table 2.

3. Results and discussion

The results obtained are discussed under the following headings.

3.1. Labelling efficiency of fluoroquinolones with ^{99m}Tc

In this study, three fluoroquinolones were labelled with ^{99m}Tc and their activity was checked against three bacterial stains. The radiochemical purity was checked with the help of paper chromatography in acetone and instant thin layer chromatography (ITLC) in saline. For all the radio labelled antibiotics, the labelling efficiency was more than 95%, which shows promising chemical purity for the present study. Labelling efficiency of three antibiotics (Table 1) indicates that minute amount of free TcO_4^{-1} (pertechnetate) and prominent amount of colloid formation results during complex formation. All the three quinolones showed similar behaviour for both paper and

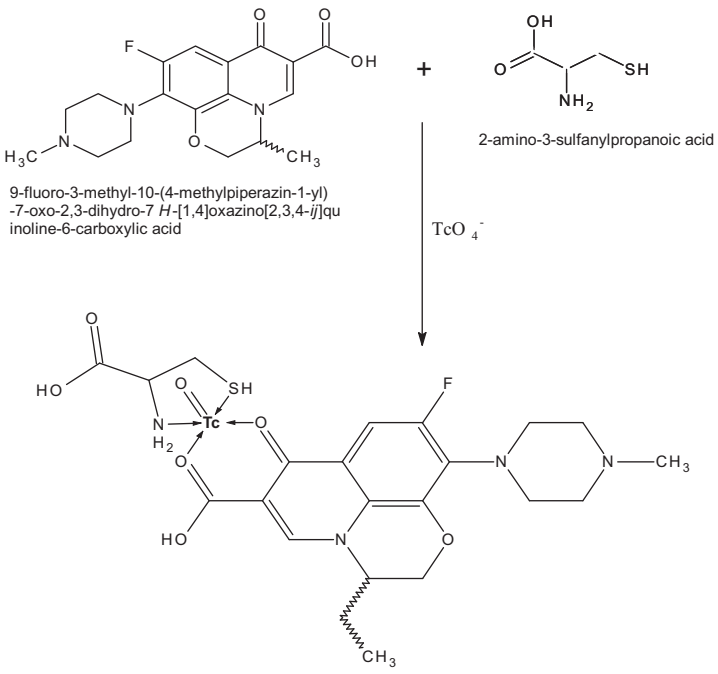

Figure 4 Complex formation of ^{99m}Tc-ofloxacin.

Table 1 Labelling efficiency of ^{99m}Tc with fluoroquinolones.

Sr. no	Antibiotic	Labelling efficiencies (%)	Colloid	Free TcO_4^{-1}
1	Ciprofloxacin	>96	2.27 ± 0.12	0.80 ± 0.03
2	Enorfloxacin	>95	3.45 ± 0.10	1.50 ± 0.02
3	Ofloxacin	>97	2.05 ± 0.09	0.95 ± 0.02

ITLC. The colloidal remains at the origin, while free TcO_4^{-1} moved along the solvent front. The labelling of the fluoroquinolones was determined by the formula:

$$\% \text{ of labelled fluoroquinolones} = 100 - (\% \text{ activity of colloids} + \% \text{ activity of } TcO_4^{-1}) \quad (2)$$

3.2. Binding assay of labelled fluoroquinolones with bacteria

The stains of bacteria used in this research were commonly infection causing pathogens. The overnight culture of bacteria's was incubated and approximately, 1.0×10^8 cfu/mL was conformed by optical density. Table 2 shows negligible binding efficiency of free ^{99m}Tc with bacterial stains at three different intervals, as compared to binding efficiencies of ^{99m}Tc-flouroquinolones.

3.3. Activity of ^{99m}Tc-ciprofloxacin in live and killed bacteria

^{99m}Tc-ciprofloxacin showed an un-expected behaviour of accumulation in both live and killed bacteria. The activity of ^{99m}Tc-ciprofloxacin in live *E. coli* after 30 min, 1.0 h and 4.0 h was 37, 27 and 34%, respectively, with an average of 33%, while in killed *E. coli* its activity was 46, 39 and 40% with an average of 42%, which is about 10% more than that of live *E. coli*. The activity of ^{99m}Tc-ciprofloxacin in live *Salmonella* after 30 min, 1.0 h and 4.0 h was 22, 34 and 29%, respectively, with an average of 28%. In killed salmonella, its activity was 43, 37 and 47%, respectively, with an average of 42%, it is also 14% more than live *Salmonella*. The activity of ^{99m}Tc-ciprofloxacin in *Ps. aeruginosa* after 30 min, 1.0 h and 4.0 h was 46, 42 and 37%, respectively, with an average of 42%, while in killed *Ps. aeruginosa*, its activity was 37, 39 and 37%, respectively, with an average of 38%, which is 4% less than the live *Ps. aeruginosa*. We may say that ^{99m}Tc-ciprofloxacin binds with both live and killed bacteria under the study. Our finding contradicts with the other workers[9,10], who state that fluoroquinolones are thought not to bind to dead as well as in non-microbial inflammatory processes. The behaviour of ^{99m}Tc-ciprofloxacin indicates that it not only participates in binding with gyrase but also with other parts of the pathogens as well.

3.4. Activity of ^{99m}Tc-ofloxacin in live and killed bacteria

The behaviour of ^{99m}Tc-ofloxacin was different from that of ^{99m}Tc-ciprofloxacin. The activity of ^{99m}Tc-ofloxacin in live *E. coli*, *Salmonella* and *Ps. aeruginosa* was higher than those of killed ones. The activity of ^{99m}Tc-ofloxacin in live *E. coli* after 30 min, 1.0 h and 4.0 h was 34, 41 and 41%, respectively with an average of 39%, while in killed *E. coli* its activity was 5, 19 and 13% with an average of 9%, which is 30% less than that of live *E. coli*. Similarly the activity of ^{99m}Tc-ofloxacin in live *Salmonella* was 46, 49 and 46% after 30 min, 1.0 h and 4.0 h respectively, with an average of 47%. However, its activity in killed *Salmonella* was 6, 10, and 17% with an average of 11%. This value (36%) is less than live cell activity. The activity of ^{99m}Tc-ofloxacin in *Ps. aeruginosa* live cells was 10, 31 and 46% with an average of 29% after 30 min, 1.0 h and 4.0 h respectively. Its activity in dead cells was 8, 31 and 46% with an average of 28%. This figure is also less than 1% to that

Table 2 Activity of free $^{99m}Tc^{-1}$ and labelled fluoroquinolones on live and killed bacteria after different time intervals.

Labelled compound	Live or killed stains	Bacterial species	Activity (%) after 30 min	Activity (%) after 1.0 h	Activity (%) after 4.0 h	Average activity (%)
Free $^{99m}Tc^{-1}$	Live stains	*E. coli*	0.11	0.02	0.05	0.06
		Salmonella	0.11	0.12	0.04	0.09
		Ps. aeruginosa	0.02	0.01	0.04	0.02
	Killed stains	*E. coli*	0.00	0.19	0.07	0.09
		Salmonella	0.00	0.12	0.06	0.06
		Ps. aeruginosa	0.05	0.12	0.11	0.09
^{99m}Tc-ciprofloxacin	Live stains	*E. coli*	37	27	34	32.67
		Salmonella	22	34	29	28.33
		Ps. aeruginosa	46	42	37	41.67
	Killed stains	*E. coli*	46	39	40	41.67
		Salmonella	43	37	47	42.33
		Ps. aeruginosa	37	39	37	37.67
^{99m}Tc-ofloxacin	Live stains	*E. coli*	34	41	41	38.67
		Salmonella	46	49	46	47.00
		Ps. aeruginosa	10	31	46	29.00
	Killed stains	*E. coli*	5	19	13	12.33
		Salmonella	6	10	17	11.00
		Ps. aeruginosa	8	31	46	28.33
^{99m}Tc-enorfloxacin	Live stains	*E. coli*	45	48	44	45.67
		Salmonella	34	47	38	39.67
		Ps. aeruginosa	21	16	14	17.00
	Killed stains	*E. coli*	44	36	13	31.00
		Salmonella	49	27	19	31.67
		Ps. aeruginosa	29	35	26	30.00

of live cell activity. As stated above, this difference in the activities of ^{99m}Tc-ofloxacin in all three types of pathogens and the activity of ^{99m}Tc-ofloxacin is almost two to three folds more in live cells than those of killed ones. There is much more possibility of binding of ^{99m}Tc-ofloxacin to gyrase enzyme and our finding supports the findings of other workers.[9,10]

3.5. Activity of ^{99m}Tc-enorfloxacin in live and killed bacteria

The activity of ^{99m}Tc-enorfloxacin after 30 min, 1.0 h and 4.0 h in live *E. coli* was 45, 48 and 44%, respectively, with an average of 46%, while its activity in killed *E. coli* was 44, 36 and 13%, respectively, with an average of 31%. This activity indicates a decreasing behaviour with time. The activity of ^{99m}Tc-enorfloxacin in *Salmonella* was 34, 47 and 38% with an average of 40% after 30 min, 1.0 h and 4.0 h respectively. The activity of ^{99m}Tc-enorfloxacin in killed *Salmonella* was 49, 27 and 19%, respectively, and it again showed a decreasing activity trend with time, with an average of 32%. The activity of ^{99m}Tc-enorfloxacin in live *Ps. aeruginosa* was 21, 16 and 14% after 30 min, 1.0 h and 4.0 h respectively, with an average of 17%. The activity of ^{99m}Tc-enorfloxacin in *Ps. aeruginosa* was about 50% less than that of other live pathogens. The activity of ^{99m}Tc-enorfloxacin in dead *Ps. aeruginosa* after 30 min, 1.0 h and 4.0 h was 29, 35 and 26% with an average of 30%. Overall, in case of ^{99m}Tc-enorfloxacin, the activity in dead cells was once again prominent. Therefore, we may conclude that it is not necessary that all fluoroquinolones do not act with dead pathogens as they bind with gyrase enzyme as well as with other microbial stuffs.[11–14]

The relative high activities of ^{99m}Tc-ciprofloxacin in both live and dead pathogens suggests that it may be used for imaging all three types of microbes, while ^{99m}Tc-ofloxacin may be used for imaging live *E. coli* and *Salmonella* and to lesser extent live *Ps. aeruginosa* (29% activity). ^{99m}Tc-enorfloxacin showed excellent activity in live and dead cells except live *Ps. aeruginosa* with activity of only 17%. ^{99m}Tc-enorfloxacin activity in both *E. coli* and *Salmonella* live and a dead cell was moderate. *In-vitro* studies of these complexes indicate that they may be used for imaging specific infection.

4. Conclusion

Our study indicates that on an average, ^{99m}Tc-ciprofloxacin showed 33% and 42% activity in live and killed *E. coli*, 28% and 43% activity in live and killed *Salmonella* and 42% and 38% activity in live and killed *Ps. aeruginosa*. The accumulation of ^{99m}Tc-ciprofloxacin was higher in dead cells of *E. coli* and *Salmonella*, and live *Ps. aeruginosa*. The accumulation of ^{99m}Tc-ofloxacin in live and dead *E. coli* was 39% and 12% respectively, while in *Salmonella* it was 47% and 11% in live and dead pathogens. The activity of ^{99m}Tc-ofloxacin in *Ps. aeruginosa* live and killed was 29% and 28% respectively. These results indicate that ^{99m}Tc-ofloxacin accumulates more than three times in live cells. ^{99m}Tc-enorfloxacin activity in both *E. coli* and *Salmonella* live and dead cells was moderate except for live *Ps. aeruginosa* where its activity was found to be only 17%. ^{99m}Tc-ofloxacin may be used for imaging all three live pathogens, while ^{99m}Tc-ofloxacin and ^{99m}Tc-enorfloxacin may be used for imaging both live and dead pathogenic infections. All these labelled compounds showed reasonable activity even after 4 h. All these properties indicate that these compounds may be tested for imaging infections.

Conflict of interest

There is no conflict of interest between the authors.

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