



Dose-Dependent Internalization and Externalization Integrity Study of Newly Synthesized ^{99m}Tc -Thymoquinone Radiopharmaceutical as Cancer Theranostic Agent

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Abstract

Thymoquinone (TQ) is a bioactive phytochemical isolated from *Nigella sativa* and has been investigated for biochemical and biological activities in both in vitro and in vivo models. It is best known for its anticancer activities. Thymoquinone accomplishes anticancer activities through targeting multiple cancer markers including PPAR- γ , PTEN, P53, P73, STAT3, and generation of reactive oxygen species at the cancer cell surface. The radiolabeling of TQ with γ - and β -emitter radionuclide could be used as cancer diagnostic or therapeutic radiopharmaceutical, respectively. In this study, we are reporting the radiolabeling of TQ with technetium-99m (^{99m}Tc), stability in saline and blood serum, internalization and externalization of ^{99m}Tc -TQ using rhabdomyosarcoma cancer cells line. The quality control study revealed more than 95% labeling yield and stable in blood serum up to 4 hours. In vitro internalization rate was recorded $27.08\% \pm 0.95\%$ at 1 hour post 2 hours internalization period and comparatively slow externalization. The results of this study are quite encouraging and could be investigated for further key preclinical parameters to enter phase I clinical trials.

Keywords

thymoquinone, phytochemical, rhabdomyosarcoma, theranostic potential, radiopharmaceuticals

Introduction

Cancer is a major threat to humanity throughout the globe and is listed as the second leading cause of deaths after myocardial infections.¹ It is reported that there are seven cancer causing hallmarks which include selective growth and proliferative advantage, alter stress response which favor overall survival, vascularization, invasion and metastasis, metabolic rewiring, an abetting microenvironment, and immune modulation.² These different mechanisms translate into a variety of cancers, which includes cancer of tissues or skin that covers internal organs such as lung, breast, prostate, stomach, and colon cancers, and sarcomas which include cancer of bone or muscle.³ These hallmarks provide a roadmap to understand the remarkable diversity of cancers and to design a strategy to diagnose and treat them.^{3,4}

Nuclear medicine techniques (NMT), since its development has proved itself the best diagnostic strategy to locate the origin of fever-of-unknown and hard to diagnose human diseases. High

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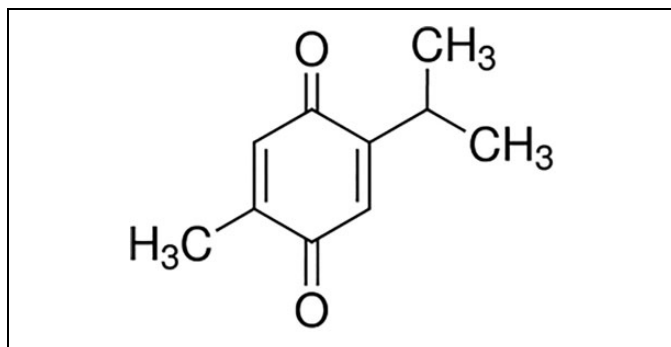


Figure 1. Structure of thymoquinone.

radiolabeling yield (>95%), promising biological stability, and high accumulation potential of radiopharmaceutical at diseased cells are the basic parameters of developing new nuclear medicine imaging agent. However, for the development of cancer therapeutic radiopharmaceuticals, the efficacy of therapy largely depends on the trafficking of radiopharmaceutical in and out from the diseased cells.⁵ The radiopharmaceuticals which based on targeting cancerous activities, mainly arrest the cancerous mechanism in cancer cells and show slow/delayed externalization.⁶ The phenomenon of rapid internalization and delayed externalization of radiopharmaceuticals, makes the bases of successful nuclear medicine therapy.⁷ The phenomenon of internalization is either mediated by cell receptors or cell membrane endocytosis.⁵ The receptor-mediated path is the main pathway by which macromolecules are entered from the extracellular matrix to the cells through endocytosis.⁶ This phenomenon also refers to an important regulatory mechanism of signaling of messages through the cell membrane by G-protein-coupled receptors.⁸ The radiopharmaceuticals based on synthetic or biomolecules could be assessed for their theranostic potential by recording the rate of internalization and externalization in disease cells.⁹

Thymoquinone, the structure is shown in Figure 1, is recognized as an efficient chemotherapeutic natural product since its first extraction from plant body in 1961s. It has been reported that TQ is an effective natural product for therapy of rhabdomyosarcoma (RMS) which is a skeletal muscle malignancy and it is common in most parts of the world.¹⁰ The aim of this study was to investigate the theranostic potential of technetium-99m (^{99m}Tc) labeled TQ using best known preclinical studies such as internalization and externalization assay using RMS cancer cell line. Further, the fate of externalized fraction was also analysed through high-performance liquid chromatography (HPLC) to assess the possible radiocytotoxic effect.

Materials and Methods

Materials

All the materials and chemicals used for this study were of analytical grade. Rhabdomyosarcoma cell line was obtained

from National Institute of Health (NIH), Islamabad, and was subcultured in photomedicine research laboratory at Pakistan Institute of Engineering & Applied Sciences, Islamabad. Cell culturing medium (Eagle's Minimum Essential Medium [MEM]) was obtained from Institute of Biomedical & Genetic Engineering at KRL Hospital, Islamabad, having 10% fetal bovine serum (FBS) and 1% of MEM of nonessential amino acids with 1% penicillin-streptomycin as antimicrobial agent. Stannous chloride dihydrate ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$), genestic acid, and TQ were purchased from Sigma-Aldrich (Steinheim, Germany). The ^{99m}Tc was eluted from locally produced fission based PAKGEN ⁹⁹Mo/^{99m}Tc generator system, obtained from Pakistan Institute of Nuclear Science and Technology, Islamabad.

Radiolabeling of TQ With ^{99m}Tc

Radiolabeling of TQ with ^{99m}Tc was carried out following the method developed earlier.¹¹ Briefly, to the 1 mL solution, containing 200 µg of TQ, added 1 mL solution of reducing agent that was prepared by dissolving 25 mg $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 0.1 mL concentrated HCl at ambient temperature and then diluted to 1 mL with doubly distilled water. Then added 550 MBq ^{99m}TcO₄⁻¹ radioactivity, eluted with 0.9% saline solution from ⁹⁹Mo/^{99m}Tc generator, to the reaction vial. The pH of the reaction mixture was adjusted to 5 with the help of 0.05 M HCl or NaOH and the reaction mixture was then incubated at room temperature for 1 hour with periodic gentle shaking.

Quality Control Studies

The radiochemical purity and potential radioactive impurities, that is, free ^{99m}TcO₄⁻¹ and hydrolyzed ^{99m}Tc was analyzed with the help of instant thin layer chromatography impregnated with silica gel (ITLC-SG) and paper chromatography. The both strips were analyzed using 2π-scanner (Dunnschicht-Scanner II, 2B 2723 Berthold, Germany, Vorschub Scanning).

Paper Chromatography Analysis

An aliquot of 2 µL was spotted at the baseline of Whatman 3MM paper strip (14 × 1.5 cm dimension) and developed with acetone solvent as mobile phase. In this system, the free ^{99m}TcO₄⁻¹ eluted with mobile phase to solvent front ($R_f = 0.9-1$), while the ^{99m}Tc-TQ and hydrolyzed technetium fraction remained at origin ($R_f = 0.0-0.1$).

Instant Thin Layer Chromatography Impregnated With Silica Gel Analysis

An aliquot of 2 µL was spotted at the baseline of ITLC-SG strip (14 × 1.5 cm dimension) and developed with 0.05 M NaOH solution as mobile phase. In this system, the ^{99m}Tc-TQ and free ^{99m}TcO₄⁻¹ eluted with mobile phase to the solvent front ($R_f = 0.9-1$), while the hydrolyzed technetium fraction remained at origin ($R_f = 0.0 - 0.1$).

From the analysis of the both chromatography procedures, using the expressions written below, the percentage of radiochemical purity of $^{99m}\text{Tc-TQ}$ and potential impurities (free $^{99m}\text{TcO}_4^{-1}$ and hydrolyzed technetium fraction) were calculated.

$$\text{Free } ^{99m}\text{TcO}_4^{-1}(\%) = \frac{\text{Activity at solvent front } (R_f = 0.9-1)}{\text{Total activity counted over ITLC strip}} \quad (\text{A})$$

$$\begin{aligned} \text{Hydrolyzed technetium } (\%) \\ = \frac{\text{Activity at base line } (R_f = 0.0-0.1)}{\text{Total activity counted over ITLC strip}} \quad (\text{B}) \\ ^{99m}\text{Tc-TQ } (\%) \end{aligned}$$

$$= \text{Total activity counted over ITLC strip} - (\text{A} + \text{B})$$

High-Performance Liquid Chromatographic Analysis

The labeled compound was also assessed with HPLC connected with NaI γ -ray and UV-visible detector. The HPLC analysis was carried out with D-200 Elite HPLC system having C-18 column as stationary phase; isocratic system using water:methanol:2-propanol (50:45:5) solution as mobile phase was selected for the analysis at a flow rate of 1.0 mL min^{-1} . Analysis time was set to 28 minutes and the detection wavelength was set at 254 nm.

In Vitro Saline and Blood Serum Stability of $^{99m}\text{Tc-TQ}$

The stability of $^{99m}\text{Tc-TQ}$ radiopharmaceutical was tested in saline for shelf life and in blood serum for biological stability. After filtration of $^{99m}\text{Tc-TQ}$ complex mixture, the radiochemical was diluted to 2 mL with 0.9% saline solution and incubated in sterile conditions. Similarly, 200 μL $^{99m}\text{Tc-TQ}$ complex mixture was mixed with 3 mL freshly harvested blood serum and incubated in an incubator at 37°C . To record the percentage of an intact radiochemical percentage, an aliquot of 2 μL was spotted at chromatography strips at 30 minutes, 1, 2, and 4 hours. The strips were then run either in acetone or 0.05 M NaOH solution mobile phase and analyzed for percent intact $^{99m}\text{Tc-TQ}$ with 2π -scanner.

Rhabdomyosarcoma Cell Line Culturing

For in vitro and in vivo evaluation of $^{99m}\text{Tc-TQ}$; RMS cells were grown to the appropriate confluency of 80% in MEM supplemented with nonessential amino acids and 1% FBS in cell culture flask. Culture flask was placed in CO_2 incubator having humidified air containing 5% CO_2 at 37°C . Penicillin-streptomycin (1% of total medium volume) was added into the culture media as antimicrobial agent. The cells were subcultured (split) at ratio 1:2 every 3 to 4 days.

Cell Subculturing Procedure

Cell culturing flasks were continuously assessed after 2 to 3 days to obtain more than 80% confluent cells for subculturing. On obtaining more than 80% confluent cells, the cells were

subcultured into new cell culturing flasks. Briefly, centrifuged the cell culture at $150 \times g$ for 5 minutes in Falcon tubes and again transferred into the culture flask. Then 0.5 mL trypsin was added into Falcon tubes so that the cells can be detached from the interior wall of the tube which then aspirated to culture flask. The flask was then placed into the incubator for 2 to 3 minutes, removed, gently shaken to break the clumps of cells, and viewed under trinocular inverted microscope for complete separation of cells followed by the addition of 5 mL MEM (10%) by pipetting out and in. Finally, 2.5 mL cell suspension was aspirated into new flasks and maintained the volume of each flask up to 10 mL by adding 7.5 mL media in each flask. All the flasks were then placed in CO_2 incubator under cell culturing conditions to use for further experimental work.

Internalization Assay

For internalization assay, 1 million/mL RMS cells were seeded onto 6-well plates 48 hours before the experiment and allowed to be grown to confluency in CO_2 incubator under cell culture conditions. The assay was performed by following the protocol reported by Naqvi et al⁵ with slight modification; briefly, the cell cultured suspension was aspirated into 1.8 mL Eppendorf tubes and centrifuged at $1000 \times g$ for 5 minutes at 4°C , decant the supernatant, washed twice with internalization media (MEM supplemented non-essential amino acids and 1% [V/V] FBS), added 1.2 mL internalization media into each tube, homogenized, and finally transferred onto 6-well plates (1 plate was used for 2 time points). Similar volume (1.2 mL) of internalization media was also added into 3 wells for control and then incubated all the plates for >1 hour at 37°C . At the end of the incubation, ~ 3 to 4 pmol of $^{99m}\text{Tc-TQ}$ complex in 150 μL phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) was added to each well with and without cells. The wells without RMS cells were used as control to measure the total radioactivity added. Three plates were then incubated for 10, 30, 60, 90, or 120 minutes at 37°C . At the end of the incubation period, the internalization reaction mixture was quickly transferred to eppendorf tube followed by transferring about 300 μL ice-cold internalized media after washing each well. The cell was centrifuged at $400 \times g$ for 5 minutes, the supernatant was decanted, and again added 1.5 mL ice-cold internalized media; centrifuged and then decanted the supernatant into previously collected supernatant to count the unbound activity. The cell pellet was then subjected twice to 1 mL 0.05 M glycine-HCl acid wash buffer by dissolving the pellet in buffer, centrifuging, and retaining the supernatant to count the surface-bound activity. Finally, the internalized activity was counted by putting the Eppendorf tubes having cell pellets into the NaI well-type γ -counter detector. Following the γ -photon counting, the pellet was again dissolved in culture media and transferred to 6-well plate for incubating at cell culture conditions in CO_2 incubator to look into externalization of internalized radiochemical.

Externalization Assay

For externalization assay, similar to internalization assay 48 hours before the experiment, 1 million/mL RMS cells were seeded onto three 6-well plates (3 wells for each time point). On the day of the experiment, the cell culture suspension from each well was transferred to 1.8 mL Eppendorf tubes and centrifuged at $1000 \times g$ for 5 minutes at 4°C , decanted the supernatant, washed twice with internalization media (MEM supplemented nonessential amino acids and 1% [V/V] FBS), added 1.2 mL internalization media into each tube, homogenized, and finally transferred onto 6-well plates. Similar volume (1.2 mL) of internalization media was also added into 3 wells for control and then incubated all the plates for more than 1 hour at 37°C . At the end of the incubation, ~ 3 to 4 pmol of $^{99\text{m}}\text{Tc-TQ}$ complex in 150 μL PBS/1% BSA was added to each well and again incubated the plates for 90 minutes for internalization (constant time point for internalization and varied time point for externalization). After incubation, the cell solution was transferred to Eppendorf tubes, centrifuged, rinsed the pellet with 1 mL internalized media, decanted the internalized media after centrifuging, then performed twice 1 mL 0.05 M glycine-HCl buffer ($\text{pH} = 2.8$) acid washing to detach any surface-bound activity. The pellet was rinsed with 1 mL of PBS/1% BSA and suspended in 1.2 mL prewarmed (at 37°C) internalized media and transferred onto 6-well plates. The plates were then incubated for 15, 45, 90 and 180 minutes. The externalization process was quenched by quick transferring the cell suspension into Eppendorf tubes and centrifuging. The supernatant media was collected in γ -counting tubes. The cell pellet was subjected to twice washing with 1 mL ice-cold internalized media and the washing media was also collected into respective γ -counting tubes to measure total externalized activity. The cells were then also subjected to radiocounts to measure the retained activity. The similar study was also performed to record externalized fraction for 1 hour (varied time internalization and constant time point for externalization) time point for 30, 60, 120 and 240 minutes internalized activity.

Cell Microscopy

The imaging of RMS cells were performed during seeding onto 6-well plates, after acid wash, and internalization and externalization process to record any change in morphology of the cells using Nikon optishot trinocular microscope connected to a Nikon UFX camera control unit (Japan Optics). The cell imaging was carried out by adding 1 mL of 0.04% (wt/vol) *Trypan Blue* into acid-washed RMS cells followed by gently shaken and incubation for 5 minutes. At the end of incubation period, a 10 μL aliquot was placed on an hemocytometer and taking the image with microscope.

Results

The percent yield of radiochemical and radioactive impurities was calculated through the counts obtained from

radiochromatogram analysis using 2π -scanner. The radiolabeling reaction yielded $>95\%$ $^{99\text{m}}\text{Tc-TQ}$ and $<5\%$ free $^{99\text{m}}\text{TcO}_4^{-1}$ and hydrolyzed impurities. The results of 2π -scanner analysis are shown in Figure 2. The radiochemical purity was further analyzed with HPLC as shown in Figure 3.

Stability Study of $^{99\text{m}}\text{Tc-TQ}$

Figure 4 shows the stability of $^{99\text{m}}\text{Tc-TQ}$ complex in physiological saline and in freshly harvested human blood serum. The complex was found quite stable in both mediums at room temperature and physiological temperature, respectively. In saline and blood serum conditions at 4 hours post incubation (PI) more than 93% intact $^{99\text{m}}\text{Tc-TQ}$ complex was recorded.

Rhabdomyosarcoma Cell Line Culturing

Rhabdomyosarcoma cells were seeded onto 96-well plates with 1×10^4 cells per well. The cultured cells adhere to the surface of well are considered alive and the those floating in the media considered dead. Further, the clumps of floating cells were larger than the adherent cells.^{12,13} The dead cells show more heterogeneity than the adherent cells and also show diversity in heterogeneity in cellular responses. This behavior is an indicator of heterogeneity in functions among the cellular population against different drug sensitivity. The appearance of the cultured cell line was seen by using phase-contrast microscopy.

Internalization of $^{99\text{m}}\text{Tc-TQ}$

Time-dependent internalization curve of $^{99\text{m}}\text{Tc-TQ}$ in RMS cells revealed a rapid endocytosis of radioligand at 37°C as shown in Figure 5. About 25% to 27% internalization plateau was reached within 45 to 60 minute incubation and then showed constant plateau over 120 minutes. At 30 minute incubation $9.78\% \pm 0.94\%$ of total $^{99\text{m}}\text{Tc-TQ}$ added activity was internalized, $2.12\% \pm 0.74\%$ activity was found bound to cell surface that was increased to $27.08\% \pm 0.98\%$ and $5.78\% \pm 1.15\%$ by incubating the cells up to 120 minutes, respectively, as shown in Figure 5. The washout activity, the activity that was not internalized or some of the internalized activity that got externalization into internalization medium (nonsurface bounded), at particular time point is shown in Figure 6.

Externalization of Internalized $^{99\text{m}}\text{Tc-TQ}$ Activity

The externalized activity of internalized $^{99\text{m}}\text{Tc-TQ}$ activity was recorded in 2 ways; in the first way, the $^{99\text{m}}\text{Tc-TQ}$ activity was allowed to internalize up to 90 minutes and then measured the externalized activity at 15, 45, 90 and 180 minutes time points as shown in Figure 7A. In the second way, the $^{99\text{m}}\text{Tc-TQ}$ complex was allowed to internalize at varied time points, that is 30, 60, 120, and 240 minutes PI and the externalized fraction was counted at 1 hour time point as shown in Figure 7B.

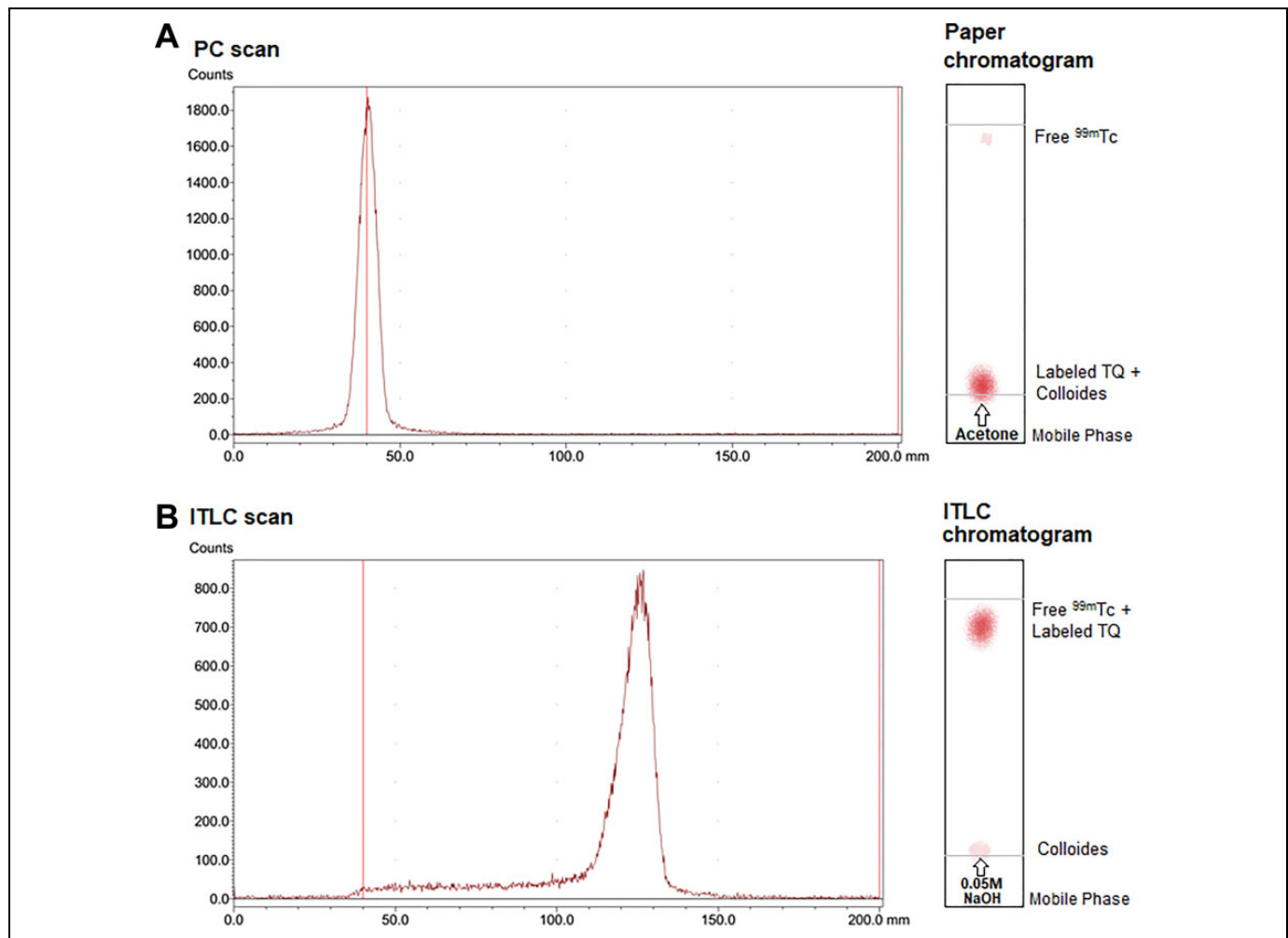


Figure 2. Paper (A) and ITLC (B) chromatograms to calculate radiochemical purity of ^{99m}Tc -TQ radiopharmaceutical synthesis. ITLC indicates instant thin layer chromatography; TQ, thymoquinone.

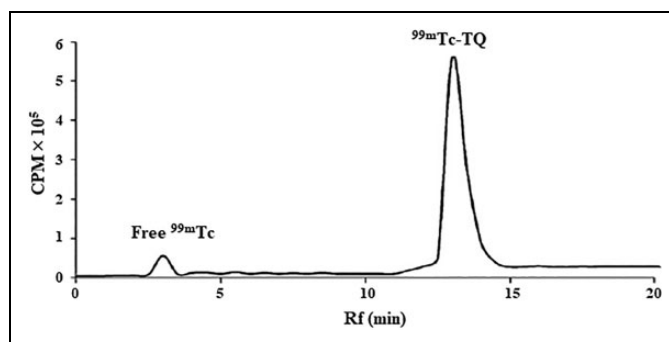


Figure 3. High-performance liquid chromatography analysis of ^{99m}Tc -TQ.

Chromatography Analysis of Externalized Fraction

The externalized activity was also analyzed at selected time points to analyze the degradation pattern of externalized fraction using HPLC, which showed both intact and degraded products. However, the intact fraction was low as compared to degraded fractions as shown in Figure 8.

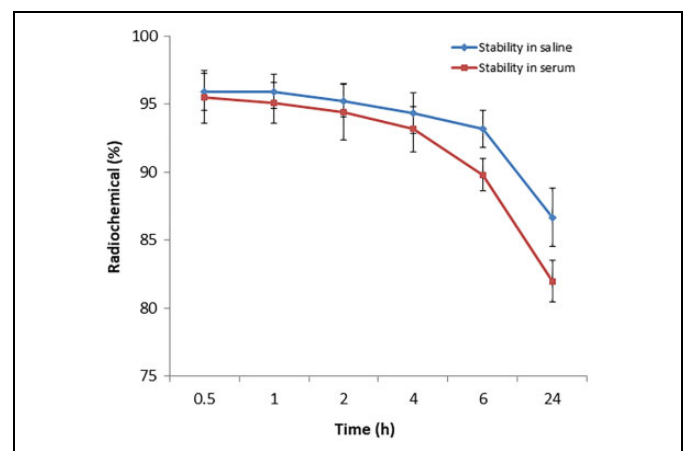


Figure 4. Stability of ^{99m}Tc -TQ in injectable saline and blood serum.

Cell Microscopy

The appearance of the cell line was imaged with phase-contrast microscopy. Before and after treating the seeded cells with

^{99m}Tc -TQ, the appearance of the cells is shown in Figure 9. The incubation span of the cell line with ^{99m}Tc -TQ affected the physiological appearance. The intact cells remain adherent to

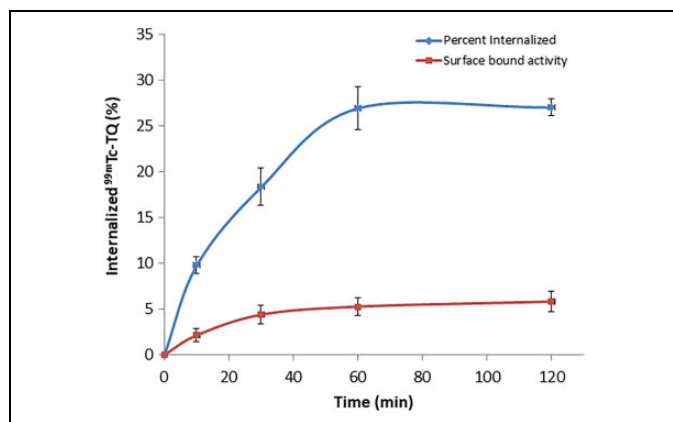


Figure 5. Internalized and cell surface-bound activity of ^{99m}Tc -TQ.

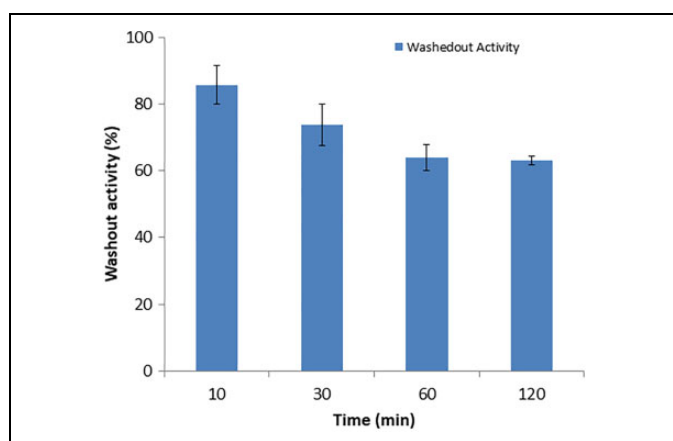


Figure 6. Washout activity of ^{99m}Tc -TQ from rhabdomyosarcoma cell surface.

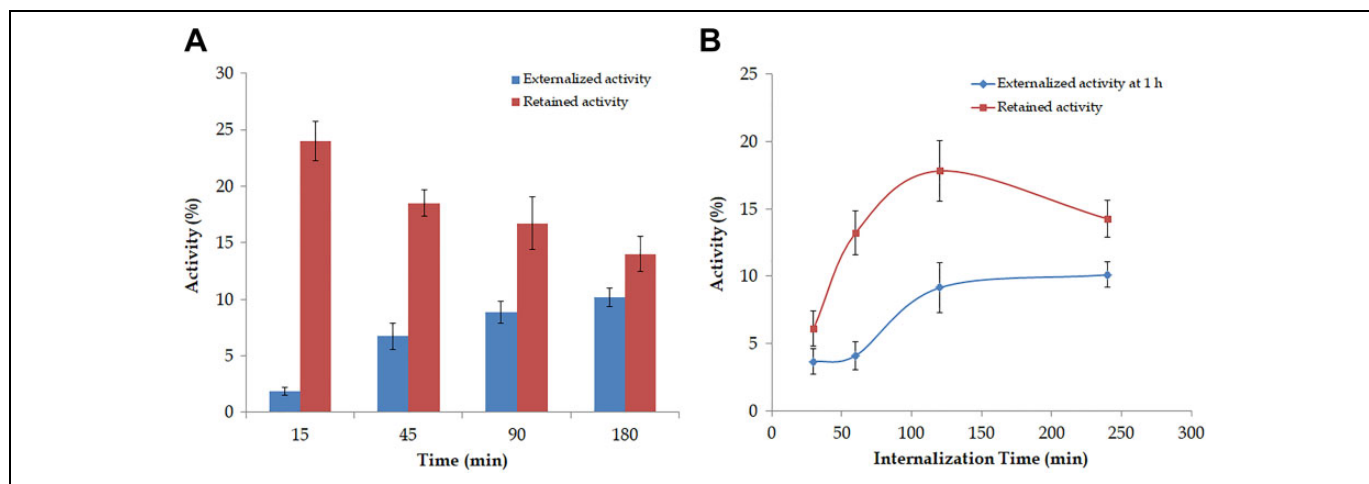


Figure 7. Internalization (A) and externalization (B) study.

the surface of cell culture plate, while the dead cells were found floating in internalization medium which increased with the passage of time (by incubating with ^{99m}Tc -TQ) as compared to the fraction without ^{99m}Tc -TQ.

Discussion

Chromatography analysis using paper, ITLC, and HPLC revealed more than 95% radiochemical yield and less than 5% radioactive impurities that mainly comprises of colloidal particles and free $^{99m}\text{TcO}_4^{-1}$. Good radiochemical yield (i.e. >90%) is a prerequisite for safe diagnosis and therapy of diseases in oncology set-up.¹⁴ The colloidal particles that exceed in size from 0.22 μm may block the blood capillaries and consequently lead to nonspecific accumulation and radiotoxicity,¹⁵ while the free $^{99m}\text{TcO}_4^{-1}$ lead to nonspecific distribution throughout the body making high background activity and made confusion during locating the area of interest.¹⁶ The least radioactive impurities greatly contribute in scintigraphy imaging and therapeutic outcome of newly developed radiopharmaceuticals. In the current study, the labeling of ^{99m}Tc with TQ was carried out due to its unique anticancer activity.¹⁷ The radiolabeling reaction resulted in >95% ^{99m}Tc -TQ yield that makes it a quite suitable candidate for the diagnosis of a variety of cancers. The radiochemical shelf life study revealed $\geq 93.18\% \pm 1.39\%$ intact ^{99m}Tc -TQ at 6 hour post-labeling in injectable saline solution and $\geq 93.17\% \pm 1.19\%$ intact ^{99m}Tc -TQ was calculated at 4 hour post-labeling period in freshly harvested human blood serum which shows ^{99m}Tc -TQ is quite stable in clinical and physiological conditions. It bears promising biological stability and could be safely administered into the animals for preclinical studies and patients for phase-I clinical trails.¹⁸⁻²¹

The cytotoxicity and cell trafficking of radiochemicals are most important and necessary parameters which are strictly studied for the development of radiopharmaceutical in addition to biodistribution and scintigraphy studies. In vitro cell trafficking, that is, internalization and externalization of

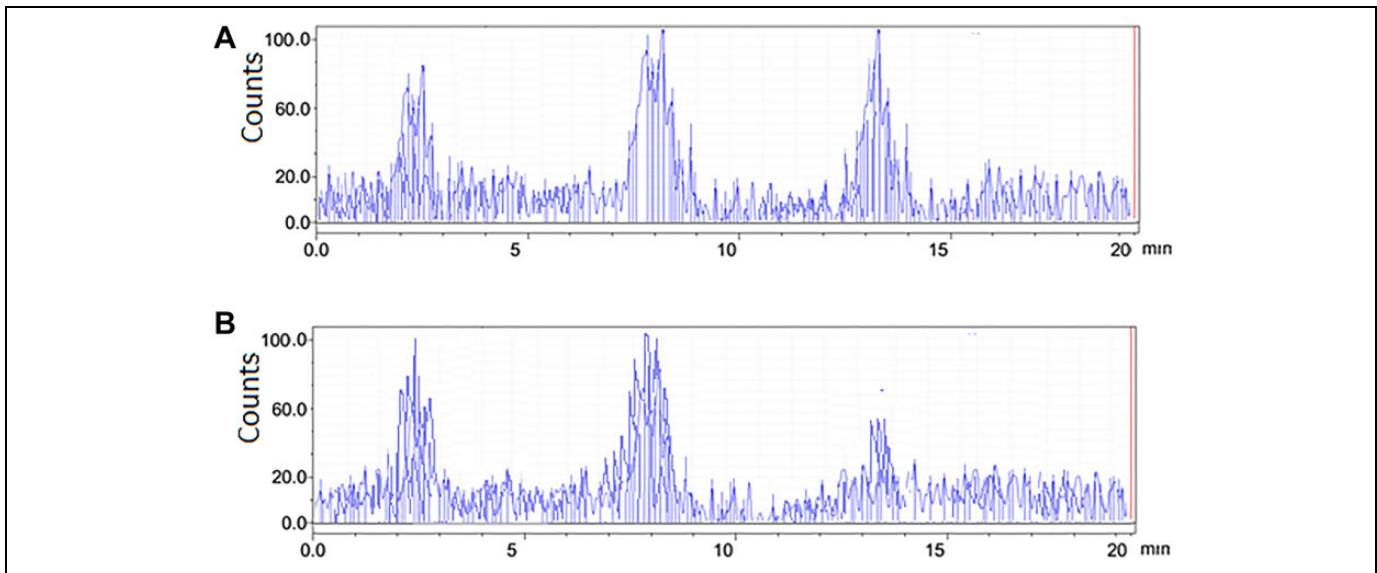


Figure 8. High-performance liquid chromatography analysis of externalized fraction at 30 minutes (A) and 180 minutes (B).

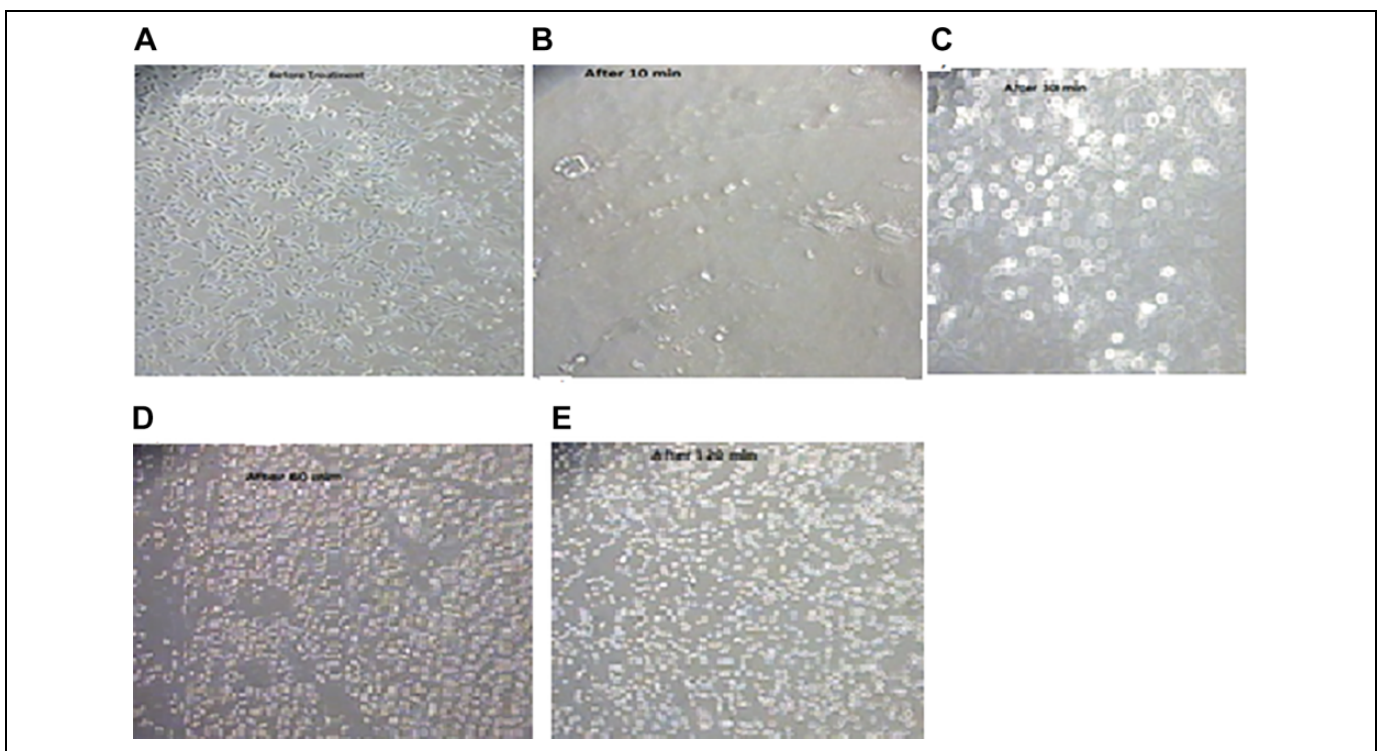


Figure 9. Rhabdomyosarcoma cancer cell line microscopy while performing internalization and externalization. Seeded cells before treatment were in a triangular shape (A) and cells that have been incubated with labeled thymoquinone for 10 minutes (B), 30 minutes (C), 60 minutes (D), and 120 minutes (E) have different physiological behavior.

radiolabeled compound into and out of cancer cells is generally used as a tool to investigate the effect of cell environment on the fate of radiopharmaceuticals.²² Generally, rapid internalization rate into cancer cells accompanied by a slow externalization rate is needed for promising imaging and therapeutic results. In vitro rate of internalization, which is mediated by

cell membrane endocytosis and cell surface-bound activity was studied with RMS cancer cells line at 10, 30, 60 and 120 minutes PI time points. The results, as shown in Figure 5, revealed $9.78\% \pm 0.94\%$ internalization and $2.12\% \pm 0.74\%$ surface-bound activity at 10 minute PI period which increased to $26.98\% \pm 2.34\%$ and $5.23\% \pm 0.94\%$ at 60 minute PI period,

respectively. No significant increase in internalization of ^{99m}Tc -TQ was recorded at 120 minute time point. Although, the cell membrane mediated endocytosis is a slow process as compared to receptor-mediated endocytosis which in most cases reach to 90% internalization at 30 minute PI period, but it showed a promising value of internalized ^{99m}Tc -TQ. TQ have no specific receptor at the surface of RMS cells due to which cell membrane lipid bilayer interaction with ^{99m}Tc -TQ appeared to be the main mechanism to engulf the radiotracers into the cell cytoplasm. Only small percent of activity remained bound at cell surface and rest was washed-out, which was not internalized or bound to the surface (Figure 6).

Externalized fraction of internalized activity was counted at a varied and constant post-internalized period. In case of varied externalized time points, at each successive time point increase in the externalized fraction was recorded, that is, at 15 minutes $1.89\% \pm 0.34\%$ activity of $26.98\% \pm 2.34\%$ internalized activity was externalized which increased to $10.17\% \pm 0.85\%$ at 180 minutes externalized time point (Figure 7A). In case of constant externalized period, the results showed that at 1 hour post 30 minutes internalization ($9.78\% \pm 1.14\%$ of incubated activity) $2.66\% \pm 0.94\%$ was externalized which increased to $10.11\% \pm 1.31\%$ at 1 hour post 120-min internalized activity which was $27.08\% \pm 1.45\%$ (Figure 7B). In both models, one thing was common; the ratio of internalized and externalized activity at a particular time point almost showed similar trend. A promising amount of internalized activity with slow externalization behavior advocate the potential of ^{99m}Tc -TQ for imaging of cancer cells.⁶ Chemotherapeutic drugs and variety of receptor-mediated radiopharmaceuticals were subjected and approved for clinical practice due to strong invasion into cancer cells.^{6,23}

The nature of externalized radioactive fraction obtained at 30 and 180 minutes was studied with the help of HPLC. The chromatogram mainly showed 3 peaks, the intact ^{99m}Tc -TQ, degraded fraction, and free $^{99m}\text{TcO}_4^{-1}$. The chromatogram obtained at 30 minutes (Figure 8A) showed the intact ^{99m}Tc -TQ fraction eluted at 13.2 minutes, the peak at 8 minutes showed uncharacterized degraded radioactive fraction, and the peak eluted at 2.27 minutes reflected free $^{99m}\text{TcO}_4^{-1}$ in addition to one other small peaks eluted at 5 minutes. The externalized fraction collected at 180 minutes also showed a similar pattern (Figure 8B) but free and degraded radioactive fraction was recorded with increased counts, while the intact ^{99m}Tc -TQ at 13.2 minutes showed very poor counts. This indicates that up to 180 minutes, either the intact ^{99m}Tc -TQ invaded the maximum cancer cell machinery or degraded into fragments. However, the counts of free and radioactive fragments are quite low at both time points which reduce the chance of nontargeted accumulation and consequently radiotoxicity. Further, the coherence in the degradation pattern of 30 and 180 minutes externalized activity showed that newly developed radiopharmaceutical has specific and simple degradation pattern and could be tested for preclinical evaluation.

Conclusion

The radiopharmaceuticals are developed for imaging and therapeutic purposes of a variety of diseases. Our study showed $\geq 95\%$ radiochemical yield, $93.18\% \pm 1.39\%$ intact ^{99m}Tc -TQ at 6 hours post labeling in injectable saline solution, and $\geq 92\%$ intact complex at 4 hours PI in blood serum. Microscopy study revealed the minimal effect of ^{99m}Tc -TQ on cell line and cell trafficking of the complex is favorable. Results obtained from certain key parameters including radiochemical yield, shelf life, blood serum stability, radiotoxicity, and targeted diseased cell specificity depicted by accumulation percentage, internalization into diseased cells, and externalization studies revealed ^{99m}Tc -TQ is strong candidate for further investigations at preclinical and clinical levels.

Authors' Note

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Declaration of Conflicting Interests

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