3. Biological evaluation of synthetic palmitoyl ester and amide derivatives

In this study the synthesized ester, amide palmitoylated derivatives and standard NSAIDs like aspirin and paracetamol were evaluated for their pharmacological activities like analgesic, anti-pyretic, anti-inflammatory and anti-ulcer. Histopathology and anti-microbial studies were also performed. Test compounds and positive control were administered orally in the form of a suspension (soya oil as vehicle). Palmitoyl salicylic acid (PSA) parent compound is salicylic acid (AA) and AA is a structural analogue of N-PAA, hence their activities are compared to standard reference aspirin. Whereas, PPAP parent compound is para amino phenol (PAP) and N-PPABA is a structural analogue of para amino benzoic acid therefore their activities are compared to standard reference paracetamol.

3. 1. Experimental animals and laboratory maintenance

Wistar albino rats (150-230 g) and mice of both sexes were selected as test animals for experimental evaluation. The selected animals were housed in acrylic cages under standard environmental conditions at 25 ± 2 °C at a relative humidity of 45-55 %, in a well ventilated room maintained at a 12:12 h light: dark cycle and were fed a standard rodent diet and water ad libitum. All the animals were acclimatized for a week. Animal experiments were carried out according to the guidelines of the Committee for the Purpose of Control of Experiments on Animals (Ref. GPC/CPCSEA/08/2006-07) with approval of the Institutional Animal Ethics Committee, Government College of Pharmacy, Bangalore, India.

All the compounds (200 mg kg⁻¹ body mass) and standard reference NSAID; aspirin, paracetamol (100 mg kg⁻¹ body mass) and omeprazole (20 mg kg⁻¹ body mass) were suspended in 1ml soya oil and administered orally using an animal feeding needle. The control group received appropriate volume of vehicle (1ml soya oil, oral).
3. 2. Pharmacological Screening

All the synthesized compounds were screened for the following pharmacological activities.

3. 2. 1. Assessment of the anti-inflammatory activity

The anti-inflammatory activity of the test compounds was assessed by the carrageenan induced paw edema method (Amir and Kumar, 2005). Albino wistar rats of either sex were weighed and divided into seven groups containing six animals each. Among these groups one was kept as control, other as standard and rests as test groups for synthesized derivatives. A mark was made on left paw just beyond knee joint of each animal of all groups, so that every time the paw can be dipped in the plethysmometer up to the mark to ensure constant paw volume. The control group was administered soya oil orally, standard group received aspirin and paracetamol. The test groups were orally administered with synthetic palmitoylated derivatives that were suspended in soya oil. After 30 min carrageenan solution (1% w/v) was injected by sub planter route to all the groups. After the administration of carrageenan solution the paw volume of control, standard and tests groups were noted. The quantity of edema formed was measured for three hours using plethysmograph. The anti-edematous effects of the compounds were estimated as percentage inhibition of edema in comparison with control (Griswold et al, 1985).

\[\text{Percentage inhibition} = \frac{\text{Control} - \text{Treated} \times 100}{\text{Control}}\]

3. 2. 2. Assessment of the anti-pyretic activity

Lipopolysaccharide (LPS) induced pyrexia was used to evaluate the anti-pyretic activity of the synthesized compounds (Sirakarn et al, 2010). The body temperature of each rat was recorded by measuring the rectal temperature at predetermined time intervals. Fever
was induced by injecting aqueous suspension of sterilized bacterial LPS (s.c) 20% of 20 ml/kg body weight (Santos et al, 1985). The rats were allowed to remain quiet in the cage for sometime. A thermister probe was inserted 3-4 cm deep into the rectum of animal after fastening the tail, in order to record the basal rectal temperature. After recording temperature animals were returned to their housing cages. After 18 h of LPS injection, rats were again restrained in individual cages to record their rectal temperature. Immediately the test and standard compounds were administered orally at their respective doses. Rectal temperature of all the rats was recorded at 18 h immediately and before administration of test compounds and again before (pre treatment) 30 min and at 30 min, 1, 2, 3 and 4 hours after administration of drug (Smith and Hambourger, 1985).

3. 2. 3. Assessment of the analgesic activity

Analgesia evaluation was done by hot plate, tail immersion and acetic acid induced writhing tests.

3. 2. 3. 1. Analgesic activity by the hot plate method

Male and female albino mice showing reaction time of 10 sec at 55 ± 1 °C thermal stimulus were selected. The time for hind paw licking or jumping on heated plate of analgesiometer was taken as reaction time after 30, 60 and 90 min intervals. Aspirin and paracetamol were used as test standards (Woolfe and Mac Donald, 1994).

3. 2. 3. 2. Analgesic activity by the tail immersion method

Mice were placed in a restrainer cage after giving oral dose of the test, control and synthesized drugs. The animals were placed in such a way that their tails hangs out. In this position the tail (5 cm) dips into the water bath maintained at 55±1°C. The reaction times (time taken to withdraw tail) were recorded after every hour for four hours (Plummer et al, 1994).
3. 2. 3. 3. Analgesic activity by the acetic acid induced writhing test

The analgesic activity was evaluated using acetic acid induced writhing in albino mice (Ark et al, 2004). Acetic acid solution of 0.3 % (v/v) was used as writh inducing agent. Mice were divided into six groups of six animals each. Group one served as control, group two and three received standard drugs and other groups received test drugs. All the drugs were prepared as homogenous suspensions in saline (0.3 % NaCl) and administered orally. Test compounds were administered orally, one hour prior to acetic acid injection. The writhes that occurred in 30 min were recorded. Analgesic activity was calculated as the percentage inhibition of abdominal constrictions (Fadl and Omar, 1998).

\[
\% \text{ Analgesic activity} = \left( n - n' / n \right) \times 100
\]

Where \( n \) - mean no of writhes of control group

and \( n' \) - mean no of writhes of test group.

3. 3. Assessment of the anti-ulcer activity

Gastrointestinal toxicity of the palmitoylated ester and amide derivatives were assessed by the measuring ulcer index adopting the following two approaches.

3. 3. 1. Ethanol induced gastric ulcer (cytoprotective mechanism)

Male Albino wistar rats weighing 150 - 200 g were divided into seven groups. The animals were fasted for 48 h with free access to water and orally administered with test compounds and omeprazole p.o. (per oral-drug doses given to animals 10 mg, 1 mL/200g of 99% alcohol ethanol). Animals were sacrificed after one hour of alcohol administration. The stomach was cut open along the greater curvature and pinned on a soft board (Thirunavukkarasu et al, 2009). The gastric mucosa was examined for ulcers. Ulcer scores were observed and ulcer index was calculated.
3.3.2. Assessment of the anti-ulcer activity by swimming stress method (proton pump inhibition mechanism)

The induction of ulcers due to swimming stress was assessed by forced swimming of rats in the glass cylinder (height 45cm, diameter 25cm) containing water to the height of 35cm maintained at 25°C for 3 h. Animals were fasted for 48 h prior to experiments and divided into groups with six animals each. Control group received soya oil, standard control group received 20 mg/kg body weight of omezaprazole. Other groups received 200mg/kg of palmitoylated derivatives. After administration of drugs, all the animals were allowed to swim in water for 3 h and later sacrificed after exposure to chloroform in a dessicator. The stomachs were isolated and cut open along the greater curvature and pinned on a soft board. The gastric mucosa was examined for ulcers (Marijana et al., 2009).

3.3.3. Evaluation of the histopathology of drug induced ulcers

Histological evaluation was performed on the glandular stomach; the stomach was washed thoroughly with saline, dehydrated in gradual ethanol (50-100%), cleared in xylene and embedded in paraffin. Sections (4-5mm) were prepared and then stained with hematoxylin and eosin (H-E) dye for microscopic observation and photography (magnification 100 X).

3.4. Determination of the anti-microbial activity of palmitoyl derivatives

3.4.1. Microorganisms

The test microorganisms used for the anti-microbial activity screening were four bacteria Staphylococcus aureus, Escherichia coli, Salmonella paratyphi A, Salmonella paratyphi B and two fungi Candida albicans and Aspergillus niger. The bacterial strains were cultured on Nutrient Agar (NA) and fungi were grown on Sabouraud Agar (SA) medium.
3.4.2. Anti-bacterial activity of palmitoyl derivatives

The bacterial strains were sub cultured on NA (beef extract 3.0 g, peptone 5.0 g, NaCl 5.0 g, agar 15.0 g, pH 7.2). The above components were dissolved in one liter distilled water and sterilized at 121°C for 15 min. After inoculation of the sterilized medium the flasks were incubated at 37±1°C for 24 h and then used for further studies. Compounds were evaluated for their anti-bacterial activity by agar diffusion method. Palmitoylated derivatives (5mg/ml) were dissolved in dimethyl formamide (DMF). Ciprofloxacin (1mg/ml) and ampicillin (1mg/ml) were used as positive controls and DMF was used as blank. Bacterial lawn on Nutrient agar was bored by using sterile borer of 6mm radius to get wells. Solutions of (100 µl) each of ciprofloxacin, ampicillin, blank (DMF) and compound were added to the wells and incubated for 24 h at 37±1°C. The diameter of the zones of inhibition around each of the well was taken as measure of the anti-bacterial activity. Zone of inhibition was observed by zone reader scale (Shirin et al, 2006).

3.4.3. Anti-fungal activity of palmitoyl derivatives

The fungal strains were sub cultured on SA (Peptone 10g, Dextrose 40g and agar 15g at pH 5.6). The above components were dissolved in one liter distilled water and sterilized at 121°C for 15 min. After inoculation of the sterilized medium with fungus, the flasks were incubated at 37±1°C for 48 h and then used for further studies. Compounds were evaluated by the agar diffusion method. Palmitoylated derivatives were dissolved in dimethyl sulphone (DMSO) to get the concentration of 5mg/ml. Fluconazole 1mg/ml was used as positive control and DMF was used as blank. Agar plates containing organisms were bored by using sterile borer of 6 mm radius. Solution of fluconazole, blank (DMSO) and compound 100 µl was added to well and incubated for 24 h at 37±1°C. The diameter of the zones of inhibition around each of the well was taken as measure of the anti-bacterial activity. Zone of inhibition was observed by zone reader scale (Hersh et al, 1991).
3. 5. Enzymatic and chemical stability studies of the palmitoylated derivatives

The chemical and enzymatic stability plays an important role in drug discovery and development. Stability evaluation of drug is the key to drug quality as it determines the efficacy. Plasma stability of drug is of great importance as drug becomes less effective as it undergoes degradation. Unstable compounds tend to have rapid clearance and short half-life, resulting in poor in vivo performance. The objective of this study is to determine the enzymatic and chemical hydrolysis of synthesized palmitoyl ester and amide derivatives.

3.5.1. Hydrolysis rate determination of synthesized palmitoylated derivatives

In vitro hydrolysis studies of synthesized palmitoylated derivatives were carried out in simulated gastric fluid (SGF) at pH 1.2, simulated intestinal fluid (SIF) at pH 7.4 and SIF + 80% human plasma at pH 7.4 (Nielsenw and Bundgaard, 1998; Arun and Ashok, 2010). A solution of 10 mg of palmitoylated derivatives was prepared in 90 mL of SIF (pH 7.4) or SGF (pH 1.2). An aliquot of 15 mL of this solution was withdrawn repeatedly at regular intervals and kept in test tubes maintained at 37 ± 0.5 °C. At a definite time interval (0.5 h, 1–8 h), an aliquot was withdrawn from different test tubes and was transferred to micro centrifuge tubes, followed by addition of methanol to make up the volume. The tubes were placed in a freezing mixture in order to arrest further hydrolysis, followed by vortexing at high speed for 5min. After vortexing, the tubes were centrifuged at high speed (3000 rpm) for 5 min. 5 mL of clear supernatant obtained from each tube was read by a spectrophotometer at 230 nm. The kinetics of hydrolysis and half-life (t1/2) were determined. The rate of hydrolysis was calculated using the equation.
\[ K_H = (2.303/t) \log (a/a-x) \]

where \( k_H \) represents the hydrolysis constant,
\( t \) is the time in min,
\( a \) is the initial conjugate concentration,
\( x \) is the amount of conjugate hydrolyzed,
\((a-x)\) is the amount of the remaining,
conjugate

3.6. Acute toxicity and maximum tolerance studies

The acute toxicity potential of the test compounds was determined by selecting albino wistar rats of either sex (250-300 g). The animals were divided into groups, each group containing six animals. The dosage was administered orally as a single dose of 2000 mg kg \(^{-1}\) body weight. The animals were continuously observed for acute toxicity such as increased or decreased motor activity, ataxia, tremors, convulsions, sedation, lacrimation etc. All the tested animals survived without any side effects. Hence, 1/10\(^{th}\) of maximum dose was considered safe for further studies (Health effect guidelines, 2002). After 24 h animals were sacrificed, stomach, intestine, and liver were inspected under the magnifying lens for any ulcer hemorrhagic spots.

3.7. Statistical analysis of the data

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by student's t-test to find correlation between the control group and groups of animals treated with test and standard compounds in various pharmacological assays. Data are expressed as mean \( \pm \) SEM.
3. 8. Results

3. 8.1. Anti-inflammatory effects of palmitoyl derivatives

The anti-inflammatory potentials of ester derivative PSA and amide derivatives N-PAA, PPAP and N-PPABA in various animal models by the carrageenan induced paw edema method exhibited significant (***P< 0.05) anti-inflammatory activities (fig.3.1 and 2). These results suggest that the palmitoyl derivates exhibit anti-inflammatory effects. Palmitoyl ester PSA reduced edema by 33.36%, aspirin 24.27%. Whereas palmitoyl amide N-PAA reduced edema by 10.636 %, PPAP 4.5 %, paracetamol 24.27 % and N-PPABA 36.36 % at the end of 3h of treatment. The data indicates that anti-inflammatory effect of PSA and N-PPABA is more potent than paracetamol, the anti-inflammatory effect of palmitoyl derivative was started from 1\textsuperscript{st} h and retained till 3\textsuperscript{rd} h , whereas for PPAP it was persistent till 4\textsuperscript{th} h , may be related to inhibition of inflammation mediators.

3. 8.2. Anti-pyretic activity of palmitoyl derivatives

The subcutaneous injection of bacterial LPS suspension markedly elevated the rectal temperature. Palmitoyl esters; PSA and N-PAA decreased the rectal temperatures. The anti-pyretic effects of N-PAA and PSA were observed from 1\textsuperscript{st} h and maintained upto 4\textsuperscript{th} h whereas aspirin showed significant activity only in 1\textsuperscript{st} h (fig. 3.3). Similarly the anti-pyretic effect of palmitoyl amide N-PPABA was maintained upto 4\textsuperscript{th} h , paracetamol showed significant activity only in initial two hours. Whereas PPAP failed to show any significant anti-pyretic activity (fig.3.4).
Fig. 3. 1. The anti-inflammatory effect of palmitoyl salicylic acid and palmitoyl anthranilic acid in animal models by the carrageenan induced paw edema method

Fig. 3. 2. The anti-inflammatory effect of amide derivatives palmitoyl para amino phenol and palmitoyl para amino benzoic acid in animal models by the carrageenan induced paw edema method
Fig. 3. 3. Anti-pyretic activity of palmitoyl salicylic acid and palmitoyl anthranilic acid in rats

Fig. 3. 4. Anti-pyretic activity of palmitoyl para amino phenol and palmitoyl para amino benzoic acid in rats

3. 8. 3. Analgesic activity of palmitoyl derivatives
The acetic acid induced writhing response in mice was carried out to examine the action on central and peripheral action of drugs. As shown in (fig. 3.5a, 3.5b) it was found that palmitoyl derivatives PSA and \( N \)-PAA inhibited the writhing response by 59.23% and 79.44% respectively and \( N \)-PAA potentiated the analgesic activity of aspirin (59.92%). Similarly Palmitoyl amide PPAP and \( N \)-PPABA inhibited writhing response by 75.95% and 75.26%. \( N \)-PPABA potentiated the analgesic activity of paracetamol.

The thermal hyperalgesia as measured by tail immersion test indicated, persistent activity initiating from \( 1^{st} \) -3\(^{rd} \) h for all the derivatives. PSA and PPAP are more efficient than aspirin and paracetamol. \( N \)-PAA and \( N \)-PPABA also confers significantly enhanced and persistent analgesic activity (fig.3.5c, 3.5d).

The evaluation of mechanical hyperalgesia by the hot plate method showed that PSA, \( N \)-PAA, PPAP and \( N \)-PPABA show significantly enhanced and consistent analgesia (30-90 min) comparable to that of reference drugs (fig. 3.5e, 3.5)
Fig. 3. 5 (a). Analgesic activity of palmitoyl salicylic acid and palmitoyl anthranilic acid determined by the acetic acid induced writhing method

Fig. 3. 5 (b). Analgesic activity of palmitoyl para amino phenol and palmitoyl para amino benzoic acid determined by the acetic acid induced writhing method
Fig. 3. 5 (c). Analgesic activity of palmitoyl salicylic acid and palmitoyl anthranilic acid determined by tail withdrawal reaction time method

Fig. 3. 5 (d). Analgesic activity of palmitoyl para amino phenol and palmitoyl para amino benzoic acid determined by tail withdrawal reaction time method
Fig. 3. 5(e). Analgesic activity of palmitoyl salicylic acid and palmitoyl anthranilic acid as determined by the hot plate method.

Fig. 3. 5(f). Analgesic activity of palmitoyl para amino phenol and palmitoyl para amino benzoic acid as determined by the hot plate method.
3.8.4. Anti-ulcer activity of palmitoyl derivatives

Gastrointestinal toxicity of the synthesized palmitoylated derivatives was measured and compared with the standard by measuring the ulcer index and percentage of inhibition of ulcer formed by ethanol induced ulcer method and swimming induced ulcer method. Results are shown in Table 3.1.

Table 3.1: List the anti-ulcer activity of palmitoyl ester and amide derivatives in rats

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Treatment</th>
<th>Ethanol induced ulcer</th>
<th>Swimming stress induced ulcer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ulcer index</td>
<td>% inhibition</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>5.67 ± 0.21</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>standard</td>
<td>1.92 ± 0.6112</td>
<td>67.08*</td>
</tr>
<tr>
<td>3</td>
<td>PSA</td>
<td>1.25 ± 0.4031</td>
<td>78.57*</td>
</tr>
<tr>
<td>4</td>
<td>N-PAA</td>
<td>0.83 ± 0.2108</td>
<td>85.3*</td>
</tr>
<tr>
<td>5</td>
<td>PPAP</td>
<td>0.83 ± 0.2108</td>
<td>85.3*</td>
</tr>
<tr>
<td>6</td>
<td>N-PPABA</td>
<td>0.75 ± 0.250</td>
<td>86.77*</td>
</tr>
</tbody>
</table>

3.8.5. Histopathological studies
The drug induced histological observations are summarized as follows. The microscopic investigations of gastric tissues and tissue samples of control group of rats showed normal histological findings (fig. 3.6 a). The micrographs obtained for the standard omeprazole group revealed a focal erosive area in gastric mucosa and a zone (clear zone) in the basal regions of the gastric glands. This zone was parallel to the surface of the stomach lumen. In this zone, the structures of the gland were destroyed. They had disintegrated from the basal lamina and fallen into the lumen. The nuclei of these cells became smaller and dense, and their cytoplasms were stained as dark eosinophilic bodies. Small hemorrhagic areas and patches of inflammatory cell infiltrations were present in the lumen of the glands and lamina propria. The omeprazole treated group has shown superficial ulcer (fig. 3.6 d). Normal histological findings were displayed for PSA, N-PAA, PPAP and N-PPABA revealing that the derivatives are not producing any ulceration in the gastric region (fig. 3.6 (e) - 3.6 (h)).
Fig. 3.6 (a). Micrograph (X100) of normal gastric mucosa (control)

Fig. 3.6 (b). Micrograph (X100) showing induced ulcers in gastric mucosa (ethanol fed)
Fig. 3. 6(c). Micrograph (X100) induced ulcers in gastric mucosa
(swimming stress)

Fig. 3. 6(d). Micrograph (X100) induced ulcers in gastric mucosa
(Omezaprazole treated)
Fig. 3. 6(e). Micrograph (X100) of gastric mucosa not showing ulcers (palmitoyl salicylic acid treated) and mucosa appearing normal

Fig. 3. 6(f). Micrograph (X100) of gastric mucosa not showing ulcers (palmitoyl anthranilic acid treated) and mucosa appearing normal
Fig. 3.6 (g). Micrograph (X100) of gastric mucosa not showing ulcers (palmitoyl para amino phenol treated) and mucosa appearing normal.

Fig. 3.6 (h). Micrograph (X100) of gastric mucosa not showing ulcers (palmitoyl para amino benzoic acid treated) and mucosa appearing normal.
3.8.6. Anti-microbial activity of palmitoylated derivatives

The results of the anti-microbial efficacy of the synthesized palmitoylated derivatives are expressed in (Table 3.2, 3.3). All the compounds possess anti-microbial activity, among them PPAP and N-PPABA have variable activity against Gram positive and Gram negative bacterial strains. When all the synthesized compounds tested with Gram positive *Staphylococcus aureus*, N-PPABA found to be highly sensitive (10 mm, 28 mm) in comparison with reference standard ampicillin (18 mm), whereas sensitivity compared to reference standard ciprofloxacin is less. As far as the Gram negative organisms are concerned, all the derivatives showed less antimicrobial potency against *E.coli*, *Salmonella paratyphi A*, *Salmonella paratyphi B* in comparison with standard ampicillin and ciprofloxacin. None of the derivatives were found to be active against *Candida albicans* and *Aspergillus niger*.

Table 3.2: The anti-bacterial activities of palmitoylated derivatives

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Compound</th>
<th>Zone of inhibition (in mm)</th>
<th>Staphylococcus aureus</th>
<th>E. coli</th>
<th>Salmonella paratyphi A</th>
<th>Salmonella paratyphi B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PSA</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>N-PAA</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PPAP</td>
<td>9</td>
<td>7</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>N-PPABA</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Standard 1 (Ciprofloxacin)</td>
<td>20</td>
<td>15</td>
<td>22</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Standard 2 (Ampicillin)</td>
<td>18</td>
<td>19</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Anti-fungal activity of palmitoyl derivatives
<table>
<thead>
<tr>
<th>Sl no</th>
<th>Compound</th>
<th>Zone of inhibition (in mm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Candida albicans</em></td>
<td><em>Asperagus nigrus</em></td>
</tr>
<tr>
<td>1</td>
<td>PAA</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>PSA</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>PPAP</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>PPABA</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Standard (Fluconazole)</td>
<td>14</td>
<td>10</td>
</tr>
</tbody>
</table>

### 3. 8.7. Kinetics of stability studies

The kinetics of chemical and enzymatic hydrolysis of the palmitoylated ester and amide prodrugs were studied at 37°C in aqueous buffer solutions SGF, pH 1.3 and SIF pH 7.4 as well as in SIF 80% human plasma. The reactions were monitored by UV spectrophotometer for the decrease in ester and amide concentration vs. time. The rate constant ($k_{obs}$) for hydrolysis and the corresponding half-lives for each of the studied prodrug
are listed in Table 4.1. The half-life ($t_{1/2}$) of PSA in SIF was found to be 0.8 h and that of PAA 24.8 h. Satisfactory hydrolysis of the palmitoyl derivatives were observed in SGF and very encouraging hydrolysis in SIF + 80 % human plasma. The latter is due to the presence of amidase and esterases (carboxyl esterases) in plasma (Arun and Theja et al, 2009).

<table>
<thead>
<tr>
<th>Compound</th>
<th>SGF pH 1.3</th>
<th>SIF pH 7.4</th>
<th>SIF+80% Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{obs}$ ($h^{-1}$)</td>
<td>$t_{1/2}$ (h)</td>
<td>$k_{obs}$ ($h^{-1}$)</td>
</tr>
<tr>
<td>PSA</td>
<td>0.1034</td>
<td>6.7</td>
<td>0.160</td>
</tr>
<tr>
<td>N-PAA</td>
<td>0.026</td>
<td>26.8</td>
<td>0.028</td>
</tr>
<tr>
<td>PPAP</td>
<td>0.044</td>
<td>15.8</td>
<td>0.036</td>
</tr>
<tr>
<td>N-PPABA</td>
<td>0.016</td>
<td>43.0</td>
<td>0.846</td>
</tr>
</tbody>
</table>

Table 3.4: Kinetic properties of palmitoylated derivatives
The results suggest that all the synthesized prodrugs are sufficiently stable at pH 1.3, which is considered as a non-enzymatic simulated gastric fluid (SGF), so that no appreciable hydrolysis to the free acids might occur in the stomach. Similarly, the chemical stability of the palmitoyl derivatives pH 7.4 ($t_{1/2}$ - 0.8 - 24.8 h) suggests that they are absorbed almost unchanged from the intestine. The kinetic data (Table: 4.1) show that the synthesized derivatives are more susceptible to enzymes in plasma. This observation may be due enrichment of plasma with esterases. It is noteworthy that the susceptibility for enzymatic hydrolysis was found to be affected by the type of NSAID attached to lipid moiety. Salicylic acid with the palmitoyl moiety was found to be the most labile. In contrast, the amide prodrugs $N$- PAA, PPAP and $N$- PPABA were the least susceptible due to presence of amide bond.

3. 9. Discussion

Inflammation process can be considered as an event of the immune response (Mario et al, 2010) through which tissue damage occurs. The latter is accompanied by the release of several biochemical mediators such as histamine, bradykinin, platelet-activating factor and a group of lipid material known as leukotrienes (LTs) and prostaglandins (PGs). These mediators are responsible for inflammation. They cause the swelling and redness of the inflamed area (due to vasodilation and increased capillary permeability).

A good anti-inflammatory agent must have high lipophilic properties and the presence of a strong acidic functional group (http://cpharm.vetmed.vt.edu/vm8784/NSAIDS/nsaids.htm). Hence palmitoyl esters: PSA and PAA with both strong lipophilic and acid characteristics show significant anti-inflammatory
actions may be due to inhibition of inflammation mediators in various animal models. Paracetamol without lipophilicity, acidity and PPAP without acidic group have less anti-inflammatory actions. On the other hand, aspirin with poor lipophilicity, strong acid functional group and PPABA with high lipophilicity and strong acidic group produces significant anti-inflammatory actions.

The subcutaneous injection of sterilized bacterial LPS suspension markedly elevated the rectal temperature after 24 h of administration due to prostaglandin synthesis (Simmons, 2008). Exogenous pyrogen LPS, is a cell wall component of gram-negative bacteria. An immune-protein called lipopolysaccharide binding protein (LBP) binds to LPS. The LBP-LPS complex then binds to the CD14 receptor of a nearby macrophage. This binding results in the synthesis and release of various endogenous cytokine factors, such as interleukin 1 (IL-1), interleukin 6 (IL-6), and the tumor necrosis factor alpha. In other words, exogenous factors cause release of endogenous factors which in turn, activate the arachidonic acid and releases PGE2 (Simmons et al, 2000). Synthesized palmitoyl ester and amide derivatives inhibit synthesis and release of PGE2, thus elucidating anti-pyretic action similar to other NSAID’s.

The abdominal constriction (writhing) is related to sensitization of noniceptive receptors to prostaglandins. It’s therefore possible that palmitoyl ester and amide produce analgesic effect may be due to inhibition of synthesis of prostaglandins (http://hdl.lib.byu.edu/1877/1333; http://contentdm.lib.byu.edu/IRMath).

The mechanical hyperalgesia observed by hot plate method was found to be suitable for evaluation of central but not peripheral acting analgesics (Safieh et al, 1996). The validity of this test has been shown even in presence of substantial impairment of motor performance (Tsuchiya et al, 2005).
The thermal hyperalgesia as measured by tail immersion test indicates that palmitoyl
derivatives may be producing their effect both peripherally (writhing test) and centrally (tail
immersion and hot plate tests) (Safich et al, 1996).

The ulcerogenic properties of NSAIDs stem from the fact that they are organic acids,
which can irritate the gastric mucosa by their inhibitory effects on prostaglandin biosynthesis
(Francis et al, 1996). Prostaglandins also play a role in ethanol induced ulcers. Ethanol
induced gastric damage is possibly through leukotriene production through 5-
lipoxygenase pathway. It has been shown that drugs which are effective against ethanol induced gastric
lesions can posses gastric mucosal membrane protective action. The protective effect of
palmitoyl derivatives may be due to their action against 5-lipoxygenase pathway. The
cytoprotective action stimulates prostaglandin synthesis which in turn protects gastric mucosa
(Malairajan et al, 2008). Water immersion stress is one of the best models of stress in rats to
induce ulcer. The model provides emotional as well as physiological stress to the animal. All
derivative showed significant ulcer inhibition ($P< 0.001$), may be due to anti-
secretary, cytoprotective and proton pump inhibition mechanism. However further studies are
required to isolate active molecules responsible for such activity (Malairajan et al, 2008).
Thus, synthesized Palmitoyl ester and amide prodrugs have minimized the ulcerogenic effect
of NSAID’s by enhancing the defensive factors so that the normal balance between offensive
and defensive factors is achieved. The histopathological findings reveal that there is limited
or no ulcer formation in stomach by the palmitoylated derivatives.

The microbial analysis suggest that all the palmitoylated derivatives possess anti-
microbial activity, among them PPAP and $N$-PPABA have potent activity against Gram
positive and Gram negative bacterial strains. None of the palmitoylated derivatives showed
anti-fungal activity.
Essential requisites for a prodrug designed for oral delivery are its chemical stability at pH values simulating the gastric fluids and its ability to readily release the parent drug after absorption. The results in Table: 3.4 reveal that all the synthesized prodrugs are sufficiently stable at pH 1.3, which is considered as a non-enzymatic simulated gastric fluid (SGF), so that no appreciable hydrolysis to the free acids might occur in the stomach. Similarly, the chemical stability of the palmitoylated derivatives pH 7.4 (t½ = 0.8 - 24.8 h) suggests that they are absorbed almost unchanged from the intestine. The kinetic data show that the synthesized derivatives are more susceptible to plasma than in SGF and SIF. This observation may be due to enrichment of plasma with esterases which is consistent with previously reported investigations (Arun et al, 2009). The rates of hydrolysis of the palmitoylated derivatives in plasma are markedly accelerated compared with those in aqueous buffers. It is noteworthy that the susceptibility for enzymatic hydrolysis was found to be affected by the type of NSAID attached to lipid moiety (Fadl and Omar, 1998). Aspirin prodrug PSA with ester bond was found to be the most labile. In contrast, the amide prodrugs N-PAA, PPAP and N-PPABA were the least susceptible ones due to lesser electrophilicity of the carbon - nitrogen bond (Surender et al, 2010). According to the in vitro stability studies, the synthesized ester and amide derivatives were sufficiently stable at SGF pH 1.2. Hence, hydrolysis was not expected in the stomach. In addition, the chemical stability of the compounds at SIF pH 7.8 suggested that they can be absorbed from the intestines almost intact. The chemical stability of the reported palmitoyl ester and amide derivatives at pH values simulating gastric and intestinal fluids revealed that the synthesized compounds might have increased absorption compared to parent NSAIDs. Moreover, it is well evidenced that the direct contact mechanism appears to play a major role in the production of gastrointestinal lesions upon administration of NSAIDs. Therefore, the chemical stability of the reported compounds indicate that stable palmitoyl ester and amide derivatives also have a potentially
improved therapeutic index compared to contemporary parent drugs. Furthermore, the enzymatic stability of the palmitoyl ester and amide derivatives after 8 h of incubation in plasma establishes evidence that they may act \textit{in vivo} without prior hydrolysis of the ester and amide bond and palmitoyl amides are more stable than palmitoyl esters.

4. Conclusion

The observed increase in the anti-inflammatory, anti-pyretic, analgesic and anti-ulcer activities and the stability in enzymatic and chemical studies are attributed to the presence of long lipophilic lipid chain and strong acidic functional group. The synthesized compounds inhibit prostaglandin synthesis.