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Glyceride derivatives as potential prodrugs: synthesis, biological activity and kinetic studies of glyceride derivatives of mefenamic acid

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In order to reduce the gastrointestinal side effect, of mefenamic acid, its carboxylic group was condensed with the hydroxyl group of 1,2,3-trihydroxy propane 1,3- dipalmitate/stearate to give **3a** and **3b**. These compounds were evaluated for their gastric toxicity, anti-inflammatory activity by the carageenan induced paw oedema test and analgesic activity by the acetic acid induced writhing method. The release of mefenamic acid from the esters **3a** and **3b** was studied at pH 3, 4, 5 and 7.4 with direct analysis by reverse phase HPLC using acetonitrile : acetate buffer (0.1 M, pH 3.5) : methanol (40 : 25 : 35) at 1 mL/min. The prodrugs showed less hydrolysis at pH 5 compared to pH 7.4 indicating that the prodrugs do not dissociate at stomach pH but release mefenamic acid at pH 7.4 in adequate amounts. The hydrolysis studies were also performed in rat plasma. A higher plasma concentration of mefenamic acid was observed in animals treated with **3a** and **3b** compared to the animals treated with the parent drug, and even after 8 h the concentration of mefenamic acid was 2 times higher. The peak plasma concentration of mefenamic acid in animals treated with mefenamic acid was attained in 1.5 h compared with 2 h in the case of prodrugs treated animals. The prodrugs showed less gastric ulceration compared to mefenamic acid at 100 mg/kg, a severity index of 1.10, 1.22 being observed with **3a**, **3b** and with mefenamic acid a severity index of 2.37 was observed. The prodrugs showed better anti-inflammatory activity compared to the parent drug and analgesic activity comparable to the parent drug. These findings suggest that the prodrugs **3a** and **3b** synthesized might be used as biolabile prodrugs of mefenamic acid with increased bioavailability and less gastrointestinal side effects.

1. Introduction

The gastric side effects related to the use of NSAIDs are generally attributed to local and/or systemic mechanisms. The direct or local mechanism involves local irritation produced by the acidic group of the NSAIDs and the systemic mechanism involves the inhibition of synthesis of cytoprotective prostaglandins (Guyton and Hall 1998; Vane et al. 1998; Cioli et al. 1979; Shanbhag et al. 1992). A possible approach to solving this problem to a considerable extent is derivatization of the carboxylic function of the NSAIDs to produce prodrug forms with adequate stability at the acidic pH of the stomach, thus preventing local irritation of the stomach mucosa, and also capable of releasing the parent drug spontaneously or enzymatically in the system following their absorption (Bodor 1997; Wang et al. 2002; Mahfouz et al. 1999; Ingram et al. 2001). The utility of glycerides as a promoiety in the design of prodrugs of carboxylic acids relates to the absorption of natural triglycerides, thereby increasing stability in the stomach and increasing overall absorption of the drug. Glyceride prodrugs of niflumic acid are also reported to have increased in their permeability to the brain (El Kihel et al. 1996). In this paper we report on glyceride derivatives of mefenamic acid as prodrugs, and their biological activity and hydrolysis studies.

2. Investigations, result and discussion

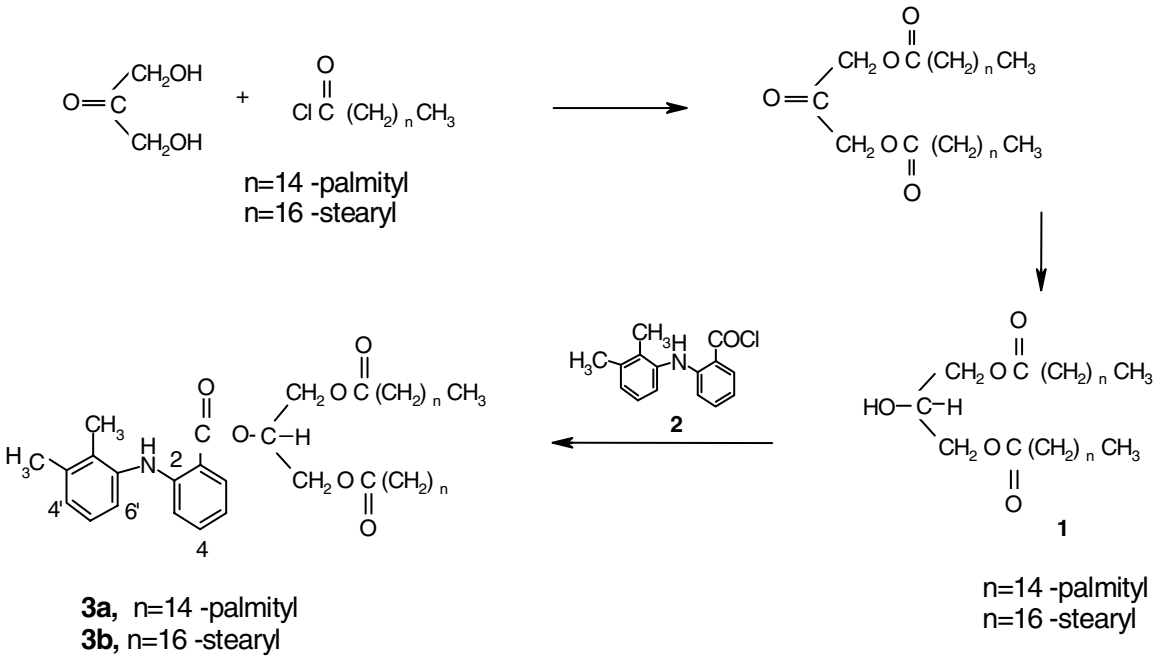
The prodrugs were obtained by the condensation of 2-hydroxy-1,3-bis (alkanoyl) glyceride with the acid chloride of mefenamic acid as shown in the Scheme. The compounds synthesized were evaluated for their antiinflammatory, and analgesic activity and gastrointestinal irritation. An *in vivo* and *in vitro* hydrolysis study in rats and at different pH values using HPLC system was also carried out (Yen Sun et al. 2003).

2.1. Hydrolysis studies

2.1.1. In vitro hydrolysis studies

Hydrolysis kinetics of the synthesised glyceride prodrugs **3a** and **3b** were studied in aqueous buffer solution at pH 7.4 (Fig. 1). Under the experimental conditions the target compounds hydrolyzed to release the parent drug as evidenced by HPLC analysis. At constant pH and temperature the reaction displayed strict Ist order kinetics. The rate

Scheme



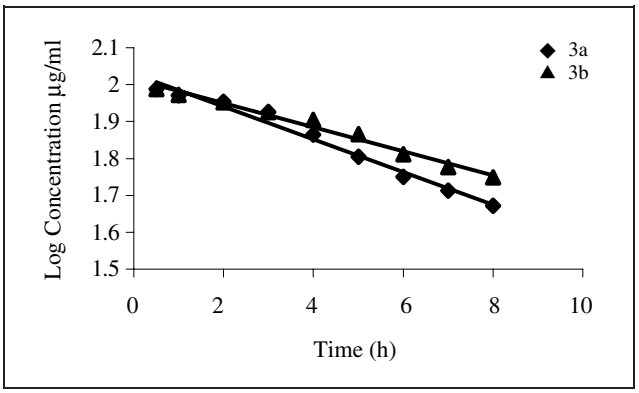


Fig. 1: First order hydrolysis plot of **3a** and **3b** at pH 7.4

Table 1: Pseudo first order rate constants $k_{(obs)}$ and the corresponding $t_{1/2}$ for the hydrolysis of 3a and 3b in buffer solutions at 37 °C

pH	**3a**		**3b**	
	$k_{obs} \times 10^{-4}$	$t_{1/2}$	$k_{obs} \times 10^{-4}$	$t_{1/2}$
3	4.1	28.1	3.5	33
4	1.7	67.94	2.0	48
5	1.6	72.18	2.06	56.06
7.4	1×10^{-3}	11.5	7.4	15.60

pH = 3, 4 and 5 Acetate buffer; pH = 7.4 Phosphate buffer

constant (K_{obs}) and the corresponding half-lives ($t_{1/2}$) for the respective prodrugs were calculated from the linear regression equation correlating the log concentration of the residual prodrug v/s time. The $t_{1/2}$ of **3a** and **3b** at pH 7.4 were found to be 11.5 and 15.6 h suggesting a slow and sustained release in the body and therefore effectiveness for a longer duration (Table 1).

To examine the degradation of the glyceryl prodrugs at pH values corresponding to the stomach, pH 3, 4 and 5

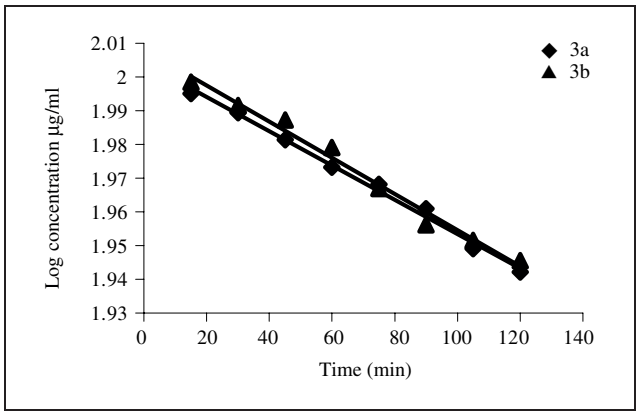


Fig. 2: First order hydrolysis plot of **3a** and **3b** at of pH 3

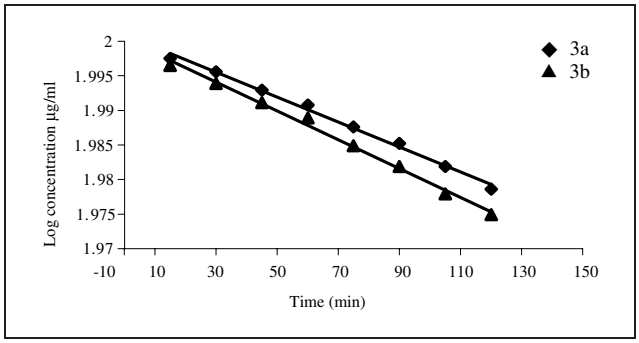


Fig. 3: First order hydrolysis plot of **3a** and **3b** at pH 4

were selected, because the mean fasting stomach pH of adults is approximately 2 and increases to 4–5 following ingestion of food. NSAIDs are not recommended to be taken in the fasting state; consequently pH 3, 4 and 5 were selected to mimic the appropriate clinical range. An assay time of 2 h was selected, after which time stom-

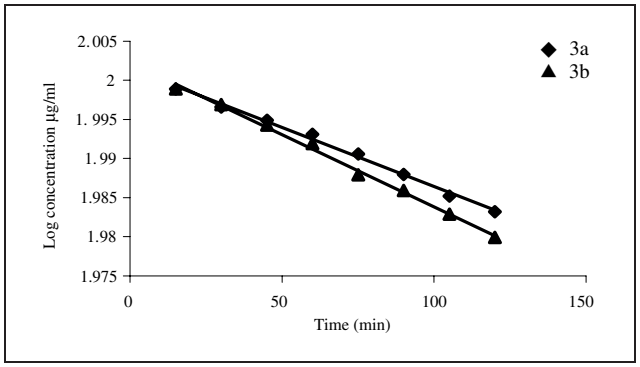


Fig. 4: First order hydrolysis plot of **3a** and **3b** at pH 5

ach emptying would normally be effectively complete (Hunt and Spurrel 1951). Each experiment was carried out in quadruplicates. The findings are shown in (Fig. 2, 3 and 4).

2.1.2. *In vivo hydrolysis studies*

The release of mefenamic acid was also studied in rats. Mefenamic acid, **3a** and **3b** were given orally to the rats at a dose of 20 mg/kg and after different time intervals, the concentration of mefenamic acid was estimated in plasma. A comparison of the plasma concentration-time curves of mefenamic acid indicated that the esters rapidly hydrolyzed to the parent drug. Maximum plasma concentration of the liberated parent drug from the glyceride prodrugs was attained in 2 h compared with 1.5 h for the parent drug treated animals. At all times the concentration of mefenamic acid in the prodrug treated animals was relatively higher compared to the animals receiving the parent drug. Furthermore, 8 h after administration of the prodrugs **3a** and **3b,** the concentration of mefenamic acid was approximately 2 times higher compared to the parent drug treated animals, indicating a sustained release. In conclusion, the *in vivo* evaluation study indicated that glyceride prodrugs of mefenamic acid might be considered as potential biolabile prodrug forms of mefenamic acid (Fig. 5).

2.2. *Ulcerogenic activity*

The ulcerogenic liability of the prepared prodrugs **3a** and **3b** was tested in comparison to the parent drug mefenamic acid following single dose oral administration in rats at three different doses (Cioli et al. 1979). Gross observation of the stomach revealed obvious widespread haemorragic spots in the mefenamic acid treated animals compared to

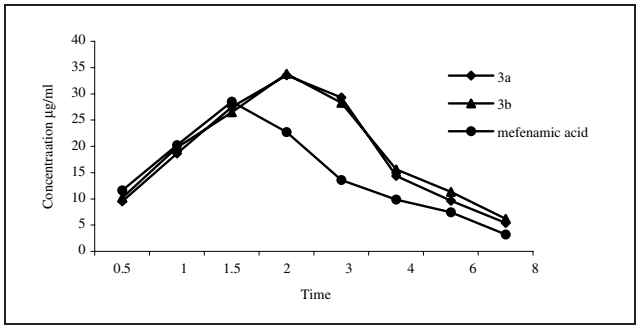


Fig. 5: Plasma concentration of mefenanic acid in rats after the single dose administration of mefenamic acid, **3a** and **3b**

Table 2: Percentage of animals with gastric ulcers and severity index after treating with 3a, 3b and mefenamic acid

Treatment	Dose (mg/kg)	Percentage of animals with ulcers	Severity index
3a	25	10*	0.16
	50	25*	0.55
	100	35*	1.10
3b	25	15*	0.20
	50	28*	0.86
	100	42*	1.22
Mefenamic acid	25	28*	0.75
	50	42*	1.10
	100	100*	2.37

'Severity index' mean score of treated group minus mean score of control, * p value < 0.001 compared to control

the prodrug treated animals. The prodrug treated groups showed intact mucosal layers and were identical to the receiving-group vehicle only. The ulcerogenic dose of the prodrugs was double that of the parent drug. These findings indicate that the prodrugs **3a** and **3b** are significantly less irritating to the gastric mucosa than the parent drug mefenamic acid. The results are given in Table 2.

2.3. *Anti-inflammatory activity*

The anti-inflammatory activity was calculated as percentage inhibition of oedema. The inhibition of swelling in carrageenan-induced oedema in rats (Winter et al. 1962) brought about by oral administration of the drugs is given in Table 3. The percentage of swelling and inhibition was calculated using equations 1 and 2

$$\text{Swelling } (\%) = [(V_t - V_o/V_o] \times 100 \qquad (1)$$

$$\text{Inhibition } (\%) = \{[(V_t - V_o)_{control} - (V_t - V_o)_{treated}]/ (V_t - V_o)_{control}\} \times 100 \qquad (2)$$

(V_t and V_o relates to the average volume in the hind paw of the rats ($n = 6$) before any treatment and after anti-inflammatory agent treatment respectively. All the results were expressed as mean ± SEM. Statistical evaluation was performed using analysis of variance followed by the t-test for sub-group comparison.

The prodrugs showed good inhibition of oedema compared to the parent drug. An inhibition of 47.2 and 48.3% was shown by **3a** and **3b** as compared to 44.55% by mefenamic acid.

2.4. *Analgesic activity*

The analgesic activity was calculated as percentage inhibition of writhing (Seigmund et al. 1957]. Compounds **3a**

Table 3: Percentage of swelling and inhibition caused by 3a, 3b and mefenamic acid in carrageenan induced paw edema in rats 3 h after drug administration

Treatment	Mean paw volume (± SEM)	% Swelling	% Inhibition
3a	0.315 ± 0.06*	44.89	47.2
3b	0.308 ± 0.03*	44.47	48.3
Mefenamic acid	0.341 ± 0.08*	58.4	44.55
Control	0.615 ± 0.040*	90.04	–

* p value < 0.001 compared to control

Table 4: Mean number of writhings and percentage protection by 3a, 3b and mefenamic acid in mice against acetic acid induced writhing

Treatment	Mean No. of writhings (± SEM)	% Protection
3a	9.8 ± 1.39*	58.29
3b	10.2 ± 1.49*	56.59
Mefenamic acid	8.8 ± 1.27*	62.55
Control	23.5 ± 2.13	–

* p value < 0.001 compared to control

and **3b** showed comparable inhibition of in writhing to the mefenamic acid. The percentage inhibition and severity index in mice brought about by administration of the drugs is given in Table 4. The percentage inhibition was calculated using eq. (3).

$$\text{Protection } (\%) = 100 - [\text{no. of writhings with test/no. of writhings with control} \times 100] \quad (3)$$

2.5. Conclusion

In conclusion the glyceride prodrugs **3a** and **3b** containing mefenamic acid were successfully synthesised. The prodrugs released the parent drug mefenamic acid quantitatively at pH 7.4 but were resistant to hydrolysis at pH 3, 4 and 5 indicating that the prodrugs do not break down under acidic conditions. *In vivo* studies in rats showed a prolonged release of mefenamic acid. The plasma concentration of mefenamic acid was high all the time in prodrug treated animals compared with the animals receiving mefenamic acid and a sustained release of mefenamic acid occurred with glyceride prodrugs. Both the prodrugs showed good anti-inflammatory and analgesic activity, comparable to the parent drug, with fewer gastric ulcers. The minimal ulcerogenic dose of the prodrugs was found to be twice as high as that of mefenamic acid and in equimolar quantities the prodrugs were consistently less ulcerogenic than their parent drug mefenamic acid.

3. Experimental

3.1. Materials

Except for dihydroxy acetone, which was purchased from E. Merck KGaA, Germany, all other reagents were obtained from E. Merck (India) Ltd. Mefenamic acid was obtained from Blue Cross Labs Ltd. India. All the solvents used in these studies were dried and distilled before use. Wistar rats of either sex weighing between 150–200 g and Swiss albino mice of either sex weighing between 25–30 g were procured from Animal House, Jamia Hamdard. TLC of the synthesised compounds was carried out in a petroleum hexane : ethylacetate (5 : 1) solvent system. The TLC spots were located by exposure to iodine vapors.

Melting points are uncorrected and were recorded in a liquid paraffin bath using open-end capillaries. ^{1}H NMR spectra were recorded on a Bruker Spectrospin Avance DP × 200, at 300 MHz in $CDCl_3$. Mass spectra were recorded on a JEOL 5 × 102/DA-6000 mass spectrometer and FT-IR spectra were recorded on a Perkin Elmer spectrometer. HPLC analysis of mefenamic acid was recorded on a Shimadzu Model LC-10ATVP (Japan) system containing a quaternary pump, UV detector and c18 reverse phase column.

Dissolution testing was carried out with a SR8 Plus dissolution test apparatus, Hanson Research, USA. Samples were filtered with 0.45 μm Millipore filters and eluted with acetonitrile : acetate buffer (0.1 M, pH 3.5) : methanol (40 : 25 : 35) at 1mL/min. The eluent was monitored at 232 nm by a Shimadzu Model SCL-10AVP, Japan UV detector.

3.2. Chemistry

3.2.1. Synthesis of 1,2,3-trihydroxy-1, 3-dipalmitate and 1,2,3-trihydroxy-1, 3-distearate

These compounds were synthesized by the method of Bently and Mc Crae (1969).

3.2.2. Preparation of acid chloride of mefenamic acid

The acid chloride was prepared by following the general procedure mentioned in Vogel's Textbook of practical organic chemistry (Vogel 1987).

3.2.3. Preparation of glyceride derivative (**3a**)

3a was prepared by dissolving 1,3-dipalmityl glyceride (5.7g, 0.010 mol) in 50 mL of freshly distilled CH_2Cl_2. Dry pyridine (0.95 g, 0.012 mol) and mefenamic acid chloride (2.84 g, 0.011 mol) were added at once, and the reaction mixture was stirred for 40 h at room temperature and treated with 100 mL of water. The aqueous layer was decanted and extracted with 2 × 25 mL of CH_2Cl_2. The CH_2Cl_2 extracts were combined, washed with 1% HCl and water, dried over anhydrous sodium sulphate and evaporated to dryness. The solid mass so obtained was crystallized from petroleum ether, m.p. 75–78 °C, yield 74%, which was TLC pure, R_f 0.71. It was characterized on the basis of ^{1}H NMR, I.R. and mass spectral data.

^{1}H NMR δ (ppm) $CDCl_3$– 0.88 (m, 6 H, 2 × CH_3 of fatty acid), 1.25 (m, 24 × CH_2), 1.62 (m, 4 H, 2 × CH_2 β to CO), 2.16 (s, 3 H, aromatic CH_3), 2.30 (m, 7 H, 2 × CH_2 α to CO + aromatic CH_3), 4.08 (m, 5 H, 2 × CH_2 + CH of glycerol), 6.68 (m, 2 H, H-3, 6), 7.13 (m, 2 H, H-4′, 5′), 7.27 (m, 2 H, H-4, 5), 8.02 (m, 1 H, H-6). I.R. (KBr/ν_{max} cm^{-1}) 2915.05, 2849.39 (CH), 1716.65 (C=O), 1263.23 (CO–O ester), 803.88 (1,2,3-substituted 3 ortho hydrogens). m/z 789 (M^+) molecular ion peak was not observed, 551, 536, 313, 283, 281, 239, 224.

3.2.4. Preparation of glyceride derivative (**3b**)

3b was prepared following the same procedure as for **3a** except that 1,3-distearyl glyceride (5.7 g, 0.010 mol) was used, m.p. 136–138 °C, yield: 64%. It was TLC pure, R_f 0.67 and was characterized on the basis of ^{1}H NMR, I.R. and mass spectral data.

^{1}H NMR δ (ppm) $CDCl_3$– 0.87 (m, 6 H, 2 × CH_3 of fatty acid), 1.25 (m, 28 × CH_2), 1.43 (m, 4 H, 2 × CH_2 β to CO), 2.14, 2.29 (s each, 2 × 3 H, 2 × aromatic CH_3), 2.37 (m, 4 H, 2 × CH_2 α to CO), 4.16 (m, 5 H, 2 × CH_2 + CH of glycerol), 6.73 (m, 2 H, H-3, 6), 7.13 (m, 2 H, H-4′,5′), 7.30 (m, 2 H, H-4,5) 8.02 (m, 1 H, H-6). I.R. (KBr/ν_{max} cm^{-1}) 2916.01, 2848.48 (CH), 1718.04 (C=O), 1265.01 (CO–O ester), 805.08 (1,2,3-substituted 3 ortho hydrogens). m/z 845 (M^+) molecular ion peak was not observed, 607, 567, 341, 311, 309, 267, 224.

3.3. Hydrolysis studies

3.3.1. In vitro hydrolysis studies

The hydrolysis kinetics of prodrugs **3a** and **3b** were studied at pH 7.4, 3, 4 and 5 using acetate and phosphate buffers. The total buffer concentration was 0.02 M and constant ionic strength of 0.5 M for each sample was maintained by adding KCl. The mixture was equilibrated at 37 °C for 1 h. To this mixture 100 mg of each sample was added and the mixture agitated by an overhead stirrer. At selected time intervals of 15, 30, 45, 60, 75, 90, 105 and 120 min, 0.1 mL of the solution was removed and diluted with mobile phase up to 10 mL and 20 μL of this solution was injected for direct analysis by HPLC. At pH 7.4 the samples were withdrawn at time intervals of 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h.

3.3.2. In vivo hydrolysis studies

Rats weighing between 150–200 g were divided into 4 groups of 6 animals each. The rats were used to compare the bioavailability of both the prodrugs with that of mefenamic acid following oral administration. In group I each animal received a dose of 20 mg/kg of mefenamic acid in the form of aqueous suspension in sodium CMC (0.5% w/v), while in groups II and III each animal received the prodrugs in a dose molecularly equivalent to mefenamic acid by following the same technique as in the case of mefenamic acid i.e. as sodium CMC suspension in the same proportions. At appropriate time intervals upto 8 h, blood samples were withdrawn from rats into heparinized tubes and centrifuged for 15 min at 4000 rpm to separate plasma. To 0.05 mL of plasma, 1.5 mL of acetonitrile was added and precipitated proteins were separated by centrifugation at 4000 rpm. The clear supernatant liquid (20 μL) obtained was used directly for analysis. The mean concentration of mefenamic acid at the respective time intervals was taken.

3.4. Ulcerogenicity

Gastrointestinal toxicity was determined by the method of Cioli et al. (1979). The activity was carried out at three different doses viz. 25 mg, 50 mg, and 100 mg. The animals were divided into groups of 6 animals each. Group I served as control and received vehicle only. Groups II, III and IV received mefenamic acid 25, 50 and 100 mg/kg respectively. Other groups were given test compounds **3a** and **3b** in doses molecularly equivalent to 25, 50 and 100 mg/kg of pure mefenamic acid respectively. The animals were fasted for 8 h prior to a single dose of each of the standard and test compounds and sacrificed 17 h later during which time food and

water were available. The gastric mucosa of the rats was examined by means of a 4 × binocular magnifier. The lesions were counted and divided into large (greater than 2 mm in diameter), small (1–2 mm) and punctiform (less than 1 mm)

For each stomach the severity of mucosal damage was assessed according to the following scoring system

0 – no lesions or up to five punctiform lesions
1 – more than five punctiform lesions
2 – one to five small ulcers
3 – more than five small ulcers or one large ulcer
4 – more than one large ulcer

The mean score of each treated group minus the mean score of the control group was considered the 'severity index' of gastric damage.

3.5. Anti-inflammatory activity

The anti-inflammatory activity was evaluated using carrageenan induced oedema of rat paws according to Winter et al. (1962). Rats were divided into 4 groups of 6 animals each. Group I served as the control group, group II received mefenamic acid 20 mg/kg, group III and IV received prodrugs **3a** and **3b** respectively, where the dose was molecularly equivalent to the free drug. Drugs were administered as a homogenous suspension in aqueous solution of sodium CMC (0.5%w/v) orally. Thirty minutes after administration, each rat received in its left hind paw a subplantar injection of 1% carrageenan in normal saline (0.1 mL/rat). The measurement of the hind paw volume was carried out using a Ugo Basile Plethysmometer before any treatment (V_o) and 3 h after (V_t) the administration of the drugs.

3.6. Analgesic activity

Analgesic activity was carried out using the acetic acid induced writhing method in Swiss albino mice (Siegmund et al. 1957). A 1% v/v solution of acetic acid was used to induce writhings. Test compounds were administered orally 3 h prior to acetic acid injection. The number of writhings in 10 min with the control and test compounds were counted and compared. Analgesic activity was measured as percentage decrease in writhing in comparison to control. Mice were divided into 4 groups of 6 animals each. Group I served as a control group without any drug, group II received mefenamic acid 20 mg/kg, and groups III and IV received prodrugs **3a** and **3b** respectively, where the dose was molecularly equivalent to the free drug. Drugs were administered as a homogenous suspension in an aqueous solution of sodium CMC (0.5%w/v) orally. Acetic acid was administered intraperitoneally at 1 mL/100g body weight of the animal.

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