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Synthesis, hydrolysis studies and pharmacodynamic profile of novel colon-specific mutual prodrug of Aceclofenac with amino acids

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Abstract

The colon specific drug delivery of non steroidal anti-inflammatory drugs involves targeting the drug to the colon, thereby lowering the required dose, reducing the systemic side effects, and thus resulting in a more effective therapy system. The gastric side effect of aceclofenac is due to the presence of free carboxylic group. The present study aimed at reducing the side effects of aceclofenac by masking the carboxylic acid group with methyl esters of amino acids through the formation of amide linkage. The amino acids like histidine, alanine, tyrosine and glycine were chosen as promoiety because they had broad spectrum of anti-inflammatory, cytoprotective and immunomodulatory properties and therefore would synergize the effect of prodrug. The structures of synthesized prodrugs were confirmed by elemental analysis, IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy. *In vitro* reconversion of prodrugs carried out in simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and simulated colonic fluid (SCF) showed that the prodrugs remained intact in SGF and SIF, except SCF. In SCF, the rat fecal content containing colonic enzyme (amidase) hydrolyzed the amide linkage of synthesized prodrugs and free aceclofenac was released Marked reduction of ulcer index and an increase in anti-inflammatory activities were observed for the prodrugs and proved to be better in action in the colon as compared to the parent drug.

Keywords: NSAIDs, aceclofenac, amide prodrug, mutual prodrugs, pharmacokinetics, ulcerogenicity

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Introduction

Colon specific drug delivery is a new approach in the treatment of chronic disorders like inflammatory bowel disease, infectious diseases and colon cancer. Colon is gaining the importance for systemic absorption of protein and peptide drugs because of less intimidating environment prevailing in colon compared with stomach and small intestine. Colon is also found to be a promising site when delay in the absorption is desirable from therapeutic point of view for the treatment of rheumatoid arthritis, nocturnal asthma and angina pectoris. Because of this unique physiological characteristics of colon, drug delivery can be achieved in different ways, including pH dependent approaches utilizing the changes in pH along the GI tract [1], coated dosage forms [2], time-controlled release systems [3], pressure-controlled colon delivery systems [4], coating drugs with bacterially degradable polymers [5] and prodrug based drug delivery system [6].

Among the number of NSAIDs available for colon specific drug delivery systems, Aceclofenac (AC), a newer derivative of diclofenac, is considered as one of the emerging NSAIDs [7, 8]. However, the problems after long term administration of these drugs, such as gastric irritation and ulceration of the GI mucosa, are 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 [9-11]. The use of prodrugs to provisionally hide the acidic group of NSAIDs by amino acids has been proposed as an approach to reduce these side effects. A drug with free carboxylic acid group can be derivitized into corresponding esters or amides of amino acids, so as to alter the physical properties of a parent drug with one or more of hydrolase enzyme serving as the in vivo reconversion sites. In addition, amino acids are non toxic in moderate doses, have healing effect on gastric toxicity, possess site specificity and shows marked anti inflammatory activity against carrageen induced hind paw edema in rats. In the present study, amino acids like histidine, alanine, tyrosine and glycine were evaluated as carriers for colon specific drug delivery, with the expectation to get non toxic prodrugs with minimized GIT disturbance while maintaining the useful anti-inflammatory activity as well as ulcerogenicity. Various microfloral enzymes (amidase) present in the rat fecal content helps in the release of AC by hydrolysis of amide linkage, without producing any xenobiotic substance within GIT. The study involves the synthesis of various methyl ester derivatives of amino acids with AC and to carry out a study on the physicochemical characteristics, hydrolysis kinetics, pharmacological activities and histopathology to find out its suitability for colon specific drug delivery.

Results and Discussion

The synthesized prodrugs of AC were confirmed by IR, ^{1}H NMR, ^{13}C NMR and mass spectral methods. Moderate to high solubility in various organic solvents, were observed for the prodrugs, indicating lipophillic behaviour. The hydrolytic studies of the prodrugs were carried out in simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and simulated colonic fluid (SCF). The half life of prodrugs AC1, AC2, AC3 and AC4 in SCF was observed as 4.5 h, 4.35 h, 4.25 h and 4.6 h respectively. The amount of AC regenerated on hydrolysis of AC1, AC2, AC3

and AC4 in SIF was 70, 67, 74 and 72 % respectively whereas 92, 95, 94 and 97 % of AC was regenerated respectively on hydrolysis of prodrugs in SCF. None of the prodrugs showed significant hydrolysis in SGF. Satisfactory hydrolysis in SIF as well as very interesting hydrolysis in SCF was observed and the latter is due to the presence of colonic enzyme (amidase) in fecal matter. The study revealed that the colonic micro flora produces a variety of enzymes, including azoreductase, various glycosidases and amidases, which were not present in the stomach or small intestine. Therefore, enzyme dependent drug release, which relies on the existence of enzyme-producing microorganisms in the colon, could be used to deliver drug to the colon after enzymatic cleavage of degradable carrier bonds and premature drug release does not occur in this case. The hydrolytic kinetics study revealed that all the prodrugs followed first-order kinetics. The prodrugs showed comparatively very low protein binding in comparison to standard drug whose value is 75 %. This increases the availability of prodrugs for hydrolysis in colonic fluid and the required dose will be less.

Pharmacological investigations of the synthesized prodrugs were done for anti-inflammatory activity and ulcerogenicity. The dose of prodrug administered was equivalent to 10 mg/kg body mass of the parent drug. The anti-inflammatory activities obtained after 2 h and 6 h of administration of standard drug AC were found as 62 and 45 %. The anti-inflammatory activity after 2 h of administration of prodrugs AC1, AC2, AC3 and AC4 was 58, 64, 65 and 58 % respectively while after 6 h of administration 67, 75, 78 and 73 % respectively were observed. The maximum anti-inflammatory activity of all the prodrugs were observed at 6 h and remained practically constant up to 8 h. It was observed that the anti-inflammatory activity of free AC decreased with time while that of its prodrugs increased with time. The latter is due to their higher bioavailability compared to the parent drug. The statistical significance testing using one way analysis of variance of data showed that the anti-inflammatory activity of the prodrugs were significant in comparison with AC.

The ulcer index of the synthesized prodrugs was recorded to observe the extent of gastrointestinal side effects. The mean ulcer index of standard drug AC was found as 28. The ulcer index of the prodrugs was found much lesser in comparison with the standard drug. The minimized side effect obtained in the prodrug might be due to the inhibition of direct contact of carboxylic acid group of the drug to the gastric mucosa which is mainly responsible for the damage.

A normal histological finding was observed for the samples of the control group rats. Small hemorrhagic areas and patches of inflammatory cell infiltrations were present in the lumen of the glands and lamina propria when treated with parent drug, but normal histological findings were displayed for all the prodrugs. This reveals that the prodrugs are not producing any ulceration in the gastric region.

Materials and Methods

The amino acids namely histidine, alanine, tyrosine and glycine were obtained from M/s Hi-Media Ltd., India and drug AC was obtained as gift sample from Alkem Laboratories, India. 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, India and are uncorrected. The elemental analysis was performed using Carlo-Erba Model 1108 Analyzer (Italy). ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO on NMR spectrophotometer (Bruker DRX 300, USA). Chemical shifts are expressed as δ (ppm) values. Mass spectra were recorded on mass spectrophotometer (Jeol SX-102 (FAB), Japan). The hydrolysis data and drug content determination were performed by Elico UV Spectrophotometer, India.

Synthesis of aceclofenac prodrugs

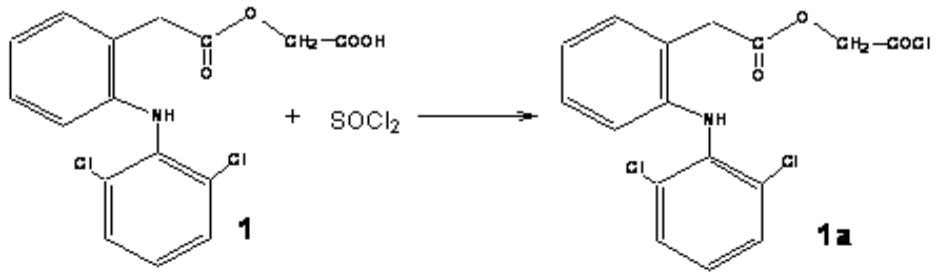
Aceclofenac is 2-[2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxyacetic acid and the synthesis of prodrugs was carried out by Schotten Baumann method [12].

Step 1: Synthesis of aceclofenac acid chloride: Aceclofenac (**1**) of 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 coloured crude aceclofenac acid chloride (**1a**).

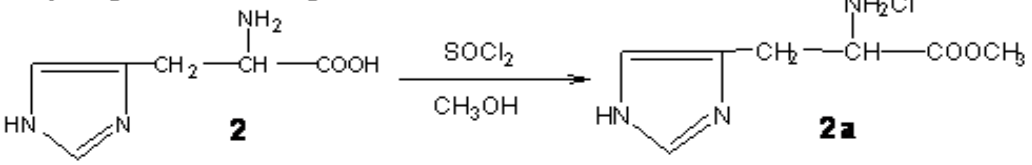
Step 2: Synthesis of methyl ester hydrochlorides of histidine, alanine, tyrosine and glycine: Freshly distilled thionyl chloride (0.05 M) was slowly added to methanol (100 ml) with cooling and histidine (**2**) (0.1 M) was added to it. The mixture was refluxed for 6-8 h at 60-70 ^{o}C with continuous 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 at 5:1 ratio followed by pure ether and dried under vacuum to get pure histidine methyl ester hydrochloride (**2a**). The same procedure was followed to synthesize methyl ester hydrochlorides of alanine, tyrosine and glycine respectively.

Step 3: Synthesis of prodrugs of aceclofenac with methyl esters of histidine, alanine, tyrosine and glycine: Ice cold, aqueous sodium hydroxide solution (5 %) was taken in 250 ml beaker and methyl ester of histidine hydrochloride (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. Aceclofenac acid chloride (0.01 M) was added in small portions with continuous stirring for 7-8 h. The solid that separated out was filtered off. The crude prodrug was re-crystallized from methanol. The schematic representation of synthesize of histidine conjugated aceclofenac (AC1) is shown in Scheme 1. The same method was adopted to synthesize alanine, tyrosine and glycine derivatives of AC and the structures of alanine conjugated aceclofenac (AC2), tyrosine conjugated aceclofenac (AC3) and glycine conjugated aceclofenac (AC4) are shown in Scheme 2.

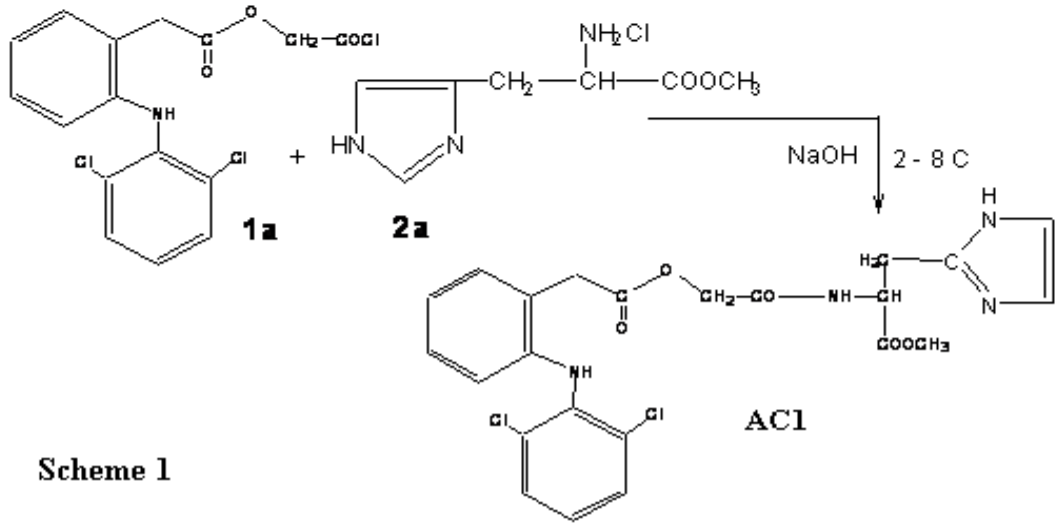
Step I: Synthesis of aceclofenac acid chloride



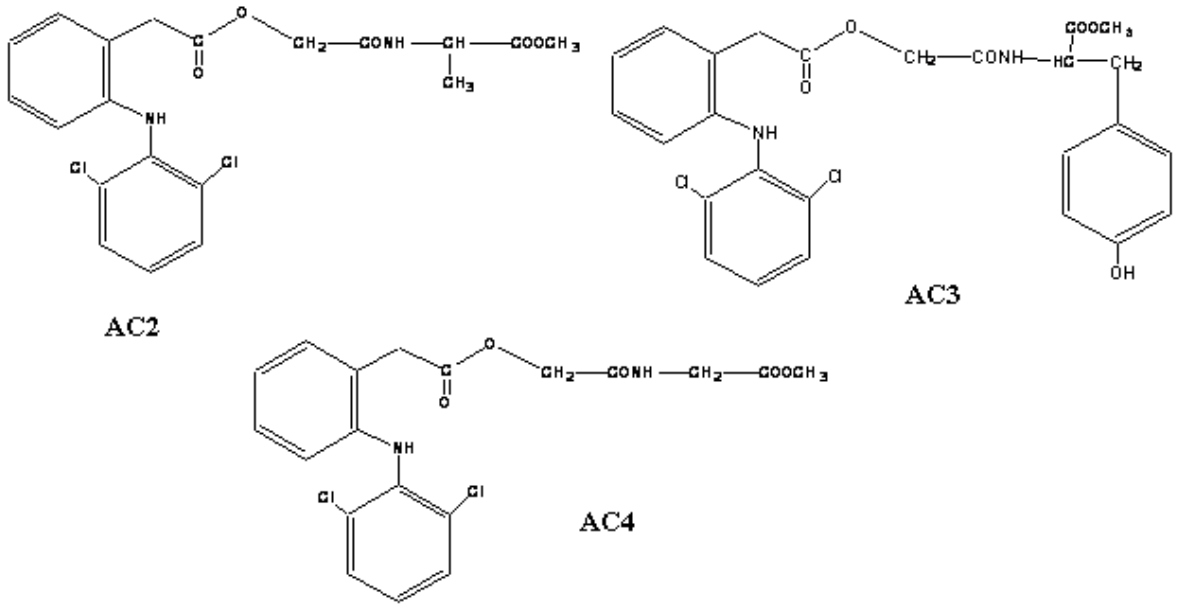
Step II: Synthesis of methyl ester of histidine



Step III: Synthesis of prodrug: conjugation of aceclofenac acid chloride with methyl ester of histidine



Scheme 1



Characterization of the synthesized prodrugs

The purity of the synthesized compounds was determined by TLC and the physicochemical parameters are given in Table 1. The structures were confirmed by elemental analysis, ^{1}H-NMR, ^{13}C-NMR, mass and IR spectral methods and are shown in Table 2. The results of elemental analysis of synthesized prodrugs were in all cases within ± 0.4 % of the theoretical values.

Table 1: Physicochemical properties of synthesized prodrugs of Aceclofenac

Prodrug	Molecular formula	Molecular weight	Colour	Melting* point (^{o}C)	Yield (%)	R_f # value	Protein binding (%)	Elemental Analysis		
									Calculated (%)	Found (%)
AC1	$C_{23}H_{22}Cl_2N_4O_5$	504.65	Brown	191-192	65	0.56	64.0	C	54.96	55.11
								H	4.37	4.53
								N	13.88	13.98
AC2	$C_{20}H_{20}Cl_2N_2O_5$	348.23	Brown	195-196	97	0.55	71.65	C	53.79	53.46
								H	4.59	4.45
								N	15.98	15.67
AC3	$C_{26}H_{24}Cl_2N_2O_5$	514.19	Brown	194-196	96	0.55	68.05	C	60.70	60.54
								H	4.66	4.65
								N	13.61	13.36
AC4	$C_{19}H_{18}Cl_2N_2O_5$	424.08	Brown	196-197	45	0.57	72.0	C	53.77	53.99
								H	4.25	4.65
								N	16.51	16.80

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

Protein binding studies [13]: 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 cellophane membrane (molecular weight cut off in the range of 10000-12000 Da obtained from Hi-Media, India) 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 ^{o}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 AC was estimated using spectrophotometer at 230 nm.

Table 2: Spectral data of synthesized prodrugs

Prodrug	Mass (m/z)	IR (KBr, cm^{-1})	^{1}H NMR (δ, ppm) (DMSO)	^{13}C NMR (δ, ppm) (DMSO)
AC1 N-[β-immidazole-3 yl α (methyl propionate)] 2 [((2, 6 dichloro phenyl) amino phenyl acetoxy acetamide)]	504 (M+)	3395 (NH), 2860 (CH str.), 1765 (C=O), 1580 (amide), 1270 (C-O ester)	9.77 (1H, NH), 8.32 (4 H, ArH), 7.28 (1H, NH), 4.97 (2H, CH=CH), 3.72 (2H, OCH_3), 3.68 (2H, CH_2CH_3), 2.35 (1H, CH in ring)	35.2, 36.2, 47.8, 51.9, 63.2, 63.2, 118.6, 120.0, 125.2, 127.0, 127.1, 127.1, 127.3, 128.4, 128.4, 128.9, 128.9, 135.3, 139.6, 139.6, 166.4, 169.2, 172.1
AC2 N-(methyl propionate) 2 [((2, 6 dichloro phenyl) amino phenyl acetoxy acetamide)]	348 (M+)	3365 (NH), 1780 (C=O), 1640 (amide), 1560 (amide II), 1490 (C-H), 1255 (C-O ester)	8.32 (4 H, ArH), 7.28 (1H, NH), 6.69-6.98 (2H,CH=CH), 4.97 (2H, CH=CH), 3.68 (2H, OCH_3), 4.35 (2H, CH_2CH_3), 3.72 (2H, $COOCH_2$), 1.48 (1H, CH in ring)	17.3, 35.2, 47.8, 51.9, 63.2, 118.6, 120.0, 125.2, 127.0, 127.4, 127.6, 128.4, 128.9, 135.3, 139.6, 139.6, 128.9, 168.6, 171.2, 172.9
AC3 N-[β-(para hydroxy phenyl propionate)]-2 [(2,6 dichloro phenyl) amino phenyl acetoxy acetamide)]	514 (M+)	3432 (NH), 3080 (CH str.), 1729 (C=O), 1610 (amide I), 1494 (amide II), 1179 (C-O ester)	9.11(1H, NH) , 8.33 (4H, ArH), 7.1(1H, NH), 6.51 (2H, CH=CH), 4.43 (2H, OCH3), 3.96 (2H, CH2CH3), 2.51 (1H, CH in ring), 1.23 (1H, CH in ring)	139.6, 128.9, 120.0, 128.9, 139.6, 135.3, 127.0, 127.1, 128.4, 118.6, 127.0, 125.3, 35.3, 171.2, 63.2, 168.6, 47.8, 172.9, 51.9, 47.8, 139.6, 135.3, 139.6, 126.5, 128.9, 129.1
AC4 N-[methyl acetate]-2 [(2,6 dichloro phenyl) amino phenyl acetoxy acetamide)]	424 (M+)	3334 (NH), 3070 (CH str.), 1725 (C=O), 1611(amide I), 1452 (amide II), 1311 (C-O ester)	9.77 (1H, NH), 8.32 (4H, ArH), 7.73 (1H, NH), 6.77 (2H,CH=CH), 3.51 (2H, OCH_3), 3.89, (2H, $COOCH_3$), 2.51(1H, CH in ring), 1.23 (1H, CH in ring)	134.5, 136.6, 127.6, 123.4, 125.3, 140.6, 128.2, 126.1, 126.1, 114.6, 127.3, 125.2, 35.2, 171.2, 63.2, 168.6, 47.8, 172.9, 51.9

Hydrolysis rate determination of synthesized prodrugs [14]: In vitro hydrolysis studies of synthesized prodrugs were carried out in SGF, SIF and SCF. A solution of 10 mg of prodrug was prepared in 90 ml of SIF or SCF. An aliquot of 15 ml of this solution was withdrawn repeatedly and kept in test tubes maintained at 37 ± 0.5 ^{o}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 followed by addition of methanol to make up the volume. The tubes were placed in freezing mixture in order to arrest further hydrolysis, followed by vortexing at high speed for 5 min. After vortexing, the tubes were centrifuged at high speed (3000 rpm) for 5 min. A 5 mL of clear

supernatant obtained from each tube was measured on UV spectrophotometer for the amount of free aceclofenac released after the hydrolysis of prodrugs in SGF, SIF and SCF at 230 nm. The kinetics of hydrolysis was monitored by increase of free drug concentration with time and order of reaction and half life 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. The comparative hydrolysis pattern of AC1, AC2, AC3 and AC4 in SIF and in SCF are shown in Fig. 1. The first order hydrolysis plot of AC1, AC2, AC3 and AC4 in SCF are shown in Fig. 2.

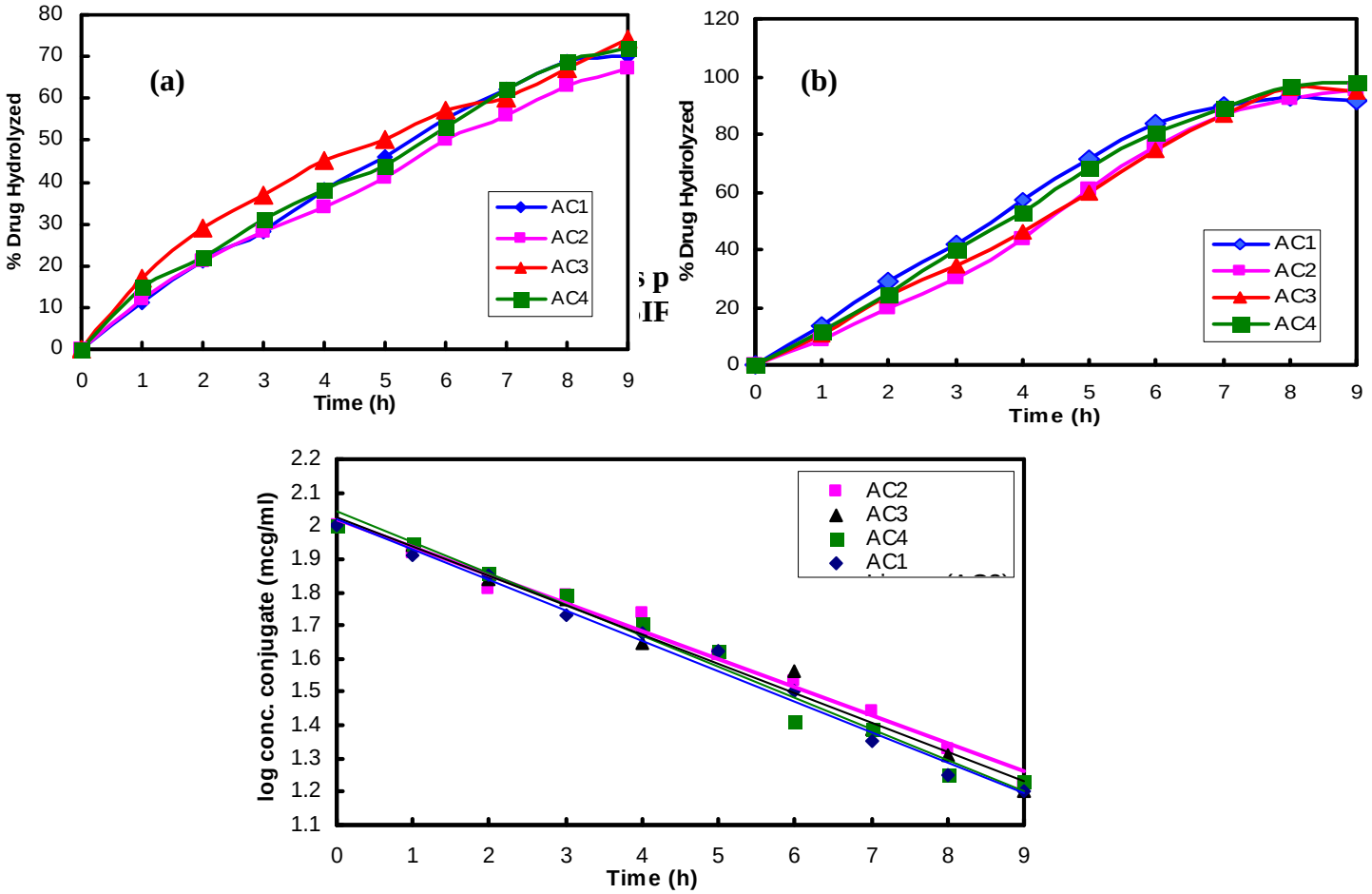


Fig. 2: First order hydrolysis plot of AC1, AC2, AC3 and AC4 in SCF

Pharmacological evaluations

Aceclofenac as well as the synthesized prodrugs were evaluated for anti-inflammatory activity, ulcerogenicity, histopathology and a comparison study with AC was performed. Test compounds and standard drugs were administered in the form of a suspension (1 % carboxymethylcellulose

as a vehicle) by oral administration for anti-inflammatory study and intraperitoneally as suspension in 2 % (m/v) acacia for ulcerogenicity. Wistar albino rats of four groups, including a control and a standard group, each with six animals were selected. The selected animals were housed in acrylic cages at standard environmental conditions at 25 ± 2 °C, relative humidity of 45–55 %, in a well ventilated room maintained at 12: 12 h light: dark cycle, fed with standard rodent diet and water *ad libitum*. All the animals were acclimatized for a week before experiment. All animal experiments were carried out according to the guidelines of the Committee for the Purpose of Control of Experiments on Animals and approval of the Institutional Animal Ethics Committee, SreeVidyanikethan College of Pharmacy, Tirupati was obtained.

Anti-inflammatory activity [15]: The anti-inflammatory activity of synthesized prodrugs was determined by hind paw edema method utilizing carrageen as phlogistic agent (0.1 ml, 1%). The initial volume of right hind paw of wistar rats (100-200 g) was measured by plethysmometer without administration of the prodrug. The prodrug of 10 mg/kg body weight was administered orally in 1% suspension of sodium carboxymethylcellulose. After 30 min of administration of prodrug, carrageenan (0.1 ml) was injected into the planter surface of right hind paw of each animal. 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 standard and control.

Percent anti-inflammatory activity was calculated using the formula

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

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

The paw volume of obtained during various time intervals are shown in Table 3 and the anti-inflammatory activity (%) is shown in Fig.3.

Table 3: Comparative paw volume of AC1, AC2, AC3 and AC4 with AC

Prodrug code	Paw Volume before administration[a]	Paw Volume after drug administration[a]		
		2 h	4 h	6 h
Control[b]	0.60 ± 0.002	0.9 ± 0.028	1.1 ± 0.055	1.6 ± 0.047
AC	0.65 ± 0.004	0.50 ± 0.052	0.66 ± 0.062	1.0 ± 0.053
AC1	0.67 ± 0.019	0.38 ± 0.060	0.40 ± 0.050	0.53 ± 0.07
AC2	0.69 ± 0.040	0.32 ± 0.018	0.35 ± 0.054	0.40 ± 0.058
AC3	0.67 ± 0.006	0.31 ± 0.049	0.32 ± 0.045	0.34 ± 0.016
AC4	0.66 ± 0.011	0.38 ± 0.061	0.34 ± 0.663	0.43 ± 0.054

[a]Each value represents the mean ± SD (n = 6). Significance levels $p < 0.05$ for all values as compared with the respective control. [b]Control animals were administered orally 1 % Carboxymethylcellulose.

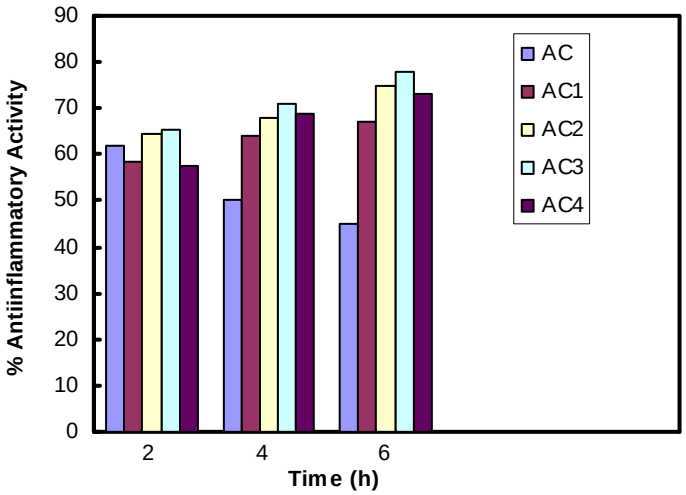


Fig. 3: Comparative anti-inflammatory activity of AC1, AC2, AC3 and AC4 with AC

Ulcer index (UI): Gastrointestinal toxicity of the synthesized prodrugs was measured and compared with the drug by measuring ulcer index [16, 17]. The control group was administered only 2 % (m/v) acacia suspension intraperitoneally. For the purpose, male albino rats were selected, weighing between 130-150 g, and were divided into six groups each comprising of three rats including a control and a standard group. Measured volume of the suspension containing dose equivalent to 10 mg/kg of body weight of AC was administered orally to the test group daily for 5 days.

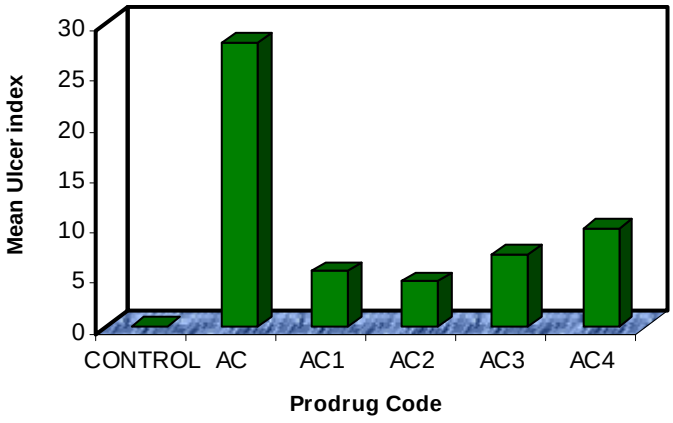


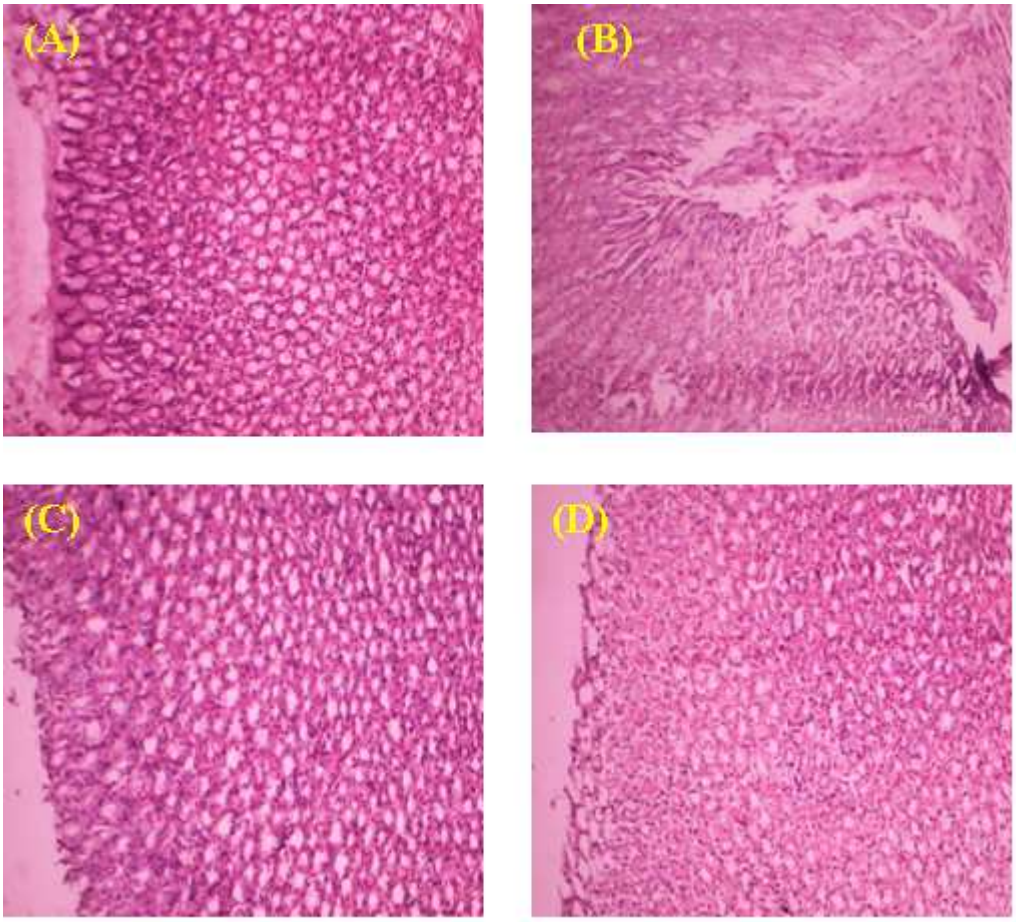
Fig. 4: Comparative ulcer index of AC1, AC2, AC3 and AC4 with AC

The rats (130-150 g) 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 ulcerative index was calculated by

severity of gastric mucosal lesions which are graded as grade1 = less than1 mm erosions, grade 2 = 1-2 mm erosions and grade 3 = More than 2 mm 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.
A comparative study of the ulcer index of AC1, AC2, AC3 and AC4 with AC was performed and is shown in Fig. 4.

Histopathological studies: The histopathological studies of stomach of rats [18] were carried out using haemotoxylin and eosin stain at Pathology Department, Sri Venkateswara Veterinary University, Tirupati, India. The stomach tissues were removed from the rats and fixed in 10 % normal saline for at least 48 h. These were then processed routinely and the tissues were embedded in paraffin wax. Histological sections were cut at 5-6 µm and stained with routine haematoxylin and eosin. These were then examined by a consultant histopathologist. The lesions observed were assessed for the following mucosal atrophy, the presence of inflammatory cells in the wall, oesinophils, lymphocytes and plasma cells. Photomicrographs of representative lesions at various magnifications were taken on Zeiss optical microscope (Germany), Stemi 2000-C, with a resolution of 10x45X, attached with trinocular camera and shown in Fig. 5.



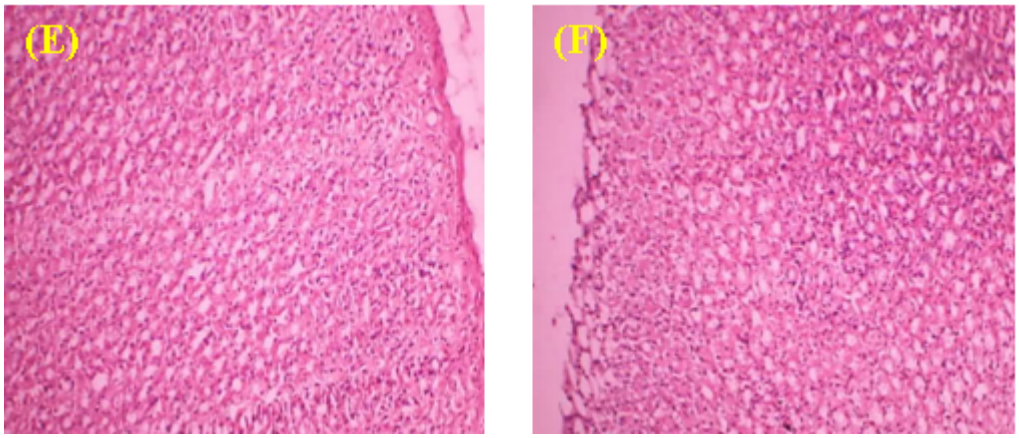


Fig. 5: Histopathological studies of prodrugs
(A) Healthy control **(B)** Ulcer control showing mucosal injury characterized by AC and massive mucosal infiltration of inflammatory cell **(C)** Treated with AC1 **(D)** Treated with AC2 **(E)** Treated with AC3 **(F)** Treated with AC4

Conclusion

The histidine, alanine, tyrosine and glycine containing aceclofenac amide prodrugs were successfully synthesized and the structures were confirmed by IR and NMR spectral analysis. The prodrugs showed excellent pharmacological response and encouraging hydrolysis rate in SCF were observed. The less protein binding of the prodrugs increased its availability for hydrolysis in colonic fluid and results in less dose requirement. Increased anti-inflammatory activity as well as reduced ulcerogenicity of the prodrugs in comparison to that the parent drug was observed. On the basis of the above observations, it is concluded that these prodrugs can be successfully applied in the colon in attaining the goal of minimized gastro intestinal toxicity without loss of desired anti-inflammatory activity of the drug. The histopathological findings reveled that there is limited ulcer formation in stomach by the prodrugs. In summary, a novel colon-specific drug-delivery system has been developed based on drug-amino acid conjugation and it provides a convenient, most beneficial and safer way for the delivery of drugs.

Statistical analysis: Statistical analysis of the pharmacological activity of the synthesized prodrugs on animals was evaluated using a one-way analysis of variance (ANOVA). Student's t-test was applied for expressing the significance and the experimental data are expressed as mean ± SD (standard deviation).

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