177Lu-DOTATATE Peptide Receptor Radionuclide Therapy: Indigenously Developed Freeze Dried Cold Kit and Biological Response in In-Vitro and In-Vivo Models

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Abstract
Somatostatin receptors (SStR) based 177Lu-DOTATATE therapy is known as one of the highly effective neuroendocrine tumors (NETs) treatment strategy. Development of DOTATATE freeze-dried kit for imaging and therapy of SStR positive NETs is a prime goal in neuroendocrine cancer research. The present work describes the development of 177Lu-DOTATATE freeze dried cold kit for indigenous needs, through technology development fund (TDF) program offered by Higher Education Commission (HEC) Pakistan. The parameters for freeze dried kit production was optimized and tested the stored lyophilized cold kits for different time intervals after labeling with 177Lu radioisotope. The effect of ligand to radionuclide ratio, pH and reaction time at 90°C was recorded. Five times greater molar concentration of ligand, pH 5 and 30 min reaction time were the effective reaction conditions for maximum radiochemical yield. The radiolabeling yield at 1 day, 1-week and 4-week post storing period showed ~100% radiochemical yield. The biodistribution study using rat model depicted the absence of non-targeted accumulation while glomerular filtration rate also explains the rapid renal washout. Cytotoxicity study showed quite favorable results for subjecting the radiopharmaceutical to clinical practice in Pakistan.

Keywords
177Lu-DOTATATE, neuroendocrine tumors, NET, SStR, radiopharmaceuticals

Introduction
Molecular imaging technique (MIT) is well known to oncology setup due to its efficiency in imaging, staging and fixing the malignant diseases. MITs such as single photon emission computed tomography (SPECT) and positron emission tomography (PET) are frequently practiced at oncology centers using target specific radiolabeled biological molecules for diagnosis of deep-seated infections and malignancies.1 Gamma or positron emitter radionuclide such as technetium-99m (99mTc), indium-111 (111In), gallium-68 (68Ga) and fluorine-18 (18F) labeled biological molecules are used for imaging process,2-5 while beta or alpha emitter radionuclide such as leutitium-177 (177Lu), yttrium-90 (90Y), rhenium-188 (188Re), actinium-225 (225Ac) are used for therapeutic procedures.6-9 A variety of radiolabeled regulatory peptides are in clinical trials and few are in clinical practice for imaging and therapy of NETs. NET imaging and therapy bases on peptide-receptor affinity.

NETs form a heterogeneous group of neoplasms that have ability to grow at any place of human body with the secretion of peptides and neuroamines. The gastroenteropancreatic endocrine tumors are the most common (~67.5%) among NETs including pancreas carcinoma (30%-40%), the small-intestine (15%-20%) and the rectum cancers.
(5%-15%) followed by bronchopulmonary tumors (25.3%). An exclusive feature of these cancers/tumors is the overexpression of SStR which act as NET markers and the principle targets of radionuclide-labeled somatostatin peptide analogues for therapy. Somatostatin is a naturally occurring hormone commonly known as growth hormone-inhibiting factor. It is cyclic peptide hormone bearing disulphide cyclic linkage with either 14 (SS-14) or 28 amino-acids (SS-28). The former form predominant in brain while latter sequence produced by intestinal enteroneuroendocrine cells. These receptors also express in the adrenals, and the pancreas. It mainly regulates the endocrine system via interaction with G-protein coupled SStR which mainly comprises of 5 subtypes (SStR1-5). There are many neuroendocrine origin tissues that show overexpression of SStR in its malignancy state. NETs, predominantly characterized by the overexpression of any of the 5 subtypes of SStR.

Molecular imaging and therapy, based on targeting the SStR is justified with the coherence between the structure of the somatostatin analogue and SStR subtype. Highly attractive somatostatin analogue that target SStR2 with its maximum efficiency (≈100%) is [DOTA0, Tyr3, Thr]-octreotide (DOTATATE). SStR2 is overexpressed in a majority of NETs, including small-cell lung carcinomas (SCLCs), breast tumor tissues, and pancreatic cancer.

In order to formulate freeze dried cold kit of DOTATATE, the radiochemical preparation was assessed at different reaction conditions and test the effect of ligand-radioactivity ratio, pH and reaction time to obtain maximum labeling yield. The optimized reaction conditions at which ≈100% labeling yield of $^{177}$Lu-DOTATATE was obtained was used to prepare freeze dried kits. Brief description of freeze-dried DOTA-TATE kit preparation is summarized as follow; DOTATATE acetate was dissolved in ultrapure filtered water to prepare the stock solution of $^{177}$LuCl$_3$ salt was carried out at Pakistan Atomic Research Reactor-1 (PARR-1), PINSTECH, Islamabad with sufficiently good specific activity required for research purposes and animal study.

**Material and Methods**

**Materials**

All the chemicals were of analytical grade and purchased from Sigma-Aldrich, Fisher Scientific and Alfa Aesar. Whatman 3 chromatographic paper was purchased from Agilent Germany. [DOTA0, Tyr3, Thr]-octreotide were synthesized in GL-Biochem laboratory, China under sterile conditions. The $^{177}$Lu radionuclide in the form of $^{177}$LuCl$_3$ was obtained from Pakistan Atomic Research Reactor-1 (PARR-1), PINSTECH, Islamabad with sufficiently good specific activity required for research purposes and animal study.

**Formulation of Freeze Dried DOTATATE kit**

In order to formulate freeze dried cold kit of DOTATATE, the radiochemical preparation was assessed at different reaction conditions and test the effect of ligand-radioactivity ratio, pH and reaction time to obtain maximum labeling yield. The optimized reaction conditions at which ≈100% labeling yield of $^{177}$Lu-DOTATATE was obtained was used to prepare freeze dried kits. Brief description of freeze-dried DOTA-TATE kit preparation is summarized as follow; DOTATATE acetate was dissolved in ultrapure filtered water to prepare the stock solution having concentration 1µg/µL and stored in 50 µL aliquots. A solution of gentisic acid was prepared by dissolving 40 mg of gentisic acid in 2 mL of 0.4 M acetic acid buffer in dark. Stock solution of ascorbic acid was prepared by dissolving 210 mg of ascorbic acid in 2 mL 0.4 M acetic acid buffer having pH 4.8 and stored the solution in dark. 50 µg of DOTATATE (1µg/µL in water) was taken in to an amber vial and added 5 mg of gentisic acid along with 26 mg of ascorbic acid from stock solution. The resulting solution was mixed thoroughly and pH was adjusted to 5. The solution was passed through 0.22 µm millipore filter and lyophilized for 24 h. The kits were stored at 4°C in refrigerator.

**Production of $^{177}$LuCl$_3$**

Production and radiochemical processing of $^{177}$Lu in the form of $^{177}$LuCl$_3$ salt was carried out at Pakistan Atomic Research Reactor-1(PARR-1), PINSTECH, Islamabad, Pakistan. The $^{177}$LuCl$_3$ obtained from PARR-1 was used for preparation and optimization of cold kit parameters. Typically, 100 µg of isotopically enriched Lu$_2$O$_3$ target (52% in $^{176}$Lu) was dissolved in concentrated HCl to get LuCl$_3$. The resulting solution was then dried in a quartz tube in desiccators and reconstituted with ultrapure water—dried it again, sealed the tube in aluminum sheet for irradiation. The sample was irradiated at a thermal neutron flux of $1.5 \times 10^{14}$ n cm$^{-2}$ s$^{-1}$ for a period of 18 h. The irradiated target was allowed to cool for 12 h and dissolved in gently warm HCl solution (pH 3-4) followed by cooling to room temperature and filtering through 0.22 µm millipore filter.
paper to obtain pure $^{177}$LuCl$_3$. Thus obtained $^{177}$LuCl$_3$ could be used for therapeutic dose of $^{177}$Lu-DOTATATE.

**Labeling of DOTATATE Kit With $^{177}$Lu**

The freeze dried cold kit of DOATATAE was reconstituted with sterile water and diluted to pH 5 followed by the addition of $^{177}$LuCl$_3$ solution. The mixture was heated to 90°C for 35 min to label the DOTATATE with $^{177}$Lu. Different labeling reaction was performed by increasing DOTATATE concentration such as 1,2,3,4, or 5 times as compared to $^{177}$Lu.

**Quality Control Studies**

Following the preparation of $^{177}$Lu-DOTATATE and cooling at room temperature, the radiochemical mixture was subjected for quality control analysis using chromatography process.

**Paper Chromatography Analysis**

The radiochemical purity of $^{177}$Lu-DOTATATE was determined by paper chromatography. The analysis was carried out by spotting an aliquot of 1 μL reaction mixture at 1.5 cm from one end of the 14 cm long chromatography paper strip (Whatman 3). The strip was developed using 50% acetonitrile aqueous solution. After drying the strip, it was scanned through Radio-TLC scanner having flat type NaI(Tl) detector. The $R_f$ value was calculated through software B-SCAN.

**Stability of $^{177}$Lu-DOTATATE Freeze Dried Kits**

The radiochemical yield of $^{177}$Lu-DOTATATE which reconstituted using freeze dried cold kits which have been stored for 24 h, 1 week and 4 weeks, was investigated by incubating the radiopharmaceutical at room temperature for predefined time intervals through paper chromatography. The percent yield after radiolabeling reaction revealed that DOTATATE analogue remained stable in one month storing period.

**Biodistribution Studies**

Biodistribution pattern of $^{177}$Lu labeled FDK of DOTATATE was studied in normal wistar rats each weighing 40-50 g in a group of 3-5. The $^{177}$Lu-DOTATATE solution was further diluted with saline to 25 MBq activity/mL. An aliquot of 200 μL of $^{177}$Lu-DOTATATE solution was then administration in animals through the tail vein of rats. For each time point, 3 animals were injected. The animals were given chloroform anesthesia prior to sacrifice at 2 h, 24 h, 48 h, and 72 h post-injection. Immediately, after animal sacrifice, blood was collected through cardiac puncture and counted the radioactivity using well-type NaI(Tl) scintillation counter. Different organs of the sacrificed animals were also excised, saline-washed, dried over filter paper, weighed and counted the radioactivity. The percentage of injected activity (%IA) accumulated in various organs was calculated. The activity excreted was indirectly determined from the difference between total injected activity (IA) and %IA accounted for all the organs.

**Glomerular Filtration Rate**

Glomerular filtration rate (GFR) study was performed using protocol as described previously published reports. Briefly, an aliquot of 200 μL of $^{177}$Lu-DOTATATE was administered into the ear vein of a group of 3 New Zealand white rabbits.
(kept under starvation conditions) early morning on the day of experiment. The GFR was analyzed with built-in software in SPECT camera software, and the urine activity was also calculated simultaneously with GFR counting.

**Hematology, Cytotoxicity and Histopathology Studies**

Cytotoxicity of $^{177}$Lu-DOTATATE against the normal biological tissues was evaluated by injecting the labeled peptide to the New Zealand white rabbit each weighing about 2-3 Kg. The cytotoxicity in animals were studied in following 3 groups; i) using over-dose of the $^{177}$Lu-DOTATATE in which 555 MBq was injected and sacrificed after 3 weeks (21 days) postinjection period, ii) using therapeutic dose of $^{177}$Lu-DOTATATE according to weight of the rabbit and completed the 3 doses each after 1 month, and iii) injecting maximum dose of the cold DOTA- TATE. The pre-injection (Pr-I) and post-injection (Ps-I) rabbits’ blood samples were collected for biochemical and hematology studies to analyze the systemic effect on blood parameters in all 3 conditions. All the animals were sacrificed and specific vital organs were excised. The tissues of heart, kidney and Lungs were taken and fixed with neutral formalin (10%), embedded in paraffin, and then manually sectioned with a microtome to obtain 4–5 mm-thick paraffin sections. Dewaxed sections were then stained with hematoxylin and eosin (H&E) method and observed under the light microscope for cytotoxicity and histopathology results.

**Results**

**Quality Control Parameter Study and Radiochemical Yield**

The radiochemical yield greatly affected by ligand to radionuclide ratio, pH and reaction time. In order to develop freeze dried DOTATATE kit and to achieve maximum radiochemical yield, and consequently maximum therapeutic advantages; the radiolabeling of $^{177}$Lu with DOTATATE were studied in detail using different reaction conditions such as ligand to radionuclide ratio in which ligand concentration was increased from 1 to 5 times as compared to $^{177}$Lu (Figure 1), pH in the range of 3.5 to 6.5 units (Figure 2) and 10 to 50 min reaction time (Figure 3) at 90°C reaction temperature. At each set of reaction conditions the reaction mixture was tested for radiochemical yield using paper chromatography. At optimized reaction conditions, the radiochromatogram of reaction mixture was developed in 2 mobile systems as shown in Figure 4.

**Stability of DOTATATE Freeze Dried Kit**

Shelf life stability of DOTATATE freeze-dried kit was assessed after 24 h, 1 week and 4 weeks of its preparation by labeling with $^{177}$Lu. The results of radiochemical yield of labeled freeze dried kits are shown in Figure 5.

**Biodistribution Study**

Biodistribution of $^{177}$Lu-DOTATATE was assessed in wister rates. Briefly, at 2, 24, 48 and 72 h post injection of 200 µL of an aliquot of $^{177}$Lu-DOTATATE through tail vein, the rats were anesthetized, sacrificed, organ excised, washed and subjected for radioactivity counts. The data were analyzed and summarized in the form of graph as shown in Figure 6.
GFR Study

GFR study was conducted to test the effect of indigenously developed $^{177}$Lu-DOTATATE kit on renal function. Renal filtration rate was noted at regular intervals that was analyzed through built-in GFR software in SPECT camera. The results of GFR study are shown in Figure 7.

Hematology, Cytotoxicity and Histopathology Study

Table 1 shows the hematology results of $^{177}$Lu-DOTATATE administrated animal model. Due to the radionuclide involvement in cancer therapeutic procedures, it is more likely that the disturbance of blood parameters by ionizing radiations may take place. Further, the ionizing radiations can also cause cytotoxicity in animal body. The results of cytotoxicity study using histopathology are shown in Table 1 and Table 2; while the effect on tissues of different key organs are shown in Figure 8.

Discussion

Currently, $^{177}$Lu-DOTATATE is gaining breathtaking attention in therapy of NETs followed by $^{68}$Ga-DOTATATE mediated PET imaging for initial diagnosis, and selection of patients for PRRT. The agent is under different clinical trials to get approval from respective authorities in different regions of world. In Pakistan, we have also developed the freeze-dried kit of DOTATATE for imaging and therapy of NETs at affordable cost.

The quality control analysis for developing freeze-dried kit at different ligand to radionuclide ratio, pH and reaction time indicate 5 times higher ligand ratio as compared to radionuclide facilitate 100% labeling yield at pH 5 and 30 min reaction time at 90°C temperature. Less than this value increases the free radioactivity. Varying the pH and reaction time directly affect the labeling yield. At optimized reaction conditions 100% labeling yield guarantied the promising efficacy of therapeutic procedure. The freeze-dried kit was tested at 24 h, 1-week and 4-weeks of post-preparation/storing period. The results indicate consistency in radiochemical purity and stability of the kit which are the primary requirements for freeze dried kit to provide at remote areas or to store for number of week to utilize on demand for therapeutic procedures. The $^{177}$Lu-labeling with freeze dried kit in intervals of weeks, analyzed using
chromatography, the results show the promising ability to bind with $^{177}$Lu with maximum labeling yield.

The $^{177}$Lu-DOTATATE kit was subjected to biodistribution in animal model to record the accumulated activity in different body organs. All organs showed normal uptake which gradually washed-out from organ tissue. The normal uptake phenomenon of $^{177}$Lu-DOTATATE is mainly due to its selectivity for target which indicated the efficacy of freeze dried kit as well. Kidneys, however showed slightly more accumulation of $^{177}$Lu-DOTATATE and the blood creatinine level remain in the limit. Previously, reported data showed slight increase (11.1\%) of blood creatinine level along with renal accumulation.\(^{24}\) The whole biodistribution pattern, however not showed unusual accumulation which expresses that there were no overexpressed SST\(\)R cells/tissues.\(^{27,28}\) The GFR results showed the peak time 8.1 and 9.8 min, percentage uptake 56.5\% and 67.23\% and GFR value 34.67 mL/min was calculated. The excretion rate of $^{177}$Lu-DOTATATE indicated the normal renal filtration which is the success point of $^{177}$Lu-DOTATATE therapy.\(^{30}\)

Cytotoxicity study of $^{177}$Lu-DOTATATE kits studied after 21st day of its preparation, by collecting the blood of $^{177}$Lu-DOTATATE administrated animal at Pr-I and Ps-I time points. The complete blood profile results indicated non-significant difference between Pr-I and Ps-I blood analysis. The minute difference in blood parameters is mainly due to ionizing potential of $\beta$-radiations, however it could be tolerating. The histopathology, study also showed the physiological changes as exist in hematology study of blood. The cytotoxicity was further analyzed by studying liver and kidney functioning parameters. The values of serum bilirubin remained within normal limit in Pr-I and Ps-I $^{177}$Lu-DOTATATE and free $^{177}$LuCl\(_3\) administration. Other parameters; serum ALP, serum uric acid, serum urea and serum creatinine remained in-range after administrating the $^{177}$Lu-DOTATATE and free $^{177}$LuCl\(_3\), while serum ALT (SGPT) and serum AST (SGOT) increased in

| Sr. No. | Parameter | $^{177}$Lu-DOTATATE (20 \(\mu\)g) | $^{177}$LuCl\(_3\) | Normal range |
|---------|-----------|-------------------------------|-----------------|--------------|
| 1. | WBC /\(\mu\)l | 4800 | 4700 | 8600 | Pr-I | Ps-I | Pr-I | Ps-I | 4000-11000 |
| 2. | RBC m/\(\mu\)l | 4.0 | 3.89 | 5.47 | 6.22 | 4.5-6.0 |
| 3. | Hemoglobin g/dl | 11.6 | 11.4 | 12.9 | 12.3 |
| 4. | Hematocrit % | 30.0 | 32.3 | 35 | 36.4 | 40-50 |
| 5. | MCV fl | 51 | 52.8 | 63.9 | 58.6 | 76-96 |
| 6. | MCH pg | 21 | 19.4 | 23.6 | 19.8 | 27-31 |
| 7. | MCHC g/dl | 32 | 31 | 36.9 | 32.3 | 32-36 |
| 8. | Platelets/\(\mu\)l | 490000 | 489000 | 311000 | 545000 | 140000-450000 |

Differenita Count

| Sr. No. | Parameter | $^{177}$Lu-DOTATATE | $^{177}$LuCl\(_3\) | Normal range |
|---------|-----------|-----------------|----------------|--------------|
| 9. | Neutrophils% | 70 | 75 | 62 | 76 | 50-75 |
| 10. | Lymphocytes% | 25 | 20 | 31 | 16 | 20-45 |
| 11. | Monocytes% | 1 | 3 | 4 | 5 | 02-08 |
| 12. | Eosinophils% | 1 | 2 | 3 | 3 | 0-6 |

| Sr. No. | Liver & kidney function parameters | $^{177}$Lu-DOTATATE | Free $^{177}$LuCl\(_3\) | Normal range |
|---------|-----------------------------------|-----------------|----------------|--------------|
| 1. | Serum Bilirubin (total) (mg/dl) | 0.5 | 0.7 | 0.5 | 0.7 | ~ 1.0 |
| 2. | Serum ALT(SGPT) (\(\mu\)/L) | 39 | 68 | 80 | 84 | ~ 46 |
| 3. | Serum AST(SGOT)(\(\mu\)/L) | 22 | 82 | 30 | 71 | ~ 35 |
| 4. | Serum ALP (\(\mu\)/L) | 134 | 111 | 98 | 114 | Adults <258 children <600 |
| 5. | Serum uric acid (mg/dl) | 3.2 | 4.2 | 5.1 | 3.3 | ~ 3.5-7 |
| 6. | Serum urea (\(\mu\)/L) | 42 | 3.7 | 38 | 32 | ~ 10-50 |
| 7. | Serum creatinine (\(\mu\)/L) | 1.1 | 0.8 | 1.1 | 0.7 | ~ 0.6-1.2 |
either case of administration, $^{177}$Lu-DOTATATE or free $^{177}$LuCl$_3$. Serum ALT(SGPT) was increased to 68 $\mu$L in case of $^{177}$Lu-DOTATATE Ps-I and 84 $\mu$L in case of free $^{177}$LuCl$_3$ Ps-I analysis. The Serum ALT(SGPT) limit in blood is $\sim$ 46. The other liver parameter, serum AST(SGOT) also showed increase in its level i.e. 82 $\mu$L in case of $^{177}$Lu-DOTATATE Ps-I and 71 $\mu$L in case of free $^{177}$LuCl$_3$ Ps-I as compared to its higher limit, $\sim$ 35 $\mu$L. The increase in both liver markers indicate minor liver damage. It can be considered that some damaging effect may possible due to $\beta$-radiations. Moreover, the liver was the most common site of NET metastasis (91.7%) which also indicate the presence of SSTR which facilitate the accumulation of DOTATATE and hence the damaging. The injection of free $^{177}$Lu dose equivalent to the $^{177}$LuDOTATATE dose, however showed comparable values. The histopathology analysis showed non-significant alteration in tissue morphology except some changes were seen in liver slides.

**Conclusion**

The radiochemical yield, stability study of freeze-dried kit, biodistribution, GFR values, and cytotoxicity results showed good development, however it needs further improvement to make the kit of world health standards and to submit the experimental data to “Animal and Human Ethics Board” of institute for further guidelines and experimentation to conduct Phase-I & II trials.

**Authors’ Note**

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

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**Figure 8.** Histopathology images of control, free $^{177}$Lu and $^{177}$Lu-DOTATATE administrated kidney, liver and heart tissues.
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