

# Radiolabeling of benzylpenicillin with lutetium-177: Quality control and biodistribution study to develop theranostic infection imaging agent

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**Abstract:** Benzylpenicillin acts through binding with beta-lactamase enzyme and inhibiting the bacterial cell wall biosynthesis. Therefore, the radiolabeling of benzylpenicillin with lutetium-177 is expected to serve as a theranostic agent for deep-seated bacterial infections. The radiolabeling of benzylpenicillin resulted ~93% radiochemical yield at optimized reaction conditions. Radiochemical purity analysis was tested with the help of Whatman No. 2 paper and instant thin layer chromatography. Biodistribution study with healthy New Zealand white rabbit revealed moderate accumulation in different organs. Kidneys are the major organs, showed not more than 4.57±0.89% injected dose per gram organ (ID/gm organ) at 1 h time point and 3.48±1.11% ID/gm organ at 6 h time point. The accumulation of tracer agent in liver was found in the range of 7.42±2.42% to 9.09±2.76 ID/gm organ. The glomerular filtration rate studies revealed rapid clearance – omitting the chance of nephrotoxicity. The radiolabeling yield, biodistribution and glomerular filtration rate results revealed <sup>177</sup>Lu-benzylpenicillin could be a potential candidate to diagnose the deep-seated bacterial infection.

**Keywords:** Benzylpenicillin, infection imaging, radiopharmaceuticals, nuclear medicine, lutetium-177.

## INTRODUCTION

World health organization (WHO) and medical science declared the infectious diseases, a wide threat for humanity. Sub-optimal diagnosis of deep-seated bacterial infections in term of infection site, nature and severity of infection, leads to misuse and over-dose administration of antibiotics. Excessive and sub-optimal use of antibiotic develops the antibiotic resistance and drug related chronic side effects of gastric region, which ultimately forms the ulceration to sub-mucosa. Therefore, accurate diagnosis for causative agent is necessary milestone in deep-seated bacterial infections. Neither biochemical parameters nor symptomatic diagnosis without using the instruments to identify the deep-seated bacterial infections leads to excessive use of drugs for therapy. The instrumental modalities such as ultrasound, magnetic resonance imaging (MRI) and computed tomographic (CT) scan can diagnose the deep-seated bacterial infection in term of location, area and volume. Although MRI and CT scan are potential diagnostic modalities with 100% accuracy – but the only demerit of these two techniques are that, they are limited to morphological and entomological changes which appear after a long-time of initiation of disease (wang *et al.*, 2013). Further availability and cost per scan is another issue with MRI and CT scan.

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Nuclear medicine technique for diagnosis use target specific radio labeled agents (radiopharmaceutical) and scanning the whole body with single photon emission computed tomography (SPECT) camera is unique regarding diagnosis of disease at molecular level stage – that is far early than morphological state of disease. A number of biological active molecules were labeled with gamma emitter radionuclides for imaging purpose of deep-seated bacterial infections at molecular level such as <sup>99m</sup>Tc-sulfadiazin (Ahmed *et al.*, 2017), <sup>99m</sup>Tc-metronidazole (Iqbal *et al.*, 2017), <sup>99m</sup>Tc-amoxicillin (Ilem *et al.*, 2016), <sup>99m</sup>Tc-enrofloxacin (Siaens *et al.*, 2004), <sup>99m</sup>Tc-sitafloxacin (Qaiser *et al.*, 2010), <sup>99m</sup>Tc-cefepime (Motaleb *et al.*, 2011), <sup>99m</sup>Tc-cefzolin (El-Tawoosy *et al.*, 2013), <sup>99m</sup>Tc-ciprofloxacin (Britton *et al.*, 2002). <sup>99m</sup>Tc-ciprofloxacin was remained the agent of choice for bacterial infection but due to decrease in infection specificity other agents were also investigated (Auletta *et al.*, 2016). The main reasons of non-specificity of <sup>99m</sup>Tc-ciprofloxacin and how it could be overcome was discussed in a review article (Naqvi & Darlica *et al.*, 2017).

Benzylpenicillin is beta-lactamase sensitive penicillin antibiotic. Its bactericidal action takes place by binding with beta-lactamase enzyme and inhibiting the bacterial

cell wall biosynthesis. Therefore beta-lactamase might be a good target of  $^{177}\text{Lu}$ -benzylpenicillin for imaging and therapeutic purposes of deep-seated bacterial infections. The choice of lutetium-177 ( $^{177}\text{Lu}$ ) is based on the emission of gamma photon (for diagnosis) and beta photon (for therapeutic) of promising energies (Dash *et al.*, 2015). The aim of this study is to develop  $^{177}\text{Lu}$  labeled benzylpenicillin for imaging and therapeutic radiopharmaceutical of deep-seated bacterial infections.

## MATERIALS AND METHODS

Benzylpenicillin were purchased from Abbott (Sweden). Lutetium-177 was obtained from Pakistan Institute of Nuclear Science and Technology (PINSTECH), Islamabad, Pakistan in the form of  $^{177}\text{LuCl}_3$  for labeling process. Sodium hydroxides (NaOH), ammonium hydroxide ( $\text{NH}_4\text{OH}$ ), were obtained from Merck (Germany). New Zealand white rabbits were obtained from National Intuition of Health (NIH) Islamabad. Instant thin layer chromatographic sheets impregnated in silica gel (ITLC-SG) were obtained from Agilent (USA) for quality control analysis. The animal ethics committee of the institute gave an ethical approval for the biodistribution study. All other chemicals used were of analytical reagent grade and were also purchased from Merck (Germany).

### Radiolabeling of benzylpenicillin with $^{177}\text{Lu}$

Radiolabeling of benzylpenicillin was carried out in the cationic solution of  $^{177}\text{LuCl}_3$ . Different parameters such as benzyl penicillin as ligand, DTPA as co-ligand, pH, reaction temperature and time were assessed to obtain maximum labeling yield. The benzyl penicillin was obtained in mega unit vials (1mega unit=600 mg benzyl penicillin). For maximum yield the ligand quantity (24000-168000 units),  $^{177}\text{Lu}$  (18.5-111 MBq), pH (2-8) and reaction time (10-40 min) were tested. However, the amount of co-ligand (DTPA) was taken constant (5 $\mu\text{g}$ ). The pH of the reaction mixture was adjusted with the help of 0.05N NaOH or 0.01N HCl solutions. The total volume of the reaction mixture was taken 2mL in all experiments.

### Quality control of $^{177}\text{Lu}$ -benzyl penicillin

To assess the radiochemical purity (RCP), quality control analysis was carried out using Whatman paper # 2 and ITLC-SG chromatographic strips (2 $\times$ 14 cm size). For analysis ~1-2  $\mu\text{L}$  aliquot of reaction mixture was spotted at the base line of the both strips.

### Paper chromatographic analysis

Paper chromatographic analysis was carried out using a mixture of ammonium hydroxide: water: methanol (1:2:2) as mobile phase. In this system  $^{177}\text{Lu}$ -benzylpenicillin was travelled along solvent front ( $R_f=1$ ), while colloid remained at base line ( $R_f=0$ ).

### Instant thin layer chromatographic analysis

ITLC-SG analysis was carried out using 50mM EDTA solution in 0.1M sodium acetate solution as mobile phase. The  $^{177}\text{Lu}$ -benzylpenicillin remained at base line ( $R_f=0$ ) while other impurities eluted with solvent front ( $R_f=1$ ). The radiolabeling experiment and QC analysis was repeated thrice to confirm the reproducibility of the radiolabeled complex at  $25\pm 2^\circ\text{C}$  and the results were reported as mean ( $n=3$ )  $\pm$  standard deviation (S.D.).

### In vitro stability of $^{177}\text{Lu}$ -Benzylpenicillin

To determine the *in vitro* stability of the  $^{177}\text{Lu}$ -benzylpenicillin, the reaction mixture was incubated at room temperature for 6 h and stability of the complex was analyzed by spotting ~1-2  $\mu\text{L}$  aliquot at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0h time points at chromatographic strips.

### Biodistribution study

Biodistribution studies were accomplished thrice at different days using New Zealand white rabbits. On the day of experiment the rabbits were anesthetized with intramuscular injection of diazepam (5mL) followed by laying the rabbit on hard board with all four legs spread out. The legs were fixed with surgical tap. The rabbit was then injected with 500 $\mu\text{L}$  solution of  $^{177}\text{Lu}$ -benzylpenicillin (~74MBq) into rear ear vein. The biodistribution was recorded at 1, 4 and 6 h post injection through SPECT gamma camera.

### Glomerular filtration rate analysis

Glomerular filtration rate (GFR) analysis was carried out by using the method reported by Levey *et al.*, (1993). Briefly, prior to GFR study the rabbit was kept on fasting with drinking water for overnight to increase the flow rate of urine. At early of the day the rabbit was injected with 300  $\mu\text{L}$  solution of  $^{177}\text{Lu}$ -benzylpenicillin and regular uptake and excretion of activity by kidneys were recorded with SPECT gamma camera interfaced with GFR study software installed dedicated computer.

## RESULTS

### Radiochemical yield

Radiochemical purity of  $^{177}\text{Lu}$ -benzylpenicillin, in the presence of impurities i.e. free  $^{177}\text{Lu}$  and  $^{177}\text{Lu}$ -DTPA was analyzed with paper chromatographic and ITLC-SG analysis using different mobile phase systems. The percent (%) yield of  $^{177}\text{Lu}$ -benzylpenicillin and other impurities were calculated by using the following expressions;

$$^{177}\text{Lu}\text{-benzylpenicillin (ITLC strip)} = \frac{\text{Radioactivity counts at } R_f 0-0.1}{\text{Total radioactivity counts at strip}}$$

$$\% \text{ colloid/free } ^{177}\text{Lu}^{3+} \text{ (PC strip)} = \frac{\text{Radioactivity counts at } R_f 0-0.1}{\text{Total radioactivity counts at strip}}$$

$$\% \text{ yield of } ^{177}\text{Lu}\text{-DTPA} = 100 - (\% ^{177}\text{Lu}\text{-benzylpenicillin} + \% \text{ colloid/free } ^{177}\text{Lu}^{3+})$$

### Quality control parameter study

Typically, amount of ligand, radioactivity and pH are the main parameters that affect the radiochemical yield of the radiopharmaceutical. The optimization of reaction parameters involves continuous testing with gradual change in reaction conditions and then monitoring the radiochemical yield with chromatographic analysis.

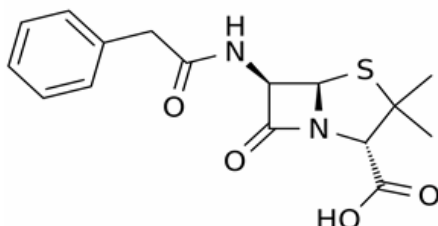


Fig. 1: Benzyl penicillin structure

### Effect of benzyl penicillin amount on radio labeling

The amount of benzylpenicillin was optimized using a series of radiochemical reactions at constant radioactivity and varying the pH from physiological pH. It was recorded that 120000 unit of benzylpenicillin showed maximum radiochemical yield when 75 Mbq  $\text{LuCl}_3$  was incubated at pH 6 (near to physiological pH). At these conditions the reaction yielded >93% radiochemical product. Fig. 2a shows that at lower concentration i.e. below 120000 unit benzylpenicillin the reaction produced poor radiochemical yield and slightly decreasing trend was recorded when benzylpenicillin concentration was increased than optimum amount.

### Effect of radionuclide activity

Fig. 2b shows the trend of radiochemical yield at varied amount of radionuclide activity. The radionuclide activity is directly related to radionuclide concentration. Radioactivity is easier to measure as compared to concentration in case of radionuclide material. It is obvious from fig. 2b that 75 MBq  $^{177}\text{LuCl}_3$  is an optimal radioactivity that produce maximum radiochemical yield in the presence of 120000 units of benzylpenicillin and 6 pH. At low and higher than 75 MBq activity the yield was reduced from maximum yield.

### Effect of pH

Fig. 2c shows the effect of pH of radiochemical yield. After optimized reaction conditions the effect of pH was noted. The pH affected significantly the radiolabeling yield of  $^{177}\text{Lu}$ -benzylpenicillin. The maximum radiochemical yield was recorded at pH 6 while at more acidic and greater than 6 pH the radiochemical yield was found less than optimum-yield i.e. 93%.

### Effect of reaction time

Reaction time is the optimum time required to complete the chemical reaction and in case of  $^{177}\text{Lu}$  labeling with

benzyl penicillin it is 35 min at which maximum number of  $^{177}\text{Lu}$  make complex with benzyl penicillin as shown in fig. 2d. At 10 min reaction period we recorded ~50% radiochemical yield which increased to >93% at 35 min.

### Radiochemical shelf-life stability

Fig. 3 shows the stability trend of radiochemical that is actually the extension of effect of incubation time on reaction yield. Once the maximum radiochemical yield was obtained the stability of the radiochemical appears as an important factor. We noted the radiochemical was sufficiently stable up to 4h i.e. at 4h time point ~90% radiochemical was recorded. However, at 5 and 6 h time point it was degraded to 88 and 80%, respectively.

### Biodistribution study

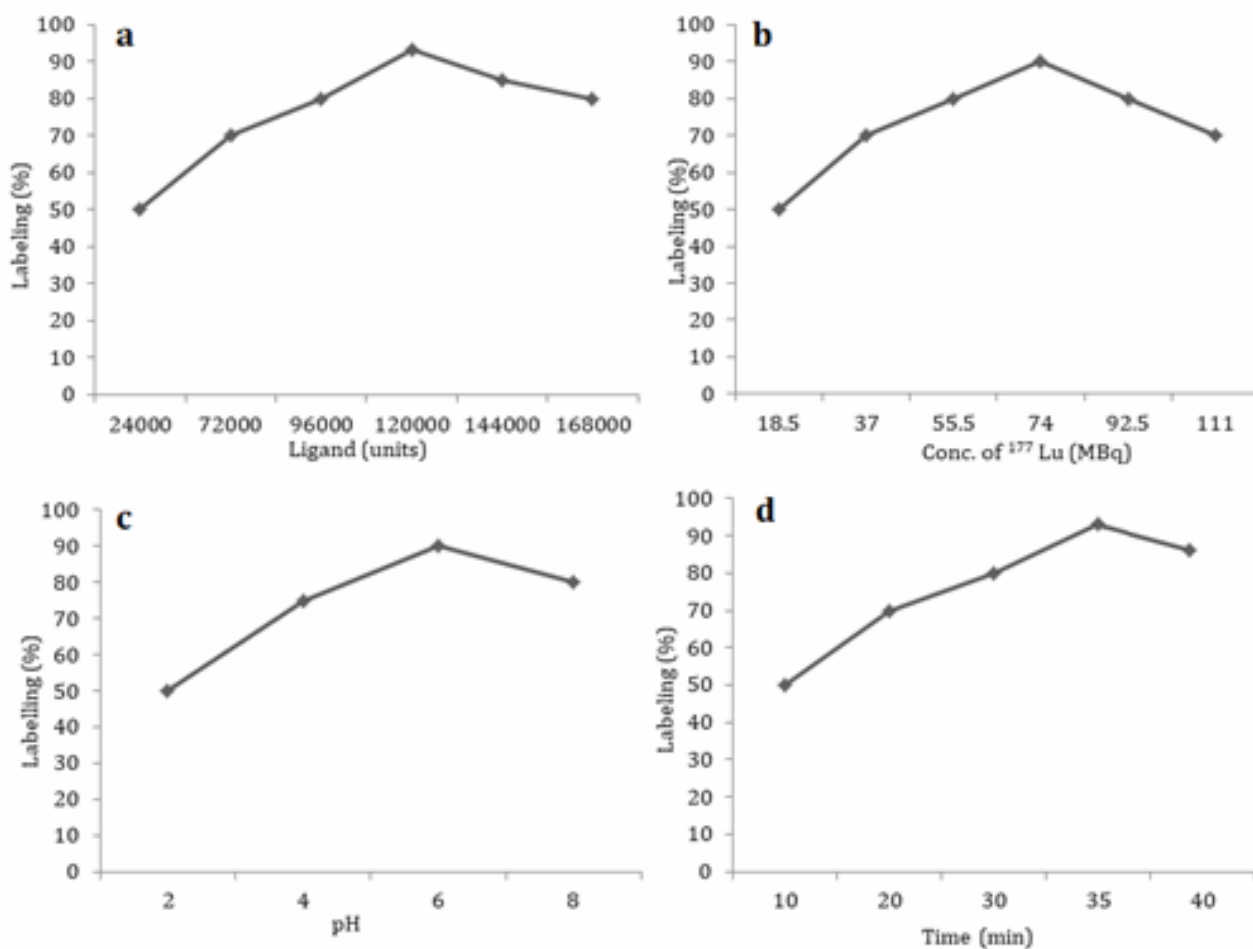
The biodistribution of  $^{177}\text{Lu}$ -benzylpenicillin was measured as % ID/gm organ in New Zealand white rabbits using SPECT gamma camera to measure the counts in key body organs. After 6h study the rabbits were sacrificed to measure the weight of heart, kidneys, lungs, spleen, and stomach for calculating % ID/gm organ. All organs showed the uptake of  $^{177}\text{Lu}$ -benzylpenicillin at 1h time point - the kidneys showed uptake  $3.70 \pm 0.89$  to  $4.57 \pm 1.16\%$  while the heart and stomach showed the tracer uptake  $3.63 \pm 1.08\%$  and  $3.84 \pm 0.91\%$  at 1h, respectively. At subsequent time points i.e. 4 and 6 h time point slow wash out of radioactivity was noted as shown in table 1.

### In-vivo Bio-kinetics and scintigraphic study

Glomerular filtration rate was recorded to measure the renal function after administration of  $^{177}\text{Lu}$ -benzylpenicillin. The rabbit at fasting overnight with drinking water was anesthetized with diazepam injection and laid down the SPECT camera – administrated with 300 $\mu\text{L}$  solution of  $^{177}\text{Lu}$ -benzylpenicillin and the renal filtration was recorded using dynamic imaging mode of SPECT camera. The renal filtration and bladder accumulation pattern is shown in fig. 4 along with scintigraphic image of the rabbit. The scintigraphic image indicated the uptake of  $^{177}\text{Lu}$ -benzylpenicillin in key body organs in compliance with biodistribution study.

## DISCUSSION

Nuclear medicine imaging technique (NMIT) has astonishing potential to detect hard-to-diagnose abnormalities in the patient's body. NMIT not only helps in diagnosing the disease but also offers the 3D view of infected organs that helps in dose adjustment for patients. Before the advent of nuclear medicine protocol for bacterial infection diagnosis; bacterial infection in history remained a big threat to human nations all over the world. With the development of technetium-99m ( $^{99m}\text{Tc}$ ) labeled white blood cell and then subsequently  $^{99m}\text{Tc}$ -ciprofloxacin, deep-seated bacterial infection imaging



**Fig. 2:** Quality control parameter study – effect of ligand, radioactivity, pH and reaction time on radiochemical yield

procedure appeared as a powerful diagnostic strategy. However, later on poor specificity of <sup>99m</sup>Tc-ciprofloxacin tempered the early enthusiasm. It was mainly due to bacterial resistance or some mechanistic factors as reported recently in a review (Naqvi & Drlica, 2017). In parallel variety of antibiotics and biological active molecules were labeled with <sup>99m</sup>Tc for bacterial infection imaging, most of them failed in preclinical trails or in clinical trials.

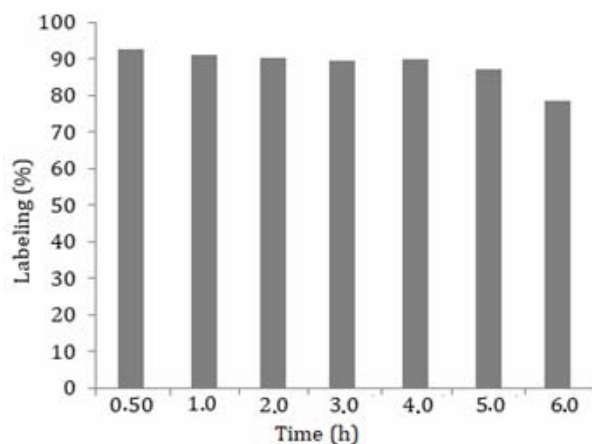
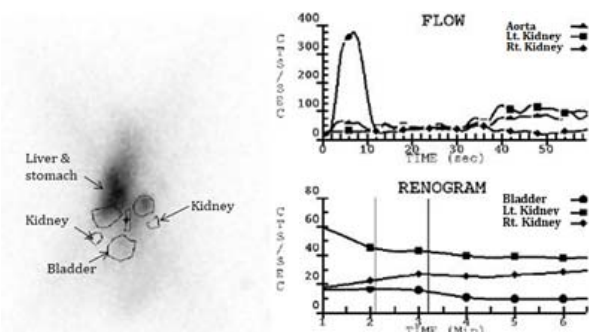
The development of <sup>177</sup>Lu-benzylpenicillin was carried out for dual functioning i.e. imaging and burning the bacteria at infection site. The labeling with <sup>99m</sup>Tc only helps in imaging process as <sup>99m</sup>Tc ( $t_{1/2}=6.06$ h) radionuclide emits gamma radiation ( $E_{\gamma}=140$  KeV) that have no ability to kill the bacteria (Alberto & Abram, 2011). On the other hand <sup>177</sup>Lu ( $t_{1/2}=6.73$  days) offers good affinity to bind with open chain compound to form the complex as compared to closed chain compound (Yousefnia *et al.*, 2010) and emits two types of photons (gamma photons with  $E_{\gamma} = 112$  keV (6.4%) and 208 keV (11%); and beta photon with  $E_{\beta\max} = 497$  keV) due to

which it can be used for imaging and therapeutic procedures (Rasheed *et al.*, 2016).

The radiolabeling yield of <sup>177</sup>Lu-benzylpenicillin (>93%) is adequate for nuclear imaging procedure and in good agreement with previously reported radiolabeled antibiotics for infection imaging (Rasheed *et al.*, 2016). The other possible impurities i.e. free <sup>177</sup>LuCl<sub>3</sub> and <sup>177</sup>Lu-DTPA do not impose lethal side-effect at administrated radiopharmaceutical concentration. The <sup>177</sup>Lu-DTPA is already used as an agent to calibrate renal function of patients in oncology and it excretes cery rapidly through kidneys. Therefore, at optimum radiolabeling conditions the <sup>177</sup>Lu-benzylpenicillin is safe and could be investigated for pre-clinical and clinical evaluation. Other than optimum conditions we found high concentration of free <sup>177</sup>LuCl<sub>3</sub> that may cause sever radiotoxic effect. The complex stability up to 5h also revealed that the <sup>177</sup>Lu-benzylpenicillin is safe to administrate as most of nuclear medicine imaging procedures have been completed within 2-3h.

**Table 1:** Biodistribution of  $^{177}\text{Lu}$ -benzylpenicillin in different body organs of rabbit

Body Organ	% Injected Dose / Gram Organ		
	1 hour	4 hours	6 hours
Heart	3.63 ± 1.08	3.14 ± 0.9	2.46 ± 1.03
Right kidney	3.70 ± 0.89	3.17 ± 1.12	2.90 ± 0.67
Left kidney	4.57 ± 1.16	4.05 ± 1.08	3.40 ± 1.06
Lungs	4.86 ± 1.31	4.21 ± 1.45	3.48 ± 1.11
Liver	9.09 ± 2.76	8.37 ± 2.55	7.42 ± 2.42
Spleen	2.79 ± 0.67	2.70 ± 0.78	2.61 ± 0.83
Stomach	3.84 ± 0.91	3.27 ± 0.88	2.95 ± 0.79

**Fig. 3:** Shelf-life stability of the  $^{177}\text{Lu}$ -benzylpenicillin**Fig. 4:** Scintigraphic image and renogram demonstrating the renal filtration of radioactivity

The in-vivo biodistribution study of novel  $^{177}\text{Lu}$ -benzylpenicillin in healthy rabbit model revealed the agent has no extra-affinity for any organ. Heart, spleen and stomach showed minimal uptake. However, liver showed significant uptake  $9.09 \pm 2.76$ ,  $8.37 \pm 2.55$  and  $7.42 \pm 2.42\%$  ID/gm organ at 1, 4 and 6h time points, respectively. The high uptake in liver is mainly due to the fact that liver commonly facilitate metabolic activities and most of the foreign bodies metabolize in liver before excretion (Vallabhajosula *et al.*, 2010). GFR study is an important protocol to assess the renal function while treating the patients. In nuclear medicine it is studied to record the radiopharmaceutical accumulation in glomerular tubules of kidneys and renal filtration rate.

Normally in healthy human subject renal filtration rate is 60mL/min; however, it varies from patient to patient (Rasheed *et al.*, 2016).  $^{177}\text{Lu}$ -benzylpenicillin in rabbit showed slightly slow filtration (48mL/min) as shown in fig. 4. Comparatively slow filtration rate might be due to the abnormality of the renal function that results in increased affinity of radiotracer with glomerular walls. These circumstances may impose renal toxicity in case of high dose of radiopharmaceutical administration; however administration of low dose (as in common practice) does not impose renal radiotoxicity.

## CONCLUSION

Benzylpenicillin showed more than 93% radio labeling yield with  $^{177}\text{Lu}$  at optimum conditions at minimal concentration of impurities. Stability study, biodistribution results using healthy rabbits and GFR results indicated the tracer agent has potential to subject it for preclinical and clinical studies for bacterial infection imaging procedure.

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