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LABELING AND EVALUATION OF FREEZE-DRIED OFLOXACINE KITS FOR ^{99m}TC LABELING

Mahmoud A. Mahdy
Department of Pharmaceutics, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

ABSTRACT

Ofloxacine was labeled with ^{99m}Tc. The labeling reaction was carried under the specified conditions. The yield of the labeled ofloxacine was determined using TLC and HPLC and it was higher than 95%. The radioactive preparation is able to localise in both bacterial and sterile inflammations induced by Escherichia coli and turpentine oil, which suggest that its accumulation is due to increased blood flow together with enhanced vascular permeability. Also the freeze-dried kits were designed for direct labeling technique. The solution of Sn-ofloxacine was sterilized by 0.22μm millipore filtration and dispensed in a laminar flow hood (5 ml/vial), then the vials were introduced in the lyophilizer. The process of lyophilization was continued for 24 h. At the end of the cycle, the vials were closed under nitrogen. The prepared ofloxacin freeze-dried kits have high radiochemical purity ≥ 95% and it was stable for at least 8 h. after labeling.

INTRODUCTION

Fluoroquinolones represent a new group of compounds that exhibit a very high activity against a broad spectrum of bacteria. ^{18}F-quinolones as Fleroxacine[1] and Tosufloxacine[2] which have three fluorine atoms in its native structure, have been labelled with ^{18}F by fluorine exchange and proposed as tracers for PET measurements in the study of the pharmacokinetics of the quinolones in normal and infected animals and humans. Among the marketed quinolones ofloxacine (9-fluor-2,3-dihydro-10-(4-methyl-1-yl)-7-methyl-7-oxo-7 H-pyrido [1,2,3-de]-1,4-benzoxacine-6-carboxylic acid), a new quinolone Fig. (1) with activity against both gram-negative and gram-positive organisms. Owing to its biological properties ofloxacine has been described as a promising agent for the treatment of different kinds of infections. Vinganmen et-al have recently developed a new technetium-99m radiopharmaceutical (^{99m}Tc infection) based on ciprofloxacine[3-5]. The prepared freeze-dried kits was evaluated in respect to the following parameters: moisture content, radiochemical purity, sterility, apyrogeneicity, cardiac toxicity and in-vivo distribution.

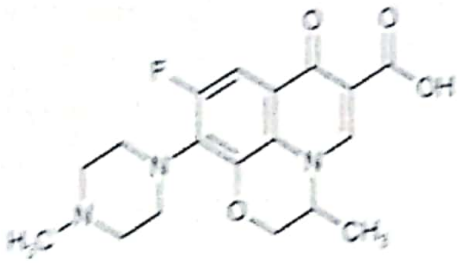

Fig. (1) The chemical structure of ofloxacine

EXPERIMENTAL

Chemicals:

Ofloxacine was purchased from Memphis Pharmaceutical Co. the chemical structure is presented in Fig. (1). The purity of ofloxacine was assayed using HPLC system with RP-18 column and all the chemicals and reagents used in the preparation of ofloxacine freeze-dried kits were of analytical grade.

- Thioglycolate media for aerobic and anaerobic microorganisms: OXOID LTD., Basingst-oke, Hants, England.
- Soyabean-casein digest media for moulds and fungus: BDH chemicals LTD. Poole, England.

Animals:

Mice and rabbits were used in the present work. The first was used for biological distribution study and the second was used for pyrogen testing.

Mice: Albino type weighing 20-25g were divided in groups containing 3 mice each.

Rabbits: Chamshella type, each weighing 2-4 Kg were divided in groups each consists of 3 healthy rabbits. The temperature of rabbits was rectally recorded at 2 h intervals for 1-3 days before use and rabbits with temperature below 38.8°C or higher than 39.9°C were rejected.

Radiolabeling of ofloxacine:

Ofloxacine was radiolabeled with ^{99m}Tc. To a suitable amount of ofloxacine, a suitable amount of Sn^{2+} and 0.5-1.5 ml (37-185 Mbq) of ^{99m}Tc-sodium pertechnetate solution were added and the reaction mixture was then boiled at 100 °C for 15 min.

Preparation of Sn-ofloxacine for freeze drying:

All the stock solutions in this experiment are prepared just prior to kit preparation.

1- 100 mg of ofloxacine was dissolved in approximately 75 ml 0.5 M phosphate buffer pH 7, purged with N_2 gas, then the solution was cooled.

2- 5 ml of stannous chloride solution, (1 mg/ml) was added.

3- The pH of the solution was adjusted to 7 by dropwise addition of 2 M NaOH and the solution was completed to 100 ml.

4- The solution was sterilized by millipore filtration in a laminar flow.

5- The sterile solution was dispensed into 1ml quantities in sterile vials and fitted with sterile rubber closure.

6- The vials were transferred to the freeze drier and lyophilized for 24h.

7- The vials were stoppered under dry sterile nitrogen gas.

8- The vials were stored at 4-6°C.

Determination of moisture content:

The moisture content of the freeze-dried kit was determined by the drying method according to the Egyptian pharmacopoeia[6].

Determination of Tin (II) content:

The freeze-dried kit of ofloxacine was analyzed for Tin(II) content by photometric assay technique[7]. The standard curve was constructed by drawing absorbance via standard Tin(II) concentration ranging from 5 to 100 µg. After 15 min the intensity of the oragne color was measured at 460 nm against blank solution.

Test for sterility, apyrogenicity and undue toxicity:

The test for sterility and undue toxicity were carried out according to the British pharmacopoeia[8]. The test for apyrogenicity was carried out according to the Egyptian pharmacopoeia[9].

Determination of radiochemical purity:

The radiochemical purity of ofloxacine freeze-dried vials was determined using HPLC RP-18 and TLC systems. A spot from ^{99m}Tc-ofloxacine solution was applied, then the sheet was developed in an ascending manner in a closed jar filled with N_2 gas, to prevent oxidation of the labeled compound. The developing solvents, acetone and 0.9% NaCl, were also bubbled with N_2 for the same purpose. The sheet after complete development, was dried and cut into sections 1cm each. Then the sections were counted in a well type γ-counter. The percent of free pertechnetate was determined from the distribution of activity on the TLC as free pertechnetate moves with the solvent front, R_f =1.0, while reduced hydrolyzed technetium, RH-^{99m}Tc' stays at the starting line with R_f =0.00 when either 0.9% Nacl or acetone was used.

HPLC analysis:

^{99m}Tc-ofloxacine complex was analyzed using HPLC equipped with UV and radiometric detector. The UV detector was set at 294 nm and a senstivity of 0.002 absorbance unit full scale. 5µl of the reaction mixture was injected to the RP-18 reverse phase column and the elution was done using acetonitrile: 0.05 M phosphate buffer pH 3:0.01 M tetrabuty1 ammonium bromide (1:4:1) and adjust the final pH to 3 by the addition of 1 M phosphoric acid which was pumped through the column at flow rats 0.5 ml/min.

A radiochromatogram for the reaction mixture was shown in Fig (2), which shows the presence of two peaks. The first peak of ^{99m}Tc-ofloxacine was appeared at the fraction 5 with retention time 5 min and the second peak was appeared at the fraction 31 with retention time, T_r 31 min which is the location of $^{99m}TcO_4^-$.

Animal experiments:

Infection models:

Groups of 6 female Albino mice, weighting approximately 25 gm each, were injected with 100µl ($2x10^6$ organism) suspension of E. coli in the right lateral thigh muscle to produce focal infection. Twenty four hour later, when gross swelling was apparent in the infected thigh the ^{99m}Tc-ofloxacine (100 µl) was I.V. administered to each animal. At 4 and 24 h following injection. The animals were sacrificed. Both thighs (target and non - target) were dissected and counted and target to non-target thigh radioactivity ratio was then determined.

Sterile abscess / inflammation:

Sterile abscess/inflammation was induced by injecting 100 µl of turpentine oil intramuscularly in the right lateral thigh muscle. At 1 to 8 days after the turpentine oil administration, ^{99m}Tc-ofloxacine (100 µl) was i.v. administered to each animal. At 4 and 24 h following injection, the animals were sacrificed. Both thighs (target and non - target) were dissected and counted and target to non-target thigh radioactivity ratio was then determined. The results were expressed as percent of injected dose per organ (% I.D/organ). Mean values and standard deviations were determined, the activity was calculated assuming that blood, bone and muscle were assumed to be 7, 10 and 40% respectively of the total body weight[10].

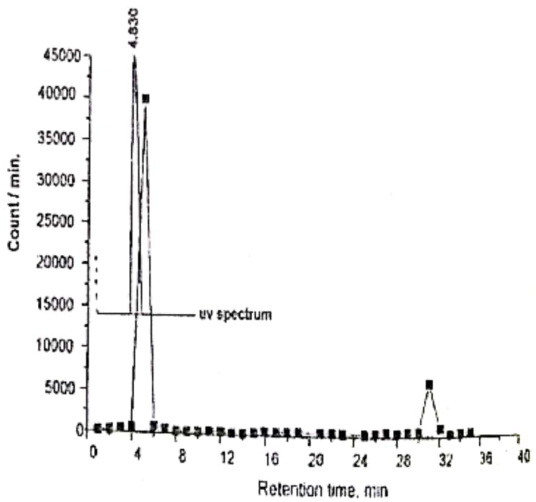

Fig. (2): HPLC & UV profiles of ^{99m}Tc-ofloxacine [conditions: Rp-18 reverse phase column, mobile phase acetonitrile: 0.05 M phosphate buffer pH3: 0.01 M tetrabutyl ammonium bromide (1:4:1) and adjust the final pH to 3 with 1 M phosphoric acid at flow rate 0.5 ml/min.]

RESULTS AND DISCUSSION

1- Effect of substrate concentration:

The dependence of the percentage of the labeling yield of ofloxacine concentration has been studied and the results are shown in Table 1 which clearly shows that by increasing substrate content the yield was increased to a maximum (98%), at 1mg ofloxacine. Increasing ofloxacine concentration above 1mg, the radiochemical yield decreased by small amount with an increase of the reduced hydrolyzed technetium.

Table (1): Effect of ofloxacine amount on the percent yield of ^{99m}Tc-ofloxacine (x mg ofloxacine, 50µg Tin(II), 20 min reaction time at 100 °C at pH 7)

Substrate amount, mg	% Radiochemical species		
	RH-^{99m}Tc^{III}	$^{99m}TcO_4^-$	^{99m}Tc-ofloxacine
0.1 mg	13.5 ± 1.3	1.5 ± 0.3	85 ± 1.1
0.2 mg	8.1 ± 1.0	0.9 ± 0.1	91 ± 0.9
0.5 mg	5.2 ± 0.5	0.8 ± 0.1	94 ± 0.8
1.0 mg	2.0 ± 0.0	0.0 ± 0.0	98 ± 0.5
2.0 mg	4.5 ± 0.7	0.5 ± 0.0	95 ± 0.7

Mean ± S.D. (Mean of three experiments)

2- Effect of Tin (II) content:

The chemical form of ^{99m}Tc available from the ^{99}Mo/^{99m}Tc generator is sodium pertechnetate, $^{99m}TcO_4^-$ which is non - reactive and doesn't label any compound. Prior to preparation of ^{99m}Tc - radiopharmaceutical, reduction of $^{99m}TcO_4^-$ from the 7+ state to a lower valence state, 3+, 4+ or 5+, is required. Various reducing agents can be used but the most commonly used is the stannous chloride in an acidic medium. The concentrations of tin (II) 5, 10, 20, 50, 100 µg were added to the solutions containing 1mg ofloxacine, then ^{99m}Tc eluate was added. The results shown in Table 2 indicate that 50 µg tin (II) is an optimum quantity, which gives a high radio chemical yield > 95%, at pH 7.

Table (2): Effect of Tin (II) content on the percent yield of ^{99m}Tc- ofloxacine

(1 mg ofloxacine, x µg Tin (II), 20 min reaction time at 100 °C at pH 7)

Tin content, µg	% Radiochemical species		
	RH-^{99m}Tc and Sn colloid	$^{99m}TcO_4^-$	^{99m}Tc-ofloxacine
5 µg	9.2 ± 0.4	28.3 ± 1.2	62.0 ± 2.1
10 µg	7.5 ± 0.5	25.3 ± 1.1	67.2 ± 1.9
20 µg	3.2 ± 0.0	24.8 ± 1.0	72.0 ± 1.0
50 µg	0.5 ± 0.0	4.0 ± 0.1	95.0 ± 0.8
100 µg	10.4 ± 0.3	5.5 ± 0.2	85.0 ± 1.2

Mean ± S.D. (Mean of three experiments)

3- Effect of pH of the reaction mixture:

Table 3, shows the pH dependence of the yield of ^{99m}Tc-ofloxacine when $^{99m}TcO_4^-$ is reduced with 50µg of tin (II) in the presence of 1 mg ofloxacine. The ^{99m}Tc-ofloxacine complex was greater than 95% at pH 7 after 20 min reaction time. At pH 4 or less the complex yield decreased rapidly up to 15%.

Table (3): Effect of pH of the reaction mixture on the percent yield of ^{99m}Tc-ofloxacine

(1mg ofloxacine, 50 µg Tin(II), 20 min reaction time at 100 °C at different pH values)

PH	% Radiochemical species		
	RH-^{99m}Tc^{III}	$^{99m}TcO_4^-$	^{99m}Tc-ofloxacine
1	29.0 ± 0.9	55.5 ± 0.8	15.5 ± 0.1
4	25.5 ± 1.0	49.5 ± 1.1	25.0 ± 0.2
7	0.5 ± 0.0	3.6 ± 0.5	95.9 ± 0.8
9	11.0 ± 1.0	9.0 ± 0.9	80.0 ± 0.7
11	35.2 ± 1.0	16.3 ± 1.0	48.5 ± 1.0

Mean ± S.D. (Mean of three experiments)

4- Effect of reaction temperature:

The labeling was carried out using 1mg ofloxacine, at pH 7, after 20 min reaction time, at different temperatures to attain the optimum temperature at which maximum labeling yield was obtained. Table 4 illustrates that by increasing the reaction temperature, the radiochemical yield was increased until a maximum of 97.2% at 100 °C after 20 min, while at 200 °C the reaction yield was equal to 91%.

Table (4): Effect of reaction temperature on the percent yield of ^{99m}Tc-ofloxacine

(1mg ofloxacine, 50 µg Tin(II), 20 min reaction time, at different reaction temperatures, at pH 7)

Temperature	% Radiochemical species		
	RH-^{99m}Tc^{III}	$^{99m}TcO_4^-$	^{99m}Tc-ofloxacine
25 °C	35.0 ± 1.2	25.5 ± 1.1	40.0 ± 0.1
45 °C	16.7 ± 0.9	18.3 ± 0.9	65.0 ± 1.2
60 °C	3.9 ± 0.1	11.1 ± 0.8	85.0 ± 0.8
100 °C	0.3 ± 0.0	2.5 ± 0.1	97.2 ± 0.5
200 °C	7.2 ± 0.3	1.8 ± 0.0	91.0 ± 0.3

Mean ± S.D. (Mean of three experiments)

5- Effect of reaction time:

Table 5 illustrates the relationship between reaction time and the yield of ^{99m}Tc- ofloxacine. It is clear that the radiochemical yield was increased from 75% to 97.5% with increasing reaction time from 1 min to 20 min at 100°C, using 1 mg ofloxacine.

Table (5): Effect of reaction time on the percent yield of ^{99m}Tc-ofloxacine

(1mg ofloxacine, 50 µg Tin (II), at 100 °C, at pH, 7, at different reaction time).

Time/min	% Radiochemical species		
	RH-^{99m}Tc^{III}	$^{99m}TcO_4^-$	^{99m}Tc-ofloxacine
1 min	13.8 ± 0.9	16.2 ± 1.2	75.0 ± 1.0
5 min	5.9 ± 0.3	13.0 ± 1.1	81.1 ± 1.1
10 min	3.3 ± 0.1	9.5 ± 0.5	87.2 ± 1.0
20 min	0.5 ± 0.0	2.0 ± 0.0	97.5 ± 1.1
30 min	4.9 ± 0.1	5.6 ± 0.0	90.5 ± 1.0

Mean ± S.D. (Mean of three experiments)

Evaluation of the freeze - dried ofloxacin kit:

1- Moisture content:

The moisture content of the ofloxacine freeze-dried vials depends on the condition of freeze drying process. Increasing the time of drying and product temperature cause of a removal of water vapor from the sample. The process of lyophilization was continued for 24 h. The percent of moisture content was determined according to the Egyptian pharmacopoeia, it was found that ofloxacine freeze - dried vials have 0.1% moisture content and this is in a good agreement with the reported limit[11].

2- Tin II content:

Standard solutions of tin (II) were prepared and standard curve was plotted spectrophotometrically, Fig (3). Data on the evaluation of tin (II) content and its percentage found in the prepared ofloxacine freeze-dried vials were presented in Table 6. The results of this analysis clearly show very small loses of tin (II) during preparation and freeze-dried process which doesn't affect the percent of the yield of ^{99m}Tc-ofloxacine complex as will be indicated.

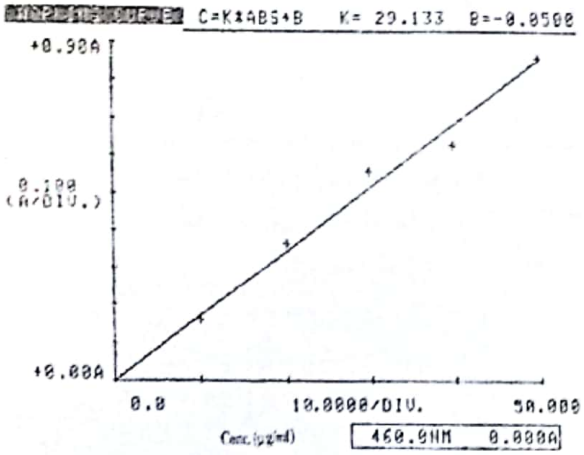

Fig. (3): The standard curve for tin (II) determined spectrophotometry

Table (6): Percent of tin (II) amount found in the freeze-dried ofloxacine kits at the end of freeze-dried cycle.

Kit type	The amount of tin (II), µg		% Tin (II) found
	Added	Found	
Ofloxacine Kit	50	48 ± 0.9	96

Mean ± S.D. (mean of three experiments)

3- Sterility, apyrogenicity and undue toxicity:

All injectable materials must be sterile, free from pyrogenic substances and have no toxicity to the human. Sterility means that the radiopharmaceuticals do not contain any kind of viable microorganisms. Most of radiopharmaceutical preparations are heat sensitive and are sterilized by filtration through 0.22 µm millipore filter, all the prepared ofloxacine freeze-dried kits were subjected to sterility test using both thioglycolate and soyabean - casein media. The kits were found sterile since no growth was detected in both culture media.

Most pyrogen are heat stable, filterable and soluble substances that exist as a result of contamination by bacteria, viruses, yeast, molds or occasionally chemicals. The usual pyrogen is the products of gram - negative bacterial cell wall, so called endotoxins that are polysaccharides. The test for pyrogen was carried out according to the Egyptian pharmacopoeia. It was found that no rabbits show an individual rise in temperature of 0.6°C or more above its normal temperature. Thus the in-house prepared ofloxacine freze-dried vials were pyrogen free since analytical grade chemicals and freshly prepared solutions were used in the preparation steps.

Radiopharmaceuticals should be prepared from the highest quality chemicals and the purest radioisotopes. However, it is possible that in some instances, a toxic substance may be incorporated or be formed. Thus, in the quality control, a test for undue toxicity is performed on all oral and parenteral preparation. This test was carried out according to the Egyptian pharmacopoeia procedure and the obtained data referred that the product is non toxic.

4- In - vitro stability:

The in-vitro stability for both solution and kit form, after labeling with technetium -99m was determined using ITLC/Saline or acetone system. The results of this study was presented in Table 7, which clearly show that the yield of ^{99m}Tc ofloxacine are not less than 95%.

Table (7): The in-vitro stability of the ^{99m}Tc-ofloxacine complex prepared before and after kit formulation

Type	Radio-chemical Species	% Radioactivity at different times post labeling		
		15 min	4 H	8 H
Solution Form	$^{99m}TcO_4^-$	1.5 ± 0.3	1.6 ± 0.3	2.0 ± 0.5
	^{99m}Tc-ofloxacine	96.5 ± 1.2	96.1 ± 1.1	95.5 ± 1.2
Kit Form	$^{99m}TcO_4^-$	1.4 ± 0.3	1.5 ± 0.3	1.9 ± 0.4
	^{99m}Tc-ofloxacine	96.1 ± 1.1	95.8 ± 1.2	97.0 ± 1.1

Mean ± S.D. (Mean of three experiments)

5- Biodistribution pattern:

The tissue distribution of the labeled ofloxacine preparation was calculated as percentage of the injected dose per organ (% I.D /organ) in normal and infected mice. the data presented in Table 8 shows that ^{99m}Tc-ofloxacine is rapidly distributed after i.v. administration as shown by the value of the renal elimination. The radioactivity ratios between the target to non-target thigh obtained at 4 and 24 h after administration was equal to 2.2 and 2.5% respectively in case of E.coli and to 3.0 and 2.7% respectively in case of turpentine oil. Based on biological data, it is observed that ^{99m}Tc-ofloxacine was taken up by both bacterial and sterile inflammations. This finding led us to admit that the accumulation detected could be related to increased blood flow to the area together with enhanced vascular permeability, which facilitate the passage of the tracer to the extra vascular space.

Table (8): Biodistribution data percent injected total organ for ^{99m}Tc-ofloxacine 4 and 24 h after i.v. administration in normal infected and inflamed model.

Organ	Normal		*E. coli*		Turpentine	
	Time/hour					
	4 h	24 h	4 h	24 h	4 h	24 h
Blood	6.8±0.8	5.2±0.3	5.1±0.8	3.5±0.0	3.6±0.4	2.6±0.3
Bone	4.1±0.3	3.2±0.2	4.9±0.7	2.7±0.1	5.5±1.0	5.1±0.9
Muscle	3.2±0.2	1.9±0.2	3.3±0.5	2.1±0.0	3.8±0.8	2.7±0.5
Liver	8.4±1.0	7.5±0.9	8.0±1.0	4.5±0.5	8.1±1.2	6.2±1.1
Spleen	0.1±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.0
Heart	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0
Lung	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	1.1±0.1	1.2±0.0
Kidney	8.1±0.0	5.5±0.3	8.0±1.0	4.0±0.2	8.7±1.2	5.1±1.0
Stomach	8.4±1.0	6.1±0.4	7.1±1.0	5.0±0.3	7.5±1.0	6.1±0.9
Urine	9.6±1.1	36.5±0.4	21.1±1.1	36.0±0.3	30.0±1.2	41.5±1.1
Normal thigh	0.7±0.1	0.5±0.0	0.8±0.2	0.2±0.0	1.3±0.1	0.7±0.0
Infected thigh	--	--	1.8±0.3	0.5±0.0	4.7±1.0	1.9±0.2
Infected/Normal	--	--	2.2±0.2	2.5±0.3	3.0±0.4	2.7±0.4

Mean ± S.D. (Mean of six experiments)

CONCLUSION

The labeling of ofloxacine with technetium-99m under the designed conditions was done successfully with a yield higher than 95%. Biodistribution studies in inflammed mice have shown that ^{99m}Tc-ofloxacine is rapidlydistributed after I.V. administration. The radioactive preparation is able to localize in both bacterial and sterile inflammations induced by E.coli and turpentine oil. However its accumulation may be due to an increased blood flow together with an enhanced vascular permeability.

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ترقيم وتقييم الأوفلوكساسين فى الصورة المجفدة للترقيم بالتكنسيوم-٩٩م

محمود عبد الغنى مهدى

قسم الصيدلانيات – كلية الصيدلة – جامعة الزقازيق – الزقازيق – مصر

تم ترقيم مادة الأوفلوكساسين بالتكنسيوم-٩٩م حيث أجرى تفاعل الترقيم تحت ظروف معينة وتم استخدام كروماتوجرافيا الطبقة الرقيقة وكروماتوجرافيا السوائل ذات الضغط العالى فى تعيين النقاوة الراديوكيميائية لمعقد الأوفلكساسين-تكنيسيوم ٩٩م وكانت النسبة أكبر من ٩٥%. سبتت كفاءة هذا المعقد فى التمركز باماكن الإلتهابات المعقمة أو الناتجة عن استخدام بكتيريا الإشريشياكولاى ويعزى هذا التمركز إلى إزدياد سريان الدم ونفاذية الأوعية الدموية.

تم صياغة هذه التركيبة فى صورة مجفدة وذلك بتعقيم محلول أوفلوكساسين قصديروز بتمريره من خلال مرشحات بكتيرية ذات قطر ٠,٢٢ ميكروميتر ثم تعبئته فى زجاجات بنسلين معقمة (١ مل/زجاجة) وذلك داخل دولاب الهواء المعقم ثم نقلها إلى جهاز التجفيد. استغرقت عملية التجفيد ٢٤ ساعة حيث تم إغلاق الزجاجات فى نهاية الدورة تحت ضغط موجب لغاز النيتروجين وكانت الزجاجات المحتوية على المادة المجفدة ذات نقاوة راديو كيميائية ≤ ٩٥% وذات درجة ثبات عالية لمدة ثمانى ساعات بعد إضافة التكنيسيوم-٩٩م.