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Local and Systemic Gastrointestinal Side effects of Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

By

Tahereh Khazaeinia

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Spring, 2000

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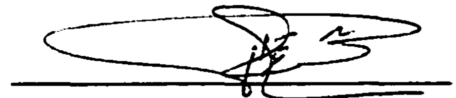

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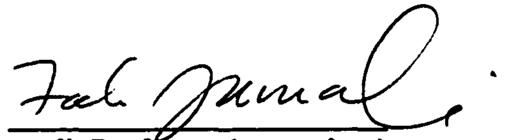

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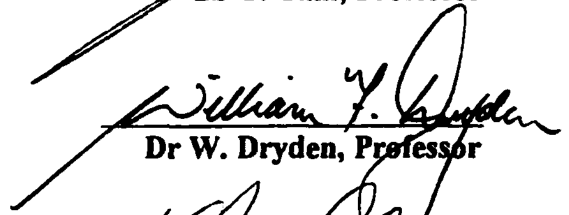

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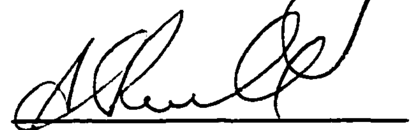

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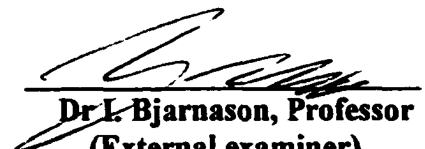

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This thesis is dedicated to:

My brother, G.H. Khazaeinia, for believing in me

and for his constant encouragement, support,

and understanding through my education.

I am forever indebted to him.

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ABSTRACT

The most frequent and important adverse effect of nonsteroidal anti-inflammatory drugs (NSAIDs) is in the gastrointestinal (GI) tract. The objective of this research was to evaluate the importance of the direct local versus general systemic effect in inducing GI toxicity. This was achieved by studying the pattern of increased GI permeability as a surrogate marker of GI toxicity caused by different formulations of either ibuprofen or diclofenac in rat. In addition, the pharmacokinetic and induced GI permeability of diclofenac acid associated with dipalmitoyl phosphatidyl choline (DPPC) were examined and compared with either diclofenac acid or its sodium salt.

Both immediate and sustained release preparations of ibuprofen increased upper and lower GI permeability with no shift of toxicity to the site of drug release. Immediate and sustained release preparations of diclofenac sodium similarly increased upper GI permeability. The induced toxicity in the lower GI tract of sustained release diclofenac lasted longer than that of immediate release preparation. Up to 1 h post-dose diclofenac-DPPC complex and diclofenac acid, in contrast to diclofenac sodium, did not induce a significant increase in upper GI permeability. In the lower GI tract, the induced increased permeability was significant for all formulations. Therefore, for up to 1 h diclofenac associated with DPPC has less toxicity in the upper GI tract. However, the protective effect of the association with DPPC is not pronounced in the lower GI tract.

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Ibuprofen-induced increased GI permeably appears to be independent of the type of formulation, and is mainly due to its systemic effect. Ibuprofen may be a suitable candidate for sustained release formulations since its effect may be prolonged without the danger of a shift of side effects from the upper to the lower GI tract. On the other hand, diclofenac induced toxicity is formulation dependent and is due to both systemic and local effects. There was no particular advantage for using the sustained release formulation. In fact, in the rat, the sustained release preparation of diclofenac caused more increased intestinal permeability than the immediate release preparation. Diclofenac-DPPC complex was devoid of significant toxicity in the upper GI tract, up to 1h post-dose. However, the protective effect of DPPC did not last. NSAIDs are heterogeneous in causing GI toxicity. The induced GI toxicity is due to direct local and/or systemic effects. In developing a sustained release formulation of NSAIDs, knowledge of the extent of contribution of the local exposure to the overall GI toxicity is essential.

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ABBREVIATIONS

°C	Temperature in degree Celsius
^{51}Cr-EDTA	^{51}Cr -labeled ethylendiaminetetraacetic acid
5-HETE	5-hydroxyeicosatrienoic acid
5-HPETE	5-hydroperoxyeicosatrienoic acids
5-LO	5-lipoxygenase
^{99m} Tc-DTPA	^{99m}Tc-diethylenetriaminopentaacetate
AA	Arachidonic acid
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
$AUC_{0\text{-}t}$	Area under the plasma concentration time curve from zero to time t
cAMP	Cyclic adenosine monophosphate
CD	Clusters of differentiation
C_{max}	Maximum plasma drug concentration after single oral dose
CNS	Central nervous system
COX-1	Cyclooxygenase-1, prostaglandin H synthase-1
COX-2	Cyclooxygenase-2, prostaglandin H synthase-2
CPC	Calculated partition coefficient
CSF	Colony stimulating factors
DCBM	Double-contrast barium meal

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DPPC	Dipalmitophosphatidylcholine
DSC	Differential scanning calorimetry
EDRF	Endothelia derived relaxing factor
g	Gram(s)
g	Centrifugal force
GI	Gastrointestinal
GM-CSF	Granulocyte/macrophage-CSF
GTP	Guanine triphosphate
h	Hour(s)
HPLC	High performance liquid chromatography
ICAM	Intercellular adhesion molecule
IFN	Interferon
IFN-γ	Interferon-gamma
IL-1	Interleukin -1
IL-10	Interleukin -10
IL-13	interleukin -13
IL-1ra	Interleukin-1 receptor antagonist
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin -2
IL-4	Interleukin -4
IL-6	Interleukin -6
IL-8	Interleukin-8
ILs	Interleukins

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IV	Intravenous
Kg	Kilogram(s)
L	Liter
LDH	Lactic dehydrogenase
LT	Leukotrine
LTB4	Leukotriene B4
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LTs	Leukotrienes
M- CSF	Macrophage colony stimulating factor
M	Molar
Min	Minute(s)
ml	Mililiter(s)
MPC	Measured partition coefficient
NO	Nitric oxide
NO-NSAIDs	Nitric oxide donors NSAIDs
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
NSAIDs	Nonsteroidal anti-inflammatory drugs
p	Probability of rejecting the null hypothesis when it is true
PAF	Platelet activating factor
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol

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PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H synthase
PGI_2	Prostaglandin I2
PGs	Prostaglandins
r^2	Correlation coefficient
s.c.	Subcutaneous
SEM	Standard error of mean
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
t_{max}	Time to maximal plasma concentration following an oral dose
T_m	Phase transition temperature
TNF	Tumor necrosis factor
U	Unit(s)
UV	Ultraviolet
vs	Versus

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CHAPTER 1

INTRODUCTION

AND

HYPOTHESES

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Arthritis (inflammatory joint disease) is one of the major types of joint disease. This condition is characterized by inflammatory damage or destruction in the synovial membrane or articular cartilage, and by systemic signs of inflammation (such as fever, leukocytosis, malaise, anorexia or hyperfibrinogenemia). Rheumatoid arthritis is the most common systemic inflammatory disease manifested clinically by inflammation of the joints and connective tissues (Goetzl et al., 1971). The drugs used to treat patients with inflammatory disorders are a heterogeneous group with differing chemical and physical properties. The first choice of treatment for most patients is nonsteroidal anti-inflammatory drugs (NSAIDs). The slow-acting anti-rheumatic drugs, such as gold, hydroxychloroquine or methotrexate, are usually added to an NSAID. Penicillamine and sulfasalazine may also be used for rheumatoid arthritis. Corticosteroids are reserved for short-term management of patients with severe limitations of their daily activities while they are waiting for a therapeutic response to slow-acting anti-rheumatic drug, or for patients who fail all other treatments (Schuna et al., 1993).

1.1. THERAPEUTIC USE

NSAIDs are a group of medications with powerful analgesic and anti-inflammatory activities. They are used mainly in the treatment of acute and chronic painful disorders of the locomotor system, inflammation, pain, fever and the prevention of thrombotic events. In the future, NSAIDs may be used more

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widely for the prevention of colonic tumors and polyps, as well as in Alzheimer's disease.

Some NSAIDs have been used extensively in the treatment of patients with migraine, especially in children. In adults, their efficacy is less clear. This is probably due to their poor absorption during a migraine attack as result of a delayed gastric emptying rate. Regular low-dose aspirin® is an effective agent in the prophylaxis of migraine (Buring et al., 1990, and O'Neil et al., 1978), and significantly reduces the recurrence rate (20%) (Buring et al., 1990).

The antipyretic effect of NSAIDs may be due to inhibition of prostaglandin (PG) release in the central nervous system (Vane 1976). In addition, the central action of NSAIDs may be mediated in part by endogenous opioid peptides, or NSAIDs may block the release of serotonin by central secretion of bradykinin (Jurna et al., 1990).

Aspirin and other NSAIDs may reduce the occurrence or progression of colorectal cancers and polyps, and perhaps of other gastrointestinal (GI) tumors (Heath et al., 1994, Rosenberg 1995, Muscat et al., 1995, Thun et al., 1995, and Harris et al., 1995). The basal level of prostaglandin E_2 (PGE_2) in homogenized human colon tumor tissue is higher than that of surrounding normal mucosa (Bennett et al., 1977). The levels of PGE_2 synthesized by the tumors and present in venous blood correlate with the extent of invasion of surrounding tissue, but not with the sites or number of distant metastases (Marnet 1995). Therefore, arachidonic acid products may correlate to colon carcinogenesis by the induction of mutations, enhancement of proliferation, stimulation of

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metastasis, or inhibition of the host immune response (Marnet 1995). NSAIDs, by inhibition of cyclooxygenase activity, reduce the number of animals with tumors, as well as the number of tumors per animal. Non-intestinal tumors may be affected. Animal studies also showed that NSAIDs have anti-tumor effects on the mammary gland (Harris et al., 1995). Furthermore, the potencies of NSAIDs as inhibitors of human colon carcinoma cell proliferation correlated well with their affinities for the alpha-subunit of trifunctional protein of the long chain fatty acid oxidation pathways. Therefore, inhibition of long chain fatty acid oxidation via binding of NSAIDs to the alpha-subunit of trifunctional protein may contribute to the inhibitory effects of NSAIDs on colorectal carcinoma cell growth (Baldwin et al., 1998).

Aspirin may be used to prevent cardiovascular diseases, or their progression. It irreversibly inactivates cyclooxygenase in blood platelets (FitzGerald et al., 1991). Low dose aspirin (75 mg/day) reduces the number of postoperative strokes (Lindbald et al., 1993). The use of low dose aspirin is safe and effective in reducing cerebrovascular events after carotid endarterectomy (Lindbald et al., 1993).

NSAIDs may play a protective role in Alzheimer's disease (McGeer and McGeer 1998). Abnormal processing of the amyloid precursor protein (APP) is thought to contribute to the formation of amyloid plaques in Alzheimer's disease. It has been reported that IL-1 beta increased the maturation of APP, and caused increased processing of the full- length APP isoforms. Indomethacin, by inhibiting cyclooxygenase and possibly lipoxygenase pathways, may block these

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effects (Dash et al., 1995). Patients taking NSAIDs perform better on mental tests (Rich et al., 1995).

1.2. ADVERSE EFFECTS

The adverse effects of NSAIDs may have a number of mechanisms, including; 1) idiosyncratic allergic reactions; 2) propensity for serious repercussions in overdose; 3) physiologic changes, eg., inhibition of prostaglandins (PGs) in parts of the body other than joints (GI, pulmonary and renal toxicity); 4) immunologic abnormalities; and 5) enterohepatic recirculation of certain NSAIDs (O'Brien et al., 1985). The most frequent and important adverse effects induced by NSAIDs are in the GI tract. Renal adverse effects are the next most common. Dermatologic effects are also relatively frequent, followed by central nervous system reactions (Brooks et al., 1991).

1.2.1. HEPATIC TOXICITY

Aspirin and other NSAIDs induce reversible and mild hepatotoxicity in patients with systemic lupus erythematosus (Seaman et al., 1974) and juvenile rheumatoid arthritis (Rich and Johnson 1973). There are mild forms of hepatitis, hepatocellular necrosis and ultrastructural changes in the hepatocytes (Seaman et al., 1974 and Iancu et al., 1976). The cytotoxic NSAIDs decrease hepatocellular adenosine triphosphate (ATP) content. The NSAID-induced decrease in ATP, probably by their uncoupling effects on mitochondrial oxidative phosphorylation, is involved in the hepatotoxicity of the NSAIDs (Masubuchi et

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al., 1998). Clinical manifestations of hepatotoxicity range from anorexia and vomiting, to jaundice and hepatomegaly.

"Liver function tests" may be abnormal: NSAIDs enhance serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase, lactic dehydrogenase (LDH), and total bilirubin. In some instances NSAID-associated hepatic dysfunction may be sufficiently severe to prolong the patient's prothrombin time.

1.2.2. DERMATOLOGIC TOXICITY

Most of the NSAID-associated dermatologic toxicities are mild and reversible as soon as drug is stopped. Rarely, NSAIDs cause fatal reactions, including erythema multiforme, Stevens-Johnson syndrome, or toxic epidermal necrolysis. This is especially the case for some phenylpropionic acids, which have been reported to cause photosensivity reactions (O'Brien et al., 1985). Nonphotosensitivity vesiculobullous reactions induced by piroxicam have also been reported (Stern et al., 1984). Generally, dermatologic toxicity occurs with the NSAIDs with a longer half-life (O'Brien et al., 1985 and Adams 1987).

1.2.3. PULMONARY TOXICITY

From 2 to 20% of adult asthmatics have nasal polyps and hypersensitivity to aspirin, as well as to other NSAIDs. This is apparently induced by the inhibition of synthesis of PG (Samster and Beers 1967). Asthmatic patients may

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experience rhinitis, wheezing and bronchospasm, which may be fatal (O'Brien et al., 1985).

1.2.4. RENAL TOXICITY

Prostaglandins modulate many of the physiologic activities of the kidney, including renal blood flow, glomerular permeability, renin release, tubular ion transport and water exchange (Zambraski 1995). NSAIDs, by inhibition of cyclooxygenase, can cause reversible impairment of glomerular function, sodium retention, edema, hypertension, interstitial nephritis and papillary necrosis, acute renal failure and hyperkalemia (Remuzzi and Remuzzi 1995 and Kleinknecht 1995). The risk factors for NSAID-induced renal toxicity are a past history of renal disease and hepatic disease. Thus, caution must be used when administering NSAIDs in these settings.

1.2.5. CENTRAL NERVOUS SYSTEM TOXICITY

Adverse effects of NSAIDs in central nervous system (CNS) include headache, drowsiness and alterations in mood. The more lipid-soluble NSAIDs readily penetrate the CNS, and may have greater central effects (Netter et al., 1985). Nausea associated with NSAIDs has its basis in the CNS, rather than in the gastrointestinal tract.

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1.2.6. GASTROINTESTINAL TOXICITY

Gastrointestinal (GI) events are the most frequently recognized adverse effects of NSAIDs. They range from abdominal discomfort, to life-threatening bleeding. Serious GI side effects with NSAIDs occur more commonly in the elderly (especially women) than in younger patients. These side effects are also more likely to be fatal in older patients (Committee on Safety of Medicines, 1986).

1.2.6.1. Gastroduodenal

NSAIDs commonly may induce complications such as abdominal discomfort, gastric erosions and peptic ulceration with perforation or hemorrhage on the upper GI tract. The overall ratio of gastric ulceration to duodenal ulceration is approximately 2:1 in chronic NSAID users (Zeidler 1992). About 15 to 20% of patients on NSAIDs develop gastric or duodenal ulcer, and 3% of this group experience hemorrhage or perforation (Lanza 1993). Upper GI mucosal lesions range from trivial injury and superficial erosions, to significant and potentially serious peptic ulcers (Hirschowitz 1994). Dyspepsia may be present in 19% of the patients with a normal mucosa. On the other hand, no dyspeptic symptoms were seen in 91% of patients with abnormal endoscopic findings. Therefore, dyspepsia and other abdominal symptoms do not serve as good indicators of mucosal damage in these patients. Thus, the diagnosis of serious GI pathology often is not made until the patients are seriously ill. After taking

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NSAIDs, acute gastric erosions and petechiae are seen at gastroscopy (20%). There is no correlation between endoscopic evidence of mucosal irritation and clinical symptoms. Particular risk factors for the development of NSAID-induced peptic ulceration are age (60 years or older), past history of peptic ulceration, cigarette smoking, concomitant corticosteroid therapy, alcohol use, and high dose and/or multiple NSAID therapy (Agrawal 1991). Sometimes, the asymptomatic lesions may heal spontaneously (adaptation). Gastric adaptation to aspirin injury involves enhanced cell proliferation which appears to be mediated by increased expression of spasmolitic peptide (a member of trefoil peptides) and transforming growth factor alpha (TGF-α). Rapid up-regulation of COX-2 expression following single and repeated aspirin insults may represent a compensatory response to suppression of prostaglandin generation by this NSAID (Konturek et al., 1998).

1.2.6.2. Small intestine

Recently, it has been shown that the GI side effects of NSAIDs are not limited to the upper region of the tract. Indeed, NSAID-induced abnormalities at the lower GI tract are not uncommon. However, the importance of the toxic effect of NSAIDs on the lower GI tract is often overshadowed by the readily detected adverse effects on the gastroduodenal region (Bjarnason et al., 1993). Up to 70% of patients on long term NSAIDs have evidence of increased intestinal permeability, inflammation, and bleeding in the small and large intestines (Bjarnason et al., 1987a, 1987b, and Sigthorsson et al., 1998). The macroscopic

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appearance of small bowel lesions induced by NSAIDs includes single or multiple erosions or ulcers (Agrawal 1993), leading to hemorrhage, perforation (Langman et al., 1985), or stricture requiring surgery (Bjarnason et al., 1988). NSAID -induced strictures may be broad- based or diaphragm- like in the mid small intestine (Hershfield 1992). NSAID use is associated with an increased incidence of small intestinal ulceration (Agrawal 1993). The maximal effect of NSAIDs is on the midileum (Baerjee and Peters 1990). Inflammation, in contrast to diaphragm formation, is reversible once NSAID therapy is ceased. NSAID-induced intestinal blood loss may lead to iron deficiency anemia (Collins and Adu Toit 1987). Iron deficiency anemia and abdominal cramps and/or severe diarrhea or constipation following long-term slow-release NSAID therapy indicate damage induced by NSAIDs in the small bowel or proximal colon.

The presence of bacterial flora in the near-neutral pH enviroment of the intestine is the major factor in producing ulceration. Antibiotic treatment or administration of NSAIDs to germ-free animals reduces the ulcerogenic effects of NSAIDs (Kent et al., 1969).

1.2.6.3. Colon

The adverse effects of NSAID therapy in the large intestine have been reported from experimental studies, epidemiological data, and from a number of case reports (Aabakken and Osnes 1989c and Halter et al., 1993). NSAID-induced colitis is a significant clinical problem. Many of the patients developing colitis are taking mefenamic acid or flufenamic acid (fenmate colitis). Colitis may

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be detected at an early stage by tests for fecal occult blood. Among elderly patients, bleeding is sometimes assumed to come from diverticula or ischemic bowel disease, although no specific site may be identified. About 10% of patients with newly diagnosed colitis may be related to NSAID administration, and subjects taking NSAIDs are five times as likely as the general population to develop colonic inflammation (Gibson et al., 1992). There is enhanced mucosal permeability, increased tissue myeloperoxidase levels and reduced body weight gain, analogous to idiopathic inflammation bowel disease. The extent of colitis ranges from proctitis to left-sided colitis to pancolitis (Bjarnason et al., 1993). Colitis induced by other NSAIDs, unlike fenmate colitis, is usually symptomatic within days of starting NSAID therapy. After discontinuing the drug, diarrhea stops within days, usually with full histological recovery (Bjarnason et al., 1993).

Ulcerative proctocolitis is associated with taking NSAIDs (Pearson et al., 1983 and Ravi et al., 1986). Several types of colitis have been reported due to NSAID therapy, including; pseudomembranous colitis (diclofenac) (Gentric Pennec 1992), eosinophilic colitis (naproxen) (Bridges et al., 1990), and collagenous colitis (by other NSAIDs) (Giardiello et al., 1990). NSAIDs may also reactivate idiopathic inflammatory bowel disease (ulcerative colitis and Crohn's disease) within few days after starting NSAID therapy (Kaufman and Taubin 1987).

NSAIDs may induce perforation and hemorrhage in the large intestine. The patient with one of these serious complications is more than twice as likely to be on NSAID therapy (Langman et al., 1985). Isolated ulcers and multiple,

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concentric stenoses of the ascending colon have been found in middle aged or elderly patients on long-term NSAID therapy. The lesions are in the vicinity of ileocecal valve, and in most cases the severity of the colitis decreases towards the hepatic flexure (Halter et al., 1993). The characteristics of NSAID-induced lesions in the colon are submucosal fibrosis and low-grade mucosal inflammation (Monahan et al., 1992). The concentric stenoses is similar to diaphragm disease of the small intestine. Slow-release NSAIDs also induce lesions and diaphragms in the ascending colon (Halter et al., 1993 and Huber et al., 1991). The diaphragms have macroscopic and microscopic similarities with congenital duodenal diaphragms, but their location is different (Boyden et al., 1967). NSAID-induced colonic strictures may lead to large bowel obstruction and secondary cecal perforation (Robinson et al., 1995).

Colonic diverticular disease may be present in over one third of people over 60 years, but complicated disease requiring surgery occurs less frequently. However, the most serious complication, generalized peritonitis, is quite uncommon (Krukowski and Matheson 1984). Complicated diverticular disease may be as commonly associated with NSAID therapy (Corder 1987 and Wilson et al., 1990) as is gastroduodenal haemorrhage. Diverticular disease alone is not an absolute contraindication for the use of NSAIDs, but there should be a very good reason for prescribing NSAIDs in these patients (Campbell and Steele 1991).

An increased risk of perforation of the small and large bowel, not necessarily related to diverticular disease, has been found in patients who were

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on NSAID therapy (Langman et al., 1985). NSAIDs impair the ability of the colon to limit or cease inflammatory processes occurring within a diverticulum. Alternatively, NSAIDs may mask symptoms so that patients present with more advanced disease (Corder 1987).

1.3. MECHANISM OF ADVERSE EFFECTS OF NSAIDS ON GI TRACT

Arachidonic acid (AA) is a constituent of cell membrane phospholipids. AA is released in the response to inflammatory stimuli, and its release from phospholipid by phospholipase A_2 is inhibited by glucocorticosteroids. The three major enzymatic pathways of the metabolism of free AA include prostaglandin endoperoxide H synthases (cyclooxygenase, prostaglandin G/H synthases), lipoxygenases, and cytochromes P 450 (Eberhart and Dubois 1995). Prostaglandin H synthases (PGHS) catalyze two separate reactions: 1) a cyclooxygenase reaction, in which AA is converted to prostaglandin G_2 (PGG_2); and 2) a peroxidase reaction, in which PGG_2 undergoes a two-electron reduction to produce prostaglandin H2 (PGH_2). PGH_2 is an unstable intermediate, which is further metabolized to the different forms of biologically active prostanoids (prostaglandin and thromboxane) depending on the type of cell or tissue (Goppelt-Struelbe 1995). The synthesized prostanoids leave the parent cell via carrier-mediated diffusion (Kanai et al., 1995). They then act locally in a paracrine and autocrine manner via G protein-linked receptors, and modulate

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cell and tissue responses in physiological and pathophysiological processes (Smith 1992).

1.3.1. INHIBITION OF CYCLOOXYGENASE

The cyclooxygenase and peroxidase activities overlap within the PGHS (Smith and DeWitt 1995). Mammalian cells contain at least two related but unique PGHS isozymes, referred to as constitutive PGHS-1 (COX-1) and inducible PGHS-2 (COX-2). The two isozymes are about 60% identical (Smith 1992). COX-1 is expressed constitutively in most tissues. COX-1 produces prostaglandins (PGs) that regulate normal gastric function, as well as vascular homeostasis (DeWitt et al., 1993). Orriginally COX-2 was thought to be expressed only in response to cytokines, growth factor or tumor promoters. COX-2 produces prostaglandins involved in inflammation and cell growth (DeWitt et al., 1993). Anti-inflammatory steroids have little or no effect on COX-1, but inhibit the COX-2 (Mertz et al., 1994).

According to the mode of their inhibition of PGHSs, NSAIDs may be classified into three groups: 1) simple, competitive; 2) competitive, time-dependent, reversible; and 3) competitive, time-dependent, irreversible inhibitors of PGHS (Lora et al., 1998). However, there is considerable variation amongst the different NSAIDs in the inhibition of COX-1 versus COX-2 (Mitchell et al., 1993). By inhibiting the activity of the enzyme cyclooxygenase, NSAIDs suppress prostaglandin (PG) synthesis. PGs are physiological modulators of gastrointestinal defence (gastric acid, pepsin, mucus and bicarbonate secretion,

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mucosal blood flow and leukocyte adherence). Furthermore, there is a reasonable correlation between the ability of an NSAID to suppress PG synthesis, and its ability to induce gastrointestinal lesions (Rainsford and Willis 1982).

1.3.1.1. Gastric acid and pepsin

The regulation of gastric acid secretion, and maintenance of the structural and functional integrity of the gastric mucosal barrier, may be influenced by local production of PGs in the stomach. Therapeutic doses of indomethacin enhance basal and histamine- stimulated gastric secretion in normal volunteers, probably by inhibiting systemic PG biosynthesis. The role of indomethacin on gastric mucosal damage probably is a result of the enhanced gastric acid output brought about by cyclooxygenase inhibition in the parietal cell (Levine and Schwartz 1984). This effect is quite variable from person to person (Feldman and Colturi 1984).

NSAIDs enhance proteolytic activity of pepsin on gastric mucus by decreasing the integrity and viscosity of mucus (Kaufman 1989). NSAIDs probably increase acid secretion by stimulating the gastric H^+, K^+-ATPase, which is the pump responsible for acid secretion by the stomach. The proton pump inhibitors, such as omeperazol, are concentrated in the canaliculus of the secreting parietal cell, where they are converted to sulfonamide forms. These inhibit ATP hydrolysis and H^+ transport, resulting in effective, long-lasting regulation of acid secretion (Hirschowitz et al., 1995). It has been reported that

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40mg/day omeperazole protected 85% of subjects from extensive gastric erosions or ulceration induced by NSAIDs, and duodenal injury was eliminated by omeprazole co-therapy (Scheiman 1994). It is not yet reported how effective will be the lower doses of omeperazole or the comparative advantages of a proton pump inhibitor versus a synthetic prostaglandin (such as misoprostol) in gastroduodenal mucoprotection.

1.3.1.2. Bicarbonate secretion

Secretion of bicarbonate into the unstirred layer within the gastric gel mucus adjacent to the epithelium may inhibit the diffusion of hydrogen ions from the gastric lumen into epithelium (Allen and Garner 1980). This unstirred layer may also trap hydrogen ion secreted by the parietal cells and delay its diffusion into the juices in the gastric lumen. For example, the diffusion coefficient for hydrogen ions through a known thickness of mucus is one quarter the diffusion coefficient through an equivalent thickness of unstirred solution (Williams and Turnberg 1980).

Bicarbonate production by the stomach and duodenum is an active, energy-mediated process dependent on mucosal blood flow (Flemstrom and Garner 1982), or as a result of passive diffusion (Hudson et al., 1992). This nonparietal cell alkaline secretion is an important constituent of the pH-mucus alkaline microenviroment, and may play a role in mediating the cytoprotective properties of PGs. Several PGs have been shown to elicit the secretion of gastric bicarbonate in a dose-dependent fashion (Flemstrom and Garner 1982).

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It is also possible that active bicarbonate secretion occurs independent of endogenous PGs (Garner et al., 1979). The NSAIDs reduce active bicarbonate secretion, both *in vivo* and *in vitro* in various experimental animals, by inhibition of PG synthesis. They also reduce the magnitude of the mucus pH gradient. It is likely that indomethacin, as well as other NSAIDs, may induce duodenal mucosal injury, at least in part by reducing mucosal PG production and epithelial bicarbonate (Selling et al., 1987). In several animal studies, this reduction has been reversed by PGs (Flemstrom and Garner 1982).

Aspirin reduces the pH gradient across the rat gastric mucus layer, probably by inhibiting bicarbonate secretion. In humans, indomethacin did not inhibit gastric bicarbonate secretion (Forsell et al., 1985). However, indomethacin decreased duodenal bicarbonate secretion in human duodenal mucosa, and there was a direct relationship between mucosal bicarbonate and PG production (Selling et al., 1987).

Because intrarectal indomethacin produces a similar response to oral administration of NSAIDs, the suppression of mucosal bicarbonate secretion is likely to be due to its systemic rather than topical effect (Selling et al., 1987). However, because the conjugates of indomethacin and the drug itself undergo some enterohepatic cycling, the response to intrarectal administration may still be due in part to its topical effect.

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1.3.1.3. Mucus

The secretion of gastric mucus and bicarbonate create a pH gradient across the adherent mucus gel. This represents the first line barrier against acid damage (Lewis and Piper 1975). PGs elicit the copious secretion of a matrix of glycoprotein molecules, which form a gel. This gel is joined together by physical noncovalent interactions, making it particularly suitable to support hydrogen and bicarbonate ion gradients (Allen and Garner 1980). However, the pH of the cell membrane is slightly alkaline (approximately 7.3), even though the luminal gastric juice has a pH often less than 2. This gradient represents the pH-mucus alkaline microenviroment. A number of studies (Rainsford 1978, Rees and Turnberg 1982) have shown that any one of a variety of NSAIDs inhibits the synthesis of PGs which would otherwise be responsible for mucus production (Lewis and Piper 1975).

1.3.1.4. Mucosal blood flow

Many PGs such as PGI_2, PGE possess potent vasodilatory properties and enhance gastric mucosal blood flow. Adequate mucosal blood flow is one of the most important gastrointestinal protective factors. There should be a balance between the luminal hydrogen ion concentration and mucosal blood flow, because if the mucosal blood flow is decreased, vascular endothelial cell injury will develop (Richti and Shearburn 1977). Several mechanisms have been proposed to explain the means by which enhanced mucosal perfusion could

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protect the gastric epithelium against injury and necrosis. These include: 1) the maintenance of adequate oxygen availability and energy sources to ensure the efficiency of intracellular aerobic metabolites; and 2) the promotion of the more rapid elimination and buffering of back diffused hydrogen ions that have gained access to the lamina propria.

The microvascular injury produced by NSAIDs may be related to the propensity of these drugs to inhibit endothelial cell PG synthesis. This injury may also depend on the capacity of the capillaries in different species to adapt to injury on repeated dosage of these drugs (Ainsford 1983). The fall in gastric mucosal blood flow induced by indomethacin, in doses sufficient to inhibit PG formation in the mucosa, could suggest a role for endogenous PG (or some other product of the PG cyclooxygenase system) in the local regulation of the microcirculation. Cyclooxygenase inhibition by indomethacin may lead to the production of vasoconstrictor metabolites from PG precursors. Alternatively, indomethacin may have inherent vasoconstrictor activity (Flemstrom and Garner 1982), so that small changes could reflect intense focal ischemia. Such areas likely would be the sites of subsequent erosions, especially in the presence of other noxious stimuli such as acid. Moreover, indomethacin delays ulcer healing by reducing blood flow around the ulcerated area (Hirose et al., 1991).

1.3.1.5. Leukocyte adherence to the vascular endothelium

The NSAID-induced neutrophil adherence to the vascular endothelium may occur because of inhibition of endothelial production of prostacyclin.

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Prostacyclin has anti-adhesive effects on circulating leukocytes, and stimulates neutrophil production of leukotriene B4 (LTB4). PGs exert inhibitory effects on various neutrophil functions, including leukotriene synthesis. LTB4 produced by neutrophils likely stimulate the functional up-regulation of the adhesion glycoprotein CD11/CD18. Depletion of neutrophil circulation or treatment with anti-CD18 result in the prevention of NSAID-induced mucosal injury (Wallace et al., 1991).

Endothelial adhesion molecules (ICAM-1, P-selectin) and leukocyte adhesion molecule (CD18) have an important role in indomethacin-induced leukocyte adherence and mucosal injury. The role of E-selectin in these processes is unclear. Anti ICAM-1, anti p-selectin, anti CD18 and anti E-selectin reduce mucosal injury. Only anti E-selectin did not affect indomethacin-induced leukocyte adherence (Wallace et al., 1993).

1.3.2. ENHANCED LIPOXYGENASE ACTIVITY

Another explanation of the NSAIDs-induced gastric mucosal damage is that inhibition of cyclooxygenase can cause diversion of AA metabolism through 5-lipoxygenase (5-LO) to leukotrienes (LTs) and peroxidative derived oxyradical species (Rainsford 1987). There are at least two potentially important consequences of this diversion of AA metabolism: 1) the enhanced production of vasoactive leukotrienes and 5-hydroperoxyeicosatrienoic acids (5-HPETE); and 2) the generation of oxygen radicals during the conversion of 5-HPETE to 5-hydroxyeicosatrienoic acid (5-HETE) in this pathway. Both of these steps could

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be responsible for the vascular and other elements of mucosal cell injury. The evidence for the effects of NSAIDs causing diversion of AA, leading to enhanced lipoxygenase activity, comes from studies showing that inhibitors of 5-LO (Gerkens et al., 1977, and Peskar et al., 1986) and LT antagonists decrease the gastric mucosal damage induced by NSAIDs (Konturek et al., 1988).

1.3.2.1. Leukotrienes

Leukotrienes display pro-inflammatory properties. For example, LTB4, in addition to stimulating aggregation of neutrophils (Ford-Hutchinson et al., 1980), superoxide production (Gyllenhammer 1989), expression of adhesion molecules CD11a/CD18 (Shames and Goetzel 1993) and CD11b/CD18 (Sengelov et al., 1993), promotes leukocyte adherence to vascular endothelium (Hoover et al., 1984 and Gimbrone et al., 1984). The leukocyte adhesion to the endothelium correlates with increased LTB4 concentrations, and adhesion is prevented by inhibitors of the synthesis of leukotrienes (Asako et al., 1992).

Leukotriene C4 (LTC4) could mediate gastric mucosal damage both by its vasoconstrictive actions, as well as its effects on vascular permeability promoting vascular stasis and subsequent reduction in tissue perfusion (Hudson et al., 1993). Leukotriene D4 (LTD4) may also increase neutrophil adherence and microvascular permeability (Goetzl et al., 1983). NSAID treatment in patients with arthritis is associated with increased gastric mucosal synthesis of LTB4, but there are no significant changes in synthesis of LTC4 related to

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NSAIDs (Hudson et al., 1993). This suggests a direct interaction between NSAIDs, the synthesis of LTB4, and mechanisms of subsequent mucosal injury.

However, some data fail to support the hypothesis of augmented leukotriene B4 synthesis following inhibition of PG synthesis by NSAIDs (Wallace et al., 1990a). For example, indomethacin appeared to affect both the cyclooxygenase and lipoxygenase pathway and shifted the balance from protective PG to the pro-ulcerogen LTC4 (Peskar 1991). MK-886, a selective LT biosynthesis inhibitor, corrected this imbalance but failed to inhibit the mucosal damage due to indomethacin. Moreover, both aspirin and indomethacin apparently decreased the *ex-vivo* production of LTC4 (Peskar et al., 1988). These contradictory findings can be explained by many factors: technical problems (Rainsford 1989); mechanisms other than substrate diversion; various eicosanoids arising from different cellular sources; and substrate diversion only occurring in a cell capable of synthesizing LTB4 but not LTC4 (Hudson et al., 1993).

1.3.2.2. Cytokines

Cytokines are a group of peptides with immunoregulatory effects. Cytokines are made by cells that affect the function of other cells. These peptides are different from the growth factors that modulate the proliferation of non-immune cells. However, there is considerable functional overlap between the cytokines and growth factors. Cytokines can be classified as interleukin (IL),

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interferon (IFN), tumor necrosis factor (TNF-α and TNF-β), and hemopoietic growth factor (colony stimulating factor, CSF) (Whicher and Evans 1990).

IL-1, TNF-α, and IL-8 are often grouped together as pro-inflammatory cytokines to distinguish them from the so-called anti-inflammatory cytokines. The anti-inflammatory cytokines include interleukin-1 receptor antagonist (IL-1 ra), IL-4, IL-10 and IL-13 (Dinarello et al., 1993). The most important functions of cytokines are their local effects, modulating the function of adjacent cells (paracrine), or the cell that secrets them (autocrine). There are also significant effects of some cytokines (IL-1, TNF-α and IL-6) on distant organs (endocrine).

Binding of cytokine to the cytokine receptor induces new activities in the cell, such as growth, differentiation or death. The cytokine receptors contain a complex of seven transmembrane domains that interact with GTP-binding proteins. GTP-binding proteins regulate critical enzymes in different signal transduction pathway. Examples of GTP-binding proteins include phosphoinositase C (which leads to mobilization of calcium and activation of protein kinase C) and adenylate cyclase (which catalyzes the conversion of ATP to cAMP). The IL-1 receptor is linked to adenylate cyclase and to protein kinase A, presumably via GTP-binding protein in some cells. cAMP is likely a second messenger (Mitchel 1989) in this process.

A single cytokine may show completely opposing effects under different circumstances. Therefore, it is important to know that cellular regulation by cytokines depends on the circumstances under which the cell is living at a particular time. *In-vitro* experiments with single cytokines or even simple

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combinations of cytokines may be misleading, and measurements of single cytokines cannot show the whole mechanism of action. Furthermore, in many cases there is duplication of function between cytokines. For example, IL-1 shares activities with platelet-derived growth factor (PDGF) on smooth muscle cells, with macrophage colony stimulating factor (M- CSF) on hemopoietic cells, with IL-6 on the induction of acute-phase proteins, and with TNF-α in inducing fever (Whicher and Evans 1990), neutrophila, decreased iron and zinc plasma level (Dinarello 1989). Thus, a change in the level of certain cytokines in a disease process must be interpreted with caution.

Many cytokines are produced by cells within the lamina propria (Madara and Stafford 1989). Conversely, the intestinal epithelial cells express and/or respond to several broadly active cytokines including IL-1, IL-6, TNF, IFN-γ (Brugnara et al., 1986 and Madara and Stafford 1989). The pro-inflammatory cytokines play an important role in the pathogenesis of gut inflammation (Fiocchi 1993). Blockade of IL-1 through the administration of IL-1 ra significantly reduces both colonic inflammation and tissue damage (Cominelli et al., 1992).

1.3.2.2.1. IL-1

IL-1 consists of two structurally related polypeptides, IL-1α and IL-1β, both with a broad spectrum of biological activities. The interleukin-1 receptor antagonist (IL-1 ra) inhibits the activities of IL-1. Most nucleated cells appear able to produce IL-1 when injured, but macrophages produce the largest

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amounts and are probably the key producers in inflammation (Giri et al., 1985). IL-1 stimulates the release of pituitary hormones, and prostaglandin production. IL-1 has also been implicated in the destruction of the beta cells in the β islets of Langerhans, the growth of acute and chronic myelogenous leukemia cells, inflammation associated with arthritis and colitis, and the development of atherosclerotic plaques (Dinarello 1993). IL-1 increases IL-2 production, and promotes T-cell proliferation via increased production of IL-4 (Ho et al., 1987).

IL-1, along with TNF and IL-6, modulate the acute phase response to inflammation. This is an endocrine function that exerts both pro-inflammatory and anti-inflammatory effects, and facilitates the production and recycling of molecules participating in the inflammatory events (Dinarello 1984). IL-1 potentiates the effects of colony stimulating factor (CSF) in inducing hemopoiesis, and IL-1 may be useful for accelerating recovery after chemotherapy (Neta and Oppenheim 1988).

IL-1 reduces the gastric damage caused by indomethacin or aspirin (Wallace 1990b), and may increase the capacity of the gastric mucosa in rats to synthesize PGE_2 by induction of cyclooxygenase gene expression (Robert et al 1991). In addition, IL-1 decreases gastric acid secretion (Uehara et al 1989), inhibits the migration of neutrophils in response to LTB4 (Wallace et al., 1992a), and alters the secretion of mediators from neutrophils (Smith et al 1985).

IL-1 may protect the mucosa through its ability to inhibit the release of pro-inflammatory mediators (e.g., platelet activating factor) and to promote the release of anti-inflammatory mediators (e.g., nitric oxide) (Wallace et al., 1992b).

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IL-1 can also inhibit the ability of neutrophils to respond to chemotactic stimuli and prevent LTB4-induced neutropenia (Wallace et al., 1992a).

1.3.2.2.2. IL-6

IL-6 synthesis occurs in a large number of different tissues. IL-6 has an accessory role in T-cell activation. IL-6 synergizes with IL-3, macrophage-CSF (M-CSF), or granulocyte/macrophage-CSF (GM-CSF) to enhance the growth of hemopoietic progenitor cells. IL-6 is induced by other cytokines such as IL-1, platelet-derived growth factor (PDGF), and GM-CSF (Whicher and Evans 1990). IL-6 may have an important role in corticosteroid release during inflammation: it stimulates release of corticotropin by cultured pituitary cells (Besedovsky et al., 1986), and corticosterone by cultured rat adrenal cortical cells (Salas et al., 1990).

1.3.2.2.3. TNF

TNF is one of the best examples of cytokines functions of which are modulated, induced, and even reversed in the presence of other cytokines. TNF-α, a product of monocytes, and TNF-β, a lymphocyte product, are two cytokines with cytotoxic and anti-tumor properties. TNF-α has a wide range of pro-inflammatory effects, which are similar to and may synergise with those of IL-1. IL-1 induces synthesis of TNF, and vice versa. TNF has a direct action on inflammatory cells including neutrophil degranulation and oxygen radical production (Klebanoff et al., 1986).

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TNF interacts with a high affinity membrane receptor present on the cells of most tissues. This interaction involves activation of phospholipase A2, possibly by a GTP-binding protein, in a manner analogous to the IL-1 receptor (Kull et al., 1985).

1.3.2.2.4. Interferon

The interferons (IFN) are a family of anti-viral cytokines. Interferon-α (IFN-α) is a mononuclear cell product, interferon-β (IFN-β) comes primarily from fibroblasts and epithelial cells, and interferon-γ (IFN-γ) is from T cells. IFN-γ induces the synthesis of IL-1 and TNF, resulting in subsequent production of IL-2 and IFN-γ by T cells.

NSAIDs up-regulate TNF, IFN-γ and IL-2 and IL-12 production. In contrast, NSAIDs down regulate IL-6 and IL-4 expression (Tsuboi et al., 1995). The long-term effect of aceclofenac and diclofenac on the production of a series of inflammatory mediators was studied in a patient with severe knee osteoarthritis. IL-1 beta production decreased at 180 days with both drugs in the group of high producer patients. In a few patients with high basal mononuclear cell TNF-α production, this also decreased on treatment for 180 days with NSAIDs. In the remaining low TNF-α-producing patients, TNF-α production tended to increase. IL-6 synthesis was not affected by aceclofenac while it was diminished by diclofenac. The decrease in IL-6 in all treated patients was significantly correlated with a worsening of the clinical condition (Gonzalez et al., 1994).

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1.3.2.3. Nitric oxide

Nitric oxide (NO) is an important vasodilator and anti-aggregation mediator. NO plays a central role in the physiology of the GI tract, and its response to aggressive factors. In the GI tract, NO is generated in different cell types in the mucosa, submucosa, and muscular layers (Nichols et al., 1993) by three isoforms of nitric oxide synthesis (NOS). The three types of NOS include: type 1 (brain NOS), type 2 (inducible NOS), and type 3 (endothelial NOS). These NOS isoforms may also be classified into two major types (Moncada et al., 1991), constitutive and inducible. NO also may be also produced nonenzymatically in the GI tract by the reduction of nitrite under acidic conditions (Anggard 1994) or by luminal anaerobic microorganisms which use nitrite and nitrate for respiration (Goretski et al., 1990).

Under normal conditions, the calcium-dependent constitutive NOS is present in neural (Barry et al., 1994), endothelial (Nichols et al., 1993) and epithelial tissue (M'Rabet-Touil et al., 1993). The inducible NOS is up-regulated by infection or by cytokines in the gut (Tepperman et al., 1993). In response to chemoattractant cytokines, such as IL-8 produced by activated enterocytes (Eckmann et al., 1993), leukocytes penetrate the vascular wall and infiltrate the mucosa. The leukocytes activated by cytokines express high concentrations of type 2 NOS (McCall et al., 1989), which may contribute to the total NO release from the gut.

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It is important to consider that NO, as a free radical, is highly lipophilic and diffuses freely across membranes (Anggard 1994). Thus, the production and inhibition of NO within one cell population may be expected to have complex effects due to its impact on adjacent tissues. (Stark and Szurszewski 1992).

NO protects the GI mucosa from aggressive factors by 1) increasing the mucosal blood flow (Anggard 1994), 2) inhibiting the accumulation of inflammatory neutrophils via the down- regulation of surface expression of the leukocyte adhesion molecule, CD11/CD18 (Miller et al., 1994), 3) inducing mucus secretion in the stomach (Browm et al., 1993), 4) preventing mast cell activation (Kanwar et al., 1994), and 5) inhibiting the release of certain inflammatory mediators, such as platelet activating factor (PAF) and histamine (Kanwar et al., 1994). On the other hand, excessive NO may directly produce mucosal injury: high concentration of NO; 1) disrupts the actin cytoskeleton, 2) inhibits ATP formation, 3) dilates cellular tight junctions and thereby increases permeability.

Inhibition of NO synthesis augments gastric damage induced by NSAIDs, while NO and NO donor NSAIDs (NO-NSAIDs) reduce the severity of damage in experimental ulcer models (MacNaughton et al., 1989 and Kitagawa et al., 1990). Therefore, NO-NSAIDs exhibit markedly reduced ulcerogenic effects in the rat and rabbit stomach after both single and repeated dosing (Wallace et al., 1994). The NO-NSAIDs suppress PG synthesis in the stomach and peripheral sites of inflammation as effectively as the parent NSAID (Wallace et al., 1994).

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In vitro these compounds have been shown to exert effects on COX -1 and COX-2 comparable with those of the parent NSAIDs (Mitchel et al., 1994). NO-generation from NO-NSAIDs maintains gastric blood flow and prevents neutrophil adherence to the vascular endothelium (Wallace et al., 1994), but it has not yet been established if these effects are attributable to NO generation. Significant increases in plasma nitrate-nitrite levels following NO-NSAIDs administration suggest that these compounds do release NO, although it should be noted that at anti-inflammatory doses, NO-NSAIDs do not affect systemic blood pressure (Wallace et al., 1994). The NO-NSAIDs are also capable of accelerating tissue repair (Elliot et al., 1995).

1.3.2.4. Free radicals

Oxygen free radicals derived from neutrophil leukocytes in damaged gastric mucosal microcirculation are among the most potent ulcerogenic factors related to microvascular derangement. The short-term damaging effect of free radicals is thought to be due to their interaction with unsaturated fatty acids in phospholipids leading to membrane disruption (Freeman and Crapo 1982). Moreover, free radicals (superoxide), which may directly contribute to tissue necrosis, accelerate the inactivation of the endothelium derived relaxing factor (EDRF) (Gryglewski et al., 1986) subsequently identified as NO, responsible for maintaining mucosal microcirculation.

Oxyradicals are generated in the conversion of 5-HPETE to 5-HETE as a consequence of enhanced 5-LO activity by NSAIDs. Evidence for the

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involvement of oxyradicals in NSAID-induced mucosal injury has been derived from experiments in which antioxidants and reducing agents have been shown to have protective effects against NSAID-induced gastric mucosal injury (Rogers et al., 1988). The free radical scavengers abolish indomethacin-induced gastric damage (Del Soldato et al., 1985). Aspirin-induced damage can be reduced by superoxide dismutase or catalase (Pihan et al., 1987).

Superoxide anions might be produced by activation of xanthine oxidase by the increased concentration of AMP relative to ATP and ADP (Granger et al., 1981). Alteration in AMP relative to ATP and ADP arises from the mitochondrial uncoupling effects of NSAIDs (Glarborg et al., 1976a and 1976b), and from the perturbed oxygenation of mucosal tissue by altered blood flow (Gerkens et al., 1977). Some evidence of xanthine oxidase being involved in aspirin-induced gastric mucosal damage was shown by experiments in which allopurinol and a wide range of xanthine oxidase inhibitors exhibited protective effects against aspirin-induced gastric ulcers in rats (Scarpignato 1995).

1.3.3. TIGHT JUNCTIONS

Epithelia are made up of cells joined together by cell junctions. The two routes of movement for solutes across single-layered epithelia are 1) a **transcellular route** that consists of two barriers, an apical and a basolateral cell membrane; and 2) a **paracellular pathway (shunt path)** that can be considered as either a single barrier, the tight junction, or as two barriers, the tight junction and the intercellular space (Powell 1981). Most if not all of the paracellular

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permeability between epithelial cells behaves as if the barrier includes pores with a distinct preference for cations and a size limit for solutes. This preference for cations may be explained by the negative charge nature of paracellular pathways that discourage the passive movement of anions. On the other hand, anion-selective paracellular paths have been found in the rabbit colon and frog skin.

When considering the gastrointestinal tract, it may be useful to classify epithelia in three categories: leaky (gallbladder and small intestine), moderately leaky or moderately tight (colon and gastric antrum), and tight (gastric fundus and esophagus) (Powell 1981). However, some recent findings indicate that rat colon is more permeable to inulin than is the small intestine (Ma et al., 1995). The higher colonic permeability may be caused by differences in mucosal surface area, a solvent drag effect, and by differences in net water fluxes of the colon and small intestine.

There are different types of tight junctions (Figure 1). **Gap junctions** connect adjacent cells through a hexagonal array of cylindrical tubules, and allow the passage of small molecules from cell to cell. There are two types of **desmosomes**: Spot desmosomes (macula adherence) join cells together at specific locations, like spot welds. They protect cells against mechanical stress. Belt desmosomes (intermediate junction or zonula adherens) attach to microfilaments, completely encircle the cells, contract in response to ATP, Ca^{2+} and Mg^{2+} ions, and allow the cell membrane active movement.

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Tight junctions (limiting junctions or zonula occludens) are near the apices of cells. They seal off completely the space between the cells, and attach each cell to its neighbor. Tight junctions act as a diffusion barrier. They are composed of rows of integral membrane protein that form fibrils or strands that meet at the intercellular space to hold cell membranes together.

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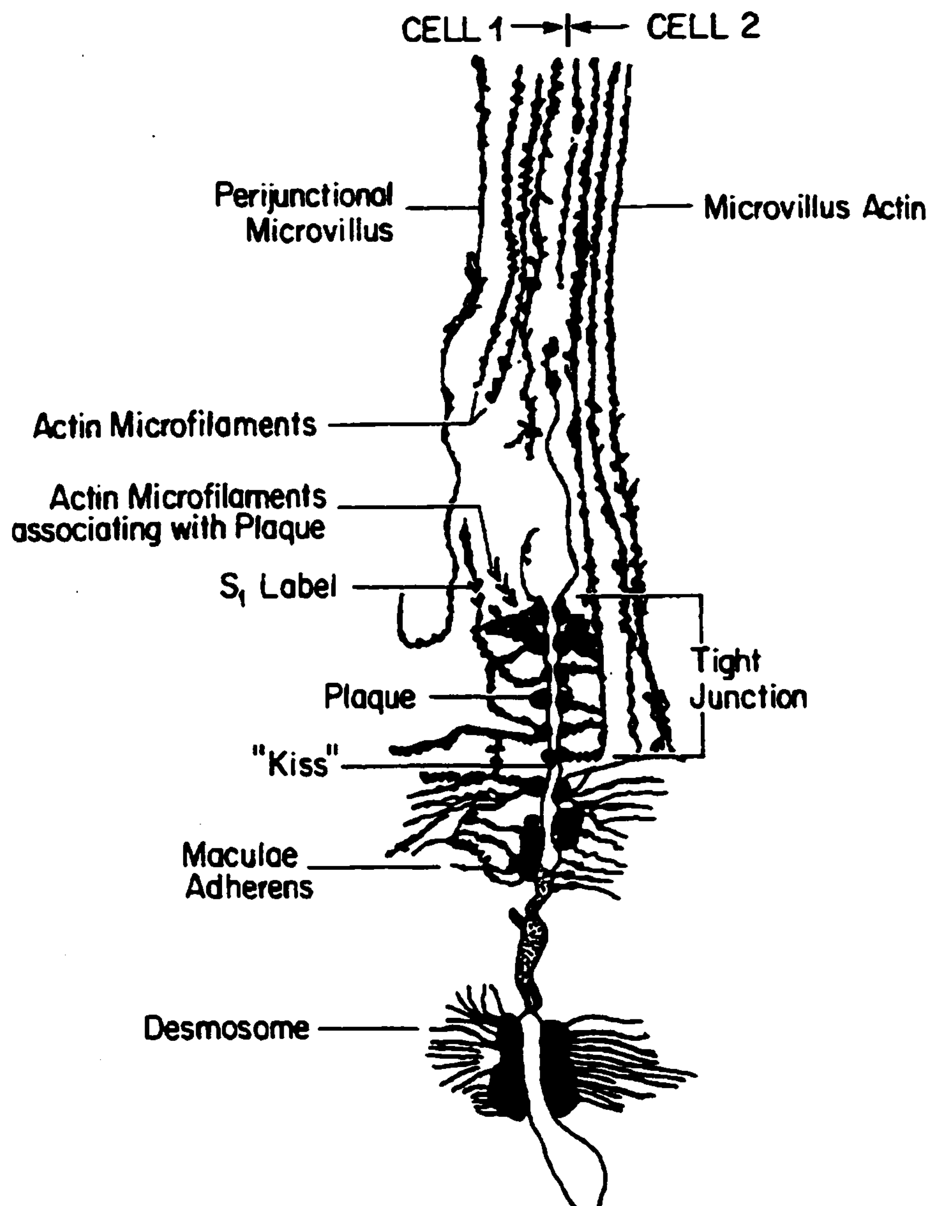

Figure 1: Labled sketch of naked cytoskeleton in zone of ideally sectioned absorptive cell tight junction. Electron-dense plaques intimately associate with intrajunctional 'kisses' on 1 side and with cytoskeletal elements on other (from Madara 1987).

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There is a correlation between the number of fibrils and paracellular resistance (Claude 1978). In general, junctions of tight epithelia are composed of several strands, whereas junctions of leaky epithelia have one or two strands.

The transmembrane proteins associate with similar proteins on neighboring cells to seal the paracellular space. At the tight junction (Figure 2), the paracellular seal is formed by the contact of the transmembrane proteins occludin, which is directly bound to ZO-1 and ZO-2. These cytoplasmic plaque proteins play an important role in organizing the paracellular seal. The expression of ZO-2 is restricted exclusively to tight junctions, whereas ZO-1 is also observed at some types of cadherin based junctions. Conceivably, occludin dissociates from the plaque protein (ZO-1) in some cases in which the paracellular barrier is disrupted (Anderson et al., 1995), such as following the ligation of the common bile duct of rats (Fallon et al., 1995).

Adherence junctions (zonula adherence) are formed by the transmembrane protein cadherin, which associates directly with the cytoplasmic protein α, β, and γ-catenine (Figure 2). Cytoskeletal elements such as actin filaments, containing myosin II, are under the apical junction complex. Most of the actin is concentrated directly under the adherens junction where several actin binding proteins (α-actinin, vinculin and radixine) (Figure 2) are located (Anderson et al., 1995). Although less prominent, actin filaments from the perijunctional ring also attach directly to the cytoplasmic surface of tight junction contacts where occludin is located (Hirokawa and Tilney 1982). Any perturbation

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of the perijunctional actin (for example using actin-disrupting drugs such as cytochalasin) disrupts the paracellular barrier (Stevenson and Begg 1994).

The ATP-dependent movement of perijunctional actin-myosin ring in non-muscle cells is regulated by the intracellular signaling messengers (including diacyloglyceryl, Ca^{2+}, cAMP, and lipid second messengers such as arachidonate), and by leukotriene control of the perijunctional actin-myosin ring (Hall 1994). For example, elevation of intracellular Ca^{2+} may contract the perijunctional actin and increase paracellular permeability (Yamaguchi et al., 1991). Therefore, tight junctions are not static in either structure or function (Madara et al., 1987). Junctional lability may be controlled by some cytoskeletal events such as alteration in perijunctional structure (Madara 1983).

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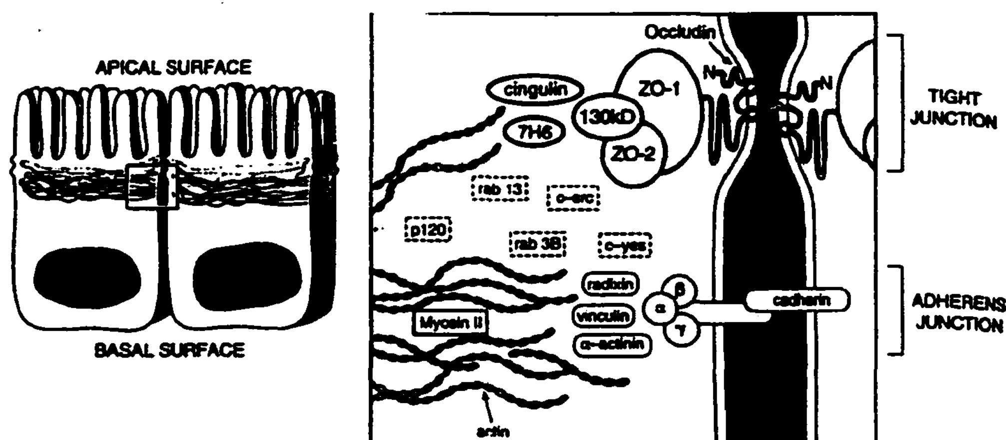

Figure 2: *Left*: tight junctions are positioned as continuous contacts at apical-lateral borders between enterocytes. Boxed region is expanded at right. *Right*: hypothetical model of protein interactions at tight junction and adherens junction of 2 simple columnar epithelial cells. The intercellular barrier at the tight junction is formed by homotypic contacts of the transmembrane protein occludin, which is bound on the cytoplasmic surface directly to ZO-1. The ZO-1/ZO-2 heterodimer binds an uncharacterized 130-kDa protein. Binding interactions of cingulin and the 7H6 antigen are presently undefined. Adherens junctions are formed by homotypic association of the transmembrane protein cadherin, which associates directly with the cytoplasmic proteins α-, β-, and γ-catenin. Several regulatory 'signaling' proteins are localized within the apical junction complex, including c-Src, c-Yes, zeta-protein kinase C, Rab 13, and Rab3b. A thick band of perijunctional actin is positioned under the adherens junction with connection to tight junction plaques (from Anderson et al., 1995).

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There is uncoupling of mitochondrial oxidative phosphorylation following NSAIDs absorption. Following a single dose of indomethacin in rats (Hayler et al., 1991) extensive mitochondrial swelling, as well as distention of the endoplasmic reticulum and the intercellular space has been reported. There was also an increase in the activity of succinate dehydrogenase, alpha-glucosidase (Hayler et al., 1991), and citrate synthase in the segments of duodenum, jejunum and ileum of rats (Somasundaram et al., 1992). This pathogenic change in mitochondrial structure occurs within one hour of NSAID administration, and leads to reduced ATP levels. The decreased ATP levels result in loss of control of the integrity of intercellular junctions with increased permeability (Bjarnason et al., 1993). Another consequence of the uncoupling process is an efflux of calcium and hydrogen ions from the mitochondria, which further depletes ATP stores.

1.4. DETECTION OF MUCOSAL INJURY

Any diagnostic test must be sensitive, specific, safe, and cost effective. In addition, reproducibility of the results and patient convenience and comfort are important factors. Practical considerations such as how long the test takes, how soon the patient can be scheduled, and how soon the results are available, often determine which investigations are used.

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1.4.1. ENDOSCOPY

Endoscopy is used to evaluate the mucosal surface of the esophagus, stomach and duodenum. A variety of factors are necessary for safe and effective endoscopy to be performed. These include well-designed and maintained equipment, a properly trained endoscopist, well-trained and clinically experienced assistants, thorough mechanical cleaning and high-level disinfection of endoscopoic accessories. It is also important for the endoscopist to observe the patient during and after the endoscopic procedure.

Endoscopy is a sensitive and specific method to detect mucosal injury. Its sensivity and specificity for gastric ulcer are 85-95% and 100%, respectively. Its sensivity and specificity for duodenal ulcer are 77-99% and 100% (Dooley et al., 1984). Although endoscopy is well correlated with severe gastroduodenal problems, it has poor correlation for mild gasteroduodenal problems. Endoscopy in comparison with barium meal has a sensivity of 92% versus 54% (Dooley et al., 1984). Endoscopy also has more specificity (100% versus 91%). This superior sensivity and specificity of endoscopy remains when patients with subtle mucosal lesions are excluded from the analysis (Dooley et al., 1984).

Endoscopy is an important tool that permits direct visualization and biopsy of the upper GI mucosa. It is used to evaluate many signs, symptoms and laboratory abnormalities in patients who range from being well to critically ill. Diagnosis by means of endoscopy are better than double-contrast barium meal (DCBM), since it has the support of histology and cytology, and is able to make

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diagnostic use of the colour change of the mucosa in certain inflammatory states.

Contraindications for endoscopy include suspected perforation of a viscus, an uncooperative patient, shock, respiratory distress, or severe injury to the hypopharynx. Upper GI endoscopy is an expensive procedure with the possibility of rare adverse effects such as cardiopulmonary complications, infection, aspiration, drug reaction, bleeding and perforation. These may be clinically serious, particularly in elderly person (Kahn et al., 1988).

1.4.2. GASTROINTESTINAL RADIOLOGY

In the past radiographic studies were the principal diagnostic procedures available for the routine diagnosis of the upper GI tract disease. The overall error rate for single-contrast barium meal has ranged from 18% to 54% (Dooley et al., 1984). The double-contrast barium meal (DCBM) provided the correct diagnosis in 70% of patients, compared with 96% for the endoscopy. The accuracy of the DCBM in routine use does not appear to be significantly different from that reported for the single contrast barium meal. Review of the films by more than one radiologist provides a more accurate final diagnosis, with elimination of some perceptive errors (Dooley et al., 1984).

The accuracy of DCBM is related to the degree of patient cooperation, the ability of the technique to show subtle mucosal lesions, and perceptual and technical difficulties. DCBM is accurate for gross lesions of the esophagus but not for esophagitis or small varices or growths. It is not accurate in the detection

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of subtle gastric lesions, particularly small gastric ulcers or gastritis (Dooley et al., 1984). By the use of low viscosity, high concentration barium and gaseous distention, it is possible to perform routine DCBM in which mucosal surface views are shown. This leads to the detection of smaller lesions and to greater accuracy in distinguishing benign from malignant disease (Kreel et al., 1973). Biphasic examination combines the benefits of both double contrast and single contrast techniques (Dekker and Op den Orth 1988) to get more accurate diagnosis.

The DCBM is a safe procedure with a low complication rate. On the other hand, the DCBM technique is especially unreliable for the examination of the anterior walls of the gastric corpus, antrum and duodenal bulb. The DCBM is time-consuming, and requires more films than a conventional barium examination (Kreel et al., 1973). Barium sulfate in the gut can significantly degrade the image of many other diagnostic examinations. Barium introduces a reflective interface, causing artifactual shadows that obscure anatomy on an ultrasound image, and barium residue inhibits endoscopic inspection of the gut mucosa. Therefore, in many instances the administration of barium contrast media should be withheld until all other examinations are accomplished.

In the past, DCBM was the diagnostic procedure of choice in the field of gastroduodenal disease. At present time endoscopy is a sensitive and specific test, which represents the standard procedure for the detection of mucosal injury. On the other hand, noninvasive permeability tests may offer a better

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diagnostic method that can potentially detect injury, otherwise difficult to visualize with endoscopy.

1.4.3. PERMEABILITY TESTS

On the basis of patient symptoms, laboratory reports, physical signs, or the result of GI radiology, endoscopy may be indicated. Many patients taking NSAIDs are asymptomatic, and are not subjected to endoscopy until complications, such as upper GI bleeding occur. Endoscopy is not suitable as a screening test for NSAID-associated mucosal damage, because it is subjective, time-consuming, invasive, expensive, and available only in specialized centers. Furthermore, endoscopy cannot access most of the small intestine.

Permeability tests are used to assess the mucosal injury by measuring urinary excretion of orally administered probes. These tests are inexpensive, noninvasive, sensitive, specific, quantitative, convenient, and can be used for assessment of both the upper and lower GI tract. Permeability tests are a novel way of screening the GI tract for mucosal injury.

The ideal permeability probe has certain specific physiochemical properties including water solubility, first -order kinetics of permeation, nontoxic, nondegradable, and not metabolized before, during, or after permeating the GI tract. The permeability probes should not be present naturally in urine (or somehow could be distinguished and measured), urinary excretion should be complete following intravenous (IV) administration (without systemic loss), and

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measurement of the probe in urine should be sensitive, accurate, and easy (Chadwick et al., 1977a and 1977b).

1.4.3.1. Gastroduodenal permeability

Sucrose is a disaccharide, which has been suggested as a suitable permeability probe for determination of upper GI toxicity induced by NSAIDs (Meddings et al., 1993). As a disaccharide, it is effectively excluded by the healthy gastric epithelium. On the other hand, sucrose is rapidly degraded by sucrase in the small intestine to its monosaccharide constituents, glucose and fructose. The activity of sucrase is maintained even with extensive small intestinal damage. Intact sucrose can be absorbed only from damaged upper GI tract, primarily the stomach. The unabsorbed sucrose, passing through the small intestine, is rapidly degraded. Therefore, the presence of intact sucrose in the urine indicates absorption of sucrose from damaged upper GI tract. This property makes sucrose a suitable permeability probe for the upper GI tract (Meddings et al., 1995). Less than 1% of the administrated dose of sucose is measurable as the unmetabolized disaccharide in the urine of the control animal (Meddings et al., 1993).

The detection of accumulated sucrose in urine after oral doses reflects the gastroduodenal permeability, and is proportional to the degree of gastric damage. Even severe and extensive small intestine damage distal to the duodenum is not associated with increased urinary excretion of intact sucrose (Meddings et al., 1993). The sucrose permeability correlates well with the

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degree of endospically-detectable damage. It yields specificity of 96.6% and sensitivity of 69% for severe gastritis (presence of more than 10 erosions or mucosal hemorrhages) and 84% for gastric ulcer (lesions having demonstrable depth, with surface at least 5 mm). The sucrose permeability test is insensitive to esophageal and duodenal disease. For example, only 11.1% of esophageal and 23.8% of duodenal disease demonstrated to be present on endoscopy, are detected by the sucrose permeability test (Suherland et al., 1994). In dogs the sucrose permeability test is a sensitive test, with a good correlation of urinary sucrose excretion and gastric damage score (Figure 3) (Meddings et al., 1995). However, in humans, only 15.8% of persons with mild gastritis (presence of less than 10 scattered erosions or mucosal damage) have an abnormal sucrose test. Thus, the sucrose test is not sufficiently sensitive to be useful in human to detect mild gastritis (Suherland et al., 1994).

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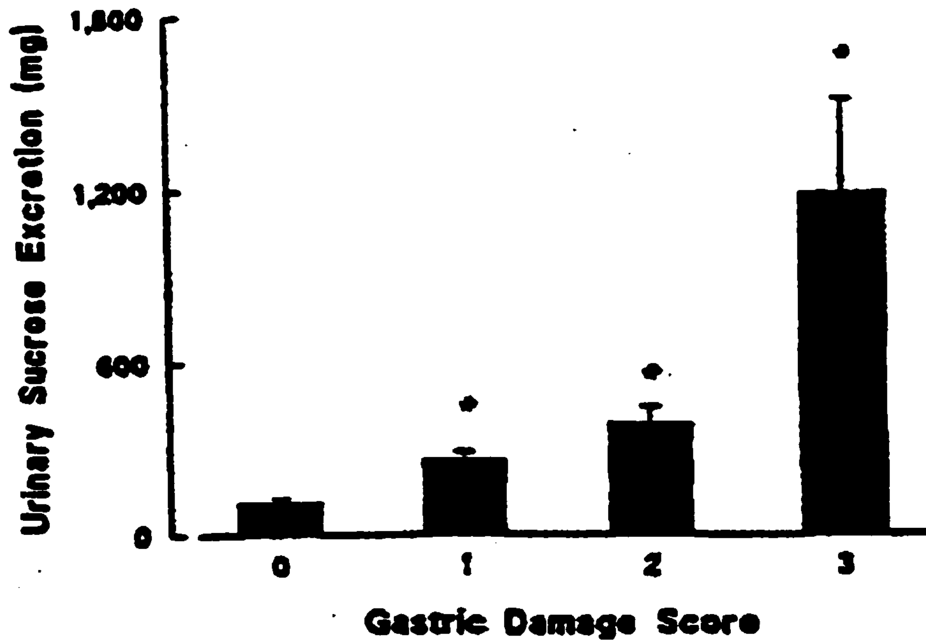

Figure 3: Sucrose permeability. For all endoscopic evaluations of a particular score, mean sucrose permeability is presented with the SEM. Normal stomach was given a grade of 0. Grade-1 damage was given as presence of fewer than 10 erosions or hemorrhagic lesions; grade 2 indicated presence of more than 10 such lesions; and grade 3 was defined as presence of a gastric ulcer. A significant correlation was observed between endoscopic grade and sucrose permeability. * $p<0.05$ grade 0 (from Meddings et al., 1995).

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Determination of increased sucrose permeability is useful, simple, inexpensive, noninvasive, and readily accepted by patients. Increased sucrose permeability is useful in predicting the presence of clinically significant gastric disease, and following patients at risk of developing upper GI disease such as NSAID- induced gastropathy (Meddings et al., 1995). Furthermore, healing of the gastric lesions can also be monitored by sequential measurements of sucrose permeability. Sucrose permeability decreased more rapidly than the disappearance of gastric ulcers, suggesting that this technique is more sensitive to the presence of generalized mucosal damage than it is to the presence of endoscopically visible ulceration (Meddings et al., 1995).

Sucrose permeability has a sensivity of 84% in gastric ulceration, which is reasonable for a screening test, and compares favorably to that reported for upper GI radiology. In comparing double contrast barium studies with endoscopy, it has been reported that the sensitivity of radiographic techniques for gastric ulcer is 44-91% with specificity of 93-99% (Dooley et al., 1984). The sensivity of endoscopy for gastric ulcers is 85-95% with specificity of 90-100% (Dooley et al., 1984). The sensitivity of GI radiography is insignificantly different from those found with sucrose permeability, close to a 70% sensitivity of severe gastritis and 84% sensitivity of gastric ulcer are also reasonable and probably superior to radiographic techniques (Suherland et al., 1994).

Gastric damage in animal models can be also detected by measuring increased sucrose permeability (sensitivity 84%). The rat is a suitable model for studies of GI permeability, and may be useful in the preclinical screening of

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NSAID preparations as well as in the detection of other GI abnormalities (Davies et al., 1994).

1.4.3.2. Intestinal permeability

NSAIDs damage tight junctions, which leads to increased intestinal permeability. NSAIDs may increase permeability in the small intestine directly during absorption, systemically after absorption, and/or by re-exposure after biliary excretion. Concentration of an active NSAID above a threshold, which is unique for each NSAID, either after ingestion or biliary excretion, is needed to increase small intestine permeability (Bjarnason et al., 1992). Intestinal permeability seems to be a more sensitive index of gut toxicity than is the area of ulceration when comparing NSAID potency. The measurement of intestinal permeability is easier, less time consuming than other methods, and presents a useful indicator of NSAID-induced intestinal permeability (Ford et al., 1995).

The common probes for assessment of intestinal permeability are various polymers of polyethylene glycol, monosaccharides (L-rhamnose and mannitol), lactulose, ^{51}Cr -labeled ethylendiaminetetraacetic acid (^{51}Cr-EDTA), ^{99m}Tc-diethylenetriaminopentaacetate (^{99m} Tc-DTPA), as well as using a disaccharide and monosaccharide together to obtain a specific index of intestinal permeability.

There are two pathways for crossing the intestinal mucosa: paracellular (the permeation of lactulose and ^{51}Cr-EDTA across the intestine occurs exclusively through the intercellular junction), and transcellular. Transcellular

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pathway can be divided into two subgroups, transcellular 'aqueous' (L-rhamnose), and transcellular 'lipid' (poly ethylene glycol 400) (Maxton et al., 1986). The possible paracellular versus transcellular route of polyethylene glycol (PEG) permeation is dependent on its possible lipid solubility and its geometric shape. The above pathways are concluded from the study, which has shown hyperosmolarity has different effects on the permeation of different probes. Hyperosmolarity increases the permeation of PEG 400, lactulose and ^{51}Cr-EDTA, whereas it does not increase the urinary excretion of L-rhamnose (Maxton et al., 1986 and Bjarnason et al., 1994). The precise mechanism of the osmotically induced, increased intestinal permeability is uncertain (Bjarnason et al., 1994). It is possible that the hyperosmolar solution may affect the permeation by the separation of obliterated intervillous spaces and increasing the total available mucosal surface area (Wheeler et al., 1978). Alternatively, the hyperosmolar solution may induce the influx of fluid and increase the intestinal surface exposed to the hyperosmolar solution (Laker and Menzies 1977). The sensitivity of lactulose and ^{51}Cr-EDTA increases with hyperosmolarity (Bjarnason et al., 1994). It is important, however, when using permeability probe solutions that both control and test conditions have the same osmolarity.

1.4.3.2.1. Ethylene glycol polymers

Polyethylene glycols (PEG) have the general formula HO (CH2CH2)$_n$ OH with an n value from 4 to about 150. The most widely used PEG as an intestinal probe is PEG 400, with a molecular mass of about 194-502 (Bjarnason et al.,

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1995). Five to 10 grams of PEG 400 is taken orally after an overnight fast in a fixed volume of water (150 ml), and urine samples are collected for 5-6 hours. Extraction of PEG polymers is time consuming, but separation and quantitation is readily achieved by gas or high-pressure liquid chromatography.

Whereas PEG 400 meets many criteria for being an ideal probe, it has a rather unpleasant taste, as well as variable and low urine excretion (25.9-68.5%) after IV administration (systemic loss) (Maxton et al., 1986). The use of PEG as permeability probes continues to be questionable (Bjarnason et al., 1995).

There is a significant correlation between the permeation of lactulose and ^{51}Cr-EDTA, but not between PEG 400 and either L-rhamnose, lactulose, or ^{51}Cr-EDTA (Maxton et al., 1986). Therefore, ^{51}Cr-EDTA and lactulose are both sensitive markers of permeability, with ^{51}Cr-EDTA giving the most reproducible results (Ford et al., 1995).

1.4.3.2.2. L-Rhamnose and Mannitol

L-rhamnose and mannitol are both used as intestinal probes. Although mannitol has many properties of an ideal probe, it has small metabolic losses after IV administration, and exists naturally in the urine of normal subjects. The presence of natural mannitol in urine may be due to diet, endogenous production, or release from dietary fiber by the action of colonic bacteria. In addition, mannitol is susceptible to degradation by intestinal bacteria, particularly in patients with small intestine bacteria contamination (Flaker et al.,

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1982). Mannitol has little affinity for facilitated transport systems, and is generally considered to be a transcellular marker *in vivo*. It was included to be a marker for physiological changes other than NSAID-induced variations in mucosal paracellular permeability, such as altered gastrointestinal motility and renal function (Maxton et al., 1986).

L-rhamnose resists metabolic degradation in humans. However, recovery of L-rhamnose after IV administration is less than that of lactulose or ^{51}Cr-EDTA (71.5% versus 92.7% of lactulose and 97.4% of ^{51}Cr-EDTA). Mannitol and L-rhamnose cross healthy human GI mucosa more than lactulose and ^{51}Cr-EDTA (Maxton et al., 1986) and its absorption is about 40 times greater. Therefore, these are not ideal permeability probes.

1.4.3.2.3. Lactulose

Lactulose is a water-soluble disaccharide, which has characteristics of an ideal probe. This molecule is not degraded within the small intestine, and appears to cross the epithelium at sites of damage or through the paracellular pathway (Maxton et al., 1986). The percentage recovery of lactulose after intravenous injection is complete (Maxton et al., 1986). The excretion rate of lactulose correlates with small intestine damage in diseases such as celiac (Menzies 1974), Crohn's disease (Sanderson et al., 1987a and 1987b), and NSAID-enteropathy (Bjarnason et al., 1992).

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Increasing the amount of lactulose more than 5 g decreases the analytical sensitivity. Intestinal permeability of lactulose is similar to ^{51}Cr-EDTA, but less reproducible (Ford et al., 1995).

1.4.3.2.4. L-Rhamnose-Mannitol or Lactulose-Mannitol

Urinary excretion of a single oral probe is dependent on several pre- and postmucosal factors other than mucosal integrity. Some variables such as GI motility, blood flow, renal function may affect the results of the permeability tests. In order to increase the sensivity and specificity of the permeability test, the urinary excretion ratio of two probes may be used to obtain a specific index of intestinal permeability. The ratio of a disaccharide (lactulose) and a monosaccharide (mannitol) or two monosaccharides (L-rhamnose-mannitol) are usually determined. The lactulose-mannitol ratio is consistent with small intestinal damage (Meddings et al., 1993). However, the analysis of these sugars is time consuming and tedious. Furthermore, as discussed below (1.4.3.2.5), there are other reasons why a single probe may be suitable, especially when the single probe is not a disaccharide or a monosaccharide.

1.4.3.2.5. ^{51}Cr-EDTA and ^{99m} Tc-DTPA

^{51}Cr-EDTA and ^{99m} Tc-DTPA are widely used to test intestinal permeability. The radiolabeled probes are administered after an overnight fast (Bjarnason et al., 1995), and are measured easily and quantitatively in urine. It is more convenient to use ^{51}Cr-EDTA as a probe than ^{99m} Tc-DTPA, since ^{51}Cr-EDTA has

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a 27 day half-life, and the stock solution can be used for about 40 days. In contrast, doses of ^{99m} Tc-DTPA have to be prepared individually, and urine samples must be analysed quickly after collection (Bjarnason et al., 1995).

^{51}Cr-EDTA is a hydrophilic molecule, which is poorly absorbed in healthy small intestine. Unlike sugar probes, ^{51}Cr-EDTA is resistant to bacterial degradation in the lower intestine, and is suitable for assessing permeability in colonized regions of the gut. To test intestinal permeability changes-induced by NSAIDs in animal models, usually 10 μCi ^{51}Cr-EDTA is administered following the dose of NSAIDs. Collected urine samples are counted by a gamma counter for 1 minute in a window scanning within a range of 0-2 Mev. Relative permeability is determined by calculating the activity present in each urine sample as a percent of the administered dose after correcting for background radiation (Davies et al., 1994). The sensivity of analysis of ^{51}Cr-EDTA was 0.03% of the administered dose per liter of urine, and the precision was between 1 and 6%. The percentage recovery of ^{51}Cr-EDTA was almost complete (97.4%) (Bjarnason et al., 1995).

Lactulose and ^{51}Cr-EDTA are the most sensitive markers of NSAIDs-induced permeability changes, and give similar results, although ^{51}Cr-EDTA permeation measurements are more reproducible (Ford et al., 1995). Standard deviation for ^{51}Cr-EDTA ranges from 38% for 200 mg/kg (S+) ibuprofen to 61 % for drug vehicle only, whereas standard deviation of lactulose permeation for these treatment are 85% and 96%, respectively. This is because the enzymatic assay of lactulose introduces more error into the determination of marker

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permeation (Ford et al., 1995). The ^{51}Cr-EDTA permeability changes caused by NSAIDs correlate well with ulceration and GI blood loss (Ford et al 1995) (Figure 4).

^{51}Cr-EDTA is the prefered intestinal permeability probe. ^{51}Cr-EDTA has poor absorption, after oral dose about 0.5-1.1% of ^{51}Cr-EDTA is excreted in urine, and is resistant to bacterial degradation. Using ^{51}Cr-EDTA as a permeability probe is very easy, convenient, inexpensive, noninvasive, and results are quantitative and more reproducible. Therefore, ^{51}Cr-EDTA is a suitable and sensitive probe, which can be used as a single probe, for permeability changes induced by NSAIDs.

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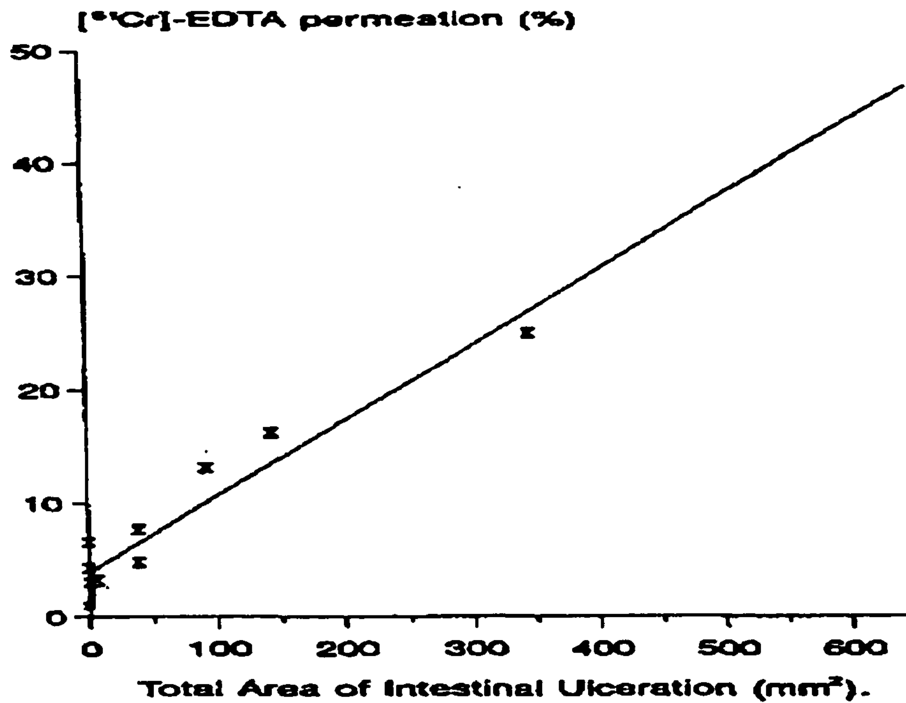

Figure 4:Graph of intestinal permeability (urine recovery of ^{51}Cr-EDTA as a percentage of oral dose) against area of intestinal ulceration (mm^2) for piroxicam (from Ford 1995).

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1.4.4. INTESTINAL INFLAMMATION

NSAID-induced permeability changes lead to inflammation of the small intestine (NSAID-induced enteropathy) in two-thirds of patients on long-term treatment with NSAIDs. The intestinal inflammation may persist for up to 16 months after withdrawal of NSAID. The mechanism of inflammation is not known. Possibly by increasing the intestinal permeability, NSAIDs may expose the mucosa to luminal bacteria as well as other luminal content which may have aggressive effects and produce inflammation (Bjarnason et al., 1987a). Although the permeability changes are seen within hours of taking NSAIDs, the inflammation may take longer to develop. However, there have been no prospective or sequential studies to see how quickly inflammation occurs (Bjarnason et al., 1993). After NSAID withdrawal, permeability changes return to normal quickly in the absence of inflammation. On the other hand, permeability changes accompanied by inflammation persist with the inflammation. The long-standing abnormalities of intestinal permeability are seen in celiac and Crohn's disease (Bjarnason et al., 1994). The inflammation may be associated with bleeding, protein loss, ileal dysfunction, and strictures (Bjarnason et al., 1993).

The clinical grading system for patients with idiopathic inflammatory bowel disease (Saverymuttu et al., 1983b) is:

Mild: patients with mild symptoms, bowel frequency once or twice daily, no recent weight loss, and minimal abnormality or normal appearance at sigmoidoscopy in the patients with proctitis (Saverymuttu et al., 1983b).

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Moderate: patients with intermittent abdominal pain, bowel frequency three to five times a day, weight loss less than 3 kg, sigmoidoscopy showing either a granular or reddened mucosa in patients with proctitis.

Severe: patients with continuous abdominal pain, bowel frequency more than five times per day, weight loss greater than 3 kg (Saverymuttu et al., 1983b).

With using ^{111}In-labeled neutrophils, it is possible to localize by scintigram and to measure by gamma counter the intestinal inflammation in patients on NSAIDs. The presence of leucocytes in the feces indicates infiltration of leucocytes in the gut, and is characteristic of acute inflammation of the intestine. Once the inflammation is established, ^{111}In-labeled leucocyte excretion may be increased for up to 16 months after discontinuing NSAID therapy (Saverymuttu et al., 1983b). All commonly used NSAIDs are associated with inflammation, and most of the patients with inflammation have received a number of different NSAIDs in the past.

After injection of ^{111}In-labeled neutrophils, feces are collected for 4 days for the determination of indium 111 excretion. Gamma counting of feces permits a quantitative assessment of the leucocyte excretion. The fecal excretion of indium 111 increases progressively with disease severity (Saverymuttu et al., 1983b). The 4-day fecal excretion of indium 111 has an upper limit of 1% of the dose in control patients (Saverymuttu et al., 1983b). Fecal excretion of indium 111 by the NSAID-treated patients with rheumatoid arthritis is significantly different from that of the patients with irritable bowel syndrome or untreated rheumatoid arthritis (Bjarnason et al., 1987a). Patients with irritable bowel

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syndrome or rheumatoid arthritis who have not received NSAIDs show no evidence of inflammation and have normal excretion values. About 60-70% of patients on NSAIDs have increased fecal excretion of ^{111}In, which is the defining feature of NSAID-induced inflammation. Endoscopy shows a prevalence of damage similar to that seen with the leucocyte studies. The damage ranges from mucosal red spots, erosions with surrounding villous atrophy, to frank ulcers (Morris et al., 1992).

Abdominal scintigrams are also obtained at 0-4 h (early) and 20 h (late) after injection of labeled cells. The labeled cells localize within 4 hours to areas of active inflammation in over 90% of patients with inflammatory bowel disease (Saverymuttu et al., 1983b). The reason the cells are not visualized within 4 hours is that the inflammation is of low activity and diffuse, and combined with the mobility of the small intestine, there is insufficient indium radioactivity for early localization. All patients show the distribution of radioactivity in spleen, liver, and bone. The control patient shows no accumulation of indium 111 in the intestine on early or late scintigrams, whereas the majority of the patients with inflammatory bowel disease show early localization of indium 111. Early scans are positive in all patients with moderate or severe inflammatory bowel disease, but it is not sensitive (false negative) in patients with mild inflammation (Saverymuttu et al., 1983b). The low indium 111-leukocyte density reflects low-grade inflammation, the movement of the small intestine, and the high back-ground due to bone uptake. All these factors may contribute to the lack of visualization (Saverymuttu et al., 1983a) in patients with mild inflammation. The

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clarity and quality of the scans varies with the activity of disease. In general, the more active the disease the clearer the scans (Bjarnason et al., 1987a) .

There is a significant correlation between fecal excretion of indium 111 and clinical inflammation disease activity, which supports the suggestion that the presence of fecal indium 111 may be specific for intestinal inflammation and suitable for objective assessment of the inflammatory activity (Saverymuttu et al., 1983b). Therefore, the 4 -day fecal excretion of ^{111}In provides an objective, sensitive index of inflammation of the whole of the GI tract, giving information not available by other techniques (Saverymuttu et al., 1983b). It has been thoroughly validated and is the technique of choice to assess intestinal inflammation and the efficacy of medical treatment in patients with inflammatory bowel disease. The fecal excretion of indium 111-labeled leukocytes over 4 days is a noninvasive, comfortable, quantitative, and safe assessment of intestinal inflammation (Bjarnason et al., 1987b). Also, with using scintigrams, even in acutely sick patient, inflammation can be localized rapidly within three to five hours after injecting the labeled cells (Saverymuttu et al., 1983b). The sensivity of the scintigram to indium 111 is as good as radiology and similar to colonoscopic and histopathological assessment. False positive scans are not observed in the control patients (Saverymuttu et al., 1983b).

1.4.5. INTESTINAL BLEEDING

NSAID-induced enteropathy is associated with intestinal bleeding. There is no close relationship between upper endoscopic results and evidence of

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intestinal bleeding in NSAID-treated patients, even when chronic blood loss has led to an iron deficiency anemia (Collins and Adu Toit 1987) which is so common in rheumatic patients. The absence of a good correlation is likely due to the limitation of endoscopy for esophagus, stomach, and duodenum evaluations, whereas intestinal bleeding occurs more in the small intestine and colon.

Simultaneous injection of ^{111}In-labeled neutrophils and technetium (^{99m}Tc) -labeled red cells are used to assess intestinal bleeding in patients on NSAID therapy. High labeling efficiency of ^{99m}Tc is confirmed by the lack of thyroid uptake in studied patients (Bjarnason et al., 1987b). The refined ^{99m}Tc -labeled red cell technique has more labeling efficiency to overcome the problem of false positive scans due to gastric secretion of unbound ^{99m}Tc. ^{99m}Tc activity is distributed in liver, kidneys, abdominal aorta and iliac arteries. All the patients with an abnormal ^{111}In-labeled neutrophil scintigram have identical localization of ^{99m}Tc -labeled red cells. The identical localization of ^{99m}Tc and ^{111}In on the late scintigrams indicates that the small intestinal inflammation due to NSAIDs was associated with bleeding, which may explain iron deficiency and positive occult blood tests in patients treated by NSAIDs (Bjarnason et al., 1987b).

To date, no study has compared a nuclear scan technique to histologic findings in order to assess the sensivity and specificity of the NSAID-induced enteropathy (Kwo and Tremaine 1995).

Since, the ^{99m}Tc- labeled red cell technique is not quantitative, ^{51}Cr-labeled erythrocytes may also be used to quantify the intestinal blood loss (Chafetz et al., 1976). After IV injection of ^{51}Cr-labeled erythrocytes, chromium

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enters the gut lumen and 4-day fecal recovery is a valid quantitative marker for GI bleeding. The results are expressed as cumulative blood loss over 4 days. The upper limit of normal GI blood loss is less than 4 ml over this period (Bjarnason et al., 1987b). There is a significant correlation between the intestinal inflammatory activity and the amount of blood loss, which indicates that bleeding originates from the inflammation sites (Bjarnason et al., 1993). However, the magnitude of inflammation is greater than blood loss, and the small intestine is the site of mild chronic blood loss in patients on NSAID therapy. The intestinal blood loss (1-10 ml/day) leads to iron-deficiency anemia, which may also be affected by other factors: limitation of food intake because of NSAID-induced dyspepsia or cachexia; lack of reduction of ingested iron to the ferrous state because of relative achlorhydria; inability to increase iron absorption in small intestine in response to iron deficiency (Bjarnason et al., 1993).

1.5. PROTECTION OF GI FROM NSAIDS DAMAGE

NSAIDs exert their GI side effect through both local and systemic availability at the GI tract (Schoen and Vender 1989). The direct local effect may be due to the local exposure after oral administration and also secondary to biliary excretion into the GI tract. The post absorption systemic effect can be manifested after all routes of administration even via non-oral routes (e.g. intravenous and rectal suppositories) (Ligumsky et al., 1990).

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NSAIDs belong to a variety of chemical classes (table 1). The physiochemical properties of NSAIDs may influence their local effect on the GI tract. Lipid solubility and degree of ionization (Table 1) may contribute to gastric absorption of acidic NSAIDs, and, as a result, to GI toxicity (McCormack and Brune 1987). Most of these drugs are weak acids with ionization constants (pK_a) ranging from 3 to 5. The nonionized proportion of NSAID is important because it determines the distribution of drug (Brune and Graft 1978). Lipid soluble weak organic acids (e.g., aspirin and many NSAIDs) in the acidic condition of the stomach are in the nonionized form, which freely diffuses into the mucosal cells. Inside the cell where the pH is higher, the drugs are in the ionized form and become trapped. Therefore, the high concentration of drugs inside the cells can change the permeability of cell membranes and cause influx of hydrogen ions from the lumen into the mucosal cells, resulting in mucosal damage (Davenport 1965). Furthermore, it has been suggested that uncoupling of oxidative phosphorylation or inhibition of electron transport by NSAIDs may be an early pathogenic event of the direct local effect (Somasundaram et al., 1997).

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Table 1: NSAIDs by chemical class, and their pK_a. MPC and CPC are measured and calculated partition coefficient, respectively (Hansch et al., 1990).

	pKa	MPC	CPC
CARBOXYLIC ACIDS			
Salicylic acid & esters			
Aspirin	3.5	1.19	1.10
Benorylate		2.15	2.18
Diflunisal	3.0	4.44	4.42
Fendosal	3.1		7.90
Acetic acids			
Phenylacetic			
Diclofenac	4.50	4.40	4.77
Fenclofenac	4.53	4.80	4.87
Carbo- and heterocyclic			
Etodolac			
Indomethacin	4.50	4.27	4.23
Tolmetin	3.50	2.79	2.46
Propionic acids			
Ibuprofen	4.4	3.50	3.68
Naproxen	4.2	3.18	2.82
Flurbiprofen	3.9	5.25	5.58
Fenoprofen	4.5	0.83	3.82
Fenbufen	4.51	3.62	3.21
Indoprofen	5.8	2.77	2.74
Fenamic acids			
Flufenamic	3.9	5.25	5.58
Mefenamic	4.2	5.12	5.34
Meclofenamic			6.50
Niflumic			4.99
Tolfenamic			5.70
ENOLIC ACIDS			
Pyrazolones			
Oxyphenbutazone	4.7	2.72	2.50
Phenylbutazone	4.4	3.16	3.17
Oxicams			
Piroxicam	4.6	0.26	
Sudoxicam		1.64	
Isoxicam			
Tenoxicam			
NONACIDIC COMPOUNDS			
Nabumetone			2.77

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Other factors, which add to the complexity of the local effects of NSAIDs, are biliary excretion and enterohepatic circulation. Usually NSAIDs with high biliary excretion have more GI toxicity, and there is a correlation between enterohepatic circulation and GI toxicity (Reuter et al., 1997).

Several approaches, discussed below, have been used to decrease GI toxicity.

1.5.1. PRODRUGS

A prodrug is defined, with some exception as a pharmacologically inactive chemical compound, which is metabolized into an active therapeutic agent in the body. Many prodrugs are obtained by chemically modifying known compounds, which have excellent actions but also potential undesirable properties. The purpose for modifying compounds into prodrugs include: 1) improved stability; 2) improved solubility; 3) reduction in incidence and degree of adverse effects; 4) improved absorption; 5) faster action; 6) specific local effects; and 7) improved formulation (Mizushima 1982). An ideal prodrug should; 1) be readily break down following absorption to release the parent drug; 2) have adequate water solubility and lipophilicity to be absorbed after oral administration; and 3) be stable toward GI enzymes before absorption from the GI tract (Bundgaard et al., 1987).

In order to develop a safe and effective NSAID with little or no GI side effects, several prodrugs of NSAIDs have been designed. These prodrugs may be absorbed from the GI tract in inactive forms, and then converted mostly in the

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liver to active metabolites (Dandona and Jeremy 1990). Also prodrugs may mask the acidic group of NSAIDs in order to prevent direct contact of the acidic drug to the GI mucosa. They then release the active and acidic drug after absorption, away from the stomach, which presents anti-inflammatory effects (Shanbhag et al., 1992). The effectiveness of any prodrug depends on the relative importance of local effects versus systemic effects of the NSAID. A prodrug, with less contribution of local effects in the induced GI toxicity, is unlikely to be a safe drug. Sulindac after absorption is converted to active metabolites that have anti-inflammatory activity. Shortly after absorption, sulindac is reversibly reduced to sulindac sulfide, its active metabolite (Duggan et al., 1977). When sulindac was introduced, it was stated to have a low incidence of GI side effects (Graham et al., 1985). With longer periods of clinical experience, however, GI complications with sulindac have been similar to those of other NSAIDs (Henry et al., 1996). Nabumetone is a nonacidic prodrug NSAID (Dandona and Jeremy 1990) that is converted to its active metabolite, 6 methoxy naphthalene acetic acid (6MNA), after absorption. Both nabumetone and 6MNA are equally potent as inhibitors of COX-1 and COX-2 in blood, and both were only minimally COX-2-selective (Cryer and Feldman 1998). However, 6MNA was 40 times more potent a gastric COX (COX-1) inhibitor than nabumethone. Thus, the 6MNA metabolite of nabumetone may lead to GI mucosal PG depletion and predispose to ulcer formation. In fact, the incidence of peptic ulcer complications in patients using nabumetone was comparable with that observed for other examined NSAIDs (Inman et al., 1990). Thus, it appears

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that even though the prodrug nabumetone may offer reduced direct GI toxicity, its systemic effect still presents a significant risk of serious GI complications.

1.5.2. ENTERIC COATED AND SUSTAINED RELEASE PREPARATIONS

Altering the type of NSAID formulation in the hope of achieving superior anti-inflammatory properties with reduced GI toxicity is a common strategy. It has been assumed that sustained release preparations of those NSAIDs that are rapidly eliminated from the body may provide more convenient treatment regimens, fewer incidences of GI toxicity due to lowered peak plasma concentrations and decreased local effect in the upper GI tract (Mascher 1989, and Idkaidek et al., 1998). However, these formulations may increase the exposure of the active substance to the lower GI tract where the adverse reactions are usually more risky and life threatening and it is more difficult to diagnose (Halter et al., 1993, and Day 1983). In the rat, gastroduodenal permeability of sustained release flurbiprofen (Davies and Jamali 1997) and tiaprofenic acid (Vakily et al., 1999) is significantly less than that of regular release formulation, due to exposure of gastroduodenum to a relatively lower concentration of the drug. On the other hand, sustained release formulations of the latter drugs induce more increased permeability in distal intestine. This suggests a shift in the site of toxicity of these NSAIDs from the upper to the lower GI tract. Similarly a retrospective study of NSAID users has revealed that the GI toxicity of sustained release NSAIDs may, indeed, be more frequent than that of regular release (Figueras et al., 1994).

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1.5.3. ASSOCIATION WITH DIPALMITOPHOSPHATIDYLCHOLINE (DPPC)

It has been reported that associating of NSAIDs with DPPC blocks the GI damage induced by NSAIDs (Lichtenberger et al., 1995). While lipid permeability, antipyretic, and anti-inflammatory activity of the complex is apparently increased (Lichtenberger et al., 1995). Pre-associating a number of NSAIDs with dipalmitophosphatidylcholine (DPPC), a zwitterionic phospholipid, i.e., a phospholipid having both a positive and negative charge, prevents the increase in surface wettability of mucus gel layer and maintains the hydrophobic barrier integrity of the mucous gel layer (Lichtenberger et al., 1995). Zwitterionic phospholipids are synthesized in surface mucus cells of the stomach. These phospholipids are also present in discrete submucosal glands of the GI tract (Kao and Lichtenberger 1991).

1.5.4. COMPLEXATION OF NSAID WITH CYCLODEXTRIN

Cyclodextrins, which are units of glucose linked together, have been used as complexation agents with NSAIDs (Acerbi et al., 1988). The 3 main types of cyclodextrins are α (6 glucose units), β (7 glucose units), and γ (8 glucose units). Complexation of flurbiprofen with cyclodextrins (preferably β-cyclodextrin) has been found to reduce ocular irritancy (Rainsford 1990). Recent study showed that complexation of tiaprofenic acid with diethyl- β-cyclodextrin did not cause damage in the upper GI but induced significant damage in the lower GI tract (Vakily et al., 1999).

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1.5.5. NO DONORS NSAID

NO donors might be potent anti-inflammatory agents. NO is an important vasodilator and anti-aggregator mediator (refer to section 1.3.2.3.). Both endogenous and exogenous NO have beneficial effects in acute inflammatory processes. NO and NO donors were known to maintain gastric mucosal blood flow, reduce leukocyte adherence, function as antioxidants, depress mast cell reactivity, as well as reduce thrombosis.

Numerous NSAIDs have been synthesized with a moiety that generates NO with the ability to suppress PG synthesis (Reuter et al., 1994). The NO-releasing NSAIDs also have a higher dissociation constant (pK_a) for ionization and would be less liable to gastric mucosal trapping.

Compared with conventional NSAIDs, it has been reported that NO-releasing NSAIDs cause very little injury to the GI tract (Wallace et al., 1994). This reduction in GI damage has been observed after both single and repeated dosing (Wallace et al., 1994). Several NSAIDs, including diclofenac, naproxen, and flurbiprofen have been coupled to a NO-releasing moiety. These NO-NSAIDs inhibit both COX isoforms with the same potency as the parent compound and retain anti-inflammatory, antithrombotic, and analgesic activity, but they have markedly decreased gastric toxicity (Del Soldato et al., 1999, Wallace et al., 1994, Davies et al., 1997). Also NO-NSAID can accelerate healing of pre-existing ulcers (Elliot et al., 1995). Although these data suggest that mechanisms of physiologic redundancy may be beneficially manipulated,

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the clinical value of such manipulation needs to be more fully explored in human.

1.5.6. SELECTIVE COX-2 INHIBITORS

It has been suggested that the anti-inflammatory actions of NSAIDs be due to inhibition of COX-2, whereas the GI side effects are due to inhibition of COX-1. Therefore, a selective inhibitor of COX-2 was expected to suppress prostaglandin synthesis at sites of inflammation with sparing of constitutive prostaglandin in the GI tract (Xie et al., 1992, Mitchell et al., 1993, and Vane 1994). Therefore, such a drug could have a good anti-inflammatory effect without side effects on the GI tract. However, it is important to emphasize that just as all the effects of NSAIDs cannot explained by their COX inhibition, all the function of the COX isoforms cannot be explained by the COX hypothesis (Wolfe 1998). COX-2 has physiological functions in different organs:

1.5.6.1. Renal effects

Recent studies determined that COX-2 has a physiological function in the postnatal development of the mouse kidney (Dinchuk et al, 1995 and Morham et al., 1995) and in glomerular hemodynamics in humans (Komhoff et al., 1997). The clinical development of a specific COX-2 inhibitor drug, flosulide, was terminated because of renal toxicity (Emery 1996).

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1.5.6.2. Reproduction effects

COX-2 is important in female reproduction physiology. Increased expression of COX-2 appearance is required for ovulation, fertilization, implantation, and decidualization in mice (Chakraborty et al., 1996 and Lim et al., 1997). Also, COX-2 may be involved in the process of parturition (Hirst et al., 1995). The latter effect has led some investigators to use a selective COX-2 inhibitor in the management of preterm labor (Sawdy et al., 1997). Unfortunately, there is limited information about the effects of COX-2 inhibition on the fetus. Although, COX-2 is induced by cytokines, it is also constitutively expressed by certain organs during fetal development (Hanna et al, 1997, Hirst et al., 1995, Peri et al., 1995). In addition, it has been reported that COX-2 plays a significant role in regulating the tone of the fetal lamb ductus arteriosus (Clyman et al., 1999). Therefore, it was recommended that caution should be exercised in prescribing COX-2 inhibitor in pregnant women (Clyman et al., 1999).

1.5.6.3. Hepatic effects

There is a single case report about fulminant hepatic failure and massive hepatic necrosis due to nimesulide, a selective COX-2 inhibitor NSAID, (McCormick et al., 1999).

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1.5.6.4. Cardiovascular effects

COX-2 has a dual action with both inflammatory and anti-inflammatory aspects (Mitchell and Evans 1998). COX-2 is induced in most cell types after exposure to cytokines, particularly IL-1β. Once induced at the site of inflammation (e.g., venules), the prostanoid formed by COX-2 mediates swelling and pain. Thus, at this site, COX-2 is an inflammatory enzyme and an appropriate target for inhibitors. However, when COX-2 is induced in the smooth muscle component of large capacity vessels (e.g., coronary artery) the production of prostacyclin inhibits proliferation, cytokine release, and adhesion receptor expression. In these circumstances, the induction of COX-2 represents a defense mechanism for damaged vessels, inhibition of which may exacerbate disease, such as atherosclerosis (Mitchell and Evans 1998). Therefore, it has been suggested that COX-2 can produce the same beneficial effects as COX-1. Thus the highly selective COX-2 inhibitors could have important side effects, particularly in the cardiovascular system (Mitchell and Evans 1998).

1.5.6.5. Gastrointestinal effects

It is well known that treatment with conventional NSAIDs causes a delay in the healing of gastric ulcers. This delay is associated with a reduction of the increased PG production in ulcerated tissues in rats and humans (Szelenyi et al., 1982, Wang et al., 1989, Levi et al., 1990, Lancaster-Smith et al., 1991). Exogenous PGE_2 prevents the indomethacin-induced delay in ulcer healing in

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rats (Wang et al., 1989). COX-2 has a predominant role in gastric ulcer healing. COX-2 expression is induced by gastric ulceration in rats and that the level of COX-2 mRNA decreases with ulcer healing. In contrast, COX-1 mRNA levels remain constant during ulcer healing. PGE_2 production was significantly elevated in ulcerated tissue when compared with that in normal tissue and this increased production returned to the normal level in parallel with ulcer healing (Shigeta et al., 1998). This change in PGE_2 production was well correlated with COX-2 mRNA expression. Therefore, COX-2 contributes to the elevation of PGE_2 production in the ulcerated tissue. On the other hand, a selective COX-2 inhibitor, NS-398, caused a significant delay in the ulcer healing by preventing maturation of the ulcer base and angiogenesis through the inhibition of COX-2 activity in rats. This effect is more evident if a COX-2 inhibitor is used in the early phase of the healing process (Shigeta et al., 1998).

The effects of indomethacin, diclofenac, and L-745,337, a selective COX-2 inhibitor, have been assessed in the chronic ulcer rat model (Schmassmann 1998). L-745, 337 caused a profound delay of gastric ulcer healing, with histologic signs of NSAID toxicity. Moreover, chronic administration of L-745,337 caused a dose-independent intestinal perforation that was comparable to that seen with diclofenac (Schmassmann 1998). Overall, it has been reported that none of the currently marked NSAIDs even those that are COX-2 selective do not guarantee GI-sparing features (Cryer et al., 1998).

In spite of studies to date for less GI toxicity of the selective COX-2 inhibitors, some questions remain unanswered regarding the ability of these new

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NSAIDs to inhibit the endogenous prostanoids that are biologically important. Therefore, the confirmation of the COX-2 safety awaits more extensive future clinical evaluation.

1.6. RATIONALE

Finding an effective and safe NSAID by modification of the physiochemical and pharmacological properties of the drug has not been completely successful. The effectiveness of these approaches depends on the relative importance of local effects versus systemic effects of the NSAID. Therefore, a better understanding of the pathogenesis of GI mucosal injury may help to prevent or decrease the incidence and severity of NSAIDs induced mucosal injury. In addition, knowledge of the mechanism of GI toxicity, whether caused by the direct local damage and/or after the systemic absorption, is of importance to the development of NSAIDs formulations.

For these studies, two drugs from two different groups of NSAIDs were selected: ibuprofen and diclofenac sodium. Ibuprofen is a propionic acid derivative with pK_a=4.4, and partition coefficient about 3.5, and diclofenac sodium is a substituted phenyl-acetic acid derivative with pK_a equal to 4.5, and partition coefficient about 4.5. In contrast to humans, rats excrete about 12% of a dose as unchanged ibuprofen in bile (Dietzel et al., 1990). In the rat, approximately 40% of an intravenous dose of diclofenac sodium undergoes enterohepatic circulation over 8 hours (Tabata et al., 1995). Also, it has been reported that NSAIDs associated with zwitterionic phospholipids may reduce GI

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toxicity (Lichtenberger et al., 1995). However, there is not sufficient data about the NSAIDs pharmacokinetics administered as these preparations. Furthermore, little is known about the effects of the NSAID-phospholipid complexes on the upper and the lower GI tract.

1.7. HYPOTHESES

1. Ibuprofen and diclofenac sodium-induced GI toxicity could be due to both local and systemic effects (figure 5).
2. Furthermore, local effects are formulation dependent.
3. The induced GI toxicty of sustained release formulation of ibuprofen and diclofenac is equal to that of the immediate release.
4. NSAID associated with DPPC has less toxicity in the upper and the lower GI tract.

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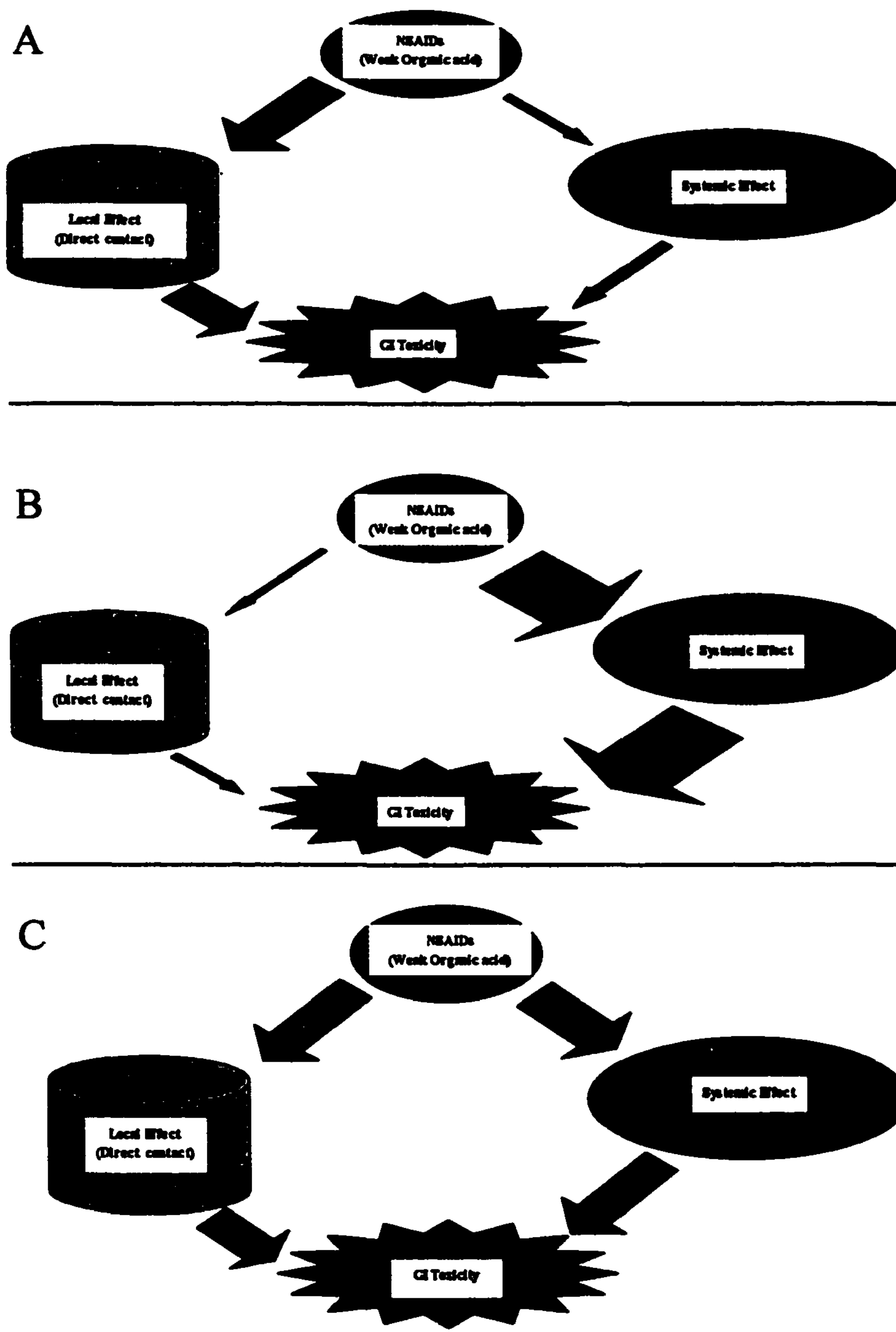

Figure 5: Mechanisms of NSAIDs-induced GI toxicity involving mainly local effects (A), mainly systemic effects (B), and both local and systemic effects (C).

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1.8. OBJECTIVES

1- To elucidate the mechanisms of induced GI toxicity by ibuprofen and diclofenac sodium

2- To examine the effects of altered site of drug release on the GI toxicity of ibuprofen and diclofenac sodium

3- To investigate the effects of association of NSAID with phospholipid on the pharmacokinetics, and the upper and lower GI toxicity. Local effect is contributed to overall induced GI toxicity of the selected NSAID.

Modification of NSAID release may alter the direct contact of induced GI toxicity but have little influence on the systemic effect. Therefore, it might be possible to delineate the mechanism of induced GI toxicity of NSAIDs by using different formulations and routes of administration. In this study, upper (sucrose) and lower (^{51}Cr-EDTA) GI permeability changes were used as the surrogate marker of NSAID induced GI toxicity in the rat model (Davies, et al., 1994 and Davies, et al 1995).

1.9. RESEARCH PLANS

1.9.1. IBUPROFEN

1.9.1.1. The effect of formulation on the GI permeability

1.9.1.1.1. Immediate *v.s.* sustained released ibuprofen

1.9.1.1.2. Ibuprofen lysinate v.s. immediate release

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1.9.1.2. The effect of dose and route of administration on the GI permeability

1.9.1.2.1. 100 mg/kg vs 200 mg/kg

1.9.1.2.2. Oral vs *sc*

1.9.2. DICLOFENAC SODIUM

1.9.2.1. To evaluate and compare the dissolution rate of immediate and sustained release diclofenac sodium preparations.

1.9.2.2. To evaluate and compare the pharmacokinetics of immediate and sustained release diclofenac sodium preparations.

1.9.2.3. To evaluate and compare immediate and sustained release preparations of diclofenac sodium on the upper and lower GI permeability.

1.9.3. ASSOCIATION WITH DPPC

1.9.3.1. To associate NSAID with DPPC and to confirm the association by differential scanning calorimeter (DSC).

1.9.3.2. To evaluate and compare the pharmacokinetics of NSAID, and NSAID – DPPC complex.

1.9.3.3. To evaluate and compare the upper and lower GI permeability of NSAID, and NSAID-DPPC complex.

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CHAPTER 2

EXPERIMENTAL

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2.1. MATERIALS

Racemic ibuprofen powder B.P/U.S.P. was from Winthrop Laboratories (Newcastle, England). Sustained release granules of ibuprofen were from Apotex (Weston, Canada). Ibuprofen lysine (Dolormin®) was purchased from Woelm Pharma GmbH & Co (Eschwege, Germany). Diclofenac sodium powder, Trinder's Reagent, naphthoxy acetic acid sodium, and lysine were acquired from Sigma (St. Louis, MO, USA). Sustained release tablets of diclofenac sodium 75 mg were obtained from Novopharm (Scarborough, Canada). Methylcellulose, D-glucose, and potassium dihydrogen orthophosphate were purchased from BDH Chemicals (Edmonton, Canada). Methoxyflurane was supplied by Janssen Pharmaceutica (Mississiauga, Canada). Sucrose was ordered from Aldrich Chemical Company Inc (Milwaukee, WI, USA). ^{51}Cr-EDTA was acquired from Dupont NEN (Wilmington, DE, USA). ELISA assay plates were purchased from Fisher Scientific (Edmonton, Canada). All solvents and reagents were of HPLC and analytical grade.

2.2. FORMULATIONS

2.2.1. IBUPROFEN FORMULATIONS

Immediate release ibuprofen preparation was made by suspending ibuprofen powder in 1 % methylcellulose. Oral doses were administered to rats by oral gavage. Dry sustained release ibuprofen granules and crushed ibuprofen

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lysinate were introduced into stomach by a flexible plastic tube attached to oral gavage followed by 0.3 ml water. Water, methylcellulose, or lysine was used as placebo in control rats. Subcutaneous (*sc*) ibuprofen solution was prepared by dissolving ibuprofen powder in 1ml ethanol, diluted and its pH adjusted to 9 with sodium bicarbonate. The *sc* vehicle was used in control rats.

2.2.2. DICLOFENAC-SODIUM FORMULATIONS

Immediate release preparation of diclofenac sodium was prepared by suspending diclofenac sodium powder in 1% methylcellulose and administered to rats by oral gavage. One piece of gently crushed sustained release diclofenac sodium tablets were used as sustained release formulation and intubated into the stomach by a flexible plastic tube attached to an oral gavage followed by 0.3 ml water.

2.2.3. DICLOFENAC ACID, DICLOFENAC-DPPC COMPLEX FORMULATIONS

Diclofenac acid was prepared by acidification of aqueous solution of diclofenac sodium, extraction into chloroform, and recrystallization. The diclofenac-DPPC complex was prepared by associating diclofenac acid and an equimolar concentration of DPPC. A suspension of the complex in water was used as the diclofenac-DPPC complex. Diclofenac sodium and diclofenac acid suspension in 1 % methylcellulose were used as diclofenac sodium and diclofenac acid preparations, respectively. Rats were dosed by gavage.

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2.3. DISSOLUTION RATE STUDY

The USP paddle method was used to measure the dissolution rate of immediate release and sustained release diclofenac sodium preparations in 500 ml water at 37° C and 100 rpm for 1 h (Liu CH, et al., 1995). Subsequently 400 ml phosphate buffer (17.1g sodium phosphate and 5.6 ml concentrated hydrochloric acid / 400 ml) was added to raise the pH to 6.8. The pH was adjusted to 6.8±0.1 with either hydrochloric acid or sodium hydroxide solution if necessary. Serial samples were collected and analyzed for diclofenac using spectrophotometer at 276 nm.

2.4. DICLOFENAC ASSAY

Several HPLC methods have been described for determination of diclofenac in plasma (Hases et al., 1995, and Mohamed et al., 1994) as well as in urine (Avegerinos et al., 1993). However, the range of examined concentrations was small such as 0.005 to 2 μg/ml (Hases et al., 1995), 0.01 to 10 μg/ml (Mohamed et al., 1994). In addition, most of these methods need more than 0.1 ml plasma (Mohamed et al., 1994).

In order to evaluate the pharmacokinetic parameters of diclofenac, it was necessary to develop a sensitive and specific HPLC assay for determination of diclofenac in small volume of rat plasma (0.1 ml). Plasma concentrations of diclofenac sodium were quantified by a reverse phase HPLC method at ambient

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temperature. Briefly, to 100 μl of rat plasma, 25 μl of internal standard (naphthoxy acetic acid sodium, 10 μg/ml), 10 μl of pure phosphoric acid and 1 ml chloroform were added. The contents of the tubes were vortexed for 1 min, then centrifuged (Dynamic II Centrifuge, Clay Adams, USA) at 1800 *g* for 5 min. The organic layer was transferred to a clean test tube, and evaporated to dryness. Following evaporation of the organic layer, the residue was reconstituted in 200 μl of HPLC water and 50-100 μl of aqueous phase injected into the HPLC system.

HPLC system consisted of a M-45 solvent delivery system (Waters Associate, Inc., Milford, MA, USA), a SPD-10A UV-Vis variable wavelength detector (Shimadzu Cooperation, Kyoto, Japan), a SIL-9A autoinjector (Shimadzu analytical instruments division, Kyoto, Japan), and a 10 cm ×4.6 mm i.d. Partisil ODS-3 analytical column (Whatman, USA) with a C18 pre-column (Resolve C18 Guard-Pak, Waters Corporation, Milford, MA, USA).

The mobile phase consisted of acetonitrile-phosphate buffer (33:67 v/v). Phosphate buffer was prepared by dissolving 6.8g KH_2PO4 (0.05M), 1ml triethanolamine, and 3 ml 2M sulfuric acid in 1000ml water (pH=4). The mobile phase was filtered through a Millipore 0.5-um filter, type FH (Milford, MA, USA). The flow rate was 1 ml/min and detection was performed at 276 nm.

Under the chromatographic conditions internal standard and diclofenac were eluted at 5.8 and 32 min, respectively. Between the elution of internal standard and diclofenac four metabolites of the drug were separated. The developed method was successfully applied for the determination of the

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pharmacokinetic parameters of diclofenac in rats up to 8 h following receiving a single oral dose of the drug (15 mg/kg).

Typical chromatograms of (a) blank rat plasma, (b) rat plasma at time zero after oral administration of diclofenac are shown in Figure 6. The chromatograms of plasma samples taken at 1.5 h after oral administration of diclofenac sodium (15 mg/kg) as immediate release (a), and sustained release (b) preparations are shown in Figure 7. The Figures demonstrate the specificity of the method by the lack of interference at the retention times of internal standard and diclofenac.

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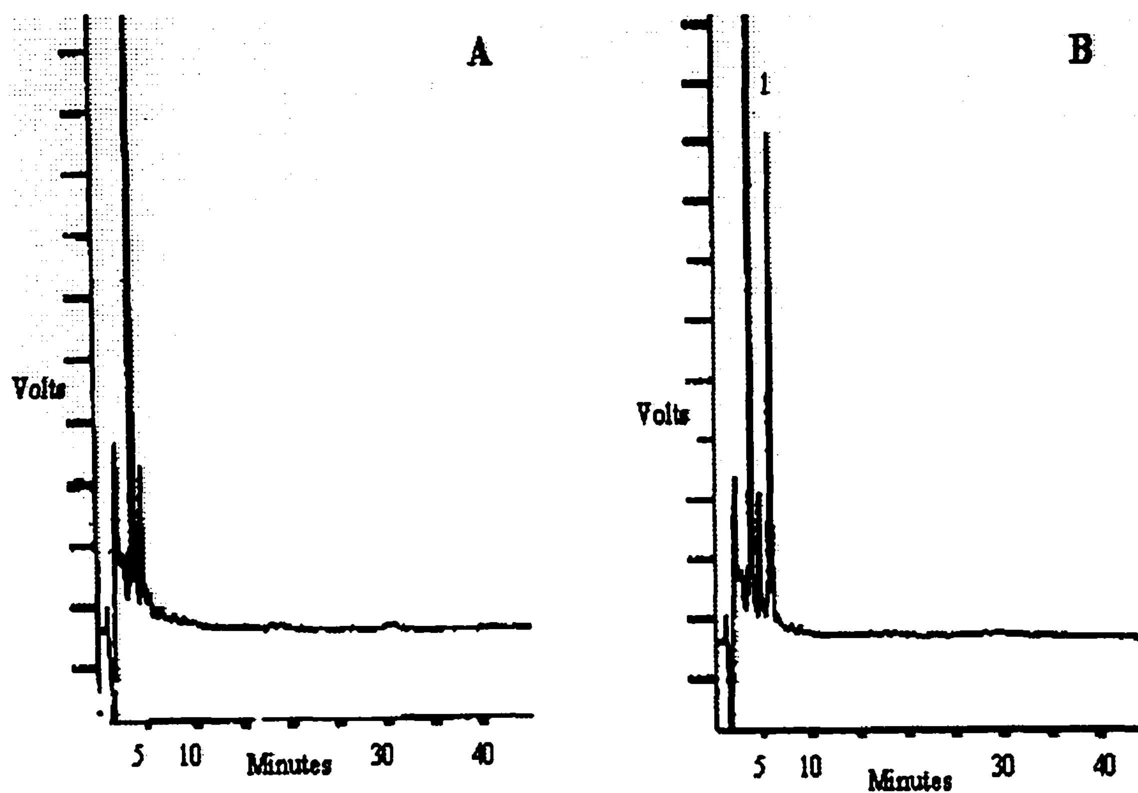

Figure 6: Chromatograms of blank rat plasma (A), and rat plasma at time zero following diclofenac administration (B). Peak 1 is internal standard (naphthoxy acetic acid sodium).

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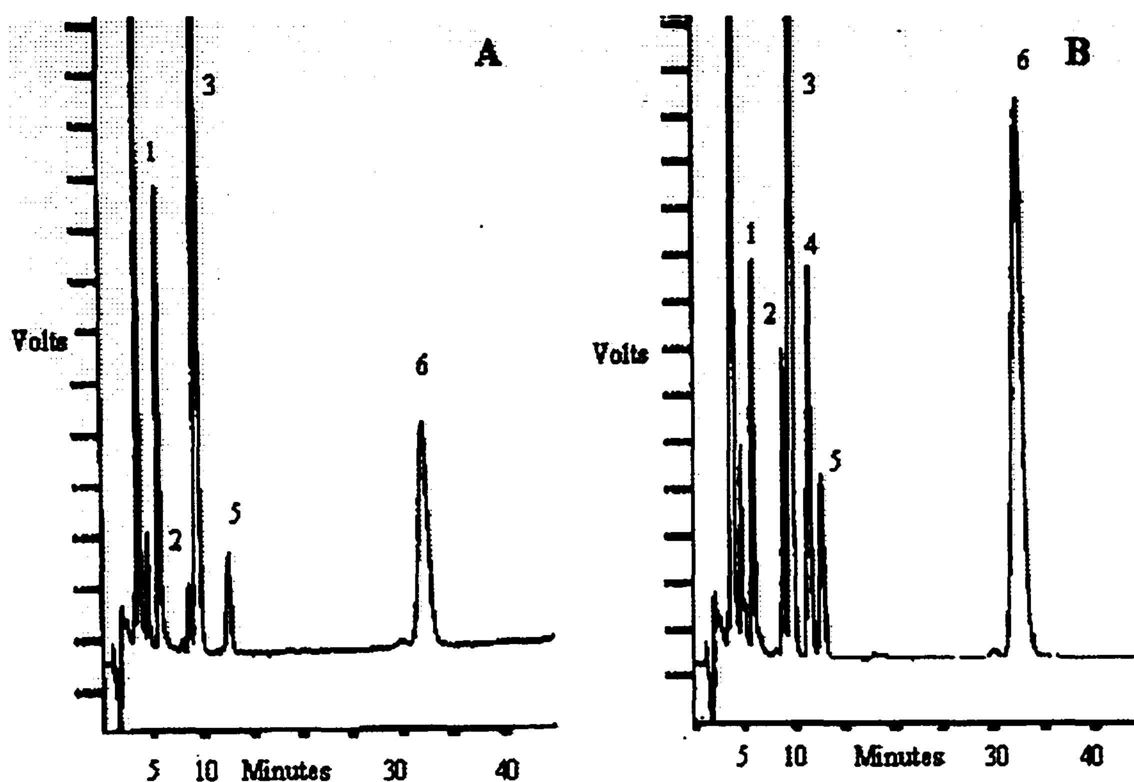

Figure 7: Chromatograms of plasma sample taken at 90 min after a single oral dose of diclofenac sodium as immediate release (A), and sustained release preparations. Peak 1 (5.8 min)=internal standard (naphthoxy acetic acid sodium), peak 2 (8.91 min)=metabolite 1, peak 3 (9.73 min)=metabolite 2, peak 4 (11.53 min)=metabolite 3, peak 5 (12.79 min)=metabolite 4, and peak 6 (32.58 min)=diclofenac.

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All peaks were sharp and symmetrical with good baseline resolution, hence facilitating accurate measurement of the peak height ratio. The calibration curves were plotted by the peak height ratios of diclofenac/internal standard versus their respective concentration of diclofenac. The mean standard plot of diclofenac over the concentration range 0.025 to 20 μg/ml in plasma is depicted in Figure 8. The average slope of four standard curves prepared at four different days was 0.271±0.011 ml/μg (mean± STD), and an interday coefficient variation (CV%) of 4.1, indicating good inter-day reproducibility. Least square regression analysis of the calibration curve consistently gave linear response over the examined concentration range of diclofenac sodium (0.025-20 μg/ml) with a correlation coefficient (r^2) of greater than 0.9997. The validation of the method was evaluated by analyzing diclofenac in spiked plasma samples at low (0.05 μg/ml), intermediate (1 μg/ml), and high (10 and 20 μg/ml) concentrations of diclofenac sodium. The percent error was less than 10 percent in each occasion. The sensitivity of assay defined as the minimum concentration that can be quantified with a statistically acceptable coefficient of variation (5%) was 50 ng/ml. The minimum detectable concentration was 25 ng/ml that gives half of the response of 50 ng/ml, but the related CV was higher than 10%.

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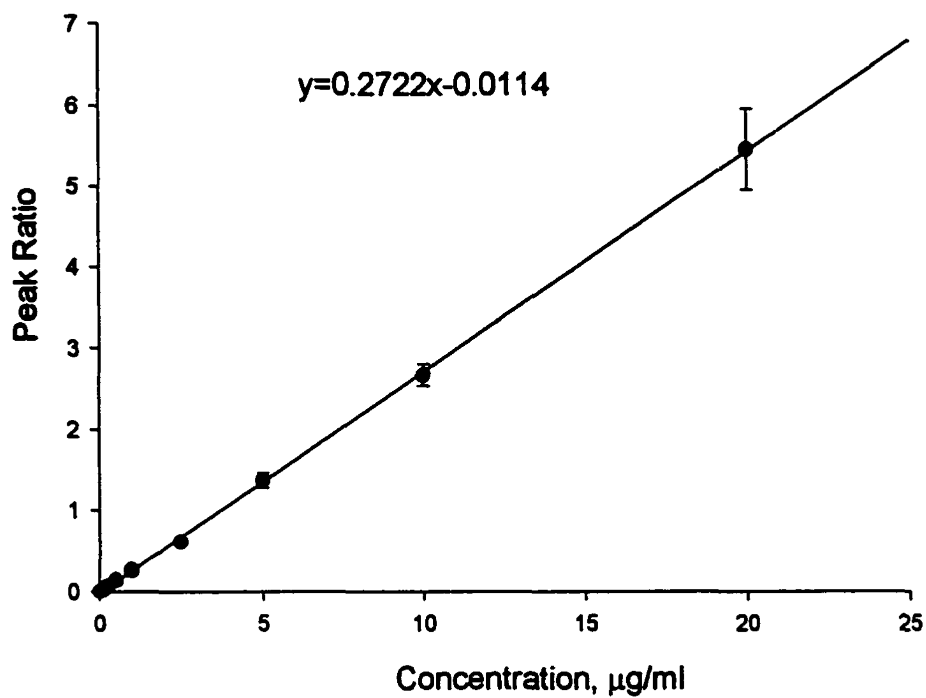

Figure 8: A typical standard curve of diclofenac was constructed using spiked blank rat plasma (n=4), $r^2>0.9997$. Error bars represent standard deviation.

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By using this method (Figure 7) there were no interference due to endogenous plasma components or the metabolites at the retention times of diclofenac and the internal standard. However, the run time of each sample was longer than other methods that quantify the drug alone (Mohamed et al., 1994). The results of the quantification of diclofenac metabolites are not reported, but typical plots of the peak ratio of drug and metabolites versus time following immediate and sustained release preparations are shown in Figure 9.

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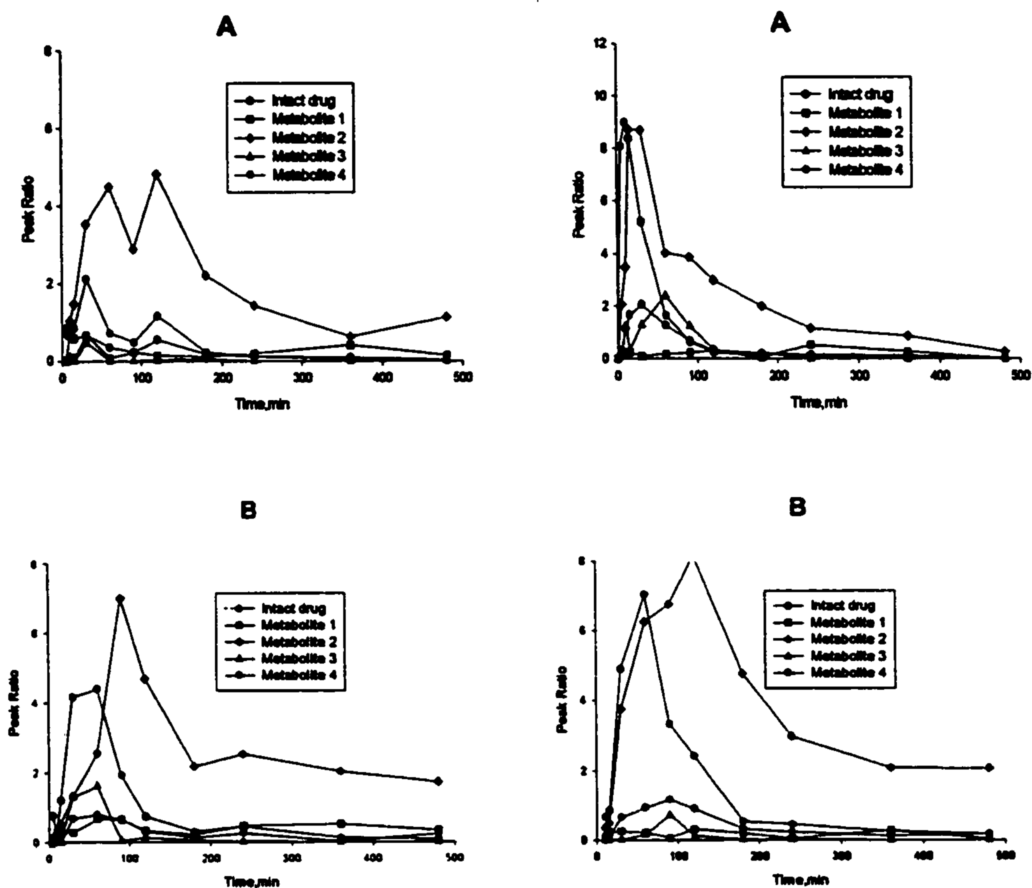

Figure 9: The peak ratio of diclofenac and its four metabolites versus time following administration 15 mg/kg either immediate (A) or sustained release (B) in representive individual rats.

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2.5. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

The thermograms of the diclofenac acid, diclofenac sodium, diclofenac-DPPC complex were recorded on a Seiko SSC/5200 differential scanning calorimeter (Plymouth Meeting, Pennsylvania, USA). The thermal behavior was studied by heating 2.0±0.2 mg of the samples in a covered sample pan under nitrogen gas flow and the investigation was carried out over the temperature range 25-200 ° C with a heating rate of 10 ° C/min.

2.6. ETHICS APPROVAL

All experiments were approved by the Animal Care Committee of the University of Alberta, Canada.

2.7. ANIMALS

2.7.1. ANIMALS HANDLING

Adult male rats (250-350 g) were of the Sprague- Dawley strain of Rattus Norvegicus and raised by the University of Alberta Animal Colony Facility. Animals were fasted over night and during the experiment had free access to water. The rats were housed at ambient temperature (25° C) in individual metabolic cages (Fisher Scientific, Edmonton, Canada).

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2.7.2. ANIMAL SURGERY

Male Sprague-Dawley rats (n=5/group) were anesthetized with methoxyflurane. A combination of PE-10 (Dow Corning Corp, Midland, MI, USA) and PE-50 tubing (Clay Adams, Parrispanny, NJ, USA) catheters was used to implant into right jugular vein for the collection of blood samples. Animals recovered overnight before drug administration.

2.8. PHARMACOKINETIC STUDY

The rats (n=5/group) received single oral dose (equivalent to 15 mg/kg of diclofenac sodium) of one of following formulations; immediate release diclofenac sodium, sustained release diclofenac sodium, diclofenac acid, diclofenac -DPPC complex preparations. Blood samples (0.2 ml) were taken at 0, 0.08, 0.17, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8hr post dose. The whole blood samples were collected in polypropylene vials (Fisher Scientific, Edmonton, Canada) containing heparin powder (about 10 IU heparin/tube), and immediately centrifuged on a Fisher Model 235A microcentrifuge (Fisher Scientific, Edmonton, USA) for 3 min at 2500g. The plasma was separated and kept frozen at –20 °C until analyzed.

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2.9. GI PERMEABILITY

Rats were housed at ambient temperature (25 °C) and humidity in individual metabolic cages with wire mesh floors allowing for separate collection of urine and feces. Animals were fasted overnight and during the experiment with free access to water. On the day of experiment each rat received a single dose of examined drug. In order to measure upper and lower GI permeability simultaneously, 1ml of the permeability probe syrup containing 1g sucrose and 10 μ Ci ^{51}Cr EDTA were given orally through an 18 gauge 5 cm curved feeding needle (Harvard Apparatus) attached to a 1 ml syringe. The administration times of permeability probes were selected according to the release profile of the examined formulations. Urine was collected from 0 to 8 h following the administration of permeability probes. An 8 h collection period is shown to be adequate (Ford et al., 1995, Davies et al., 1994). After each collection period, each urine collection tray was rinsed with 10 ml of tap water, which was then transfered to individual scintillation vials where volume was recorded.

2.9.1. EFFECT OF IBUPROFEN ON GI PERMEABILITY

2.9.1.1. The effect of formulation on the GI permeability

2.9.1.1.1. Immediate v.s. sustained released ibuprofen: Three groups (n=6/group) of rats (300-350 g) were dosed orally with 100 mg/kg of immediate release, sustained release of ibuprofen or placebo. GI permeability changes

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were determined at 1, 3, and 8 h postdose of either drug or placebo. Control rats received either 1 % methylcellulose or water.

2.9.1.1.2. Ibuprofen lysinate v.s. immediate release: Rats (n=6/group) with weight of 300-350 g were dosed orally with 100 mg/kg ibuprofen as either ibuprofen lysinate or immediate release. GI permeability changes were assessed after 1h post dose of either drug or placebo. Control rats received either 1 % methylcellulose or 75 mg/kg lysine.

2.9.1.2. The effect of dose and route of administration on the GI permeability

Rats (n=6/group) with weight of 250-300 g were dosed with ibuprofen 100 and 200 mg/kg either orally or *sc*. GI permeability changes were assessed at the time for maximum effect (Sattari and Jamali 1994) i.e., after 1h post dose. Control rats received either 1 % methylcellulose or *sc* vehicle.

2.9.2. EFFECT OF DICLOFENAC SODIUM ON GI PERMEABILITY

The rats (n= 6/group) were dosed 10 mg/kg orally with immediate and sustained release of diclofenac sodium. Water or 1 % methylcellulose was used as placebo in control rats. GI permeability changes were assessed at 1, 2, 3, 5, and 8 h post-dose of drug or placebo. The time post-dose was selected according to the profile release of the examined formulations and the contribution of enterohepatic circulation to the induced GI toxicity.

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2.9.3. EFFECT OF DICLOFENAC-DPPC COMPLEX ON GI PERMEABILITY

Rats (n= 6/group) were dosed (equivalent to 10 mg/kg diclofenac sodium) orally with diclofenac sodium, diclofenac acid, diclofenac acid-DPPC complex. Control rats received placebo (either DPPC or 1 % methylcellulose) in each group. GI permeability changes were assessed at 1, and 3 h post-dose of drug or placebo.

2.10. GI PERMEABILITY ASSAYS

2.10.1. ASSAY OF SUCROSE

The analysis of sucrose in urine involved an indirect spectrophotometric measurement via differences in glucose concentrations before and after acid cleavage of the urine samples (Davies et al., 1995). Briefly, calibration curves of sucrose and glucose within 0-100 µg/ml concentration range were prepared by adding sucrose and glucose solutions to rat urine (final volume =100 µl). Since sucrose was not reactive to the Trinder's Reagent, first it was completely cleaved to fructose and glucose using 25 µl of 2M H_2SO4 followed by a brief vortex-mix and 10 min incubating in a boiling water bath. Forty µl of 2M NaOH was then added followed by Sorensons Phosphate buffer (pH=7±0.1) q.s.0.5 ml. Sorensons buffer was prepared by adding 41.3 ml of 1/15 M monopotassium phosphate and 58.7 ml of 1/15 M disodium phosphate. One ml of Trinder's Reagent was then added to each urine sample and incubated in room

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Reagent was then added to each urine sample and incubated in room temperature for 18 min. Then, 100 µl of samples were put in ELISA plates and absorbance was determined on an ELISA plate reader (Cayman Chemical, Ann Arbor, MI, USA) with a 492 nm lens.

2.10.2. ASSAY OF ^{51}Cr-EDTA

Urine samples were counted directly by Minaxi Auto-Gamma 5000 Counter (Packard, Meriden, CT, USA) for 1 min in a counting window scanning within a range of 0-2 Mev. Two standards of 100 µl of the administered ^{51}Cr EDTA was counted with every set of urine samples. Relative permeability was determined by calculating the activity percent in each urine sample as a percent of the administered dose after correcting for background radiation (Davies et al., 1994).

2.11. ANALYSIS OF DATA

2.11.1. PHARMACOKINETIC ANALYSIS

Pharmacokinetic parameters (t_{max}, and C_{max}) were delineated according to a non-compartmental model, in which no assumption for a specific compartment model is required. The linear trapezoidal method was used to calculate area under the concentration-time curve (AUC_{0-t}).

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2.11.2. PHARMACODYNAMIC ANALYSIS

Relative permeability changes were assessed by calculating the percent of administered dose of sucrose and the activity of ^{51}Cr-EDTA excreted in urine over 8h. In some cases, the upper and lower GI permeability changes were expressed as percent increased permeability from baseline values. AUC of the increased permeability *vs* time was measured using the trapezoidal rule.

2.11.3. STATISTICAL ANALYSIS

Differences between two means were determined by Student's unpaired t-test. One- way ANOVA followed by Duncan's multiple comparison test have used to determine difference between multiple means. A p value of <0.05 was considered significant. Unless stated otherwise, data are presented as mean ± standard error (SEM).

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CHAPTER 3

MECHANISM OF INDUCED GI TOXICITY OF IBUPROFEN

This chapter has been published in part as an abstract: Khazaeinia T and Jamali F. Gastrointestinal toxicity of ibuprofen assessed using GI permeability as a surrogate marker in the rat: Effect of dose, formulation and route of administration. Pharm Res., 13, S-417, 1996.

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3.1. RESULTS

Both sustained and immediate release preparations of ibuprofen significantly increased upper GI permeability at 1 h post-dose as compared with control rats (Figure 10). At 3 h post-dose only sustained release formulation induced significant increase in the upper GI permeability. The immediate release preparation on the other hand, significantly increased lower GI permeability at 1 h, while sustained release preparation did so at 3h (Figure 11). Following both preparations the upper and lower of GI toxicity returned to baseline level in 8 h.

Ibuprofen lysinate induced similar significant ($p<0.05$) increased upper (Figure 12) and lower (Figure 13) GI tract permeability as compared with control rats, which received either 1 % methylcellulose or lysine (Figure 12 and 13).

Increased permeability was dose dependent after both *p.o.* and *s.c.* Administration of 100 and 200 mg/kg of ibuprofen subcutaneously induced a significant permeability increase in upper (Figure 14) and lower GI tract (Figure 15) permeability when compared to control rats. The increased upper and lower GI permeability following 100 mg/kg *sc,* but not 200 mg/kg, was significantly greater than those after the oral administration (Figure 15).

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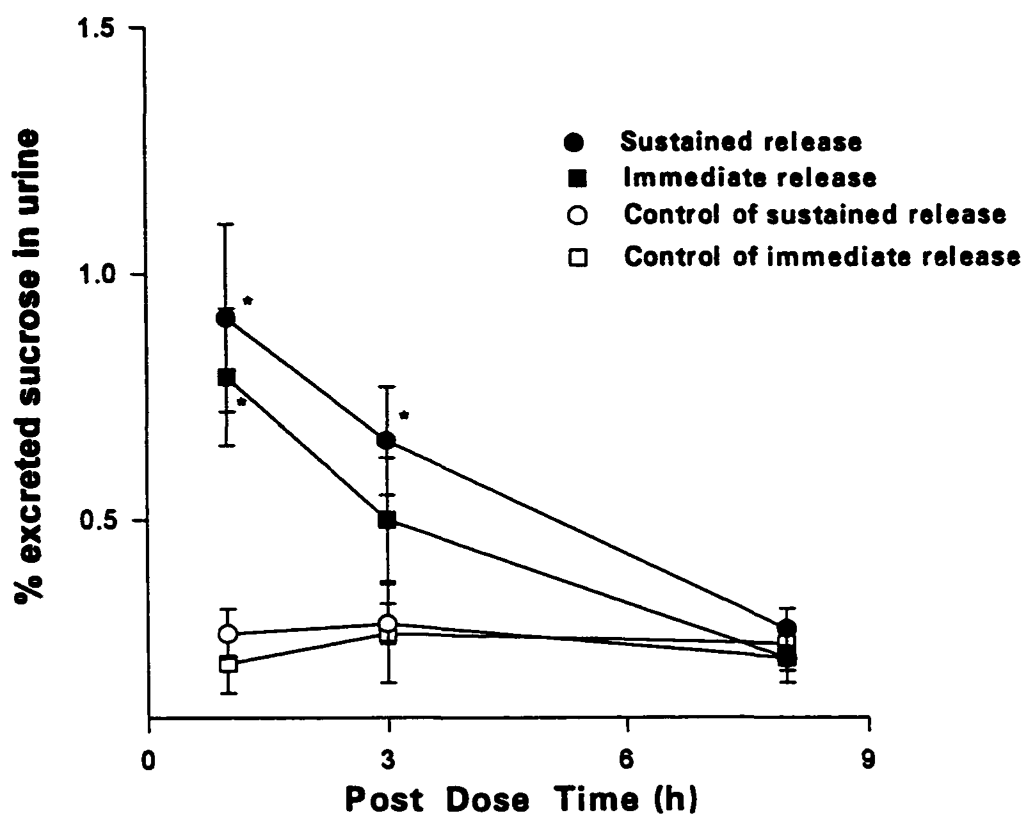

Figure 10: Sucrose (gastroduodenal) permeability at 1, 3, and 8h post-dose of 100 mg/kg ibuprofen as immediate release, and sustained release preparations (*, significantly different from control, $p<0.05$). Control immediate release and sustained release animals received placebo. Error bars represent standard error of the mean.

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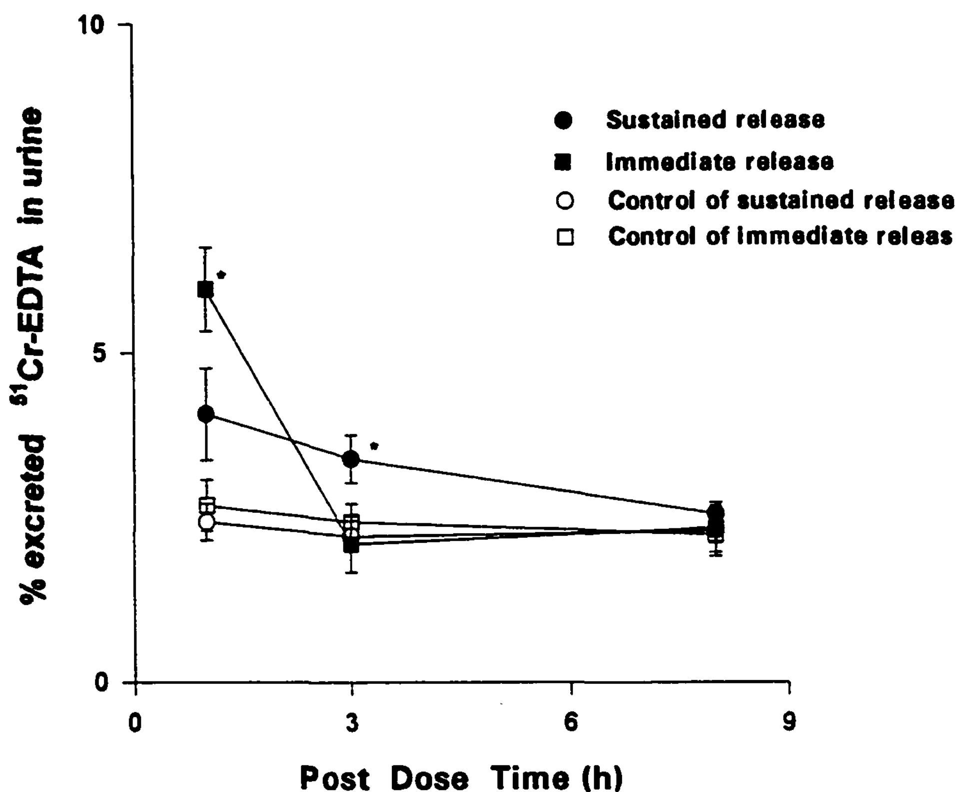

Figure 11: ^{51}Cr-EDTA (lower GI tract) permeability at 1, 3, and 8h post-dose of 100 mg/kg ibuprofen as immediate release, and sustained release preparations (*, significantly different from control, $p<0.05$). Control immediate release and sustained release animals received placebo. Error bars represent standard error of the mean.

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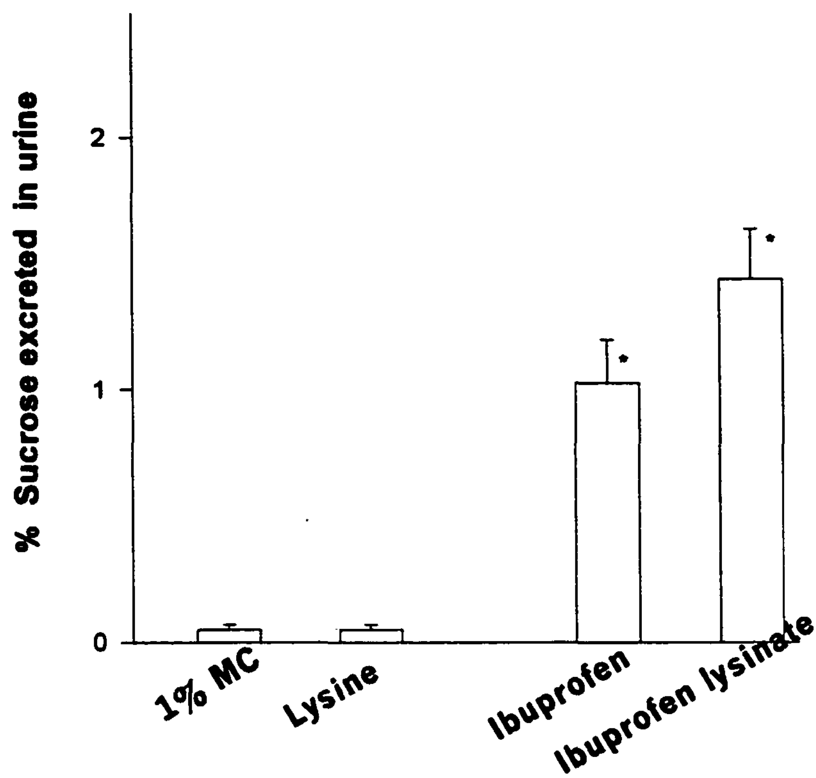

Figure 12: Sucrose (gastroduodenal) permeability at 1 h post-dose of 100 mg/kg ibuprofen as immediate release, and ibuprofen lysinate preparations (*, significantly different from control, $p<0.05$). Control rats received 1 % methylcellulose or lysine. Ibuprofen and ibuprofen lysinate are not significantly different. Error bars represent standard error of the mean.

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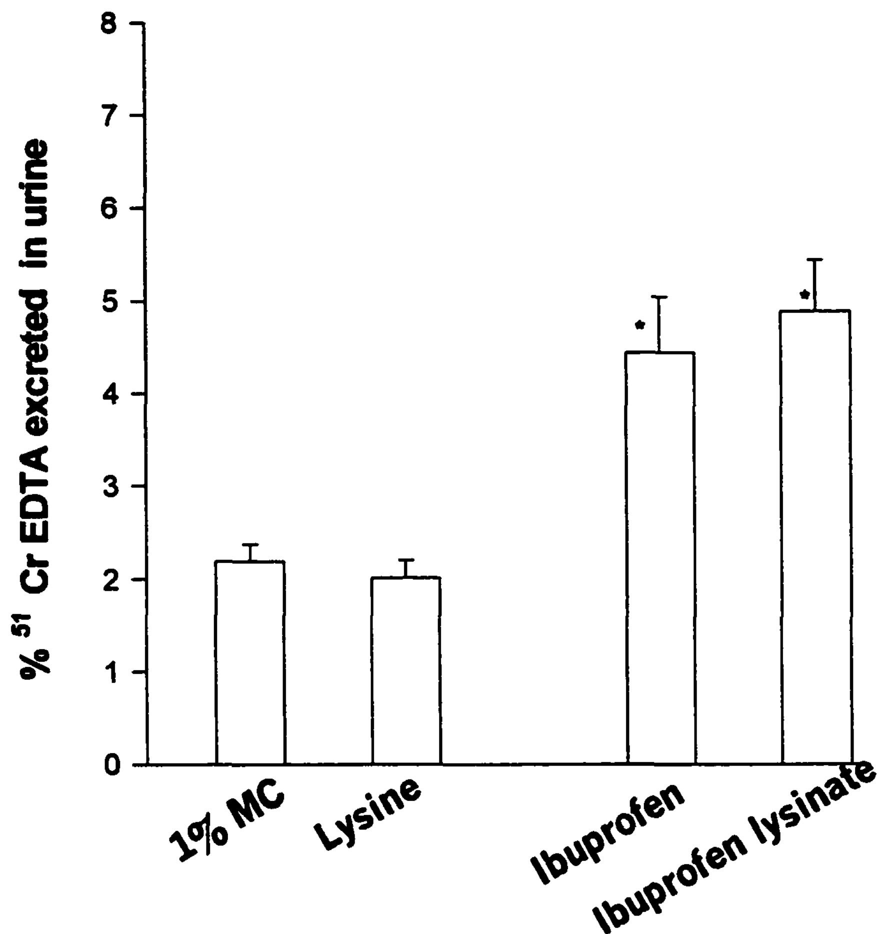

Figure 13: ^{51}Cr-EDTA (lower GI tract) permeability at 1 h post-dose of 100 mg/kg ibuprofen as immediate release, and ibuprofen lysinate preparations (*, significantly different from control, $p<0.05$). Control rats received 1 % methylcellulose or lysine. Ibuprofen and ibuprofen lysinate are not significantly different. Error bars represent standard error of the mean.

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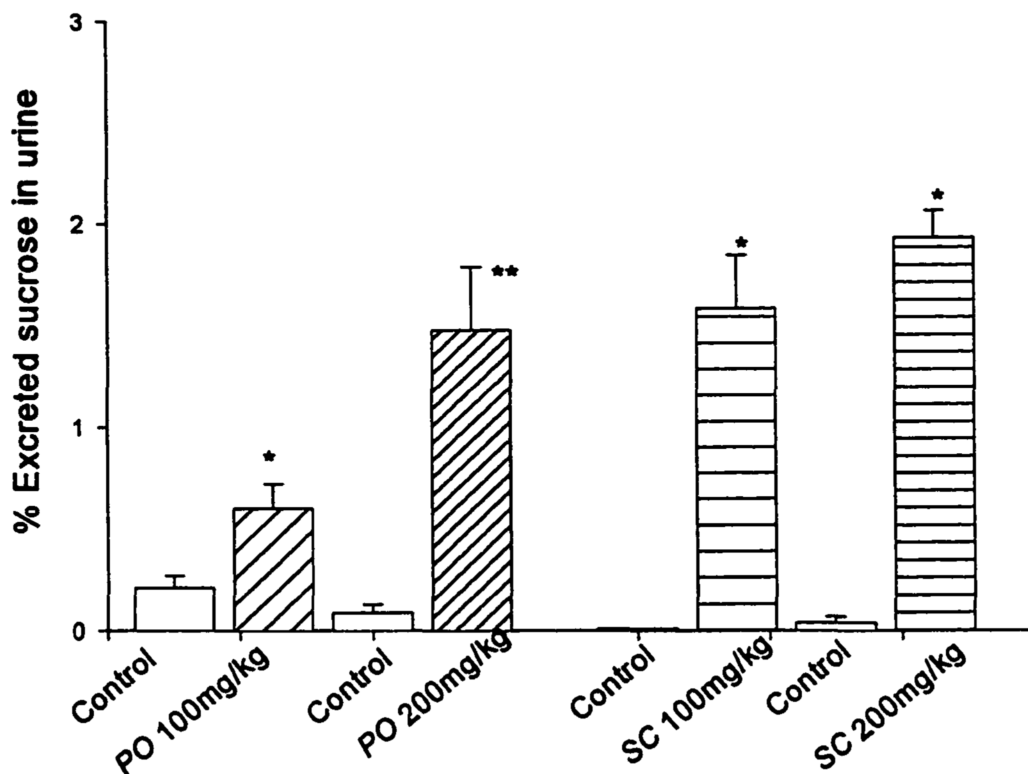

Figure 14: Effect of route of administration (oral and *sc*) and the dose (100 and 200 mg/kg) on the gastroduodenal (sucrose) permeability of ibuprofen at 1 h post-dose (*, significantly different from paired control. **, significantly different from paired control and 100 mg/kg dose, $p<0.05$). Error bars represent standard error of the mean.

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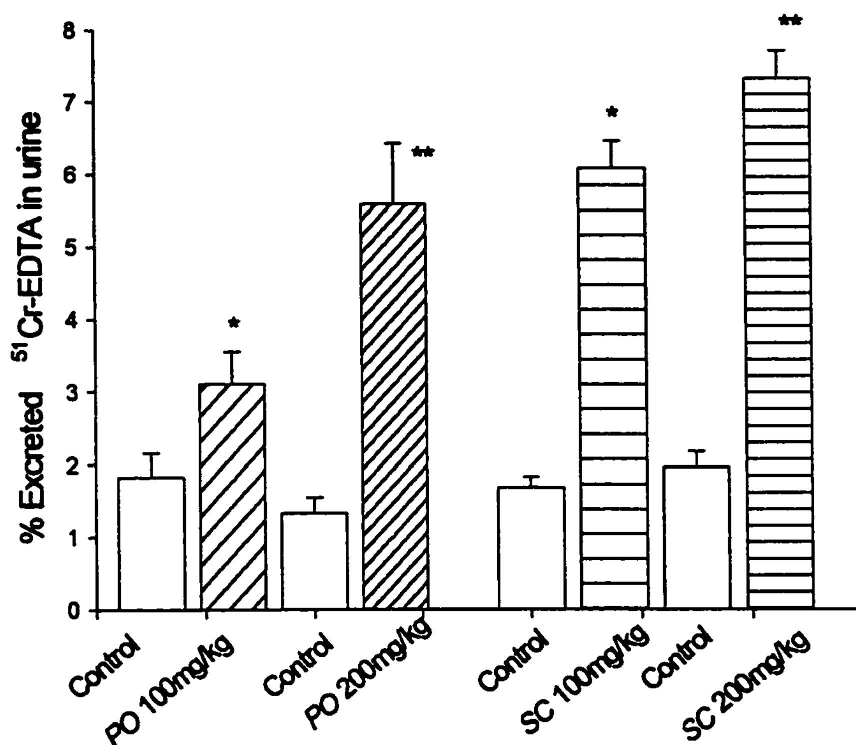

Figure 15: Effect of route of administration (oral and *sc*) and the dosage (100 and 200 mg/kg) on the lower GI tract (^{51}Cr-EDTA) permeability of ibuprofen ibuprofen at 1 h post-dose (*, significantly different from paired control. **, significantly different from paired control and 100 mg/kg dose, $p<0.05$). Error bars represent standard error of the mean.

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3.2. DISCUSSION

Ibuprofen is a 2-phenylpropionic acid derivative with powerful analgesic and anti-inflammatory activities used extensively in the treatment of osteoarthritis and rheumatoid arthritis, and as an analgesic.

A considerable number of different preparations of ibuprofen are being consumed every day. Attempts have been made to improve the efficacy and safety profile of ibuprofen by altering its absorption kinetics such as preparation of sustained release formulation or ibuprofen lysinate (Geisslinger et al., 1989, and Martin et al., 1990). Racemic ibuprofen lysinate, a water-soluble salt of ibuprofen, was developed in Europe to shorten the onset of the therapeutic effect. For example a faster absorption rate has been observed following the lysine salt as compared to acid (Geisslinger et al, 1989, Martin, et al, 1990).

Altered NSAID release may influence the toxicity due to the direct contact but should have no influence on the toxicity that is contributed by the systemic inhibition of the prostaglandin synthesis. Therefore, it might be possible to delineate the mechanism of induced GI toxicity of NSAID by using different formulations and route of administrations. The pattern of the increased GI permeability as a surrogate marker of induced GI toxicity (Davies, et al., 1994 and Davies, et al., 1995) caused by different preparations of ibuprofen as well as oral *vs sc* route of administration was studied.

In this study three different ibuprofen formulations, i.e., immediate release powder, sustained release granules, ibuprofen lysinate crushed tablets with

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different release profile were used to assess the contribution of the local effect to the overall induced toxicity. Previously it has been reported that the time to release 50 % of ibuprofen powder and ibuprofen sustained release granules used in this experiment to be 6 and 42 min, respectively (Sattari and Jamali 1994). Consequently, in the rat the time to reach peak concentration (t_{max}) of immediate release powder and sustained release granules were approximately 1 and 2.5 h, respectively (Sattari and Jamali 1994). Furthermore, in humans, ibuprofen lysinate has a faster dissolution, hence a more rapid absorption (t_{max}=0.55 h) than ibuprofen (t_{max}=0.89 h) (Geisslinger et al, 1989 and Martin et al, 1990). Therefore, the pattern of the release of ibuprofen and subsequent local effect on the GI tract of ibuprofen following administration of ibuprofen lysinate, ibuprofen powder, and sustained release ibuprofen is different. It has been reported that the lysine salt has better GI tolerability as opposed to that of free acid (Martin et al, 1990). The extent of sucrose permeability (i.e., gastroduodenal toxicity) after administration of all examined ibuprofen products was generally similar suggestive of a less importance for the local toxicity caused in the upper GI tract. Gradual absorption of sustained release seems to yield sufficient blood S-ibuprofen to produce GI effect. This effect was expected to be greater after immediate release at the initial stage of absorption. However due, perhaps, to wide variation in sucrose permeation no difference between the two formulations at 1 h post-dose was noticed (Figure 10). The only difference between the immediate and sustained release formulations was that the effect of the latter on sucrose permeability persisted longer than the former. At three hour

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post dose, the permeability following the immediate release, but not after sustained release formulation returned to the baseline (Figure 10). The difference between the two formulations at 3h was, however, insignificant. This minor difference between formulations could be a reflection of the more sustained systemic presence of the sustained release formulation. The lack of local effect was further confirmed by administration of ibuprofen via *sc* route. The increased permeability in the upper GI tract following 100 mg/kg *sc* administration of ibuprofen was higher than oral administration. Similarly, in the lower GI tract, equal doses of *sc* ibuprofen (100 and 200 mg/kg) induced higher levels of permeability than to those of oral doses. The significant increased permeability following *sc* administration might be due to more complete absorption of the drug. These finding suggested that there was not a substantial local effect in the events leading to increased GI permeability.

There was no shift of increased permeability from gastroduodenum to the lower intestine following administration of sustained release ibuprofen. Indeed, similar to the observation made following sucrose administration, ibuprofen lysinate, ibuprofen powder, and ibuprofen sustained release caused comparable increased permeability in lower GI tract. The only minor difference between the immediate and sustained release formulations was a longer onset of activity for the latter (Figure 11) which may be explained by the delayed absorption of the sustained release formulation. These results are in agreement with the observations made for ibuprofen in human by using endoscopy and ^{51}Cr-labeled erythrocyte to assess GI toxicity (Aabakken et al., 1989b). The incidence of

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serious mucosal damage in the GI tract resulting in the overt fecal microbleeding was measured by ^{51}Cr-labeled erythrocyte following a plain and a sustained release ibuprofen formulation. The authors, however, did not assess the site of induced side effect (Aabakken et al., 1989b), the lower GI tract. Similarly, the induced GI toxicity of enteric-coated naproxen was comparable to regular formulation (Aabakken et al., 1989a).

In conclusion, the induced GI toxicity by ibuprofen was significant regardless of the route of administration. Oral doses were not more toxic than *sc* doses indicating a lack of local effect. Rapidly absorbed, immediate release and sustained release preparations demonstrated similar permeability patterns indicating no shift of toxicity to the site of drug release and further suggesting the lack of local toxicity. Therefore, ibuprofen effect on the GI permeability is mainly systemic. Hence, sustained release formulations of ibuprofen may offer the advantages of prolonged efficacy without increasing the GI toxicity. This should not be extrapolated to other NSAIDs e.g., flurbiprofen, tiaprofenic acid, and diclofenac for which different toxicity patterns have suggested.

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CHAPTER 4

MECHANISM OF INDUCED GI TOXICITY OF DICLOFENAC

This chapter has been published in part as an abstract: Khazaeinia T and Jamali F. Formulation dependent gasterointestinal toxicity of diclofenac following administration of immediate and sustained release formulation in rats. Pharm Res, 14(11), s-368 (2469), 1997.

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4.1. RESULTS

4.1.1. DISSOLUTION RATE AND PHARMACOKINETIC STUDIES OF DICLOFENAC

In order to substantiate the release rate of crushed sustained release tablet, dissolution rate and pharmacokinetic studies were carried out.

The immediate release preparation released diclofenac immediately while the sustained release preparation took approximately 1 h for 50% dissolution (Figure 16). The profile of plasma concentration versus time curve (Figure 17) indicates that the time to reach maximum concentration (t_{max}) of sustained release preparation was significantly longer than the immediate release formulation (80.0±24.1 min *vs* 16.0±4.7 min). The C_{max} of sustained release (15.0±2.4 µg/ml) was not significantly different from the immediate release (17.6±6.3 µg/ml). However, the plot of partial AUC depicts a significantly delayed of absorption for the sustained release perpetration (Figure 18). The maximum partial AUC occurred at 0.5-1 h for the immediate release and at 2-4 hr for sustained release preparation. The inter-formulation difference was significantly different. No significant difference in the bioavailability was observed between the formulations ($AUC_{0\text{-}8h}$, 1408 ±232 *vs* 1048±263 µg.min/ml, for sustained and immediate release preparations, respectively).

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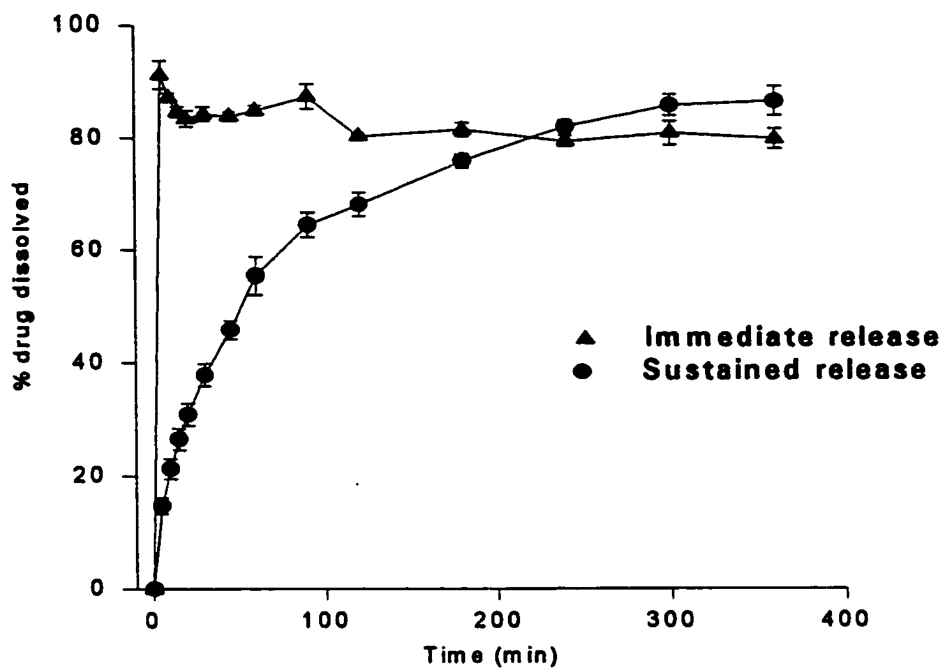

Figure 16: Dissolution rate plots of immediate (▲) and sustained release (●) preparations of diclofenac sodium.

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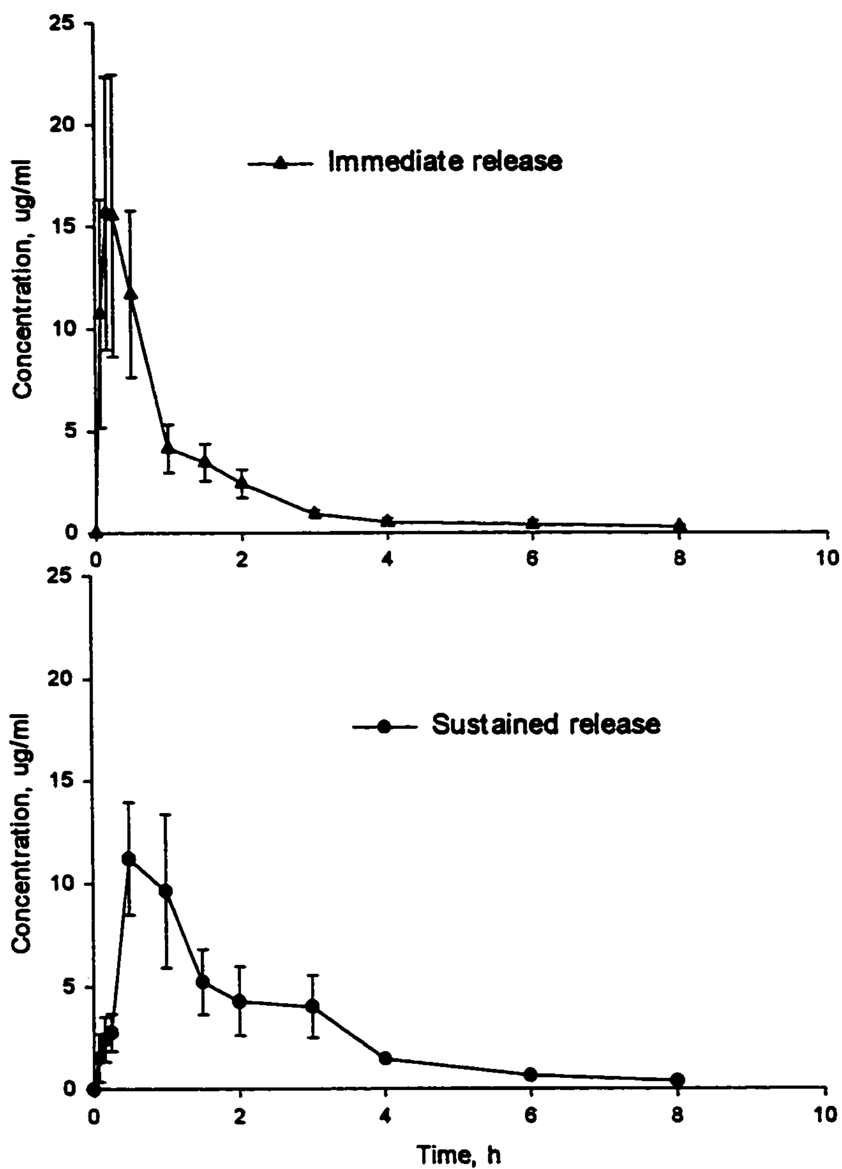

Figure 17: Diclofenac sodium plasma concentration vs time plot following administration of immediate (▲) and sustained release (●) preparations.

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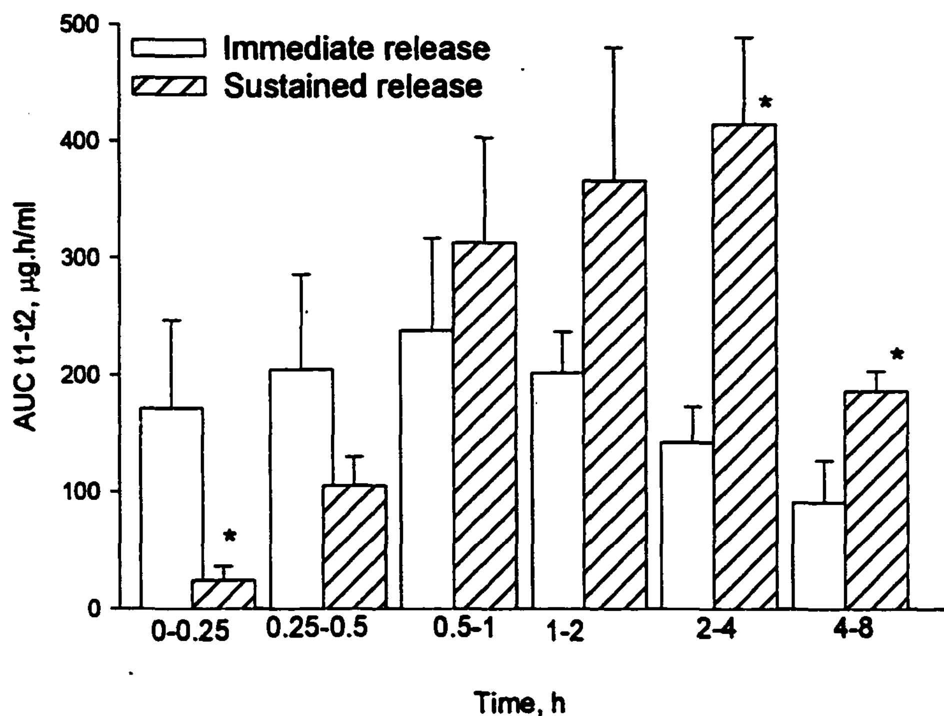

Figure 18: Partial AUC of diclofenac sodium following administration of immediate and sustained release preparations. (*, significantly different from immediate release preparation, $p<0.05$). Error bars represent standard error of the mean.

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4.1.2. GI PERMEABILITY STUDY

The assessment of upper GI toxicity measured as increased sucrose permeability (Figure 19) indicated a significant increase following the immediate release preparation at 1 h post-dose, but not after the sustained release formulation. At 2, 3 and 5 h post-dose, both preparations had significantly increased permeability. At 8 h, both preparations returned to baseline.

In the assessment of lower GI toxicity with ^{51}Cr EDTA (Figure 20), immediate release preparation was more toxic at 1, 2, and 3h post-dose which increased permeability significantly, but later on, it decreased almost to baseline regions. On the other hand, sustained release preparation did not show any increase in permeability at 1 h, but at 2, 3, and 5h post-dose there was a significant increase in lower GI permeability. Also, at 8 h post-dose, there was a significant increase in permeability induced by sustained release preparation, which was indicative of delayed and increased toxicity.

AUC of percent increased sucrose permeability from baseline *vs* time of immediate and sustained released preparations were not significantly different. In contrast, on the lower GI tract, there was a significant difference between two preparations (Figure 21).

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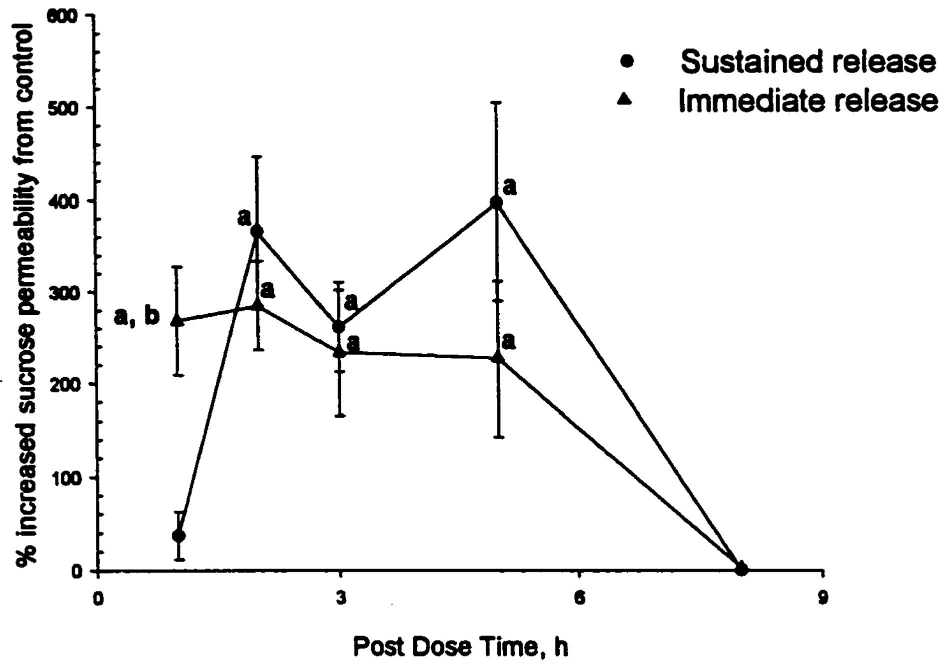

Figure 19: The time courses of percent increase of the upper GI permeability (sucrose) from baseline (control rats) of 15 mg/kg diclofenac sodium as immediate (▲) and sustained (●) release preparations. (a, significantly different from control. b, significantly different from sustained release preparation). Control rats received 1% methylcellulose or water. Error bars represent standard error of the mean.

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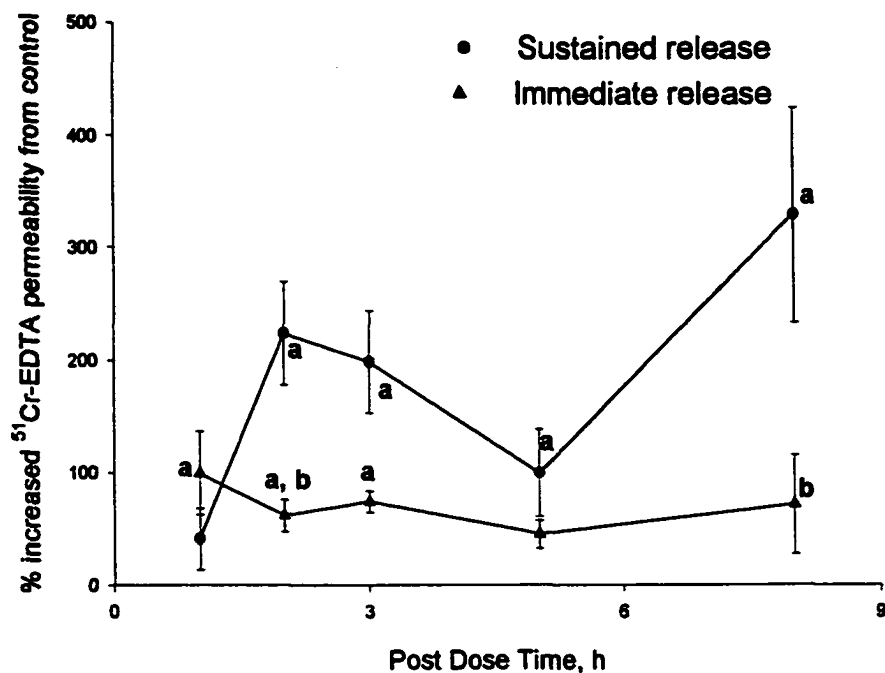

Figure 20: The time courses of percent increase of the intestinal permeability (^{51}Cr-EDTA) from baseline (control rats) of 15 mg/kg diclofenac sodium as immediate (▲) and sustained (●) release preparations. (a, significantly different from control. b, significantly different from sustained release preparation). Control rats received 1% methylcellulose or water. Error bars represent standard error of the mean.

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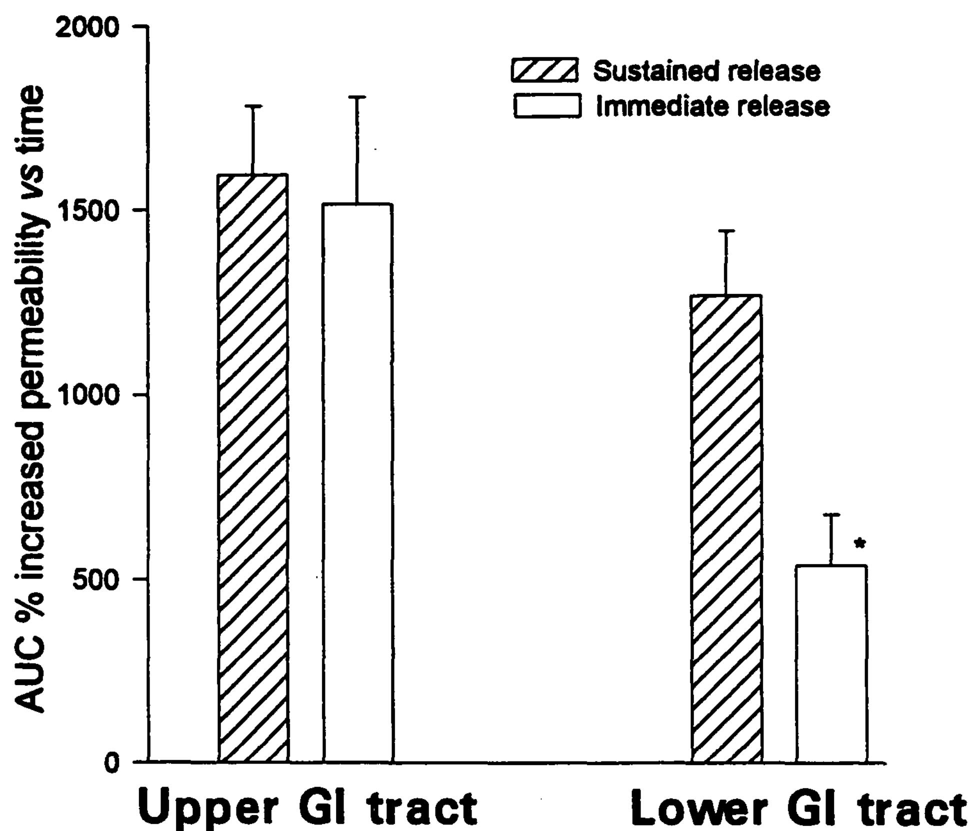

Figure 21: Area under the effect (increased permeability) vs time in upper and lower GI tract following administration diclofenac sodium as immediate and sustained release preparations.

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4.2. DISCUSSION

Diclofenac sodium, a substituted phenylacetic acid derivative, non-steroidal anti-inflammatory drug (NSAID), is commonly used in the management of rheumatic disorders and other chronic inflammatory disease (Zuckner 1986). In addition, due to its analgesic action, it may also be used for acute pain, such as dental (Matthews, et al, 1984), renal colic (Lundstam et al, 1982), post-operative and post-traumatic pain (Kantor 1986).

Diclofenac sodium induced gastrointestinal (GI) toxicity is one of the most severe side effects associated with the use of this drug (Figueras et al., 1994). Similar to other NSAIDs, the induced GI toxicity may range from upper abdominal discomfort to rare but potentially life-threatening effects in the lower GI tract. These conditions are particularly evident in the elderly. It has been assumed that sustained release preparations of NSAIDs with short half-lives, such as diclofenac, may provide more convenient treatment regimens, due to their prolonged effect. Further, lower peak plasma concentrations are expected to reduce the incidences of GI toxicity (Mascher 1989, and Idkaidek et al., 1998). Diclofenac sodium is eliminated rapidly by hepatic metabolism with a short half-life of about 1-2 hours in human (Todd and Sorkin 1988), therefore, it is a suitable drug for sustained release preparations. However, sustained release preparation actually increases the exposure of the lower GI tract to the drug. In case of some NSAIDs, this prolonged exposure may increase the side effects in

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the distal intestine, where the adverse reactions may be even more serious and life threatening (Bjarnason et al., 1993, Davies and Jamali 1997, Davies 1999).

Rationale for the use of sustained release preparation of NSAIDs includes 1) prolongation of the anti-inflammatory and analgesic effects, and 2) reduction of adverse effects through minimized exposure of the upper GI tract to the drug and lower peak plasma concentrations (Mascher 1989, and Idkaidek et al., 1998). However, it has been shown that the GI side effects of NSAIDs are not limited to the upper region of the tract. Indeed, NSAID-induced abnormalities at the lower GI tract are not uncommon. Increased intestinal permeability, inflammation, bleeding, ulceration, and strictures are the serious side effects due to long term NSAIDs therapy (Bjarnason et al., 1987a and 1987b). However, the importance of the toxic effect of NSAIDs on the lower GI tract is often overshadowed by the readily detected adverse effects on the gastroduodenal region (Bjarnason et al., 1993). Diclofenac is available on the market as enteric coated and sustained release formulations. However, a retrospective study of NSAID users raised doubts about the relative safety of sustained release and enteric-coated diclofenac in practice (Figueras et al., 1994). It has been reported that the percent incidence of GI toxicity attributed to sustained and enteric-coated diclofenac (52.1%) was significantly greater than that attributed to plain diclofenac (37.5%)

A better understanding of the effect of the pharmaceutical formulation on the NSAIDs pathogenesis on the GI mucosal injury may help to prevent or decrease the incidence and severity of the toxicity. Diclofenac may exert its GI

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toxicity through direct contact, distribution to the mucosa upon systemic availability, and biliary excretion. Although, lipid solubility and degree of ionization of acidic NSAIDs may contribute to their GI toxicity (McCormack and Brune 1987), it has been suggested that uncoupling of oxidative phosphorylation or inhibition of electron transport by NSAIDs may be an early pathogenic event of the direct local effect (Somasundaram et al., 1997). The involvement of systemic effect of diclofenac induced GI toxicity has been supported by subcutaneous administration of diclofenac to the rat (Skeljo et al., 1993). Indeed, rats dosed subcutaneously with diclofenac showed an even higher incidence of gastric damage compared to those receiving oral doses This was probably due to receiving a slightly higher systemic dose subcutaneously than that orally because diclofenac undergoes approximately 20% first pass metabolism after the oral administration (Peris-Ribera et al., 1991). Biliary excretion and enterohepatic circulation may also contribute to the direct local effects of NSAIDs. Usually NSAIDs with high biliary excretion have more GI toxicity (Reuter et al., 1997). Some NSAIDs such as indomethacin (Beck et al., 1990) and diclofenac (Fukuyama et al., 1994) undergo biliary excretion as conjugated metabolites which, in turn, are hydrolyzed in the intestine to the parent drug. In the rat, approximately 40% of an intravenous dose of diclofenac sodium undergoes enterohepatic circulation over 8 hours (Tabata et al., 1995). This results in further exposure of the gut to the toxin and also prolongation of apparent half-life of the drug. Both these outcomes may promote GI toxicity.

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In this study two diclofenac sodium formulations with different release profiles were used to simultaneously assess the effects of altered site of drug release and the contribution of the local effect to the overall induced GI toxicity up to 8 h post-dose in the upper and lower GI tract. Since both formulations undergo extensive enterohepatic recirculation, the observed difference in the GI toxicity profiles may be due to the local effect. Despite the limited drug release in the upper GI (Figure 16, 17, and 18) the gastroduodenal sucrose permeability following administration of immediate release and sustained release preparations are almost similar (Figure 19, and 21). Therefore, the induced upper GI damage of the diclofenac sodium may be attributed primarily to systemic effects. The sustained release formulation, on the other hand, was more potent in increasing distal intestinal permeability as compared with immediate release formulation (Figure 20, and 21). Since, our data indicates that the sustained release formulation is mainly released in the lower GI tract (Figure 17, and 18), its enhanced intestinal permeability as compared with the immediate release formulation in the lower GI tract indicates damage due to prolonged local exposure at the site of release, i.e., distal intestine.

NSAIDs are heterogeneous in their effect on the GI tract. The mechanisms of some of the NSAIDs may be mainly due to their direct local effects (e.g. aspirin) (Brodie and Hooke 1971). Therefore, the change of conventional formulation of aspirin to enteric-coated preparation reduced the GI damage (Hoftiezer et al., 1980). On the other hand, the mechanism of induced GI toxicity of other NSAIDs (e.g. ibuprofen) is mainly systemic and the route of

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administration as well as the type of formulations does not have significant effects on the GI toxicity (Khazaeinia and Jamali 1996). Moreover, the mechanism of induced GI toxicity of some NSAIDs is due to both local and systemic effects (e.g. flurbiprofen, and tiaprofenic acid) (Davies and Jamali 1997, and Vakily et al., 1999) with a shift of the toxicity of the modified release preparations from the upper to the lower GI tract.

The results of this study are in accordance with the other study that compared the lower GI permeability of conventional and sustained release preparations of diclofenac sodium. It has been reported that ^{51}Cr-EDTA permeability in human is significantly enhanced following one-week administration of a sustained release diclofenac formulation but not after a regular release formulation (Choi et al., 1995).

In conclusion, diclofenac induced toxicity was due to both systemic and local effects. Sustained release preparations of diclofenac induced toxicity in both the upper and the lower GI tract, and the induced lower GI toxicity of this preparation was more than immediate release. Assessment of the diclofenac toxicity on the entire GI tract revealed that there was no particular advantage for using sustained release formulation. In fact, in the rat, sustained release preparation of diclofenac caused more increased intestinal permeability than an immediate release preparation.

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CHAPTER 5

THE COMPARISON GI TOXICITY OF DICLOFENAC ACID, DICLOFENAC SODIUM, DICLOFENAC-DPPC COMPLEX

This chapter has been published in part as an abstract: Khazaeinia T and Jamali F. The effect of association of diclofenac acid with sodium and phospholipid on the gastrointestinal (GI) toxicity. Pharm Res, 1(1), s-331 (2666), 1998.

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5.1. RESULTS

5.1.1. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

In order to substantiate the association of diclofenac acid with DPPC, DSC analysis was performed on diclofenac acid, DPPC, and the diclofenac-DPPC complex. The results confirmed the association of diclofenac acid and DPPC by changing the position of peaks of diclofenac acid and DPPC in diclofenac-DPPC complex thermogram (Figure 22). The disappearance of the DPPC, and diclofenac acid peaks from the complex indicates complete interaction between diclofenac acid and DPPC. In order to confirm the amount of diclofenac acid per milligram of complex, a known concentration of diclofenac-DPPC complex in cethyl trimethylammonium bromide was prepared and authenticated in comparison with the standard curve.

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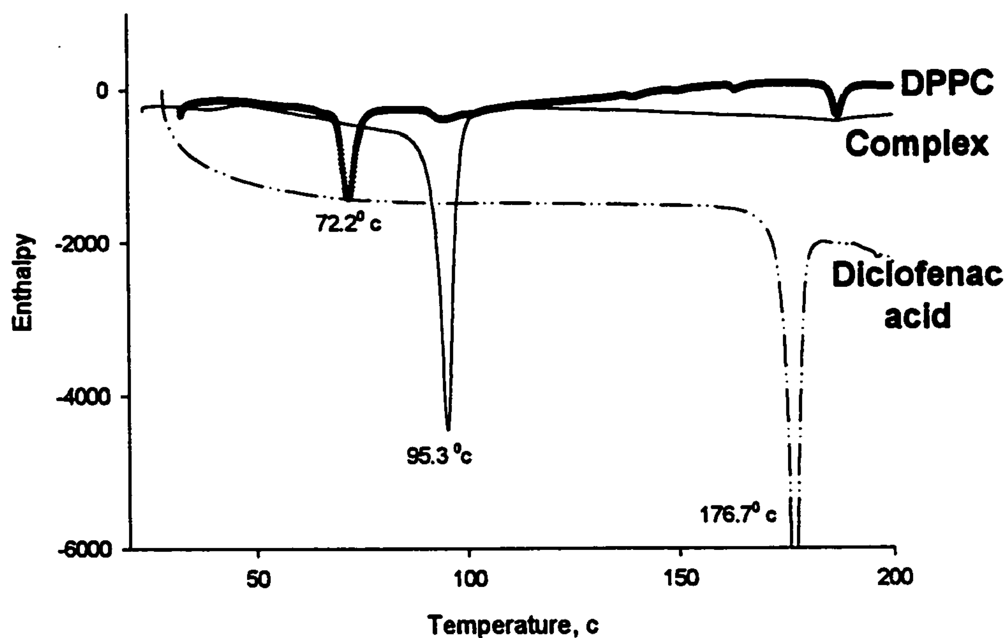

Figure 22: DSC thermograms of diclofenac acid, DPPC, diclofenac acid-DPPC complex.

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5.1.1. PHARMACOKINETICS

As depicted in figure 23, the maximum diclofenac plasma concentration (C_{max}) and the time of its attained (t_{max}) following diclofenac sodium and diclofenac-DPPC complex were similar (17.6±6.3 μg/ml, 16.2±4.7 min, versus 13.2±2.3 μg/ml, 17.0±3.4 min, respectively). These values were significantly different for diclofenac acid (4.2±0.6 μg/ml, 79.2±32.2 min). In addition, the areas under the plasma concentration-time curve up to 1h (AUC_{0-1h}) of diclofenac sodium (613±228 μg.min/ml) and diclofenac-DPPC complex (377±57 μg.min/ml) were significantly higher than that of diclofenac acid (149±36 μg.min/ml). The AUC_{0-3h} of diclofenac acid and the complex were similar (354±48 and 498±65 μg.min/ml, respectively) while the AUC_{0-3h} of diclofenac sodium (915±236 μg.min/ml) was significantly higher than the other formulations. However, there was no significant difference among the AUC_{0-8h} of the formulations (Figure 24).

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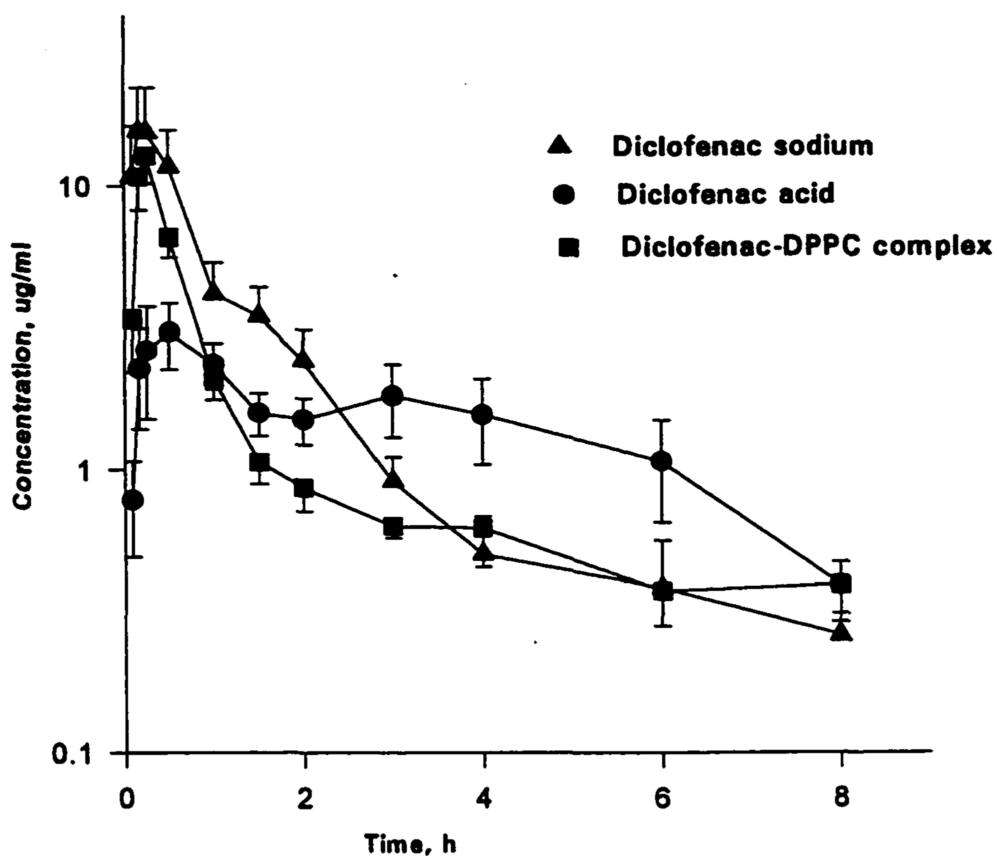

Figure 23: Concentration vs time plot following administration of diclofenac sodium, diclofenac acid, diclofenac-DPPC complex.

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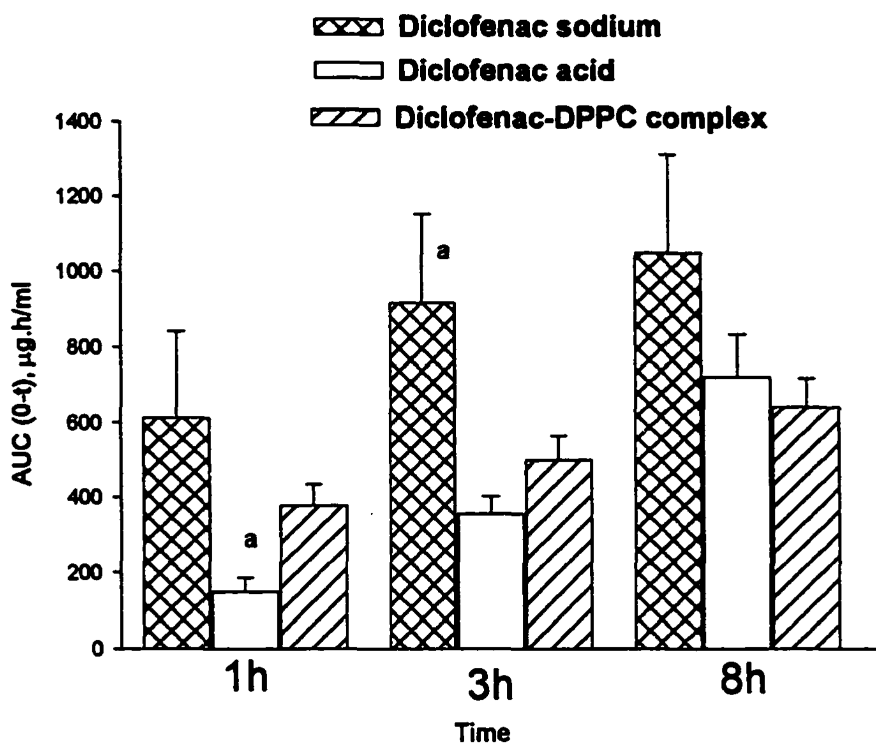

Figure 24: The partial AUC of diclofenac sodium, diclofenac acid, and diclofenac-DPPC complex. (a, significantly different from other preparations, $p<0.05$). Error bars represent standard error of the mean.

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5.1.3. GI PERMEABILITY

As depicted in figure 25 diclofenac-DPPC complex similar to diclofenac acid alone did not significantly increase permeability at 1 h post-dose while diclofenac sodium induced a significant elevated the upper GI permeability. At 3 h all formulations significantly increased upper GI permeability but the induced toxicity by diclofenac sodium and diclofenac-DPPC complex significantly exceeded that of diclofenac acid (Figure 25).

In the lower GI tract, the induced increased permeability was significant at 1, and 3 h post-dose for all formulations (Figure 26). There was no significant difference among formulations.

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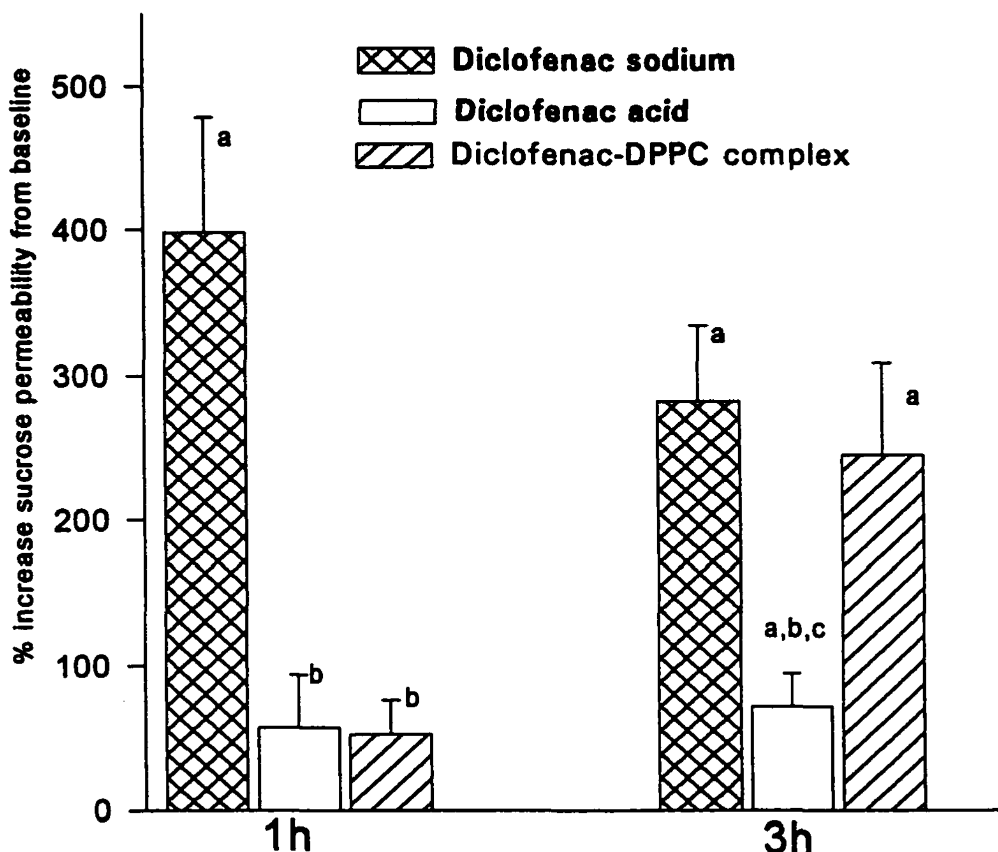

Figure 25: The percent increase upper GI permeability at 1, and 3h postdose following administration of diclofenac sodium, diclofenac acid, and diclofenac-DPPC complex. (a, significantly different from the baseline. b, significantly different from diclofenac sodium. c, significantly different from diclofenac-DPPC complex, $p<0.05$). Error bars represent standard error of the mean.

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Figure 26: The percent increase lower GI permeability at 1, and 3h postdose following administration of diclofenac sodium, diclofenac acid, and diclofenac-DPPC complex. (a, significantly different from the baseline, $p<0.05$.). Error bars represent standard error of the mean.

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5.2. DISCUSSION

Diclofenac, is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs) (Peloso 1996) for its analgesic, and anti-inflammatory indications. Unfortunately, similar to other NSAIDs, diclofenac is associated with serious and sometimes fatal gastrointestinal (GI) side effects, including ulceration, and hemorrhage especially in elderly (Figueras et al., 1994).

Our previous studies demonstrated that local effects of diclofenac are also attributed to induced GI toxicity in addition to the effects caused by the systemic and enterohepatic recirculation (Khazaeinia and Jamali, 1997). Therefore, due to contributions of local effects, modification of diclofenac formulations may reduce GI toxicity.

One focus of development of a NSAID with reduced GI damage is the association of NSAIDs with phospholipid (Lichtenberger et al., 1995). The presence of an adsorbed layer of surface-active phospholipids on the top of the mucus that covers the surface epithelium is suggested to protect the GI tissues by making a hydrophobic layer between the epithelium and the luminal contents (Goddard et al, 1990, and Lichtenberger et al., 1983). In addition, the phospholipid layer increases mucosal resistance to luminal acidity by repelling the diffusion of hydrogen ions (Hills and Kirwood 1992). There are a number of lipids that appear to be covalently and nocovalently associated with mucus glycoprotein (Sloniany et al., 1987, Sloniany et al., 1991, and Lichtenberger et al. 1995). Phosphatidylcholine with the dipalmitoyl species represents the most

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potent surface-active phospholipid (Lichtenberger et al., 1983). It has been suggested that ionic binding between DPPC and NSAIDs shields the NSAID from undergoing pH-dependent changes in charge hence the complex remains lipophilic even as the intragastric pH approached neutrality (Lichtenberger et al., 1996).

It is well known that prostaglandins (PGs) induce secretion of surface-active phospholipids by gastric mucus cells and submucosal glands of proximal duodenum (Hills and Kirwood 1992 and Lugea et al., 1994). It seems that NSAIDs by suppression of PGs production, and subsequently the secretion of surface-active phospholipids may decrease mucosal hydrophobicity. Furthermore, NSAIDs by attachment to the phospholipid layer may weaken the interaction between the phospholipid molecules. Due to enhanced disattachment of phospholipids from the mucus surface, the phospholipid layer becomes more fluid thus loses some of its hydrophobic property (Kivinen et al., 1994).

Diclofenac is a weak acid (pK_a=4.0) with a partition coefficient of 13.0 in octanol/phosphate buffer (pH=7.4) (Menasse et al., 1978). The solubility of diclofenac under physiological conditions ranges from 17.8 mg/L water at neutral pH to less than 1 mg/L at acidic pH (Chiarini et al., 1984, and Fini et al., 1986). Sodium diclofenac has a solubility of 1113 mg/L in water (Fini et al., 1986). The association of diclofenac with zwitterionic phospholipids, which may be both electrostatic and hydrophobic in nature, renders the phospholipids more water-soluble and the NSAID to become more lipid-soluble (Lichtenberger et al.,

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1996). It has been reported that the diffusion of NSAIDs across lipidic membranes and into target cells is accelerated when it is present as a complex with DPPC (Lichtenberger et al., 1996). Our results (Figure 24) suggest that both diclofenac sodium and diclofenac-DPPC complex showed higher drug concentrations than diclofenac acid due to higher solubility, which was demonstrated in the $AUC_{0\text{-}1h}$ and the $AUC_{0\text{-}3h}$.

It has been reported that NSAIDs have the ability to decrease the normal hydrophobic properties of the mammalian stomach (Lichtenberger et al. 1995). The gastric mucosa has nonwettable hydrophobic surface characteristics which protects the epitelium from the luminal acid (Hills et al., 1983). Hydrophobicity is found to be different in the esophagus, antrum, proximal and distal duodenum, and the colon (Hills et al., 1983). Furthermore, the surface hydrophobicity in the stomach is higher than in the proximal duodenum (Lugea et al., 1997). The hydrophobicity in the GI tract is due to appreciable quantities of phosphatidycholines and other surface-active phospholipids which are found in material removed from the mucosal surface of the stomach and other locations in the GI tract (Hills et al., 1983).

The surface mucus cells have the capacity to synthesize, store and secrete the phospholipids into the mucus gel layer, a process that can be modulated by prostaglandins (Kao and Lichtenberger 1993). Phosphatidyl-cholines, including the palmitoyl derivative, represent a very prominent component of the mucus phospholipids, and its mucosal concentration appears to be associated with the integrity of the protective barrier (Lichtenberger et al.,

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1983 and Lichtenberger 1995). NSAIDs appear to decrease mucosal hydrophobicity due, perhaps, to their ability to suppress prostaglandin synthesis. In addition, they may chemically associate with phospholipids and destabilize them from the mucus gel layer (Lichtenberger et al., 1995). Such a biophysical transition would increase the stomach's wettability to luminal acid, resulting in an increase in the back diffusion of luminal acid into the mucosa and the development of erosions and ulcers. It has been reported that NSAIDs chemically pre-associated with zwitterionic phospholipids have limited interaction with intrinsic phospholipids. Since pre-associated NSAIDs with zwitterionic phospholipids no longer have binding sites available to make a complex with the intrinsic phospholipids of the mucus gel layer of the GI tract, thereby, the protective hydrophobic properties of the tissue is preserved (Lichtenberger et al., 1995, and 1996). In this study diclofenac acid associated with DPPC had less toxicity in the upper GI tract only up to 1 h. In addition, in the lower GI tract the complex exhibited no protective effect. These may be due to the systemic effect and/or enterohepatic circulation of diclofenac. Similar to local exposure following oral administration, enterohepatic circulation may reduce hydrophobicity of the GI tract. Enterohepatic circulation results in further exposure of the GI tract to diclofenac (Fukuyama et al., 1994). In addition, the importance of enterohepatic circulation on the induced GI toxicity might be due to the effect of bile on the hydrophobicity of GI tract. Oral administration of bile alone has the ability to significantly decrease hydrophobicity of the gastric mucosa. Furthermore, the administration of bile from rats pretreated with

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diclofenac also reduced gastric hydrophobicity, and the effect was significantly different from that induced by bile from rats pretreated with saline (Lugea et al., 1997). Subcuteneously administration of diclofenac to rats significantly altered the surface hydrophobicity of both gastric and duodenal mucosa (Lugea et al., 1997), and the amount of phospholipids was reduced significantly both in stomach and in duodenum 3 h after treatment with parenteral diclofenac (Lugea et al., 1997). However, in rats with a ligated bile duct, diclofenac did not induce any significant change in gastric or duodenal surface hydrophobicity (Lugea et al., 1997). It seems that under the conditions of continuous enterohepatic recirculation of diclofenac molecules, there are local interactions between the drug and surfactant phospholipids on the GI tract (Lugea et al., 1997). Other explanation for the lack of protective effect of the complex observed in this study is the presence of enzymes such as lipase in the lower GI tract that may dissociate the.drug from the complex.

Overall, the results of this study demonstrated that diclofenac acid was less toxic than diclofenac sodium and diclofenac-DPPC complex. This may be due to a lack of early rise in drug concentration due to slower dissolution, which was demonstrated in the $AUC_{0\text{-}1h}$ and the $AUC_{0\text{-}3h}$. The $AUC_{0\text{-}1h}$ of diclofenac acid was significantly lower than those of diclofenac sodium and diclofenac-DPPC complex. Also, the $AUC_{0\text{-}3h}$ of diclofenac sodium was significantly higher than those of diclofenac acid and diclofenac-DPPC complex. On the other hand, the lack of toxicity of diclofenac-DPPC complex on the upper GI tract at 1h was not likely due to a less local exposure of the GI tract to the drug, since the $AUC_{0\text{-}1h}$ of

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diclofenac-DPPC complex was not significantly different from diclofenac sodium. It seems that only at 1h post-dose, the diclofenac-DPPC complex was a safer drug with respect to the upper GI tract. The protective effect of DPPC did not last likely due to the effect caused by systemic effects and/or enterohepatic circulation of diclofenac. However, the induced toxicity of diclofenac-DPPC complex in the lower GI tract was similar to diclofenac sodium, probably due to the dissociation of the complex in the lower GI tract.

In conclusion, in the upper GI tract, up to 1 h post-dose, diclofenac-DPPC complex was devoid of significant toxicity. However, in the lower GI tract, the complex did not have a superior safety profile than diclofenac sodium.

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CHAPTER 6

SUMMARY, CONCLUSION, AND REFERENCES

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6. SUMMARY AND CONCLUSION

NSAIDs remain the principal medications for symptomative relief in patients with rheumatoid arthritis, osteoarthritis, and other rheumatic diseases. With their analgesic and anti-inflammatory properties, NSAIDs have improved the quality of life for millions of arthritis patients over the world.

The number of new formulations or modification of available NSAIDs continues to increase. Despite subtle differences in pharmacokinetics, these drugs are commonly associated with GI toxicity. The induced GI toxicity ranges from abdominal discomfort, gastric erosions and peptic ulceration with perforation or hemorrhage in upper GI tract to increased intestinal permeability, Inflammation, bleeding (Bjarnason et al., 1987a, 1987b, and Sigthorsson et al., 1998), ulceration (Agrawal 1993), and stricture (Hershfield 1992) requiring surgery in lower GI tract. Because of widespread use of these drugs, GI toxicity has become an important public health concern.

A number of methods have been developed to screen the GI injuries. Recently, studies have demonstrated that utilization of specific probes can assess changes in membrane permeability thus providing an effective way of assessing GI mucosal damage (Meddings et al., 1993, Davies et al., 1994, Ford et al., 1995, and Davies et al 1995). By measuring urinary excretion of orally administered probes, the severity of the mucosal injury becomes evident. Sucrose has been shown to be effective in determining upper GI toxicity induced by NSAIDs (Meddings et al., 1993). The disaccharide's limited absorption

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increased when the gastroduodenal tract is damaged. It is rapidly degraded by sucrase in the small intestine to its monosaccharide constituents, glucose and fructose. On the other hand, ^{51}Cr-EDTA is a stable hydrophilic molecule that its poor absorption is enhanced when small intestine is damaged. Due, perhaps, to the low residence time in the upper GI tract and limited surface area of the region, permeability of ^{51}Cr-EDTA takes place merely in the small and large intestine. Hence ^{51}Cr-EDTA presents a non-invasive indicator of NSAID-induced intestinal toxicity (Ford et al., 1995). In the present studies, permeability changes were used as the surrogate marker of NSAID induced GI toxicity in the rat model (Davies et al., 1994 and Davies et al 1995). The permeability tests had sufficient sensitivity to simultaneously screen upper and lower GI toxicity of different NSAID formulations. The wide range of values found in the present study is indicative of the degree of variability inherent in measurment of GI permeability. This wide range variation has also been seen in other studies (Davies et al., 1994, Davies et al 1995, Ford et al., 1995, and Meddings et al., 1995).

Many approaches have been used to inhibit or decrease the severity of GI toxicity of NSAIDs. These approaches include designing of prodrugs (Shanbhag et al., 1992, Dandona et al., 1990), preparation of enteric coated and sustained release formulations (Hoftiezer et al., 1980, and Trondstad et al., 1985), cyclodextrine-NSAID complexes (Acerbi et al., 1988), nitric oxide- donor NSAIDs (Wallace et al., 1994), selective COX-2 inhibitors (Mitchell et al., 1993), and association of NSAID with dipalmitoyl-phosphatidylcholine (Lichtenberger et

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al., 1995). In the present studies two of the mentioned appraoches, sustained release preparation as well as assosiation of NSAID with DPPC were investigated.

It has been reporeted that NSAIDs exert their GI side effect through both local and systemic availability at the GI tract (Schoen and Vender 1989). The direct local effect may be due to the local exposure after oral administration and also secondary to biliary excretion into the GI tract. The post absorption systemic effect can be manifested after all routes of administration even via non-oral routes (e.g. intravenous and rectal suppositories) (Ligumsky et al., 1990). However, NSAIDs are heterogeneous in their induced GI toxicity. Therefore, Knowledge of the mechanisms responsible for NSAID-associated GI toxicity is necessary to decrease the incidence of these events. In the present studies, the mechanisms of the GI toxicity of ibuprofen and diclofenac sodium were delineated. Altered release may influence the toxicity due to the direct contact but should have no effect on the toxicity that is contributed by the systemic inhibition of the prostaglandin synthesis. Therefore, it might be possible to delineate the mechanism of induced GI toxicity of NSAID by using different formulations and route of administrations.

In order to define the mechanism of ibuprofen induced GI toxicity different preparations of ibuprofen, i.e., immediate release powder, sustained release granules, ibuprofen lysinate crushed tablets as well as route of administration were used. Iboprufen induced GI toxicity is mainly due to systemic effect. Rapidly absorbed, immediate release and sustained release preparations of

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ibuprofen demonstrated similar permeability patterns indicating no shift of toxicity to the site of drug release and suggesting the lack of local toxicity. The lack of contribution of local effect in the induced toxicity was further confirmed by the administration of ibuprofen via *sc* route. Oral doses were not more toxic than *sc* doses. Therefore, sustained release formulation of ibuprofen may offer the advantages of prolonged efficacy without increasing the GI toxicity.

Diclofenac induced GI toxicity is due to both systemic and local effects. Sustained release preparations of diclofenac induced toxicity in both the upper and the lower GI tract, and the induced lower GI toxicity of this preparation was more than immediate release. Since both formulations undergo extensive enterohepatic recirculation, the observed difference in the GI toxicity profiles may be due to the local effect. Furthermore, assessment of the diclofenac toxicity on the entire GI tract at different post-dose revealed that there was no particular advantage for using sustained release formulation. In fact, in the rat, sustained release preparation of diclofenac caused more increased intestinal permeability than an immediate release preparation. It appears that sustained release formulation of those NSAIDs with contribution of local effect to induced GI toxicity is more toxic than immediate release formulation.

The difference between the induced GI toxicity of ibuprofen and diclofenac could be due to the fact that NSAIDs are different in delivering local effect. One explanation can be the difference between these two NSAIDs on uncoupling of oxidative phosphorylation and the extent of contribution of local effect (Somasundaram et al., 1997).

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Although, it has been reported that NSAIDs associated with zwitterionic phospholipids may reduce GI toxicity (Lichtenberger et al., 1995), there was not sufficient data about the NSAIDs pharmacokinetics administered as these preparations. Furthermore, little was known about the effects of the NSAID-phospholipid complexes on the upper and the lower GI tract.

Our studies demonstrated that local effects of diclofenac is also attributed to induced GI toxicity in addition to the effects caused by the systemic and enterohepatic recirculation. Therefore, due to contributions of local effects, modification of diclofenac formulations may reduce GI toxicity. The present study demonstrated that diclofenac acid associated with DPPC had less toxicity than diclofenac sodium in the upper GI tract up to 1 h. However, there was no significant difference between diclofenac acid and diclofenac-DPPC complex at 1h. It is to be noted that this protective effect did not last in the upper GI tract after 1 h. The induced upper GI permeability of diclofenac-DPPC complex was simillar to diclofenac sodium and higher than that of diclofenac acid. In lower GI tract, however, the complex exhibited no protective effect. The induced lower GI permeability was simillar amoung three preparations. Diclofenac-DPPC complex, however, showed higher drug concentrations than diclofenac acid probably due to higher drug concentrations than diclofenac acid probably due to higher solubility, which was demonstrated in the $AUC_{0\text{-}1h}$ and the $AUC_{0\text{-}3h}$.

In conclusion;

1. NSAIDs are heterogenous in their induced GI toxicity. The mechanism of induced GI toxicity of ibuprofen and diclofenac is different.

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2. Ibuprofen induced GI toxicity is mainly due to systemic effect.

3. Diclofenac induced GI toxicity is due to both systemic and local effects. The local effect is formulation dependent.

4. The induced GI toxicity of sustained release formulation of ibuprofen is not significantly different of that from immediate release formulation.

5. Sustained release formulation of ibuprofen has the advantages of prolonged efficacy without increasing the GI toxicity.

6. The induced upper GI toxicity of sustained release formulation of diclofenac sodium is similar to that of immediate release formulation, while the induced lower GI toxicity is significantly higher than that of immediate release formulation.

7. Diclofenac associated with DPPC has less toxicity up to 1 h in the upper GI tract. However, the protective effect of the association with DPPC is not prominent in the lower GI tract.

8. In assessing NSAID induced GI toxicity, the effect on the entire GI tract, at different times following drug administration must be considered.

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