Development of ^{99m}Tc-5-fluorouracil as a potential tumor diagnostic agent

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Abstract: 5-Fluorouracil is a well know drug for chemotherapy of various types of cancer. In the present study, we radiolabeled 5-fluorouracil with ^{99m}Tc for a diagnostic study of cancer. After successful labeling of the drug we performed an animal study to evaluate the potential of this radiopharmaceutical as a tumor diagnostic agent. The results showed 98.1 ± 1.2 % labeling efficacy of 5-fluorouracil with ^{99m}Tc. The *in vitro* stability of the radiolabeled drug at room temperature at 4 hr of post-labeling was >96.5 ± 0.4 %. The binding of the radiolabeled drug with plasma proteins was 66.6 ± 3%. Partition coefficient results showed that this drug is hydrophilic in nature. Biodistribution study in rabbit models displayed faint uptake in liver. Both kidney and bladder were prominent as excretory route of the labeled drug. Bioevaluation was performed in Swiss Webster mice having naturally developed tumor. Mice were dissected, uptake of drug in various organs was studied and results showed prominent uptake in liver and tumor. Tumor was further investigated by histopathological study.

Keywords: 5-Fluorouracil, radiolabeling, biodistribution, tumor, diagnosis.

INTRODUCTION

5-Fluorouracil is a pyrimidine analog that belongs to antimetabolite family of pharmaceuticals. It is used as an anticancer drug in the chemotherapeutic treatment of various types of cancer as bowel, stomach, pancreatic, breast and gullet (oesophagus) cancer (Gunnar *et al*., 1996).

5-Fluorouracil is involved in metabolic activation of RNA to 5-fluoro-2'-deoxyuridine-5-monophosphate; it inhibits the enzymatic activity of thymidylate synthetase that is an important enzyme for DNA synthesis (Heidelberger, 1981). 5-Fluorouracil is converted into various cytotoxic metabolites inside the cell which are then incorporated into RNA and DNA, it causes the cell apoptosis and cell cycle arrest by suppressing the cell's ability for synthesizing DNA. It inhibits the function of the exosome complex in DNA and RNA, exosome complex is an exoribonuclease and its activity is essential for cell survival (Jain, 1997). It reaches the solid tumors through blood supply and penetrates into the tumor via extravascular space to come across all cancer cells in significant concentration to cause lethal toxicity. The rate of drug metabolism, diffusion through tissue and binding to tissue components, greatly affects the distribution of drugs in tumors (Hicks *et al.*, 1997; Jain, 1990; Wilson *et al.*, 1999).

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In the present study, radiolabeling of 5-fluorourail has been performed by using $Na^{99m}TcO_4$, to use it as a potential candidate for diagnosis of cancer. The intention was to deliver high dose of radiation to selected malignant sites in targeted tumor, while minimizing the radiation dose to surrounding healthy cells. As 5-fluorouracil has already been in use for chemotherapeutic treatment of cancer, its pharmaceutical characteristics are well known. We, hereby, report a very simple and easy method of its radiolabeling that is performed under physiological conditions.

MATERIALS AND METHODS

All chemicals used for this research were of analytical grade. 5-Fluorouracil, stannous chloride and L-cysteine hydrochloride monohydrate were purchased from Aldrich, USA. ^{99m}Tc generator was taken from Pakistan Institute of Nuclear Science and Technology (PINSTECH, Islamabad), Pakistan. Saline was purchased from Ostuka, Pakistan. Rabbits were obtained from the animal house/laboratory of the Institute of Nuclear Medicine and Oncology (INMOL), Lahore, Pakistan. Swiss Webster mice, weighing approximately 28 g with naturally developed tumors, were obtained from the Animal House at School of Biological Sciences (SBS), University of the Punjab, Lahore, Pakistan.

Kit formation

10 mg 5-fluorouracil was dissolved in 10 ml of distilled water with continuous stirring. 5 mg of L-cysteine

hydrochloride monohydrate was added in it. Afterwards, 200 µg $SnCl_2.2H_2O$ was added and pH was maintained at 7 by using 5N NaOH, 1N NaOH and 0.1N NaOH. The product was passed through a 0.22 µm membrane filter. 1 ml/kit of resultant solution were dispensed in sterilized serum vials.

Radiolabeling and quality control

10 mCi/0.5 ml of $Na^{99m}TcO_4$ eluted from ^{99}Mo-^{99}Tc generator was added to the kit (1 ml) and incubated at room temperature for 15 min. Radiochemical purity of the ^{99m}Tc-labeled 5-fluorouracil was studied by using two simple chromatographic techniques, e.g., Instant Thin Layer Chromatography (ITLC) and Paper Chromatography (3MM Whatman paper). The idea was to determine the percentage fraction of radioisotope bound to ligand, hydrolyzed portion, and amount of free pertechnetate. Acetone was used as a mobile phase for paper chromatography and saline was used for ITLC. Small aliquots from the reconstituted kit were spotted on the respective strips. The strips, after elution, were cut in fractions of 1 cm and counted for radioactivity in a well type gamma scintillation counter.

In vitro stability at room temperature

In vitro stability of the 5-fluorouracil radiocomplex with ^{99m}Tc was studied at room temperature at time intervals of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hr. The purpose to determine the *in vitro* stability was to detect any dissociation of the complex at room temperature. For this purpose, ITLC-SG and Paper Chromatography (PC) were again used as standard techniques. The percentage dissociation of the complex at a particular time interval was detected by the percentage of free pertechnetate (%) at that time. In case of significant loss of metal-complex stability, it is not advisable to use the radiopharmaceutical for clinical applications. Data are shown in fig. 1.

Protein binding

5 mCi of $Na^{99m}TcO_4$ was added to the 5-fluorouracil kit and incubated at room temperature for 15 min. 5 ml of fresh human blood was taken in the tube and radiolabeled kit was added into the blood sample and incubated at room temperature for 1 hr. The reaction was terminated by placing tube in water bath pre-set at 37ºC for 10 min. Contents of the tube were centrifuged at 3000 rpm for 10 min for separation of serum and blood cells. Serum was collected in a tube and equal volume of 10% TCA (trichloro acetic acid) solution was added, which was used as a precipitating agent for the serum proteins. After shaking for 10 min, the mixture was centrifuged at 3000 rpm for 10 min. Residue was separated from supernatant and both layers were counted for radioactivity.

Partition coefficient

200 µl phosphate buffer of pH 6.6, 7.0 and 7.6 was taken in separate vials and same quantity of octanol was added in each vial. 200 µl of radiolabeled kit was added in each vial. These vials were sealed and shaken for 10 min. After vortex mixing, two separate layers were obtained. They were separated and counted for radioactivity. Same procedure was repeated by using saline instead of phosphate buffer.

Biodistribution study in normal rabbit

For bioevaluation of the radiolabeled pharmaceutical as an imaging agent, the study was carried out in normal rabbit models. Valium injection was used as an anesthetic drug. The kit was prepared under sterilized conditions and 300 µl (3 mCi) from it was injected intravenously into the rear ear vein. The scanning was executed on a dual head SPECT gamma camera to study the uptake of radiopharmaceutical and its route of excretion for different intervals of time.

Biodistribution study in tumor bearing mice

For studying the tumoral uptake of drug, we injected 200 µl/mCi of the radiolabeled drug intravenously in the tail of mouse. After 1.5 hr, the animal was dissected and percent uptake of drug in various organs was calculated by using scintillation gamma counter.

Histopathology of the tumor

After dissection, the tumor samples from the mice were subjected to HE stains and CK-specific immunostaining. For this purpose, paraffin-embedded blocks were prepared from the tumor tissue and 4 µm thick serial sections were developed from the blocks. Streptavidin-biotin method was used to perform immunohistochemical staining with a murine monoclonal antibody, CAM 5.2, against CK of low molecular weight. The dehydrated and dewaxed constituents were heated for 10 min in a microwave oven for recovery of antigens in the specimens. The samples were incubated with 3% hydrogen peroxide in 100% methanol to block the endogenous peroxidase. The tissue constituents were then incubated over night at 4°C with the primary antibody CAM 5.2 at 25 µg per ml. The second antibodies and biotinylated antibodies were applied against mice immunoglobulin and constituents were incubated with peroxidase-labeled streptavidin. Products were depicted with diaminobenzidine as the chromogen, and for the visualization of lymphocytes, constituents were counterstained with methyl green. Tris-buffered saline was used rather than primary antibody for negative controls.

RESULTS

Quality control of radiolabeled 5-fluorouracil

During the radiolabeling of the drug, three species were formed, the bound ^{99m}Tc complex, free pertechnetate ($^{99m}TcO_4^-$) and reduced or hydrolyzed $^{99m}TcO_2$, which were separated by PC and ITLC, respectively. In PC,

$^{99m}TcO_4^-$ had an *Rf* of 0.8 – 0.9, while the ^{99m}Tc-ligand complex and the reduced or hydrolyzed $^{99m}TcO_2$ appeared at *Rf* = 0.00 – 0.01. The reduced or hydrolyzed fraction was separated from ^{99m}Tc-ligand complex by using saline, in which case the ^{99m}Tc-ligand complex and free $^{99m}TcO_4^-$ appeared at *Rf* = 0.9 – 1.0, and reduced or hydrolyzed fraction ($^{99m}TcO_2$) was detected at *Rf* = 0.00 – 0.01. The overall labeling yield of ^{99m}Tc-ligand complex, as calculated by these methods, was more than 98.1 ± 1.2%.

In vitro stability of radiolabeled drug

In vitro stability of the radiolabeled 5-fluorouracil was evaluated as a function of time by determining the amount of free pertechnetate and colloid formed with constant intervals of time up to 4 hr. It was observed that the labeling efficacy remained > 96.5 ± 0.4 % after 4 hr with minor fractions of free and colloid formation (fig. 1). The high labeling efficacy shows the validity of labeling technique with the radiometal. These data indicate that the radiometal complex should be quite suitable for its further evaluation as a potential scintigraphic agent.

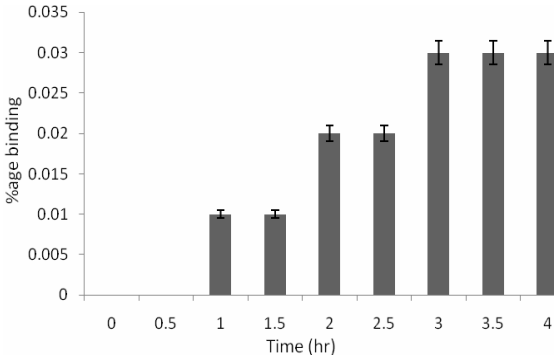


Fig. 1: Graphical presentation of increase in colloids formation (%) with time.

Protein binding

In vitro protein binding of ^{99m}Tc labeled 5-fluorouracil in human blood was observed to be 66.6 ± 3%. This feature might be of assistance to retain the drug in blood and decreasing the chance of any pharmacological change in body (Hull *et al.,* 1988).

Partition coefficient

^{99m}Tc labeled 5-fluorouracil was evaluated for its hydrophilic and lipophilic characteristics. Results indicate that ^{99m}Tc labeled 5-fluorouracil shows maximum binding in hydrophilic layer (phosphate buffers of pH 6.6, 7.0, 7.6), thus showing that the radiolabeled compound is hydrophilic in nature. Data are shown in the Table 1.

Biodistribution in normal rabbit and tumor bearing mice

The uptake pattern of radiolabeled drug in animal models was evaluated by performing the biodistribution study in normal rabbit and mice bearing naturally developed tumors. It was observed that in the rabbit model, the ^{99m}Tc labeled 5-fluorouracil initially showed the tracer uptake in liver (fig. 2), which was then excreted from heart and liver within 1 hr. High uptake of radiolabeled drug in tumor presents its significant application in diagnosis of cancer. Mice were dissected at 90 min post injection, each organ collected in a separate test tube and counted for radioactivity in gamma scintillation counter. I.D/G in each organ is shown in table 2.

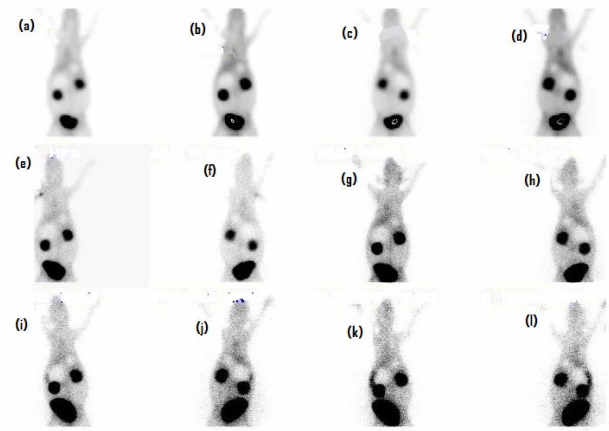


Fig. 2: Biodistribution study of radiolabeled 5-fluorouracil in rabbit at various time intervals, post injection (p.i.), **(a)** A posterior view at 7 min p.i. from a dynamic study of 15 min, **(b)** A posterior view at 15 min p.i. from a dynamic study of 15 min, **(c)** An anterior view at 7 min p.i. from a dynamic study of 15 min, **(d)** An anterior view at 15 min p.i. from a dynamic study of 15 min. **(e)** Static view in posterior posture at 1 hr p.i. **(f)** Static view in anterior posture at 1 hr p.i. **(g)** Static view in posterior posture at 2 hr p.i. **(h)** Static view in anterior posture at 2 hr p.i. **(i)** Static view in posterior posture at 3 hr p.i. **(j)** Static view in anterior posture at 3 hr p.i. **(k)**

Table 1: Binding of radiometal-drug complex (%) in phosphate buffers and octanol

Sample		Radiolabeling in hydrophilic layer (% ± 1)	Radiolabeling in lipophilic layer (% ± 0.2)
Hydrophilic solvent	Lipophilic solvent		
Phosphate buffer pH = 6.6	Octanol	99.7	0.3
Phosphate buffer pH = 7.0	Octanol	99.1	0.9
Phosphate buffer pH = 7.6	Octanol	99.6	0.4
Saline	Octanol	99.8	0.2

Static view in posterior posture at 4 hr p.i. **(ll)** Static view in anterior posture at 4 hr p.i.

Table 2: I.D/G (injected dose per gram) of radiolabeled ^{99m}Tc-5-flourouracil in various organs of mice

Organ	I.D/G
Brain	0.06 ± 0.02
Liver	25.04 ± 1
Heart	0.07 ± 0.05
Spleen	0.09 ± 0.05
Kidney	2.46 ± 1
Bladder	0.21 ± 0.05
Blood	0.01 ± 0.005
Lungs	0.02 ± 0.005
Tissue	0.39 ± 0.05
Tumor	21.03 ± 1
Tail (injection site)	51.01 ± 1

Histopathology data

Fig. 3A shows the data of tumor tissues after staining with immunostaining specific for cytokeratin (CK) and hematoxylin-eosin (HE). Histopathologic analysis revealed that sex-cord stromal tumor of testis of diverse histopathology type might be present. Afterwards, immunohistochemical staining for cytokeratin (fig. 3B), which is a marker of luminal epithelial cells, was applied. The cytokeratin (CK) findings were marked as negative because cells remained unidentifiable after immunostaining specific for CK.

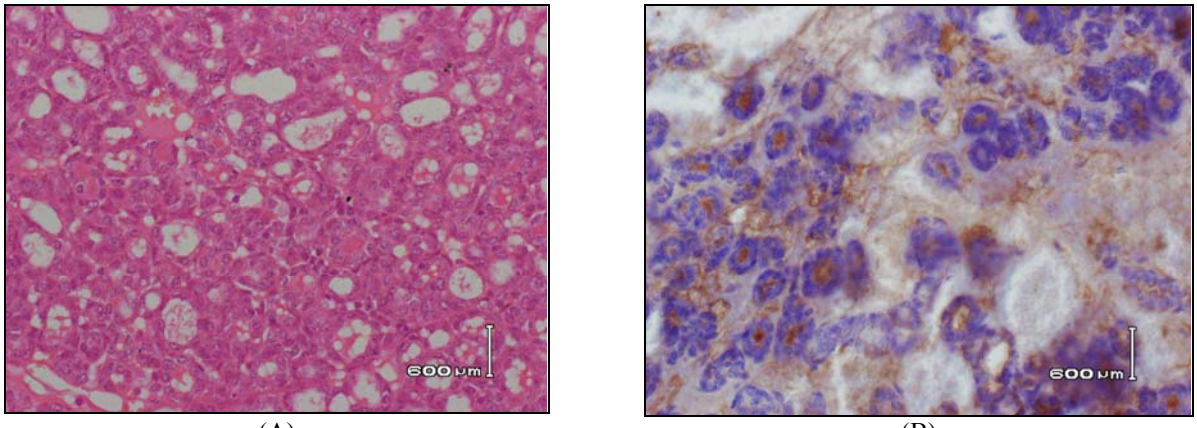
DISCUSSION

Since the chemotherapeutic drug 5-fluorouracil (5FU) is used for treatment of various type of cancers, so the present study was conducted to evaluate the potential of this drug as a diagnostic radiopharmaceutical in normal and tumor-bearing animal models. We have labeled 5FU by using the stannous chloride reduction method with technetium-99m (^{99m}Tc). The retention of the radiolabeled drug in the liver might be due to 5FU metabolism in the liver. The high uptake of ^{99m}Tc-5FU in the tumors showed the possibility of its application in cancer diagnosis.

Interaction of radiolabeled drug with blood proteins highly affects the pharmacokinetic characteristics such as volume of distribution, metabolism and excretion of drug, and accordingly, the dosage (Rolan, 1994). Binding of radiolabeled drug with blood proteins is a very important parameter for the measurement of effectiveness of chelating moiety to coordinate the radiometal. There is transchelation procedure involved in this process in which radiometal transchelates to blood proteins, particularly albumin. So it is important to study the *in vitro* blood protein binding with radiolabeled drug before it is applied to any organism. Most of the drugs bind to blood proteins or many other biological materials like glycoprotein, lipoprotein, erythrocytes, albumin and α, β and γ-globulins. Such type of binding decreases the concentration of drug in plasma. The free or unbound drug is responsible for the side effects and pharmacological activities in body (Nix *et al.*, 2004; Scaglione *et al.*, 1998; Zeitlinger *et al.*, 2004).

Percentage binding of drug with blood proteins tells us the efficacy of drug in the body. The bounded drug remains in the blood stream and the free portion of the drug is extracted and called active part of the drug which may cause pharmacological changes. This parameter is considered more important when patient might be on other medication. Because certain proteins are already saturated, this may affect the binding of new drug, thus changing its pharmacological effect.

Partition coefficient is an important parameter to be studied for evaluation of any drug. Hydrophilic and lipophilic characteristics of drugs highly affect the



(A) (B)

Fig. 3: Immunohistochemistry data of tumor tissue samples obtained after dissection of mice, **A**), Sex-cord stromal tumor of testis after staining with hematoxylin-eosin (HE), which was **B)** unidentifiable after immunostaining specific for cytokeratin (CK), thus showing cytokeratin negative.

pharmacodynamics properties of drugs. The lipophilic characteristic of the drug affects their binding to the receptor targets (Eisenberg *et al.*, 1986; Miyamoto *et al.*, 1993). One drawback of lipophilic drug is that it tends to be toxic because of longer retention and wider distribution in body. While hydrophilic drugs show rapid clearance from body, it is highly recommended to make a drug with high hydrophilic properties (Pliska *et al.,* 1996).

Biodistribution study was performed in normal rabbit models for evaluating the distribution of drug in various organs. *In vivo* stability of drug was observed by absence of activity in stomach and thyroid. The retention of radiolabeled drug in liver might be due to its metabolism in liver. It was observed that the ^{99m}Tc labeled 5-fluorouracil initially showed the tracer uptake in liver (fig. 2), which was then excreted from heart and liver within 1 hr. The most probable route of excretion observed for this drug was through the kidney and bladder and no evidence of hepatobiliary excretion was found. These findings support the earlier findings in which 5-FU exhibited a high intracellular catabolite gradient. Prominent uptake was observed in the liver and the tumor specimens. It shows that uptake of a radiotracer is dependent on various factors, like the nature of the complex, pH, blood flow, plasma concentration, etc., thus, indicating a slow transfer of charged metabolites formed across the cell membrane. Although, the present study did not provide any explanation of high uptake in liver, it can be proposed that the present radiocomplex might be taken inside the liver cells and that its degradation leads to the formation of the ^{99m}Tc-complex which is not being easily eliminated out of the cell. In other tissues, two different trends of change in activity have been observed (Visser *et al*., 1996).

CONCLUSION

In conclusion, the data obtained from the present study show selectivity of ^{99m}Tc-labeled 5-FU towards the solid tumors. However, further investigations are necessary to increase the target to nontarget ratio.

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