Development and Characterization of Regorafenib loaded Liquid Suppository for Rectal Delivery

M.Phil Thesis

by

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2023

Development and Characterization of Regorafenib loaded Liquid Suppository for Rectal Delivery

Thesis Submitted by

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to

Department of Pharmacy,

In Partial Fulfillment of the Requirements for the Degree of

Master of Philosophy

in

Pharmacy (Pharmaceutics)

Department of Pharmacy
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Islamabad, Pakistan
2023

AUTHOR'S DECLARATION

I, Aiman Saleem hereby declare that my thesis titled **"Development and characterization of regorafenib loaded liquid suppository for rectal delivery"** submitted at Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad for the award of degree of Master of Philosophy in **Pharmacy (Pharmaceutics)** is the result of research work carried out by me under the supervision of Dr. Fakhar ud Din during the duration of 2021-2023. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship. I am aware of the terms copyright and plagiarism. I will be responsible for any copyright violation found in this work.

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THIS RESEARCH WORK IS DEDICATED TO MY PARENTS AND SIBLINGS FOR THEIR ENDLESS LOVE, SUPPORT AND ENCOURAGEMENT THROUGHOUT MY PURSUIT FOR EDUCATION. A SPECIAL FEELING OF GRATITUDE TO MY FRIENDS WHOSE ALWAYS ENCOURAGE ME TO DO MY BEST.

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Acknowledgments

All praises to Allah Almighty, the most merciful and beneficial, the creator of universe. All respect to Holy Prophet (صَلَّى اللهُ عَلَيْهِ وَسَلَّمَ) the most intelligent and intellectual man who ever lived. By the grace of Allah , I have completed my research work.

I am very thankfull to my parents for their continuous support throughout my educational career.This journey was impossible without their love and support. I am thankfull to my supervisor Dr. Fakhar Ud Din for his wise suggestions, counseling, appreciation, kindness and for the facilities he provided me during my research work.

I am greatful to the Chairman of Deparment of Pharmacy, Quaid-i-Azam university, Prof Dr. Ihsan ul Haq for the facilities , he provided me during my research.

I am sincerely thankful to all the faculty members of Saulat Institite of Pharmaceutical Sciences and Drug Research, QAU for encouragement and kind recommendation.

I acknowledge the facilitation of Prof. Han Gon Choi, Hanyang University Ansan, South Korea, in providing utilizable and technical support.

I am thankful to the Higher Education Commission of Pakistan, for funding this research work.

I would like to extend my gratitude to my lab seniors, Zakir Ali, Fatima Zahid, Kanwal Shabbir, Saba Sohail, Sibgha Batool and my batchmates Uswa Shafique, Mohsin Fawad, and Shah Faisal Ghani, to all my seniors and juniors of department for their assistance, opinion and precious time for discussion. I would like to acknowledge all the clerical staff, laboratory staff and administration staff for helping me.

Aiman Saleem

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Abbreviations	**Descriptions**
AIC	Akiake Information Criterion
AUC	Area Under Curve
BCS	Biopharmaceutical Classification System
BMI	Body Mass Index
CC	Colon Cancer
Cmax	Maximum Plasma Concentration
CRC	Colorectal Cancer
CSC	Cancer Stem Cells
CT	Computed Tomographic
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
FDA	Food and Drug Administration
FGFR1	Fibroblast Drowth Factor Receptor 1
FTIR	Fourier Transform Infrared Spectroscopy
GIT	Gastrointestinal Tract
GIST	Gastrointestinal Stromal Tumors
H	Hour
h^{-1}	Per Hour
HCC	Hepatocellular Carcinoma
H&E	Hematoxylin and Eosin
HFSR	Hand-Foot Skin Reaction
HPLC	High Pressure Liquid Chromatography
IV	Intravenous
K_{el}	Elimination Rate Constant

MSC	Model Selection Criterion
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-2H-Tetrazolium Bromide
NIH	National Institute of Health
P188	Poloxamer 188
P407	Poloxamer 407
PBS	Phosphate Buffer Saline
RG	Regorafenib
Rpm	Revolution per Minute
T80	Tween® 80
Tmax	Maximum Time for Peak Drug Concentration
TAM	Tumor Associated Macrophages
PM	Physical Mixture
$t_{½}$	Half-Life
µg/ml.h	Microgram per Milliliter. Hour

Abstract

Regorafenib (RG), a multi-targeting kinase inhibitor, has recently been used in the colorectal cancer (CRC) treatment. However, its clinical effectiveness is severely constrained by its poor aqueous solubility, low oral bioavailability, extensive hepatic metabolism, and serious side effects like Hand and foot skin reaction (HFSR), rashes, fatigue, stomatitis, diarrhea, dizziness and hypertension. Therefore, it is necessary to investigate alternative administration routes in order to increase RG efficacy. The aim of this research was to develop RG loaded liquid suppository with enhanced bioavailability and reduced toxicity. RG loaded liquid suppository was optimized using Design Experts® software, with various quantities of drug (RG), polymers [poloxamer 407 (P407), and poloxamer 188 (P188)], surfactant [tween® 80, (T80)] and distilled water. Physicochemical characterization and *in vitro* drug release behavior of the RG loaded liquid suppository were investigated followed by *in vitro* cell lines study. Additionally, pharmacokinetics, *in vivo* safety and *in vivo* localization studies carried out following rectal administration in Sprague-Dawley rats. Results showed that increased P407 and T80 concentrations significantly lowered the temperature and time for gelation, while the strength and mucoadhesion of gel was meaningfully enhanced. However, RG concentration increased didn't show significant effect on all these parameters. RG loaded liquid suppository displayed a significantly improved *in vitro* release, augmented *in vivo* localization, and enhanced bioavailability, when compared with the RG suspension. *In vitro* cell lines data demonstrated that unlike normal saline treated Caco-2 and HCT 116 cell lines, the RG loaded liquid suppository and RG suspension has significantly reduced the cancer cell burden, which was also observed through their lower IC_{50} values. Histopathology study confirmed the formulation's safety for rectal administration, whereas RG suspension instigated severe damage to the rectal tissues. Concluded from the results that RG loaded liquid suppository has a great potential to target CRC with enhanced drug localization, improved bioavailability and no toxicity at the application's site.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. The Definition of Cancer

Cancer is a disorder in which body cells grows in uncontrolled manner. It can also migrate to other parts of the body. Cancer cells has the ability to occur at any part of the body. Body cells by cell division process typically develop and divide in order to developed new body cells. Old cells die from damage or ageing, and then new ones take their place. When this process failed to occus, cells that are damaged or abnormal may proliferate and grow in an uncontrolled manner (Abbas *et al.,* 2018). These cells are capable of developing into tumours, which are tissue lumps. There are two different types of tumors, cancerous or not cancerous (benign). By the process of metastasis, cancerous tumors can spread to other parts of the body to form new tumors, by invading nearby tissues or by spreading to distant parts in the body. The term "malignant tumor" can also refer to cancerous tumors. However, many cancers develop solid tumors, while cancers related to blood like leukaemias typically do not (Boutry *et al.,* 2022). The invasion or spread of benign tumors to nearby tissues is not observed. Cancerous tumors occasionally reoccur after its removal, while benign tumors typically don't. However, benign tumors have the potential to be quite large in size sometimes. Some of them, like benign tumor of brain, can be fatal or cause severe symptoms (Wang *et al.,* 2018).

1.2. Colorectal cancer (CRC)

The term CRC refers to a type of cancer in which abnormal cells develops itself gradually and forms tumor or tissue growth on the layer of the colon or rectum (Arai *et al.,* 2019). The chances of metastasis of cancer cells to other region of the body increases if polyp (abnormal growth) eventually develops into tumor. It can form a tumor on the colon or rectum lining and then spread to blood vessels or lymph nodes (Marley *et al.,* 2016; Valastyan *et al.,* 2011).

1.2.1. Epidemiology

CRC is the 2nd most lethal cancer and the 3rd most prevalent malignancy, causing approximately 0.9 million fatalities and 1.9 million cases globally in 2020 (Xi *et al.,* 2021). With 53 200 projected deaths from CRC in 2020, it is the 3rd most common cancer mortality cause for both men and women in the United States (Biller *et al.,* 2021). CRC is the 2nd leading cause of cancer related disease and mortality in men and women in Europe. Out of 4 million newly diagnosed cases of cancer each year, 0.5 million cases are related to CRC. Additionally, more than 0.25 million fatalities each year are also directly related to CRC (Dyba *et al.,* 2021). Almost 2% of CRCs are related to autoimmune diseases, and 3–5% of CRCs are hereditary in origin. In contrast, more than 90% of CRC cases are genetically caused by a person's lifestyle and environment, (Schmoll *et al.,* 2012; Li *et al.,* 2021), such as high tobacco and alcohol intake or, a low-fiber and high-fat diet, obesity, and inactivity. A central part of CRC pathogenesis also involves in the alteration of the microbiome of intestine and the growth of tumor from non-malignant tumour (Brockmueller *et al.,* 2023).

1.2.2. Pathophysiology

The hallmark of cancer development in epithelial cells of colon is facilitated by the genetic and environment factors that causes CRC (Hanahan *et al.,* 2000; Hanahan *et al.,* 2011). The genetic and epigenetic mutations gradually accumulated and activated the oncogenes and deactivated the suppressor genes of tumors. By this method hallmarks of cancer are developed. Most of the initial colonic neoplastic tumors, including aberrant crypt foci (ACF), adenomas, and sessile serrated adenoma, have been found to have diminished stability of the genome and/or its epigenome. This loss is like a key pathophysiological and molecular factor in the CRC development (Colussi *et al.,* 2013). In oncogenes and tumor suppressor genes, the stability loss epigenome and genome speeds up the mutations and epigenetic changes. As a result of which colon cells transformed to malignant through the clonic expansion cycle that favous the most aggressive and malignant type cells (Lengauer *et al.,* 1998). According to a widely accepted paradigm, the majority of CRC develop from stem cells or cells that resemble them at the base of the colon crypts (Zeki *et al.,* 2011). According to this model, these cells develop cancer stem cells (CSC), which are necessary for the development and upkeep of a tumor, as a result of mutations in oncogenes and tumor suppressor genes (Kuipers *et al.,* 2015).

1.2.3. Risk factors

1.2.3.1. Environmental factors

According to numerous studies, if a person adopting a "Westernised lifestyle" the risk of CRC development increases in them (Stigliano *et al.,* 2014; Bishehsari *et al.,* 2014). This phrase refers to a combination of factors that have been linked to a higher risk of CRC, including obesity, sedentary lifestyles, and diets high in meat, calories, fat, and fiber (Bishehsari *et al.,* 2014; Harris, 2016). The risks of CRC are also increased by tobacco smoking and alcohol consumption. Research has since shown that people who drink maximum drinks 4/day have a higher risk (52%) of getting this disease than those who don't drink or only do so occasionally (Pelucchi *et al.,* 2011). The alcohol effects on the synthesis of folate may be reflected in this carcinogenic process's mechanism. Particularly, acetaldehyde is produced when alcohol enters the colon through microbial metabolism, and it breaks down folate in living organisms (Harris, 2016). A lack of folate can result in chromosome destruction, uracil misappropriation, and other imbalances in deoxyribonucleic acid (DNA) precursors, all of which can contribute to the development of cancer, because folate is necessary for the synthesis and repair of DNA. By promoting proliferation of colon cell and apoptosis reduction, hyperinsulinemia raises the risk of CRC (Lopez Morra *et al.,* 2014).

1.2.3.2. Genetic factors

CRC has significant genetic causes in addition to the aforementioned environmental factors. The genetics role in the prevention and development of CRC has been clarified by numerous subsequent findings. The LAMB1, 20q13, and CHDH1, which are located on chromosomes 7q31, 20q13, and 16q22, respectively, have been linked to a higher risk of developing CRC, according to studies by the UK inflammatory Bowel Disease (IBD) GC and the WTCCC. Additionally, this study was the first to discover a genetic connection between IBD and CRC (Barrett *et al.,* 2009). Further investigation revealed that the genetic variants strong linkage disequilibrium rs6017342, miR-196a2, and the C allele of strong linkage disequilibrium rs11614913 all contributed to an increased CRC risk (Wu *et al.,* 2015; Zhang *et al.,* 2014). The rs4939827 strong linkage disequilibrium on the SMAD7 gene has been associated with a reduced patient survival rate, demonstrating that genes not only influence the incidence of CRC but also its mortality (Zhang *et al.,* 2014).

1.2.3.3. Effect of gene-environment interactions on CRC

The risk of CRC is influenced by the interaction between gene and environment. The outcome of CRC, for instance, may be influenced by the interaction between exercise and specific genes. PTGS2-positive individuals were found to have a correlation between level of physical activity and CRC survival, but PTGS2-negative individuals did not (Yamauchi *et al.,* 2013). Likewise, among patients in which p27 is expressed, those who engaged in physical activity had colon cancer (CC) mortality rates that were 68% lower than those who did not. But in people who did not express p27, did not receive these benefits of exercise (Meyerhardt *et al.,* 2009).

CRC risk has also been found to be influenced by the interactions between genetic and environmental factors, including aspirin use, obesity, vitamin D , and polyunsaturated fatty acids intake and alcohol consumption. For those who are CTNNB1-negative, the risk of CRC increases as body mass index (BMI) rises but no such association is seen in CTNNB1-positive patients (Morikawa *et al.,* 2013). However, those patients who were CTNNB1-positive, and also obese with a maximum BMI of 30, greater CRC survival was observed, whereas for patients which are non obese, are not dependent on the CTNNB1 status for survival (Morikawa *et al.,* 2013).

For those who have the wild-type BRAF gene, taking aspirin regularly has been linked to a lower risk of developing CRC, and those who have the mutated PIK3CA gene have been shown to have a longer survival time. Those with wild-type PIK3CA genotypes or mutated BRAF genotypes did not exhibit these associations (Nishihara *et al.,* 2013; Liao *et al.,* 2012). Similarly, those patients in which 15-PGDH gene is highly expressed were also found to have a lower risk of CRC with regular aspirin intake, but no such relationship was discovered in those with low 15-PGDH expression (Fink *et al.,* 2014). According to research, those who regularly consume alcohol but have low IGF2 DMR0 methylation have a higher risk of developing CRC than those who have higher IGF2 DMR0 methylation capacity (Nishihara *et al.,* 2014).

1.2.4. Signs and symptoms of CRC

CRC may not show symptoms in the early stages. Symptomatic problems typically begin once they have spread. CRC symptoms can vary depending on where the tumor is located and include (WebMD 2022):

- Changes in bowel habits, such as persistent diarrhea or constipation that doesn't go away
- Having the urge to poop immediately or having the sensation that you can't completely empty your bowels (tenesmus).
- Constriction in the rectum
- Bleeding in the rectum
- Dark blood stains in stools
- Slender, long, "pencil stools"
- Bloating or discomfort in the abdomen
- Lethargy
- Appetite loss and weight loss without a known cause
- Pain in pelvic region
- Anemia as a result of intestinal bleeding

1.2.5. Diagnosis

A diagnosis of CRC is made either as a result of evaluation of a patient who presents with symptoms or the results of screening. Numerous symptoms, such as bloodly stools, changes in bowel habits, and pain in abdominal region, can be linked to the disease. Other signs and symptoms include exhaustion, weight loss, and anemia-related signs and symptoms like pallor and dyspnea. Several people are diagnosed with CRC at the preclinical stage as a result of the widespread introduction of population screening. The preferred method of investigation for symptomatic patients is a colonoscopy, but there are other endoscopic techniques that are also available or being developed.

1.2.5.1. Colonoscopy

The most accurate way to diagnose CRC is through a colonoscopy. It is highly accurate method for diagnoses and can determine where the tumor is located. This method significantly enables simultaneous biopsy sampling and provides samples for both histological diagnosis and molecular profiling. Additionally, colonoscopy can be used for both diagnostic and therapeutic purposes. This is the only screening procedure wihich is used for both (Morris *et al.,* 2015; Pox *et al.,* 2012).

1.2.5.2. Capsule endoscopy

With capsule endoscopy, almost the entire gastrointestinal tract (GIT) can be examined without the need for traditional endoscopy with the help of wireless capsule device. This device can be swallowed by the screenee (Spada *et al.,* 2012). Adenomas and CRC can be diagnosed with the help of capsule endoscopy (Milluzzo *et al.,* 2019).

1.2.5.3. Computed tomographic (CT) colonography

In order to obtain the colon picture from inside, low dose of CT scanning technique is used by CT colonography. This technique is well known as a diagnostic tool for CRC. For the detection of CRC, CT colonography has been shown to have a 96% sensitivity (Pickhardt *et al.,* 2011).

1.2.5.4. Biomarkers of CRC

Due to the ability to analyse patient samples in batches, molecular detection of CRC offers a non-invasive test that is appealing to both patients and clinicians. The ideal molecular marker should continuously release into the bowel lumen or circulation, be highly specific for advanced adenomas and cancer, distinguish them from other lesions, and disappear or diminish after the lesion is removed or treated. It's true that tests using DNA, RNA, and proteins, in the blood, stool, and urine have been developed, but they haven't always been successful (Church *et al.,* 2014).

1.2.6. Treatments

Most commonly used methods for CRC treatment are given below,

1.2.6.1. Surgery

Surgery is the trusted source for the treatment of CC in its early stage. Polypectomy is used to remove cancerous polyp when cancer is present in polyp. Colectomy is a type of colon surgery in which some part or all of the colon is removed (Burch, 2021).

During the process of surgery, a surgeon will remove cancerous part of colon, along with some of the surrounding area.

In order to decrease the chances of cancer spreading to other area, surgeon may also remove surrounding lymph nodes. Depends on the stoma extent, surgeon will reattach the undamaged colon portion or will formed a stoma. Basically stoma refers to surgical opening in the abdomen wall. Waste materials passes through this opening into a bag, due to which the need for the lower part of the colon is eliminated and this process is known as colostomy.

There are some other types of surgery which includes:

- Endoscopy: In this method, surgeon will remove some small, localized cancerous cells. Flexible, thin tube will be inserted by surgeon in which light and camera is also attached. In order to remove cancer tissues, there will also have an attachment.
- Laparoscopy: In this method, a surgeon will make many small cuts in the abdominal region. This may be a best to remove polyps which are larger in size.
- Palliative surgery: In case of the advanced or untreatable cancer, palliative surgery is the best option. The aim of this surgery is to relieve symptoms. A surgeon will try to relieve any colon blockage and reduce pain, bleeding, and other symptoms.

1.2.6.2. Radiation therapy

In radiation therapy, high energy γ-rays are concentrated on cancerous cells in order to destroy them. An external radiotherapy may be used by oncology team, in which machine are used outside of the body which expels these rays.

Whereas in internal radiation thearpy, radioactive materials will be implanted in the form of a seed by a doctor near the cancerous site. Radium is a metal, which emits gamma rays. Radiation therapy may be recommended by a doctor as a standalone treatment in order to shrink a tumor or destroy cancerous cells.

Radiation therapy shows some common side effects such as, nausea, vomiting, fatigue, diarrhea, mild changes to skin that resembles to sunburn or suntan, loss appetite and weight.

1.2.6.3. Therapies using medication

Cancer can also be treated by using some medications, that targets the cancerous tissues, destroy them and stop them from spreading.

Following are the types of therapies which are used to treat CRC:

- Chemotherapy
- Targeted therapy
- Immunotherapy

1.2.6.3.1. <u>Chemotherapy</u>

In chemotherapy, drugs will be administered by cancer care team, these drugs will interfere with the division of cancerous cells. Chemotherapeutic drugs will destroy and kill cancerous cells by the protein and DNA disruption which are needed by the cells for development. In chemotherapy, cells that are rapidly dividing (cancer cells as well as healthy ones) are targeted. Healthy cells usually recover from the damaged induced by chemotherapy, while cancer cells do not. Chemotherapy usually takes place in cycles thus between doses body gets time to recover.

Chemotherapy is recommended by a cancer specialist, or oncologist, to treat CRC, before the surgical removal of tumors, in order to shrink tumor so that it can easily removed. Also, it is recommended after surgery in order to kill tumor cells that are remaining.

Some common side effects caused by chemotherapy are nausea, vomiting, hair loss and fatique.

1.2.6.3.2. <u>Targeted therapy</u>

Targeted therapy is used to target some specific genes of cancer, proteins that contributes to cancer growth and survival, or the cancerous tissue environment. This therapy is very useful because any damage to healthy cells are avoided, also it blocks the development and metastasis of tumors.

Following are the some targeted therapies which are used for CRC treatment,

a. Anti angiogenesis therapy

The process by which new blood vessels formed inside the body is known as as angiogenesis. It is because tumor cells needed nutrients for the development and metastasis, which is delivered by these blood vessels. So the target of anti angiogenesis therapy is to “starve” the cancer cells, for which they block angiogenesis process.

Some of the common examples of anti-angiogenesis therapy are,

RG , Bevacizumab, Ziv-aflibercept and Ramucirumab.

b. Epidermal growth factor receptor (EGFR) inhibitors

Drugs such as Panitumumab and Cetuximab are used as EGFR inhibitors. They are effective in stopping and slowing the development of CRC by blocking EGFR (Cancer.Net, 2022).

1.3. Regorafenib (RG)

RG is an orally administered, belongs to a class of medication known as multiple kinases inhibitor (Müller *et al.,* 2021a). The action of abnormal protein that gives signals to cancerous cells for multiplication, is blocked by the RG (Boovizhikannan *et al.,* 2022). RG is preferred in the treatment of CRC. CRC begins in the large intestine or rectum. RG is used in those patients who have been treated with certain other medicines but treatment fails to cure the disease and cancer starts to spread to other regions in the body (Rizzo *et al.,* 2020). Gastrointestinal stromal tumors (GIST) is also treated by RG in those patients who are treated with certain other medicines but they are not fully cured. It was given approval in February 2013 for patients with locally advanced, not resectable GIST tumor who had formerly received treatment with sunitinib and imatinib (Bekaii-Saab, 2018). In GIST, cancerous cells grows in the stomach, intestine, or esophagus. RG is also a drug of choice in patients suffering from hepatocellular carcinoma (HCC; a type of liver cancer) (Ettrich *et al.,* 2018). It was approved to be used in HCC on April 2017, in patients who had previously treated with sorafenib (Bekaii-Saab, 2018).

The RG is approved by Food and Drug Administration (FDA) as a treatment of choice for CRC which was previously treated with chemotherapy, on September, 2012 (Aljubran *et al.,* 2019). RG and sorafenib is structurally resemble to each other except that RG have fluorine atompresent in the central phenyl ring (Figure 1.1) (Wilhelm *et al.,* 2011; Wilhelm *et al.,* 2004). When compared to sorafenib, RG have similar but distinct biochemical profile. Addionally, RG is pharmacologically more potent (Strumberg *et al.,* 2012).

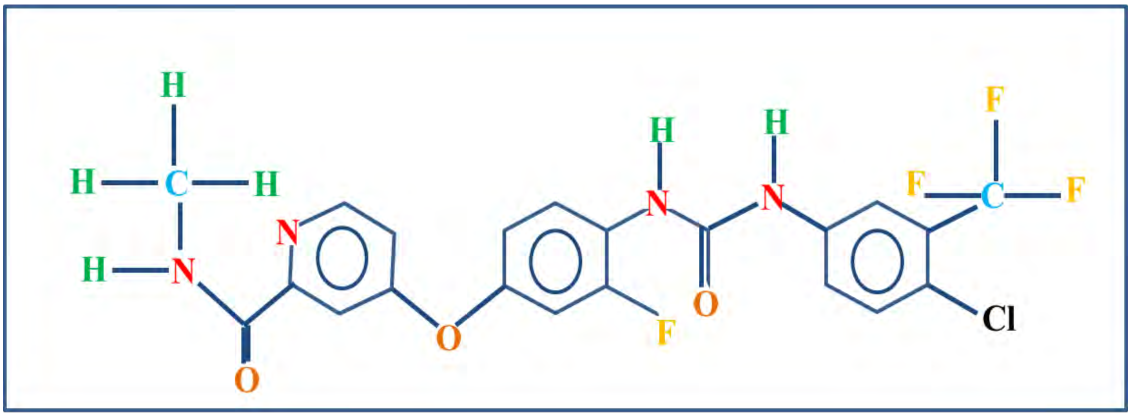

Figure 1.1. Chemical structure of regorafenib.
Note: *Image developed on power point.*

1.3.1. Physicochemical properties of RG

According to the Biopharmaceutics classification system (BCS), RG solubility in water is poor and it is classified as class II (Müller *et al.,* 2021a). Small molecule multikinase inhibitor RG received FDA approval in 2012 for the first time to treat metastatic CRC at a dose of 40 mg per tablet and a therapeutic single dose of 4 tablets, taken once daily (Goel, 2018).

The physical properties of RG is represented in Table 1.1. RG is hydrophobic in nature (Liu *et al.,* 2022). RG is sparingly soluble in acetone (CH_3COCH_3) and poorly soluble in water. In methanol (CH_3OH), acetonitrile (C_2H_3N), ethanol (CH_2CH_3OH), and ethyl acetate ($C_4H_8O_2$), it is barely soluble. It is monohydrate (Elamarthi, 2022).

Table 1.1. Physical properties of regorafenib.

Sr.no.	Physical properties		References
1	**Form**	White powder	(Shiri *et al.,* 2022)
2	**Melting point**	206.0 to 210.0 °C	(Müller *et al.,* 2021b)
3	**Water Solubility**	Less than 1 µg/mL	(Müller *et al.,* 2021a)

The chemical properties of RG such as its chemical name, molecular formula and weight is given in Table 1.2.

Table 1.2. Chemical properties of regorafenib.

Sr.no.	Chemical properties		References
1	**Chemical name**	4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]-3-fluorophenoxy]-*N*-methylpyridine-2-carboxamide.	(Majithia *et al.,* 2016)
2	**Molecular formula**	$C_{21}H_{15}ClF_4N_4O_3$	(Yi-Kun Wang *et al.,* 2018)
3	**Molecular weight**	482.8	(NCBI, 2023)
4	**Log P**	5	(Müller *et al.,* 2021a)
5	**Pka value**	10.5	(Hu *et al.,* 2017)

The medication should be taken orally as a whole tablet with water each day following a low-fat meal, which is defined as one with less than 600 calories and less than 30% of those calories coming from fat (Elamarthi, 2022). Pharmacokinetic parameters such as absorption, metabolism and elimination of RG after its administration into the body are illustrated in Table 1.3.

Table 1.3. Pharmacokinetic properties of regorafenib.

Sr.no.	Pharmacokinetic Parameters		References
1	**Absorption**	AUC = 70.4 µg.*h/mL* , Cmax = 2.5 µg/mL; Tmax = 4 h	(Xia *et al.,* 2023)
2	**Protein binding**	highly bound (99.5%)	(Zopf *et al.,* 2016)
3	**Metabolism**	metabolized by CYP3A4 and UGT1A9.	(Kojima *et al.,* 2021)
4	**Half-life**	28 h	(Ettrich *et al.,* 2018)
5	**Route of elimination**	71% excreted in feces (47% as parent compound, 24% as metabolites) and 19% excreted in urine	(Gerisch *et al.,* 2018)

Note: *Cmax: maximum plasma concentration, Tmax: time to reach maximum concentration, AUC: area under curve.*

1.3.2. RG as multitargeting kinase inhibitor

RG is an anticancer drug acting by multi-targeting kinase inhibitor. It is approved for the metastatic CRC treatment. Through many mechanisms of action, the main therapeutic action of RG involves anti-angiogenesis process and recreation of tumor microenvironment (Arai *et al.,* 2019). Additionally, it has been reported that RG completely inhibited metastasis by tyrosine kinase inhibition. It can be done through immunoglobulin (Ig) and epidermal growth factor (EGF) homology domain 2 (TIE2) signals, that contributing to the metastasis inhibition. This may decrease the tumor associated macrophages (TAMs) infiltration in CRC (Abou-Elkacem *et al.,* 2013; Zhao *et al.,* 2017). RG is a multikinase inhibitor which targeted the oncogenic kinases and angiogenic tumor microenvironment that includes fibroblast growth factor receptor 1 (FGFR1), RAF (Rapidly Accelerated Fibrosarcoma), RET (Ret Proto-Oncogene), KIT, BRAF (v-raf murine sarcoma viral oncogene homolog B1), and vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR3, VEGFR1 (Krishnamoorthy *et al.,* 2015). Regorafenib effectively blocks the stromal and angiogenic receptors that stimulate neovascularization of tumor, lymphatic vessel formation and stabilization and are crucial components of the tumor microenvironment, that favours the metastasis and tumor development (Wilhelm *et al.,* 2011).

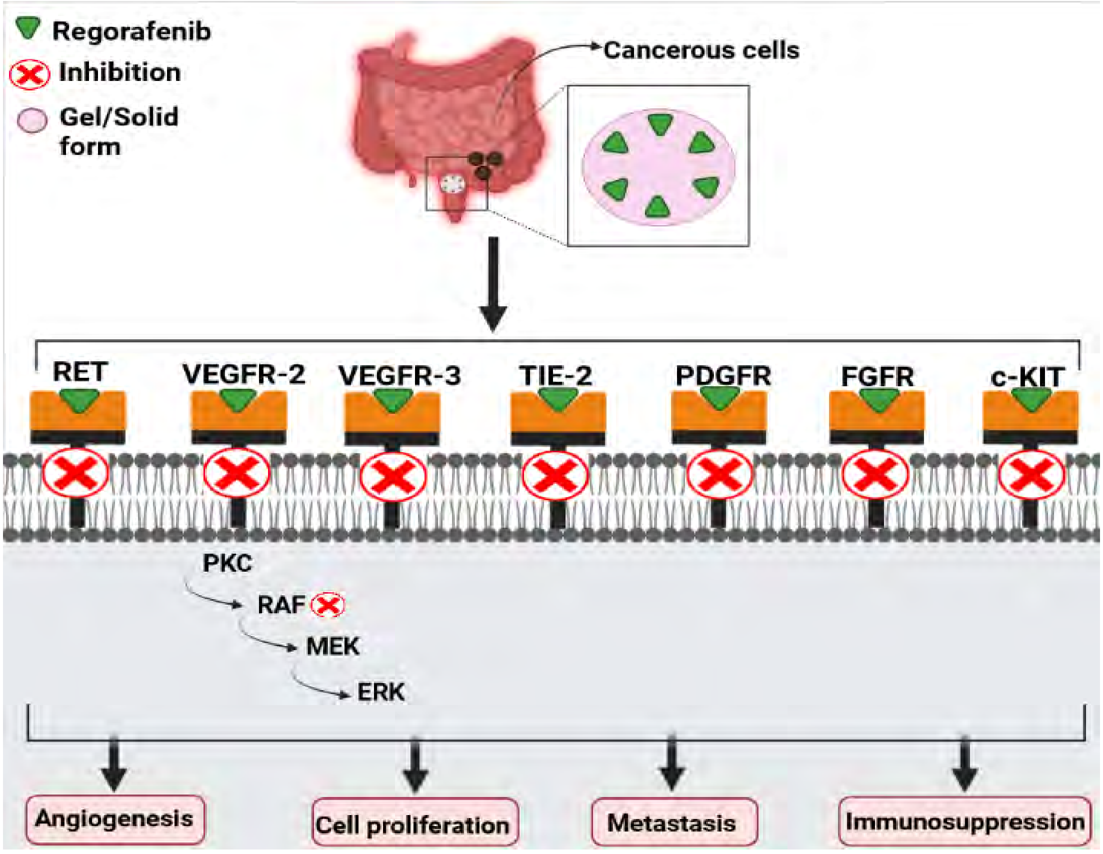

Figure 1.2. Graphical representation of the regorafenib mechanism of action for targeting colorectal cancer.
Note*: Image developed on BioRender.com.*

1.3.3. Problems associated with RG

The therapeutic efficacy of RG is drastically limited because of its poor oral bioavailability and its low aqueous solubility (Jia *et al.,* 2021). Moreover, it undergoes extensive hepatic first pass metabolism (Fu *et al.,* 2018). Additionally, oral administration of RG has the potential to cause significant toxicities and drug interactions, which could result in early treatment termination (Venu *et al.,* 2018). RG also shows some serious side effects i.e. Hand-foot skin reaction (HFSR), stomatitis, rashes, fatigue, hypertension, dizzeness and diarrhea (Krishnamoorthy *et al.,* 2015). In HFSR, bruises appeare on the palms and soles which are very painful and erythematic. It is commonly caused by dose modification or discontinuation of treatment (Majithia *et al.,* 2016).

1.4. Rectal Route of Administration

Drug administration through oral route is the most easiest, favourable and preferred route for many therapeutic drugs delivery including the anti-cancer drugs. However, there are certain limitations with the oral route of drug administration. These limitations includes, low water solubility and bioavailability of the drug, GI environment which may be harsh on drug, limited permeability of drug across the GI membrane, or drug may undergo high hepatic first pass metabolism, and multidrug efflux transporter (Hu *et al.,* 2012; Jun *et al.,* 2012). Specifically, the lipophilic drugs bioavailability through the oral route administration, is still a major concern. Thus, to enhance the bioavailability of drugs and to improve its therapeutic efficacy, the urge to develop an appropriate delivery system for drugs, is increasing day by day. In this respect, rectal route of drug administration through the suppository may be a feasible alternative to oral route of administration. It will successfully address the bioavailability issues and improve the drug's therapeutic potential (Xuan *et al.,* 2010).

Generally the treatment of CRC includes surgery, chemotherapy and radiotherapy. For chemotherapy, the most commonly routes of drug administration is either oral route or intravenous (IV) route. When anti-cancer drugs are administered through these routes, they may undergo extensive GI and hepatic first pass metabolism (Rizzo *et al.,* 2020). The metabolism of anti-cancer drugs produce a toxic effect and it can damage the liver. A rectal route of administration was developed in order to decrease the GI side-effects, avoid the hepatic first-pass metabolism, and to eliminates the undesirable effects produce by meals on the absorption of drugs (Lin *et al.,* 2012).

1.5. Difference between Solid and Liquid Suppository

Solid suppository is administered through conventional rectal route, and patient shows low compliance to it because it often causes patient discomfort. Whereas, a solid suppository is non-mucoadhesive in nature, it may reach to the colon's end due to which drug carried in suppository undergoes first pass effect (Bialik *et al.,* 2022). Additionally, as a result of quick melting or softening of solid suppository inside the rectum, drug is released from it in un sustained manner (Lin *et al.,* 2012).

In order to address these problems associated with solid suppository, it is desirable to formulate liquid suppository. Some of the desirable characteristics of liquid suppository are, they convert into gel form at the body temperature i.e 37 °C, also possess suitable gel strength. Due to the optimum gel strength liquid suppository does not leak out from the anus after rectal administration (Bialik *et al.,* 2022). Due to the suitable bioadhesive force, liquid suppository may not reach to the end of colon and thus drug carried in suppository not undergoes first pass effect (Lin *et al.,* 2012). Also, liquid suppository is safe for rectal administration as it do not cause any damage to the rectum and it reduces the feeling of a external body as compared to the solid suppository (Pásztor *et al.,* 2011; Purohit *et al.,* 2018).

1.6. Liquid Suppository as Thermosensitive Rectal Gels

Liquid suppository is a rectal gel with a thermosensitive behavior. The TS behavior of the liquid suppository is the result of the base materials which are used in the liquid suppository formulation. It is a polymer which is thermosensitive in nature and it converts liquid into gel form at the body temperature i.e 37 °C (Purohit *et al.,* 2018; Özgüney *et al.,* 2014). The administration of liquid suppository is easy and it quickly converts to gel form, because of the temperature deviations they undergo a sol-to-gel conversion (ud Din *et al.,* 2015).

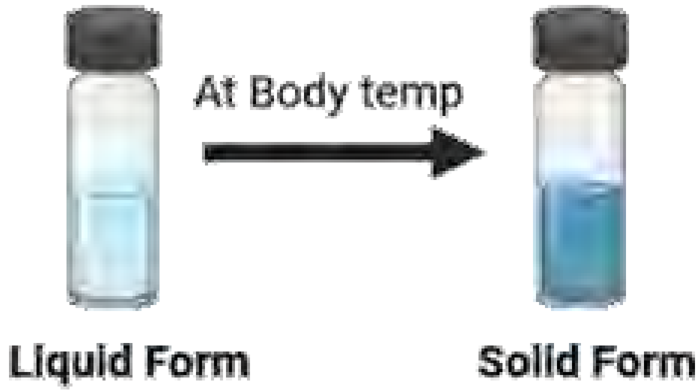

Figure 1.3. Illustration of the conversion phenomenon of liquid suppository from liquid phase to gel phase at body temperature.

1.6.1. Composition of liquid suppository

1.6.1.1. Poloxamer

Poloxamers, which are the triblock copolymers of poly(oxyethylene-oxypropylene-oxyethylene), are the most widely used base for thermosensitive liquid suppositories. They are made of two blocks of polyoxyethylene which is hydrophilic in nature, with a central block of polyoxypropylene which is hydrophobic surrounding it (Kassab *et al.,* 2014). For the thermosensitive gelling properties, Poloxamer 407 (P407) and poloxamer 188 (P188) were selected for the liquid suppository formulation. P407 and P188 were also known for its low irritation, low toxicity, excellent drug release property, excellent solubility in water, and they are also compatible with a wide range of excipients. The incorporation of P188 into P407 solution is used for the modulation of phase transition temperature. This is used to formed a gel which is temperature-sensitive with an appropriate temperature for the phase transition. The transition temperature is higher in case of poloxamer mixture solutions, as compared to P407. A suitable gelation temperature is not possible in case of P407 or P188 alone (Chen *et al.,* 2013; Fakhar ud din *et al.,* 2019). Reverse thermal gelation is a phenomenon that occurs in poloxamer solutions, causing them to remain solutions at room temperatures (25 °C) and at higher temperatures (30–35 °C), it converts into gel form. The Poloxamers transition temperature is typically concentration-dependent. Poloxamer solutions remain liquid below body temperature and change into a semi-solid substance above 30 °C temperature (Barakat, 2009).

1.6.1.2. Tween

Surfactants which are non ionic in nature are also used in the manufacturing of the thermosenstive liquid suppository formulations. These surfactants produce a positive effect on the drug's release and can also act as wetting agents (Fontan *et al.,* 1991). Tween is added i.e most commonly Tween® 80 (T80) is used in the gel formulations in order to increase its viscosity, mucoadhesive strength and gelation temperature, gelation time is decreased (Teaima *et al.,* 2020). The impact of T80 on the gel properties is shown by the mechanism in which it strengthen the hydrogen bonding between the poloxamers (i.e P188 and P407) inside the gel matrix (Fakhar ud *et al.,* 2019). Due to the inert nature of non-ionic surfactants such as T80, it results in no damage effect on the mucous membranes. Also, T80 is a safe option with no side effects in the rectal administration (Yeo *et al.,* 2013).

Herein, the graphical representation of mechanism of action of RG loaded liquid suppository is demonstrated in Figure 1.4. The RG loaded liquid suppository is in solution form at room temperature and when administered through stomach sonde needle in to rectum, it converts into gel form at physiological temperature. Inside the rectum it will adhere to the mucous membrane, and RG will bind to the receptors that are exposed on the surface of the cancer cells.

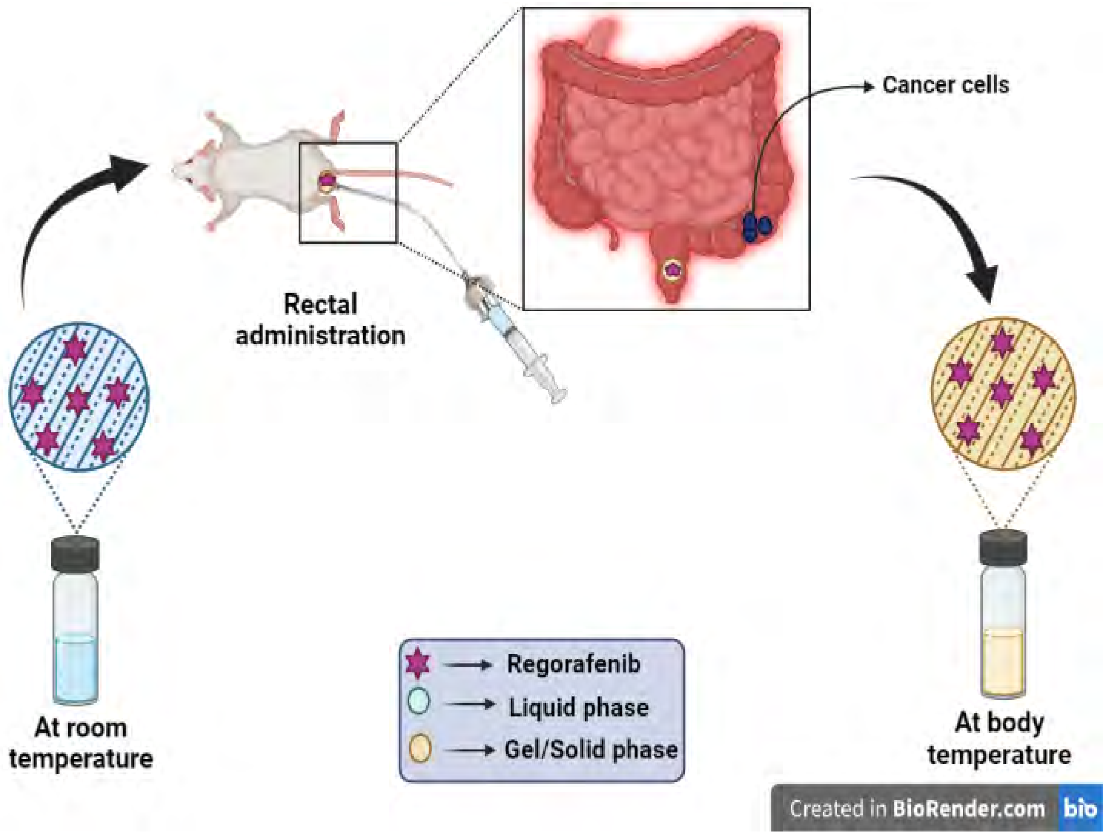

Figure 1.4. Graphical representation of the thermosensitive behavior of regorafenib loaded liquid suppository in targeting colorectal cancer.
Note: *Image developed on BioRender.com.*

1.7. Problem Statement

Clinical effectiveness of RG is extremely limited because of it poor water solubility due to which its oral bioavailability is low (Jia et al., 2021), undergoes extensive hepatic metabolism (Fu et al., 2018) and shows some serious side effects i.e HFSR, rashes, stomatitis, fatigue, hypertension, dizzeness and diarrhea (Krishnamoorthy et al., 2015). Therefore, dissolution enhancement and bioavailability improvement along with the nontoxic profile of RG is needed in order to achieve maximum therapeutic action against CRC. Additionally, investigation of the new routes of administration may improve the drug efficacy.

1.8. Rationale of the Study

The rationale of this research was to developed a RG loaded liquid suppository, which prevent the drug from the hepatic metabolism, increase its bioavailability and also safe rectal delivery was ensured. Also, it solved all the problems associated with the solid suppository by the formulation of liquid suppository.

1.9. Aim and Objectives

1.9.1. Aim

Aim of the study was to formulate and evaluate the potential of liquid suppository system for the rectal administration of RG.

1.9.2. Objectives

- Preparation and optimization of the RG loaded liquid suppository
- Characterization of the RG loaded liquid suppository
- Evaluation of the *in vitro* drug release and *in vitro* cell lines viability of the RG loaded liquid suppository and its comparison with RG suspension.
- Pharmacokinetic evaluation of the RG loaded liquid suppository and its comparison with RG suspension.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Regorafenib (RG) was kindly gifted by Hanyang University (Ansan, South Korea). Poloxamer (P188) and poloxamer 407 (P407) were purchased from Sigma-Aldrich (Heidenheim, Germany). Tween® 80 (T80) was gifted from Vision Pharmaceuticals, Islamabad, Pakistan. All additional chemicals of pure analytical grading were used, without any further refinement.

2.1.2. Instrument and equipments

HPLC (S-200, PerkinElmer®, Waltham, USA), Hot plate stirrer (Eisco Scientific, North America), Pharmaceutical refrigerator (MPR-161D H, Panasonic, Japan), Light microscope (E400, Nikkon, Tokyo, Japan), Dissolution tester (VISION-6 Classic, Chatsworth,CA, USA), Water distillation apparatus (GmbH IM50, Irmeco, Germany),Weighing balance (PA 214C, Ohaus corporation, USA), pH meter (pH 7110, inoLab, Germany), water bath (Memnert; WNB-7; Germany), Bath sonicator (elmasonic GmbH, E60H, Germany), Centrifuge machine (Hermle Z-206A, MK,Germany), Oven (Memmert, Germany), Brookfield DV-I Prime viscometer (Middleboro, MA 02346, USA), FTIR spectrometer (Nicolet-6700, Thermo Scientific, USA), Dialysis membrane (MW-3500 Da, Spectrum Laboratories Inc., USA), Thermometer, Glass vials, Volumetric flasks, Eppendorf tubes, falcons, stirrers, petri dish and beakers (Sigma Aldrich, USA).

2.1.3. Animals

Sprague-Dawley rats weighing 250 ± 20 g were brought from National Institute of Health (NIH) Islamabad, Pakistan, following approval from the Bio ethical committee of the Quaid-i-Azam University, under approval number of BEC-FBS-QAU2022-413. The rats were then placed under standard conditions according to the Animal Welfare Act of NIH policy, ARRIVE (Version 2) and academic press (Edition 8) guidelines. Moreover, drinking facilities and animal standard food were provided. Temperature and relative humidity were set at 25 ± 4 °C and 65 ± 6%

respectively. Prior to the animal studies, rats were starved for 8-12 h to keep their rectum clean, however easy water access was given.

2.2. Methods

2.2.1. Buffer solution preparation

2.2.1.1. Phosphate buffer saline (PBS) pH 7.4

In order to prepare PBS of pH 7.4, 8.0 g of sodium chloride (NaCl), 2.38 g of di-sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) and 0.19 g of potassium di-hydrogen phosphate (KH_2PO_4) was dissolved in a 800 mL distilled water in a 1000 mL flask. To make the final volume upto 1000 mL, distilled water was added. Then, adjust the pH to 7.4 with the help of sodium hydroxide (NaOH) or hydrochloric acid (HCl). This method was adopted from US pharmacopoeia (Hua *et al.,* 2010).

2.2.2. Method of preparation of RG loaded liquid suppository

Cold method was used for preparing liquid suppository (Jadhav *et al.,* 2009). RG loaded liquid suppository was made in three stages. First, P407 and P188 were dissolved with moderate stirring in sterile distilled water using cold water bath system to maintained temperature. The resulting mixture was then left at 4 °C for the night in order to formed a clear poloxamers solution. The second step involved mixing specific amounts of RG and T80 while stirring continuously. Then finally, to the poloxamers solution, the drug and T80 mixture was gradually added while gently stirring at 4 °C using water bath, to formed a clear and transparent RG loaded liquid suppository solution (Fakhar ud din *et al.,* 2019; Mushtaq *et al.,* 2022).

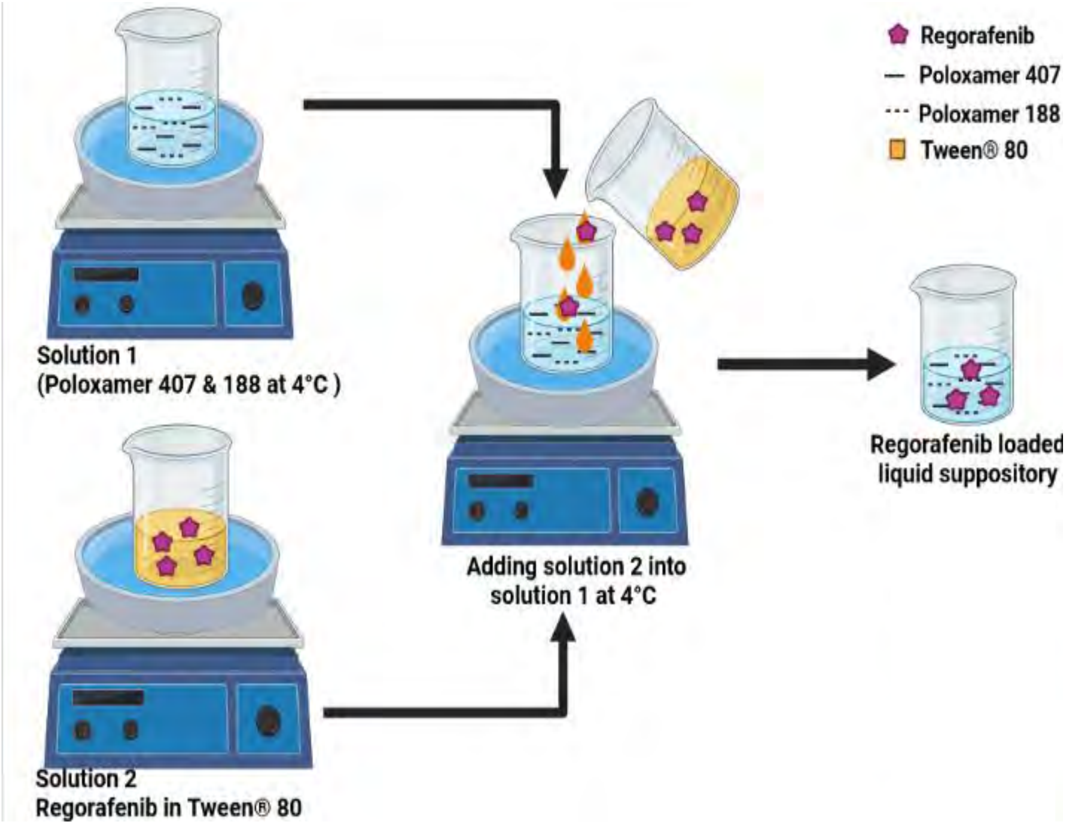

Figure 2.1. Method of preparation of the regorafenib loaded liquid suppository.
Note: *The proposed diagram was drawn on biorender.*

2.3. Optimization of RG Loaded Liquid Suppository by Design Expert® Software

After the pre-optimization study by hit and trial method (as shown in Table 3.1), Box-Behnken design was utilized for the optimization of RG loaded liquid suppository using 12^{th} version of Design Expert® software. Total 14 runs were developed using this software design, keeping P407, T80 and RG as independent variables while temperature and time required for gelation, mucoadhesive force and gel strength were kept as dependent variables. The levels of the dependent and independent variables are shown in Table 2.1. Formulations were prepared for all experimental runs suggested by the Box-Behnken design, and the corresponding characteristics of the RG loaded liquid suppository such as the temperature, time for gelation, gel mucoadhesion and its strength were noted.

Table 2.1. Levels of independent and dependent variables in Box-Behnken design.

Variables	**Levels**	
	Low (-1)	High (+1)
Independent variables (g)		
Poloxamer 407	5	17
Tween® 80	5	15
Regorafenib	0.25	2.25
Dependent variables		
Gelation temp (°C)	Minimize	
Gelation time (min)	Minimize	
Gel strength ($\times 10^2$mPa.s)	Maximize	
Mucoadhesive force ($\times 10^2$dyne/cm^2)	Maximize	

Note: *Poloxamer 188 was kept constant.*

2.4. Characterization of RG Loaded Liquid Suppository

2.4.1. Gelation temperature

For the determination of gelation temperature, liquid suppository (4 g) and a magnetic stirrer (radius 10 × 3 mm) were placed inside a glass vial (10 mL). The glass vial was put inside the glass container with a wide mouth that was set above magnetic stirring hot plate. After that, a thermometer was placed inside the vial, in order to measure the gelation temperature of liquid suppository. Then, hot plate was used for increasing the temperature by 1 °C per min from 25 °C. A constant stirring speed of 60 rpm was set on the magnetic plate. When the magnetic bar inside the liquid suppository stopped rotating as a result of gelation, then temperature was noted as a gelation temperature on the thermometer. Each experiment was carried out three times (Mushtaq *et al.,* 2022; Fakhar ud din *et al.,* 2019).

2.4.2. Gelation time and gel strength

The gel strength of the liquid suppository is its viscosity at temperature 36.5 °C, and the gelation time is how long it takes for a liquid suppository to turn from solution into gel form. In order to determine the liquid suppository's gel strength and time required for gelation, a Brookfield viscometer (Middleborough, MA 02346, USA) was used. A water bath was used to maintain the system's temperature. The sample cup's ports were connected to the water bath in which the temperature was controlled. The temperature of the water bath was set at 36.5 ± 0.5 °C. The speed knob on the viscometer was set to the required rpm. After adjusting the gap setting, the test sample was added. The gel strength was determined by taking average of the viscometer readings (Fakhar ud din *et al.,* 2019; Jiao *et al.,* 2023). The amount of time the liquid suppository needed to change from solution to gelled form was noted and is referred to as the gelation time (Yeo *et al.,* 2013).

2.4.3. Mucoadhesive force

This study was conducted using Sprague-Dawley (260 ± 20 g). Before starting experiment, rats were kept in starvation condition but they were provided with excessive water for drinking. After 12-20 h of the starvation time period, for the ongoing investigation, the rats' rectums were removed after sacrificing them. The mucoadhesive force was evaluated using a reformed balance. Two glass vials; fixing one vial on pan using adhesive tape that are adjustable, and the other was suspended from the reformed balance, were taken. After that, two distinct sections of rat rectum tissues were put on each vial. Then, the RG loaded liquid suppository was put on tissue, positioned on the adjustable glass vial. Next, the adjustable vial raised to a point until the two vials connected to each other. The weight was increased on the vial that is hanged from reformed balance, until vials were separated from one another. The mucoadhesive force also known as detachment force, is the smallest amount of weight or the force which is required to causes the separation of the vials (Jiao *et al.,* 2023; Fakhar ud din *et al.,* 2019)

Mucoadhesive force in (dyne/cm²) was measured by using formula given below,

$$Detachment\ force\ \left(\frac{dyne}{cm^2}\right) = (m)\ X\ \left(\frac{g}{A}\right)$$

Where,

A = Area of tissues that are exposed

m = Weight required for seperation of vials (g)

g = Gravitional acceleration [980 cm/s²]

Measurements were repeated three times and rectum tissue was changed for each measurement (Jadhav *et al.*, 2009).

2.4.4. Drug content

Three equal portions (Upper, middle and lower) of liquid suppository were taken and then content of the drug in each portion was analyzed in order to measure the drug content uniformity inside the liquid suppository (Palakurthi *et al.,* 2023). In a 100 mL volumetric flask, 1 mL solution was taken from the formulation and added to 70 mL of PBS (pH 7.4). After 10 mins of sonication, the final volume was made up to 100 mL. The solution was then filtered by passing it through a filter (0.45 µm pore size) after 2 mins of shaking (Montazam *et al.,* 2020; Alkufi *et al.,* 2019). HPLC was used for the quantification of drug. The apparatus was equipped with the C-18 column (250 × 4.6 mm, Shiseido, Tokyo, Japan) and a UV detector. The composition of mobile phase was 30:70 v/v of potassium di-hydrogen phosphate (KH_2PO_4, 0.5%, pH 3.5) and acetonitrile. The phosphoric acid (H_3PO_4, 50%) was used for the pH adjustment of KH_2PO_4. A flow rate adjustment was made to 1 mL/min. Each sample was mixed with 20 µL mobile phase and then vortex for 1 min. Injection volume into the analytical column was 20 µL of each sample. All samples were analyzed at 260 nm at ambient temperature. Analysis was performed in triplicate. The calibration curve equation was utilized to determined the amount and uniformity of drug within the formulation (Fujita *et al.,* 2016).

2.4.5. Physicochemical evaluation

The color, transparency, grittiness, and gelation of RG loaded liquid suppository were examined .

2.5. Analysis of Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectrometer (Biotech Engineering Management, UK) was used to record the RG's FTIR spectrum, which was then analyzed using the transmittance method in the spectral range (400 and 4000 cm^{-1}). At 37 °C, the liquid suppository solidified into a gel and was then completely dried using freeze drying (FDU-1200, Eyela). Before investigation, the obtained dry powder was grounded in an agate motor at a ratio of 1:99 with KBr and then it was compressed into a disc. For the purpose of examining drug and excipients potential interaction within the formulation, FTIR compatibility studies were conducted at room temperature (Alkufi *et al.,* 2019; Jadhav *et al.,* 2009).

2.6. *In Vitro* RG Release Study

Dissolution tester apparatus was utilized for the *in vitro* study of drug release. This study was performed to compare and evaluate the release of RG suspension and RG loaded liquid suppository. A dialyzing tube was filled with both the test formulations, each of which contained 25 mg of RG. Both ends of the tube was tied, and then it was placed inside a beaker containing dissolution medium (PBS pH 7.4). Dissolution medium (900 mL) was added in each 1000 mL beaker of the dissolution tester, that was shaken horizontally at 70 rpm and the temperature was set at 36.5 ± 0.5 °C. To keep the sink conditions constant, 3 mL aliquots of the medium were taken out at each time period and were replaced with equivalent volumes of the corresponding PBS. Then HPLC was used to quantify the RG concentration which was calculated using a standard calibration curve, as discussed above. Moreover, to analyze the release kinetics, different models of kinetics were applied (Kim *et al.,* 2021; Khaleeq *et al.,* 2020).

2.6.1. Drug release kinetics

i. Zero order model

In zero order kinetics model, the drug is continuously released from the drug delivery system where amount of drug remains constant throughout the drug delivery process. To analyze the release kinetics of drug, release data was plotted according to the following equation (Dash *et al.,* 2010; Gouda *et al.,* 2017).

$$Q_t = Q_o + K_o t$$

Where,

Q_o = Initial concentration of drug (time t=0)

Q_t = Total quantity of drug release (time t)

K_o = Rate constant of zero order

t = Time

ii. First order model

First order release kinetics implies to the release process in which there is direct relationship between the drug release rate and drug dissolved concentration (Jahromi *et al.*, 2020). To determined the correlation of obtained data with the first order model, log % of remaining drug and time were plotted by the following equation;

$$\log C = \log C_o - K_1 \frac{t}{2.303}$$

Where,

C = Remaining drug concentration at time t

C_o = Initial concentration of drug at time t

K_1 = Rate constant of first order

iii. Higuchi model

This model describe the drug release from non-erodible matrix system as a process of drug diffusion based on the ficks law. The data obtained was plotted as percentage cumulative release of drug versus time square root represented by the following equation given below (Dash *et al.*, 2010; Higuchi, 1963).

$$ft = Q = A\sqrt{D(2C - Cs)Cst}$$

$$ft = Q = K_H \text{ x } t_{½}$$

Where

Q = Amount of drug release

C = Initial concentration of drug

A = Surface area

Cs = Solubility of drug in the matrix media

D = Drug diffusivity from matrix media

t = Time

K_H = Rate constant of higuchi diffusion

iv. Hixon-Crowell model

This model describe the release properties of system involving a change in the diameter and surface area of the particles. Such process can be explained by the following equation (4) (Rehman *et al.,* 2020).

$$W_o^{⅓} - Wt^{⅓} = K_{HC}t$$

Where,

W_o = Initial drug concentration in dosage form

Wt = Remaining drug concentration in dosage form

K_{HC} = Hixon-Crowell release constant

v. Korsmeyer-Peppas model

This model is used to determine the diffusion type involved in release of drug. The following equation is used to calculate the drug's release and dissolution from the thermosensitive system (Talevi *et al.,* 2022).

$$\frac{Mt}{M} = Ktn, \log\left[\frac{Mt}{M}\right] = \log k + n \log\ [t]$$

Where,

Mt/M = Drug fraction released at time t,

K = Thermosensitive system constant

n = Drug release mechanism

The n and k values were obtained from log [Mt/M] versus log [t]. The higher the k value indicates that drug release will takes place more quickly. The zero-order release kinetics are indicated by the value of n = 1. If n value is greater than 0.5 and less than 1, it corresponds to non-fickian diffusion and if n is equal to 0.5, it represents fickian release model which is higuchi model (Fakhar ud din *et al.* 2019; Pathak *et al.* 2016, Tan *et al.* 2014).

2.7. Stability Study

The stability study of optimized RG loaded liquid suppository was performed over a period of 6 months at temperature 2-8 ± 2 °C and 25 ± 2 °C and relative humidity (RH) 65 ± 5% storage condition. International conference of harmonization (ICH) guidelines was used for performing this study. The freshly prepared optimized RG loaded liquid suppositories were placed at their respective temperature conditions. After specific time intervals, from each storage conditions, sample was taken and analyzed for temperature and time required for gelation, gel strength, and mucoadhesion, in order to find out any changes in their properties during the respective storage time period (Keny *et al.,* 2010; Choi *et al.,* 1998b).

2.8. *In Vitro* Cytotoxicity Assay

In vitro cytotoxicity analysis of RG loaded liquid suppository was performed in comparison to the normal saline and RG suspension, by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Two CRC cell lines were used i.e. HCT 116 and Caco-2. In a 96 well plate, 8 × 10^3 cells were seeded in each well and then it was placed in an incubator at 37 °C temperature for 24 h. The cells in each well were then subjected to different concentrations (0.5-100 µg/mL) of RG liquid suppository, normal saline, and RG suspension. Each well's final volume was 200 µL, and it was incubated for 24 h. The cytotoxicity of RG on the Caco-2 and HCT 116 cell lines was assessed using the tetrazolium dye MTT and PBS. MTT (20 µL) and PBS (5 mg/mL) were added into each well, which were then incubated at 37 °C for 4 h. Formazen was produced due to MTT reduction. Then, in order to dissolve MTT formazan crystals, 200 µL of DMSO were added to each well. After 10 mins of shaking, microplate reader was utilized for measuring the absorbance at 570 nm. Cell viability percentage was calculated and then compared to un-treated cells. Each experiment was carried out three times (Afshar *et al.,* 2020; Napolitano *et al.,* 2015).

$$\%\ Cell\ viability = \left(\frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells}\right) X\ 100$$

2.9. Pharmacokinetic Study

2.9.1. Administration and blood collection

Total twelve Sprague-Dawley rats, divided into two groups (six rats in each group) were used in the pharmacokinetic study. One group was treated with RG loaded liquid suppository and other group was treated with RG suspension. As discussed earlier they were kept fasted for 12 h followed by anesthetization using isoflurane. They were restrained only during the cannulation procedure and remained conscious during the study. A poly ethylene tube was cannulated into right femoral artery of each group. Optimized RG loaded liquid suppository was administered to one group and RG suspension was given to another group, in the rectum, with the help of stomach sonde needle, 4 cm above the rectum. RG was administered at 5 mg/kg dose. To avoid any leakage, rectum of the rats treated with RG suspension was closed with the help of glue. Approximately, 300 µL of the blood were taken after specific time period, such as 30 mins, 1, 2, 4, 6, 12, and 24 h. It was then centrifugated at 12000 rpm for 10 mins. Plasma was obtained which was kept at -20 °C temperature, until quantification of drug concentration in plasma (Mushtaq *et al.,* 2022; Fakhar ud din *et al.,* 2019).

2.9.2. Blood sample analysis

Plasma samples were kept at room temperature to melt them before analysis. Acetonitrile solution almost 250 µL with 50 µg/mL of sorafenib (as internal standard) were mixed with specified amount of plasma (150 µL). Then centrifugation of the mixture was done at 12000 rpm for 10 mins. Supernatant almost 10 µL was finally taken for analysis using HPLC as discussed above (Xing *et al.,* 2021, Din *et al.,* 2017).

2.9.3. Statistical analysis of pharmacokinetic daga

WinNonlin software was utilized for the non compartmental analysis to determine area under the curve (AUC). Moreover, the maximum time (T_{max}) required for maximum plasma concentration (C_{max}) were measured directly from the plasma concentration. Student's t-test was used to obtain the significanct level of the data ($p < 0.05$).

2.10. Histopathology of Rectum

The rats were sacrificed after the completion of pharmacokinetic analysis. One rat from each group, RG loaded liquid suppository and RG suspension treated, was analyzed for histopathology study. One untreated (normal) rat was also used for comparison. Briefly, the corresponding group representative rats rectum were removed and photomicrographs of rectal tissues were taken. After obtaining rectal tissues, they were washed with saline solution (3 times). Then, were placed in 10% formaldehyde embedded in paraffin. For histopathological observations, such as inflammatory cell infiltrations or tissues damaged, 2 to 4 µm fragments of the rectum were separated. It was then dyed with Hematoxylin and Eosin (H&E) at the appropriate time. After H&E staining, tissues were carefully inspected under a light microscope for any obvious inflammation or damage. The results of rectal tissues photomicrographs of the formulation were compared with the untreated (control) group (Khan *et al.,* 2021; Jiao *et al.,* 2023).

2.11. *In Vivo* Localization of Suppository

This study was carried out on rats to identify the localization of the applied liquid suppository. A plastic syringe with a stomach sonde needle attached was used to inject a liquid suppository into the rectum. It contains methylene blue dye (1%) for color identification inside the rectum. After administration, the rectum was sectioned at specific time period such as 10 mins, 2, 4, and 6 h. The blue colour indicated where the liquid suppository was located inside the rectum (Lin *et al.,* 2012; Lo *et al,* 2013). Moreover, threshold of the gelation, which is the least gel strength at which liquid suppository administered and did not leaked from rectum, was also checked in these rats for 30 mins of administration (Yeo *et al.,* 2013).

2.12. Statistical Significance

All analysis was carried out in triplet times (n=3) or sextuplicate (n=6). Also, results were shown with standard deviation (±). For statistical analysis, Design-Expert® software (version 12), Sigma plot (version 15), ORIGIN pro software version 2018, GraphPad-Prism-9.5.1, Microsoft Excel 365, DD solver 1.0, PK Solver 2.0, were used. P-value less then 0.05 was calculated through t-test.

CHAPTER 3

RESULTS

3. RESULTS

3.1. Formulation of RG Loaded Liquid Suppository

RG loaded liquid suppository was successfully prepared by cold method. It was colorless as shown in Figure 3.1.

Figure 3.1. Image of the regorafenib loaded liquid suppository at physiological temperature.

3.2. Pre-Optimization of RG Loaded Liquid Suppository

As, pre-optimization study, liquid suppository was prepared and were optimized by trial-and-error method as shown in Table 3.1. The formulation was optimized by varying the concentration of P407, T80 and drug to analyze their effects on different parameters of formulation such as temperature, time for gelation, strength and mucoadhesion of gel. Readings for each formulations were noted in triplicate.

Table 3.1. Pre-optimization study of regorafenib loaded liquid suppository.

Sr No.	P188/ P407 (g)	T80 (g)	RG (g)	Gelation temp (°C)	Gelation time (min)	Gel strength (×10²mPa.s)	Mucoadhesive force (×10²dyne/cm²)
1	15/5	-	-	76.37 ± 0.2	10.71± 0.2	59.71 ± 0.4	8.91 ± 0.7
2	15/7	-	-	63.21 ± 0.3	8.52± 0.6	72.43 ± 1.3	10.45± 0.9
3	15/9	-	-	45.21 ± 0.6	5.28± 0.4	86.51 ±1.6	12.34± 1.2
4	15/11	-	-	39.21 ± 0.5	3.54± 0.8	98.64 ± 1.9	15.81± 0.5
5	15/11	5	-	35.21 ± 0.2	2.51± 0.2	104.9 ± 0.7	17.69± 1.2
6	15/11	10	-	32.46 ± 0.7	1.32± 0.3	115.4 ± 0.5	24.13± 0.6
7	15/11	15	-	29.45 ± 0.5	0.74± 0.9	128.1 ± 0.9	32.97± 0.2
8	15/11	10	0.5	32.12 ± 0.4	1.29± 0.9	118.7 ± 1.2	25.07± 0.6
9	15/11	10	1.0	31.95 ± 0.3	1.27± 0.5	118.4 ±0.1	25.65 ± 0.6
10	15/11	10	1.5	31.50 ± 0.4	1.01± 0.4	122.5 ± 0.7	26.96 ± 0.2

***Note:** All the data is represented with ± standard deviation, n=3: g: gram*

3.2.1. Analysis of Box-Behnken design

Quadratic model was the best suitable model for temperature, time of gelation and gel strength, however; linear model was suitable for mucoadhesive force. Optimization results are shown in Table 3.2. Design expert® suggested run 11 and 12 as the optimized formulations with 15 g P188, 11 g of P407, 10 g T80 and 1.25 g of RG. The results demonstrated that the recommended optimized formulation had gelation temperature of 31.1 ± 0.2 °C and 31.5 ± 0.2 °C , gelation time was 1.02 ± 0.2 min and 1.09 ± 0.2 min , gel strength was 121 0.7 ×10² mPa.s and 122.4 ± 0.7 ×10² mPa.s and mucoadhesive force was 26.4 ± 0.2 ×10² dyne/cm² and 26.96 ± 0.2 ×10² dyne/cm². All values were within the desired ranges.

Table 3.2. Box-Behnken design experimental runs and resultant responses for optimization of regorafenib loaded liquid suppository.

Sr No.	P188 (g)	P407 (g)	T80 (g)	RG (g)	Gelation temp (°C)	Gelation time (min)	Gel strength (×10² mPa.s)	Mucoadhesive force (×10² dyne/cm²)
1	15	11	15	2.25	29.17 ± 0.5	0.89 ± 0.2	133.5 ± 0.5	35.12 ± 0.9
2	15	17	10	0.25	25.13 ± 0.3	0.35 ± 0.2	143.8 ± 0.3	43.19 ± 0.5
3	15	17	10	2.25	25.76 ± 0.3	0.41 ± 0.1	144.2 ± 0.7	43.52 ± 0.3
4	15	11	5	0.25	37.02 ± 0.7	3.31 ± 0.5	101.9 ± 0.2	18.94 ± 0.8
5	15	5	10	0.25	70.81 ± 0.2	9.26 ± 0.7	59.83 ± 0.2	9.32 ± 0.2
6	15	5	10	2.25	69.13 ± 0.4	9.11 ± 0.6	60.14 ± 0.9	9.89 ± 0.1
7	15	11	5	2.25	36.61 ± 0.2	3.02 ± 0.5	103.1 ± 0.7	18.42 ± 0.1
8	15	17	15	1.25	22.19 ± 0.2	0.11 ± 0.1	152.9 ± 0.5	54.17 ± 0.9
9	15	17	5	1.25	28.16 ± 0.5	0.73 ± 0.4	136.7 ± 0.7	38.17 ± 0.3
10	15	5	15	1.25	66.52 ± 0.3	8.56 ± 0.9	69.41 ± 0.7	11.21 ± 0.7
11	15	11	10	1.25	31.1 ± 0.5	1.02 ± 0.2	121 ± 0.4	26.4 ± 0.6
12	15	11	10	1.25	31.5 ± 0.3	1.09 ± 0.2	122.4 ± 0.3	26.96 ± 0.8
13	15	5	5	1.25	74.51 ± 0.9	10.1 ± 0.7	53.82 ± 0.8	6.86 ± 0.4
14	15	11	15	0.25	29.42 ± 0.4	0.85 ± 0.5	132.7 ± 0.6	34.93 ± 0.3

Note: *All the data is represented with ± standard deviation, n=3: g: gram*

For statistical optimization of RG loaded liquid suppository, regression analysis of Box-Behnken design is presented in Table 3.3.

Table 3.3. Regression analysis for statistical optimization of regorafenib loaded liquid suppository.

Regression analysis of gelation temp, gelation time, gel strength and mucoadhesive force						
Responses	**R²**	**Adjusted R²**	**Predicated R²**	**Adequate precision**	***p*-value**	**F-value**
Gelation temp (°C)	0.8378	0.7891	0.6865	10.8985	0.0003	17.21
Gelation time (min)	0.8295	0.7784	0.6799	10.7895	0.0004	16.22
Gel strength (×10² mPa.s)	0.9933	0.9781	0.8924	24.2550	0.0006	65.65
Mucoadhesive force (×10² dyne/cm²)	0.9789	0.9726	0.9542	36.9662	0.0001	154.88

3.2.2. Effect of independent variables on gelation temperature

According to the findings of the statistical analysis, it is clear that changing the concentration of independent variables such as P407 and T80 showed pronounced impact on gelation temperature with p-value < 0.0001, whereas drug (RG) showed insignificant impact with a p-value of 0.18. The three dimensional response surface graphs depicted a decrease in the gelation temperature (74.51-22.19 °C) , when the concentrations of P407 (5-17 g), and T80 (5-10 g) were increased, while keeping the amount of RG constant at 1.25 g as shown in Figure 3.2 (I). On the other hand, Figure 3.2 (II) indicated that increased T80 and RG (0.25-2.25 g) concentrations has reduced the gelation temperature, when the P407 quantity was kept constant at 11 g. Similarly, a decreased gelation temperature was observed, when conecntrations of P407 and RG were increased, keeping the T80 constant at 10 g, as demonstrated by the three dimensional graph in Figure 3.2 (III). Model terms (A, B, AC, A^2, B^2, and C^2) with p-values under 0.05 were significant. The model's significance was further supported by the F-value of 3815.71. In comparison to pure error, the lack of fit (2.07) was insignificant. The difference between the adjusted R^2 0.9996 and the predicted R^2 0.9983 was < 0.2, as indicated in Table 3.3, therefore they were reasonably consistent. It was suggested that this model might be utilized to explore the design space by the ratio of signal to noise, which was measured with adequate precision and had a value of 162.57. The following regression equation illustrates the relationship between the independent variables;

$$Gelation\ temperature = +31.30 - 22.47A - 3.63 - 0.2138C + 0.5050\ AB + 0.5775\ AC + 0.0400BC + 15.60\ A^2 + 0.9463\ B^2 + 0.8087\ C^2$$

Linear coefficients are A, B, C whereas, two factor interacting coefficients are AB, BC, AC. In total 14 runs of the Design Expert® formulations shown in Table 3.2 , the minimum and maximum gelation temperature were 22.19 ± 0.2 °C and 74.51 ± 0.9 °C, respectively.

3.2.3. Effect of independent variables on gelation time

It is clear from the statistical analysis, that there was an inverse relation between independent variables (P407, T80) and time required for gelation, as by increasing the concentrations of P407 and T80 there was decrease in gelation time with p-value of 0.0001 and 0.0054 respectively, whereas, drug (RG) had a non-significant impact with a p-value of 0.79. The three dimensional response surface graphs of gelation time are shown Figure 3.3 (I). It can be seen that the gelation time was significantly reduced (10.09-0.11 min) as the concentrations of P407 (5-17 g) and T80 (5-10 g) were increased and the RG concentration was kept constant at 1.25 g. Similarly, gelation time was decreased when the concentrations of T80 and RG (0.25-2.25 g) was increased, keeping the P407 concentration constant at 11 g (Figure 3.3 (II)). Also, when the concentration of the T80 was kept constant at 10 g and the concentrations of P407 and RG were increased, gelation time was reduced Figure 3.3 (III). Model terms (A, B, A^2) with p-values under 0.05 were significant. Additionally, the F-value of 116.36 suggested the model's significance. In comparison to pure error, the insignificant lack of fit (102.37) was considered good. The difference between the adjusted R^2 0.9876 and the predicted R^2 0.9393 was < 0.2, indicating that they were reasonably consistent and used to explore the design space by the ratio of signal to noise, which was measured with adequate precision (28.708). The following regression equation mention below illustrates the relationship between all independent variables;

$$Gelation\ time = +1.06 - 4.43 - 0.8425\,B - 0.0425\,C + 0.2275\,AB + 0.0525\,AC + 0.0825\,BC + 3.29\,A^2 + 0.5262\,B^2 + 0.4362\,C^2$$

The gelation time was found between 0.11 ± 0.1 min and 10.09 ± 0.7 min in Design Expert® formulations as demonstrated in Table 3.2.

3.2.4. Effect of independent variables on gel strength

The statistical analysis of Design Expert® formulations, demonstrating that by changing independent variables concentrations such as P407 and T80 showed pronounced impact on gel strength with p-value < 0.0001 and 0.0033 respectively, on the other hand, drug (RG) showed non-significant impact with a p-value of 0.86. As shown via the three dimensional response surface graph in Figure 3.4 (I), gel strength was significantly increased from 53.82 to 152.9 $\times 10^2$ mPa.s when the concentrations of P407 (5-17 g), and T80 (5-10 g) were increased and the concentration of RG was kept constant at 1.25 g. Likewise, a meaningfully enhanced gel strength was observed as the concentrations of T80 and RG (0.25-2.25 g) were increased and the concentration of P407 was kept constant at 11 g Figure 3.4 (II). Moreover, Figure 3.4 (III) demonstrated increased gel strength with the increased P407 and RG concentrations and quantity (10 g) of T80 constant. The p-values under 0.05 were significant in model terms (A, B, A^2). The F-value of 64.92 further supported model's significance. The lack of fit value (36.96) was insignificant which is mandatory to fit the model. The values of adjusted R^2 0.9779 and the predicted R^2 0.8919 were reasonably consistent with less than 0.2 difference. The given model might be used to explore the design space, which was based on adequate precision calculation and had a value of 24.146. The regression equation describes the relationship between the independent variables, is given below;

$$Gel\ strength = +121.70 + 41.80\,A + 11.62\,B + 0.3387\,C + 0.1525AB + 0.0225AC - 0.1000\,C - 17.15\,A^2 - 1.34\,B^2 - 2.56\,C$$

The minimum gel strength was 53.82 ± 0.8 ×10^2 mPa.s and maximum gel strength was 152.9 ± 0.5 ×10^2 mPa.s as given in Table 3.2.

3.2.5. Effect of independent variables on mucoadhesive force

Figure 3.5 showed the the three dimensional response surface graphs of mucoadhesive force. It was indicated that the concentration of independent variables such as P407 and T80 showed direct impact on mucoadhesive force (i.e. by increasing its concentration mucoadhesive force increases) with a p-value of < 0.0001, whereas, the drug (RG) showed non-significant impact with a p-value of 0.93. As shown in Figure 3.5 (I), increased concentrations of P407 (5-17 g) and T80 (5-10 g) augmented the mucoadhesive force (6.86 to 54.17 ×10^2 dyne/cm^2) when the concentration of RG was kept constant at 1.25 g. Similarly, improved mucoadhesive force was observed with increased concentration of the T80 and RG (0.25-2.25 g) when the P407 was kept constant at 11 g (Figure 3.5 (II)). Also, it was noted that increased concentrations of the P407 and RG and a constant amount (10 g) of T80 meaningfully enhanced the mucoadhesive force (Figure 3.5 (III)). Model terms (A, B) with p-values under 0.05 were significant. The model's significance was strongly supported by the F-value of 154.11. The adjusted R^2 0.9725 and the predicted R^2 0.9541 were reasonably consistent. The adequate precision with a value of 36.608, suggested that this model might be used to explore the design space by the ratio of signal to noise. The lack of fit (43.79) was not significant. The relationship between the independent variables was demonstrated by the regression equation as mention below;

$$Mucoadhesive\ force = +26.94 + 17.72\,A + 6.63\,B + 0.0713C$$

3.3. Drug Content

The optimized liquid suppository contained 99.43 ± 0.3% of the loaded drug (1.25 g), which shows suitability of the liquid suppository as drug carrier. The test was carried out three times, and the content uniformity was confirmed to be uniform.

3.4. Physicochemical Evaluation

The optimized RG loaded suppository was clear and transparent with uniform homogeneity as shown in Figure 3.6.

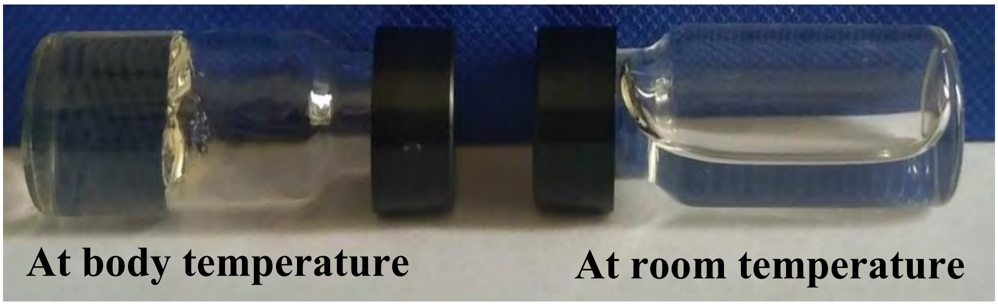

Figure 3.6: Illustration of the gelation of liquid suppository.

3.5. FTIR

The FTIR spectra of RG, P188, P407, physical mixture (PM) and RG loaded liquid suppository are represented in Figure 3.7. Pure RG spectrum was characterized by N–H peaks at 3389/3350/3305 cm^{-1} and C=O stretching vibration at 1720/1656 cm^{-1}. FTIR spectrum for P188 showed characteristic bands of C-H stretching at 2880 cm^{-1}, O-H bending at 1310 cm^{-1} and C–O stretching at 1111 cm^{-1}, whereas for P407 O-H stretching at 3483 cm^{-1}, C–H stretching at 2882 cm^{-1}, O–H bending at 1340 cm^{-1} and C–O stretching at 1120 cm^{-1} in its FTIR spectrum. Although O-H band is generally reported above 3000 cm^{-1}, but the peaks observed at 1300 region in case of the P407 and P188 suggest the existence of OH bending (δ-OH) (Asselin *et al.* 1971, Ahmed *et al.* 2023).

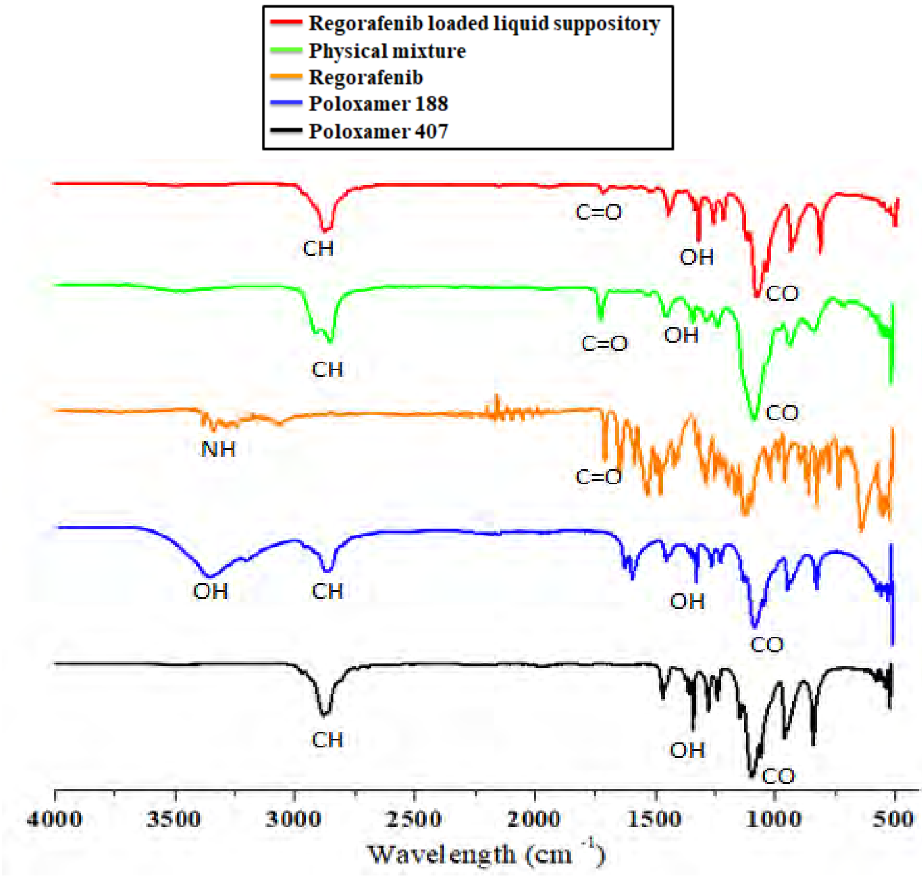

Figure 3.7: Fourier transform infrared (FTIR) spectra of poloxamer 188, poloxamer 407, regorafenib, physical mixture, and regorafenib loaded liquid suppository.

In PM and RG loaded liquid suppository, all these bands remained unchanged up to some extent indicating the compatibility and no chemical interaction between different constituents of liquid suppository.

3.6. Standard Curves of RG

RG standard curve was prepared in PBS 7.4. Serial dilution method was used for the dilutions. Then each dilution was analyzed for its absorbance at 260 nm by HPLC. Table 3.4 shows the concentrations and its absorbance in PBS 7.4. While Figure 3.8 outlined the calibration curve of RG.

Table 3.4. Absorbance value of regorafenib at different concentrations in PBS 7.4.

Concentrations (µg/mL)	Mean absorbance
30	2.04
15	1.03
7.5	0.52
3.75	0.26
1.875	0.15
0.937	0.09
0.468	0.04

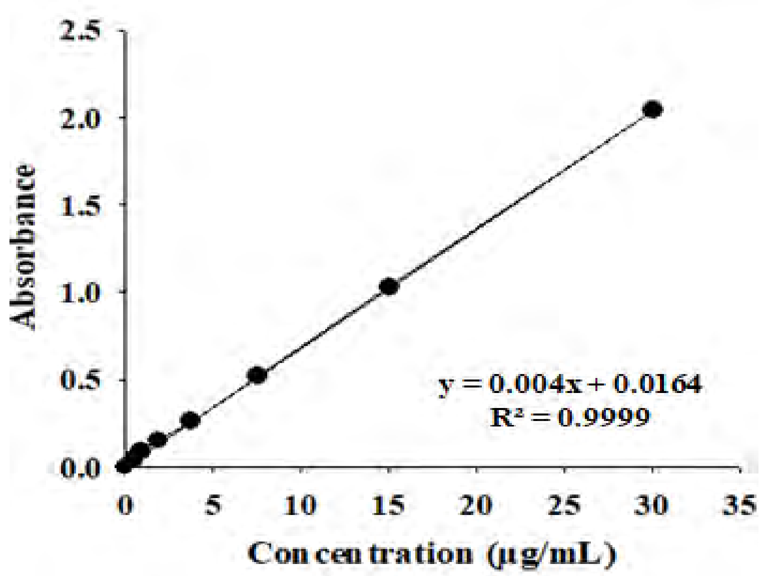

Figure 3.8: Calibration curve of regorafenib at pH 7.4.

3.7. *In Vitro* RG Release Study

In vitro study of RG release from the liquid suppository was determined and compared with RG suspension. Sigma plot software was used for the graphical representation of results (% cumulative release vs time) as shown in Figure 3.9. The results indicated a significantly improved release profile of RG from liquid suppository in comparison to its suspension. At 2 h, the cumulative release of RG from liquid suppository and suspension was 56% and 8%, respectively. However, at 6 h, complete drug released was observed from liquid suppository, and from RG suspension only 17% RG was released.

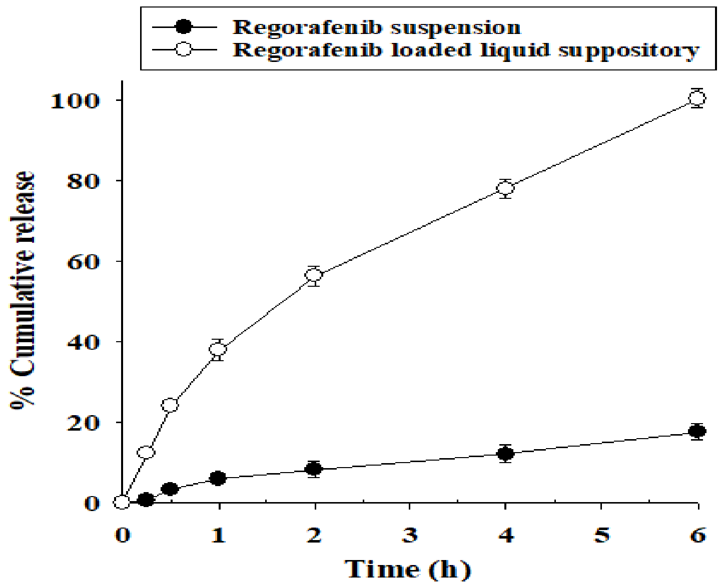

Figure 3.9. *In vitro* release profile of regorafenib loaded liquid suppository and its comparison with RG suspension at pH 7.4

3.7.1. Kinetic models for drug release

Several kinetic models were implemented to the *in vitro* release data of RG from suspension and liquid suppository in order to determine the kinetic models for RG release with the help of DD solver. Zero and first order, Hixson-Crowell, Higuchi, and Korsmeyer-Peppas kinetics models were applied and Rad², AIC and MSC values were calculated, as presented in Table 3.5. The highest value of Rad², MSC and lowest value of AIC for RG release showed that the first order has been followed by RG loaded liquid suppository and Higuchi model by RG suspension as illustrated in Table 3.5.

Table 3.5. Rad², AIC and MSC values for kinetic modeling of regorafenib loaded liquid suppository.

Kinetic models	Regorafenib loaded liquid suppository			Regorafenib suspension		
	Rad²	AIC	MSC	Rad²	AIC	MSC
Zero order	- 0.27	98.66	- 0.44	0.68	59.19	0.95
First order	0.98	56.65	3.75	0.77	55.69	1.30
Higuchi	0.70	83.96	1.02	0.98	33.95	3.47
Hixson-Crowell	0.81	79.45	1.48	0.74	56.92	1.17
Korsemeyer Peppas	0.85	77.81	1.64	0.97	35.95	3.27

Difference between predicated and observed values of RG loaded liquid suppository in first order kinetic model and RG suspension in Higuchi model at pH 7.4 is indicated in Figure 3.10A & B respectively.

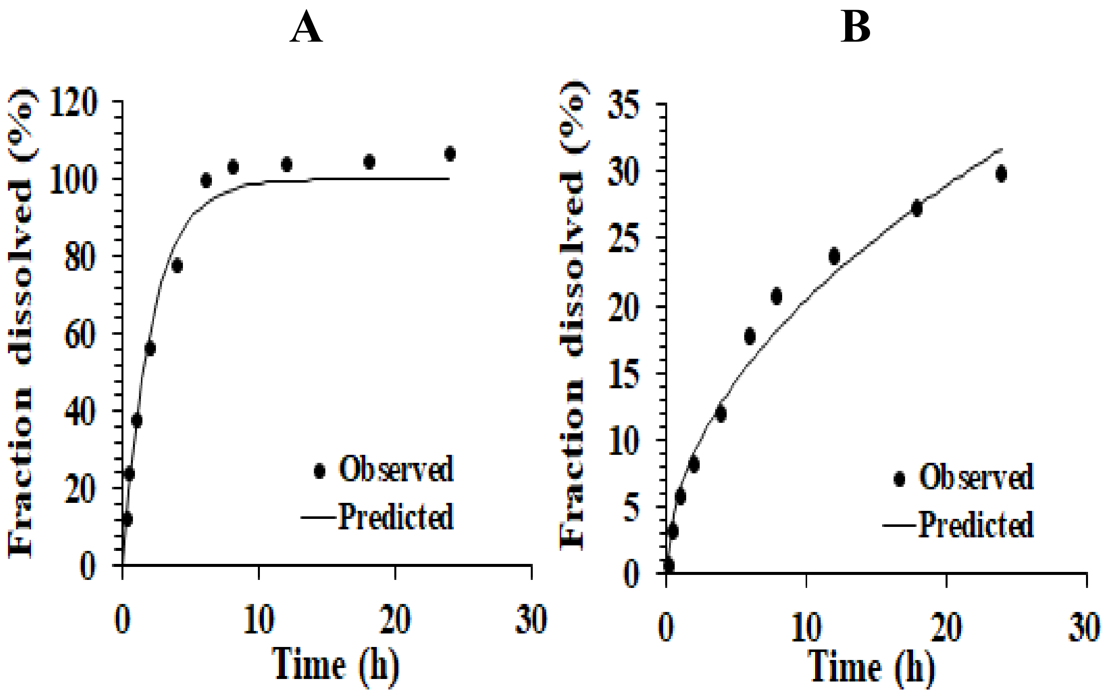

Figure 3.10. Difference between predicated and observed values of regorafenib at (A) First order kinetic model, and (B) Higuchi model at pH 7.4.

Table 3.6. Cell viability assay of normal saline, regorafenib suspension and regorafenib loaded liquid suppository at various concentrations on Caco-2 cell line.

Sr.No.	Conc. (µg/mL)	Normal saline	Regorafenib suspension	Regorafenib loaded liquid suppository
1	0.5	100.01 ± 0.1	92.13 ± 3.42	89.55 ±3.18
2	1	100.03 ± 0.3	87.459 ± 3.40	84.91 ± 3.67
3	2.5	100 ± 0.01	82.0281 ± 4.17	79.35 ± 3.12
4	3	99.5 ± 1.15	78.37 ± 3.42	74.21 ± 3.59
5	5	97.7 ± 1.25	71.37 ± 3.61	68.44 ± 3.29
6	12.5	96.54 ± 1.82	65.032 ± 3.10	62.82 ± 3.35
7	25	95.9 ± 1.93	44.318 ± 3.97	41.16 ± 3.01
8	50	95.06 ± 2.92	27.682 ± 3.16	24.45 ± 3.03
9	75	95.21 ± 2.91	18.021 ± 3.49	15.982 ± 3.14
10	100	93.33 ± 3.04	9.96 ± 3.12	6.561± 1.02

Note: *All the data is presented with ± standard deviation, n=3: µg: microgram, mL: milliliter*

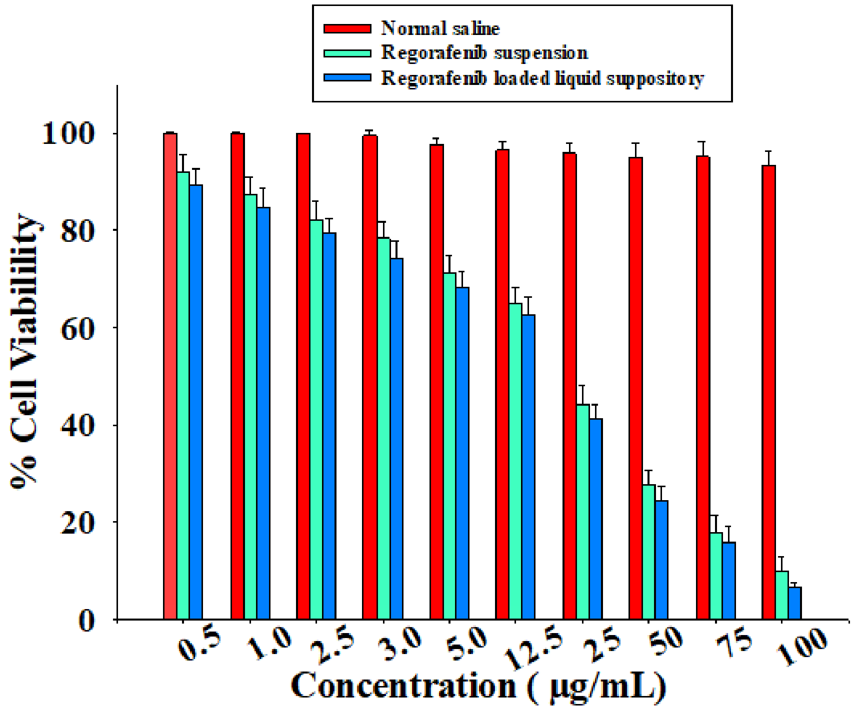

Figure 3.15: Cell viability study of the regorafenib loaded liquid suppository on Caco-2 cell lines and its comparison with normal saline and regorafenib suspension.

3.9.2. HCT 116 cell line

The MTT assay performed on the HCT 116 cell line showed that in case of the normal saline solution, the cells viability at 100 µg/mL concentration was 100.01 ± 0.10 and at 0.5 µg/mL concentration was 94.01 ± 2.04, indicaing its non-toxic behavior. On the other hand, RG suspension showed 90.13 ± 3.42 cells viability at 0.5 µg/mL concentration and 7.16 ± 1.91 cells viability at 100 µg/mL concentration. However, in case of the RG loaded liquid suppository, there was a significant decrease in the cells viability with increase in concentrations, i.e. 87.55 ± 3.18 to 5.56 ± 1.92 at 0.5 µg/mL and 100 µg/mL concentrations respectively as shown in Table 3.7. RG suspension and RG loaded liquid suppository exhibited maximum decrease in the cells viability, demonstrating its significant potential in eliminating the cancer cells in comparison to normal saline as shown in the Figure 3.16.

Table 3.7. Cell viability assay of normal saline, regorafenib suspension and regorafenib loaded liquid suppository at various concentrations on HCT 116 cell line.

Sr.No.	Conc. (µg/mL)	Normal saline	Regorafenib suspension	Regorafenib loaded liquid suppository
1	0.5	100.01 ± 0.1	90.13 ± 3.42	87.55 ± 3.18
2	1	100.03 ± 0.2	85.45 ± 3.40	81.91 ± 3.67
3	2.5	100 ± 0.01	79.02 ± 4.17	76.35 ± 3.12
4	3	99.5 ± 1.15	74.37 ± 3.42	72.21 ± 3.59
5	5	97.7 ± 1.25	69.37 ± 3.61	67.44 ± 3.29
6	12.5	96.54 ± 2.82	59.03 ± 3.10	56.82 ± 3.35
7	25	95.9 ± 2.93	42.31 ± 3.97	39.916 ± 3.01
8	50	95.06 ± 2.82	25.68 ± 3.16	21.45 ± 3.03
9	75	94.61± 2.61	14.02 ± 3.49	12.982 ± 3.14
10	100	94.013 ± 2.04	7.16 ± 1.91	5.561 ± 1.92

***Note**: All the data is represented with ± standard deviation, n=3: µg: microgram, mL: milliliter*

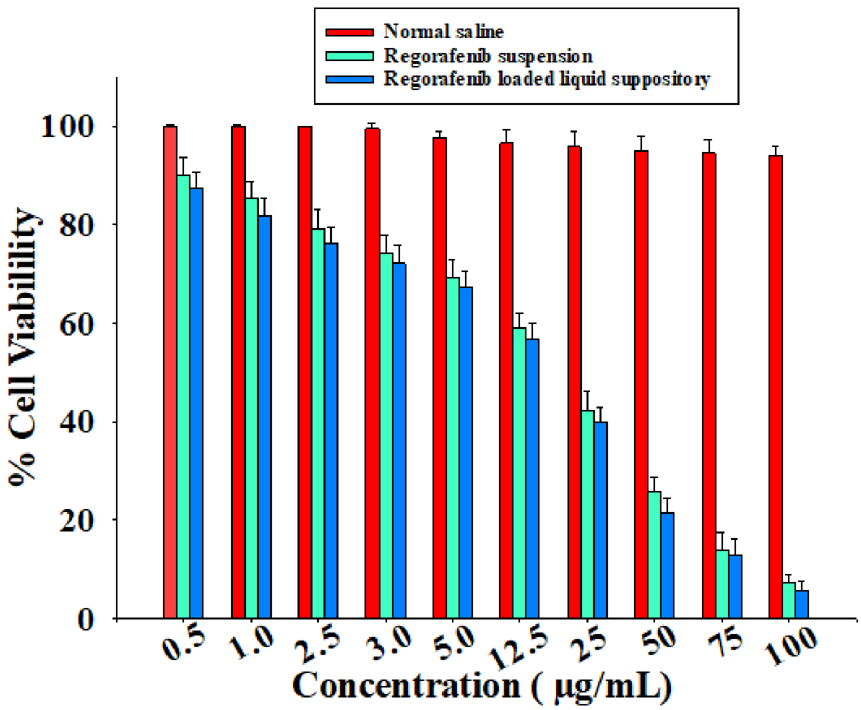

Figure 3.16: Cell viability study of the regorafenib loaded liquid suppository on HCT 116 cell lines and its comparison with normal saline and regorafenib suspension.

Half maximal inhibitory concentration (IC_{50}) were calculated and presented in Table 3.8. In case of the Caco-2 cell line data, IC_{50} for normal saline was 20899 ± 2.41 µg/mL, for RG suspension was 16.36 ± 1.13 µg/mL and for RG loaded liquid suppository was 13.53 ± 1.84 µg/mL. However, in HCT 116 cell line data, IC_{50} calculated for normal saline was 25360 ± 3.18 µg/mL, for RG suspension was 13.32 ± 1.32 µg/mL and for RG loaded liquid suppository was 11.34 ± 2.11 µg/mL.

Table 3.8: IC_{50} values of normal saline, regorafenib suspension and regorafenib loaded liquid suppository on Caco-2 cell lines and HCT 116 cell lines.

Caco-2 cell line		**HCT 116 cell line**	
Formulation	**IC_{50} ± S.D (µg/mL)**	**Formulation**	**IC_{50} ± S.D (µg/mL)**
Normal saline	20899 ± 2.41	Normal saline	25360 ± 3.18
RG suspension	16.36 ± 1.13	RG suspension	13.32 ± 1.32
RG loaded liquid suppository	13.53 ± 1.84	RG loaded liquid suppository	11.34 ± 2.11

Note: *All the data is represented with ± standard deviation (S.D), n=3. IC_{50}: half maximal inhibitory concentration, µg: microgram, mL: milliliter.*

3.10. Pharmacokinetic Study

The pharmacokinetic profiles of RG loaded liquid suppository and RG suspension is given in Figure 3.17 whereas, Table 3.9 represents additional detailed parameters. The RG loaded liquid suppository showed a significantly improved bioavailability profile when compared with the RG suspension at all the time points of the study. As shown, a meaningfully improved (five times) AUC (205.42 ± 21.04 µg.h/mL) was observed in the RG loaded liquid suppository treated rats group, when compared with RG suspension, which had a lower AUC of 38.97 ± 12.34 µg.h/mL. Moreover, the C_{max} value of the RG loaded liquid suppository was 24.18 ± 0.19 µg/mL, clearly higher than the RG suspension C_{max} value of 4.72 ± 0.11 µg/mL. The higher C_{max} value of RG loaded liquid suppository may indicate a stronger therapeutic action of RG. However, T_{max} values of both formulations were similar (T_{max}=2) and the difference between $t_{½}$ and K_{el} values of liquid suppository and suspension was not significant.

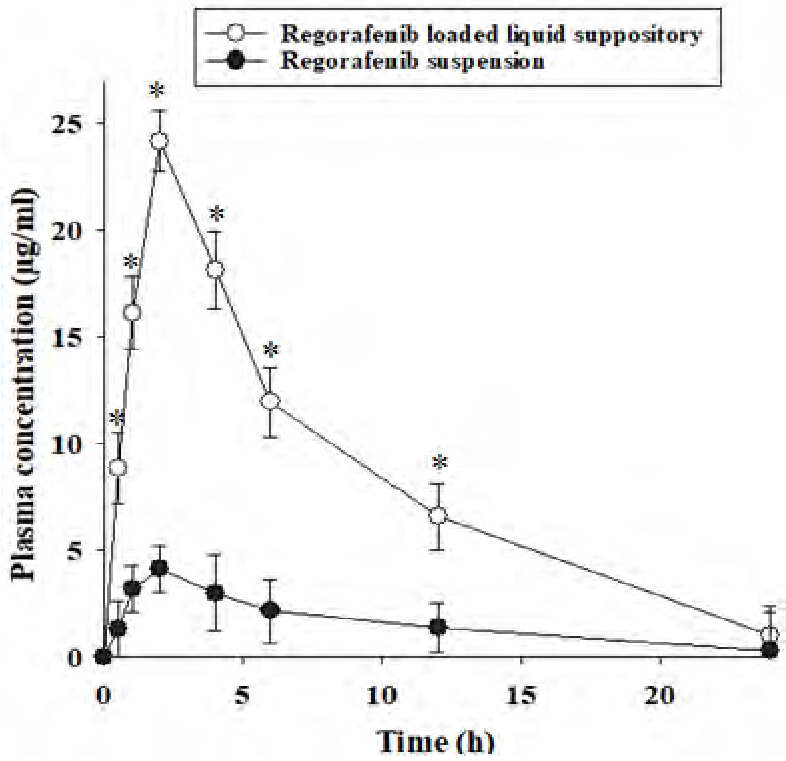

Figure 3.17: Pharmacokinetic data of regorafenib loaded liquid suppository and its comparison with regorafenib suspension.
Note*: p-value less then 0.05 symbolized by *.*

Table 3.9: Pharmacokinetic parameters of regorafenib loaded liquid suppository and its comparison with regorafenib suspension.

Parameters	Regorafenib suspension	Regorafenib loaded liquid suppository
C_{max} (µg/mL)	4.72 ± 0.11	24.18 ± 0.19
AUC (µg·h/mL)	38.97 ± 12.34	205.426 ± 21.04
T_{max} (h)	2.00 ± 0.31	2.00 ± 0.45
$t_{½}$(h)	5.46 ± 1.52	5.81 ± 1.97
K_{el} ($x10^{-2}$ h^{-1})	0.12 ± 0.14	0.11 ± 0.17

Note: *All the data is presented with ± standard deviation, n=3,* * Symbolizes p< 0.05 compared to suspension. *Cmax: maximum plasma concentration, AUC: area under curve, Tmax:time to reach maximum concentration, $t_{½}$: Elimination half-life, K_{el}: elimination constant , µg: microgram, mL: milliliter, h: hour.*

1.1 Histopathology

RG loaded liquid suppository-treated rectal tissues in rats were examined for the histopathology study using H&E staining, and the results were compared to control rectal tissues (untreated) and RG suspension. The histopathology study data are shown in Figure 3.18. As demonstrated by Figure 3.18A, the untreated control group rectal tissue, in which glandular architecture integrity is intact and no tissue inflammation and infiltration is seen. Figure 3.18B illustrated that RG suspension irritated the rectal mucosa, glandular architecture integrity is compromised and tissues is inflamed and infiltrated. While RG loaded liquid suppository is safe to the rectal mucosa as it shows no effects on the rectal tissues as shown by Figure 3.18C, just like untreated control group (Figure 3.18A).

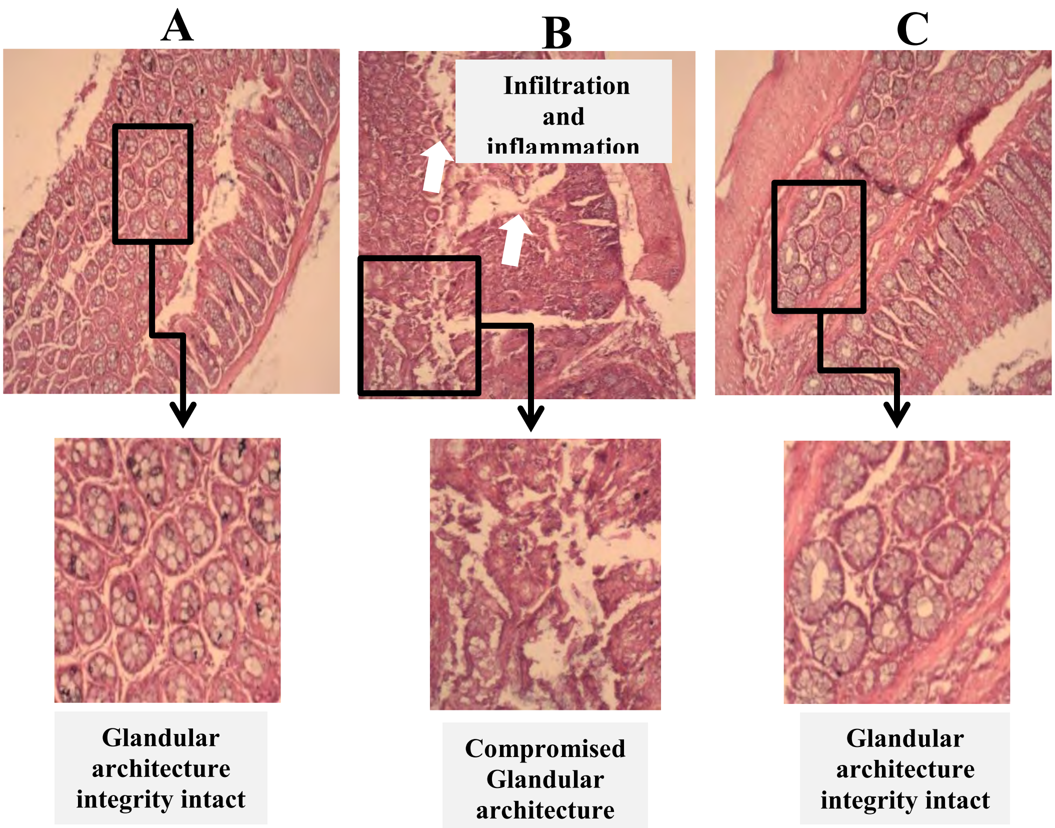

Figure 3.18. Histopathological examination of rats rectal mucosa.
Note: *(A) Untreated control group (B) RG suspension treated group (C) RG loaded liquid suppository treated group. Image was accessed at 40X.*

3.11. *In Vivo* Localization of Suppository

RG loaded liquid suppository containing methylene blue dye (1%) was inserted into the rats rectum, and at various time intervals, their localization in the rats rectum were observed. The liquid suppository was distinctly visible inside the rectum represented by dark blue color, 10 min after administration (Figure 3.19A). Liquid suppositories formed mucoadhesive gels that were light blue in color and occupied same positions in the rectum at 2 and 4 h after administration as illustrated in Figure 3.19B & 9C. Then after 6 h of administration (Figure 3.19D), liquid suppository was represented by dulled blue color inside the rectum. Although, the location of suppositories were not, changed noticeably yet the liquid suppository vanishes over the 6 h time period.

Moreover, this study also demonstrated that RG liquid suppository, with the gel strength 122.4 ± 0.3 $\times 10^2$ mPa.s which is between the two thresholds and gelation temperature 30 to 36 °C, when administered into the rat's rectum, didn't leak out.

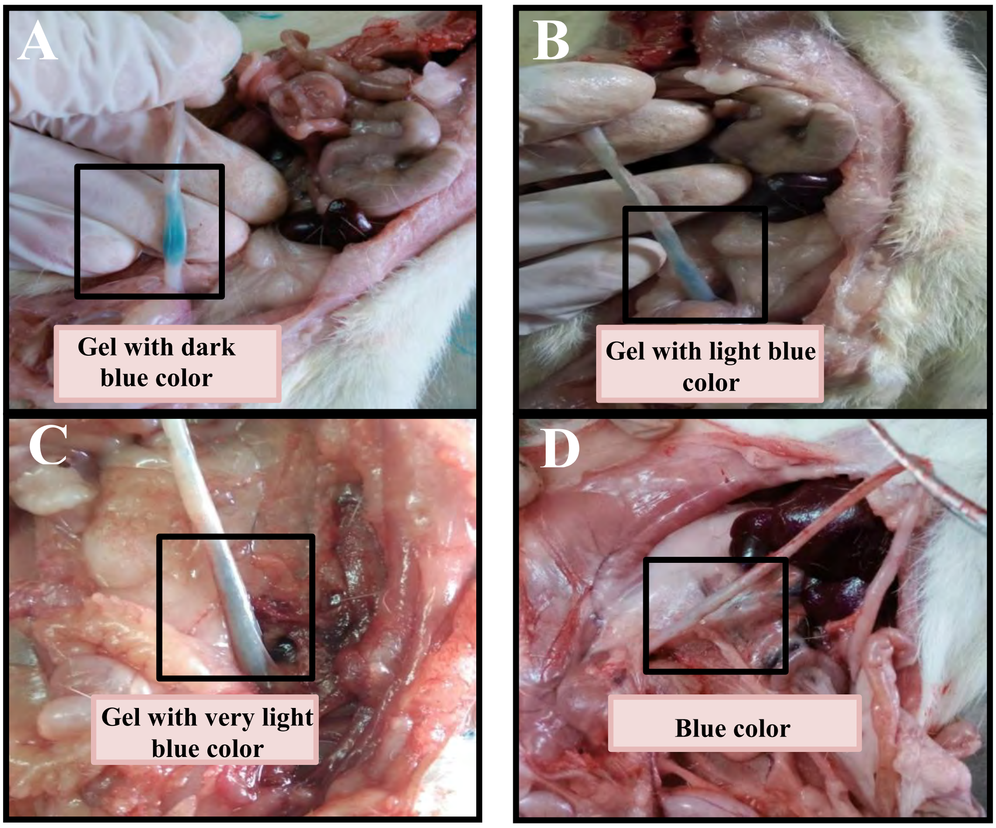

Figure 3.19. Localization of regorafenib liquid suppository inside the rectum of the rats.
Note: *At (A) 10 mins (B) 2 h (C) 4 h and (D) 6 h after administration, respectively, the rectum was sectioned.*

CHAPTER 4

DISCUSSION

4. DISCUSSION

Liquid suppository system is usually prepared from thermosensitive polymers, so that they may remain in liquid form at room condition and may convert into get form at physiological temperature. Thus, poloxamers, P407 and P188 were selected as gelling ingredients in the liquid suppository formulation. P407 and P188 are well known for biocompatibility, safety, water solubility and their compatibility with a wide range of excipients (Din *et al.,* 2021). The incorporation of P188 into P407 solution is used for the modulation of phase transition temperature. Their combination was used because, it is very difficult to obtain thermosensitive behavior (gelation temperature) with or P188 alone (Chen *et al.,* 2013; Fakhar ud din *et al.,* 2019). Moreover, T80 was chosen as a solubilizing agent, to enhance the RG solubility (Yong *et al.,* 2004). Also, with the addition of T80, a highly viscous gel formed, which may be responsible for the increased gel strength and mucoadhesive properties as well as the shorter gelation time and lower gelation temperature. As RG is a preffered in the treatment of CRC, so it was selected for targeted therapy of CRC (Ettrich *et al.,* 2018).

The Design Expert® software was used to construct factorial Box-Behnken design experiments in order to determine variables of the best formulation. The Box-Behnken design experiment was chosen because it required minimum number of experimental runs. The amount of P407, T80, and drug (RG) was chosen as an independent variable, and its impact was assessed in terms of gelating temperature and time required for gelation, gel mucoadhesion and its strength. Design Expert® software developed an experimental design matrix consisting of 14 runs. The best model for gel strength, its gelling temperature and time, was found to be the quadratic model whereas, linear model was the best fitted model for mucoadhesion force. Regression analysis for each dependent variable is shown in Table 3.3. It was evident from the statistical analysis (ANOVA) that changing the concentration of independent variables had a significant impact on the dependent variables. Minimum temperature and time required for gelation, and maximum strength, and mucoadhesion of gel, were the point prediction parameters. Using the results of the point prediction, formulation 11, 12, was chosen as the most suitable formulation.

The regression equation showed that P407 had inverse effect on gelation temperature and time (Figure 3.2 & 3.3) whereas, direct effect on mucoadhesive force and gel strength (Figure 3.4 & 3.5). Increasing P407 concentration results in decreased gelation temperature and time whereas, gel mucoadhesion and its strength increased. The increase in P407 concentration, may be correlated with the gelation temperature reduction, which is dependent on the volume and micelles number at elevated temperature (Escobar Chavez *et al.,* 2006). Change in the configuration of poloxamer solution explains the phenomenon of temperature-sensitive gelation. Poloxamer molecules arranged in a well-zigzag configuration which upon increase in temperature, changed into tightly pack configuration resulting into gel formation (Chan *et al.,* 2017; Fakhar ud din *et al.,* 2019). As the P407 concentration increases, gel strength also increases because the network model in the gel come close to each other and arranged in a tightly pack manner (Escobar Chavez *et al.,* 2006). Different types of hydrophilic, hydroxyl (-OH) and carboxyl (COOH) groups are present in the poloxamer molecules, while oligosaccharide chains are present in the rectum mucosal lining (Ruiz Pulido *et al.,* 2021). As the concentration of P407 increases, more hydrophilic groups are available for attachment to oligosaccharide chains present in the rectum lining, which may improve the liquid suppository mucoadhesion to the rectal mucosa (Chen *et al.,* 2012). Morever, increase in the density and more compact lattice structure development also causes increase in the mucoadhesion (Fakhar ud din *et al.,* 2019). This phenomenon results in strong mucoadhesive force.

Likewise, the T80 had inverse effect on gelation temperature and time as indicated in Figure 3.2 & 3.3, yet, it has direct effect on mucoadhesion of the gel and its strength, of the RG loaded liquid suppository, illustrated in Figure 3.4 & 3.5. It was anticipated that by increasing concentration of T80 between the molecules of poloxamer matrix may reinforce the hydrogen bonding inside the poloxamer cross linking gel, which in turn could affect the rheological properties of the liquid suppository (Choi *et al.,* 2011). As previously reported, T80 through hydrogen bond expansion, improves the gel strength and mucoadhesive forces of the liquid suppository (Yeo *et al.,* 2013; Diaz Salmeron *et al.,* 2021). With the addition of T80, a highly viscous gel formed, which may be responsible for the increased gel strength and mucoadhesive properties as well as the shorter gelation time and lower gelation temperature (Yeo *et al.,* 2013). However, the drug (RG) loading in the liquid suppository showed insignificant effect on the gelating temperature, time, mucoadhesion of gel, and its strength.

The content of the drug present inside the suppository was determined to be within the formulation's suitable range. This study confirms that the liquid suppositories manufacturing method was able to produce suppository with uniformly distributed drug content. Solubility of the RG increased due to P407, P188 and T80 as a result of which clear and transparent formulation was produced (Keny *et al.,* 2010; Tran *et al.,* 2019). The FTIR spectrum of RG, P188, P407, PM and RG loaded liquid suppository was recorded. The FTIR spectrum of RG loaded liquid suppository and its PM confirmed that there was no interaction between RG, P 407 and P188.

In vitro drug release study demonstrated that the RG release from the liquid suppository was six times increased when compared to its suspension. The possible reason behind this could be due to the enhanced dissolution of RG within the liquid suppository system decorated with poloxamer and T80, and its temperature sensitive behavior (Jiao *et al.,* 2023). Poloxamer facilitates the solubilization of hydrophobic molecules, promoting their complete dissolution (Tran *et al.,* 2019). In liquid suppository, the drug is effectively dissolved and exists in a liquid state at 25 °C which changes to gel form at 36.5 °C. At low temperatures, a hydration layer surrounds the poloxamer molecules; however, as the temperature rises, the hydrogen bonding between the aqueous solvent and the copolymer's hydrophilic chains is broken. The polyoxypropylene blocks' hydrophobic interactions are favoured by this desolvation (Giuliano *et al.,* 2018). The gelation process is promoted by this phenomenon. This temperature sensitive behavior causes better dissolution of the incorporated drug, resulting in increased release of drug from liquid suppository (Bialik *et al.,* 2021). On the other hand, RG suspension being hydrophobic in nature couldn't improve drug solubilization, due to which RG release declines from suspension (Fakhar ud din *et al.,* 2019). Therefore, it can be concluded that the drug's bioavailability may be improved by using a liquid suppository system, which increased its solubility. Several kinetic models were implemented to the *in vitro drug* release profile of RG from liquid suppository and suspension and the results showed that the first order has been followed by RG loaded liquid suppository and Higuchi model by RG suspension as illustrated in Table 3.5. The first order indicated that the release rate of RG from liquid suppository is dependent on the concentration. Whereas, the Higuchi model (1963) described the drug release from an impermeable matrix (RG suspension), as the square root of a process that is time-dependent. (Thakur, 2022).

RG loaded liquid suppository was found stable as per the ICH guidelines for at least 6 months. Results showed that when compared to the initial values, the RG loaded liquid suppository had not significantly changed during the evaluation period. A sufficient amount of surfactants, such as poloxamer and T80, may have contributed to its excellent stability (Din *et al.,* 2017). As a result, it was concluded that both room and refrigerator temperature, were suitable temperature for storing the RG loaded liquid suppositories.

To investigate the antitumor effectiveness of RG loaded liquid suppository in a dose-dependent response, *in vitro* cell lines studies were conducted and the results were compared with RG suspension and normal saline. More than 90% of cells were found viable at various concentrations of normal saline, demonstrating its biocompatibility and non-toxic nature. Moreover, it demonstrated a no antitumor effect of the normal saline. Unlikely, RG suspension and RG loaded liquid suppository exhibit significantly augmented cytotoxic effect due to their effective antitumor potential (Anwer *et al.,* 2022). Even if no significant difference between the RG loaded liquid suppository and RG suspension was exhibited, yet RG loaded in liquid suppository has promising antitumor effect. The localized delivery of RG from liquid suppository also enhanced the RG passive targeted transport into tumor cells, through leaky capillary fenestrations of tumor cells (Lo *et al.,* 2013). Poloxamers were found to have the ability to reverse the effects of multidrug resistance in tumors (Zhang *et al.,* 2011). This phenomenon explained the pronounced tumor cells growth inhibition in CRC after rectal administration of RG in liquid suppository. Furthermore, IC_{50} values were calculated through software GraphPad-Prism-9.5.1 using non-linear regression equation. IC_{50} values presented in Table 3.8, showed a significant decrease for RG suspension and RG loaded liquid suppository in comparison to the normal saline. This significant reduction in IC_{50} values indicated an enhanced antitumor potential of RG, specifically when loaded in liquid suppository.

Pharmacokinetics data exhibited a significant improved RG bioavailability loaded in liquid suppository in comparison to the RG suspension. An increased AUC of the liquid suppository showed that the five times more of RG was available for its therapeutic effects over the extended period of time. Moreover, improved C_{max} values indicated that comparatively greater quantity of the RG was available at the time when maximum concentration was achieved. The possible reason behind this may be the appropriate composition of the liquid suppository, matrix of the gel, and physiological characteristics, which enhanced the solubilization of hydrophobic drug (Jiao *et al.,* 2023). Also, the poloxamers thermosensitive behavior, which ensures release of the RG following rectal administration, may also contributed to these improved pharmacokinetic parameters (Akl *et al.,* 2019). Besides, no leakage was observed as a result of the liquid suppository quick gelation inside the rectum, improved gel strength, and mucoadhesion with the rectal mucosa (Mushtaq *et al.,* 2022). Also, liquid suppository and suspension gave same time ($T_{\max}$) to reach the maximum concentration however C_{max} of RG was higher in case of liquid suppository than suspension. The reason behind this difference could be the mucoadhesive nature of liquid suppository (Mushtaq *et al.,* 2022).

The purpose of histopathology study was to ensure the safety of liquid suppository. Histopathology of the rat's rectum showed that no morphological damage or inflammation to the rectal tissue was caused by the RG loaded liquid suppository (Figure 3.19C). The rectum morphology was intact as observed in the untreated (control) rectal tissues (Figure 3.19A). However, when treated with RG suspension, the rectal tissues showed significant signs of morphological damage or inflammation (Figure 3.19B). The reason behind this toxicity caused by RG suspension may be due to the drug's direct interaction with the rectum. However, the protective behavior of the poloxamer solution contributed to the safety of RG loaded liquid suppository. Another factor contributing to the decrease in the anticancer agent's toxic profile may be the drug's loading inside the poloxamer gel which will prevent its abrupt release and direct contact with the mucosal lining (Jiao *et al.,* 2023; Mushtaq *et al.,* 2022). This study verified the formulation's safety for rectal administration.

The liquid suppository localization *in vivo* indicated by the blue color inside the rat rectum is illustrated in Figure 3.20. The liquid suppository's color changed from dark to light over the course of 10 mins, 2, 4, and 6 h, with the blue color eventually fading away. However, its location did not alter significantly over time inside the rectum. The results show that the gelled liquid suppository's mucoadhesive force was sufficient to keep it in the rats' rectum for at least 6 h. Possible reason behind these mucoadhesion could be attributed to the hydrogen bonding between carboxyl

(COOH), hydrophilic, and hydroxyl (-OH) groups of the poloxamer and the oligosaccharide chains of mucosal lining of rectum (Jiao *et al.,* 2023; Yuan *et al.,* 2012). Suppository's faded blue color after 6 h of administrations indicating that liquid suppository retained its position inside the rectum for at least 6 h (Lin *et al.,* 2012).

The lowest gel strength at which the liquid suppository administered into the rectum without its leakage during the first 30 min of administration was referred to as the threshold of gelation. The liquid suppository remained in the upper part of the rectum and did not leaked out because it gelled more quickly and had ideal gel strength. The liquid suppository with maximum gel strength that could easily be injected into rats' rectum is known as the maximal threshold of gel strength. Whereas, the liquid suppository with minimum gel strength that could not leaked out from rats rectum is known as the minimal threshold of gel strength (Choi *et al.,* 1998a). Also, a liquid suppository needs to have a temperature range between 30 °C and 36 °C for gelation (Bialik *et al.,* 2021). If the liquid suppository's gelation temperature is less than 30 °C, gelation will take place at room temperature, due to which the production, handling, and administration of it becoming difficult. Similarly, if gelating occurs at a temperature greater than 36 °C, the liquid suppository will be in liquid form at body temperature, which increases the chances of its leakage from the rectum (Choi *et al.,* 1998a; Yeo *et al.,* 2013).

CONCLUSIONS

- The RG loaded liquid suppository was prepared in accordance with the specifications for an ideal liquid suppository, including the suitable temperature and time required for gelation, optimum mucoadhesion and gel strength.
- The importance of the system is its thermosensitive nature, as at room temperature it is in solution form and transforms into gel at body temperature after rectal administration.
- *In vitro* drug release study showed that liquid suppository system demonstrated improvement in the drug release in contrast to the drug suspension.
- According to the pharmacokinetic study, AUC and C_{max} values indicated that RG loaded liquid suppository improved the bioavailability of the drug and thus enhanced the therapeutic effect, when compared with the RG suspension.
- Histopathology study of the rat's rectum confirmed the formulation's safety for rectal administration as compared to RG suspension which caused severe damage to the rectal tissues.
- In comparison to normal saline, both RG suspension and RG loaded liquid suppository exhibit significantly more cytotoxic effect due to their effective antitumor potential.
- Therefore, concluded from the outcomes, liquid suppository with enhanced bioavailability, safe rectal delivery and improved drug dissolution can be successfully used as a delivery system for the drugs belong to BCS class II. However, *in vivo* antitumor studies are recommended for the evidence of the concept, which will be performed in the next phase of the study.

FUTURE PROSPECTIVES

- *In vivo* antitumor analysis will be performed to confirm the antitumor effect of RG loaded liquid suppository in animal model.
- The developed formulation of RG can be compared with the marketed product.

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Annexure I: Approval from Bioethics Committee

قائدِاعظم یونیورسٹی

QUAID-I-AZAM UNIVERSITY

Faculty of Biological Sciences
Bioethics Committee

No. #BEC-FBS-QAU2022-413 Dated: 28-07-2022

Miss Aiman Saleem
C/O Dr. Fakhar Ud-Din
Department of Pharmacy,
Faculty of Biological Sciences,
Quaid-i-Azam University, Islamabad
45320, Pakistan

Subject: - **“Development and characterization of Regorafenib loaded liquid suppositories for rectal delivery.”**

Dear Miss Aiman Saleem,

We wish to inform you that your subject research study has been reviewed and is hereby granted approval for implementation by Bio-Ethical Committee (BEC) of Quaid-i-Azam University, Your study has been assigned protocol **#BEC-FBS-QAU2022-413.**

While the study is in progress, please inform us of any adverse events or new, relevant information about risks associated with the research. In case changes have to be made to the study procedure, the informed consent from and or informed consent process, the BEC must review and approve any of these changes prior to implementation.

Sincerely,

Prof. Dr. Sarwat Jahan
Department of Zoology

cc:
Dean, F.B.S

Annexure II: Turnitin Similarity Index Report

Aiman Thesis 13-8-2023 Similarity

ORIGINALITY REPORT

9%	6%	6%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to University of Hong Kong Student Paper	1 %
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