ORIGINAL PAPERS

Adv Clin Exp Med 2013, **22**, 3, 319–325
ISSN 1899–5276

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Synthesis of ^{99m}Tc Labeled Temafloxacin Complex and Biodistribution in Male Wistar Rats Artificially Infected with *Streptococci pneumonia*

Synteza kompleksu temafloksacyny znakowanego ^{99m}Tc i biodystrybucja u samców szczurów szczepu Wistar sztucznie zakażonych szczepami *Streptococcus pneumoniae*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Radiotracers techniques are offering a unique way to diagnosis deep tissue infection in its early stages. The radiotracers including radio-antibiotics have shown promising results in the early diagnosis of infection and its discrimination from infectious foci but wide ranges of microorganisms still poses threats.
Objectives. Synthesis of Technetium-99m (^{99m}Tc)-temafloxacin (TMC) complex for the localization of in vivo *Streptococci pneumoniae* infection in the early stages.
Material and Methods. The ^{99m}Tc-TMC complex was prepared by adding 50 µg of stannous chloride ($SnCl_2$) with 37 MBq (0.5 mL) of sodium pertechnetate ($Na^{99m}TcO_4^-$) at a pH of 5.2. Then 1 mg of the TMC was added to the mixture followed by incubation at room temperature for 10 min. The same procedure was repeated by changing the amount of the $SnCl_2$ from 50 to 250 µg along with the TMC from 2 to 5 mg and $Na^{99m}TcO_4^-$ from 74 to 185 MBq. In higher concentrations of cysteine the stability of the ^{99m}Tc-TMC complex was evaluated. In vitro *Streptococci pneumoniae* uptake was investigated to validate the accuracy and preciseness of the ^{99m}Tc-TMC complex. In vivo uptake of the ^{99m}Tc-TMC complex was evaluated in ten (10) normal male Wistar rats (MWR) (140–160 g) divided into two groups (I and II).
Results. Maximum stability of 98.00 ± 0.34% at 30 min after reconstitution was observed by mixing 2.5 mg of TMC, 100 µg of $SnCl_2$ with 74 MBq of the $Na^{99m}TcO_4^-$. The stability of the complex remained 90% up to 4 hours. In serum the complex showed stability up to 16 hours. A saturated in vitro binding was noted with live *Streptococci pneumoniae*. In the infected region (left thigh) of the MWR, almost five times higher uptake was observed as compared to the inflamed and normal muscles.
Conclusions. The above results confirm the suitability of the ^{99m}Tc-TMC complex as a potential *Streptococci pneumoniae* infection localizing agent (**Adv Clin Exp Med 2013, 22, 3, 319–325**).

Key words: ^{99m}Tc-Temafloxacin, *Streptococci pneumoniae,* infection localization.

Streszczenie

Wprowadzenie. Techniki wykorzystujące radioznaczniki oferują unikalny sposób rozpoznawania zakażenia tkanek głębokich w jego początkowej fazie. Radioznaczniki, w tym antybiotyki, wykazały obiecujące wyniki w wczesnej diagnostyce infekcji i odróżnianie jej ognisk zakaźnych, ale szeroki zakres drobnoustrojów nadal stwarza zagrożenia.
Cel pracy. Synteza kompleksu technetu 99m (99mTc) i temafloksacyny (TMC) w celu umiejscowienia *in vivo* wczesnego zakażenia szczepami *Streptococcus pneumoniae.*
Materiał i metody. Kompleks ^{99m}Tc-TMC został przygotowany przez dodanie 50 µg chlorku cyny (SnCI2) do 37 MBq (0,5 ml) nadtechnecjanu sodu ($Na^{99m}TcO_4^-$) przy pH 5,2. Następnie 1 mg TMC dodano do mieszaniny, a następnie inkubowano w temperaturze pokojowej przez 10 minut. Tę samą procedurę powtórzono, zmieniając

ilość $SnCl_2$ od 50 do 250 ug, TMC od 2 do 5 mg, $Na^{99m}TcO_4^-$ od 74 do 185 MBq. W większych stężeniach cysteiny oceniono stabilność kompleksu ^{99m}Tc-TMC. Badano wychwyt szczepów *Streptococcus pneumoniae in vitro* w celu sprawdzenia dokładności kompleksu ^{99m}Tc-TMC. Wychwyt kompleksu ^{99m}Tc-TMC oceniano *in vivo* u dziesięciu (10) zdrowych samców szczurów Wistar (MWR) (140–160 g), które podzielono na dwie grupy (I i II).
Wyniki. Maksymalną stabilność 98,00 ± 0,34% 30 minut po rozpuszczeniu obserwowano przez zmieszanie 2,5 mg TMC, 100 ng SnCl2 z 74 MBq w $Na^{99m}TcO_4^-$. Stabilność kompleksu pozostała na poziomie 90% do 4 godzin. W surowicy kompleks wykazał stabilność do 16 godzin. Nasycone wiązania *in vitro* stwierdzono u żywych *Streptococcus pneumoniae*. Z zakażonego obszaru ciała (lewe udo) MWR zaobserwowano prawie pięciokrotnie większy wychwyt w porównaniu do mięśni zdrowych i zmienionych zapalnie.
Wnioski. Powyższe wyniki potwierdzają przydatność kompleksu ^{99m}Tc-TMC jako środka do umiejscawiania potencjalnego zakażenia *Streptococcus pneumoniae* (**Adv Clin Exp Med 2013, 22, 3, 319–325**).

Słowa kluczowe: ^{99m}Tc-temafloksacyna, *Streptococcus pneumoniae*, umiejscawianie zakażenia.

Deep soft tissue bacterial infection is currently the focal point of the investigators for rapid and more accurate diagnosis of infectious foci and its discrimination from inflammation during early stages [1]. The role of modern diagnostic facilities like ultrasonography (USG), computerized tomography (CT) and magnetic resonance imaging (MRI) have proven inadequate in the early diagnosis of infections and its differentiation from non-infectious foci. However, radionuclide imaging technology has shown promising results in radio tracing of deep tissue infections [2, 3].

Nowadays, radiolabeled antibiotics have played a significant role in the early diagnosis of infection and its discrimination from inflammation. The reported technetium-99m (^{99m}Tc) radiolabeled antibiotics, including authors' newly developed ones, have shown promising radio-yield for a maximum period of time. The reported radiolabeled antibiotics have shown significantly higher stability in saline, serum, saturated *in vitro* binding with bacteria and higher uptake in infected muscle as compared to the inflamed and normal muscle, target to non-target ratio [4–10].

Temafloxacin (TMC) (Fig. 1a) [1-(2,4-Difluorophenyl)-6-fluoro-7-(3-methylpiperazin-1-yl)-4-oxoquinoline-3-carboxylic] is a broad-spectrum fluorinated quinolone antibiotic representing helpful *in vivo* and *in vitro* pharmacokinetics. TMC, similar to other quinolone antibiotics, has demonstrated higher activity against a wide variety of bacteria including *Staphylococcus aureus, Moraxella catarrhalis, Haemophilus influenzae, Legionella pneumophila and Klebsiella pneumoniae*. TMC has shown almost eight times higher antibiotic activity against *Streptococci pneumoniae* and *Streptococcus pyogenes* [11, 12].

In the current investigation the authors exploit the elevated antibacterial affinity of TMC for the radio-diagnosis of the infection caused by *Streptococci pneumoniae* after labeling it with technetium-99m (^{99m}Tc) using stannous chloride as reducing agent. The labeled TMC was further characterized in terms of radiochemical stability in saline, serum, in vitro binding with *Streptococci pneumoniae* and biodistribution in a male Wistar rat (MWR) model artificially infected with live and heat killed *Streptococci pneumoniae*.

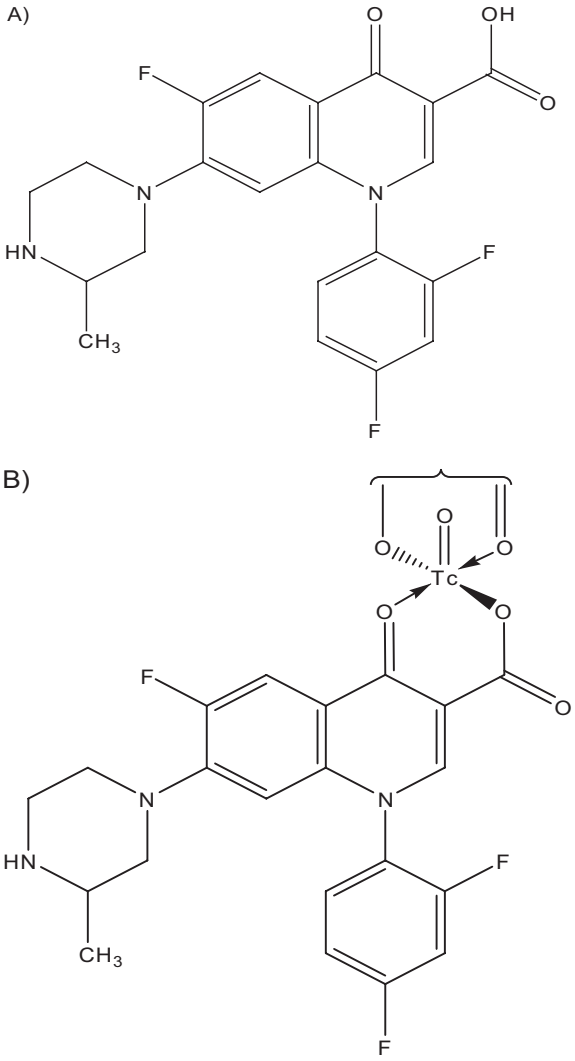

Fig. 1. A) Chemical structure of the temafloxacin (TMC); B) Speculated structure of the ^{99m}Tc-TMC complex

Ryc. 1. A) Struktura chemiczna temafloksacyny (TMC), B) hipotetyczna struktura kompleksu ^{99m}Tc-TMC

Material and Methods

Temafloxacin (TMC) (Shanghai Sciencya Biotechnology Co., Ltd. Shanghai, China), Thin Layer Chromatographic (TLC) Strips (Merck) and all the other chemicals and solvents of analytical grade (Sigma). RP-HPLC (Shimadzu, Japan) well counter and scalar count rate meter (Ludlum, USA), dose calibrator (Capintech, USA) and gamma camera GKS-1000 (GEADE Nuclearmedizin system, Germany).

Radiolabeling of Temafloxacin (TMC)

Ten nitrogen filled sterilized vials were taken and 25 to 250 μg in 25 μg increments of stannous chloride was injected through insulin syringe into each, followed by addition of 0.5 to 5.0 mCi in 0.5 mCi increments of $Na^{99m}TcO_4^-$ in sequential order. Thereafter, to the above mixture, 0.5 to 5.0 mg in 0.5 mg increments of TMC was added. The pH of the mixture was adjusted to 5.5 with a 0.01N HCl solution. Subsequently, the preparations were incubated for 10 minutes at room temperature followed by filtration through a Millipore filter.

HPLC Radiocharacterization

Radiolabeled TMC (^{99m}Tc-TMC complex) was radio-characterized with HPLC using the previously reported method [7]. Briefly, to the main Shimadzu HPLC unit fitted with UV detector (operating at 254 nm) and C-18 (4.6 × 150 mm) column, 5 μL of the ^{99m}Tc labeled TMC was injected. Thereafter, a mobile phase of a mixture of triethylaminophosphate (TEAP) and methyl alcohol (MA) (MA:TEAP) was employed for 20 min with a flow rate of 1 mL/min., engaged for 0–3 min (100:00%), 3–6 min (75:25%), 6–8 min (53:47%), 8–10 min (25:75%), 10–12 min (00:100%) and 12–15 min (50:50%). The radio-eluents received during the elution were separately collected in clean vials and counted for radioactivity in each vial for determination of radiochemical stability using a well counter interface with count rate meter.

Stability in Serum and Challenge Assay

TLC was used to determine the stability of the ^{99m}Tc labeled TMC using the previously reported method [7]. Briefly, at 37°C the labeled TMC (0.2 mL) was incubated for 16 hours with 1.8 mL of serum. After that 1 μL aliquots were withdrawn from the incubated mixture and evaluated for stability using (CH_2Cl_2:CH_3OH (9:1) (v/v) as a developing solvent.

Additionally, the ^{99m}Tc-TMC radio-stability was verified in a challenge assay. An equimolar solution of the ^{99m}Tc-TMC was incubated with the increasing amount of cysteine (1×10^7 mol/L) at 37°C. Thereafter, aliquots were taken out from the incubated mixture for stability determination using (CH_2Cl_2:CH_3OH (9:1) (v/v) as a developing solvent.

Binding with *Streptococci pneumoniae*

In vitro binding of ^{99m}Tc-TMC with *Streptococci pneumoniae* was evaluated using the reported method [13]. Briefly, 0.2 mL of the ^{99m}Tc-TMC was vortexed with 0.1 mL of the sodium phosphate buffer (SPB) followed by addition of 0.8 mL (50% v/v) 0.01 M acetic acid containing 1×10^8 colony forming units (CFU) of *Streptococci pneumoniae*. The mixture was incubated at 4°C for 1 hour with a pH of 5. After 1 hour the mixture was centrifuged for 10 min at 2000 rpm. Thereafter, the supernatant from the mixture was removed and re-suspended in 2 mL SPB followed by centrifugation at 2000 rpm for 10 min. The supernatant was again removed and the level of activity in the pellets was measured using a well counter interface with count rate meter.

Biodistribution in Rats

In vivo distribution of the ^{99m}Tc-TMC in different organs of the MWR artificially infected with live and heat killed *Streptococci pneumoniae* was investigated at 30, 60, 90 and 120 min post injection (p.i). Ten (10) normal MWR (140–160 g) were divided into two groups (I and II). Intramuscularly, 0.2 mL of sterile turpentine oil was injected into the left thigh of each MWR of group I and II. Thereafter, in 0.2 mL saline, 2×10^8 live organisms of the *Streptococci pneumoniae* were injected into the right thigh of each MWR of group I and heat killed into group II. After 18 hours of the I.M. injections, 0.2 mL of the freshly prepared ^{99m}Tc-TMC was injected through the ear vein into each MWR of group I and II. Both groups of MWR were sacrificed in accordance with the approved rules of the Nuclear Medicine Research Laboratory (NMRL), University of Peshawar. In the blood, liver, spleen, stomach, intestines, kidneys, infected, inflamed and normal muscles of the MWR, percent uptake was determined using a well counter interface with count rate meter.

Results and Discussion

Temafloxacin (TMC) (Figure 1A) was tagged with ^{99m}TcO using the method reported earlier to give ^{99m}Tc-TMC as given in Figure 1B. The basic structure of the complex (Figure 1B) can be speculated by comparison with the reported properties of the TcN core [14].

Radiochemistry of ^{99m}Tc-TMC Complex

Technetium have a number of oxidation states but the +V state is the most frequent in TcN complexes. The two sets of molecular orbitals (MO) will be the highest occupied non-bonding MO (HOMO) and the lowest two unoccupied degenerate π* antibonding orbitals (LUMO) together can account for the arrangement of the square pyramidal structure of the TcN complexes.

From the crystal structural studies on TcN complexes, it has been shown that when the four coordinated atoms are π-donor Lewis bases, then square pyramidal (sp) geometry is preferred [15]. The speculated structure of ^{99m}Tc-TMC (Figure 1B) with bidentate ligand will have a square pyramidal geometry with ^{99m}Tc-TMC:Ligand ratio of 1:2.

HPLC Radiocharacterization

Figure 2 the HPLC radiochromatogram of the ^{99m}Tc-TMC complex gave two clearly different peaks at 3.2 and 11.1 min of retention. The HPLC peak at 3.2 signifies the unlabeled free ^{99m}Tc and that at 11.1 represents the ^{99m}Tc-TMC complex.

The ^{99m}Tc-TMC complex showed stability in normal saline up to 240 min after its reconstitution with a minimum de-tagging as shown in Figure 3. After 30 min of reconstitution, the level of bound activity was 98.00 ± 0.34%. The radiochemical stability profile of the ^{99m}Tc-TMC complex was found promising by mixing 2.5 mg TMC, 100 μg stan-

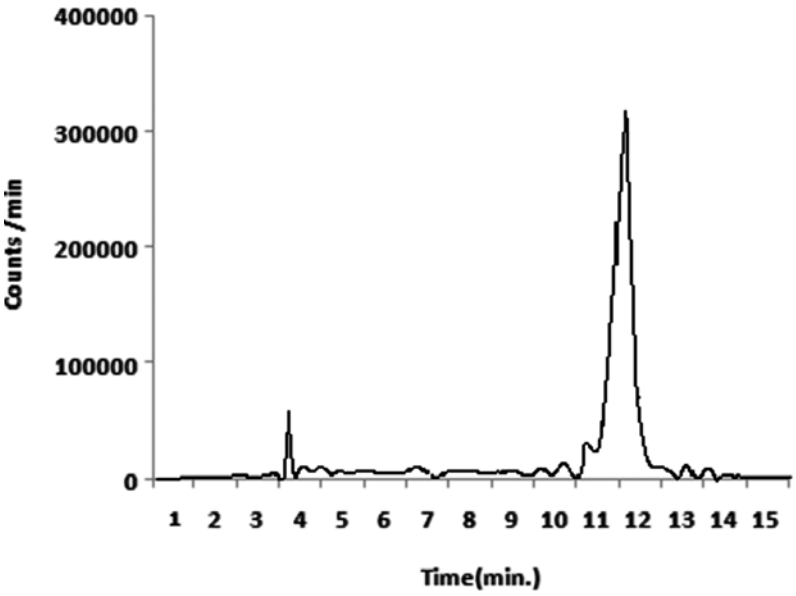

Fig. 2. HPLC radiochromatogram of the ^{99m}Tc-TMC complex

Ryc. 2. Radiochromatogram HPLC kompleksu ^{99m}Tc-TMC

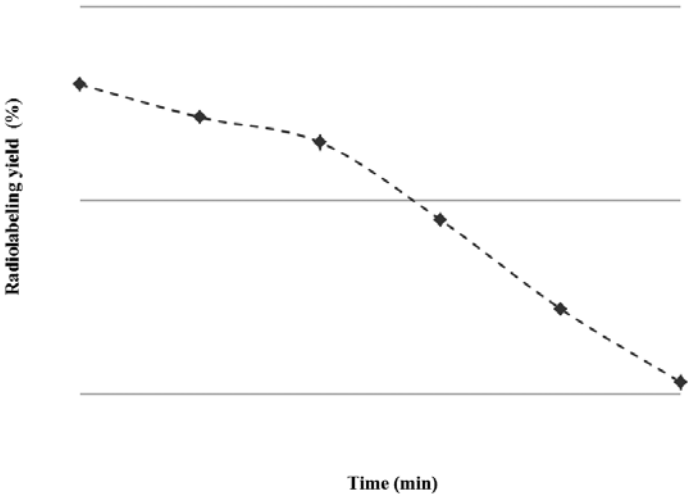

Fig. 3. Stability of the ^{99m}Tc-TMC complex in normal saline

Ryc. 3. Stabilność kompleksu ^{99m}Tc-TMC w soli fizjologicznej

nous chloride and 2 mCi of the $Na^{99m}TcO_4^-$. The minimum stability observed at 240 min after reconstitution was 90.30 ± 0.22%.

Stability in Serum and Challenge Assay

The ^{99m}Tc-TMC complex showed stability up to 16 hours after incubation with serum at 37 °C. The stability profile of the ^{99m}Tc-TMC complex is given in Figure 4A. Overall the free activity (side product appeared due to de-tagging) within 16 hours was 15.65%.

The challenge assay profile of the ^{99m}Tc-TMC complex suggested stability as shown in Figure 4B. In increasing concentrations of the cysteine (1 x 10^7 mol/L), the compound decreased to 8.85%.

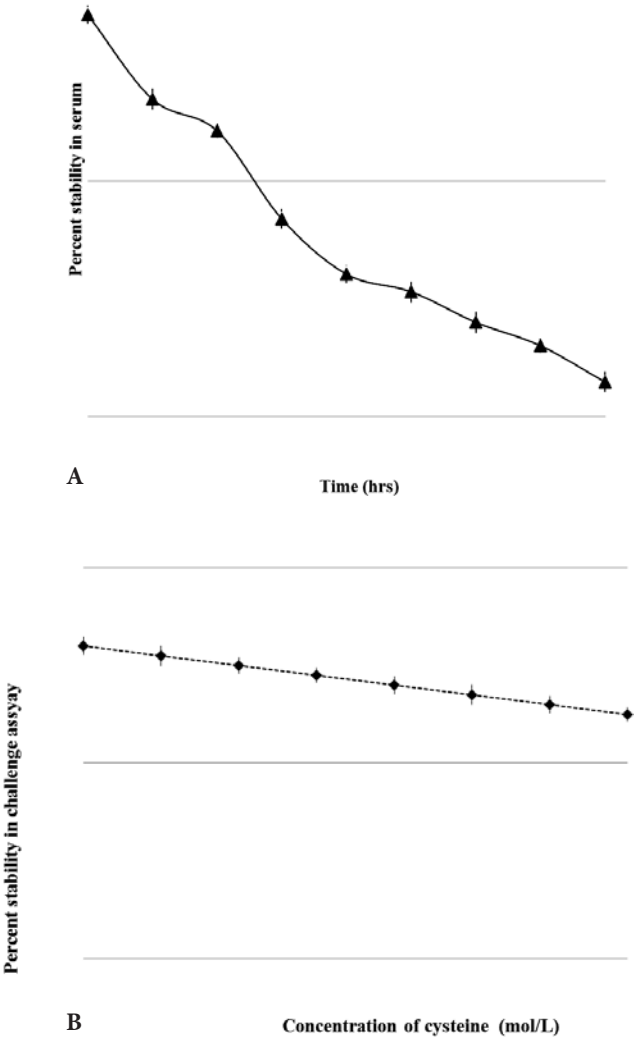

Fig. 4. A. Stability of the ^{99m}Tc-TMC complex at 37°C in serum up to 16 h. **B.** Challenge assay test of stability of the ^{99m}Tc-TMC complex

Ryc. 4. A. Stabilność kompleksu ^{99m}Tc-TMC w temperaturze 37°C w surowicy do 16 godz. **B.** Test stabilności kompleksu ^{99m}Tc-TMC

Binding with *Streptococci pneumoniae*

The ^{99m}Tc-TMC complex showed saturated in vitro binding with *Streptococci pneumoniae* as shown in Figure 5. It was observed that at 90 min of incubation 62.65 ± 0.44% of the ^{99m}Tc-TMC complex was taken up by the *Streptococci pneumoniae.*

Biodistribution in Rats

The percent uptake of the ^{99m}Tc-TMC complex in different organs of the MWR artificially infected with live and heat killed *Streptococci pneumoniae* at 30, 60, 90 and 120 min post p.i. is given in Table 1. It was observed that the level of ^{99m}Tc-TMC complex in the blood, liver, spleen, stomach and intestine in the gaining was high which decreased with time and appeared in the kidneys and infected muscle of the MWR infected with live *Streptococci pneumoniae.* In group I MWR, the level of activity in the blood, liver, spleen, stomach and intestine was decreased from 18.55 ± 0.28 to 3.80 ± 0.40%, 13.55 ± 0.34 to 4.00 ± 0.40%, 8.35 ± 0.38 to 4.15 ± 0.44% and 8.10 ± 0.42 to 4.00 ± 0.00% within 120 min of p.i respectively. A similar profile of the ^{99m}Tc-TMC complex was seen in group II. However, the activity of the ^{99m}Tc-TMC complex in infected muscle was different from the inflamed and normal muscles in group I and II. The level of activity in the infected muscle of group I was almost five times higher than the inflamed and normal muscles, while in group II, the activity was almost similar in infected, inflamed and normal muscles. The ratios of the infected to normal, inflamed to normal and infected to inflamed

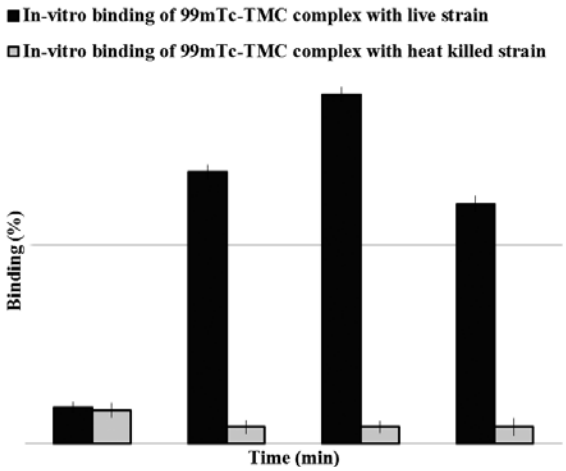

Fig. 5. *Streptococci pneumoniae* in vitro binding with ^{9m}Tc-TMC complex at 30, 60, 90 and 120 min.

Ryc. 5. Wiązania Streptococcus pneumoniae in vitro z kompleksem ^{9m}Tc-TMC w 30., 60., 90. i 120. minucie

Table 1. Biodistribution of the ^{99m}Tc-TMC complex in the blood, liver, spleen, stomach, intestines, kidneys, infected, inflamed and normal muscles of *Streptococci pneumoniae* infected MWR model

Tabela 1. Biodystrybucja kompleksu ^{99m}Tc-TMC we krwi, wątrobie, śledzionie, żołądku, jelitach, nerkach, zakażonych, zmienionych zapalnie i zdrowych mięśniach zakażonych *Streptococci pneumoniae* w modelu MWR

Organs/ tissues – gm (Narządy/ tkanki – gm)	Uptake in different p.i. – min (Wychwyt w różnych p.i. – min)							
	live *S. aureus*				heat killed *S. aureus*			
	30	60	90	120	30	60	90	120
Infected muscle (Mięsień zakażony)	6.15 ± 0.44	9.95 ± 0.30	13.15 ± 0.38	11.25 ± 0.00	3.00 ± 0.32	3.50 ± 0.38	3.50 ± 0.40	3.00 ± 0.36
Inflamed muscle (Miesień zmieniony zapalnie)	3.00 ± 0.38	3.50 ± 0.30	3.00 ± 0.42	3.00 ± 0.34	3.00 ± 0.42	3.50 ± 0.36	3.00 ± 0.30	3.00 ± 0.42
Normal muscle (Zdrowy mięsień)	2.50 ± 0.40	3.00 ± 0.30	2.50 ± 0.36	2.50 ± 0.00	2.50 ± 0.38	3.00 ± 0.34	2.50 ± 0.40	2.50 ± 0.42
Blood (Krew)	18.55 ± 0.28	10.20 ± 0.36	8.15 ± 0.00	3.80 ± 0.40	18.44 ± 0.22	10.25 ± 0.32	8.20 ± 0.44	3.55 ± 0.38
Liver (Wątroba)	13.55 ± 0.34	10.25 ± 0.30	8.40 ± 0.00	4.00 ± 0.40	13.50 ± 0.36	10.10 ± 0.34	8.15 ± 0.44	4.15 ± 0.40
Spleen (Śledziona)	8.35 ± 0.38	6.90 ± 0.34	5.65 ± 0.36	4.15 ± 0.44	8.20 ± 0.40	7.10 ± 0.34	5.45 ± 0.00	3.95 ± 0.38
Kidney (Nerka)	9.00 ± 0.40	14.20 ± 0.34	18.50 ± 0.36	24.50 ± 0.00	8.65 ± 0.38	14.60 ± 0.34	19.00 ± 0.36	23.6 ± 0.44
Stomach & intestines (Żołądek i jelita)	8.10 ± 0.42	7.15 ± 0.30	6.80 ± 0.38	4.00 ± 0.00	8.15 ± 0.30	6.90 ± 0.38	6.65 ± 0.40	3.80 ± 0.38

muscles is given in Figure 6. The level of activity was initially found to be low in the kidneys and it went up from 9.00 ± 0.40 to 24.50 ± 0.00% within 120 min p.i. Group II also showed a similar profile. The disappearance of activity from the circulatory system and appearance in the urinary system established the normal path of excretion.

The authors concluded that ^{99m}Tc-TMC complex was synthesized and characterized in terms of radiochemical stability in saline and serum, *in vitro* binding with live and heat killed *Streptococci pneumoniae* and biodistribution in male Wistar rat (MWR) models. The complex showed a maximum radiochemical stability of 98.00 ± 0.34% at 30 min. In serum, the complex was found stable at 37°C up to 16 hours and saturated *in vitro* binding with live *Streptococci pneumoniae* was noted. Based on the above results, the suitability of the ^{99m}Tc-TMC complex as a potential *Streptococci pneumoniae* infection localizing agent is confirmed.

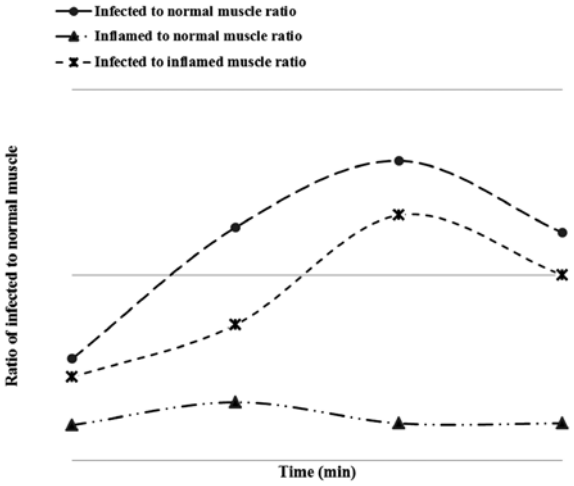

Fig. 6. Ratio wise distribution of the ^{99m}Tc-TMC complex in MWR model

Ryc. 6. Rozkład kompleksu ^{99m}Tc-TMC w modelu MWR

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Conflict of interest: None declared

Received: 15.06.2011
Revised: 29.11.2012
Accepted: 13.06.2013