UNIVERSITI PUTRA MALAYSIA

ANTI-INFLAMMATORY AND ANTI-PYRETIC EFFECTS OF FREE AND LIPOSOME-ENCAPSULATED PIROXICAM IN RATS

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Piroxicam (4-hydroxy-2-methyl-N-(pyridine-2-yl)-2H-1,2-benzothiazine-3-carboxamide-1,2-dioxide), an oxicam derivative, is a well known non-steroidal anti-inflammatory drug (NSAID) discovered by Pfizer Laboratories and registered in 1982 (Starek & Krzek, 2009; Baudrimonta *et al*. 1995). It has been reported that piroxicam is one of the most potent NSAID with a long plasma elimination half-life of about 50 hours (Rasetti-Escargueil & Grange, 2005; Canto *et al*., 1990).

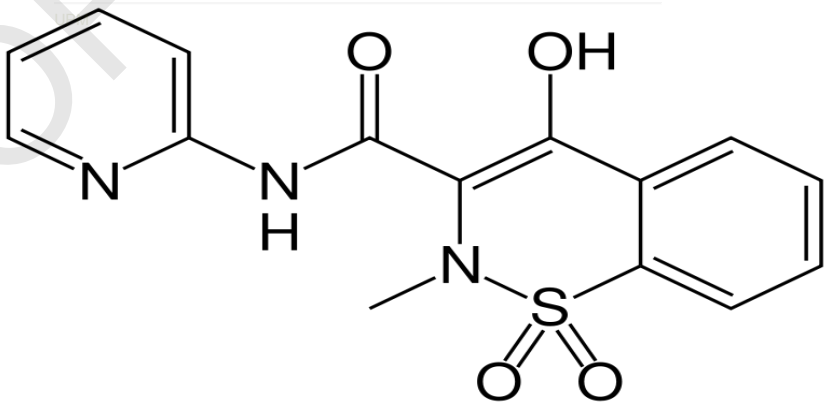

Figure 1.1: Structure of Piroxicam (adapted from Sauceau *et al*., 2008).

Piroxicam, an Oxicam, is a nonselective cyclooxygenase (COX) inhibitor that at high concentrations also inhibits polymorphonuclear leukocyte migration, decrease oxygen radical production, and inhibits lymphocyte function (Bertram, 2007). It has been used for acute or long-term of a wide variety of human diseases including rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, acute gout, acute muscular and skeletal disorders as well as various pain disorders (Baudrimonta *et al*., 1995).

According to the Biopharmaceutic Drug Classification System (BCS) proposed by Amidon *et al*., piroxicam classified under class II drug with low solubility and high permeability. Its pharmacokinetic pattern is characterized by slow and gradual absorption via the oral route and a long half-life of elimination, rendering a prolonged therapeutic action but also a delayed onset of anti-inflammatory and analgesic effect.

Recently, considerable attention has been focused on the improvement of bioavailability and clinical efficacy of poorly water-soluble, lipophilic drugs given orally. Numerous techniques have been used to improve the oral bioavailability hence the efficacy of these drugs by enhancing their solubility in water (Yuksel *et al*., 2003). In order to enhance the solubility of piroxicam and to improve bioavailability, piroxicam was dissolve in a lipophilic vesicle which acts as a delivery system.

As one of the most promising lipid-based nanoparticle technologies, liposomal delivery system is particularly attractive to achieve these objectives (Hens & Romero, 2006). The cooperation of these lipid vesicles with the drug results in physiological changes such as slowing the gastric emptying time, increase in the fluidity and permeability of the epithelial membrane as well as increase uptake into the lymphatic transport system (Schilling & Metra, 1990; Story, 1991).

Liposomes are broadly defined as lipid bilayers surrounding an aqueous space, which formed by phospholipids dispersed in water (Kozubek *et al.,* 2000). The common feature of classical liposomes, i.e., made preferentially of phospholipids and of vesicles made of amphiphilic molecules, was their ability to form dynamic lamellar structures with barrier properties separating the interior of the vesicles from the outside medium (Kozubek *et al.,* 2000). They are generally considered non-toxic, biodegradable and non-immunogenic. Associating a drug with liposomes markedly changes its pharmacokinetics and lowers systemic toxicity; furthermore, the drug is prevented from early degradation and/or inactivation after administration (Kozubek *et al*., 2000).

Liposome-encapsulated drugs have been shown in various studies to exhibit improved therapeutic effects and superior pharmacological properties than those observed with conventional formulations (Vasudevan & Sreekumari, 2005). Thus, the purpose of this study was to determine the therapeutic effects of piroxicam encapsulated with liposomes in rats.

1.2 Objective

1.2.1 General objective

To compare the anti-inflammatory and anti-pyretic efficacy of free and liposome-encapsulated piroxicam in rats.

1.2.2 Specific objective

- To determine the acute anti-inflammatory efficacy of liposome-encapsulated piroxicam compared to free piroxicam using carrageenan-induced paw edema test.
- To determine the chronic anti-inflammatory efficacy of liposome-encapsulated piroxicam compared to free piroxicam using Cotton Pellet-induced granuloma test.
- To determine the anti-pyretic efficacy of liposome-encapsulated piroxicam compared to free piroxicam using Brewer's yeast-induced hyperthermia test.

1.3 Hypothesis

Liposome-encapsulated piroxicam exhibit higher therapeutic effects compared to piroxicam alone when administered to experimental rats.

CHAPTER 2

LITERATURE REVIEW

2.1 Piroxicam

Piroxicam is one of the most potent non-steroidal anti-inflammatory drugs. It is water-insoluble drugs that can be ionized at a physiological pH. This drug can be ionized as a zwitterions with two pKa values (pKa_1 = 1.86 and pKa_2 = 5.46) (Jinno *et al*., 2000). A zwitterionic drug possesses a large intramolecular multipole moment due to its multiplicity of oppositely charged groups therefore, drugs that having this properties have a low solubility in polar and non-polar media, as well as a low lipopolicity (Gwak *et al*., 2005).

Piroxicam is classified under class 2 with a low solubility and high permeability based on the Biopharmaceutics Classification System (Lipka and Amidon, 1999). Based on a study conducted by Tagliati *et al*., it takes more than 2 hours for piroxicam to reach the maximum concentration, indicating the slow absorption rate after being administered orally.

In various animal models, piroxicam inhibits cell migration into an inflamed site whereas in in-vitro, piroxicam inhibits both superoxide anion production and

lysosomal enzyme release from human neutrophils and also inhibits IgM-rheumatoid factor production by human lymphocytes (Ando and Lombardino, 1983). Extensive clinical trials in over 66,000 patients have demonstrated the high efficacy and excellent toleration of piroxicam in rheumatoid arthritis, osteoarthritis, gout, various musculoskeletal disorders and pain of varied etiology (Ando and Lombardino, 1983).

Side effects necessitating discontinuation of piroxicam therapy occur in only 5% of patients, with gastrointestinal side-effects being the most manifested, occurring in less than 1-4% of patients, followed by skin, cardiovascular and central nervous system which generally occur in less than 1% of patients (Baudrimont *et al.,* 1994). However, a studied conducted by Ando *et al*. in 1983 revealed the possible nephrotoxic effects of long term (3 months) administration of piroxicam at a very high doses to rats induces morphological alterations of the proximal tubules and renal papilla.

2.2 Liposome

The characteristics of liposome which exhibit several properties, such as physico-chemical properties and biological characteristic, which enable specific interaction with biological membranes and various cells (Lasic, 1992), it is very useful in various application, such as application in basic science, medicine, bioengineering, cosmetics and agro-food industry (Lipowsky and Sackmann, 1995).

Application of liposomes in pharmacology and medicine can be divided into therapeutic and diagnostic applications of liposomes containing drugs or various markers, and their use as a model, tool, or reagent in the basic studies of cell interactions, recognition processes, and of the mode of action of certain substances (Lasic, 1992).

Great range of delivery system in enhancing effectiveness of drugs in the treatment or prevention of disease is the aim of numerous research groups worldwide. It covers therapies such as those for cancer, microbial infections, hormones and enzymes deficiencies and gene malfunction, as well as vaccines (Lasic and Papahadjopoulos, 1998). Studies showed that liposomes exhibit different biodistribution and pharmacokinetics than free drug molecules, and in several cases this can be used to improve the therapeutic efficacy of the encapsulated drug molecules (Lipawsky and Sackmann, 1995).

The main objective of using liposomes as drug carriers is to achieve selective localization of active drug in disease sites such as tumors and inflammation sites, and this can be obtained through either passive or active targeting (Barenholz, 2001). The passive targeting is a process by which the physical properties of the liposomes together with the microanatomy of the target tissue determine drug selective localization, while active targeting requires, in addition to the ability to reach the disease site, that a homing device (antibody or receptor ligand) will be attached to the liposome surface so that the liposomes can recognize the 'sick' cells and bind to them (Bearenholz, 2001).

Amphothericin B is a drug of choice for antifungal therapies, unfortunately the drug itself is very toxic and its dosage is limited due to neuro- and nephro-toxicity. These toxicities are often correlated to the size of the drug molecule or its complex and obviously liposome encapsulation prevents accumulation of drug in these organs and drastically reduces toxicity (Lopez-Berenstein *et al.,* 1985).

Many different liposome formulations of various anti-cancer agents were shown to be less toxic than free drug (Gabizon, 1989). In some anticancer therapy cases, such as systemic lymphoma, the effect of liposome-encapsulated andriamycin showed enhanced efficacy due to the sustained release effect, i.e. longer presence of therapeutic concentrations in the circulation (Storm *et al*., 1987).

Liposomes can also be used to deliver drugs into the lung (McCalden, 1990), and it is most often done by inhalation of liposome aerosol which can be used either for the treatment of various lung disorders, infections, asthma, or using lungs as a drug depot for the systemic delivery (Lipowsky and Sackmann, 1995).

In ecology, liposomes offer improvements in bio-reclamation and various monitoring and analytical-diagnostic applications. Due to the surfactant action, liposomes also improve the coagulation and sinking of oil spread on the water surface or its cleaning up with floating booms (Gatt *et al.,* 1991) as for example, the Environmental Protection Agency is testing liposomes' ability to deliver nutrients to oil spills to speed up the degradation (Dutton, 1993).

2.3 Inflammation

There are many definitions about inflammation reaction stated by researchers. A definition stated by Sherwood (2004), inflammation is a response to infection, antigen challenge or tissue injury that is designed to eradicate microbes or irritants and to potentiate tissue repair, whereas Cuzzocrea (2005) stated that inflammation occurs as a defensive response, which induces profound physiological adaptations triggered in an attempt to limit tissue damage and remove the pathogenic insult.

Cuzzocrea also mentioned that these mechanisms involve a complex series of events including dilatation of arterioles, venules and capillaries with increased vascular permeability, exudation of fluids including plasma proteins and leukocyte migration into the inflammation area. Excessive inflammation may, however, lead to cellular adaptation and can, if severe, cause physiological injury, organ dysfunction and death (Sherwood and Toliver-Kinsky, 2004).

It is caused by the release of chemicals or mediators from tissue and migrating cells which includes the prostaglandins (PGs), leukotrines (LTs), histamine, bradykinin, platelet- activating factor (PAF) and interleukine-1 (Vane and Botting, 1987), and later studies includes cytokines and nitric oxide as part of the mediators (Sherwood and Toliver-Kinsky, 2004).

Inflammation is characterized by the immediate infiltration of a specific site or lesion with polymorphonuclear cells (PMNs), followed by monocytes and finally

lymphocytes (Cuzzocrea, 2005). Specific adhesion molecules and mediators play an important role in recruiting PMN's into the specific site of inflammation. Activated PMN's play a crucial role in the destruction of foreign antigens and the breakdown remodeling of injured tissue (Cuzzocrea, 2005). Inflammation can be divided into two major categories, acute and chronic, based on timing and pathological features.

2.4 Acute inflammations

Acute inflammation is typically of relatively short duration (hours to days) and is characterized by vasodilatation, the exudation of protein-rich fluid (plasma) and a migration of cells (primarily neutrophils) into site of injury (Splettstoesser and Schuff-Werner, 2002) and, in some cases, activation of the coagulation cascade (Carraway *et al.,* 2003).

Locally, the acute inflammatory response includes capillary vessel vasodilatation (congestion), exudation of plasma proteins (edema), leukocyte adherence to endothelium, chemo-attraction and local activation of leukocytes, release of numerous mediators, elimination of foreign substances (phagocytosis), elimination of recruited cells (apoptosis) and healing of tissue (Cavaillon & Duff 1999).

The classical feature of acute inflammation is vasodilatation and it is clinically characterized by redness and warmth at the site of injury. The vasodilatory response occurs in order to facilitate the local delivery of soluble mediators and

inflammatory cells, and it is primarily mediated by nitric oxide (NO) and vasodilatory prostaglandins (Sherwood and Toliver-Kinsky, 2004).

Edema formation is another early sign of inflammation. This condition is caused by the transvascular flux of protein-rich fluid from the intravascular compartment into the interstitium as a result of the actions of histamine, bradykinin, leukotrines, complement components, substance P and platelet-activating factor (PAF) (Friendl *et al.,* 1989).The barrier functions of small blood vessels are markedly altered by the factors and the permeability of capillaries and venules are increased for both water and protein (Demling *et al*., 1984)

The acute inflammation response has two main functions which are destroy and eliminate infective causative agent, and restore the tissue to useful function. There are four probability outcome of acute inflammation which is complete resolution, healing by organization (scarring), abscess formation (suppuration), and progression to chronic inflammation.

2.5 Chronic Inflammation

Chronic inflammation characterized by a prolonged duration (weeks to months to year) in which active inflammation, tissue destruction and attempts at tissue repair are occurring at the same time (Liew, 2003). The typical features of chronic inflammation are the infiltration of mononuclear cells, such as macrophages, lymphocytes, and plasma cells (Mairir *et al.,* 2004), and fibrosis formation (Davies *et*

al., 2003). Examples of chronic inflammatory diseases condition include rheumatoid arthritis, systemic lupus erythematosus, silicosis, atherosclerosis and inflammatory bowel disease.

Chronic inflammation usually occur due to (1) persistent infections by microbes that are difficult to eradicate, (2) immune-mediated inflammatory diseases or hypersensisitivity diseases which under certain condition can lead to autoimmune disease, and (3) prolonged exposure to potentially toxic agents (Abbas *et al*., 2007).

Macrophages are the dominant cells in chronic inflammation condition. This type of cells is derived from circulating blood monocytes after their emigration from the bloodstream (Abbas *et al.,* 2007). Macrophages play a key role in chronic inflammation as they do in the latter stages of acute inflammation. The preferential accumulation of macrophages in chronic inflammation is at least partly a result of the profile of adhesion molecules displayed by the endothelium in damaged area (Bass and de Boulay, 1997). Macrophages can produce range of substances. Particularly important in chronic inflammation are the 'pro-fibrosis' molecules, such as growth factors and certain cytokines (Bass and de Boulay, 1997).

2.6 Mediators of inflammation

The inflammatory process is associated with the formation of many mediators including prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and the pro-inflammatory cytokines including interleukin-1 (IL-1), IL-8

and tumor necrosis factor (TNF) (Cuzzocrea, 2005). They are released by the body when there is tissue injury, and produce those changes seen in acute inflammation (Speactor and Willoughby, 1963).

2.6.1 Histamine

Histamine or 2-(4-imidazole) ethylamine, formed from histidine, is released when mast cells and basophils degranulate in response to various immunological (lgE or cytokines) or nonimmunological stimuli (Barnes *et al*, 1998). It was synthesized as a curiosity by Wndaus and Vogt (1907) and later extracted from putrefying mixtures by Ackerman (1910). Dale et al. assumed histamine to be responsible for anaphylaxis and Eppinger (1913) demonstrated that histamine produced a reaction in human skin similar to that seen in insect bites.

Lewis (1927) proposed that histamine was released by a variety of injurious stimuli and thought the substance might be the H-substance responsible for triple response. The main functions of histamine are vascular dilatation and smooth muscle constriction (Dale and Laidlaw, 1918).

2.6.2 Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter released as an inflammatory mediator by platelets and murine mast cells. It sensitizes nociceptors to thermal stimuli and to bradykinin application (Lang *et al.*, 1990; Rueff and Dray, 1993). Besides, serotonin is found capable of

increasing vascular permeability, dilating capillaries and producing contraction of nonvascular smooth muscle (Speactor and Willoughby, 1963).

2.6.3 Arachidonic acid (AA) Metabolites

Arachidonic acid, also called eicosanoids, is a 20-carbon polyunsaturated fatty acid (with four double bonds) derived primarily from dietary linoleic acid and present in the body mainly in its esterified form as a component of cell membrane phospholipids (Abbas *et al*., 2007), and the metabolism of arachidonic acid by Cyclooxygenase will produce variety of prostaglandins and thromboxane A_2 and by Lypooxygenase which produce leukotrienes (Karmazyn, 1989).

Prostaglandins are one group of hormone-like substances present in a wide variety of tissues and body fluids. Prostaglandin has many subtypes which are prostaglandin E_2 (PGE_2), PGD_2, $PGF_2\alpha$, and PGI_2 (prostacyclin). PGD_2 is the major metabolite of the COX pathway in mast cells; along with PGE_2 and $PGF_2\alpha$; it causes vasodilation and potentiates edema formation (Abbas *et al.*, 2007). PGE_2 also augments pain sensitivity to a variety of other stimuli and interacts with cytokines to cause fever (Mitchell and Cotran, 2003).

Leukotrienes are formed from 5-lipoxygenases via lipooxygenase pathway of arachidonic acid metabolism. The first leukotriene generated is called leukotriene A4 (LTA_4), which in turn gives rise to LTB_4 or LTC_4.

LTB_4 is produced by neutrophils and some macrophages whereas LTC_4 and its subsequent metabolites, LTD_4 and LTE_4, are produced mainly in mast cells (Abbas *et al.*, 2007). LTB_4 is a potent chemotactic agent and causes neutrophil chemotaxis. (Pettipher *et al*, 1993) LTB_4 also synergizes with PGE_2 in increasing vascular permeability. LTD_4 and LTE_4 cause vasoconstriction, broncospasm, and increased vascular permeability (Abbas *et al*., 2007).

2.6.4 Cytokines

Cytokines are polypeptide products of many cells types including red bone marrow cells, leucocytes, fibroblasts, macrophages, and endothelial cells (Abbas *et al*., 2007). Some cytokines are pro-inflammatory, whereas others are anti-inflammatory, and yet others have both categories of effects (Moalem *et al.*, 2005). Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), produced by activated macrophages, mast cells and endothelial cells, are the major cytokines that mediate inflammation.

IL-1 induces proliferation and activation of T and B lymphocytes, macrophages, endothelial cells, synovial cells, and epithelial cells. It also acts on hypothalamus to cause fever. TNF-α stimulates accumulation of neutrophils and macrophages at sites of inflammation and stimulates their killing of microbes; stimulates macrophages to produce IL-1; and functions as an endogenous pyrogen to induce fever (Tortora Grabowski, 2003). In addition, this mediator sensitizes neutrophils and monocytes to produce

reactive oxygen and nitrogen intermediates (Punjabi *et al*., 1992; Tracey *et al*., 1986).

2.6.5 Chemokines

The chemokines are a family of small (8-10kD), structurally related proteins that act primarily as chemoattractants for different subsets of leukocytes (Sallusto and Mackay, 2004)). Chemokines that are produced transiently in response to infection or inflammatory stimuli will recruit particular cell populations, such as neutrophils, eosinophils, or lymphocytes) to sits of inflammation (Kerry, 2003).

2.6.6 Nitric oxide

Nitric oxide (NO), also known as endothelial-derived relaxation factor, is a short lives, soluble, free radical gas that is synthesized by three distinct nitric oxide synthase (NOS) (Abbas *et al*., 2007). NO plays multiples roles in inflammation, including relaxation of vascular smooth muscle (vasodilation), interacts with reactive oxygen species to be cytotoxic to certain microbes, tumor cells and other tissue cells, and reduction of leukocyte recruitment at inflammatory sites (Laroux, 2001).

2.7 Non-steroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a heterogeneous class including aspirin and various other nonselective and selective inhibitors of

cyclooxygenase (COX) (Patricia *et al*, 2004). The primary mechanism of NSAIDs in the treatment of inflammation is the inhibition of COX, which exists in 2 forms. COX-1 has been shown to regulate many normal physiologic functions, and COX-2 mediates the inflammatory response (Fung & Kirschenbaum, 1999*)*. NSAIDs act by inhibiting the activity of the COX enzyme that catalyzes the conversion of arachidonic acid to prostaglandin H_2, the initial step in the formation of prostaglandin and thromboxane (Vane, 1971; Smith and Willis, 1971; DeWitt, 1991). Inhibition of COX enzyme eliminates direct prostaglandin induced hyperalgesia and attenuates the sensitising effects on other inflammatory mediators (Vane *et al.*, 1994).

Aspirin is the best known and most widely used NSAID. Aspirin are anti-inflammatory, anti-pyretic and analgesic. Tissue injury is associated with release of various mediators including prostaglandins and leads to inflammation. NSAIDs prevent this by inhibiting COX, key enzyme of prostaglandin. Thus, NSAID is less effective in non-flamed tissue (Hawthorn and Redmond, 1998). Prostaglandins have an effective role in the gastro-intestinal mucosa. The action of aspirin on these prostaglandins will causes peptic erosion or ulceration.

2.8 Principles of Anti-inflammatory Assays

2.8.1 Carrageenan-induced Paw-edema Test

Carrageenan, a phlogistic agent, is known to activate Hageman factor thus causing inflammation (Schwartz and Kellermeyer, 1969; Yamamoto and Nozaki-Taguchi, 1997; Zelen'ko, 1994).

Liberation of bradykinin can be seen following the injection of carrageenan into the rat paw, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates (Ueno *et al.,* 2000).

Carrageenan-induced paw edema is a biphasic phenomenon (Vinegar *et al.*, 1969). The early phase, which occurs after injection of the phlogistic agent, has been attributed to the action of histamine, serotonin, and bradykinin on vascular permeability (Vinger *et al.*, 1987). The second, accelerating phase of edema is also a complement-dependent reaction which shown as a result of over production of prostaglandin-like substances (Vinegar *et al.*, 1969; Di Rosa, 1974).

2.8.2 Cotton Pellet-Induced Granuloma Test

Cotton pellet-induced granuloma formation is a typical feature of an established chronic inflammatory reaction and can serve as a subchronic and chronic inflammatory test model for investigation of anti-arthritic substances (Spector, 1969). This model has been employed to assess the transudative and proliferative components of chronic inflammation. The fluid adsorbed by the pellet greatly influences the wet weight of the granuloma whereas the dry weight correlates well with the amount of granulomatous tissue formed.

This method is widely used to evaluate the transudative and proliferative components of the chronic inflammation. Swingle and Shideman (1972) has shown that non-steroidal anti-inflammatory agents such as aspirin give only slight inhibition whereas steroidal anti-inflammatory agents have a strong inhibition on both the transudative and proliferative phase. The moist weight of the cotton pellet correlates with the transuda, whereas the dry weight of the pellet correlates with the amount of the granulomatous tissue (Olajide *et al.*, 1999 and Olajide *et al.*, 2000). This effect did not appear to have any relation to the events involved with cellular migration to injured sites.

2.9 Principles of Anti-Pyretic Assay

2.9.1 Brewer's Yeast-Induced Hyperthermia Test

Fever originates by central nervous system (CNS) activities, but neither exogenous nor endogenous pyrogens are able to cross the blood brain barrier (A.-S. Bhat *et al*., 2005). Research has been done to determine the freely diffusible mediator for the genesis of fever in response to yeast administration.

In Brewer's yeast-induced pyrexia in rodents, yeast cell wall products deposited subcutaneously act as exogenous pyrogens and stimulate the immune cells (macrophages and lymphocytes) to generate endogenous pyrogens in the form of cytokines in the circulation that go to anterior hypothalamus to alter the set point for body temperature (Miiler, 2000).

Cyclooxygenase (COX) enzyme is activated by pyrogens, which converts arachidonic acid to prostaglandins (PG), and PG subtype, PGE_1, is thought to be increased in the hypothalamus (Tiwari *et al*., 2010). Li *et al*. (2001) and Zhang *et al.* (2003) stated that COX-2 isoform mediates febrile response of mice to interleukin-1-beta and COX-2 has predominant role in lipopolysaccharide-induced fever in rats.

CHAPTER 3

METHODOLOGY

3.1 Liposome preparation

Two types of commercially available pro-liposomes, namely Pro-lipoTM Duo and Pro-lipoTM C, were used respectively to encapsulate the model drug (piroxicam) in accordance to the manufacturer's instructions with some modifications. The proportion by weight of pro-liposome: piroxicam aqueous solution (hydration): distilled water (dilution) was 1:2:5.

A pre-determined amount of piroxicam was first completely dissolved in suitable solvent (DMSO). This stock piroxicam solutions were prepared freshly daily, protected from direct light and discarded after use. Next, 400µl of the drug solution with known concentration was added gradually into 2g pro-liposome in a beaker with moderate stirring (±100 rpm) for 60 minutes. The mixture was then hydrated with 3.6ml distilled water which was added gradually to form a concentrated liposomal suspension.

These liposomal suspensions was stirred continuously for a pre-determined period of stirring (hydration) time before further diluted with 10ml distilled water with continuous stirring for another 30 minutes. Preparation of all liposomal samples was carried out at room temperature. All formulations (distinctive by different type of pro-liposome, drug concentration and/or duration of hydration time used during preparation process) were produced in three batches respectively to ensure their reproducibility.

3.2 Animals

Male Sprague–Dawley rats (200–350 g) were used for anti-inflammatory assay. The animals were housed in standard cages (6 rats in each standard cages) at room temperature (22 ± 2 °C) under a 12 h light/12 h dark cycle in the animal house of Faculty of Medicine and Health Science, Universiti Putra Malaysia. The animals had free access to standard pellet diet and water *ad libitum.* The room humidity was maintained in 70-80%.

The animals were acclimatized for at least three days before subjection to experiments. The experimental procedures were carried out in strict compliance with the Institutional Animal Ethics Committee regulations and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

3.3 Anti-Inflammatory Assays

3.3.1 Carrageenan-Induced Paw Edema Test

The Carrageenan-induced paw edema was assessed by the method described by Winter *et al*. with a slight modification. Rats were divided into 8 groups (n=6) and receive piroxicam, Px (0, 0.3, 3, and 30 mg/kg) or liposome-encapsulated piroxicam, LPx (0, 0.3, 3, and 30 mg/kg). The treatment was administrated via oral route. 30 minutes post-treatment, intraplantar administration of 0.005 ml of 1% Carrageenan suspension was injected into the right hind paw.

Paw volume was measured before (V_0) and at 1, 2, 3, 4 and 5 hour (V_f) following the carrageenan administration using a plethysmometer. The degree of inflammation was quantified by measuring the volume displaced by the paw between the final volume (V_f) and initial volume (V_0). The percentage of anti-inflammatory or inhibition was calculated using the formula below:

$$\text{Percentage edema} = \frac{V_T - V_0}{V_0}$$

$$\text{Percentage inhibition} = \frac{(V_T - V_0)_{\text{control}} - (V_T - V_{0)\text{ treated}}}{(V_T - V_0)_{\text{control}}} \times 100$$

3.3.2 Cotton Pellet-Induced Granuloma Test

The method of Gupta *et al.* was employed, with slight modifications. Cotton pellets, weighing around 30 mg each, were autoclaved for 2 hours. The autoclaved cotton pellets were implanted aseptically at intrascapular distance under the skin on the shaved backs of anesthesized rats.

Rats were divided into 8 groups (n=6) and receive piroxicam, Px (0, 0.3, 3, and 30 mg/kg) or liposome-encapsulated piroxicam, LPx (0, 0.3, 3, and 30 mg/kg). Treatments were given once a day for a period of 7 days and the rats were sacrificed on the 8th day. The moist cotton pellets, surrounded y granuloma tissue were dissected out and weighed, then dried at 60ºC overnight before weighed again for its final dry mass.

The percentage of inhibition, indicated by its ability to reduce the development of granuloma tissue, was calculated using the equation below:

$$\left(\frac{T_C - T_T}{T_C}\right) \times 100$$

where T_C = weight of granuloma tissue of control group; T_T = weight of granuloma tissue of treated group.

3.4 Anti-Pyretic Assay

3.4.1 Brewer's Yeast-Induced Hyperthermia Test

Hyperthermia was induced in rats by the method described by Teotino *et al.* with slight modifications. 10 ml/kg of 20% aqueous suspension of Brewer's yeast which prepared daily were sub-cutaneously injected into 2 groups of rats. The rectal temperatures were recorded initially and at 18 hour.

Animals with elevated body temperature were orally given with piroxicam, Px (0, 0.3, 3, and 30 mg/kg) or liposome-encapsulated piroxicam, LPx (0, 0.3, 3, and 30 mg/kg) after 18 hour yeast injection. The body temperature was measured at 1 hour intervals up to 5 hours after administration of treatment drug.

3.5 Statistical Analysis

The results were expressed as Mean ± S.E.M. The statistical analysis was performed by analysis of variance (ANOVA). Difference between each groups are further analyzed by Student's *t*-test and Dunnet's multiple comparison test. P values less than 0.005 ($P<0.005$) were considered as indicative of significance. All statistical analyses were carried out using the SPSS 16 for windows.

CHAPTER 4

RESULT

4.1 Carrageenan-Induced Paw Edema

Results obtained from the carrageenan-induced paw edema test are shown in Table 4.1. Doses of piroxicam (Px) 3.0 and 30 mg/kg and liposome-encapsulated piroxicam (LPx) with the dose of 0.3, 3.0, and 30 mg/kg exhibited significant ($P<0.05$) anti-inflammatory activity. Minimum significant inhibitory properties of 40.0% was achieved in 3 mg/kg of Px at the first hour and gradually increased with the increment in concentration of the dose. The first effect of encapsulated piroxicam can be seen at the fifth hour following treatment with 3.0 mg/kg of LPx. 30 mg/kg of encapsulated piroxicam shown to have greater swelling inhibitory compared to free piroxicam. The effect can be seen from the final three hour of the treatment.

Table 4.1: Carrageenan-induced Paw Edema Test: Mean Hind Paw Volume

Treatment	Dose (mg/kg)	Edematous Paw Volume, ml, mean ± SEM (n = 6)					anti-inflammation, %				
		1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
Px	0 (control)	0.48±0.07	0.43±0.09	0.55±0.09	0.5+0.10	0.5+0.08	-	-	-	-	-
	0.3	0.29+0.05	0.21+0.03	0.33+0.05	0.24+0.05	0.29+0.06	40.0	50.0	39.1	52.4	42.9
	3	0.29+0.04*	0.26+0.7	0.26+0.06*	0.24+0.05	0.26+0.04*	40.0	38.9	52.2	52.4	47.6
	30	0.29+0.05	0.17+0.06*	0.21+0.03*	0.24+0.03*	0.17+0.04*	40.0	61.1	60.9	52.4	66.7
LPx	0	0.46+0.47	0.36+0.08	0.45+0.08	0.38+0.05	0.38+0.11	0.0	16.7	17.4	23.8	23.8
	0.3	0.24+0.03*	0.29+0.05	0.26+0.04*	0.26+0.04	0.24+0.04*	50.0	33.3	52.2	47.6	52.4
	3	0.19+0.03*	0.21+0.07	0.29+0.04*	0.21+0.05*	0.12+0.04*#	60.0	50.0	47.8	57.1	76.2
	30	0.17+0.02*	0.10+0.03*	0.07+0.05*#	0.12+0.02*#	0.05+0.03*#	65.0	77.8	86.9	76.2	90.5

* = t-test ($P<0.05$, statistically different from control)

= t-test ($P<0.05$, statistically different compare to group at the same dosage)

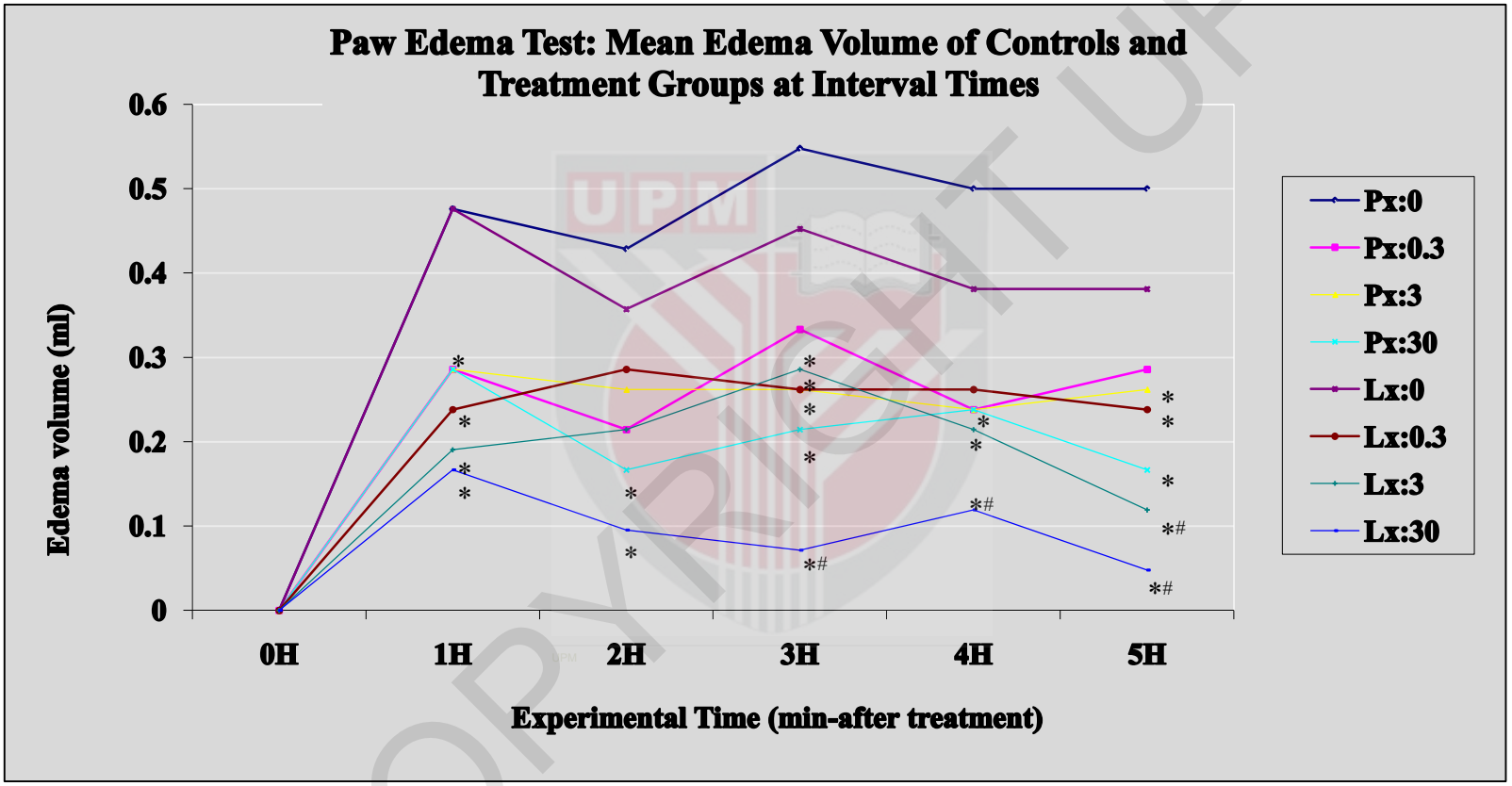

Figure 4.1: Paw-Edema Test: Percentage of Inhibition of Paw-edema Among Treatment Groups at Interval Times

4.2 Cotton Pellet-Induced Granuloma Test

The anti-inflammatory activity of piroxicam (Px) and liposome-encapsulated piroxicam (LPx) against a chronic model of inflammation is shown in table 4.2. The significant inhibitory activity can only be seen in higher treatment dose. Treatment with Px at the dose of 30 mg/kg and LPx (3 and 30 mg/kg) shown to have a significant anti-inflammatory activity when compared to control, as indicated by the increment of percentage inhibition. Liposome-encapsulated piroxicam also exhibit greater inflammatory activity when compared to free piroxicam at the same doses. Treatment with LPx 3 mg/kg showed a significant 36.4% and 49.3% of inhibition for both transudative and granuloma weight respectively. Effect of LPx at the dose of 30 mg/kg can only be seen in the reduction of granuloma weight with 51.8% inhibition.

Table 4.2: Cotton pellet Granuloma Model Test: Increase in Mean Weight of Pellet

Treatment	Dose (kg/mg)	Weight of Cotton Pellet, mg, mean ± SEM (n=6)			
		Transudative	Inhibition, %	Granuloma	Inhibition, %
Px	0	429.9	-	90.8±11.9	-
	0.3	376.9	12.3	78.9±12.4	13.1
	3	352.1	18.1	76.3±3.7	15.9
	30	296.8	31*	61±5.6	32.9*
LPx	0	428.1	0.4	82.3±5.9	9.4
	0.3	353	17.9	69±3.5	24
	3	273.4	36.4*#	46.1±7.1	49.3*#
	30	243.9	43.3*	43.8±5.1	51.8*#

* = t-test ($P<0.05$, statistically different from control)

= t-test ($P<0.05$, statistically different compare to group at the same dosage)

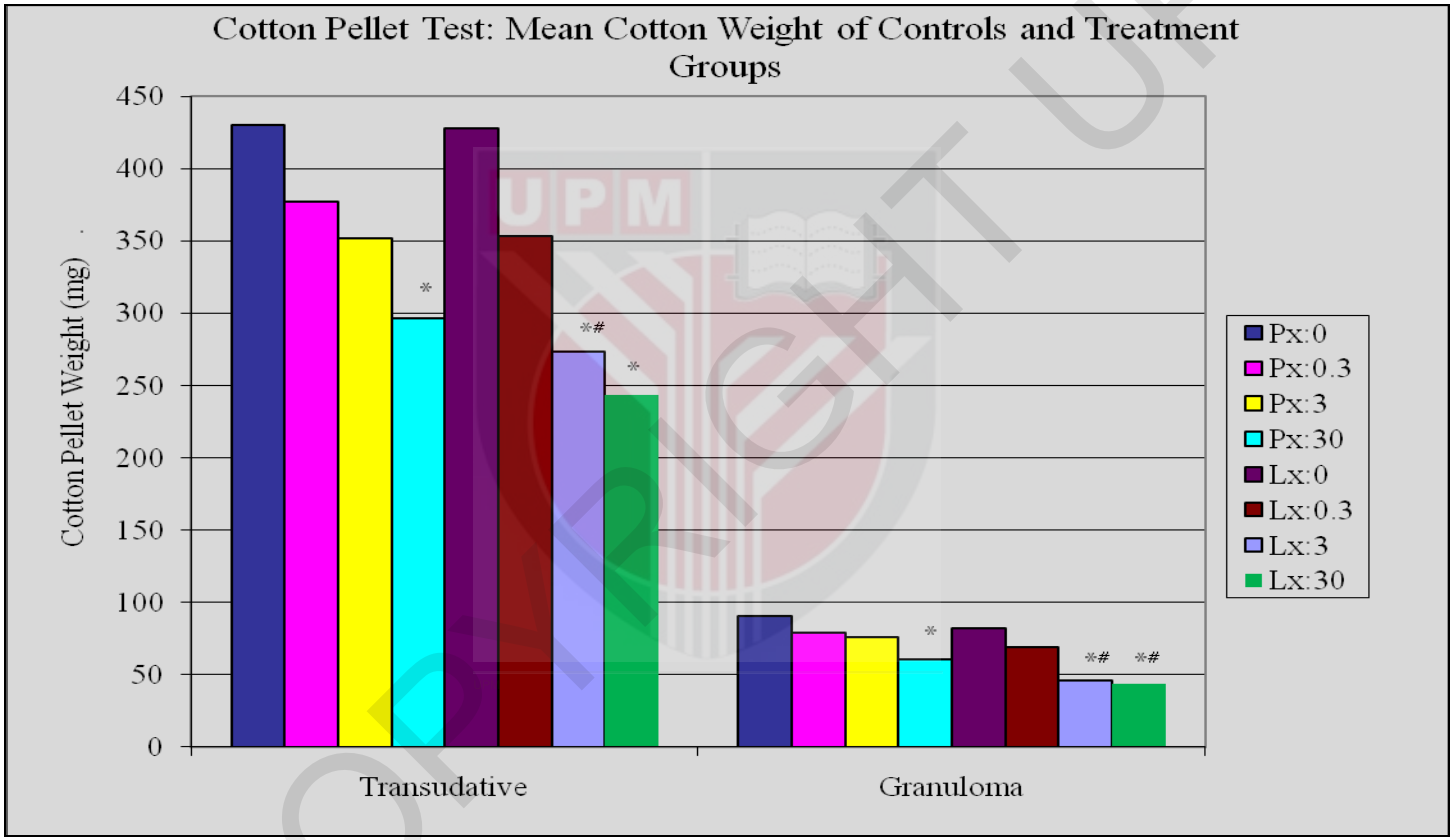

Figure 4.2: Cotton Pellet-Induced Granuloma Model Test: Increase in Mean Weight of Pellet

4.3 Brewer's Yeast-Induced Hyperthermia Test

The treatment using both piroxicam (Px) and liposome-encapsulated piroxicam (LPx) at the highest dosage (30mg/kg) caused a significant lowering in rectal temperature of hyperthermic rats. Based on table 4.3, 30mg/kg of LPx is shown to have longer anti-pyretic properties as the effect of treatment can be seen from the first hour until 5th hour following treatment. Moreover, treatment with LPx at lower dosage, 3mg/kg, also lowered the rectal temperature.

Table 4.3: Means rectal temperature of control and treated groups at interval time.

Treatment	Dose (mg/kg)	Rectal Temperature (°C) after yeast injection, mean ± SEM (n=6)						
		-18h	0h	1h	2h	3h	4h	5h
Px	0 (control)	37.48±0.33	38.07±0.30	37.83±0.15	37.78±0.16	37.53±0.07	37.75±0.17	37.57±0.17
	0.3	37.10±0.10	37.85±0.11	37.62±0.08	37.62±0.14	37.63±0.17	37.35±0.11	37.27±0.16
	3	37.1±0.19	37.82±0.25	37.68±0.13	37.37±0.28	37.53±0.26	37.40±0.16	37.2±0.26
	30	37.50±0.24	37.98±0.26	37.48±0.20	37.30±0.20	36.88±0.24*	36.62±0.09*	36.88±0.13*
LPx	0	37.20±0.22	37.80±0.18	37.83±0.21	37.80±0.14	37.78±0.10	37.52±0.16	37.47±0.09
	0.3	37.06±0.2	37.7±0.04	37.78±0.17	37.72±0.18	37.27±0.20	37.02±0.17*	37.07±0.19
	3	37.23±0.25	37.73±0.16	37.52±0.09	37.22±0.14*	37.13±0.12*	36.93±0.16*	36.950±0.06*
	30	37.60±0.19	37.73±0.12	37.32±0.11*	37.23±0.16*	36.85±0.11*	36.52±0.13*	36.58±0.12*

* = t-test ($P<0.05$, statistically different from control)

= t-test ($P<0.05$, statistically different compare to group at the same dosage)

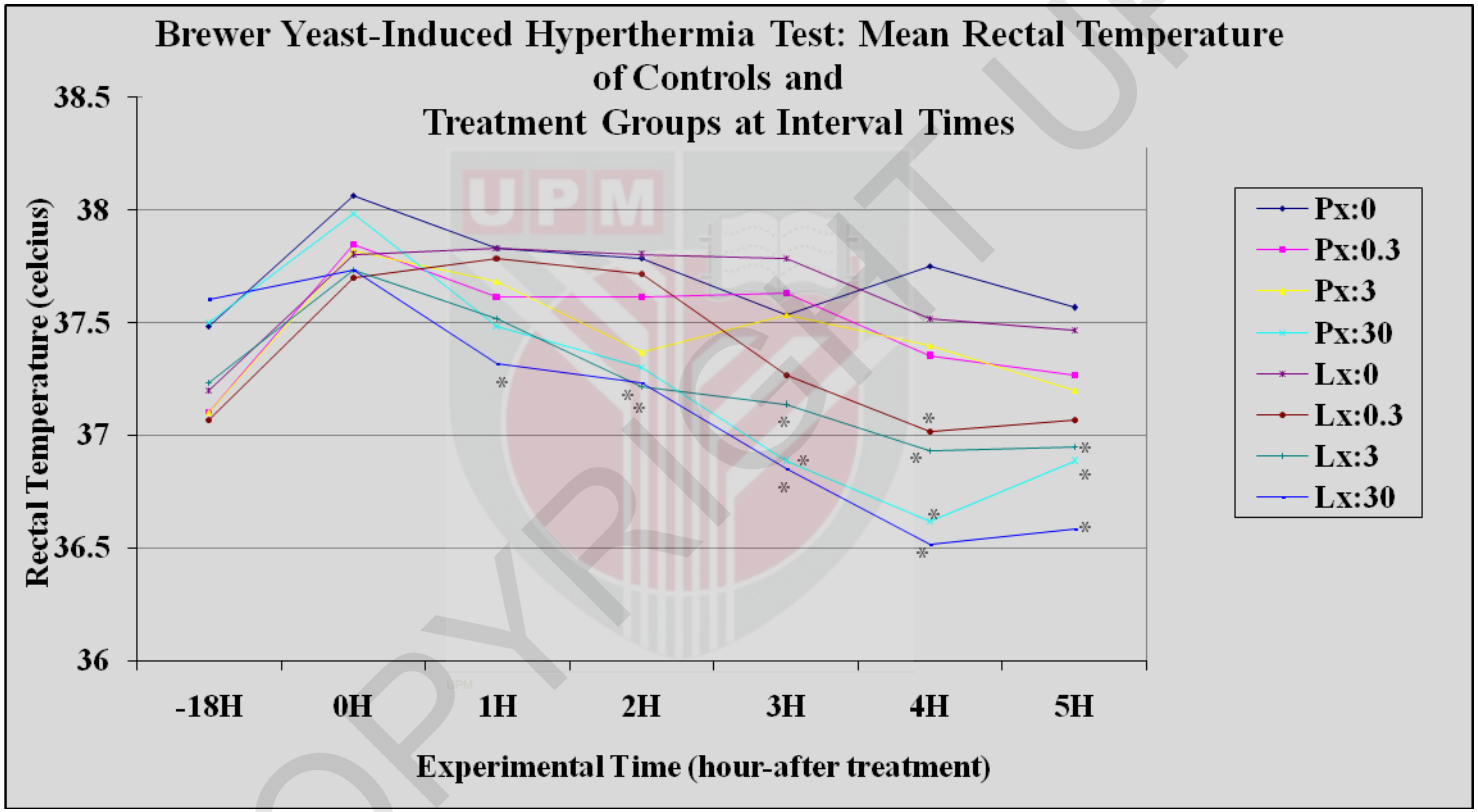

Figure 4.3: Mean Rectal Temperature of Controls and Treatment Groups at Interval Times

CHAPTER 5

DISCUSSION

Liposomes can dissolve molecules that are sparingly water soluble and act as microreservoirs with controllable leakage characteristic. The growing use of liposome as drug delivery systems requires potential alteration to those features of the bilayers that may affect the barrier properties of the skin in order to improve bioavailability and therapeutic index (Canto et al., 1999). In this study, the anti-inflammatory and anti-pyretic effect of free piroxicam and liposome-encapsulated piroxicam were evaluated in three experimental models, the Carrageenan-induced paw edema test, Cotton pellet-induced granuloma test, and Brewer's yeast-induced hyperthermia test.

Based on the study conducted, the results obtained showed that liposome has the ability to alter the therapeutic effect of piroxicam. The entrapment of piroxicam in liposomes produced an enhancement of the anti-inflammatory effect compared with free drug (Canto et al., 1999). This study revealed that oral administration of liposome-encapsulated piroxicam reduced carrageenan-induced paw edema in a dose-dependent manner. Free piroxicam inhibit the development of paw edema at the

dose of 3 and 30 mg/kg. When compared to control, 3 mg/kg of piroxicam inhibit the inflammation at the first, third and fifth hour, whereas 30 mg/kg of piroxicam inhibit paw edema development from the second hour following carrageenan injection with a maximum 66.7% of edema inhibition.

On the other hand, liposome-encapsulated piroxicam showed a better anti-inflammatory activity, as it can be seen on table 4.1, where 0.3 mg/kg of encapsulated piroxicam inhibit the paw edema development at the first, third, and fifth hour following induction, the same effect seen when administered with 3 mg/kg of free piroxicam. This indicates that anti-inflammatory effect can be seen at a lower treatment dosage. Liposome-encapsulated piroxicam at the dose of 3 and 30 mg/kg also showed the same anti-inflammatory effect with free piroxicam.

Regarding the possible mechanisms involved, it has been suggested that several inflammatory mediators play a role, e.g. complement, histamine, kinins, prostaglandins (PGs) and pro-inflammatory cytokines (Di Rosa et al., 1971 and Hirschelmann and Bekemeier, 1981). Histamine and serotonin are usually responsible for eliciting the immediate response of inflammation in rats whereas the kinins and prostaglandins mediate the more prolonged delayed-onset responses (Di Rosa et al., 1971 and Goetzel, 1980). On the other hand, the delayed phase of carrageenan-induced edema results mainly from the potentiating effects of prostaglandins on mediator release, especially bradykinin.

In comparison to free piroxicam, 3 and 30 mg/kg of liposome-encapsulated piroxicam exhibit higher anti-inflammatory effect. Treatment with 3 mg/kg of encapsulated piroxicam showed a significant higher paw edema inhibition at the fifth hour with 76.2% of inhibitory effect compared to 3 mg/kg of free piroxicam which possess 47.6%. 30 mg/kg of encapsulated piroxicam showed a better inhibitory property as the significant different can be seen from the third hour until the final measurement (5^{th} hour). The inhibitory effect of 30 mg/kg encapsulated piroxicam was 86.9%, 76.2% and 90.5% compared to free piroxicam with only 60.9% 52.4% and 66.7%. These results showed that the therapeutic effect of piroxicam is increased by the encapsulation of piroxicam which directly supports the idea of Noble *et al.* (2004) who stated that the main advantage of using liposome to deliver drug is that they offer the potential to improve the therapeutic index.

The Cotton-pellet-induced granuloma test model has been employed to assess the transudative and proliferative components of chronic inflammation. During the repair process of inflammation, there are proliferations of macrophages, neutrophils, fibroblasts and angiogenesis, which are the basic sources of forming a highly vascularised reddish mass, termed granulation tissue (Swingle, 1974 and Bhattacharya et al., 1992).

In the experiment conducted, only 30 mg/kg of piroxicam and 3 and 30 mg/kg of liposome-encapsulated piroxicam shown to have a significant ($P<0.005$) anti-inflammatory activity when compared to control group. The inhibition of

granuloma and transudative weight by 30 mg/kg encapsulated piroxicam was the highest, with 51.8% and 43.3% respectively. In comparison with free piroxicam, 3 mg/kg of encapsulated piroxicam exhibit greater therapeutic effect in reducing both transudative and granuloma weight. The putative mechanism associated with the treatment's activity may be inhibition of the synthesis of many mediators involved in the formation of fibrovascular tissue, including chemokines, cytokines and eicosanoids (Lukacs et al. 1994 and Moore et al. 1998).

Increased body temperature is known as one of the symptom of the body against an inflammatory stimulation. In this study, Brewer's yeast-induced hyperthermia in rat model was used to evaluate the anti-pyretic activity of free and liposome-encapsulated piroxicam. Cyclooxygenase (COX) enzyme is activated by pyrogens, which converts arachidonic acid to prostaglandins (PG), and PG subtype, $PGE_{1,}$ is thought to be increased in the hypothalamus (Tiwari et al., 2010). Li *et al*. (2001) and Zhang *et al.* (2003) stated that COX-2 isoform mediates febrile response of mice to interleukin-1-beta and COX-2 has predominant role in lipopolysaccharide-induced fever in rats.

The therapeutic effect of 3 and 30 mg/kg liposome-encapsulated piroxicam obtained were better than that of free piroxicam when both compared to control. The result also suggested that hyperthermic rats responded better to 30 mg/kg of encapsulated piroxicam as the rectal temperature showed a significant reduction from as early as the first hour following treatment. Effect of free piroxicam can only be

seen at the dose of 30 mg/kg, reducing rectal temperature from the third to fifth hour following treatment. This showed that the treatment has some influence on prostaglandin biosynthesis because prostaglandin is believed to be a regulator of body temperature (Milton, 1982). Unfortunately, however, there is no significant different obtained when to comparing between free and encapsulated piroxicam. This may be due to the fact that the anti-pyretic of piroxicam is sufficient, so encapsulation of the drug does not produce any significant increase in therapeutic effects.

The enhancement of the anti-inflammatory effect obtained when piroxicam was encapsulated in liposome probably is related to the permeation increase due to the amphiphile of the liposome bilayers and to the slow release of the drug from the liposomes (Canto et al., 1999). This enables piroxicam to last longer in the locality of its pharmacological effect (Mezei, 1994). The drug will be released from the liposomes if the bilayer is destroyed by organic fluids (Weiner et al., 1989). Liposomes can act as a reservoir system, similar to a slow-release vehicle (Fattal and Puisieux, 1996), enabling more uniform and prolonged release of the drug (Lasic, 1993). So, a high topical concentration of the drug can be maintained when compared with conventional vesicles (Masini et al., 1993).

CHAPTER 6

CONCLUSION AND RECOMMENDATION

Based on the study conducted, it can be concluded that the liposome-encapsulated piroxicam exhibit greater anti-inflammatory and anti-pyretic effects when compared to free piroxicam. The encapsulation of piroxicam prolonged the treatment effect and enabled the reduction of the dose, with increased topical anti-inflammatory activity, with a possibility to decrease the systemic side effects. Liposome-encapsulated piroxicam were shown to produce faster anti-pyretic effect on hyperthermic rats.

The mechanism of action for the better therapeutic effect by liposome-encapsulated piroxicam could be evaluated in future studies. This liposomal drug delivery system could also be applied and studied using different type of modal drugs in order to improve their therapeutic effects as well as reducing the incidence and severity of adverse effects.

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APPENDICES

Appendix A: Raw Data from Carrageenan-Induced Paw Edema Test

Group 1: 0 mg/kg Piroxicam (control)							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	183	NA	0.428571	0.142857	0.285714	0.285714	0.285714
2	162	NA	0.285714	0.285714	0.428571	0.285714	0.428571
3	206	NA	0.571429	0.571429	0.571429	0.428571	0.428571
4	154	NA	0.571429	0.571429	0.714286	0.571429	0.857143
5	215	NA	0.714286	0.714286	0.857143	1	0.571429
6	157	NA	0.285714	0.285714	0.428571	0.428571	0.428571
Mean	179.5	NA	0.47619	0.428571	0.547619	0.5	0.5
Stdev	26.22022	NA	0.173009	0.221313	0.21028	0.267261	0.196915
SEM	-	-	0.07063	0.09035	0.08585	0.10911	0.08039

Group 2: 0.3 mg/kg Piroxicam							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	247	NA	0.285714	0.285714	0.428571	0.142857	0.285714
2	220	NA	0.428571	0.142857	0.428571	0.285714	0.285714
3	225	NA	0.285714	0.142857	0.285714	0.285714	0.285714
4	150	NA	0.142857	0.142857	0.285714	0.142857	0.142857
5	165	NA	0.142857	0.285714	0.142857	0.142857	0.142857
6	226	NA	0.428571	0.285714	0.428571	0.428571	0.571429
Mean	205.5	NA	0.285714	0.214286	0.333333	0.238095	0.285714
Stdev	38.60958	NA	0.127775	0.078246	0.116642	0.116642	0.156492
SEM	-	-	0.05216	0.03194	0.04762	0.04762	0.06389

Group 3: 3.0 mg/kg Piroxicam							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	180	NA	0.285714	0.285714	0.142857	0.285714	0.285714
2	140	NA	0.142857	0.285714	0.142857	0.142857	0.142857
3	190	NA	0.285714	0.428571	0.142857	0.285714	0.285714
4	195	NA	0.428571	0.428571	0.428571	0.428571	0.428571
5	150	NA	0.285714	0.142857	0.285714	0.142857	0.142857
6	160	NA	0.285714	0	0.428571	0.142857	0.285714
Mean	169.1667	NA	0.285714	0.261905	0.261905	0.238095	0.261905
Stdev	22.45366	NA	0.090351	0.167006	0.140456	0.116642	0.107539
SEM	-	-	0.03689	0.6818	0.05734	0.04762	0.0439

Group 4: 30 mg/kg Piroxicam							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	255	NA	0.142857	0	0.142857	0.142857	0.142857
2	200	NA	0.428571	0.285714	0.142857	0.285714	0.285714
3	275	NA	0.285714	0.142857	0.285714	0.285714	0.142857
4	215	NA	0.428571	0.285714	0.285714	0.285714	0.285714
5	300	NA	0.285714	0.285714	0.285714	0.285714	0.142857
6	223	NA	0.142857	0	0.142857	0.142857	0
Mean	244.6667	NA	0.285714	0.166667	0.214286	0.238095	0.166667
Stdev	-	-	0.127775	0.140456	0.078246	0.073771	0.107539

Group 5: 0 mg/kg Liposome-encapsulated Piroxicam							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	240	NA	0.428571	0.142857	0.142857	0.285714	0.142857
2	180	NA	0.285714	0.142857	0.428571	0.285714	0.142857
3	160	NA	0.571429	0.571429	0.571429	0.428571	0.714286
4	130	NA	0.571429	0.428571	0.714286	0.571429	0.428571
5	160	NA	0.571429	0.571429	0.571429	0.428571	0.714286
6	220	NA	0.428571	0.285714	0.285714	0.285714	0.142857
Mean	181.6667	NA	0.47619	0.357143	0.452381	0.380952	0.380952
Stdev	41.19061	NA	0.116642	0.196915	0.21028	0.116642	0.280912
SEM	-	-	0.4762	0.08039	0.08585	0.04762	0.11468

Group 6: 0.3 mg/kg Liposome-encapsulated Piroxicam							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	210	NA	0.285714	0.428571	0.285714	0.142857	0.285714
2	220	NA	0.285714	0.285714	0.285714	0.285714	0.142857
3	255	NA	0.285714	0.428571	0.428571	0.428571	0.428571
4	228	NA	0.142857	0.142857	0.142857	0.285714	0.142857
5	245	NA	0.285714	0.285714	0.285714	0.142857	0.285714
6	240	NA	0.142857	0.142857	0.142857	0.285714	0.142857
Mean	233	NA	0.238095	0.285714	0.261905	0.261905	0.238095
Stdev	16.7332	NA	0.073771	0.127775	0.107539	0.107539	0.116642
SEM	-	-	0.03012	0.05216	0.0439	0.0439	0.04762

Group 7: 3.0 mg/kg Liposome-encapsulated Piroxicam							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	189	NA	0.285714	0.571429	0.428571	0.428571	0.285714
2	205	NA	0.142857	0.142857	0.285714	0.142857	0
3	257	NA	0.142857	0.142857	0.285714	0.142857	0
4	179	NA	0.142857	0.142857	0.285714	0.142857	0.142857
5	203	NA	0.142857	0.142857	0.285714	0.142857	0.142857
6	191	NA	0.285714	0.142857	0.142857	0.285714	0.142857
Mean	204	NA	0.190476	0.214286	0.285714	0.214286	0.119048
Stdev	27.67671	NA	0.073771	0.174964	0.090351	0.119523	0.107539
SEM	-	-	0.03012	0.07143	0.03689	0.0488	0.0439

Group 8: 30 mg/kg Liposome-encapsulated Piroxicam							
Rat no.	**Weight**	**Edema volume (Vt-V0)**					
		0H	**1H**	**2H**	**3H**	**4H**	**5H**
1	259	NA	0.142857	0	0	0	0
2	213	NA	0.142857	0.142857	0.142857	0.142857	0
3	224	NA	0.142857	0.142857	0.142857	0.142857	0
4	185	NA	0.285714	0.142857	0.142857	0.142857	0.142857
5	163	NA	0.142857	0.142857	0.142857	0.142857	0
6	162	NA	0.142857	0	-0.14286	0.142857	0.142857
Mean	201	NA	0.166667	0.095238	0.071429	0.119048	0.047619
Stdev	38.09987	NA	0.058321	0.073771	0.119523	0.058321	0.073771
SEM	-	-	0.02381	0.03012	0.0488	0.02381	0.03012

Appendix B: Raw data from Cotton Pellet-induced Granuloma test

Group 1: 0 mg/kg Piroxicam (control)						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	332	30	549.1	113.7	435.4	83.7
2	314	30	428.6	100	328.6	70
3	256	30	827.4	178.2	649.2	148.2
4	287	30	529.4	112.7	416.7	82.7
5	208	30	434.9	99.5	335.4	69.5
6	308	30	534.9	120.8	414.1	90.8
Mean	-	30	550.7167	120.8167	429.9	90.81666667
Stdev	-	0	145.3182	29.31589	116.3946391	29.31589444
SEM	-	-	-	-	47.5179	11.9682

Group 2: 0.3 mg/kg Piroxicam						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	397	30	306.7	59.5	247.2	29.5
2	338	30	504.3	128.2	376.1	98.2
3	309	30	665.5	144.7	520.8	114.7
4	276	30	463.5	103.6	359.9	73.6
5	285	30	426.4	93.5	332.9	63.5
6	261	30	548.8	124	424.8	94
Mean	-	30	485.8667	108.9167	376.95	78.91666667
Stdev	-	0	120.5867	30.29108	91.705327	30.2910823
SEM	-	-	-	-	37.4385	12.3663

Group 3: 3.0 mg/kg Piroxicam						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	236	30	396	96.5	299.5	66.5
2	256	30	527.3	115.4	411.9	85.4
3	264	30	437.9	102	335.9	72
4	299	30	434.7	98	336.7	68
5	236	30	527.3	118.7	408.6	88.7
6	294	30	427.1	107.4	319.7	77.4
Mean	-	30	458.3833	106.3333	352.05	76.33333333
Stdev	-	0	55.40723	9.178162	47.08374454	9.178162489
SEM	-	-	-	-	19.2219	3.7470

Group 4: 30 mg/kg Piroxicam						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	254	30	461.3	96	365.3	66
2	259	30	326.9	76.1	250.8	46.1
3	250	30	324.3	77.6	246.7	47.6
4	255	30	359.5	113.2	246.3	83.2
5	320	30	412.5	88.4	324.1	58.4
6	319	30	441.7	94.4	347.3	64.4
Mean	-	30	387.7	90.95	296.75	60.95
Stdev	-	0	59.0922	13.69463	55.07092699	13.69463399
SEM	-	-	-	-	22.4826	5.5908

Group 5: 0 mg/kg Liposome-encapsulated Piroxicam						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	305	30	595.9	118.2	477.7	88.2
2	297	30	603.9	123.4	480.5	93.4
3	290	30	563.4	112.6	450.8	82.6
4	256	30	555.3	112.3	443	82.3
5	255	30	454.1	94.3	359.8	64.3
6	228	30	455.5	98.7	356.8	68.7
Mean	-	30	538.0167	109.9167	428.1	79.91667
Stdev	-	0	67.05987	11.25156	56.02107	11.25156
SEM	-	-	-	-	22.8705	5.9001

Group 6: 0.3 mg/kg Liposome-encapsulated Piroxicam						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	283	30	499.2	103.9	395.3	73.9
2	289	30	439	97.5	341.5	67.5
3	269	30	487.2	102	385.2	72
4	248	30	353.4	82.5	270.9	52.5
5	218	30	495	106.4	388.6	76.4
6	302	30	438.3	101.6	336.7	71.6
Mean	-	30	452.0167	98.98333	353.0333	68.98333
Stdev	-	0	55.48789	8.591022	47.39585	8.591022
SEM	-	-	-	-	19.3493	3.5073

Group 7: 3.0 mg/kg Liposome-encapsulated Piroxicam						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	286	30	274.7	55	219.7	25
2	254	30	393.6	89.9	303.7	59.9
3	278	30	406.8	87.8	319	57.8
4	294	30	404.6	88.9	315.7	58.9
5	321	30	364.1	81.8	282.3	51.8
6	303	30	252.9	53.1	199.8	23.1
Mean	-	30	349.45	76.08333	273.3667	46.08333
Stdev	-	0	68.41616	17.3082	51.31587	17.3082
SEM	-	-	-	-	20.9496	7.0660

Group 8: 30 mg/kg Liposome-encapsulated Piroxicam						
Rat no.	**BW**	**Initial**	**Wet**	**Dry**	**Transudative**	**Granuloma**
1	277	30	294.6	77.7	216.9	47.7
2	255	30	402.6	93.3	309.3	63.3
3	244	30	246.6	54.5	192.1	24.5
4	242	30	364.1	72.1	292	42.1
5	236	30	320.8	72.7	248.1	42.7
6	352	30	277.5	72.4	205.1	42.4
Mean	-	30	317.7	73.78333	243.9167	43.78333
Stdev	-	0	57.5768	12.44193	48.01268	12.44193
SEM	-	-	-	-	19.6011	5.0794

Appendix C: Raw data from Brewer's Yeast-induced Hyperthermia test

Group 1: 0 mg/kg Piroxicam (control)								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	232	37.7	38.5	37.9	37.3	37.4	37.7	36.9
2	287	38.4	38.8	37.3	37.3	37.3	37.6	37.5
3	127	38.4	38.8	38.2	38.1	37.7	38.2	38
4	151	36.6	37.2	37.5	37.9	37.7	38.2	38
5	150	37.1	37.3	38.2	38	37.5	37.7	37.4
6	203	36.7	37.8	37.9	38.1	37.6	37.1	37.6
Mean	191.67	37.48	38.07	37.83	37.78	37.53	37.75	37.57
Stdev	60.69	0.81	0.73	0.37	0.38	0.16	0.41	0.41
SEM	-	0.33	0.29	0.15	0.16	0.07	0.17	0.17

Group 2: 0.3 mg/kg Piroxicam								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	197	36.8	37.4	37.7	38	37.4	37.5	37.5
2	232	37	38.1	37.6	37.7	37.5	37.2	37.4
3	275	37.1	37.9	37.3	37.4	37.7	37.5	36.5
4	191	37.5	37.8	37.9	38	37.8	37.7	37.3
5	180	37.2	38.1	37.7	37.4	38.3	37.2	37.3
6	225	37	37.8	37.5	37.2	37.1	37	37.6
Mean	216.67	37.1	37.85	37.62	37.62	37.63	37.35	37.27
Stdev	34.92	0.24	0.26	0.20	0.34	0.41	0.26	0.39
SEM	-	0.09	0.11	0.08	0.14	0.17	0.11	0.16

Group 3: 3 mg/kg Piroxicam								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	205	37.3	38.3	37.5	36.3	36.8	36.9	36.4
2	242	36.4	37	37.8	37.7	37.3	37.7	37.4
3	202	36.7	38.3	38.2	38.3	38.6	37.7	38.3
4	178	37.5	38.3	37.5	37.2	37.1	37.3	36.8
5	252	37.6	37.9	37.8	37.6	37.9	37.8	37.3
6	194	37.1	37.1	37.3	37.1	37.5	37	37
Mean	212.17	37.1	37.82	37.68	37.37	37.53	37.4	37.2
Stdev	28.74	0.47	0.61	0.32	0.67	0.64	0.39	0.65
SEM	-	0.19	0.25	0.13	0.28	0.26	0.16	0.26

Group 4: 30 mg/kg Piroxicam								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	187	38.1	38.6	38.1	37.8	37.4	36.8	37.1
2	220	38.1	38.6	38.1	37.5	37.4	36.6	37.1
3	210	37.6	37.5	37.2	36.6	36.1	36.2	36.7
4	243	37.5	38.4	37	37.2	36.5	36.7	36.3
5	199	37.1	37.7	37.4	37.8	37.4	36.6	37.1
6	223	36.6	37.1	37.1	36.9	36.5	36.8	37
Mean	213.67	37.5	37.98	37.48	37.3	36.88	36.62	36.88
Stdev	19.63	0.58	0.64	0.49	0.49	0.58	0.22	0.32
SEM	-	0.24	0.26	0.20	0.20	0.24	0.09	0.13

Group 5: 0 mg/kg Liposome-encapsulated Piroxicam								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	180	37.3	37.2	36.9	37.5	37.4	37	37.3
2	195	38.2	38.5	38.1	37.9	38	37.8	37.5
3	202	36.8	38	37.7	37.4	37.6	37.6	37.6
4	238	37	37.8	37.9	37.7	37.9	37.1	37.4
5	225	37.2	37.5	38.4	38.3	37.8	38	37.8
6	188	36.7	37.8	38	38	38	37.6	37.2
Mean	204.67	37.2	37.8	37.83	37.8	37.78	37.52	37.47
Stdev	22.41	0.54	0.44	0.51	0.33	0.24	0.39	0.22
SEM	-	0.22	0.18	0.21	0.14	0.10	0.16	0.09

Group 6: 0.3 mg/kg Liposome-encapsulated Piroxicam								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	156	36.1	37.8	37.1	37	36.4	36.2	36.4
2	210	37.1	37.7	37.9	37.8	37.3	37.3	37.1
3	216	37.3	37.6	38.2	38.2	37.7	37	37.5
4	220	37.5	37.7	37.9	38.1	37.7	37.1	37.4
5	205	37.2	37.6	37.5	37.6	37.5	37.4	37.4
6	184	37.2	37.8	38.1	37.6	37	37.1	36.6
Mean	198.5	37.07	37.7	37.78	37.72	37.27	37.02	37.07
Stdev	24.33	0.49	0.09	0.41	0.43	0.50	0.43	0.46
SEM	-	0.20	0.04	0.17	0.18	0.20	0.17	0.19

Group 7: 3.0 mg/kg Liposome-encapsulated Piroxicam								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	181	36.7	37.3	37.3	36.9	37	36.7	37
2	160	36.6	37.6	37.6	37.1	37.4	36.6	36.8
3	220	37.5	37.7	37.8	37.5	36.9	37.1	37.2
4	205	36.8	37.6	37.4	37.8	36.9	37.2	36.8
5	202	38.1	38.5	37.7	37	37.6	37.5	37
6	212	37.7	37.7	37.3	37	37	36.5	36.9
Mean	196.67	37.23	37.73	37.52	37.22	37.13	36.93	36.95
Stdev	22.21	0.62	0.40	0.21	0.35	0.29	0.39	0.15
SEM	-	0.25	0.16	0.09	0.14	0.12	0.16	0.06

Group 8: 30 mg/kg Liposome-encapsulated Piroxicam								
Rat no.	**Weight**	**-18**	**0H**	**1H**	**2H**	**3H**	**4H**	**5H**
1	238	38.3	37.7	37.1	37.1	37.1	36.2	36.6
2	226	38	37.8	37.2	37.5	37	36.9	36.7
3	195	37.1	37.5	37.2	36.8	36.6	36.3	36.3
4	220	37.5	38.3	37.6	37.7	36.6	36.7	36.6
5	230	37.1	37.5	37.1	36.8	36.6	36.2	36.3
6	200	37.6	37.6	37.7	37.5	37.2	36.8	37
Mean	218.17	37.6	37.73	37.32	37.23	36.85	36.52	36.58
Stdev	17.11	0.48	0.30	0.26	0.39	0.28	0.32	0.26
SEM	-	0.19	0.12	0.11	0.16	0.12	0.13	0.11