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SYNTHESIS, PHARMACOLOGICAL ACTIVITY AND HYDROLYTIC BEHAVIOUR OF MUTUAL PRODRUGS OF IBUPROFEN

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ABSTRACT: For reducing the gastrointestinal (GI) toxicity associated with ibuprofen (IBU), its carboxylic group was masked by synthesizing its mutual prodrugs with propyphenazone by direct coupling and by using spacer technique (amino acid was taken as a spacer). The structures of synthesized prodrugs were confirmed by ^{1}H-NMR, ^{13}C-NMR, Mass and FT-IR spectral methods, and their purity were established by elemental analysis. The mutual prodrugs were evaluated for their drug release behavior in enzyme-free simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4). The release of free ibuprofen from prodrugs showed negligible hydrolysis at gastric pH in SGF as compared to SIF where they undergo significant hydrolysis and thus release IBU in adequate amounts following first-order kinetics. Both IBU prodrugs were retaining anti-inflammatory activity intact and exhibited better analgesic activity along with much-reduced ulcerogenic. Prodrug IP1, however, showed better analgesic activity and negligible ulcerogenic tendency than IP2, and hence it could be considered as a better candidate for prodrug among the two.

INTRODUCTION: Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed drugs, and consumption is projected to increase because of the aging population and more widespread use in cardiac and cerebrovascular disease [1]. NSAIDs are generally prescribed for pain management in musculoskeletal or osteoarticular pathologies and rheumatic diseases, very common diseases in the general population.

The therapeutic efficacy of currently available NSAIDs is significantly limited by associated GI toxicity, which causes a higher incidence of morbidity in the long term NSAID users. However, both traditional NSAIDs and second-generation NSAIDs (cyclooxygenase-2 inhibitors) can lead to very expensive and serious adverse events.

Gastrointestinal, cardiovascular, and renal complications associated with NSAIDs are dose-dependent [2]. These GI complications are believed to be determined from the mixed effect of irritation caused by blockage of prostaglandin biosynthesis in the GI tract, as the endogenous prostaglandins are known to have cytoprotective action on the gastric mucosa and direct action of free carboxylic groups in NSAIDs [3].

The recent failure of the selective COX-2 inhibitors leaves the compelling need for effective NSAIDs with improved safety NSAIDs [4]. Therefore, it is necessary to consider NSAID-induced toxicity as a serious public health problem contributing significantly to the morbidity and mortality of patients receiving these drugs. For many years, several attempts have been made to develop reversible derivatives or prodrugs of NSAIDs containing carboxylic acid function to depress upper gastrointestinal (GI) irritation and bleeding [5].

In recent years, there has been an increasing interest in the design and development of mutual prodrugs, which involves combining two different pharmacophores with similar pharmacological activities to give synergistic action. For example, the mutual prodrug of etodolac and glucosamine solves not only the formulation problem of Etodolac (lower aqueous solubility, BCS class II drug) but also increased synergistic anti-inflammatory and antiarthritic activity with lower toxicity and less ulcerogenic activity than the parent drug [6].

Similarly, Mutual prodrug of ketoprofen and glucosamine have also been synthesized with synergistic action [7]. Codrug or mutual prodrug is an approach where various effective drugs, which are associated with some drawbacks, can be modified by attaching with other drugs of same or different categories directly or via a linkage. More appropriately, one can say combining two different pharmacophores with similar or different pharmacological activities elicit synergistic action or help to target the parent drug to specific site/organ/cells, respectively. This approach is commonly used to improve physicochemical, biopharmaceutical, and drug delivery properties of therapeutic agents [8, 9]. Even though ibuprofen (IBU) is very potent and widely used among other clinically used NSAIDs, literature is abundant with its gastric and other side effects because of the presence of the free carboxylic group. The frequent medication of IBU, however, is well known to cause serious GI damage, like other NSAIDs. Also, IBU has a short plasma half-life of 1-2 hours, which further necessitates frequent administration to maintain therapeutic drug doses [10-12]. Many IBU prodrugs, *i.e.*, glyceride derivatives [11], ethylenediamine, and benzathine conjugates [13],

amide derivatives [14], poly (HEMP) conjugates [15], acrylic-type derivatives [16], N-hydroxymethyl-succinimide-/isatin esters[17], anhydride derivatives [18], *etc.* and some mutual prodrugs such as IBU-anthraquinone [19], IBU-sulpha drugs [20], IBU-paracetamol, and IBU- salicylamide [21], *etc.* were reported in the literature.

Recently, IBU has been conjugated with different antioxidants (menthol, thymol, and eugenol) having antiulcerogenic activity with the objective of obtaining NSAIDs- antioxidant mutual prodrugs as gastrosparing NSAIDs devoid of ulcerogenic side effects [22]. Mahdi and co-workers have also reported the synthesis of mutual prodrugs of IBU by coupling with two different sulfa drugs sulfathiazole and sulfadiazine using a glycolic acid spacer (- OCH_2COO-) to reduce the ulcerogenic side effects of IBU by esterification of the free carboxyl group of the IBU that is responsible for the local irritation [23].

After going through vast literature survey and earlier reports, a possible way to solve GI side effects of IBU was found to derivatize the carboxylic functional group of the IBU to produce mutual prodrug with adequate stability at the acidic pH of the stomach and releasing the drug in a sustained manner predominantly at the intestinal pH in order to avoid direct contact of free carboxyl group of the drug to the gastric mucosa. Propyphenazone is a non-acidic pyrazole drug and has a good analgesic and antipyretic activity with no anti-inflammatory activity.

The current work is targeted at the concept of designing drug through the conjunction of two different pharmacophores having similar pharmacological activities.

In the view of this background, the present study was conducted to design, synthesis, and preliminary kinetics study of mutual prodrugs of IBU with propyphenazone to get NSAIDs with lesser ulcerogenic side effects while retaining the anti-inflammatory and analgesic activity.

MATERIALS AND METHODS: Propy-phenazone was obtained as a gift from Vani Pharma Labs Limited, Hyderabad, AP, India and drug IBU was obtained as a gift sample from Jarkar Pharmaceuticals, HSIDC Industrial Complex Rai,

Sonepat, Haryana. The other reagents and solvents used were of analytical grade. The starting compound 3-bromomethyl-propyphenazone was prepared in a pure crystalline form according to the reported method [24].

The melting points of synthesized compounds were determined in open capillary using Decibel melting point apparatus and recorded in ºC without correction. The reactions were monitored by TLC on precoated silica gel G plates using iodine vapors as a detecting agent. The infrared spectra were recorded by PERKIN ELMER spectrophotometer using potassium bromide pellet technique and sodium chloride cells for liquid samples. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on Bruker Avance II 400 NMR Spectrophotometer using tetramethylsilane as an internal standard.

^{1}H NMR spectra were recorded with $CDCl_3$ as a solvent, and the chemical shift data were expressed as values relative to TMS (Chemical shift δ in ppm). Mass spectra of the compounds were obtained using LC-MS (SHIMADZU-2010 AT, Software class VP). Elemental analyses were performed at the Analysis center, Chemistry department, Faculty of Science, Delhi University. The hydrolysis data and drug content determination were performed by a UV-Visible Spectrophotometer Pharma Spec-1700 (SHIMADZU).

General Procedure for the Synthesis of Mutual Prodrugs of Ibuprofen (IBU): Mutual prodrugs of IBU were prepared by two methods:

a. Without spacer (IP1)
b. With spacer (IP2)

Synthesis of IBU–propyphenazone (Ibu-Propy) mutual prodrug without spacer (IP1).

Synthesis of 3-bromomethyl propyphenazone (BMP): 3-bromomethyl propyphenazone, $C_{14}H_{17}BrN_2O$, was synthesized from propyphenazone and bromine, according to Lucius (1907) with some modifications to increase yield and purity as follows; About 10 g propyphenazone was dissolved in 25 mL dichloromethane (DCM) in 100 mL round bottom flask fitted with dropping funnel; it was kept over an ice bath, and bromine solution (6.95 g; 2.23 mL bromine dissolved in 5

mL CH_2Cl_2) was added drop-wise and very slowly with the aid of stirring for 1 h.

The brown color of the bromine faded rapidly upon its addition. The temperature was maintained 10-15 ºC throughout the reaction. The reaction progress was monitored by TLC using ethyl acetate (EA): hexane (2:1) ratio. An aliquot of 30 mL 10% cold aqueous sodium carbonate solution was added with vigorous shaking.

The organic phase was separated and dried with Na_2SO_4, filtered, and concentrated under vacuum at 40 ºC until the volume was about 10 mL. The reaction mixture was cooled to room temperature, and about 15 mL diethyl ether was added. This solution was left to stand in the stoppered flask at room temperature in the dark. The colorless crystals which formed were separated and washed with cold diethyl ether (about 5 ºC).

The obtained crystals were dried overnight in vacuum desiccators over anhydrous calcium chloride, in the dark to yield 11.8 g (87.86%) of pure dried crystals of BMP. The modifications made to the reported method included, (a) The use of cold sodium carbonate aqueous solution to avoid formation of any 3-hydroxymethyl-propyphenazone; (b) Using diethyl ether instead of dichloromethane to get more yield of BMP crystals; and (c) Using cold diethyl ether for crystallization instead of dichloromethane at room temperature to get more pure crystals of BMP.

Synthesis of Potassium Salt of IBU: Potassium tertiary butoxide (0.224 g, 2 mmol) was taken in two-necked round bottom flask, fitted to a guard tube. To this, 3.0 mL of toluene was added and stirred well until it completely dissolves. To the above reaction mixture 0.412 g (2 mmol) IBU was added, little by little with continuous stirring. As soon as the addition is complete round bottom flask was stoppered and stirred for some more time until a clear solution is obtained. The solvent was evaporated on a rotary evaporator to get 0.34g (69.67%) hygroscopic white powder of potassium salt of IBU.

Synthesis of IBU-propyphenazone ester by Coupling of BMP and Potassium Salt of IBU (IP1): A solution of BMP (0.309 g, 1 mmol) and potassium salt of IBU (0.488 g, 2 mmol) in 5.8 mL

of dimethylformamide (DMF) was refluxed on an oil bath at 110 ºC. The reaction mixture was continuously stirred throughout the process. The progress of the reaction was monitored by TLC using EA: Hexane (2:1). The reaction mixture was diluted with 100 mL of water and extracted with ethyl acetate. Ethyl acetate layer was collected washed with water and dried over anhydrous sodium sulphate. Finally, the solvent was evaporated using rotary evaporator to yield 0.56g (65.02%) of pure ester prodrug. Synthesis of IBU–propyphenazone (Ibu-Gly-Propy) mutual prodrug with spacer (IP2)

Preparation of 3- Hydroxymethyl Propyphenazone (HMP): BMP (0.309 g, 1 mmol) was refluxed with 5.0 mL of water. The reaction progress was monitored by TLC, using EA: Hexane (2:1). Once the reaction is completed, the reaction mixture was cooled, extracted with dichloro-methane (DCM), and the DCM layer was collected. It was washed with water and dried over anhydrous sodium sulfate. The concentration of the DCM layer resulted in white hydroxyl derivative. Recrystallization with hot water resulted in 0.21g (85.36%) pure white crystals of HMP.

Preparation of glycinyl-3-hydroxymethyl propyphenazone (Gly-HMP) To an ice-cold solution of Boc-glycine (1.75 g, 10 mmol) in 30 mL, DCM was added HMP (2.46g, 10 mmol), dimethylaminopyridine (50 mg), and dicyclohexylcarbodiimide (DCC, 2.27 g). The reaction mixture was stirred at 4 ºC for 1 h, and kept overnight at room temperature. The precipitated dicyclohexylurea (DCU) was separated by filtration, and the filtrate was washed with cooled 0.05 N HCl and followed by a saturated solution of NaHCO$_3$ and finally with brine, dried over anhydrous Na$_2$SO$_4$, and evaporated under reduced pressure. The product was then treated with 10 mL of 6 N HCl in dioxane and stirred for 1 h followed by evaporation under vacuum at room temperature. The resulting product was triturated with 100 mL of dry ether and left for 2 h in the refrigerator. The precipitated product was filtered and vacuum dried to yield 2.4 g (79.21%) crystals of Gly-HMP.

Preparation of IBU – Glycine – 3 - hydroxypropyphenazone (IP2): A solution of CDI (1 g, 6.16 mmol) in anhydrous DMF (5 mL) was added drop-wise, at 4 ºC to a solution of IBU (1 g) dissolved in anhydrous DMF (10 mL). To the cold mixture, an equimolar solution of Gly-HMP in dry DMF (20 mL) was added drop-wise. The reaction mixture was stirred for 10 min at 4 ºC, for 3 h at room temperature and then evaporated under vacuum. The residue was purified by column chromatography (hexane/ethyl acetate; 2: 1) to yield 1.52 g (64.04%) of pure compound IP2.

3 - (bromomethyl) - 1, 2 – dihydro - 4 -isopropyl-2-methyl-1-phenylpyrazol-5-one (BMP): Yield – 87.86%; Mp (°C) 98-100 ºC; R$_f$ value – 0.67; IR (cm^{-1}): 3023.15 (aromatic C-H str.), 2969.26 (aliphatic C-H str.), 633.83 (C-Br str.), 1593.71, 1489.41 (C=C, phenyl nucleus); ^{1}H-NMR (δ (ppm)): 7.247-7.480 (m, 5H, Ar-H), 2.818-2.888 (m, 1H, -CH of -CH(CH$_3$)$_2$), 3.075 (s, 3H, CH$_3$N), 4.329 (s, 2H, CH$_2$Br), 1.334 (d, 6H, CH$_3$ of – CH(CH$_3$)$_2$, J= 6.9 Hz); ^{13}C-NMR (δ (ppm): 21.78 (CH$_3$ of –CH(CH$_3$)$_2$), 38.84 (CH of –CH(CH$_3$)$_2$), 39.91 (N-CH$_3$), 209.14 (CO), 125.61-136.56 (Ar-carbons), 42.35 (CH$_2$ of CH$_2$Br); Mass (m/z): 309 (M$^+$); Elemental analysis: Calculated: C, 54.38%; H, 5.54%; N, 9.06% Found: C, 54.41%; H, 5.50%; N, 9.10%.

1, 2 - dihydro-3-(hydroxymethyl)-4-isopropyl -2-methyl-1-phenylpyrazol-5-one (HMP): Yield – 85.36%; Mp (°C) 102-104 ºC; R$_f$ value – 0.28; IR (cm^{-1}): 2927.94 (aliphatic C-H str.), 3298.44 (O-H str.), 1344.23 (O-H in plane bending), 659.90 (O-H out of plane bending), 1492.04 (C=C, phenyl nucleus); ^{1}H-NMR (δ (ppm): 7.248- 7.462 (m, 5H, Ar-H), 2.176 (s, 3H, CH$_3$ of – CH$_3$N), 2.794-2.879 (m, 1H, -CH of -CH(CH$_3$)$_2$), 4.521 (s, 2H, -CH$_2$OH), 1.278 (d, 6H, CH$_3$ of –CH(CH$_3$)$_2$, J= 6.9 Hz), 1.991 (s, 1H, -OH); ^{13}C-NMR (δ (ppm)): 20.13 (CH$_3$ of –CH(CH$_3$)$_2$), 37.56 (CH of – CH(CH$_3$)$_2$), 39.73 (N-CH$_3$), 212.22 (CO), 128.11-131.26 (Ar-carbons), 58.05 (CH$_2$ of CH$_2$OH); Mass (m/z): 246 (M$^+$); Elemental analysis: Calculated: C, 68.27%; H, 7.37%; N, 11.37%; Found: C, 68.31%; H, 7.33%; N, 11.39%.

(2, 3 – dihydro – 4 - isopropyl – 1 - methyl-3-oxo-2 phenyl-1H-pyrazol-5-yl) methy l - 2 - (4-isobutylphenyl) propanoate (IP1): Yield – 65.02%; Mp (°C) 164-166 ºC; R$_f$ value – 0.58; IR (cm^{-1}): 1734.60 (C=O str. of ester), 2958.76

(aliphatic C-H str.), 1152.12 (C-O-C str.), 1014.33 (O-C-C, bond of ester from 1° alcohol), 1457.04 (C=C, phenyl nucleus), 699.29 (Aromatic C-C out of plane bending), 759.82 (Aromatic C-H out of plane bending); ^{1}H-NMR (δ (ppm)): 7.072-7.255 (m, 4H, Ar-H), 7.301-7.397 (m, 5H, Ar-H), 4.536 (s, 2H, CH_2 of -COOCH$_2$-), 3.648-3.769 (m, 1H, -$CH(CH_3)COO$), 2.836 (d, 2H, CH_2 of – $CH_2CH(CH_3)_2$, J=7.5 Hz), 2.425-2.449 (m, 1H, -CH of –$CH(CH_3)_2$), 1.488 (d, 6H, CH_3 of – $CH(CH_3)_2$, J=7.2 Hz), 2.851 (s, 3H, CH_3 of – CH_3N); ^{13}C-NMR (δ (ppm): 22.09 (CH_3 of Ar-$CH(CH_3)_2$), 21.13 (CH_3 of –$CH(CH_3)_2$), 29.62 (CH of –$CH(CH_3)_2$), 39.04 (N-CH_3), 164.36 (CO of pyrazole), 175.64 (COO), 123.07-139.37 (Ar-carbons), 78.50 (CH_2 of -CH_2OCO), 40.08 (CH of Ar-$CH(CH_3)CO$), 18.52 (CH_3 of Ar-$CH(CH_3)CO$); Mass (m/z): 434 (M^+); Elemental analysis: Calculated: C, 74.62%; H, 7.89%; N, 6.45%, Found: C, 74.56%; H, 7.92%; N, 6.40%.

(2, 3 – dihydro - 4- isopropyl – 1 – methyl - 3-oxo-2-phenyl-1H-pyrazol-5-yl)methyl – 2 - (2-(4-isobutylphenyl) propanamido) aectate (IP2): Yield – 64.04%; Mp (°C)- oily liquid; R_f value – 0.78; IR (cm^{-1}): 1723.29 (C=O str. of ester), 2959.01 (aliphatic C-H str.), 1099.65, 1176.23 (C-O-C str., ester), 1059.83 (O-C-C, bond of ester from 1° alcohol), 1457.14 (C=C, phenyl nucleus), 3294.18 (N-H str. of amide), 1637.61 (C=O str., amide), 1502.33 (N-H bend, 2° amide), 855.92 (C-N str.), 664.35 (Aromatic C-C out of plane bending), 759.63 (Aromatic C-H out of plane bending); ^{1}H-NMR (δ (ppm)): 7.211-7.473 (m, 5H, Ar-H), 6.887-7.080 (m, 4H, Ar-H), 7.973 (s, 1H, NH), 2.850 (d, 2H, CH_2 of –$CH_2CH(CH_3)_2$, J=9.9 Hz), 4.519 (s, 2H, CH_2 of -COOCH$_2$-), 2.850 (s, 3H, CH_3N), 0.894 (d, 6H, -CH_3 of –$CH_2CH(CH_3)_2$, J=6.6 Hz), 2.414-2.437 (m, 1H, -CH of -$CH(CH_3)_2$), 1.258 (d, 6H, -CH_3 of –$CH(CH_3)_2$, J=7.2 Hz), 2.154-2.293 (m, 1H, CH of – $CH_2CH(CH_3)_2$); ^{13}C-NMR (δ (ppm)): 21.32 (CH_3 of –$CH(CH_3)_2$), 29.12 (CH of –$CH(CH_3)_2$), 39.64 (N-CH_3), 166.21 (CO of pyrazole), 171.51 (CONH), 41.11 (NH-CH_2-COO), 169.27 (COO), 117.87-138.58 (Ar-carbons), 69.03 (CH_2 of -CH_2OCO), 18.06 (CH_3 of -$CH(CH_3)CONH$), 42.60 (CH of -$CH(CH_3)CONH$), 45.71 (CH_2 of $CH_2CH(CH_3)_2$); Mass (m/z): 491 (M^+); Elemental analysis: Calculated: C, 70.85%; H, 7.59%; N, 8.55% Found: C, 70.91%; H, 7.51%; N, 8.49%.

***In-vitro* Hydrolysis Studies of IBU Prodrugs:** The hydrolysis study was carried out using Veego dissolution apparatus (VDA-8DR, USP standard). The test was conducted at 37 ± 0.5 ℃ using apparatus II and pedal speed rotation of 100 rpm. Two hydrolysis media were used: 900 mL of non-enzymatic simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4).

An accurate amount of 100 mg of the prodrug was used for the study. From the matrix, aliquots of 1 ml were withdrawn at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 hours and were immediately replaced with 1.0 mL of fresh hydrolysis media equilibrated at 37 ± 0.5 ℃.

Free IBU which was supposed to be released after the hydrolysis of prodrugs extracted with 5 mL methanol. The methanol layer was estimated on UV spectrophotometer for the amount of free IBU released after hydrolysis of prodrugs in SGF and SIF. The kinetics of hydrolysis was monitored by the increase of free drug concentration with time and order of reaction, and half-life ($t_{1/2}$) were also calculated.

The rate of hydrolysis was calculated using equation, k = (2.303/t) log (a/a-x) where k represents hydrolysis constant, t is the time in min, 'a' is the initial concentration of prodrug, x is the amount of prodrug hydrolyzed and (a-x) is the amount of prodrug remaining.

***In-vivo* Evaluation:** The prodrugs prepared were screened for analgesic and anti-inflammatory activity as compared with that exerted by IBU and HMP, separately. Male Swiss mice (25-30 g) and male Wistar rats of Albino strain (150-200 g) were used. All the animals were obtained from Disease Free Small Animal House CCSHAU, Hisar.

The selected animals were housed in polypropylene cages at standard environmental conditions at 22 ± 2 °C, the relative humidity of 45–55 %, in a well-ventilated room maintained at 12: 12 h light: dark cycle, fed with standard pellet diet and water *ad libitum*. All the animals were acclimatized for a week before the experiment. All animal experiments were carried out according to the guidelines of the Committee for Control of Experiments on Animals and approval of the Institutional Animal Ethics Committee, Maharshi

Dayanand University, Rohtak, Haryana (Establishment Reg. no. 134/99/CPCSEA) was obtained. The animals fasted with free access to water for 12 h before the tests. The tested compounds were prepared for oral administration in aqueous 0.5% carboxymethylcellulose (CMC) solution.

The experimental protocol was approved by the Institutional Animal Ethics Committee (Endst. No. CBT/2010/604-1609, dated 29-10-2010). All the results were expressed as Mean ± Standard error (SEM). Data were analyzed using one-way ANOVA followed by Tukey-Kramer multiple comparisons test. p-values < 0.05 were considered as statistically significant.

Analgesic Activity: The analgesic activity was assessed by acetic acid-induced writhing [25]. Mice were divided into five groups (n=6 in each group). Group I served as control (received 0.5% w/v CMC (10 mL/kg) only). Group II and III received standard drugs *i.e.* IBU (20 mg/Kg body weight, 96.90 µmol/kg) and HMP (23 mg/Kg body weight, 96.90 µmol/kg), Group IV and V received prodrug IP1 (42.07 mg/Kg body weight, 96.90 µmol/kg) and IP2 (47.60 mg/Kg body weight, 96.90 µmol/kg), where the dose was molecular equivalent to the free drug. The control, standard, and test drugs were given by oral route while 1% v/v acetic acid solution was injected intraperitoneal to mice. Mice were pretreated with drug vehicle or standard or test drugs 1 h before acetic acid injection.

The writhing response was induced by an intraperitoneal injection of 1% v/v acetic acid solution. The mice were placed in separate boxes under observation immediately after acetic acid injection and a total number of writhes, which was a parameter of chemically induced pain (i.e., constriction of the abdomen, turning of trunk and extension of hind limbs), was counted for 10 min. The analgesic effect was expressed as percent reduction of writhes in comparison with the control. The percentage protection was calculated by the following formula:

% Protection = 100 - (No. of writhes in test/No. of writhes in control) $\times$ 100

Anti-Inflammatory Activity: The carrageenan-induced rat hind paw edema method [26] was used to evaluate the acute anti-inflammatory activity of the prodrugs. Rats were divided into control, standard, and test groups of six animals each. Pretreatment initial paw volumes of all animals were measured using a mercury plethysmometer. The control group was given only an appropriate volume of 0.5% CMC. The standard group received IBU and HMP (equivalent dose, 96.90 µmol/kg) respectively.

To the test group, prodrugs (IP1 and IP2) were administered orally using similar doses as employed in the analgesic activity. One hour after treatment, edema in the left hind paw of the rat was induced by injection of 0.1 mL of 1% (w/v) carrageenan solution in normal saline solution (0.9% w/v). The paw was marked with ink at the level of lateral malleolous and immersed in mercury up to this mark.

The relative change in paw volume was determined by measuring the paw volume immediately after injection and at 1, 2, 3, 4, 6, and 8 h intervals following the carrageenan administration. The percent inhibition of edema, as an indication of anti-inflammatory activity was compared with the controls. The percentage inhibition of swelling was calculated using the following formula:

$$\text{Inhibition (\%)} = (V_t\text{-}V_o)_{control} - (V_t\text{-}V_o)_{treated} \times 100 / (V_t\text{-}V_o)_{control}$$

V_o and V_t relates the average volume in the hind paw of rats (n=6) before any treatment and after anti-inflammatory agent treatment, respectively.
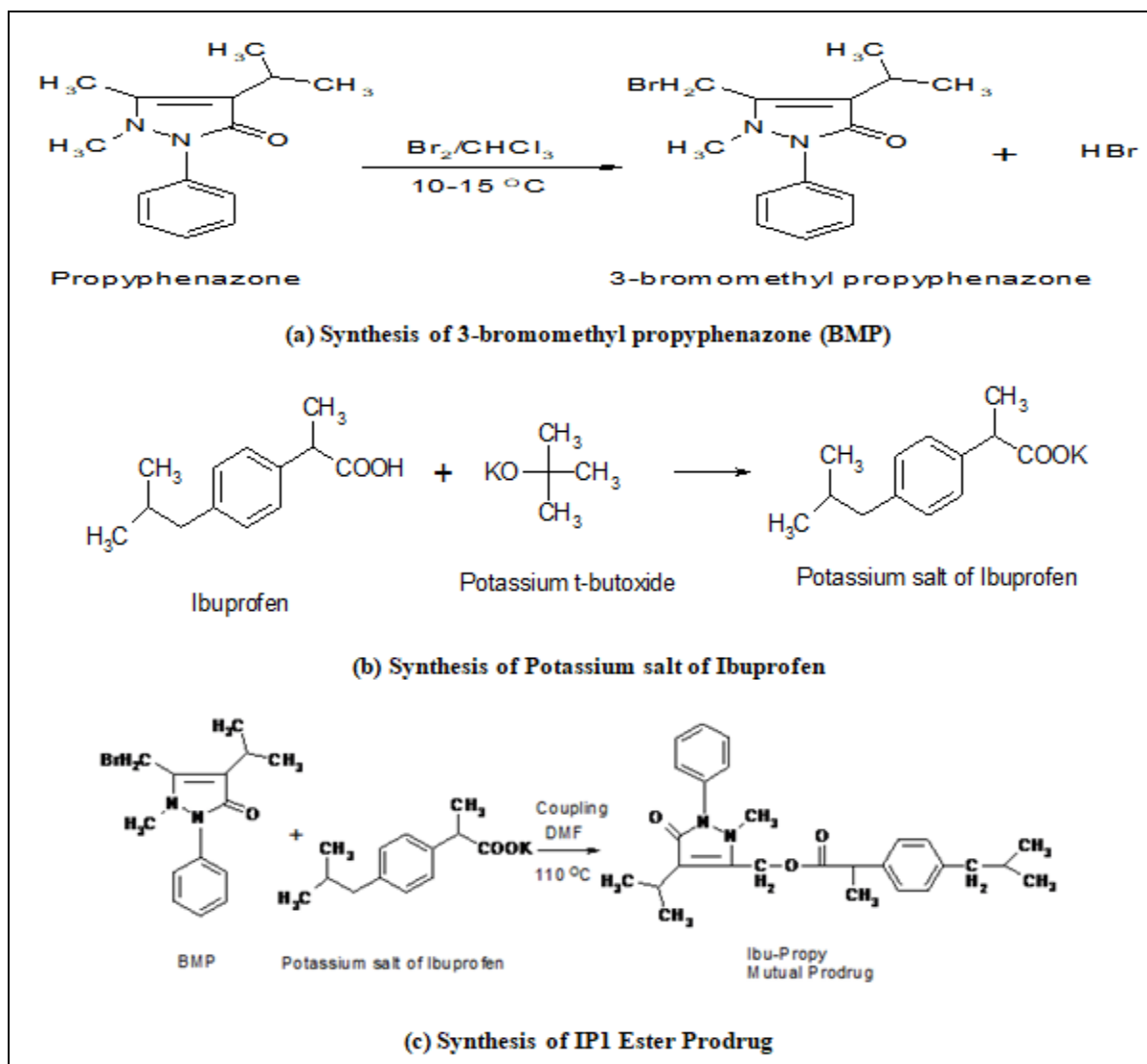
Ulcerogenic Activity: Ulcerogenesis test was performed according to the method of Kunchandy *et al.* [27] Wistar rats were divided into five groups consisting of six animals in each group. All animals fasted for 12 h before the administration of the drug.

The first group served as control and received *p.o.* administration of the vehicle (0.5% CMC) only. Group II and III received IBU and HMP (in equivalent doses, 96.90 µmol/kg) respectively. Group IV and V received equivalent doses of prodrugs IP1 (42.07 mg/kg, 96.90 µmol/kg) and IP2 (47.60 mg/kg, 96.90 µmol/kg) separately. All the test drugs or standard of the vehicle were administered orally to rats over a period of seven successive days.

All the rats fasted for 24 h on the 8th day. The animal was sacrificed with excessive anesthesia. The stomach was removed, opened along the greater curvature, and washed gently in running tap water. The gastric mucosa of the rat was examined using a magnifying lens and compared with that after ibuprofen administration. For each stomach, the mucosal damage was assessed according to the following scoring system: 0.0 - normal colored stomach; 0.5 - pink to red coloration of stomach; 1.0-spot ulcer; 1.5 - haemorrhagic streak; 2.0 - number of ulcers < 5; 3.0 - number of ulcers > 5; 4.0 - ulcers with bleeding.

The mean score of each treated group minus the mean score of the control group was regarded as the severity index of gastric mucosal damage.

RESULTS AND DISCUSSION: Bromopropyphenazone (BMP) was prepared by bromination of propyphenazone according to Meister, Lucius, Bruning in Hoechst, 1907, with some modifications to increase yield and purity. IBU-propyphenazone ester (IP1) was synthesized by coupling of BMP and potassium salt of IBU by refluxing at 110 °C, as shown in **Scheme 1**.

(a) Synthesis of 3-bromomethyl propyphenazone (BMP)

(b) Synthesis of Potassium salt of Ibuprofen

(c) Synthesis of IP1 Ester Prodrug

SCHEME 1: GENERAL STEPS FOR THE SYNTHESIS OF IBU-PROPY PRODRUG (IP1)

Bromopropyphenazone (BMP) was used for the preparation of hydroxymethyl-propyphenazone (HMP) by reflux with water. To avoid or minimize the steric hindrance effect on the hydrolysis of IBU-HMP ester prodrug (IP1), a spacer was introduced. The spacer chosen was glycine. N-Protected glycine coupled with HMP by DCC followed by N-deprotection and coupling with IBU using CDI, yielded IBU amide prodrug (IP2) as illustrated in **Scheme 2**.
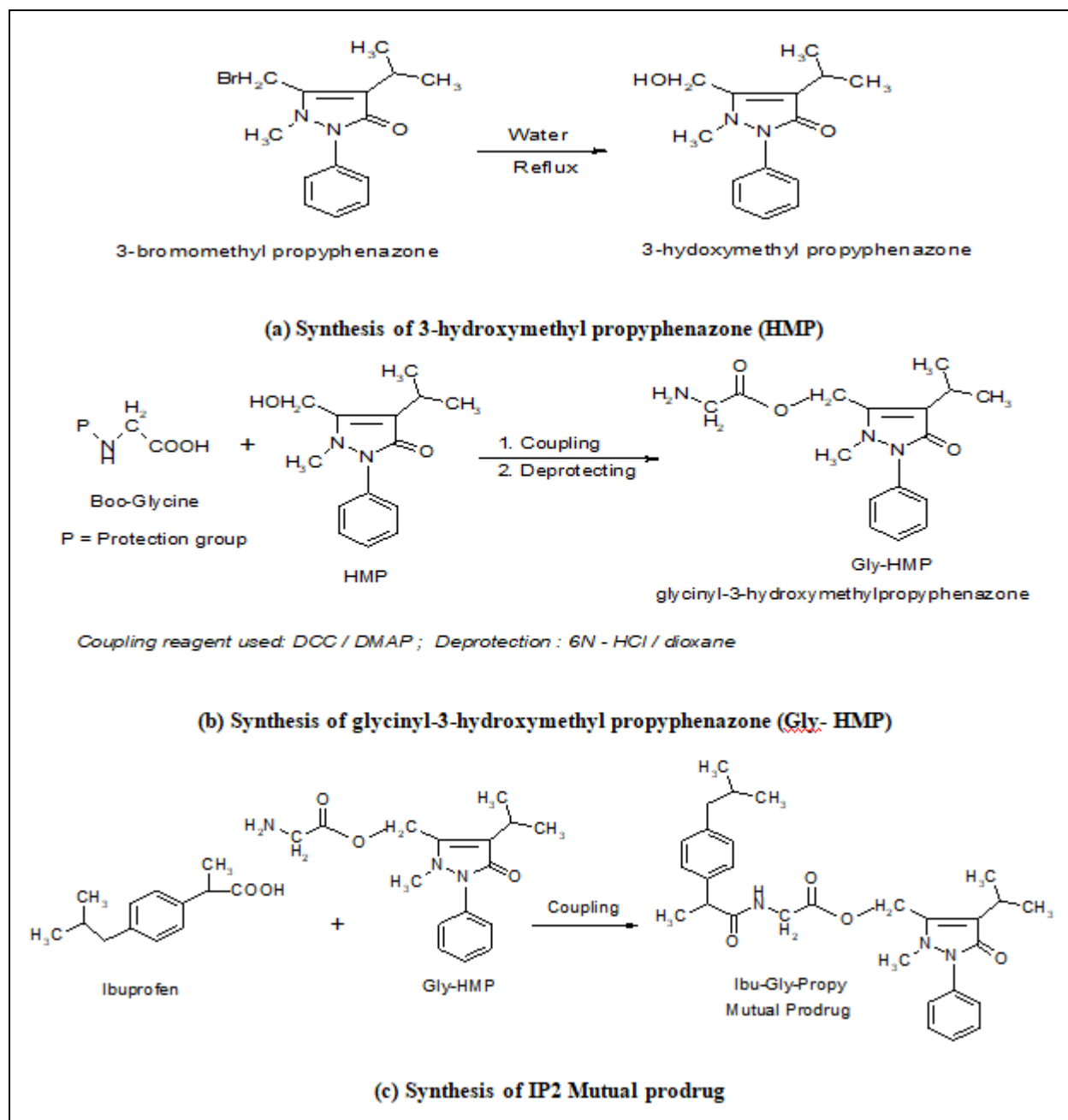
SCHEME 2: GENERAL STEPS FOR THE SYNTHESIS OF IBU-GLY-PROPY PRODRUG (IP2)

The physicochemical properties were determined and shown in **Table 1**. Carbonyl diimidazole (CDI) is a useful coupling reagent that allows one-pot amide formation. Acyl carboxy imidazole and imidazole were initially formed but readily reacted together to yield the activated species as the acyl imidazole. Practically, the acyl imidazole was preformed for 1 h, and then the amine was added. This reaction, which generates imidazole *in-situ*, does not need an additional base and is even compatible with HCl salts of the amine.

TABLE 1: PHYSICOCHEMICAL PROPERTIES OF SYNTHESIZED PRODRUGS

Prodrug Code	Molecular formula	Mol. wt.	Colour	m.p.* (ºC)	% Yield	$R_f^\#$ value	Log P
IP1	$C_{27}H_{34}N_2O_3$	434	White	164-166	65.02	0.58	5.54
IP2	$C_{29}H_{37}N_3O_4$	491	Brown	Liquid	64.04	0.78	4.39

*Uncorrected; #Ethyl Acetate: n-Hexane (2:1)

In **Scheme 2**, the formation of Gly-HMP is an example of Steglich esterification. It is a variation of an esterification with dicyclohexylcarbodiimide (DCC) as a coupling reagent and 4-dimethyl

aminopyridine (DMAP) as a catalyst. The reaction mechanism involved two steps. In the first step, the carboxylic acid reacted with DCC to an O-acyl isourea, which was more reactive than the free acid and in a second step the alcohol of HMP attacks this intermediate, forming DCU and corresponding ester. The physicochemical properties were determined and shown in **Table 1**. The yields of prodrugs were good. The structures of prodrugs formed were confirmed by ^{1}H-NMR, ^{13}C-NMR, Mass, and FT-IR spectral methods. The purity was determined by TLC. The results of elemental analysis of synthesized prodrugs were in all case within 0.4% of theoretical values and were in confirmation of the desired structure.

IR stretching band ranging from 1740-1723 cm^{-1} indicated the formation of an ester linkage (C=O str.). The presence of C-O str. (ester linkage) was obtained in a range of 1020-1275 cm^{-1}. IR stretching band at 633.83 cm^{-1} in BMP indicated the presence of bromo group, whereas, IR stretching band at 3298.44 cm^{-1} indicated the presence of hydroxyl group in HMP. Presence of aliphatic 1° alcohol in HMP was indicated by the presence of OH in-plane bending at 1344.23 cm^{-1} and OH out of plane bending at 659.90 cm^{-1} respectively. Presence of phenyl nucleus in the synthesized compounds was indicated by the presence of a skeletal stretching band of phenyl nucleus at 1590-1480 cm^{-1}.

The appearance of the multiplet signal around δ 6.997-7.973 ppm depicted the presence of aromatic protons. The appearance of the singlet signal at δ 1.991 ppm in HMP confirmed the presence of the aliphatic hydroxyl group. Doublet signal observed

in the range δ 1.258-1.488 ppm indicated the presence of isopropyl group -CH$_3$ of –CH (CH$_3$)$_2$ in both prodrugs.

Presence of isopropyl group (-CH of -CH(CH$_3$)$_2$) in all synthesized compounds is also indicated by multiplet signals around δ 2.414-2.449 ppm. In ^{13}C NMR spectra, the signals for aromatic carbons had a spread from δ 117.87 to 139.37. Signals for other carbons of parent structures were observed at about δ 21.22 (CH$_3$ of –CH (CH$_3$)$_2$), δ 29.37 (CH of –CH (CH$_3$)$_2$), 172.45 (COO), 165.27 (CO of pyrazole), 73.76 (CH$_2$ of -CH$_2$OCO) and 39.37 (N-CH$_3$). Additional peaks for NH-CH$_2$-COO and CONH were observed at δ 41.11 and δ 171.51 respectively. Further, the elemental analysis and mass spectra also supported the formation of title compounds.

***In-vitro* Hydrolysis of Ester:** One of the crucial requirements for a prodrug to be used, they should show good stability in aqueous solutions and in gastrointestinal fluid, and it should be readily hydrolyzed following gastrointestinal absorption to release the parent drug [27]. Since, the carboxylic group of IBU is essential for the therapeutic action, prodrugs of prolonged action were designed in a form which the biologically active moiety can be released in its original state with time.

Therefore the release of IBU from its prodrugs was studied *in-vitro* to evaluate the possible period in which the drug could be available from different prodrugs. The comparative patterns of hydrolysis of these prodrugs in SGF and SIF are shown in **Fig. 1** and **2**, respectively.
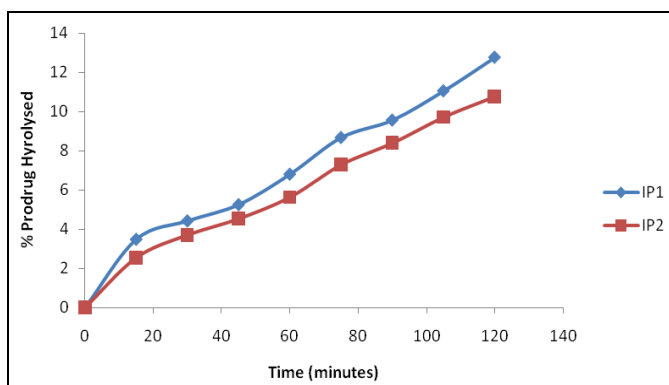

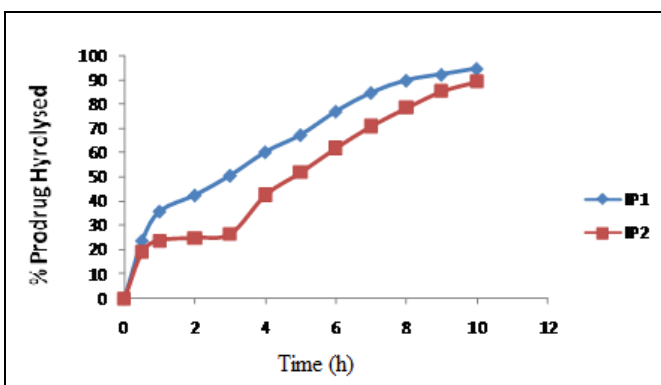**FIG. 1. COMPARATIVE PATTERN OF HYDROLYSIS OF IP1 AND IP2 PRODRUGS IN SGF (pH-1.2)**

FIG. 2. COMPARATIVE PATTERN OF HYDROLYSIS OF IBU-PROPY(IP1) AND IBU-GLY-PROPY (IP2) PRODRUGS IN SIF (pH-7.4)

The amount of IBU regenerated on hydrolysis of IP1 and IP2 in SGF (pH-1.2) was found as 26.29 and 24.02% respectively and that in SIF (pH-7.4) was found as 94.82 and 89.52% respectively throughout 10 h. The results of the hydrolytic kinetics study revealed that both followed first-order kinetics. But these prodrugs showed negligible hydrolysis in acidic medium (pH 1.2) for 2 h. From these results, this is confirmed that the release of IBU should occur predominantly at higher pH of the intestine. This may be because ester hydrolysis is a reversible reaction in acidic pH, and alkaline pH it is irreversible and complete [21].

The predominant release of IBU from its prodrugs at pH 7.4 indicates the potential of the prodrug to reduce the gastric complications caused by direct contact of the free carboxyl group of the drug to the gastric mucosa. Kinetic parameters for hydrolysis of mutual prodrugs at 37 ºC are shown in **Table 2**. The corresponding half-lives for IP1 and IP2 were

found to be 14.70 and 17.14 h (in SGF, pH 1.2) and 2.28 and 3.45 h (in Phosphate buffer, pH 7.4) respectively. The half-lives and the rate constants for prodrug hydrolysis **Table 2** indicated that esterification of the carboxylic group of IBU rendered its prodrugs more stable at pH 1.2, but less stable at pH 7.4.

TABLE 2: KINETIC PARAMETERS FOR HYDROLYSIS OF MUTUAL PRODRUGS IP1 AND IP2 AT 37 ºC

Prodrug code	K_{obs} $(h^{-1})^a$	$t_{1/2}$ $(h)^a$	K_{obs} $(h^{-1})^b$	$t_{1/2}$ $(h)^b$
IP1	0.047	14.70	0.30	2.28
IP2	0.040	17.14	0.20	3.45

[a] In SGF (pH-1.2), [b] In SIF Phosphate buffer (pH- 7.4)

***In-vivo* Biological Evaluation:** Analgesic, anti-inflammatory and ulcerogenic activities of the prodrugs were studied in comparison to equivalent doses (96.90μmol/kg) of IBU and HMP. Results of anti-inflammatory activity by IBU and its mutual prodrugs in terms of the difference in paw volume and percentage inhibition at various time intervals are presented in **Table 3** and **4,** respectively.

TABLE 3: PAW VOLUME OF TREATED GROUPS (IBUPROFEN AND ITS MUTUAL PRODRUGS) AT VARIOUS TIME INTERVALS

Treatment	Dose (mg/Kg)	Change in Paw Volume (mL) (Mean ± SEM)					
		1 h	2 h	3 h	4 h	6 h	8 h
Control	-	0.375 ±0.0106	0.45 ±0.034	0.525 ±0.0091	0.475 ±0.011	0.35 ±0.012	0.125 ±0.0065
IBU	20.00	0.221 ±0.0085[***]	0.161 ±0.0057[***]	0.121 ±0.0122[***]	0.118 ±0.0073[***]	0.108 ±0.007[***]	0.0785 ±0.007[***]
HMP	23.00	0.257 ±0.0075[***Δ]	0.241 ±0.0081[***Δ]	0.314 ±0.0089[***Δ]	0.327 ±0.014[***Δ]	0.267 ±0.012[***#]	0.11 ±0.009[Δ]
IP1	42.07	0.309 ±0.007[***#]	0.204 ±0.0063[***]	0.123 ±0.010[***]	0.1 ± 0.010[***]	0.1 ±0.01[***]	0.05 ±0.005[***Δ]
IP2	47.60	0.325 ±0.0076[**#]	0.2 ±0.012[***]	0.105 ±0.009[***]	0.105 ±0.009[***]	0.025 ±0.0025[***#]	0.015 ±0.003[***#]

Oedema is expressed as mean change in paw volume ± SEM
N= 6 animals. [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ as compared to control. [Δ]$p<0.05$, [ΔΔ] $p<0.01$, # $p<0.001$ as compared to ibuprofen

TABLE 4: % INHIBITION OF IBUPROFEN AND ITS MUTUAL PRODRUGS AS COMPARED TO CONTROL

Treatment	Dose (mg/Kg)	% Inhibition					
		1 h	2 h	3 h	4 h	6 h	8 h
Control	-	0.00	0.00	0.00	0.00	0.00	0.00
IBU	20.00	40.96	64.28	76.95	75.16	69.14	37.2
HMP	23	31.47	46.44	40.19	31.16	23.71	12
IP1	42.07	17.63	54.77	76.66	78.95	71.43	61.20
IP2	47.60	13.33	55.55	80	77.89	92.85	88.00

IBU exhibited maximum anti-inflammatory effect (76.95%) at 3rd h whereas second parent drug HMP showed its maximum effect (46.44%) at 2nd h. Both mutual prodrugs showed better maximum inhibition and for a longer time as compared to both parent drugs. IP1 ester prodrug displayed

maximum activity (78.95%) at 4th h whereas IP2 prodrug displayed its maximum activity (92.85%) at 6th h. IP2 prodrug showed very high % inhibition, *i.e.* 80.00, 77.89, 92.85 and 88.00% during 3rd, 4th, 6th, and 8th h respectively.

The comparative pattern of paw volume of treated groups and anti-inflammatory activity (% Inhibition) at various time intervals are shown in **Fig. 3** and **4,** respectively.
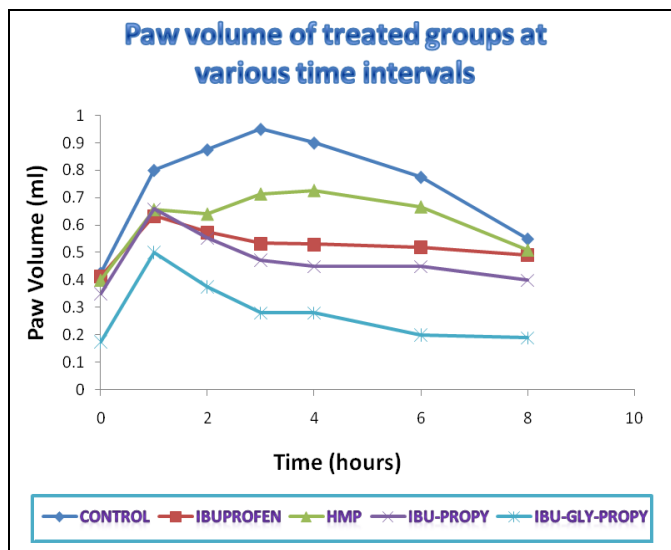

FIG. 3: COMPARATIVE PATTERN OF PAW VOLUME OF TREATED GROUPS AT VARIOUS TIME INTERVALS
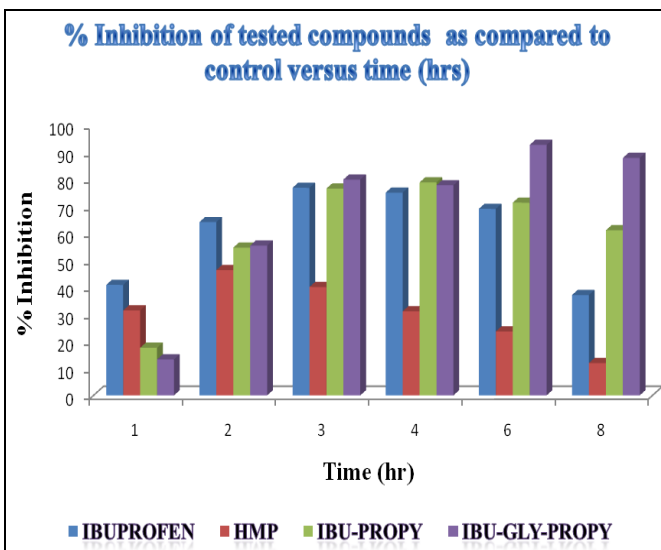

FIG. 4: COMPARATIVE ANTI-INFLAMMATORY ACTIVITY (% INHIBITION) AT VARIOUS TIME INTERVALS

The results showed that the mutual prodrug synthesized with spacer technique using glycine amino acid as a spacer was found to be more effective than prodrug made by direct esterification of parent drugs. The anti-inflammatory activity of prodrugs significantly improves over time.

This means that the prodrug parse is devoid of anti-inflammatory activity, and the observed latent activity results from hydrolysis to the parent drugs. Both prodrugs exhibited highly significant results (p<.001) as compared to control and also possessed significantly better activity as compared to IBU.

The analgesic activity was determined by acetic acid-induced writhing method, and the results are shown in **Table 5**. The results indicated that IP1 showed significant reduction (86.67%, p<0.001) in writhing response produced by acetic acid as compared to parent drug IBU (54.43%) whereas IP2 prodrug didn't produce statistically significant percentage protection (56.67%, p not less than 0.05) as compared to parent drug IBU. The animals which were treated with IBU, HMP, and their mutual prodrugs showed significant (p<0.001) analgesic activity when compared with control animals.

TABLE 5: ANALGESIC ACTIVITY AND ULCEROGENIC POTENTIAL OF IBUPROFEN AND ITS MUTUAL PRODRUGS

Treatment	Dose (mg/ Kg)	Analgesic activity		The average number of ulcer score ± SEM
		Mean number of writhing ± SEM	% Protection	
Control	-	30.00 ± 1.29	-	0.00 ± 0.00
IBU	20.00	$13.7 \pm 0.92^{***}$	54.43	$2.25 \pm 0.250 ***$
HMP	23.00	$6.5 \pm 0.76^{***\#}$	78.33	$1.25 \pm 0.214^{\Delta\Delta ***}$
IP1	42.07	$4.0 \pm 0.58^{***\#}$	86.67	$0.25 \pm 0.118^{\#}$
IP2	47.60	$13.0 \pm 0.58^{***}$	56.67	$0.67 \pm 0.211^{\#}$

n= 6 animals, ***p < 0.001 as compared to control. $^{\#}$p <0.001, $^{\Delta\Delta}$p <0.01, $^{\Delta}$p<0.05 as compared to ibuprofen

The ulcerogenic potential of synthesized prodrugs was tested in comparison to the parent drugs following oral administration for 7 days in rats, and the results are shown in **Table 5**. Screening for ulcerogenic activity showed that synthesized compounds had fewer tendencies (p<0.001) to form ulcer when compared to that of the parent drugs. Photographs illustrated the gastric mucosal injury in **Fig 5**.

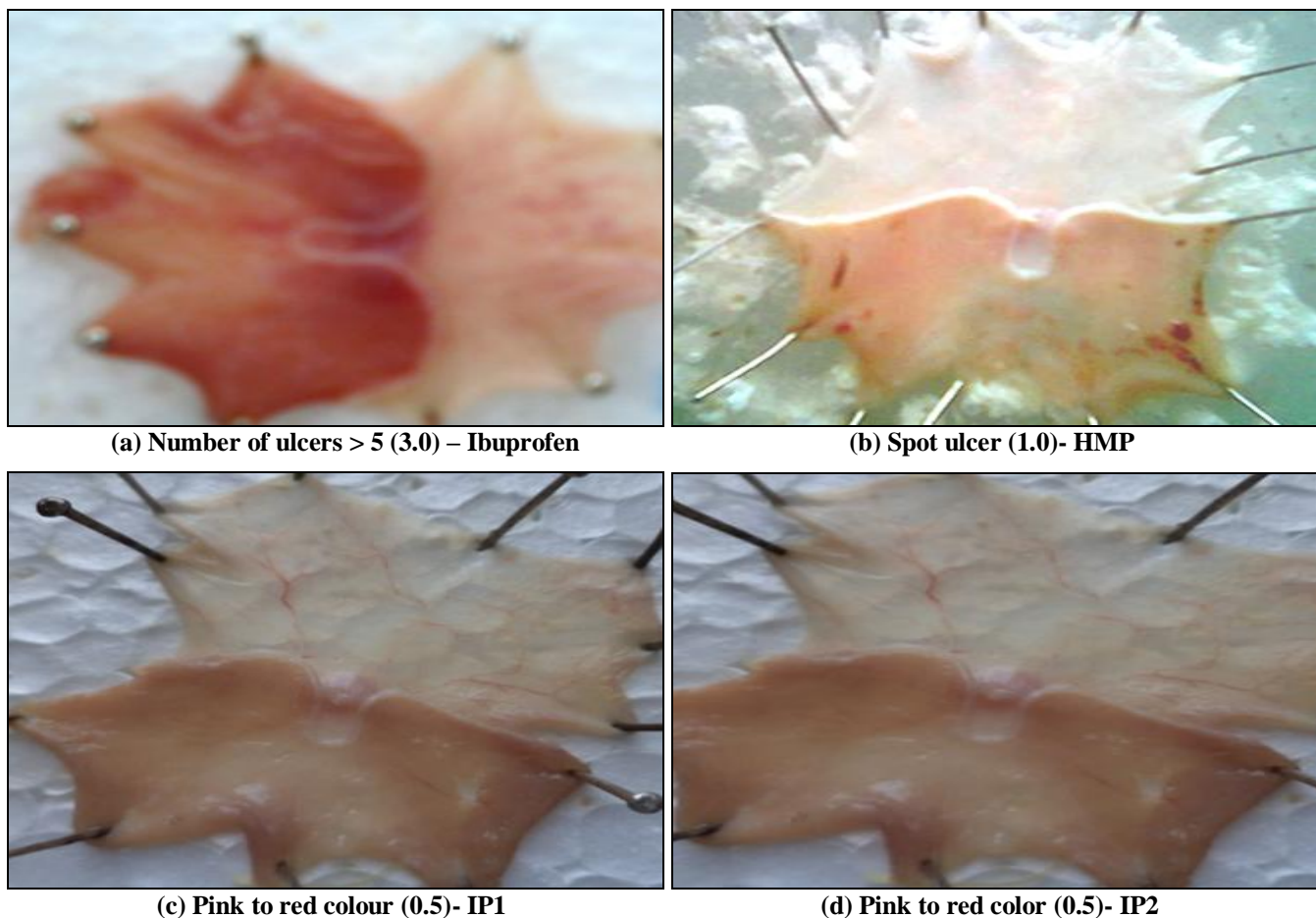**(a) Number of ulcers > 5 (3.0) – Ibuprofen**

(b) Spot ulcer (1.0)- HMP

(c) Pink to red colour (0.5)- IP1

(d) Pink to red color (0.5)- IP2

FIG. 5: ILLUSTRATION OF THE MUCOSAL INJURY IN RATS FOLLOWING ORAL ADMINISTRATION OF IBUPROFEN, HMP, AND THEIR MUTUAL PRODRUGS

CONCLUSION: The mutual prodrugs of IBU were successfully synthesized, and structures were confirmed based on spectral analysis. Both prodrugs showed encouraging hydrolysis rate in SIF and excellent pharmacological response. The *in-vitro* and *in-vivo* evaluation of synthesized prodrugs revealed improvement in the therapeutic index of parent drugs. The derivatives were characterized by prodrug profile, adequate chemical stability, and reduced ulcerogenic liability. Based on above observations, it is concluded that both IBU prodrugs were retaining anti-inflammatory activity intact and exhibited better analgesic activity along with much-reduced ulcerogenicity, but prodrug IP1, however, showed better analgesic activity and negligible ulcerogenic tendency than IP2 and hence it could be considered as a better candidate for prodrug among the two.

CONFLICT OF INTEREST: Nil

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