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Research Article

SYNTHESIS, HYDROLYSIS AND PHARMACODYNAMIC PROFILES OF NOVEL PRODRUGS OF MEFENAMIC ACID

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ABSTRACT

Mefenamic acid (MA) suffers from general side effects of NSAIDs, owing to the presence of free carboxylic group. The study was aimed to retard the adverse effects of gastrointestinal origin. Prodrugs of MA were synthesized by amidation with methyl esters of aminoacids like histidine and tryptophan. Purified prodrugs were characterized by m.p., TLC, solubility, protein binding, elemental analysis, UV, ^{1}H-NMR, ^{13}C-NMR, mass and FT-IR. Synthesized prodrugs were also subjected to hydrolysis studies, anti-inflammatory and analgesic activities as well as ulcer index. Marked reduction of ulcer index and comparable anti-inflammatory activity were obtained in both cases as compared to MA. The new prodrugs shows excellent pharmacological response and encouraging hydrolysis rate both in SIF and SIF+ 80% human plasma. These findings suggested that both the prodrugs are better in action as compared to the parent drug and are advantageous in having less gastrointestinal side effects.

Keywords: Anti-inflammatory activity, Mefenamic acid, Amide prodrug, Mutual prodrugs, Pharmacokinetics

INTRODUCTION

Mefenamic acid (MA) is 2-[(2, 3-dimethylphenyl) amino] benzoic acid, belonging to the family of N-aryl anthranilic acid. It is one among the widely used NSAIDs having both anti-inflammatory and analgesic activities. The main side effects of MA include GIT disturbance, peptic ulceration and gastric bleeding. These gastroenteropathies are generally believed to be resulted from the direct contact effect, which can be attributed to the combination of local irritation produced by the free carboxylic group in the molecular structure and by local blockage of prostaglandin biosynthesis in the GI tract. Therefore, the development of new NSAIDs without these side effects has long been awaited. The use of prodrugs to provisionally hide the acidic group of NSAIDs has been proposed as an approach to reduce or suppress the GI toxicity due to the direct contact effect.

Literature reveals that many efforts had made to synthesis prodrugs via masking carboxylic acid group by forming ethyl ester, methyl ester, glycolamide ester and amide prodrug using various aminoacids[1,2,3]. However no attempts were made to develop amide prodrugs of NSAIDs using aminoacid, which has been utilized as a major tool with other NSAIDs. The advantages of using aminoacids for this purpose are owing to their characteristics like normal dietary constituent, non toxic in moderate doses, healing effect on gastric toxicity, marked anti inflammatory activity and site specificity. In this background, the present research aims to synthesize the amide prodrug of MA with methyl esters of histidine and tryptophan, and a study on their various physicochemical characters, hydrolysis kinetics, anti inflammatory activity, analgesic activity and ulcer index as prodrugs.

EXPERIMENTAL

Materials

The aminoacids L-histidine and L-tryptophan were obtained from M/s Hi-Media Ltd., Mumbai, India and drug mefenamic acid was obtained as gift sample from Alkem Laboratories, Mumbai. The other reagents and solvents used were of analytical grade.

The reactions were monitored by TLC on pre coated silica G plates using iodine vapour as detecting agent. The melting points were recorded using melting point determination apparatus by Sigma Instrument, Chennai and are uncorrected. The elemental analysis was performed using Carlo-Erba Model 1108 Analyzer (Italy) and found values are ±0.4% of theoretical values unless otherwise noted. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO on a Bruker DRX 400 Fourier Transform Spectrometer with TMS as internal standard. Chemical shifts are expressed as δ (ppm) values. Mass spectra were recorded on Thermo Finnigan trace DSQ GC-Mass Spectrometer. The hydrolysis data and drug content determination were performed by Elico UV Spectrophotometer.

Synthesis of prodrugs

Mefenamic acid (MA) is 2-[(2, 3-Dimethylphenyl) amino] benzoic acid and the synthesis of prodrugs was carried out by Schotten Baumann technique[4,5].

Step 1: Synthesis of methyl ester hydrochlorides of L-histidine and L-tryptophan: Freshly distilled thionyl chloride (0.05 M) was slowly added to methanol (100 ml) with cooling and amino acid (0.1 M) was added to it. The mixture was refluxed for 6-8 h at 60-70^{o}C with continuous stirring on magnetic stirrer. Excess thionyl chloride and solvent was removed under reduced pressure giving crude amino acid methyl ester hydrochloride. It was titrated with 20 ml portion of cold ether at 0^{o}C until the excess of dimethyl sulphate was removed. The resulting solid product was collected and dried under vacuum. It was re-crystallized from hot methanol by slow addition of 15-20 ml ether followed by cooling at 0^{o}C. The crystals were collected on next day and washed twice with ether methanol mixture (5:1) followed by pure ether and dried under vacuum to give pure amino acid methyl ester hydrochloride.

Step 2: Synthesis of mefenamic acid chloride: Mefenamic acid (0.05 M) was dissolved in minimum amount of chloroform and freshly distilled thionyl chloride (0.05 M) was added slowly to it. The mixture was refluxed for 15 h at 60-70^{o}C with continuous stirring on magnetic stirrer. The viscous liquid was immediately poured on petridish and was vacuum dried to give yellow colour crude mefenamic acid chloride.

Step 3: Synthesis of prodrugs of mefenamic acid with methyl esters of L-histidine and L-tryptophan: Ice cold, aqueous sodium hydroxide solution (5%) was taken in 250 ml beaker and methyl ester of L-histidine and L-tryptophan hydrochlorides (0.05 M) was added to it. The reaction mixture was mechanically stirred for 30 min at room temperature, after which the beaker was transferred to an ice bath kept on mechanical stirrer, maintaining the temperature at 10^{o}C. Mefenamic acid chloride (0.01 M) was added in small portions with continuous stirring for 7-8 h. The solid that separated out was filtered using vacuum pump and dried. The crude prodrug was re-crystallized from methanol.

Chemistry

The amide prodrugs of MA were synthesized with methyl esters of histidine and tryptophan by Schotten-Baumann technique to obtain N-[β-immidazole-3 yl α (methyl propionate)] 2 [(2, 3 dimethyl phenyl amino benzamide)] (MA1) and N-[β-indole-3 yl α (methyl propionate)] 2[(2, 3 dimethyl phenyl amino benzomide)] (MA2) respectively and is illustrated in Scheme

1. The physicochemical properties were determined and shown in Table 1. The yields of prodrugs were good and the structures were established by elemental analysis, ^{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 desired structure.

Table 1: Physicochemical properties of synthesized prodrugs

Prodrug Code	Molecular formula	Mol. wt. calculated	Colour	Melting* point (°C)	% Yield	R_f # value	% Protein binding	Elemental Analysis	
								Calculated (%)	Found (%)
MA1	$C_{22}H_{24}N_4O_3$	392	Yellow	165	64.90	0.54	65.10	C 67.35 H 6.12 N 14.29	67.25 6.15 14.32
MA2	$C_{27}H_{27}N_3O_3$	441	Yellow	198	97.09	0.56	70.15	C 73.47 H 6.12 N 9.52	73.54 6.20 9.85

* Uncorrected # acetone: chloroform:acetic acid: water is 3:2:1:4

MA1: N-[β-immidazole-3 yl α (methyl propionate)] 2 [((2, 6 dichloro phenyl) amino phenyl acetoxy acetamide)]

IR (KBr, cm^{-1}): 3359 (NH str. of amide), 3276 (aromatic CH str.), 2946, 2909 (aliphatic CH str.), 1738 (C-O str. of ester), 1661 (amide I), 1438, 1376 (CH bend, aliphatic), 1269 (C-O str. of ester); **^{1}H NMR (DMSO, δ(ppm)):** 7.28-7.88 (m, 5H, aromatic ring), 6.69 (d, 1H, CH in ring), 5.63 (d, 1H, CH in ring), 3.82 (t, 2H, CH_2 in ring), 2.83-2.96 (m, 2H, CH_2 in ring), 8.83 (br, NH),7.20 (q, 2H, OCH_3), 1.21 (t, 3H, OCH_3), 9.10 (br, NH ring), 7.18-7.24 (m, 3H, ring of imidazole nucleus), 4.79-4.82 (m, 1H); **^{13}C NMR (DMSO, δ(ppm)):** 139.6, 135.3, 139.6, 128.9, 128.9, 123, 17.3, 19.6, 127, 127.1, 128.4, 118.6, 127, 125.7, 171.2, 17.8, 51.9, 64.2, 139.6, 128.9, 126.3, 35.2; **Mass (m/z):** 392 (M+).

MA2: N-[β-indole-3 yl α (methyl propionate)] 2 [(2, 3 dimethyl phenyl amino benzomide)]

IR (KBr, cm^{-1}): 3423 (NH str. of amide), 3052 (aromatic CH str.), 2921, 2892 (aliphatic CH str.), 1727 (C-O str. of ester), 1655 (amide I), 1450, 1380 (CH bend, aliphatic), 1260 (C-O str. of ester); **^{1}H NMR (DMSO, δ(ppm)):** 7.28-7.81 (m, 5H, aromatic ring), 6.55 (d, 1H, CH in ring), 5.61 (d, 1H, CH in ring), 3.73 (t, 2H, CH_2 in ring), 2.94-2.98 (m, 2H, in CH_2 in ring), 8.76 (br, NH),4.13 (q, 2H, OCH_3), 1.27 (t, 3H, OC H_3) 7.18-7.24 (m, 4H, aromatic ring of indole nucleus), 4.79-4.82 (m, 1H, $COCH_3$); **^{13}C NMR (DMSO, δ(ppm)):** 135.3, 139.6, 128.9, 120, 128.9, 17.3, 17.8, 139.6, 127, 127.8, 128.4, 118.6, 127, 125.2, 171.2, 63.2, 172.9, 51.9, 35.2, 139.6, 128.9, 120.6, 128,139,135.6, 134.4, 128.6; **Mass (m/z):** 441 (M+).

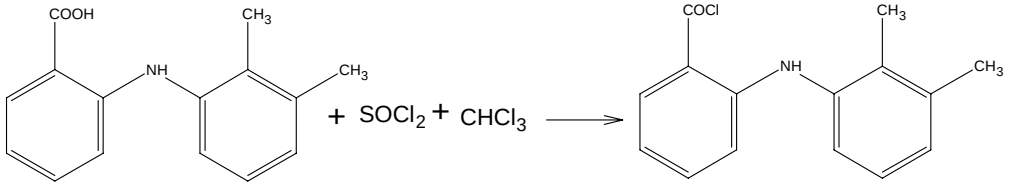
Solubility

Approximately 5 mg of prodrug was dissolved in 5 ml of each solvent at 37 ±1°C in glass test tubes. The solvents used were 0.1 N NaOH, 0.1 N HCl, ethanol, methanol, ether, ethyl acetate, chloroform, acetone, DMF and water. Test tubes were gently shaken and solubility was observed. In case of any

observed insoluble fractions, the known amount of solvent was further added to ascertain the solubility of the compound.

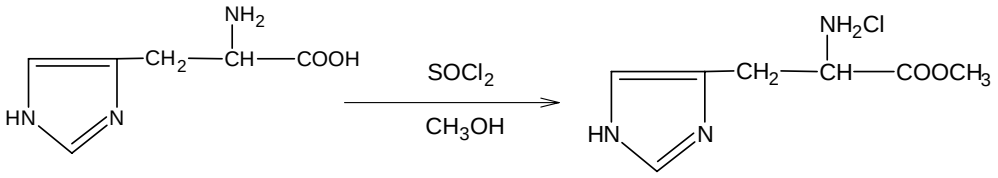
Scheme 1: Structures of newly synthesized compounds of MA1 and MA2.

Step I: Synthesis of mefenamic acid chloride



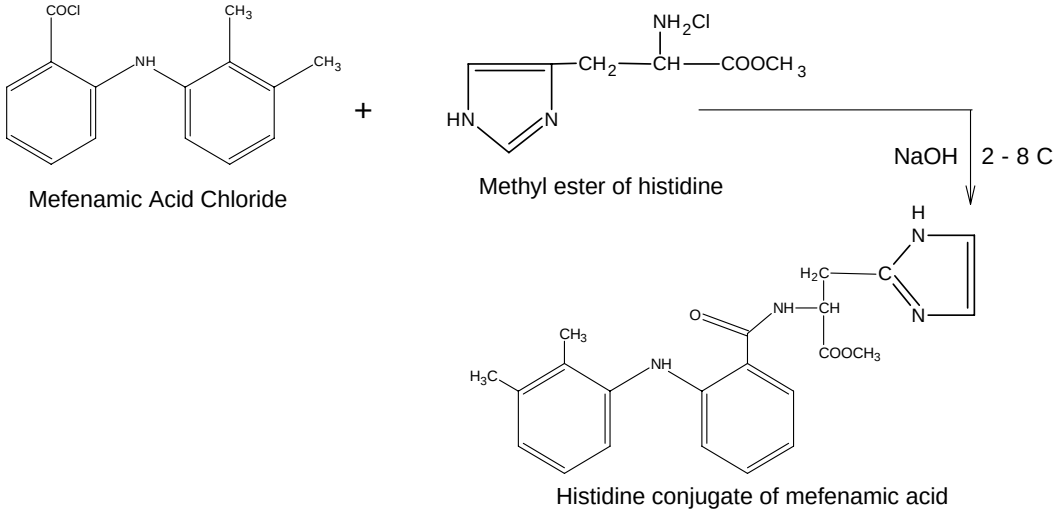
Mefenamic Acid — Mefenamic Acid Chloride

Step II: Synthesis of methyl ester of histidine



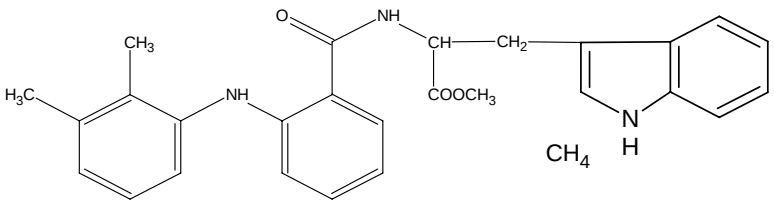
Histidine — Methyl ester of histidine

Step III: Synthesis of prodrug: Conjugation of mefenamic acid chloride with methyl ester of histidine



Mefenamic Acid Chloride — Methyl ester of histidine

Histidine conjugate of mefenamic acid



Tryptophan conjugate of mefenamic acid

Protein binding studies[6]

A solution of synthesized prodrug (10 mg/ml) was made in phosphate buffered saline (PBS, pH 7.4). A 100 ml of this solution was taken in a beaker. The prepared membrane was first washed with distilled water and then with buffer solution (pH 7.4). It was tied at the opening end of dialysis tube; the dialysis tube containing (6%) egg albumin was dipped into the drug solution and covered. The whole assembly was placed on a magnetic stirrer and switched at low revolutions per minute. The temperature was maintained at 37±0.5°C. After every 1 h, 1 ml of the PBS containing drug solution was replaced with fresh 1 ml of PBS. Withdrawn sample was diluted further with 1 ml phosphate buffer and the concentration of the prodrug was estimated using spectrophotometer at 230 nm.

***In vitro* hydrolysis studies**

In vitro hydrolysis studies of synthesized prodrugs MA1 and MA2 were carried out in simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4) and SIF+80% human plasma (pH 7.4)[7,8]. A solution of 10 mg of prodrug 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 and kept in test tubes maintained at 37±0.5°C. At a definite interval of time (0.5 h, 1-8 h) an aliquot was withdrawn from different test tubes and was transferred to micro centrifuge tubes. Free mefenamic acid which was supposed to be released after the hydrolysis of prodrugs extracted with two 5 ml portion of methanol. The methanol layer was estimated on UV spectrophotometer for the amount of free MA released after hydrolysis of prodrugs in SGF, SIF, SIF+80% human plasma. The kinetics of hydrolysis was monitored by 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.

Anti-inflammatory activity

The anti-inflammatory activity was evaluated using carrageenan-induced oedema of rat paw[9]. Albino rats (100-200g) were divided into four groups of six animals each. Group 1 served as control group, group II received mefenamic acid 20 mg/kg, group III and IV received prodrug MA1 and MA2 respectively, where the dose was molecularly equivalent to the free drug. The initial volume of right hind paw of albino rat was measured by plethysmometer without administration of drug. The drug was administered orally in 1% suspension of sodium CMC. After 30 min of drug administration, carrageenen (0.1 ml, 1% w/v solution in normal saline) was injected into the planter surface of right hind paw of each animal as phlogistic agent. The volume of right hind paw of albino rats was measured after 2, 4 and 6 h. The mean difference in the volume of the right hind paw of rats was compared with control and standard. Percent anti-inflammatory activity is calculated by

$$\% \text{ inhibition} = (1-Vt/Vc) \times 100$$

where Vc – mean relative change in paw edema volume in control group and Vt - mean relative change in paw edema volume in test group. 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.

Analgesic activity

The analgesic activity of synthesized prodrugs was determined by thermal stimulus using tail flick method[10, 11]. Analgesiometer was used for the determination of pain threshold of albino rats. Cold water was circulated through the water jackets of the instrument to avoid heating of the area around the hot wire. Rats (100-200g) were divided into four groups, each comprising of six rats. The rat was placed in a holder through which the tail of the rat was protruded out. The reaction time was recorded at 30min, 1, 2, 3 and 4h after the treatment and cut-off time was 9 sec. The normal reaction time, i.e. the time taken to flick the tail was noted. Animals showing delayed response were rejected. The prodrug (dose of each prodrug was equivalent to 20 mg/kg body weight) was administered orally in 1% suspension of sodium CMC. The percentage of analgesic activity is calculated as.

$$\% \text{ Analgesic activity} = [(T_2-T_1)/(Tc-T_1)] \times 100$$

where T_1 - the reaction time (s) before administration of prodrug and T_2 -the reaction time (s) after administration of prodrug and Tc - cutoff time in sec.

Ulcerogenic activity

Gastrointestinal toxicity of the synthesized prodrugs was measured and compared with the drug by measuring ulcer index[12, 13]. For the purpose, male albino rats were selected, weighing between 130-150 g, the rats were divided into four groups each comprising of six rats, including a control and a standard group. The prodrug was suspended in 10 ml of 2% w/v suspension of acacia. Measured volume of the suspension containing dose equivalent to 20 mg/kg of body weight of MA was administered orally to the test group daily for 5 days. The rats were fasted after the administration of last dose, thereafter they were sacrificed by decapitation and the stomach was removed, opened and washed with distilled water. The lesions on the gastric mucosa were counted by visual examination using a binocular magnifier. Ulcers greater than 0.5 mm were recorded. The ulcer index was calculated by severity of gastric mucosal lesions which are graded as grade1 = less than1mm erosions, grade 2 = 1-2mm erosions and grade 3 = More than 2mm erosions. The UI was calculated as

UI= [1× (number of lesions of grade 1) + 2× (number of lesions of grade 2) + 3× (number of lesions of grade 3)]/10.

RESULTS AND DISCUSSION

Physicochemical properties and hydrolytic studies

The synthesized prodrugs of MA were subjected to solubility, physicochemical characterization and hydrolytic studies. The greater solubility of the standard drug MA is mainly due to the presence of the free carboxylic acid, which forms sodium salt and makes the compound ionic. The prodrugs showed moderate to high solubility, compared to MA, in various solvents of chloroform, ethanol and acetone indicating their lipophillic behaviour. The comparative patterns of hydrolysis of these prodrugs in SIF and SIF+80% human plasma (pH 7.4) are shown in Figs 1 and 2 respectively. The amount of MA regenerated on hydrolysis (in SIF, pH 7.4) of MA1 and MA2 was found as 81 and 76.5 % respectively and that in SIF+80% human plasma was found as 94 and 96

% respectively. The prodrugs showed no hydrolysis in SGF, satisfactory hydrolysis in SIF and the encouraging hydrolysis rate in SIF+80% human plasma due to the presence of amidase in plasma. The results of the hydrolytic kinetics study revealed that both MA1 and MA2 followed first order kinetics and is shown in Fig. 3. The protein binding of prodrugs MA1 and MA2 was found as 65.10 and 70.15% respectively while that of the standard drug was 89.10 %. This increases the availability of prodrugs for hydrolysis in plasma and the required dose will be less.

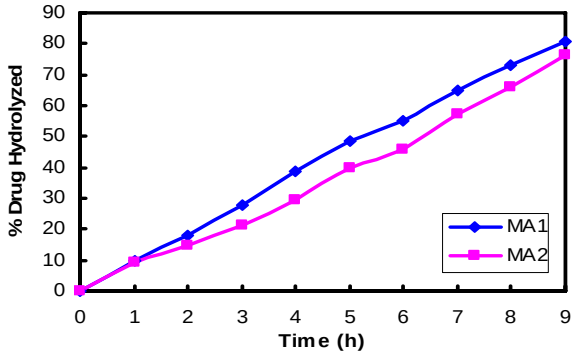


Fig. 1: Comparative pattern of hydrolysis of MA1 and MA2 in SIF.

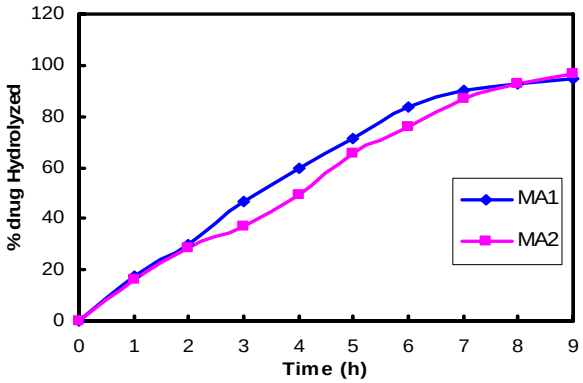


Fig. 2: Comparative pattern of hydrolysis of MA1 and MA2 in SIF and 80%plasma

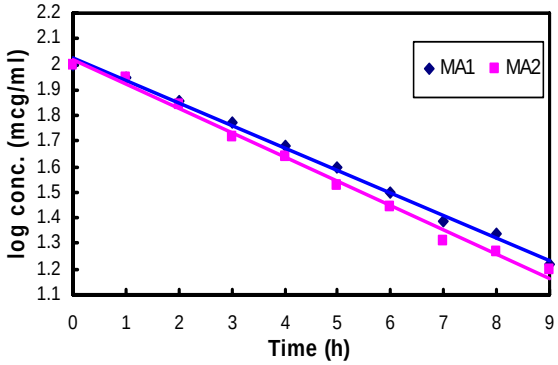


Fig. 3: First order hydrolysis plot of MA1 and MA2

Pharmacological evaluation

The anti-inflammatory activity was calculated as percentage inhibition of oedema. The inhibition of swelling in carrageenan-induced oedema in rats bought about by oral administration of drugs is shown in fig. 4. After 6 hrs of administration of drug, the prodrugs MA1 and MA2 showed good inhibition of oedema of 70 and 62% respectively as compared to 40% by MA.

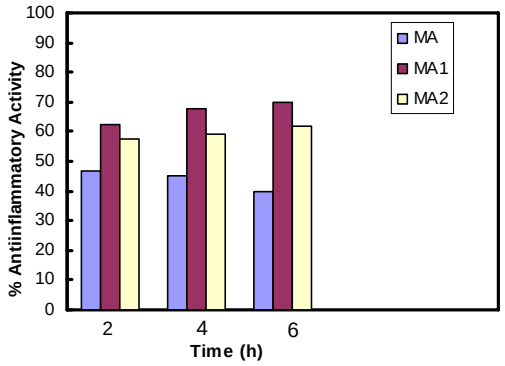


Fig 4: Comparative anti-inflammatory activity of MA, MA1 and MA2.

The analgesic activity was determined by tail flick method and the result was shown in Fig. 5. The prodrugs MA1 and MA2 showed decreased analgesic activity of 61 and 58% respectively while MA showed 74% after 4 hrs of administration of drug. The results thus confirm that the prodrugs showed insignificant analgesic activity when compared with mefenamic acid.

Ulcerogenic liability of synthesized prodrug MA1 and MA2 was tested in comparison to the parent drug mefenamic acid following single dose oral administration in rats at 3 different doses and the results are shown in Fig 6. Gross observation of the stomach revealed obvious wide spread hemorrhagic spots in the mefenamic acid treated animals compared to the prodrug treated animals. The prodrug treated group showed intact mucosal layers and were identical to the receiving groups vehicle only. The ulcerogenic dose of the prodrug was double that of the parent drug. These findings indicate that the prodrugs MA1 and MA2 are significantly less irritating to the gastric mucosa than mefenamic acid.

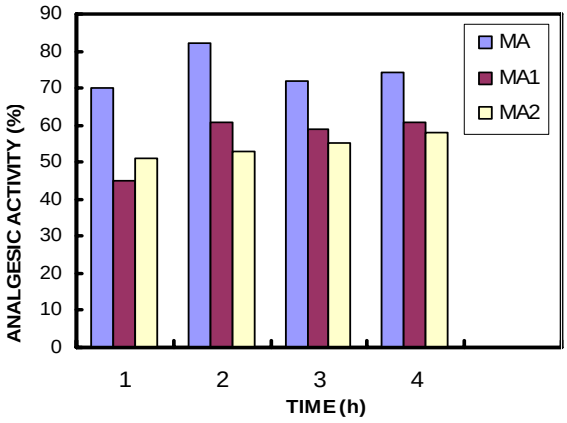


Fig 5: Comparative analgesic activity of MA, MA1 and MA2.

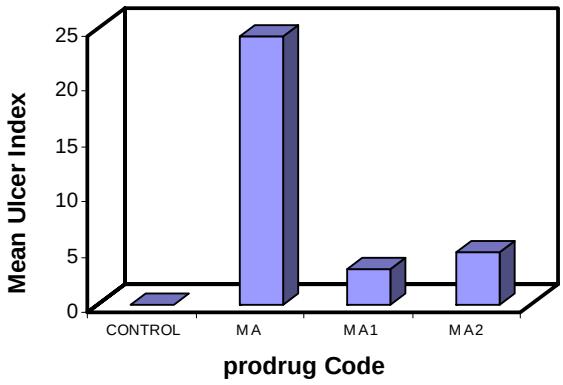


Fig 6: Comparative ulcer index of MA, MA1 and MA2.

CONCLUSIONS

The histidine and tryptophan containing mefenamic acid amide prodrugs were successfully synthesized and the structures were confirmed based on their spectral analysis. Both MA1 and MA2 showed excellent pharmacological

response and encouraging hydrolysis rate were attained in SIF+80% of human plasma. The less protein binding of the prodrugs increased its availability for hydrolysis in plasma and thus results in less dose requirement. Increased anti-inflammatory and reduction in ulcer index of the prodrugs were observed when compared to the parent drug. On the basis of the above observations, it is concluded that these prodrugs can be successfully applied in attaining the goal of minimized gastro intestinal toxicity without loss of desired anti-inflammatory of the drug.

Animal experiments

All animal experiments were carried out according to the guidelines of the Committee for the Purpose of Control of Experiments on Animals (Reg. No. 930/a/06/CPCSEA) and approval of the Institutional Animal Ethics Committee was obtained.

Statistical analysis

Experimental data are expressed as mean ± SD. Student's t test was applied for expressing the significance and P value less than 0.05 was considered as significant

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