IN-VITRO SCREENING, FORMULATION AND EVALUATION OF INTESTINAL P-GLYCOPROTEIN MODULATION ACTIVITY OF SELECTED CANDIDATES

By

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Under the guidance of,

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Dedicated To My

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Abbreviations	Expansions
P-gp	Permeability Glycoprotein
%	Percentage
%CDR	Percentage cumulative drug release
°C	Degree Centigrade
Abs	Absorbance
BP	British Pharmacopoeia
Cm	Centimeter
C_{max}	Maximum concentration
Conc	Concentration
CDR	Cumulative drug release
FTIR	Fourier Transform infrared
GIT	Gastro intestinal tract
Hrs	Hours
MDR	Multi Drug Resistance
ICH	International Conference on Harmonization
IP	Indian Pharmacopoeia
Kg	Kilogram
mcg/µg	Microgram
mg	Milligram

min	Minute
mL	Milliliter
mm	Millimeter
nm	Nanometer
pH	Negative logarithm of hydrogen ion concentration
BA	Bioavailability
P^{ka}	Dissociation constant
RH	Relative humidity
BCS	Biopharmaceutical Classification System
BE	Bioequivalence
dQ/dT	Amount transported-versus-time plot
P_{app}	Apparent permeability co-efficient
CO_2	Carbon dioxide
KRS	Krebs's Ringers solution
USP	United State Pharmacopoeia
UV	Ultraviolet
Vs	Verses
w/v	Weight by volume
w/w	Weight by weight

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

P-glycoprotein (P-gp) is an energy dependent transporter protein located in the apical membrane of intestinal mucosal cells. It limits the bioavailability of various orally given drugs by transporting back to the intestinal lumen. Agents which inhibit such drug transport are called as P-gp inhibitors which tend to increase the bioavailability of most of orally given drugs. The objective of the study was to evaluate the P-gp inhibition activity of selected herbal extracts choosing Ciprofloxacin as a substrate by *in-vitro* everted sac method by using rat intestine. The ethanolic extracts of pepper, turmeric, zinger, drumstick, cumin and pomegranate juice were investigated for P-gp inhibition activity on intestinal absorption of ciprofloxacin. Transport of drug across the intestine in presence and absence of test sample was estimated at frequent interval of time using UV Spectrophotometer at 276nm. Amongst the samples, the 20 mg extracts of pepper, cumin, pomegranate juice showed 3 folds and extracts of zinger and turmeric showed 2 fold increases in drug absorption indicating the P-gp inhibition activity. However the extract of drumstick did not show any significant change in the drug absorption. Tablets were prepared using drug and extracts by wet granulation method. Pre and post compression parameters of the compressed tablets were found to be in the limits of IP. FTIR studies indicated no interaction between drug and extracts and drug and excipients. Stability studies at $40^0 \pm 2\ ^0C$ / 75% $\pm$ 5 % RH for 2 months showed no physical and chemical changes. The above results indicated that the absorption of P-gp substrates can be enhanced by the use of extracts of pepper, zinger, turmeric, and cumin and pomegranate juice individually or separately.

Key words P-glycoprotein, P-gp inhibitors, Ciprofloxacin

Chapter 1

Introduction

INTRODUCTION

Drug absorption is the process of movement of unchanged drug from the site of administration to systemic circulation. The rate limiting barriers for the drug absorption of orally administered drugs are aqueous solubility and intestinal permeability. Drug absorption is considered to be complex transfer process across the intestinal lining, which includes any of the mechanisms a) Transcellular/intracellular transport, b) Paracellular /intracellular transport c) Vesicular transport

a) Trans-cellular/ Intracellular transport is the passage of drugs across the epithelium. It is the common pathway for drug transport. The various Trans cellular transport processes involved in drug absorption includes Passive transport process which does not require energy other than that of molecular motion to pass through the lipid bilayer. Passive transport of drug can include passive diffusion, Pore transport, Ion- pair transport, Facilitated- or carrier mediated diffusion. Active transport is another way of drug transport process which requires energy in the form of ATP to move drug molecule from extracellular to intracellular milieu.
b) Paracellular/Intracellular transport is one through which drug transports between the junctions of GI epithelial cells. Low molecular weight molecules take this route for transport.
c) Vesicular or corpuscular transport (endocytosis) includes the transport of substance within vesicles into a cell and the complete mechanism is done across the cell membrane. This transport can be Pinocytosis (cell drinking) where liquid molecules will be taken up by the vesicles and Phagocytosis (cell eating) where vesicles take up the solid particles inside and digest.(1)

Brief overview of the Gastro intestinal tract

Oral administration of drug leads to stomach region which is highly acidic of pH is 1.2. In this region most of the acidic drugs get absorbed as these drugs available in unionized form. Gastric emptying mainly affects the drug absorption process. Faster gastric emptying favors the absorption of basic drugs and absorption of acidic drugs decreases. In other words, slow gastric emptying favors the absorption of acidic drugs. From stomach, drug enters small intestine region where the pH is towards basic side (6.8-7.0). Small intestine provides largest surface area for absorption of food as well as drugs. Most of the drugs absorb passively in this region. Small intestine is structurally divided into three parts viz duodenum jejunum and ileum. Structural and functional units of small intestine are called as microvilli and villie which are responsible drug absorption and providing largest surface area by folding in many folds. In this region most of the basic drugs, drugs transported via active transport and carrier mediated transport is facilitated. From small intestine, drug enters to colon region where natural micro flora is present. Many drugs also absorb in this region as its pH is 6.8 and this colon is highly perfused tissue.(2)

Physiology of the small intestine

The human small intestine is approximately 2±6 m and is loosely divided into three sections: duodenum, jejunum, and ileum, which comprise 5%, 50%, and 45% of the length (Ganong, 1995). Approximately 90% of all absorption in the gastrointestinal tract occurs in the small intestinal region. The intestinal surface of the small intestine has surface projections that increase the potential surface area for digestion and absorption. Macroscopic valve like folds, called circular folds, encircling the inside of the intestinal lumen is estimated to increase the surface area of the small intestine

threefold. Villi increase the area 30-fold and the microvilli increase it by a factor of 600. Thus, such unique structures lead to a tremendous increase in surface area available for absorption in the small intestine.

The major role of the small intestine is the selective absorption of major nutrients and to serve as a barrier to digestive enzymes and ingested foreign substances. The epithelial cells in the intestinal region are a heterogeneous population of cells that include enterocytes or absorptive cells, goblet cells that secrete mucin, endocrine cells, paneth cells, M cells, tuft, and cup cells. The most common epithelial cells are the enterocytes or the absorptive cells. This cell is responsible for the majority of the absorption of both nutrients and drugs in the small intestine. It is polarized with distinct apical and basolateral membrane that are separated by tight junctions. Thus, the bulk of absorption takes place in the small intestine by mechanisms such as passive diffusion (paracellular and transcellular), carrier-mediated process (facilitated and active), and endocytosis.(3)

Permeability-glycoprotein (P-gp) is an energy dependent transporter protein located in the apical membrane of intestinal mucosal cells. It is believed that it may limit the bioavailability of many orally administered drugs, by transporting them back into the intestinal lumen following their absorption by the enterocytes(4). P-gp is associated with the multidrug resistance in tumor cell, but also expressed in a variety of normal human tissues including liver, brain, kidney, gastrointestinal tract, intestine, adrenal gland, blood-brain barrier, placenta, blood-testis, blood-ovarian barriers and in various tumors. At the intestinal level, P-gp is located on the apical membrane of the mature intestinal cells and acts as a pump that transports drugs back into the lumen as they are absorbed across the intestinal mucosa(5).

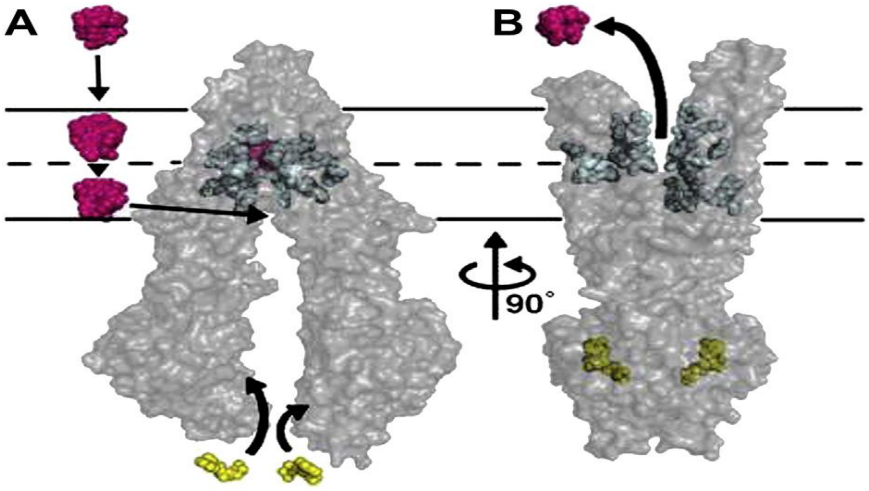


Fig.1 Model of substrate transport by permeability glycoprotein (P-gp). (A) Substrate partitions into the bilayer from outside the cell to the inner leaflet and enters the internal drug-binding pocket through an open portal. The residues in the drug-binding pocket (cyan spheres) interact with inhibitors and substrates in the inward-facing conformation. (B) Adenosine triphosphate (ATP) (yellow) binds to the nucleotide-binding domains, causing a large conformational change presenting the substrate and drug-binding site(s) to the outer leaflet/extracellular space.

P-gp transport system

The x-ray crystallographic structure for P-gp is depicted in Figure 1, showing ATP binding to produce conformation changes resulting in the extrusion of a substrate to the extracellular space. The P-gp transporter is found in the luminal membrane of the small intestine and blood-brain barrier and in the apical membranes of excretory cells such as hepatocytes and kidney proximal tubule epithelia. Expression on intestinal epithelial cells is responsible for efflux that limits cellular uptake and absorption into enterocytes, whereas expression on the canalicular surface of hepatocytes and renal tubular cells enhances the elimination of drugs into the bile and urine. P-gp expression

in the blood-brain barrier plays an important role in limiting the entry of various drugs into the central nervous system

P-gp mediates the export of drugs from cells located in the small intestine, blood-brain barrier, hepatocytes, and kidney proximal tubule, serving a protective function for the body against foreign substances. Intestinal absorption, biliary excretion, and urinary excretion of P-gp substrates can therefore be altered by either the inhibition or induction of P-gp. A wide spectrum of drugs, such as anticancer agents and steroids, are known P-gp substrates and/or inhibitors, and many cardiovascular drugs have recently been observed to have clinically relevant interactions as well. Cardiovascular drugs with narrow therapeutic indexes (e.g., anti arrhythmic agents, anticoagulant agents) have demonstrated large increases in concentrations when co administered with potent P-gp inhibitors, thus increasing the risk for drug toxicity. Therefore, dose adjustment or use of alternative agents should be considered when strong P-gp-mediated drug-drug interactions are present.(6)

It was recently identified that drug efflux pumps like P-glycoprotein (P-gp) are playing major role in altering the pharmacokinetics of various drugs and particularly associated with poor bioavailability in co-ordination with gut wall metabolism. Though much work has been pursued in the process of establishing the role of P-gp in multidrug resistance (MDR) in cancer cells, only recently it is gaining importance in absorption enhancement due to its selective distribution at the site of drug absorption. Thus, a deep insight and thorough understanding of P-gp, its physiological and biochemical role in effluxing drugs is worthwhile, in order to have an opportunity to improve the bioavailability of drugs restricted by P-gp. Role of other transporters such as multidrug resistance protein (MRP) 1–6 and BCRP at different biological barriers

was also proved significant in determining pharmacokinetics of various clinically important drugs.(7)

Role of P-gp in drug absorption

The most convenient route of administration of drugs is the peroral route because of the high level of patient safety and acceptance, and relatively low production cost. On the other hand, an oral drug will be therapeutically efficient only if it is absorbed from the gastrointestinal tract and can gain access to the systemic circulation in sufficient quantities. The bioavailability of a drug is defined as the fraction of the dose that appears intact in the systemic circulation. Major efforts are invested by drug companies to assess new drug candidates to ensure they have acceptable bio availabilities.

The absorption of drugs from the intestinal lumen is a very complex process influenced by both physicochemical properties of the drug and physiological factors of the gastrointestinal tract. Drug transport across intestinal epithelial is mediated basically by one or several of the following processes: passive diffusion (transcellular or paracellular transport), carrier-mediated influx or efflux transport. In passive transcellular transport, the drug is able to transverse the enterocyte depending on its membrane permeability proper- ties. In passive paracellular transport, the drug is transported via water-filled pores between the cells (this route is generally restricted to small hydrophilic molecules). In small intestine, a variety of influx carriers that transport nutrients and endogenous compounds are capable of transporting drugs with sufficiently similar molecular structures. On the other hand, efflux transporters such as P-gp, multidrug-resistance-associated protein 2 (MRP2; ABCC2) and breast cancer resistance protein (BCRP; ABCG2), expressed on the apical membrane of intestinal

epithelial in humans and rodents, may limit the net absorption of the drugs by pumping them back to the gut lumen.

P-gp is able to recognize a variety of structurally and pharmacologically unrelated neutral and positively charged hydrophobic compounds, whereas the range of MRP2 and BCRP substrates seems to be somewhat narrower. Therefore, P-gp seems to be the efflux transporter which could have the most relevant role in limiting the intestinal absorption of many drugs. Once a P-gp substrate drug crosses the apical membrane of the enterocyte, a portion will be effluxed back to the intestinal lumen by P-gp and another part of the drug could be subjected to intestinal first pass metabolism by cytochrome P-450 (CYP) enzymes, predominantly CYP3A, which is relatively abundant in the intestinal mucosa. In addition, enzymatic processes such as phase II conjugation may also occur. Consequently, the net amount of drug absorbed into the mesenteric blood circulation is the difference between the amount absorbed by the influx process and the sum of the amount extruded by efflux transport (P- gp) together with the amount metabolized by enzymes. This fraction of drug absorbed enters into the liver via the portal vein and may be subjected to hepatic first pass elimination. The molecule may be metabolized by hepatic enzymes, trans- ported from the hepatocyte into the bile by P-gp or continue to the systemic circulation by the hepatic vein distributing in the organism. The unchanged drug (as well as the result- ing metabolites) secreted into the bile will be secreted into the duodenum. In the intestine, the compound could be reabsorbed or eliminated in the faeces.(8)

P-glycoprotein substrates

P-gp transporter has a diverse array of substrates which vary not only in size and structure but also in several chemical properties. Since the primary criterion

subjecting a substrate to P-gp efflux is its interaction with the bilayer lipid membrane, a wide range of cationic, lipophilic and planar drugs become the protein substrates inspite of their structural dissimilarity. This explains the vast spread structural specificity or in the practical sense, the non-specific nature of P-gp. However, there is one unifying structural feature that is commonly shared among all the substrates of P-gp, and that is they all possess spatially separated hydrophilic and hydrophobic moieties. The P-gp substrates reconstitute most of the clinically efficient agents. Anticancer drugs, various pharmacotherapeutic agents that act on central nervous system, cardio vascular system and antimicrobials are substrates to this efflux protein.(9)

P-glycoprotein inhibitors

Screening studies for P-gp–drug interactions identified a number of clinically important drugs as P-gp substrates, which are as diverse as anthracyclines (doxorubicin, daunorubicin), alkaloids (reserpine, vincristine, vinblastine), specific peptides (valinomycin, cyclosporine), steroid hormones (aldosterone, hydrocortisone) and local anaesthetics (dibucaine). Even dye molecules (Rhodamine 123) and pharmaceutical excipients exhibited P-gp substrate activity. Few of them were identified to inhibit P-gp, setting off an opportunity in MDR reversal. Improved clinical efficacy of various drugs observed by P-gp inhibition, especially drug subjected to MDR, lead to the design and development of modulators, which specifically block P-gp efflux and having improved toxicity profiles. P-gp inhibitors are gaining recognition to improve bioavailability by inhibiting P-gp in intestine, brain, liver and kidneys, which has been hypothesised and emphasized by many researchers in recent years.(10)

Based on the specificity and affinity, P-gp inhibitors are classified to three generations. First-generation inhibitors are pharmacological actives, which are in clinical use for other indications but have been shown to inhibit P-gp. These include calcium channel blockers such as verapamil; immunosuppressants like cyclosporin A; anti-hypertensives, reserpine, quinidine and yohimbine; and antiestrogens like tamoxifen and toremifena. The usage of these compounds is limited by their toxicity due to the high serum concentrations achieved with the dose that is required to inhibit P-gp. A great deal of research by industrialist and academicians in the direction of improving toxicity profile resulted in secondand third-generation inhibitors that specifically modulate P-gp. Second-generation modulators are agents that lack the pharmacological activity of the first-generation compounds and usually possess a higher P-gp affinity. However, inhibition of two or more ABC transporters leads to complicated drug–drug interactions by this class of compounds, which include non-immunosuppresive analogues of cyclosporin A, PSC 833; D-isomer of verapamil, dexverapamil; and others such as biricodar (VX-710), GF120918 and MS-209. On the other hand, several other novel third-generation P-gp blockers are under development, however, primarily with the purpose to improve the treatment of multidrug resistant tumours and to inhibit P-gp with high specificity and toxicity. Modulators such as LY335979, OC144093 and XR9576 are identified to be highly potent and selective inhibitors of P-gp with a potency of about 10-fold more than the first and second-generation inhibitors.(11)

Pharmacokinetic advantages by P-gp inhibition

Several studies have demonstrated the possible use of P-gp inhibitors that reverse the MDR phenotype-associated P-gp-mediated efflux in an attempt to improve the efficiency of chemotherapeutic agents and pharmacokinetic and pharmacodynamic

profiles of a number of challenging molecules. This concept also offers new opportunities to overcome drug–drug interactions exhibited by a combination of P-gp substrates/inhibitors; and in developing clinically useful oral formulations of drugs which are, as a consequence, of poor oral absorption are administered only by parenteral routes. Further, P-gp inhibitors may also influence absorption, distribution, metabolism and elimination of P-gp substrates in the process of improving pharmacokinetics. P-gp inhibition may reduce the clearance of intravenously administered substrates as a consequence of increased reuptake from intestinal lumen and/or a diminished intestinal secretion and increased renal reuptake.

Examples for improved pharmacokinetics of P-gp substrates with coadministration of P-gp inhibitors

P-glycoprotein substrates	**P-gp modulator**	**Experimental model**	**Pharmacokinetic effect**
First-generation inhibitors			
Digoxin(a)	Verapamil	Single-pass perfusion in rats	Increase in absorption rate
Doxorubicin	Cyclosporine	Cancer patients	Inhibitor dose-dependent permeability enhancement
Second-generation inhibitors			
Doxorubicin	PCS 833(b)	Cancer patients	~50% increase in AUC
Paclitaxel	R-Verapamil	Cancer patients	Delayed mean paclitaxel clearance and increased peak concentration Enhanced BA
Paclitaxel(c)	GFI20918	mdr1ab(−/−)	

		knockout mice and wild-type mice	More than 50% decrease in paclitaxel clearance 1.9- and 4.5-fold increase in BA in rats and mice, respectively
Paclitaxel(d)	VX-710	Cancer patients	
Paclitaxel	MS-209	Rats and mice	
Third-generation inhibitors			
Doxorubicin	LY335979	Cancer patients	~25% increase in BA at doxorubicin dose of 60mg/m2 and ~15% increase at a dose of 75mg/m2
Paclitaxel	OC144093	Cancer patients	~1.5-fold increase in AUC and ~2-fold increase in Cmax
Docetaxel(e)	R101933	Cancer patients	Pharmacokinetics did not alter in the presence of inhibitor but the faecal excretion of docetaxel decreased significantly 44% increase in AUC
Doxorubicin	XR9576	Cancer patients	

a-Absorption rate of digoxin varied at different segments of GIT at different concentrations of verapamil.

b-Drugs like doxorubicin are transported by P-gp and MRP2, thus non-specific inhibitors (second-generation compounds) when coadministered gives large increase in Cmax and AUC.

c-VX-710 showed decrease in paclitaxel clearance with a maximum tolerated dose of paclitaxel (<80mg/m2) that is roughly half of standard dose of 175mg/m2.

d-Pharmacokinetics of paclitaxel in mdr1ab(−/−) knockout mice was not altered by GF120918 whereas a significant increase in oral bioavailability (8.5–40.2%) was observed in wild-type mice.

e-Docetaxel clearance decreased from 2.5% to less than 1%(12)

Permeability

The permeability class boundary is based indirectly on the extent of absorption (fraction of dose absorbed, not systemic BA) of a drug substance in humans and directly on measurements of the rate of mass transfer across human intestinal membrane. Alternatively, nonhuman systems capable of predicting the extent of drug absorption in humans can be used (e.g., in vitro epithelial cell culture methods). In the absence of evidence suggesting instability in the gastrointestinal tract, a drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be 90% or more of an administered dose based on a mass balance determination or in comparison to an intravenous reference dose.

Determination of permeability

The permeability class of a drug substance can be determined in human subjects using mass balance, absolute BA, or intestinal perfusion approaches. Recommended methods not involving human subjects include in vivo or in situ intestinal perfusion in

a suitable animal model (e.g., rats), and/or in vitro permeability methods using excised intestinal tissues, or monolayers of suitable epithelial cells. Chemical structure and/or certain physicochemical attributes of a drug substance (e.g., partition coefficient in suitable systems) can provide useful information about its permeability characteristics.

1. Pharmacokinetic Studies in Humans

a. Mass Balance Studies

Pharmacokinetic mass balance studies using unlabeled, stable isotopes or a radiolabeled drug substance can be used to document the extent of absorption of a drug. Depending on the variability of the studies, a sufficient number of subjects should be enrolled to provide a reliable estimate of extent of absorption. Because this method can provide highly variable estimates of drug absorption for many drugs, other methods described below may be preferable.

b. Absolute Bioavailability Studies

Oral BA determination using intravenous administration as a reference can be used. Depending on the variability of the studies, a sufficient number of subjects should be enrolled in a study to provide a reliable estimate of the extent of absorption. When the absolute BA of a drug is shown to be 90% or more, additional data to document drug stability in the gastrointestinal fluid is not necessary.

2. Intestinal Permeability Methods

The following methods are described in the guidance to determine the permeability of a drug substance from the gastrointestinal tract: (1) in vivo intestinal perfusion studies in humans; (2) in vivo or in situ intestinal perfusion studies using suitable animal

models; (3) in vitro permeation studies using excised human or animal intestinal tissues; or (4) in vitro permeation studies across a monolayer of cultured epithelial cells.

In vivo or in situ animal models and in vitro methods, such as those using cultured monolayers of animal or human epithelial cells, are considered appropriate for passively transported drugs. The observed low permeability of some drug substances in humans could be caused by efflux of drugs via membrane transporters such as P-glycoprotein (P-gp). When the efflux transporters are absent in these models, or their degree of expression is low compared to that in humans, there may be a greater likelihood of misclassification of permeability class for a drug subject to efflux compared to a drug transported passively. Expression of known transporters in selected study systems should be characterized. Functional expression of efflux systems (e.g., P-gp) can be demonstrated with techniques such as bidirectional transport studies, demonstrating a higher rate of transport in the basolateral-to-apical direction as compared to apical-to-basolateral direction using selected model drugs or chemicals at concentrations that do not saturate the efflux system (e.g., cyclosporin A, vinblastine, rhodamine 123). An acceptance criterion for intestinal efflux that should be present in a test system cannot be set at this time. Instead, this guidance recommends limiting the use of nonhuman permeability test methods for drug substances that are transported by passive mechanisms. Pharmacokinetic studies on dose linearity or proportionality may provide useful information for evaluating the relevance of observed in vitro efflux of a drug. For example, there may be fewer concerns associated with the use of in vitro methods for a drug that has a higher rate of transport in the basolateral-to-apical direction at low drug concentrations but exhibits linear pharmacokinetics in humans.

For application of the BCS, an apparent passive transport mechanism can be assumed when one of the following conditions is satisfied:

• A linear (pharmacokinetic) relationship between the dose (e.g., relevant clinical dose range) and measures of BA (area under the concentration-time curve) of a drug is demonstrated in humans

• Lack of dependence of the measured in vivo or in situ permeability is demonstrated in an animal model on initial drug concentration (e.g., 0.01, 0.1, and 1 times the highest dose strength dissolved in 250 ml) in the perfusion fluid

• Lack of dependence of the measured in vitro permeability on initial drug concentration (e.g., 0.01, 0.1, and 1 times the highest dose strength dissolved in 250 ml) is demonstrated in donor fluid and transport direction (e.g., no statistically significant difference in the rate of transport between the apical-to-basolateral and basolateral-to-apical direction for the drug concentrations selected) using a suitable in vitro cell culture method that has been shown to express known efflux transporters (e.g., P-gp)(13)

In vitro methods for assessment of permeability

Quantification of the drug absorption across gastrointestinal tract involves considering various physiological and physicochemical parameters. Physiological factors include gastric juice and bile, precipitation at the absorption site, chemical, enzymatic and bacterial degradation etc. Physicochemical factors include the particle size, shape, molecular size, lipophylicity, pKa etc. No methods can assess all these aspects simultaneously, with the exception of studies in intact animals or in humans. But whole animal studies suffer from many drawbacks: first, they are unsuitable for

screening large number of compounds at experimental stage. Second, they pose ethical difficulties if the pharmacological effects and side effects insufficiently well defined. As result verity of alternative methods are developed to assess the permeation characteristics of the drug. Few biological methods are discussed below.

1. Brush border membrane vesicles (BBMV)

2. Isolated intestinal cells technique

3. Intestinal rings technique

4. Everted intestinal sacs technique

5. Simultaneous diffusion and absorption study

6. Cultured cells

1. Brush border membrane vesicles (BBMV)

In this approach, cell homogenates or intestinal scrapings are treated with the CaCl2, precipitation method using centrifugation. The final pellet contains the luminal wall bound proteins and phospholipids which contain most of the brush border enzymatic and carrier activity. Resuspension of the pellet in buffer results in the formation of vesicles. These vesicles are mixed with the permeant in buffer and filtered after a fixed time; the amount of permeant taken by the vesicles is then determined. This technique is adopted to measure the transcellular transport. This technique is also useful for mechanistic studies of drug absorption.

2. Isolated intestinal cells technique

Isolated cells from the intestine of the animal or human can be used as uptake system in the assessment of oral bioavailability. The procedures used to isolate mucosal cells

can be divided into fundamentally in two categories: an in situ procedure, in which the tissue is perfused with enzyme solutions that release the cells; and an ex -vivo approach, in which the cells are treated by chelating agents or by enzymatic means. Freshly isolated cells are immediately suspended in Krebs – Henseleit buffer solution with glucose and kept in ice for 15 min, during which they are bubbled with carbogen (95% O2 / 5 % CO2).

3. Everted intestinal rings technique

In this method, a section of the intestine is isolated immediately after anesthetizing the animal, washed in ice cold buffer to remove debris and digestive products, and tied at one end with a piece of suture; now, the closed end carefully pushed through the intestine using glass rod, resulting in eversion of the intestine which is then cut into small rings of 3-4 mm wide. These slices are incubated in a water bath. After designated time interval the tissue slice is taken out of the solution, blotted dry, and weighed, and then dissolved or processed for assay. Tissue eversion is performed to maximize the contact between tissue and solute. These everted segments are claimed to be viable for a period of 30-60 min, if they are maintained before use in a physiological buffer containing glucose. The uptake in slices or rings is determined as a function of time. The permeability P0 is calculated from the flux (J0), the concentration of the incubation medium (C) and a conversion factor for exposed tissue (<cf>) in cm2/g for tissue / surface area from the equation: J0 = P0 C <cf>, where P0 is expressed in cm/s. This technique is simple and easy to perform and offers more flexibility in the experiment. This technique also possess few drawbacks; the transport of solute into the slices includes all areas accessed by incubation solution not only through luminal membrane; connective tissue and muscle tissue are also exposed to drug solution and included in the calculation of ‘uptake’. Another

drawback is paracellular and transcelluar mechanism cannot be distinguished in this method.

4. Everted Intestinal Sac Techniques

In the everted intestinal sac technique, a 2 to 4 cm section of the intestines tied off at the one end and everted using a glass rod or a thread. As with the everted slices and rings, the mucosa becomes the outer side of the sac and is in contact with incubation media, but in contrast with the rings, only the mucosa is in contact with permeant. The sac is filled with buffer and put in a flask with oxygenated (95% O2/ 5% CO2) buffer containing the compound under investigation. At the end of the experiment the sac is cut opened at one end and serosal fluid is collected. Viability of the sac can be monitored during the experiment by measuring the transport of a marker (Trypan blue dye) The transport decreases significantly within the first few minutes, if the animal is killed by cervical dislocation before removal of the segment. However the deterioration can be avoided if the animal is anesthetized properly before isolation of the intestines, and euthanized only after harvesting the segment. The everted sac method is an inexpensive technique that is also relatively simple and allows several experiments to be performed using tissue from just one intestine. One advantage is the low serosal volume compared with the area for absorption, which is especially advantageous with low soluble drugs or low concentration of drugs. This method has been a useful tool for studying mathematical aspects of absorption and influence absorption enhancement. Also, by preparing the segment form different parts of the intestine, the absorption from different sites, such as small intestine and colon can be compared.

5. Diffusion cells using tissue

In this method, small sections of intestine (2 cm) are clamped between two glass chambers filled with buffer and nutrients such as glucose, at a temperature of 37^0C.The buffer solutions at both sides of the membrane are gassed continuously with carbogen, to maintain the viability of the tissue and to ensure reproducible hydrodynamics (good mixing). A modification of the original method was published, using smaller acrylic chambers, allowing diffusion experiments with smaller amounts of compounds. In this method carbogen bubbling is applied to increase viability and improve hydrodynamics. The permeability is calculated from the equation given below

Papp = dC/dt X (V/A X C), where dC/dt is the change in concentration in the receptor compartment per unit time, V is the receptor volume and A is the area available for diffusion or transport. Papp is usually expressed in centimeters per second (cm/s).(14)

Turmeric

Turmeric (*Curcuma longa*) is a rhizomatous herbaceous perennial plant of the ginger. It is native in southeast India, and needs temperatures between 20 and 30°C and a considerable amount of annual rainfall to thrive. Plants are gathered annually for their rhizomes, and propagated from some of those rhizomes in the following season. The wide range of turmeric health benefits come mainly from its main ingredient, curcumin. This widely researched component of turmeric is highly therapeutic and is used in various drugs and pharmaceutics mainly because of its immunity boosting and anti-oxidant properties.

Black Pepper

Black pepper(*Piper nigrum)* is a perennial vine. Its involvement in increasing the absorption of nutrients in the body and also novel applications like helping to fight against colon cancer.

It has also anti-inflammatory, thermogenic, growth stimulatory, anti-thyroid and chemo preventive activities. It is inhibition of hepatic drug metabolism, enhancing pentobarbitone induced hypnosis, bioavailability of oxyphenyl butazone, hepatoprotective activity, inhibition of lipid peroxidation during experimental inflammation, antifertility and radio protective effects. It has been found to inhibition of human CYP3A4, P-glycoprotein and enzymes important for metabolism and transport of xenobiotics and metabolites.

Drumstick

Drumstick(*Moringa oleifera)* is the most widely cultivated species of the genus *Moringa*, which is the only genus in the family Moringaceae. English common names include: moringa, drumstick tree (from the appearance of the long, slender, triangular seed-pods), horseradish tree (from the taste of the roots, which resembles horseradish), Ben tree, or benzoil tree (from the oil which is derived from the seeds). It is a fast-growing, drought-resistant tree, native to the southern foothills of the Himalayas in northwestern India, and widely cultivated in tropical and subtropical areas where its young seed pods and leaves are used as vegetables. It can also be used for water purification and hand washing, and is sometimes used in herbal medicine.

Medicinal properties:

- Antihypertensive, diuretic and cholesterol lowering activities
- Antibacterial and antifungal activities
- Antitumor and anticancer activities

Cumin

Cumin (*Cuminum cyminum*) locally known as 'zeera' is a flowering plant in the family Apiaceae. It is commonly used as a condiment and flavoring in many eastern dishes. Cumin is known for its antioxidant properties. The most important chemical component of cumin fruits is essential oil content, ranging from 2.5% to 4.5% which is pale to colorless depending on age and regional variations. Studies of the chemical composition of cumin oil from different countries showed the presence of the following components: α-pinene (0.5%), Myrcene (0.3%), limonene (0.5%), 1-8-cineole (0.2%), p-menth-3-en-7- ol (0.7%), p-mentha-1, 3-dien-7-ol (5.6%), caryophyllene (0.8%), β-bisabolene (0.9%), β-pinene (13.0%), P-cymene (8.5%), β-phellandrene (0.3%), D-terpinene (29.5%), cuminic aldehyde (32.4%), cuminyl alcohol (2.8%), β-farnesene (1.1%) together with much smaller quantities of α-phellandrene, α- terpinene, cis and trans sabinene, Myrtenol, α-terpineol and phellandral. In addition to volatile oil cumin also contains nonvolatile chemical components including tannins, oleoresin, mucilage, gum, protein compounds and malates.

Ginger

Ginger *(Zingiber officinale)* contains 80.9% moisture, 2.3% protein, 0.9% fat, 1.2% minerals, 2.4% fibre and 12.3% carbohydrates. The minerals present in ginger are

iron, calcium and phosphorous. It also contains vitamins such as thiamine, riboflavin, niacin and vitamin C. The composition varies with the type, variety, agronomic conditions, curing methods, drying and storage conditions.

In the fresh ginger rhizome, the gingerols were identified as the major active components and gingerol [5-hydroxy- 1-(4-hydroxy-3-methoxy phenyl) decan-3-one is the most abundant constituent in the gingerol series.The volatile oil consists of mainly mono and sesquiter– penes; camphene, beta-phellandrene, curcumene, cineole, geranyl acetate, terphineol, terpenes, borneol, geraniol, limonene, linalool, alpha-zingiberene (30-70%), beta-sesquiphellandrene (15-20%), beta-bisabolene (10- 15%) and alpha-farmesene.

Uses

- To improve blood circulation.
- To lower blood glucose in the treatment of diabetes.
- To treat migraine headache.

Pomegranate juice

Pomegranate juice contains considerable amounts of sugars, organic acids, amino acids and phenolic compounds such as gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, oand p-coumaric acids, catechin and quercetin as well as enzymes, proteins, pectins and insoluble complexes as colloids (polysaccharides). The phenolic constituents give color, astringency and bitterness to the pomegranates juice. It also contains potassium besides that is rich in some essential vitamins as folate and vitamin K. The most added value of pomegranate is its large content in phenolic compounds. Moreover, phenolic bioactives have been reported to be the major antioxidants of pomegranate.

The above mentioned herbal drugs are chosen as potential candidates for screening the P-gp inhibition activity by everted sac technique using rat intestine. Further, identified P-gp inhibitors would be incorporated in the dosage form followed by its evaluation.

Chapter 2

Objectives

OBJECTIVES

The main objectives of this project is to

1. To screen P-gp inhibition activity of selected candidates by *in-vitro* sac method for the determination of permeability of a model drug using rat/ goat or sheep intestine models.
2. To incorporate the identified P-gp inhibitor in the dosage form and study the P-gp inhibition activity.
3. To study the drug interaction studies and stability studies.

Chapter 3

Review of Literature

REVIEW OF LITERATURE

- **Eric Le Ferrec at al.,** This is the report of the forty-sixth of a series of workshops organised by the European Centre for the Validation of Alternative methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of in vitro tests and their potential uses, and make recommendations about the best ways forward. In addition, other topics relevant to the Three Rs (reduction, refinement and replacement) concept of alternatives to animal experiments have been considered in several ECVAM workshops. The principal aims of the workshop were to seek a consensus on the cur- rent models of the intestinal barriers and ways to screen for the movement of drugs across this barrier, and to make useful recommendations for the promotion of the Three Rs in this area. The panel of techniques used for the prediction of the relevant parameters is very large, including in vivo and in situ methods.(15)
- **Li M et al.,** demonstrated that P-gp is located at apical membrane of the epithelial cells and it transports drugs back into the gut lumen. Studies in animals and human have indicated that P-gp plays a major role in limiting drug absorption and consequently oral bioavailability.(16)
- **Neerati P et al.,** showed that P-gp can decrease the intestinal permeability and plasma concentrations of phenytoin by effluxing it back to intestine. P-gp

inhibitory activity of verapamil resulted significant improvement in intestinal permeability and AUC, peak plasma concentrations (Cmax) of phenytoin. The observed effect may be beneficial in a way to reduce the pharmacoresistance of phenytoin by using any safe P-gp inhibitors to improve its oral bioavailability.(17)

- **Schinkel AH** as he studied on P-Glycoprotein, a gatekeeper in the blood–brain barrier. He found on Absence of functional P-glycoprotein in the blood–brain barrier leads to highly increased brain penetration of a number of important drugs. Depending on the pharmacological target of these drugs in the central nervous system (CNS), this can result in dramatically increased neurotoxicity, or fundamentally altered pharmacological effects of the drug. Given the variety of drugs affected by P-glycoprotein transport, it may be of tremendous therapeutic value to apply these insights to the development of drugs that should have either very poor or very good brain penetration, whichever is preferred for pharmacotherapeutic purposes. The clinical application of P-glycoprotein blockers should also be considered in order to improve the blood–brain barrier permeability of certain drugs that currently display insufficient brain penetration for effective therapy.(18)
- **Fromm MF** showed that P-glycoprotein has very broad substrate specificity, it determines disposition of a broad variety of drugs. Moreover, induction and inhibition of P-glycoprotein are new mechanisms for drug interactions in humans. Very recently, systematic screens of the MDR1 gene have identified multiple single nucleotide polymorphisms. Some of those appear to be associated with altered transporter function and expression. This review discusses the currently available data on the influence of MDR 1

polymorphisms on P-glycoprotein tissue expression, drug disposition, treatment outcome and disease risk.(19)

- **Quevedo MA et al.,** studied on the Zidovudine (AZT) was the first drug approved for the treatment of Acquired Immunodeficiency Syndrome (AIDS) in humans, and although its clinical efficacy has been demonstrated, suboptimal pharmacokinetic aspects still remain a concern. To assess the basis of its highly variable oral bioavailability, this work deals with the study of AZT intestinal absorption by applying the gut sac technique. Permeation through the rat jejunum and ileum segments was analyzed at different drug concentrations and gut regions, with higher apparent permeability coefficients (Papp) being found for the proximal regions of the small intestine compared to distal ones. Bi-directional permeation assays demonstrated that AZT is subjected to efflux mechanisms in distal regions of small intestine, which are blocked by verapamil (VER), thus demonstrating a P-glycoprotein (P-gp) mediated mechanism. The efficiency of AZT efflux increased in the distal ileum as consequence of exposure to AZT, with the amount of drug permeating from the mucosal to the serosal side diminishing after 35 min. Molecular modelling techniques were applied to analyze the binding mode of AZT to P-gp, which was compared to that of VER and AZT-Ac, a novel prodrug of AZT. The energy required for their salvation was found to constitute a critical feature in their binding to this efflux protein.(20)
- **Zhang Cet al** showed that one of the potential mechanisms interesting researchers is over-expression of P- glycoprotein (P-gp, also known as ABCB1 or MDR1) in endothelial cells of the blood–brain barrier (BBB) in epilepsy patients. P-gp plays a central role in drug absorption and distribution

in many organisms. The ex- pression of P-gp is greater in drug-resistant than in drug-responsive patients. Some studies also indicate that several AEDs are substrates or inhibitors of P-gp, implying that P-gp may play an important role in drug resistance in refractory epilepsy. In this article, we review the clinical and laboratory evidence that P- gp expression is increased in epileptic brain tissues and that AEDs are substrates of P-gp in vitro and in vivo. We discuss criteria for identifying the substrate status of AEDs and use structure–activity relationship (SAR) models to predict which AEDs act as P-gp substrates.(21)

- **Eichhorn T et al.,** P-glycoprotein belongs to the family of ATP-binding cassette (ABC) transporters. It functions in cellular detoxification, pumping a wide range of xenobiotic compounds, including anticancer drugs out of the cell. In cancerous cells, P-glycoprotein confers resistance to a broad spectrum of anticancer agents, a phenomenon termed multidrug resistance. An attractive strategy for overcoming multidrug resistance is to block the transport function of P-glycoprotein and thus increase intracellular concentrations of anticancer drugs to lethal levels. Efforts to identify P-glycoprotein inhibitors have led to numerous candidates, none of which have passed clinical trials with cancer patients due to their high toxicity. The search for naturally inhibitory products from traditional Chinese medicine may be more promising because natural products are frequently less toxic than chemically synthesized substances. In this review, we give an overview of molecular and clinical aspects of P-glycoprotein and multidrug resistance in the context of cancer as well as Chinese herbs and phytochemicals showing inhibitory activity towards P-glycoprotein.(22)

- **Borska S et al.,** P-glycoprotein (P-gp) is one of the ABC transporters responsible for the resistance of several tumours to successful chemotherapy. Numerous agents are capable of interfering with the P-gp-mediated export of drugs but unfortunately most of them produce serious side effects. Some plant polyphenols, including the flavonol quercetin (Q), manifest anti-neoplastic activity mainly due to their influence on cell cycle control and apoptosis. Reports are also available which show that Q may intensify action of cytostatic drugs and suppress the multidrug resistance (MDR) phenomenon. The study aimed at determination if Q sensitizes cells resistant to daunorubicin (DB) through its effect on P-gp expression and action. The experiments were conducted on two cell lines of human pancreatic carcinoma, resistant to DB EPP85-181RDB and sensitive EPP85-181P as a comparison. Cells of both lines were exposed to selected concentrations of Q and DB, and then membranous expression of P-gp and its transport function were examined. The influence on expression of gene for P-gp (*ABCB1*) was also investigated. Results of the studies confirmed that Q affects expression and function of P-gp in a concentrationdependent manner. Moreover it decreased expression of *ABCB1*. Thus, Q may be considered as a potential modulator of P-gp.(23)
- **Hussain K et al.,** discussed in detail about the everted sac technique. They specified the simple and reproducible method for intestinal segment eversion using simple glass rod and also described the mounting of evereted sac using the apparatus. Viability of the intestinal segment was reported by measuring the transport of glucose across the intestine for one hour. Furthermore, they investigated the transport of marketed human insulin preparation across rat intestine repeatedly. They concluded that various parameters such as pH,

solvent nature, ionic strength and excipients of the marketed preparation would be responsible for their reproducible results.(24)

- **Wang et al.,** stated that the *in-vitro* everted gut sac technique is frequently used to study the permeability and absorption kinetics of drugs. The everted intestinal sac model can provide information on drug absorption mechanisms by testing drug content absorbed through the intestinal mucous membrane.(25)
- **Balimane PV et al.,** proposed that passive and active transport can be studied by everted sac method and it is ideal for studying the absorption mechanism of drugs. Moreover, the everted gut sac model has an additional analytical advantage compared with other *in-vitro* models because the sample volume on the serosal side is relatively small and drugs accumulate faster.(26)
- **Sadeghi AMM et al.,** demonstrated that the insulin loaded nanoparticles showed much less permeation across the Caco-2 cell monolayer in comparison to the free-soluble polymers. Mass balance transport studies revealed that a substantial amount of the nano particles has been entrapped into the Caco-2 monolayer or attached to the cell surface. It can be stated that while free-soluble polymers can reversibly open the tight junctions and increase the permeation of insulin, the nano particles had basically only a low effect on the opening of the tight junction and the paracellular transport of insulin across the Caco-2 cell monolayer. These data convincingly showed that nano particles consisting of chitosan and its quaternary ammonium derivatives loaded with insulin are less effective in facilitating paracellular transport across Caco-2 cell monolayers than the corresponding free polymers.(27)
- **Beig A et al.,** worked that the apparent solubility of the lipophilic drug carbamazepine was measured in systems containing various levels of the co-

solvent PEG-400. The corresponding permeability was then measured by PAMPA assay and in the rat jejunal perfusion model. Thermodynamic activity was maintained equivalent in all permeability studies (50% saturation). PEG-400 increased carbamazepine solubility in a concentration-dependent fashion. Decreased carbamazepine intestinal permeability with increased apparent solubility was observed in both PAMPA and rat perfusion models.(28)

- **Simmons NL et al.,** demonstrated that Madin-Darby canine kidney (MDCK) dog-renal epithelial cell-line is a model for renal tubular P-glycoprotein mediated secretion. The apical membrane is rendered effectively impermeable to archetype substrates such as vinblastine by a combination of low intrinsic permeability and the operation of ATP-dependent export from cytosol to the apical (lumen) solution. Since the MDCK cell-line possesses features characteristic of the distal/collecting duct, this suggests a possible role for tubular expression at distal sites in limiting back-diffusion and trapping of moderately lipophilic P-glycoprotein substrates within the renal medulla after secretion in the proximal tubule and subsequent concentration resulting from tubular fluid reabsorption.(29)
- **Bharadwaj RK et al.,** provided a strong evidence that a major constituent of pepper inhibits function of human P-glycoprotein and CYP3A4. This is in line with a limited number of clinical studies showing an effect of pepper or piperine intake on drug disposition. Authors suggested for further studies in humans are needed to clarify the impact of this nutrient on disposition of orally administered substrates of P-glycoprotein, CYP3A4, and possible other drug-metabolizing enzymes.(30)

- **Tatiraju D et al.,** stated that black cumin (*Cuminum cyminum*) is a carminative, estrogenic, anti-nociceptive, anti-inflammatory, anti-oxidant and anti- microbial. The bioenhancer chemical constituent present in cumin is 3', 5-dihydroxyflavone-7-O-D-galactouronide-4'-O-D- glucopyranoside. The effective dose of the bioenhancer extract is in the range of 0.5- 25 mg/kg body weight. Percentage enhancement of bioavailability for rifampicin is 250%, for cycloserine is 89%, for ethionamide is 78%.(31)
- **Langguth P et al.,** showed that grapefruit juice (GFJ) is known to affect the pharmacokinetics of various drugs, presumably mainly via inhibition of oxidative metabolism. In order to evaluate the effect of GFJ on P-glycoprotein-related transport processes, measurements of transport characteristics through Caco-2 monolayers and *in- vivo* drug absorption studies were performed with the transported, yet not metabolized model compound talinolol.(32)
- **Ampasavate C et al.,** stated that the a-b transport of daunorubicin was increased by curcumin, demethoxycurcumin and bisdemethoxycurcumin while the b-a transport was significantly decreased by curcumin and demethoxycurcumin. However, calcein-AM uptake into the human P-gp overexpression cell line, LLC-GA5-COL300, was increased by curcumin and demethoxycurcumin in a concentration-dependent manner but not affected by bisdemethoxycurcumin. These results show that curcumin and demethoxycurcumin could inhibit P-gp but bisdemethoxycurcumin may modulate the function of other efflux transporters such as MRP. Therefore this information may indicate the impact of *Curcuma longa* and *Curcuma sp.*

‘‘khamin-oi’’ on pharmacokinetics of orally administered drugs that are P-gp substrates.(33)

- **Elvira Escribano ferrer et al.,** have presented the study of intestinal permeabilities (Peff) of five model drugs: furosimide, piroxicam, naproxen, ranitidine and amoxicillin in the *in situ* intestinal perfusion technique in mice and compare them with corresponding rat and human in vivo Peff values. The mouse method correctly assigned the BCS permeability classification of a given drug and correlation between mouse permeability data and the fraction of an oral dose absorbed in humans was achieved.(34)

3.1 DRUG PROFILE

3.1.1Ciprofloxacin hydrochloride

Ciprofloxacin Hydrochloride contains not less than 98.0percent and not more than 102.0percent of $C_{17}H_{18}FN_3O_3 \cdot HCl$.

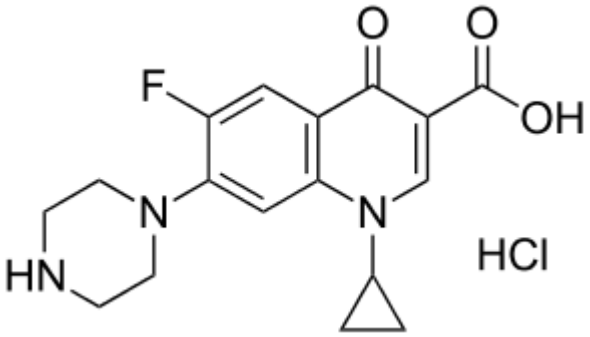


Fig 2 Structure of Ciprofloxacin

Empirical formula: $C_{17}H_{18}FN_3O_3$

Chemical name: 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid.

CAS number: 85721-33-5

Molecular mass: 367.8

Solubility: Soluble in water, slightly soluble in methanol, vary slightly soluble in ethanol and practically insoluble in acetone, ethyl acetate.

Description: It is a faintly yellowish to light yellow crystalline substance

Physical State: Solid

pK: pK_a: 6.43 (Predicted), pK_b: 8.68 (Predicted**)**

Melting point: 311-320°C

Bioavailability: 69%

Peak Serum concentration: 2.4µg/ml

Oral Dose: 250-500 mg

Half life: 4 hours

Pharmacokinetics:(35)(36)(37)(38)

Absorption:

Ciprofloxacin given as an oral tablet is rapidly and well absorbed from the gastrointestinal tract after oral administration. The absolute bioavailability is approximately 70% with no substantial loss by first pass metabolism. Ciprofloxacin maximum serum concentrations and area under the curve are shown in the chart for the 250 mg to 1000 mg dose range.

Dose (mg)	**Maximum Serum Concentration (µg/mL)**	**Area Under Curve (AUC) (µg•hr/mL)**
250	1.2	4.8
500	2.4	11.6
750	4.3	20.2
1000	5.4	30.8

Distribution:

The binding of ciprofloxacin to serum proteins is 20 to 40% which is not likely to be high enough to cause significant protein binding interactions with other drugs. Oral administration of ciprofloxacin is widely distributed throughout the body. Ciprofloxacin is present in active form in saliva, nasal and bronchial secretions, mucosa of the sinuses, sputum, skin blister fluid, lymph, peritoneal fluid, bile, and prostatic secretions. Ciprofloxacin has also been detected in lung, skin, fat, muscle, cartilage, and bone. The drug diffuses into the cerebrospinal fluid (CSF); however, CSF concentrations are generally less than 10% of peak serum concentrations.

Metabolism:

Metabolism occur through liver including CYP1A2 and also Four metabolites have been identified in human urine which together account for approximately 15% of an oral dose. The metabolites have antimicrobial activity, but are less active than unchanged ciprofloxacin.

Excretion:

The serum elimination half-life is approximately 4 hours. Approximately 40 to 50% of an orally administered dose is excreted in the urine as unchanged drug. After a 250 mg oral dose, urine concentrations of ciprofloxacin usually exceed 200 μg/mL during the first two hours and are approximately 30 μg/mL at 8 to 12 hours after dosing. The urinary excretion of ciprofloxacin is virtually complete within 24 hours after dosing. The renal clearance of ciprofloxacin, which is approximately 300 mL/minute, exceeds the normal glomerular filtration rate of 120 mL/minute.

Mechanism of action:

Ciprofloxacin is a broad-spectrum antibiotic active against both Gram positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type

II topoisomerase, and topoisomerase IV, enzymes necessary to separate bacterial DNA, thereby inhibiting cell division.

Indication(39)(40)**:**

1. Urinary tract infection
2. Lower respiratory tract infection
3. Acute sinusitis
4. Skin and skin structure infection
5. Bond and joint infection
6. Complicated intra-abdominal infection
7. Infectious diarrhoea
8. Typhoid fever
9. Sexual transmitted disease
10. Pheonephritis in children

Side effects:

Common adverse reactions involve the GI tract, with 3% to 17% of patients reporting mostly mild nausea, vomiting, and/or abdominal discomfort. Diarrhea and antibiotic-associated colitis have been unusual.

CNS side effects involve mild headache and dizziness; have been seen in 0.9% to 11% of patients. Rarely, hallucinations, delirium, and seizures

Interactions(41)

1. Ciprofloxacin + Antacid (Aluminum hydroxide, magnesium hydroxide)**:** To reduce the absorption.
2. Ciprofloxacin + Calcium salt: To reduce the absorption.
3. Ciprofloxacin + Cyclosporine : Increase the risk of nephrotoxicity
4. Ciprofloxacin + Ferrous salt: To reduce the absorption

5. Ciprofloxacin + Ibuprofen: Increase the risk of convulsion
6. Ciprofloxacin + warfarin: To enhance the anticoagulant effect
7. Ciprofloxacin + Zinc sulphate: To reduce the absorption

Contraindications:

- Concomitant administration with tizanidine is contraindicated.
- Anyone with a history of hypersensitivity to any member of the quinolone class of antimicrobial agents, including ciprofloxacin, or any of the product components is contraindicated.

3.1.2 Verapamil Hydrochloride (42)(43)(44):

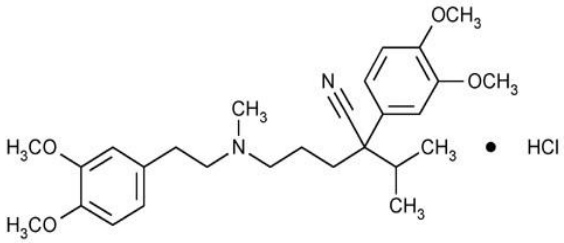
Chemical Name – -{3-[[2-(3, 4-dimethoxyphenyl) ethyl] methyl amino] propyl]-3, 4- dimethoxy –a-(1-methyl ethyl benzeneacetonitrile hydrochloride

Generic name – Verapamil Hydrochloride

Molecular formul- $C_{27}H_{18}N_2O_4.HCL$

Molecular weight – 451.9

Fig 3 Structural formula



Description -:

Color – white or almost white, colorless crystalline powder

Odor - odorless

Solubility –Verapamil is highly soluble in water (80-90 mg/ml), ethanol >100mg/m and methanol >100mg/Ml but practically insoluble in ether

pKa –6.5

Melting point- 140º-144ºC

Category – Calcium channel antagonist

Indications - in treatment of Hypertension

Ischemic heart diseases – chronic stable angina pectoris myocardial infarction arrhythmias (ventricular & supraventricular)

Mechanism of action :

Verapamil inhibits the entry of calcium ions in to cardiac and smooth muscles. As a calcium ion influx inhibitor (calcium entry blocker or calcium ion antagonist), Verapamil is believed to exert its effect by selectively inhibiting transmembrane influx of calcium ions in cardiac muscle, coronary and systemic arteries and in cells of the intracardiac conduction system. Verapamil blocks the transmembrane influx of calcium through the slow channel (calcium ion antagonism) without significantly affecting transmembrane influx of sodium ions through the fast channel. Verapamil exerts antihypertensive effects by inducing vasodilation and reducing peripheral vascular resistance usually without reflex tachycardia. Verapamil is a potent smooth muscle relaxant with vasodilator properties, and a depressant of myocardial contractility

Pharmacokinetics :

Verapamil Hydrochloride is well absorbed following oral administration. The peak plasma concentration is reached after 1-2 hrs. The sustained release formulations may have slightly low bioavailability and peak plasma concentration occurs between 4-6 hrs. The absorption of sustained release formulation may be affected by presence of food Verapamil Hydrochloride undergoes presystemic metabolism which is

stereospecific. It distributes widely and rapidly in the body. The plasma protein binding is 90%. Half life of Verapamil Hydrochloride is 4-6hrs. and volume of distribution is 4-7 L/Kg. Verapamil is majorly metabolized by O-demethylation and N- dealkylation to the equally active metabolites, which are rapidly metabolized by conjugation. Major metabolite of Verapamil is Nor verapamil (20% activity). About 3% of administered dose of Verapamil is excreted unchanged while about (70%) is excreted in urine as metabolised form and (15%) in faeces.

Drug-Drug interactions of Verapamil hydrochloride-:

Potentially hazardous interactions-

Verapamil Hydrochloride increases plasma digoxin concentration by 50% in normal volunteers and 100% in patients by reducing its plasma clearance.

ß-blockers **-**The combination of Verapamil Hydrochloride with ß-blockers may result in severe myocardial depression. Intravenous combinations may produce complete heart block

Class I Anti -arrhythmics :The combination may result in cardiac depression Verapamil Hydrochloride may reduce the clearance of Qunidine, it may also produce complete atrioventricular block.

Minor interactions –

1. Drugs which induce hepatic enzymes such as Phenobarbital, Phenytoin and Rifampin increase the pre systemic metabolism of orally administered Verapamil hydrochloride.
2. Verapamil increases the levels of free Carbamazepine, Cyclosporin, Theophylline and Lithium in blood.
3. Cimetidine increases Verapamil Hydrochloride concentration in blood.

Interaction with food

Administration of Verapamil with meals slows absorption and slightly decreases its relative bioavailability slightly.

Contraindications -:

Bradycardia – Verapamil Hydrochloride is contraindicated in patients with bradycardia and in conditions of AV block.

Hypotension – Verapamil Hydrochloride should be avoided in patients with very low blood pressure.

Heart Failure- Verapamil Hydrochloride has negative inotropic effect, hence it will worsen the condition.

Storage- Verapamil Hydrochloride is supposed to be stored in air tight containers. Store in airtight containers.

Shelf Life : 2 years

Dosage forms –:

Oral – film coated tablets

Parenteral - i.v infusion

Dosage regimen -:

Orally

In the treatment of arrhythmia 40 – 120 mg thrice daily In the treatment of angina 80-120 mg, thrice daily, In the treatment hypertension; 80-120 mg in 2-3 divided doses.

Parentrally,

Slow I.V infusion 5-10 mg/ml.

Marketed preparations -:

Tablets-

Calaptin 40mg, 80 mg, 120 mg (Nicholas Piramal India Ltd. Mumbai)

Calaptin - SR 120 mg, 240 mg (Nicholas Piramal India Ltd. Mumbai)

Vasopten 40 mg, 80 mg, 120 mg (Torrent Pharmaceuticals Ltd. Ahmedabad)

Injection

2ml ampoule 2.5mg/ml (Nicholas Piramal India Ltd.)

3.2 POLYMER PROFILE:

3.2.1 POLYVINYL PYRROLIDONE(45)

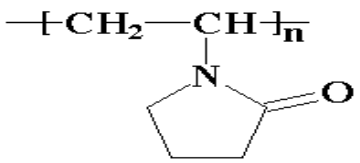


Fig 4: Chemical structure of Polyvinylpyrrolidone

Synonym: plasdone k-30, luviskol k-30, plasdone, povidone, pvp p, pvp k-30; pvp; polyvinylpyrrolidone; poly(1-(2-oxo-1-pyrrolidinyl)ethylene); povidone k-30; poly(n-vinylbutyrolactam); poly(1-vinylpyrrolidinone)

Chemical name: poly (1-vinyl-2-pyrrolidinone)

Chemical formula: $(C_6H_9NO)_n$

Functional category: suspending agent; tablet binder

Molar mass: 2.500-2.5000.000g.mol-1

Density: 1.2g/cm^3

Melting point: 150-1800C

Boiling point: 1930C

Description: it is a fine, white to creamy-white colored, odorless, hygroscopic, amorphous powder.

Incompatibility: reactive with oxidizing agents.

Solubility: soluble in cold water, soluble in chloroform, alcohol, chlorinated hydrocarbons, amines, nitroparaffin's, lower weight fatty acids.

Application: PVP k series can be used as film forming agent, viscosity enhancement agent, lubricator and adhesive. In tableting, PVP solutions are used as binders in wet granulation process. PVP is also added to powder blends in the dry forms and granulated *in-situ* by addition of water, alcohol or hydroalcoholic solutions. PVP solutions may also be used as coating. It is also used as a suspending, stabilizing or viscosity- increasing agents in topical and oral suspensions and solutions.

Stability and storage conditions: PVP darkens to some extent on heating at 150oC, with a reduction in aqueous solubility. PVP may be stored under ordinary conditions without undergoing decomposition or degradation. It stored in an airtight container in a cool place, dry place.

Safety: when consumed orally, PVP may be regarded as essentially nontoxic since it is not absorbed from the gastrointestinal tract or mucous membranes. PVP has no irritant effect on the skin and causes no sensitization.

3.2.2 MAGNESIUM STEARATE(46)

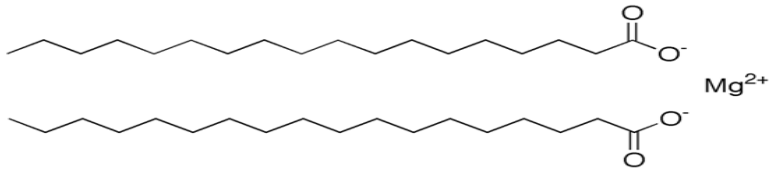


Fig 5: Chemical structure of magnesium stearate

Synonym: Magnesium octadeconate, Octadecanoic acid magnesium salt, stearic acid magnesium salt.

Chemical name: Octadecanoic acid magnesium salt

Functional category: Tablets and capsules lubricant

Description: It is a fine, white, precipitated or milled, impalpable powder with low bulk density. Insoluble in water, powder shows a faint odor of stearic acid, tasteless. The powder is greasy to touch and readily adhere to skin.

Applications in pharmaceutical formulations and technology:

It was extensively used in cosmetic formulations, food and pharmaceutical formulations. It is primarily used as a lubricant in tablets and capsule fabricating processes at a concentration of 0.25-5.0% also used to prepare barrier creams

Stability and storage: It is a stable chemical substance. It shall be stored in a well closed, air tight container in a cool and dry place

Incompatibilities: It is incompatible with strong acids, iron salts and should be avoiding mixing with strong oxidizing agents. It should not be included in the formulations containing aspirin, some vitamins, and most of the alkaloidal salts.

Safety: It is one of the mostly used pharmaceutical excipient as it is an non-toxic when ingested through oral route. Upon consumption of large amount produces laxative effect and can irritates mucosal layer of G.I.T.

3.2.3 TALC(47)

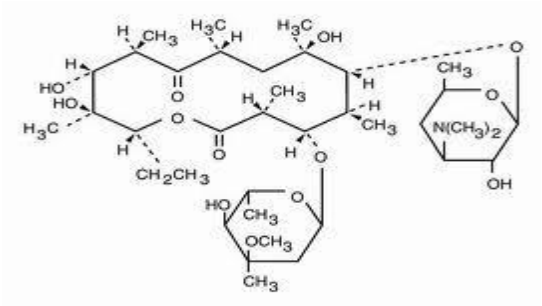


Fig 6: Chemical structure of talc

Synonyms: Magsil star, powdered talc, Purified French chalk, Purtalc, steatite, Soapstone

Empirical Formula: $Mg_6 (Si_2O_5)_4(OH)_4$

Functional Category: Anti-caking agent, Glidant, tablet and capsule diluents, tablet and capsule lubricant.

Description: Talc is a very fine, white to grayish-white colored, odorless, impalpable, unctuous, crystalline powder. It adheres readily to the skin, it soft to the touch, and free from grittiness.

Applications in Pharmaceutical Formulation or Technology:

Talc was once widely used in oral solid dosage formulations as a lubricant and diluents, although today it is less commonly used. However, it is widely used as a dissolution retardant in the development of controlled-release products. Talc is also used as a lubricant in tablet formulations) in a novel powder coating for extended release pellets and as an adsorbent.

Stability and Storage Conditions: Talc is a stable material and may be sterilized by heating at 1600 C for not less than 1 hour. It may also be sterilized by exposure to ethylene to ethylene oxide or gamma irradiation. Talc should be stored in a well-closed container in a cool, dry place

Safety: Talc is mainly used in tablet and capsule formulation. Oral ingestion talc is not absorbed systemically and regarded as a nontoxic material.

3.2.4 LACTOSE(48)

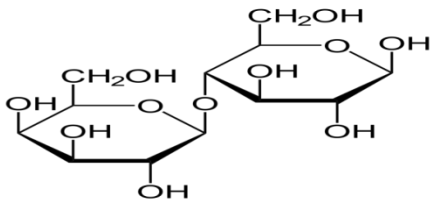


Fig 7: Chemical Structural of lactose

Formula weight: $C_{12}H_{22}O_{11}$

Molecular weight: 342.30

Description: Lactose occurs as white to off-white crystalline particles or powder. Several different brands of anhydrous lactose are commercially available which contain anhydrous β-lactose and anhydrous α-lactose. Anhydrous lactose typically contains 70–80% anhydrous β-lactose and 20–30% anhydrous α-lactose.

Functional Use: Binding agent, directly compressible tablet excipient, lyophilization aid, tablet and capsule filler.

Solubility: Soluble in water; sparingly soluble in ethanol (95%) and ether.

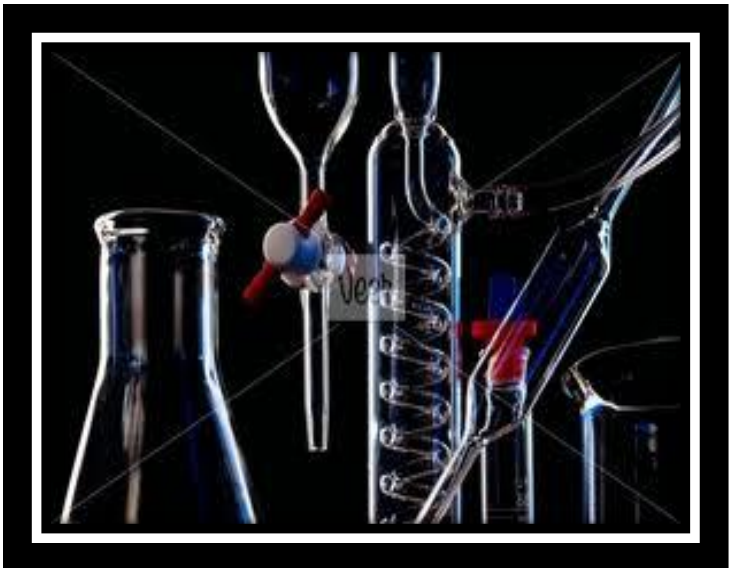
Applications in Pharmaceutical Formulation or Technology

Anhydrous lactose is widely used in direct compression tablet applications and as a tablet and capsule filler and binder. Anhydrous lactose can be used with moisture-sensitive drugs due to its low moisture content.

Safety:

Lactose is diluent and filler-binder in oral capsule and tablet formulations. It may also be used in intravenous injections. Adverse reactions to lactose are largely due to lactose intolerance, which occurs in individuals with a deficiency of the intestinal enzyme lactase, and is associated with oral ingestion of amounts well over those in solid dosage forms.

Chapter 4



Materials & Methods

4.1 MATERIALS

4.1.1 Drugs /Chemicals

Table 1: Materials and instruments used in the experiment are of laboratory grade.

DRUG	**MANUFACTURER**
Ciprofloxacin	Biocon Lab, Bangalore
Verapamil hydrochloride	Yarrow chem products, Mumbai
Turmeric (*Curcuma longa*)	Local market
Black Piper (*Piper nigrum)*	Local market
Drumstick (*Moringa oleifera*)	Local market
Zinger (*Zingiber officinale*)	Local market
Cumin (*Cuminum cyminum*)	Local market
Pomegranate juice	Local market
Sodium chloride (NaCl)	Karnataka fine chem., Bangalore
Potassium chloride (KCl)	Qualingens fine chemicals, Mumbai
Magnesium sulphate ($MgSO_4$)	S d fine chem. Ltd, Mumbai, India
Potassium dihydrogen phosphate (KH_2PO_4)	S.D. Fine Chem. Ltd, Mumbai, India
Sodium bicarbonate ($NaHCO_3$)	S.D. Fine Chem. Ltd, Mumbai, India
Calcium carbonate ($CaCl_2$)	S.D. Fine Chem. Ltd, Mumbai, India
Glucose	S.D. Fine Chem. Ltd, Mumbai, India
Water	Purified water by reverse osmosis Process

4.1.2 Equipments or instruments used

Table no: 3 List of Equipments

EQUIPMENT	MODEL/COMPANY
UV- Visible spectrophotometer	Shimadzu UV-1800, Japan
Electronic balance	Acculab, Europe, Germany
Hot air oven	Kadavil electro mechanical Ind., Kerala
Water bath	Riche / Riche, Bangalore
Aerator	Atlels, Mumbai
Glassware	Borosil, Technoscientific, Bangalore
Dissolution apparatus	Lab India, Mumbai, India
FTIR	Thermo Nicolet
Tablet hardness tester	Monsanto hardness tester
Friability test apparatus	Roche friabilator
Multi tablet Punching machine	LAB PRESS, CIP Machinaries Ltd. Ahmedabad
Stability chamber	LABTOP, SKY Lab Instruments & Engineering Pvt. Ltd.

4.2 Standard graphs

4.2.1 Ciprofloxacin

Ciprofloxacin is freely soluble in water. An absorption maxima of Ciprofloxacin was determined by scanning 200-400nm using 10µg/ml concentration after correcting the baseline. The λmax of the drug was found to be 278 nm.

4.2.2 Standard Curve for Ciprofloxacin

100 mg of Ciprofloxacin was accurately weighed and dissolved in 100 ml of water to prepare first stock solution (1mg/ml). 10ml of first stock solution was taken and diluted to 100 ml with the same solvent to prepare II stock solution (100mcg/ml). The aliquots of stock solution

II was further diluted with water to get 1, 2, 3, 4, 5 and 6µg/ml of the final solution. The absorbance was measured in a UV spectrophotometer at 278nm against water as blank.

(C) Standard graph in Physiological salt solution (Krebs Ringer solution (KRS))

100 mg of Ciprofloxacin was accurately weighed and dissolved in 100 ml of KRS to prepare first stock solution (1mg/ml). 10ml of first stock solution was taken and diluted to 100 ml with the same solvent to prepare II stock solution (100mcg/ml). The aliquots of stock solution II was further diluted with KRS to get 1, 2, 3, 4, 5 and 6µg/ml of the final solution. The absorbance was measured in a UV spectrophotometer at 278nm against KRS as blank.

4.2.2 Verapamil hydrochloride

(A) Determination of absorption maxima

Verapamil hydrochloride is freely soluble in water. Absorption maxima of Verapamil hydrochloride was determined by scanning 200-400nm using 10µg/ml concentration after correcting the baseline. The λmax of the drug was found to be 276 nm.

(B) Standard graph in water

100 mg Verapamil hydrochloride of was accurately weighed and dissolved in 100 ml of water to prepare first stock solution (1mg/ml). 10ml of first stock solution was taken and diluted to 100 ml with the same solvent to prepare II stock solution (100mcg/ml). The aliquots of stock solution II was further diluted with water to get 1, 2, 3, 4, 5 and 6µg/ml of the final solution. The absorbance was measured in a UV spectrophotometer at 276nm against water as blank.

(C) Standard graph in KRS

Verapamil hydrochloride 100 mg of was accurately weighed and dissolved in 100 ml of KRS to prepare first stock solution (1mg/ml). 10ml of first stock solution was taken and diluted to 100 ml with the same solvent to prepare II stock solution (100mcg/ml). The aliquots of stock

solution II was further diluted with KRS to get 1, 2, 3, 4, 5 and 6µg/ml of the final solution. The absorbance was measured in a UV spectrophotometer at 276nm against KRS as blank.

4.2.3 Estimation of Verapamil hydrochloride by oxidimetry

Aliquots of pure drug solution containing 0.1-0.7 mcg/ml were transferred into a series of seven 10ml calibrated flasks. To each flask 1ml of 0.2M H2SO4 acid solution was added followed by 0.5ml of KMnO4 solution. The contents were mixed and the flasks were heated for 10min. These were cooled and 1ml of Rhodamine-B solution was added to each flask, diluted to the mark with water and the absorbance of solution was measured at 557nm. A standard graph was prepared by plotting the absorbance versus the concentration of drugs and computed from the regression equation derived using Beer's law.

4.3 Screening of P-Glycoprotein modulation activity

4.3.1Plant extracts

4.3.1 Preliminary preparation

Powdered roots of *Curcuma longa,* dried roots of *Zinger officinalae,* dried seeds of *Piper nigrum,* and *Cuminum cyminum* were purchased from the local market of Bellur and dried at 40^0C for 4 hours in hot air oven, finely powdered and packed in self sealing plastic cover until further use. Fresh leaves of *Moringa oleifera* were collected from BG Nagara in Mandya district. Leaves were separated, dried in cool place of laboratory for 5 days, finely powdered and packed in self sealing plastic cover until further use. Fresh juice of seeds of Pomegranate was used during the experiments.

4.3.2Extraction

Dried powders were extracted with 99% ethnol using a Soxhlet extractor apparatus. The 100g power was put in soxhlet thimble and put into a Soxhlet thimble tube. 1000 ml of ethanol was added to a Soxhlet flask, and then extracted at 60°C until the extract was not clear or about 8

hour. The ethanol was removed under pressure using a rotator evaporator. Then dried residue crude extract were stored in dark bottle a 4°C.

4.3.2 Composition & preparation of KRS

Krebs-Ringer's solution of composition (g/L-1): NaCl 6.9g, KCl 0.35g, $MgSO_4.7H_2O$ 0.29g,KH_2PO_4 0.16g, $NaHCO_3$ 2.1g,$CaCl_2$ 0.28g and 0.2% glucose (pH 7.3).

All ingredients except $CaCl_2$ were dissolved in 600 ml of water in 1000ml volumetric flask.$CaCl_2$ was dissolved in 100ml water in 250ml beaker. This solution was added to the abovesolution and made up to 1000ml using purified water.

Note: Turbidity appears, if all ingredients were dissolved at once in water. Hence CaCl2 solutionshould be added at last.

4.3.3 Preparation of stock solutions using KRS

Accurately 100mg of model drug (Ciprofloxacin) was weighed and dissolved in KRS and made up to 100ml using 100ml volumetric flask. This stock solution was used for further experiments.

4.4 Animals

Male albino rats weighing 170-220g on standard feed and tap water were used throughout study and were maintained in standard lab conditions (22 ± 3^0C) with 12 h light and 12 h dark exposure. Animals were fasted overnight before scarification.

4.5. Everted rat intestine apparatus

The design of the glass apparatus used for the present study is depicted in Fig. 1a. The apparatus consists of two cylindrical glass tubes; one (110×17 mm) joined to other (48×17 mm) via J-shaped tapering end. Both the tubes are held together by a glass joint on the upper end. On the lower ends of both tubes a bulge is given for proper mounting of tissue. The dimensions of the apparatus (110×49×17mm) are such that it can be conveniently set up in a 250.0ml glass beaker. After mounting the everted intestinal segment on the apparatus and

setting it in the beaker; the inside of the glass tubes serve as the mucosal compartment and the beaker serves as the serosal compartment.

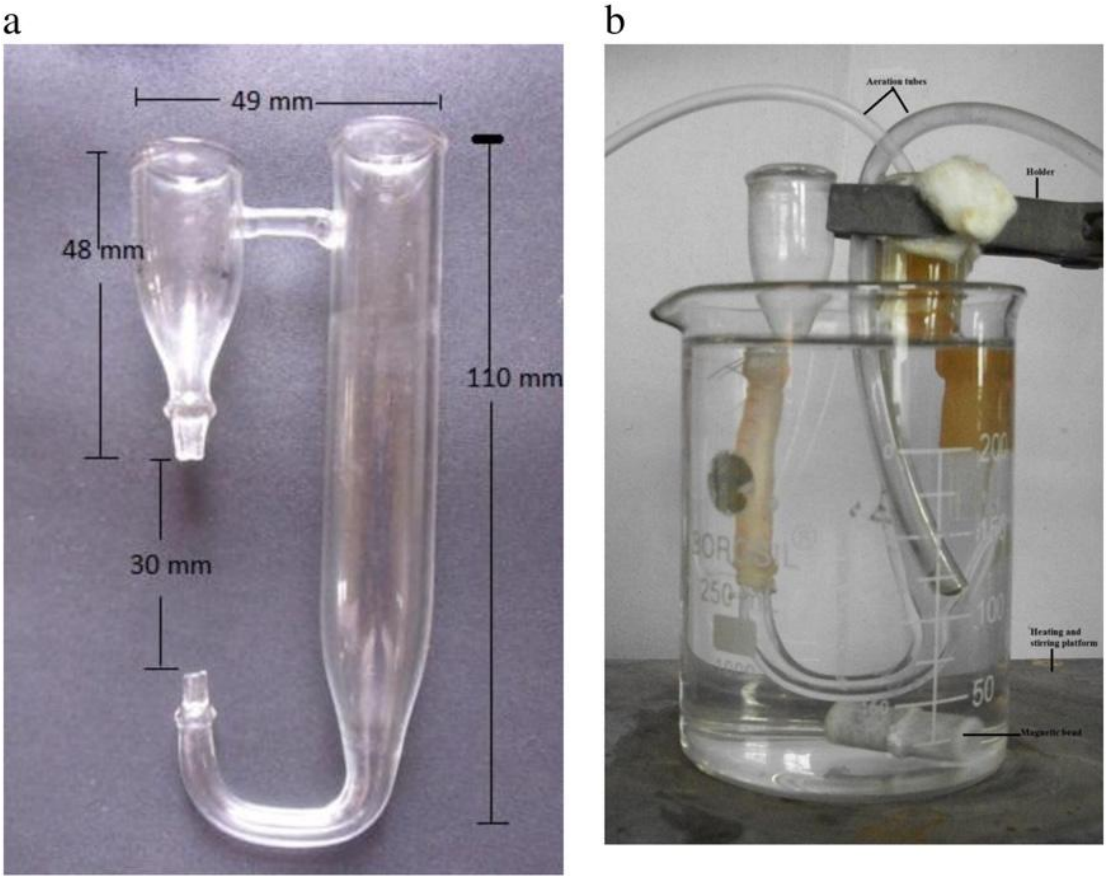


Fig. 8. Photographs of the apparatus (a) with dimensions; and (b) complete set up.

4.5.1 Permeability markers:

Phenol red was used as permeability marker as it was reported to have zero permeability. These markers ensure that the intestine is intact and leak proof throughout the experiment. Furtherindicators such as thymol blue, crystal violet and eosin did not show any permeation frommucosal fluid to serosal fluid during our three hours of experiment. Further studies are desired toconsider these indicators as permeability markers.

4.5.2 Preparation of everted intestinal sac

The everted intestinal sac techniqueis a suitable and universal *in-vitro* model for the study of intestinal transference of the drugs.After being fasted overnight with free access to water, the

rats were anesthetized by chloroform.Their abdomen opened with a middle incision.The underlying mesenterium and fatwere removed, and the sacs were carefully everted with a glass rod (2.5mm diameter).A 7 cm segment,5–6 cm distant to the ileocaecal junction was excised, and it was removedof the mesenteric attachments carefully without damagingthe intestinal architecture. The intestinal segment was transferredto a petri dish containing Kreb's medium The tissue medium was continuously bubbled with a gas mixture of 95% $O_2$and 5%CO_2.

4.6 Test samples (suspected candidates for P-gp inhibition)

Table 4: The following samples were screened for P-gp inhibition activity

Sl.no.	Name	Concentration
1.	Verapamil hydrochloride (standard)	0.5 & 1mg/ml
2.	Turmeric (*Curcuma longa)*	0.5 & 1mg/ml
3.	Black Piper (*Piper nigrum)*	0.5 & 1mg/ml
4.	Drumstick (*Moringa oleifera)*	0.5 & 1mg/ml
5.	Zinger (*Zingiber officinale*)	0.5 & 1mg/ml
6.	Cumin (*Cuminum cyminum*)	0.5 & 1mg/ml
7.	Pomegranate juice	0.5 & 1mg/ml

Optimized experimental procedure using everted intestinal sac was adopted to determine the flux (dQ/dt) and Permeability coefficient (Paa) for the drug transport in presence and absence of test samples. Parameters such as evertion of intestine, choosing suitable length, operating temperature, aeration technique, and duration of the experiment, tissue viability and addition of indicators were standardized by trial and error method.

An everted segment of 3cm was chosen and tied both sides to the modified apparatus as shown in the figure 8. Blank KRS was filled inside the apparatus (serosal side) and Ciprofloxacin solution (0.5 or 1.0 mg/ml) was filled in the outer part (Mucosal side) along with Phenol red as an indicator. Drug transport from mucosal side to serosal side is estimated by sampling the drug solution from serosal side periodically with an interval of 5 min. Further the drug concentration is determined at 276 nm after suitable dilution. The experiment was repeated with the addition of test extracts / standard drug in the mucosal side

along ciprofloxacin. Two sets of experiments were done using the same test samples with two different concentrations of drugs in mucosal side (0.5 and 1.0 mcg/ml) and compared the results. As Phenol red is impermeable and stays in mucosal side always. Appearance of traces of Phenol red in the serosal solution indicates the puncture of the intestine and can be detected by measuring the absorbance at 560nm.

4.7 Sampling

1ml from the serosal fluid was withdrawn at every 5 min intervals in 10 ml volumetric flaskusing glass syringe. Serosal fluid loss was compensated by replacing 1ml blank KRS solutioninto the serosal fluid. Volume of the sample withdrawn was made up to 10ml using blank KRS.

4.8 Permeability determination

The apparent permeability (Papp) value was calculated according to the following equation:

$$\textbf{Papp} = \frac{dQ}{dt} \times \frac{1}{A * Co}$$

Where Papp is apparen permeability co-efficient (10-6cm/sec), dQ/dT the cumulative amount ofdrug (Q) appearing in the acceptor (serosal) compartment as a function of time, and was obtainedfrom the slope of the linear portion of the amount Transported-versus-time plot, A is surface areaof the intestine (cm2) Co is the initial concentration of drug in the donor compartment (µg/ml).After each experiment, intestinal segment is cut open and surface area is determined bymeasuring length and breadth.

4.9 Histology studies

In order to notice the effect of samples on the intestine, these histological studies were carried out. Here a small segment of everted intestine is allowed with the test samples, drug samples alone and in combination for 30 min interval. The samples were separated and kept in 10%

formalin solution and submitted to department of histology, AIMS, BG Nagara. Samples were fixed on glass slides suing various stains and observed the changes using photomicro-graphed under a confocal microscope.

4.10 PREPARATION OF CIPROFLOXACIN TABLETS BY WET GRANULATION METHOD

All the ingredients were passed through sieve no 40 separately and weighed. Weighed ingredients were transferred into mortar and mixed for 15 minutes. After mixing thoroughly the granules are pass through the sieve and subjected for drying. The granules were evaluated for various pre-compression parameters like bulk volume, tapped volume, bulk density, tapped density and angle of repose.

After compression they were evaluated for appearance, diameter, tablet weight, thickness, hardness, and friability, uniformity of dispersion, weight variation, content uniformity and dissolution profile. Stability studies were also carried out.

4.11 EVALUATION OF BLENDED CHARACTERISTICS OF CIPROFLOXACIN

Evaluation of Granules(49)(50)

Angle of Repose:

The angle of repose of granules was determined by the funnel method. The accurately weighed granules were taken in a funnel. The height of the funnel was adjusted in such a way that the tip of the funnel just touched the apex of the heap of the granules. The granules were allowed to flow through the funnel freely onto the surface. The diameter of the powder cone was measured and angle of repose was calculated using the following equation

$$\mathbf{Tan\ \theta = h/r}$$

Where, ***h***and ***r*** are the height and radius of the powder cone respectively.

Bulk Density (Db): It is the ratio of total mass of powder to the bulk volume of powder. It was measured by pouring the weighed powder into a measuring cylinder and the volume was noted. It is expressed in gm/mland is given by

Db= Mass powder/Volume

Tapped density (D_t): It is the ratio of total mass of powder to the tapped volume of powder. The tapped volume was measured by tapping the powder to constant volume. It is expressed in gram/ml and is given by

$$D_t = M/V_t$$

Where, **M** - Mass of the powder

V_t – Tapped volume of the powder.

Compressibility index (I) and Hausner's ratio: Carr's index and Hausner's ratio measure the propensity of granule to be compressed and the flow ability of granule. Carr's index and Hausner's ratio were calculated using following formula.

$$C.I = (D_t - D_b)100/D_t$$

Where, D_t – Tapped density of the powder

D_b – Bulk density of the powder

4.12 FORMULATION DEVELOPMENT CIPROFLOXACIN TABLETS

Table 5. Selected excipient for prototype formulation

Sl NO	EXCIPIENT	FUNCTION
1	Lactose	Thickening agent
2	PVK_{30}	Binding agent
3	Talc	Glidant
4	Magnesium stearate	Lubricant

Table 6: Formulations containing & various concentrations of excipients

INGREDIENTS	WEIGHT OF ONE TABLET (MG/TAB)
Ciprofloxacin	300
Pepper	50
Curcumin	50
Ginger	50
Drum stick	50
Cumin	50
Pomegranate Juice	50
Lactose	50
PVK_{30}	q **.s**
Talc	30
M. stearate	20
Total	700

4.13 EVALUATION OF CIPROFLOXACIN TABLETS

The matrix tablets prepared were evaluated for the following parameters:

1. Weight variation
2. Hardness
3. Friability
4. Drug content
5. *In-vitro* Dissolution Studies
6. Stability Studies

Weight Variation Test

To study weight variation, 20 tablets of each formulation were weighed using an electronic balance and the test was performed according to the official method.

Table 7: IP standards of Uniformity of weight

Sl. No	Avg Wt of Tablet	% of Deviation
1	≤ 80 mg 10	10
2	>80mg- 250mg	7.5
3	≥250	5

Hardness and Friability

For each formulation, the hardness and friability of 6 tablets were determined using the Monsanto hardness tester (Cadmach, Ahmedabad, India) and the Roche friabilator (Campbell Electronics, Mumbai, India) respectively.

The percent friability calculated as follows

$$\%F = \frac{W_1 - W_2}{W_1} \times 100$$

Drug Content:

Five tablets were weighed and triturate, from that transfer an accurately weighed portion of the powder equivalent to about 100mg of Ciprofloxacin in a 100ml volumetric flask containing buffer solution and then concentration is measured at λ max 278 nm.

4.9 *IN-VITRO* DISSOLUTION STUDIES

The *in-vitro* dissolution studies were performed using the USP-II (Paddle) dissolution apparatus at 50 rpm. The dissolution medium consisted of 900ml of phosphate buffer pH 6.8, maintained at 37±0.50C. An aliquot (5ml) was withdrawn at specific time intervals and drug

content was determined by UV-visible spectrometer at 278nm. The study was performed in triplicate.

4.14 KINETIC ANALYSIS OF *IN-VITRO* RELEASE RATES OF ORAL RELEASE TABLETS OF CIPROFLOXACIN

The results of *in-vitro* release profile obtained for all the formulations were plotted in modes of data treatment as follows:-

1. Zero- order Kinetic model – Cumulative % drug released versus Time.
2. First- order Kinetic model – Log cumulative % drug remaining versus Time.
3. Higuchi's model- Cumulative percent drug released versus square root of time.
4. Korsmeyer equation / Peppa's model- Log cumulative percent drug released versus log time.

Zero order kinetics:

Zero order release would be predicted by the following equation:-

$$\mathbf{A_t = A_0 - K_0 t}$$

Where,

A_t= Drug release at time't'.

A_0= Initial drug concentration

K_0=Zero-order rate constant (hr^{-1}).

When the data is plotted as cumulative % drug release versus time, if the plot is linear then the data obeys zero- order release Kinetics, with a slope equal to K^0.

First order Kinetics:

First order release would be predicted by the following equation:-

$$\mathbf{Log\ C = log\ C_0 - K_t / 2.303}$$

Where,

C = Amount of drug remained at time't'.

C_o = Initial amount of drug.

K = First – order rate constant (hr^{-1}).

When the data is plotted as log cumulative % drug remaining versus time yields a straight line, indicating that the release follows first order kinetics. The constant 'K' can be obtained by multiplying 2.303 with the slope values.

Higuchi's model:

Drug release from the matrix devices by diffusion has been described by following Higuchi's classical diffusion equation.

$$\mathbf{Q = [D_{\varepsilon} / \bar{\iota} \ (2A - \varepsilon C_S) C_S t]^{1/2}}$$

Where,

Q = Amount of drug released at time't'.

D = Diffusion co-efficient of the drug in the matrix.

A = Total amount of drug in unit volume of matrix.

C_S = The solubility of the drug in the matrix.

ε = Porosity of the matrix.

$\bar{\iota}$ = Tortuosity.

When the data is plotted according to equation i.e. cumulative drug release versus square root of time yields a straight line, indicating that the drug was released by diffusion mechanism. The slope is equal to 'K' (Higuchi's 1963).

Korsmeyer equation/ Peppa's model:

To study the mechanism of drug release from the sustained – release matrix tablets of losartan potassium, the release data were also fitted to the well – known exponential equation (Korsmeyer equation/ peppa's law equation), which is often used to describe the drug release behavior from polymeric systems.

$M_t / M_a = Kt^n$

Where,

M_t / M_a = the fraction of drug released at time 't'.

K = Constant incorporating the structural and geometrical characteristics of the drug/ polymers system.

N = Diffusion exponent related to the mechanism of the release.

Above equation can be simplified by applying log on both sides,

Log M_t / M_a = Log K + n log t

When the data is plotted as log of drug released versus time, yields a straight line with a slope equal to 'n' and the 'K' can be obtained from y- intercept. For Fickian release 'n' = 0.5 while for anomalous (non- Fickian) transport 'n' ranges between 0.5 and 1.0. The result of *in- vitro* drug release study of all the formulation as shown below.

Table 8: Mechanism of Drug Release as per Korsmeyer Equation/ Peppa's Model

S. No	'n' value	Drug release
1.	0.45	Fickian release
2.	0.45 <n<0.89	Non- Fickian release
3.	n>0.89	Class II transport

4.15 STABILITY STUDIES

Stability of a pharmaceutical product may be defined as the capability of a particular formulation, in a specific container, to remain within its physical, chemical, therapeutic and toxicological specifications throughout its shelf life.

ICH specifies the length of study and storage conditions.

Long Term testing: $25^0C \pm 2^0C$ / 60% RH ± 5% for 12 months

Accelerated Testing: $40^0C \pm 2^0C$ / 75% RH ± 5% for 6 months

4.11.1 Method

The optimized formulation was subjected for two month stability study according to ICH guidelines**.** The selected formulations were packed in aluminum foils, which were in wide mouth bottles closed tightly. They were then stored at 40ºC / 75% RH for 2 months. Then samples were evaluated for their content and *in vitro* dissolution studies.

Chapter 5

Results

RESULTS

Table 9: Calibration data of Ciprofloxacin in water.

Sl.No	Concentration (µg/ml)	Absorbance (276nm)
1	1	0.066
2	2	0.139
3	3	0.216
4	4	0.278
5	5	0.336
6	6	0.419

Figure 9: Standard graph of Ciprofloxacin in water.

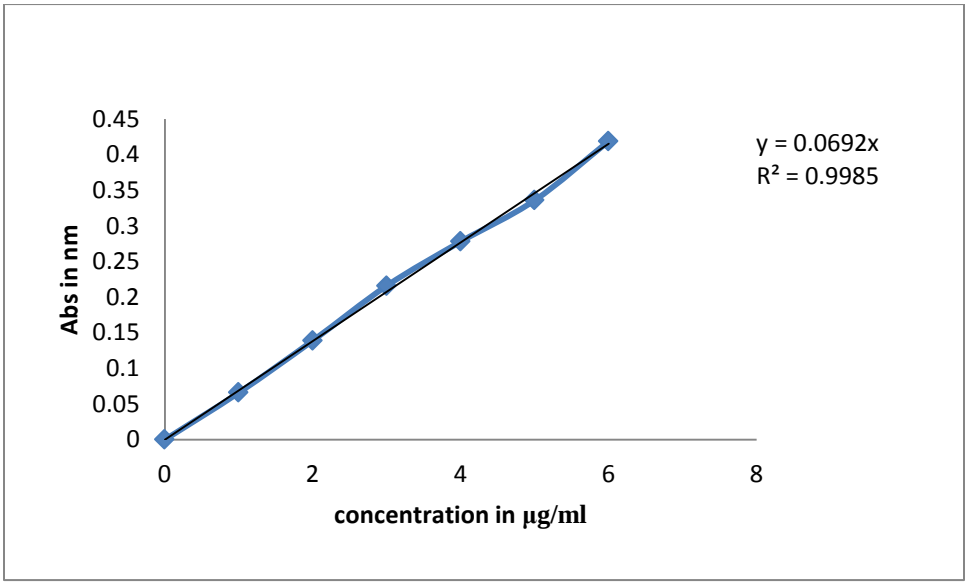


Table 10: Calibration data of Ciprofloxacin in KRS

Sl.No	Concentration (µg/ml)	Absorbance (276nm)
l	1	0.068
2	2	0.132
3	3	0.202
4	4	0.264
5	5	0.34
6	6	0.409

Figure 10: Standard graph of Ciprofloxacin in KRS

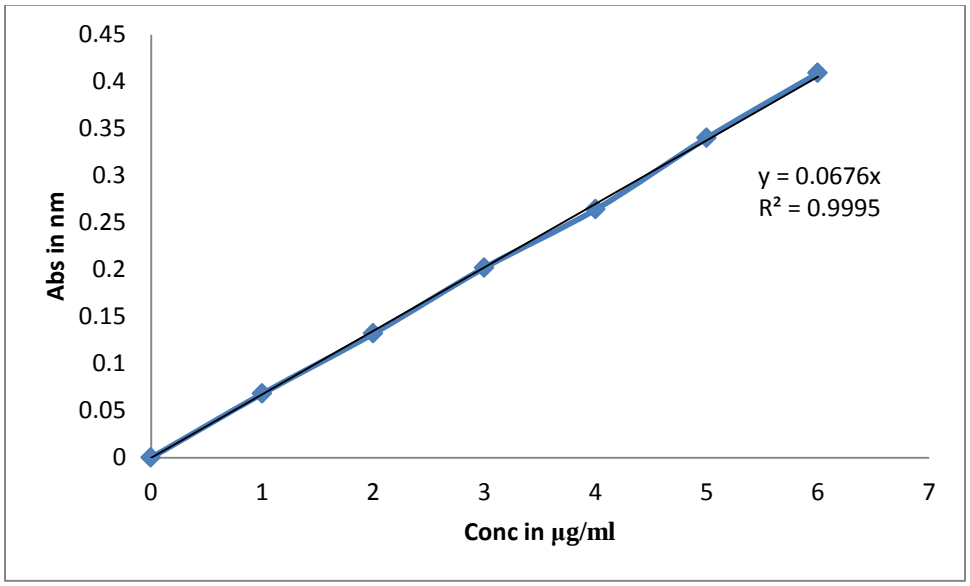


Table 11 Calibration data of Verapamil hydrochloride in water

Sl.No	Concentration (µg/ml)	Absorbance (278nm)
l	1	0.11
2	2	0.202
3	3	0.315
4	4	0.428
5	5	0.519
6	6	0.61

Figure 11: Standard graph of Verapamil hydrochloride in water

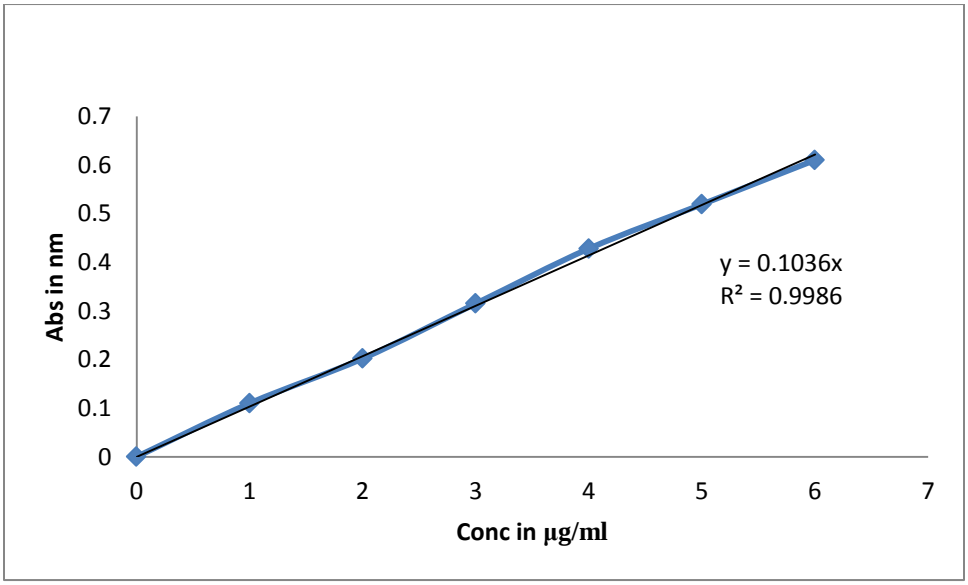


Table 12 Calibration data of Verapamil hydrochloride in KRS

Sl.No	Concentration (µg/ml)	Absorbance (278nm)
l	1	0.073
2	2	0.139
3	3	0.201
4	4	0.268
5	5	0.337
6	6	0.415

Figure 12: Standard graph of Verapamil hydrochloride in KRS

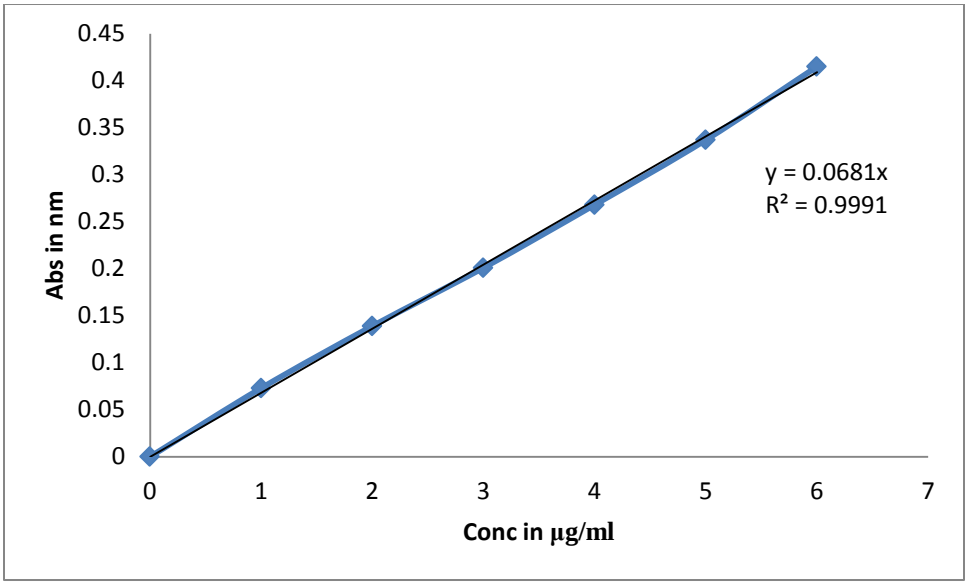


Table 13 Estimation of Verapamil hydrochloride by oxidometry

Sl.No	Concentration (µg/ml)	Absorbance (557nm)
l	0.1	0.067
2	0.2	0.084
3	0.3	0.102
4	0.4	0.119
5	0.5	0.137
6	0.6	0.154
7	0.7	0.171

13 Graph showing estimation of Verapamil hydrochloride by oxidometry

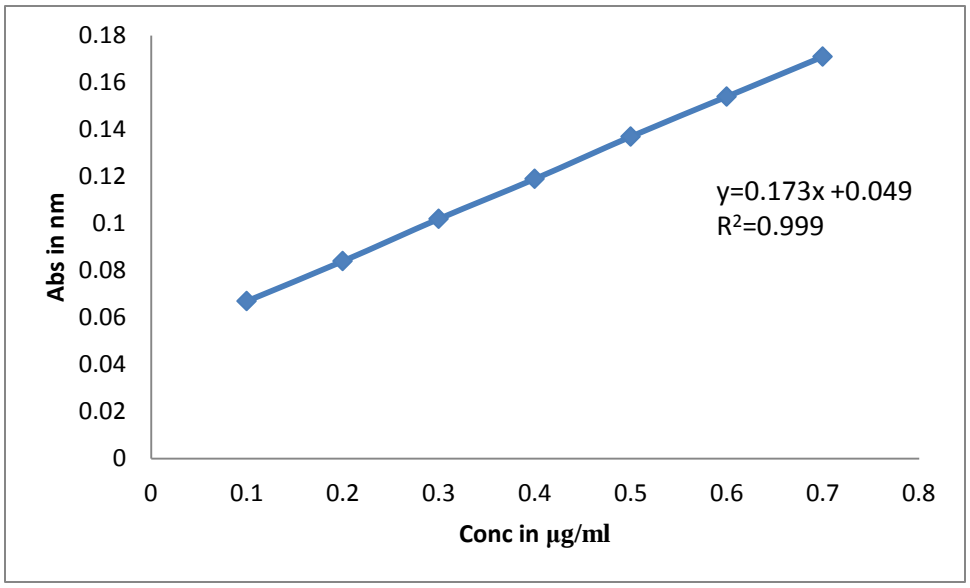


Table 13 Rate of diffusion and Permeability coefficient of Ciprofloxacin and Ciprofloxacin &Verapamil hydrochloride combination

	Ciprofloxacin(100mg)		Ciprofloxacin +Verapamil hydrochloride*		Ciprofloxacin(50mg)		Ciprofloxacin +Verapamil hydrochloride*	
Time (min)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)
0	0	0	0	0	0	0	0	0
5	0.266	0.09	0.311	0.13	0.155	0.12	0.177	0.12
10	0.333	0.12	0.422	0.17	0.244	0.18	0.266	0.18
15	0.422	0.15	0.555	0.23	0.377	0.28	0.377	0.26
20	0.466	0.17	0.844	0.35	0.488	0.37	0.511	0.35
25	0.555	0.20	0.933	0.39	0.555	0.42	0.44	0.44
30	0.844	0.30	1.177	0.49	0.666	0.50	0.8	0.55

***Verapamil was estimated by oxidimetry the value found to be 0.049**

Table 14 Surface area of intestinal segment and Mucosal Concentration (µg/ml) details

Sample name	Length (cm)	Breadth(cm)	Surface area(cm^2)	Mucosal Concentration(µg/ml)
Ciprofloxacin(100mg)	3.12	0.9	2.80	1
Cipro+ Verapamil hydrochloride	3.03	0.8	2.42	1
Ciprofloxacin(50mg)	3.32	0.8	2.64	0.5
Cipro+Verapamil hydrochloride	3.25	0.9	2.92	0.5

Figure 14 Graph showing effect of Verapamil hydrochloride on the absorption of Ciprofloxacin A(100mg) B(100mg)

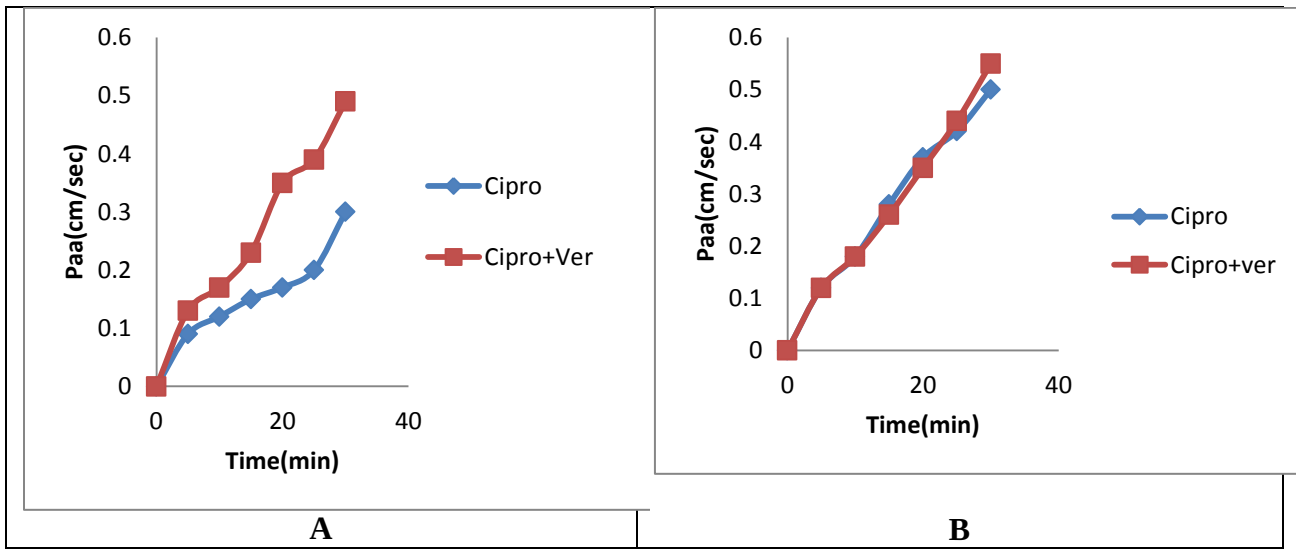


Table 15 Rate of diffusion and Permeability coefficient of Ciprofloxacin and Ciprofloxacin & drum stick combination

	Ciprofloxacin(100mg)		Ciprofloxacin+Drum stick		Ciprofloxacin(50mg)		Ciprofloxacin+Drum stick	
Time (min)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)
0	0	0	0	0	0	0	0	0
5	0.266	0.09	0.622	0.22	0.155	0.12	0.2	0.17
10	0.333	0.12	0.688	0.25	0.244	0.18	0.266	0.22
15	0.422	0.15	0.8	0.28	0.377	0.28	0.4	0.34
20	0.466	0.17	0.888	0.32	0.488	0.37	0.488	0.41
25	0.555	0.20	1.066	0.38	0.555	0.42	0.577	0.49
30	0.844	0.30	1.222	0.44	0.666	0.50	0.688	0.58

Table 16 Surface area of intestinal segment and Mucosal Concentration (µg/ml) details

Sample name	Length (cm)	Breadth(cm)	Surface area(cm^2)	Mucosal Concentration(µg/ml)
Ciprofloxacin(100mg)	3.12	0.9	2.80	1
Cipro+Drum stick	3.15	0.9	2.82	1
Ciprofloxacin(50mg)	3.32	0.8	2.64	0.5
Cipro+Drum stick	3.4	0.7	2.38	0.5

Figure 15 Graph showing effect of Drum stick on the absorption of Ciprofloxacin A(100mg) B(50mg)

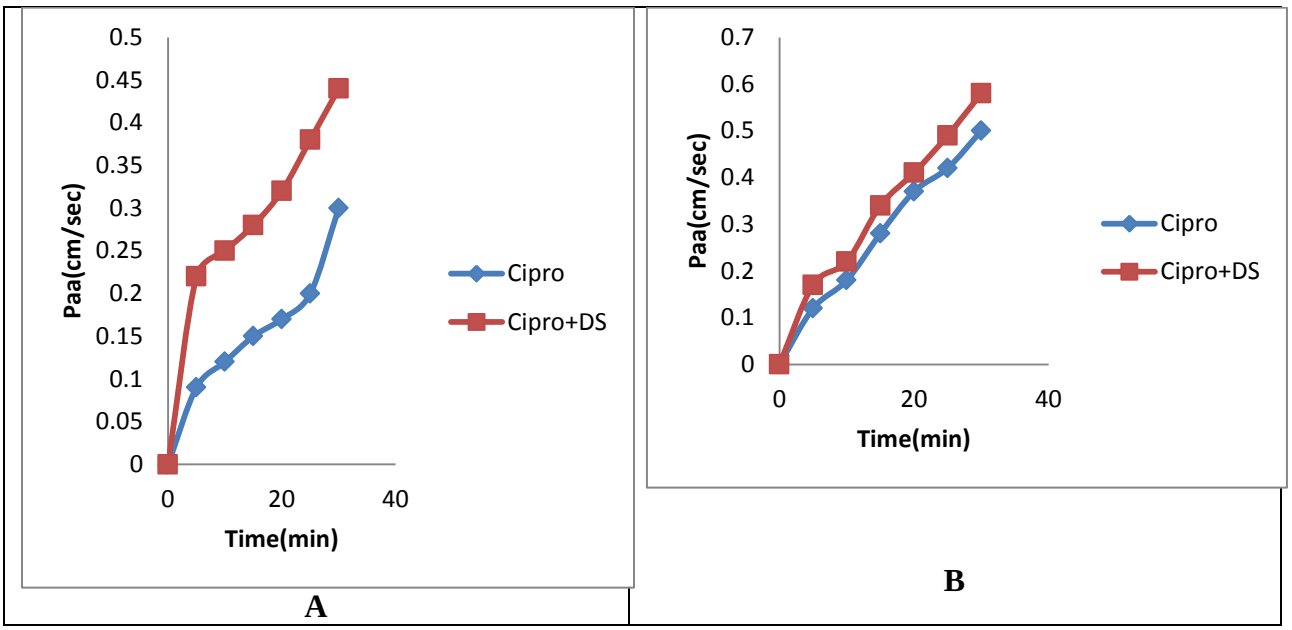


Table 17 Rate of diffusion and Permeability coefficient of Ciprofloxacin and Ciprofloxacin & Piperine combination

	Ciprofloxacin(100mg)		Ciprofloxacin+Piperine		Ciprofloxacin(50mg)		Ciprofloxacin+Piperine	
Time (min)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)
0	0	0	0	0	0	0	0	0
5	0.266	0.09	1.133	0.44	0.155	0.12	0.8	0.59
10	0.333	0.12	1.555	0.61	0.244	0.18	1.088	0.80
15	0.422	0.15	1.733	0.68	0.377	0.28	1.177	0.87
20	0.466	0.17	2.044	0.80	0.488	0.37	1.488	1.10
25	0.555	0.20	2.244	0.88	0.555	0.42	1.8	1.33
30	0.844	0.30	2.622	1.02	0.666	0.50	2.133	1.57

Table 18 Surface area of intestinal segment and Mucosal Concentration (µg/ml) details

Sample name	Length (cm)	Breadth(cm)	Surface area(cm^2)	Mucosal Concentration(µg/ml)
Ciprofloxacin(100mg)	3.12	0.9	2.79	1
Cipro+Piperine	3.2	0.8	2.56	1
Ciprofloxacin(50mg)	3.32	0.8	2.64	0.5
Cipro+Piperine	3.01	0.9	2.70	0.5

Figure 16 Graph showing effect of Piperine on the absorption of Ciprofloxacin A(100mg) B(50mg)

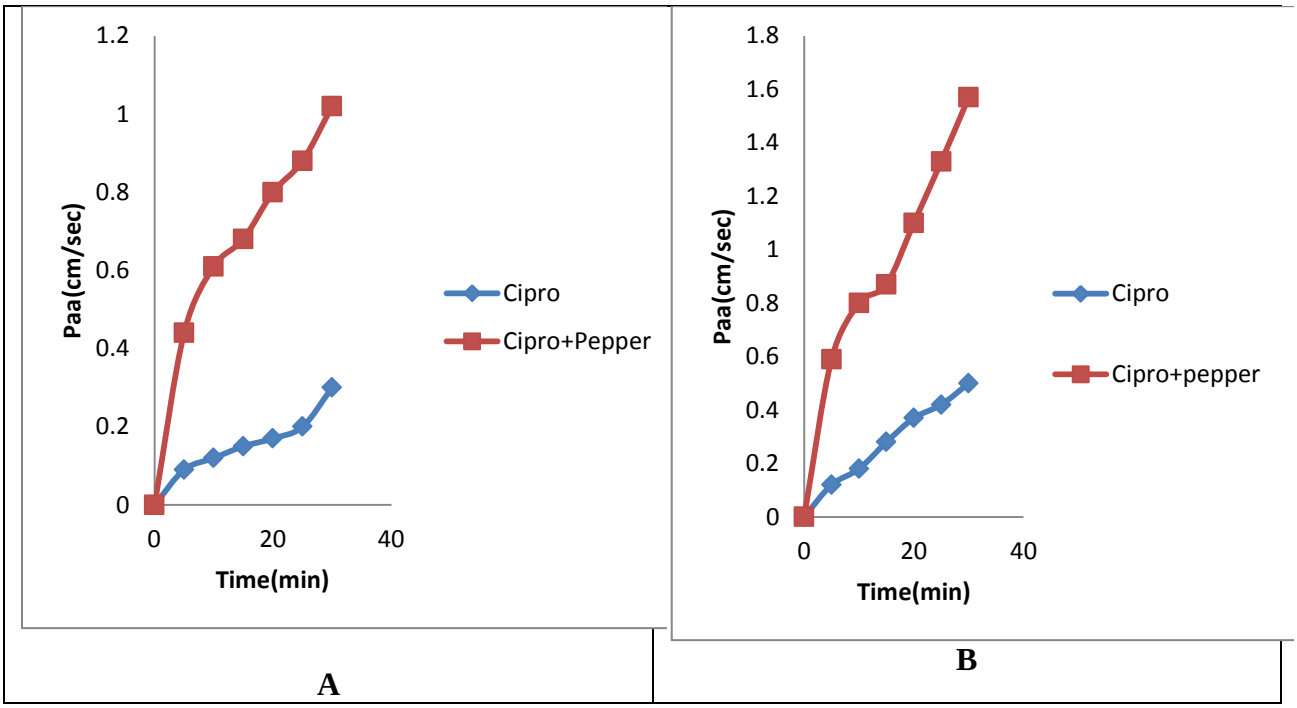


Table 18 Rate of diffusion and Permeability coefficient of Ciprofloxacin and Ciprofloxacin & Curcumin combination

	Ciprofloxacin(100mg)		Ciprofloxacin+Curcumin		Ciprofloxacin(50mg)		Ciprofloxacin+Curcumin	
Time (min)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)
0	0	0	0	0	0	0	0	0
5	0.266	0.09	0.911	0.39	0.155	0.12	0.577	0.44
10	0.333	0.12	1.066	0.45	0.244	0.18	0.733	0.56
15	0.422	0.15	1.222	0.52	0.377	0.28	0.822	0.62
20	0.466	0.17	1.511	0.64	0.488	0.37	1.066	0.81
25	0.555	0.20	1.644	0.70	0.555	0.42	1.355	1.03
30	0.844	0.30	1.977	0.84	0.666	0.50	1.533	1.17

Table 19 Surface area of intestinal segment and Mucosal Concentration (µg/ml) details

Sample name	Length (cm)	Breadth(cm)	Surface area(cm^2)	Mucosal Concentration(µg/ml)
Ciprofloxacin(100mg)	3.12	0.9	2.79	1
Cipro+Curcumin	3.35	0.7	2.31	1
Ciprofloxacin(50mg)	3.32	0.8	2.64	0.5
Cipro+Curcumin	3.29	0.8	2.63	0.5

Figure 17 Graph showing effect of Curcumin on the absorption of Ciprofloxacin A(100mg) B(50mg)

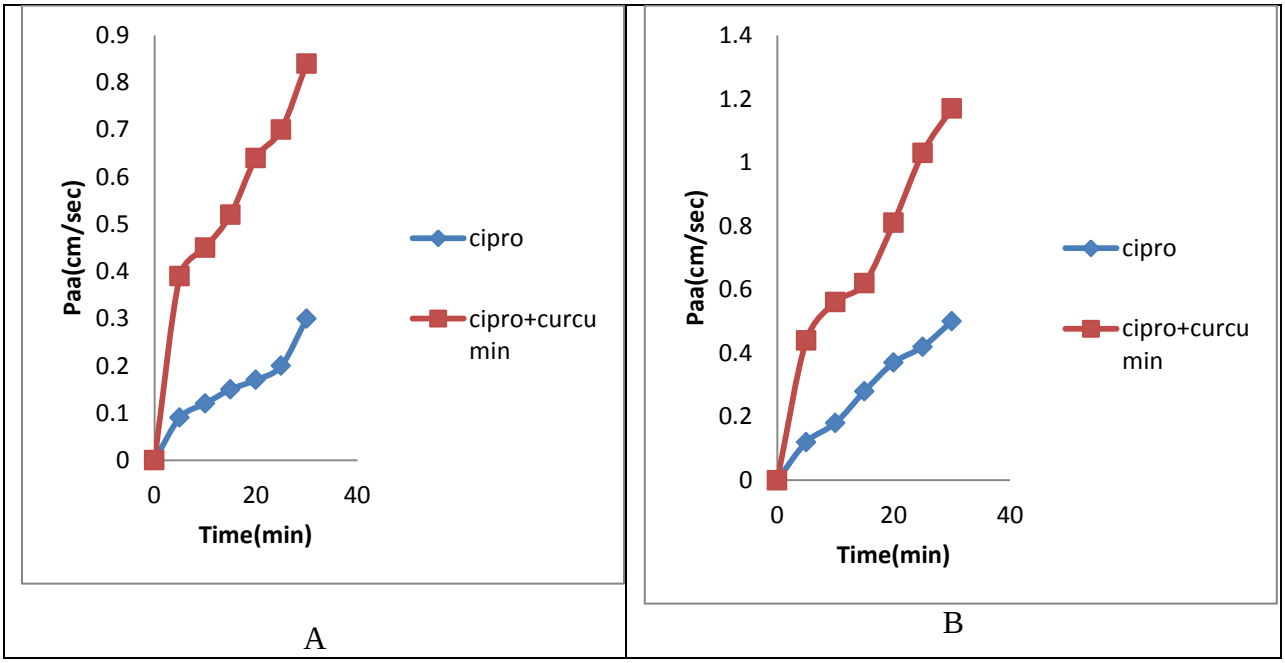


Table 20 Rate of diffusion and Permeability coefficient of Ciprofloxacin and Ciprofloxacin & Ginger combination

	Ciprofloxacin(100mg)		Ciprofloxacin+Ginger		Ciprofloxacin(50mg)		Ciprofloxacin+Ginger	
Time (min)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)
0	0	0	0	0	0	0	0	0
5	0.266	0.09	0.422	0.18	0.155	0.12	0.266	0.19
10	0.333	0.12	0.622	0.26	0.244	0.18	0.4	0.28
15	0.422	0.15	0.8	0.33	0.377	0.28	0.555	0.39
20	0.466	0.17	0.911	0.38	0.488	0.37	0.688	0.49
25	0.555	0.20	1.088	0.45	0.555	0.42	0.844	0.60
30	0.844	0.30	1.377	0.57	0.666	0.50	1	0.71

Table 21 Surface area of intestinal segment and Mucosal Concentration (µg/ml) details

Sample name	Length (cm)	Breadth(cm)	Surface area(cm^2)	Mucosal Concentration(µg/ml)
Ciprofloxacin(100mg)	3.12	0.9	2.79	1
Cipro+Ginger	3.01	0.8	2.42	1
Ciprofloxacin(50mg)	3.32	0.8	2.64	0.5
Cipro+Ginger	3.15	0.9	2.83	0.5

Figure 18 Graph showing effect of Ginger on the absorption of Ciprofloxacin A(100mg) B(50mg)

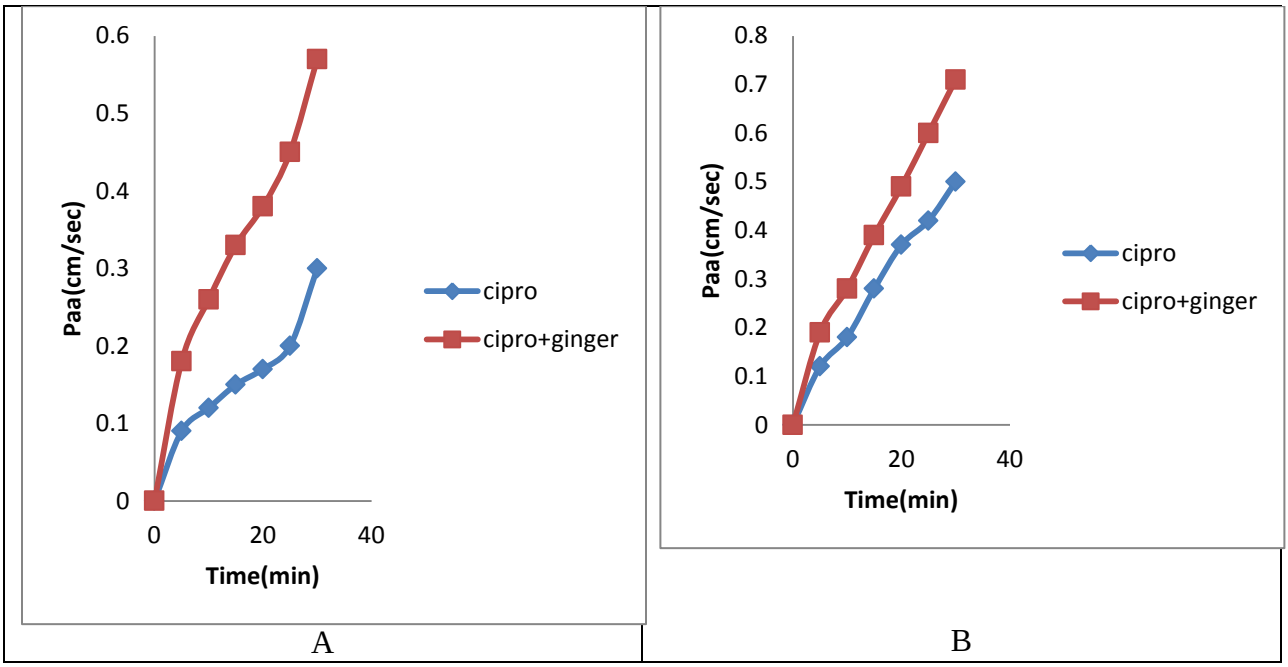


Table 22 Rate of diffusion and Permeability coefficient of Ciprofloxacin and Ciprofloxacin & Cumin combination

	Ciprofloxacin(100mg)		Ciprofloxacin+Cumin		Ciprofloxacin(50mg)		Ciprofloxacin+Cumin	
Time (min)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)
0	0	0	0	0	0	0	0	0
5	0.266	0.09	0.777	0.34	0.155	0.12	0.422	0.30
10	0.333	0.12	1.377	0.60	0.244	0.18	0.622	0.44
15	0.422	0.15	1.577	0.68	0.377	0.28	0.822	0.59
20	0.466	0.17	1.755	0.76	0.488	0.37	0.955	0.68
25	0.555	0.20	1.955	0.85	0.555	0.42	1.066	0.76
30	0.844	0.30	2.155	0.93	0.666	0.50	1.222	0.87

Table 23 Surface area of intestinal segment and Mucosal Concentration (µg/ml) details

Sample name	Length (cm)	Breadth(cm)	Surface area(cm^2)	Mucosal Concentration(µg/ml)
Ciprofloxacin(100mg)	3.12	0.9	2.79	1
Cipro+Cumin	3.3	0.7	2.31	1
Ciprofloxacin(100mg)	3.32	0.8	2.64	0.5
Cipro+Cumin	3.11	0.9	2.77	0.5

Figure 19 Graph showing effect of Cumin on the absorption of Ciprofloxacin A(100mg) B(50mg)

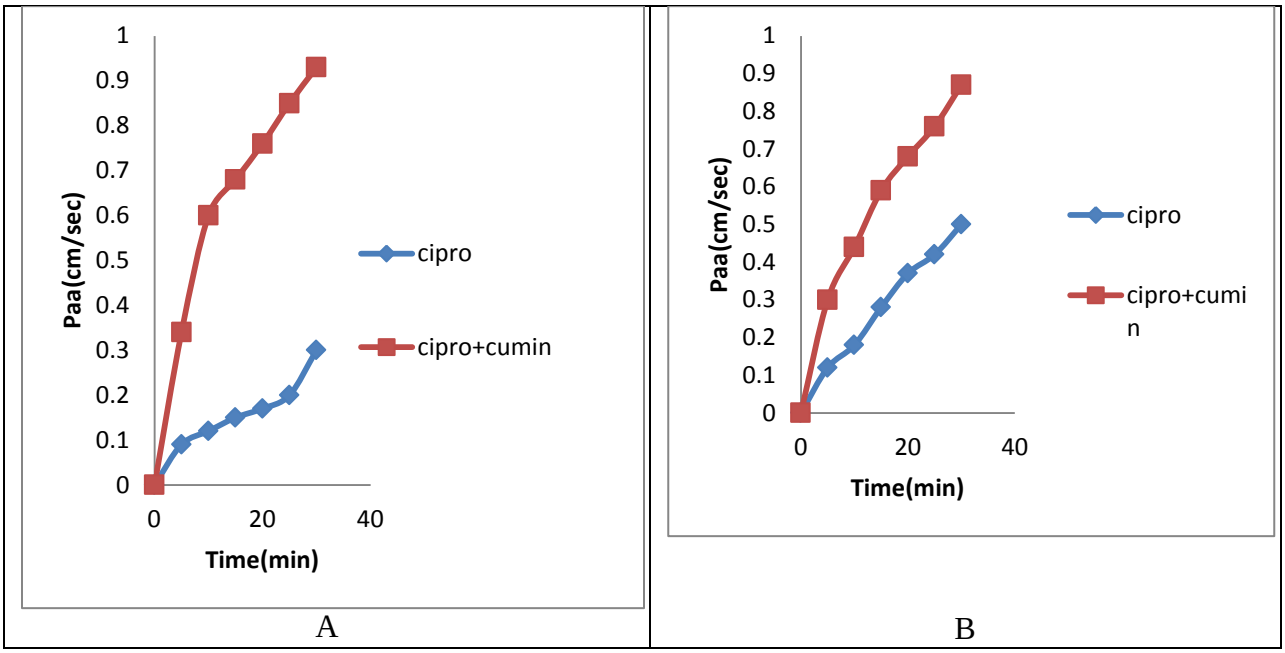


Table 24 Rate of diffusion and Permeability coefficient of Ciprofloxacin and Ciprofloxacin & Pomegranate juice combination

	Ciprofloxacin(100mg)		Ciprofloxacin+Pomegranate juice		Ciprofloxacin(50mg)		Ciprofloxacin+Pomegranate juice	
Time (min)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)	dQ/dt mg/min	Paa (cm/sec)
0	0	0	0	0	0	0	0	0
5	0.266	0.09	0.933	0.39	0.155	0.12	0.355	0.30
10	0.333	0.12	1.444	0.60	0.244	0.18	0.622	0.52
15	0.422	0.15	1.6	0.66	0.377	0.28	0.822	0.69
20	0.466	0.17	1.844	0.76	0.488	0.37	0.955	0.80
25	0.555	0.20	2.111	0.87	0.555	0.42	1.244	1.04
30	0.844	0.30	2.4	0.99	0.666	0.50	1.377	1.15

Table 25 Surface area of intestinal segment and Mucosal Concentration (µg/ml) details

Sample name	Length (cm)	Breadth(cm)	Surface area(cm^2)	Mucosal Concentration(µg/ml)
Ciprofloxacin(100mg)	3.12	0.9	2.79	1
Cipro+Pomegranate juice	3.02	0.8	2.41	1
Ciprofloxacin(50mg)	3.32	0.8	2.64	0.5
Cipro+Pomegranate juice	3.41	0.7	2.38	0.5

Figure 20 Graph showing effect of Pomegranate juice on the absorption of Ciprofloxacin A(100mg) B(50mg)

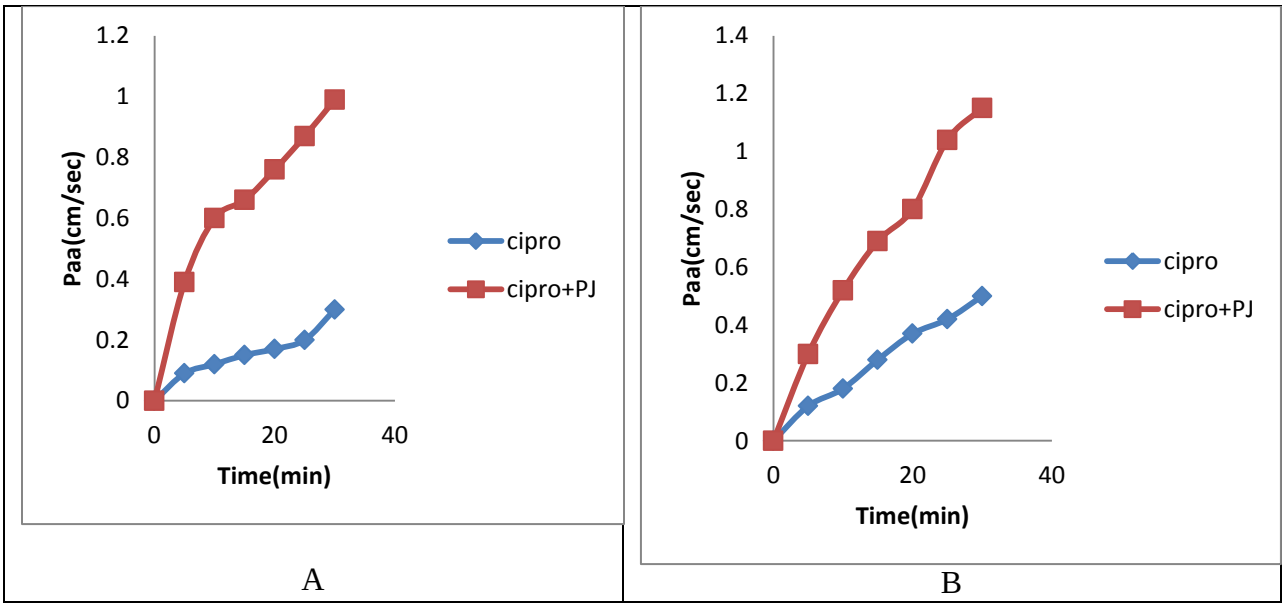


Table 26 Effect of all P-gp inhibitors on the intestinal absorption of Ciprofloxacin(100mg)

Time	Cipro	Cipro+Ver	Cipro+DS	Cipro+Piperine	Cipro+Curcumin	Cipro+Ginger	Cipro+Cumin	Cipro+PJ
0	0	0	0	0	0	0	0	0
5	0.09	0.13	0.22	0.44	0.39	0.18	0.34	0.39
10	0.12	0.17	0.25	0.61	0.45	0.26	0.6	0.6
15	0.15	0.23	0.28	0.68	0.52	0.33	0.68	0.66
20	0.17	0.35	0.32	0.8	0.64	0.38	0.76	0.76
25	0.2	0.39	0.38	0.88	0.7	0.45	0.85	0.87
30	0.3	0.49	0.44	1.02	0.84	0.57	0.93	0.99

Figure: 21 Graph showing comparative effects of P-gp inhibitors on the intestinal absorption of Ciprofloxacin

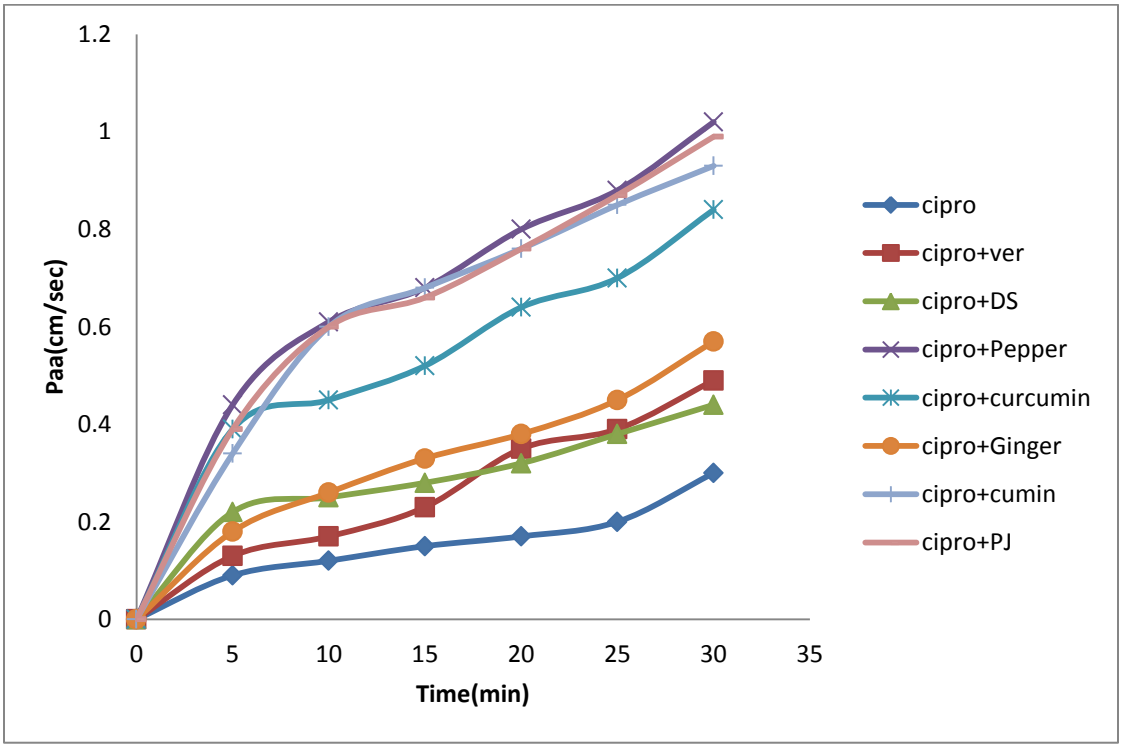


Table 27 Effect of all P-gp inhibitors on the intestinal absorption of Ciprofloxacin (50mg)

Time	Cipro	Cipro+Ver	Cipro+DS	Cipro+Piperine	Cipro+Curcumin	Cipro+Ginger	Cipro+Cumin	Cipro+PJ
0	0	0	0	0	0	0	0	0
5	0.12	0.12	0.17	0.59	0.44	0.19	0.19	0.3
10	0.18	0.18	0.22	0.8	0.56	0.28	0.28	0.52
15	0.28	0.26	0.34	0.87	0.62	0.39	0.39	0.69
20	0.37	0.35	0.41	1.1	0.81	0.49	0.49	0.8
25	0.42	0.44	0.49	1.33	1.03	0.6	0.6	1.04
30	0.5	0.55	0.58	1.57	1.17	0.71	0.71	1.15

Figure: 22 Graph showing comparative effects of P-gp inhibitors on the intestinal absorption of Ciprofloxacin

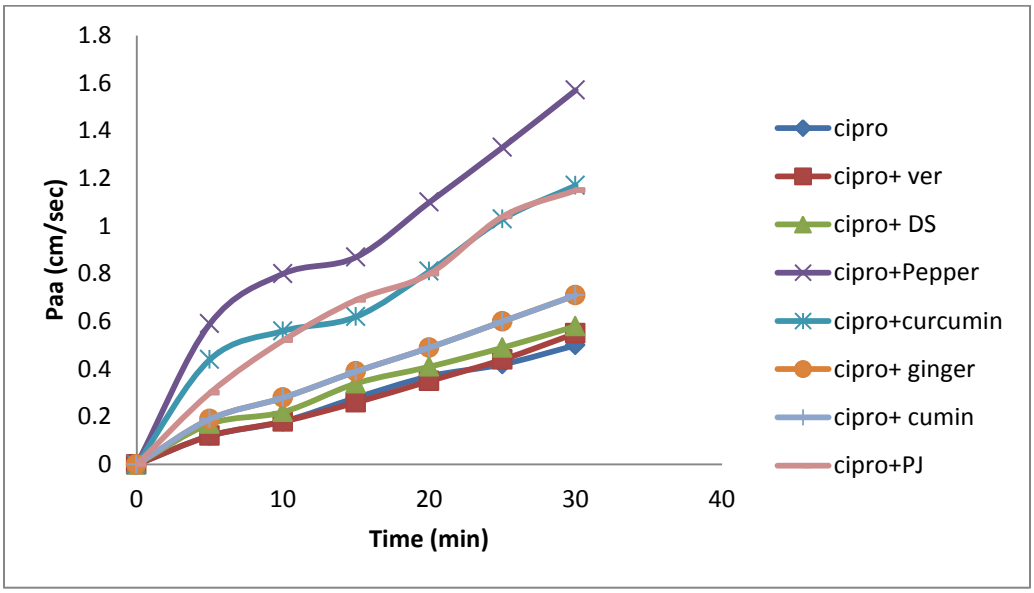
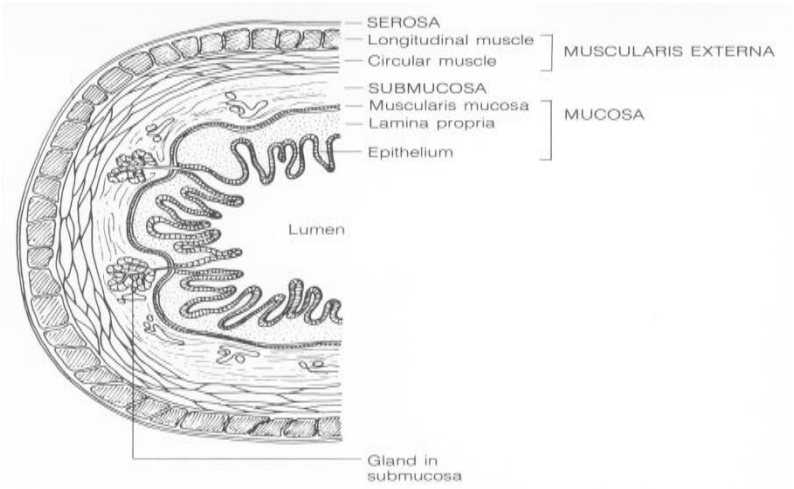


Fig 23 Structure of Small intestine



Histological studies of rat intestine

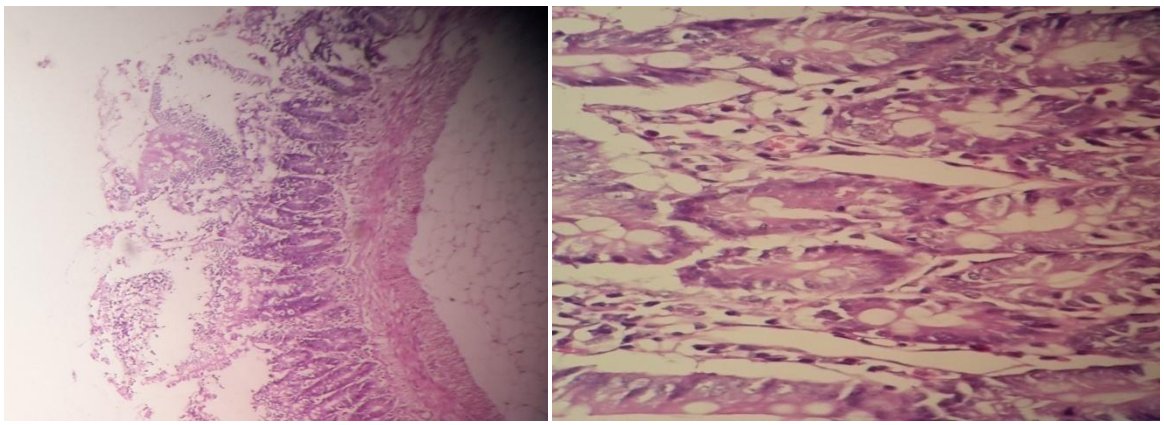


Fig 24 Ciprofloxacin

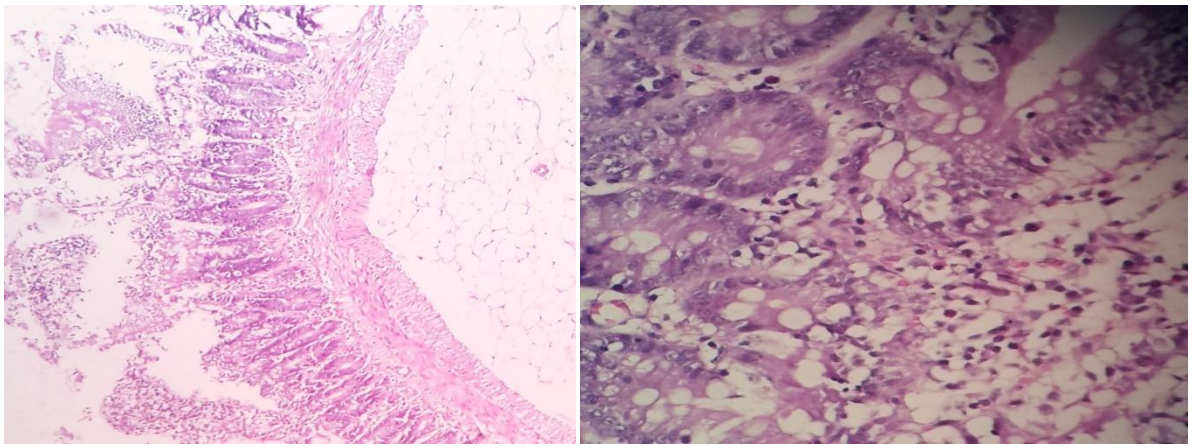


Fig 25 Verapamil

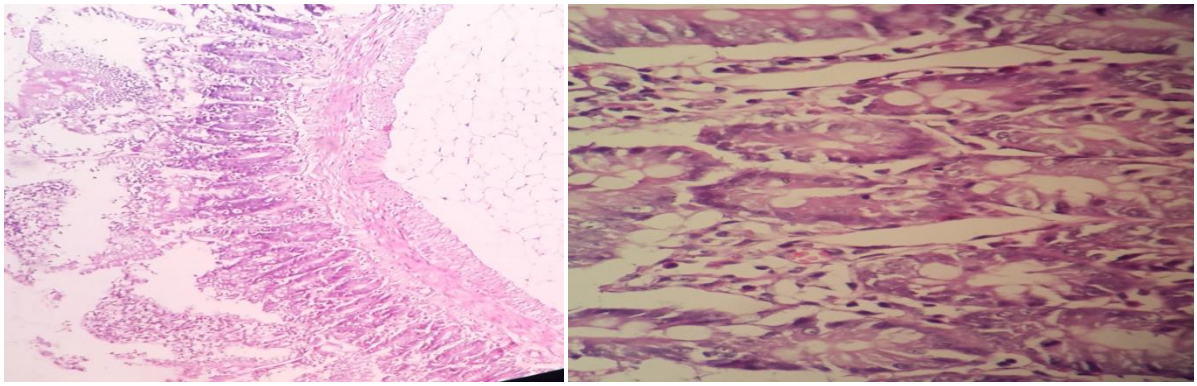


Fig 26 Pepper

Compatibility study

Table 29: Compatibility study of drug and excipient using FTIR

Peaks (cm^{-1})	**Groups**	**Peak assignment**
3500-3450	Hydroxyl group	O-H stretching vibration, intermolecular H-bonded
3000-2950	Aromatic, cyclic enes	υ=CH and Ar-H
1750-1700	CO group of acid	C=O stretching vibration
1650-1600	Quinolines	δN-H bending vibration
1450-1400	Carbonyl group	υC-O
1300-1250	Hydroxyl group	δO-H bending vibration
1050-1000	Fluorine group	C-F stretching

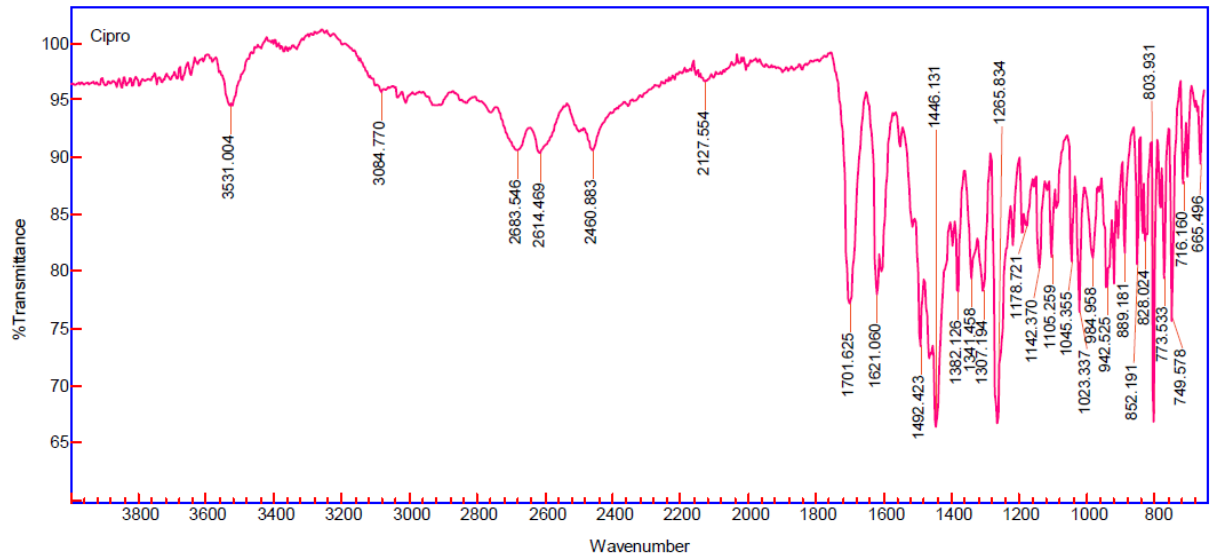


Fig 27:FT-IR spectra of Ciprofloxacin

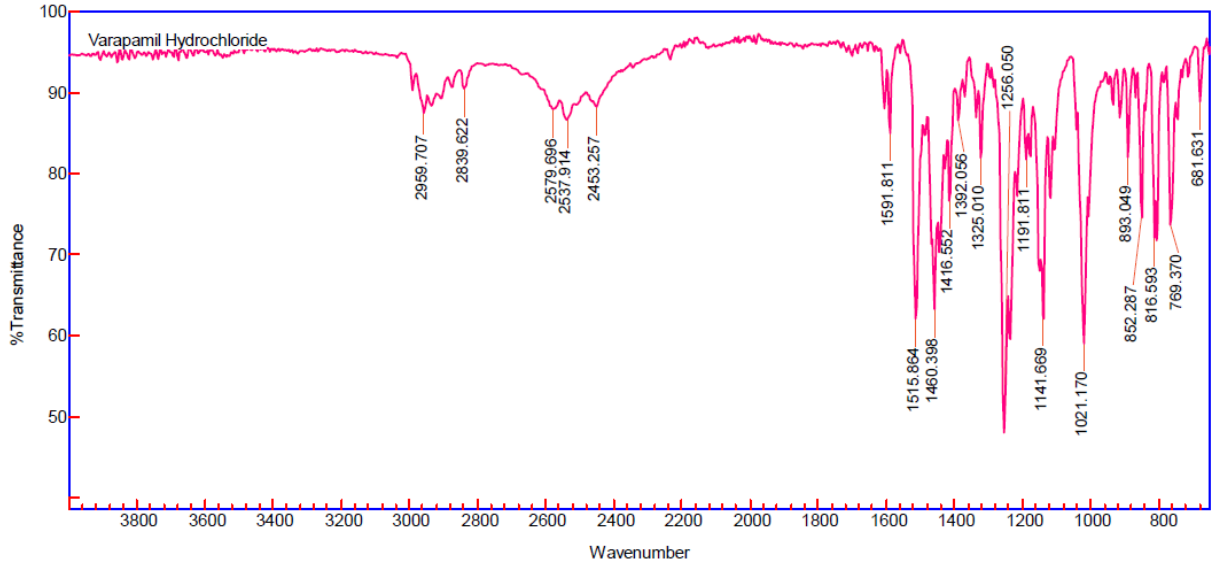


Fig 28:FT-IR spectra of Verapamil hydrochloride

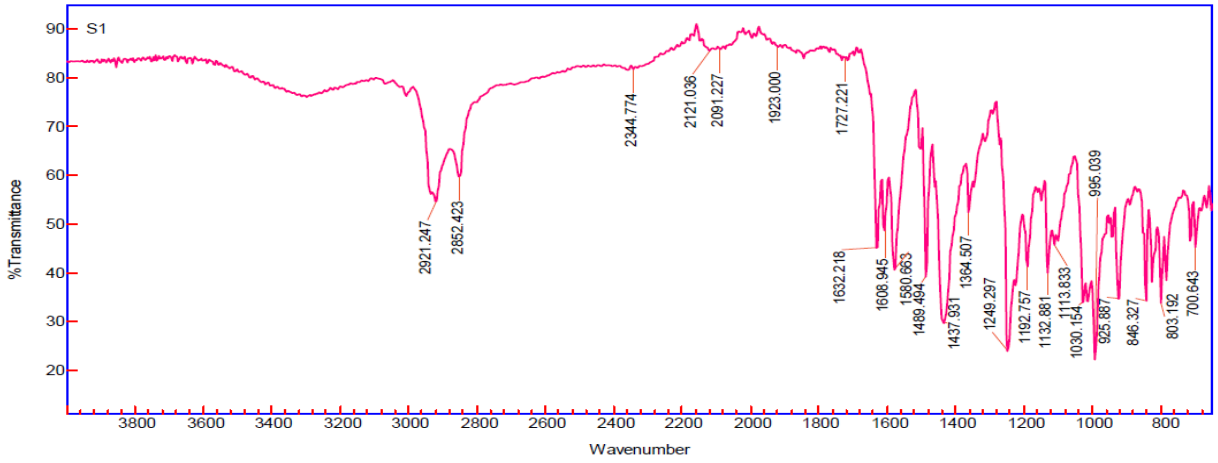


Fig 29: FT-IR spectra of pepper extract

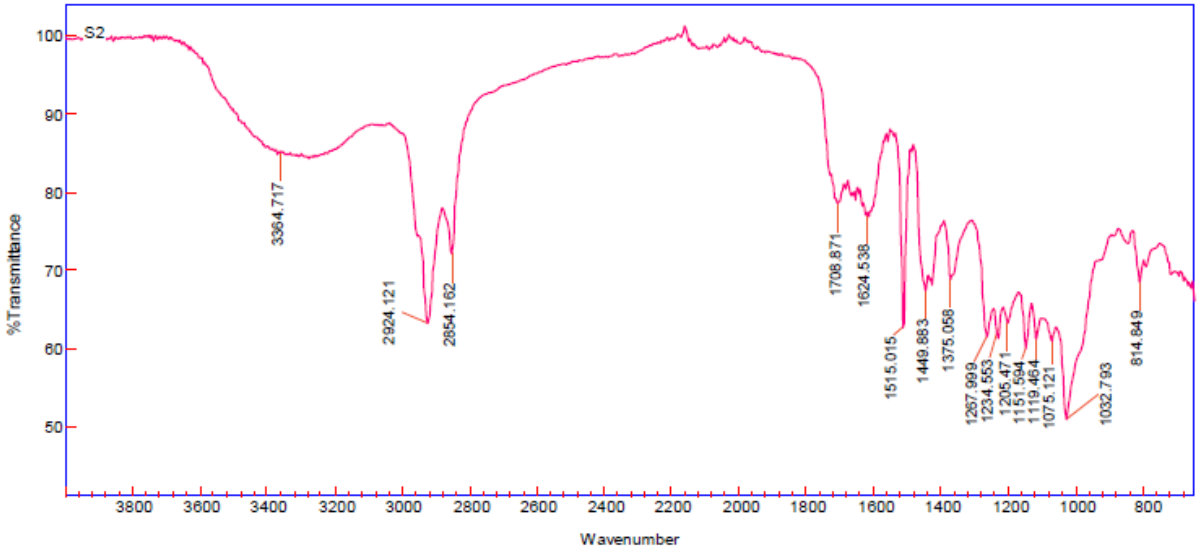


Fig 30: FT-IR spectra of zinger extract

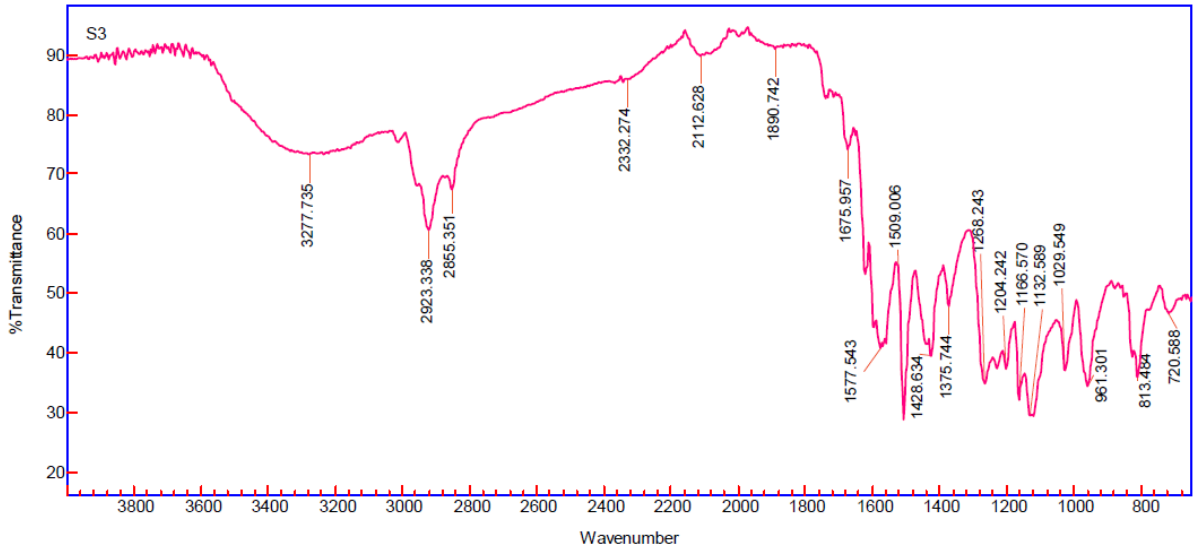


Figure 31: FT-IR spectra of turmeric extract

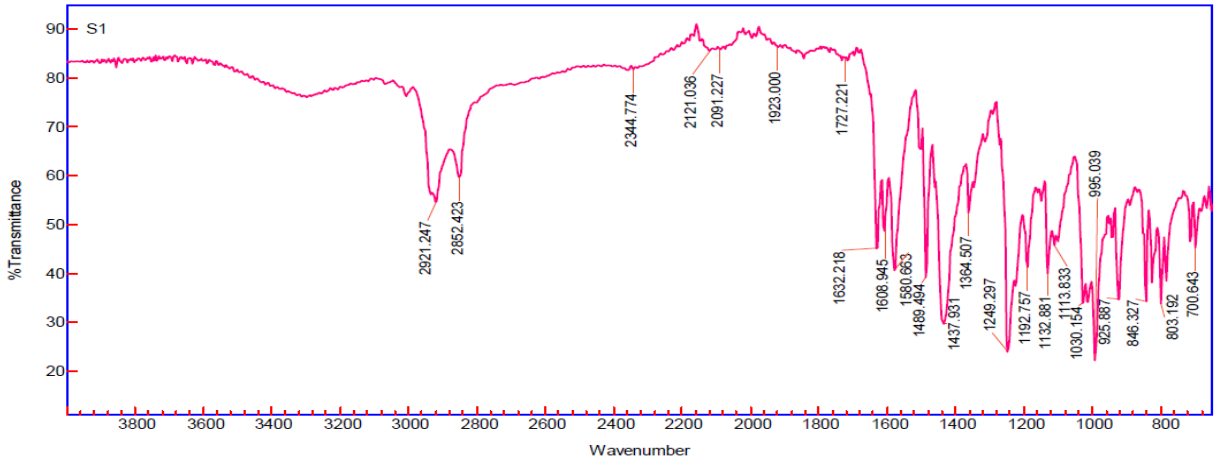


Figure 32: FT-IR spectra of cumin extract

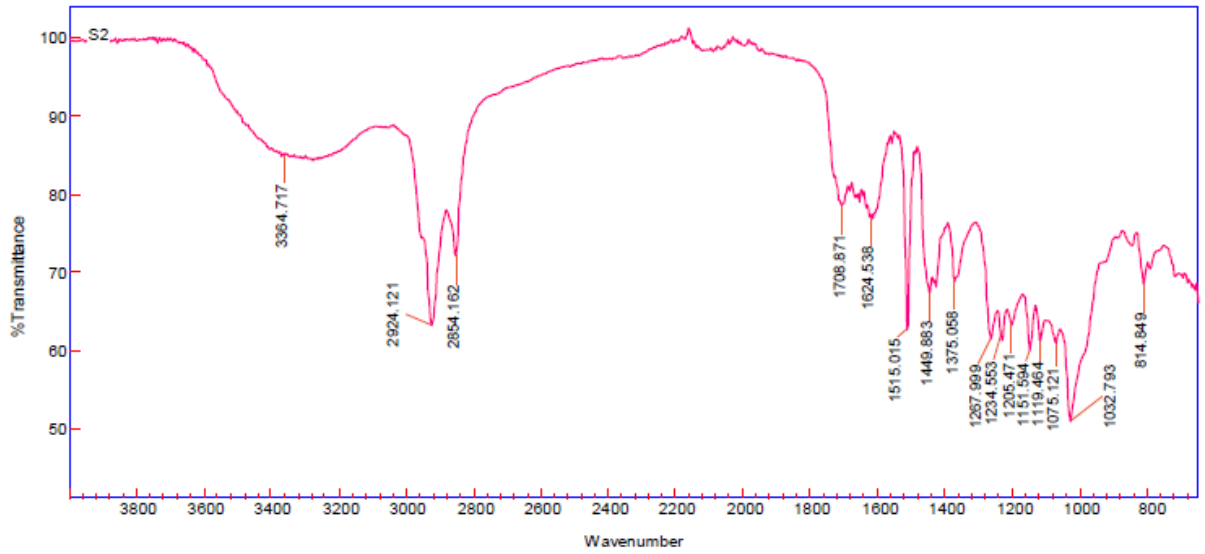


Figure 33: FT-IR spectra of Pomegranate juice

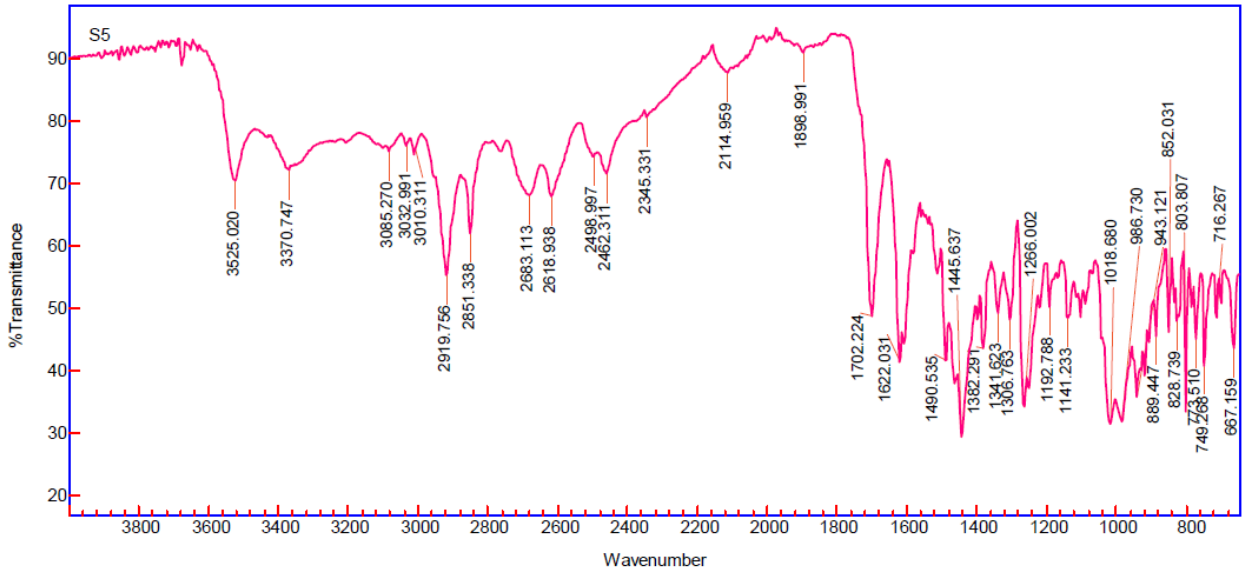


Figure 34: FT-IR spectra of combination of ciprofloxacin, verapamil hydrochloride,pepper, zinger, turmeric, cumin and pomegranate juice

Table 30: FT-IR Characteristic peak of Pure drug (Ciprofloxacin), sample and Drug with Sample

SL. No.	Functional Group	IR Range (cm^{-1})	IR Observed Peaks							
			Pure drug	Verapamil hydrochloride	Pepper	Turmeric	Zinger	Cumin	Pomegranate juice	Mixture
1	O-H	3500-3400	3531	-	-	3277.7	3364.71	3333.2	3387.36	3525.02
2	Ar-H ,υ=CH	3000-2950	2930	2948.25	2921.24	2923.3	2924.12	-	-	2919.75
3	C=O	1750-1700	1701.6	1718.2	1727.22	-	1708.87	-	1738.55	1702.22
4	δN-H	1650-1600	1621.0	-	1632.21	1675.9	1624.53	-	-	1622.03
5	δC-O	1450-1400	1442.4	-	1437.93	1428.6	1449.88	-	-	1445.63
6	δO-H	1300-1200	1307.1	-	1249.29	1268.2	1267.95	-	-	1266.02
7	C-F	1050-1000	1045.3	-	1030.15	1029.5	1075.12	-	-	1018.68

5.3 EVALUATION OF BLENDED CHARACTERISTICS OFCIPROFLOXACIN

Table 31 FORMULATION

Bulk density gm/ml (mean± sd)	0.496±0.004
Tapped density gm/ml (mean± sd)	0.686±0.017
Carr's index (mean± sd)	14.81±1.38
Haunser's ratio (mean± sd)	1.46±0.025
Angle of repose (°)	23.6±0.93

5.4. POST- COMPRESSION EVALUATION PARAMETERS

Table 32:Post- compression parameter results

Weight Variation mean± sd (g)	0.721±0.003
Hardness mean± sd (kg/cm^2)	4.13±16
Friability mean± sd (%)	0.19±0.010
Drug contain mean± sd (%)	99.58±0.70
Thickness mean± sd (mm)	3.01± 0.02
Disintegration time mean± sd (sec)	14± 0.97

5.5*IN-VITRO*DRUG RELEASE STUDIES:

Table 33: Cumulative % of drug release

Time in minutes	% of cumulative Release
0	0
15	29.68
30	61.32
45	75.59
60	87.14
75	92.88
90	99.15

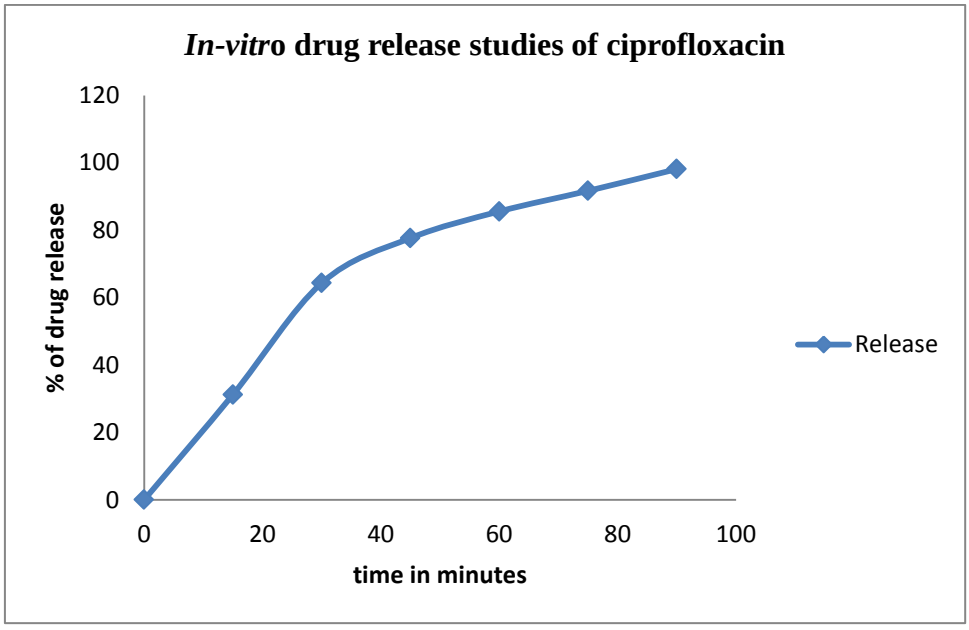


Figure 11. Cumlative % drug release of formulations

Table 34:Mathematical modelling and drug release kinetics of Ciprofloxacin

Drug release kinetics (R^2)				Release exponential(n)
Zero order	First order	Higuchi	Korsmeyer	
0.901	0.924	0.958	0.972	0.926

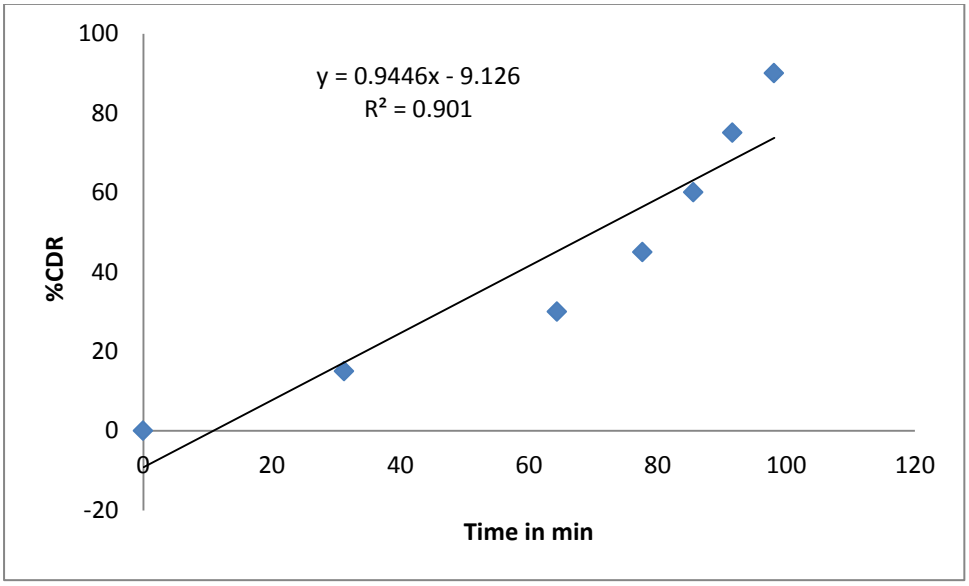


Figure35. Zero Order release profile of formulations

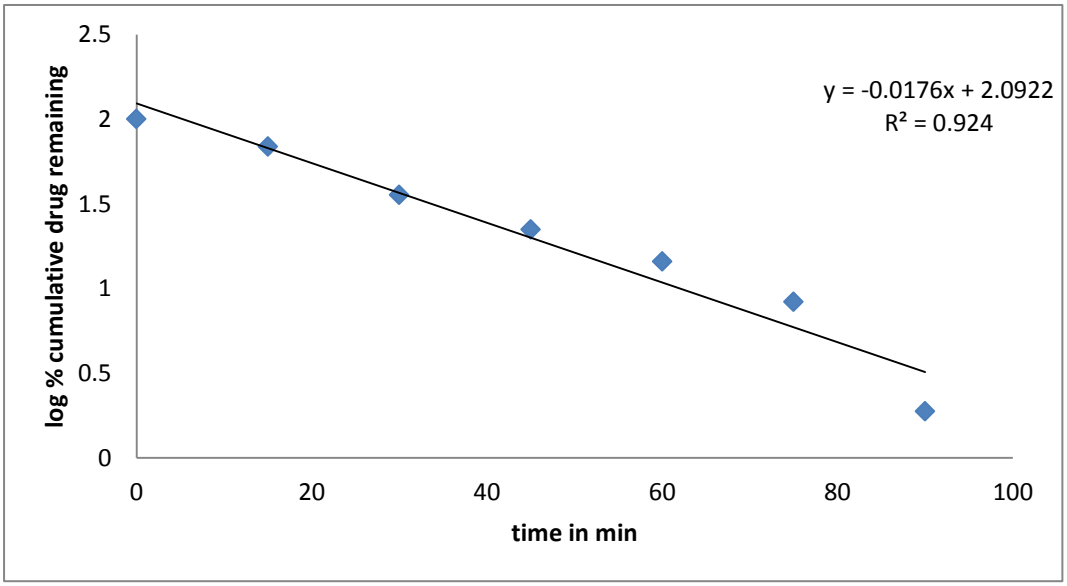


Figure 36: First Order release profile of formulations

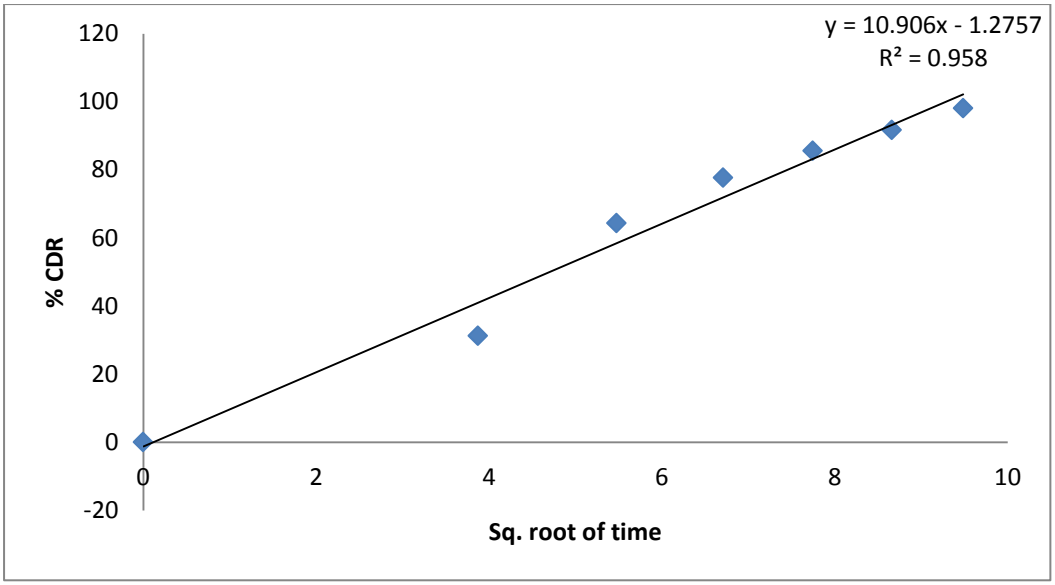


Figure 37: Higuchi release profile of formulations

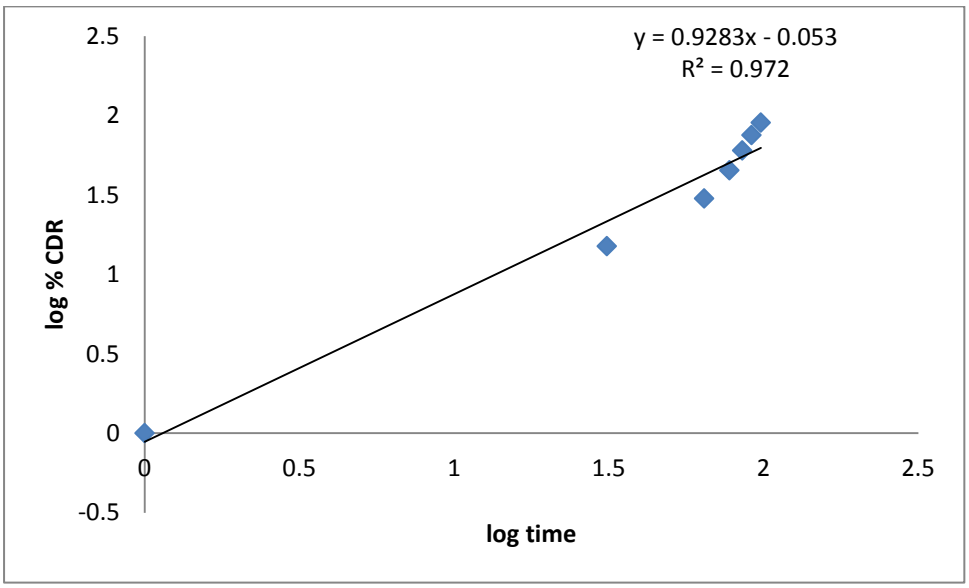


Figure 38:Korsmeyer release profile of formulations

5.7 STABILITY STUDIES:

After the stability studies the formulations were subjected to content estimation and the results shown in table

Table 35: Drug content data of stability study

S NO.	TIME IN DAY	FORMULATIONS (Content estimation in %)
1	15	99.25
2	30	99.69
3	45	98.88
4	60	99.37

After the stability studies the formulations were subjected to *in vitro* dissolution and the results shown in table

Table 36.*In-vitro* drug release of tablets stability study of formulation

Time in min	Before stability studies	After stability studies
0	0	0
15	29.68	30.01
30	61.32	62.57
45	75.59	76.48
60	87.14	86.98
75	92.88	91.52
90	99.15	98.97

Chapter 6

Discussion

DISCUSSION

Permeability-glycoprotein (P-gp) is an energy dependent transporter protein located in the apical membrane of intestinal mucosal cells. P-gp is associated with the multidrug resistance in tumor cell, but also expressed in a variety of normal human tissues including liver, brain, kidney, gastrointestinal tract, intestine, adrenal gland, blood-brain barrier, placenta, blood-testis, blood-ovarian barriers and in various tumors. At the intestinal level, P-gp is located on the apical membrane of the mature intestinal cells and acts as a pump that transports drugs back into the lumen as they are absorbed across the intestinal mucosa. Inhibition of P-gp is said to increase the bioavailability of variety of drugs as well as food nutrients by inhibiting the back efflux into the intestine.

This project is designed primarily to screen the candidates which possess P-gp inhibition property using everted rat intestine model. Secondary objective of this project is to formulate and evaluate selected P-gp inhibitor into a tablet dosage form along with the substrate. Six plants or its extracts based on the folklore use and literature were chosen to screen for P-gp inhibition activity. Verapamil, a known P-gp inhibitor and Ciprofloxacin, a known P-gp substrate are chosen as a model drugs for the experiment.

Ciprofloxacin and Verapamil were found to water soluble; absorption maxima of both drugs were determined in distilled water and Krebs Ringer solution (KRS) and were found to be 276nm and 278 nm respectively. Calibration curves of both the drugs were prepared (1-6 mcg/ml) in both the solutions and linearity was seen with 0.99+ regression coefficient. As the absorption maxima of both the drugs were in the same range (276 and 278 nm), in order to avoid bios in drug estimation when both drugs were present, a

separate oxidometric titration method for the estimation of Verapamil was adopted. Graph was found to be linear with 0.99+ regression coefficient.

Powdered roots of *Curuma longa* and *Zingibar officinale,* dried powdered seeds of *Piper nigrum and cumin and* dried powdered leaves *of Moringa olifera,* were extracted using 99% ethanol and concentrated using rotator evaporator. Fresh juice of pomegranate was taken during the experimentation. Verapamil with Ciprofloxacin was used as reference standard (known P-gp inhibitor).

Optimized experimental procedure using everted intestinal sac was adopted to determine the flux (dQ/dt) and Permeability coefficient (Paa) for the drug transport in presence and absence of test samples. Parameters such as evertion of intestine, choosing suitable length, operating temperature, aeration technique, and duration of the experiment, tissue viability and addition of indicators were standardized by trial and error method.

An everted segment of 3cm was chosen and tied both sides to the modified apparatus. Blank KRS was filled inside the apparatus (serosal side) and Ciprofloxacin solution (0.5 or 1.0 mg/ml) was filled in the outer part (Mucosal side) along with Phenol red as an indicator. Drug transport from mucosal side to serosal side is estimated by sampling the drug solution from serosal side periodically with an interval of 5 min. further the drug concentration is determined at 276 nm after suitable dilution. The experiment was repeated with the addition of test extracts / standard drug in the mucosal side along ciprofloxacin. Two sets of experiments were done using the same test samples with two different concentrations of drugs in mucosal side (0.5 and 1.0 mcg/ml) and compared the results. As Phenol red is impermeable and stays in mucosal side always. Appearance of

traces of Phenol red in the serosal solution indicates the puncture of the intestine and can be detected by measuring the absorbance at 560nm.

Results indicated that all test samples and Verapamil showed better absorption than ciprofloxacin alone in both concentrations. However the Paa was shown in the following decreasing order in combination with ciprofloxacin

Pepper extract > Pomegranate Juice > Cumin extract > Turmeric extract > Zinger extract > Verapamil> *Moringa olifera* extract > ciprofloxacin alone

Permeation was increased at least 1.5 times to 3.5 folds. Amongst the samples, Pepper extract, Pomagranate juice, Cumin extract, showed more than 3 folds; Turmeric extract, more than 2 folds; Zinger extract and Verapamil showed more than 1.5 folds increase in the permeation in 30 min. These results showed that the test samples are Bio-enhancers and are potential candidates to improve the bioavailability of most of the drugs. In order to notice the effect of test samples on the mucosal side of the everted intestine, Histological studies are carried out. In this study, samples were examined for any change in epithelium, lamina propria, sub mucosal cells of mucosal layer and changes in muscularies externa. Pepper extract, curcumin extract, drumstick extract showed slight changes in the goblet cells however no significant change was noticed in muscularies externa. In rest of the samples sight variation was observed in the arrangement of epithelial cells indicating no significant interaction.

From the above results, Ciprofloxacin tablets were prepared and evaluated by incorporating the bio-enhancers by wet granulation method. Ciprofloxacin (300mg) extracts of Pepper, Turmetic, Zinger, cumin and drumstick (50mg each) tablets were compressed along with the other excipients such as lactose (diluents), PVP-K 30 (binder),

magnesium sterate and talc (flow property enhancers). Drug -extract and drug-excipient intraction was determined by FTIR studies. Pomagrante Juice, if added in the tablet formulation, resulted stink smell after 3-4 days of compression, hence pomegranate juice was omitted though it showed a potential bio-enhancer property. Granules were prepared and evaluated. Flow property of the granules was found to good. The bulk density, tapped density and cars index was found to be 0.496 gm/ml, 0.686 mg/ml and 14.81 respectively. The Hunsers ratio and angle of repose was 1.46 and 23.6^{o} respectively. Tablets were punched and post compression parameters were evaluated. The hardness of the tablet was about 4 Kg and friability was below 0.18 %. The drug content was above 98% well within the specified limit. Disintegration time was found to below 15min. *In vitro* release studies showed 90 % release in 75 min.

The release kinetics of tablet was fitted into zero, first, Higuchi and Korsmeyer models where 'r' was for first order release, zero order and Higuchi's was 0.924, 0.901 and 0.958. It was understood to be predominant Higuchi's release pattern. Further, to understand the drug release mechanism, the data were fitted into Peppas exponential model $M_t/M_a=Kt^n$, where M_t/M_a is the fraction of drug released after time 't' and 'K' is kinetic constant and 'n' is release exponent which characterizes the drug transport mechanism. The values 'n' was in the 0.928. The formulation indicating Class II transport release mechanism ('n' values is n>0.89).

FTIR studies indicated no significant interaction between drug- extracts and drug-excipients. Ciprofloxacin showed characteristic peaks at 3531.004m^{-1} due to hydroxyl group O-H stretching vibration, intermolecular H-bonded, 2930.54cm^{-1} due to Aromatic, cyclic enes, 1701.62 cm^{-1} due to CO group of acid peak assignment C=O stretching

vibration, at 1621.06 indicate quinolines because δN-H bending vibration, at 1492.42 indicate carbonyl group because υC-O, at 1307 .19 peak assignment by δO-H bending vibration and at 1023.33 indicate fluorine group due to C-F stretching. Moreover, same peaks were observed for the mixture of drug with different herbal extract Pepper, Turmeric, Zinger, and cumin. Hence, it was found that all the herbal extract used in formulations were compatible with Ciprofloxacin.An accelerated stability study as per ICH norms was performed for the formulation for 60 days at, 40^0C/75% RH. The stability of the tablet was found be in limit and observed no change in the physical appearance, release nature and drug content.

In summary, bio-enhancers can be included along with the drug to improve bio – availability. Further certain food influences the absorption of most of the dugs hence drug food interaction must be considered while prescribing the dose.

Chapter 7

Conclusion & Summary

SUMMARY AND CONCLUSION

Permeability-glycoprotein (P-gp) is located on the apical membrane of the mature intestinal cells and acts as a pump that transports drugs back into the lumen as they are absorbed across the intestinal mucosa. Inhibition of P-gp is said to increase the bioavailability of variety of drugs as well as food nutrients by inhibiting the back efflux into the intestine.

The primary objective of the study is to screen the candidates which possess P-gp inhibition property using everted rat intestine model. Secondary objective of this project is to formulate and evaluate selected P-gp inhibitor into a tablet dosage form along with the substrate.

- Powdered roots of *Curuma longa* and *Zingibar officinale,* dried powdered seeds of *Piper nigrum and cumin and* dried powdered leaves *of Moringa olifera,* were extracted using 99% ethanol and concentrated using rotator evaporator. Fresh juice of pomegranate was taken during the experimentation. Verapamil with Ciprofloxacin was used as reference standard (known P-gp inhibitor).
- Verapamil, a known P-gp inhibitor and Ciprofloxacin, a known P-gp substrate are chosen as a model drugs for the experiment.
- Calibration curves of Ciprofloxacin and Verapamil drugs were prepared (1-6 mcg/ml) in water and krebs ringer solution and linearity was seen with 0.99+ regression coefficient.
- As the absorption maxima of both the drugs were in the same range (276 and 278 nm), in order to avoid bios in drug estimation when both drugs were present, a separate oxidometric titration method for the estimation of Verapamil was adopted. Graph was found to be linear with 0.99+ regression coefficient.

- Experimental Parameters such as evertion of intestine, choosing suitable length, operating temperature, aeration technique, and duration of the experiment, tissue viability and addition of indicators were standardized by trial and error method.
- Flux (dQ/dt) and Permeability coefficient (Paa) for the drug transport was determined in presence and absence of test samples
- Results indicated that all test samples and Verapamil showed better absorption than ciprofloxacin alone in both concentrations.
- Paa was shown in the following decreasing order in combination with ciprofloxacin **Pepper extract > Pomegranate Juice > Cumin extract > Turmeric extract > Zinger extract > Verapamil> *Moringa olifera* extract > ciprofloxacin alone**
- Permeation was increased at least 1.5 times to 3.5 folds.
- Amongst the samples, Pepper extract, Pomagranate juice, Cumin extract, showed more than 3 folds; Turmeric extract, more than 2 folds; Zinger extract and Verapamil showed more than 1.5 folds increase in the permeation in 30 min.
- The results showed that the test samples are Bio-enhancers and are potential candidates to improve the bioavailability of most of the drugs.
- Histological studies showed few changes in epithelium, lamina propria, sub mucosal cells of mucosal layer and changes in muscularies externa.
- Pepper extract, curcumin extract, drumstick extract showed slight changes in the goblet cells
- No significant change was noticed in muscularies externa.
- Ciprofloxacin tablets were prepared and evaluated by incorporating the bio-enhancers by wet granulation method.

- Ciprofloxacin (300mg), extracts of Pepper, Turmetic, Zinger, cumin and drumstick (50mg each) tablets were compressed along with the other excipients such as lactose (diluents), PVP-K 30 (binder), magnesium sterate and talc (flow property enhancers).
- FTIR studies depicts no Drug -extract and drug-excipient intraction was found.
- Pomagrante Juice, if added in the tablet formulation, resulted stink smell after 3-4 days of compression, hence pomegranate juice was omitted though it showed a potential bio-enhancer property
- Pre compression and post compression parameters were found be good and were within the limit of pharmacopeia.
- An accelerated stability studies at for 60 days at, 40^0C/75% RH indicated no significant change in physical appearance, release nature and drug content.
- In summary, bio-enhancers can be included along with the drug to improve bio – availability.
- Further certain food influences the absorption of most of the dugs hence drug food interaction must be considered while prescribing the dose.

Chapter 8

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