Evaluation of Topical Formulations of Selective Cyclooxygenase-2(COX-2) Inhibitors for Analgesic and Anti-inflammatory Efficacy as well as Plasma Concentration Profile

THESIS

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DOCTOR OF PHILOSOPHY

By

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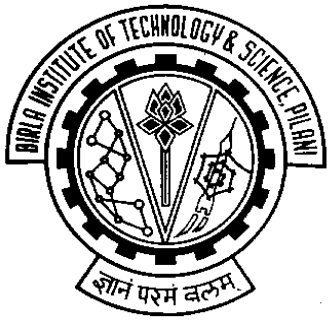

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CERTIFICATE

This is to certify that the thesis entitled "**Evaluation of Topical Formulations of Selective Cyclooxygenase-2(COX-2) Inhibitors for Analgesic and Anti-inflammatory Efficacy as well as Plasma Concentration Profile**" which is submitted by **Shirumalla Raj Kumar,** ID No. **2001PHXF008** for award of Ph.D. degree of the institute, embodies original work done by him under my supervision.

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Shirumalla Raj Kumar

List of Tables

List of Figures

List of Abbreviation

APPROVe	: Adenomatous Polyp Prevention on Vioxx
AUC	: Area under the curve
CABG	: Coronary Arteries Bypass Grafting
CLASS	: Celecoxib Long-Term Arthritis Safety Study
cm^2	: Centimeter square
C_{Max}	: Maximum plasma concentration
CNS	: Central Nervous System
Conc.	: Concentration
COX	: Cyclo-oxygenase
COX-1	: Cyclo-oxygenase-1
COX-2	: Cyclo-oxygenase-2
COX-3	: Cyclo-oxygenase-3
CPMP	: Committee for Proprietary Medicinal Products of the EMEA
cps	: Centi poise
DP	: Prostaglandin D_2 receptor
EDGE study	: Etoricoxib Diclofenac Gastrointestinal Evaluation
EGF	: Epidermal Growth Factor
EMEA	: European Medicines Agency
EP	: Prostaglandin E_2 receptor
ERK	: Extracellular signal-Regulated Kinase
FAP	: Familial Adenomatous Polyposis
FDA	: Food and Drugs Administration, USA.
FGF	: Fibroblast growth factor
FP	: Prostaglandin $F_{2\alpha}$ receptor
G	: Gram
GFR	: Glomerular Filteration Rate
GI	: Gastrointestinal
Hrs	: Hours
IASP	: International Association for the Study of Pain
IL-1β	: Interleukin 1β

IPA	: Isopropyl alcohol
IP	: Prostacyclin receptor
µm	: Micrometer
µM	: Micromolar
mg	: Milligram
min	: Minutes
ml	: Millli liter
NSAIDs	: Non-Steroidal Anti-inflammatory Drugs
OA	: Osteoarthritis
OTC	: Over the counter
PDGF	: Platelet-Derived Growth Factor
PG	: Prostaglandin
PGE_2	: Prostaglandin E2
PGI_2	: Prostaglandin I2 (Prostacyclin)
RA	: Rheumatoid arthritis
RCS	: Rabbit Aorta Contracting substance (Thromboxane A2)
RRL	: Ranbaxy Research Laboratories
SEM	: Standard error
SPID	: Sum Pain Intensity Difference Score
sPLA2	: Soluble Phospholipase A2
TAMC	: Total Aerobic Microbial Count
TARGET	: Therapeutic Arthritis Research and Gastrointestinal Event Trial
TGA	: Therapeutic Goods Administration, Australia
TGF-α	: Tumor Necrotic Factor-α
TIS	: Total Irritation Score
TOTPAR	: Total Pain Relief Score
TP	: Thromboxane receptor
TPA	: Tetradecanoyl phorbol acetate
TxA_2	: Thromboxane A2
TxB_2	: Thromboxane B2
TYMC	: Total Combined Yeasts/Moulds count
USA	: United States of America

USP	: United States Pharmacopoeia
UV	: Ultraviolet
VIGOR	: Vioxx Gastrointestinal Outcomes Research
WOMAC	: Western Ontario McMaster Osteoarthritis Questionnaire
w/w	: Weight by weight

Abstract

Selective cyclo-oxygenase-2 (COX-2) inhibitors have been in use as analgesic and anti-inflammatory agents for over a decade. In addition they have been shown to have beneficial effect in cancer chemotherapy. However, COX-2 inhibition has been associated with an increased risk of cardiovascular as well as other adverse events in several clinical trials. Delivery of coxibs by topical route can be useful to avoid the adverse drug effects, without losing the beneficial effects of the coxibs. Topical formulations of Rofecoxib and Celecoxib were prepared, evaluated for its acceptability, efficacy and safety. These were tested for physical appearance, pH, spreadability, drug content uniformity and *in vitro* diffusion. Emulsion gel formulations were prepared containing 1-3% Rofecoxib or 3-5% Celecoxib (w/w) along with optimized amounts of Carbopol 940, Labrasol and Ethanol. Use of sub-micronised Rofecoxib/ Celecoxib in the above formulation (FG14/ FG30, respectively) resulted in improved *in vivo* efficacy. Selected formulations FG14 and FG30 were evaluated in models of acute and chronic inflammation as well as hyperalgesia. Comparison of systemic exposure following topical as well as oral dosing by determining plasma concentrations of Rofecoxib/ Celecoxib in test animals, determination of skin irritation, gastic ulceration or other systemic adverse effect potential after chronic exposure and determining of *ex-vivo* COX-1 and COX-2 inhibition potential in rat blood were conducted to study the safety of topical gels. 1% (w/w) Rofecoxib gel, FG14 and Celecoxib gel containing 5% (w/w) of the drug, FG30, was significantly ($p < 0.05$) more effective in inhibiting inflammation and hyperalgesia associated with inflammation, compared to placebo gel, in acute and chronic model. However, these gel formulations were not effective in centrally mediated model such as, phase –I pain in formalin induced pain model and rat tail flick model. Topical administration of Rofecoxib/ Celecoxib used in the formulation minimizes the risk of systemic effects, as shown by much lower systemic exposures than oral drug at equiactive doses; hence, this may be the alternative to oral preparations. In addition 28 day application of the gels was well tolerated with no effect on skin as well as systemic adverse effects. Determination of *ex-vivo* inhibition of COX -1 and COX-2 in these animals also revealed an improvement in safety window with topical administration

Table of Contents

1.0 LITERATURE SURVEY AND RESEARCH OBJECTIVES

1.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed medications for the inflammation of arthritis and other body tissues, such as in tendinitis and bursitis. NSAIDs are taken regularly by approximately 30 million people worldwide with a worldwide market of US$ 30.5 billion in 2008.

NSAIDs were first introduced in more than hundred years ago and till now more than thirty NSAIDs have been developed, such as phenybutazone, indomethacin, ibuprofen, naproxen, piroxicam, and nabumetone. In 1971, Sir John vane proposed that NSAIDs suppress prostaglandin syntheses by antagonising the action of Cyclooxygenase (COX) (Vane, 1971).

In 1990s two isoforms of COX were identified, COX-1 and COX-2. COX-1 in most tissues is constitutively expressed and functions to maintain normal physiological processes while COX-2 is predominantly induced following inflammatory stimuli and play an important role in mediating it (Reed *et al.*, 1996). Thus COX-1 enzyme of the stomach ensure the natural mucus lining which protects the inner stomach, so when COX-1 enzyme is blocked, the protective mucus lining of the stomach is also reduced, which can cause stomach upset, ulceration, and bleeding from the stomach and intestines. However, blocking COX-2 enzyme, which is responsible for inflammation, reduces inflammation. Since the COX-2 enzyme does not play a role in the normal function of the stomach or intestinal tract, medications which selectively block COX-2 do not present the risk of injuring the stomach or intestine.

The major side effects of nonselective NSAIDs as explained above are to the gastrointestinal system. Some 10 - 50% of patients are unable to tolerate NSAID treatment because of side effects, including abdominal pain, diarrhea, bloating, heartburn, and upset stomach (dyspepsia). Approximately 20% of patients on long- term NSAID treatment develop ulceration of the stomach and duodenum (Jackson and Hawkey, 2000). Even though many of these patients with ulcers do not have symptoms and are unaware of their ulcers, they are at risk of developing serious ulcer complications such as bleeding or perforation of the stomach. Thus the newer NSAIDs such as Celecoxib and Rofecoxib (coxibs), having selective COX-2 inhibitory activity were introduced as an effective and safer alternative to the widely used nonselective NSAIDs. Although, introduced with a limited indication specifically for treating

signs and symptoms of osteoarthritis, rheumatoid arthritis, and dysmenorrheal and managing acute pain mainly in patients with high risk of gastrointestinal side effects. As initial clinical data suggested better GI tolerance for coxibs as compared to nonselective NSAIDs, their usage increased considerably not only in high risk groups but more so in individuals at low risk for GI side effects **(**Becker, 2005). The high usage profile of coxibs in general population brought to light several adverse pharmacological effects associated with NSAIDs and specifically related to COX-2 inhibitors usage. Based on series of published reports, it now appears that at best, coxibs cause significant ulceration at about half the rate of conventional NSAIDs (Bombardier *et al*., 2000) with a heightened risk in patients with history of gastric bleeding. A series of discoveries have strongly suggested a crucial role of COX-2 in GI mucosal defense and repair.

COX-2 inhibitors also inherit the renal effects of traditional NSAIDs, particularly in patients considered at risk of adverse renal effects related to use of NSAIDs such with renal impairment or in condition of dehydration or high salt intake (Brater, 1999).

There have been some reports of adverse psychiatric effects associated with COX-2 owing to the role of COX-2 in generation of prostaglandins in central nervous system (Coulter, 2002).

Apart from these adverse effects the most notorious adverse effect reported is the effect on the cardiovascular system. This cardiovascular risk differs, to some degree, across agents, and does appear to be dose related. The relationship between cardiovascular risk and duration of therapy has also emerged as an important factor. Early risk, from the perspective of pathobiology, may differ from long-term risk. The mechanism of cardiovascular risk appears to be multifactorial and relates to sites of COX-2 synthesis, expression within the vasculature, and related local consequences of an imbalance between thromboxane A2 and prostacyclin. Considered collectively, increased platelet aggregation, hypertension, endothelial cell dysfunction, impaired angiogenesis, and destabilization of the atherosclerotic plaque matrix are important contributors to the "prothrombotic environment" (Grosser *et al.*, 2006). Several randomised Clinical trials, of prolonged treatment with coxibs have demonstrated these effects **(**Bresalier *et al*.**,** 2005**;**Mukherjee *et al*. 2001;Bombardier *et al.*, 2000;Silverstein *et al*., 2000**)** which eventually lead to withdrawal of several selective COX-2 inhibitors from clinical use and revised labeling for the rest. The risk of complications is higher in elderly patients, rheumatoid arthritis sufferers, patients taking blood thinning medications

(anticoagulants such as Coumarins and heparin) or prednisone (cortisone medication), and patients with heart disease or a prior history of bleeding ulcers.

However, delivery of coxibs by topical route can be useful to avoid the adverse drug effects, without losing the beneficial effects of the Coxibs. Topical drug delivery offers s102everal advantages such as: (i) avoidance of the gastric route, reducing the potential for both degradation of the drug and gastric irritation; (ii) high therapeutic tissue *vs* plasma concentration can be obtained thereby achieve therapeutic concentrations in the tissues subjacent to the site of application while maintaining low plasma concentrations thus reduce the risk of serious adverse events related to elevated plasma concentrations; (iii) improve patient compliance with drug administration; (iv) may be cost effective on long term.

1.2 Non-steroidal Anti-Inflammatory drugs: History, mechanism, pharmacology and clinical issue

1.2.1 Developments of NSAIDs: A Historical prospective

Salicylic acid and salicylates are constituents of several plants that have long been used as medicaments. Salicylic acid was chemically synthesized in 1860 in Germany and its ready supply led to extended use as an external antiseptic, as an antipyretic and in the treatment of rheumatism. Subsequently, Bayer made a more palatable form of salicylate, acetylsalicylate or aspirin, to treat severe rheumatism (Vane *et al*., 1990). By the early 1900s, the main therapeutic actions of aspirin (and sodium salicylate itself) were recognized as being antipyretic, anti-inflammatory and analgesic. Over time, several other drugs were discovered that shared some or all of these actions, including antipyrine, phenacetin, acetaminophen (paracetamol), phenylbutazone, and more recently the fenamates, indomethacin, ibuprofen, and naproxen. As a result of their similar therapeutic actions, these drugs tended to be regarded as a group and generally became known as the ‘‘aspirin-like drugs’’. Furthermore, as these drugs were clearly distinct from the glucocorticosteroids (the other major group of agents used to treat inflammation) they were also classed as the ‘‘Nonsteroidal Anti-Inflammatory Drugs’’ (NSAIDs) (Flower, 1974).

Alongside the development of Aspirin, Pyrazolone class of NSAIDs was also developed independently. In fact the earliest Pyrazolones- Phenazone and Amidopyrine were developed before Aspirin and were great commercial success until Aspirin was launched. The main side

effects are agranulocytosis, which led to development of newer Pyrazolone - Phenylbutazone and Oxyphenbutazone after the II world war. They had better efficacy than aspirin in treating acute inflammatory events but were more toxic to bone marrow than their predecessors, so are relegated to treat acute inflammation (Boynton *et al*., 1988).

In the late 1950s mefenamic acid, a Fenamate was identified. Mefenamic Acid and its successors Flufenamic acid, Diclofenac and Meclofenamate are powerful anti-inflammatory agents but are associated with diarrhea and occasional development of colitis which has limited their use only in rare case when other NSAIDs fail**.** In 1963, Indomethacin was identified from indole derivatives. Indomethacin had superior anti-inflammatory activity and has become standard for comparison. It has become drug of choice for ankylosing spondylitis, psoriatic arthritis and Reiter's syndrome. Side effects are rare, prominently related to CNS. Other Indoles launched are Sulindac, which had lower toxicity. The indole ring was replaced by pyrrole ring to develop Tolmetin and Zomepirac. After the spectacular success of Indomethacin in 1960s, Ibufenac was launched but was withdrawn because of serious hepatotoxicity (1% incidence). It was superseded by Ibuprofen, a propionic acid derivative which showed better-tolerance was considered safer as an OTC preparation. Other members in this class were Flubiprofen, Fenoprofen, Ketoprofen and Carprofen. These were followed by Oxicams which offered potent activity and better half-life. *Piroxicam* introduced in 1979 has half-life of 45 hrs, with once daily dosing (Boynton *et al*., 1988).

Despite the diversity of their chemical structures, these drugs share the same therapeutic properties and similar side-effect profile. When a chemically diverse group of drugs all share not only the same therapeutic qualities (which in themselves do not have much connection with each other) but also the same side-effects, it is fairly certain that the actions of these drugs are based on a single biochemical intervention.

1.2.2 NSAIDs and their mechanism of action

Before 1971, little was known about the mechanism of action of NSAIDs except that they produced an anti-inflammatory effect that was qualitatively and quantitatively different from the more potent anti-inflammatory glucocorticosteroids. Piper and Vane presented experimental evidence to show that the release of rabbit aorta contracting substance (RCS) from guinea pig isolated lungs during anaphylaxis was blocked by aspirin (Piper and Vane,

1969). This and other clues led Sir John Vane to propose that NSAIDs act by suppression of prostaglandin syntheses (Vane, 1971). Two other papers appearing in the same issue lent support to his finding and extended it considerably (Smith and Willis, 1971; Ferreira *et al*., 1971). All three studies, originating from the same department, demonstrated that aspirin reduced prostaglandin release which resulted in pharmacological responses.

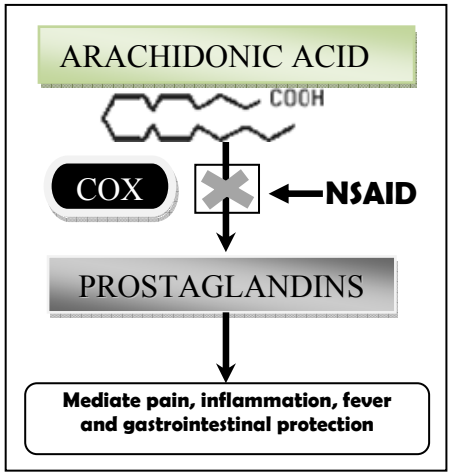


Fig 1. Mechanism of action of NSAID's

1.2.3 Cyclooxygenase Biology

A homogeneous, enzymatically active cyclooxygenase (COX) or prostaglandin endoperoxide synthase was isolated in 1976 (Hemler *et al.*, 1976). This membrane-bound hemo- and glycoprotein was found in greatest amounts in the endoplasmic reticulum of prostanoid-forming cells (Smith, 1986). The glycoprotein was shown to exhibit COX activity— both cyclized arachidonic acid and added the 15-hydroperoxy group to form prostaglandin G_2. The hydroperoxy group of prostaglandin G_2 is known to be reduced to the hydroxy group of prostaglandin H_2 by a peroxidase that utilizes a wide variety of compounds to provide the requisite pair of electrons. Both COX and hydroperoxidase activities were contained in the same dimeric protein molecule.

Given the broad role prostaglandin's play in normal human physiology, systemic suppression of PGs lead to unwanted side-effects. In particular, even short exposure to NSAIDs causes gastric and renal side effects. As many as 25% individuals using NSAIDs experience some type of side effects and as many as 5% develop serious health consequences. The different effects of PGs can be explained by considering their varied chemistry, the diversity of PG receptors and modulation of PG synthesis by local and their upstream and downstream effects. Fig. 2 depicts the various roles and effects of PGs in different organs.

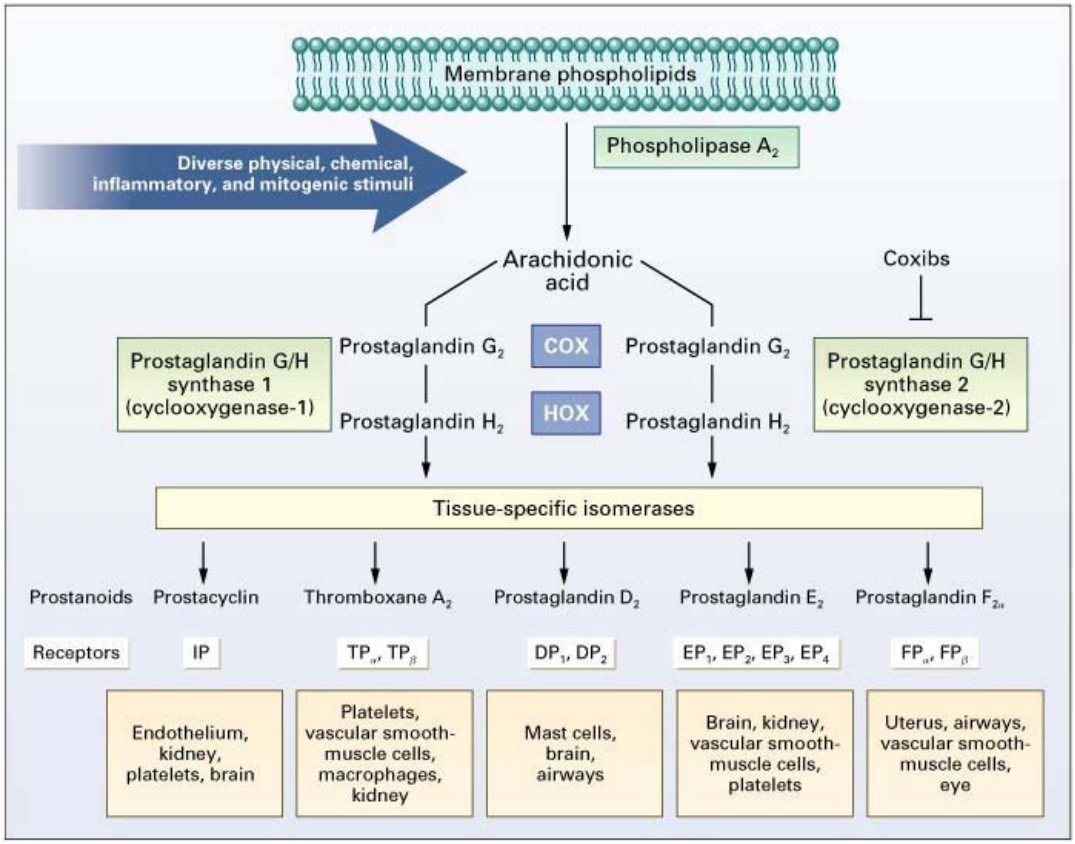


Fig 2. Production and Actions of Prostaglandins and Thromboxane.

Arachidonic acid, a 20-carbon fatty acid containing four double bonds, is liberated from the sn2 position in membrane phospholipids by phospholipase A_2, which is activated by diverse stimuli. Arachidonic acid is converted by cytosolic prostaglandin G/H synthases, which have both cyclooxygenase (COX) and hydroperoxidase (HOX) activity, to the unstable intermediate prostaglandin H_2. The synthase enzymes are colloquially termed cyclooxygenases and exist in two forms, cyclooxygenase-1 and cyclooxygenase-2. Coxibs selectively inhibit cyclooxygenase-2. Prostaglandin H_2 is converted by tissue-specific isomerases to multiple prostanoids. These bioactive lipids activate specific cell-membrane receptors of the superfamily of G-protein–coupled receptors. Some of the tissues in which individual prostanoids exert prominent effects are indicated. IP denotes prostacyclin receptor, TP thromboxane receptor, DP prostaglandin D_2 receptor, EP prostaglandin E_2 receptor, and FP prostaglandin $F_{2\alpha}$ receptor (FitzGerald and Patrono, 2001).

Since the discovery of cyclooxygenase, they were believed to be expressed constitutively with constant levels in individual tissues and prostaglandin synthesis was believed to increase in inflammation because of increased release of precursor. However, cyclooxygenase activity increases in inflammation but can be prevented by corticosteroids. From these clues, two different approaches identified a new inducible isoform (COX-2). Investigators studying cell growth signaling pathways identified a unique, inducible gene product related to the known COX sequence (Herschman, 1996). Concurrently, a different cyclooxygenase protein was detected in monocytes stimulated by interleukin 1 (Fu *et al*., 1990). A molecular program,

designed to identify inducible immediate-early-response genes, yielded one with considerable sequence homology with the known (COX-1) gene (Kujubu *et al.*, 1991). Both immuno-precipitation of this COX variant with an anti-COX antibody, as well as the production of an antibody that precipitated only the COX-2 isoform, allowed the identification of two different COX isoforms. It was subsequently determined that the COX-1 and COX-2 proteins are derived from distinct genes (Reed *et al.*, 1996).

Taken together, these early studies revealed that while both enzymes carry out essentially the same catalytic reaction and have similar primary protein structures, many of the inflammatory, inducible effects of COX appeared to be mediated by the newly discovered COX-2, while many of the 'housekeeping' effects of COX appear to be mediated by COX-1. This functional role for each isoform is consistent with their tissue expression patterns: nearly all normal tissues were found to express COX-1 with low to undetectable levels of COX-2. However, COX-2 is constitutively expressed in the brain and kidney of rodents. Other differences between COX-1 and COX-2 include differences in utilization of arachidonic acid substrate pools as well as in mRNA stability (Kutchera *et al*., 1996; Reddy and Herschman, 1996).

Based on these finding a the "COX-2 hypothesis" was proposed, that at equally effective doses, selective COX-2 inhibitors will cause less serious gastrointestinal (GI) adverse effects than traditional nonselective NSAIDs. The effective clinical development of COX-2 – selective inhibitors would thus rest on two assumptions:

a) COX-2 inhibition is necessary and sufficient for analgesic/anti-inflammatory efficacy
b) COX-1 inhibition is largely responsible for the serious GI toxicity of conventional NSAIDs.

The regions regulating gene expression of COX-1 and COX-2 show little similarity. For example, the promoter and enhancer regions regulating COX-2 contain a variety of response elements that have been shown to explain, at least in part, its inducibility by hormones, growth factors, phorbol esters, cAMP, inflammatory factors and cytokines. Much less is known about the elements involved in regulating COX-1 gene expression, although studies have reported induction of COX-1 in some circumstances involving differentiation of macrophages. (Hoff *et al.,* 1993; Murakami *et al*., 1994; Ueda *et al*., 1997; Smith *et al*., 1993)

The amino acid sequence of COX-2 complementary DNA showed 60% homology to the sequence of COX-1, with the size of the messenger RNA for COX-2 approximating 4.5 kilobases (kb) and that of COX-1 being 2.8 kb. However, both enzymes have a molecular mass of 71 kDa as well as similar active sites for their natural substrate and for blockade by NSAIDs.

Another major difference between COX-1 and COX-2 appears to be in their ability to use different substrate pools. For example, in both fibroblasts and immune cells, COX-2 was able to utilize endogenous arachidonic acid whereas COX-1 was not. In these systems, COX-1 requires exogenous substrate. Soluble PLA2 can produce an alternative source of substrate for COX-1 and Herschman (1996) has suggested that in some tissues the release of sPLA2 from neighboring cells might provide the primary regulation of COX-1 activity.

X-ray crystallography of the 3-D structure of COX-1 and COX-2 has helped to demonstrate how NSAIDs work, isoforms specificity can be achieved and guide drug discovery. COX-1 and COX-2 are very similar enzymes consisting of a long narrow channel with a hairpin bend at the end. Both the isoforms are membrane-associated so arachidonic acid released from damaged membranes adjacent to the opening of the enzyme channel, which is largely hydrophobic, is sucked in, twisted around the hairpin bend, two oxygens are inserted, and a free radical extracted, resulting in the five-carbon ring that characterises prostaglandins (Fig 3). X-ray crystallography studies have also shown that NSAIDs act on both COX isoforms by hydrogen bonding to a polar arginine nucleotide present at position 120. Selectivity of many drugs for COX-2 enzyme is dependant on a single amino acid difference at position 523. In COX-1 an isoleucine molecule exists while in COX-2 a valine molecule exists (smaller by a single methyl group). This smaller valine molecule gives access to a side-pocket, which is thought to be the site of binding of many selective drugs (Hawkey, 1999).

The kinetics of COX-2 inhibition is also different from those of COX-1. COX-1 inhibition is instantaneous and competitively reversible as would be expected from a process based on hydrogen bonding. COX-2 inhibition is time-dependent with selectivity developing over 15-30 minutes and is thereafter essentially irreversible. Fig 3. Shows the prostaglandin synthesis and inhibition in COX-1 and COX-2.

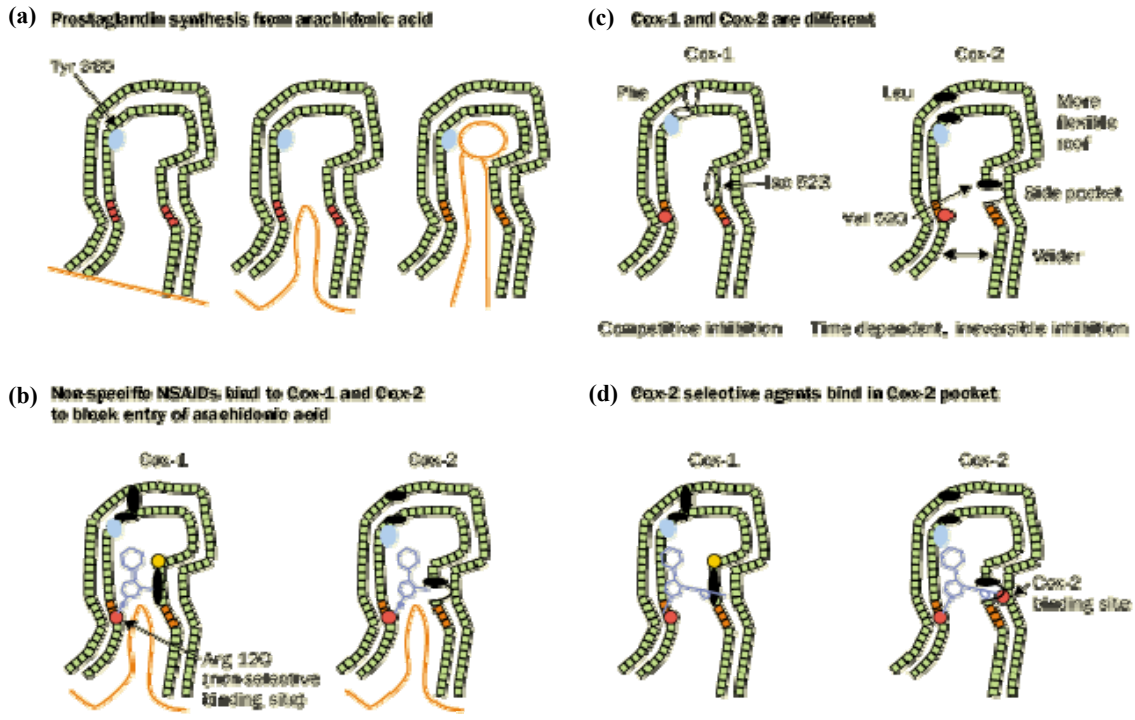


Fig 3. Prostaglandin synthesis and inhibition in COX-1 and COX-2.

(a) Initial stages of prostaglandin synthesis is illustrated, (b) Binding mode of standard NSAIDs to arginine 120 to inhibit prostaglandin synthesis by direct blockade of cyclo-oxygenase channel, (c) Differences between COX-1 and COX-2 bind pockets and (d) shows specific blockade of COX-2 enzyme (Hawkey, 1999).

As newer COX-2 inhibitors have been developed based on the COX-2 hypothesis that the development of COX-2 inhibitors would lead to potentially gastro-safe NSAIDs is being debated and some have described as flawed concept (HawKey, 1999). Firstly, most NSAID use is for conditions not regarded as inflammatory; despite this, COX-2 inhibitors are analgesic in such conditions, although their potency is debated. Secondly, the human gastrointestinal tract contains many cells that can express COX-2, such as macrophages, neutrophils, myofibroblasts and endothelial cells. The gut can mount inflammatory responses and express COX-2. In particular, the active gastritis caused by *Helicobacter pylori* is associated with COX-2 expression which raises the possibility that COX-2 inhibitors would inhibit the synthesis of protective prostaglandins in these circumstances. Fortunately, however, it seems that COX-1 remains the predominant source of gastric mucosal prostaglandins even with *H pylori* infection (Jackson *et al.*, 2000).

In summary, the COX-1 and COX-2 genes are regulated by two independent and quite different systems even though the enzymatic reaction they catalyze is identical. To better

understand, how these systems function in health and disease, how they are regulated and how they interact we have to understand biology of COX-1 & 2 in a greater detail.

1.2.4 COX-1 Biology

As noted above, COX-1 has been localized in nearly all tissues under basal conditions. From this type of tissue expression pattern, one would expect that COX-1's major function is to provide PG precursors for homeostatic regulation. One important site of COX-1 function is the blood platelet, where the enzyme is responsible for providing precursors for thromboxane synthesis (Schafer, 1995). Teleologically this makes sense, since platelets, which do not have nuclei, cannot produce an inducible enzyme in response to activating conditions. Rather, platelets carry a supply of COX-1. In the presence of an NSAID like aspirin, platelets are prevented from generating thromboxane during activation and fail to complete successful aggregation, inhibiting their thrombogenic potential. In the adjacent vascular endothelium, PGs play a different role. The release of eicosanoids by activated platelets is thought to provide both a substrate and stimulus for the generation of prostacyclin (PGI_2) by the endothelium. This compound stimulates vasodilatation counteracting the vasoconstrictor thromboxane.

COX-1 function in two other physiological systems leading to vasodilatation in the presence of contractile conditions. In both kidney and stomach, normal physiological stimuli are associated with dramatic changes in blood flow. During times of lowered blood volume, the kidney releases angiotensin and other factors to maintain blood pressure by systemic vasoconstriction (Palmer and Henrich, 1995). At the same time, angiotensin provokes PG synthesis in the kidney. COX-1 is expressed in the vasculature, glomeruli and collecting ducts of the kidney, and it appears to be important in producing the vasodilating PGs which maintain renal plasma flow and glomerular filtration rate during conditions of systemic vasoconstriction. In the presence of NSAIDs, this protective response fails leading to renal ischemia and damage in susceptible individuals (Zambraski, 1995). Similarly, in the gastric antrum, NSAID use leads to ischemia followed by mucosal damage and ulceration (Trevethick *et al.,* 1995). The enzyme blocked by NSAIDs is thought to be COX-1 (Wallace, 1997) that produces PGs, which alter blood flow in the microcirculation of the gastric mucosa.

Therefore, COX-1 acts in a variety of settings to produce homeostatic or basal levels of PGs. In some cases, induction of PLA2 is responsible for increased PG synthesis. In others, COX-1 levels are modulated (i.e., macrophages stimulated to differentiate). It is not clear whether all cells and tissues with COX-1 rely on exogenous supplies of substrate but this situation would allow for a variety of cooperative mechanisms tying COX-1 activity to neighboring physiological requirements.

1.2.5 COX-2 Biology

One of the first studies conducted after the discovery of two isoforms of COX was a screen of existing NSAIDs for those that had differential effects on inhibition of COX-1 vs. COX-2 and some were found to have a 20- to 70-fold selective preference. As a result, studies were done using differential inhibition of COX-1 or COX-2 activities to sort out the relative contributions of these isoforms under a variety of experimental conditions. While initial studies upheld the concept that COX-2 is mainly an inflammatory, inducible enzyme but subsequent research revealed additional functions (Williams and DuBois, 1996). The various organ systems and disease states where COX-2 appears to have functional significance are described below.

1.2.5.1 Renal function

Prostaglandins are known to serve as important physiologic modulators of vascular tone and sodium and water homeostasis in the mammalian kidney including modulation of glomerular hemodynamics, tubular reabsorption of sodium as well as water and regulation of renin secretion. While COX-1 has long been recognized to be involved in normal kidney function, COX-2 is now seen to have a distinct role. Localization studies have found COX-2 in both the macula densa of the rat kidney (Harris *et al*., 1994) and the interstitial cells of the medulla (Guan *et al*., 1997). The macula densa plays an important role in mediating the interaction among glomerular filtration, proximal reabsorption, and regulation of renin release (Harris, 1996), which in turn is responsible for sodium balance and fluid volume. Although, PGE_2 has been reported to inhibit chloride reabsorption in the ascending limb of Henle (Stokes, 1979) chronic sodium deprivation was found to increase COX-2 levels in the region of the macula densa, and COX-2-generated prostanoids may be important mediators of renin production and tubuloglomerular feedback. Mapping of PG receptors in the kidney (Breyer *et al*., 1996) does show differential locations of receptors specific for different PGs indicating that

differential synthesis of specific types of prostaglandins may be responsible for separating the effects of COX-1 and COX-2.

In addition to the multiple roles played by PGs in the adult kidney, COX-2 null mice show severe disruption of kidney development (Dinchuk *et al*., 1995; Morham *et al*., 1995). The localization and level of COX-2 expression in the human kidney is currently unknown but this information will be essential in evaluating the role of COX-2 in human kidney function. NSAIDs are known to have multiple effects on kidney function and specific COX-2 inhibitors should be useful in dissecting the role of PGs generated from the COX-2 pathway in normal renal physiology.

1.2.5.2 Nerve and brain function

COX-2 seems to have some role in regulating brain function. PGs have long been known as mediators of fever, of inflammatory reactions in neural tissue and more recently of brain function. The recognition that each of these processes involves induction of PG synthesis has led to an appreciation of the role COX-2 plays in the PG-mediated functions. While NSAIDs are commonly used to control fever, the actual mechanism of fever induction has only recently been described. Intraperitoneal injection with lipopolysaccharide (LPS) causes a marked fever response in rats. In an elegant dissection of molecular and tissue interactions, Matsumura and colleagues have shown how COX-2 induction in brain endothelial cells temporally parallels the fever response (Matsumura *et al*., 1997). This leads to the synthesis of PGs, which then act on temperature-sensing neurons in the preoptic area. In turn, selective COX-2 inhibition can effectively block fever (Taniguchi *et al*., 1997). Communication between local inflammatory sites and the brain endothelium is mediated by cytokines such as IL-1, which can directly induce COX-2 expression in these cells (Cao *et al*., 1997). These investigators have also shown induction of COX-2 expression in other parts of the brain but these areas are not directly associated with the fever pathway.

A separate inflammatory pathway is one mediated by microglial cells, a type of tissue-specific macrophage that lies dormant until needed for defense or tissue remodeling (Bauer *et al*., 1997). While known as a source of PGs during inflammatory states, the microglial cell does not show induction of COX-2 in response to cytokines, in strong contrast with other inflammatory cells. Instead, the microglial COX-2 response is limited to direct LPS exposure, an event that would only occur by direct bacterial infection of the brain. Thus, the microglial

defensive response is segregated from systemic inflammation by its limited repertoire of inducers.

This segregation of the brain from systemic inflammatory inducers is important because COX-2 also plays a central role in neural development and adaptation. During earlier stages of brain development, neural genes and proteins are developmentally induced and play a major role in the maturation process. During later stages of maturation, however, environmental influences as represented by neural responses and synaptic activity play an increasingly important role in determining brain structure. It is in these final stages of development and brain modeling that COX-2 becomes active in a manner that coincides with the imprinting of environmental influences (Kaufmann *et al*., 1997).

COX-2 remains an important modulator of neural response throughout adult life. COX-2 levels increase dramatically after seizures and N-methyl-D aspartate-mediated activity (Yamagata *et al*., 1993). The sites of induction are the postsynaptic dendritic arborizations (Kaufmann *et al*., 1996) of specific excitatory neurons located in the major processing centers of the brain. The actual role of COX-2 and PGs in these sites is not yet understood but associations between COX-2 induction and neural degeneration after glutamate stimulation (Tocco *et al*., 1997), seizures and spreading depression waves (Miettinen *et al*., 1997) suggest that COX-2 may play more of a role in the selective loss of neural connections than in their formation.

1.2.5.3 Maintenance of gastrointestinal integrity

The intestinal epithelium is a tissue that undergoes constant regeneration in response to both insult and normal use. The use of NSAIDs causes a variety of problems in the gastrointestinal tract including irritation and ulceration of the stomach lining (Roth, 1996). Radiation exposure leads to intestinal epithelial cell death, leaving crypt cells to regenerate the population. In animal studies COX-2 is not induced after exposure to radiation and its presence is not essential for crypt cell survival (Cohn *et al*., 1997). Under these circumstances, COX-1 appears to play a major role, as it does in the stomach in maintaining proper glandular architecture. Gastrointestinal epithelium is also the target of numerous infectious and parasitic organisms. In response to infection or invasion, COX-2 expression is induced in epithelial cells (Eckmann *et al*., 1997) which leads to increased PG production. The PGs then stimulate chloride and fluid secretion from the mucosa which flushes bacteria

from the intestine. In addition, COX-2 is expressed during inflammation and wound healing and in animal models treatment with COX-2 inhibitors can exacerbate inflammation and inhibit healing. Nevertheless, COX-2 selective inhibitors appear to be associated with less gastrointestinal damage than conventional NSAIDs (Simon, 1997). Clinical trials evaluating compounds that are highly selective for COX-2 have also indicate a paucity of gastrointestinal side effects. In the Vioxx Gastrointestinal Outcomes Research (VIGOR) study (Bombardier *et al*., 2000) treatment with Rofecoxib at twice the approved maximal dose for long-term use resulted in significantly lower rates of clinically important upper gastrointestinal events and complicated upper gastrointestinal events seen with a standard dose of Naproxen. Moreover, the incidence of complicated upper gastrointestinal bleeding and bleeding from beyond the duodenum was significantly lower among patients who received Rofecoxib. In the Celecoxib Long-Term Arthritis Safety Study (CLASS) (Silverstein *et al*., 2000), incidences of symptomatic ulcers and/or ulcer complications were not significantly different in patients taking Celecoxib versus NSAIDs who were also taking concomitant low-dosage aspirin, indicating that the use of low-dose aspirin may abrogate the gastrointestinal-sparing effects of Celecoxib. By contrast, analysis of non-aspirin users alone demonstrated that Celecoxib at a dosage 2- to 4-fold greater than the maximum therapeutic dosages was associated with a significantly lower incidence of symptomatic ulcers and/or ulcer complications compared with NSAIDs.

Although these data point toward a significantly improved risk-benefit ratio of COX-2 specific inhibitors in terms of gastrointestinal safety compared with traditional NSAIDs, it is noteworthy, however, that specific COX-2 inhibitors are associated with some dyspepsia with an incidence less than that seen with NSAIDs but higher than with placebo (Langman *et al*., 1999).

Another important finding of the past years was the observation that COX-2 may influence ulcer healing and the associated angiogenesis. In accord with this concept, COX-2 has previously been shown to be induced in tissue on the edges of ulcers (Mizuno *et al*., 1997). Moreover, in animal studies, selective COX-2 inhibitors have been demonstrated to retard ulcer healing (Schmassmann *et al*., 1998). As a consequence, it will be necessary to understand whether effective ulcer healing occurs in patients with NSAID-associated ulcers switched to specific COX-2 inhibitors. It has been shown that both COX-2 selective and nonselective NSAIDs inhibit angiogenesis through direct effects on endothelial cells

involving inhibition of mitogen-activated protein kinase activity and interference with extracellular signal-regulated kinase (ERK) nuclear translocation (Jones *et al*., 1999). Remarkably, interference of NSAIDs with angiogenesis involved both prostaglandin-dependent and prostaglandin-independent components.

As with patients with pre-existing ulcers, the clinical implications of *Helicobacter pylori*-associated induction of COX-2 expression in patients who are on specific COX-2 inhibitors has also been studies. Investigations suggest that COX-2 may modulate the inflammatory process of the mucosa as well as alterations in epithelial cell growth in gastritis (Fu *et al*., 1999). Increased COX-2 levels have been detected in mononuclear and fibroblast cells in the lamina propria in *H. pylori*-positive gastritis. In another study the expression of COX-2 in the antral mucosa was reduced after successful eradication of *H. pylori*, implying that the expression of COX-2 is a direct response to bacterial infection (McCarthy *et al.*, 1999).

It has been shown that Salmonella infection of cultured intestinal epithelial cells gives rise to a rapid induction of COX-2, an associated rise in PGs and acceleration in chloride secretion that can be blocked by an anti-PGE antibody. With the response of infected cells, uninfected cells also acquired a state of increased fluid secretion, demonstrating the paracrine nature of the PG response. This model shows the essential features of the PG response: upregulation of COX and PG synthesis, physiological adaptation to infection and coordination of a protective response (Eckmann *et al*., 1997).

1.2.5.4 Ovarian and uterine function

One of the earliest noted sites of PG accumulation was amniotic fluid (O'Brien, 1995), and one of the first known biological responses to a prostaglandin was the rhythmic contraction of the uterine myometrium. These associations with pregnancy and labor led to the recognition of PGs as a major effector in induction of labor. Prenatal indomethacin is sufficient to block PG production leading to premature duct closure and disruption of fetal circulation (Hammerman, 1995).

Ovulation, the process by which oocytes are released from the preovulatory follicle in the ovary is accompanied by induction of prostaglandin synthesis as a consequence of the LH surge. This marked response led to the first observation of COX-2 induction during a normal physiological event (Richards *et al*., 1995). One of many molecular events associated with ovulation (Richards, 1994), the induction of COX-2 is necessary for the successful rupture of

the follicle probably mediating directly the generation or activation of proteolytic enzymes necessary for this process (Tsafriri, 1995). Induction of COX-2 can also be modulated by the gonadotropins LH and FSH and by TGF-α, IL-1β or other cellular signaling pathways (Morris, and Richards, 1996).

After fertilization, COX-2 again plays a role, this time during implantation of the embryo in the uterine endometrium. During the pre-implantation period, uterine COX-1 (Chakraborty *et al*., 1996) and the PGE_2 receptors EP1, EP3 and EP4 (Yang *et al*., 1997) may modulate preparation of the uterus for interaction with the embryo and COX-2 (Chakraborty *et al*., 1996) and the EP2 receptor (Lim, and Dey, 1997) appear to mediate the embryo-uterine interactions during implantation. COX-2 null mice show multiple failures in reproductive function, including ovulation, fertilization, implantation and decidualization, underscoring the multiple roles of PGs during these processes (Lim *et al*., 1997). Finally, at the completion of pregnancy, PGs again act in the ovary and uterus to help mediate the delivery process. As mentioned above, PGs have long been known to stimulate uterine contraction. In mice, the production of PGF2 by the fetal and/or uterine tissues signals the ovary to induce luteolysis (Sugimoto *et al*., 1997) leading to a decline in maternal progesterone and the induction of oxytocin receptors in the myometrium. This induction in turn increases the myometrial response to oxytocin and brings on parturition.

1.2.5.5 Bone

The role of PGs in bone metabolism is not only complex, but also contradictory. For example, while PGs were initially characterized by stimulating bone resorption in culture, human and animal responses to PGs often include stimulation of bone formation (Kawaguch *et al*., 1995). Collagen synthesis by osteoblasts can be both stimulated (Woodiel *et al*., 1996) or inhibited by PGs. Mechanical stress on bone cells leads to an increase in PG synthesis suggesting that immobilization would be characterized by low PG levels (Klein-Nulend *et al.,* 1997*).* Nevertheless, immobilization is associated with PG-mediated bone loss, an effect that is slowed by NSAIDs.

While little is known of how PG synthesis affects the balance between bone loss and formation, COX-2 induction in osteoblasts is reported to be essential to the acute stress response in a bone remodeling system (Pilbeam *et al*., 1997).

1.2.5.6 Inflammation and arthritis

The inducibility of COX-2 activity and the central role of this induction in the amplification of inflammation have been fully appreciated only in late 1990s (Needleman and Isakson, 1997). Evidence provided by animal models of inflammatory arthritis strongly suggests that increased expression of COX-2 is responsible for increased PG production seen in inflamed joint tissues (Anderson *et al*., 1996). COX-2 induction has been observed in both human osteoarthritis-affected cartilages (Amin *et al*., 1997) as well as in synovial tissue taken from patients afflicted with rheumatoid arthritis (Kang *et al*., 1996). Cell culture experiments utilizing primary cells derived from human synovial tissue or cell types (e.g., monocytes) important in inflammatory processes have been critical to an understanding of factors involved in modulating this induction. The pro-inflammatory agents IL-1β, TNF-α and LPS, as well as the growth factors TGF-ß, EGF, PDGF and FGF have all been shown to induce COX-2 expression in this system. On the other hand, the anti-inflammatory cytokines IL-4 and IL-13, as well as the immunosuppressive glucocorticoids, were shown to decrease COX-2 levels (Crofford, 1997). Although the synovial tissues of patients with osteoarthritis express lesser amounts of COX-2, primary explant cultures of human osteoarthritis-affected cartilage spontaneously express large amounts of COX-2 and PGs (Amin *et al*., 1997). Nitric oxide, another important inflammatory modulator has been shown to regulate PG production in osteoarthritic cartilage, though not in synovial cells. Whether this modulation attenuates or enhances COX activity remains controversial (Amin *et al*., 1997; Manfield *et al*., 1996).

1.2.5.7 Pain

Local tissue injury and inflammatory diseases like osteoarthritis are associated with increased PGs and pain receptors are known to be sensitized to lower levels of stimulus by PGs (Dray and Urban, 1996). Thus, the action of COX at the site of injury or inflammation is hyperalgesic and the pain-relieving action of NSAIDs at the local site is easily explained by this mechanism. In addition, PGs are thought to act in the spinal cord to facilitate the transmission of pain responses, though there is little known about how they might do this (Yamamoto and Nozaki-Taguchi, 1996). COX-2 is induced in both local and central sites (Beiche *et al*., 1996). Intrathecal injection of both the COX-2 specific inhibitor NS-398 and the nonspecific NSAID Indomethacin suppressed a formalin-mediated pain response (which measures a central response) but neither suppressed a high temperature-induced pain response (i.e. a local response) (Yamamoto and Nozaki-Taguchi, 1996). In contrast, systematically administered Meloxicam, an NSAID more specific for COX-2 than COX-1,

suppressed the inflammatory pain response locally (Laird *et al*., 1997) without affecting central pain transmission. In neither of these studies was the drug introduced into both sites to allow an internal comparison, but together they show that COX-2 can act both locally and centrally to mediate pain. In fact, the COX-2 specific inhibitor Celecoxib was shown in short-term human studies to effectively suppress the pain associated with dental work, osteoarthritis, or rheumatoid arthritis without causing any significant gastroduodenal lesions (Seibert *et al*., 1994).

1.2.5.8 Alzheimer's disease

Epidemiological studies have shown a connection between the COX pathway and Alzheimer's disease. In Baltimore Longitudinal Study of Ageing (Stewart *et al*., 1997) with 1686 participants, the risk of developing Alzheimer's disease was significantly reduced among users of NSAIDs, particularly when NSAIDs were taken for two years or more. The apparent protective effect of NSAIDs suggests that COX might be involved in neurodegenerative mechanisms. A role for COX-2 in this process has been established by several lines of evidence and has been reviewed by Pasinetti, (2001). In Alzheimer's disease, COX-2 is up-regulated in brain areas related to memory (hippocampus, cortex) with the amount of COX-2 correlating with the deposition of β-amyloid protein in the neuritic plaques. β-Amyloid is thought to be elaborated as part of an inflammatory process in which activated microglia, the predominant source of COX-2-dependent prostanoids, participate. Elevation of COX-2 expression in hippocampal neurons during the early phase (mild dementia) of Alzheimer's disease dementia is considered to favor the later inflammatory neurodegenerative process. Moreover, emerging evidence suggests that COX-2-derived prostanoids potentiate glutamate excitotoxicity, thereby accelerating neurodegeneration. Accordingly, primary neuron cultures derived from transgenic mice with neuronal overexpression of human COX-2 are more susceptible to excitotoxic and synthetic aggregated β-amyloid-mediated neuronal death (Pasinetti, 2001). However, the precise role of COX-2 in Alzheimer's disease remains to be clarified in further mechanistic studies. Ongoing studies (e.g., large National Institutes of Health-supported trials) under way are evaluating whether selective COX-2 inhibitors may control the destructive progression of Alzheimer's disease.

On the other hand, the anti-thrombotic activity of PGs may be important for protection against Alzheimer’s disease (AD). De la Torre (1997) has hypothesized that AD is caused by the development of tortuous and flow-impeded capillaries in the brain. This would

presumably promote intravascular coagulation leading to ischemic damage in the brain that could promote the development of AD. It seems likely that clinical trials of COX-2 specific NSAIDs will be started before a role of COX-2 in AD is proven but the mechanistic studies already under way will provide insight and direction for further developments.

1.2.5.9 Cancer

In several population-based studies use of aspirin and other NSAIDs have shown a 40–50% decrease in relative risk of colorectal cancer (Giovannucci *et al.,* 1994; Smalley and DuBois, 1997). Clinical trials with NSAIDs in patients with Familial Adenomatous Polyposis have clearly demonstrated that NSAID treatment caused regression of pre-existing adenomas (Giardiello *et al*., 1995). Studies in a variety of animal models (both genetic and carcinogen-induced) of colon cancer have also indicated a significant reduction in tumor multiplicity by NSAID treatment (Williams *et al*., 1997). In fact, some of these studies have shown as much as 80–90% reduction in tumor burden (Kawamori *et al.*, 1998).

Initial attempts to determine the molecular basis for these observations found that both human and animal colorectal tumors express high levels of COX-2 whereas the normal intestinal mucosa has low to undetectable COX-2 expression (Kutchera *et al*., 1996; Eberhart *et al*., 1994; Kargman *et al*., 1995; Sano *et al*., 1995). These findings led to the hypothesis that COX-2 may be playing a role in colon cancer growth and progression. Subsequent experiments appear to support this view. For example, the effects of a highly selective COX-2 inhibitor (SC-58125) were tested on two different cell lines only one of which had a high level of COX-2 expression and activity. It was observed that the inhibitor decreased cell growth in both *in vitro* and *in vivo* assays only in the COX-2-expressing cell line (Sheng *et al*., 1997). Other work in cell culture models has shown that COX-2 expression contributes significantly to the tumorogenic potential of epithelial cells by increasing adhesion to extracellular matrix and making them resistant to apoptosis (Tsujii and DuBois, 1995). These phenotypic changes were shown to be reversible by treatment with a highly selective COX-2 inhibitor (Kawamori *et al*., 1998). It has also been shown that cyclooxygenase may play a vital role in the regulation of angiogenesis associated with neoplastic tumor cells (Tsujii *et al*., 1998). Hence, COX inhibitors may block the growth of blood vessels into developing tumors.

Genetic evidence supporting a role for COX-2 in the development of intestinal neoplasia has also been reported. Oshima et al. (Oshima *et al*., 1996) assessed the development of intestinal

adenomas in Apc716 mice (a model in which a targeted truncation deletion in the tumor suppresser gene APC causes intestinal polyposis) in a wild-type and homozygous null COX-2 genetic background. The number and size of polyps were reduced dramatically (six- to eightfold) in the COX-2 null mice compared with COX-2 wild-type mice. In addition, treatment of the Apc716 mice with a novel COX-2 inhibitor, Merck Frosst tricyclic, reduced polyp number more significantly than the non-selective NSAID, sulindac (Oshima *et al*., 1996).

Whether NSAIDs block tumor progression solely by blocking PG synthesis is a matter of debate. Several studies have shown that NSAIDs can act through mechanisms that are independent of their ability to inhibit COX (Piazza *et al*., 1995; Thompson *et al*., 1997; Hanif *et al*., 1996). Most of this work has been done in cell culture models where effects are only seen at fairly high concentrations of drug (200–1000 μM). Recently, animal studies have been reported that demonstrate the ability of sulindac sulfone to have protective effects against mammary tumors (Thompson *et al*., 1997). Sulindac sulfone is a metabolite of the NSAID sulindac which lacks the ability to inhibit COX directly. Therefore, some evidence indicates that COX-independent pathways also play an important role in the cancer chemopreventive properties of NSAIDs and it is likely that both COX-dependent and independent pathways are involved.

Several studies have further indicated that COX-2 overexpression is not necessarily unique to cancer of the colon, but may be a common feature of other epithelial cells. Increased COX-2 levels have been identified in lung, breast, gastric, and prostate cancer, as well as in pancreatic adenocarcinomas (Prescott and Fitzpatrick, 2000). On the this basis, it is conceivable that specific COX-2 inhibitors might be used as adjuvant in the treatment of tumors, as well as in cancer prevention.

1.2.6 COX-3 Biology

Chandrasekharan *et al*., (2002) first reported COX-3 as a variant of COX-1 which is inhibited by acetaminophen and other analgesic/antipyretic drugs. The argument for existence of this variant stems from fact that acetaminophen often categorized as a nonsteroidal anti-inflammatory drug, yet in clinical practice and in animal models possesses little anti-inflammatory activity although it inhibits pain and fever and is one of the world's most popular analgesic/antipyretic drugs. This variance in its pharmacology gave rise to the

concept that variants of COX enzymes exist that are differentially sensitive to this drug and that acetaminophen acts centrally. Additionally, acetaminophen at therapeutic concentrations, in whole cells or homogenates failed to show sufficient inhibition of the two isoforms of COX known indicating that neither of the isozyme is a good candidate for the site of action of acetaminophen.

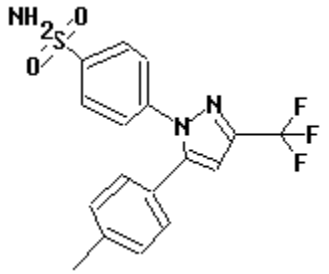
Human *COX-3* is a 5·2 kb transcript encoded on chromosome 9 that is expressed in specific tissues with highest levels in the brain (reaching 5% of the total amount of COX-1 mRNA) and in the heart (Chandrasekharan *et al*., 2002). COX-3 shares all of the catalytic features and important structural features of COX-1 and -2. It also appears to enter the endoplasmic reticulum where it is glycosylated and its glycosylation is required for activity.

COX-3 was also found to differ in its sensitivity to inhibition by a selection of NSAIDs. Diclofenac was the most potent inhibitor of COX-3 and Diclofenac, aspirin, and ibuprofen preferentially inhibited COX-3 over COX-1 and COX-2. Thalidomide and caffeine, both have analgesic properties, did not inhibit COX-3. Chandrasekharan *et al*., (2002) have shown that COX-3 possesses COX activity that differs pharmacologically from both COX-1 and COX-2 but is more similar to COX-1.

COX-3 inhibition by acetaminophen is not specific and only weak and therefore does not completely solve the mystery of acetaminophen being analgesic without affecting COX-1 or COX-2. This finding opens up the search for further COX variants or paracetamol-inducible antipyretic and analgesic-acting proteins. COX-3 does not appear to be the solitary elusive target of acetaminophen. Therefore more research is needed to answer the analgesic activity of COX inhibition. The limited number of drugs tested so far allows no final conclusions. Another interesting observation is that selective COX-2 drugs have no effect on COX-3. COX-2-selective drugs are suspected to be less analgesic than non-selective COX-1 or COX-2 inhibitors like ketoprofen (Mazario *et al*., 2001) naproxen or diclofenac. An important future aspect of human COX-3 research might be the promise of a new target for analgesic drug discovery, enriching the oversimplified COX-1 and COX-2 debate.

1.3 Cyclo-oxygenase-2 Inhibitors

1.3.1 Celecoxib (SC 58635, YM 177, Celebrex, Celecox, Niflam, Onsenal)



Chemical Name: Benzenesulfonamide, 4-[1,1,1-trifluoromethyl- 5-(4-methylphenyl) pyrazol-1-yl]

Molecular Formula: C17 H14 F3 N3 O2 S

CAS Number: 169590-42-5; 194044-54-7

Originator Companies: Pfizer
Licensee Companies: Astellas Pharma; Roemmers

Highest Development Phase: It has been launched worldwide for Osteoarthritis, Rheumatoid arthritis and Pain.

Drug Development Phase
Celecoxib is in different phases of development for additional indications as under.

Indication	Phase	Country
Dysmenorrhoea	Launched	Spain
Familial adenomatous polyposis	Launched	USA
Postoperative pain	Launched	USA
Ankylosing spondylitis	Registered	European Union, USA
Dysmenorrhoea	Registered	USA
Familial adenomatous polyposis	Registered	European Union
Juvenile rheumatoid arthritis	Registered	USA
Back pain	Preregistration	Japan
Barrett's oesophagus	Phase-III	USA
Bladder cancer	Phase-III	USA
Colorectal cancer	Phase-III	USA
Dental pain	Phase-III	Japan
Pain	Phase-III	Japan
Postoperative pain	Phase-III	Japan
Skin cancer	Phase-II	USA
Non-small cell lung cancer	Phase-II	USA
Prostate cancer	Phase-II	USA
Cervical dysplasia	Clinical-Phase-Unknown	USA
Oesophageal cancer	Clinical-Phase-Unknown	USA
Breast cancer	Preclinical	USA
Cachexia	Preclinical	USA

Introduction

Celecoxib is a specific cyclooxygenase 2 (COX-2) inhibitor. It was originally discovered by Searle (now Pfizer). Celecoxib is launched as an orally administered therapy for the treatment of osteoarthritis, rheumatoid arthritis, pain, dysmenorrhoea and familial adenomatous polyposis. In addition, it is approved in the US and EU for the treatment of ankylosing spondylitis and has been submitted for review in Japan for the treatment of back pain. It is in

clinical trials for the treatment of Barrett's oesophagus, bladder cancer, colorectal cancer, pain, non-small cell lung cancer, skin cancer, cervical dysplasia, prostate cancer and oesophageal cancer.

Mechanism of Action: Cyclooxygenase 2 inhibitors

Pharmacodynamics: Analgesic effects; inhibition of crypt formation in rat colon; anti-inflammatory and antirheumatic effects; significantly reduces polyp numbers in patients with FAP; abrogates the increase in prostaglandin-E_2 levels; down-regulates cyclo-oxygenase 2 and biological parameters associated with tumour cell proliferation and angiogenesis

Comparative Efficacy: ≥ Ketoprofen (Ankylosing spondylitis), = Aspirin (Postoperative pain), = Diclofenac (Rheumatoid arthritis), = Naproxen (Osteoarthritis), = Naproxen (Rheumatoid arthritis)

Pharmacokinetic Characteristics:
t_{max} (h): 1.8 - 2.1, 2.0 - 5.8 (Children)
$t_{½\beta}$ (h): 9.1 - 10.5, 3.0 - 10.1 (Children)
Linear Kinetics: Yes
Frequency of administration: bid
Route of elimination: unspecified
Route of administration: PO

Physico-chemical properties:
Experimental LogP/Hydrophobicity – 3.9
pKa – 11.1 (weakly acidic)
Solubility in various vehicles at 25°C (Sathesh Badu *et al.,* 2007)

Solvent / Vehicle	Solubility (mg/ml)
Water	0.007
0.15 M Sodium chloride	0.0582
Phosphate buffer solution (0.1 M) pH 7.4	0.0035
Ethanol	0.0648
Propylene glycol	0.0688
Polyethylene glycol 400	1.0740
Tween 80 (10%)	0.3837

Key development decisions

Health Canada announced restrictions on the use of Celecoxib in April 2005. The drug is not to be used in patients who have had a heart attack or stroke, have experienced serious chest pain related to heart disease or who have had serious heart problems such as congestive heart failure. The drug is to be used at the lowest possible dose for the shortest necessary period

and is to be used only to treat pain and inflammation of arthritis and certain types of acute pain (Media Release: 7 Apr 2005. Available from: URL: http://www.hc-sc.gc.ca)

In February 2005, the joint Arthritis Drugs/Drug Safety & Risk Management Advisory Committee of the FDA recommended that marketing of Celecoxib should continue. The committee stated that Celecoxib 200 mg/day is at the low end of risk for cardiovascular events; however, the committee recommended a "black box" warning regarding cardiovascular risk (Media release: 18 Feb 2005. Available from: URL: http://www.fdaadvisorycommittee.com)

In August 2005, the FDA finalised prescribing instructions for Celecoxib for all approved uses including a boxed warning regarding potential cardiovascular and gastrointestinal risks (Media release: 1 Aug 2005. Available from: URL: http://www.pfizer.com).

The EMEA concluded its review of selective COX-2 inhibitors in June 2005; the conclusion was that Celecoxib, like other selective COX-2 inhibitors, has an increased risk of thrombotic adverse reactions. The CHMP recommended appropriate contraindications (ischaemic heart disease, cerebrovascular disease, and peripheral arterial disease) and precautions (Media Release: 27 Jun 2005. Available from: URL: http://www.emea.eu.int).

Alzheimer's disease: The National Institute of Aging in the US was conducting the Alzheimer's Disease Anti-Inflammatory Prevention Trial (ADAPT) in individuals aged ≥70 years with a family of history of the disease and no memory problems or symptoms of dementia themselves. The aim of the 7-year placebo-controlled study initiated in 2001 was to see whether Celecoxib 200mg bid or naproxen 220mg bid could delay or prevent the onset of Alzheimer's disease. In September 2002, the interest group, Public Citizen asked the US government to halt the trial because of potential side effects from both drugs (Media Release: 4 Sep 2002. Available from: URL: http://www.reutershealth.com). The trial continued until December 2004 when in the interests of public safety, the NIH suspended the use of Celecoxib and naproxen in the 2400 subject trial, in part because of findings reported from a National Cancer Institute trial which showed an increased risk of cardiovascular events in patients taking Celecoxib, compared with placebo. However, no significant increase in cardiovascular risk was seen with Celecoxib in the ADAPT trial although an apparent increase was seen with naproxen (Media Release: 20 Dec 2004. Available from: URL:

http://www.nia.nih.gov; The National Institutes of Health, Washington, District of Columbia, USA)

Trials such as ADAPT were initiated because of evidence that inflammation may play a role in the neurodegenerative process of Alzheimer's disease, and the use of NSAIDs may be associated with reduced occurrence of the disease. However, it has been discovered that some NSAIDs encourage β-amyloid production. Research indicates that those NSAIDs that may have beneficial effects in Alzheimer's disease are those that inhibit γ-secretase, and naproxen and Celecoxib are not among those.

Celecoxib has been approved in the US for the relief of the signs and symptoms of juvenile rheumatoid arthritis in patients ≥2 years (Media Release: 15 Dec 2006. Available from: URL: http://www.fda.gov).

Skin cancer: studies in mice show that Celecoxib dramatically reduces numbers of ultraviolet-B-induced tumors, suggesting that the drug may hold promise as a preventative of skin cancer (Inpharma 1347: 20 Jul 2002). A phase II/III study of Celecoxib is in progress for the prevention of skin cancer in patients with actinic keratoses.

Adverse Events

General adverse events: The Committee for Proprietary Medicinal Products (CPMP) of the EMEA has reviewed the COX-2 inhibitors Celecoxib, Etoricoxib, Parecoxib, Rofecoxib and Valdecoxib for gastrointestinal and cardiovascular safety. The CPMP concluded that the benefit-risk balance for these products is positive, but recommended adding warnings concerning risk of severe skin and hypersensitivity reactions, and the need for caution in patients with underlying gastrointestinal and cardiovascular risks(Media Release: 21 Nov 2003. Available from: URL: http://www.emea.eu.int).

Common adverse events included generalised skin rash, headache, upper respiratory tract infection, diarrhea, dyspepsia, nausea, dizziness, somnolence and abdominal pain.

Gastrointestinal tolerance relative to other NSAIDs: The results of the Celecoxib Long-term Arthritis Safety Study (CLASS) supported the overall safety of Celecoxib. In CLASS study, the combined incidence of symptomatic ulcers or perforation, gastric outlet obstruction or

bleeding associated with Celecoxib was significantly lower than with comparator NSAIDs. However, the rate of ulcer complications, the primary endpoint of the study only showed a non-significant trend in favour of Celecoxib; the annualised incidence rates of ulcer complications alone for Celecoxib and comparator NSAIDs were 0.76% and 1.45%, respectively (Silverstein *et al*., 2000). Similar improved gastrointestinal protections were observed in several short and long term clinical studies.

Cardiovascular tolerance: Patients with Alzheimer's disease who received treatment with Celecoxib had four times the heart attack risk as compared to those receiving placebo as shown above.

The Committee for Proprietary Medicinal Products reaffirmed the safety of Celecoxib in May 2004. Results from a study conducted in elderly patients showed Celecoxib had no increase in hospital admissions for congestive heart failure versus patients on placebo. Patients taking Rofecoxib experienced an 80% increase in hospitalisation for congestive heart failure. Patients treated with non-selective NSAIDS had a 40% increase in hospitalizations (Mamdani *et al*., 2004).

In a multicentre, randomised, double-blind, parallel-group study, 811 hypertensive elderly patients with osteoarthritis received either Celecoxib 200mg once-daily or Rofecoxib 25mg once-daily for 6 weeks. The study was partly funded by Pharmacia Corporation and Pfizer. Rofecoxib compared with Celecoxib was associated with a greater incidence of oedema (10% *vs* 5%) and a higher incidence of systolic BP increases of >20mm Hg. Four patients who received Rofecoxib developed congestive heart failure. The incidence of all other adverse events was similar in both groups (Whelton *et al*., 2001).

To determine whether treatment with the COX-2 inhibitors Rofecoxib and Celecoxib is associated with new-onset hypertension in community practice, US researchers conducted a retrospective case-control study in 17 844 Medicare beneficiaries aged $\geq$ 65 years. During 1999 to 2000, 3915 patients were diagnosed with and began treatment for hypertension and four controls were selected for each case. Rofecoxib use was found to be associated with a significantly increased relative risk (RR) of new-onset hypertension, compared with Celecoxib, nonselective NSAIDs or no NSAID treatment. In contrast, Celecoxib was not associated with an increase in relative risk of new-onset hypertension. There did not appear to

be a clear dose or duration relationship between use of either Rofecoxib or Celecoxib and new-onset hypertension although long duration Rofecoxib was associated with a slightly higher risk than short duration when compared with nonselective NSAIDs, with a similar trend when compared with Celecoxib. In addition, in patients with renal disease, liver disease or congestive heart failure, Rofecoxib treatment was associated with a higher relative risk of new-onset hypertension than Celecoxib (Solomon *et al*., 2004)

Isolated case reports of hepatotoxicity with use of Celecoxib especially in patients with known allergy to sulfa have been reported. Stopping the Celecoxib treatment resulted in improved liver function (Nachimuthu *et al*., 2000).

In addition case reports of renal papillary necrosis (Celecoxib 200mg twice daily for 6 months) auditory hallucinations (Celecoxib 200mg twice daily for 8 day); vision disorders and thrombosis have also been reported.

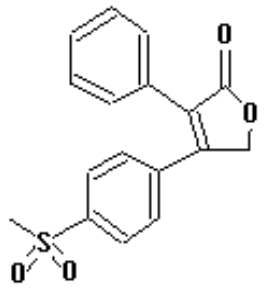
Drug interaction: Minimal. Therapy with Celecoxib and Warfarin requires monitoring of anticoagulant activity; administering Celecoxib with cytochrome P450 2C9 inhibitors requires caution; therapy with Celecoxib and lithium requires close monitoring of lithium plasma levels. Acute renal failure can occur in patients treated with Celecoxib in combination with an ACE inhibitor and a diuretic. This effect is similar to that previously reported with conventional NSAIDs in combination with ACE inhibitors and diuretics.

Indications (approved /under development)

Alzheimer's disease, Ankylosing spondylitis, Back pain, Barrett's oesophagus, Bladder cancer, Breast cancer, Cachexia, Cervical dysplasia, Colorectal cancer, Dental pain, Dysmenorrhoea, Familial adenomatous polyposis, Juvenile rheumatoid arthritis, Non-small cell lung cancer, Oesophageal cancer, Osteoarthritis, Pain, Postoperative pain, Prostate cancer, Rheumatic disorders, Rheumatoid arthritis, Skin cancer.

Patent information -The US patent exclusivity for Celecoxib expires in 2013.

1.3.2 Rofecoxib (Ceoxx, MK 966, Vioxx)



Chemical Name: 4-[4-(Methylsulfonyl)phenyl]-3-phenylfuran-2(5*H*)-one
Molecular Formula: C17 H14 O4 S
CAS Number: 162011-90-7

Originator Companies: Merck & Co
Licensee Companies: Laboratorios Phoenix
Other Companies: Banyu; CollaGenex Pharmaceuticals; Merck Frosst

Highest Development Phase: Withdrawn from worldwide market on 1st October 2004, due to safety concerns of an increased risk of cardiovascular events.

Introduction

Rofecoxib is a selective cyclo-oxygenase 2 (COX-2) inhibitor which has been developed by Merck & Co. as an anti-inflammatory and analgesic agent. It is a potent inhibitor of COX-2 and does not affect platelet thromboxane B2 synthesis. Rofecoxib is administered orally, once daily. However, Merck & Co. announced a voluntary withdrawal of Rofecoxib from the US and worldwide market on 1 October 2004 due to safety concerns of an increased risk of cardiovascular events (Media Release cited: 30 Sep 2004. Available from: URL: http://www.merck.com).

Rofecoxib was launched for the treatment of osteoarthritis, rheumatoid arthritis, pain and dysmenorrhoea. It was approved for the treatment of migraine and juvenile rheumatoid arthritis. Clinical trials were in progress in the following indications: colorectal cancer and sporadic adenomas, preterm labour, and Alzheimer's disease. Rofecoxib has also been investigated in familial adenomatous polyposis.

Mechanism of Action: Cyclo-oxygenase 2 inhibitors

Pharmacodynamics: Analgesic effect, antipyretic, antiarthritic and anti-inflammatory activity; prevents polydevelopment in FAP-prone mice

Comparative Efficacy: = Diclofenac (Osteoarthritis), = Diclofenac (Pain), = Ibuprofen (Osteoarthritis), = Ibuprofen (Pain), = Nabumetone (Osteoarthritis), = Naproxen (Dysmenorrhoea), = Naproxen (Pain), = Naproxen (Rheumatoid arthritis)

Pharmacokinetic Characteristics:
Bioavailability oral (%): 93
t_{max} (h): 2 - 3
$t_{½\ \beta}$ (h): 17
Protein binding (%): 87
Linear Kinetics: Yes
Route of elimination: hepatic
Route of administration: PO

Physico-chemical properties:
Experimental LogP/Hydrophobicity – 3.2
pKa – 10.1 (weakly acidic)
Solubility in various vehicles at 25°C (Sathesh Badu *et al.,* 2007)

Solvent / Vehicle	Solubility (mg/ml)
Water	0.0004
0.15 M Sodium chloride	0.0582
Phosphate buffer solution (0.1 M) pH 7.4	0.0035
Ethanol	0.0648
Propylene glycol	0.0688
Polyethylene glycol 400	1.0740
Tween 80 (10%)	0.3837

Key development decisions

Merck & Co. withdrew Rofecoxib worldwide on 1 October 2004 due to concerns of an increased risk of cardiovascular events including heart attack and stroke. No further development of the drug is proceeding (Media Release: 30 Sep 2004. Available from: URL: http://www.merck.com). Recent studies of Rofecoxib in combination with Astaxanthin showed that Astaxanthin eliminated the adverse effects of Rofecoxib on LDL oxidation preventing cardiotoxicity (Media Release: 28 Jun 2006. Available from: URL: http://www.cardaxpharma.com.).

In February 2005, the joint Arthritis Drugs/Drug Safety & Risk Management Advisory Committee of the FDA recommended that the risk-benefit profile for Rofecoxib supports marketing in the US. However, the committee stated that if the drug is re-introduced, stronger warnings should be applied (including a "black box" warning regarding cardiovascular risk), and only second-line or third-line use should be considered. Re-introduction should also be limited to a 12.5mg dose; previously, Rofecoxib was marketed in 25-50mg doses. Merck & Co. may consider re-introducing Rofecoxib to the market (Media Release: 18 Feb 2005. Available from: URL: http://www.fdaadvisorycommittee.com.).

Adverse Events

General adverse events: The Committee for Proprietary Medicinal Products (CPMP) of the EMEA has reviewed the COX-2 inhibitors Celecoxib, Etoricoxib, Parecoxib, Rofecoxib and Valdecoxib for gastrointestinal and cardiovascular safety. The CPMP concluded that the benefit-risk balance for these products is positive but recommended adding warnings concerning risk of severe skin and hypersensitivity reactions and the need for caution in patients with underlying gastrointestinal and cardiovascular risks (Media Release: 21 Nov 2003. Available from: URL: http://www.emea.eu.int.).

In a randomised, multicentre study, 672 patients with osteoarthritis who had experienced pain recurrence after withdrawal from NSAID treatment received once-daily, orally administered Rofecoxib 5mg (n = 149), 12.5mg (144), 25mg (137), 50mg (97) or placebo (145) for 6 weeks. Rofecoxib was well tolerated at all doses. For both Rofecoxib and placebo, the most commonly reported adverse events were diarrhoea, headache, insomnia, oedema and upper respiratory infection.

In an investigational study in patients with acute migraine headaches, the most common adverse events were dry mouth and dizziness. The overall rate of adverse events was similar in patients treated with Rofecoxib 25mg and placebo.

Cardiovascular adverse events: To determine whether treatment with the COX-2 inhibitors Rofecoxib and Celecoxib is associated with new-onset hypertension in community practice, US researchers conducted a retrospective case-control study in 17844 Medicare beneficiaries aged ≥ 65 years. During 1999 to 2000, 3915 patients were diagnosed with and began treatment for hypertension and four controls were selected for each case. Rofecoxib use was found to be associated with a significantly increased relative risk of new-onset hypertension, compared with Celecoxib, nonselective NSAIDs or no NSAID treatment. In contrast, Celecoxib was not associated with an increase in relative risk of new-onset hypertension. There did not appear to be a clear dose or duration relationship between use of either Rofecoxib or Celecoxib and new-onset hypertension although long duration Rofecoxib was associated with a slightly higher risk than short duration, when compared with nonselective NSAIDs, with a similar trend when compared with Celecoxib. In addition, in patients with renal disease, liver disease or congestive heart failure, Rofecoxib treatment was associated with a higher relative risk of new-onset hypertension than Celecoxib (Solomon *et al*., 2004).

Gastrointestinal adverse events: Rheumatologists in the UK have stated that good postmarketing surveillance of Rofecoxib is "*vital*". They also state that much more data are required to demonstrate whether Rofecoxib launched in the UK in June 1999 is associated with a lower incidence of gastrointestinal toxicity compared with other NSAIDs. There is concern regarding the inhibition of COX-2 as this enzyme has important physiological effects in various organs. Furthermore, no data exist comparing the tolerability of Rofecoxib with etodolac or meloxicam, 2 NSAIDs that have a degree of COX-2 selectivity.

Merck & Co. claims that Rofecoxib has an endoscopic ulcer rate similar to placebo and is seeking to have this claim included in labelling for the compound. At a meeting of the US FDA Arthritis Advisory Committee where Rofecoxib was recommended for approval for the treatment of osteoarthritis, Merck stated that combined results of 2 trials indicated that the ulcer rate seen with the 50mg dose of Rofecoxib narrowly missed equivalence with placebo. However, concern was expressed about the unusually high ulcer rate found in the placebo group in 1 of the studies (10%).

Gastrointestinal tolerance relative to other NSAIDs: A meta-analysis of 8 clinical trials comparing Rofecoxib with the traditional NSAIDs ibuprofen, diclofenac and nabumetone in patients with osteoarthritis showed that the cumulative incidence of confirmed perforations, ulcers and bleeding was significantly lower after treatment with Rofecoxib compared with the other NSAIDs. In a study carried out by researchers for the Vioxx Gastrointestinal Outcomes Research (VIGOR) study group, patients with rheumatoid arthritis received Rofecoxib 50 mg/day (n = 4047) or naproxen 500mg twice daily (4029) for 0.5-13 months (median 9 months). Compared with the naproxen group the relative risks of GI events in the Rofecoxib group were 0.5 (95% CI 0.3-0.6) for confirmed upper GI events, 0.4 (0.2-0.8) for complicated confirmed upper GI events, 0.4 (0.2-0.7) for complicated upper GI bleeding and 0.5 (0.2-0.9) for bleeding beyond the duodenum. In a comparative gastrointestinal safety study in 1516 patients with osteoarthritis, Rofecoxib 25 and 50 mg/day had an ulceration rate similar to that of placebo (4.7%, 8.1% and 7.3% of patients, respectively) at 12 weeks. In comparison, 28.5% of ibuprofen recipients had ulcers at 12 weeks. After 24 weeks, patients receiving Rofecoxib 25, 50 mg/day or ibuprofen had ulceration rates of 9.7, 13.5 and 46.4%, respectively.

Helicobacter pylori status did not affect ulcer development in either Rofecoxib or ibuprofen

recipients.

Effects on renal function: The effects of Rofecoxib and the nonselective cyclo-oxygenase inhibitor Indomethacin on renal function in healthy elderly subjects were investigated and both Rofecoxib and Indomethacin tended to reduce urinary sodium and potassium excretion but not all changes differed significantly. Same renal precautions should be observed with both selective and nonselective COX inhibitors.

Isolated cases of renal failure, nephritis and pulmonary oedema have been report with Rofecoxib therapy.

Effects on ovulation: In a double-blind, randomised study, women were assessed over 2 menstrual cycles while receiving Rofecoxib (n = 6) or placebo (7). Rofecoxib recipients were found to have a greater mean maximum preovulatory follicle diameter and a longer delay until signs of follicle rupture occurred than placebo recipients (30 *vs* 21mm, and > 48h in 4 patients *vs* > 36h following peak luteinising hormone level, respectively). The delay in follicle rupture seen in Rofecoxib recipients was characteristic of luteinised unruptured follicle syndrome. Following discontinuation of Rofecoxib the patients' menstrual cycles returned to normal.

Drug interaction:

Drug interactions are minimal. Does not affect cytochrome P450 enzymes; Rofecoxib at concentrations ≤ 100 µmol/L *in vitro* did not significantly inhibit CYP1A2, 2D6, 2E1, 2C9, 2C19, 2E1 and 3A4/5. Rofecoxib has no effect on serum concentration of oral contraceptives; antacids do not affect plasma concentrations of Rofecoxib; does not affect digoxin pharmacokinetics or aspirin pharmacodynamics; clinically non-significant interactions with rifampicin, methotrexate and warfarin. Rifampicin 600 mg/day decreased plasma Rofecoxib levels by 50%; coadministration of Rofecoxib with methotrexate increased plasma methotrexate levels 23% and reduced renal clearance of methotrexate; in healthy volunteers, coadministration of Rofecoxib with warfarin increased prothrombin time 8%. These interactions were not considered to be clinically significant.

Patient Benefits: Effective in the treatment of osteoarthritis and rheumatoid arthritis and for

the treatment of acute pain; lower incidence of gastrointestinal adverse events than other, nonselective NSAIDs; lower incidence of gastrointestinal complications resulting in fewer gastrointestinal procedures and hospitalizations

Patient Limitations: Efficacy in certain types of postoperative pain unclear; potential cardiovascular risk in some patients; not recommended in patients with moderate or severe hepatic insufficiency or patients with pre-existing asthma

Pharmacodynamics

In vitro, Rofecoxib inhibited the cyclo-oxygenase (COX)-2-dependent production of prostaglandin E_2 in human osteosarcoma cells and Chinese hamster ovary cells expressing COX-2 with respective IC_{50} values of 26 and 18 nmol/L. Rofecoxib inhibited purified human recombinant COX-2 time-dependently with an IC_{50} value of 0.34 μmol/L. The drug inhibited purified human recombinant COX-1 non-time-dependently with an IC_{50} value of 26 μmol/L. In human whole blood, Rofecoxib inhibited lipopolysaccharide-induced COX-2-dependent prostaglandin E_2 production and COX-1-dependent thromboxane B_2 synthesis after blood coagulation with respective IC_{50} values of 0.53 and 18.8 μmol/L. COX-1 IC_{50}/COX-2 IC_{50} selectivity ratios for inhibition of COX-2, relative to COX-1, were 36, 6.6, 2, 3 and 0.4 for Rofecoxib, Celecoxib, meloxicam, diclofenac and Indomethacin, respectively (Summary basis of Approval - Rofecoxib).

In vivo, in rodent models, Rofecoxib inhibited carrageenan-induced paw oedema, carrageenan-induced paw hyperalgesia and lipopolysaccharide-induced pyresis with respective ID_{50} values of 1.5, 1.0 and 0.24 mg/kg. Rofecoxib inhibited adjuvant-induced arthritis in rodents with an ID_{50} value of 0.74 mg/kg/day. Rofecoxib inhibited adjuvant-induced damage to cartilage and bone structure (Summary basis of Approval - Rofecoxib).

In an animal model of familial adenomatous polyposis (FAP), mice that were so prone to precursors of polyps that some of the lesions were likely to develop *in utero*, were treated with Rofecoxib or vehicle. After 8 weeks, around 200 tumours were present in untreated mice whereas there was a 55% reduction in the number of tumours in mice given Rofecoxib at an equivalent of 25 mg/day. Furthermore, these mice had an 80% reduction in the numbers of

polyps > 1mm. Animals receiving a dosage of Rofecoxib that was subclinical for achieving anti-inflammatory effects had a reduction of about 35% in the number of polyps.

In a randomised and partially blinded study, 76 female volunteers received one of the following: placebo, Rofecoxib 12.5mg 4 times daily, Rofecoxib 25mg 4 times daily, diclofenac 50mg 3 times daily, ibuprofen 800mg 3 times daily, naproxen sodium 550mg twice daily or meloxicam 15mg 4 times daily, for 5 days with a morning dose on day 6. Blood was collected at predose on day 6 and at 2, 4 and 8h postdose; urine was also collected to determine COX-1 generated 11-dehydro thromboxane B_2. The average inhibition *ex vivo* in blood of lipopolysaccharide-stimulated prostaglandin E_2 production (a measure of COX-2 activity) relative to baseline was 2.4%, 66.7%, 69.2%, 77.5%, 93.9%, 71.4% and 71.5% for placebo, Rofecoxib 12.5mg, Rofecoxib 25mg, Meloxicam, Diclofenac, ibuprofen and naproxen sodium, respectively. The corresponding values for average inhibition of thromboxane B_2 relative to baseline were 3.9%, 9.0%, 5.1%, 53.3%, 49.5%, 88.7% and 94.9%, respectively. Rofecoxib had no significant effect on urinary 11-dehydro thromboxane B_2, relative to placebo, whereas Diclofenac and Meloxicam caused a reduction.

Table 1. Other COX-2 inhibitors

S.No.	Name	Originator	Indication	Current status
1	Etoricoxib	Merck & Co.	Dental pain, Dysmenorrhoea, gout, Musculoskeletal pain, Osteoarthritis, RA.	Launched (except USA)
2	Valdecoxib	Pfizer (Licensee-Astellas Pharma, Japan)	Osteoarthritis, Pain and Rheumatoid arthritis	Phase – II (Launched by Pfizer; but withdrawn in May 2005)
3	Paracoxib	Pfizer	Pain	Pre-registration
4	Lumaricoxib	Novartis	Osteoarthritis, Pain and Rheumatoid arthritis	Launched
5	GW 406381	GlaxoSmithKline	Pain	Phase-III
6	CS 502	Daiichi Sankyo	Inflammation and Pain	Phase-II
7	CS 706	Daiichi Sankyo	Inflammation and Pain	Phase-II
8	Cimicoxib	Uriach	Depression	Phase-II
9	Research prog.: P 61 - Phytopharm	Phytopharm	Asthma, Inflammation, IBS	Preclinical
10	FR 140423	Astellas Pharma	Inflammation and pain	Preclinical

1.4 Issues with COX-2 inhibition

1.4.1 Effect on gastrointestinal mucosa

Conventional nonspecific nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with panoply of adverse gastrointestinal (GI) events ranging in severity from dyspepsia to endoscopic lesions which in turn can range from mucosal haemorrhage and erosions to frank ulceration, complicated by bleeding, perforation and death.

Newer NSAIDs include nabumetone, meloxicam, etodolac and nimesulide, all of which are associated with a lower rate of GI adverse events than their predecessors. However, it is the introduction of the cyclooxygenase-2 (COX-2)–specific inhibitors such as Celecoxib, Rofecoxib etc., that promised to greatly improve GI tolerability. A prediction of improved GI tolerability is based on the hypothesis that two of the COX isoforms are found in the GI tract; COX-1 is primarily constitutive and essential for maintaining mucosal integrity whereas COX-2 is associated with the inflammatory response. The high incidence of adverse GI events evident with nonspecific NSAIDs is primarily a consequence of inhibition of both COX-1 and COX-2. The development and introduction of Rofecoxib and Celecoxib has essentially provided proof for this concept.

Proof has come from several key studies.

- Rofecoxib does not inhibit prostaglandin E2 synthesis, a product of COX-1, in human gastric mucosal biopsies whereas the nonspecific NSAID, naproxen, clearly does.
- Faecal blood loss of ^{51}CR-labelled red blood cells and studies of small bowel macromolecular permeability show no significant differences between placebo and Rofecoxib [at 2 to 4 times the therapeutic dose required for osteoarthritis (OA)] in contrast with increased blood loss and bowel permeability observed with ibuprofen and Indomethacin, respectively.
- Short term endoscopy studies performed over 7 days revealed erosions/ulcers in 12% of healthy volunteers receiving Rofecoxib (at 10 to 20 times the therapeutic dose) compared with rates of 8, 94 and 71% with placebo, aspirin (acetylsalicylic acid) and ibuprofen, respectively (Scott and Lamb, 1999).

The relevance of such short-term studies to clinical outcomes has been much debated. Thus, two 6-month endoscopy studies were conducted in high-risk patients with OA (15% had a history of previous ulcer complicated by bleeding and perforation and 12% had erosions at baseline endoscopy). Rofecoxib 25 and 50mg (2 to 4 times the therapeutic dose) was associated with ulcers in 5 and 8% of patients, respectively, and was not significantly different from placebo (7%) over the first 12 weeks, whereas significantly more ulcers were seen with ibuprofen (2400 mg/day) at 28%. The incidence of ulcers observed at 24 weeks did not increase significantly with Rofecoxib (10 and 13%, respectively) over that seen at 12 weeks, whereas the incidence of ulcers with ibuprofen increased to 46% **(**Langman *et al.,* 1999). However, it is now clear that, at best, coxibs cause significant ulceration at about half the rate of conventional NSAIDs (Bombardier *et al*., 2000), while also exhibiting significant toxicity in the renal and cardiovascular systems (Cheng & Harris, 2004).

The advent of the selective COX-2 inhibitor has stimulated considerable research into the role of this enzyme in gastric mucosal defense and the associated impact on human health and disease.

The lining of the GI tract is exposed regularly to a wide range of potentially damaging substances including those that we ingest (alcohol, aspirin, etc.) and endogenous secretions (acid, bile salts). Perhaps most notable about the GI mucosa is not simply its ability to resist damage by these substances but also its ability to repair itself in case of damage. PGs, particularly PGE_2 and PGI_2, play a very important role in modulating GI mucosa defense and repair **(**Wallace and Granger, 1996; Wallace and Ma, 2001**).** The primary effects of these PGs with respect to mucosal defense are stimulation of mucus and bicarbonate secretion and maintenance of mucosal blood flow. Thus, inhibition of PG synthesis by NSAIDs is one of the primary mechanisms through which this class of drugs produces injury in the GI tract (Wallace, 1997). NSAIDs reduce mucus and bicarbonate secretion as well as reduce mucosal blood flow. These drugs also trigger an increase in adhesion of leukocytes (most notably neutrophils) to the vascular endothelium in the GI microcirculation which has been shown to be an early and critical event in the pathogenesis of NSAID-induced mucosal ulceration (Wallace, 1997).

Early studies of selective COX-2 inhibitors in healthy rats and mice confirmed the predictions of the COX-2 hypothesis that these drugs would not produce gastric injury. Since

mice with targeted disruption of COX-2 did not develop spontaneous gastric lesions, the production of the PGs that mediate gastric mucosal defense, at least under normal conditions, was attributed to COX-1 activity. Surprisingly, COX-1 knockout mice also did not spontaneously develop gastric lesions, despite negligible gastric PG synthesis, but were susceptible to lesion formation when given an NSAID **(**Langenbach *et al*., 1995**).** Other studies demonstrated that suppression of gastric COX-1 activity in the rat with a selective inhibitor (SC-560) failed to elicit gastric damage (Wallace *et al*., 2000). When both COX-1 and COX-2 were inhibited, as would occur with conventional NSAIDs, gastric damage was elicited (Wallace *et al.*, 2000). A similar situation was observed in models of NSAID-induced small intestinal injury; that is, selective inhibition of COX-1 or COX-2 did not result in injury, but suppression of both isoforms of COX led to significant damage (Tanaka *et al*., 2002). It can therefore be concluded that PGs derived from both COX-1 and COX-2 contribute to mucosal defense. Indeed, there is good evidence that the two COX isoforms may influence different components of mucosal defense: suppression of COX-1 accounts for the reduction in mucosal blood flow that is observed following NSAID administration while suppression of COX-2 accounts for the increase in leukocyte adherence to vascular endothelium that is observed following NSAID administration (Wallace *et al.,* 2000). Thus COX-2 enzyme seems to have a role in GI mucosa to resist and respond to luminal insults.

Emerging evidence indicates that COX-2 plays an expanded role in modulating resistance to luminal irritants when other mediators of mucosal defense are pharmacologically or genetically depressed. For example, nitric oxide is an important mediator of many components of mucosal defense (Wallace & Miller, 2000). Studies in the rat have demonstrated that when nitric oxide synthesis is inhibited, administration of selective COX-2 inhibitors results in significant gastric damage (Ehrlich *et al*., 2004).

Sensory afferent nerves also contribute significantly to the ability of the GI mucosa to resist injury, mainly by regulating mucosal blood flow. When sensory afferent nerves are chemically ablated, administration of selective COX-2 inhibitors results in formation of hemorrhagic lesions (Ehrlich *et al*., 2004). Thus, at the molecular level, mucosal resistance to luminal irritants involves significant cross-talk among COX-2-derived signals and other endogenous signaling pathways.

Further there is convincing evidence for physiological and beneficial roles for COX-2 in maintenance of mucosal integrity in more distal parts of the GI tract. For example, COX-2 has been suggested to be an essential factor in immune tolerance**.** Newberry *et al*. (1999; 2001**)** reported that lamina propria stromal cells constitutively express COX-2 and produce PGE_2 via this enzyme in a continuous manner. Given the known immunomodulatory effects of PGE_2, it was suggested that COX-2 contributes to ongoing downregulation of the intestinal immune response. Further evidence of COX-2 involvement in maintenance of mucosal integrity was the report that COX-2 knockout mice spontaneously developed peritonitis, presumably related to deterioration of intestinal barrier function (Morham *et al.,* 1995). These consequences of either chronic suppression of COX-2 activity or absence of COX-2 expression may be related to a role for products of this enzyme in mediating GI epithelial proliferation. No doubt, further studies will delineate the molecular mechanisms behind beneficial roles of COX-2 in maintenance of mucosal integrity during responses to luminal irritants along the GI tract. It is reported that products of cyclooxygenase-2 contribute to mucosal defense. Acetylation of COX-2 by aspirin has been shown to result in the generation of 15(R)-epi-lipoxin A4 (ATL), which exerts protective effects in the stomach. In gastritis, it is possible that lipoxin A4 or ATL makes a greater contribution to mucosal defense in both the normal and inflamed stomach (Souza *et al.*, 2003). COX-2-derived ATL may also mediate, at least in part, the well-recognized ability of the stomach to adapt to repeated exposure to aspirin. In humans and animals, many studies have demonstrated that the gastric mucosa also becomes progressively more resistant to injury during chronic ingestion of aspirin (Wallace, 1997). In parallel with this increased resistance to damage, there is marked upregulation of COX-2 expression in the gastric mucosa, and increased generation of ATL (Fiorucci *et al*., 2003). Inhibition of ATL generation, with a COX-2 inhibitor, reversed this adaptive response, returning the susceptibility of the stomach to injury to its basal level (Fiorucci *et al*., 2003).

Inflammation is a key element of mucosal defense. It is aimed at limiting entry of foreign material and microbes to the systemic circulation, as well as facilitating the repair of damaged tissue. Dysregulated inflammation can itself cause significant damage to host tissue. For instance, a dysregulated inflammatory response is thought to contribute to ulcer formation associated with use of NSAIDs, infection with *Helicobacter pylori* and in IBD. Resolution of inflammation is therefore a crucial process for restoring homeostasis, and one in which COX-2 plays a key role. The importance of COX-2-derived PGs in resolving

peripheral inflammation was shown by **(**Wallace and Devchand, 2005). They showed that when carrageenan was injected into the hindpaw of normal mice, significant inflammation was induced, including significant edema formation and it had resolved within 24–48 hrs. When carrageenan was injected into the hindpaw of COX-2-deficient mice, a similar acute inflammatory response was observed. However, even 7 days later the inflammatory response including granulocyte infiltration and edema was still evident (Wallace *et al*., 1998). Thus, in the absence of COX-2 the acute inflammatory response to carrageenan was dysregulated. This important role of COX-2 and its metabolites, in resolution of inflammation has been observed in a range of inflammatory models. These metabolites appeared to produce the resolution via induction of apoptosis of infiltrating neutrophils and macrophages.

An anti-inflammatory role of COX-2 has been observed in studies of the colon. There is a rapid and substantial upregulation of COX-2 after induction of colitis in rats. This is accompanied by a sharp increase in colonic generation of PGD2, which has a dampening effect on granulocyte infiltration (Ajuebor *et al*., 2000). Consistent with these observations, treatment with selective COX-2 inhibitors during the early phase of experimental colitis leads to enhancement of granulocyte infiltration and, if continued for several days, to further penetration of ulcers deeper into the wall of the bowel and, eventually, to perforation and death (Reuter *et al*., 1996).

It has also been shown that COX-2 enzyme also plays an important role in mucosal defense in *H. pylori*-associated inflammation. This infection is associated with significantly elevated expression of COX-2 in the stomach and treatment with COX-2 inhibitors has resulted in significantly more damage (Tatsuguchi *et al*., 2000).

Selective COX-2 inhibitors, like conventional NSAIDs have also been shown to, significantly delay gastric ulcer healing (Mizuno *et al*., 1997; Schmassmann *et al*., 1998; Halter *et al*., 2001; Ma *et al*., 2002; Perini *et al*., 2003; Schmassmann, 1998). Damage to the GI epithelium likely occurs on a daily basis, but true ‘ulcers’ (which penetrate deeper than the mucosal layer) only rarely develop. Superficial damage to the mucosa can be healed in a few hours or days. When damage penetrates into the submucosa and muscularis, repair can take several weeks or months, and involves formation of granulation tissue at the ulcer base, formation of new blood vessels (angiogenesis) and and re-establishment of the glandular architecture. As outlined above, COX-2 expression in the normal stomach is low. However, at the margins of

ulcers, COX-2 expression is very strong (Mizuno *et al.,* 1997). It is at the margins of ulcers that epithelial proliferation primarily occurs, which is critical for reestablishment of glands. COX-2 is also strongly expressed in endothelial cells in the ulcer bed (Mizuno *et al*., 1997), which is the site of new vessel growth. Therefore, COX-2 inhibition by selective COX-2 inhibitors or conventional NSAIDs that also suppress COX-2 activity, significantly delay gastric ulcer healing. Both selective and nonselective NSAIDs inhibit angiogenesis through direct effects on endothelial cells. The inhibition of ulcer healing associated with inhibition of COX-2 activity may be in part related to effects on serum levels of growth factors that regulate angiogenesis. Growth factors released from platelets and contained within serum can profoundly affect ulcer healing (Ma *et al*., 2001). When gastric ulcers are induced in rats, a shift in the serum and platelet levels of growth factors occurs such that the balance between pro- and antiangiogenic factors is tilted in favour of promotion of angiogenesis, thereby assisting ulcer healing (Ma *et al*., 2001). When rats with pre-established gastric ulcers were treated with a selective COX-2 inhibitor (Celecoxib) or a conventional NSAID (Flurbiprofen), the balance of pro- and antiangiogenic factors in serum was altered in the opposite direction, favoring inhibition of angiogenesis (Ma *et al*., 2001). Moreover, both Celecoxib and Flurbiprofen significantly inhibited ulcer repair in this model.

In conclusion, selective COX-2 inhibitors were developed as anti-inflammatory drugs with GI sparing activity. However, a series of discoveries have strongly suggested a crucial role of COX-2 in GI mucosal defense and repair. While expressed in low levels in the healthy GI tract, COX-2 nonetheless contributes significantly to mucosal immunity and to the ability of the mucosa to resist injury induced by luminal irritants. The COX-2 gene rapidly responds to stress, and the downstream products of this enzyme are potent lipid mediators that enhance resistance to injury and regulate dynamics of both inflammation and resolution. Emerging evidence implicates COX-2 in mediating some of the long-term consequences of inflammation in the GI tract, including the generation of symptoms in conditions such as irritable bowel syndrome, and the predisposition to cancer in individuals with colitis.

1.4.2 Effect on Renal function.

Renal effects of Coxibs are based on their primary pharmacologic mechanism of action—inhibition of PG synthesis. These effects are relatively mild and rare in healthy individuals but can be serious in patients whose renal function is PG dependent. Patients with contracted

effective intravascular fluid volume as a result of congestive heart failure, cirrhosis, diuretic use, or restricted sodium intake, are at an increased risk for coxib-related changes in renal function.

Both COX isoforms are present constitutively in the human kidney. COX-1 is found in the glomerulus and afferent arteriole, while COX-2 is located in the podocytes, thick ascending limb of the loop of Henle, macula densa and afferent arteriole (Fig 4A) (Nantel *et al.*, 1999; Schnermann and Briggs, 1999).

(A) (B)

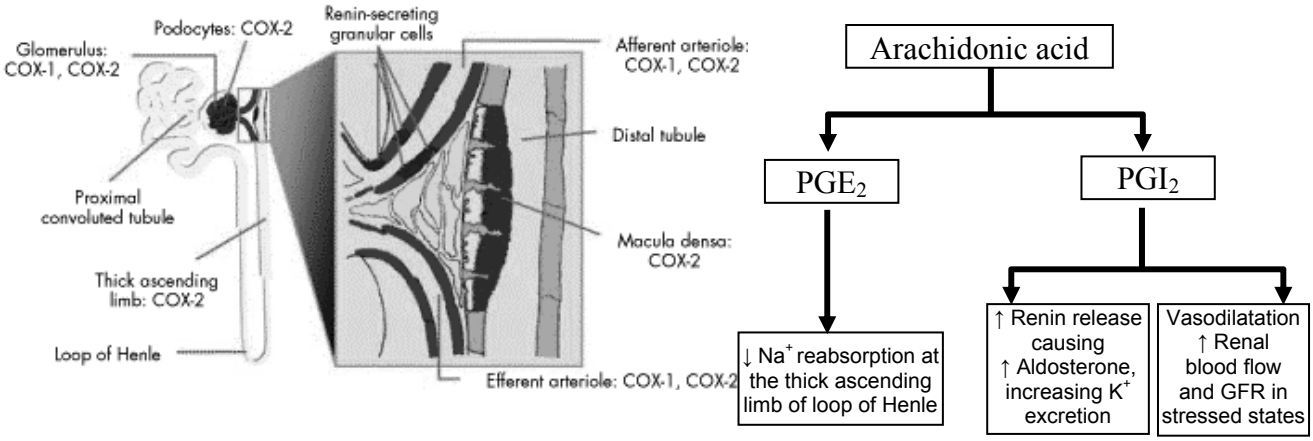


Fig 4. Distribution of COX-1 and COX-2 and role of prostaglandins in the kidney (Schnermann and Briggs, 1999)

These distinct areas of distribution correlate with the different effects on renal function displayed by COX-1 and COX-2. Prostaglandins produced by COX-1 primarily affect renal homeostasis, and promote dilation of the renal vascular bed, lowering of renal vascular resistance and increases in renal perfusion (Weir and Froch, 2000). In contrast, prostaglandins produced by COX-2 have diuretic and natriuretic effects (Fig 4B).

Under physiological conditions, prostaglandins do not play a major role in the maintenance of basal renal function. However, in situations where renal hemodynamics is compromised, such as in subjects with volume depletion, prostaglandins have a profound effect on the maintenance of renal perfusion and Glomerular filteration rate (GFR) by virtue of their vasodilatory effects. In these prostaglandin dependent states, NSAID-mediated prostaglandin inhibition can result in acute renal failure (Brater, 1999).

The localization of COX-2 in these cortical regions suggests a role in the production of PGs regulating release of renin. COX-2 is also expressed in the medullary interstitial cells, which are thought to be involved in regulation of tubular absorption and possibly in regulation of

medullary blood flow. In the human renal cortex, COX-2 is predominantly expressed intraglomerularly in podocytes, suggesting a role in regulation of glomerular hemodynamics through contraction of podocytes (Harris and Breyer, 2001; Harris, 2000). In the renal cortex, the expression of COX-2 is upregulated in response to decreased sodium intake, volume depletion, renal artery stenosis, active lupus nephritis, partial renal ablation, and therapy with inhibitors of the renin–angiotensin system—angiotensin-converting enzyme inhibitors and angiotensin II type 1 (AT1) receptor blockers.

In the medulla, COX-2 expression decreases with salt depletion and increases with a high-salt diet or dehydration. This increased expression of COX-2 under conditions of hypertonicity may have a cytoprotective role. These results have been confirmed in an animal model. In rabbits treated with a COX-2 inhibitor, water deprivation was associated with the presence of apoptotic patches of renal medullar interstitial cells **(**Hao *et al.*, 2000). No apoptosis was seen in renal medulla of rabbits that were treated with the COX-2 inhibitor alone or in those that underwent water deprivation and no pharmacologic treatment. Therefore, it seems that inducible expression of COX-2 in renal medulla is an important survival factor under conditions of hypertonic stress. PGs are also known to play a critical role in maintaining medullary renal blood supply and renal salt excretion, particularly under conditions of volume depletion (Qi and Breyer, 2000). Increased expression of COX-2 after dehydration and results from preliminary animal studies are consistent with an important role of this isozyme in maintaining renal medullary perfusion in the setting of volume depletion.

PGs synthesized by COX-2 seem to be a part of the feedback mechanism regulating activity of the renin– angiotensin system. Angiotensin-converting enzyme inhibition causes an increase in plasma renin, which can be significantly diminished by simultaneous treatment with a COX-2 inhibitor. (Cheng *et al*., 1999) Experiments with genetically altered mice confirmed that the effect of angiotensin II inhibition on renin release is mediated by COX-2 (Cheng *et al*., 2001). In wild-type mice, administration of angiotensin-converting enzyme inhibitors leads to increased expression of COX-2 and renal renin. In knockout mice with deletions of the COX-2 gene, the expression of renal renin is not significantly affected by angiotensin-converting enzyme inhibition (Cheng *et al*., 2001). In addition to the effects on the renin–angiotensin system, COX-2–synthesized PGs may have a direct role in modulating sodium reabsorption in the cortical thick ascending limb region. In summary, COX-2 expression in the renal cortex seems to be inhibited by angiotensin II and stimulated under

conditions of low sodium intake. If the activity of the renin–angiotensin system is diminished or insufficient to maintain electrolyte balance, increased COX-2 expression and synthesis of PGs activate expression and release of renin, leading to increased activity of angiotensin II and aldosterone and resulting in increased tubule reabsorption facilitating reestablishment of intravascular volume homeostasis. Once this is achieved, the expression of COX-2 is reduced through inhibition by angiotensin II, and renin production and release are reduced

Similar results were also observed in several clinical trials. In elderly patients on salt restricted diet, treatment with Rofecoxib resulted insignificant reductions in the GFR and in sodium excretion which was similar to the comparator standard Indomethacin. (Catella-Lawson *et al.*, 1999; Swan *et al.*, 2000). A single 400-mg dose of Celecoxib significantly decreased GFR and renal plasma flow, demonstrating again that COX-2 inhibition with this agent can affect renal function under conditions of increased renal dependence on PGs (*FDA Celebrex Advisory Committee*). In other studies, both Celecoxib and naproxen produced transient reductions in urinary sodium excretion over the first 3 days of treatment. Another study examined the effects of Celecoxib on renal function of patients with renal insufficiency. A total of 71 patients with stable chronic renal insufficiency were randomized to receive Celecoxib (200 mg bid), naproxen (500 mg bid), or placebo for 7 days (*FDA Celebrex Advisory Committee*). After 7 days of treatment, statistically significant decreases in GFR were observed among patients receiving Celecoxib and among those receiving naproxen. Sodium excretion was transiently reduced with both active treatments. Therefore, the available evidence from these small pharmacodynamic trials indicates that the renal effects of coxibs may be similar to those of traditional NSAIDs, particularly in patients considered at risk of adverse renal effects related to use of NSAIDs.

1.4.3 Effect on Central nervous system.

Less common but clinically relevant adverse effects associated with NSAID use include impairment of the CNS and particularly, occurrence of neuropsychiatric symptoms. (Jiang and Chang, 1999; Clark and Ghose, 1992) These neuropsychiatric symptoms include changes in cognition, mood and even precipitation or exacerbation of pre-existing psychiatric conditions. Several cases of NSAID associated neuropsychiatric symptoms have been reported in the medical literature in the past decades, but this subject has not been evaluated systematically and no predisposing factors have been identified for these adverse events.

In recent years, several national reports described psychiatric adverse events associated with use of the newer selective COX-2 inhibitors. In 1999, 26 cases of psychiatric adverse drug events related to Celecoxib were reported to the Canadian Adverse Drug Reaction Monitoring Program. [Celecoxib (Celebrex): 1 year later (2000). *Canadian ADR Newsletter* 10: 1-3]. In New Zealand in 2002, the Intensive Medicines Monitoring Program received 13 reports of cases of acute psychiatric events related to COX-2 inhibitor use (Celecoxib, 11 cases; Rofecoxib, 2 cases) (Coulter, 2002). Five of the reports were of confusion, two of depression and three of hallucination. Exacerbation of manic depressive psychosis was also reported in one case. Anxiety and abnormal thinking were reported once. In this report, most of the patients were elderly, and the psychiatric events rapidly resolved upon withdrawal of the COX-2 inhibitor in each case. Finally, in 2003 the Australian Adverse Drug Reactions Advisory Committee reported 142 (5% of the total number of adverse drug reactions reported for the drug) cases of acute neuropsychiatric events associated with Celecoxib and 49 (8%) with Rofecoxib. [Acute neuropsychiatric events with Celecoxib and Rofecoxib (2003). *Aust Adv Drug Reactions Bull*; 22 (1): 3]. The most common events with Celecoxib were confusion, somnolence and insomnia. As a proportion of the total reports, hallucination has been reported more commonly with Rofecoxib than with Celecoxib. In addition to the population studies, some reports of cases of psychiatric adverse events have also been reported (Lantz and Giambanco, 2000; Macknight and Rojas-Fernandez, 2001).

It is not yet clear how NSAIDs and more specifically COX-2 inhibitors can precipitate the onset of psychiatric symptoms. NSAIDs block the synthesis of prostaglandins by inhibiting the activity of COX. This enzyme has shown to have a key role in the CNS, particularly in thermoregulation and pain. COX-2 is the most abundant COX isoform found in the CNS, and it has been identified in neocortex, hippocampus, amygdala, limbic cortices and in nuclei adjacent to the third ventricle, both in neurones and in the non-neuronal cells (Vane *et al.*, 1998). COX-2 is upregulated by normal or by abnormal (convulsive) nerve activity, and it is preferentially localised in distal dendrites and dendritic spines, which are cellular specialisations involved in synaptic signaling (Kaufmann *et al.*, 1997). These findings suggest a role for prostaglandins in CNS transmission and raise the possibility that selective COX-2 inhibition may modulate CNS function.

A further question regarding the role of selective COX-2 inhibitors in the onset of psychiatric symptoms are raised by a study showing a beneficial antipsychotic effect of these drugs when

used in association with antipsychotics to treat patients with schizophrenia (Muller *et al*., 2002).

In conclusion, psychiatric symptoms are a rare but relevant complication of NSAID use. This effect is probably a consequence of impairment in the neurotransmission modulated by prostaglandins when NSAIDs are used by susceptible individuals. These drugs have to be used with caution in high-risk individuals with preexisting psychiatric illness and caution may also be advisable in the postpartum period. To date, reports of NSAID-related psychiatric adverse events have most commonly involved Indomethacin and selective COX-2 inhibitors. Whether this reflects a greater incidence of such events with these drugs or is related to other factors such as usage and reporting patterns is unknown.

1.4.4 Effect on Cardiovascular system.

The role of COX-2 inhibition in cardiovascular diseases is complex and is not fully understood. It was reported that coxibs like Celecoxib and Rofecoxib suppressed the formation of prostaglandin I_2 in healthy volunteers (FitzGerald, 2003). Prostaglandin I_2 had previously been shown to be the predominant cyclooxygenase product in endothelium, inhibiting platelet aggregation, causing vasodilatation, and preventing the proliferation of vascular smooth-muscle cells in vitro. However, it was assumed that prostaglandin I_2 was derived mainly from COX-1, the only cyclooxygenase species expressed constitutively in endothelial cells. This assumption later proved incorrect, since studies in mice and humans showed that COX-2 was the dominant source. The individual cardiovascular effects of prostaglandin I_2 *in vitro* contrast with those of thromboxane A_2 the major COX-1 product of platelets, which causes platelet aggregation, vasoconstriction, and vascular proliferation.

While the traditional NSAIDs inhibited platelet aggregation *ex vivo* transiently at the time of peak action, the coxibs had no such effect, compatible with the absence of COX-2 from mature human platelets (Patrignani *et al*., 1999). Unlike the non-selective NSAID comparators in these studies — ibuprofen and Indomethacin —Celecoxib or Rofecoxib only inhibited PGI_2 leaving the COX-1–derived TxA_2 unaffected. Thus, the cardiovascular effects of TxA_2 would be expected to be exaggerated with the use of coxibs. However, as PGI_2 was known to act as a general restraint on any recognized stimulus to platelet activation, it was not suggested that upsetting a notional "balance" between these 2 prostanoids was likely to be

the mechanism of drug action. Correspondingly, variation in other endogenous mediators, such as NO, would be expected to modulate the impact of COX-2 inhibition on cardiovascular function. Given that similar observations were made with several coxibs, it appeared that this effect was mechanism based, rather than an off-target effect restricted to 1 compound. These effects may differ among structurally distinct COX-2 inhibitors with different levels of COX-1 or COX-2 selectivity, but evidence for a differential cardiovascular effect is limited.

Despite the presence of only COX-1 in endothelial cells under static conditions *in vitro* (Creminon *et al*., 1995), PGI_2 — a dominant product of endothelium (Moncada *et al*., 1977) — appeared largely to derive from COX-2 under physiological conditions in humans. Topper *et al.,* (1996) had found that subjection of endothelial cells in culture to laminar shear upregulated COX-2 expression. Thus, induction of COX-2 was likely to have occurred in response to blood flow under physiological conditions *in vivo*. Studies that led to approval of the coxibs were too short and too small in subject number to have excluded a risk of myocardial infaction or stroke attributable to this hypothesis.

Before examining the cardiovascular hazards with COX-2 inhibitors any further, it would be important to understand the variables involved. They include (i) the actual degree of selectivity attained at the vascular interface *in vivo* and its relevance. Although assays in whole blood *in vitro* suggest a clear segregation between the degrees of selectivity attained by the drugs under consideration, there are substantial inter-individual differences in drug response (Fries *et al., 2006*) and consequent overlap in the degree of selectivity attained *in vivo*. Selectivity for COX-2 can be viewed as a continuous variable within the class of NSAIDs. Indeed, some non selective NSAIDs — diclofenac, nimesulide, meloxicam— express average selectivity for COX-2 similar to that of Celecoxib in human whole blood *in vitro* (FitzGerald and Patrono, 2001). Sufficient concentration of any selective COX-2 inhibitor becomes nonselective as it begins to inhibit COX-1, at least in vitro (FitzGerald and Patrono, 2001; Brune and Hinz, 2004). (ii) More prolonged the drug exposure (determined by dose, duration of action, and duration of treatment), the more likely an adverse consequence. (iii) Concordant administration of low-dose aspirin, which favors inhibition of COX-1 (McAdam, *et al.*, 2000), would be expected to mitigate but not abolish the hazard. The degree and duration of simultaneous inhibition of the 2 COX enzymes would also be expected to influence the existence of a cardiovascular hazard from NSAIDs. (iv) $IP^{-/-}$ mice are more

responsive to thrombogenic stimuli but they do not develop spontaneous thrombosis (Murata *et al*., 1997). Thus, a clinical or genetic predisposition to thrombosis would favor emergence of a drug-related cardiovascular event.

Apart from the question posed by clinical pharmacology, the first evidence consistent with the hypothetical cardiovascular hazard emerged in the Vioxx Gastrointestinal Outcome Research (VIGOR) study (Bombardier *et al.*, 2000), in which a 2-fold change in the incidence of serious GI adverse events between Rofecoxib and the NSAID naproxen coincided with a 5-fold divergence in the incidence of myocardial infarction (20 versus 4 events). This study was conducted with a high dose (50 mg/day) of Rofecoxib in patients in whom low-dose aspirin was precluded. Most of the patients suffered from RA, a disease associated with an odds ratio of a myocardial infarction roughly 50% higher than in patients with osteoarthritis or no arthritis (Watson *et al*., 2003). These results generated considerable controversy; some researchers claimed that Rofecoxib was neutral and that the result reflected a cardioprotective effect of naproxen, based on its extended duration of action (VanHecken *et al.*, 2000), permitting this mixed inhibitor of COX-1 and COX-2 to behave like aspirin.

The corresponding outcomes study of Celecoxib (Celecoxib Long-term Arthritis Safety Study [CLASS]) was published in a highly unorthodox manner (Silverstein *et al*., 2000). Partial presentation of the data seemed to suggest that high-dose (800 mg/day) Celecoxib had caused fewer GI adverse effects than its nonselective NSAID comparators; however, this turned out not to be the case when the full data set was revealed (Juni *et al*., 2002). This study, conducted with, on average, a shorter-lived, less selective COX-2 inhibitor than Rofecoxib, also demonstrated no difference in the incidence of cardiovascular events. Around 20% of the patients took aspirin, and much was made of the apparent divergent incidence of GI adverse effects on ibuprofen versus Celecoxib in a post hoc analysis of nonaspirin users. Perhaps aspirin had masked the GI advantage of Celecoxib. However, if so, it may also have masked the cardiovascular hazard. A similar underpowered and retrospective analysis suggests that cardiovascular events occurred more often with Celecoxib than with ibuprofen in nonaspirin users. Interestingly, the incidence of both GI and cardiovascular events on diclofenac and Celecoxib appeared to be similar (FitzGerald, 2003).

In summary, the number of events reported in the VIGOR study was small. However, if the estimate of the difference between the 2 treatment groups was reliable, this was larger than might be expected from an "aspirin-like" effect of naproxen; clearly it was compatible with

the coincidence of a cardiovascular hazard from Rofecoxib and some protection from naproxen.

Later**,** Mukherjee *et al*. (2001) also suggested that the use of coxibs may be associated with an increased risk of cardiovascular events, particularly myocardial infarction. This analysis compared the incidence of myocardial infarction in patients receiving Rofecoxib in the VIOXX Gastrointestinal Outcomes Research (VIGOR) study (Bombardier *et al.*, 2000) and those receiving Celecoxib in the Celecoxib Long-term Arthritis Safety Study (CLASS) (Silverstein *et al*., 2000) with the incidence in healthy patients receiving placebo in four older aspirin primary prevention trials. The incidence of myocardial infarction was reported to be comparable in patients receiving Rofecoxib in the VIGOR study and Celecoxib in the CLASS study (0.74 vs. 0.80, respectively), but significantly lower in the placebo group (0.52) employed in the analysis. They also reported that the incidence of cardiovascular events was 2.38 times more common with Rofecoxib than with naproxen in the VIGOR study, while there was no significant difference between Celecoxib and the comparator NSAIDs in the CLASS study. It is apparent that, though the incidence of myocardial infarction with Rofecoxib and Celecoxib was similar, it was substantially higher than naproxen. Following this paper several author reported detection of cardiovascular hazard associated with 50 mg/d Rofecoxib, but most failed to do so with lower doses such as that (25 mg/d) used in the randomized controlled-outcome trial. However, the most significant study of them was the Adenomatous Polyp Prevention on Vioxx (APPROVe) study that subsequently led to the withdrawal of the drug (Bresalier *et al*., 2005). The study was designed to test the hypothesis that COX-2 inhibitors could prevent recurrent colonic polyps, showed increased cardiovascular toxicity with a relative risk of 1.92 fold for Rofecoxib. The significantly increased relative risk became apparent after 18 months of treatment; during the first 18 months, the event rates were similar in the two groups. These results primarily reflect a greater number of myocardial infarctions and ischemic cerebrovascular events in the Rofecoxib group. Most observational studies and overview analyses of the small, short studies that provided the basis for drug approval also failed to detect a hazard from Celecoxib (Kimmel *et al*., 2005) and valdecoxib (White *et al*., 2004). Pharmacoepidemiology alone did not clearly discriminate between a hazard peculiar to Rofecoxib and a mechanism-based effect.

The situation was clarified by the emergence of information from four published placebo-controlled trials. The pattern of the clinical information was consistent with the proposed mechanism. For reasons discussed above, a prothrombotic clinical substrate would favor the rapid emergence of adverse cardiovascular events in a relatively small study. An example of such a setting is coronary artery bypass grafting (CABG), which is characterized by intense hemostatic activation (Cannata *et al*., 2004). Two placebo-controlled studies of valdecoxib (Ott *et al.,* 2003; Nussmeier *et al.,* 2005), anteceded by its intravenous prodrug parecoxib, were performed in patients undergoing CABG. Despite their small study sizes (462 and 1636 patients, respectively) and short duration (10 and 14 days of treatment, respectively), pooled analysis of the 2 quite similar studies suggests that parecoxib/valdecoxib elevate the combined incidence of myocardial infarction and stroke by 3-fold in this population (Furberg *et al*., 2005). Although the patients were prescribed aspirin, the timing of its administration relative to the incidence of the vascular events is unclear. CABG is also a setting of apparent "aspirin resistance" (Zimmermann *et al*., 2003). These studies are compatible with the rapid emergence of a cardiovascular hazard based on suppression of COX-2–derived PGI_2 in a population with preexisting, intense hemostatic activation. Similarly, one would anticipate that a less pronounced prothrombotic substrate, such as the patients with RA in the 9-month VIGOR trial, might reveal a hazard more gradually. The rapidity with which a cardiovascular risk might manifest would reflect in part the intensity of a genetic or environmental predisposition to thrombosis.

APPROVe (Bresalier *et al*. 2005) and Adenoma Prevention with Celecoxib (APC), two studies in patients with colonic adenomata, presumed initially to be at low risk of cardiovascular events, revealed the gradual emergence of a cardiovascular risk attributable respectively to Rofecoxib (Bresalier *et al*. 2005) and Celecoxib (Solomon *et al*., 2005) after dosing for more than 1 year. Supportive of this being a true drug-related effect, the hazard in patients taking Celecoxib 200 mg, bid and 400 mg, bid appeared to be dose related (Solomon *et al*., 2005). This has resulted in introduction of new restriction in its use. Several comparative studies of COX-2 inhibitors and NSAIDs failed to detect a discriminant incidence of cardiovascular events. However, in each case, these studies were substantially underpowered to exclude this possibility. These include the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET) (Schnitzer *et al.*, 2004), which comprised two 1-year long, comparator studies of lumiracoxib, with ibuprofen and naproxen. While cardiovascular events tended to be higher in the lumiracoxib group, the study included

patients mostly at low risk, and the power of the comparisons was undermined (Topol and Falk, 2004). Furthermore, TARGET was not designed to establish noninferiority of cardiovascular risk among the treatment groups; thus, it had no predefined upper confidence interval for relative risk (Jones *et al.*, 1996) and used an intention-to-treat analysis. While it has been suggested that the pharmacokinetics of lumiracoxib favor a transient exposure in the vascular compartment with prolonged availability in the joint space (Scott *et al.*, 2004), 400 mg/dl of lumiracoxib exceeds considerably the dose necessary to inhibit COX-2 at the time of peak drug action. Given at this dose, it has a prolonged systemic pharmacodynamic half-life, depressing PGIM excretion to a similar extent and for a similar duration as Rofecoxib (Tacconelli *et al*, 2004).

In summary, while the number of cardiovascular events in all of the relevant individual RCTs addressing this issue is small, however substantial clinical evidence has emerged in last two-three years which is remarkably compatible with a unitary mechanism for which there is comprehensive biological plausibility. The clinically concordant evidence includes the following: (a) the easiest detection of a signal in epidemiological studies for a long time use of a highly selective COX-2 inhibitor, Rofecoxib, given at a high dose (50 mg/day); (b) the rapid emergence of a signal in 2 relatively small RCTs of valdecoxib in a setting of intense hemostatic activation and likely aspirin resistance; (c) the intermediate time to detection of a hazard in RA patients in the VIGOR study in whom hemostatic activation and risk of thrombosis is considerably less than in those individuals that have undergone CABG but exceeds that in patients without arthritis; (d) the similarity of the overview analyses of etoricoxib versus naproxen to what was observed in VIGOR and evidence in trials to date consistent with a cardiovascular hazard from this drug; (e) the delayed emergence of a hazard in 2 RCTs of prolonged treatment with Rofecoxib and Celecoxib, which is compatible with risk transformation in patients initially at low risk of cardiovascular disease; and (f) the evidence of hazard involving 3 structurally distinct selective COX-2 inhibitors — belying the notion that this is an off-target effect of Rofecoxib. These new studies have resulted in withdrawal of several selective COX-2 inhibitors from clinical use and restriction of their labels for other.

1.4.5 Conclusions

Cycloxygenase-2 inhibitors were introduced as an effective and safer alternative to the widely used nonselective NSAIDs. Although, introduced with a limited indication specifically for

treating signs and symptoms of osteoarthritis, rheumatoid arthritis, and dysmenorrheal and managing acute pain mainly in patients with high risk of gastrointestinal side effects. As initial clinical data suggested better GI tolerance for coxibs as compared to nonselective NSAIDs, their usage increased considerably not only in high risk groups but more so in individuals at low risk for GI side effects (Becker, 2005). The high usage profile of coxibs in general population brought to light several adverse pharmacological effects associated with NSAIDs and specifically related to COX-2 inhibitors usage. Based on series of published reports, it now appears that at best, coxibs cause significant ulceration at about half the rate of conventional NSAIDs (Bombardier *et al*., 2000) with a heightened risk in patients with history of gastric bleeding. A series of discoveries have strongly suggested a crucial role of COX-2 in GI mucosal defense and repair.

COX-2 inhibitors also inherit the renal effects of traditional NSAIDs, particularly in patients considered at risk of adverse renal effects related to use of NSAIDs such with renal impairment or in condition of dehydration or high salt intake.

There have been some reports of adverse psychiatric effects associated with COX-2 owing to the role of COX-2 in generation of prostaglandins in CNS.

Apart from these adverse effects the most notorious adverse effect reported is the effect on the cardiovascular system. The risk differs, to some degree, across agents, and does appear to be dose related. The relationship between cardiovascular risk and duration of therapy has also emerged as an important factor. Early risk, from the perspective of pathobiology, may differ from long-term risk. The mechanism of cardiovascular risk appears to be multifactorial and relates to sites of COX-2 synthesis, expression within the vasculature, and related local consequences of an imbalance between thromboxane A2 and prostacyclin. Considered collectively, increased platelet aggregation, hypertension, endothelial cell dysfunction, impaired angiogenesis, and destabilization of the atherosclerotic plaque matrix are important contributors to the “prothrombotic environment.” Several randomised Clinical trials, of prolonged treatment with coxibs have demonstrated these effects which eventually lead to withdrawal of several selective COX-2 inhibitors from clinical use and revised labeling for the rest.

However, delivery of coxibs by topical route can be useful to avoid the adverse drug effects, without loosing the beneficial effects of the Coxibs. Topical drug delivery offers several advantages such as: (i) avoidance of the gastric route, reducing the potential for both degradation of the drug and gastric irritation; (ii) high therapeutic tissue *vs* plasma concentration can be obtained thereby achieve therapeutic concentrations in the tissues subjacent to the site of application while maintaining low plasma concentrations thus reduce the risk of serious adverse events related to elevated plasma concentrations; (iii) improve patient compliance with drug administration; (iv) may be cost effective in the long term. Additionally, COX-2 inhibitors have been shown to have beneficial effect in cancer chemotherapy. COX-2 is shown to be involved in skin tumor promotion (Muller-Decker *et al.*, 1998), and topically applied COX-2 inhibitors have been reported to have beneficial effect in skin tumors in animal models (Pentland *et al.*, 1999). Therefore, topically application of Coxibs will not only be useful as an analgesic and anti-inflammatory agent for management of acute pain and for treating signs and symptoms of osteoarthritis, rheumatoid arthritis but additionally have useful role in cancer chemotherapy.

1.5 Topical drug delivery: Basic principles

Topical application of a wide range of pharmaceutical agents is a useful alternative to more traditional routes of drug administration. Major advantages include: (i) avoidance of the gastric route, reducing the potential for both degradation of the drug and gastric irritation; (ii) a reduction in first pass metabolism by the liver; (iii) high therapeutic tissue *vs* plasma concentration can be obtained thereby achieve therapeutic concentrations in the tissues subjacent to the site of application while maintaining low serum concentrations thus reduced risk of serious adverse events related to elevated plasma concentrations; (iv) improve patient compliance with drug administration; (v) may be cost effective in the long term; (vi) non-invasiveness, etc.

There is an increasing number of pharmaceutical products being registered and marketed for topical application, although limited information is available about the percutaneous penetration of the active ingredient/s or their vehicles in the intended species. It is not only important to show efficacy of formulations by topical route but also characterize the transdermal pharmacokinetics for development of an efficient topical formulation.

The skin is the largest organ of the body, accounting for more than 10% of body mass. It has important protective and homeostatic roles and is generally regarded as a critical protective barrier to the external environment. The extent of absorption through the epidermis, dermis and systemically availability becomes important (Walters and Roberts, 2002) when we consider that drugs are applied to the skin for: (i) local effects (e.g., corticosteroids for dermatitis); (ii) transport through the skin for systemic effects (e.g., fentanyl, nicotine, oestradiol and testosterone patches); (iii) surface action (e.g., sunscreens and anti-infectives), and (iv) targeting subjacent tissue (e.g., non-steroidal anti-inflammatory agents [NSAIDs] for muscle inflammation). Investigation of human skin has revealed that the major resistance to drug penetration is the outermost layer, the stratum corneum (SC). Several theories have been proposed for drug passage through the stratum corneum into the viable epidermis and dermis, including the "bricks and mortar" theory, representing keratinocytes held together by a lipid bilayer (Michaels *et al*., 1975; Menon and Elias, 1997). However, differences in skin thickness, density of appendages (hair follicles and glands), vascularity and metabolic enzymes mean that different regions of skin in the same individual display different pharmacokinetics of percutaneous drug penetration. Therefore, it is important to understand structure of the skin, the factors that effect the penetration through the skin and methods to enhance/ modulate the penetration through the skin to achieve desired pharmacokinetic profile of the topical formulation.

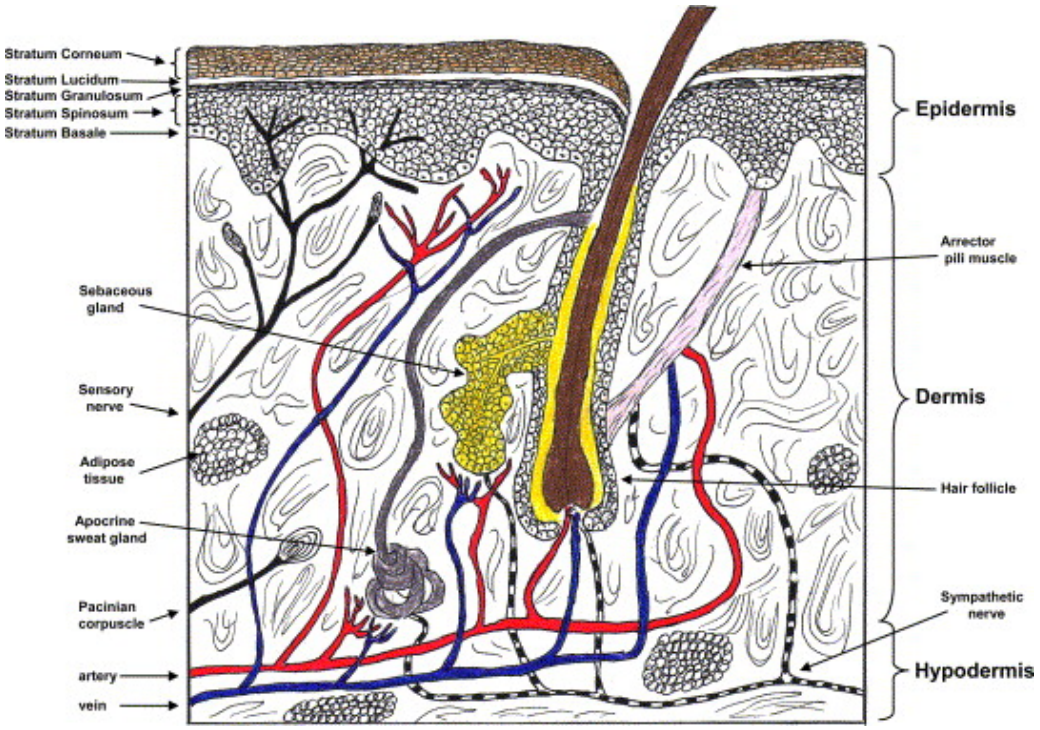


Fig 5. The typical structure of mammalian skin *(Walters and Roberts, 2002)*

1.5.1 Overview of the anatomy and function of the skin

The skin consists of the epidermis, the dermis and the subcutis or subcutaneous tissue which anchors the skin to underlying tissues. Each layer is physically and functionally distinct with appendages, including hair follicles, sweat ducts and sebaceous glands, bridging between the layers and the skin surface (Fig. 5).

1.5.1.1 The epidermis

The cells of the epidermis can be classified as keratinocytes or non-keratinocytes comprising melanocytes (cells producing melanin to control pigmentation of the skin, Langerhans cells (dendritic antigen-presenting cells) which have a role in skin sensitisation and keratinocyte proliferation and Merkel cells, found in the basal region and function as sensory receptors for the peripheral nervous system (Walters and Roberts, 2002).

Five layers of keratinocytes are generally recognized (Fig. 5) and represent different stages of differentiation, ranging from the proliferating cells of the stratum basale to the terminally differentiated keratinocytes of the stratum corneum. Keratinocytes are anchored to the basement membrane by hemidesmosomes and to other keratinocytes by desmosomes. The cornfield cells of the stratum corneum, keratinocytes undergo a total turnover every two to three weeks (Walters and Roberts, 2002).

Keratinocytes are surrounded by a continuous lipid phase known as the intercellular lipid bilayers. These are comprised primarily of cholesterol (27%), ceramides (41%) and free fatty acids (9%). These sphingolipids are arranged as multiple lamellar structures and the arrangement led to the popular ‘bricks and mortar’ model of the stratum corneum. During desquamation, as the keratinocytes migrate towards the stratum corneum, phospholipids are replaced with sphingolipids which constitute the majority of the long-chain, saturated fatty acids. This creates a highly lipophilic barrier which prevents excessive water loss to the environment and protects against the transdermal penetration of drug molecules (Walters and Roberts, 2002).

1.5.1.2 The Dermis

The dermis is a vascularized collagen-rich connective tissue containing mucopolysaccharides, primarily proteoglycans, collectively known as the ground substance. The main cell type is the fibroblast, which produces connective tissue. Mast cells are contained within the dermis,

although the number of cells and the contents of the granules (histamine, heparin or serotonin), vary depending on region of the body. Similar inflammatory and immune cells are found in the dermis as in the epidermis.

Temperature regulation is mediated by adipose cells and by arteriovenous anastomoses which permit direct shunting of up to 60% of skin blood flow between arteries and veins, by-passing the fine capillaries of the upper layers of the dermis. The nerve supply is extensive, functioning to respond to stimuli from the external environment, including perception of pain, pressure and temperature. Lymphatics are important within the dermis to regulate interstitial pressure, mobilise defence mechanisms and remove wastes (Walters and Roberts, 2002). The lymphatics also appear important in the clearance of larger molecules, such as interferon, from the dermis (Cross and Roberts, 1993).

1.5.1.3 Subcutis

The subcutis or hypodermis is mostly composed of adipocytes with fewer fibroblasts, endothelial cells and macrophages. It connects to the dermis via collagen and elastin fibers, while anchoring the skin to underlying muscle. Its major role is to carry the vessels and nerves that supply the skin (Walters and Roberts, 2002).

1.5.1.4 Skin appendages

The stratum corneum is interrupted at regular intervals by appendages penetrating through from the dermis, particularly hair follicles (with associated sebaceous glands) and sweat glands (apocrine and eccrine). The number and type of appendages vary with body region. Hair follicles function to protect the epidermis, while lubrication is provided by the sebum. Eccrine glands are important to body cooling, while the apocrine glands release a lipid secretion and may function as a vestigial secondary sex gland (Walters and Roberts, 2002).

1.5.2 Penetration of molecules through the skin

1.5.2.1 Epidermal transport

The skin is generally regarded as a physiological barrier yet pharmacological agents will penetrate into and through the skin. Studies examining the characteristics of solutes with respect to the ultrastructure of the stratum corneum have revealed that the tortuous intercellular diffusion of solutes along a concentration gradient was the pathway through the

stratum corneum following topical application (Bunge *et al*., 1999). This led to the acceptance of the 'bricks and mortar' model of solute penetration (as shown in the Fig 5), with penetration through the stratum corneum achieved by drugs moving within intercellular lipids between keratinocytes. The speed of the solute movement through the skin, including the stratum corneum, is known as the diffusivity of the drug, and is limited by the binding of the drug to keratinocytes, the viscosity of the intercellular environment and the tortuosity of the pathway (Roberts *et al*., 2002). Transport via a transcellular pathway is unlikely because it would require repeated partitioning of the solute between lipophilic and hydrophilic compartments, including the almost impenetrable intracellular matrix of the keratinocytes. Studies have shown that all solutes are transported through a lipid pathway with resistance to passage of lipophilic solutes arising from the dermis and not the keratinocytes (Roberts *et al*., 2002).

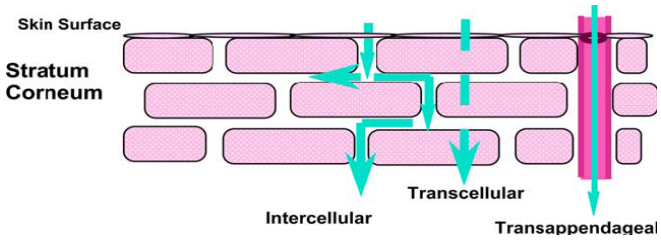


Fig 6. Possible routes of solute transport through the epidermis (*Roberts et al., 2002*).

An obvious issue arising from a model of solutes penetrating along intercellular lipids between keratinocytes is that there are two extremes of environmental characteristics. These are hydrophilic characteristics formed by the polar head groups of the bilayer formation, while the wider section within the bilayer is lipophilic due to the presence of long non-polar chains. It was found, however, that the permeability of very polar solutes through the stratum corneum was almost constant, while permeability for lipophilic solutes changed with the degree of lipophilicity. The degree of lipophilicity (log P) of any solute is determined by its relative tendency to partition between octanol and water (log P = logo/w), with a log P between 1 and 3 considered ideal to facilitate penetration through the stratum corneum. Therefore, the ideal solutes for topical delivery can be described as non-ionic, reasonably lipophilic and, particularly, of small molecular weight.

1.5.2.2 Appendageal transport

The hair follicles and sweat glands have openings that effectively bypass the stratum corneum barrier to the underlying dermal structures. The role of appendages in transdermal

drug penetration has been controversial, possibly related in part to the relatively sparse hair cover in humans and animals, such as the pig, and the small fraction of total surface area they constitute. Studies using human skin suggested that a follicular or 'shunt' pathway may be important immediately following topical drug application, but, because of its larger surface area, the intercellular pathway becomes dominant (Scheuplein, 1967).

1.5.2.3 Skin Metabolism

Drugs penetrating through skin may be subjected to both Phase I and Phase II metabolic processes, leading to a first-pass effect as parent drug and/or metabolite reach dermal vasculature. This is more relevant in case of topical delivery of drugs for systemic activity. For example, the bioavailability of topically applied nitroglycerine has been shown to be $56.6 \pm 5.8\%$ when compared to intravenous administration (Wester *et al*., 1983). These metabolic enzymes, including hydroxylases, deethylases, hydrolases, esterases and peptidases, reside in skin and appendages, mostly at the epidermal layer (Liu *et al*., 1994). The metabolic activity of skin may however be useful to activate pro-drugs, which can be formulated to maximise transdermal penetration, with active drug molecules available for circulation, such as a number of topically applied corticosteroid formulation (Hotchkiss, 1998).

1.5.3 Techniques to study transdermal drug penetration

1.5.3.1 In vitro techniques

In vitro techniques can be applied to the skin from humans or any suitable animal species and may become one of the most important tools in predict the permeability in commercial formulations intended for human use.

A) *Diffusion cells*

The primary technique of *in vitro* transdermal drug studies involves skin harvested from the target species (human or animal) and mounted within a two-chambered diffusion cell. The stratum corneum side of the skin is orientated towards the donor chamber, while the underside is exposed to the receptor chamber. The diffusion cells are maintained at 32–35°C to keep the skin at a physiological temperature (Franz, 1975). The effect of variation in the physicochemical properties of the drug on diffusion kinetic can be easily investigated. Due to ethical issue related to the use of human or animal skins, several artificial membranes have been developed to study the *in vitro* release characteristics of the topical formulations.

Several reports have however been critical of this technique as they fail to demonstrate a correlation between *in vitro* and *in vivo* release/permeation characteristics (Degim *et al.*, 1998).

B) *Isolated perfused porcine skin flap*

A useful in situ model developed to investigate transdermal drug penetration is the Isolated Perfused Porcine Skin Flap (IPPSF) described by Riviere *et al.,* 1986**.** The pig was chosen as a model of human penetration kinetics due to structural similarities in the two membranes. Briefly, a flap of skin is dissected from the abdomen of an anaesthetised pig with a remaining pedicle both attaching the flap and maintaining vascularity of the tissue. Two days later, the flap is harvested from the pig and maintained in an environment controlled for temperature and humidity, with a modified perfusate replacing blood within the vasculature serving to maintain tissue viability and as a mechanism to collect residues of drug penetrating through the skin into the dermis. This model approaches the physiological integrity of the *in vivo* system, yet permits control of several confounding variables, such as skin blood flow and reliance on systemic rather than local drug concentration.

1.5.3.1 In vivo techniques

Ideally drug absorption parameters should be studied under the same circumstances as it will be applied. *In vivo* models can be relatively expensive and difficult to manipulate for accurate control of various physiological parameters. However, many interesting studies have generated valuable transdermal kinetic data and demonstrated marked species differences in transdermal penetration of several drugs.

A) *Whole animal studies*

Many of the early studies of transdermal drug penetration used some form of whole animal model. The drug/formulation of interest was applied topically on the living animal and the rate of drug movement through full thickness skin was assessed by measuring parent drug and/or metabolites in the systemic vasculature or in urine. A problem with this approach is that certain drugs or a percentage of all drugs may not reach the dermal vasculature and would therefore be unavailable for systemic recovery. Importantly, it has been recently demonstrated that drug concentrations in the tissue and synovial fluid following topical administration of methylsalicylate could not be predicted by systemic drug plasma concentration (Mills *et al*., 2005).

Several techniques have been proposed specifically to measure local drug concentrations using whole animal models. Biopsies of skin will provide an indication of drug penetration to various layers of skin through to the dermis, but only at the time-point that the sample was collected. However, multiple skin biopsies are ethically impractical, plus the physical collection of a skin biopsy will affect the local environment (altered skin blood flow and extravasation of plasma and inflammatory cells), making subsequent biopsies meaningless. Tape stripping, where adhesive tape is applied to the skin then removed, has provided some useful data relating to drug deposition within progressively deeper layers of the SC. Researchers have shown that continued application and removal of the adhesive tape (up to 20 'strips') will gradually remove all layers of the SC including any drug bound within the cell layer (Cross *et al*., 2003)

B) *Microdialysis*

A recent and highly innovative addition to the techniques used to study cutaneous drug delivery is microdialysis. This technique involves placing a microdialysis probe into the tissue of interest, such as the dermis and/or subcutis. The probe consists of a semi-permeable membrane forming a thin hollow tube which is slowly perfused (typically 0.1–5 μL/min) with a physiological solution (perfusate) which equilibrates with the extracellular fluid of the surrounding tissue. Small substances, including drug molecules, can pass into the membrane as long as they are smaller than the molecular weight cut-off value of the membrane (typically 10,000 D) and these molecules can then be measured in the outflow (dialysate) from the probe continuously over time course of the study following topical application. The advantages of microdialysis are that it is relatively non-invasive and, since the probe functions as an artificial blood vessel, it is possible to monitor topical drug penetration continuously with detailed real-time chronology (Anderson *et al*., 1996; Benfeldt *et al*., 1999).

1.5.4 Factors affecting transdermal drug movement

1.5.4.1 Molecular considerations

The convenience of topical drug administration has led researchers to investigate a wide range of compounds for potential therapeutic application to skin. The specific ideal characteristics of pharmacological agents formulated to penetrate through normal skin are: low molecular weight (500 Da), few atoms available for hydrogen bonding, log P

(lipophilicity) < 2.6 and a low melting point. Outside this range, reduced permeation kinetics are expected, however for sufficiently potent drugs or drugs targeted into hair follicles, sufficient delivery is feasible. Alternately, addition of suitable pharmaceutical excipients may also improve the permeability (Roberts *et al.*, 2002).

1.5.4.2 Vehicle and formulations

The majority of commercial formulations applied to the skin, either intentionally or inadvertently, contain a number of substances, such as inert excipients, solvents, preservatives, fragrances and stabilizers, collectively known as the 'vehicle'. The active drug is contained within the vehicle, either dissolved or as an emulsion or suspension, at a known concentration. The activity of the commercial formulation is based on movement of the active ingredient from the vehicle into and through the skin.

Two primary factors determine the rate and extent of active drug leaving the vehicle and moving through the skin. The first is the relative and absolute solubility of the drug in the two phases, vehicle and skin. The relative solubility determines the partition coefficient which, in turn, determines the likelihood of the drug being taken up into the stratum corneum from the vehicle, whereas the absolute solubility determines the total amount that can be contained within the SC (Roberts *et al.*, 2002). The second factor is the diffusivity. Vehicles must therefore be sufficiently soluble to contain the active drug in an aesthetically acceptable form (i.e., no granules), yet the drug must simultaneously be sufficiently soluble in the stratum corneum lipids and be able to diffuse through these intercellular lipids to reach the site of intended action.

Penetration through the skin can only be increased by altering the characteristics of the solute, such as: (i) increasing the diffusivity of the solute in the stratum corneum; (ii) affecting the partitioning of solute between stratum corneum lipids and other constituents of stratum corneum, or (iii) increasing the solubility of the solute in the intercellular lipids (Roberts *et al.*, 2002). It is well recognized, however, that many vehicles not only interact with the applied drug, but also interact with the skin, with subsequent altered penetration of drug and vehicle, dependent on these interactions.

1.5.4.3 Integrity of the skin

In some skin diseases such as xerosis, progressive loss of the stratum corneum will greatly diminish the barrier function of skin (Quatresooz and Pierard, 2007). Extraction of

intercellular lipids with various solvents also caused a significant reduction in the barrier function of the stratum corneum. Similarly, delipidation of skin by acetone significantly increased the in vivo transdermal penetration of salicylate measured using microdialysis (Benfeldt *et al*., 1999). Altering lipid content and fluidity could be seen as one strategy to enhance transdermal permeability.

1.5.4.4 Skin blood flow

The flow of blood in the upper dermis acts as a sink to remove solutes that have penetrated through the upper skin layers (Roberts *et al.,* 2002). Interruption of cutaneous blood flow due to vasculitis or any other inflammatory condition in the cutaneous vessels will therefore reduce local clearance of solute and, consequently, initiate a peripheral accumulation of active drug below the site of application. One obvious consequence of a topically applied drug reaching the underlying vasculature is that the drug, any metabolites and, possibly, the vehicle constituents, will be available for systemic absorption and distribution. This may be the intended target of the drug, particularly if other routes of systemic administration are less than ideal. However, concentrations of pharmacological agents in the systemic circulation may predispose to toxicity and adverse reactions which may be dose related. Also, any drug interactions likely to be encountered due to co-administration of certain drugs will also are likely to occur in one or both of the agents are administered topically and systemic administration occurs. Consequently, variation in cutaneous blood flow, including regional variation in different body sites, may have important implications following topical drug administration.

1.5.4.5 Site of application

The decision of where on the body to apply a topical medication is determined by whether direct application to a local skin site is required (e.g., an anti-inflammatory cream to the inflamed skin or a NSAID applied over a sore muscle or joint) or purely at a site of convenience when absorption for systemic effect is intended (e.g., nicotine patches). One problem with the later approach is that many drugs may show significant regional differences in transdermal penetration, which may be related to differences in the thickness, hair follicle density of other physiological parameters of different skin sites and/or in the degree of cutaneous blood flow.

1.5.4.6 Density of appendages

Early considerations of the contribution of hair follicles, sweat and sebaceous glands to drug penetration in humans suggested that due to their occupying a relatively small percentage (0.1%) of skin surface, they were unlikely to contribute significantly to transdermal drug penetration. More recent studies have disagreed with this assumption and shown that appendages may indeed act as a shunt through the upper layers of skin for some compounds (reviewed by Schaefer and Lademann, 2001). Overall, it would appear that the concentration and type of appendages may assist transdermal drug penetration more than previously thought; this may have greater relevance when applying topical substances to animals and may thus be a contributing factor in regional differences in transdermal drug penetration.

1.5.4.7 Skin hydration

The effect of hydration of the skin is well recognized, with the stratum corneum absorbing up to 10 times its dry weight when soaked in water (Idson, 1978). Keratinocytes swell as they absorb water into the intracellular keratin matrix, disrupting the organized layers of the stratum corneum. Stratum corneum permeability has been shown to increase rapidly with water uptake then reach a steady-state of diffusion (Roberts and Walker, 1993). One technique to enhance skin hydration is to apply an occlusive dressing which captures insensible water loss, effectively hydrating the skin.

1.5.5 Methods to enhance transdermal penetration

1.5.5.1 Chemical penetration enhancers

Penetration enhancers are substances that can partition into, and interact with skin constituents (mainly the intercellular lipid fraction) and induce a temporary and reversible decrease in skin barrier properties. Similar to hydration, penetration enhancers possibly interact with some components of the skin to increase fluidity in the intercellular lipids, possibly inducing swelling of keratinocytes and/or leaching out of structural components, reducing the barrier function of the stratum corneum. It has been suggested that penetration enhancers may increase by up to approximately 100-fold skin permeability to macromolecules (approximately 1–10 kDa), including heparin, luteinising hormone releasing hormone (LHRH) and oligonucleotides, without inducing skin irritation (Karande *et al*., 2004). Permeation enhancers fall into two major categories: those that impact diffusion across the stratum corneum and those that alter partitioning into the stratum corneum. The

former class generally comprises a long alkyl chain capable of intercalating with the long chains of the intercellular lipids, in addition to a polar head group that is capable of interacting with the lipid polar head groups. This serves to disrupt the ordered nature of the skin lipids, increasing the fluidity and hence assisting permeation of the drug. The latter class of permeation enhancers works by affecting the solubility properties of the skin, thereby increasing the solubility of the drug within the stratum corneum.

1.5.5.2 Physical means of enhancing transdermal penetration

There are several techniques where electrically generated currents or energy fields can assist in transdermal drug penetration. The value of these techniques lies in enhancing the transdermal penetration of larger polar molecules that may not normally be suitable for topical application and reducing the lag time of topically applied products, such as local anaesthetics. Some of the available techniques are as follows:

A) *Ultrasound*

Ultrasound assists transdermal drug penetration because low frequency energy waves disturb the SC layers by cavitation. Early attempts relied on physiotherapy wave frequency settings which focus on deeper muscle structures, while lower frequency ultrasound (20 kHz) can enhance drug penetration by up to 1000-fold (Mitragotri *et al*., 1995). There have been some successful reports using ultrasound to enhance the penetration of insulin, erythropoietin and interferon through human (Mitragotri *et al*., 1995) and NSAID by rabbit skin (Meshali *et al.,* 2008).

B) *Iontophoresis*

Iontophoresis uses a small electrical current (0.5 mA/cm2) applied between two electrodes in contact with the skin to drive a charged molecule (although neutral molecules can also be enhanced through electro-osmosis) through the barrier (Banga *et al*., 1999). The efficiency of iontophoresis depends on the polarity, valency and mobility of the drug molecule, plus the electrical cycle and formulation containing the drug. Iontophoresis has been reported to enhance the delivery through the stratum corneum of proteins (Mitragotri *et al.,* 1995) and oligonucleotides, and other small molecules (Banga *et al*., 1999). A potential drawback with iontophoresis is that the hair follicle has the least resistance through the skin and electrical current passing via this route may irreversibly damage growing hair (Cullander and Guy, 1991).

C) *Electroporation*

Electroporation involves the application of short (μs or ms) electrical pulses (100–1000 V/cm) to the skin (Prausnitz et al., 1993). This creates transient aqueous pores through the stratum corneum, which permits drugs to penetrate more readily (Prausnitz, 1999). Electroporation has been used to enhance the transport of vaccines, liposomes and microspheres. It has also been shown to deliver physostigmine as therapy for organophosphate poisoning (Rowland and Chilcott, 2000). Similar to iontophoresis, skin can be damaged using electroporation and further study is required for more widespread use (Prausnitz, 1999).

D) *Particle-mediated epidermal delivery (PMED)*

Particle mediated epidermal delivery uses particles of gold, coated with DNA or protein, that are accelerated into the epidermis by a similar device to that used to deliver DNA and protein vaccines (Chen *et al*., 2002 and Dean *et al*., 2005). These particles make contact with the dense network of epidermal antigen presenting cells which results in antigen presentation to the systemic immune system by the transfected antigen presenting cells (Chen *et al*., 2002 and Dean *et al*., 2005). Local keratinocytes will also becomes transfected and will then express and secrete antigen which is picked up by resident antigen presenting cells (Dean *et al*., 2005).

In conclusion, the ease of administration of topical formulations encourages compliance and avoiding of undesirable adverse effects makes it an attractive route of administration for COX-2 inhibitors. Use of suitable excipients to improve penetration so as to ensure sufficient drug concentrations in the therapeutic tissue with low systemic bioavailability will provide viable clinical use for the COX-2 inhibitors.

1.6 Emulsion-gels

Emulsion-gels are oil-in-water systems containing a gum solution as thickened aqueous outer phase. These systems are also defined to have a jelly-like consistency, are transparent, clear, homogenous, optically isotropic and thermodynamically stable. These gels have been designate by many terms. Among the most common are micro-emulsion gel, transparent emulsion gel, clear resonant gel, cream gel, viscous isotropic phase, ringing gel, oleo-hydrogel, viscosized microemulsion and emulgel. There is no agreement as to whether these gels have to be considered as microemulsions, as solubilized systems, as stabilized emulsions or as a cubic liquid crystalline phase.

The structure and formation of emulsion gels, according to widely accepted theories, explain that the surface-active agents form spherical miscelles upon dispersion in water at concentrations above the critical miscellar concentration. The HLB of the emulsified mixture allows formation of miscelles with a hydrophilic polar surface and a lipophilic non-polar center. Emulsifier molecules, depending on their concentration, build either infinite cylinders with a liquid hydrocarbon chain core arranged in a two dimensional hexagonal array (i.e., the hexagonal liquid crystalline phase) or lamellar structures (i.e., the lamellar liquid crystalline phase). The oil phase may be said to be in a "solublized" state, surrounded by the surfactant. This is in contrast with a conventional emulsion or a cream, wherein the oil forms an entirely separate phase. There is further no agreement as to what should be the concentration of surfactant to achieve the desired formulation. Studies are reported wherein more than one emulsifier with a high HLB have been proposed for the formation of such systems. In contrast, it has also been reported that, a single emulsifier suffices for the formation of liquid crystalline phases. *In vitro* experiments carried out indicate a higher rate of drug release from these types of formulations as compared to conventional ones. The results also attribute the drug release to partitioning of the drugs (at least for those examined) into a lipidic phase (Murdan *et al*., 1999).

Advantages of Emulsion-gels

1. They have better application property in comparison to classical formulations-such as creams and ointments.
2. They have a faster and more complete release of the drug from the vehicle to the skin and therefore higher efficacy.
3. They exhibit improved spreadability and thus are convenient for application on larger

skin area.

4. It is convenient to apply emulsion-gels on hairy skin due to absence of greasiness and lack of residue upon application.
5. It is convenient to apply emulsion-gels on seborrhoic skin due to its cooling effect.

Provosot *et al.,*(1990) have studied the gel structure in transparent oil water gels by differential scanning calorimetry. The binary, ternary and quaternary mixtures were prepared using Cetiol HE, Eumulgin, isopropyl palmitate and water. The thermal behavior of the single component and some of their binary emulsifier water and emulsifier oil-water mixtures was also studied. They showed that the mechanism underlying the formation of transparent oil water gels involved an interaction of isopropyl palmitate with one or both hydrated emulsifiers. The results also indicated that some of the water is tightly associated with both the surfactants through hydrogen bonding. They concluded that the three dimensional network in transparent oil water gels is based on hydrogen bonds linking neighbouring structural associations of hydrated emulsifier molecules.

In another study, percutaneous absorption of Indomethacin from transparent oil in water gels was compared with absorption from a hydrophilic gel and from a spray formulation. The area under the curve and Cmax values in plasma were significantly higher for transparent oil in water gels in comparison with other formulations after single application. After multiple applications the transparent oil in water gels induced a larger increase in AUC in comparison with other formulations. The pH of the aqueous phase of transparent oil in water gels did not significantly influence the bioavailability **(**Vos *et al*., 1991). However, this was further evaluated in another study by Vos and Kinget, (1994) in which they have studied Indomethacin release as a function of pH of gel's water phase. In this study, Indomethacin partitioning between I-dodecanol and water, with variation of pH of aqueous phase was studied. Apparent partition coefficient determinations and flame photometric measurements showed that both Indomethacin and its sodium salt, as the ion pair, migrate into I-dodecanol layer. The release studies showed a linear relationship between the initial drug concentration and release rate. As pH was increased, the release rate was higher and at very high pH values, a change in the slope of the curve around the pKa and plateau in high pH range was observed. It was concluded that Indomethacin may partition into a lipophilic phase mainly in unionized form and partly as ion pair. Ion pair formation being naturally abundant plays an important role in the percutaneous absorption of Indomethacin.

Rhee et al., (1991) have reported oleo-hydrogel preparations of ketoprofen with enhanced skin permeability. The effect of pH of the gel on release of ketoprofen was examined. The pH dependence for the permeation of ketoprofen was not significant. In addition, influence of polymer concentration on permeation rates of ketoprofen was also studied. The steady state flux decreased exponentially as a function of polymer concentration. The concentration of ketoprofen had an influence on release rates. The diffusion constant of ketoprofen decreased exponentially as the drug concentration increased in the hydrogel. A permeation enhancer was utilized in the formulation that increased the steady state flux of ketoprofen. The relationship between in vitro permeation and in vivo skin permeability of ketoprofen was investigated. It was observed that a very high correlation existed between the log of *in vivo* penetration rate and *in vitro* steady state flux.

Another important aspect of transdermal delivery of drugs is the particle size of the active pharmaceutical ingredient in the gel formulation which influences percutaneous absorption. This is more important in case of insoluble drugs. There are several studies in the literature which show that increasing the surface area of a particulate drug, poorly soluble in water, by decreasing the particle size improves the rate of drug dissolution resulting in an improved permeability and percutaneous efficacy (Friedman *et al*., 1995). Friedman *et al*., (1995) have studied a sub-micron emulsion vehicle for enhanced transdermal delivery of steroidal and non steroidal anti-inflammatory drugs. Drug loaded sub-micron emulsion was prepared by high pressure homogenization and consisted of 20% w/w of oil. The anti-inflammatory activity of medicated topical sub-micron emulsion was compared with regular topical cream formulations, being measured by the carrageenan induced paw oedema rat model. Both steroidal and non-steroidal anti-inflammatory drugs exhibited more activity in comparison to conventional ones. The sub-micron emulsion delivery system when tested for primary irritation in humans, showed low irritancy and excellent human acceptance.

1.7 Animal Models for screening analgesic and anti-inflammatory activity

1.7.1. Animal models of Pain

Introduction

The absence of verbal communication in animals is undoubtedly an obstacle to the evaluation of pain. Rodents are used in almost all animal models of pain. The essential mechanisms that make it possible for an organism to react to a stimulus, which might endanger its existence (including sensory perception), exist throughout the animal kingdom, except perhaps in arthropods and particularly in insects (Eisemann *et al*., 1984; Walters, 1994).

Zimmermann (1986) defined pain that it could be applied to animals: "an aversive sensory experience caused by actual or potential injury that elicits progressive motor and vegetative reactions, results in learned avoidance behavior, and may modify species specific behavior, including social behavior".

The larger issues related to use of animal in pain research is the ethical problem. As with any biomedical research involving animals, pain research presents ethical problems at two levels. From a general point of view, investigators have to follow the recommendations of ethics committees and, notably, those of international scientific review boards so as to ensure a given level of physiological well being in the animal. Indeed, if the animal is miserable or in a state of stress in which neurovegetative reactions are exacerbated, it is clear that scientific observations will not be valid from a physiological point of view. Thus, it is not only for moral reasons but also for scientific reasons that some rules have to be observed (Zimmermann, 1983).

Because no test of nociception meets all these criteria, the choice of which test to use has to be a compromise. Before describing these tests, it is worth noting that in general, they can be divided into two overall categories depending on whether it is a threshold or a supraliminal response to a given stimulus that is being measured. Note that both these categories permit one to investigate only one point on the stimulus-response curve is it the threshold or an arbitrary point further up the curve. As a result, they allow only a rough appreciation of the gain of the process (Tjølsen and Hole, 1997). For the main part, the models involve rodents, most often the rat or mice.

Since our objective was to evaluate topical efficacy of the formulations we have restricted ourselves to acute cutaneous and acute visceral pain, it is useful to classify the animal models used on the basis of the physical characteristics of the stimuli. We therefore successively consider tests based on the use of short-duration stimuli (in the order of seconds) and then those based on the use of longer-duration stimuli (in the order of minutes). The former relate to pains of cutaneous origin, with physical stimuli (thermal, mechanical, electrical) applied to small areas, often at increasing intensities. The latter relate to pains of cutaneous or visceral origin, with chemical stimuli (algogenic substances) being applied usually subcutaneously or intraperitoneally. In addition, one can add to the latter category tests based on the distension of hollow organs (visceral mechanical stimulation); such stimuli last for intermediate periods of time but may not be of much use in screening topical pharmacological agents.

1.7.1.1 Use of Short-Duration Stimuli ("Phasic Pain")

A) *Tests Based on the Use of Thermal Stimuli*

1) Tail Flick test

There are two variants of the tail-flick test. One consists of applying radiant heat to a small surface of the tail. The other involves immersing the tail in water at a predetermined temperature. Although apparently similar, these two alternatives are actually quite different at a physical level: the cutaneous temperature varies with the square root of time in the first case and more rapidly in the second.

i) The Tail-Flick Test Using Radiant Heat

The application of thermal radiation to the tail of an animal provokes the withdrawal of the tail by a brief vigorous movement. It is the reaction time of this movement that is recorded (often referred to as "tail-flick latency"). This is achieved by starting a timer at the same time as the application of the heat source. By using a rheostat, the intensity of current through the filament and therefore of radiant heat emission can be controlled in such a way that one can empirically predetermine the time until the withdrawal of the tail. This is usually between 2 and 10 sec. (most commonly between 2 and 4 sec.). A photoelectric cell stops the timer and switches off the lamp at the moment the tail is withdrawn. A lengthening of the reaction time is interpreted as an analgesic action. It is advisable not to prolong the exposure to radiant heat

beyond 10 to 20 sec.; otherwise the skin may be burned. The advantages of this method are its simplicity and the small inter-animal variability in reaction time measurements under a given set of controlled conditions.

The reaction time of the tail movement varies with the intensity (power) of the source of radiant heat: when it is more intense, the temperature slope is steeper and, consequently, the reaction time is shorter and the movement is greater. Equally, the reaction time varies with the surface area stimulated: when the area increases, the reaction time decreases (Le Bars *et al.,* 2001). However, this reaction time also varies with the site stimulated; paradoxically, it decreases when the stimulus is applied to increasingly distal parts of the tail (Ness *et al.*, 1987) even though the pathway for the afferent signals is longer. One can demonstrate that the tail-flick is a spinal reflex in that, at least in its shorter latency form, it persists after section or cold block of upper parts of the spinal cord (Irwin *et al.*, 1951; Sinclair *et al.*, 1988). As with all reflexes, it is subject to control by supraspinal structures.

From a pharmacological point of view, there is a consensus that this test is truly efficient only for revealing the activity of opioid analgesics. In this context, it is adequate for predicting their analgesic effects in humans (Le Bars *et al.,* 2001)

ii) The Tail-Flick Test Using Immersion of the Tail.

The use of immersion of the tail is apparently a variant of the test described above. The most obvious difference is that the area of stimulation is far greater. Immersion of an animal's tail in hot water provokes an abrupt movement of the tail and sometimes the recoiling of the whole body. Again, it is the reaction time that is monitored (Le Bars *et al.,* 2001).

2) The Paw Withdrawal Test.

In principle, this test is entirely comparable to the tail-flick test but offers the advantage that it does not involve the pre-eminent organ of thermoregulation in rats and mice, i.e., the tail. With the aim of studying hyperalgesic phenomena resulting from inflammation, Hargreaves *et al.* (1988) had an inspired idea for supplementing the model of Randall and Selitto (1957): radiant heat was applied to a paw that had already been inflamed by a subcutaneous injection of carrageenan or by exposure to ultraviolet rays (Perkins *et al*., 1993). One advantage in these tests is that heat is applied (to the plantar surface of the foot) of a freely moving animal.

However, there is a disadvantage in that the position of the leg becomes a factor since the background level of activity in the flexors varies with the position of the animal.

3) The Hot Plate Test.

This test consists of introducing a rat or mouse into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode or a boiling liquid. A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered to be supraspinally integrated responses.

As far as analgesic substances are concerned, the paw-licking behavior is affected only by opioids. On the other hand, the jumping reaction time is increased equally by less powerful analgesics such as acetylsalicylic acid or paracetamol, especially when the temperature of the plate is 50°C or less (Ankier, 1974) or if the temperature is increased in a progressive and linear fashion, e.g., from 43 to 52°C at 2.5°C/min (Hunskaar et al., 1985). The specificity and sensitivity of the test can be increased by measuring the reaction time of the first evoked behavior regardless of whether it is paw-licking or jumping, or by lowering the temperature.

4) Tests Using Cold Stimuli.

Cold is very rarely used to test acute pain. On the other hand, it is more common to test cold allodynia in animal models of neuropathies. The techniques are directly inspired by those that use heat by contact i.e. by immersion of the tail or a limb or placing the animal on a cold surface.

B) *Tests Based on the Use of Mechanical Stimuli*

The application of a noxious mechanical stimulus can be progressive or coarse. Responses produced by noxious mechanical stimuli are graded in relation to the intensity and/or duration of the stimulus, from reflexes up through vocalizations ultimately to complex motor behaviors. The stimulus is stopped as soon as a response is obtained. The type of mechanical stimulus used has the disadvantage of activating low-threshold mechanoreceptors as well as nociceptors. Consequently, the stimulus is not specific. There are also technical difficulties in applying mechanical stimuli, especially in freely moving animals. In addition, when

mechanical stimuli are truly nociceptive, they are likely to produce changes in the tissues (sensitization or actual lesions). (Le Bars *et al.,* 2001).

The preferred sites for applying nociceptive mechanical stimuli are the hind paw and the tail. Tests using gradually increasing pressures are preferred over constant pressure application. In the course of such a test, a pressure of increasing intensity is applied to a punctiform area on the hind paw or, far less commonly, on the tail. In practice, the paw or tail is jammed between a plane surface and a blunt point mounted on top of a system of cogwheels with a cursor that can be displaced along the length of a graduated beam (Green *et al.*, 1951). These devices permit the application of increasing measurable pressures and the interruption of the test when the threshold is reached. The measured parameter is the threshold (weight in grams) for the appearance of a given behavior. When the pressure increases, one can see successively the reflex withdrawal of the paw, a more complex movement whereby the animal tries to release its trapped limb, then a sort of struggle, and finally a vocal reaction.

If the first of these reactions is undoubtedly a proper spinal reflex, the last two clearly involve supraspinal structures. This type of mechanical stimulation has a certain number of disadvantages: 1) it is sometimes difficult to measure the intensity of the stimulus with precision; 2) repetition of the mechanical stimulus can produce a diminution or conversely an increase in the sensitivity of the stimulated part of the body in the latter case, this carries the risk that the tissues may be altered by inflammatory reactions that could call into question the validity of repeated tests; 3) the necessity of applying relatively high pressures which explains the weak sensitivity of the method and the relatively small number of substances that have been shown to be active by this test; and 4) a non-negligible level of variability of the responses (Le Bars *et al.,* 2001).

With the aim of improving the sensitivity of the test, Randall and Selitto (1957) proposed comparing thresholds observed with a healthy paw and with an inflamed paw. The inflammation was induced beforehand by a subcutaneous injection into the area to be stimulated of substances such as croton oil, beer yeast, or carrageenan, the last of these being the most commonly used today. Even though it was found that the sensitivity of the method was improved, it was to the detriment of its specificity because two different pharmacological effects, analgesic and anti-inflammatory could be confused. It is therefore quite difficult to state that there has been analgesic or even "antalgesic" activity. However, a comparison in the

same animal of responses triggered from a healthy and an inflamed paw allows this problem to be overcome: nonsteroidal anti-inflammatory drugs (NSAIDs) are inactive on the former but do increase the (lowered) vocalization threshold when pressure is applied to the latter (Winter and Flataker, 1965).

C) *Tests Based on the Use of Electrical Stimuli*

The application of electrical stimuli has the advantages of being quantifiable, reproducible, and noninvasive and of producing synchronized afferent signals. However, it also has serious disadvantages. 1) electrical stimuli are not a natural type of stimulus like those encountered by an animal in its normal environment, 2) intense electrical stimuli excite in a non differential fashion all peripheral fibers, including large diameter fibers, which are not directly implicated in nociception, as well as fine Aδ and C fibers, which mediate sensations of cold and hot as well as nociceptive information; 3) this type of stimulation completely short-circuits peripheral receptors, thus preventing any study of peripheral transduction mechanisms with these methods; 4) there are difficulties introduced by variations in the impedances of the tissues being stimulated, although these can be minimized by the use of a constant current stimulator and the monitoring of the voltage as well as the current of the applied stimulus **(**Le Bars *et al.,* 2001**).**.

1.7.1.2 Tests Based on the Use of Long Duration Stimuli ("Tonic Pain")

Basically, these tests involve using an irritant, algogenic chemical agent as the nociceptive stimulus. They differ from the vast majority of other tests in that they abandon the principle of determining the nociceptive threshold and involve a quantitative approach to the behavior observed after the application of a stimulus with a potency that is going to vary with time. They can be thought of as a kind of model for tonic pain. However, they are not models for chronic pain because their duration is only in the order of some tens of minutes.

A) *Intradermal Injections –The formalin test*

The most commonly used substance for intradermal injections is formalin (the "formalin test"). The term *formalin* usually means a 37% solution of formaldehyde. Less commonly used are hypertonic saline, ethylene diamine tetra-acetic acid, Freund's adjuvant, capsaicin, and bee sting.

A 0.5 to 15% solution of formalin injected into the dorsal surface of the rat forepaw provokes a painful behavior that can be assessed on a four-level scale related to posture: 0, normal posture; 1, with the injected paw remaining on the ground but not supporting the animal; 2, with the injected paw clearly raised; and 3, with the injected paw being licked, nibbled, or shaken (Dubuisson and Dennis, 1977). The response is given a mark, and the results are expressed either continuously per unit of time or at regular time intervals when several animals are observed sequentially. Each level on this scale can be weighted to optimize the test. This method has also been used in the mouse, cat, and monkey. The measured parameter can also be the number of licks or twitches of the paw per unit of time, the cumulative time spent biting/licking the paw, or even a measure of the overall agitation of the animal obtained by a strain gauge coupled to the cage. Such specific behaviors resulting from an injection of formalin can be captured automatically by a camera attached to a computer; in this way, the effects of a pharmacological substance on such motor activity can be identified, analyzed, and uncoupled from antinociceptive effects (Le Bars *et al*., 2001).

In the rat and the mouse, intraplantar injections of formalin produce a biphasic behavioral reaction. This behavior consists of an initial phase, occurring about 3 min after the injection, and then after a quiescent period, a second phase between the 20th and 30th minutes. The intensities of these behaviors are dependent on the concentration of formalin that is administered (Rosland *et al*., 1990; Aloisi *et al*., 1995). The first phase results essentially from the direct stimulation of nociceptors, whereas the second involves a period of sensitization during which inflammatory phenomena occur. Thus, the second phase cannot be interpreted as a consequence of the first; it clearly also originates from peripheral mechanisms.

Opioid analgesics seem to be antinociceptive for both phases, although the second is more sensitive to these substances. In contrast, NSAIDs such as Indomethacin seem to suppress only the second phase, especially when the formalin is injected in high concentrations (Yashpal and Coderre, 1998).

B) *Intraperitoneal Injections of Irritant Agents (the "Writhing Test")*

The intraperitoneal administration of agents that irritate serous membranes provokes a very stereotyped behavior in the mouse and the rat which is characterized by abdominal contractions, movements of the body as a whole (particularly of the hind paws), twisting of dorsoabdominal muscles, and a reduction in motor activity and motor incoordination. The test

is sometimes called the abdominal contortion test, the abdominal constriction response, or the stretching test, but more commonly it is known as the "writhing test". Generally the measurements are of the occurrence per unit of time of abdominal cramps resulting from the injection of the algogenic agent. These behaviors are considered to be reflexes and to be evidence of visceral pain; however, it would probably be wiser to call it peritoneovisceral pain. Indeed, given the well established fact that the parietal peritoneum receives a somatic innervation, it is possible that the pain may not be visceral at all. However, the pain is probably similar to that resulting from peritonitis (Le Bars *et al*., 2001). Unfortunately, the frequency of cramps decreases spontaneously with time (Michael-Titus and Costentin, 1988) to such an extent that it is impossible to evaluate the duration of action of an analgesic on a single animal.

This model has the advantage that it can be used to study the efficacy of weak analgesics. On the other hand, they lack specificity. Indeed, these tests work not only for all major and minor analgesics, but equally for numerous other substances, including some that have no analgesic action, e.g., adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors, and neuroleptics. Thus, a positive result with this test does not necessarily mean there is analgesic activity. Nevertheless, because all analgesics inhibit abdominal cramps, this method is useful for sifting molecules whose pharmacodynamic properties are unknown (Le Bars *et al*., 2001). Although the writhing test has a poor specificity, it is sensitive, and predictive, as shown by the correlation between ED_{50} values obtained in rats using this test and analgesic doses in humans (Dubinsky et al., 1987).

Intraperitoneal injections of algogenic substances have also been used in nonbehavioral models of nociception, i.e., models in which the animal is anesthetized. For example, changes in mean arterial blood pressure and intragastric pressure have been used as indicators of nociceptive responses to intraperitoneal bradykinin in anesthetized rats (Holzer-Petsche, 1992; Griesbacher et al., 1998).

1.7.2 Animal models of Inflammation

Inflammation was characterized two thousand years ago by Celsius by the four Latin words: Rubor, calor, tumor and dolor. Inflammation has different phases: the first phase is caused by an increase of vascular permeability resulting in exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes from the blood into the tissues

and the third one by granuloma formation. Accordingly, anti-inflammatory tests have to be divided into those measuring acute inflammation, subacute inflammation and chronic repair processes. In some cases, the screening is directed to test compounds for local application. Predominantly, however, these studies are aimed to find new drugs against polyarthritis and other rheumatic diseases. Since the etiology of polyarthritis is considered to be largely immunological, special tests have been developed to investigate various immunological and allergic factors. According to these phases, pharmacological methods have been developed. Methods for testing acute and subacute inflammation are described below.

1.7.2.1 Acute inflammation models

A) Ultraviolet erythema in guinea pigs

The test was first described by Wilhelmi (1949). Although, the skin protects the body from environmental, physico-chemical and microbial insults, it is the organ itself directly exposed to the deleterious effects of ultraviolet (UV) light irradiation. The exposure of the skin to UV light in the 280–320 nm range, results in both an acute inflammatory response characterized by erythema and edema and chronic photoaging as well as carcinogenesis (Hruza and Pentland, 1993).

From the observations of the UV-exposed skin, there seems to be at least the following three sequential and overlapping events taking place in UVB-induced inflammation. (Hruza and Pentland, 1993) The first is the early vasodilatory phase, where PGE_2 and nitric oxide (NO) are known to be involved in the induction of vascular dilatation. (2). The second is the inflammatory phase, where various types of cells and mediators participate in the development of the erythematous changes; and (3) the last is the regressive phase consisting of an anti-inflammatory event, where several mediators and cells have been demonstrated to be involved.

B) Vascular permeability model.

The test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by a phlogistic substance. Mediators of inflammation, such as histamine, prostaglandins and leukotrienes are released following stimulation e.g. of mast cells. This leads to a dilation of arterioles and venules and to an increased vascular

permeability. As a consequence, fluid and plasma proteins are extravasated and edemas are formed. These effects are counteracted by H1 - antihistaminics, inhibitors of Arachidonic acid metabolism and by leukotriene receptor antagonists. In addition, membrane-stabilizing drugs are able to reduce capillary permeability. Vascular permeability is increased by intracutaneous injection of the mast cell-degranulating compound 48/80. The increase of permeability can be recognized by the infiltration of the injected sites of the skin with the vital dye Evan's blue.

Several modifications to these methods have been reported. Shionoya and Ohtake (1975) described a simple method for extraction of extravasated dye (Evans blue) in the skin. Watanabe *et al*., (1984) used fluorescein isothiocyanate labeled bovine serum albumin as tracer to measure vascular permeability in the carrageenan air pouch of rats.

C) The oxazolone-induced ear edema model

The oxazolone-induced ear edema model as first described by Evans *et al*., (1971) in mice is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration.

Most of the frequently used test systems for drug-induced topical anti-inflammatory activity measure changes in skin inflammation. This is assessed by skin erythema and edema after challenge of animals sensitized with a model allergen (Meingassner and Stütz, 1992; Duncan, 1994). Although such assays have been, and continue to be, of proven value, they are not without limitations. The time-consuming tests measure 'subjective' and rather unspecific end-points and require large numbers of test animals. Therefore, it is appropriate that attempts are made to design reliable alternative test methods with short-term protocols which measure objective parameters. Contact hypersensitivity has served as a useful model for the primary activation of T cells in skin and skin-associated lymphoid tissue, such as local draining lymph nodes (Kimber and Weisenberger, 1989; Enk and Katz, 1995). During induction of contact hypersensitivity, antigen-presenting cells of the skin take up antigen and migrate to local lymph nodes where they present relevant antigen determinants together with co-stimulatory molecules to T cells and induce antigen-specific T-cell activation (Baker *et al*., 1989; Kripke *et al*., 1990; Enk and Katz, 1995). Furthermore, T cells play an important role in

inflammatory skin diseases, such as atopic dermatitis and psoriasis (Bos *et al*., 1994; Cooper, 1994; Bata-Csorgo *et al.,* 1995).

Several contact allergens such as oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), TNCB (2,4,6-trinitro-1-chlorobenzene) and DNFB (2,4-dinitrofluorobenzene) etc., have commonly used to induce contact hypersensitivity and screen drugs for anti-inflammatory potential (Harada et al., 2006; Bhol, and Schechter, 2005).

The method is suitable for both steroidal and non-steroidal compounds as well as for the evaluation of various topical formulations. However, this test is time-consuming and measures 'subjective' and rather unspecific end-points and require large numbers of test animals.

D) Croton-oil ear edema in rats and mice

The method has been developed mainly as a bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied steroids by Tonelli *et al.,* (1965). The method is useful for evaluation of anti-inflammatory topical steroids especially in the modification when thymus weight is determined simultaneously. The method also can be used for topically applied nonsteroidal anti-inflammatory drugs. Application of an irritant substance to the skin or to a mucous membrane is followed by an inflammatory reaction. Kiernan, (1977) showed the involvement of axon reflexes, mast cells and various humoral mediators in chemically induced acute inflammation. Apart from Croton oil, several irritants have been used to induce inflammation of skin, such as benzoic acid, TPA (tetradecanoyl phorbol acetate), Arachidonic acid etc.

E) Paw edema in rats

Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil(R), sulfated polysaccharides like carrageenan or naphthoylheparamine. The effect can be measured in several ways. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls. Some irritants induce only a short lasting

inflammation whereas other irritants cause the paw edema to continue over more than 24 hours.

Various devices have been developed for plethysmography of the paw. Winter *et al.* (1963) used mercury for immersion of the paw. Alpermann and Magerkurth (1972) described an apparatus based on the principle of transforming the volume being increased by immersion of the paw into a proportional voltage using a pressure transducer. Webb and Griswold (1984) reported a sensitive method of measuring mouse paw volume by interfacing a Mettler DeltaRange top-loading balance with a microcomputer. Several authors used a commercially available plethysmometer from UGO Basile, Varese, Italy.

The paw edema method has been used by many investigators and has been proven to be suitable for screening purposes as well as for more in depth evaluations. Dependent on the irritant steroidal and nonsteroidal anti-inflammatory drugs, antihistaminics and also, to a lesser degree, serotonin antagonists are active in the paw edema tests. Since so many different irritants have been used by the various investigators the results are often difficult to compare.

F) Pleurisy tests

Pleurisy is a well known phenomenon of exudative inflammation in man. In experimental animals pleurisy can be induced by several irritants, such as histamine, bradykinin, prostaglandins, mast cell degranulators, dextran, enzymes, antigens, microbes, and nonspecific irritants, like turpentine and carrageenan. Carrageenan induced pleurisy in rats is considered to be an excellent acute inflammatory model in which fluid extravasation, leukocyte migration and the various biochemical pa-rameters involved in the inflammatory response can be measured easily in the exudate. In this model the irritant is injected into the pleural cavity through this incision. 72 hrs later the animals are sacrificed and pleural cavity is exposed and is levaged with 1 ml heparinized Hank's solution. The levaged fluid is then measured for total volume along with several other parameters such as measuring the white blood cell number in the exudates using a Coulter counter or a hematocytometer, determination of lysosomal enzyme activities, determination of fibronectin, determination of PGE_2, cytokines, etc. The pleurisy model has been accepted as a reliable method to study acute and subacute inflammation allowing the determination of several parameters simultaneously or successively. The activity of steroids as well as of non-steroidal drugs can be measured.

1.7.2.2 Proliferative phase methods

A) *Cotton wool granuloma*

The method has been described first by Meier *et al.,* (1950) who showed that foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histologically giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan. The average weight of the pellets of the control group as well as of the test group is calculated.

B) *Glass rod granuloma*

The glass rod granuloma as first described by Vogel in 1970, reflects the chronic proliferative inflammation. Of the newly formed connective tissue not only wet and dry weight, but also chemical composition and mechanical properties can be measured.

C) *PVC sponge granuloma*.

The sponge implantation technique was described first by Saxena (1960) for short term experiments but was used subsequently to study the formation of granulomata using long-term implantation. Sponges used for intradermal implantation are prepared from polyvinyl foam sheets (thickness 5 mm). Discs punched out to a standard size and weight (10.0 ± 0.02 mg) using a 13 mm cork borer, soaked in sterile 0.9% saline in which either drugs, antigens or irritants have been suspended. Post implantation, sponges are either recovered within 24 hrs (in acute study) or upto a period of 3 weeks (chronic study). In acute study fluid phase of sponge exudates are analysed for, e.g. protein content, enzyme levels and biological mediators such as prostaglandins as well as for leukocyte migration. For studying the chronic phase of inflammation besides dry weight, hexosamine, indicating glycosaminoglycane content, and hydroxyproline, indicating collagen content, can be determined.

1.7.2.3 Immunological Models

Chronic inflammatory disorders such as polyarthritis and other rheumatic diseases are considered to have etiology which is largely immunological. Therefore, special tests have

been developed to investigate various immunological and allergic factors. Improvement in our understanding of inflammatory pathways has also led to discovery of several new targets and factors mediating these inflammatory pathways. Screening of new compounds for efficacy against these targets also requires special tests with immunological bases. A range of different models have been developed to evaluate and understand various aspects of the inflammatory conditions and diseases. Some of these have been briefly discussed below.

A) Adjuvant arthritis in rats

Adjuvant arthritis in rats has been described by Pearson and Wood (1959) exhibiting many similarities to human rheumatoid arthritis. Injection of complete Freund's adjuvant into the rat paw induces inflammation as primary lesion with a maximum after 3 to 5 days. Secondary lesions occur after a delay of approximately 11 to 12 days which are characterized by inflammation of non-injected sites (hindleg, forepaws, ears, nose and tail), a decrease of weight and immune responses. The procedure has been modified by several authors in order to differentiate between anti-inflammatory and immunosuppressive activity (e.g. Perper *et al.,* 1971). Anti-inflammatory compounds do not inhibit secondary lesions, which are prevented or diminished by immunosuppressive agents.

NSAIDs are effective on primary lesions when dosage is started on the day of injection of the irritant. They are not effective on the secondary lesions. In contrast, immunosuppressants like cyclophosphamide or any of anti-inflammatory agents such as p38 Mitogen activated protein kinase inhibitors and corticosteroids, inhibited the secondary lesions.

Evidence was given that adjuvant arthritis in the rat is associated with chronic pain (Colpaert, 1987), although, the measure of pain in this model is based on the behavioral responses. Kazuna and Kawai (1975) used rats with established lesions to test analgesics in the arthritic flexion pain test. The method is claimed to be specific by detecting only central analgesics and nonsteroidal anti-inflammatory drugs but not other classes such as CNS-depressant or antihistaminic drugs.

Several modifications to the general method have been reported. Brackertz *et al.,* (1977) established antigen-induced arthritis in the mouse by immunization with methylated bovine serum albumin in complete Freund's adjuvant with B pertussis vaccine. A streptococcal cell wall-induced arthritis in rats has been described by Wilder *et al.,* (1982).

B) Passive cutaneous anaphylaxis

Passive cutaneous anaphylaxis is an immune reaction of the immediate type. By passive immunization of rats in the skin with rat anti-ovalbumin serum and a challenge 2 days later with ovalbumin at the same skin area antigen-antibody complexes are formed in the mast cells inducing release of mediators. This results in vasodilatation, increase in permeability of the vessel walls and leakage of plasma. To make the allergic reaction visible, Evan's blue dye is administered along with the antigen. Evan's blue dye is attached to the albumin fraction of plasma, producing a blue spot. This blue spot indicates that an anaphylactic reaction has taken place in the skin.

C) Arthus type immediate hypersensitivity

The immune complex induced Arthus reaction comprises inflammatory factors that have been implicated in the acute responses in joints of rheumatic patients. Complement and polymorphonuclear cells (neutrophils) are activated via precipitating antigen-antibody complexes leading to an inflammation, characterized by edema, hemorrhage and vasculitis. Arthus reaction of the immediate type becomes maximal 2–8 hours after challenge. In this model Wistar or Sprague-Dawley rats are sensitized by i.m. administration of ovalbumin suspension. 7 days later the rats are challenged by injection of highly purified ovalbumin in the left hind paw. Swelling of the paw occurs which reaches a maximum after a few hours. The footpad thickness can be measured by calipers.

D) Delayed type hypersensitivity

This is another model to screen immune mediated inflammatory processes in animals. Delayed type hypersensitivity is a reaction of cell mediated immunity and becomes visible only after 16–24 hours. The same methods as for testing immediate type hypersensitivity can be used with the end point measurement 24hrs after the secondary challenge.

1.8 Objectives of the current research project

Ever since their launch in late 1990s selective Cyclooxygenase 2 (COX-2) inhibitors have hogged the limelight. Initially, for their reported ability to spare the gastrointestinal adverse effects associated with traditional non-steroidal anti-inflammatory drugs, which resulted in considerably increased usage in not only high risk groups but more so in individuals at low risk for GI side effects (Becker, 2005). The high usage profile of coxibs in general population brought to light several adverse pharmacological effects associated with NSAIDs and specifically related to COX-2 inhibitors usage. Based on series of published reports, it now appears that, coxibs cause significantly less ulceration than conventional NSAIDs (Bombardier *et al*., 2000) but with a heightened risk in patients with history of gastric bleeding. A series of discoveries have strongly suggested a crucial role of COX-2 in gastrointestinal mucosal defense and repair.

COX-2 inhibitors also inherit the renal effects of traditional NSAIDs, particularly in patients considered at risk of adverse renal effects related to use of NSAIDs such with renal impairment or in condition of dehydration or high salt intake (Brater, 1999).

Apart from these adverse effects the most notorious adverse effect reported is the effect on the cardiovascular system. This cardiovascular risk differs, to some degree, across agents, and does appear to be dose related. The relationship between cardiovascular risk and duration of therapy has also emerged as an important factor. Early risk, from the perspective of pathobiology, may differ from long-term risk. The mechanism of cardiovascular risk appears to be multifactorial and relates to sites of COX-2 synthesis, expression within the vasculature, and related local consequences of an imbalance between thromboxane A2 and prostacyclin. Considered collectively, increased platelet aggregation, hypertension, endothelial cell dysfunction, impaired angiogenesis, and destabilization of the atherosclerotic plaque matrix are important contributors to the “prothrombotic environment.” Several randomised Clinical trials, of prolonged treatment with coxibs have demonstrated these effects which eventually lead to withdrawal of several selective COX-2 inhibitors from clinical use and revised labeling for the rest.

However, delivery of coxibs by topical route can be useful to avoid the adverse drug effects, without losing the beneficial effects of the Coxibs. Topical drug delivery offers several advantages such as: (i) avoidance of the gastric route, reducing the potential for gastric irritation; (ii) high therapeutic tissue *vs* plasma concentration can be obtained thereby achieve therapeutic concentrations in the tissues subjacent to the site of application while maintaining low plasma concentrations thus reduce the risk of serious adverse events related to elevated plasma concentrations; (iii) improve patient compliance with drug administration; (iv) may be cost effective in the long term.

Topical formulations of NSAIDs have been used effectively across the world e.g. Ketoprofen, Felbinac, Ibuprofen, Piroxicam, Diclofenac, Indomethacin, etc. Several randomised placebo controlled trials have demonstrated that the topical non-steroidals are significantly more effective than placebo for pain relief (Moore *et al*., 1998; Mason *et al*., 2004). In some studies topical preparations have also been shown to produce efficacy similar to those seen with oral analgesics in moderate or severe pain (Åkermark and ForsskÅhl, 1990; Hosie, 1993; Vanderstraeten and Schuermans, 1990; Tugwell *et al*., 2004). Browning and Johson (1994) had shown that reduction in oral NSAIDs dose by a factor of 2 and supplementing with topical gel of Piroxicam resulted in similar efficacy as full dose of oral treatment and topical gel alone.

Topical NSAIDs having demonstrated clinical efficacy in several studies as described above, it is important to understand the adverse effect liability of such formulations. As discussed above topically applied non-steroidal anti-inflammatory drugs are advocated as they show lower incidence of adverse effects due to relatively lower plasma concentrations of the drugs after topical application. However, several studied have reported incidence of adverse effects although to a lesser extent as compared to oral treatment. The major adverse effects observed with topical NSAIDs were local skin reactions (~85% of all adverse effects). However other studies have shown association between the use of topical non-steroidal anti-inflammatory drugs and severe gastrointestinal adverse events, including dyspepsia, abdominal pain, diarrhoea, nausea, upper gastrointestinal bleeding and perforation (Tugwell *et al*., 2004; Evans *et al*., 1995). Figueras and co workers (1994), have also reported incidence such as duodenal ulcer, gastrointestinal bleeding, diarrhoea, dyspnoea etc., in Spanish patients with topical NSAIDs. The relative occurrence of these incidences was low as compared to oral

treatment but some of these local or systemic adverse effects of sufficient severity have also resulted in withdrawal from the study (Moore *et al*., 1998). Therefore, topical administration of COX-2 inhibitors with proven improved gastrointestinal adverse effect profile will be beneficial. With this intension the current project was undertaken to develop a topical gel formulation of COX-2 inhibitors (Celecoxib and Rofecoxib) and evaluate their efficacy and safety profile. The research work was carried out in the following stages for achieving this broad objective:

- Section of appropriate formulation excipients based on pre-formulation studies
- Optimisation of process variables and physical characteristics of the formulation such as drug dispersion, addition of sensitive excipients, effect of Freeze-Thaw cycle, appearance, viscosity, pH etc.
- Evaluation and optimisation of active drug concentration, it's *in vitro* release character and stability.
- *In vivo* evaluation of efficacy in models of pain and inflammation
    - Carrageenan Induced rat paw edema test (anti-inflammatory activity)
    - Duration of action studies in Carrageenan Induced rat paw edema test to determine the onset and duration of action
    - Comparison of topical efficacy with oral activity of the coxibs in the carrageenan induced paw edema model.
    - Carrageenan Induced hyperalgesia test in rats
    - Complete Freund's adjuvant-induced chronic paw oedema in rats
    - Phenyl-p-benzoquinone induced writhing test in mice
    - Formalin-induced phase I and phase II pain models.
    - Tail flick method in rats
- Evaluation of safety of the selected formulation by
    - Systemic exposure studies using suitable analytical methods to determine plasma levels of active drug in rats so as to compare the levels obtained after topical application with levels obtained after oral administration, at equiactive doses.
    - Acute toxicity in rats (LD_{50} studies)
    - 28 day toxicity test along with skin irritation test and effect on gastric mucosa.
        - *Ex vivo* effect on COX-1 and COX-2 inhibition.

2.0 MATERIALS AND METHODS

2.1 Materials: Reagents

Table 2. List of reagents used in the studies.

Items	Source
Acetic acid	Sigma Chemical Co., USA
Acetonitrile (AR grade)	Ranbaxy Fine Chemical Ltd. India
Acetonitrile (HPLC-grade)	Ranbaxy Fine Chemical Ltd. India
Carboxypolymethylene (Carbopol 940)	Libraw Pharma, India
Carrageenan	Sigma Chemical Co., USA
Celecoxib	Vorin Labs. Ltd., India
Dichloromethane (HPLC-grade)	Ranbaxy Fine Chemical Ltd. India
Diclofenac Sodium	Ranbaxy labs Ltd., India
Ethanol	Changshu Yangyuan Chemical, China
Ether	Qualigens Fine Chemicals, India
Formaldehyde	Ranbaxy Fine Chemical Ltd. India
Formic acid (AR grade)	Ranbaxy Fine Chemical Ltd. India
Heparin Sodium	Biological E. Ltd., India
Incomplete Freund's adjuvant	Sigma Chemical Co., USA
Isopropyl Alcohol	Ranbaxy Labs. Ltd, India
Ketoprofen	Ranbaxy Labs. Ltd, India
Lipopolysaccharide	Sigma Chemical Co., USA
Methanol (HPLC-grade)	Qualigens Fine Chemicals, India
Mycobacterium butyricum desiccated	Difco Laboratories, USA
n-hexane (HPLC-grade)	Qualigens Fine Chemicals, India
Nimesulide	Sigma Chemical Co., USA
Oil of Lemon lime (S 3770)	Bush Boake Allen India Ltd, India
ortho-phosphoric acid (85% w/w - AR grade)	Qualigens Fine Chemicals, India
Paraffin wax	International Group Inc., USA
Pentobarbitone sodium	Celegen , New jersey , USA
Phenoxyethanol	Aldrich Chemical Co.,
Phenyl-p-benzoquinoline	Sigma Chemical Co., USA
Polyethylene glycol (PEG 400)	BASF , Germany
Polyethylene glycol –8 Glyceryl Caprylate (Labrasol)	Gattefosse Corp, France
Polyoxyethylene-polyoxypropylene Copolymer (Poloxamer 407, Lutrol)	BASF , Germany
Polyoxyl 40 hydrogenated Castor oil (*Cremophor RH40*)	Libraw Pharma, India
Polyvinyl pyrrolidone (povidone)	BASF , Germany
Propylene glycol	BASF , Germany
Prostaglandin E2 – ELISA Kit	Assay Designs, USA
Rofecoxib	Vorin Labs. Ltd., India

Sodium acetate	Ranbaxy Fine Chemical Ltd. India
Sodium chloride	Ranbaxy Fine Chemical Ltd. India
Sodium hydroxide	Ranbaxy Fine Chemical Ltd. India
Sodium lauryl sulphate	Sigma Chemical Co., USA
Sterile Saline	Claris Lifesciences Limited, India.
Tetrahydrofuran (AR grade)	SD Fine Chemicals, India
Thromboxane B_2 - ELISA Kit	R & D systems, USA.
Triethanolamine	Qualigens Fine Chemicals, India
Xylene	Qualigens Fine Chemicals, India

2.2 Equipments

Table 3. List of equipments used in the studies.

Items	**Source**
Analgesiometer- (Randall-Sellito)	UGO Basile, Italy
Animal Weighing Balance	Sartorius AG, Germany
Biofuge Refrigerated centrifuge	Kendro Lab. Products, USA
Brookfield digital viscometer	Brookfield Engineering lab, Inc, U.S.A
Cyclomixer	Remi equipments, India
Deep Freezer (-20^{0}C)	Kendro Lab. Products, USA
Digital Balance	Sartorius AG, Germany
Digital pH meter- Orion 410Aplus	Thermo Electron Corporation, USA
Dyno Mill	W. A. Bachofen, Switzerland
ELISA plate reader-POLARstar OPTIMA	BMG Labtech GmbH, Germany.
Franz Diffusion Cell (HDT-10)	Copley Scientific Limited, UK
Hair clipper	Wahl Clipper Corporation, USA
High Performance Liquid Chromatography (HPLC) with SIL-10Ad*vp* auto injector and SPD-10A*vp* UV Detector and LC Solution software for Processing	Shimadzu Scientific Instruments, USA.
Hot air oven	Narang Scientific Works, India
Magnetic stirrer	GPC Medical Ltd. India
Mastersizer 2000 (Particle size analyser)	Malvern Instruments, U.K.
PE Sciex LC-MS/MS system along with Shimadzu SIL-HTC autosampler and API 3000 MS/MS detector	Shimadzu Scientific Instruments, USA.
Plethysmometer	UGO Basile, Italy
Rotary microtome-HM-355S	Microme, Germany
Single pan balance	Sartorius AG, Germany
Spin tissue processor-STP120	Microme, Germany
Tail Flick Apparatus	UGO Basile, Italy
Tissue embedding centre-EC-350	Microme, Germany
UV/Visible Spectrophotometer (UV1601)	Shimadzu Scientific Instruments, USA
Water bath (Julabo)	Siskin Instruments Co. (P) Ltd. India

3.0 Methods

3.1 Preparation of gel formulations

3.1.1 Preparation of Rofecoxib Gels

3.1.1.1 Preparation of micronised gels formulation

Polyethylene glycol, propylene glycol, polyethylene glycol-8 glyceryl caprylate and phenoxyethanol were stirred well to form dispersion. Rofecoxib (micronised with particle size distribution of - d(0.1)=1.67±0.03; d(0.5)=3.17±0.03; d(0.9)=7.38±0.12µm) was then added slowly under continuous stirring. Stirring was continued till a uniform dispersion was formed. Carboxyvinyl polymer (Carbopol 940) was further dispersed in the resultant dispersion following which a portion of water was added. Ethanol, fragrance and a solution of triethanolamine was then dispersed by constant stirring. The weight was made upto 100g with purified water and the resultant mixture was thoroughly agitated until a homogenous composition was obtained

3.1.1.2 Preparation of sub-micronised gels formulation

Similarly, Rofecoxib gel formulations with sub-micron particle size were prepared. For this purpose, 0.5% w/w polyoxyl 40 hydrogenated castor oil was dissolved in warm purified water and Rofecoxib (15% w/w) was dispersed in this aqueous solution under constant stirring. The resultant suspension was milled in a bead mill (Dyno mill) to attain sub-micronized drug particle (with particle size distribution of - d(0.1) = 0.08 ± 0.002; d(0.5) = 0.131 ± 0.001; d(0.9) = 0.204 ±0.003µm) . This drug powder was then used to formulate the sub-micronised Rofecoxib gel formulation by the protocol described above.

3.1.2 Preparation of Celecoxib Gels

3.1.2.1 Preparation of micronised gels formulation

Polyethylene glycol, propylene glycol, polyethylene glycol-8 glyceryl caprylate and phenoxyethanol were stirred well to form dispersion. Celecoxib (micronised with particle size distribution of - d(0.1)=1.95±0.08; d(0.5)=7.07±0.05; d(0.9)=15.9±0.26µm) was then added slowly under continuous stirring. Alternately 0.5% w/w polyoxyl 40 hydrogenated castor oil was dissolved in warm purified water and Celecoxib was dispersed in this aqueous solution under constant stirring. Polyethylene glycol, propylene glycol, polyethylene glycol-8 glyceryl caprylate and phenoxyethanol were then added under constant stirring to the dispersion.

Stirring was continued till a uniform dispersion was formed. Carboxyvinyl polymer (Carbopol 940) was further dispersed in the resultant dispersion following which ethanol, fragrance and a solution of triethanolamine was then dispersed by constant stirring. The weight was made upto 100g with purified water and the resultant mixture was thoroughly agitated until a homogenous composition was obtained.

Alternately to prepare formulations with Poloxamer 407, Polyoxyethylene-polyoxypropylene copolymer (poloxamer 407) was heated to 60-70^{o} C in purified water. This was cooled to 5^{o} C. The temperature of the formed gel was brought to room temperature and was added in place of Carbopol 940 in the above formulation.

3.1.2.2 Preparation of sub-micronised gels formulation

Similarly, Celecoxib gel formulations with sub-micron size range were prepared. For this purpose, Sodium lauryl sulphate (0.5% w/w) and polyvinyl pyrrolidone (povidone K30 1.w/w) were dissolved in warm purified water. Celecoxib (8% w/w) was dispersed in this aqueous solution and the resultant suspension was milled in a bead mill (Dyno mill) to attain sub-micronized drug particle (with particle size distribution of - d(0.1) = 0.078 ± 0.001; d(0.5) = 0.130 ± 0.002; d(0.9) = 0.239 ±0.005μm). This drug power was then used to formulate the sub-micronised Celecoxib gel formulation by the protocol described above.

After preparation of the gel formulations, measurements of pH were done using Systronics digital pH meter. Concentrations of each of the ingredients used in the formulation are reported in Table 1 (Rofecoxib gels) and 2 (Celecoxib gels).

3.1.3 Particle size determination

Particle size distribution was determined by photon correlation spectroscopy, using a Mastersizer 2000 (Malvern Instruments, UK). Light scattering was monitored at 25°C. A solid state laser diode was used as light source. The samples were suitably diluted with distilled water to make a polydispersion and then placed in quartz cuvette for particle size analysis.

3.2 Physico-chemical characterisation of prepared formulations

3.2.1 Determination of Drug Content

Drug concentration was determined using HPLC as described below.

3.2.1.1 Method for the Estimation of Rofecoxib in gel formulations

A) *Instrumentation*

- Shimadzu LC-10AD*vp* pump
- Shimadzu SIL-10Ad*vp* auto injector
- Shimadzu SPD-10A*vp* UV Detector
- L C Solution software for Processing

B) *Preparation of Reagents*

Phosphoric acid buffer (Solution A)

1 mL of ortho-phosphoric acid (85% w/w - AR grade) was accurately transferred in 1000 mL of water, filtered and degassed the solution before use.

Organic phase

Tetrahydrofuran (AR grade) and Acetonitrile (HPLC-grade) were mixed in the ratio of 1:2 v/v. Mix well and degassed before use.

Mobile phase

700 mL of Phosphoric acid buffer (Solution A) was transferred into a 1000-mL reagent bottle; 300 mL of organic phase was added and mixed well.

Dilution solution

Milli Q Water and Acetonitrile (HPLC-grade) were mixed in the ratio of 1:2 v/v and degassed before use.

B) *Standards Stock Solution*

Rofecoxib stock solution

About 50 mg of Rofecoxib working standard was weighed accurately and transferred into a 50-mL volumetric flask. It was dissolved in sufficient diluent, sonicated and volume was made up with the same to produce a solution of 1 mg/mL of Rofecoxib. Final concentration of Rofecoxib was corrected, accounting for its potency and the actual amount weighed.

Further, 5 ml of this solution was diluted to 50mL with diluent, filtered through 0.45µm or finer porosity membrane filter and stored in refrigerator below 12º C, protected from light.

C) *Sample Preparation*

About 2.5gm of Rofecoxib gel was weighed accurately and transferred into a 250-mL volumetric flask. It was dissolved in sufficient diluent, sonicated and volume was made up with the same to produce a solution of 0.1 mg/mL of Rofecoxib. It was then filtered through 0.45µm or finer porosity membrane filter.

D) *Chromatographic Conditions*

A summary of chromatographic and mass spectrometric conditions is as follows:

Column	: Kromasill C-18, (250 x 4.6 mm), 5µm
Flow	: 1.5 mL/minute
Mobile phase	: Phosphoric acid buffer: Organic phase
	70 30
Detection	: UV-272nm
Injection Volume	: 20 µL
Column oven temperature	: 40^0C
Sample cooler temperature	: 10^0C ± 0.2^0C
Retention times	: Rofecoxib – 13 minutes

E) *Chromatograms were considered successful only if,*

1. The trailing factor for Rofecoxib peak is not more than 1.5
2. USP tangent for Rofecoxib peak is not <3000.
3. The relative standard deviation of area counts for 5 replicate injections for Rofecoxib peaks is not more than 1.0%.

F) *Procedure*

Injected the 20µL of sample /standard solution, in triplicate into chromatograph and record the chromatogram. The major peaks were noted down from the chromatographic report. The retention time for Rofecoxib peak was 13 min. The calculations were done using the following formula:

$$\text{Rofecoxib (\% w/w)} = \frac{AT}{AS} \times \frac{DS}{DT} \times P \times \frac{100}{1000}$$

Where

AT = Average area counts for Rofecoxib peak in the chromatogram of sample solution
AS = Average area counts for Rofecoxib peak in the chromatogram of standard solution
DS = Dilution factor of Rofecoxib standard solution, in mg/mL.
DT = Dilution factor of sample solution, in gm/mL
P = Percent potency of Rofecoxib working standard

3.2.1.2 Method for estimation of Celecoxib in gel formulations

A) *Instrumentation*

- Shimadzu LC-10AD*vp* pump
- Shimadzu SIL-10Ad*vp* auto injector
- Shimadzu SPD-10A*vp* UV Detector
- L C Solution software for Processing

B) *Preparation of Standard Stock Solutions*

Celecoxib stock solution

500 mg of Celecoxib working standard was transferred into a 100-mL volumetric flask, sonicated to dissolve in sufficient 'dilution solution' and made up the volume with the same. The above final concentration of Celecoxib was corrected for accounting for its potency and the actual amount weighed. Further 5 ml of this solution was diluted to 50mL with diluents, filtered through 0.45µm or finer porosity membrane filter and stored in refrigerator below 12°C, protected from light.

C) *Preparation of Reagents*

Mobile phase

A mixture of water (HPLC-grade) and acetonitrile (HPLC-grade) was prepared in a ratio of 55:45 and used as mobile phase. The mixture was degassed before use.

Dilution solution

50 mL of acetonitrile (HPLC-grade) was transferred to a 100-mL reagent bottle; 50 mL of Milli Q water was added and mixed well.

D) *Sample Preparation*

About 2.5gm of sample was accurately weighed and transferred to a 250 mL volumetric flask. 150 mL of diluent was added and was shaken till the gel dissolves. It was then

sonicated for 1 min and the volume was made up with diluent. This was filtered through 0.45µm or finer porosity membrane filter.

E) *Chromatographic Conditions*

Column	: Zorbax TMS (4.6 mm x 50 mm) 5µm
Mobile phase	: Water: Acetonitrile (55:45)
Flow rate	: 1.5 mL/minute
Detection	: UV at 254nm
Injection volume	: 20 µL
Run time	: 10min.
Retention times	: 3 minutes

F) *Chromatograms were considered successful only if*

1. The trailing factor for Celecoxib is not more than 1.5
2. The column efficiency determined for Celecoxib peak is not less than 4000 theoretical plates.
3. The relative standard deviation of area counts for 5 replicate injections for Celecoxib peak is not more than 1.0%.

G) *Procedure*

Injected the sample solution, in triplicate into chromatograph and recorded the chromatogram. The retention time for Celecoxib peak was 3 min. The calculations were done using the following formula:

$$\text{Celecoxib (\% w/w)} = \frac{AT}{AS} \times \frac{DS}{DT} \times \frac{P}{100} \times \frac{100}{1000}$$

Where

AT = Average area counts for Celecoxib peak in the chromatogram of sample solution

AS = Average area counts for Celecoxib peak in the chromatogram of standard solution under condition described above

DS = Dilution factor of Celecoxib standard solution, in mg/mL

DT = Dilution factor of sample solution, in gm/mL

P = Percent potency of Celecoxib working standard

3.2.2 Determination of viscosity

The viscosities of the formulations were determined at 25 ± 1^{o} C using Brookefield type RVT series viscometer, with a 0.5 inch helipath and T-spindle (size E) rotating at 2.5 rpm.

3.2.3 Determination of Spreadability

The spreadability of emulsion- gels was evaluated at ambient conditions. The spreading diameter (ϕ) of 1 ± 0.1 gm of emulsion-gel placed between two horizontal glass plates (16 x 16 cm) was measured after 1 minute (mass of upper plate 125 ± 1 g). Following classification was adopted: fluid gel (ϕ) > 70 mm; semifluid gel - 70 to 55 mm; semi stiff gel 55 to 47 mm; stiff gel - 47 to 40 mm and very stiff gel (ϕ)<40 mm. (Lardy *et al*., 2000)

3.2.4 Effect of Freeze-Thaw cycle

To study the effect of freeze thaw cycles on physical stability, gels were kept at -4° C for 24 hrs then kept at 4-8° C for further 24 hours. This constituted one Freeze-Thaw cycle. Gels were evaluated after three consecutive freeze-thaw cycles.

3.3 *In Vitro* Studies

3.3.1 Drug Release and Permeation Studies

Diffusion through dialysis membrane: Diffusion studies were carried out using modified Franz diffusion cells consisting of two compartments, a donor and a receptor, separated by a cellulose acetate nitrate membrane (0.45µ). A thin layer of the test formulation was uniformly applied while a mixture of isopropyl alcohol (IPA) and water was used as medium to maintain the sink conditions in the receptor compartment. In case of Celecoxib gel formulation the ratio of IPA: Water was 55:45 while for Rofecoxib gel formulation it was 80:20 and maintained at constant temperature of 32°±1°C by circulating water bath. The samples were withdrawn from the receptor compartment at predetermined time intervals and replaced with an equal volume of mixture. The samples were analysed for Rofecoxib (272nm)/Celecoxib (254nm) content using a UV spectrometry (Shimadzu Scientific Instruments, USA).

3.3.2 Accelerated stability studies

Accelerated stability studies were carried out for optimized formulation of Rofecoxib and Celecoxib. The formulations were packed in containers, capped securely and kept at 40± 2°C/

75 ± 5% RH for 90 days. The drug content of the formulations was analysed on 15th, 30th, 60th and 90th day. The physicochemical parameters like pH, viscosity, spreadability were also analysed on these days upto 90th day.

Microbial limit test were also carried out on all the samples. For this purpose Microbial limit tests described in European Pharmacopoeia (sec 2.6.12 and 2.6.13 –Microbial examination of non-sterile products) was followed. Briefly, for Total aerobic microbial count (TAMC) and Total Combined Yeasts/Moulds count (TYMC), 10gm of the gel was suspended in 100ml of buffered sodium chloride-peptone solution (pH 7.0) and 1 ml of this suspension was added to petri-dish. To this 15 ml of casein soya bean digest agar medium (for TAMC) or Sabouraud-dextrose agar medium (for TYMC) was added and were maintained at 45º C. Casein soya bean digest agar plates were incubated at 30-35º C for 3-5 days and Sabouraud-dextrose agar was incubated at 20-25º C for 5-7 days. At the end of the incubation period the number of colony forming units were counted and expressed as CFU/gm of sample gel. To neutralize the anti-microbial agent in the gels 0.5% soya lecithin along with polysorbate was added.

For test of specified micro-organisms 10gm of the gel was suspended in 100ml of buffered sodium chloride-peptone solution (pH 7.0). 10 ml of this preparation was added to casein soya bean digest broth, mixed and incubated at 30-35º C for 24 hrs. 24 hrs later, the suitable amounts of this broth was sub-cultured further as described in European Pharmacopoeia (sec 2.6.13 –Microbial examination of non-sterile products) to determine the presence of *Escherichia coli, Salmonella, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Antimicrobial preservatives are substances added to nonsterile dosage forms to protect them from microbiological growth or from microorganisms that are introduced inadvertently during or subsequent to the manufacturing process. Preservative efficiency test was performed as per the 'Antimicrobial Effectiveness Testing' section of United States Pharmacopoeias, to evaluate the efficiency of preservative used. For this purpose 6 tubes of 20 gm gel was mixed with 6 x 10^5 microorganisms, using the cultures of following microorganisms per tube: *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). One tube was used as control. These tubes were incubated for 28 days at 22.5 ± 2.5º C. 1 gm samples were withdrawn on days 0, 7, 14 as well as on day 28. 1 gm sample was serially diluted with diluent (buffered sodium

chloride-peptone solution, pH 7.0, with polysorbate 80 and 0.5% soya lecithin) and 1ml of these diluted samples were plated with 15 - 20 ml of media as described above and incubated for 5 days. At the end of 5 days of incubation cfu were counted. The samples were considered compliant if the bacterial load showed not less than 2.0 log reduction from the initial count on 14^{th} day, no increase from the 14 day's count on 28^{th} day and the Yeast and Molds load showed no increase from the initial calculated count at 14 and 28 days.

3.4 *In vivo* Studies

3.4.1 Preparation of animals

Rats and mice used for the *in vivo* studies were procured from the Experimental Animal Facility, Ranbaxy Research Laboratories (RRL), Gurgaon, Haryana and were acclimatised for 48 hrs prior to the study. Animals were housed up to 5 animals per cage in polypropylene cages with paddy husk as bedding. Animal room temperature was maintained at 24 ± 2^{o}C with controlled illumination to provide a light dark cycle of 12 hrs. All animals were having free access to drinking water and to standard pellet laboratory animal diet (2018S from Harlan Teklad). All the experiments were conducted as per the guidelines of the Institutional animal ethics committee and those of the Government of India.

3.4.2 Carrageenan induced Paw edema model in rats

3.4.2.1 Animals

Wistar Rats (200 ± 20 gm) were procured from the Experimental Animal Facility, RRL Gurgaon and were acclimatised for 48 hrs prior to the study. Each treatment group comprised of 6-10 animals. Animals were used only once in the study.

3.4.2.2 Treatment

All the gel formulations were evaluated and ED_{50} for each formulation was determined. For ED_{50} determination, each formulation was studied atleast at 4 dose levels (for FG5 to FG10 doses were 20, 60, 200 and 600 mg/paw, for FG11 to FG14 as well as FG22 to FG25 the doses were 6.7, 20, 60, 200 and 600 mg/paw and for FG26 to FG30 the doses were 12, 40, 120 and 400 mg/paw), with each dose level having atleast 6 animals each. Animals were randomly allocated to different groups. Control animals received highest dose of respective

gel formulation without the active drug. Two standard marketed gel formulations were also screened in this model. These were Diclofenac Gel 1% w/w - (Novartis, India; B.No. 04093E) and Nimesulide Gel -1 % w/w (Panacea Biotec Ltd., India; B. No. 601728).

3.4.2.3 Method of application

The gel was applied on the plantar surface of the paw by index finger and was rubbed 25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min. (Chi and Jun, 1990). To prevent animals from licking the paws after the application of gel formulation, Elizabethan collars were used for 3 hrs post drug application.

3.4.2.4 Experimental Procedure

Required quantity of the gel was weighed and applied on the right hind paw of the rat as mentioned above. In control group, control gels were applied on the hind paw as mentioned above. Three hours after the treatment, the basal paw volume (in mL) of the treated hind paws was measured using a water displacement Plethysmometer (UGO Basile, Italy). Immediately after this, 0.1ml of 1 % w/v carrageenan in saline was injected sub-plantarly in the treated paw. Three hrs after carrageenan injection, paw volume of hind paws was again measured. Change in paw volume was determined by following equation:

$$Change\ (\Delta)\ in\ paw\ volume = V\text{-}Vi$$

Where V is the paw volume, 3 hours post-carrageenan injection and Vi is the initial paw volume.

The paw swelling in the group of the treated rats was compared with that of the control rats and percent inhibition of edema formation was determined using the following equation:

$$\%\ inhibition = \left\{1 - \frac{(mean\ change\ in\ paw\ volume\ with\ test)}{(mean\ change\ in\ paw\ volume\ with\ vehicle)}\right\} \times 100$$

Average values in test group were compared with respective control groups using one way analysis of variance followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

ED_{50} value was obtained by regression analysis of concentration and percent protection data using Graphpad prism software (GraphPad Software, USA).

3.4.3 Carrageenan induced hyperalgesia

In the above study analgesic effect of the selected gel formulation (Rofecoxib gel FG14 - dosed 6.7, 20, 60, 200 and 600 mg/paw and Celecoxib gel FG30 dosed 12, 40, 120, 400 and 600 mg/paw) was also evaluated. For this purpose separate set of animals were recruited and studied.

Effect of thermal hyperalgesia was measured using the procedure described by Jain *et al*. (2001). The mean paw withdrawal latency of the carrageenan-injected paw when dipped in water bath maintained at 47±0.5° C was measured. The baseline latency of paw withdrawal from thermal source was established three times, 5 min apart and averaged. A cut-off time of 15s was imposed to avoid injury to the paw. The mean paw withdrawal latency (L3h) 3 hrs after carrageenan administration in vehicle and drug-treated animals was measured. Change in the paw withdrawal latency (L0h–L3h) was calculated as a measure of hyperalgesia. Anti-hyperalgesic activity was expressed as percent inhibition of hyperalgesia and was calculated by taking the values in the control group as 0% inhibition.

Another group of animals was used to measure the nociceptive mechanical threshold, expressed in grams, using an Analgesiometer (UGO Basile, Italy) as described by Randall and Selitto (1957). The test was performed by applying noxious pressure to the inflamed paw. By pressing a pedal that activated a motor, the force was increased at a constant rate in a linear fashion. When the animal displayed pain by withdrawal of the paw or vocalization, the pedal was immediately released, and the nociceptive pain threshold was read on the scale. A cut-off of 500 g was used to avoid potential tissue injury. The mean paw withdrawal threshold (T3h) 3 hrs after carrageenan administration in vehicle and drug-treated animals was measured and the change in the paw withdrawal latency (T0h–T3h) was calculated as a measure of hyperalgesia. The antihyperalgesic activity is expressed as percent inhibition of hyperalgesia and was calculated by taking the values in the control group as 0% inhibition.

3.4.4 Duration of action studies

For duration of action studies, the above mentioned procedure was followed and the study was performed 1, 3, 6, 12, 18 and 24 hrs post gel formulation application at one concentration of 600 mg of gel/paw for 1% Rofecoxib and 400mg of gel/paw for 5% w/w Celecoxib gel formulation.

3.4.5 Comparison with oral dosing

In another study Rofecoxib or Celecoxib were prepared in PEG400 and was given perorally by intragastric administration as a gavage using oral feeding needle in overnight fasted rats. The doses studied were 1, 3, 10, 30 and 100 mg/kg of Rofecoxib/ Celecoxib. One hour post dosing animals were challenged with 0.1ml of 1% w/v carrageenan as described above and effect on paw edema was evaluated 3 hours post carrageenan challenge. ED_{50} values were obtained and compared with values obtained after topical administration.

3.4.6 Formalin induced Phase-I and Phase-II pain model in rats

3.4.6.1 Animals

The study was conducted on male Wistar rats, weighing between 180-220 gm. Animals were obtained from Experimental Animal Facility of Ranbaxy Research Laboratories. Rats were group housed in polypropylene cages (not more than five rats per cage) and maintained at a temperature of 24 ± 2^{o}C with controlled illumination to provide a light dark cycle of 12 hrs. All animals were having free access to drinking water and to standard pellet laboratory animal diet (Golden Feeds, New Delhi). Each treatment group comprised of 6-8 animals. Animals were used only once in the study.

3.4.6.2 Treatment

2 short listed gels formulations, 1 each of Rofecoxib (FG14) and Celecoxib (FG30), were evaluated in this model. They were studied at 4 dose levels (FG14 and FG30 dosed 20, 60, 200 and 600 mg/paw) with each dose level having at least 6 animals. Animals were randomly allocated to different groups. Control animals received highest dose of respective gel formulation without the active drug. Two standard gel formulations were also screened in this model-Diclofenac gel formulation (FG31) and Nimesulide gel formulation (FG32) at doses of 20, 60, 200 and 600 mg/paw.

3.4.6.3 Method of application

The gel was applied on the plantar surface by index finger and was rubbed 25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min. (Chi and Jun, 1990). To prevent animals from licking after the application of gel formulation, Elizabethan collars were used for 3 hrs post drug application.

3.4.6.4 Experimental Procedure

On the day of experiment 3 hrs after application of test/vehicle/standard gel formulations, each rat received 50µL of 2.5% formalin solution intraperitoneally in plantar surface of the right paw. (Farsam *et al.,* 2000). Pain behaviour was quantified by counting the time spent in lifting, favouring, licking, flinching, shaking and biting the injected paw. The rat was observed for 60 min after the injection of formalin and the amount of time spent licking the injected hindpaw was recorded. The first 5 min post formalin injection is known as the early phase and the period between 15–60 min as the late phase. Scores of 6-8 animals of each treatment group were averaged for early phase and late phase and expressed as mean ± S.E.M. (Standard error of mean). Percentage inhibition of formalin effect was calculated using the following formula:

$$\%\, inhibition = \left\{1 - \frac{(mean\ response\ time\ with\ test)}{(mean\ response\ time\ with\ vehicle)}\right\} \times 100$$

Average values in test group were compared with respective control groups using one way analysis of variance followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

3.4.7 Phenyl-p-benzoquinone induced Writhing test in Swiss mice

3.4.7.1 Animals

The study was conducted on male Swiss mice, weighing between 25-30 gm. Animals were obtained from Experimental Animal Facility of Ranbaxy Research Laboratories. Each treatment group comprised of 6-8 animals. Animals were used only once in the study.

3.4.7.2 Treatment

2 short listed gels formulations, 1 each of Rofecoxib (FG14) and Celecoxib (FG30), were evaluated in this model. They were studied at 4-5 dose levels (FG14 – dosed 10, 30, 60, 100 and 300 mg/mice; FG30 - dosed 10, 30, 100 and 300 mg/kg) with each dose level having at least 6 animals. Animals were randomly allocated to different groups. Control animals received highest dose of respective gel formulation without the active drug. One standard gel formulations (Diclofinac gel, FG31) was also screened in this model.

3.4.7.3 Method of application

The gel was applied on the abdominal surface by index finger and was rubbed 25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min. (Chi and Jun, 1990).

To prevent animals from licking the abdomen after the application of gel formulation, hand made Elizabethan collars were used for 3 hrs post drug application.

3.4.7.4 Experimental Procedure

On the day of experiment 3 hrs after application of test/vehicle/standard gel formulations, each mouse received 10 ml/kg of PQ (Phenyl-p-benzoquinone) solution intraperitoneally (0.02 % in 5% ethanol, 2 mg/kg), (Pong *et al.*, 1985). The number of occurrences of characteristic writhing responses, such as stretching, twisting a hind leg inward and contraction of the abdomen, was counted from 5 to 15 min after PQ administration. The number of counts for an individual mouse from a treatment group was expressed as writhing score. Scores of 6-7 mice of each treatment group were averaged and expressed as mean ± SE (Standard error). Percentage inhibition from PQ effect was calculated using the following formula:

$$\% \text{ inhibition} = \left\{1 - \frac{(\text{Mean writhing scores with test})}{(\text{Mean writhing scores with vehicle})}\right\} \times 100$$

Average values in test group were compared with respective control groups using one way analysis of variance followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

3.4.8 Tail flick test in rats

3.4.8.1 Animals

Wistar Rats (200 ± 20 gm) were procured from the experimental animal facility, Ranbaxy Research Labs., Gurgaon and were acclimatised for 48 hrs prior to the study. Rats were housed up to 4 animals per cage.

3.4.8.2 Treatment

2 short listed gels formulations, 1 each of Rofecoxib (FG14) and Celecoxib (FG30), were evaluated in this model. They were studied at 4 dose levels (20, 60, 200 and 600mg/rat tail) with each dose level having at least 6 animals. Animals were randomly allocated to different groups. Control animals received highest dose of respective gel formulation without the active drug. 1 standard marketed gel formulation was also screened in this model. Pentazocine was used as method control

3.4.8.3 Method of application

The gel was applied on the surface of the tail by index finger and was rubbed 25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min. (Chi and Jun, 1990) To prevent animals from licking the tail after the application of gel formulation, Elizabethan collars were used for 3 hrs post drug application.

3.4.8.4 Experimental Procedure

Gel was weighed and applied on the surface of the tail of the rat as mentioned above. In vehicle control group control gels were applied on the tails as mentioned above. 1 hour after the treatment, animals were placed on the tail flick apparatus (UGO Basile, Italy) in such a way that the animal was restrained on the apparatus with its tail resting on the path of radiant heat emitting infra-red light beam. After the animal had settled, the light beam was switched on to exert a heat source on the proximal third of the tail. A timer attached to this switch was also switched on automatically. As soon as the threshold of pain was reached animal flicks the tail away from the direction of light beam which switches-off the light beam and the timer. The time from initiation of light beam to the tail flick was recorded as reaction time. Only animals showing a basal reaction time of 2.5 - 5 sec were included in the study. A cutoff time of 12 sec was used to prevent any damage to the rat tail.

The reaction time of the treated rats was compared with that of the control rats and percent inhibition of reaction time was determined using the following equation:

$$\%\, inhibition = \left\{1 - \frac{(mean\ reaction\ time\ with\ test)}{(mean\ reaction\ time\ with\ vehicle)}\right\} \times 100$$

Average values in test group were compared with respective control groups using one way analysis of variance followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

3.4.9 Complete Freund's adjuvant-induced chronic paw oedema in rats

3.4.9.1 Animals

Wistar Rats (200 ± 20 gm) were procured from the experimental animal facility, Ranbaxy Research Laboratories, Gurgaon and were acclimatised for 48 hrs prior to the study. Each treatment group comprised of 6-8 animals. Animals were used only once in the study.

3.4.9.2 Treatment

2 short listed gels formulations, 1 each of Rofecoxib (FG14) and Celecoxib (FG30), were evaluated in this model. They were studied at 4 dose levels (FG14 at doses of 20, 60, 200 and 600mg/paw and FG30 at doses of 12, 40, 120 and 400 mg/paw) with each dose level having at least 6 animals. Animals were randomly allocated to different groups. Control animals received highest dose of respective gel formulation without the active drug. One standard gel formulation (FG32-1% Nimesulide gel at a dose of 600 mg/paw) was also screened in this model.

3.4.9.3 Method of application

The gel was applied on the surface of the paw by index finger and was rubbed 25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min. (Chi and Jun, 1990). This procedure was followed twice daily. To prevent animals from licking the paws after the application of gel formulation, handmade Elizabethan collars were used for 3 hrs post drug application.

3.4.9.4 Experimental Procedure

On day 1, paw volume of the animals was determined using water displacement plethysmograph (UGO Basile, Italy). Weighed amount of test/standard gels were applied on the paw of the rats as mentioned above. In vehicle control group, control gels were applied as mentioned above. 1 hr. after the treatment, animals were injected 0.1 ml of complete Freund's adjuvant, in the plantar region of right paw, containing 1mg of *Mycobacterium butyricum* (Difco Laboratories, USA) desiccated, suspended in incomplete Freund's adjuvant (Sigma Chem. Co., USA). The treatment gels were applied to respective treatment groups twice daily for 14 days. Paw volume was recorded at 0 min. (just before adjuvant injection - which served as basal paw volume) then at 18 hrs and finally on day 14. Paw edema at 18 hrs post injection was taken as acute phase of inflammation, while paw edema on day 14 was taken as index of chronic inflammation (Gupta *et al*., 1996).

Pain threshold of effected paws was determined by the number of squeak vocalizations induced by five consecutive gentle flexions of the right ankle joint at 3 sec interval (Noguchi *et al*., 2005). The change in paw volume at acute and chronic inflammation phase as well as pain response in test gel treated group was compared with that of the control rats and percent inhibition was determined using the following equation:

$$\%\, inhibition = \left\{1 - \frac{(mean\ response\ in\ test\ group)}{(mean\ response\ in\ vehicle\ group)}\right\} \times 100$$

Average values in test group were compared with respective control groups using one way analysis of variance followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant

3.5 Safety Studies

3.5.1 Systemic penetration studies

Systemic penetration studies using HPLC to determine plasma levels of active drug at ED_{50} dose in rats and compare it with levels obtained with oral administration.

3.5.1.1 Animals

The study was conducted on male Wistar rats, weighing between 250±25 gm. Animals were obtained from Experimental Animal Facility of Ranbaxy Research Laboratories, Gurgaon, Haryana. Each treatment group comprised of 8-10 animals. Animals were used only once in the study.

3.5.1.2 Treatment

34 rats were randomly assigned to 4 treatment groups. Group I and III were administered Rofecoxib and Celecoxib respectively at dose of 10mg/kg. p.o., in overnight fasted animals**.** Group II and IV were treated with Rofecoxib and Celecoxib gels respectively at dose of 100 mg of gel applied on the shaven dorsum in an area of 2.5 x 2.5 cm^2. Gels were applied by index finger and was rubbed 25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min.

3.5.1.3 Blood sample collection and processing

Blood was collected from retro orbital plexus into chilled tubes containing heparin at 0.5, 1, 1.5, 2, 4, 8, 12 and 24 hrs after administration of the dose. Each animal was bled not more than 4 times and 4 blood samples were collected at each time point. Plasma was separated by centrifugation and was stored at -20° C until analysed for drug concentration.

3.5.2 Method for the Estimation of Rofecoxib in Rat Plasma

3.5.2.1 Instrumentation

- Shimadzu LC-10AD*vp* pump
- Shimadzu SIL-10Ad*vp* auto injector
- Shimadzu SPD-10A*vp* UV Detector
- L C Solution software for Processing

3.5.2.2 Standards Stock Solution

A) *Rofecoxib stock solution*

About 50 mg of Rofecoxib working standard was weighed accurately and transferred into a 50-mL volumetric flask. It was dissolved in sufficient acetonitrile and volume was made up with the same to produce a solution of 1 mg/mL of Rofecoxib. The stock solution was stored in refrigerator below 12° C, strictly protected from Light. The stock solution was diluted to suitable concentrations using 'dilution solution' for spiking in plasma to obtain calibration curve (CC) standards and quality control (QC) samples.

B) *Ketoprofen stock solution (Internal Standard-IS)*

About 0.2mg of Ketoprofen working standard was accurately weighed and transferred into a 100-mL volumetric flask. It was dissolved in 10 ml of methanol and the volume was made up with the 0.01M sodium hydroxide (NaOH) to produce a solution of 2µg/mL of Ketoprofen. The stock solution was stored in refrigerator below 12° C.

3.5.2.3 Preparation of Reagents

0.1 % Acetic acid solution (Solution A)

1000 mL of Milli Q water was transferred into a 1000-mL reagent bottle. To this 1000 µL of acetic acid (AR grade) was added and mixed well. Finally 300µl of Triethylamine was added and mixed well.

Mobile phase

650 mL of 0.1 % Acetic acid solution (Solution A) was transferred into a 1000-mL reagent bottle; 350 mL of acetonitrile (HPLC-grade) was added and mixed well.

Dilution solution

50 mL of acetonitrile (HPLC-grade) was transferred into 100-mL reagent bottle; 50 mL of Milli Q water was added and mixed well.

Rinsing solution

500 mL of Milli Q water was transferred to a 1000 mL reagent bottle, to this 500 mL of acetonitrile (HPLC-grade) was added and mixed well.

0.05M Acetate Buffer solution

41mg of sodium acetate was accurately weighed and transferred into 100ml volumetric flask and volume was made up with Milli Q water. The pH was adjusted to 4.5 with acetic acid.

3.5.2.4 Sample Preparation

To 200 µL of plasma sample, 50 µL (~ 2 µg/mL) of internal standard and 100 µL of acetate buffer solution was added and vortexed. 5ml of ethyl acetate buffer was then added and vortexed again for 1.5min. The test tubes were place on reciprocating shaker for 10 minutes at 175 rpm. 4.4 mL of supernatant organic layer was removed and evaporated to dryness at 50ºC, under nitrogen flow of 15psi. The residue was reconstituted in 200 µL of mobile phase and transferred to HPLC vials for analysis.

3.5.2.5 Chromatographic Conditions

A summary of chromatographic and mass spectrometric conditions is as follows:

Column	: Kromasill C-18, (250 x 4 mm), 10µ
Flow	: 1.00 mL/minute
Detection	: UV-272nm
Injection Volume	: 100 µL
Column oven temperature	: 40^0C
Sample cooler temperature	: 10^0C ± 0.2^0C
Retention times	: Rofecoxib – 10.0 to 10.4 minutes
	Ketoprofen – 14.5 to 14.8 minutes

Calibration curve**:** The calibration curve range for the estimation of Rofecoxib in rat plasma is 10.05 ng/mL to 1960.00 ng/mL. Sample HPLC chromatograms and standard curve are presented in Appendix –I.

3.5.3 Method for Estimation of Celecoxib in Rat Plasma

3.5.3.1 Instrumentation

- PE Sciex LC-MS/MS system
- LC-10ADvp pump

- Shimadzu SIL-HTC autosampler
- API 3000 MS/MS detector
- Analyst software for data processing

3.5.3.2 Preparation of Standard Stock Solutions

A) *Celecoxib stock solution*

About 50 mg of Celecoxib working standard was accurately weighed and transfer into a 50-mL volumetric flask. It was dissolved in sufficient 'dilution solution' to produce a solution of 1 mg/mL of Celecoxib. The above final concentration of Celecoxib was corrected to account for its potency and the actual amount weighed and stored in refrigerator below 12ºC, protected from light.

The stock solution was diluted to suitable concentrations using 'dilution solution' for spiking in plasma to obtain calibration curve (CC) standards and quality control (QC) samples.

B) *Rofecoxib stock solution (Internal Standard-IS)*

About 10 mg of Rofecoxib working standard was accurately weighed and transfer into a 10-mL volumetric flask. It was dissolved in 'dilution solution' to produce a solution of 1 mg/mL of Rofecoxib. The above final concentration of Rofecoxib was corrected to account for its potency and the actual amount weighed. It was the stored in refrigerator below 12ºC, protected from light.

The stock solution was diluted to suitable concentration (~ 1 µg/mL) using 'dilution solution' prior to be use as an internal standard for processing.

3.5.3.3 Preparation of Reagents

<u>0.1 % Formic acid solution</u>

500 mL of Milli Q water was transferred into a 500-mL reagent bottle. 500 µL of formic acid (AR grade) was added and mixed well.

<u>Mobile phase</u>

100 mL of 0.1 % formic acid solution was transferred into a 1000-mL reagent bottle; 900 mL of methanol (HPLC-grade) was added and mixed well.

<u>Dilution solution</u>

50 mL of acetonitrile (HPLC-grade) was transferred into a 100-mL reagent bottle; 50 mL of Milli Q water was added and mixed well.

Extraction solution

250 mL of dichloromethane (HPLC-grade) was transferred into a 500-mL reagent bottle; 250 mL of n-hexane (HPLC-grade) was added and mixed well.

Rinsing solution

500 mL of Milli Q water was transferred into a 1000-mL reagent bottle; 500 mL of acetonitrile (HPLC-grade) was added and mixed well.

0.1M Sodium Acetate Buffer

0.82gm of anhydrous sodium acetate was weighed and dissolved in 100 mL of milli Q-water. pH of solution adjusted to 5 ± 0.1

3.5.3.4 Sample Preparation

To 150 µL of plasma sample, 50 µL (~ 1µg/mL) of internal standard dilution and 250 µL of 0.1M sodium acetate buffer was added and vortex for 20 seconds. 4 mL of extraction solution was added and vortexed for 2 minutes followed by centrifugation at 4000 rpm for 2 minutes. 3.6 mL of supernatant organic layer was removed and evaporated to dryness at 50ºC, under nitrogen flow of 15psi. The residue was reconstituted in 400 µL of mobile phase and transferred to HPLC vials for analysis.

3.5.3.5 Chromatographic Conditions

Column : Chromolith (4.6 mm x 50 mm)

Mobile phase : 0.1 % Formic acid solution: Methanol (10:90)

Flow rate : 0.6 mL/minute

Detection : Celecoxib m/z- 382.2 (parent) and 362.5(product)
Rofecoxib m/z- 315.4 (parent) and 297.0(product)

Ion source : Turbo ion Spray

State file information for API 3000

IS (Ion spray voltage) : 5500

TEMP (Temperature) : 400

DP (Declustering potential) : 50

FP (Focusing potential) : 320

EP (Entrance potential) : 8

CE (Collision energy) : Celecoxib 38
Rofecoxib 20

CXP (Collision cell exit potential) : 18

CEM (Channel electron multiplier) : 2800

NEB (Nebuliser gas) : 13

CUR (Curtain gas) : 9

CAD (Collision gas) : 7

Sample cooler temperature : 6ºC

Injection volume : 10 µL

Retention times : Celecoxib – 1.5 to 2.0 minutes

Rofecoxib - 1.5 to 2.0 minutes

3.5.3.6 Calibration curve:

The calibration curve range for the estimation of Celecoxib in rat plasma is 4.75 ng/mL to 1899.24 ng/mL. Sample HPLC chromatograms and the standard curve are presented in Appendix –I.

3.5.4 Skin irritation studies

3.5.4.1 Animals

Wistar Rats (250 ± 25 gm) were procured from the experimental animal facility, Ranbaxy Research Labs., Gurgaon and were acclimatised for 48 hrs prior to the study. Each treatment group comprised of 6-8 animals. Animals were used only once in the study.

3.5.4.2 Treatment

2 short listed gels formulation, 1 each of Rofecoxib and Celecoxib, were evaluated in this model. They were studied at 2 dose levels with each dose level having 9-10 animals. Animals were randomly allocated to different treatment groups. Control animals received equivalent dose of respective gel formulation without the active drug. One group of animals received saline.

3.5.4.3 Method of application

An area of 2.5x 2.5 cm was depilated on the abdomen of the rats using a hair clipper under anaesthesia. The gel was applied on the depilated abdominal surface by index finger and was rubbed 25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min. (Chi and Jun, 1990)

3.5.4.4 Experimental Procedure

On day 1, animal were weighed, marked and test/ vehicle gel was applied as described above. This procedure was followed for 28 days once daily. Each day animals were observed for gross changes in vital clinical signs and body weight.

At the end of experiments, inflammatory reactions on rat skin were assessed by gross microscopic evaluation. The site of application including non-affected area on the skin from each group was excised and fixed in 10% neutral buffered formalin for at least 48 hour. These sections were subjected to histological technique following the protocol described by Bancroft and Gamble, (2002) with slight modifications. Sections were processed by Spin tissue processor-STP120 (Microme, Germany). Briefly, after fixation and overnight washing, the sections were initially treated with ascending grades of alcohol viz. 70%, 80%, 90% and 100% alcohol, cleared with xylene and finally were subjected to paraffin wax for infiltration. Paraffin embedding was done in Tissue embedding centre-EC-350 (Microme, Germany) and blocks were prepared. Tissues were then cut at 4µ thicknesses using Rotary microtome-HM-355S (Microme, Germany). Sections were stained with Hematoxylin and Eosin for histological evaluation by light microscopy. Pathological changes were graded from no changes to marked change (level 0 to 4, respectively) including the inflammation and vascular changes of epidermis, dermis and subcutis as well as degeneration of skin appendages (Wu *et al*., 2002). Total irritation score (TIS) was obtained by summation of each score and used as an index for evaluation of skin damage.

3.5.5 *Ex vivo* COX inhibition assay

In the same study *ex vivo* COX inhibitory potential was also studied. For this, blood was collected on days 28. These blood samples were evaluated as described below for TxB_2 and PGE_2 release to determine systemic COX-1 and COX-2 inhibitory potential of the topical gel application respectively.

3.5.5.1 COX-1 assay

Blood was collected in tubes without anticoagulants and allowed to clot. 500µl of blood was incubated at 37°C for 60 minutes. Reaction was stopped by cold centrifugation at 800g for 10 min at 4°C to pellet the cells. Supernatants were recovered which were frozen immediately at -20°C. Thromboxane B_2 (TxB_2) was measured (dilution 1:100) using a commercially available ELISA kit (R & D systems, USA) following the manufacturer's instructions (Gierse *et al*., 2005).

3.5.5.2 COX-2 assay

Blood was collected in heparinized tubes. To 500µL of blood, Lipopolysaccharide (LPS, 100µg/ml) was added and incubated at 37°C for 24 hrs. Samples were centrifuged at 800g for 10 minutes at 4° C to collect the plasma which were frozen immediately at -20°C. Plasma supernatant (40 µl) was precipitated with 4 volumes of methanol (160 µl) and spun at 800g for 10 min. Supernatants were recovered and diluted 1:50 in ELISA buffer for quantitation of Prostaglandin E2 (PGE_2) using a commercially available ELISA kit (Assay Designs, USA) following the manufacturer's instructions (Gierse *et al*., 2005).

3.5.5.3 Effect on gastric ulcers

At the end of the study rats were killed with overdose of thiopental sodium (150mg/kg, i.p.). Stomachs were rapidly dissected out, opened along the greater curvature, the mucosa was rinsed with normal saline and exposed for macroscopic evaluation for the presence of petechiae or frank hemorrhagic lesions. Petechiae were assigned a score of 1 and lesions were scored according to their length (a score of 5 for lesions with a length between 1 and 3 mm; a score of 10 for lesions greater than 3 mm). The sum of total scores was used for comparison. The size of the ulcer in square millimeters was considered the most objective measurement of macroscopic lesion.

3.5.6 Determination of acute toxicity (LD_{50} determination)

3.5.6.1 Animals

Swiss Albino Mice (25 ± 3 gm) were procured from the experimental animal facility, Ranbaxy Research Laboratories, Gurgaon. Animals were acclimatised for 48 hrs prior to the study. Animals were used only once in the study.

3.5.6.2 Treatment

Two short listed gels formulation, 1 each of Rofecoxib and Celecoxib, were evaluated in this model. They were studied at 3 dose levels with each dose level having 10 animals (5 males and 5 females). Animals were randomly allocated to different groups.

3.5.6.3 Method of application

An area of 1.5 x 2 cm^2 was depilated on the abdomen using a hair clipper under light anaesthesia. Gels were applied on the depilated abdominal surface by index finger by rubbed

25 times in 1 min., followed by a gap of 15 sec. and then repeated 25 times in 1 min. (Chi and Jun, 1990).

3.5.6.4 Experimental procedure

On the day of experiment the animals were weighed, marked and respective formulation gel was applied as described above. Doses of Rofecoxib and Celecoxib gels used were 1.2, 4 and 12 gm/kg. Animals were observed for any gross behavioral changes and for mortality for 24 hrs. Dose of drug showing 50% mortality was expressed as LD_{50} dose.

4.0 Results and Discussion

Celecoxib as well as Rofecoxib have high lipophilicity (Summary Basis of Approval of Rofecoxib/Celecoxib) therefore, formulating them as emulsion gels was considered. Poor percutaneous absorption is a significant problem encountered in the development of topical formulations containing drugs that are poorly soluble in water such as Celecoxib or Rofecoxib. It has been observed that by increasing surface area of a particulate drug, such as by decreasing the particle size, improves the dissolution rate of the drugs, resulting in better permeability and percutaneous efficacy (Muller *et al.*, 2001; Shudo *et al*., 2008). Therefore, initial formulations were prepared with a micronized API of both Rofecoxib and Celecoxib as indicated in the methods section.

Another important aspect of preparing a topical formulation is solubility of drug substance in the intercellular lipids (Roberts *et al.*, 2002), specially when the drug molecule is insoluble. This is improved by the addition of the solubilising / penetration enhancing agents in the formulation. It has been suggested that penetration enhancers may increase skin permeability of insoluble compounds or macromolecules including heparin, oligonucleotides etc., by up to 100-fold, without inducing skin irritation (Karande *et al*., 2004). Permeation enhancers fall into two major categories: those that impact diffusion across the stratum corneum. These have a long alkyl chain capable of intercalating with the long chains of the intercellular lipids, in addition to a polar head group that is capable of interacting with the lipid polar head groups. The other class of permeation enhancers alters partitioning into the stratum corneum by affecting the solubilising property of the skin, resulting in increased solubility of the drug within the stratum corneum. For this purpose several combinations of the solubiliser/ penetration enhancers were tried either alone or in combination

4.1 Preparation of formulation

Gel formulation of Rofecoxib and Celecoxib were prepared as described in the methods section. The following excipients were selected for pre-formulation development studies:

4.1.1 Solublisers/permeation enhancer

4.1.1.1 Propylene glycol

Propylene Glycol is clear, colourless, stable, odourless liquid and widely used as a solvent, humectant and antimicrobial preservative. At 15% w/w concentration, it acts

as humectant in topical formulations. It is miscible with water and ethanol.

4.1.1.2 Polyethylene glycols

PEG (200-600) is clear, colourless or slightly yellow coloured stable liquid. PEG 400 is widely used in variety of pharmaceutical formulation including parenteral and topical.

Polyethylene glycol 400 is stable hydrophilic substance that is essentially non irritant to the skin. PEG 400 is water soluble and easily removable from the skin, therefore useful in topical products.

4.1.1.3 Ethyl/ isopropyl alcohol

Ethyl / isopropyl alcohol are widely used as a solublizer / penetration enhancer in topical dosage form. They also act as antimicrobial agent.

4.1.1.4 Polyethylene glycol -8 glyceryl caprylate (Labrasol)

Caprylocaproyl macrogol-8 glyceride (Labrasol) is a safe, nonionic surfactant developed by Gattefosse Corp. (Saint-Priest, France). Labrasol is synthesized by an alcoholysis/esterification reaction using medium chain triglycerides from coconut oil and PEG400 as starting materials. Thus, Labrasol is comprised of a well-defined mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethyleneglycol, with the predominant fatty acids being caprylic and capric acids. Labrasol has been reported to increase the solubility of water-insoluble drugs by emulsification (Yuksel *et al.,* 2003).

The above mentioned solubliser and penetration enhancers were used and evaluated during formulation development of Rofecoxib and Celecoxib gels.

4.1.2 <u>Gelling Agent</u>:

4.1.2.1 Carbomers

Carbomers are synthetic high molecular weight polymers of acrylic acid cross linked with either allylsucrose or allyethers of pentaerythritol. They contain between 56.0 - 86.0% of carboxylic acid groups. The molecular weight of these polymers is theoretically estimated to be in the range of 7 Lacs to 3 or 4 billion. Different grades

of Carbormer like Carbomer907, 910 NF, 941 NF. 981 NF, 1342 NF, 1382 NF, 934 NF 940 NF etc. are used as gelling agent for topical formulations.

As per literature Carbopol 940 NF is an efficient thickener of all Carbopol resins and forms sparkling clear water or hydro-alcoholic gels.

4.1.2.2 Poloxamers

The poloxamers are non ionic polyoxyethylene polyoxypropylene co-polymers. Poloxamer (Lutrol F127/F 68) are white, coarse grained powder with waxy consistency. It is soluble in water, ethanol and isopropanol. It is insoluble in ether, paraffins or fatty oils. Lutrol F 127 is used as a thickening agent and gel former, as a co-emulsifier and consistency enhancer in cream and liquid emulsions. Owing to its ability to affect viscosity, Lutrol F 127 is suitable as a stabilizer for topically and orally administered suspensions.

4.1.2.3 Hydroxypropyl Cellulose:

Hydroxypropyl Cellulose is widely used in topical pharmaceutical formulations as a gelling agent

Poloxamers are known to cause stickiness during application. Carbomers are commonly used as gelling agents in topical pharmaceutical preparations and therefore were selected for further optimisation in the formulation.

4.1.3. Neutralising Agent:

Molecules of Carbopol resins in its presolvated state are tightly coiled and its performance capabilities are limited. The performance is maximised when the polymer molecules are fully un-coiled and extended. This uncoiling can be achieved by addition of a neutralizing agent / basifying agent

Inorganic bases such as Sodium Hydroxide, Potassium Hydroxide or low molecular weight amines and alkanol arnines provide satisfactory neutralisation. Some of the amine bases are like Triethanolamine (TEA), Aminomethylpropanol (AMP 95). Tromethamine (TrisAmino) and Tetrahydroxypropylethylenediamine (Neutrol TE) are effective as neutralising agents. Triethanolamine is commonly used as neutralizing agent for

carbomers, hence was selected.

4.1.4. Preservative

Methyl Paraben, Propyl Paraben, Benzyl Alcohol, Phenoxyethanol are commonly used as preservatives in the topical products. Parabens exhibit pH dependent anti-microbial action and are effective in combination, as anti-microbial agents. Phenoxyethanol is a colourless, slightly viscous with pleasant odour and exhibits pH independent anti-microbial action. Hence, Phenoxyethanol was selected for formulation development.

A total of 30 formulations of Rofecoxib and Celecoxib were prepared using different combinations of the above mentioned excipients as mentioned in Table 4 (Rofecoxib gel formulations) and Table 5 (Celecoxib gel formulations). These include the blank gels, prepared without the active drug substance.

Table 4. Rofecoxib gel formulations with varying excipient concentration

Ingredients ↓ / Formulation no. ⟶	Quantity (%)													
	FG1	**FG2**	**FG3**	**FG4**	**FG5**	**FG6**	**FG7**	**FG8**	**FG9**	**FG10**	**FG11**	**FG12**	**FG13**	**FG14**
Rofecoxib (micronized)	0.0	0.0	0.0	0.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	3.0	0.0	0.0
Rofecoxib - (sub-micronized)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0
Carboxypolymethylene (Carbopol 940)	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.0	1.0	1.0	1.4	1.4	1.4	1.4
Polyethylene glycol (PEG 400)	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Propylene glycol	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Polyethylene glycol -8 Glyceryl Caprylate(Labrasol)	5.0	5.0	5.0	10.0	5.0	5.0	5.0	10.0	5.0	0.0	5.0	10.0	5.0	5.0
Ethanol	0.0	7.5	10.0	10.0	0.0	7.5	10.0	10.0	10.0	10.0	10.0	10.0	0.0	7.5
Triethanolamine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	0.5	0.5	1.0	1.0	1.0	1.0
Phenoxyethanol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Fragrance (oil of Lemon)	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Purified water	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100
pH	*5.86*	*5.67*	*5.76*	*5.82*	*5.83*	*5.76*	*5.81*	*5.87*	*5.86*	*5.82*	*5.86*	*5.92*	*5.81*	*5.72*

FG indicates the Formulation Gel number.

Table 5. Celecoxib gel formulations with varying excipient concentration.

Ingredients ↓ **Formulation no.** →	**Quantity (%)**													
	FG15	**FG16**	**FG17**	**FG18**	**FG19**	**FG20**	**FG21**	**FG22**	**FG23**	**FG24**	**FG25**	**FG26**	**FG27**	**FG28**
Celecoxib (micronized)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	3.0	3.0	3.0	5.0	5.0	5.0
Carboxypolymethylene (Carbopol 940)	1.4	0.0	0.0	0.0	1.4	1.4	1.4	1.4	0.0	0.0	0.0	1.4	1.4	1.4
Polyoxyethylene-polyoxypropylene copolymer (Poloxamer 407, Lutrol)	0.0	20.0	20.0	25.0	0.0	0.0	0.0	0.0	20.0	20.0	25.0	0.0	0.0	0.0
Polyethylene glycol (PEG 400)	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	15.0	12.5	12.5	12.5
Propylene glycol	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Polyethylene glycol -8 Glyceryl Caprylate(Labrasol)	10.0	10.0	10.0	10.0	5.0	5.0	5.0	10.0	10.0	10.0	10.0	5.0	5.0	5.0
Ethanol	10.0	10.0	30.0	10.0	0.0	7.5	30.0	10.0	10.0	30.0	10.0	0.0	7.5	30.0
Polyoxyl 40 hydrogenated Castor oil (Cremophor RH40)	0.0	0.0	0.0	0.0	0.5	0.5	0.5	0.0	0.0	0.0	0.0	0.5	0.5	0.5
Triethanolamine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Phenoxyethanol	1.0	0.4	0.4	0.4	1.0	1.0	1.0	1.0	0.4	0.4	0.4	1.0	1.0	1.0
Fragrance (oil of Lemon)	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Purified water	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100	to 100
pH	*5.79*	*5.67*	*5.84*	*5.82*	*6.63*	*6.52*	*6.46*	*5.83*	*5.92*	*5.92*	*5.97*	*6.73*	*6.68*	*6.46*

FG indicates the Formulation Gel number.

Table 5. Celecoxib gel formulations with varying excipient concentration (continued).

Ingredients ↓ Formulation no. →	Quantity (%)			
	FG29	**FG30**	**FG31**	**FG32**
Celecoxib (sub-micronized)	5.0	5.0	0.0	0.0
Diclofenac	0.0	0.0	1.0	0.0
Nimesulide	0.0	0.0	0.0	1.0
Carboxypolymethylene (Carbopol 940)	1.4	1.4	1.4	1.4
Polyethylene glycol (PEG 400)	12.5	12.5	12.5	12.5
Propylene glycol	5.0	5.0	5.0	5.0
Polyethylene glycol -8 Glyceryl Caprylate(Labrasol)	5.0	5.0	5.0	5.0
Ethanol	0.0	7.5	7.5	7.5
Polyoxyl 40 hydrogenated Castor oil (Cremophor RH40)	0.5	0.5	0.5	0.5
Triethanolamine	1.0	1.0	1.0	1.0
Phenoxyethanol	1.0	1.0	1.0	1.0
Fragrance (oil of Lemon)	0.34	0.34	0.34	0.34
Purified water	to 100	to 100	to 100	to 100
pH	*6.81*	*6.72*	*5.81*	*6.48*

Formulation no.	**Details**
FG33	Diclofenac Gel - (Novartis; B.No. 04093E)
FG34	Nimesulide Gel -1 % w/w (Panacea Biotec Ltd.; B. No. 601728)

4.2 Optimisation studies

4.2.1 Selection of Solubilisers/ Penetration Enhancers

Solubiliser, penetration enhancers were used either alone or in combination and the results are presented in Table 6.

Table 6. Effect of solubiliser/ penetration enhancer combinations on gel formulation.

	Solubiliser/ penetration enhancers (%w/w)	Properties	Results
1	PEG 400 20%, Propylene Glycol 5%	Slightly warm sensation which was irritating to skin	Not acceptable
2	Labrasol 25%	Gel was not stable and looses consistency on 1 month/ 40° C.	Not acceptable
3	PEG 400 15%, Labrasol 10%	Slightly warm sensation which was irritating to skin	Not acceptable
4	PEG 400 15%, Labrasol 10%, Propylene Glycol 5%, Ethanol 10%	Gel turned light yellow on 1 month/ 40° C.	Not acceptable
5	PEG 400 15%, Labrasol 10%, Propylene Glycol 5%, Ethanol 7.5%	Gel turned light yellow on 1 month/ 40° C.	Not acceptable
6	PEG 400 12.5%, Labrasol 5%, Propylene Glycol 5%, Ethanol 7.5%	White to off-white thick viscous gel maintains its consistency over a period of 3 months/ 40° C	Acceptable

Gels containing PEG 400 15%, Labrasol 10%, Propylene Glycol 5% and Ethanol 7.5%, turned yellow in color upon storage at accelerated conditions. However, the problem was resolved by reducing the concentration of solubiliser/ penetration enhancer. As the concentration of the solubiliser/ penetration enhancer decreased, the available water for Carbopol hydration has increased, resulting in a good structured gel formulation. Therefore, PEG 400 12.5%, Labrasol 5%, Propylene Glycol 5%, Ethanol 7.5% was finalized for the formulations. However concentration of ethanol was varied in some gels to study the effect on *in vivo* efficacy which will be discussed later in this section.

4.2.2 Gelling agents (Carbopol 940)

Carbopol 940 was studied at different concentrations and data is given in Table 7.

Table 7. Effect of different concentrations of Carbopol 940 on gel consistency.

S. No.	Concentration of Carbopol (%w/w)	Viscosity (cps) Spindle T-C, rpm 2.5, 25 ± 1°C	Description
1	1.0	1,40,000	White to off white gel was having fluid consistency on application
2	1.2	1,58,000	White to off white gel was having slightly fluid consistency on application
3	1.4	1,72,000	White to off white, with thick viscous gel
4	1.5	1,68,000	White to off white, with thick viscous gel

A Carbopol concentration of 1.4% w/w, gel structure / skin spreadability was acceptable. There was not much change between 1.4 and 1.5%w/w. Hence, 1.4%w/w was considered for evaluation.

A few gels formulation with poloxamer (Polyoxyethylene-polyoxypropylene copolymer, Poloxamer 407, Lutrol) were also prepared for comparison in the *in vivo* models.

4.2.3 Neutralizing Agents

Carbopols (pKa 6.0±0.5) undergoes swelling in water and forms a gel at pH 5.0-7.0. To maintain a pH range of 5-7 for gel formulation, different concentrations of Triethanolamine were tried as shown in Table 8.

Table 8. Effect different concentrations of neutralizing agent Triethanolamine on Carbopol gel formulations

	Triethanolamine (% w/w)	Carbopol (% w/w)	pH
1	0.5	1.2	5.87
2	0.5	1.4	5.93
3	1.0	1.2	6.13
3	1.0	1.4	6.1

1% triethanolamine was sufficient to form a well structured gel and to maintain a pH range of 5-7. Hence 1% w/w was selected.

4.2.4 Preservative

Phenoxyethanol at different concentration was evaluated and the data is presented in Table 9.

Table 9 Evaluation of preservative efficiency of Phenoxyethanol

Parameters	Phenoxyethanol concentration (% w/w)	
	1	1.5
Microbial limits		
Total aerobic bacterial count (cfu/mL)	<10	<10
Total yeast and mould count (cfu/mL)	<10	<10
E. Coli	Absent	Absent
S.aureus	Absent	Absent
Salmonella	Absent	Absent
P. aeuriginosa	Absent	Absent
Preservative efficacy test	Complies	Complies

Phenoxyethanol at 1.5 and 1% w/w complies with preservative test and microbial limits. 1% was selected for the formulation.

4.2.5 Fragrance

Oil of Limon and Lime (S 3770, Bush Boake Allen India Ltd, India) at 0.34% was acceptable on the formulation.

4.3 Process optimisation studies

4.3.1 Drug Dispersion

Since, Rofecoxib as well as Celecoxib were hydrophobic in nature; drug dispersion in water was not homogenous and led to agglomeration. However, this problem was overcome by wetting the drug in co-solvents like PEG 400 or Cremophor RH40.

4.3.2 Addition of Carbopol 940

Direct addition of Carbopol to drug dispersion led to lump formation. Hence, alternative methods of Carbopol addition were evaluated as shown in Table 10.

Table 10. Evaluation of different methods for addition of Carbopol 940.

	Carbopol addition	Dispersion formation
1	Direct addition	Lumps are formed
2	Hot water suspension	No lumps
3	By sifting through 10 mesh	No lumps

Addition of Carbopol to drug slurry either in hot water suspension or sifting through 10 mesh did not cause lump formation. Hence, Carbopol 940 was added as a hot water suspension. Effect on Active Pharmaceutical ingredient was studied and was found to be stable.

4.3.3 Addition of Ethanol

Ethanol, being volatile in nature (boiling point 78° C), the additions were done at temperatures lower than 40° C to prevent evaporation.

4.3.4 Effect of Freeze-Thaw cycle

To study the effect of freeze thaw cycles on physical stability, gels were kept at -4° C for 24 hrs. Then kept at 4-8° C for further 24 hours. This constituted one Freeze-Thaw cycle. Gels were evaluated after three consecutive freeze-thaw cycles. After, three freeze-thaw cycles no separation, syneresis or liquification of the gels was observed and there was no change in the pH or viscosity of the gels.

4.3.5 Concentration verification

Gel formulation prepared above were analysed for concentration of active drug substance. It was observed that Rofecoxib and Celecoxib were uniformly distributed in all the formulation. Gel formulations in which active drug substance was found to be >95% of the stated value on the label were taken for further development. The concentration of all the gels is shown in the Table 11.

Table 11. Concentration verification of active drug in different gel formulation.

S.No.	Formulation Gel No.	% of API	Rofecoxib (%)	Celecoxib (%)
1	FG5	**1**	**1.03** ± 0.04	**-**
2	FG6	**1**	**1.0** ± 0.03	**-**
3	FG7	**1**	**1.03** ± 0.01	**-**
4	FG8	**1**	**0.98** ± 0.02	**-**
5	FG9	**1**	**0.97** ± 0.03	**-**
6	FG10	**1**	**1.01** ± 0.03	**-**
7	FG11	**3**	**3.04** ± 0.05	**-**
8	FG12	**3**	**3.01** ± 0.04	**-**
9	FG13	**1**	**0.99** ± 0.03	**-**
10	FG14	**1**	**1.02** ± 0.02	**-**
11	FG22	**3**	**-**	**3.1** ± 0.03
12	FG23	**3**	**-**	**2.99** ± 0.01
13	FG24	**3**	**-**	**3.04** ± 0.01
14	FG25	**3**	**-**	**3.04** ± 0.05
15	FG26	**5**	**-**	**5.09** ± 0.06
16	FG27	**5**	**-**	**5.13** ± 0.05
17	FG28	**5**	**-**	**4.98** ± 0.02
18	FG29	**5**	**-**	**5.06** ± 0.08
19	FG30	**5**	**-**	**5.04** ± 0.01

2.5 gram of formulation dissolved in 100ml acetonitrile and drug concentration was determined using HPLC as described in methods section. Data is expressed as Mean ± Standard error, with n=3.

4.3.6 Determination of Viscosity

Viscosity of all the gel formulations was determined by using Brookfield digital viscometer. The measurements were carried out using 0.5 inch helipath and T-spindle size-E, rotating at 2.5 rpm at a temperature of 25 ± 1°C. Viscosity of Carbopol gels did not show much variance as shown in Table 12. Addition of active drug also did not alter the viscosity in these gels. Increasing the concentration of alcohol tended to decrease the viscosity marginally. Poloxamer gels, on the other hand, have shown much higher viscosity as compared to Carbopol gels thus reducing their utility in the formulation development. With increasing concentration of Poloxamer, the viscosity increased considerably. Like Carbopol gels, addition of active drug did not alter the viscosity of the gels. pH or the concentration of added surfactant did not show any significant effect on the viscosity of the gels.

Table 12. Viscosity (cps-centipoise) and spreadability (cm) of different gel formulation.

S.No.	Formulation Gel No.	**Viscosity (cps)**	**Spreadability (cm)**
1	FG1	164000 ± 1732	4.63 ± 0.09
2	FG2	162000 ± 1528	4.67 ± 0.09
3	FG3	158000 ± 1000	4.80 ± 0.08
4	FG4	148000 ± 577	5.02 ± 0.06
5	FG5	156000 ± 1732	4.57 ± 0.09
6	FG6	170000 ± 289	4.62 ± 0.11
7	FG7	158000 ± 1155	4.7 ± 0.06
8	FG8	140000 ± 897	4.65 ± 0.03
9	FG9	143000 ± 1000	4.72 ± 0.09
10	FG10	150000 ± 361	4.858 ± 0.05
11	FG11	165000 ± 231	4.70 ± 0.03
12	FG12	154000 ± 231	4.82 ± 0.04
13	FG13	165000 ± 577	4.67 ± 0.07
14	FG14	172000 ± 264	4.82 ± 0.07
15	FG15	159000 ± 346	4.73 ± 0.09
16	FG16	821000 ± 1732	3.35 ± 0.03
17	FG17	941200 ± 6275	3.15 ± 0.03
18	FG18	817000 ± 1155	3.38 ± 0.06
19	FG19	162000 ± 346	4.58 ± 0.13
20	FG20	163200 ± 153	4.63 ± 0.02
21	FG21	152100 ± 153	4.82 ± 0.06
22	FG22	162000 ± 551	4.83 ± 0.03
23	FG23	853000 ± 1528	3.30 ± 0.03
24	FG24	833000 ± 1528	3.73 ± 0.04
25	FG25	1006000 ± 11015	3.12 ± 0.06
26	FG26	175000 ± 1041	4.60 ± 0.03
27	FG27	178000 ± 866	4.72 ± 0.07
28	FG28	152100 ± 351	4.95 ± 0.13
29	FG29	174100 ± 557	4.67 ± 0.12
30	FG30	165000 ± 814	4.88 ± 0.02
31	FG31	168000 ± 404	4.7 ± 0.03
32	FG32	174000 ± 603	4.73 ± 0.04

** Data expressed a Mean ± Standard error, with n=3.*

4.3.7 Determination of Spreadability

The spreadability of gels was evaluated at ambient conditions. The spreading diameter (Φ) of 1 ± 0.1 g of emulsion-gel placed between two horizontal glass plates (16 x 16 cm) was measured after 1 minute (mass of upper plate 125 ± 1 g). It was observed that in gels with

Carbopol as the gelling agent, the gels were of semi-stiff gel category and like viscosity; spreadability also was not altered much in different Carbopol gels. Increased alcohol concentration had marginally improved the spreadability. However, poloxamer gels were of very stiff gel category as shown in Table 12 and could be difficult to apply. Increase in poloxamer concentration marginally decreased the spreadability. pH or the concentration of added surfactant did not show any significant effect on the spreadability of the gels.

4.4 *In Vitro* Studies

4.4.1 Drug Release and Permeation Studies

Diffusion studies were carried out using modified Franz diffusion cells for all the gel formulations containing the active drug. Franz diffusion cells had two compartments, a donor and a receptor, separated by a cellulose acetate nitrate membrane (0.45μ). A thin layer of the test formulation was uniformly applied while a mixture of isopropyl alcohol (IPA) and water served as medium in the receptor compartment. In case of Celecoxib gel formulation the ratio of IPA: Water was 55:45 while for Rofecoxib gel formulation it was 80:20 and maintained at constant temperature of 32°±1°C by circulating water bath. The samples were withdrawn from the receptor compartment at 15, 30, 60, 120, 180, and 240 min time points. The samples were analysed for Rofecoxib/Celecoxib content.

Table 13. Diffusion of Rofecoxib and Celecoxib from the different test gels through diffusion membrane.

Test Gels	Flux (µg/ml/mm^2)					
	At 15 min	At 30 min	At 60 min	At 120 min	At 180 min	At 240 min
FG5	3.24 ± 0.11	8.19 ± 0.2	9.53 ± 0.41	12.35 ± 0.35	14.38 ± 0.53	15.43 ± 0.43
FG6	3.53 ± 0.04	8.93 ± 0.23	10.37 ± 0.6	13.25 ± 0.39	15.74 ± 0.82	16.46 ± 1.06
FG7	3.85 ± 0.09	9.12 ± 0.16	11.22 ± 1.05	13.63± 1.07	16.64 ± 0.42	17.28 ± 0.19
FG8	3.60 ± 0.25	5.90 ± 0.29	10.27± 0.31	13.79 ± 0.7	15.33 ± 0.47	17.57 ± 0.19
FG9	3.68 ± 0.19	6.51 ± 0.2	8.78 ± 0.2	13.42 ± 0.96	15.7 ± 0.76	16.75 ± 0.24
FG10	2.31 ± 0.56	4.48 ± 0.41	6.56 ± 0.38	11.47 ± 0.43	15.15 ± 0.17	16.72 ± 0.41
FG11	3.94 ± 0.24	7.10 ± 0.27	9.18 ± 0.26	14.76 ± 0.64	18.57 ± 0.43	22.26 ± 0.63
FG12	3.26 ± 0.18	6.09 ± 0.3	11.61 ± 0.64	16.15 ± 0.19	18.66 ± 1.11	23.86 ± 1.56
FG13	3.22 ± 0.16	8.25 ± 0.18	10.49 ± 0.32	14.62 ± 0.86	17.08 ± 2.48	18.77 ± 1.4
FG14	3.65 ± 0.48	9.51 ± 0.3	12.48 ± 0.42	15.57 ± 0.76	17.66 ± 0.5	21.78 ± 1.95
FG22	1.29 ± 0.02	3.54 ± 0.19	5.87 ± 0.34	7.82 ± 0.3	8.55 ± 0.41	9.12 ± 0.37
FG23	1.34 ± 0.06	4.97 ± 1.4	8.89 ± 1.32	15.11 ± 4.16	17.97 ± 3.74	20.53 ± 4.26
FG24	1.3 ± 0.14	4.76 ± 1.32	9.33 ± 1.3	15.57 ± 6.57	20.14 ± 3.97	26.34 ± 5.19
FG25	1.39 ± 0.06	5.49 ± 0.96	9.34 ± 1.62	18.78 ± 1.22	26.28 ± 4.63	30.3 ± 10.85
FG26	1.2 ± 0.06	2.27 ± 0.13	5.19 ± 0.26	8.64 ± 0.46	11.84 ± 0.66	12.59 ± 0.74
FG27	1.15 ± 0.17	2.15 ± 0.23	5.28 ± 0.14	9.17 ± 0.3	14.2 ± 0.36	15.01 ± 0.59
FG28	1.32 ± 0.15	3.22 ± 0.9	6.05 ± 0.32	9.61 ± 0.58	12.69 ± 0.98	14.52 ± 0.98
FG29	1.22 ± 0.04	2.25 ± 0.19	6.91± 0.8	10.42 ± 1.14	14 ± 0.14	18.09 ± 0.77
FG30	1.16 ± 0.06	2.25 ± 0.2	7.29 ± 0.81	10.41 ± 0.69	16.23 ± 1.79	19.06 ± 0.63

Data present as mean ± standard deviation of 3 to 4 trials.

As shown in Table 13, not much change in the diffusion profile was observed with micronised Rofecoxib gel formulation. Increasing the ethanol concentration only marginally improved the diffusion. Absence of Labrasol in FG10 also tended to delay the diffusion marginally. Reduction of particle size to sub-micron size with 7.5 % ethanol improved the release of drug in FG14, which showed the most efficient release of active drug.

Similar to Rofecoxib gels, not much change in the diffusion profile was observed with micronised Celecoxib gels as well. Gels having poloxamer as gelling agent showed improved release kinetic as shown in Table 13. Reduction of particle size to sub-micron size with 7.5 % ethanol in FG30 improves the diffusion of Celecoxib from the gel formulation. This gel shows the best release rate profile among all the Celecoxib gels.

4.4.2 Accelerated stability studies

Accelerated stability studies were carried out for the optimized formulation, FG14 and FG30. These were maintained at 4 and 40° C for 90 days. The formulations were analysed on days 15, 30, 60 and 90 for drug content. Viscosity, spreadability, microbial limits and pH were

also evaluated on day 90. In both the formulations, FG14 and FG30, none of the parameters tested showed any change from initial value as shown below.

Table 14. Stability profile of Rofecoxib 1% gel formulation (FG14)

Time points of evaluation (days)	Initial (0)	15	30	60	90
Rofecoxib concentration (% w/w)	1.02	1.03	1.00	0.99	0.96
pH (at 25° C)	5.72	5.74	5.68	5.64	5.60
Viscosity (cps, 25° C ; Spindle T-size E, 2.5 rpm, RVT model)	1,72,000	1,74,000	171000	165000	1,60,000
Microbial limits Total aerobic microbial count – TAMC (cfu/mL) Total yeast and mould count – TYMC (cfu/mL) E. Coli S.aureus Salmonella P. aeuriginosa	<10 <10 Absent Absent Absent Absent		<10 <10 Absent Absent Absent Absent	<10 <10 Absent Absent Absent Absent	<10 <10 Absent Absent Absent Absent
Preservative Efficacy test	Complies		Complies	Complies	Complies

Table 15. Stability profile of Celecoxib 5% gel formulation (FG30)

Time points of evaluation (days)	Initial (0)	15	30	60	90
Celecoxib concentration (% w/w)	5.02	5.01	5.04	4.98	4.92
pH (at 25° C)	6.72	6.70	6.72	6.68	6.58
Viscosity (cps, 25° C ; Spindle T-size E, 2.5 rpm, RVT model)	1,65,000	1,70,000	1,65,000	1,55,000	1,48,000
Microbial limits Total aerobic microbial count – TAMC (cfu/mL) Total yeast and mould count – TYMC (cfu/mL) E. Coli S.aureus Salmonella P. aeuriginosa	<10 <10 Absent Absent Absent Absent		<10 <10 Absent Absent Absent Absent	<10 <10 Absent Absent Absent Absent	<10 <10 Absent Absent Absent Absent
Preservative Efficacy test	Complies		Complies	Complies	Complies

FG 14 and FG30 studied upto 3 months did not show any significant change in any of the parameters studied and were stable for 3 months (Table 14 and 15).

4.5 *In vivo* Studies

4.5.1 Carrageenan Induced rat paw edema test (anti-inflammatory activity)

Further optimisation of the formulation required evaluation of the test formulations in a suitable *in vivo* model. Since, prostaglandins play an important role in promoting the signs and symptoms of inflammation (Vane, 1971); acute models of inflammation can be used as early screen. Carrageenan-induced paw edema model is one of the most commonly used tests for studying anti-inflammatory activity in animals (Dirig *et al*., 1998; Jett *et al*., 1999). It is very widely used to screen anti-inflammatory agents, especially after topical administration (Chi and Jun, 1990; Friedman *et al*., 1995; Gupta *et al*., 1996). Thus, for the purpose of screening anti-inflammatory activity, test/ standard gels were applied on the rat paws as described by Chi and Jun, 1990, and evaluated for effect on carrageenan induced paw edema. All the gel formulations were evaluated for *in vivo* efficacy in carrageenan induced paw edema model. Before screening of gel formulations, this model was standardize using standard marketed gels of Diclofenac Gel 1% w/w - (Novartis, India; B.No. 04093E) and Nimesulide Gel -1 % w/w (Panacea Biotec Ltd., India; B. No. 601728). Wistar rats were treated with formulation gels which were applied, topically on the rat paw as described in methods section. Three hours post gel application, the basal paw volume of the rats was recorded using water displacement plethysmometer (UGO Basile, Italy) and then were challenged with 0.1mL of 1% carrageenan solution, injected in sub-plantar region of the right hind paw. 3 hours after the carrageenan challenge the rats paw volume was again recorded and change in paw volume in treated group was compared with vehicle control animals to calculate % inhibition in carrageenan induced paw oedema in treated animals. Regression analysis was used to compute the ED_{50} for each of the gel formulation. In the initial standardization, carrageenan was challenged in 1, 2 and 3 hrs after the gel application and a more pronounced inhibition was obtained at 3hrs post dosing.

As shown in Fig 7A, Carrageenan challenge produced a paw edema with an increase in rat hind paw volume of 0.95 ± 0.09 mL. Treatment with marketed Diclofenac gel showed a dose dependent decrease in rat paw edema with a peak inhibition of 61% at a dose of 600mg gel/rat paw (it was not possible to apply more gel/rat paw). ED_{50} for Diclofinac gel was 150 mg/paw or 7.48 mg/kg as active drug which was 748 mg/kg of gel formulation.

Similarly, in Nimesulide treated animals also, a dose related decrease in carrageenan induced paw swelling was observed as shown in Fig 7B. A peak inhibition of 57 % was observed at dose of 600mg gel/paw (30mg/kg). ED_{50} for Nimesulide gel was 363 mg/paw or 18.1mg/kg as active drug, which was (1813 mg/kg) of gel formulation.

Gupta *et al*., 1996, have reported a comparative study of 1% w/w Diclofenac and Nimesulide gels in carrageenan induced paw edema model in rats. In this study application of 1% (w/w) Diclofenac and Nimesulide gels at a dose of 50 mg/rat paw showed an inhibition of 64.4% and 71.2%, respectively. In our model we saw a 40 and 33% inhibition, respectively, at a dose of 60mg. This difference in activity could be due several reasons such as difference in gel excipients which can affect the drug release, its percutaneous absorption and activity as indicated by Seed *et al*., 1997. It is also reported that gel bases may not be totally inactive and that sufficient controls gels need to be included in the *in vivo* studies to account for this effect (Seed *et al*., 1997). In this regard we had evaluated all the blank gels of all the test formulations in carrageenan induced paw edema model. For this purpose all the blank gel, i.e. without any active drug substance as mentioned in Table 4 and 5 were evaluated in this model at a dose of 600mg gel/rat (the highest dose of test gels to be tested). As shown in Fig 8 treatment with any of these blank gels (FG1, 2, 3, 4, and FG15 to FG21) did not produced any significant change in carrageenan induced paw edema in rats. Hence, these blanks could serve as placebo while screening of the test gel formulations.

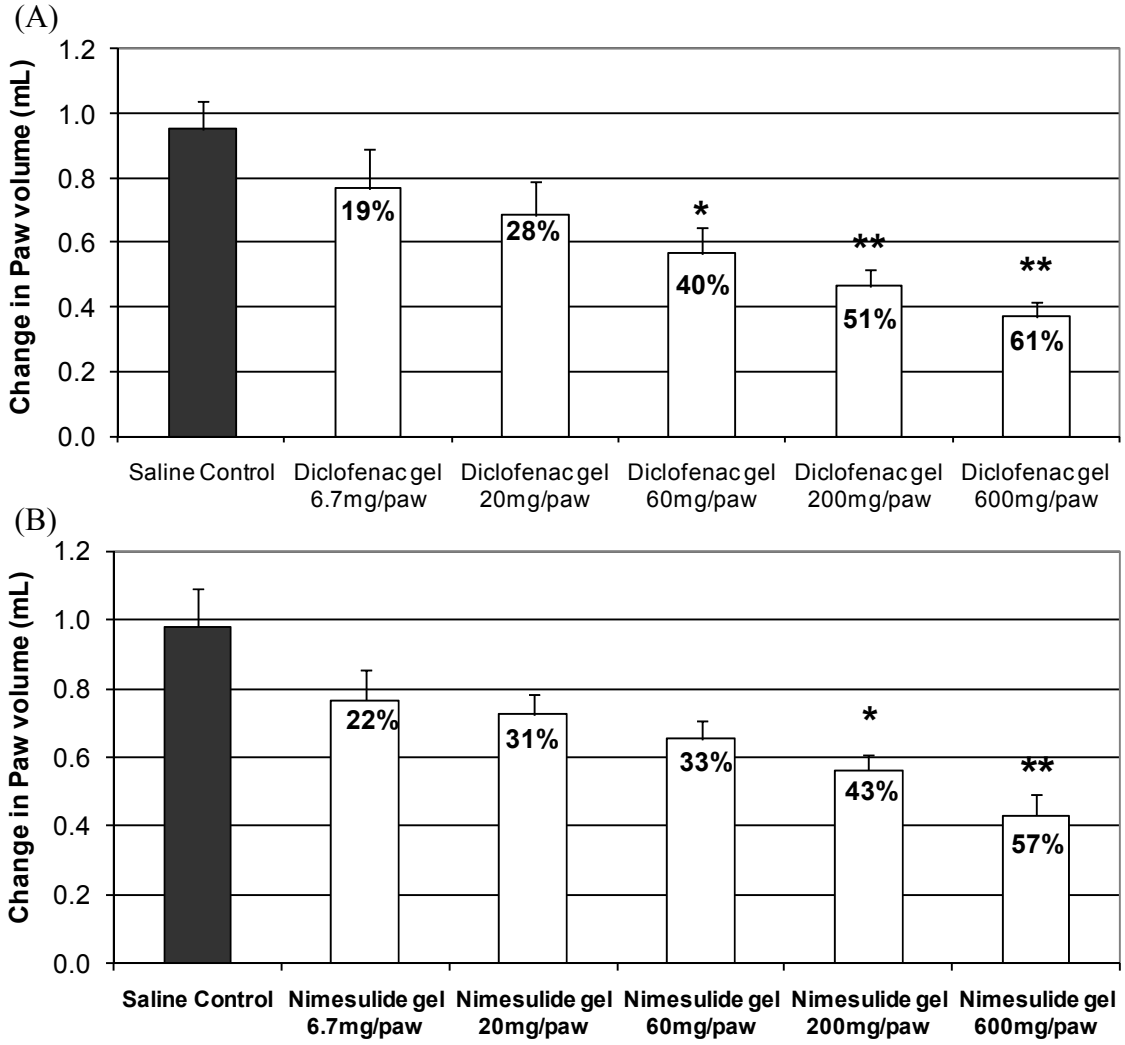


Fig 7. Effect of Standard marketed gels in carrageenan induced paw edema in rats

*Three hours after the topical application of the gel formulation, Diclofenac gel -1% w/w (**A**) or Nimesulide- 1% w/w (**B**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume three hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-8 animals per group. Data in the bars indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant). (A dose of 0.3, 1, 3, 10 and 30mg/kg is 6, 20, 60, 200 and 600mg of gel per rat of 200± 20gm body weight)*

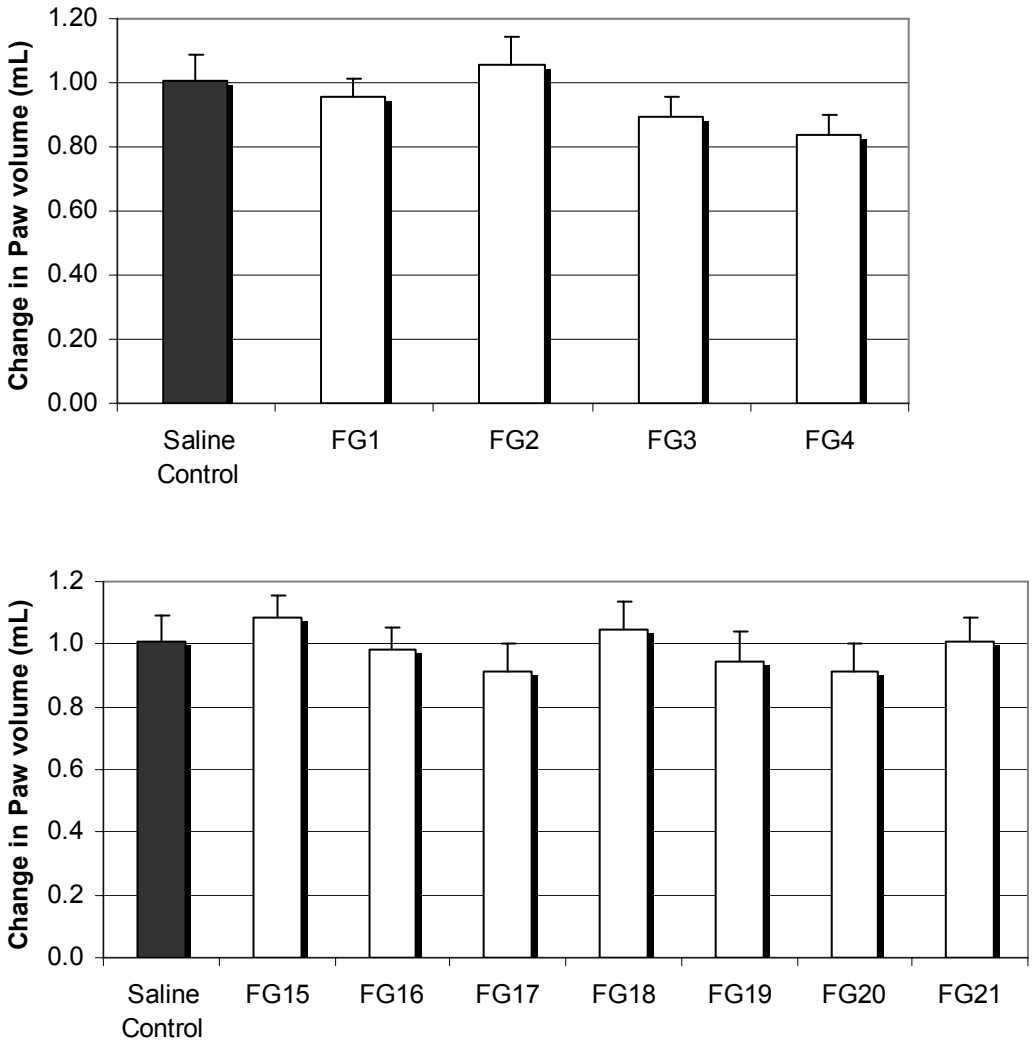


Fig 8. Effect of Placebo gels in carrageenan induced paw edema in rats

Three hours after the topical application of the gel formulation (600mg/rat), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-8 animals per group. (*), indicates statistically significant difference from respective saline control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p < 0.05$, **, $p < 0.01$ was considered significant).*

4.5.1.1 Effect of Rofecoxib gel formulation on carrageenan induced paw edema model

Rofecoxib gels formulations were prepared as mentioned in Table 4. A total of 10 formulations (FG5 to FG14) with 1 or 3% Rofecoxib were selected for *in vivo* screening. The primary objective of this study was to evaluate the *in vivo* anti-inflammatory efficacy of test gel formulation containing Rofecoxib as active drug substance. For this carrageenan induced paw edema model in Wistar rats was used. In this study, doses of 20, 60, 200 and 600 mg/paw were used, for 1 % gel and 6.7, 20, 60, 200 and 600 mg/paw for 3% gels.

FG5, FG6 and FG7 were formulated with Rofecoxib (as micronised powder) using increasing concentration of ethanol. The ethanolic content in these gels was 0, 7.5 and 10%, respectively. As shown in Table 16 and in Fig 9, treatment with FG5, FG6 and FG7, showed a dose related inhibition of carrageenan induced paw swelling. The inhibition was statistically significant at a dose of 60mg of gel/paw (10 mg/kg of active drug) for FG5 and FG6 and 200mg of gel/paw for FG7. However, these gels were less potent as compared to marketed Diclofenac gel (ED_{50} = 150 mg/paw). FG6 (with 7.5% ethanol) treated animals showed a marginal improvement in the activity with an ED_{50} value of 232 mg/paw as compared to 346 mg/paw in FG5 (with 0% ethanol). Further increase in ethanol concentration to 10% in FG7 did not improve the inhibitory activity any further. Hence a concentration of 7.5% ethanol was considered optimal for efficacy.

In the next set of 1% Rofecoxib gels i.e., FG8, FG9 and FG10, effect of different concentration of Labrasol, was studied. FG8, FG9 and FG10 had Labrasol 10, 5, and 0%, respectively. As shown in Table 16, 3 hrs post challenge, carrageenan produced 0.86 ± 0.12 mL increase in paw volume in control animals treated with blank gel-FG4. Treatment with Rofecoxib 1% gels, FG8, FG9 and FG10 produced a dose related inhibition of carrageenan induced paw edema as shown in Fig 10 with an ED_{50} of 160, 275 and >600 mg/paw of gels, respectively. FG8 with 10% Labrasol was more potent than FG10 in which Labrasol was absent indicating that inclusion of Labrasol in the gels formulation improves the activity of the gel formulation. However, as shown above in the gel optimisation studies, formulation with 10% Labrasol turns light yellow in 1 month stability studies when stored at 40° C and was considered unacceptable. A gel formulation with 5% labrasol was considered suitable.

The next parameter studied was effect of increasing the concentration of Rofecoxib in the gels. For this purpose 2 gels (FG11 and FG12) with 3% Rofecoxib were prepared and evaluated *in vivo*. As shown the Table 16 and Fig 11, treatment with FG11 and FG12 also produced a dose dependent decrease in the paw volume in the dose range of 6.7 to 600mg/paw, as compared to respective blank gel treated control animals. Increasing the dose to 600mg/paw (90mg/kg of active drug) did show any further improvement in the efficacy. The ED_{50} in FG11 and FG12 was 153 and 118 mg/paw, respectively. This effect was comparable to marketed gel formulation of Diclofenac 1% gel (ED_{50} = 160 mg/paw). However, since FG11 and FG12 were 3% gels, the gels were not considered equipotent to Diclofenac 1% gel.

To further improve the efficacy of Rofecoxib gel, Rofecoxib was milled in a Dyno mill to obtain a sub-micron particle size. The resultant Rofecoxib obtained was sub-micron (with particle size distribution of - d(0.1) = 0.08 ± 0.002; d(0.5) = 0.131 ± 0.001; d(0.9) = 0.204 ±0.003μ) and was then used to formulate the sub-micronised Rofecoxib gel formulation by the protocol as described in methods section. Two gel formulations FG13 and FG14 were prepared with 0 and 7.5% (w/w) ethanol respectively. As shown in Fig 12, Treatment with FG13 showed a dose related inhibition of carrageenan induced paw edema in a dose range of 6.7 – 600 mg/paw with a peak inhibition of 68% at a dose of 600mg/paw. The ED_{50} for FG13 gel was 120 mg/paw. Animals treated with FG14 (with 7.5% ethanol) also showed a potent and dose dependent inhibition of carrageenan induced paw edema with a peak inhibition of 71% at dose of 600mg/paw (Fig 12). FG14 showed an ED_{50} of 77 mg of gel/paw. FG14 showed most potent inhibition among all the Rofecoxib gels screened and was also comparable to 1% Diclofenac and was more potent as compared to 1% Nimesulide gel formulation. Diclofenac sodium and Nimesulide gels were also prepared in the same formulation as Rofecoxib 1% gel (FG14) and was evaluated in carrageenan induced paw edema model. Both these gels had shown comparable activity as seen in the respective marketed formulations (Table 4 and Fig 13) and showed an ED_{50} value of 188 or 330 mg of gel/paw, respectively. Hence, FG14 gel formulation of Rofecoxib was selected for further profiling *in vivo*.

This improvement in activity in FG14 with sub-micron particle size is in sync with the above discussion that increasing surface area of a particulate drug, such as by decreasing the particle size, the rate of dissolution of the drug is increased thereby resulting in better permeability and percutaneous efficacy. This phenomenon was also demonstrated by Friedman *et al*., 1995, using several topically administered steroidal and non-steroidal anti-inflammatory agents**.** However, this study for the first time demonstrates the same phenomenon in case of COX-2 inhibitors.

Table 16. Effect of Rofecoxib gel formulations in carrageenan induced paw edema model in rats

Treatment	Test gels no.	Blank gel used	Change in Paw volume (mL)						ED_{50} of gel	
			Negative control	6.7 mg/paw	20 mg/paw	60 mg/paw	200 mg/paw	600 mg/paw	mg/kg	mg/paw
Rofecoxib 1% gel	**FG5**	FG1	1.04 ± 0.06	-	0.87 ± 0.06	0.79* ± 0.09	0.54** ± 0.05	0.46** ± 0.06	1729	346
Rofecoxib 1% gel	**FG6**	FG2	0.87 ± 0.11	-	0.77 ± 0.05	0.56* ± 0.06	0.48* ± 0.06	0.38** ± 0.04	1161	232
Rofecoxib 1% gel	**FG7**	FG3	0.91 ± 0.09	-	0.76 ± 0.07	0.72 ± 0.14	0.44** ± 0.07	0.38** ± 0.06	1237	247
Rofecoxib 1% gel	**FG8**	FG4	0.86 ± 0.12	-	0.66 ± 0.08	0.5* ± 0.12	0.45* ± 0.05	0.36** ± 0.07	801	160
Rofecoxib 1% gel	**FG9**	FG4	0.86 ± 0.12	-	0.74 ± 0.07	0.69 ± 0.07	0.38** ± 0.1	0.41* ± 0.1	1373	275
Rofecoxib 1% gel	**FG10#**	FG4	0.86 ± 0.12	-	0.78 ± 0.1	0.71 ± 0.07	0.51 ± 0.14	0.47* ± 0.06	-	-
Rofecoxib 3% gel	**FG11**	FG3	0.91 ± 0.08	0.8 ± 0.03	0.63* ± 0.06	0.57** ± 0.1	0.42** ± 0.05	0.38** ± 0.05	763	153
Rofecoxib 3% gel	**FG12**	FG4	0.86 ± 0.08	0.71± 0.03	0.74 ± 0.07	0.49** ± 0.07	0.31** ± 0.07	0.35** ± 0.1	588	118
Rofecoxib 1% gel	**FG13**	FG1	0.97 ± 0.05	1.01 ± 0.08	0.79 ± 0.13	0.65* ± 0.11	0.41** ± 0.05	0.32** ± 0.06	603	121
Rofecoxib 1% gel	**FG14**	FG2	0.83 ± 0.1	0.74 ± 0.1	0.57 ± 0.04	0.48** ± 0.05	0.32** ± 0.05	0.25** ± 0.03	383	77
Diclofenac 1% gel	**FG31**	FG2	0.86 ± 0.18	0.76±0 .09	0.72 ± 0.11	0.57± 0.08	0.44*± 0.04	0.31** ± 0.05	938	188
Nimesulide 1% gel	**FG32**	FG2	0.86 ± 0.18	0.79±0 .09	0.71 ± 0.12	0.63 ± 0.17	0.48 ± 0.18	0.38*± 0.17	1651	330

Three hours after the topical application of the gel formulation, rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-12 animals per group. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p < 0.05$, **, $p < 0.01$ was considered significant). # in FG10 50 % inhibition was not attained.*

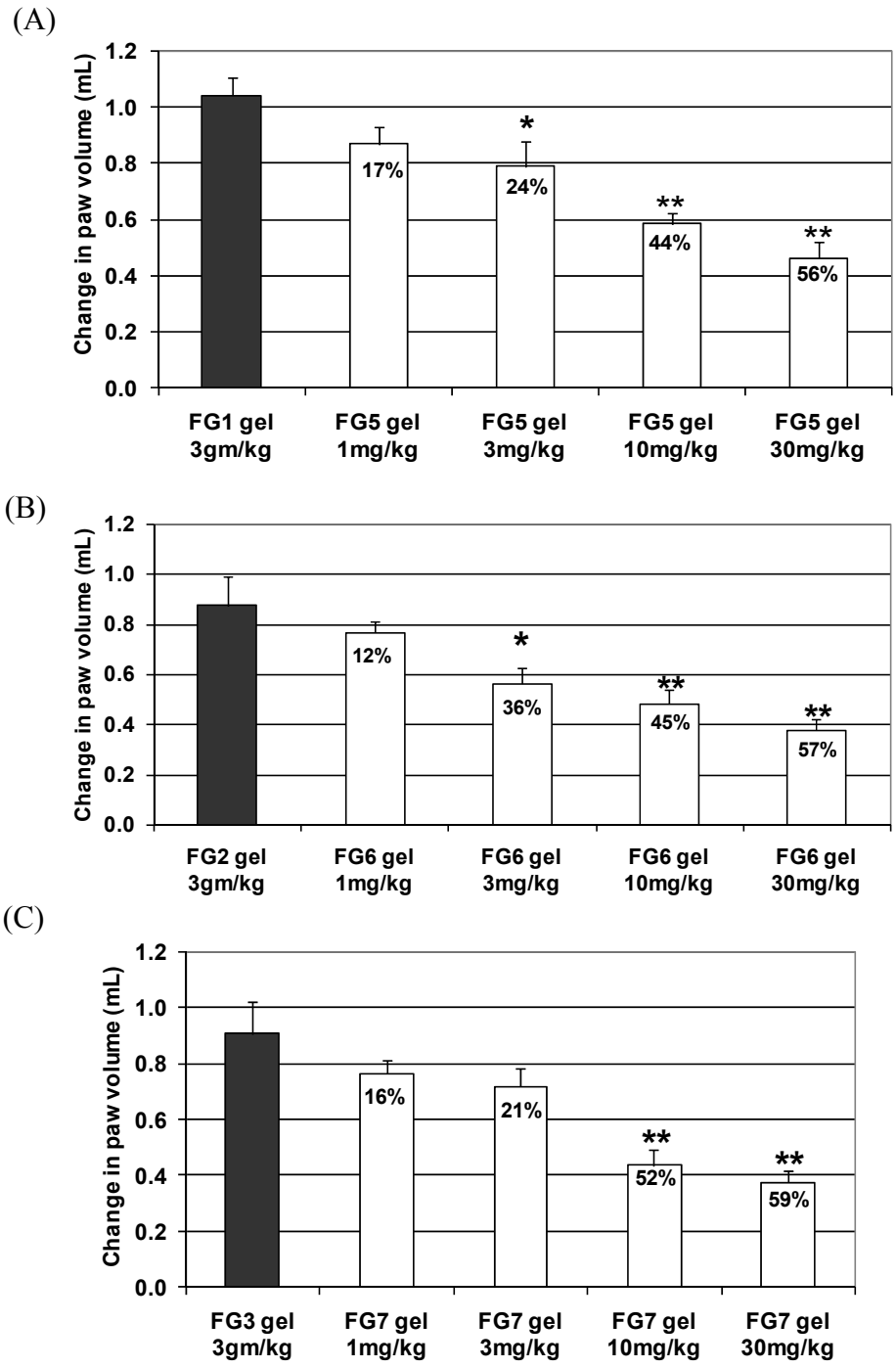
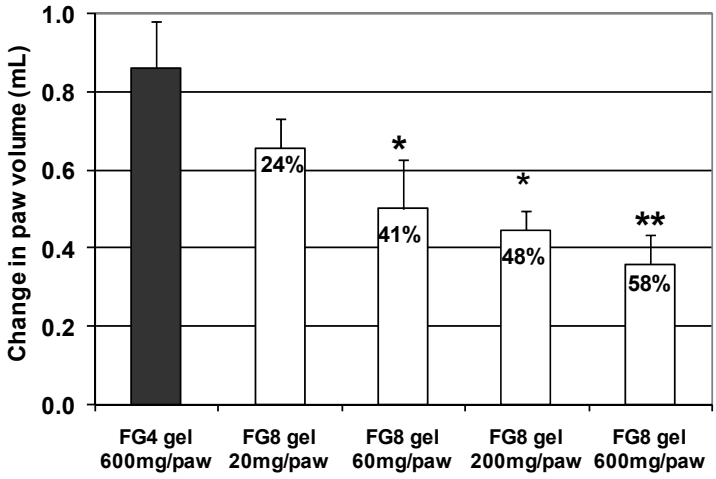


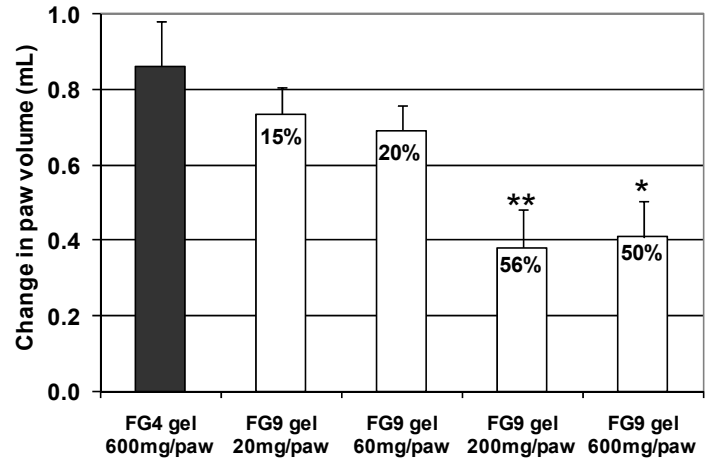
Fig 9. Effect of Rofecoxib gel formulations FG5, FG6 and FG7 on carrageenan induced paw edema model in rats

*Three hours after the topical application of the gel formulation FG5 (**A**), FG6 (**B**) or FG7 (**C**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-10 animals per group. Data in the bars indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant).*

(A)



(B)



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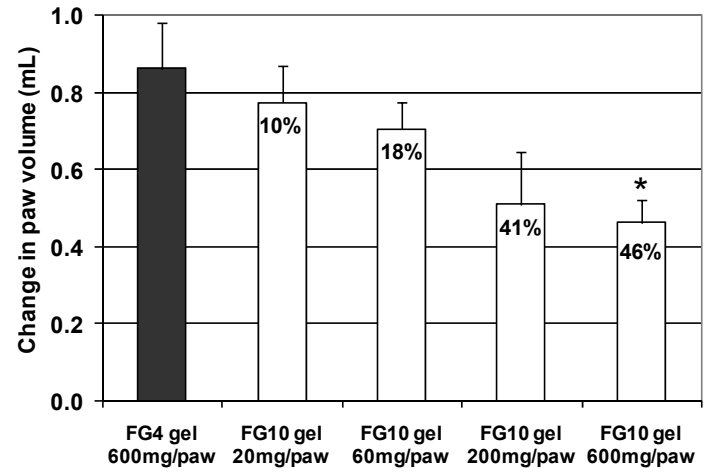


Fig 10. Effect of Rofecoxib gel formulations FG8, FG9 and FG10 on carrageenan induced paw edema model in rats

*Three hours after the topical application of the gel formulation FG8 (**A**), FG9 (**B**) or FG10 (**C**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-10 animals per group. Data in the bars indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant).*

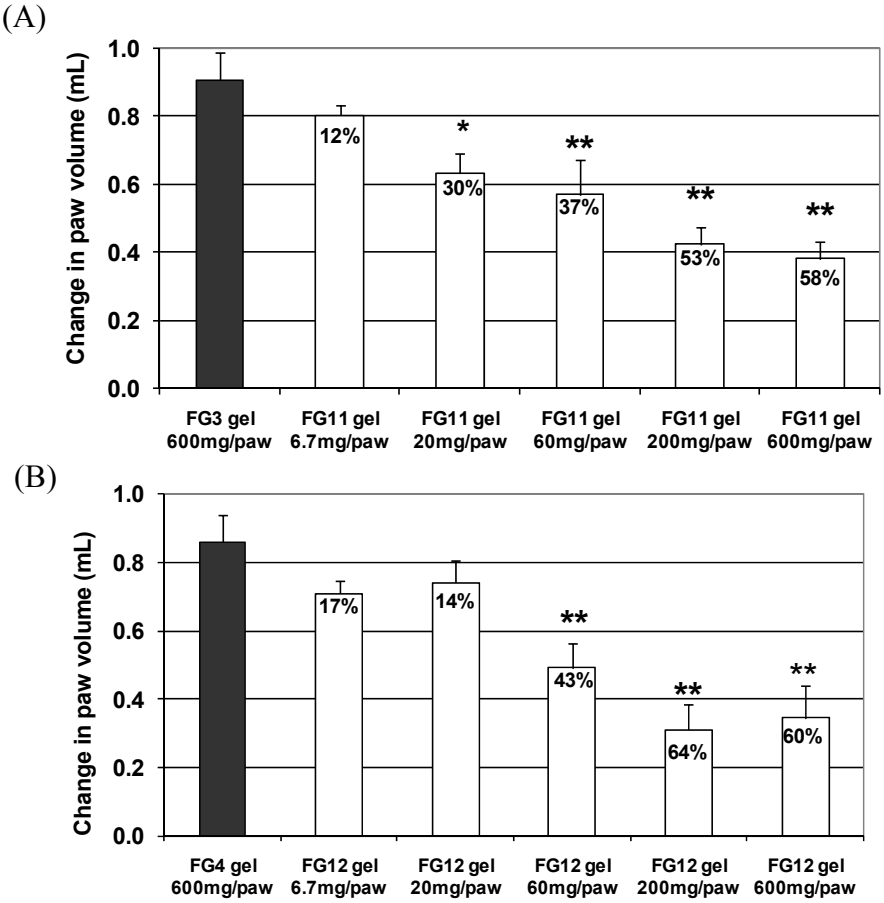
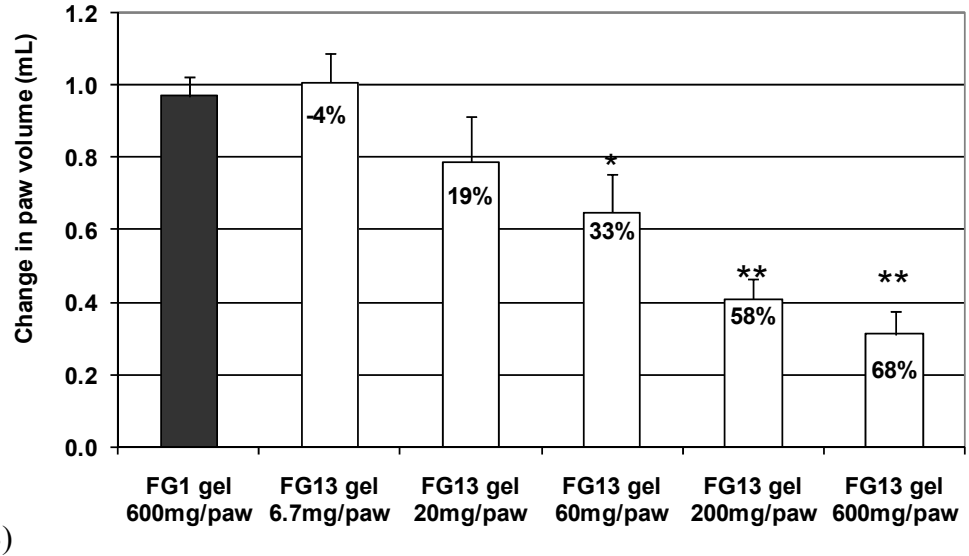


Fig 11. Effect of Rofecoxib gel formulations FG11 and FG12 on carrageenan induced paw edema model in rats

*Three hours after the topical application of the gel formulation FG11 (**A**) or FG12 (**B**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-10 animals per group. Data in the bars indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant).*

(A)



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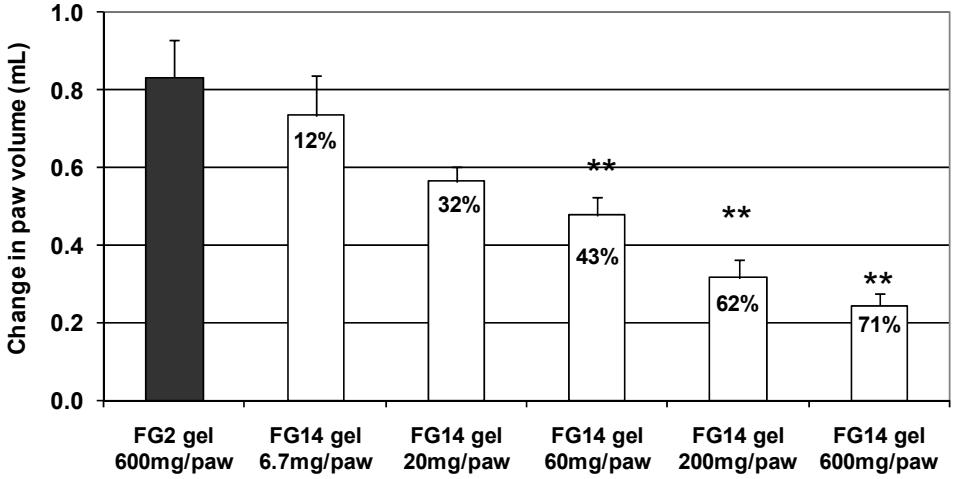


Fig 12. Effect of Rofecoxib gel formulations FG13 and FG14 on carrageenan induced paw edema model in rats

*Three hours after the topical application of the gel formulation FG13 (**A**) or FG14 (**B**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 8-12 animals per group. Data in the bars indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant).*

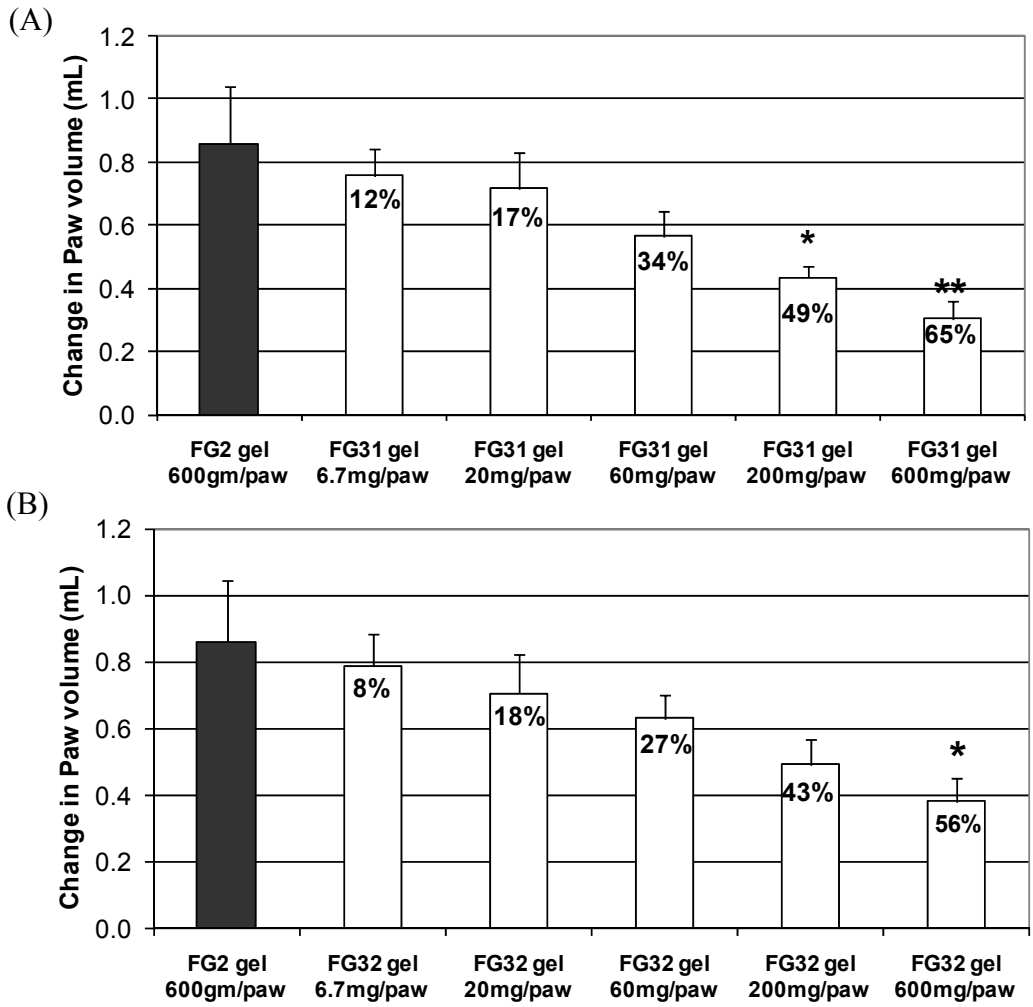


Fig 13. Effect of Diclofenac and Nimesulide gel formulations on carrageenan induced paw edema model in rats.

*Three hours after the topical application of the gel formulation Diclofenac sodium gel, FG31 (**A**) or Nimesulide gel, FG32 (**B**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-8 animals per group. Data in the bars indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (*, $p < 0.05$; **, $p < 0.01$ was considered significant).*

4.5.1.2. Effect of Celecoxib gel formulation on carrageenan induced paw edema model.

Celecoxib gels formulations were prepared as mentioned in Table 5. A total of 9 formulations (FG22 to FG30) with 3 or 5% Celecoxib, were screened for *in vivo* activity. The primary objective of this study was to evaluate the *in vivo* anti-inflammatory efficacy of test gel formulation containing Celecoxib as active drug substance. For this carrageenan induced paw edema model in Wistar rats was used. In this study, dose of 6.7, 20, 60, 200 and 600 mg/paw were used in 3% gels, which was 1, 3, 10, 30 or 90 mg/kg, body weight, while 5 % gel

formulation was tested at doses of 12, 40, 120 and 400 mg/paw which was 3, 10, 30 or 100 mg/kg.

Formulations of Celecoxib - FG22, containing Celecoxib as a micronised powder, was prepared to study the anti-inflammatory activity of 3% Celecoxib gel containing 1% w/w Carbopol 940, as gelling agent. FG22 gel was white to off white gel, having slightly fluid consistency on application. As shown in Fig 14, treatment with FG22 dose dependently inhibited carrageenan induced paw edema with a peak inhibition of 55% at a dose of 200mg/paw (30mg/kg). Increasing the dose to 600mg/paw did not increase the inhibition any further. The ED_{50} was calculated as 198mg/paw of Celecoxib, viz., 29.8 mg/kg of active drug. This formulation was found to be more potent than the Nimesulide 1% w/w gel formulation and comparable to Diclofenac gel. However, since the gel had a fluid consistency, it was difficult to apply. To further improve upon the potency and consistency of the formulation, gels with alternative gelling agent, Poloxamer 407 (Polyoxyethylene-polyoxypropylene copolymer, Lutrol) were prepared. FG23 and FG24 were prepared as 3% Celecoxib gels, with 20% w/w Poloxamer 407, having 10 and 30% ethanol, respectively. FG25 with 25% w/w Poloxamer 407 and having 10% ethanol was also prepared. Use of Poloxamer 407 as gelling agent resulted in formulation of very stiff gels as shown in Table 12, having higher viscosity and low spreadability. These properties made Poloxamer gels less attractive for development of topical gels, although they had shown better drug release kinetics in the *in vitro* studies using Franz Diffusion Cell. When evaluated in carrageenan induced paw edema model FG23 shows a dose related inhibition of carrageenan induced paw edema in rats with an ED_{50} of 297 mg of gel/paw (44.5 in terms of mg/kg body weight) (Fig 15), which was less potent as compared to Diclofenac 1% gel and 3.8 fold less than Rofecoxib gel, FG14. Increasing the alcohol content to 30% w/w improved the activity with an ED_{50} value of 194 mg/paw. Increasing the Poloxamer concentration to 25% w/w in FG25 did not improve the activity and the ED_{50} was 256 mg/paw (38.4mg/kg). Since, the consistency of poloxamer gels was not found to be suitable and hence Carbopol gels were pursued for further optimisation studies.

In the next set of gels, to improve the potency, 5% w/w Celecoxib gels were prepared with Carbopol 940 as gelling agent. Gel formulations of 5% w/w Celecoxib were FG26, FG27 and FG28 containing Celecoxib as a micronised powered and with increasing concentration of ethanol, i.e., with 0, 7.5 and 10% w/w ethanol content respectively. As shown in Table 17

and in Fig 16, treatment with FG26, FG27 and FG28, showed a dose related inhibition of carrageenan induced paw swelling. The inhibition was statistically significant at a dose of 120mg of gel/paw. In FG26, FG27 and FG28, with increasing concentration of ethanol, *viz*., 0, 7.5 and 10%, respectively, not much change in their ED_{50} values were observed as shown in Table 17.

To further improve the efficacy of Celecoxib gel, sub-micron particle size was used. For this purpose, Celecoxib (8% w/w) was dispersed in a solution of Sodium lauryl sulphate (0.5% w/w) and polyvinyl pyrrolidone (Povidone K30, 1% w/w) and the resultant suspension was milled in a bead mill (Dyno mill) to attain sub-micronized drug particle size. The resultant Celecoxib obtained was sub-micron (with particle size distribution of - d(0.1) = 0.078 ± 0.001; d(0.5) = 0.130 ± 0.002; d(0.9) = 0.239 ±0.005µm) and was then used to formulate the sub-micronised Celecoxib gel formulation by the protocol as described in the methods section. Two gel formulations FG29 and FG30 were prepared with 0 and 7.5% (w/w) ethanol respectively. As shown in Fig 17, treatment with FG29 showed a dose related inhibition of carrageenan induced paw edema in dose range of 12 – 400 mg/paw with a peak inhibition of 57% at a dose of 400mg/paw (100mg/kg). The ED_{50} for FG29 gel was 109 mg/paw (27.3 mg/kg of active drug). Treatment with FG30 (with 7.5% ethanol) resulted in a more potent and dose dependent inhibition of carrageenan induced paw edema with a peak inhibition of 64% at dose of 100 mg/kg (400mg/paw) (Fig 17), with an ED_{50} of 80 mg/paw of 5% w/w Celecoxib gel/paw i.e. 20.3 mg/kg of Celecoxib. FG30 showed most potent inhibition among all the Celecoxib gels screened and was also comparable or superior to 1% diclofenac and 1% nimesulide gel formulation. These results again demonstrate that decrease in the particle size with concurrent addition of suitable excipients can improve the activity of topically delivered drug with poor solubility and high lipophilicity.

Table 17. Effect of Celecoxib gel formulations in carrageenan induced paw edema model in rats.

Treatment	Test gel no.	Blank gel used	Change in Paw volume (mL)						ED50 of gel	
			Negative control	6.7 mg/paw	20 mg/paw	60 mg/paw	200 mg/paw	600 mg/paw	mg/kg	mg/paw
Celecoxib 3% gel	**FG22**	FG15	0.81± 0.09	0.61± 0.06	0.54± 0.07	0.51* ± 0.08	0.36 **± 0.05	0.38**± 0.09	992	198
Celecoxib 3% gel	**FG23**	FG16	0.83 ±0.07	0.82 ±0.07	0.58± 0.06	0.52* ± 0.06	0.41**± 0.05	0.39** ± 0.1	1483	297
Celecoxib 3% gel	**FG24**	FG17	0.82± 0.14	0.61 ±0.07	0.54± 0.07	0.5* ± 0.05	0.34** ± 0.02	0.43*± 0.09	969	194
Celecoxib 3% gel	**FG25**	FG18	0.91 ±0.09	0.73± 0.06	0.62 ±0.09	0.57* ± 0.08	0.45** ± 0.05	0.44* ± 0.13	1281	256
			Negative control		**12 mg/paw**	**40 mg/paw**	**120 mg/paw**	**400 mg/paw**		
Celecoxib 5% gel	**FG26**	FG19	0.80 ±0.09	-	0.61 ±0.08	0.53* ± 0.03	0.41** ± 0.04	0.35** ±0.02	742	148
Celecoxib 5% gel	**FG27**	FG20	1.02 ±0.14	-	0.74 ±0.09	0.7 ± 0.12	0.46* ± 0.04	0.45** ±0.06	667	133
Celecoxib 5% gel	**FG28**	FG21	0.72 ±0.05	-	0.78 ±0.13	0.53 ± 0.09	0.36* ± 0.03	0.3** ± 0.03	649	130
Celecoxib 5% gel	**FG29**	FG19	0.75 ±0.06	-	0.69 ±0.13	0.44 ± 0.12	0.4* ± 0.03	0.33**± 0.02	546	109
Celecoxib 5% gel	**FG30**	FG20	0.84 ± 0.1	-	0.63 ±0.09	0.5* ± 0.02	0.39** ± 0.03	0.30** ±0.06	402	80

Three hours after the topical application of the gel formulation, rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded. Data expressed as mean ± S.E.M. for 6-10 animals per group. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p < 0.05$, **, $p < 0.01$ was considered significant).*

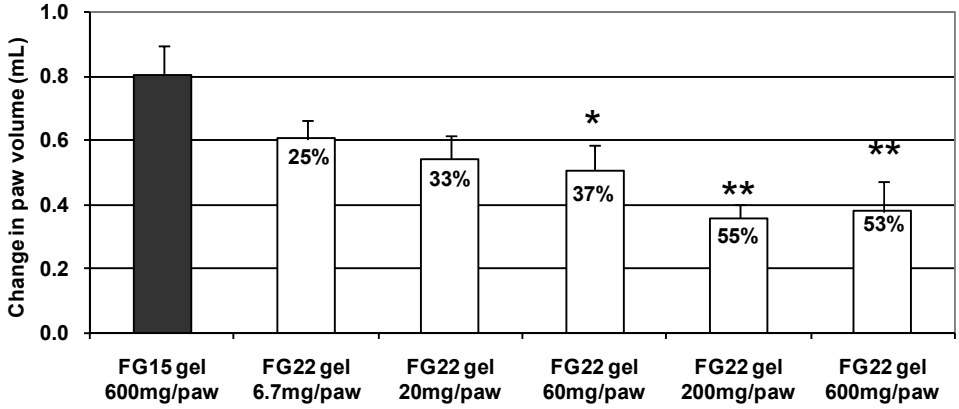


Fig 14. Effect of Celecoxib gel formulation- FG22 on carrageenan induced paw edema model in rats

Three hours after the topical application of the gel formulation, FG22, rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p < 0.05$, **, $p < 0.01$ was considered significant).*

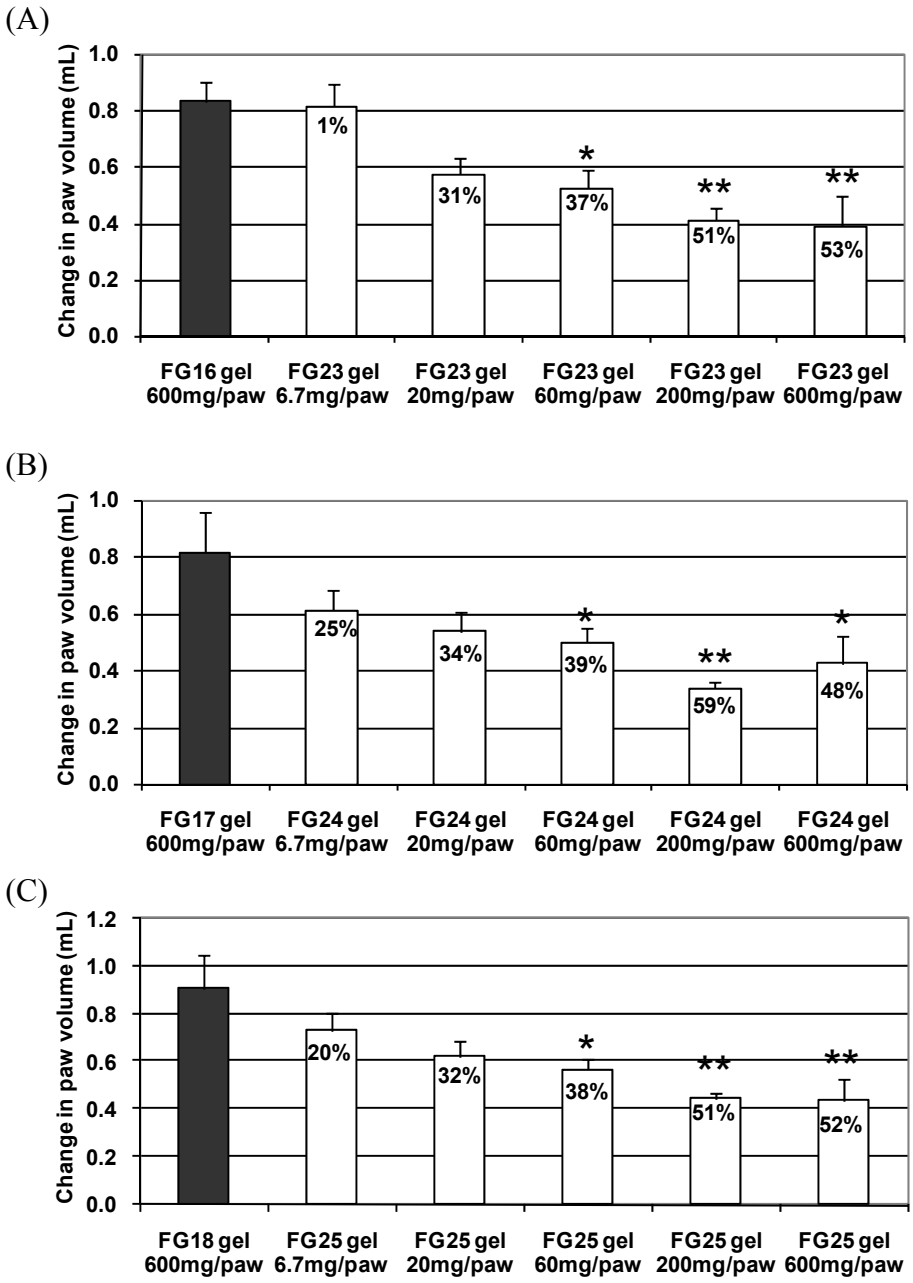


Fig 15. Effect of Celecoxib gel formulations FG23, FG24 and FG25 on carrageenan induced paw edema model in rats

*Three hours after the topical application of the gel formulation FG23 (**A**), FG24 (**B**) or FG25(**C**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant).*

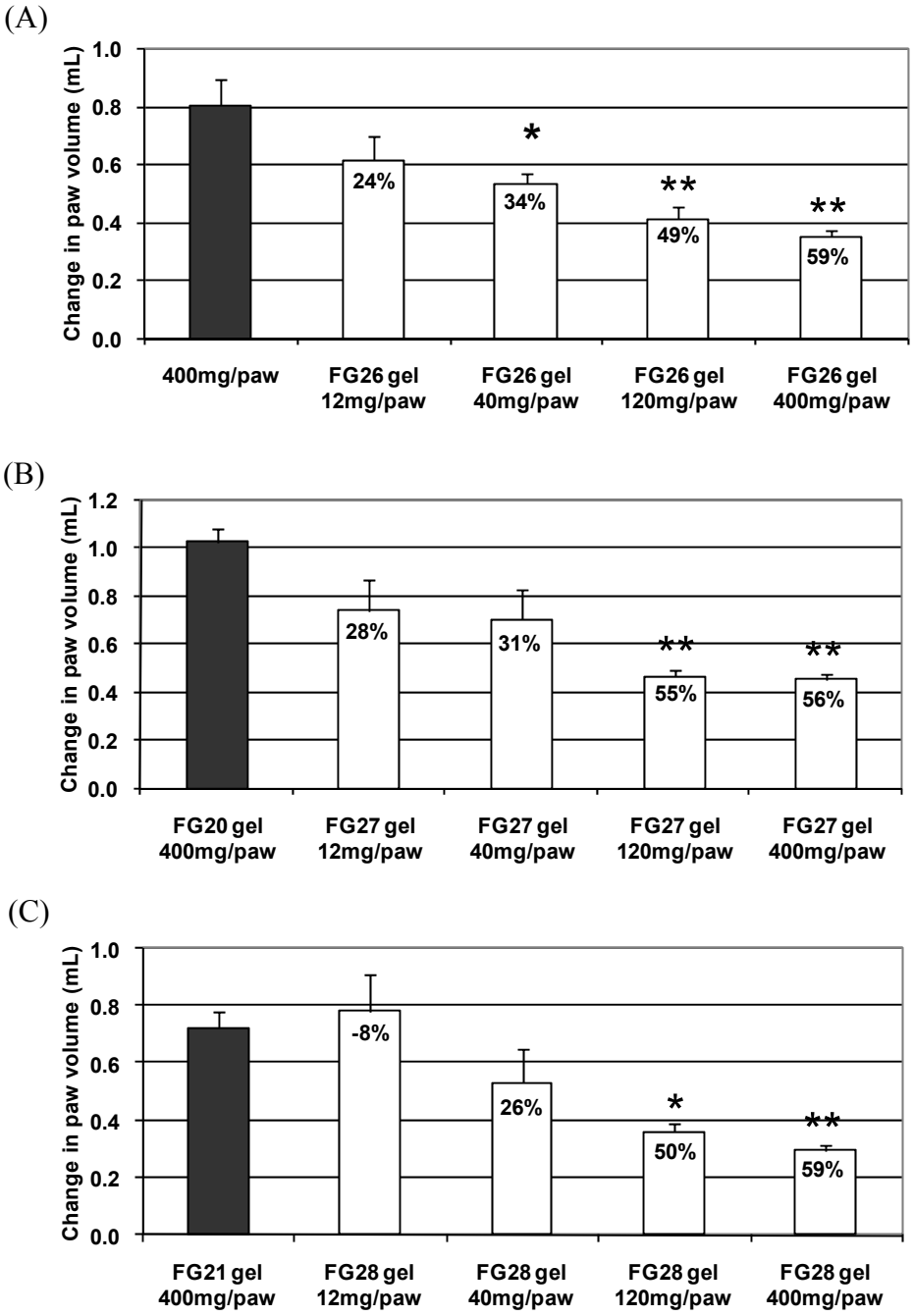


Fig 16. Effect of Celecoxib gel formulations FG26, FG27 and FG28 on carrageenan induced paw edema model in rats

*Three hours after the topical application of the gel formulation FG26 (**A**), FG27 (**B**) or FG28(**C**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant).*

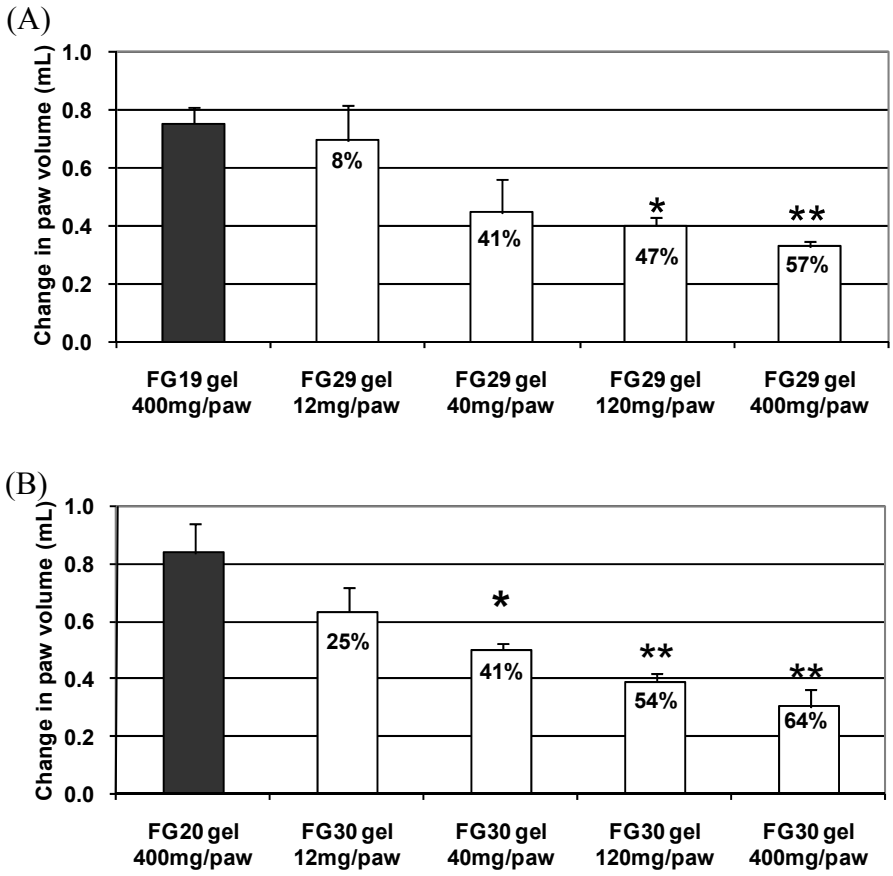


Fig 17. Effect of Celecoxib gel formulations FG29 and FG30, on carrageenan induced paw edema model in rats

*Three hours after the topical application of the gel formulation FG29 (**A**), or FG30 (**B**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-10 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (* , $p < 0.05$, **, $p < 0.01$ was considered significant).*

4.5.2 Duration of action of Rofecoxib and Celecoxib gel formulation

After evaluating the anti-inflammatory efficacy of the gel formulation in carrageenan paw edema model, two gels one each of Rofecoxib (FG14) and Celecoxib (FG30) were selected for further profiling studies. In the next set of studies, duration of action of the selected gels was evaluated. For this study 600mg of 1% w/w Rofecoxib gel – FG14 (30mg/kg Rofecoxib) and 400mg of Celecoxib gel - FG30 (100 mg/kg Celecoxib) were applied as explained in the method section on the paw. 1, 3, 6, 12 and 24 hrs after the gel application, animals were challenged by 0.1 mL of 1.0% carrageenan solution. Change in paw volume (*Vs* t_0) at 3 hrs post carrageenan challenge was recorded. The anti-inflammatory activity was expressed as percent inhibition of paw edema and was calculated by considering the values in the control group as 0% inhibition. Treatment groups were compared with control group to determine statistical significance, using unpaired student's 't' test at each time point with $p \leq 0.05$ considered statistically significant.

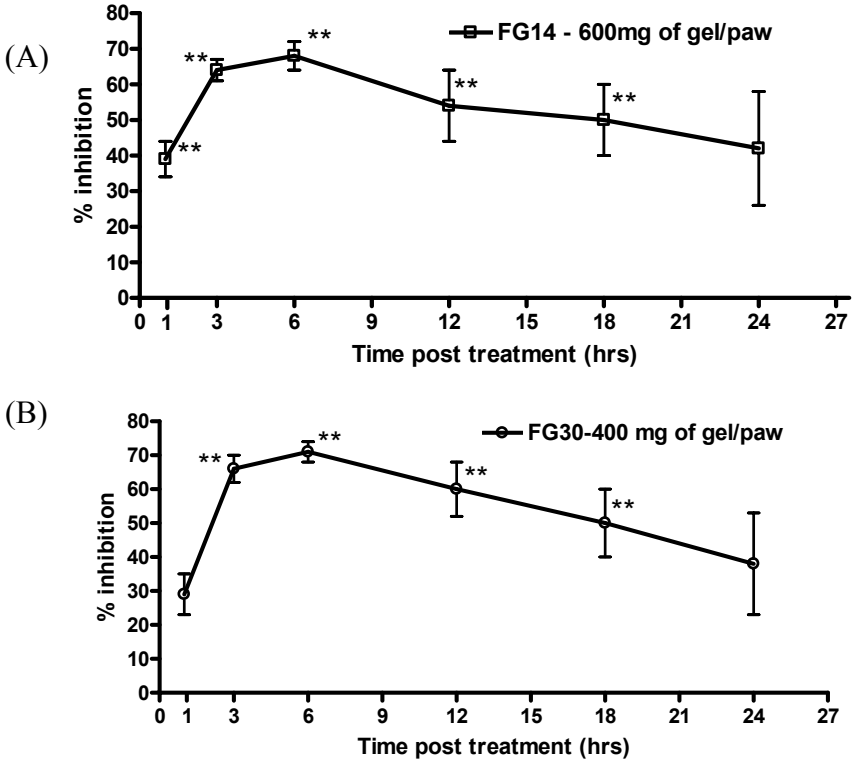


Fig 18. Duration of action of Rofecoxib and Celecoxib gel formulations on carrageenan induced paw edema model in rats.

*1, 3, 6, 12, 18 and 24 hours after the topical application of the gel formulation FG14 (**A**), or FG30 (**B**), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-10 animals per group. (*), indicates statistically significant difference from respective control group using unpaired student's 't' test at each time point tested (*, p < 0.05, **, p < 0.01 was considered significant).*

As shown in Fig 18A, treatment with FG14, Rofecoxib 1%w/w gel formulation, a significant inhibition of 39% was seen 1 hrs pretreatment. This inhibitory activity increased at 3 hrs time point and decreased at 24 hrs time point, indicating to duration of action of at least 18 hrs.

Similar to Rofecoxib gel, treatment with 5% Celecoxib gels, FG30, also showed a potent inhibition of carrageenan induced paw edema, with significant effect lasting for 18 hrs., indicating duration of action of at least 18 hrs (Fig 18B). Treatment with Celecoxib gel however, showed a delayed onset of action with a significant effect seen at 3 hrs post dosing. This is an important improvement over most marketed gel formulations of non-selective NSAIDs which normally demonstrate duration of 4-5 hrs (Gupta *et al*., 2002). This extended duration of action observed in animals if translates in human subjects can greatly improve patient compliance and acceptance.

4.5.3 Comparison with oral efficacy

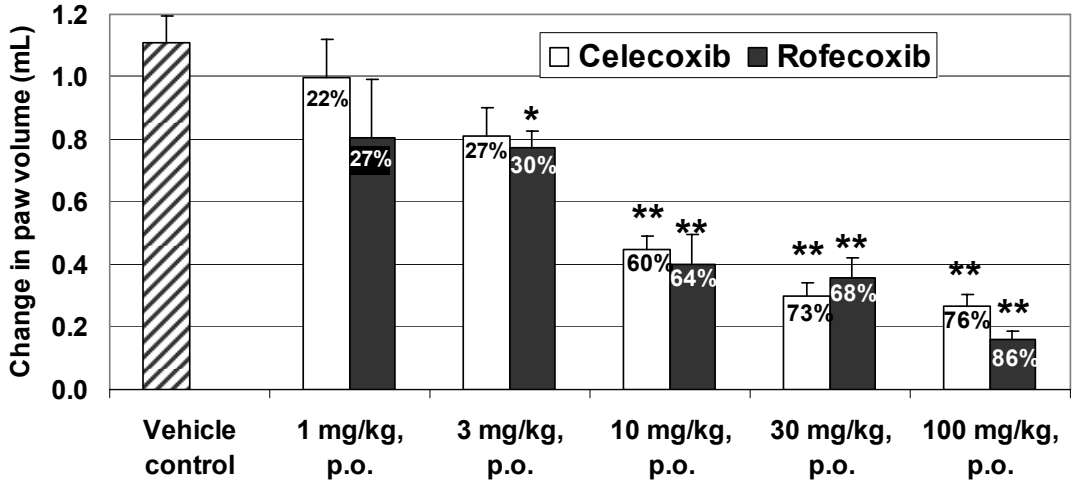


Fig 19. Effect of orally administered Celecoxib and Rofecoxib on carrageenan induced paw edema model in rats

One hours after the oral administration of Celecoxib (empty bar) or Rofecoxib (solid bar) rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw volume, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant).*

Micronised powder of Rofecoxib and Celecoxib were administered orally in overnight fasted rats at dose of 1, 3, 10, 30 and 100 mg/kg. Treatment with Celecoxib showed a dose dependent inhibition of carrageenan induced paw edema with a peak inhibition of 76% at

dose of 100mg/kg (Fig 19). The effect was statistically significant at a dose of 10mg/kg and with an ED_{50} was 7.3mg/kg.

Treatment with Rofecoxib also showed a dose related inhibition of carrageenan induced paw edema with a peak inhibition of 86% at dose of 100mg/kg (Fig 19). The effect was statistically significant at a dose of 3mg/kg and with an ED_{50} was 6.9mg/kg.

4.5.4 Carrageenan induced hyperalgesia

This anti-inflammatory activity of the Rofecoxib (FG14) and Celecoxib (FG30) gel formulations is primarily due to their ability to inhibit inducible COX-2 at the site of action and thereby inhibit prostaglandin synthesis which plays an important role in promoting the signs and symptoms of inflammation (Vane, 1971). Prostaglandins also sensitize terminal afferent C fibers in the periphery and enhance the response of C fibers to analgesic stimuli resulting in hyperalgesia (Martin *et al*., 1987; Cohen and Perl, 1990). One of the defining features of inflammatory pain is a pronounced hypersensitivity to noxious mechanical and thermal stimulation of the skin. Thus, carrageenan-induced paw edema is also most commonly used for studying effect of drugs on hyperalgesia in animals, apart from anti-inflammatory activity (Dirig *et al*., 1998; Jain *et al*., 2001).

Carrageenan injection not only induced paw edema but produced significant hyperalgesia, recorded as decrease in the paw withdrawal latency in response to mechanical stimulation 3 hrs post carrageenan injection. The basal response in vehicle treated animals treated with blank gel FG2 was 113±5gm. It was reduced significantly to 45±5.2gm, 3 hrs post carrageenan challenge. In animals treated with increasing doses of Rofecoxib gel, basal response was not different from the vehicle treated animals. Three hours post carrageenan challenge, in Rofecoxib gel treated groups a dose dependent reversal of the carrageenan induced decrease in paw withdrawal latency was observed (Fig 20) with a peak inhibition of 79 % at a dose of 600mg/paw (30mg/kg of active drug). This effect was statistically significant from a dose of 3mg/kg and was comparable to standard gel of 1% Diclofinac sodium. Consistent with previous studies where maximum hyperalgesia developed 3–4 h after carrageenan administration (Friedman *et al*., 1995; Jain *et al*., 2001; Jett *et al*., 1999) and the peak anti-inflammatory and antihyperalgesic effect was observed 3 hrs after carrageenan challenge.

In a similar assay Celecoxib gel was also evaluated and has shown a dose dependent inhibition of carrageenan induced hyperalgesia. A peak inhibition of 71 % was observed at a dose of 600mg/paw (150mg/kg of active drug) (Fig 21). This effect was statistically significant from a dose of 120 mg/paw and showed comparable potency as 1% Rofecoxib gel and standard gel of 1% Diclofinac sodium.

In same set of carrageenan challenged animals, effect on thermal hyperalgesia was also evaluated. As seen in Fig 22, rats treated with blank gel FG2 show a decrease of 8.6 ± 0.6 sec. in paw withdrawal latency following carrageenan challenge when exposed to thermal stimuli. Treatment with increasing doses of 1% Rofecoxib gel formulation showed a dose related reversal of the carrageenan induced hyperalgesia. A significant inhibition of 37% was observed at a dose of 400 mg/paw and increased to 61% at a dose of 30 mg/kg (600 mg gel/rat paw). A similar inhibition was also observed with 1% Diclofenac gel formulation (Fig 22).

In Celecoxib treated animals, unlike the effect on mechanical hyperalgesia, a significant inhibition of thermal hyperalgesia was also observed at dose of 400 mg of gel/rat paw. A peak inhibition of 57% was observed at this dose (Fig 23) which did not improve any further upon increasing the dose to 600 mg of gel/rat paw.

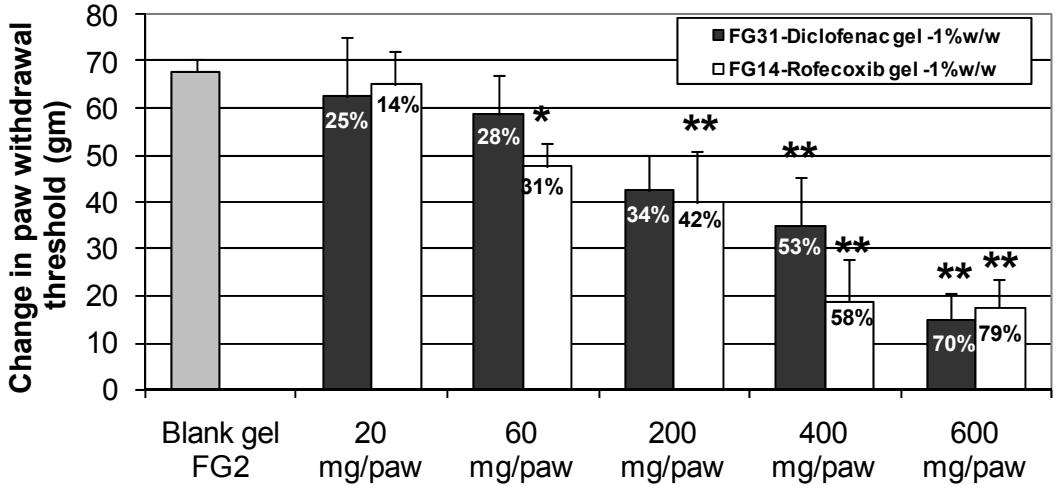


Fig 20. Effect of Rofecoxib gel formulation on carrageenan induced mechanical hyperalgesia in rats

Three hours after topical application of 1% w/w Rofecoxib gel, FG14 (empty bar) or Diclofenac sodium gel FG31 (solid bar), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw withdrawal threshold, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-10 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically

significant difference from respective control group(Blank gel FG2) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant).*

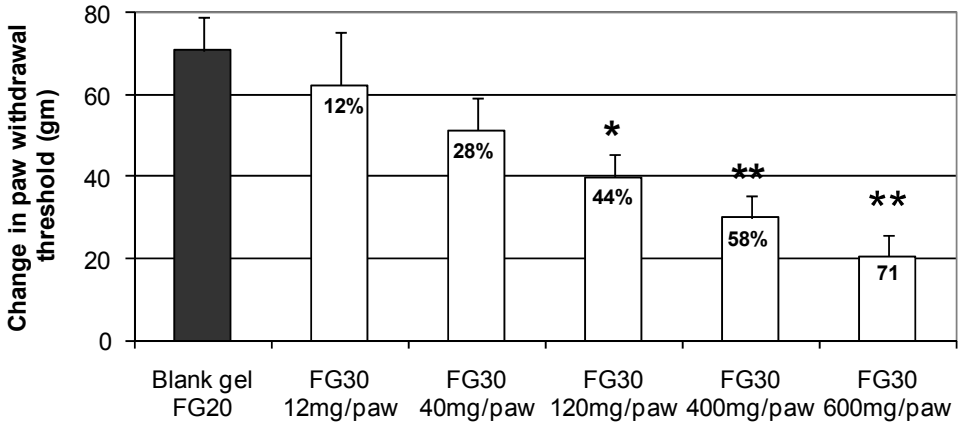


Fig 21. Effect of Celecoxib gel formulation on carrageenan induced mechanical hyperalgesia in rats

Three hours after topical application of 5% w/w Celecoxib gel, FG30 (empty bar), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw withdrawal threshold, 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 7-10 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group(Blank gel FG2) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant). (A dose of 3, 10, 30, 100 and 150mg/kg of active drug is 12, 40, 120, 400 and 600mg of gel /paw of 200± 20gm rats).*

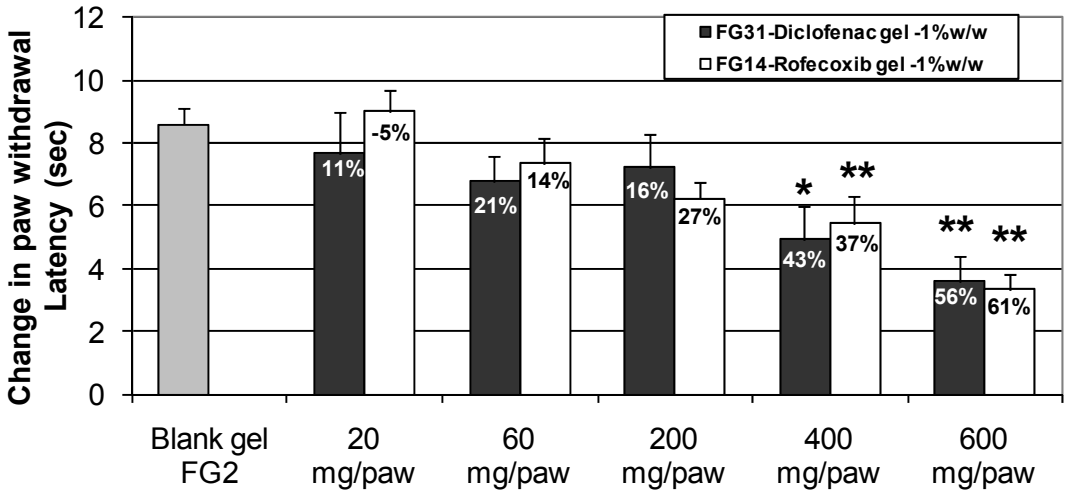


Fig 22. Effect of Rofecoxib gel formulation on carrageenan induced thermal hyperalgesia in rats

Three hours after topical application of 1% w/w Rofecoxib gel, FG14 (empty bar) or Diclofenac sodium gel FG31 (solid bar), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw withdrawal latency (sec), 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 6-10 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control. (*), indicates statistically significant difference from respective control group(Blank gel FG2) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant).*

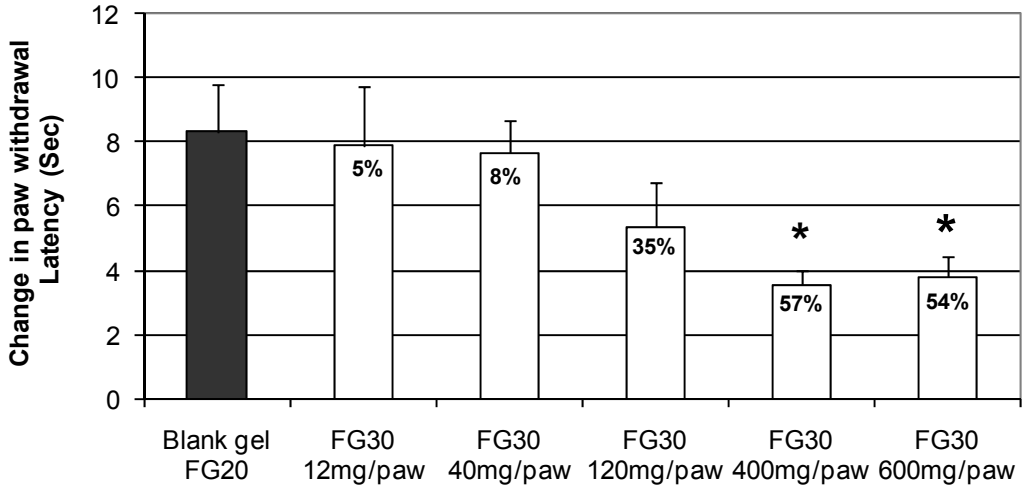


Fig 23. Effect of Celecoxib gel formulation on carrageenan induced thermal hyperalgesia in rats

Three hours after topical application of 5% w/w Celecoxib gel, FG30 (empty bar), rats were challenged with 0.1ml of 1% carrageenan in the sub-plantar region of the right hind paw. Change in paw withdrawal latency (sec), 3 hrs after carrageenan challenge was recorded and expressed as mean ± S.E.M. for 7-10 animals per group. Data in each bar indicates % inhibition in treatment group as compared to respective control. (*), indicates statistically significant difference from control group (Blank gel FG20) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant).*

These results also indicate that COX-2 plays an important role in hyperalgesia, peripherally and selective COX-2 inhibitors can reverse this effect as well as non-selective COX inhibitors. Nantel *et al*., 1999, had also shown that COX-2 levels are elevated for 3hrs after carrageenan injection in the paw, and treatment with NSAIDs reversed COX-2 induction. Although, oral COX-2 inhibitors have been shown to inhibit hyperalgesia in carrageenan induced hyperalgesia model (Smith *et al*., 1998; Gierse *et al*., 2005), we have shown the efficacy by topical route as well, thus opening the option of treating the pain stimuli topically in clinical setting using COX-2 selective inhibitors.

Formalin induced Pain model in rats

Another commonly used model of pain is formalin induced pain in rats. The time course of pain induced by formalin injection is completely different from that induced by carrageenan injection. In formalin test, the noxious response starts immediately after the formalin administration and last for a maximum of 1 hour as against duration of pain of >3 hours in carrageenan model. Administration of formalin into the paw induces a typical biphasic licking and biting response (agitation behavior) with an early Phase I which last for 1-5 min post injection and a late phase II which lasted from 10-60 min. This behavior consists of an

initial phase, occurring about 1-5 min after the injection and then after a quiescent period a second phase between the 20th and 60th minutes. (Rosland *et al*., 1990; Aloisi *et al*., 1995). The first phase results essentially from the direct stimulation of nociceptors whereas the second phase involves a period of sensitization during which inflammatory phenomena occurs. Thus, the second phase cannot be interpreted as a consequence of the first; it clearly also originates from peripheral mechanisms.

In control group treated with blank gel formulation FG2, the mean licking and biting response time in the early and late phase was 107 ± 15 sec and 264 ± 27 sec., respectively. This was not different from the early and late phase response time of 103 ± 9 and 273 ± 23 sec. respectively, obtained with FG20, the blank gel for Celecoxib gel formulation.

Opioid analgesics seem to be antinociceptive for both phases, although the second is more sensitive to these substances. In contrast, NSAIDs suppress only the second phase (Yashpal and Coderre, 1998). Therefore, this model was standardized using Pentazocine (Ranbaxy Labs Ltd., India) at a dose of 5 mg/kg, i.v. Pentazocine produced a significant inhibition of 54% in the early response and 71% inhibition of the late phase response.

Treatment with Rofecoxib, Diclofenac, Nimesulide as well as Celecoxib gels formulations in rats had no effect on the early phase upto the highest dose tested (600 mg /rat paw). This reiterates the lack of activity of NSAIDs and COX-2 inhibitor in the early phase. However, Rofecoxib gel formulation FG14 showed a dose related inhibition of the late phase response at higher doses of 200 and 600 mg of gel/ paw. The peak inhibition at the highest dose tested was 53% and was statistically significant as compared to blank gels (Fig 24A). 1% w/w Diclofenac gel in the same formulation showed an improved inhibitory profile (Fig 24B) with a peak inhibition of 70% at the highest dose tested (600mg of gel/paw). On the other hand 1% Nimesulide gel had shown a much shallow inhibitory response, showing only 42% inhibition at the highest dose tested (600mg of gel/paw) as shown in Fig 24C. Celecoxib gel formulation prepared as 5% w/w gel also shows a significant inhibition of late phase response although with at much higher dose at a dose of 200 and 600 mg of gel/ paw as shown in Fig 25.

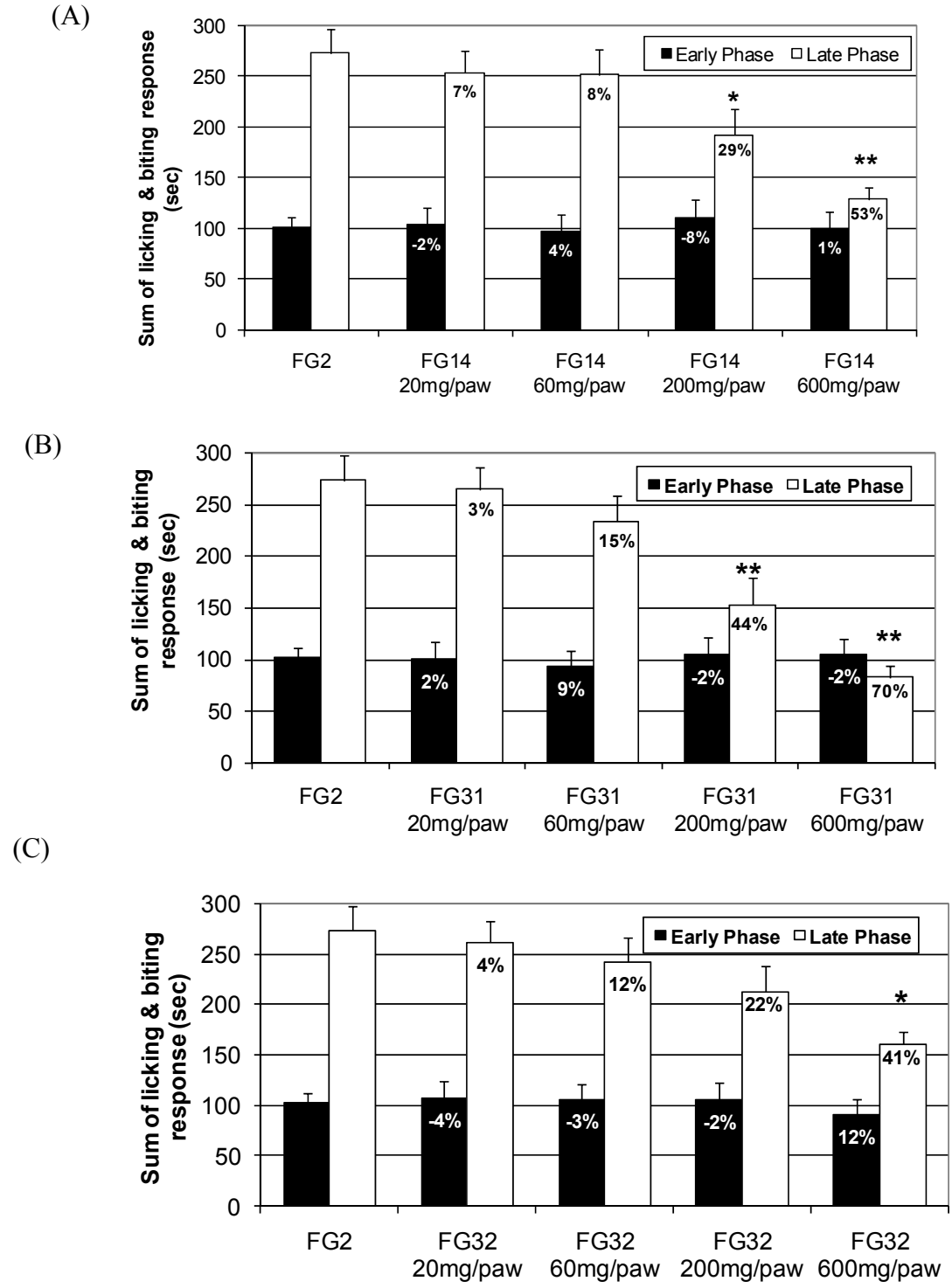


Fig 24. Effect of Rofecoxib gel formulation on formalin induced nociceptive response in rats

*Dose-dependent inhibition of the licking and biting response (indicated as nociceptive response) by (**A**) Rofecoxib gel formulation (FG14) (**B**)Diclofenac gel formulation (FG31) and (**C**) Nimesulide gel formulation (FG32) in the early phase (solid bars) and the late phase (empty bars) of the formalin test in rats. Formalin (2.5%; 50 µl/paw) was administered subplantarly 3 hrs after application of gel formulations the nociceptive response was recorded for every 5-min periods from 0 to 60 min after formalin injection. Data is presented*

as means ± S.E.M. of 6-8 animals in each group. Percent analgesic effect observed with various doses of Rofecoxib and Diclofenac gel against the formalin-induced licking and biting response is represented on the bars. (*), indicates statistically significant difference from control group (Blank gel FG2) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, p ≤ 0.05; **, p ≤ 0.01 was considered significant).*

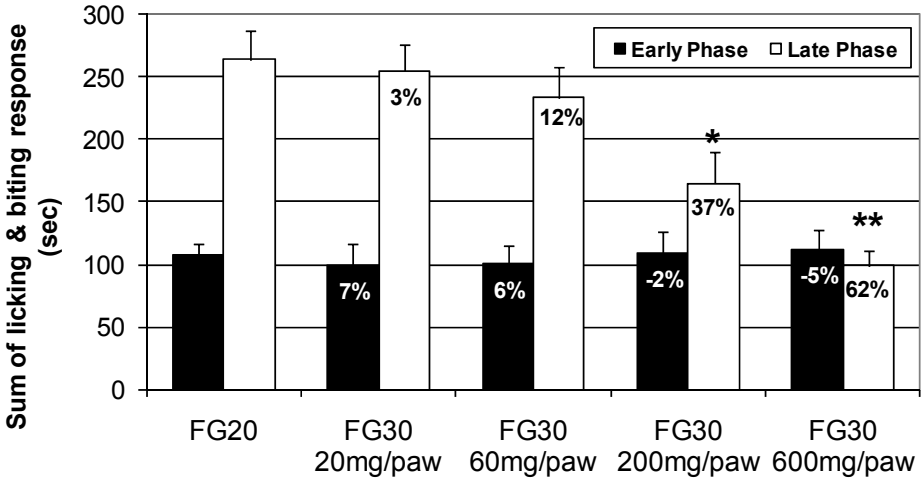


Fig 25. Effect of Celecoxib gel formulation on formalin induced nociceptive response in rats

Dose-dependent inhibition of the licking and biting response (indicated as nociceptive response) by Celecoxib gel formulation (FG30) in the early phase (solid bars) and the late phase (empty bars) of the formalin test in rats. Formalin (2.5%; 50 µl/paw) was administered subplantarly 3 hrs after application of gel formulations the nociceptive response was recorded for every 5-min periods from 0 to 60 min after formalin injection. Data is presented as means ± S.E.M. of 6-8 animals in each group. Percent analgesic effect observed with various doses of Celecoxib gel against the formalin-induced licking and biting response is represented on the bars. (*), indicates statistically significant difference from control group (Blank gel FG20) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, p ≤ 0.05; **, p ≤ 0.01 was considered significant).*

In this model Pentazocine, an opioid, showed a significant inhibition of both early as well as the late phases while NSAIDs or COX-2 inhibitors applied as topical gel formulation typically blocked only the late phase with comparable activity. A similar inhibitory response by oral Celecoxib was also demonstrated by Yamamoto and Nozaki-Taguchi, (2002). Similar to the results seen in formalin test where COX-2 inhibitors and NSAIDs fail to inhibit centrally mediated early phase, no significant inhibition in hot plate test was observed in both Rofecoxib as well as Celecoxib treated animals (Yamamoto and Nozaki-Taguchi, 1996). Thus, treatment with Rofecoxib or Celecoxib gel formulation do not seem to modify supraspinal responses and the anti-nociceptive responses are limited to peripheral or local area.

4.5.5 Phenyl-p-benzoquinone induced writhing in mice

Phenyl-p-benzoquinone induced writhing in mice is commonly used model of visceral pain. Phenyl–p-benzoquinone produces 73 ± 11 and 78 ± 4.4 writhes in animals treated with blank gel FG2 and FG20, respectively. In standard gel formulation of 1% Diclofenac gel (FG31) treated group, mean writhing responses observed were 36 ± 10 and 15 ± 5 indicating a significant inhibition of 51 and 79% at doses of 100 and 300 mg of gel/mice, respectively. Lower doses of 30 and 60 mg of gel /mice also showed inhibition of writhing responses; however the effect was not statistically significant. Treatment with Rofecoxib gel formulation (FG14) at doses of 10, 30, 60, 100 and 300 mg/mice did not elicit a significant inhibition of PQ induced writhing response in mice as shown in Fig 26.

In Celecoxib treated group a dose related inhibition of 11, 19, 45 and 53 % was observed at doses of 10, 30, 100, 300 mg/mice (20, 60, 200, 600mg/kg of active drug), respectively. The inhibitory effect was found to be significant at doses of 100 and 300mg/mice (Fig 27). Treatment beyond this dose was not practically feasible in this model.

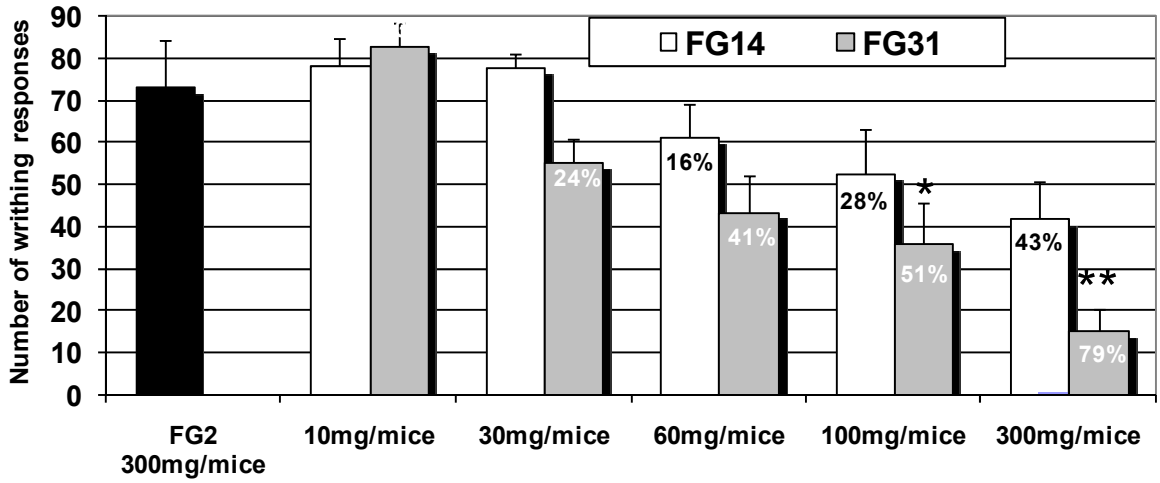


Fig 26. Effect of topical gel formulation on Phenyl-p-benzoquinone induced writhing responses in mice

Mice were treated with either blank gel formulation FG2 (solid bar) or Rofecoxib gel formulation (FG14) (empty bars) or Diclofenac gel formulation (FG31-grey bars). Three hours later, Phenyl-p-benzoquinone (PQ) induced writhing responses (i.e. stretching, twisting a hind leg inward & abdominal contraction) were counted from 5-15 min post PQ administration. Data is presented as means ± S.E.M. of 6-7 animals in each group. Percent analgesic effect observed with various doses of test or standard gel is represented on the bars. (*), indicates statistically significant difference from control group(Blank gel FG2) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant).*

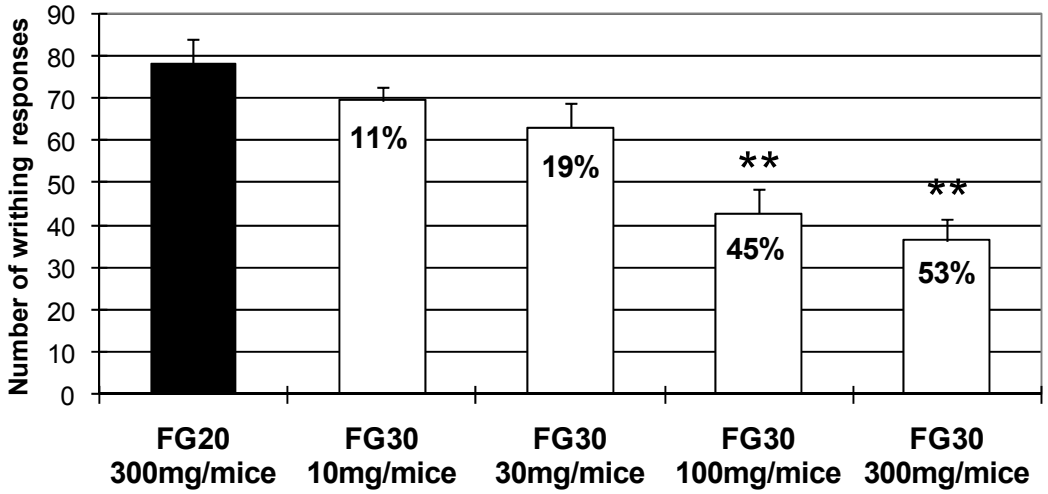


Fig 27. Effect of Celecoxib gel formulation on Phenyl-p-benzoquinone induced writhing responses in mice

Mice were treated with either blank gel formulation FG20 (solid bar) or Celecoxib gel formulation (FG30) (empty bars). Three hours later, Phenyl-p-benzoquinone (PQ) induced writhing responses (i.e. stretching, twisting a hind leg inward & abdominal contraction) were counted from 5-15 min post PQ administration. Data is presented as mean ± S.E.M. of 7 animals in each group. Percent analgesic effect observed with various doses of test or standard gel is represented on the bars. (*), indicates statistically significant difference from control group (Blank gel FG20) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$; **, $p \leq 0.01$ was considered significant).*

Rofecoxib gel formulation in this study failed to show any statistically significant inhibitory response. This could be explained on the hypothesis that prostaglandins derived from the cyclooxygenase-1 pathway but not from the cyclooxygenase-2 pathway play a role, whereby cyclooxygenase-2 inhibition is not sufficient to decrease nociceptive inputs (Padi *et al*., 2004). This supports the lack of efficacy of coxib in these tests. However, in Celecoxib gel treated group a significant inhibitory effect of 45 and 53% was found at doses of 100 and 300mg/mice, respectively. This was similar to the 54 % inhibition of acetic acid induced writhing in mice by 50mg/kg, p.o. Celecoxib (Summary Basis of Approval – Celecoxib, FDA, USA). The reasons for this activity of Celecoxib are not very clear, but could be due to relatively lower selectivity for COX-2 over COX-1.

4.5.6 Tail-flick test in rats

The selected test gels were also evaluated in tail flick test in rats using a Tail-flick apparatus. Treatment with blank gels FG2 and FG20 did not show any significant change in reaction time in rats. Treatment with method control Pentazocine 10mg/kg, i.p., resulted in a significant increase in reaction time. Treatment with test gels of FG14 and FG30 at doses of

600mg/paw did not produce a statistically significant increase in the reaction time in rats indicating a lack of centrally mediated effect of the test gels. These observations are in line with the reports that COX-2 inhibitors do not inhibit thermal-induced acute pain in the tail even when after intrathecal injected (Nishiyama, 2006; Malmberg and Yaksh, 1992).

4.5.7 Complete Freund's adjuvant-induced chronic paw edema in rats

In addition to the acute models of inflammation as well as pain Rofecoxib and Celecoxib gel formulations were also tested in chronic inflammation and pain model. In vehicle treated animals, injection of complete Freund's adjuvant (CFA) resulted in acute inflammation measured Eighteen hours later as shown in Fig 28A. In this period paw volume increased by 1.01 ± 0.1 ml in saline treated animals and was not different from the response observed in blank gel (FG2 and FG20) treated groups (Fig 28A). The acute inflammation induced by Complete Freund's adjuvant (CFA) increased further with a paw volume of 2.65 ± 0.09, and 3.18 ± 0.14 ml observed on day 7 and day 14, respectively in saline treated group. Change in paw volume observed on day 14 was taken as an index of chronic inflammation as shown in Fig 28A. Treatment groups treated with blank gel formulation of FG2 and FG20 also showed a similar inflammation as seen in saline treated group, indicating no inhibitory effect of these gel formulations.

Eighteen hours post CFA challenge, treatment with Rofecoxib gel formulation at a dose of 20, 60, 200 and 600 mg/paw, bid, showed a dose related decrease in acute inflammation, with a 15, 25, 37 and 52% inhibition respectively, as compared to blank gel (FG2) treated animals. This effect was statistically significant at a dose of 200 and 600 mg/paw dose. This inhibitory effect persisted upto day 14 with a significant inhibition of 50 and 51% observed at a dose of 200 and 600mg/paw, bid, dose group (Fig 29). Standard gel formulation of 1% Nimesulide gel FG32 applied as a topical gel on the rat paws at dose of 600mg/paw, bid for 14 days also showed significant inhibition (46%) of paw volume on day 14. This inhibitory effect was comparable to the inhibition obtained with same dose of 1% Rofecoxib gel formulation. However, treatment with 1% Nimesulide-600mg/paw, only showed 27 % inhibition of acute inflammation recorded 18 hrs post CFA challenge. Gupta *et al*., 2006 have earlier shown a similar inhibitory activity of 1% Nimesulide gel.

Treatment with Celecoxib gel formulation also resulted in a dose related inhibition of acute

inflammation, eighteen hours post CFA challenge. A significant inhibition of 34 and 52 % was recorded at doses of 120 and 400 mg/paw (active drug) bid, respectively (Fig 30). Lower doses of 12 and 40 mg/paw, bid, did not show significant inhibition. A significant inhibition was also observed on day 14 with an inhibition of 46 and 52% observed at a dose of 120 and 400 mg/paw, bid, respectively (Fig 30). This efficacy of Celecoxib gel formulation was comparable to Rofecoxib gel formulation.

In the same model effect on pain threshold was also determined in the right hind paw on day 14. Pain threshold was determined by the number of squeak vocalizations induced by five consecutive gentle flexions of the right ankle joint at 3-s intervals. In animals challenged with incomplete Freund's adjuvant vocalization frequency was 0, i.e. none of the animals produced vocalization response upon gentle flexion. In CFA control animals treated with saline a significant increase in the vocalization frequency (4.2 ± 0.48) was observed (Fig 28B). Animals challenged with CFA and treated with blank gel formulation FG2 and FG20 for 14 days also showed a significant vocalization response (4.67 ± 0.42 and 4.5 ± 0.62, respectively) which was not different from saline treated animals again indicating no inhibitory effect of these gel formulations. Treatment with Rofecoxib gel FG14 (60, 200 and 600 mg/kg), Celecoxib gel – FG30 (40, 120 and 400mg/kg) and Nimesulide gel formulation – FG32 have shown significantly decreased vocalization frequency compared with the respective blank gel-treated adjuvant induced arthritis group (Fig 31 & 32).

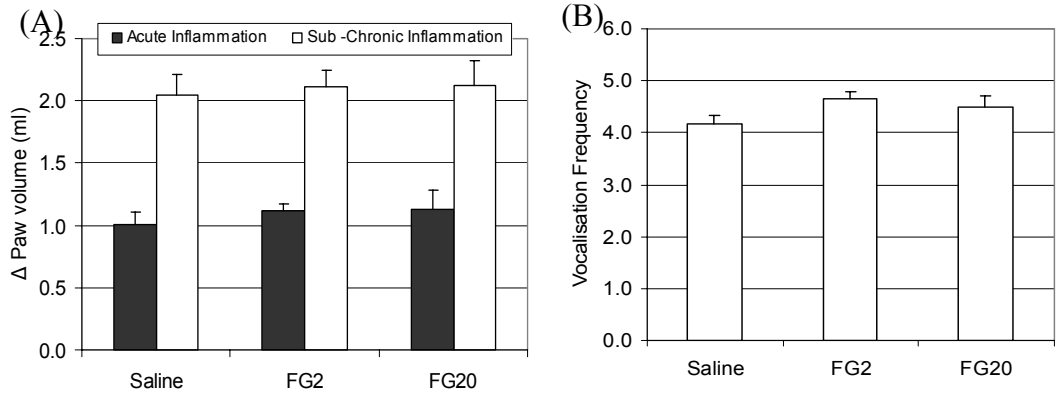


Fig 28. Effect of blank gel formulation on complete Freund's adjuvant-induced acute and sub-chronic inflammation and pain in rats

Rats were injected complete Freund's adjuvant. Eighteen hours later (acute inflammation- solid bars) and on day 14 (sub-chronic inflammation- empty bars) change in paw volume from basal were recorded. Rats were treated with topical application of blank gel FG2-600 mg/paw or FG20-600mg/paw or saline, twice daily for 14 days. Change in paw volume (mL) in acute and subchronic

*phases (**A**) were recorded and effect on joint flexion induced pain was recorded as vocalization frequency (**B**) on day 14 and expressed as mean ± S.E.M. for 6-8 animals per group.*

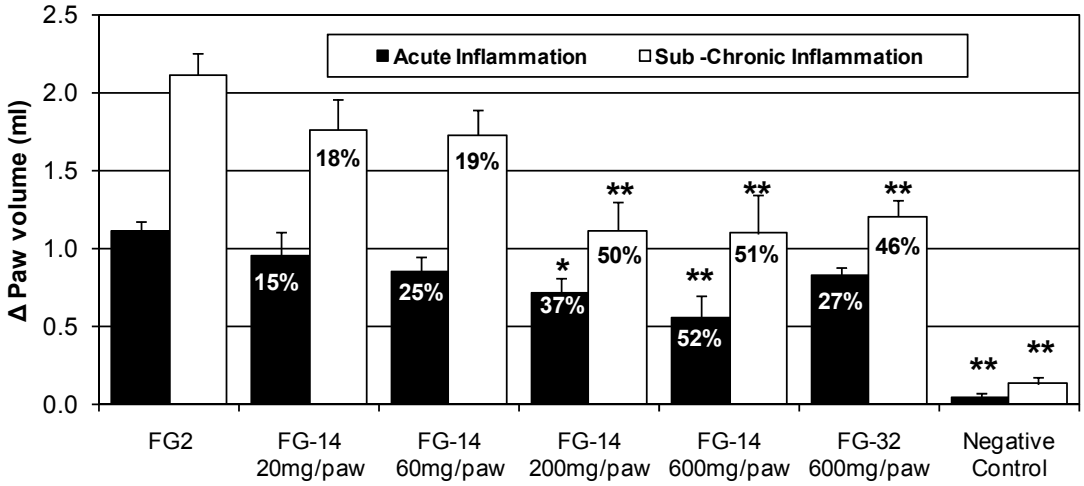


Fig 29. Effect of Rofecoxib gel formulation in complete Freund's adjuvant-induced acute and sub-chronic inflammation in rats

Rats were injected complete Freund's adjuvant. Eighteen hours later (acute inflammation- solid bars) and on day 14 (sub-chronic inflammation- empty bars) change in paw volume from basal were recorded. Rats were treated with topical application of 1% w/w Rofecoxib gel, FG14 or blank gel FG2 or 1% Nimesulide gel FG32, twice daily for 14 days. Change in paw volume (mL) in acute and subchronic phases were recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control groups. (*), indicates statistically significant difference from respective control group(Blank gel FG2) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$; **, $p \leq 0.01$ was considered significant).*

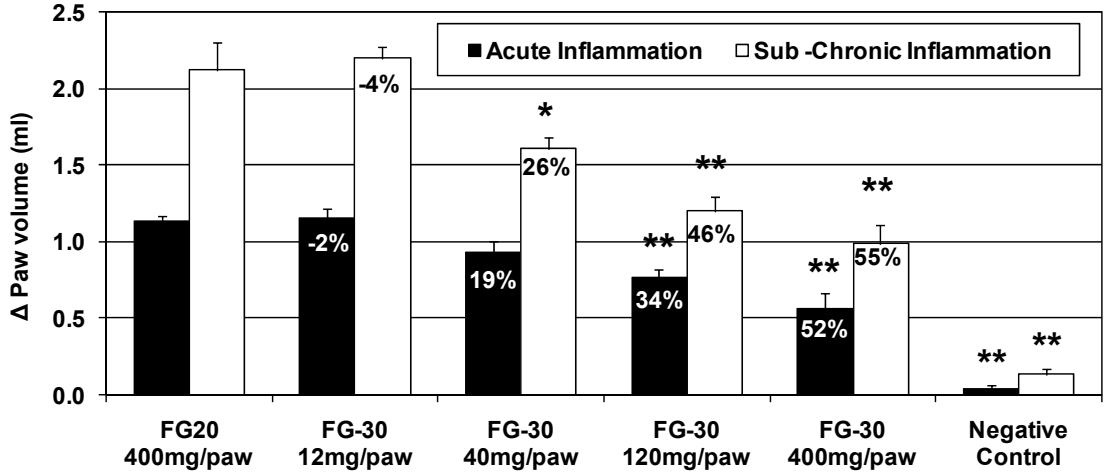


Fig 30. Effect of Celecoxib gel formulation in complete Freund's adjuvant-induced acute and sub-chronic inflammation in rats

Rats were injected complete Freund's adjuvant. Eighteen hours later (acute inflammation- solid bars) and on day 14 (sub-chronic inflammation- empty bars) change in paw volume from basal were recorded. Rats were treated with topical application of 1% w/w Celecoxib gel, FG30 or blank gel FG20 or twice daily for 14 days. Change in paw volume (mL) in acute and subchronic phases were recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control groups. (*), indicates statistically significant difference from respective control group(Blank gel FG2) using one-way analysis of

variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant).*

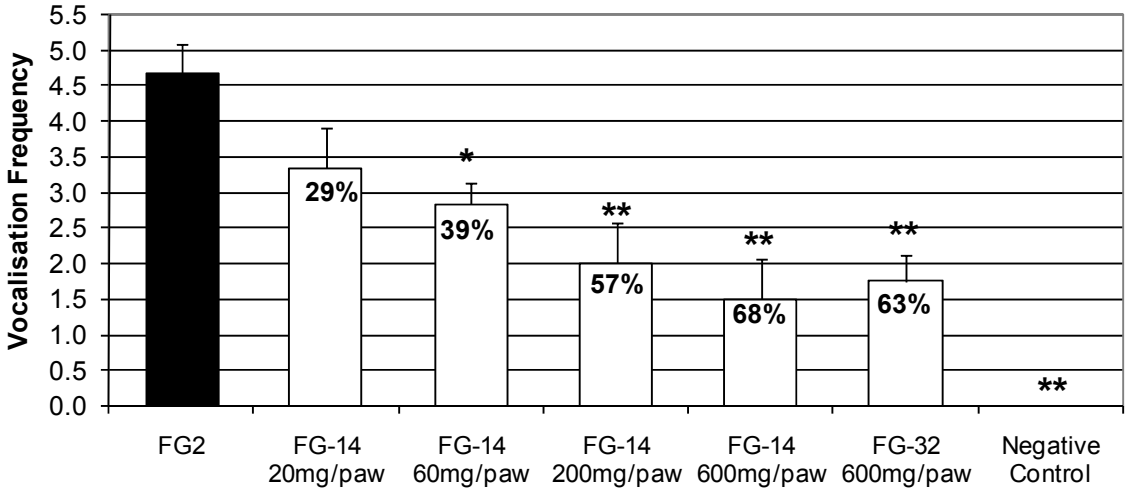


Fig 31. Effect of Rofecoxib gel formulation on joint flexion induced pain in adjuvant-induced sub-chronic inflammation in rats

Rats were injected complete Freund's adjuvant and effect on joint flexion induced pain was recorded as vocalization frequency on day 14. Rats were treated with topical application of 1% w/w Rofecoxib gel, FG14 or blank gel- FG2 or 1% Nimesulide gel-FG32, twice daily for 14 days. Data was recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control groups. (*), indicates statistically significant difference from respective control group(Blank gel FG2) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant). (A dose of 1, 3, 10 and 30mg/kg of active drug is 20, 60, 200 and 600mg of gel /paw of 200± 20gm rats).*

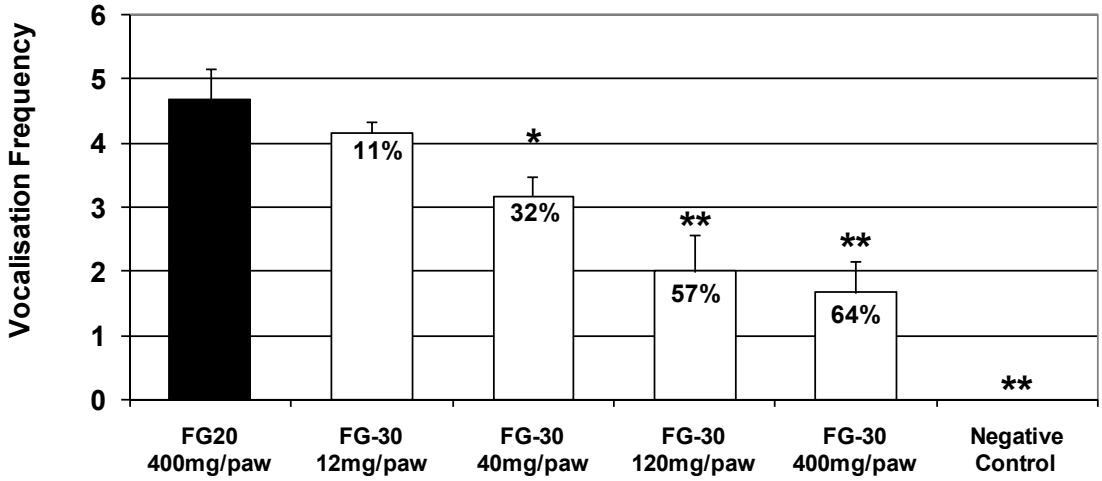


Fig 32. Effect of Celecoxib gel formulation on joint flexion induced pain in adjuvant-induced sub-chronic inflammation in rats

Rats were injected complete Freund's adjuvant and effect on joint flexion induced pain was recorded as vocalization frequency upto day 14. Rats were treated with topical application of 1% w/w Celecoxib gel, FG30 or blank gel FG20, twice daily for 14 days. Data was recorded and expressed as mean ± S.E.M. for 6-8 animals per group. Data in each bar indicates % inhibition in the treatment group as compared to respective control groups. (*), indicates statistically significant difference from respective control group(Blank gel FG20) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant). (A dose of 3, 10, 30 and 100mg/kg of active drug is 12, 40, 120 and 400mg of gel /paw of 200± 20gm rats).*

Widening the efficacy observed in acute inflammation and pain, these results indicate usefulness of Rofecoxib as well as Celecoxib gel formulation in chronic inflammatory conditions such as Rheumatoid arthritis, Psoriatic arthritis, Osteoarthritis, etc.

After demonstrating the efficacy of test gels in animal models it was important to understand the safety profile of these gel formulations to have any meaningful differentiation over oral coxibs and other NSAIDs. In this regard, the first study was designed to compare the systemic exposure achieve after oral and topical administration of Rofecoxib/Celecoxib at equiactive dose by either route.

4.6 Safety evaluation studies

4.6.1 Plasma exposure study in rats

Since, coxibs have adverse effect liability with systemic exposure, systemic penetration studies using suitable analytical methods to determine plasma exposure are important to demonstrate superior therapeutic window of topically administered drug formulation. For this purpose relative bioavailability of active drug at equipotent doses (i.e. ED_{50}) after oral and topical administration were determined in rats.

In this study male Wistar rats, 8 per treatment group, were administered Rofecoxib / Celecoxib either by oral or topical route. For oral delivery, both the drugs were suspended in 0.25% methyl cellulose and administered at a dose of 10mg/kg, p.o. (ED_{50} by oral route in our study was 6.9 and 7.3mg/kg, p.o., respectively). For topical delivery Rofecoxib gel formulation FG14 or Celecoxib gel FG30 was applied on the shaven dorsum of rats at a dose of 100mg/animal (ED_{50} of 77 and 80mg/paw in FG14 and FG30 respectively). Blood was collected at 0.5, 1, 1.5, 2, 4, 8, 12 and 24 hrs post dosing, with each animal bled not more than 4 times and 4 blood samples were collected at each time point. Plasma was separated by centrifugation and was stored at -20° C until analysed for drug concentration by method described in methods section.

Table 18. Plasma concentration profile of Celecoxib and Rofecoxib gel formulations in Wistar rats.

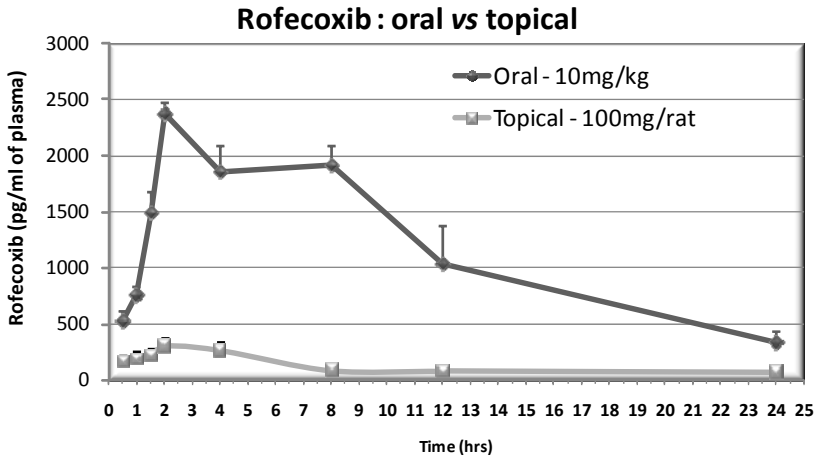
	Route of administration	Dose	C_{max} (ng/mL)	T_{max} (hrs)	$AUC_{0\rightarrow24h}$ (ng.hr/mL)	Relative bioavailability (Oral *vs* topical)
Rofecoxib	Oral	10mg/kg	2378 ± 90	2	27913	9.9
	Topical FG14 (1%w/w)	100mg/animal (4 mg/kg)	302 ± 45	2	2819	
Celecoxib	Oral	10mg/kg	2380 ± 408	2	35212	5.3
	Topical FG30 (1%w/w)	100mg/animal (20mg/kg)	357 ± 70	4	6655	

Data presented as mean ± S.E.M. with n=4 in each treatment group.

Male Wistar rats treated with Rofecoxib – 10mg/kg, orally, showed expected plasma exposure as reported in the literature. As shown in Fig 33A, detectable plasma concentration of obtained from 0.5 to 24 hrs post dosing with a C_{max} of 2378 ± 90 (ng/mL) at T_{max} of 2 hrs (Table 18). The $AUC_{(0\rightarrow24h)}$ was 27913 ng.hr/mL. Since, terminal phase was not attained in these animals, $t_{½}$ and $AUC_{(0\rightarrow\infty)}$ was not considered. Treatment with Rofecoxib gel topically also showed a detectable plasma concentration from 0.5 to 24 hrs post application. However, a much reduced exposure was observed by topical route as shown in Table 18 and Fig 33A. The relative bioavailability of oral *vs* topical showed a 10 fold decrease in the $AUC_{(0\rightarrow24h)}$ at equipotent doses, indicating a lower systemic exposure and consequently lower adverse effect liability.

Similar results were also obtained with Celecoxib treatment as well. Administration of equipotent doses by oral and topical routes resulted in 5.3 fold decrease in the systemic exposure levels after topically administration in rats as shown in Table 18 and Fig 33B. This lower systemic exposure again offers a potential to have lower adverse effect liability and provides ample opportunity for safe clinical use of these agents.

(A)



(B)

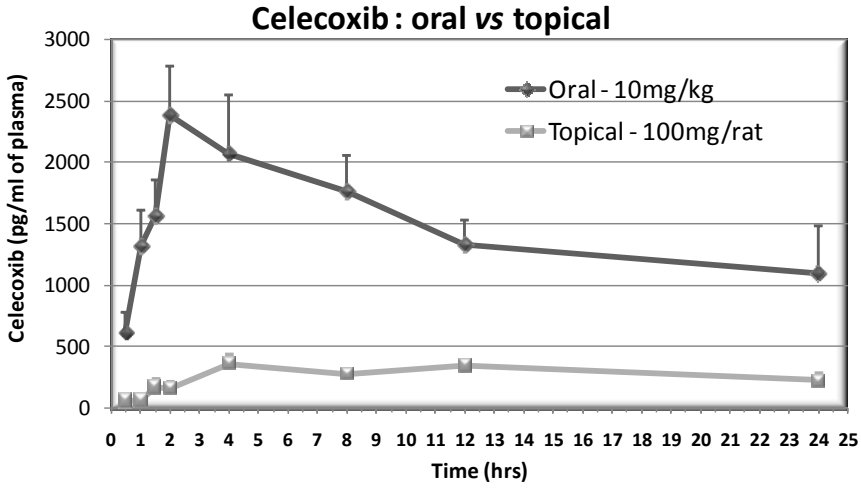


Fig 33. Plasma concentrations of Rofecoxib and Celecoxib after oral/topical administration in male Wistar rats

*Rats were treated with Rofecoxib (**A**) / Celecoxib (**B**) orally at a dose of 10mg/kg and topically (gel formulation of FG14 and FG30, respectively) at a dose of 100mg/rat. Blood was collected at 0.5, 1, 1.5, 2, 4, 8, 12 and 24 hrs post dosing, with each animal bled not more than 4 times and 4 blood samples were collected at each time point. Plasma was separated by centrifugation and was stored at -20° C until analysis for drug concentration by method described in methods section. Data is presented as mean ± SEM of 4 animals at each point.*

4.6.2 Acute toxicity in rats (LD_{50} studies)

To study the acute toxicity of the gel formulations, Rofecoxib gel (FG14) or Celecoxib gel (FG30) were applied on shaven mouse skin at doses of 30, 100 and 300mg/mouse (i.e. 1.2, 4 and 12gm/kg in ~25gm mouse). Rofecoxib gel (FG14) or Celecoxib gel (FG30) at doses of tested were well tolerated and with no mortality observed in any of the doses upto day 7. No gross behavior changes were observed at any of the doses tested except a mild decrease in the

locomotor activity in FG30 - 300 mg/mouse treated animals from 4th hour onwards which recovered at 12 hrs post treatment. The doses used were the highest possible dose that could be used for testing in mice and therefore these gels might show even better tolerance in clinical scenarios.

4.6.3 Skin irritation test and effect on gastric mucosa.

One of the major adverse effects observed with topical NSAIDs was local skin reactions (~85% of all adverse effects) (Moore *et al*., 1998; Mason *et al*., 2004). Therefore, dermal tolerance of the selected gel formulations was evaluated in a chronic study using rats. In this study gel formulation of Rofecoxib (FG14) and Celecoxib (FG30) were applied on the surface of the skin at doses of 240 and 800mg/rat (3 and 10 fold of the ED_{50} dose), topically once daily for 4 weeks on the shaven skin on the rat dorsum. At the end of the study animals were euthanized and skin tissue was collected and fixed in 10% neutral buffered formalin. Tissue samples were trimmed, processed, sectioned at 4µ thickness and stained with Hematoxylin and Eosin for histological evaluation under light microscopy. Pathological changes were graded from no changes to marked change (level 0 to 4, respectively) including the inflammation and vascular changes of epidermis, dermis and subcutis as well as degeneration of skin appendages (Wu *et al*., 2002).

Treatment with Rofecoxib gel (FG14) or Celecoxib Gel (FG30) at doses of 240 and 800mg/rat was well tolerated with no mortality observed in the entire study. No effect on body weight as compared to blank gel treated animals was observed. At the end of the study, gross examination of the skin samples treated with Rofecoxib and Celecoxib gels formulation did not reveal any abnormality. Histopathological evaluation of skin samples also did not reveal any changes in skin compared to their vehicle controls. Moreover, they follow the normal histological pattern in epidermis, dermis and subcutis with accessory skin appendages in comparison to saline control as shown in Fig 34-36. These results did not indicate any cutaneous/dermal adverse effect liability for the test gels of FG14 and FG30 upto 10 fold of the ED_{50} values.

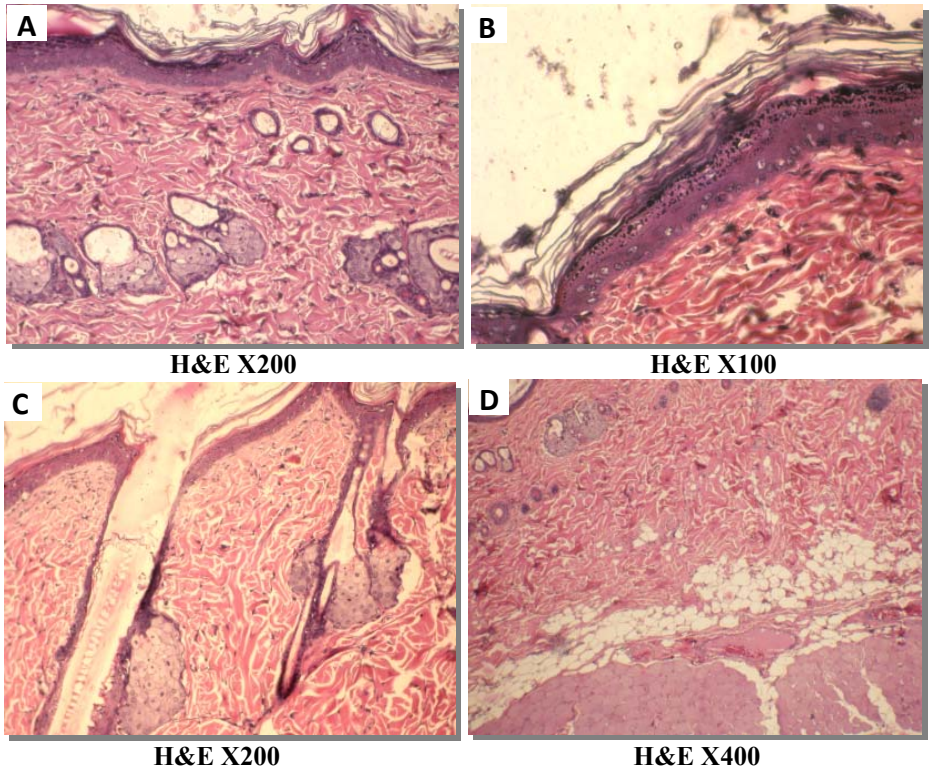
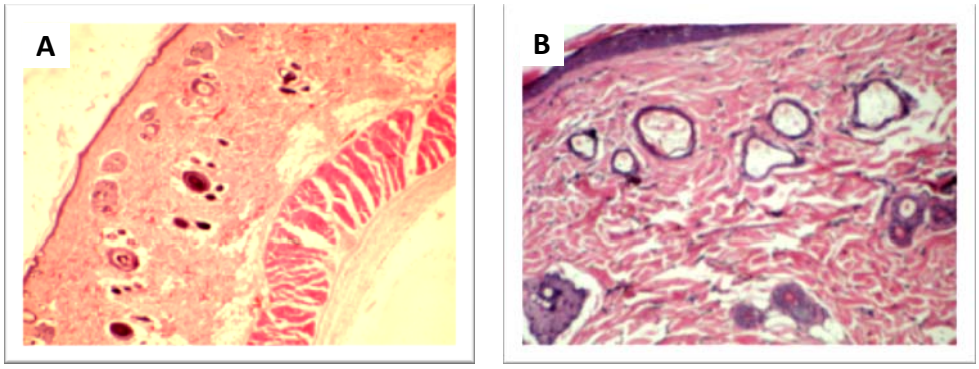
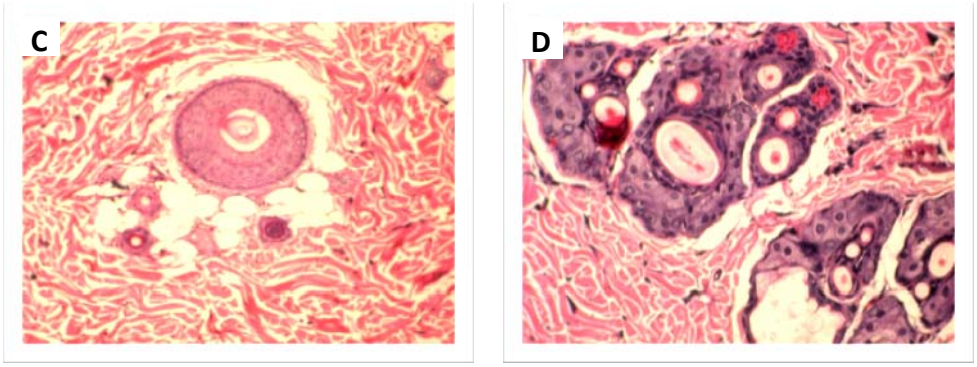


Fig 34. Photomicrograph of skin histology in control animals

*The epithelium forming the surface layer of skin is darkly stained epidermis. The lighter stained layer, the dermis, mainly consists of dense irregular connective tissue which represents the collagen with sparingly distributed elastic fibers. It is much thicker than the epidermis and showed the presence of sebaceous glands. (**A**). The most superficial part of the epidermis is formed by the stratum corneum with absence of nuclei. The stratum granulosum is a single layer of very dark and flattened cells. Polyhedral cells with clear outlines form the stratum spinosum. The stratum basale is formed by a single layer of cuboidal or columnar cells and delimits the epidermis from the dermis (**B**). Sebaceous glands are seen adjacent to hair follicles and empty their secretory product into the upper parts of the hair follicles (**C**). The hypodermis is the lightest layer visible and consists mainly of adipose tissue. Dense connective tissue strands may extend from the dermis deep into the hypodermis and anchor the skin to underlying structures (**D**)*



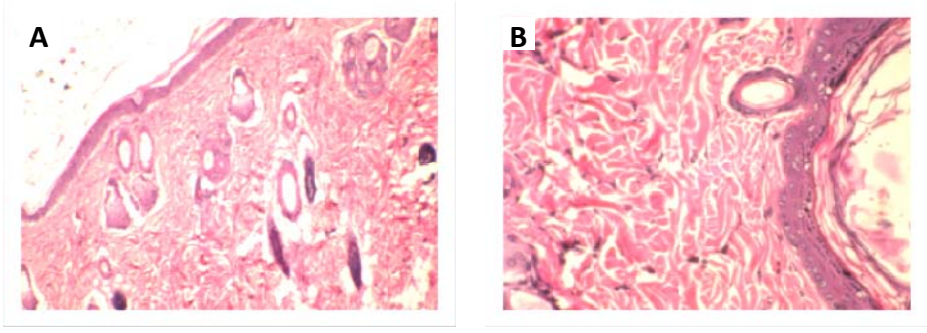
H&E X40 H&E X100



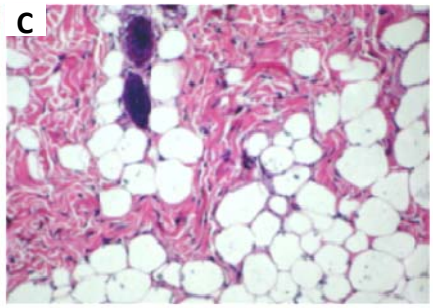
H&E X200 H&E X400

Fig 35. Photomicrograph of skin from Rofecoxib gel (FG14) treated animals

*Treatment with Rofecoxib gel (FG14) at dose 800 mg/rat topically once daily for 4 weeks did not alter the skin histology (**A**). Skin showed compact epidermis and dermis (**B**) and intact hair root in dermis (**C**) and sebaceous glands (**D**)*



H&E X100 H&E X200



H&E X200

Fig 36. Photomicrograph of skin from Celecoxib gel (FG30) treated animals

*Treatment with Celecoxib gel (FG30) at a dose of 800mg/rat topically once daily for 4 weeks did not alter the skin histology. Skin showed compact epidermis and dermis with normal skin appendages (**A**). Dermis showed intact irregular connective tissue (**B**) and hypodermis had normal distribution of adipose tissues (**C**)*

In the same assay on day 28 animals were subjected to gross pathological evaluation, hematological, biochemical and urine analysis. Gross pathological evaluation of various organs revealed no abnormal finds in any of the treatment group. No abnormal changes in the organ weight was also recorded, therefore histopathological evaluation of these organs was not done. Hematological, biochemical or urine analysis also did not show any abnormal finding. Finally the stomachs were dissected out, opened along the greater curvature and the mucosa was rinsed with normal saline, and the mucosa was macroscopically evaluation for the presence of petechiae or frank hemorrhagic lesions. No gastric lesions or ulcerations were observed in microscopic evaluation in any of the groups treated with Rofecoxib gel (FG14) or Celecoxib gel (FG30). Therefore, no toxicity liability was observed in animals upto 800mg/rat (10 times the ED_{50} dose)

4.6.4 *Ex vivo* effect on COX-1 and COX-2 inhibition.

Although no obvious toxicity was observed in 28 day study, however it is important to address the cardiovascular risks associated with the coxibs. The role of COX-2 inhibition in cardiovascular diseases is complex and is not fully understood but it appears to be multifactorial and relates to sites of COX-2 synthesis, expression within the vasculature, and related local consequences of an imbalance between thromboxane A2 and prostacyclin. It was reported that coxibs like Celecoxib and Rofecoxib suppressed the formation of prostaglandin I_2 in healthy volunteers (FitzGerald, 2003). Prostaglandin I_2 had previously been shown to be the predominant cyclooxygenase product in endothelium, inhibiting platelet aggregation, causing vasodilatation and preventing the proliferation of vascular smooth-muscle cells *in vitro*. The individual cardiovascular effects of prostaglandin I_2 *in vitro* contrast with those of thromboxane A_2 (the major COX-1 product of platelets, which causes platelet aggregation, vasoconstriction, and vascular proliferation). While the traditional NSAIDs inhibited platelet aggregation *ex vivo* transiently at the time of peak action, the coxibs had no such effect which is compatible with the absence of COX-2 from mature human platelets (Patrignani *et al*., 1999**)**. Unlike the non-selective NSAID, coxibs such as Celecoxib or Rofecoxib only inhibited PGI_2 leaving the COX-1 derived TxA_2 unaffected. Thus, the cardiovascular effects of TxA_2 would be expected to be exaggerated with the use of coxibs. However, as PGI_2 was known to act as a general restraint on any recognized stimulus to platelet activation, it may be possible to conclude - that upsetting a notional "balance" between these 2 prostanoids was likely to be the mechanism of cardiovascular adverse

effects. Although, variation in other endogenous mediators such as NO, would be expected to modulate the impact of COX-2 inhibition on cardiovascular function. Given that similar observations were made with several coxibs, it appeared that this effect is mechanism based, rather than an off-target effect restricted to one compound. These effects may differ among structurally distinct COX-2 inhibitors with different levels of COX-1 or COX-2 selectivity, but evidence for a differential cardiovascular effect is limited. Considered collectively, increased platelet aggregation, hypertension, endothelial cell dysfunction, impaired angiogenesis, and destabilization of the atherosclerotic plaque matrix are important contributors to the "prothrombotic environment." Several randomised Clinical trials, of prolonged treatment with coxibs have demonstrated these effects which eventually lead to withdrawal of several selective COX-2 inhibitors from clinical use and revised labeling for the rest.

It has been shown that COX-2 inhibition, as determined by PGE_2 levels in LPS-stimulated whole blood *in vitro*, can be used as a marker to predict drug efficacy in humans (Huntjens *et al.,* 2005). In fact, IC_{80} values have been found to correlate directly with the analgesic/anti-inflammatory plasma concentrations of different COX inhibitors (Huntjens *et al.,* 2005). Cryer and Feldman reported that the inhibitory effects of NSAIDs on gastric PGE_2 synthesis correlate with COX-1 inhibitory potency in clotting blood ($P < 0.001$) which can be used as a biomarker of gastric toxicity of COX inhibitors (Cryer and Feldman, 1998). At the same time, the extent of inhibition of whole blood COX-2 and COX-1 activities may acquaint on the impact of the drugs on cardiovascular homeostasis. In fact, 2 important concepts have to be taken into consideration: (i) due to a nonlinear relationship between COX-1 inhibition *ex vivo* and TxA_2 inhibition *in vivo*, a drug will be cardioprotective if, at the therapeutic concentrations, will cause an almost complete suppression (>95%) of platelet COX-1 activity in clotting blood (Reilly and FitzGerald, 1987); (ii) due to a linear relationship between COX-2 inhibition *ex vivo* and PGI_2 inhibition *in vivo* (Capone *et al*., 2007), the profound suppression (70–80%) of COX-2 activity required for efficacy (Huntjens *et al.,* 2005) will translate into a substantial deficit of the cardioprotective action of PGI_2 (Capone *et al*., 2007). With this background we have evaluated the ability of the topically administered gel formulation FG14 (Rofecoxib) and FG30 (Celecoxib) to show > 70-80% *ex vivo* inhibition of PGE2 and TxA_2 and affect the cardiovascular homeostasis. This study was done in blood sample drawn from the 28 day toxicity study.

Prior to *ex vivo* evaluation of rat blood samples, *in vitro* potency of the test item was determined in the same assay. For this study, Rofecoxib / Celecoxib (dissolved in DMSO - final conc.0.4%) were treated in 8 concentration starting from 300µM for COX-1 assay and 30 µM for COX-2 assay with half- log dilutions. Indomethacin was used as standard substance in this assay**.** Inhibitory activity of COX isoforms in vitro by Rofecoxib/ Celecoxib is presented in Table 19 and Fig 37.

Table 19. *In vitro* COX-1 and COX-2 inhibitory potency in rat blood.

	IC_{50}(µM)		IC_{80}(µM)	
	COX-1	COX-2	COX-1	COX-2
Rofecoxib	17.6 ± 2.6	0.78 ±0.11	76.1 ± 6.8	7.4 ± 2.6
Celecoxib	22.7 ± 2.1	0.82 ± 0.08	82.0 ± 7.1	9.1 ± 2.4
Indomethacin	0.19 ± 0.06	0.16 ± 0.05	0.96 ± 0.35	0.91 ± 0.33

Blood was collected in tubes without anticoagulants and allowed to clot, supernatants were recovered and Thromboxane B_2 (TxB_2) was measured using a commercially available ELISA kit to estimate the COX-1 inhibitory potency in rat whole blood. Blood was collected in heparinized tubes, challenged with lipopolysaccharide (100µg/ml) for 24 hrs. Prostaglandin E2 (PGE_2) was estimate using a commercially available ELISA kits in the plasma to determine COX-2 inhibitory potency. Data is presented as mean ± SEM of 4-5 experiments.

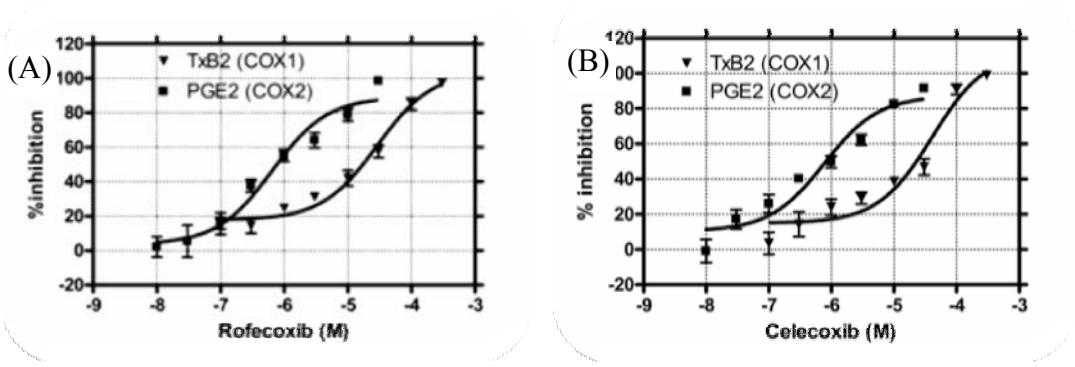


Fig 37. *In vitro* COX-1 and COX-2 inhibitory potency in rat blood

Blood was collected in tubes without anticoagulants and allowed to clot, supernatants were recovered and Thromboxane B_2 (TxB_2) was measured using a commercially available ELISA kit to estimate the COX-1 inhibitory potency in rat whole blood. Blood was collected in heparinized tubes, challenged with lipopolysaccharide (100µg/ml) for 24 hrs. Prostaglandin E2 (PGE_2) was estimate using a commercially available ELISA kits in the plasma to determine COX-2 inhibitory potency. Blood samples were either treated with Rofecoxib (A) or Celecoxib (B). Data is presented as mean ± SEM of 4-5 experiments.

Table 20. *Ex vivo* COX-1 and COX-2 inhibitory activity in rat blood.

	COX-1 (TxB_2 release in ng/ml)			COX-2 (PGE_2 release in ng/ml)		
	2hrs	4hrs	24hrs	2hrs	4hrs	24hrs
Blank gel (FG2) 800mg/rat	66.5 ± 7.2	65.6 ±19.3	62.9 ± 9.9	30.1 ± 7.6	40.7 ± 4.6	31.1 ± 1.6
Rofecoxib (FG14) 240mg/rat	65.2 ± 0.5 (2%)	54.8 ±5.6 (17%)	47.5 ± 6.6 (11%)	28 ± 2.6 (7%)	33.1 ± 5.2 (16%)	27.8 ± 2.8 (11%)
Rofecoxib (FG14) 800mg/rat	47.8 ± 4.9 (28%)	50 ± 15.7 (24%)	60.2 ± 3.5 (4%)	18.9 ± 3.4 (37%)	21.6 ± 4.9 (45%)	26 ± 4.3 (17%)
	4hrs	8hrs	24hrs	4hrs	8hrs	24hrs
Blank gel (FG20) 800mg/rat	74.4 ± 18.1	73 ± 13.9	65.7 ±22.5	41.8 ± 6.3	43.2 ± 5.4	51.7 ± 4.3
Celecoxib (FG30) 240mg/rat	68.8 ± 1.2 (7%)	61.9 ±8 (15%)	63.4 ± 10 (4%)	25.5 ± 3.7 (39%)	31.9 ± 6.6 (26%)	47.5 ± 5.6 (8%)
Celecoxib (FG30) 800mg/rat	58.0 ± 5.8 (22%)	53.9 ± 9.2 (26%)	52.2 ± 4.6 (20%)	23.0 ± 5.3 (45%)	23.5 ± 5.9 (46%)	33.1 ± 5.5 (36%)

Rats were treated with gel formulations of Celecoxib /Rofecoxib/ blank gels for 28 days. On day 28, 2, 4 and 24 hrs post treatment blood was withdrawn from Rofecoxib gel treated groups and 4, 8 and 24 hrs post treatment from Celecoxib treated animals. Blood was collected in tubes without anticoagulants and allowed to clot, supernatants were recovered and Thromboxane B_2 (TxB_2) was measured using a commercially available ELISA kit to estimate the COX-1 inhibitory activity. Blood was also collected in heparinized tubes, challenged with lipopolysaccharide (100µg/ml) for 24 hrs. Prostaglandin E2 (PGE_2) was estimate using a commercially available ELISA kits in the plasma to determine COX-2 inhibitory activity. Data is presented as mean ± SEM of 3-4 experiments. (*),indicates statistically significant difference from respective control group(Blank gel FG2/FG20) using one-way analysis of variance, followed by Dunnett's multiple comparison test (, $p \leq 0.05$, **, $p \leq 0.01$ was considered significant)*

Ex vivo inhibitory activity of the selected gel formulation of Rofecoxib (FG14) or Celecoxib (FG30) was also screened for inhibition of COX isoform (COX-1 and COX-2) in the 28day study in rats. As shown in Table 20 treatment with either Rofecoxib (FG14) or Celecoxib (FG30) did not show any significant inhibition of Thromboxane B2 release (indicative of COX-1 inhibitory activity) from platelets in rat blood collected from rats treated for 28 days upto a dose of 800mg/rat, topically. In this study, COX-2 inhibitory activity was measured in rat blood by challenging with lipopolysaccharide (100µg/ml) for 24 hrs. Prostaglandin E2 (PGE_2) released in the plasma was estimated using a commercially available ELISA kits to determine COX-2 inhibitory activity. As shown in Table 20, treatment with Rofecoxib (FG14) at doses of 240 and 800mg/rat showed a 16% and 45 % inhibition of PGE_2 release, respectively, indicative of COX-2 inhibitory activity at 10 times the ED_{50} dose. However, this effect was not statistically significant. Treatment with Celecoxib (FG30) also produced a similar trend at doses of 240 and 800mg /rat as shown in Table 20.

This indicates that the test gels of FG14 and FG30 even at 10 times the therapeutic doses, dosed for 28 days was not sufficient to show requisite 80% or more inhibition of PGE_2

required to alter the cardiovascular homeostasis in the absence of inhibitory effect on COX-1 derived thromboxane B2. This in part could be due to ~10 fold lower exposure observed with topical administration as compare to oral dosing. Taken together, these results indicate an improved safety for topical gel formulations of Rofecoxib and Celecoxib although not very conclusive. For this purpose a detailed clinical study is require to determine the improved safety for these topical gel formulations.

5.0 Conclusions

There is a huge unmet need in therapeutic management of pain and inflammation. The most commonly used agents such as NSAIDs and the newer class of selective cyclooxygenase -2 inhibitors with improved gastric tolerance have shown severe adverse effects potential resulting in withdrawal of some drugs and change in label for the remaining drugs. This provides an opportunity to explore alternative routes of administration of COX-2 inhibitors with a view to decrease the systemic exposure and thereby minimise the adverse effects. Hence, in this research project we have prepare a series of emulsion gel formulations of Rofecoxib and Celecoxib and evaluated for efficacy in animal models of pain and inflammation. We have also evaluated the safety profile of the optimized gels formulations.

In conclusion in our study we have shown

- Formulation of selective cyclooxygenase inhibitors Rofecoxib and Celecoxib as acceptable and stable emulsion gel formulation.
- Topical application of 1% w/w Rofecoxib and 5% w/w Celecoxib gel formulations resulted in significant anti-inflammatory activity in acute models of the carrageenan induced paw edema.
- Topical application of these gels also resulted in significant analgesic/anti-hyperalgesic activity.
- 1% w/w Rofecoxib and 5% w/w Celecoxib gel formulations were also effective in chronic pain and inflammation.
- Reduction in particle size to sub-micron size improved the efficacy of Rofecoxib and Celecoxib gel formulations as decreasing the particle size resulted in increased surface area of a particulate drug and increased rate of dissolution of the drug thereby resulting in better permeability and percutaneous efficacy. This phenomenon was also demonstrated by Friedman *et al*., 1995, using several topically administered steroidal and non-steroidal anti-inflammatory agents. However, this study for the first time demonstrates the same phenomenon in case of COX-2 inhibitors.
- Application of Rofecoxib and Celecoxib gel formulations resulted in long duration of activity (at least 18hrs) which can present an improved patient compliance.
- In addition to demonstrated efficacy Rofecoxib and Celecoxib gel formulations are well tolerated and show an improved safety profile.
    - A 10 fold decrease in systemic exposure at equiactive doses in rats
    - No skin irritation upon 28 days daily application

        - Gross pathological evaluation of various organs revealed no abnormal finds in any organ with either of the gel formulations at 10 times the therapeutic dose, along with a clean profile in hematological, biochemical or urine analysis.
    - Evaluation of gastric mucosa microscopically also did not reveal presence of any petechiae or frank hemorrhagic lesions after 28 days daily application.
    - Improved cardiovascular liability

COX-2 has been shown to be involved in skin tumor promotion (Muller-Decker *et al*., 1998), and topically applied COX-2 inhibitors have been reported to have beneficial effect in skin tumors in animal models (Pentland *et al*., 1999). Therefore, topical application of coxibs will not only be useful as analgesic and anti-inflammatory agent for management of acute pain and for treating signs and symptoms of osteoarthritis, rheumatoid arthritis but additionally have useful role in cancer chemotherapy. Therefore, the gel formulations of coxibs will find clinical use in

1. Acute and chronic Pain
2. Acute inflammation
3. Rheumatoid arthritis
4. Osteoarthritis
5. Psoriatic arthritis
6. Skin Cancer
7. Protection in skin irritants
8. For patients at very high risk, such as those with a previous gastrointestinal event who are taking aspirin, and those who are taking aspirin plus steroids or warfarin.

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Appendix-I

Sample Chromatograms and standard plots

LCMS- chromatograms

Fig A. Blank chromatogram without Celecoxib or internal standard (Rofecoxib)

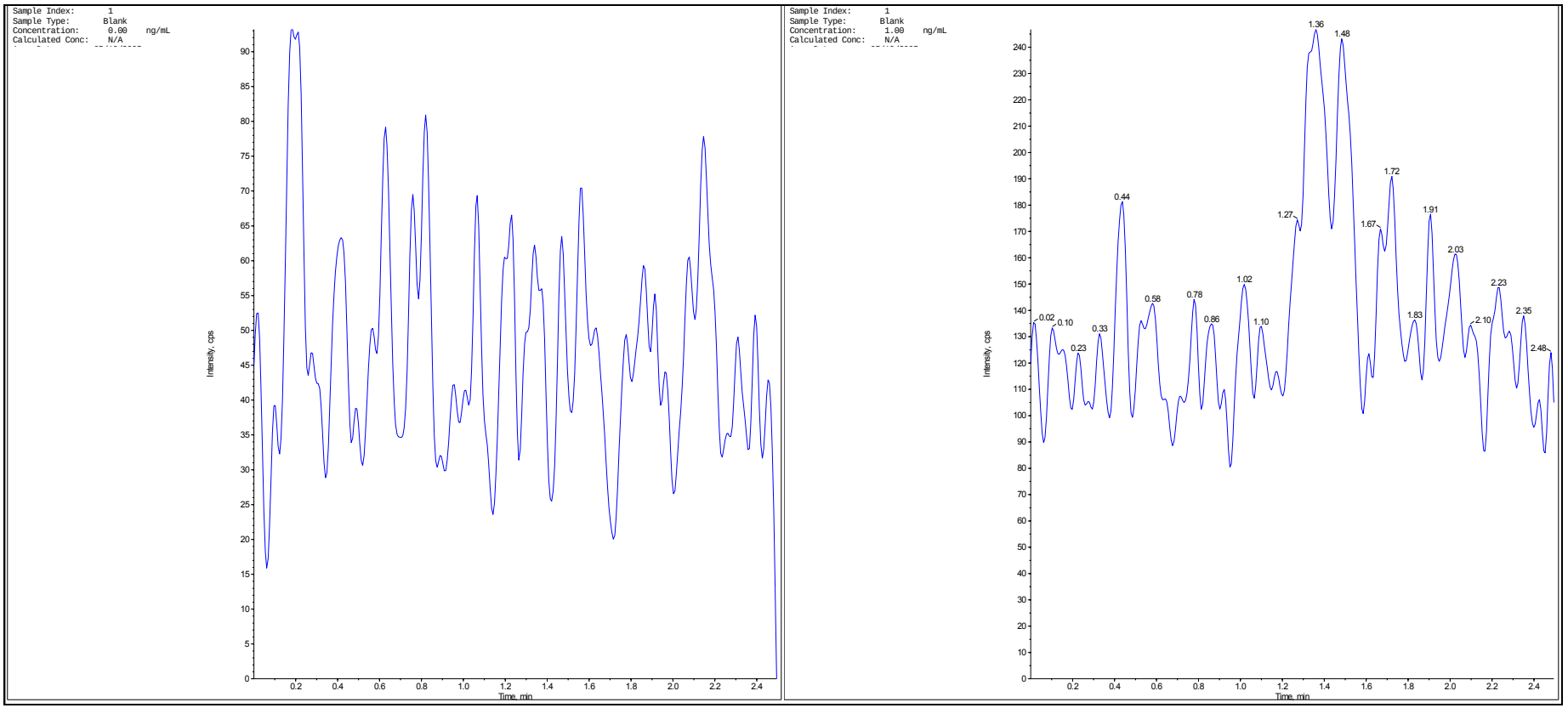


Fig B. Blank chromatogram without Celecoxib with internal standard (Rofecoxib)

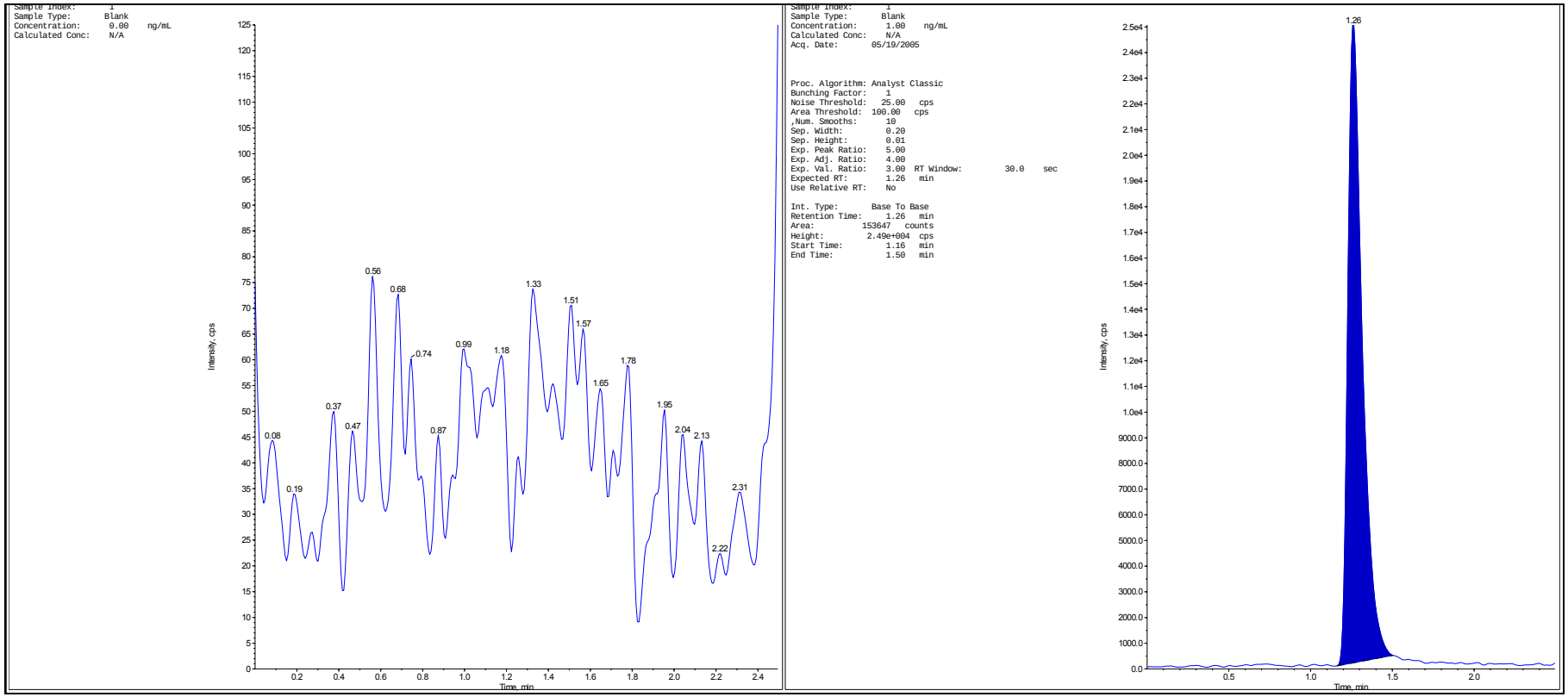


Fig C. Standard chromatogram of Celecoxib (LLOQ-4.75 ng/ml) with internal standard (Rofecoxib)

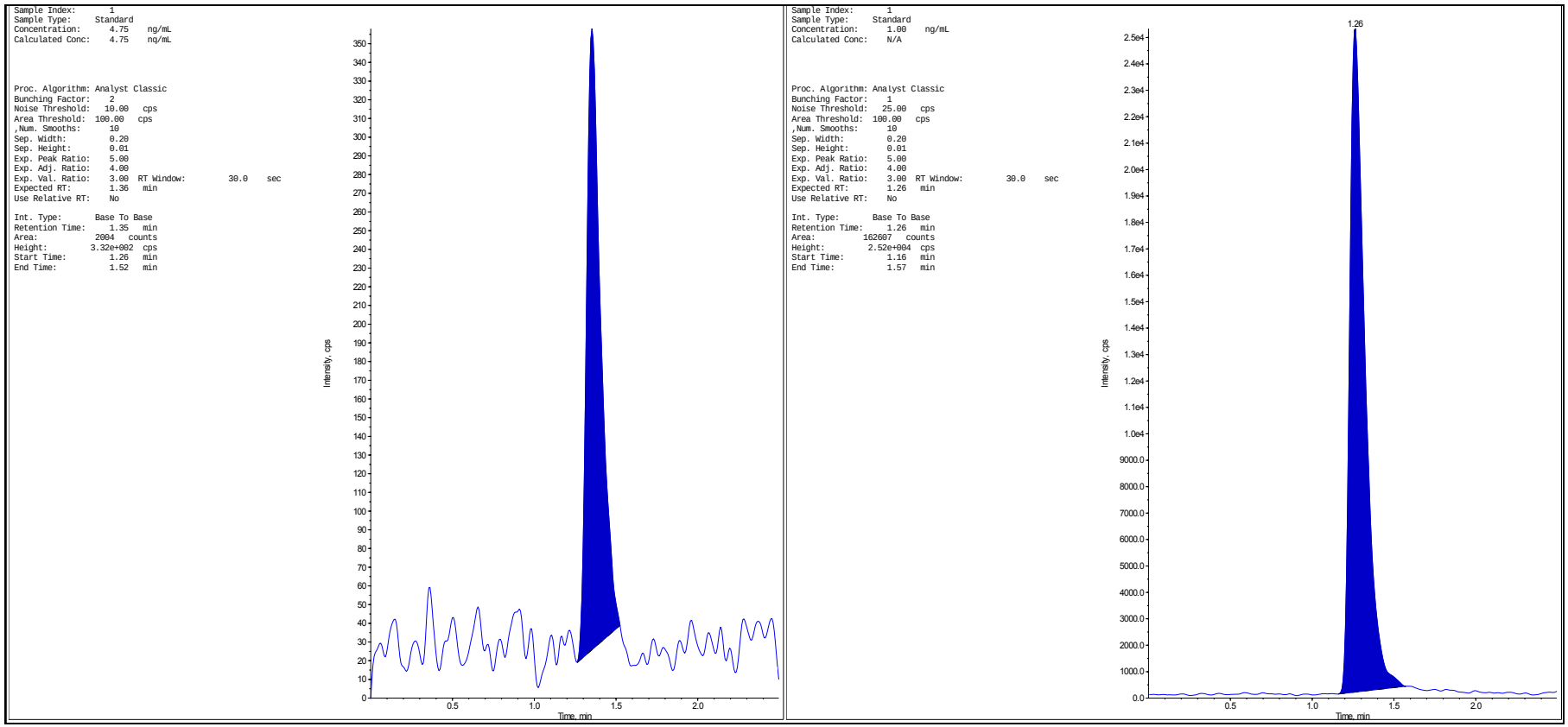


Fig D. Standard chromatogram of Celecoxib (ULOQ-1899.24 ng/ml) with internal standard (Rofecoxib)

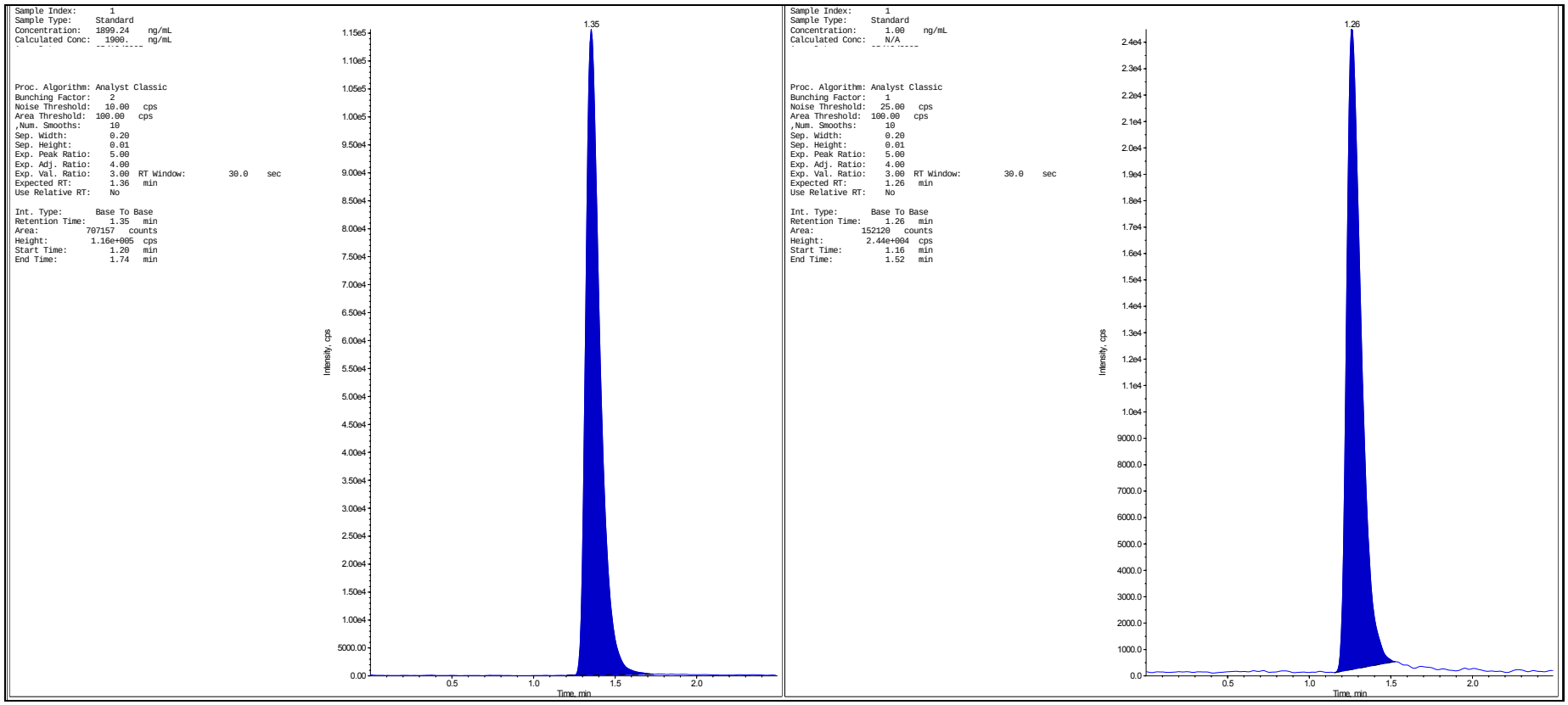


Fig E. Sample chromatogram of Celecoxib with internal standard (Rofecoxib)

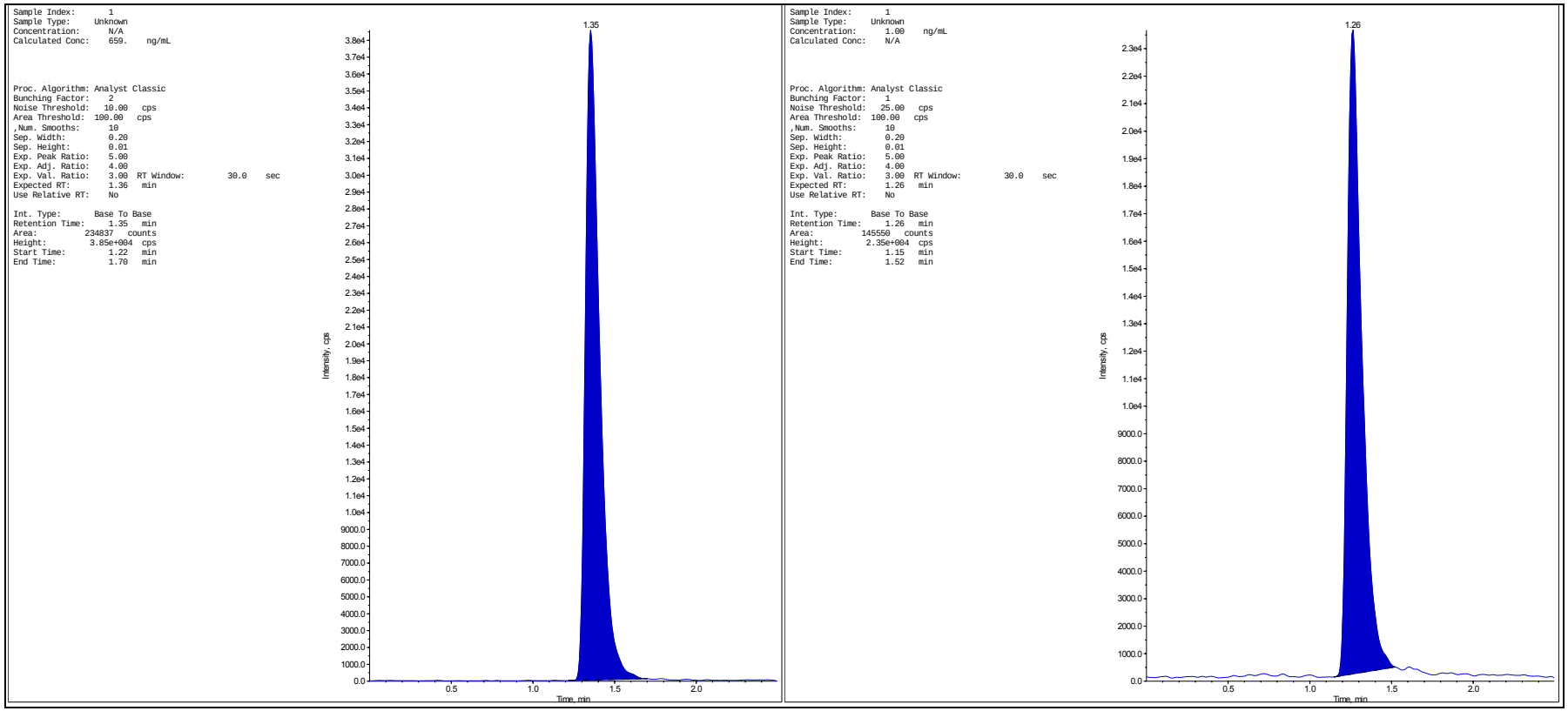
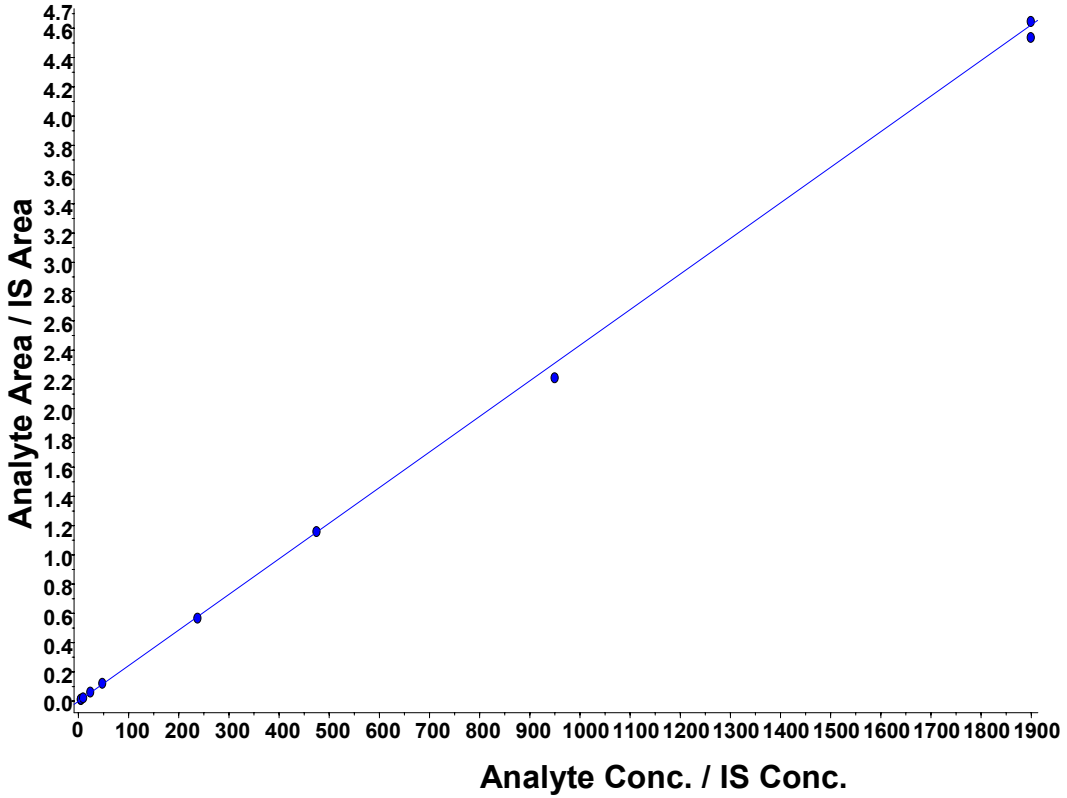


Fig F. Calibration Curve of Celecoxib in Plasma



HPLC – representative chromatograms

Fig G. Blank chromatogram without Rofecoxib or internal standard (Ketoprofen)

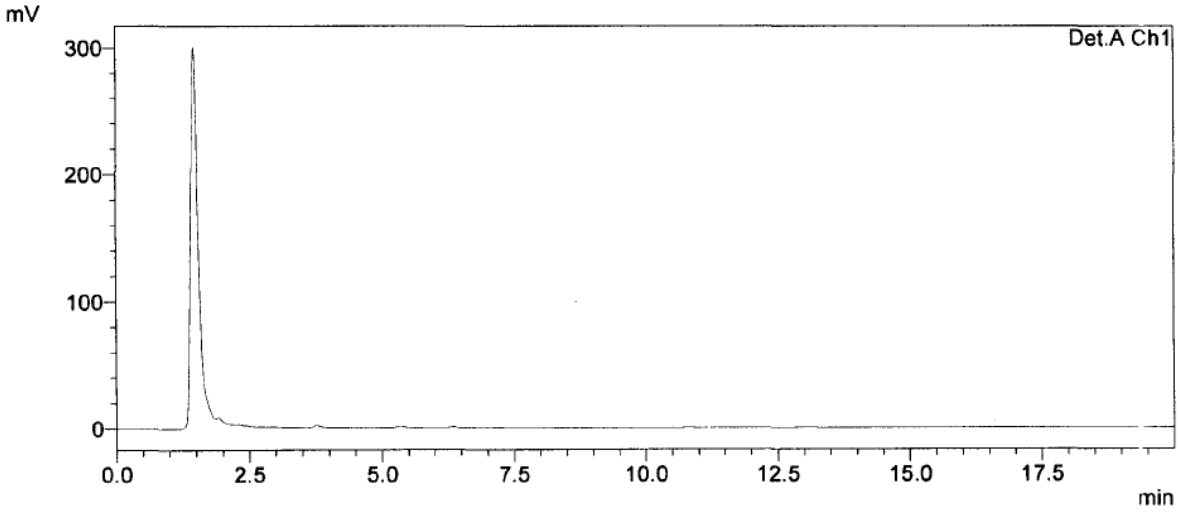
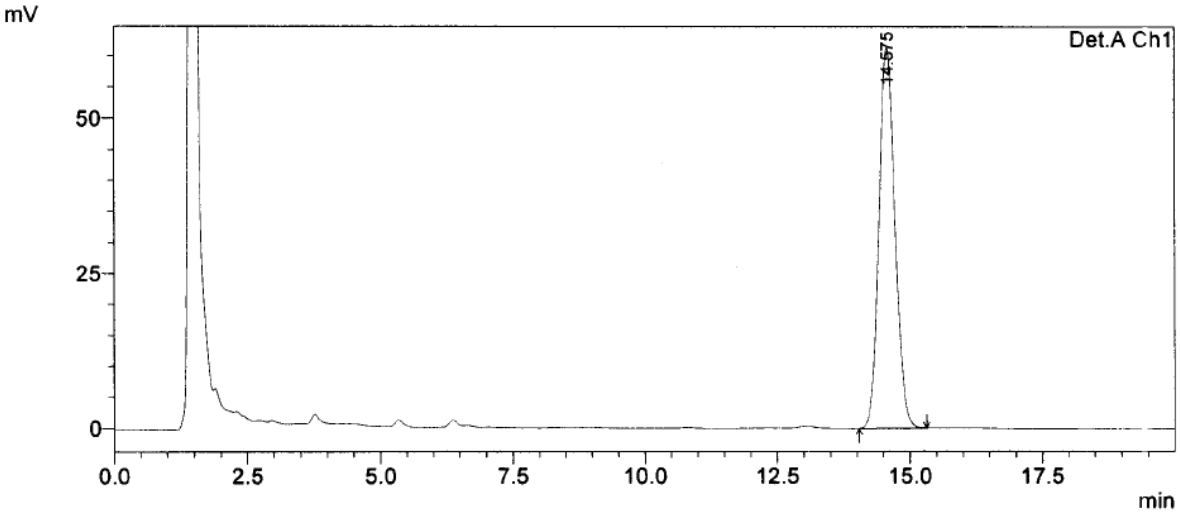
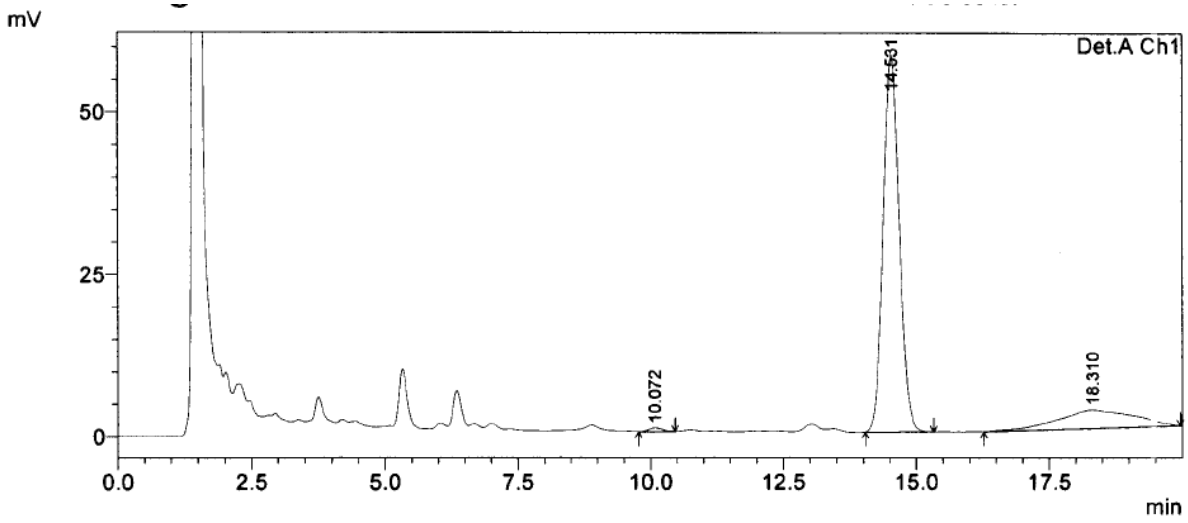


Fig H. Blank chromatogram without Rofecoxib with internal standard (Ketoprofen)



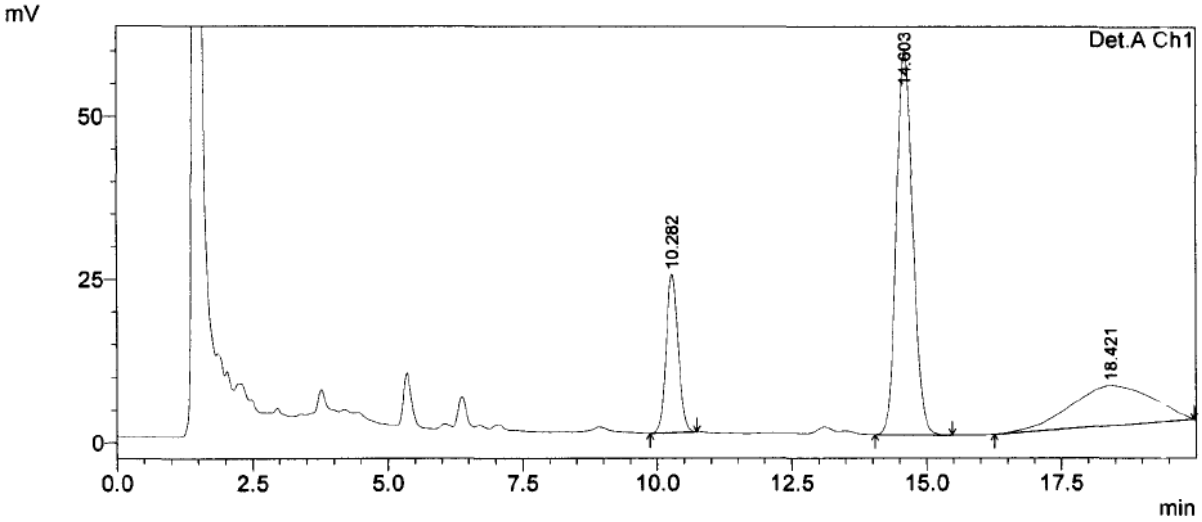
Name	Ret. Time	Area	Conc.
ROFECOXIB	-	-	-
KETOPROFEN	14.575	1295400	-

Fig I. Standard chromatogram of Rofecoxib (LLOQ) with internal standard (Ketoprofen)



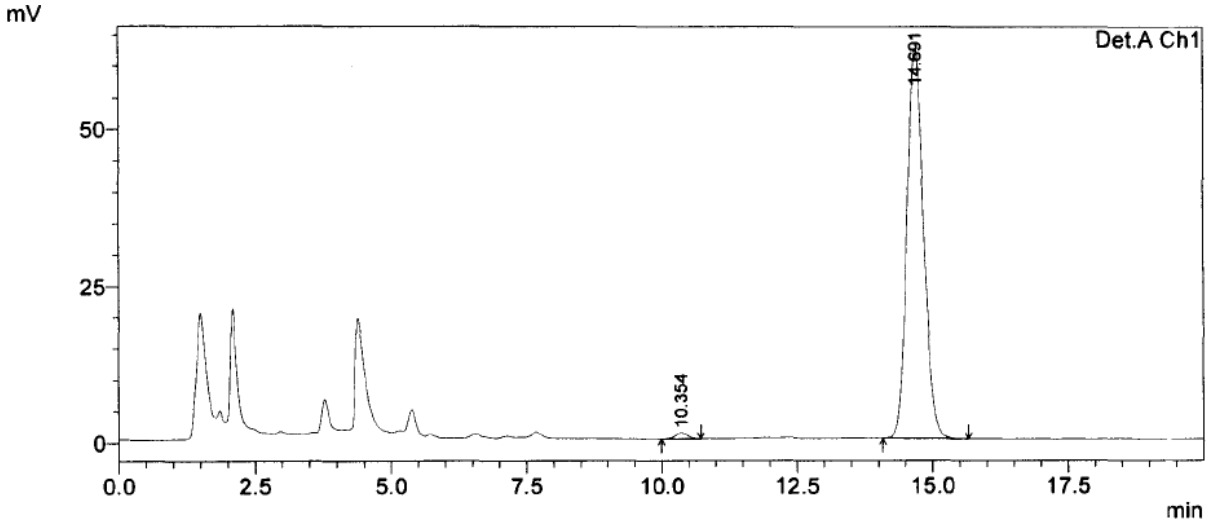
Name	Ret. Time	Area	Conc.
ROFECOXIB	10.072	8623	10.05
KETOPROFEN	14.531	1227493	-

Fig J. Standard chromatogram of Rofecoxib (ULOQ) with internal standard (Ketoprofen)



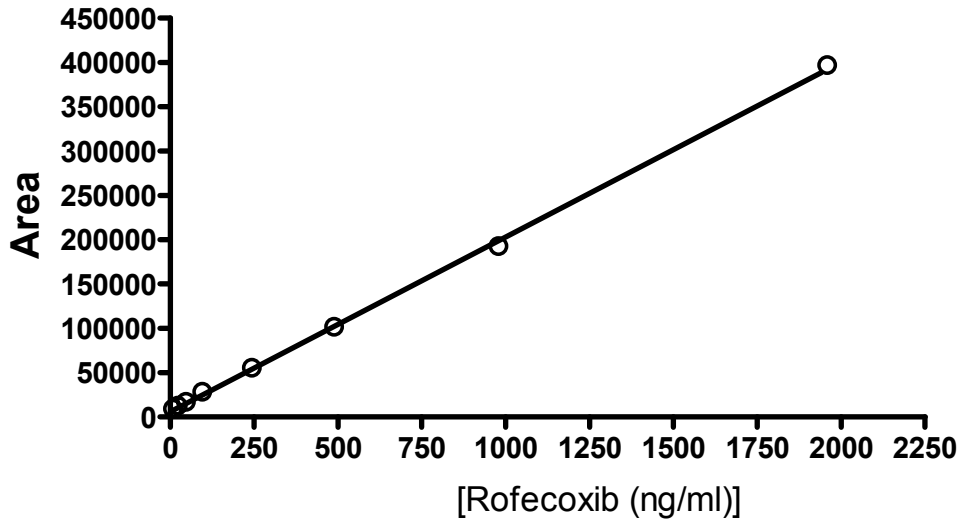
Name	Ret. Time	Area	Conc.
ROFECOXIB	10.282	396318	1960
KETOPROFEN	14.603	1253390	-

Fig K. Sample chromatogram of Rofecoxib (ULOQ) with internal standard (Ketoprofen)



Name	Ret. Time	Area	Conc.
ROFECOXIB	10.354	15232	47.292
KETOPROFEN	14.691	1253390	-

Fig L. Calibration Curve of Rofecoxib in Plasma



Appendix II

List of Publications and Presentation

- Shirumalla R.K.**,** Kumar M., Singla A. K., Arora V.K., Malik R., inventor; Ranbaxy Inc. assignee. *Process for the preparation of Pharmaceutical composition for topical delivery of cyclooxygenase-2-enzyme inhibitors***.** United States Patent US 2005/0049291 (Pub. Date 2005, March 03) (Patent Family - CN1606445, WO 03035080, EP 1448209).
- Shirumalla R.K., Ray A. Evaluation of Sub-micron Topical Formulations of Selective Cyclooxygenase-2 Inhibitors – Celecoxib, for Analgesic and Anti-inflammatory Efficacy as well as Safety Profile. Proceedings of the National Conference on Emerging Trends in Life Sciences Research, Pilani, India, 2009, March 6-7, 55-56.
- Shirumalla, R. K., Sharma, P., Dastidar, S. G., Paliwal, J. K., Kakar, S., Varshney, B., Singh Saini, G., Sattigeri, V., Salman, M. and Ray, A. (2008) Pharmacodynamic and pharmacokinetic characterisation of RBx 7796: a novel 5-lipoxygenase inhibitor. *Inflamm Res* 57, 135-143.
- Shirumalla, R. K., Naruganahalli, K. S., Dastidar, S. G., Sattigeri, V., Kaur, G., Deb, C., Gupta, J. B., Salman, M. and Ray, A. (2006) RBx 7,796: A novel inhibitor of 5-lipoxygenase. *Inflamm Res* 55, 517-527.
- Ravisankar R., Singh S.K., Ramesh S., Sinha S., Nagpal J., Raj Kumar Shirumalla, Ray A. Efficacy of dexamethasone in chronic phorbol ester-induced psoriasis-like lesions in mice. *Indian Journal of Pharmacology* 2008, 40 (supp 2): S155.

Appendix III

Biography of Dr Abhijit Ray

Dr Abhijit Ray obtained his M.Sc. and Ph.D. degree in Pharmacology from University of British Columbia, Vancouver, Canada (1986-1992). After a three year Post-doctoral experience at University of Ottawa, Ottawa, Canada, in the area of molecular pharmacology Dr Ray joined Ranbaxy in 1995. Dr Ray has grown with the organization and presently holds the position of Director and Head of Pharmacology at New Drug Discovery Research, of Ranbaxy Research Laboratories, Gurgaon, Haryana. As a member of discovery team Dr Abhijit Ray has put three molecules in human trials. He has published research article in peer reviewed journals and delivered talk in national and international symposia. He has several internationals patents to his credit.

Biography of Raj Kumar Shirumalla

Mr. Raj Kumar Shirumalla has Bachelor of Pharmacy (B. Pharm) and Master of Pharmacy (M. Pharm) from Faculty of Pharmacy, Jamia Hamdard (Hamdard University), New Delhi. He has been working in Pharmacology department, New Drug Discovery Research, Ranbaxy Research Laboratories, Gurgaon, Haryana since 1996 and is currently working as group Leader in the in vivo pharmacology laboratory. He has actively contributed to several drug discovery programs in New Drug Discovery Research, Ranbaxy Research Laboratories which have resulted in discovery of compound currently in clinical development. He has published research articles in peer reviewed journals and presented papers / posters in national as well as international conferences. He also has several patents to his credit.