

Original Research Article

Biodistribution and pharmacokinetic studies on topically-delivered technetium-99m-labeled 5-FU nanogel formulation for management of pre-cancerous skin lesions

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Abstract

Purpose: To prepare technetium-99m (^{99m}Tc)-labelled nanogel loaded with 5-fluorouracil (5-FU) containing synthesized gallic acid-stearylamine (GA-SA) conjugate in order to reduce its systemic toxicity and provide site-specific delivery to skin lesions.

Methods: Lipid nanocarrier-based 1 % (w/w) 5-FU nanogel containing GA-SA conjugate was successfully formulated. Parameters that included pH, viscosity and entrapment efficiency were measured. Furthermore, 1 % (w/w) 5-FU nanogel and 1 % (w/w) 5-FU commercial formulations were radiolabelled with ^{99m}Tc. The radiolabelled ^{99m}Tc-5-FU nanogel and commercial formulations were subjected to successive preclinical assessments with respect to radiochemical stability, biodistribution, and gamma scintigraphy in BALB/c mice, and pharmacokinetic studies in New Zealand albino rabbits.

Results: The entrapment efficiency of 5-FU in the nanogel preparation was 82.12 ± 1.2 %. The 5-FU nanogel formulation exhibited excellent radiolabelling efficiency (> 93 %) and high stability. Skin/blood localization ratios of 274.93 and 167.89 were obtained for topical radiolabelled drug-loaded 5-FU nanogel formulation and 5-FU commercial formulation, respectively, after 1 h of administration. Gamma scintigraphy and biodistribution studies showed that topically administered ^{99m}Tc-5-FU nanogel was distributed mostly in skin, when compared to marketed 5-FU formulation. Pharmacokinetic studies revealed low maximum activity in the blood ($C_{max} = 34.20$ µg/mL), with low intensity (AUC) for topically administered ^{99m}Tc-5-FU nanogel formulation.

Conclusion: 5-FU nanogel enhances specific delivery of 5-FU at targeted sites and decreases its toxicity in tissues distant from the site of application. The results suggest that nanogel loaded with 5-FU containing synthesized GA-SA conjugate is a novel effective approach for the treatment of skin lesions.

Keywords: 5-Fluorouracil, Skin lesions, ^{99m}Technetium, Biodistribution, Pharmacokinetics, Gamma scintigraphy

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INTRODUCTION

Actinic keratosis (AK) is a precancerous disease which occurs in the epidermal layer of the skin.

The disease which is also known as solar keratosis, occurs mostly in individuals who are chronically exposed to UV radiation [1]. It manifests as red scaly patches on the skin which

vary in size from 1 to 20 mm [2]. Actinic keratosis lesions indicate the early stage of non-melanoma skin cancer. Lesions may occur singly or in multiples, and may vary in diameter from 0.1 to 2.5 cm. Usually, AKs are considered as lesions with low potential for aggressive malignancy, which subsequently develop higher potential for spontaneous regression. An invasive AK has the potential to develop into the squamous cell carcinoma (SCC), a serious type of skin cancer [3].

Lipids present in the skin contribute to its barrier properties, and may serve as rate limiting membrane barrier for systematic absorption of active moieties. Lipid vesicles are non-toxic penetration enhancers due to their amphiphilic nature [4]. In addition, vesicles which are rich in lipids are used to carry substantial quantities of drugs across the skin through enhancement of cutaneous retention and drug permeation [5]. Nanogels are nanosized particles formed via chemically crosslinked polymer networks that swell in appropriate solvents. They have the capacity to form complexes with various DNAs, proteins and drugs, and they are also used to coat surfaces of liposomes and solid surfaces, including cells [6,7]. In contrast to other transdermal delivery agents such as creams and ointments, nanogels act as excellent carriers with stable and controlled drug release kinetics, with lower side effects [8]. Due to their tiny volumes, they have the ability to penetrate tissues through transcellular or paracellular pathways [9].

Technetium-99m (^{99m}Tc) is the most widespread radionuclide due to its versatile chemistry, cost effectiveness, low radiation dose, near-ideal energy (140 keV), short half-life (6 h), and ideal nuclear medicine imaging properties [10,11]. A previous study showed that combination therapy using 5-FU/GA-SA conjugate at a ratio of 1:1 (1 : 10 $\mu\text{g}/\text{mL}$) provided synergistic cytotoxicity in A431 skin cancer cells [12]. In continuation of the study, the present work focused on the preparation and evaluation of 5-FU nanogel containing GA-SA conjugate formulation to potentiate site-specific drug delivery and reduce systemic toxicity in the treatment of skin lesions.

EXPERIMENTAL

Chemicals

5-Fluorouracil was purchased from Ranbaxy Industries Ltd. Sodium cholate was obtained from Sigma Aldrich Pvt Ltd, Bangalore, India. Soya lecithin was purchased from Acros Organics (USA). Triethanolamine (98 %), methyl paraben, propyl paraben and propylene glycol

were products of Central Drug House Pvt Ltd, New Delhi, India, while Carbopol 934 were bought from Himedia Pvt. Ltd, Mumbai, India.

Animals

BALB/c mice weighing 20 - 25 g were used for biodistribution studies, while New Zealand albino rabbits weighing 2 - 3 kg were used for pharmacokinetics studies. All animal experiments were followed by International regulations of AAALAC [13] (Assessment and Accreditation of Laboratory Animal Care International). The experimental protocols were approved by the Institutional Animal Ethics Committee of Institute of Nuclear Medicine and Allied Sciences, New Delhi, India (approval no. INM/IAEC/16/03 INMAS).

Formulation of lipid carrier based 5-FU nanogel with GA-SA conjugate

Gallic acid (GA)/stearylamine (SA) conjugate was prepared using the method outlined in a previous report [12]. Soya lecithin (lipid, 9.5 g), sodium cholate (surfactant, 0.5 g) and GA-SA conjugate (10 g) were put in a round bottom flask and dissolved with chloroform: methanol mixture (3 : 1 v/v). The organic solvent was removed using a rotary evaporator (Model Buchi Rotavapor R-300 India) to form a lipid film on the inner side of the flask. Residual drops of organic solvent were removed using a vacuum oven (Model Hmg, India). The lipid film was hydrated with sufficient quantity of 5-FU [1% (w/v) solution in phosphate buffer saline, pH 7.4] to form lipid carrier dispersion. Then, it was probe-sonicated with Model VCX 500 (Sonics, USA) for 5 min at 30 % output frequency to form the lipid nanocarrier dispersion.

Incorporation of the prepared 5-FU lipid nanocarrier dispersion system in carbopol gel base

5-Fluoro uracil (5-FU) nanogel (liposome bearing 5-FU gel) formulations were prepared by adding the fine 5-FU lipid nanocarrier dispersion system to carbopol 934 polymer (3.5 %). The polymer was gradually mixed with distilled water. It was hydrated and allowed to swell for 5 h. The medium pH was adjusted to 5.5 using triethanolamine (98 %), followed by agitation for 1 h with mechanical stirring until a transparent gel was formed. Methyl paraben (0.1 %) and propyl paraben (0.01 %) were mixed in propylene glycol and added as preservatives. The gel was allowed to equilibrate for at least 24 h at room temperature. Then, the 5-FU lipid nanocarrier dispersion system was added to the polymer gel,

and continuously stirred until a uniform nanogel was attained.

Assessment of entrapment efficiency, viscosity and pH

The DVII + Pro Brookfield viscometer were used to measure viscosity. It contains a tiny adapter for placing the sample (Spindal SC4-18 and 13-R chamber) so as to determine the viscosity values of 5-FU nanogel at percentage torque values of 10 – 100 (Brookfield Engineering Laboratories, USA). The pH of 5-FU nanogel was directly measured at room temperature in samples using pH meter (Thermo Orion, USA). The entrapment efficiency of 5-FU nanogel was estimated after separating it from the entrapped drug. In this process, 100 mg of 5-FU nanogel preparation was hydrated with 10 mL of 0.9 % sodium chloride: methanol mixture (8: 2 w/v), and shaken manually for 5 min. The sample was centrifuged for 30 min at 5000 rpm to separate the un-entrapped drug, and the clear supernatant was gently separated from the sample. The unentrapped drug solution (1 mL) was diluted using 10 mL of saline: methanol solvent, and its UV-absorbance was measured. The resultant sediment was resuspended in 0.2 % Triton x-100 (1 mL), and the UV absorbance of the suspension was measured. The total amount of drug comprised the drug present in both sediment and supernatant [14]. The drug content was analysed using UV-1700 spectrophotometer (Shimadzu, India). The entrapment efficiency (EE) was computed as shown in Eq 1.

$$C (\%) = A/B \times 100 \dots \dots \dots (1),$$

where *A* is the quantity of drug deposited as sediment, *B* is the overall quantity of 5-FU added, and *C* is the 5-FU encapsulation efficiency.

Preparation of radiolabelled drug formulations

Direct-labelling technique with ^{99m}Tc was used for radiolabelling of 5-FU nanogel formulation and marketed formulation [15,16]. Stannous chloride dihydrate (100 µg) was added to 100 µL of 0.01 M HCl. Then, 0.5 mL of the resultant solution was mixed separately with 5-FU nanogel and 5-FU marketed formulation. The pH was adjusted to 6.5 using 0.5 M NaHCO₃. Then, ^{99m}Tc was added under sterile conditions, with constant stirring, and the mixture was kept warm in an incubator for 10 min at ambient temperature.

Radiochemical stability studies on ^{99m}Tc 5-FU nanogel

Radiolabelling efficiency was measured using instant thin layer chromatography (ITLC)– silica gel-coated fibre plates (ITLC-SG), with 100 % acetone as solvent. Approximately 2 - 3 µL of radiolabelled compound was applied at the bottom of the plate, and was developed in acetone. Furthermore, the strips were cut into small pieces of size 0.5 cm. The gamma count of each piece was measured using Caprac-R scintillation well counter, and the labelling efficiency values of 5-FU nanogel and marketed 5-FU formulation were calculated [17]. The stability of these two formulations were evaluated for 24 h at various time periods. The percentage labelling efficiency was calculated as shown in Eq 2:

$$E = 100 - D \dots \dots \dots (2)$$

where *D* is the percentage free ^{99m}Tc, and *E* is the percentage labelling efficiency

Biodistribution studies

Two groups of BALB/c mice (groups 1 and 2; 3 mice per group) were used. The mice were allowed *ad libitum* access to pellet diet and drinking water. All mice were shaved at the back 24 h before starting the experiments. Group 1 mice were administered ^{99m}Tc-5-FU nanogel, while group 2 mice were given ^{99m}Tc-5-FU marketed formulation. The two treatments were applied topically, each at a dose of 500 µL. At time intervals of 60, 120, 240 and 1440 min, blood samples were collected from each animal through cardiac puncture. Simultaneously, tissue samples were taken from the skin, heart, kidney, lung, bladder, liver, stomach and intestine. The tissues were washed twice using 0.9 % NaCl to rid them of adhering and unwanted tissue, and then weighed. The radioactivity in each tissue was measured using Caprac-R well-type gamma scintillation counter. The results were calculated in terms of percentage of applied dose per gram tissue using Eq 3 [18].

$$I = \{(F/G)/H\} \times 100 \dots \dots \dots (3)$$

where *F* is the count of the sample, *G* is the weight of the sample, *H* is the total count administered, and *I* is the percentage of administered dose per gram of tissue.

Pharmacokinetic studies

Three healthy albino New Zealand rabbits were used for this aspect of the study. Radiolabelled

^{99m}Tc-5-FU nanogel and 5-FU marketed formulations were administered topically (0.5 mL). At different time intervals (0.25, 0.5, 1, 2, 3, 4, 5 and 24 h), blood samples were collected in pre-weighed tubes and subjected to radioactivity count. The pharmacokinetic parameters (PK) of the radiolabelled formulations were obtained with PK solver 2.0 software.

Gamma scintigraphy imaging

The radiolabelled formulation of ^{99m}Tc- 5-FU nanogel and ^{99m}Tc-5-FU marketed formulation were applied topically at a dose of 500 µL on 2 cm² of the shaved dorsal area of each animal. Scintigraphic imaging was achieved using single photon emission computerized tomography (Diacam, LC 75-005, Siemens AG; Erlanger, Germany) at intervals of 2, 4 and 24 h post-administration.

Statistical analysis

Data are expressed as mean ± SD (standard deviation). Statistical analysis was carried out using Statistical Package for Social Sciences version 16.0 software (SPSS). *P* < 0.05 was set as statistically significant.

RESULTS

Physicochemical properties of 5-FU nanogel formulation

The viscosity, pH and percentage entrapment efficiency of 5-FU nanogel formulation are given in Table 1.

Table 1: Physicochemical properties of 5-FU nanogel formulation

Parameter	5-FU nanogel formulation
Viscosity (cp)	2800.9 ± 9.40
pH	6.5 ± 0.01
Entrapment of 5-FU (%)	82.12 ± 1.2

Values are expressed as mean ± SD (n = 3)

Radiochemical stability of ^{99m}Tc nanogel

The radiolabelling efficiencies of ^{99m}Tc-5-FU nanogel and 5-FU marketed formulations were higher than 93 %, as shown in Figure 1.

Biodistribution

The levels of radioactivity at various time points in heart, blood, intestine lung, bladder, liver, stomach, kidney and skin tissues are presented in Figures 2 – 3. The skin/blood ratio of the ^{99m}Tc-5-FU nanogel and ^{99m}Tc-5-FU marketed

formulations are shown in Table 2.

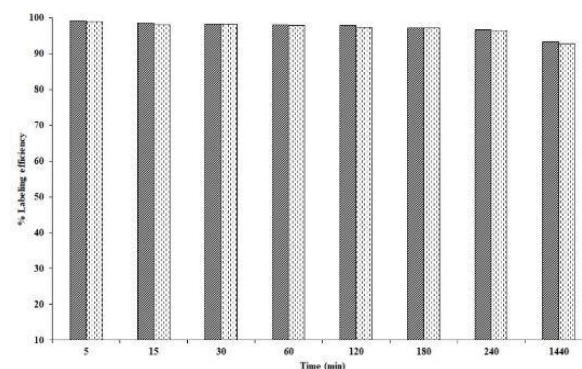


Figure 1: Radiolabelling efficiency and stability at room temperature up to 24 h. ▨ ^{99m}Tc labelled 5-FU nanogel; ▤ ^{99m}Tc labelled marketed formulation

Table 2: Skin/blood ratio of the labelled formulations of 5-FU at various time intervals

Formulation	Organ /tissue	60 min	120 min	240 min	1440 min
Nanogel	Skin/blood	274.93	259.48	293.80	1165.20
Marketed	Skin/blood	167.89	145.33	141.83	144.36

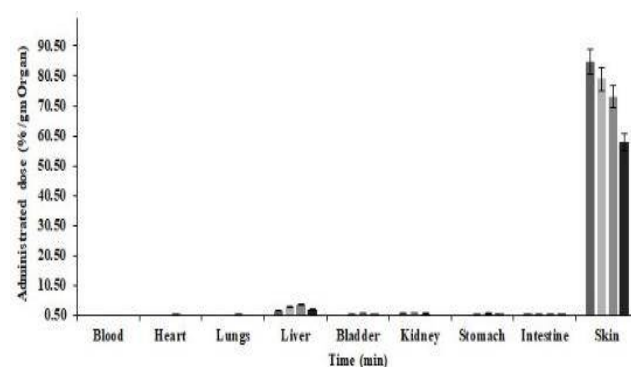


Figure 2: Biodistribution of radiolabeled 5-FU nanogel formulation. ▤ 60min; ▥ 120min; ▦ 240 min; ▧ 1440min

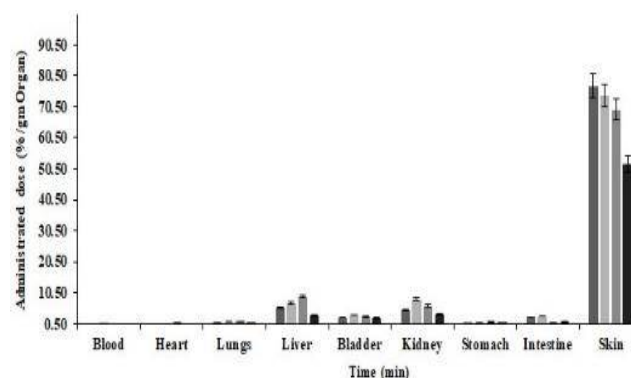


Figure 3: Biodistribution of radiolabeled 5-FU marketed formulation. ▤ 60min; ▥ 120min; ▦ 240 min; ▧ 1440min

Table 3: Pharmacokinetics of the formulations in rabbits (n = 3)

Pharmacokinetic parameter	Unit	^{99m} Tc labelled 5-FU nanogel	^{99m} Tc labelled 5-FU marketed
T _{1/2}	H	20.54	26.93
T _{max}	H	2.00	2.00
C _{max}	µg/mL	34.20	39.69
AUC _{0-t}	µg/mL*h	313.76	583.69
AUC _{0-∞}	µg/mL*h	550.01	1280.12
MRT _{0-∞}	H	27.94	38.72
CL	µg/(µg/mL)/h	0.36	0.15

Pharmacokinetics

Table 3 shows the pharmacokinetic parameters of radiolabelled 5-FU nanogel and marketed 5-FU formulations in the blood.

Gamma scintigraphy images

Figure 4 shows scintigrams in the BALB/c mice at different time intervals (2, 4 and 24 h) post-administration of radiolabelled ^{99m}Tc-5-FU nanogel and ^{99m}Tc-5-FU marketed formulations.

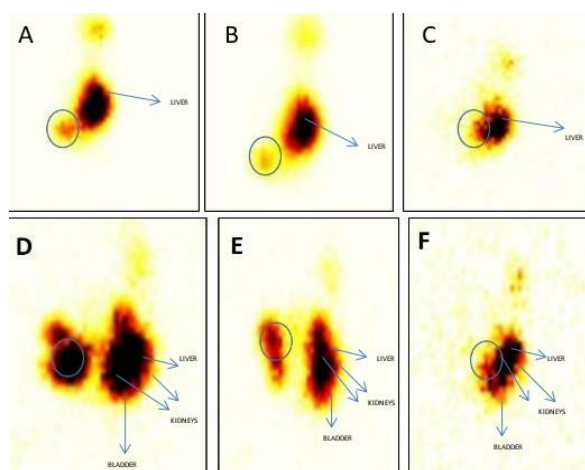


Figure 4: Scintigraphy scan of ^{99m}Tc-labeled topical 5-FU nanogel formulation imaged at (A) 2 h, (B) 4 h, and (C) 24 h post-topical administration on the dorsal side on the BALB/c mice. Scintigraphy scan of ^{99m}Tc-labeled topical 5-FU marketed formulation imaged at (D) 2 h, (E) 4 h, and (F) 24 h post-topical administration on the dorsal side on the BALB/c mice. The oval circles denote points of application of the formulation

DISCUSSION

Studies by Rajagopalan *et al* revealed that the combination of 5-FU and GA-SA conjugate led to increased synergistic cytotoxicity against skin cancer A431 cell line, without any toxic effects in normal HaCaT cell line [12]. In GA-SA conjugate, stearylamine has a long hydrocarbon chain with 18 methylene units, and GA has antioxidant [19] and anticancer properties [12]. In the present study, GA-SA conjugate was used as an

adjuvant for formulating 5-FU-loaded nanogel. In addition, sodium cholate was added as a stabilizing agent in the vesicular system, while carbopol 934 gel served as a cross-linked polyacrylic acid polymer for facilitating entrapment of the vesicles in the gel within the matrix space, thereby enhancing drug release over a long duration. Hence, the selected lipid nanocarrier-based vesicular system in gel form of 5-FU was suitable for topical administration on the skin. Viscosity (physicochemical parameter) was high in 5-FU nanogel, and pH was 6.5 ± 0.01 , which is consistent with skin pH. Thus, 5-FU nanogel is suitable as a topical formulation. The entrapment efficiency (EE) of 5-FU was also improved. The usage of highly lipophilic GA-SA conjugate and sodium cholate enhances the fluidity of vesicle membranes, thereby increasing encapsulation efficiency. At 24 h, the radiolabelling efficiency on 5-FU nanogel was more than 93 %, implying that the formulation was sufficiently stable.

The skin/blood ratio of 5-FU nanogel showed higher values for the formulated 5-FU than for the marketed 5-FU, indicating targeted delivery of the drug to the skin, thereby preventing drug entry into non-targeted locations in the body. Biodistribution studies showed that the amount of drug retained in the skin after topical administration of radiolabelled 5-FU nanogel seemed to be considerably higher even after 24 h, when compared to radiolabelled marketed 5-FU formulation. This provides further evidence of site-specific delivery of the drug. Therefore, 5-FU nanogel topical formulations have reduced systemic biodistribution and reduced toxicity.

The pharmacokinetic parameters of ^{99m}Tc-5-FU nanogel in healthy New Zealand albino rabbits showed remarkably low maximum action (C_{max}) in the plasma, low intensity (AUC) and low activity in blood, when compared to ^{99m}Tc-5-FU marketed formulation (topical).

The scintigrams revealed that high radioactivity was seen at the point of application, and at 2, 4, and 24 h post-topical administration of ^{99m}Tc-5-FU nanogel. Mice treated topically with ^{99m}Tc-5-

FU marketed formulation had high accumulation in the liver, kidney and bladder, whereas for ^{99m}Tc-5-FU nanogel (topical), only small accumulation was found in the liver, which also confirmed the results from biodistribution studies.

CONCLUSION

The study reveals that topical 5-FU nanogel possess a higher activity on the skin than a commercial formulation of 5-FU. The GA-SA conjugate increases the localization of action and stability of the formulation. These findings confirm that encapsulation of 5-FU in lipid vesicles within matrix spaces in a gel network system enhances the local action of the drug on the skin, reduces its systemic toxicity, and increases retention of drug in cutaneous epithelial cells. Thus, 1% (w/w) 5-FU nanogel preparation possesses a therapeutic potential for the management of precancerous skin lesions.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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