Applying Quality by Design Principles in the Development and Preparation of a New Radiopharmaceutical: Technetium-99m-Imatinib Mesylate

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ABSTRACT: The clinical impact and accessibility of 99mTc tracers for cancer diagnosis would be greatly enhanced by the availability of a new, simple, and easy labeling process and radiopharmaceuticals. In this study, Technetium-99m-imatinib mesylate ([99mTc]Tc IMT) was developed and prepared as a new radiopharmaceutical for breast cancer diagnosis. The effect of critical process parameters on the product quality and stability of [99mTc]Tc IMT was investigated using the quality by design concept of the ICH Q8 (Pharmaceutical Development) guideline. [99mTc]Tc IMT was subjected to in vitro cell binding studies to determine healthy and cancer cell affinity using HaCaT and MCF-7 cells, respectively. The optimal radiolabeling procedure with 1 mg of IMT, 500 μg of stannous chloride, 0.1 mg of ascorbic acid, and 1mCi 99mTc radioactivity was obtained for [99mTc]Tc IMT. The pH of the reaction mixture was adjusted to 10 and allowed to react for 15 min at room temperature. The radiochemical purity of [99mTc]Tc IMT was found to be higher than 90% at room temperature up to 6 h. Chromatography analysis revealed >85% [99mTc]Tc IMT complex formation with promising stability in saline, cell medium, and serum up to 6 h. The radiolabeled complex showed a higher cell-binding ratio to MCF-7 cells (88.90% ± 3.12) than HaCaT cells (45.64 ± 4.72) when compared to 99mTc. Our findings show that the developed preparation method for [99mTc]Tc IMT falls well within the proven acceptable ranges. Applying quality by design (QbD) principles is feasible and worthwhile for the preparation of [99mTc]Tc IMT. In conclusion, radiochemical purity, stability, and in vitro cell binding evaluation of the [99mTc]Tc IMT complex indicate that the agent can be utilized for imaging of breast cancer cells.

INTRODUCTION

Imatinib mesylate (IMT), a tyrosine kinase inhibitor, acts by competitively blocking the ATP binding site of the receptors. It has revolutionized medicine as a specific drug for the treatment of chronic myeloid leukemia.1 Except this, IMT has been used in many diseases which exemplified by various protein kinase-active cancers such as gastrointestinal stromal tumors, especially breast cancer.1−8

Diagnosis of breast cancer can be good if it is stated in early phases; however, in the presence of metastatic disease, five year survival decreased significantly. The development of new imaging agents and techniques is important for early diagnosis of breast cancer and patient survival.9,10 Nuclear medicine imaging techniques have advantages over radiological imaging in terms of higher specificity with a similar high sensitivity for cancer detection.11,12 Moreover, the use of nuclear medicine imaging in detection of tumors allows prevention of unnecessary diagnostic invasive procedures. More than 80% of all nuclear medicine, single photon emission computerized tomography (SPECT) scans are used to detect cancer with radiopharmaceuticals.13 Radiopharmaceuticals consist of two components, a pharmaceutical and radionuclide with a specific radiation.13,14 For diagnostic purposes, gamma emitter radionuclides are preferred in accordance with their low linear energy transfer which results in low tissue damage in the targeted organ. Among gamma emitter radionuclides, Technetium-99m (99mTc) is the most used radioisotope in the preparation of SPECT radiopharmaceuticals. Tc-99m is the decay product of Molybdenum-99 (Mo-99), which is mainly generated in research reactors, and has ideal properties such as 6 h physical half-life, low cost and energy, and ready availability.14−16 The pharmaceutical part of radiopharmaceuticals is responsible for the accumulation of the radionuclide in
the targeted tissue. High accumulation in the target tissue and low accumulation in the nontarget tissue are preferred to increase the resolution and sensitivity of the images and to reduce radiation damage in the rest of the body. To achieve the high target/nontarget ratio, the receptor specific molecules are radiolabeled for cancer imaging and therapy. Herein, IMT and 99mTc were used as pharmaceutical and radionuclide parts to prepare the (99mTc)TcIMT complex that is formed by the direct radiolabeling method (Figure 1).

There are samples of radiopharmaceuticals including 99mTc-labeled small-molecule inhibitors. The investigators at Molecular Insight Pharmaceuticals (MIP) developed a series of novel glutamate–urea (Glu–urea) amino acid heterodimeric inhibitors of PSMA for developing SPECT radiopharmaceuticals using 99mTc because it is the optimal radionuclide for developing SPECT radiopharmaceuticals, and the investigators at MIP developed 2 high-affinity small-molecule PSMA inhibitors, MIP-1404 and MIP-1405, which are successfully radiolabeled using 99mTc based on novel tricarbonyl chemistry.17,18 The QbD approach was described in the International Council for Harmonization (ICH) guidelines such as Q8 Pharmaceutical Development, Q9 Quality Risk Management, and Q10 Pharmaceutical Quality System. These systems are the fundamental guidelines for drug research.19,20

The main function of QbD is developing a method that is suitable for keeping the changes within an acceptable range in order to maintain the same product quality, instead eliminating the changes in formulation or process steps. For this purpose, experimental studies should be designed, necessary process and product assessments and measurements should be made, data analyses should be performed, and the design space should be formed.21

The aim of this study is to develop a new radiopharmaceutical that is 99mTc]TcIMT and investigate the influence of preparation conditions on the quality of the radiopharmaceutical product and its stability by application of QbD principles. For this purpose, the concentration of IMT was determined in vitro cytotoxicity studies. IMT was radiolabeled using 99mTc, and the quality control of the radiolabeled complex was performed with radioactive thin layer chromatography (RTLC). The effect of formulation parameters such as reducing agent, antioxidant agents, and incubation time on the labeling efficiency was aimed to be enlightened and obtain an optimum value for each parameter via MODDE Pro (Sartorius Stedim Data Analytics) and create design space. Furthermore, the in vitro cell-binding capacity of 99mTc]TcIMT to MCF-7 and HaCaT cells was investigated.

**RESULTS**

**In Vitro Cytotoxicity Studies.** The cell viability ratios were found to be 85.5, 89.9, 93.5, 96.3, 97.8, and 99% for 1500, 750, 350, 150, 75, and 5 μg/mL in MCF-7 cells at the end of 48 h (Figure 2). Furthermore, 1500 μg/mL IMT provides 83.5% cell viability, 750 μg/mL provides 88.9% cell viability, 350 μg/mL provides 94.7% cell viability, 150 μg/mL provides 98.5% cell viability, 75 μg/mL provides 99.1% cell viability, and 5 μg/mL provides 99.6% cell viability for HaCaT cells at the end of 48 h (Figure 2). According to the results, while the IMT concentration increased, cell viability % decreased. An IMT concentration of 350 μg/mL and below provides above 90% cell viability for MCF-7 and HaCaT cells. The IC50 values were calculated as 34.6 ± 2.7 and 25.3 ± 1.44 μM for MCF-7 and HaCaT cells, respectively. These values suggested that the concentration applied in MCF-7 and HaCaT cells was not likely to cause toxicity and further radiolabeling studies were performed with 1 mg of IMT.

**Defining Optimized Formulation.** To diagnose different types of cancer disease, new radiopharmaceuticals are developed and their standard procedure is still being studied by research groups and health authorities. The critical parameters such as the reducing agent, antioxidant agent, and incubation time for the radiolabeling process provide optimized radiopharmaceuticals and influence the quality of these products. Within the scope of QbD, modelling parameters to obtain optimized formulation were evaluated, and the results are given in Figure 3.

The design and knowledge space limit values of the inputs and outputs were calculated with multiple linear regression (MLR), and the results are shown in Figure 4. The requirement of the QbD approach such as minimum–maximum values of the design space was provided from the knowledge space. After providing a successful design space with the QbD steps, the optimum formulation was determined using MODDE pro, and the results are demonstrated in Table 1.
One formulation was prepared using these optimal parameters, and then, quality control of this formulation was performed. The optimal formulation’s contents were found to be 554.5 mg of stannous chloride, 0.0625 mg of ascorbic acid, and 8.125 min of incubation time with the predicted value of radiochemical purity.

Studies on Optimal Formulation. Radiolabeling Studies. The radiolabeling of IMT with $^{99m}$Tc was carried out. Radiochemical purity/radiolabeling efficiency of $[^{99m}\text{Tc}]\text{TcIMT}$ was analyzed by RTLC. The amounts of radioactive impurities were separated and quantified by two different solvent systems. The $R_f$ values of mobile phases and the RTLC chromatogram of $[^{99m}\text{Tc}]\text{TcIMT}$ are presented in Figure 5. The free $^{99m}\text{Tc}$ migrated forward with acetone as a solvent, while $[^{99m}\text{Tc}]\text{TcIMT}$ and reduced/hydrolyzed (R/H) $^{99m}$Tc remained at the spotting point in saline as the other solvent. The radiochemical purity of $[^{99m}\text{Tc}]\text{TcIMT}$ at 37 MBq was found to be over 90%.

Effect of the Reducing Agent on the Radiolabeling Process. The stability of the radiopharmaceuticals depends on its contents, such as the pharmaceutical part, reducing agent, and antioxidants used in the formulation. There are many studies in the preparation of new $^{99m}$Tc radiopharmaceuticals which have stannous chloride as a reducing agent. Stannous chloride also facilitated radiolabeling reactions.$^{22,23}$ In this study, the effect of stannous chloride on the radiolabeling process was examined, and ideal stannous chloride amount was found to be 500 $\mu$g. The radiochemical purity of $[^{99m}\text{Tc}]\text{TcIMT}$ with 500 $\mu$g/mL stannous chloride solution was over 90% and did not change significantly for 6 h at room temperature ($p > 0.05$) (Figure 6).

Effect of the Antioxidant Agent on the Radiolabeling Process. $^{99m}$Tc radiopharmaceuticals may have auto radiolysis during preparation, release, and storage. Decomposition of radiopharmaceuticals will decrease the targeting capability before and after administration. Therefore, it is very important to use a stabilizer to minimize the auto radiolysis. Radiolytic stabilizers are often antioxidants, such as ascorbic acid, gentisic acid, and $p$-aminobenzoic acid.$^{24}$ In this study, ascorbic acid was used as an antioxidant agent. The radiochemical purity of $[^{99m}\text{Tc}]\text{TcIMT}$ was found to be greater than 85% at 0.1 and

| Table 1. Optimum Formulation Given by the MODDE Pro |
|------------------------------------------|
| incubation time (min) | 8.125 |
| stannous chloride ($\mu$g) | 554.5 |
| ascorbic acid (mg) | 0.0625 |
| predicted value of labeling efficiency | 93.5 |

| RTLC-SG plaque |
|----------------|
| Acetone | Saline |
| Free $^{99m}\text{Tc}$ | 0.8-1.0 | 0.8-1.0 |
| R/H $^{99m}\text{Tc}$ | 0.6-0.1 | 0.0-0.1 |
| $[^{99m}\text{Tc}]\text{TcIMT}$ | 0.0-0.2 | 0.8-1.0 |

Figure 5. $R_f$ values of $[^{99m}\text{Tc}]\text{TcIMT}$ in mobile phases and the RTLC chromatogram of $[^{99m}\text{Tc}]\text{TcIMT}$ in different mobile phases: (A) acetone and (B) saline.
0.5 mg of ascorbic acid. Our results demonstrate that radiochemical purity in the presence of ascorbic acid was changed ($p < 0.05$) and 0.1 mg of ascorbic acid was used to radiolabel IMT (Figure 7).

Effect of Incubation Time on the Radiolabeling Process. The radiochemical purity of the complexes containing 500 $\mu$g of stannous chloride and 0.5 mg of ascorbic acid was examined at 5, 15, and 30 min to evaluate the effect of incubation time on the radiolabeling process. The radiochemical purity of $^{99m}$TcTcIMT was changed between sampling time intervals ($p < 0.05$) and found to be 75.4 ± 3.97% for 5 min incubation time at the end of study time (Figure 8). Over 90% radiochemical purity was achieved for $[^{99m}\text{Tc}]\text{TcIMT}$, and there was no statistical difference between the radiochemical purity values obtained at the end of the 6 h for 15 and 30 min ($p > 0.05$). The incubation time was selected as 15 min for the radiolabeling process, taking into consideration that the radiation exposure time should be kept to a minimum in ALARA principles.25

According to all results, specific activity and radiochemical yield of the radiolabeled complex were found to be 37 MBq and above 90%, respectively. The optimal radiolabeling procedure with 500 $\mu$g of stannous chloride and 0.1 mg of ascorbic acid was obtained for $^{99m}$TcTcIMT. Although $^{99m}$TcTcIMT was needed to adjust the body pH, preparations in small volume were adjusted to stabilize the pH. The pH of the radiolabeled complex was measured and found to be 10. Radiolabeling was performed with $^{99m}$Tc (37 MBq) in 0.9% sodium chloride solution (0.1 mL) and the solution was allowed to stand at room temperature for 15 min prior to radiochemical analysis. Also, the acidic (pH = 3−5) and neutral pH (pH = 7−7.4) values were studied to prepare $^{99m}$TcTcIMT but precipitation was observed in $^{99m}$Tc-TcIMT for both pH values.

Stability of $^{99m}$TcTcIMT in Different Media. The stability of $^{99m}$TcTcIMT was examined in saline, cell medium, and serum. The radiochemical purity of $^{99m}$TcTcIMT was found to be between 85 and 95% for 6 h in saline cell medium and saline ($p > 0.05$) (Figure 9).

Microbiological Analysis of $^{99m}$TcTcIMT. The absence and growth of microorganisms in the vials containing $^{99m}$Tc-TcIMT were observed visibly and clearly. Also, the gel clot test indicated that $^{99m}$TcTcIMT was pyrogen free.

Cell-Binding Studies. The cell-binding results of $^{99m}$TcTcIMT are demonstrated in Table 2. Although the cell-binding ratio of $^{99m}$Tc solution was found to be 15.77 ± 10.45, the cell-binding ratio of $^{99m}$TcTcIMT was found to be 88.90 ± 3.12% for MCF-7 cells. The highest MCF-7 cell-binding capacity was provided with $^{99m}$TcTcIMT. When the results obtained from HaCaT cells were evaluated, it was observed that the binding ratio was 45.64 ± 4.72 and 10.93 ± 1.035 for $^{99m}$TcTcIMT and $^{99m}$Tc, respectively. Also, the cell-binding capacity of $^{99m}$TcTcIMT was found to be higher than $^{99m}$Tc solution for each cell lines and time intervals.

**DISCUSSION**

The choice of radionuclide and pharmaceutical parts in the preparation of radiopharmaceuticals is significant. Although the pharmaceutical part of the radiopharmaceutical is kept in mind for obtaining desired localization, the radionuclide part should be thought in terms of the compatibility, half-life, energy emission and values, stability, stoichiometry, and molecular
size. Herein, IMT and 99mTc have created the pharmaceutical and radionuclide part of radiopharmaceuticals, respectively.

The aim of this study is to develop new radiopharmaceuticals by combining the radiolabeling process and QbD. In radiolabeling studies, varying most of the parameters investigated did result in adequate radiochemical purity and stability. However, in some experiments, unacceptable results (radiochemical purity < 90%) were obtained. Performing the labeling with different amounts of reducing and antioxidant agents, incubation time, and optimal conditions may yield a less stable product. The reducing agent is important for 99mTc radiopharmaceuticals. Although the colloidal systems form and the radiochemical purity starts to decline in the presence of high reducing agent concentrations, the free technetium ratio decreases in the presence of low reducing agent concentrations. Commonly, the stannous salts are used as reducing agents in 99mTc radiopharmaceuticals. Although technetium is a stable pertechnetate in the +7 oxidative state, radiolabeled compounds have been formulated with oxidation states from −1 to +7. Reducing agents lead to reduction of 99mTc from the +7 oxidation state to the more reactive +5 oxidation state to encourage the connection of 99mTc and the pharmaceutical part. Herein, 99mTc[99mTc]TcIMT was prepared using stannous chloride. Also, ascorbic acid is an antioxidant and used as a radiolysis stabilizer. Incubation time is another important parameter of the radiolabeling process. There are few studies about radiolabeling of IMT derivatives with the positron emission tomography (PET) radionuclide. Glekas et al. performed the synthesis of the 2-fluoroethyl analogue of imatinib (SKI696) and radiolabeled this molecule of pure IMT with 18F. IMT and SKI696 molecules (1–5 mg) with 200 μL of 18F containing 370–555 MBq of radioactivity and heat was applied on the labeling complex at 90 °C for 10–15 min. According to their results, 18F-IMT and SKI696 complexes remained stable (up to 90%) at room temperature for up to 4 h after labeling. Peng et al. synthesized two series of IMT (STI-571) analogues to develop a Bcr-Abl and c-KIT receptor-specific labeling agent for PET imaging. The radiolabeling of STI-571 with 18F and 131I was performed by the long and complex reaction. 18F-labeled STI-571 was prepared with high specific activity (75 GBq/μmol) by nucleophilic displacement and an average radiochemical purity of 12%. 131I-labeled STI-571 was prepared with high radiochemical purity (>90%). Kumar et al. developed the procedure for radiolabeling of an anticancer drug tamoxifen with 99mTc for tumor diagnosis and to evaluate the diagnostic effect of the radiolabeled compound in mice. Radiolabeling efficiency of tamoxifen with 99mTc was found to be 97%. In vitro stability of the labeled complex increased with time and reached maximum at 2 h (98.1 ± 0.17). This study indicates that 99mTc-labeled tamoxifen can be used as a potential tumor diagnostic agent with low uptake in normal tissues. In this study, IMT was labeled with 99mTc using a reducing agent and 99mTc[99mTc]TcIMT was found to be stable for 6 h. The various complexes of 99mTc may be formed by interactions between electron donor atoms and reduced technetium. In order to form bonds with technetium, the structure must contain electron donors such as oxygen, hydrogen, nitrogen, and sulfur. The possible nature of 99mTc[Tc]TcIMT binding is caused by coordination of IMT that has electron donor atoms such as nitrogen in its structure with 99mTc. The radiochemical purity > 90% was maintained for up to 6 h. In addition, the stability of 99mTc[Tc]TcIMT in human serum, cell medium, and saline was maintained for 6 h.

QbD is a scientific way that develops new pharmaceutical product development and produces scientific data quickly. The objective of QbD is rapidly yielding a more coherent pharmaceutical product with high quality in a short period. Although there are a number of studies in QbD-based nanostructured drug delivery system research,31,32 application of essential elements of the quality by design-concept from ICH Q8 is new for the preparation and development of radiopharmaceuticals.20,33,34 Herein, we combined and evaluated the QbD and preparation of 99mTc radiopharmaceuticals by the influence of changing preparation parameters on radiochemical purity and stability of 99mTc[Tc]TcIMT. The quality features and process parameters are significant to carry out desired QbD. They have been defined after obtaining 99mTc[Tc]TcIMT. The antioxidant and reducing agents and incubation time are the most fundamental features of 99mTc radiopharmaceuticals which are liable for efficacy/efficiency and stability of radiolabeled compounds.35 In this regard, these parameters were chosen as critical quality attributes. When QbD and practical acceptable ranges were evaluated together, the results obtained by both methods were not statistically different (p > 0.05) in this study. Mapping the proven acceptable ranges, by validating the preparation process for every individual parameter while keeping all other variables constant, is worthwhile for assuring the product quality and, therefore, contributes to the Good Manufacturing Practice.36

A high target to nontarget ratio of radiopharmaceuticals in the desired area has a critical role in clinical administrations. It can affect the quality of target organ images because radiopharmaceuticals can have little localization in the nontarget organs. The high specificity on the target organ is important for ideal radiopharmaceuticals; only then will it result in a high target to nontarget ratio. If it does not happen, radiopharmaceutical localization in nontarget areas can cause injury in these tissues. In vitro cell-binding studies indicated that the highest cell-binding capacity was observed with 99mTc[Tc]TcIMT for MCF-7 cells and the binding ratio of 99mTc[Tc]TcIMT to breast cancer cells was found to be higher than that of 99mTc solution. Also, the cell-binding ratio has been changed at time intervals (60 and 120 min), according to the obtained results (Table 2). Despite the exact reason not being explained, these changes may be due to the pharmaceutical and radionuclide part of 99mTc[Tc]TcIMT, affinity of 99mTc[Tc]TcIMT to cells, and time. Furthermore, we believe that these results can be a pioneer and guide for further in vivo studies.

Table 2. Cell Binding Ratio (%) of 99mTc[Tc]TcIMT and 99mTc (p < 0.05)

| time   | HaCaT cells | MCF-7 cells |
|--------|-------------|-------------|
| 99mTc  | [99mTc]TcIMT | 99mTc       | [99mTc]TcIMT |
| 60 min | 63.6 ± 1.71  | 39.29 ± 3.66 | 12.65 ± 1.90 | 62.05 ± 1.77 |
| 120 min| 10.93 ± 1.035| 45.64 ± 4.72 | 15.77 ± 10.45| 88.90 ± 3.12 |

**Note:** All values are expressed as mean ± standard deviation.
CONCLUSIONS

The radiolabeling process and QbD implementation were combined, and [99mTc]Tc-IMT was designed and prepared. A design space was formed for determining the quality target product profile by QbD, and the radiolabeling procedure was optimized using this design space. Optimal [99mTc]Tc-IMT was prepared as a result of mathematical modeling, and the quality control of [99mTc]Tc-IMT was realized. In the view of all results, a new radiolabeled complex has been developed which has predetermined quality features, high radiolabeling efficiency, and cell binding capacity for MCF-7.

EXPERIMENTAL SECTION

IMT was kindly provided by Novartis Pharma AG (Basel, Switzerland). Technetium-99m pertechnetate (Na99mTcO4) was obtained from the Department of Nuclear Medicine of Ege University. The MCF-7 and HaCaT cells were purchased from the American Type Culture Collection (ATCC).

In Vitro Cytotoxicity Studies. MCF-7 and HaCaT cells and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) were used for in vitro cytotoxicity studies. The cells (1 × 10^5/well) were seeded in 24-well plates. The cells were incubated with IMT in 0.1% dimethyl sulfoxide solutions containing IMT were taken out and 1 mg/mL MTT was added. After the 48 h incubation of IMT solutions and cells, the absorbance was measured at 540 nm to determine the viable cells.

Nonlinear regression was selected, and IC50 values were seen in absorbance values were entered as binding curves.6 The half maximal inhibitory concentration (IC50) was determined. IMT was directly labeled with any compound on direct addition. Therefore, prior to the labeling procedure, reduction of 99mTc is required to label with any compound on direct addition. Therefore, prior to the labeling procedure, reduction of 99mTc is required for converting 99mTc from the 99mTc(VII) state to a desired lower oxidation state, which can make complexes with the ligand to form the radiopharmaceutical. Different types of reduction agents are used for this reason. Herein, stannous chloride was used to perform the radiolabeling process.6 IMT (1 mg) was dissolved in 0.9% sodium chloride solution (1 mL). Reduction of 99mTc was performed with different amounts of stannous chloride in 0.01 N HCl (10, 50, 250, and 1500 μg/mL). Stannous chloride solution was added to IMT solution, under an atmosphere of bubbling nitrogen. Freshly eluted 37 mBq/0.1 mL 99mTc was added to each vial. The vials were filtered through a 0.22 μm pore size membrane filter, and incubated for 15 min at room temperature. The pH of the solution was measured. freshly eluted 37 mBq/0.1 mL 99mTc was added to each vial. The vials were filtered through a 0.22 μm pore size membrane filter, and incubated for 15 min at room temperature.

Effect of the Reducing Agent on the Radiolabeling Process. 99mTc pertechnetate was eluted from a 99Mo/99mTc generator in the 99mTc(VII) oxidation state which was not able to label with any compound on direct addition. Therefore, prior to the labeling procedure, reduction of 99mTc is required for converting 99mTc from the 99mTc(VII) state to a desired lower oxidation state, which can make complexes with the ligand to form the radiopharmaceutical. Different types of reduction agents are used for this reason. Herein, stannous chloride was used to perform the radiolabeling process.6 IMT (1 mg) was dissolved in 0.9% sodium chloride solution (1 mL). Reduction of 99mTc was performed with different amounts of stannous chloride in 0.01 N HCl (10, 50, 250, and 1500 μg/mL). Stannous chloride solution was added to IMT solution, under an atmosphere of bubbling nitrogen. Freshly eluted 37 mBq/0.1 mL 99mTc was added to each vial. The vials were filtered through a 0.22 μm pore size membrane filter, and incubated for 15 min at room temperature. The pH of the solution was measured. Freshly eluted 37 mBq/0.1 mL 99mTc was added to each vial. The vials were filtered through a 0.22 μm pore size membrane filter, and incubated for 15 min at room temperature. The radiochemical purity of the complex was determined by RTLC.

Effect of the Antioxidant Agent on Radiolabeling. IMT (1 mg) was dissolved in 0.9% sodium chloride solution (1 mL) in three groups of vials. Stannous chloride (500 μg/mL) in 0.01 N HCl was added to each individual group. The radiolabeling process was performed in the absence and presence (0.1 and 0.5 mg) of ascorbic acid. The pH of the solution was measured. Freshly eluted 37 mBq/0.1 mL 99mTc was added to each vial. The vials were filtered through a 0.22 μm pore size membrane filter, and incubated for 15 min at room temperature. The radiochemical purity of the complex was determined by RTLC.

Incubation Time. Incubation time was also determined as a critical parameter in the preparation process of radiopharmaceuticals. Studies have demonstrated that adequate labeling needs a certain amount of time.39,44

Identification of Critical Parameters in the Radiolabeling Process. Reducing Agent. Several studies concerning the usage of reducing agents such as stannous derivatives were identified in the literature.14,38–42

Antioxidant Agent. The amount of the antioxidant agent was identified as a critical parameter. The usage of ascorbic acid as an antioxidant agent is common43–45 for Tc-99m radiopharmaceuticals.

Incubation Time. Incubation time was also determined as a critical parameter in the preparation process of radiopharmaceuticals. Studies have demonstrated that adequate labeling needs a certain amount of time.39,44

Defining the Critical Ranges for the Identified Radiolabeling Process Variables. Radiolabeling Studies. Radiolabeling studies were performed in the presence of reducing (stannous chloride) and antioxidant (ascorbic acid) agents and different incubation time periods to find the optimum radiolabeling conditions. IMT was directly labeled with 99mTc. A total of 1 mg of IMT was dissolved in 1 mL of saline. The reducing agent solution (10, 50, 250, 500, and 1000 μg/mL stannous chloride in 0.01 N HCl) and the antioxidant agent (0.1 and 0.5 mg of ascorbic acid) were added to the solution. Radiolabeling was performed with freshly eluted 37 mBq/0.1 mL 99mTc. The mixture was shaken for 60 s, filtered through a 0.22 μm pore size cellulose nitrate membrane, and incubated for different time intervals (5, 15, and 30 min) at room temperature. The pH of the radiolabeled complex was examined using a pH meter, and the radiochemical purity of the complex was analyzed by RTLC.

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Stability of $^{99m}$Tc-TcIMT. In vitro stability of $[^{99m}Tc]$-TcIMT was estimated in the saline, cell medium, and serum by incubating 100 $\mu$L of the complex with 900 $\mu$L of media at room temperature. Aliquots at different time periods were applied on an ITLC-SG strip and allowed to run in 100% acetone to check any dissociation and degradation of the labeled complex. The dissociation was estimated as the % radioiodinated complex remaining after the incubation time of 6 h.

Implementation of QbD. Mathematical Modeling and Optimization. In order to achieve the optimum formulation and method which meets the required quality attributes, research studies and the data from preformulation studies were interpreted and the knowledge space of the formulation components and process parameters was established. Then, the experimental data were evaluated in a piece of software using statistical methods and response surface methodology in order to create an experimental design, model fitting to a response surface model using MLR, which was used for evaluation of the optimal formulation and process parameters, and form a design space. Also, it encompasses the multidimensional combination, interaction of input variables, and process parameters and has been shown to obtain the assurance of quality according to ICH Q8 (R2).20,21

In MODDE Pro 12, four statistical parameters such as $R^2$, $Q^2$, model validity, and reproducibility are used to establish the model accuracy which leads to the usage of the optimum formulation. A model fit with low significance has values of $R^2$ below 0.5. $Q^2$ which should be above 0.5 for a good model which refers to precision of the prediction. A value for reproducibility higher than 0.5 indicates reproducible results which can also lead to low model validity.48

Microbiological Analysis of Optimal $[^{99m}Tc]$TcIMT. Sterility Test. The radiolabeled complex was incubated with Thiglocollate broth and Tryptic Soy Broth medium at 37 $^\circ$C in the vials. The vials were controlled visibly during the sterility test.

Pyrogenicity Test. The pyrogenicity test is performed using a Pyrotell Gel-Clot formulation (Associates of Cape Cod Incorporated, Falmouth, MA, USA).

Cell Culture Studies. McCoy's 5A containing 10% fetal bovine serum was used for both cells. The cells were incubated at 37 $^\circ$C under 90% humidity and 5% CO$_2$.29,50

Cell-Binding Studies. A total of 1 $\times$ 10$^5$ cells/well were dispensed in 6-well plates and incubated for 7 days in an incubator to adhere the membrane. $[^{99m}Tc]$TcIMT (37 MBq/0.5 mL) was put into the cells and incubated for 120 min. The cell culture medium was collected at 60 and 120 min. After removal of the cell culture medium, the cells were consecutively washed with 1 mL of phosphate-buffered saline to separate free $^{99m}$Tc and trypsinized with 0.5 mL of trypsin to remove the cells. The cell medium (1 mL) was added to the trypsinized cells, and the system was centrifuged at 1000 rpm for 5 min. The activities in the tubes containing sediment cells and in the tubes containing culture medium were both counted using a gamma counter (Sesa Uniscaller). The radioactivity of cells as percentage was calculated from the following equation (eq 2)

Radioactivity of cells % = (radioactivity of cells/total radioactivity) $\times$ 100 (2)

Total radioactivity refers to the radioactivity of cells and cell medium.

Statistical Analysis. The calculation of means and standard deviations was performed on Microsoft Excel. One-way Anova was used to determine statistical significance. Differences at the 95% confidence level ($p < 0.05$) were considered significant.

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Notes

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