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Research Article

Technetium-99m labeling and freeze-dried kit formulation of levofloxacin (L-Flox): A novel agent for detecting sites of infection

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Abstract: In this study, the labeling method of levofloxacin with technetium-99m and its biological evaluation were described. ^{99m}Tc-L-Flox was synthesized via direct complexation with technetium-99m in the presence of stannous chloride dihydrate as reducing agent. The optimum amounts of the reactants are: 1–2 mg levofloxacin, 150 μg stannous chloride dihydrate and 48–1490 MBq pertechnetate. The reaction mixture was bring to pH 6 and kept at room temperature for 30 min. The labeled levofloxacin was stable for more than 8 h. The *in vivo* evaluation of ^{99m}Tc-L-Flox in man-induced inflammation models showed that this tracer was localized with different values. The live *E. Coli* model had the highest value which was 2.9%, the heat killed *E. coli* model had a value of 2.0%, and the turpentine oil model had a value of 1.2% at 24 post injection, while the non-inflamed muscle had activity of 0.5%. All the gathered biological data support the usefulness of ^{99m}Tc-L-Flox as infection imaging agent. The freeze-dried form of Sn-L-Flox was prepared and found meet all the radiochemical and biological tests.

Keywords: fluoroquinolones; technetium-99m; infections; freeze-drying

Introduction

Quinolone drugs are large and widely used class of synthetic antibacterial compounds.[1–3] First generation quinolones include naldexic acid and oxolinic acid. Subsequent generations have been modified to increase spectrum and potency. The most significant modification has been the addition of a fluorine atom at position C6 in drugs such as levofloxacin. Levofloxacin shown in Figure 1 is the 4th generation of quinolone antibiotics that has activity against a wide range of Gram-negative and Gram-positive microorganisms including *Streptococcus pneumoniae* multidrug-resistant strains. Levofloxacin showed widespread distribution into body tissues and it was stereochemically stable in plasma and urine. Levofloxacin undergoes limited metabolism in humans and excreted as unchanged drug in the urine that used safely in patients with impaired renal functions and hepatic insufficiency. Quinolones target bacterial type topoisomerase II 'DNA gyrase' in Gram-negative bacteria and DNA topoisomerase IV in Gram-positive bacteria.[2]

Infection is a major cause of mortality and morbidity in the world. The appearance of multidrug-resistant bacteria required a more advanced imaging technique. Nuclear medicine techniques are used in the context of infection localization. Inflammation imaging agents such as polyclonal and monoclonal antibodies, peptides,[4–6] cytokines,[7] and HMPAO-leukocytes[8,9] cannot discriminate between septic and sterile inflammatory sites. A novel approach using a bacterially binding radiolabeled antimicrobial agent to detect infections was introduced since 1996 by Vinjamuri and Co-workers[10] when they labeled ciprofloxacin with technetium-99m, and clinically used under the trade name 'Infecton'. Later, many fluoroquinolones antimicrobial agents were labeled with technetium-99m and evaluated as infection imaging agents.[11] During this study, levofloxacin was labeled with technetium-99m and the parameters affecting this labeling reaction were studied. The labeled levofloxacin was evaluated biologically in normal and in inflamed mice. A freeze-dried form of Sn-L-Flox was prepared and evaluated radiochemically and biologically.

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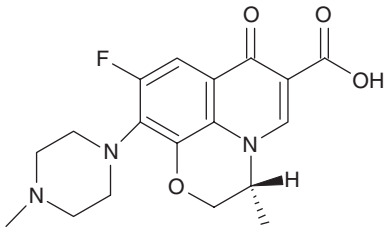

Figure 1 The chemical structure of levofloxacin (L-Flox).

Results and discussion

The presence of electron donating group such as carboxyl group and electron donating atom such as oxygen and nitrogen in the structure of levofloxacin enhance its labeling with the transition metal such as technetium-99m.

Effect of levofloxacin amount

The effect of the amount of levofloxacin on its labeling with technetium-99m was studied. The experiment was done by adding the required amount of levofloxacin solution to the reaction vial contains 150 μg stannous chloride dihydrate, followed by the addition of pertechnetate-99m and the reaction was carried out at room temperature for 30 min. The data presented in Figure 2 pointed to the ineffectiveness of this factor in the labeling process. No significant change in the radiochemical yield of ^{99m}Tc-L-Flox was observed as the result of the variation in the amounts of levofloxacin used in the range of 0.5–3.0 mg. Decrease the amount of levofloxacin to 0.1 mg leading to a lower radiochemical yield of 84%.

Effect of pH of the reaction mixture

The labeling of levofloxacin with technetium-99m was done at different pH values; 2, 4, 6, 8, and 10. The required pH value was attained using the appropriate buffer system as follows; pH 2 (0.2 M HCl/KCl), pH 4 (0.2 M CH_3COONa/CH_3COOH), pH 6 and 8 (0.2 M phosphate) and pH 10 (0.1 M $Na_2CO_3/NaHCO_3$). The results of this study are presented in Figure 3. The results clearly showed that the maximum radiochemical yield (98%) was obtained at pH 6. At acidic pH values 2 and 4, the radiochemical yields were low and equal to 60 and 80%, respectively, with the appearance of free pertechnetate as predominant species. On the other hand, at alkaline pH values 8 and 10, the radiochemical yields were relatively high (83 and

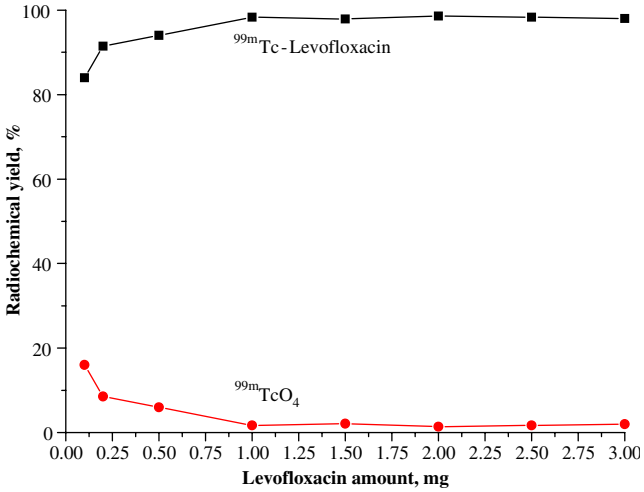

Figure 2 Radiochemical yield by levofloxacin amount in the synthesis of ^{99m}Tc-levofloxacin. Reaction conditions: *x* mg levofloxacin, 150 μg $SnCl_2.2H_2O$, ~150 MBq $^{99m}TcO_4^-$ at pH 6, the reaction mixture was kept at room temperature for 30 min.

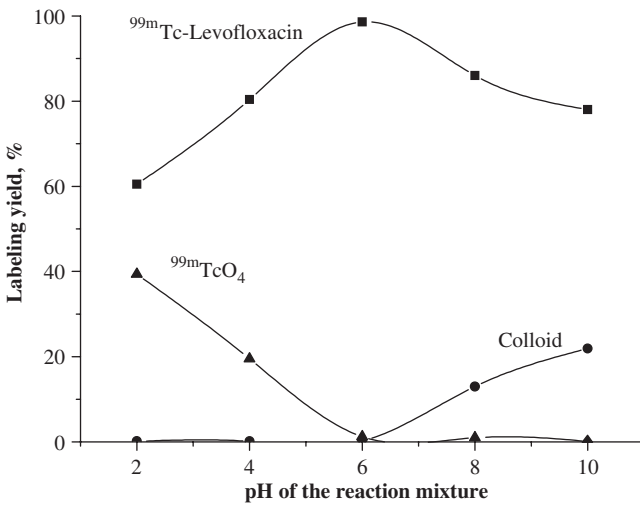

Figure 3 Radiochemical yield by pH of the reaction mixture in the synthesis of ^{99m}Tc-levofloxacin. Reaction conditions: 1 mg levofloxacin, 150 μg $SnCl_2.2H_2O$, ~150 MBq $^{99m}TcO_4^-$ at different pH values, the reaction mixture was kept at room temperature for 30 min.

80%, respectively) with the formation of colloids, which may be due to the presence of hydroxyl group in excess.

Effect of reducing agent amount

The use of stannous chloride as reducing agent in the preparation of technetium-99m radiopharmaceuticals is most widely used nowadays. In the labeling of

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levofloxacin with technetium-99m, stannous chloride dihydrate was used in the range of 25–250 μg as shown in Figure 4. At low amount of stannous chloride dihydrate, the radiochemical yield of ^{99m}Tc-L-Flox was low (~62% at 25 μg) with the appearance of free pertechnetate (~38%) indicating that the amount of reducing agent was not sufficient to reduce all the pertechnetate present in the reaction mixture. Increase the amount of the reducing agent to 150 μg leading to high radiochemical yield of 98%. Increase the reducing agent above 150 μg, leading to the appearance of colloids (12%).

Effect of the reaction time

The labeling of levofloxacin with technetium-99m was done at room temperature and in boiling water bath. The reaction was carried out at different intervals of time as cleared from Figure 5. The data presented in this figure assured that the labeling reaction was completed at room temperature (25°C) and producing 98.5% radiochemical yield of ^{99m}Tc-L-Flox after 30 min. In boiling water bath the radiochemical yield was low and did not exceed 55% at 60 min post-labeling.

Effect of pertechnetate activity on ^{99m}Tc-L-Flox stability

In nuclear medicine, the dissolution of one freeze-dried vial of Sn-L-Flox must be used for more than one patient (~370 MBq) to reduce the coast of imaging. According to this, the pertechnetate activity used in this experiment varied from 48 to 1490 MBq. The results outlined in Table 1 assured the stability of the labeled levofloxacin against the radiolysis effect of γ-ray.

On-table life time

Under the described experimental conditions, the technetium-99m labeled levofloxacin was suitable for human application along 12 h without the detection of

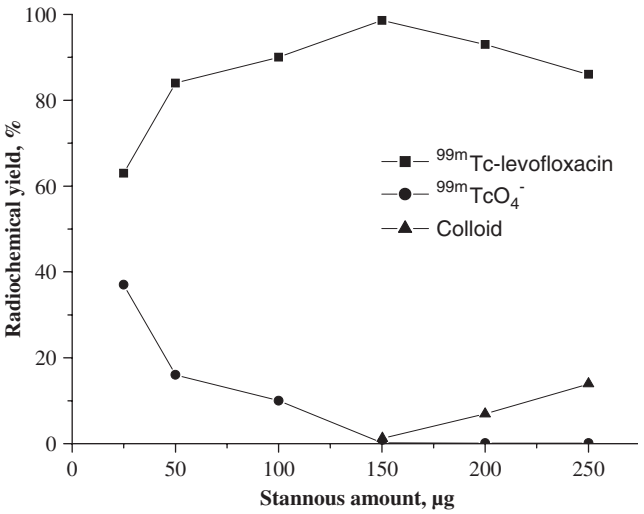

Figure 4 Radiochemical yield by stannous chloride dihydrate amount in the synthesis of ^{99m}Tc-levofloxacin. Reaction conditions: 1 mg levofloxacin, X μg $SnCl_2.2H_2O$, ~150 MBq $^{99m}TcO_4^-$ at pH 6, the reaction mixture was kept at room temperature for 30 min.

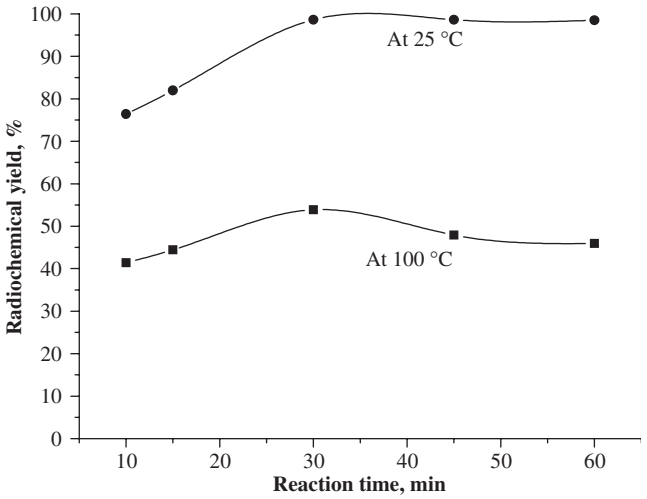

Figure 5 Radiochemical yield as a function of the reaction time in the synthesis of ^{99m}Tc-levofloxacin. Reaction conditions: 1 mg levofloxacin, 150 μg $SnCl_2.2H_2O$, ~150 MBq $^{99m}TcO_4^-$ at pH 6, the reaction mixture was kept at room temperature and in boiling water bath for different intervals of time.

Table 1 Effect of pertechnetate activity on the yield of ^{99m}Tc-L-Flox

Radiochemical species	Pertechnetate activity, (MBq)				
	48	150	740	925	1490
^{99m}Tc- L-Flox	96 ± 1.6	98.6 ± 1.5	97.5 ± 1.3	97 ± 0.7	95 ± 0.6
$^{99m}TcO_4^-$ & colloids	4 ± 0.2	1.4 ± 0.2	2.5 ± 0.5	3 ± 0.6	5 ± 0.3

Mean ± SD (mean of three experiments).
Reaction conditions: 1 mg levofloxacin, 150 μg $SnCl_2.2H_2O$, 48–1490 MBq $^{99m}TcO_4^-$ pH = 6, the reaction mixture was kept at room temperature for 30 min.

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Table 2 On-table life time of ^{99m}Tc-L-Flox

Radiochemical species	Time post-labeling (h)					
	0.5	1	2	4	8	12
^{99m}Tc-L-Flox	98.6 ± 2.3	98.5 ± 1.9	98 ± 2.1	97.5 ± 1.8	96 ± 1.3	96.5 ± 1.8
$^{99m}TcO4^{-}$	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.2
Colloids	1.2 ± 0.1	1.2 ± 0.2	1.5 ± 0.1	1.9 ± 0.1	3.3 ± 0.4	2.5 ± 0.5

Mean ± SD (mean of three experiments).
Reaction conditions: 1 mg levofloxacin, 150 μg $SnCl_2.2H_2O$, ~ 1490 MBq $^{99m}TcO_4^-$ pH = 6, the reaction mixture was kept at room temperature for 30 min.

any undesirable radiochemical species such as free pertechnetate or colloids as cleared from Table 2.

Freeze-drying cycle and quality control

Typical freeze-drying cycle is shown in Figure 6. Values for vacuum, shelf temperature, product temperature, and condenser temperature are plotted versus time for complete freeze-drying cycle of Sn-L-Flox. Actual freeze-drying was started when the product temperature has reached ≥ −40°C.

The prepared freeze-dried vials of Sn-L-Flox were evaluated for radiochemical purity, reducing agent content, moisture content, sterility, apyrogenicity, and undue toxicity. Radiochemical purity was determined using ITLC-SG/MEK system and found in the desirable range. Reducing agent was quantified utilizes UV spectrophotometric measurement of the color produced upon the reaction of tin (II) with molybdenum thiocyanate, and found equal to 130 ± 10 μg. Moisture content was determined using Karl-Fischer method and found less than 1%. Sterility, apyrogenicity, and undue toxicity meet the requirements of the Egyptian Pharmacopoeia.

Biodistribution studies

The efficacy of ^{99m}Tc-L-Flox to localize the infection sites *in vivo* was estimated in normal mice in addition to inflamed mice. The inflammations were induced via intramuscular injection of the autoclaved turpentine oil (model for sterile inflammation), live *Escherichia Coil* (*E. Coli*) (model for septic inflammation), and heat killed *E. Coli* (model for pyrogen induced inflammation) into the mice. Tracer was injected IV into the mice and kept alive in metabolic cage for different intervals of time under normal conditions. The results presented in Table 3 recorded the normal biological distribution pattern of the labeled levofloxacin in normal mice. It can be deduced from this table that the tracer was distributed effectively all over the body tissues. The conjugation of the tracer to the plasma protein was

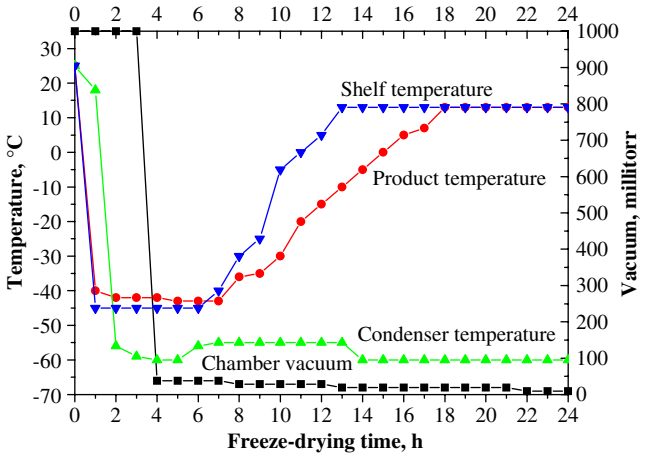

Figure 6 A typical freeze-drying cycle of Sn-L-Flox using 'CONSOL-12' freeze dryer.

high as the activity detected in the blood at 2 and 4 h post injection were 23.2 and 12.6%, respectively. As the levofloxacin excreted mainly via the kidneys in non-metabolized form, the major activity was detected in the kidneys with percentage of 16.1, 14.1 and 8.2%. The activity accumulated in the kidneys was passed to the urine giving 29.8% at 24 h post-injection. The high lung uptake (8.3% at 2 h) was due to the high penetration of the levofloxacin into the lung tissues and that lung tissues concentration was generally comparable to the plasma concentration. The biodistribution of the labeled levofloxacin in the models of inflammation are presented in Table 3. The distribution pattern of the tracer in the inflammation models were similar to large extent to that in normal mice, except in the uptake by the inflamed muscles. The uptakes of the three inflammation models were higher than the uptake of the normal muscle as shown in Figure 7. Live *E. Coli* inflammation exhibited the highest uptake, which due to the selectively uptake of the labeled levofloxacin by the topoisomerase II (DNA gyrase) that is the primary target of *E. Coli*, in addition to the uptake by the topoisomerase IV which is the second target.[12] On the other side, the uptakes by the oil and heat-killed

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Table 3 Biodistribution pattern of ^{99m}Tc-L-Flox in normal, oil-inflamed, alive *E. Coli*-inflamed, and heat-killed *E. Coli*-inflamed mice at different intervals of time

Organs and body fluids	% injected dose/organs at different intervals of time (h)											
	Normal mice			Turpentine oil			Alive *E. Coli*			Heat killed *E.Coli*		
	2	4	24	2	4	24	2	4	24	2	4	24
Blood	23.2 ± 1.5	12.6 ± 1.2	1.9 ± 0.4	21.9 ± 1.4	11.6 ± 0.9	2.0 ± 0.2	22.0 ± 1.3	14.4 ± 0.8	2.2 ± 0.4	20.8 ± 1.2	12.9 ± 0.5	2.0 ± 0.4
Heart	0.5 ± 0.2	0.3 ± 0.1	0.1 —	0.5 ± 0.1	0.3 ± 0.1	0.1 —	0.5 ± 0.1	0.3 —	0.1 —	0.6 ± 0.1	0.3 ± 0.1	0.1 —
Lungs	8.3 ± 0.5	6.4 ± 0.4	2.8 ± 0.4	7.2 ± 0.6	5.4 ± 0.3	1.7 ± 0.2	8.1 ± 0.1	5.9 ± 0.2	2.1 ± 0.1	7.1 ± 0.6	5.6 ± 0.3	1.7 ± 0.1
Spleen	1.3 ± 0.2	1.2 ± 0.1	0.1 —	1.4 ± 0.1	1.2 ± 0.1	0.2 —	1.4 ± 0.2	1.1 ± 0.1	0.2 —	1.4 ± 0.1	1.2 ± 0.1	0.2 —
G.I.T.	4.3 ± 0.2	3.9 ± 0.1	1.0 ± 0.1	4.2 ± 0.2	3.4 ± 0.2	1.2 ± 0.1	4.2 ± 0.3	3.8 ± 0.4	1.8 ± 0.1	4.1 ± 0.3	4.4 ± 0.1	1.8 ± 0.1
Liver	4.6 ± 0.3	3.4 ± 0.2	2.1 ± 0.1	4.2 ± 0.2	3.8 ± 0.3	1.9 ± 0.1	4.3 ± 0.2	3.9 ± 0.2	2.1 ± 0.1	4.2 ± 0.2	3.7 ± 0.1	2.0 ± 0.2
Kidneys	16.1 ± 1.2	14.1 ± 0.9	8.2 ± 0.9	15.9 ± 0.8	13.6 ± 0.5	8.6 ± 0.2	15.9 ± 1.3	13.8 ± 0.7	8.4 ± 0.6	15.9 ± 1.4	13.6 ± 0.7	8.6 ± 0.5
Urine	10.6 ± 1.8	26.5 ± 0.2	29.8 ± 0.5	12.4 ± 1.3	27.4 ± 0.6	32.1 ± 0.3	11.2 ± 1.3	26.3 ± 0.4	32.1 ± 0.3	12.4 ± 1.3	27.4 ± 0.6	32.1 ± 0.3

Mean ± SD (mean of three experiments).
Vial content: 1mg levofloxacin, 150 µg $SnCl_2.2H_2O$, ~ 750 MBq $^{99m}TcO_4^-$,at pH 6, the reaction mixture was kept at room temperature for 30 min.

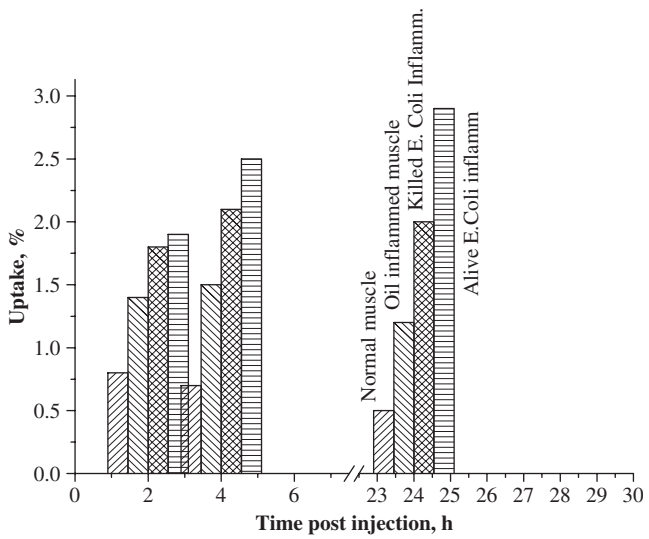

Figure 7 Histogram of the uptake of normal and oil-inflamed, heat killed ***E. Coli***-inflamed and alive ***E. Coli***-inflamed muscle at 2, 4, and 24 h post-injection.Vial content: 1 mg levofloxacin, 150 µg $SnCl_2.2H_2O$, ~750 MBq $^{99m}TcO_4^-$, at pH 6, the reaction mixture was kept at room temperature for 30 min.

E. Coli models were non-specific which attributed to the increase of the blood flow in the inflammation site.

To get a good image, the target site must be hold high activity in comparison with the blood pool (general background) and to the nearing tissues (specific background). The ratios of target to blood (T/B) and target to non-target (T/NT) were calculated and presented in Table 4. Statistical analysis was performed using the two independent sample *t*-test. A probability of less than 0.05 was considered to be significant. The differences in ratios of T/NT for live and heat killed *E. Coli* were non-significant at 2 and 4 h, while was significant at 24 h time period. The highest T/B and T/NT ratios were observed in the case of live *E. Coli* inflammation model at 24 h post-injection. This strongly supports the usefulness of ^{99m}Tc-L-Flox as infection imaging agent.

Table 4 The ratio of target muscle (T) to blood (B) and to non-target muscle (NT) of ^{99m}Tc-L-Flox at different post injection times

Ratio	Post injection times, (h)								
	Turpentine oil			Alive *E. Coli*			Heat killed *E. Coli*		
	2	4	24	2	4	24	2	4	24
T/B	0.01	0.02	0.11	0.01	0.04	0.27	0.01	0.03	0.18
T/NT	0.75	2.14	2.4	0.38	3.57	5.8	2.25	3.0	4.0

Vial content: 1 mg levofloxacin, 150 µg $SnCl_2.2H_2O$, ~ 750 MBq $^{99m}TcO_4^-$, at pH 6, the reaction mixture was kept at room temperature for 30 min.

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Experimental

Materials

Levofloxacin (-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1, 4-benzoxazine-6-carboxylic acid, L-Floxin, (-)-Ofloxacin, MF $C_{18}H_{20}FN_3O_4.1/2\ H_2O$, MW 370.38 was obtained as a gift from Amriya Pharmaceutical Industry, Alexandria, Egypt. Stannous chloride dihydrate solution was prepared by dissolving 10 mg of the crystalline salt in 0.5 ml conc. HCl by heating, then diluted to 10 ml using deoxygenated double distilled water (1 mg/ml). The solution was dispensed in '10 ml' vial capacity (1 ml each) and kept under freezing. Pertechnetate was eluted from moly generator supplied by 'ELUTEC' Brussels, Belgium. White Albino mice were used for the quantitative biodistribution studies.

Labeling of levofloxacin

Stannous chloride dihydrate solution (150 μl, 150 μg) was added to the solution of levofloxacin in phosphate buffer 0.1 M of pH 6 (0.5 ml, 1.0 mg) in a sterile vial (Wheaton type) kept under positive nitrogen gas. To this mixture, pertechnetate solution (48–750 MBq) was added and the reaction mixture was stand for 30 min at room temperature (25°C).

Determination of radiochemical yield

(a) The percentage of the colloids (reduced hydrolyzed ^{99m}Tc and stannous hydroxide colloid) were determined by filtration of the reaction mixture through 0.22 μm filter [11] using a suitable pressure and according to the following equation:

$$\%\ \text{colloid} = \frac{\text{Activity before filtration} - \text{Activity after filtration}}{\text{Activity before filtration}} \times 100 \quad (1)$$

(b) ITLC-SG/saline or ITLC-SG/MEK system was used to determine free pertechnetate and the labeled levofloxacin in the filtrate. Free pertechnetate migrate with the solvent front (R_f 0.8–1.0) while labeled levofloxacin was at the base (R_f 0.0–0.1) in both systems, and according to the following equations:

$$\%\ \text{Free pertechnetate} = \frac{\text{Activity at } R_f\ 0.8-1.0}{\text{Total activity}} \times 100 \quad (2)$$

$$\%\ \text{labeled levofloxacin} = 100 - (\%\ \text{colloid} + \%\ {}^{99m}TcO_4^-) \quad (3)$$

Biological evaluation

Sterile inflammation was induced by injection of the sterile turpentine oil (200 μl), intramuscularly (IM), into the right thigh muscle,[13] while septic inflammation was induced by IM injection of suspension of alive and heat killed *E.Coli*.[7,14] When swelling of the muscle was apparent, ^{99m}Tc-L-Flox (100 μl, 0.7 MBq) was injected intravenously (IV). Groups of three mice were used for each experiment. The mice were sacrificed by the decapitation under chloroform anesthesia at 2, 4 and 24 h post-injection. Blood sample was collected at the time of decapitation. Both thighs (right thigh muscle as target and left thigh muscle as control) and organs were dissected, weighed and their radioactivity was measured using a well-type NaI(Tl) detector connected with a single channel γ-counter (SR-7). Results were expressed as percent of the injected dose per organ or body fluid.

Preparation of Sn-L-Flox kits for freeze-drying

100 mg of L-Flox was dissolved in 80 ml of sterile saline purged with N_2 gas. 1.5 ml of stannous chloride solution (10 mg/ml) was added. The pH was adjusted to 6 using 1N NaOH, and the volume was completed to 100 ml using N_2 purged saline. The solution was sterilized by Millipore filtration in a laminar flow hood. The sterile solution was dispensed in 1 ml quantities into sterile penicillin vials and fitted with sterile rubber closures. The vials were transferred to the freeze-dryer and the process continues for 24 h. The vials were closed under dry sterile nitrogen gas and stored at 6–8°C.

Conclusion

The technetium-99m labeled levofloxacin meets the needs of radiopharmaceuticals, which are: (1) it is easy of preparation, one-step prepared kit and long shelf life; (2) it is specific imaging agent for septic inflammation, because it binds a specific target in the infectious focus; (3) it shows high and rapid accumulation in the abscess; (4) binds to a varity of microorganisms with no binding to the host cell; (5) safe for both patients with kidney and liver function impairment. In contrary to ^{99m}Tc-ciprofloxacin[15] and ^{99m}Tc-pefloxacin[11] which need boiling water bath for their preparation, ^{99m}Tc-levofloxacin was prepared at room temperature by adding pertechnetate to a freeze-dried kit (contains 1 mg levofloxacin and 150 μg $SnCl_2.2H_2O$ at pH 6). The labeled levofloxacin discriminate well between septic and sterile inflammation.

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