Development and bioevaluation of $^{99m}Tc(CO)_3$-labeled (1-azido-1-deoxy-β-D-glucopyranoside) complex as a potential tumor-seeking agent

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Abstract: The 1,2,3-triazole-containing (1-azido-1-deoxy-β-D-glucopyranoside) complex was synthesized using click chemistry approach and evaluated its potential as a tumor-seeking agent. In the present study, ^{99m}Tc-tricarbonyl labeled (1-azido-1-deoxy-β-D-glucopyranoside) radiotracer [$^{99m}Tc(CO)_3$-BM], (where BM stands for biomolecule, e.g., (1-azido-1-deoxy-β-D- glucopyranoside)) was synthesized *via* click chemistry approach and then labeled with technetium-99m through isolink kit. Radio labeled drug was tested for radiochemical purity and *in vitro* stability by chromatographic techniques. Normal distribution and tumoral uptake were studied in Swiss Webster mice. Radiochemical purity results show 97.9 ±1.5% labeling and its *in vitro* stability were studied at room temperature up to 5h. The radio labeled drug exhibited 73.6±1.1% binding with blood proteins. Normal distribution of drug shows prominent uptake in brain while in case of tumor-bearing mice, the uptake was maximum in tumor tissue and negligible amount was shown in brain. The biodistribution was further compared with 2-fluoro-2-deoxy glucose (^{18}F-FDG), which was used as a positive control. The data indicate that ^{99m}Tc-tricarbonyl labeled (1-azido-1-deoxy-β-D- glucopyranoside) radiotracer might be a feasible candidate with reasonable potential for tumor diagnosis.

Keywords: Click chemistry; 1-azido-1-deoxy-β-D- glucopyranoside; ^{99m}Tc-tricarbonyl, biodistribution; tumor

INTRODUCTION

An obligatory step for the application of metal probes in targeted imaging (using magnetic resonance, positron emission, single photon emission tomography and fluorescence) is their stable attachment to a biomolecule by means of a highly effective ligand system (Toth *et al.,* 2004; Buenzli *et al.,* 2005). This can happen using bifunctional chelating agents (BFCA), which contain a ligand system for formulation of stable chelation with the radiometal, thus developing the attachment of metal chelating moiety to the targeting molecule (Thomas *et al.,* 2008).

The radiolabeling of biologically active molecules is a vital tool for the non-invasive assessment of the newly developed drug *in vivo*. Various strategies have been reported for the synthesis of suitable bifunctional chelating system for radiolabeling with technetium-99m and rhenium-188 and their assimilation into the biomolecules. Click chemistry has various applications in target oriented synthesis (Mocharla *et al.,* 2004; Bourne *et al.,* 2004), biological conjugation (Mindt *et al.,* 2006; Seo *et al.,* 2003) and building molecular library (Lee *et al.,* 2003; O'Neil *et al.,* 2007). Most of the biologically active molecules are discovered by performing radiolabeling through click chemistry strategy (Cosyn *et al.,* 2006; Moorhouse *et al.,* 2006; Wilkinson *et al.,* 2007). The reason for significant biological activity of 1,2,3-triazole derivatives is that 1,2,3-triazole has the ability to well mimic heterocycles and peptides in geometrical shape along with interaction function (Mocharla *et al.,* 2004; Mindt *et al.,* 2006; Moorhouse *et al.,* 2006). Click chemistry is the valuable tool for rapid and efficient fictionalization of tumor targeting biomolecules for radiolabeling with ^{99m}Tc and in particular, with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ moiety. The biological efficacy of organometallic precursor $[^{99m}Tc(CO)_3(H_2O)_3]^+$(isolink kit), has been very successfully applied in healthy human volunteers (*Lipowska et al.,*2006; Taylor *et al.,* 2010) and is widely used for radiolabeling of biomolecules (Alberto *et al.,* 2005; Waibel *et al.,* 2008; De-Barros *et al.,* 2009). A varying number of tridentate ligand systems are reported for forming complexes with ^{99m}Tc tricarbonyl core (Waibel *et al.,* 2008).

In the present study, we radiolabeled (1-azido-1-deoxy-β-D-glucopyranoside) (Mindt *et al.,* 2006) with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ moiety by use of click chemistry for evaluation of its biological potential as a feasible tumor targeting drug, e.g., a subrogate in SPECT for ^{18}FDG in PET (De-Barros *et al.,* 2009; Chen *et al.,* 2006). It is one pot synthesis procedure and reaction was performed without any protecting group.

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MATERIALS AND METHODS

Materials

The chemicals used in this research work were purchased from Aldrich, USA. The technetium-carbonyl complex was developed by using isolink kits received from Mallinckrodt-Covidien, Holland. $Na^{99m}TcO_4$ was obtained from an in-house $^{99}Mo/^{99m}Tc$ generator received from Pakistan Institute of Nuclear Sciences & Technology (PINSTECH). The 2-fluoro-2-deoxy-glucose (^{18}F-FDG) was produced at our local cyclotron facility established at INMOL, Lahore. The Swiss Webster mice, weighing approximately 28g with naturally developed subcutaneous tumors, were obtained from the Animal House maintained at School of Biological Sciences (SBS), University of the Punjab, Lahore, Pakistan. Radioactivity measurements for *in vivo* distribution studies were performed by using Imaging Scanner (Bioscan Inc., USA).

Methodology

All commercial chemicals and solvents were used without further purification. The whole reaction was carried out under sterilized conditions. The apparatus was sterilized in oven pre-heated at 160°C for 120 min. The molecular mass of the product was confirmed by ESI-MS spectra. The spectra of ^{1}H and ^{13}C-NMR were recorded by using Bruker Avance AV-300 spectrometer. Chemical shift (δ) has been expressed in units of part per million (ppm). The radiolabeled kit formulation (fig. 2) was developed in a sterilized laminar flow hood. Biodistribution study was carried out in normal and tumor-bearing mice models. After biodistribution study, the type of tumor cells was further investigated by histopathological tests.

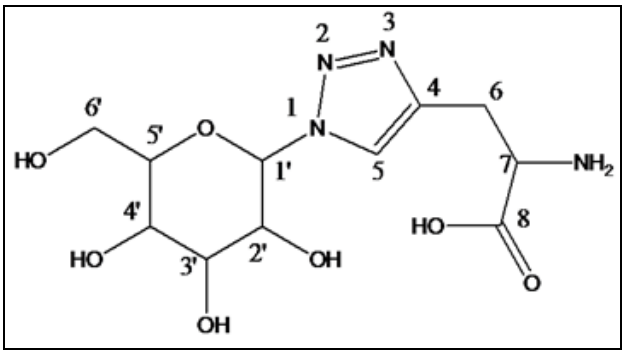

Fig. 1: Possible structure of 1-azido-1-deoxy-β-D-glucopyranoside.

Typical procedure for synthesis

This is one-step procedure that is why we called it as click chemistry. As an experimental procedure, 500 µl of 0.01M aqueous solution of propargylglycine was taken in a sterilized serum vial, followed by addition of 75 µl copper acetate of 0.01M concentration. To this reaction mixture, 150µl aqueous solution of 0.01M sodium ascorbate was added. Biomolecule coupling was performed by adding 650µl solution of (1-azido-1-deoxy-β-D-glucopyranoside) of 0.01M concentration and sealing the vial with rubber stopper and aluminum cap. The vial containing these reaction constituents was heated in a water bath filled with boiling water for 1h, following by cooling down at room temperature. The product was passed through silica gel packed flash column by using 1:1 methanol and water. The reaction yield was 1.693 mg (86.04±3%). The analytical data include: ESI MS: calculated for $C_{11}H_{18}N_4O_7$ ($M + H^+$), 318.283; found, 319.12. ^{1}H-NMR: (300 MHz, $CDCl_3$) 7.63(s, 5H) 0.58 (t, 6H, J=8.5 Hz) 2.80 (t, 6H, J=8.0 Hz) 1.78 (m, 7H) 4.54 (d, 1'H, J=8.5 Hz) 3.39 (t, 2'H, J=9.0 Hz) 3.41 (t, 3'H, J=9.0 Hz) 3.86 (dd like, 4'H, J=9.0 Hz, 1.5Hz) 3.31 (broad, 5'H) 3.68 (dd, 6'H, J=11.5 Hz, 5.5 Hz) 3.17 (dd, 6'H, J=11.5 Hz, 6.5 Hz). ^{13}C-NMR: (300MHz, D_2O) 126.4, 126.2, 29.06, 62.30, 175.43, 92.86, 71.9, 80.62, 75.56, 78.46, 63. 26.

Radiolabeling

The radiolabeling procedure involved two steps: First one was to radiolabel isolink kit with ^{99m}Tc to form $[^{99m}Tc(CO)_3(H_2O)_3]^+$ moiety (^{99m}Tc-tricarbonyl coordination complex). Second step was to radiolabel above newly synthesized product with isolink kit.

Development of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ Moiety

This precursor was developed by adding $Na^{99m}TcO_4$ generator eluent (20 mCi in 1 ml saline) in isolink kit and heating it in a water bath containing boiling water for 20 min, following by cooling to room temperature, and neutralization by adding approximately 180µl (1.0N) HCl. The pH for neutralization was determined by using pH paper.

Labeling of biomolecule with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ Moiety

Technetium-99m carbonyl complex $[^{99m}Tc(CO)_3(H_2O)_3]^+$ from the Isolink kit (prepared as mentioned above) was added in 1ml reaction mixture (synthesized in step 2.2) and heated for 1h in boiling water bath. The radiochemical purity (RCP) of the radiolabeled products was tested by chromatographic procedure, as elaborated below.

Quality control

Paper Chromatography (PC) and Instant Thin Layer Chromatography (ITLC) were used to evaluate the radiochemical purity of the radio labeled complex. To start the experimental procedure, silica gel impregnated glass fiber sheets (ITLC TM-SG, Pall Corporation, New York, NY, USA) were used as a stationary phase and Methanol/HCl (99:1) mixture, as a mobile phase. In these chromatography systems, three species namely, $^{99m}TcO_4^-$, $[^{99m}Tc(OH_2)_3(CO)_3]^+$ and $[^{99m}Tc(CO)_3$-BM], were separated. In paper chromatography, Whatman No. 1 paper was used as a stationary phase and acetone, as mobile phase. The distance traveled by solvents over the strip in each chromatographic system was 10 cm. A radio-Thin-Chromatography Imaging Scanner (Bioscan Inc.,

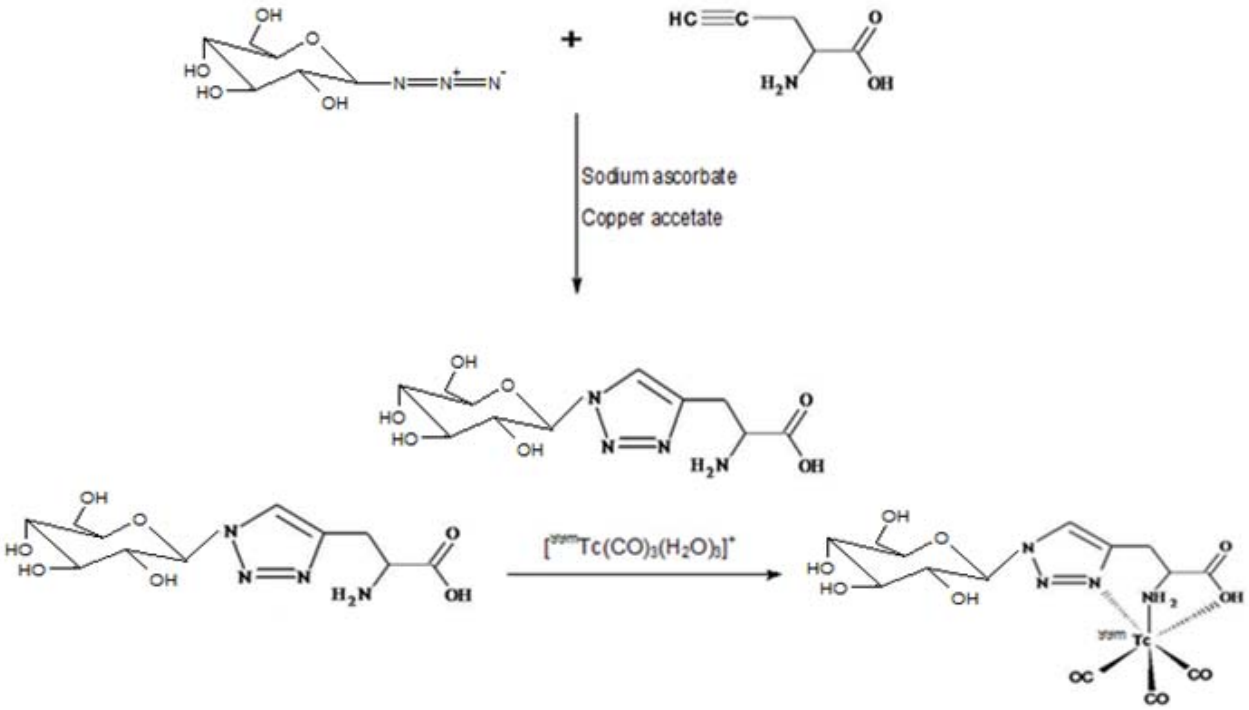

Fig. 2: Schematic diagram showing synthesis and radiolabeling of 1-azido-1-deoxy-β-D-glucopyranoside complex

USA) was used to evaluate the radioactive profile of ITLC and paper strips.

In vitro stability

Stability of radiolabeled drug was studied at room temperature at discrete time intervals of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5h, at room temperature. Change in stability was analyzed at each time interval by paper chromatography to detect any dissociation of the complex. The high labeling efficacy of radio pharmaceutical shows the validity of labeling technique with the radio metal.

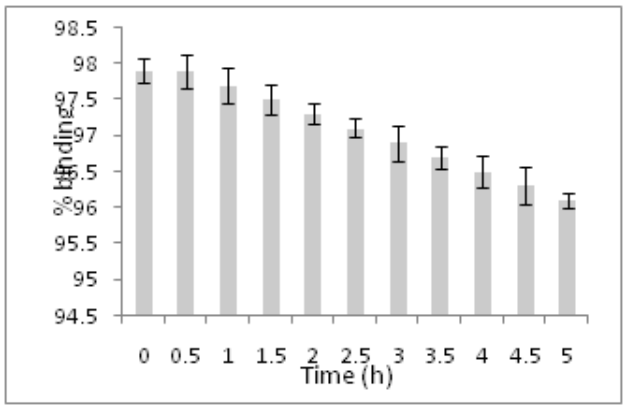

Fig. 3: Graphical presentation showing decrease in labeling efficacy (%) with time.

Protein binding

To start the experimental procedure, 3ml of fresh blood from a healthy volunteer was taken in a tube. The kit was labeled according to the procedure as mentioned before. A volume of 250µl from labeled drug was added in the blood and incubation was performed at room temperature for 1h. The reaction was terminated after incubating the tube in water bath for 10 min, pre-set at 37^{o}C. The tube was centrifuged for 10 min at 3000 rpm, followed by separation of serum and blood cells in two different layers. Equal volume of 10% trichloroacetic acid (TCA) was added into separated serum. TCA was used as a precipitating agent for the serum proteins. Tube containing serum and TCA solution was placed on the shaker for 10 min and then centrifugation was performed at 3000 rpm for another 10 min. Residue was separated from the supernatant and counted for radioactivity.

Bioevaluation in mice

For evaluating the potential of the radiolabeled drug as a feasible tumor-seeking agent, its biocompatibility was tested in normal mice models. The animal experiments were conducted in accordance with current ethical rules of INMOL Hospital, Lahore, Pakistan. For this purpose, we performed animal dissection experiments at 15 min, 60 min, 180 min and 240 min, post injection (p.i.) intervals, by sacrificing three animals at each time point. As an experimental procedure, 1mCi/200µl of radiolabeled drug was injected in tail of each normal Swiss Webster mouse, bearing weight approximately 26-28g. In the second set of experiment, we applied exactly same protocol on tumor-bearing mice. In the third set of experiment, 2-fluoro-2-deoxy-glucose (^{18}F-FDG) was applied in tumor-bearing mice, which were used as a positive control. All of the

Table 1: %ID/g of radio labeled 2-amino-3-{1-[(3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl]-1*H*-1,2,3-triazol-4-yl} propanoic acid in various organs of normal mice, at various intervals after injection

Organs	15 min	60 min	180 min	240 min
Brain	8.2±0.5	7.2±1.1	6.4±1.6	5.8±2.1
Spleen	0.6±0.1	1.5±0.3	1.6±0.5	1.3±0.8
Kidney	15.2±2.4	23.5±2.5	25.1±1.2	20.1±0.5
Liver	7.4±1.5	4.5±1.1	3.9±1.2	3.1±0.9
Lungs	4.6±1.1	4.1±0.8	3.5±0.9	2.9±0.7
Heart	1.7±0.4	1.1±0.1	0.7±0.2	0.4±0.1
Bladder	4.1±1.5	7.2±1.3	10.3±2.1	9.2±1.3
Tail	38.4±3.1	23.5±3.2	18.2±3.1	15.6±1.8

Table 2: %ID/g of radio labeled 2-amino-3-{1-[(3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl]-1*H*-1,2,3-triazol-4-yl} propanoic acid in various organs of tumor bearing mice, at various intervals after injection

Organs	15 min	60 min	180 min	240 min
Tumor	9.2±1.1	10.1±1.1	8.3±1.9	4.1±1.1
Brain	2.1±0.5	1.9±0.3	1.6±0.4	0.8±0.2
Spleen	1.2±0.4	1.3±0.3	1.1±0.3	0.9±0.3
Kidney	16.1±2.1	21.6±2.5	25.3±2.9	23.3±2.5
Liver	6.9±1.3	5.8±1.7	3.2±0.9	2.1±0.8
Lungs	5.1±0.9	4.1±0.8	3.2±0.7	2.7±0.5
Heart	1.2±0.3	0.9±0.3	0.7±0.2	0.5±0.2
Bladder	3.9±1.6	9.2±1.3	10.1±2.1	12.1±1.8
Tail	36.3±2.7	21.1±2.9	14.6±2.8	11.3±1.5

Table 3: %ID/g of 2-Fluoro-2-deoxy-glucose (^{18}F-FDG) in various organs of tumor-bearing mice, at various intervals after injection

Organs	15 min	60 min	180 min	240 min
Tumor	8.3±1.6	9.9±1.4	9.1±1.3	7.6±1.5
Brain	5.6±0.6	6.1±0.8	5.8±0.8	3.2±0.4
Spleen	1.7±0.7	2.2±0.5	1.9±0.7	1.1±0.6
Kidney	8.2±1.4	12.6±2.8	18.5±2.1	25.1±3.1
Liver	12.3±1.5	9.2±1.4	8.6±0.7	6.3±0.5
Lungs	9.4±1.5	8.5±0.6	6.4±0.5	3.1±0.8
Heart	12.4±1.3	10.5±0.8	8.2±1.5	4.2±1.3
Bladder	11.9±1.2	15.4±1.5	20.2±2.8	24.4±2.8
Tail	31.2±3.9	24.3±2.5	16.6±2.8	14.8±1.3

three experiments were performed by following exactly same protocol and dissecting five animals in each set of experiment. In all cases, the organs were weighed and counted for radioactivity.

Histopathology of tumor

Specimen (~ 2.1 x 2.1 cm diameter) from tumor of each animal, looking in smooth, reddish appearance was extracted after dissection. The sample was entirely submitted on tissue cassette. All samples were processed in the processor for 14-15h. The specimens were embedded, blocks prepared with wax and frozen. After cutting slides on microtome (in size of 3 micron), samples were prepared for staining and reporting. The blocks were cut; slides dew axed on hot plate, and dipped in xylene. The process was repeated three times, the slides dipped in 95% ethanol for 10-20 min and later in 75% ethanol for 5-7 min. The process was repeated thrice, placed slides in haematoxlyne for 15 min, followed by placement in running tab water for 10 min. Decolorization was developed by placing the slides for 1-2 second in standard acid decolorizer. The rinsing was carried out in standard ammonia solution containing 2 ml NH_3 in 8 ml water, followed by rinsing in water and placing in Eosin for 5 min. The slides were further rinsed through ethanol by using increasing concentrations of 75%, 80% and 95%. Slides were finally placed in xylene container, cleaned, put in mounting media and covered with slips.

RESULTS

Synthesis of (1-azido-1-deoxy-β-D-glucopyranoside) complex

The synthesis of (1-azido-1-deoxy-β-D-glucopyranoside) complex was performed starting from 1-azido-1-deoxy-β-D- glucopyranoside, propagylglycine, sodium ascorbate and copper accetate. Copper-catalyzed reaction was performed in aqueous media under mild reaction conditions to obtain physiologically stable product. The structure of the final product was characterized by using ESI-MS spectra, ^{1}H-NMR and ^{13}C-NMR. The ESI-MS of the product produced peak at m/z 319.12, consistent with the molecular formula $C_{11}H_{18}N_4O_7$ ($M + H^+$). fig. 1 shows the structure and numbering pattern adopted in (2-amino-3-{1-[(3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2*H*-pyran-2-yl]-1*H*-1,2,3-triazol-4-yl}) propanoic acid, for ^{1}H, and^{13}C NMR spectra.

Quality control

As described earlier, three species namely, $^{99m}TcO_4^-$, $[^{99m}Tc(OH_2)_3(CO)_3]^+$ and $[^{99m}Tc(CO)_3$-BM], were separated, in chromatographic analysis. Retention factors (R_f) in ITLC (performed with methanol/HCl, 99:1) for these three species were 1, 1 and 0, respectively. In this solvent system, only $^{99m}TcO_4^-$ migrated along the solvent front from the sample application point (R_f = 1.0) while $[^{99m}Tc(OH_2)_3(CO)_3]^+$ was settled at the baseline (R_f = 0). The labeling efficacy of $^{99m}Tc(CO)_3$-labeled 1-azido-1-deoxy-β-D-glucopyranoside complex was found to be 97.9±1.5%.

In vitro stability and protein binding

The *in vitro* stability of the drug was determined as a function of time by calculating the percentage of metal-ligand complex at constant intervals of time up to 5h. Fig. 3 shows that the labeling efficacy of the radiolabeled drug remained 96.1±1% after 5h. The high labeling efficacy of the drug shows the validity of labeling technique with the radiometal. The data indicate that the radiolabeled drug is quite suitable for its further evaluation as a potential tumor-seeking agent. *In vitro* protein binding of this novel radiopharmaceutical in human plasma protein was observed to be 73.6±1.1%

Bioevaluation

Bio evaluation of newly synthesized radiolabeled drug was studied in normal mice models for studying the distribution of radiopharmaceutical in various body organs. The mice were dissected at 15 min, 60 min 180 min and 240 min of post injection. The drug showed prominent uptake in brain at 15 min, while at 60 min study minor decrease of activity in brain was observed, which was further observed at 180 min and 240 min, post injection intervals. The data obtained from animal biodistribution experiments are shown in tables 1-3.

The tumoral uptake of radiolabeled drug was evaluated by studying its biodistribution in tumor bearing mice. High uptake of radiopharmaceutical in tumor shows its significant application in diagnosis. The uptake of the radiolabeled drug in tumoral cells depends on various factors like plasma concentration, pH, nature of the complex and blood flow. The most probable route of excretion of the drug and metabolites was through kidney and bladder. %ID/g in each organ, obtained after dissection of animal, is shown in table 2. Furthermore, the tumor tissues obtained from above study were further investigated by hispathology. After staining the tumor tissues with hematoxylin-eosin (HE), histopathological data revealed that mice were expressing neuroendocrine tumors of diverse histopathology.

DISCUSSION

The click chemistry strategy is an attractive method of choice because of high chemical yield of the product and favorable azide and alkyne reaction kinetics. The (1-azido-1-deoxy-β-D- glucopyranoside) based ligand was developed using click chemistry and specifically by combining the azide and alkyne in copper catalyzed azide-alkyne cycloaddition. Radiolabeling of the biologically active molecule is an indispensable criterion for the non-invasive evaluation of the newly synthesized drug candidates, *in vivo*. We used carbonyl method of radiolabeling. Firstly, we radiolabeled carbonyl kit with ^{99m}Tc and then allowed it to react with bifunctional chelating agent to form a radiopharmaceutical. The aim to use $[^{99m}Tc(CO)_3]^+$ core is to provide stability to the radio pharmaceutical due to its reduced size. Furthermore, it helps the basic structure to remain intact with the $[^{99m}TcO_3]^+$. Fig. 2 shows the reaction mechanism for the synthesis of (2-amino-3-{1-[(3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl]-1*H*-1,2,3-triazol-4-yl}propanoic acid and) its radiolabeling with $[^{99m}Tc(CO)_3]^+$ core.

Generally, there is transchelation of radiometal to plasma proteins, particularly, albumin so it is essential to study the *in vitro* blood protein binding with radiolabeled drug before it is applied in any *in vivo* application. Radiolabeled drug interaction with blood proteins highly affects the pharmacokinetic characteristics of the radiopharmaceuticals such as excretion of drug, volume of distribution, its metabolism and accordingly, the dosage. So, the evaluation of drug-protein interaction level becomes mandatory in most of the drug development scenarios (Rolan *et al.,* 1994; Hull *et al.,* 1988). The protein-binding assay of the radiolabeled drug is an essential parameter for measuring the effectiveness of the chelating moiety to coordinate the radio metal.

Protein binding of the radiolabeled drug tells us the efficacy of drug in the body. The drug, which shows

maximum binding with blood proteins should remain in the blood stream while free constituents of the drug should be extracted. *In vitro* protein binding of this novel radio pharmaceutical in human plasma protein was observed to be 73.6±1.1%. This feature of the drug might be helpful to retain the drug in blood by decreasing the chance of any pharmacological changes (Hull *et al.,* 1988).

The data clearly indicate that maximum uptake was shown in tumor rather than brain, as shown in table 1. These findings are in contrast with the results displayed by ^{18}F-FDG uptake in tumor cells (table 3), where in spite of significant amount of uptake in tumor, an adequate amount of activity was also detected in brain and heart which remained persistent till 240 min. Such investigations indicate that uptake of $^{99m}Tc(CO)_3$-labeled 1-azido-1-deoxy-β-D- glucopyranoside complex in tumor cells might be due to a different mechanism since it shows remarkably enhanced specificity in tumor cells with minor accumulation of activity in brain and heart. This feature exhibited by our novel radiolabeled drug could be considered as its extraordinary merit over conventional PET scans performed with ^{18}F-FDG. However, further investigations are necessary to authenticate the data and establish it further as a tumor-seeking agent of clinical significance in diagnosis of tumors.

CONCLUSION

In this study, biologically active (1-azido-1-deoxy-β-D-glucopyranoside) complex has been synthesized, radio labeled and bioevaluated to test its potential as a tumor-seeking agent. The data obtained from this study show the selectivity of biologically-active $^{99m}Tc(CO)_3$-labeled (1-azido-1-deoxy-β-D-glucopyranoside) complex towards the solid tumors. However, further investigations are necessary to increase the target to non-target ratio, of this tumor-seeking potential radiopharmaceutical.

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