Non steroidal antiinflammatory drugs (NSAIDs) are frequently used for the treatment of pain, fever and inflammation. From the last so many years several attempts have been made to develop bioreversible derivatives or prodrug of non selective NSAID containing carboxylic acid function in order to depress upper gastrointestinal irritation and bleeding (1, 2). These side effects are attributed due to either local or systemic effects. The local effect is due to local inhibition of prostaglandins synthesis in GI tract. The topical irritation by the free carboxylic group of the NSAIDs is considered as an important factor in establishing superficial erosion (3, 4). The systemic effect is due to generalized systemic action occurring after absorption following intravenous dosing (5, 6). A possible way to solve this problem is derivatization of carboxylic function of NSAIDs into ester or amide mutual prodrugs (7). Mefenamic acid is widely used to relieve pain and stiffness associated with a variety of inflammatory diseases including active anti-inflammatory arthritis. The side effects liable with this drug are gastric irritation, abdominal pain, erosion of gastroduodenal mucosa and bleeding (8). Ester prodrug forms have been reported as one of the effective mechanisms that reduce the ulcerogenic side effects of some potent NSAIDs (9-11). Paracetamol, like many phenols, has been shown to possess an antioxidant activity in vitro also reported in literature that millimolar concentrations of paracetamol inhibit lipid peroxidation artificially induced in rat liver microsomes (12-14). Also, during recent years, it has been well established that generation of reactive oxygen species (ROS) plays a significant role in the formation of gastric mucosal lesions associated with NSAIDs therapy (15-17). Based on these observations, it has been suggested that co-administration of paracetamol and mefenamic acid in pharmaceutical dosage forms may possibly decrease the risk of NSAIDs induced GI ulcerogenicity. Thus in this study, we report the synthesis and evaluation of mutual ester prodrug of mefenamic acid using paracetamol as masking group of free carboxylic group to reduce its gastrointestinal toxicity.

EXPERIMENTAL

Chemistry

Melting points were recorded in open capillary tubes and are uncorrected. UV/Visible spectrophotometer with 10 mm matched quartz cells was used for experiment. IR spectra were recorded on a Shimadzu IRAffinity-1 FTIR spectrophotometer, using DRS 8000A accessory technique. 1H NMR
spectra were recorded with Brucker Aviance II 400 MHz using CDCl₃ as the solvent. Mass spectra were recorded on Jeol Sx 102/DA-600 mass spectrometer/Data System using electron ionization (EI) technique and nitrogen analysis was done using elemental analyzer Elementar Vario EL III Carlo Erba 1108. The HPLC system Cyberlab, 20, Salo Terrace, Milbury, MA 01527, USA was used with column HISEIDO C18 column, MG 5 µm, size – 4.6 mm ID × 250 mm; injection system: 7725i (Rheodyne, USA), injection volume 20 µL. The purity of the synthesized compound was confirmed by thin layer chromatography (TLC) using silica gel G. Visualization was done using iodine vapors. The sample was monitored at 279 nm at a flow rate of 1 mL/min. The mobile phase methanol : acetonitrile (95 : 5, v/v) was used for the estimation of ester. Mefenamic acid and paracetamol were obtained

![Diagram of the synthesis of mutual prodrug](image-url)

Figure 1. Synthesis of mutual prodrug
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from Zydus Cadila, Ahmedabad, India as gift sample. Other reagents and solvents used were of analytical/spectroscopic/HPLC grade as the case desired.

Synthesis

The synthesis of mefenamic acid (MA) and paracetamol (P), mutual prodrug (MA-P) is based on Steglich esterification (18). Mefenamic acid (10 mM), 50 mL of dichloromethane and paracetamol (10 mM) were taken in 250 mL flat bottomed flask. The temperature of reaction mixture was kept at 0°C, followed by the addition of N,N'-dimethylamino.pyridine (DMAP) (10 mM) and N,N'-dicyclohexylcarbodiimide (DCC) (10 mM) added slowly in portions. The reaction mixture was stirred at room temperature for 5 h. The precipitated N,N'-dicyclohexylurea was removed by filtration. The organic layer was washed twice with 0.5 M hydrochloric acid (20 mL) and then with 5% w/v sodium bicarbonate (20 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and the solvent was removed under reduced pressure. The final product obtained as solid was recrystallized from methanol (Fig. 1).

Characterization of the synthesized prodrug

Solubility and partition coefficient

The solubility in various solvents was checked and noted. Partition coefficient of MA and MA-P were determined in n-octanol/phosphate buffer (pH 7.4). Equal volumes of phosphate buffer and n-octanol and prodrug MA-P (100 mg) were mixed and transferred into separating funnel, shaken for 2 h at room temperature and left for 1 h. The sample (1 mL) of aqueous phases was taken and extracted three times with 5 mL of chloroform. Thereafter, the respective phases were analyzed by developed HPLC assay.

Kinetics of hydrolysis in phosphate and HCl buffer

The rate of chemical hydrolysis of MA-P was determined in isotonic phosphate buffer (pH 7.4) and HCl buffer (pH 2) at 37°C. USP apparatus II (paddle type) was used during the study. Ten milligrams of prodrug were dissolved initially in 5 mL of methanol, in a 10 mL volumetric flask and it was kept at 37 ± 1°C for 10 min. Then, the content was transferred to the vessel of dissolution apparatus containing 995 mL of 0.1 M hydrochloric acid. The content was stirred at 100 rpm and aliquots of 10 mL were withdrawn at 30 min intervals for up to 3 h and replaced immediately with equal volume of fresh 0.1 M hydrochloric acid. The aliquots withdrawn were extracted three times with 5 mL of chloroform. Organic phase was further dried. The residue obtained was dissolved in methanol and diluted suitably to estimate the prodrug. The sample was filtered through 0.25 μm Whatman filter paper. The sample (20 μL) was injected in chromatographic system. The peak area was noted and the concentration of prodrug in samples was calculated from calibration curve. The same procedure was followed for hydrolysis and analysis of prodrug in the phosphate buffer (pH 7.4) study. The rate of hydrolysis was studied at 37 ± 1°C.

Kinetics of hydrolysis in human plasma

A solution of 10 mg of prodrug was prepared in methanol (5 mL) and was added to 80% human plasma 10 mL (pH 7.4, prepared by mixing 80 portion of plasma with 20 portion of phosphate buffer pH 7.4) and kept at 37 ± 0.5°C. At appropriate time intervals of 15 min for 1.30 h, sample of 200 μL was taken in Eppendorf’s tube and centrifuged at 4500 rpm for 5 min. The supernatant was extracted thrice with 5 mL of chloroform. Organic phase was evaporated to dryness after separation from aqueous phase. The residue was dissolved in methanol and diluted suitably to estimate the prodrug in the chromatographic system.

Pharmacology

All animal experiments were conducted under the conditions of the Animal Scientific Procedures. The experimental protocol was approved by the University Animal Ethical Committee of GLA University, Institute of Pharmaceutical Research, Mathura, UP India. (1260/ac/09/CPCSEA) dated 28/01/2011 and proposal number is GLAIIPR/IAEC/001. Animals are placed in colony cages at 25 ± 2°C, relative humidity of 45-55%, under a 12 h light and dark cycle; they were fed standard animal feed and water ad libitum. The synthesized prodrug, physical mixture (MA + P) along with MA were evaluated for analgesic, anti-inflammatory and ulcerogenic activity. The prodrug was compared with MA for these activities. The methods employed for this purpose were as follows.

Anti inflammatory activity

The anti-inflammatory activity was evaluated by the carrageenan induced paw edema method (19). Albino rats of Wistar strain, weighing 100-200 g, of either sex were divided into four groups of six animals each. i) vehicle (control); ii) MA (standard, 80 mg/kg b.w., 332 mM, p.o.); iii) MA-P (124 mg/kg b.w., 332 mM, p.o.); iv) MA + P (physical
mixture, 332 mM of each p.o.). The animals were fasted overnight prior to test. The tested compounds were suspended in carboxymethylcellulose (0.5%, CMC) and administered orally. Control animals were given the corresponding amount of vehicle (0.5%, CMC).

**Analgesic activity**

Analgesic activity was determined using abdominal writhing assay (20). Albino mice of either sex of Swiss strain, weighing 20-25 g, were divided into four groups of six animals each. The test compounds were suspended in carboxymethylcellulose (0.5%, CMC), administered orally before the administration (i.p.) of freshly prepared acetic acid solution (0.6%, 10 mL/kg). The number of writhes (constriction of abdomen, turning of trunk and extension of hind limbs) for each animal was recorded during 20 min period. The average number of writhes in each group of drug treated mice was compared with that of control group and degree of analgesia was expressed as % inhibition as follows:

\[
\% \text{ Inhibition} = \left(1 - \frac{N_t}{N_C}\right) \times 100
\]

where, \(N_t\) = number of writhed in drug treated mice, \(N_C\) = number of writhed in control.

**Ulcerogenic study**

To study the anti ulcer activity (21), rats were fasted overnight and divided into four groups of six animals each. Doses given were three times higher than those for anti inflammatory activity determination. Animals were treated with MA, MA-P and equivalent dose of MA + P (physical mixture). Animals were sacrificed 12 h after the treatment. The stomach was removed, opened along greater curvature, washed with saline and observed for ulcers. The ulcers were scored as: 0 = no observable damage; 1 = superficial ulcers; 2 = deep ulcers; 3 = perforation.

**Biochemical parameters**

The peripheral markers like GWM, hexosamine and proteins were calculated as follows:

- **Determination of gastric wall mucus (GWM)**
  The glandular segment of excised stomachs was taken, weighed and transferred immediately to 10 mL of 0.1% alcian blue solution (in 0.016 mmol/L sucrose solution buffered with 0.05 mL sodium acetate at pH 5). Tissue was stained for 2 h in alcian blue, and excess dye removed by two successive rinses with 10 mL of 0.25 mmol/L sucrose, firstly after 15 min then after 45 min. Dye complexed with the gastric wall mucus was extracted with 10 mL of 0.5 mmol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract was shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 rpm for 10 min and the absorbance of the aqueous layer was recorded at 580 nm. The quantity of alcian blue extracted per gram of wet glandular tissue was calculated.

- **Hexosamine assay**
  The gastric tissues were hydrolyzed in acidic medium, the hydrolysate was neutralized with 3 M sodium hydroxide and diluted to 10 mL with distilled water. To an aliquot (1 mL) acetyl acetone solution (1 mL, prepared by dissolving 1 mL of acetyl acetone in 50 mL 0.5 M sodium carbonate) was added, the solution was mixed well, and heated on a boiling water bath for 15 min avoiding evaporation. After cooling, ethanol (5 mL, 95%) was added to the mixture followed by Ehrlich’s reagent (1 mL, prepared by dissolving 0.8 g of p-dimethylaminobenzaldehyde in 30 mL of methanol and 30 mL of conc. HCl). The mixture was diluted to 10 mL with 95% ethanol, allowed to stand for 30 min, and its absorbance was read at 530 nm.

- **Protein estimation**
  To 0.1 mL of supernatant, an equal volume of 1 M sodium hydroxide and 5 mL of Bradford reagent were added. Incubation was for 5 min. Absorbance was measured at 595 nm. Quantitatively, protein was estimated from the standard curve of bovine serum albumin (BSA).

**Statistical analysis**

Statistical analysis was carried out on in vivo studies data. The ulcer index data were subjected to Student t-test (unpaired), analysis of variance (ANOVA) test, followed by Dunnett’s test for determining the levels of significance in antioxidant studies; \(p\) values < 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**Chemistry**

The chemistry underlying the scheme was that MA (1) and the DCC (2) were able to form an \(O\)-acylisourea intermediate (3), the DMAP (4) acted as stronger nucleophile, thus reacted with \(O\)-acylisourea, leading to a reactive amide (“active ester”) (5). This intermediate could not form intramolecular side products but reacted rapidly.
DMAP acted as an acyl transfer reagent, and subsequent reaction with phenol gives the ester. The m.p. and percentage yield of MA-P was found to be 136-138°C and 52.2% respectively. UV (λmax, nm, MeOH): 359, 279, IR (KBr, cm⁻¹): 3309.8 N-H stretching of amide, 3136.25 aromatic C-H stretching, 1695.79 C=O stretching of ester, 1658.78 C=O stretching of amide, 1406.11 C-N stretching, 1163-1049.25 C=O stretching. ¹H NMR (400 MHz, CDCl₃, δ, ppm): 2.32 (s, 3H, -CH₃), 3.06 (s, 6H, 2 × CH₃), 3.47 (s, 1H, Ar-NH-Ar), 6.70-6.74 (d, J = 8 Hz, 3H, Ar-H), 7.28-7.30 (d, J = 8 Hz, 4H, Ar-H), 7.32-7.57 (d, J = 8 Hz, 4H, Ar-H), 9.15 (s, 1H, -NH of amide). MS: m/z 374 (M⁺), 224 (100% abundance) (M⁺ - C₈H₈O₂N), 196 (M⁺ - C₉H₈O₃N), 150 and 134. Analysis: calcd.: C, 73.78; H, 5.92; N, 7.48%; found: C, 73.76; H, 5.91; N, 7.46%.

**Solubility and partition coefficient**

Solubility studies showed that the synthesized prodrug was found slightly soluble in 0.1 M NaOH. Prodrug was found insoluble in water and in 0.1 M HCl. It showed moderate to high solubility in various solvents such as methanol, ethanol, chloroform, dichloromethane and benzene. The greater solubility of the standard drug MA is mainly due to the presence of free carboxyl group, which forms a sodium salt and makes the compound ionic. But prodrug showed moderate to high solubility in various organic solvents, which indicates lipophilic behavior of the compound. It is known that good absorp-

### Table 1. Anti-inflammatory activities of MA, MA-P and MA + P (physical mixture).

<table>
<thead>
<tr>
<th>Group</th>
<th>Difference in paw volume (mean ± SD)*</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Vehicle (control)</td>
<td>1.59 ± 0.040</td>
<td>1.36 ± 0.041</td>
</tr>
<tr>
<td>MA</td>
<td>0.81 ± 0.040</td>
<td>0.67 ± 0.032</td>
</tr>
<tr>
<td>MA-P</td>
<td>0.83 ± 0.040</td>
<td>0.67 ± 0.050</td>
</tr>
<tr>
<td>MA + P</td>
<td>0.958 ± 0.041</td>
<td>0.81 ± 0.038</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean ± SD of six experiments; - = not determined.

### Table 2. Analgesic and ulcerogenic activities of MA, MA-P and MA + P (physical mixture).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of writhing*</th>
<th>% inhibition in writhing*</th>
<th>Ulcer index Ui ± SEM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.33 ± 2.582</td>
<td>-</td>
<td>0.341 ± 0.216</td>
</tr>
<tr>
<td>MA</td>
<td>21.00 ± 2.366</td>
<td>63.321 ± 3.844</td>
<td>10.867 ± 0.148</td>
</tr>
<tr>
<td>MA-P</td>
<td>22.83 ± 2.926</td>
<td>61.779 ± 3.785</td>
<td>5.217 ± 2.334</td>
</tr>
<tr>
<td>MA + P</td>
<td>30.00 ± 2.366</td>
<td>47.623 ± 3.813</td>
<td>8.830 ± 1.769</td>
</tr>
</tbody>
</table>

* Data are expressed as the mean ± SD of six experiments; - = not determined.

### Table 3. Effect of MA, MA-P and MA + P (physical mixture) on hexosamine, GWM and protein.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GWM (Barrier mucus) µg/g protein*</th>
<th>Hexosamine (µg/mL)*</th>
<th>Protein (µg/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>285.680 ± 3.348</td>
<td>122.323 ± 4.639</td>
<td>42.300 ± 5.773</td>
</tr>
<tr>
<td>MA</td>
<td>170.026 ± 6.980</td>
<td>139.224 ± 13.182</td>
<td>12.083 ± 4.877</td>
</tr>
<tr>
<td>MA + P</td>
<td>225.103 ± 3.737</td>
<td>141.706 ± 8.945</td>
<td>11.250 ± 5.543</td>
</tr>
</tbody>
</table>

* Data are expressed as the mean ± SD of six experiments.
tion of an orally administered drug could be obtained when apparent coefficient (log P) value is more than 2. To assess the lipophilicity, the log P of MA-P was calculated and found to be 4.49, indicating that the synthesized derivative meets the requirement for gastrointestinal absorption.

**Chemical stability**

Essential requisites for a prodrug 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. Consequently, the kinetics of prodrug synthesized was studied in aqueous buffer solutions of pH 1.2 and pH 7.4. The reactions were monitored by HPLC for the decrease in ester concentration vs. time and were found to display pseudo-first order kinetics over several half-lives. The rate constant for hydrolysis and the corresponding half life were found to be $2.433 \times 10^{-6}$ s$^{-1}$ and 79.05 h at pH 2 and $1.414 \times 10^{-5}$ s$^{-1}$ and 13.60 h at pH 7.4, respectively; this indicates stability in buffer solutions. This implicated chemical stability at pH 2 is an evidence that the compound passes unhydrolyzed. At pH 7.4, the compound showed enough stability to be absorbed intact from the intestine.

**Enzymatic hydrolysis**

The prodrug showed very encouraging hydrolysis rate in 80% human plasma (pH 7.4) and the regeneration of the active drug was found to be 80%. The rate of hydrolysis was found to be $1.8908 \times 10^{-4}$ s$^{-1}$ and the half life ($t_{1/2}$) being 61.07 min, respectively, indicating rapid hydrolysis in plasma to release the parent drug molecules.

**Pharmacological evaluation**

Pharmacological investigations of the synthesized prodrug were done for anti-inflammatory, analgesic and ulcerogenic activities. In anti-inflammatory method, carrageenan (1% w/v) was used to produce paw edema in control animals. MA, equivalent dose of MA-P and physical mixture showed significant inhibition of carrageenan induced inflammation (Table 1). The data showed that MA-P at equivalent dose showed comparable anti-inflammatory and analgesic activity. (Tables 2 and 3). Ulcerogenic index of the prodrug MA-P was found much lower in comparison to standard drug. The minimized side effects obtained for the prodrug might be due to inhibition of direct contact of carboxyl group of the drug with the gastric mucosa, which is mainly responsible for the damage.

**Biochemical evaluation**

The effects of MA, MA-P and the physical mixture (MA and P) were studied on various peripheral markers like GWM, hexosamine, and proteins (Table 3). GWM content was determined indicating that there is significant deduction in GWM when MA was given, while MA-P maintain the GWM content. Hexosamine and protein concentration got enhanced while taking MA-P whereas it had lowered with administration of MA and the physical mixture (MA and P). It can be concluded that GWM, hexosamine and protein concentration indicated that MA-P was providing cytoprotection.

**CONCLUSION**

In the present study MA-P mutual prodrug has been designed, synthesized and evaluated as safer NSAID. The derivative has been found to be chemically stable and biolabile. It exhibited retention in anti-inflammatory with significant reduced ulcerogenicity as compared to the physical mixture. This may be due to improved physicochemical properties required for enhanced bioavailability. On the basis of these observations, it can be concluded that there is advantage of giving MA and P in the form of a single molecule i.e., MA-P mutual prodrug.

**Acknowledgments**

Authors thank M/S Zydus Cadila, Ahmedabad, Gujarat, India for providing mefenamic acid as gift sample and to Vice Chancellor, GLA University, Mathura UP, India for providing the research facilities to carry out this work in the Institute of Pharmaceutical Research.

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Received: 13. 11. 2012