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Synthesis and characterization of novel dextran-conjugated macromolecules of aceclofenac

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ABSTRACT

The study involves the condensation of acylimidazole derivatives of aceclofenac (AC) with dextran 10,000 and 20,000 to obtain aceclofenac-dextran prodrugs AC10 and AC20 respectively with an aim to improve aqueous solubility, increase therapeutic efficiency and reduce the gastrointestinal side effects. The structure of synthesized prodrugs was confirmed by IR and NMR spectroscopy. The molecular weight was determined by Mark-Howink Sakurada equation and the degree of substitution was obtained as 13.3 and 16 % for the prodrugs. In vitro hydrolysis carried out in simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and simulated colonic fluid (SCF) showed faster hydrolysis in SIF and SCF. The percentage anti-inflammatory activity of AC was found as 49.56 whereas an improved value of 56.44 and 61.82 % were obtained for AC10 and AC20 respectively. The prodrugs showed improved analgesia and reduced ulcerogenicity than aceclofenac, thereby proving to be better in action than the parent drug.

KEY WORDS: Aceclofenac, polymeric prodrug, dextran, ulcer index, histopathology.

RESUMEN

El estudio se centra en la condensación de acilimidazoles derivados de aceclofenaco (AC) con dextrano 10.000 y 20.000 para obtener los profármacos de aceclofenaco-dextrano AC10 y AC20, respectivamente, con el objetivo de mejorar la hidrosolubilidad, aumentar la eficacia terapéutica y reducir los efectos secundarios gastrointestinales. La estructura de los profármacos sintetizados se ha confirmado a través de espectroscopia IR y RMN. El peso molecular ha sido determinado a través de la ecuación de Mark-Houwink-Sakurada y se ha obtenido un grado de sustitución de 13,3 y 16% para los profármacos. La hidrólisis in vitro llevada a cabo en fluido gástrico simulado (FGS), fluido intestinal simulado (FIS) y fluido colónico simulado (FCS) ha mostrado una hidrólisis más rápida en FIS y FCS. De ello ha resultado un porcentaje de actividad antiinflamatoria de AC de 49,56, mientras que para AC10 y AC20 se ha obtenido un valor aumentado de 56,44 y 61,82% respectivamente. Los profármacos han mostrado una mejor analgesia y una menor ulcerogenicidad que el aceclofenaco, por lo que se demuestra que su acción es mejor que la del fármaco base.

PALABRAS CLAVE: Aceclofenaco, profármaco polimérico, dextrano, índice de úlcera, histología patológica.

INTRODUCTION

Acetofenac (AC), a potent anti-inflammatory drug, is chemically [2-[(2, 6 dichlorophenyl) amino] phenyl] acetyl] oxy] acetic acid.^{1,2} Administration of AC by oral route causes many gastrointestinal side effects like nausea, vomiting, gastrointestinal irritation, peptic ulceration and bleeding, that limit its clinical use. Macromolecules such as antibodies, lipoproteins, lectins, proteins, polysaccharides, polypeptides, natural as well as synthetic polymers offer many applications as high molecular weight carriers for various therapeutically active compounds. Dextran serves as one of the most important polymeric carrier for a wide variety of therapeutic agents due to their excellent physicochemical properties and physiological acceptance.³⁻⁵ The pertinent literatures reveal that in most of the macromolecular or polymeric prodrug approaches, the drug is either linked by physical entrapment or chemical linkage to polymeric carriers.⁶⁻¹² The prodrugs with the polymer can temporarily mask the acidic function of AC, thereby decreasing its toxicity produced due to the direct contact effect with the gastric mucosa. The present study deals with the conjugation of dextrans of molecular weights 10,000 and 20,000 with acetofenac to produce AC10 and AC20 respectively with an aim to improve its physico-chemical properties, therapeutic efficiency and reduce GIT side effects. It is equally important to conduct a detailed pharmacological study of synthesized prodrugs, to bring out some findings that may help in its effective use.

MATERIALS AND METHODS

2.1. Materials and Instruments

The acetofenac was obtained as gift sample from Alkem Laboratories, India. Dextrans of molecular weight 10000 and 20000, and N, N-carboxyldiimidazole (CDI) was purchased from Sigma-Aldrich Chemicals Ltd, USA. Silica gel G for TLC was obtained from Sisco Laboratories, India. All other solvents and chemicals were of reagent grade and obtained from Qualinges Fine Chemicals, India. The melting point was recorded using melting point determination apparatus by Sigma Instrument, India and is uncorrected. The IR spectra were recorded using IR spectrophotometer (Shimadzu 8201 PC, Japan) in KBr phase in the range 4000 to 400 cm⁻¹. ¹H NMR spectra were recorded in DMSO on NMR spectrophotometer (Bruker DRX 300, USA). Chemical shifts are expressed as δ (ppm) values. The degree of substitution and hydrolysis studies were determined by Elico UV Spectrophotometer (India).

2.2. Synthesis of dextran prodrugs of acetofenac

Dextran prodrugs of acetofenac were prepared by

first activating the carboxylic acid group using CDI to obtain acetofenac acylimidazole (ADI), which were then condensed with dextran of different molecular weight (10000 and 20000) *in situ* to get AC10 and AC20 respectively¹³ and is shown in Scheme 1. The progress of the reaction was monitored by thin layer chromatography, which was performed on silica gel G as stationary phase and acetone: chloroform: acetic acid: water in the ratio 3:2:1:4 as mobile phase. N,N-carboxyldiimidazole is moisture-sensitive and, therefore, dry solvents were used throughout and anhydrous conditions were maintained during the experiment.

The IR and NMR spectral data of AC prodrugs are IR (KBr, max cm⁻¹): 1736 (C=O str.), 3070 (C-H str.), 736 (C-H aromatic bending), 3421 (-OH str. of polymeric -OH dextran), 1568 (str. of aromatic ring). ¹H NMR (DMSO d₆, ppm): 7.27-7.52 (m, 8H, aromatic ring), 3.89 (q, 2H, -CH₂), 1.46 (t, 3H, -CH₃), 5.30-3.63 (m, anomeric protons of glucosidic ring), 2.0- 2.49 (O-H of dextran monomer)

2.3. Characterization of the synthesized prodrugs

2.3.1. Degree of substitution

The degree of substitution of acetofenac was determined by dissolving 20 mg of the dextran prodrug in 20 ml solution of phosphate buffer (pH 7.4). The reaction mixture was maintained at 70 °C for 1h and left for 24 h for complete hydrolysis. It was then neutralized with 1N NaOH. The amount of acetofenac released during hydrolysis was extracted with chloroform and determined by UV spectrophotometer at the absorption maxima of 230 nm.

2.3.2. Molecular weight¹⁴

Intrinsic viscosities were estimated using Eq. 1. The average molecular weights were then calculated by Mark-Howink Sakurada equation (Eq 2).

$$[\eta] = [\eta_{rel-1}] / [c + 0.28 c (\eta_{rel-1})] \quad [1]$$

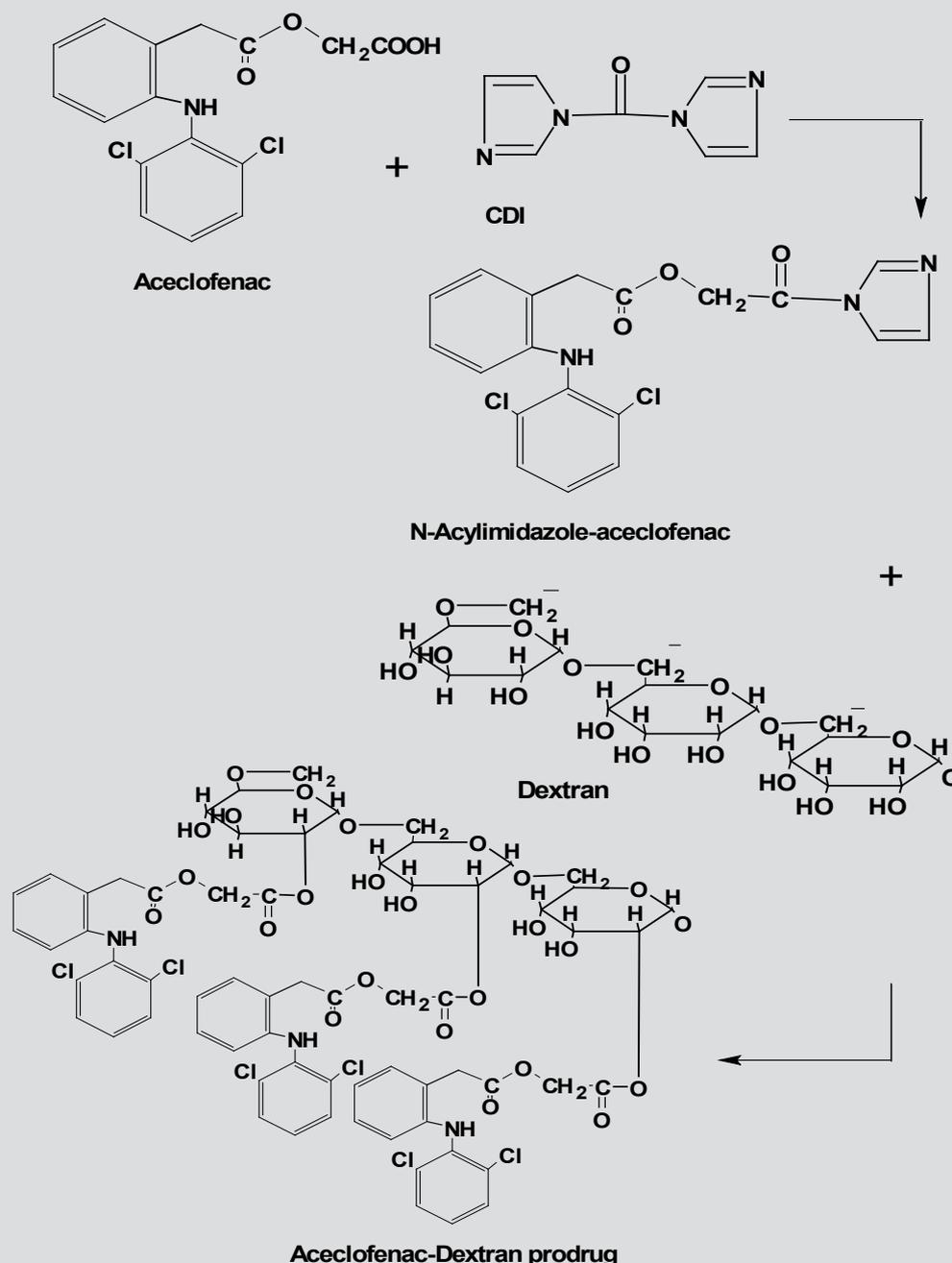
$$\log [\eta] = \log K + a \log M \quad [2]$$

where $[\eta]$ represents intrinsic viscosity, η_{rel} is the relative viscosity at concentration c (% w/v), M is the molecular weight and K and a are the constants.

2.4. In vitro hydrolysis

In-vitro hydrolysis of the dextran prodrugs was studied in simulated gastric fluid (SGF) at pH 1.2, in simulated intestinal fluid (SIF) at pH 7.4 and in simulated colonic fluid (SCF) at pH 6.8. The rate of hydrolysis of the dextran prodrugs was computed as the percentage drug hydrolysed based on the cumulative amount of drug hydrolysed divided by the total amount of drug contained in the prodrug. The rate of hydrolysis and half-life of the

Scheme 1: Synthesis of Aceclofenac-dextran prodrug



prepared prodrug were calculated using

$$r = (2.303/t) \log (b/b-x) \quad [3]$$

where r represents hydrolysis constant, t is the time in h, b is the initial concentration of prodrug, x is the amount of prodrug hydrolyzed and $(b-x)$ is the amount of prodrug remaining.

2.5. Pharmacological evaluations

AC and the synthesized prodrugs were evaluated for analgesia, anti-inflammatory activity, ulcerogenicity,

histopathology and a comparative study was performed. Test compounds and standard drugs were administered in the form of a suspension (1 % carboxy methylcellulose as a vehicle) by oral route of administration for analgesic and anti-inflammatory studies, but for ulcerogenicity studies intra peritoneally as suspension in 2 % (w/v) of acacia. Wistar albino rats of three groups, one standard group and two test groups, 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, Sree Vidyanikethan College of Pharmacy, Tirupati, India was obtained.

2.5.1. Anti-inflammatory activity

The anti-inflammatory activity was evaluated using carrageenan-induced oedema of rat paw.^{15,16} Albino rats (100-200g) were divided into three groups of six animals each. Group 1 served as standard group and received aceclofenac 2 mg/kg. The group II and group III received prodrugs AC10 and AC20 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 of prodrug, carrageenan (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 standard. The percent inhibition of paw oedema was calculated as

$$\text{Percent inhibition} = (1 - V_t / V_c) \times 100 \quad [5]$$

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

2.5.2. Analgesic activity

The analgesic activity of synthesized prodrugs was determined by thermal stimulus using tail flick method.¹⁷ Analgesiometer was used for the determination of pain threshold of albino rats. The rat (100-200 g) was placed in a holder through which the tail of the rat was protruded out. The reaction time was recorded at 1, 2, 3 and 4 h after the treatment and cut-off time was 9 s. The normal reaction

time, i.e. the time taken to flick the tail was noted. Animals showing delayed response were rejected. The prodrug was administered orally in 1% suspension of sodium CMC and compared with aceclofenac as reference. The percent analgesic activity was calculated by the formula given as

$$\% \text{Analgesic activity} = [(T_2 - T_1) / (T_c - T_1)] \times 100 \quad [4]$$

where T1 - the reaction time (s) before administration of prodrug and T2 - the reaction time (s) after administration of prodrug and Tc - cutoff time in sec.

2.5.3. Ulcerogenic activity

Gastrointestinal toxicity of the synthesized prodrugs was measured and compared with the parent drug by measuring ulcer index¹⁸. The prodrug was suspended in 10 ml of 2% w/v suspension of acacia. Measured volume of the suspension containing AC was administered orally to the test group daily for 5 days. The albino rats (100-200 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 ulcer index (UI) was calculated by severity of gastric mucosal lesions which are graded as grade 1 - less than 1mm erosions, grade 2 - 1-2 mm erosions and grade 3 - more than 2 mm erosions. The UI was calculated as:

$$\text{UI} = [1 \times (\text{number of lesions of grade 1}) + 2 \times (\text{number of lesions of grade 2}) + 3 \times (\text{number of lesions of grade 3})] / 10 \quad [6]$$

2.5.4. Histopathological studies

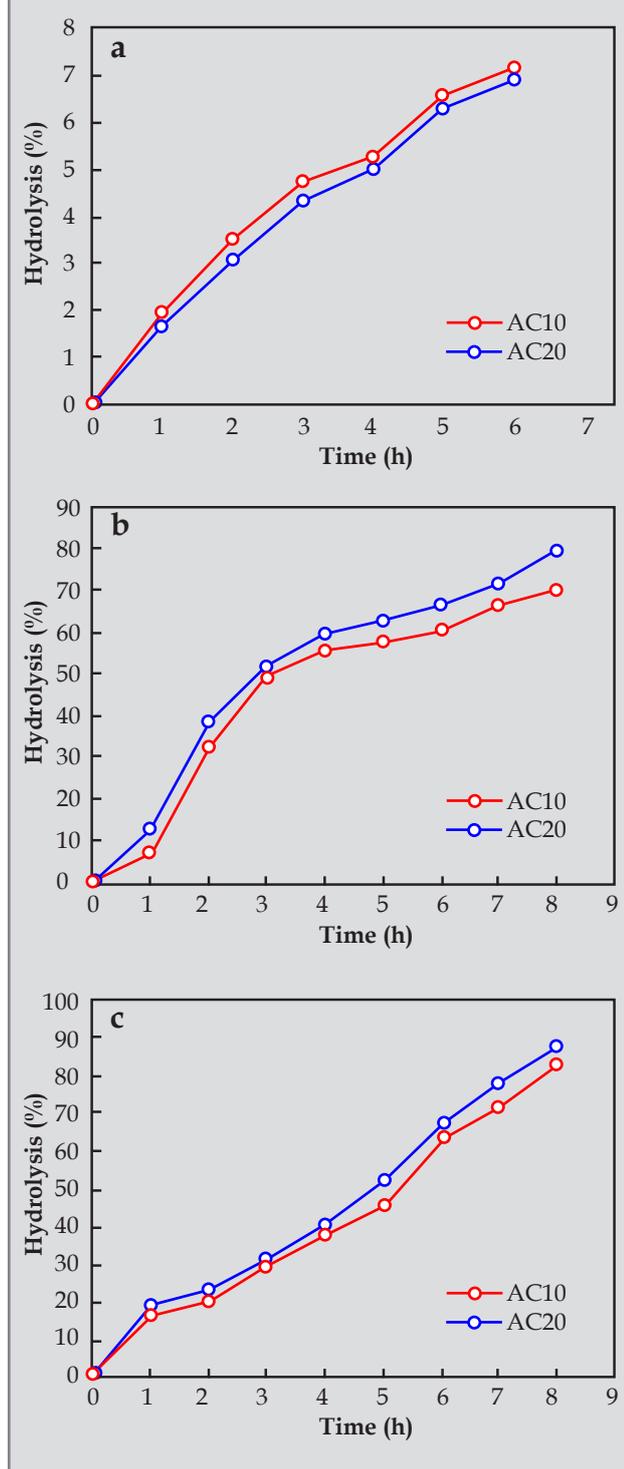
The histopathological studies¹⁹ of stomach of rats were carried out using haematoxylin and eosin stain at Pathology Department, Sri Venkateswara Veterinary University, Tirupati, India. The stomach tissues were removed from the rats and fixed in 10% normal saline for at least 48 h. These were then processed 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.

Table 1: Physicochemical properties of prodrugs

Prodrug code	Colour	M.P. (°C)*	Yield (%)	R _i # value	Degree of substitution ^a	Intrinsic viscosity	Molecular Weight	
							Calculated (%)	Found (%)
AC10	White	193-194	95.50	0.58	13.3	0.028	11250	13240
AC20	White	196-198	97.00	0.57	16	0.035	21950	25260

* Uncorrected # Acetone: chloroform: acetic acid: water in 3:2:1:4
a = amount of parent drug in mg per 100 mg of prodrug

Figure 1. Comparative pattern of hydrolysis of prodrugs in (a) SGF (b) SIF and (c) SCF



The lesions observed were assessed for the following mucosal atrophy, the presence of inflammatory cells in the wall, eosinophils, 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 10x40X,

attached with trinocular camera.

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 \pm SD (standard deviation).

RESULTS AND DISCUSSION

The synthesis of polymeric prodrugs AC10 and AC20 were carried out successfully. The prodrugs were subjected to physicochemical characterization, hydrolytic studies and pharmacological evaluation. The physicochemical properties are shown in table 1. The structure of synthesized prodrugs was confirmed by analytical and spectral data. The NMR spectra of AC prodrugs showed characteristic shifting of glucosidic ring anomeric proton signals from δ 3.63 (H-1) to δ 5.30 (H-1), H-2 proton from δ 2.0 (H-2) to δ 3.89 (H-2) which indicates the formation of an ester linkage at position C2. The disappearance of NMR signals in the range of δ 10.58 to δ 11.20 ppm for carboxylic acid group in the AC dextran prodrugs suggests that the free carboxylic acid group of drug was conjugated with hydroxyl group of dextran macromolecule and ester bond was formed. The signals of the aromatic ring of AC were found as δ 7.25 and are in agreement with the anticipated structure. The IR spectra of the AC prodrugs showed characteristic stretching at 1736 cm^{-1} and confirm the formation of ester linkage. A strong O-H stretching vibration of polymeric association at 3421 cm^{-1} and weak C-H stretching of alkane at 3070 cm^{-1} were also found. It also showed the characteristic absorption stretching at 1568 cm^{-1} for aromatic ring. An absorption maximum in phosphate buffer (pH 7.4) was observed at 230 nm which was same as that of AC. The degree of substitution was determined by UV spectrophotometry and was found as 13.3% for AC10 and 16% for AC20.

In vitro hydrolytic studies were carried out in SGF (pH 1.2), SIF (pH 7.4) and SCF (pH 6.8) and the results are summarized in Figs 1 and 2. The AC prodrugs did not show much significant hydrolysis in SGF (pH 1.2). The amount of AC regenerated by hydrolysis of AC10 and AC20 in SIF was found as 79.7 and 70.4% respectively, whereas 88 and 82% in SCF. The AC prodrugs showed much faster hydrolysis in SIF and SCF and followed first order kinetics. This encouraging hydrolysis is due to the cleavage of prodrug to drug with the help of enzymes like amidase and esterase present in the simulated fluids. The half lives of AC10 and AC20 were found to be 3.43 h and 3.48 h respectively.

Figure 2. First order kinetics plot of AC10 and AC20 in SCF (pH 6.8)

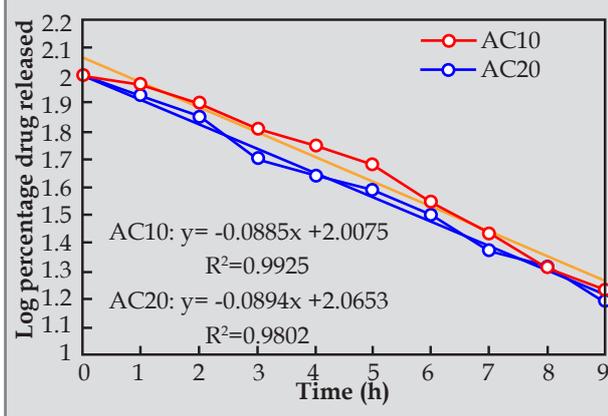


Figure 3. Comparative ulcer index of aceclofenac and its prodrugs

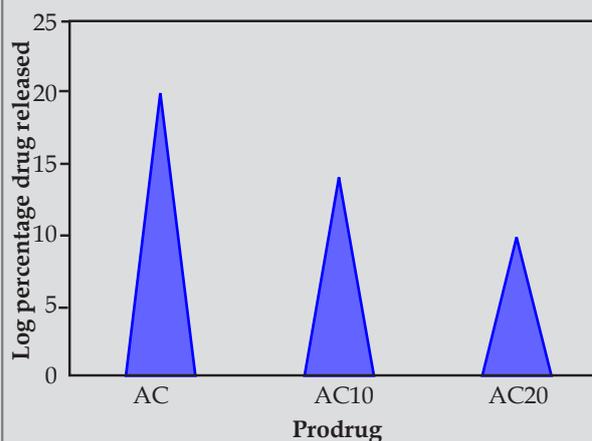
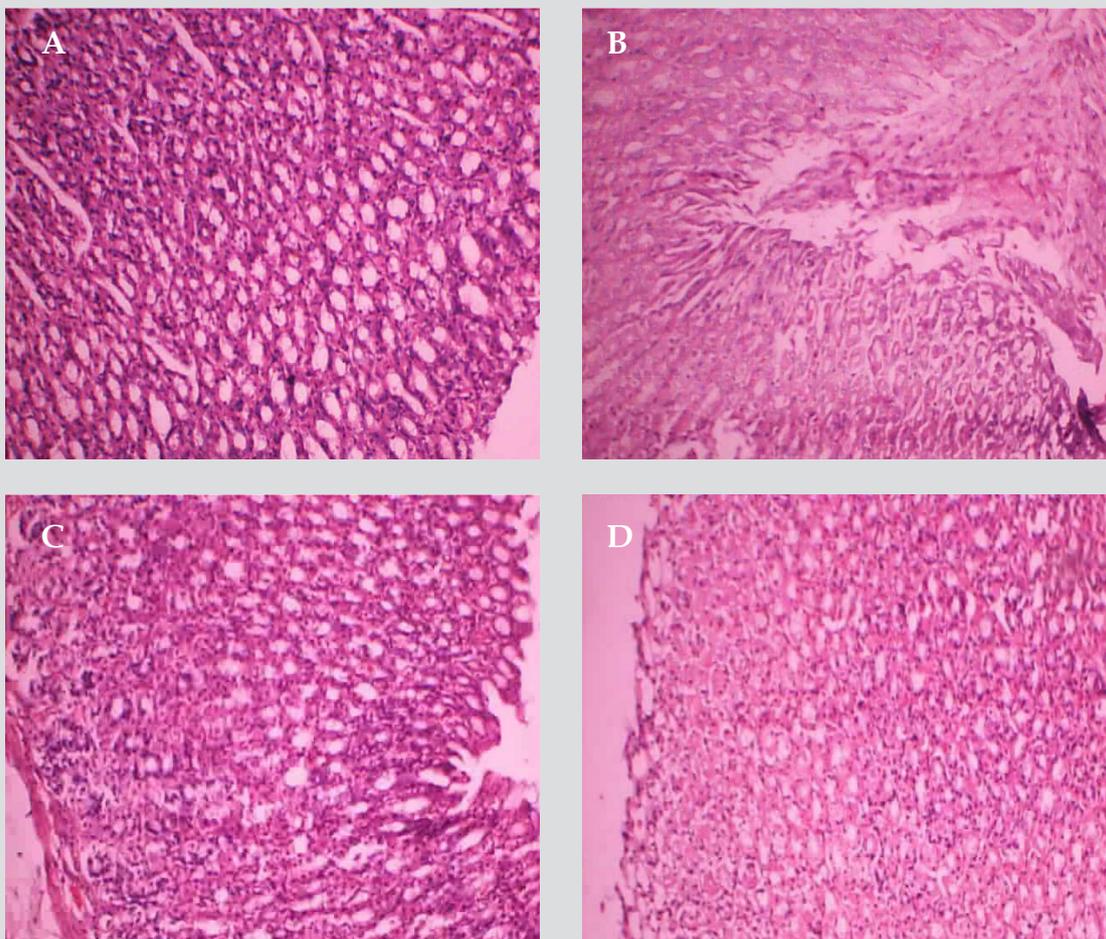


Figure 4. Histopathological findings of aceclofenac and its prodrugs on rat stomach. a) Healthy control b) Ulcer control showing mucosal injury characterized by AC and massive mucosal infiltration of inflammatory cell c) Treated with AC10 d) Treated with AC20



The synthesized prodrugs were subjected to pharmacological evaluation of anti-inflammatory activity, analgesia, ulcerogenicity and histopathology. The AC prodrugs showed improved anti-inflammatory and

analgesic activities as compared to the parent drug and are shown in table 2. After 6 h of administration of AC, the percentage anti-inflammatory activity was found as 49.56 % whereas an improved value of 56.44 and 61.82%

Table 2: Physicochemical properties of prodrugs

Group	Prodrug	Anti-inflammatory activity (%) ^b			Analgesic activity (%) ^b			
		2 h	4 h	6 h	1 h	2 h	3 h	4 h
I	ACa	42.31 ± 1.5	52.41 ± 1.2	49.56 ± 1.2	42.22 ± 1.5	55.1 ± 2.0	56.2 ± 1.1	54.41 ± 1.8
II	AC10	46.41 ± 1.0 ^{*c}	64.62 ± 1.3 ^{*c}	56.44 ± 1.3 ^{*c}	32.3 ± 1.7 ^{*c}	57.5 ± 1.3 ^{*c}	64.6 ± 2.3 ^{*c}	69.53 ± 1.9 ^{*c}
III	AC20	46.21 ± 1.2 ^{*c}	55.54 ± 1.1 ^c	61.82 ± 1.8 ^{*c}	38.1 ± 1.5 ^{*c}	45.3 ± 1.2 ^{*c}	59.1 ± 1.4 ^{*c}	75.4 ± 1.4 ^{*c}

^aStandard drug (AC) was administered orally. ^bValues were expressed as mean ± SD of 6 observations.

^cComparison between Group I Vs Group II and III.

Statistical significant test comparison was done by one way ANOVA followed by dunnet 't'

were obtained for AC10 and AC20 respectively. In case of analgesic activity, after 4 h of the administration of AC, the percentage analgesia was observed as 54.41% whereas an increased value of 69.53 and 75.4% was observed for the prodrugs. The parent drug AC has an ulcer index of 20 where as AC10 and AC20 shows reduced ulcer index of 14 and 9.8 respectively as depicted in Fig. 3.

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 both AC10 and AC20 group. This reveals that the prodrugs are not producing much ulceration in the gastric region and are shown in Fig. 4.

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