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Original article

Synthesis, characterization and pharmacological evaluation of amide prodrugs of ketorolac

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Abstract

Ketorolac (KC) suffers from the general side effects of NSAIDs, owing to presence of free carboxylic acid group. The study aimed to retard the adverse effects of gastrointestinal origin. Ten prodrugs of KC were synthesized by amidation with ethyl esters of amino acids, namely, glycine, L-phenylalanine, L-tryptophan, L-valine, L-isoleucine, L-alanine, L-leucine, L-glutamic acid, L-aspartic acid and β-alanine. Purified synthesized prodrugs were characterized by m.p., TLC, solubility, partition coefficients, elemental analyses, UV, FTIR, NMR and MS. Synthesized prodrugs were subjected for biopharmaceutical studies, analgesic, anti-inflammatory activities and ulcerogenic index. Marked reduction of ulcerogenic index and comparable analgesic, anti-inflammatory activities were obtained in all cases as compared to KC. Among synthesized prodrugs, viz. AR-11, AR-19 and AR-20 showed excellent pharmacological response and encouraging hydrolysis rate both in SIF and in 80% human plasma. Prodrugs with increased aliphatic side chain length or introduction of aromatic substituent showed enhanced partition coefficient but diminished dissolution and hydrolysis rates. Such prodrugs can be considered for sustained release purpose.

Keywords: Ketorolac; Prodrugs; Amino acid conjugates; Pharmacokinetics; Pharmacodyanamics

1. Introduction

Ketorolac (KC), one of non-steroidal anti-inflammatory drug, could not be used as up to its potential, because of its adverse reactions offered due to presence of free carboxylic acid group. The non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for indications extending from inflammation and pain to cardiovascular and genitourinary diseases. In the recent years a number of NSAIDs have been introduced into clinical practice. Hunt is on to relieve pain and inflammation with freedom of undesirable effects. Gastrointestinal side effects constitute the most frequent of all the adverse reactions of NSAIDs and often these reactions lead to GIT ulceration and hemorrhage. GI mucossal injury produced by NSAIDs is generally believed to be caused by two different mechanisms [1]. The first mechanism involves a local action composed of a direct contact while the other has indirect effect on the GI mucosa. The direct contact effect can be attributed to a combination of a local irritation produced by acidic group of NSAIDs and local inhibition of prostaglandin synthesis in the GI tract. The indirect effect can be attributed to combination of an ion trapping mechanism of NSAIDs from the lumen into the mucosa. The second mechanism is based on a generalized systemic action occurring after absorption, which can be demonstrated following intravenous dosing. Recently, considerable attention has been focused in the development of bio-reversible derivatives, by temporarily masking the acidic group of NSAIDs, as a promising mean of reducing or abolishing the GI toxicity.

In the present study well-recognized NSAID, viz. KC was selected, which suffers with the gastrointestinal side effects. Literature reveals that many efforts had been made to synthesize prodrug via masking carboxylic acid group by forming ethyl esters and [(*N*,*N*-dimethylamino)carbonyl]methyl ester

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[2,3] and amide prodrug [4] using various amines. Some alkyl esters had been synthesized for topical delivery of KC [5]. However, no attempts were made to develop amide prodrugs using amino acids, which have been utilized as a major tool with other NSAIDs [6–8]. Apart from the amino acid conjugates of NSAIDs this approach is widely adopted for synthesis of prodrugs of anticancer drugs [9]. The salient features of the usefulness of conjugation of amino acids with drugs are as follows [10]:

(i) amino acids are normal dietary constituent and they are non-toxic in moderate doses as compared to other promoities;
(ii) amino acids have healing effect on gastric toxicity;
(iii) a drug with free carboxyl group can be derivatized into corresponding esters or amide of amino acids, so as to alter the physical properties of a parent drug with one or more of the hydrolase enzymes serving as the in vivo reconversion site(s);
(iv) being a nutritional substance, the use of amino acids as a derivatizing group might also permit more specific targeting site for enzymes involved in the terminal phase of digestion;
(v) many amino acids possess marked anti-inflammatory activity against carrageenan induced hind paw edema in rats; and
(vi) by using different types of amino acids, viz. non-polar, polar, acidic and basic, the drug molecule can be made more or less polar, or more or less soluble in given solvent.

Thus present work aims to synthesize amide prodrugs of KC using amino acid ester with the expectation to get non-toxic prodrugs with minimized GIT disturbances while maintaining the useful anti-inflammatory and analgesic activities. Various proteolytic enzymes will help in release of KC by hydrolysis of peptide linkage, without producing any xenobiotic substance within serum or GIT. The list of synthesized prodrugs along with product codes, chemical names, possible trivial names and molecular structures is given in Table 1.

2. Materials and methods

2.1. Materials

All the amino acids, namely, glycine, L-phenylalanine, L-tryptophan, L-valine, L-isoleucine, L-alanine, L-leucine, L-glutamic acid, L-aspartic acid and β-alanine were procured from M/S Hi-Media Ltd., Mumbai. Drug KC was obtained as gift sample from M/S Knoll, Mumbai. Other reagents and solvents used were of analytical/spectroscopic/HPLC grade as the case desired.

2.2. Synthesis of ethyl esters of amino acid hydrochloride salts

The prodrugs were synthesized first by converting amino acids into their ethyl esters' HCl salts. Thereafter, these ethyl esters' HCl salts of amino acids were condensed with anhydride or acyl chloride of KC along with prior or simultaneous neutralization of HCl salts. Schematic representation of the reaction is given in Scheme 1.

Amino acid contains both acidic and basic groups in the same molecular and exists in the zwitterionic form. The non-availability of the free amino group in the zwitterionic form of the amino acid restricts its use in the formation of amide. Esterification of amino acid in presence of HCl produces amino acid ester hydrochloride in which neutralization of HCl, using aqueous alkali, pyridine or triethylamine, generates free amino group to react as nucleophile in the synthesis of amide [11,12].

2.3. General procedures used in acylation of amino acid ethyl ester hydrochloride

Acylation of amino acid ethyl ester hydrochloride was done as described in literature by six methods using acid anhydride of KC as acid chloride of the KC could not be formed satisfactorily and triethylamine as base, acid chloride of KC and potassium carbonate as base, acid chloride of KC and pyridine as base, DCC, drug (1:1) and triethylamine as base and acid anhydride of KC and triethylamine as base.

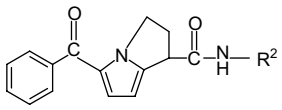
2.3.1. Method 1

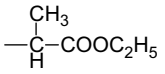
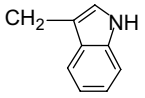
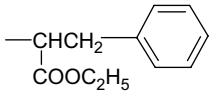
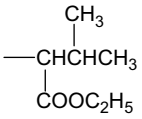
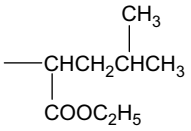
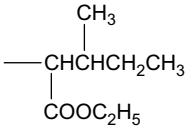
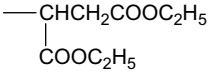
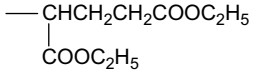
Amino acid ester hydrochloride (0.005 M) and triethylamine (2 mL) were dissolved in 10 mL of dichloromethane and stirred mechanically for 10 min to neutralize the ester hydrochloride. This solution was added slowly to the freshly synthesized acid anhydride of KC in dichloromethane at 0 °C for half an hour, followed by stirring at room temperature for 24 h. Precipitated dicyclohexyl urea (DCU) was filtered off and the solvent was removed at reduced pressure, 10 mL ethyl acetate was further added to the dried product and filtered to remove the triethylamine hydrochloride and remaining DCU. The filtrate was washed three times with 10% sodium bicarbonate and three times with water and dried over anhydrous magnesium sulphate and the solvent was removed under reduced pressure. White amorphous mass was obtained, which was dried under high vacuum. Crude material was dissolved in minimum amount of alcohol, followed by the slow addition of distilled water until further precipitation stopped. After filtration it was dried in air to get a solid product. This method was used to synthesize AR-11.

2.3.2. Method 2

KC (0.01 M) was dissolved in 40 mL dichloromethane followed by addition of DCC (0.01 M). Reaction mixture was stirred for half an hour. To this solution was added a mixture of amino acid hydrochloride (0.01 M) and triethylamine (2 mL) in 20 mL of dichloromethane in drop wise manner. Reaction mixture was stirred at 0 °C initially for 2 h followed by stirring at room temperature for whole night. Precipitated dicyclohexyl urea was filtered off. Solvent was removed at reduced pressure. Ethyl acetate (10 mL) was added to the dried product to remove the remaining DCU. Ethyl acetate

Table 1
List of synthesized prodrugs of ketorolac

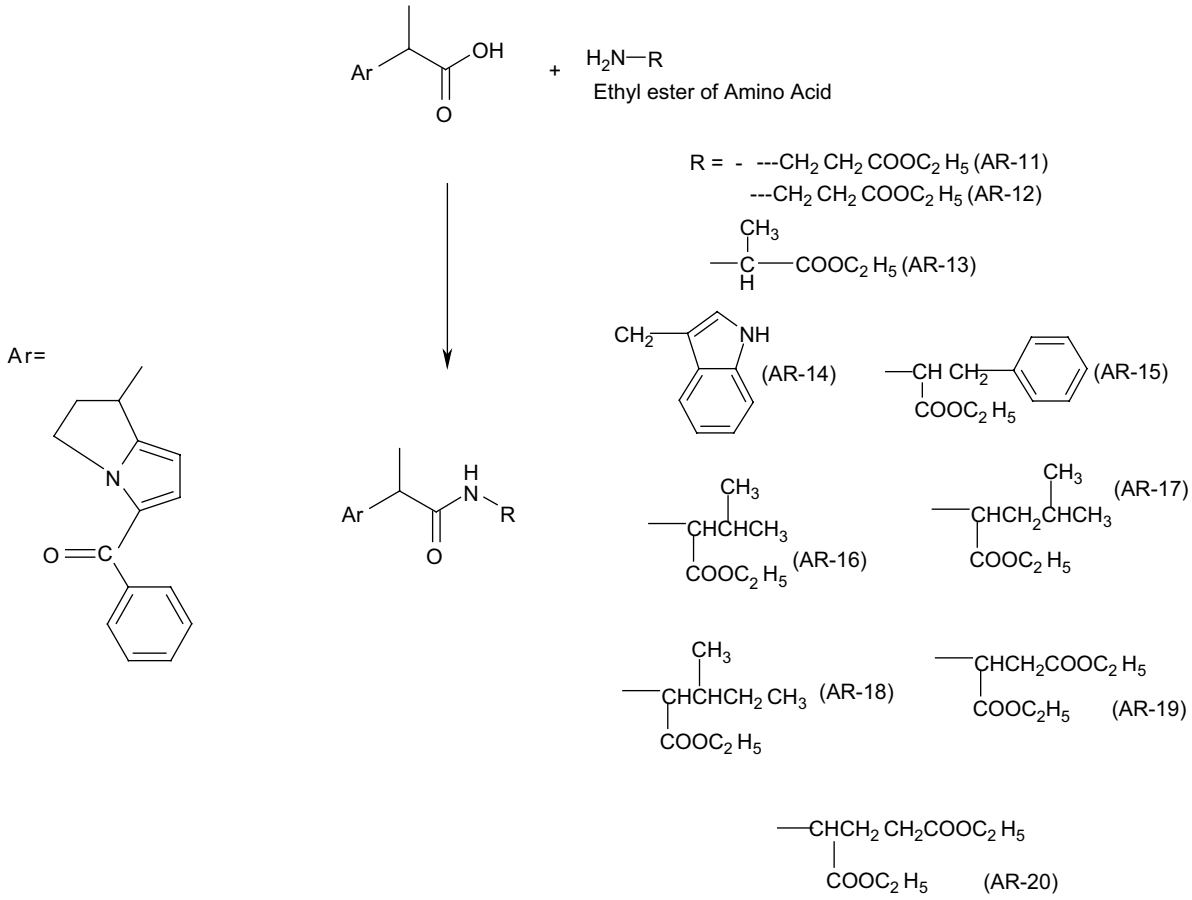


Code	Chemical name	R^2
AR-11	(±)-5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(ethylacetate)] carboxamide	$-CH_2COOC_2H_5$
AR-12	(±)-5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2-ethylpropionate)] carboxamide	
AR-13	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(3-ethylpropionate)] carboxamide	$-CH_2CH_2COOC_2H_5$
AR-14	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2-(3-(3-benzimidazole)) ethylpropionate)] carboxamide	
AR-15	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2(3-phenyl)ethylpropionate)]carboxamide	
AR-16	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2-(3-methyl)ethyl butyrate)]carboxamide	
AR-17	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2-(4-methyl)ethyl pentoate)]carboxamide	
AR-18	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2-(3-methyl)ethylpentoate)]carboxamide	
AR-19	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2-diethyl succinate)]carboxamide	
AR-20	(±)5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-[*N*-(2′-diethylglutamate)]carboxamide	

layer was washed with 10% $NaHCO_3$ and distilled water to remove the triethylamine hydrochloride and traces of alkali. Ethyl acetate layer was dried over anhydrous magnesium sulphate and filtered off. Solvent was removed at reduced pressure to get the crude product. Product was recrystallized by selective precipitation using alcohol–water mixture, dried and stored in cold condition in tightly closed container. This method was used to synthesize AR-14, AR-15 and AR-20.

2.3.3. Method 3

Amino acid hydrochloride (0.01 M), triethylamine (2 mL) and KC (0.02 M) were dissolved in 40 mL of dichloromethane. The reaction mixture was stirred at 0 °C for half an



Scheme 1. Chemical reaction adopted for syntheses of amide prodrugs of ketorolac.

hour. To this solution was added, DCC (0.01 M) in 10 mL of dichloromethane slowly in a drop wise manner. Reaction mixture was stirred for 24 h. Precipitated dicyclohexyl urea was filtered off and the solvent was distilled off at reduced pressure. Product thus obtained was again dissolved in 10 mL of ethyl acetate. White precipitate was again obtained, which was found to be a mixture of DCU and triethylamine hydrochloride. Filtrate was washed with 10% $NaHCO_3$ and distilled water in order to remove KC, triethylamine hydrochloride and traces of alkali present. Ethyl acetate layer was dried over anhydrous magnesium sulphate and filtered to get a clear solution of product in ethyl acetate. Solvent was evaporated under reduced pressure and the crude product, thus obtained, was recrystallized by dissolving it in ethyl alcohol followed by the addition of water until further precipitation stopped. Product was filtered, dried and stored in tightly closed container in cold condition. This method was used to synthesize AR-12, AR-13, AR-16, AR-17, AR-18 and AR-19.

2.4. Characterization of the synthesized prodrugs

2.4.1. Solubility

Approximately 10 mg of compound was dissolved in 0.1 mL of each solvent at 37 ± 1 °C in glass test tubes. Test tubes were gently shaken and solubility was observed. In case of any observed insoluble fraction, the known amount of solvent was further added to ascertain the solubility of the compound.

2.4.2. Partition coefficient

A 20 mg of prodrug was weighed and dissolved in 20 mL chloroform. This solution was divided in two parts and in each part was added 10 mL acidic buffer (pH 1.2) and phosphate buffer (pH 7.4) separately. The contents were thoroughly shaken for 24 h at room temperature followed by transferring in separating funnel. The chloroform layer was dried under high vacuum and the residue obtained was again dissolved in methanol (10 mL). A 20 μL of this solution was further diluted to 1000 μL with methanol. From this solution an aliquot of 250 μL was withdrawn and was mixed with 45 μL solution of acidic buffer (pH 1.2) or phosphate buffer (pH 7.4) and acetonitrile in 44:1. Volume was finally made to 1000 μL by addition of methanol. A 20 μL of this solution was filtered and injected into HPLC column (C_{18} ODS reversed phase). The mobile phase acetonitrile:water 50:50 was used for KC prodrugs. The peak area for drug as well as prodrug was observed at 311 nm for KC prodrugs using UV detector (DAD, SPD-M10A with D2 lamp).

The synthesized compounds were subjected to thin layer chromatography in order to check their purity. The prepared

plates of silica gel G adsorbents were dried and activated. The solvent system chloroform:methanol:ammonia 75:3:0.5 was used for KC. Iodine vapor was used as detecting agent. All compounds were shown as single spot. The melting points of the synthesized prodrugs were determined by open capillary tube using Toshniwal melting point apparatus and are uncorrected. The data are shown in Table 2.

The IR spectra of the compounds were obtained on IR spectrophotometer (Shimadzu 820 IPC) in KBr phase and data are shown in Table 3. The PMR spectral analyses of the synthesized prodrugs were done on NMR spectrophotometer (Bruker DRX300) using $CDCl_3$ as solvent and data are shown in Table 3. The mass was determined on Jeol SX102-FAB mass spectrometer and the results are shown in Table 3.

2.5. Biopharmaceutical evaluations

2.5.1. Plasma–protein binding studies

A solution of synthesized prodrug (20 μg/mL) was made in phosphate buffered saline (PBS, pH 7.4) [13]. 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 2 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 spectrophotometrically using spectrophotometer (Shimadzu-1601).

2.5.2. Dissolution rate studies

Dissolution test apparatus (USPXXI) was adjusted to 100 rpm speed and temperature 37 °C. Prodrug (100 mg) was suspended in 2 mL simulated gastric fluid (SGF, pH 1.2) in a dialysis bag (cut off size 5000) kept inside the basket. The basket was dipped in simulated gastric fluid (SGF, 900 mL), which was kept in the internal jar of the dissolution test apparatus. Five milliliter samples were withdrawn after every 10 min and the volume was replaced by fresh simulated gastric fluid every time. Samples withdrawn from dissolution test apparatus were subjected to HPLC. The measurements of peak area of the prodrugs were noted at 311 nm using UV detector.

The samples of synthesized prodrugs as well as drug from the dissolution study in SGF did not show their presence up to 2 h, which is considered as maximum time for any drug that remains in stomach, so after 2 h. SGF was replaced by simulated intestinal fluid (SIF, 900 mL) and the dissolution test was run again, under similar conditions of temperature and speed. The same procedure was followed for all.

2.5.3. Studies on hydrolytic behaviour of synthesized prodrugs

Hydrolytic behaviour of synthesized prodrugs was studied in SGF (pH 1.2; USP 1970), SIF (pH 7.4; USP 1970) and 80% human plasma. The method adopted for the studies is as follows.

2.5.3.1. Method for the hydrolysis rate determination. A solution of 10 mg of prodrug was prepared in acetonitrile (2 mL) and was added to 88 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 (15, 30, 45, 60, 75 and 90 min) an aliquot of 225 μL was withdrawn from different test tubes and was transferred to microcentrifuge tubes (Eppendrof's tube) followed by the 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. Clear supernatant (20 μL) obtained from each tube was then injected in to run through HPLC column (C_{18} ODS reversed phase) of HPLC instrument (Shimadzu liquid chromatograph LC-10AT). Mobile phase acetonitrile:water 50:50 was used for KC and prodrugs. Flow rate of mobile phase was kept at 1 mL/min at pressure 120–135. UV detector (DAD, SPD-M10A with D2 lamp)

Table 2
Physical constants of synthesized prodrugs of ketorolac

Prodrug code	Molecular formula	MW calculated	Melting range(°C)[a]	% Yield	Colour	R_f[b] value	% Nitrogen	
							Found	Calculated
AR-11	$C_{19}H_{20}N_2O_4$	340	144–146	84	White	0.67	8.18	8.23
AR-12	$C_{20}H_{22}N_2O_4$	354	84–86	83	Light yellow	0.73	7.82	7.90
AR-13	$C_{20}H_{22}N_2O_4$	354	116–118	54	Creamy white	0.69	7.87	7.90
AR-14	$C_{28}H_{27}N_3O_4$	469	108–110	70	Light yellow	0.8	6.19	6.15
AR-15	$C_{26}H_{26}N_2O_4$	430	84–86	88	Creamy white	0.83	6.49	6.51
AR-16	$C_{22}H_{26}N_2O_4$	382	96–98	62	Light yellow	0.79	7.29	7.32
AR-17	$C_{23}H_{28}N_2O_4$	396	92–94	71	Dirty white	0.84	7.11	7.07
AR-18	$C_{23}H_{28}N_2O_4$	396	98 with dec.	51	Dirty white	−0.83	7.01	7.07
AR-19	$C_{23}H_{26}N_2O_4$	426	98–100	63	Creamy white	0.72	6.47	6.57
AR-20	$C_{24}H_{28}N_2O_4$	440	82–84	59	Yellowish white	0.74	6.31	6.36

[a] Melting points are uncorrected.
[b] Solvent system – chloroform:methanol:ammonia 75:3:5.

Table 3
IR, ^{1}H NMR and mass spectral characterization of synthesized prodrugs of ketorolac

Prodrug code	Characteristic peaks of IR spectra	Characteristic peaks of ^{1}H NMR spectra and mass spectra
AR-11	3296 (NH str. of amide), 3062 (aromatic CH str.), 2915, 2830 (aliphatic CH str.), 1735 (C=O str. of ester), 1646 (amide I), 1578 (amide II), 1424, 1378 (CH bend, aliphatic), 1292 (C–O str. of ester)	7.29–7.86 (m, 5*H*, aromatic ring), 6.68 (d, 1*H*, CH in ring), 5.66 (d, 1*H*, CH in ring), 3.81 (t, 2*H*, CH_2 in ring), 2.56–2.86 (m, 2*H*, CH_2 in ring), 7.89 (br, *NH*), 4.17 (q, 2*H*, OCH_2CH_3), 1.28 (t, 3*H*, OCH_2CH_3) and *m/z* 340
AR-12	3312 (NH str. of amide), 3053 (aromatic CH str.), 2956, 2923, 2861 (aliphatic CH str.), 1723 (C=O str. of ester), 1654 (amide I), 1569.29 (amide II), 1424, 1378 (CH bend, aliphatic), 1292 (C–O str. of ester)	7.25–7.87 (m, 5*H*, aromatic ring), 6.59 (d, 1*H*, CH in ring), 5.69 (d, 1*H*, CH in ring), 3.79 (t, 2*H*, CH_2 in ring), 2.99–3.24 (m, 2*H*, CH_2 in ring), 7.83 (br, *NH*), 4.17 (q, 2*H*, OCH_2CH_3) 1.28 (t, 3*H*, OCH_2CH_3), 4.59–4.65 (m, 1*H*, CH–CH_3), 1.38 (d, 3*H*, CH–CH_3) and *m/z* 354
AR-13	3300 (NH str. of amide), 3020 (aromatic CH str.), 2985, 2892 (aliphatic CH str.), 1727 (C=O str. of ester), 1655 (amide I), 1605(amide II), 1450, 1380 (CH bend, aliphatic), 1260 (C–O str. of ester)	7.28–7.81 (m, 5*H*, aromatic ring), 6.55 (d, 1*H*, CH in ring), 5.61 (d, 1*H*, CH in ring), 3.73 (t, 2*H*, CH_2 in ring), 2.94–2.98 (m, 2*H*, in CH_2 in ring), 7.76 (br, *NH*), 4.13 (q, 2*H*, OCH_2CH_3), 1.27 (t, 3*H*, OCH_2CH_3) and *m/z* 356
AR-14	3359 (NH str. of amide), 3276 (aromatic CH str.), 2946, 2909 (aliphatic CH str.), 1738 (C=O str. of ester), 1661 (amide I), 1607.5(amide II), 1438, 1376 (CH bend, aliphatic), 1269 (C–O str. of ester)	7.30–7.88 (m, 5*H*, aromatic ring), 6.60 (d, 1*H*, CH in ring), 5.63 (d, 1*H*, CH in ring), 3.82 (t, 2*H*, CH_2 in ring), 2.83–2.96 (m, 2*H*, CH_2 in ring), 7.83 (br, *NH*), 7.20 (q, 2*H*, OCH_2CH_3), 1.21 (t, 3*H*, OCH_2CH_3), 10.10 (br, *NH* ring), 7.18–7.24 (m, 4*H*, aromatic ring of indole nucleus), 4.79–4.82 (m, 1*H*, $CHCH_2$) and *m/z* 470
AR-15	3306 (NH str. of amide), 3057 (aromatic CH str.), 2996, 2930 (aliphatic CH str.), 1750 (C=O str. of ester), 1653 (amide I), 1547 (amide II), 1489, 1375 (CH bend, aliphatic), 1283 (C–O str. of ester)	7.33–7.77 (m, 5*H*, aromatic ring), 6.53 (d, 1*H*, CH in ring), 5.52 (d, 1*H*, CH in ring), 3.66 (t, 2*H*, CH_2 in ring), 2.97–3.12 (m, 2*H*, CH_2 in ring), 8.21 (br, *NH*), 4.20 (q, 2*H*, OCH_2CH_3), 1.32 (t, 3*H*, OCH_2CH_3), 7.12–7.27 (m, 5*H*, aromatic ring), 4.91 (t, 2*H*, CH_2Ph) and *m/z* 430
AR-16	3299 (NH str. of amide), 3020 (aromatic CH str.), 2964, 2852 (aliphatic CH str.), 1738 (C=O str. of ester), 1655 (amide I), 1574 (amide II), 1432, 1396 (CH bend, aliphatic), 1271 (C–O str. of ester)	7.28–7.83 (m, 5*H*, aromatic ring), 6.60 (d, 1*H*, CH in ring), 5.64 (d, 1*H*, CH in ring), 3.83 (t, 2*H*, CH_2 in ring), 2.88–2.94 (m, 2*H*, CH_2 in ring), 8.12 (br, *NH*), 4.19 (q, 2*H*, OCH_2CH_3), 1.28 (t, 3*H*, OCH_2CH_3), 4.39–4.59 (m, 1*H*, $CHCH(CH_3)_2$), 3.08–3.42 (m, 1*H*, $CHCH(CH_3)_2$), 1.01 (d, 6*H*, $CHCH(CH_3)_2$) and *m/z* 382
AR-17	3315 (NH str. of amide), 3052 (aromatic CH str.), 2923, 2861 (aliphatic CH str.), 1723 (C=O str. of ester), 1653 (amide I), 1559 (amide II), 1437, 1376 (CH bend, aliphatic), 1273 (C–O str. of ester)	7.26–7.86 (m, 5*H*, aromatic ring), 6.23 (d, 1*H*, CH in ring), 6.62 (d, 1*H*, CH in ring), 3.90 (t, 2*H*, CH_2 in ring), 2.80–2.92 (m, 2*H*, CH_2 in ring), 8.02 (br, *NH*), 4.18 (q, 2*H*, OCH_2CH_3), 1.28 (t, 3*H*, OCH_2CH_3), 4.45–4.64 (m, 1*H*, $CHCH_2CH(CH_3)_2$), 1.77–1.94 (m, 2*H*, $CH_2(CH_3)_2$), 1.03 (d, 6*H*, $CH(CH_3)_2$) and *m/z* 395
AR-18	3326 (NH str. of amide), 3027 (aromatic CH str.), 2930, 2853 (aliphatic CH str.), 1736 (C=O str. of ester), 1648 (amide I), 1574 (amide II), 1430, 1400 (CH bend, aliphatic), 1270 (C–O str. of ester)	7.25–7.90 (m, 5*H*, aromatic ring), 6.61 (d, 1*H*, CH in ring), 5.56 (1*H*, CH in ring), 3.80, 2*H*, CH_2 in ring), 2.83–2.95 (m, 2*H*, in CH_2 in ring), 8.04 (br, *NH*), 4.20 (q, 2*H*, OCH_2CH_3), 1.28 (t, 3*H*, OCH_2CH_3), 4.39–4.56 (m, 1*H*, $CH(CH_3)CH_2CH_3$), 1.13–1.22 (m, 2*H*, $CHCH_2CH_3$), 0.89 (t, 3*H*, $CH(CH_3)CH_2CH_3$), 2.89–2.93 (m, 1*H*, $CH(CH_3)CH_2CH_3$), 1.08 (q, 3*H*, $CH(CH_3)CH_2CH_3$) and *m/z* 396
AR-19	3328 (NH str. of amide), 3031 (aromatic CH str.), 2960, 2906 (aliphatic CH str.), 1723 (C=O str. of ester), 1646 (amide I), 1554 (amide II), 1438, 1390 (CH bend, aliphatic), 1296 (C–O str. of ester)	7.26–7.85 (m, 5*H*, aromatic ring), 6.57 (d, 1*H*, CH in ring), 5.56 (d, 1*H*, CH in ring), 3.87 (t, 2*H*, CH_2 in ring), 2.81–2.98 (m, 2*H*, CH_2 in ring), 8.02 (br, *NH*), 4.20 (q, 2*H*, OCH_2CH_3) 1.30 (t, 3*H*, OCH_2CH_3), 2.70 (d, 2*H*, $CHCH_2$), 4.78–4.99 (m, 1*H*, $CHCH_2$) and *m/z* 425
AR-20	3296 (NH str. of amide), 3015 (aromatic CH str.), 2960, 2914 (aliphatic CH str.), 1738 (C=O str. of ester), 1661 (amide I), 1562 (amide II), 1438, 1376 (CH bend, aliphatic), 1269 (C–O str. of ester)	7.25–7.85 (m, 5*H*, aromatic ring), 6.57 (d, 1*H*, CH in ring), 5.59 (d, 1*H*, CH in ring), 3.82 (t, 2*H*, CH_2 in ring), 2.87–3.12 (m, 2*H*, CH_2 in ring), 7.85 (br, *NH*), 4.18 (q, 2*H*, OCH_2CH_3), 1.28 (t, 3*H*, OCH_2CH_3), 2.92 (t, 2*H*, $CHCH_2CH_2$), 2.21–2.64 (m, 2*H*, $CHCH_2CH_2$), 4.44–4.97 (m, 1*H*, $CHCH_2CH_2$) and *m/z* 430

was used and retention time and peak area were noted at 247 nm for KC prodrugs.

2.5.3.2. Method for the hydrolysis rate determination of prodrugs in 80% human plasma (pH 7.4). A solution of 10 mg of prodrug was prepared in acetonitrile (2 mL) and was added to 88 mL of 80% human plasma (pH 7.4, prepared by mixing 80 portion of plasma with 20 portion of phosphate buffer pH 7.4). An aliquot of 15 mL of this solution was withdrawn and kept in test tubes maintained at 37 ± 0.5 °C. At a definite interval of time (10, 20, 30, 40, 50 and 60 min) an aliquot of 225 μL was withdrawn from different boiling tubes and was transferred to microcentrifuge tubes (Eppendrof's tube). 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 (6000 rpm) for 5 min. Clear supernatant (20 μL) obtained from each tube was then injected in to run through HPLC column (C_{18} ODS reversed phase). Mobile phase acetonitrile:-water 50:50 was used for KC and prodrugs. UV detector was used and retention time and peak area were noted at 247 nm for the prodrugs.

2.6. Pharmacological evaluations

All the synthesized prodrugs along with KC were evaluated for analgesic, anti-inflammatory activities and ulcerogenic index. The prodrugs were compared with KC for these activities. The methods employed for this purpose were as follows.

2.6.1. Anti-inflammatory activity

The anti-inflammatory activity of synthesized prodrugs was determined by hind paw edema method utilizing carrageenan as phlogistic agent (0.1 mL, 1%). The animals used were *Wistar rats* (albino rats). Rats (100–200 g) were divided into seven groups, each comprising of three rats, including a control and standard group. The initial volume of right hind paw of albino rats was measured by plethysmometer, without administration of the drug/prodrug.

2.6.2. Analgesic activity

The analgesic activity of the synthesized prodrugs was determined by tail flick method using thermal stimulus. A hot water analgesiometer was used for the determination of pain threshold of *Wistar rats* (albino rats). Cold water was circulated through the water jackets of the instrument to avoid the heating of the area around the hot wire. Rats (100–200 g) were divided into seven groups, each comprising of three rats. The rat was placed in a holder through which the tail of the rat was protruded out. The normal reaction time, i.e. the time taken to flick the tail was noted. The current was adjusted so that more than 90% rats flick the tail within range of 5–9 s. Animals showing delayed response were rejected. The drug/prodrug (dose of each prodrug was calculated to equivalent to 1.2 mg/kg body weight) was administered orally in 1% suspension of sodium carboxymethylcellulose. Percent analgesia was calculated with the formula:

$$\%\text{Analgesia} = [1 - (T_2/T_1)]100$$

where T_1 is the reaction time in second before administration of drug/prodrug, and T_2 is the reaction time in second after administration of drug/prodrug.

2.6.3. Ulcerogenic index

Gastrointestinal toxicity of the synthesized prodrugs was measured and compared with the drug by measuring ulcerogenic index. For the purpose male albino rats (*Wistar rats*) were selected, weighing between 130 and 150 g, the rats were divided into 23 groups each comprising of three rats, including a control and standard group. The prodrug/drug (100 mg) was suspended in 100 mL of 2% w/v suspension of acacia. Measured volume of the suspension containing dose equivalent to 1.2 mg/kg of body weight of KC 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 2×2 binocular magnifier. Ulcers greater than 0.5 mm were recorded.

3. Results and discussion

Schematic representation of the reaction used for the synthesis is given in Scheme 1. Acid chloride of the KC could not be prepared using any of the reported methods. In all attempts a white sticky mass was observed during the reaction, possibly due to highly thermo-sensitive nature of the drug. Acylation of amino acid ester was performed using various conditions, viz. in the presence of 5% K_2CO_3 at 0–2 °C, non-aqueous acylation reaction in presence of pyridine, triethylamine and acylation reaction in presence of excess pyridine. All these methods suffer with the drawback of hydrolysis of acid chloride, slow rate of reaction and difficulty in the collection of final product due to lump formation. The acylation of amino acid ester using anhydride (formed by reaction of drug with DCC in 2:1 ratio) suffers with the problem of poor yield. When equimolar drug and DCC were used, it gave satisfactory results. Recrystallisation of the compounds was performed by selective precipitation with water–alcohol mixture. In some cases the oily products were obtained which were crystallized by induced crystallization using *n*-hexane and/or petroleum ether in the ethanolic solution of the prodrug. Purity of synthesized prodrugs was ascertained by TLC using silica gel G and physical properties are shown in Table 2. Estimation of percentage nitrogen, IR, NMR and mass spectral analyses were in confirmation of desired structure.

The synthesized prodrugs of KC were subjected to solubility, partition coefficient, dissolution and hydrolytic studies. Solubility studies showed that only KC was found highly soluble in 0.1 N NaOH. On contrary every synthesized prodrug was found slightly soluble in 0.1 N NaOH. Both drugs and all prodrugs were found insoluble in water and in 0.1 N HCl. But they showed moderate to high solubility in various solvents such as methanol, ethanol, chloroform, dichloromethane and benzene. The greater solubility of the standard drug KC is mainly due to presence of free carboxyl group, which forms sodium salt and makes the compound ionic. But all prodrugs and standard drug showed moderate to high solubility in various organic solvents, which indicate lipophilic behaviour of the compounds. FTIR, ^{1}H NMR and mass spectroscopic data of the synthesized compounds are listed in Table 3. These data are in conformity with the structure.

The partition coefficient of the drug in $CHCl_3$–PBS (pH 7.4) was found as 6.022. The partition coefficient (K) value of prodrugs of KC, viz. AR-11, AR-12, AR-13, AR-19 and AR-20 was found as 13.81, 15.61, 15.33, 18.26 and 18.08; K value of AR-14, AR-15, AR-16, AR-17 and AR-18 was found as 21.42, 23.18, 20.0, 23.09 and 23.87. The partition coefficient in $CHCl_3$–acidic buffer (pH 1.4) of KC was 75.62. The partition coefficients of prodrugs of KC, viz. AR-11, AR-12, AR-13, AR-19 and AR-20 were found as 215.9, 256.73, 250.25, 330.12 and 319.5. The partition coefficients of AR-14, AR-15, AR-16, AR-17 and AR-18 were found as 429.90, 504.05, 380.6, 640.0 and 656.8. Partition coefficient study of prodrugs showed that the major fraction of the prodrugs was partitioned towards the organic phase. It indicates enhancement in the lipophilic property, which might be favorable to biological absorption.

The dissolution studies revealed that prodrugs did not show any dissolution in SGF (pH 1.2), which might be useful in minimizing the GIT disturbances, however, this may retard absorption of the prodrugs reverse of postulation of higher

absorption due to very high partition coefficients. Prodrugs showed reduced dissolution rate as compared to KC due to enhancement in lipophilicity in SIF (pH 7.4), however, enough to get absorbed easily. The result of dissolution study showed that prodrugs AR-11, AR-12, AR-13, AR-17, AR-19, and AR-20 dissolved more than 25% in 2 h.

The protein binding of ketorolac prodrugs, viz. AR-12, AR-13 was found as 18.9 and 37.9%, respectively; and in prodrugs AR-20, AR-19, AR-17, AR-11, AR-15, AR-18, AR-16 and AR-14 protein binding was found from 45 to 79.8%. Prodrugs showed comparatively very low protein binding in comparison to standard drug. This will increase availability of the prodrug for hydrolysis in plasma and the required dose will be less.

Hydrolytic studies of the prodrugs were carried out in simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4) and in 80% human plasma (pH 7.4). The $t_{1/2}$ value of ketorolac prodrugs, viz. AR-14 was found as 178.85; the $t_{1/2}$ value of AR-17, AR-16, AR-13 and AR-12 was found between 170 and 160 min and it was found in between 154 and 128 in case of AR-18, AR-15, AR-19, AR-20 and AR-11. Hydrolytic pattern of these prodrugs is depicted in Fig. 1. Hydrolytic studies of prodrugs in 80% human plasma (pH 7.4) were carried out. The $t_{1/2}$ values of AR-17 and AR-12 were found as 61.11 and 59.20 min, respectively; $t_{1/2}$ values of AR-13 and AR-14 were found as 52.90 and 49.50 min, respectively; prodrugs AR-16, AR-11 and AR-18 showed $t_{1/2}$ values from 41 to 39 min and the $t_{1/2}$ values of AR-15, AR-20 and AR-19 were found from 35 to 27 min. Comparative pattern of hydrolysis in 80% human plasma is shown in Fig. 2.

The amount of KC regenerated on hydrolysis (in SIF, pH 7.4) of KC prodrugs, viz. AR-11 and AR-20 was found as 22.14 and 20.13%, respectively; in prodrugs AR-19, AR-15, AR-12 and AR-13 the amount of regenerated drug was found from 17.7 to 13.8%; and in prodrug AR-17, AR-14, AR-18 and AR-16 the amount of regenerated drug was found from 10.25 to 5.92%.

The amount of KC regenerated on hydrolysis (in 80% human plasma, pH 7.4) of KC prodrugs, viz. AR-19 and AR-18 was found as 64.32 and 64.17%, respectively; in prodrugs AR-15, AR-18 and AR-14 the amount of regenerated drug was found from 59.32 to 52.75% and in prodrugs AR-13, AR-14, AR-11, AR-12 and AR-17 the amount of regenerated drug was found from 39.03 to 31.66%.

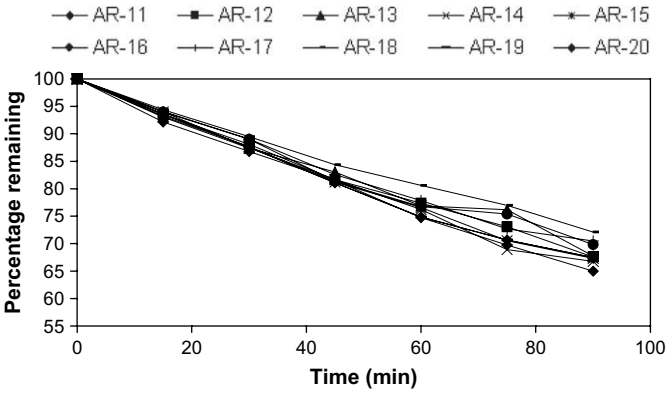


Fig. 2. Comparative pattern of hydrolysis of ketorolac amide prodrugs in 80% human plasma.

None of prodrugs showed hydrolysis in SGF (pH 1.2). Satisfactory hydrolysis was observed in SIF (pH 7.4) and the regeneration of drug was found from 14 to 63%. All prodrugs showed very encouraging hydrolysis rate in 80% human plasma (pH 7.4) and the regeneration of the active drug was found from 63 to 93%. All prodrugs followed first order kinetics. Dissolution profiles of synthesized prodrugs of ketorolac are shown in Fig. 3.

Pharmacological investigations of the synthesized prodrugs were done for anti-inflammatory, analgesic and ulcerogenic activities. In each study albino rats of either sex weighing between 100 and 200 g were used. The dose of drug/prodrug in 1% CMC suspension was given through oral route. The dose administered of prodrugs of KC was equivalent to 2.5 mg/kg body weight of KC. Anti-inflammatory, analgesic and ulcerogenic activities of the synthesized prodrugs were determined. The anti-inflammatory activities obtained after 2 and 6 h of the administration of standard drug KC were found as 64.24, and 54.21%. The percentage anti-inflammatory activity after 2 h administration of ketorolac prodrugs, viz. AR-20, AR-19 and AR-15 was found between 48.7 and 44%; prodrugs AR-18, AR-11, AR-16 and AR-13 showed anti-inflammatory

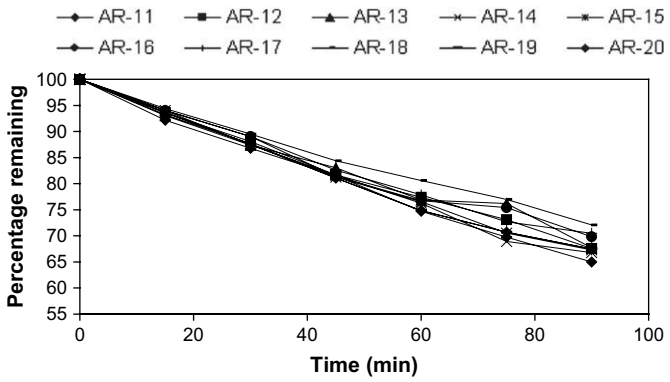


Fig. 1. Comparative hydrolytic pattern of amide prodrugs in simulated intestinal fluid of pH 7.4.

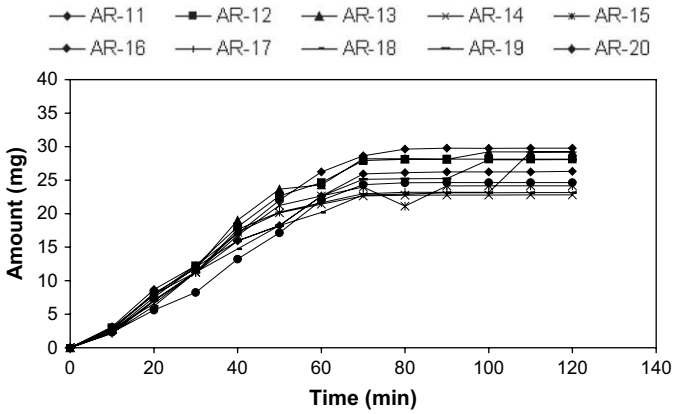


Fig. 3. Dissolution profiles of synthesized prodrugs of ketorolac.

activities from 41 to 33% and inhibition in AR-14, AR-17 and AR-12 was found from 30 and 27%. The anti-inflammatory activity of ketorolac prodrugs after 6 h administration, viz. AR-19 was found as 57.72%; in prodrugs AR-15, AR-20, AR-15, AR-11, AR-16, AR-18 and AR-13 inhibition was found from 54 to 49% and inhibition was found from 45 and 43% in AR-14, AR-17 and AR-12. The analgesic activity of synthesized prodrugs, viz. AR-20, AR-19 and AR-15 was found from 59 to 54%; prodrug AR-18, AR-16 and AR-11 showed analgesia from 47 to 36% and analgesic effect in prodrugs AR-14, AR-17 and AR-12 was found from 30 to 26% of KC. The analgesic activity of synthesized prodrugs of KC, viz. AR-20, AR-19 and AR-15 was found from 59 to 54%; prodrugs AR-18, AR-16 and AR-11 showed analgesia from 47 to 36% and analgesic effect in prodrugs AR-14, AR-17 and AR-12 was found from 30 to 26%. Ulcerogenic index of the synthesized prodrugs was recorded to observe the extent of gastrointestinal side effects. The ulcerogenic index of standard drug KC was found as 23.2. Comparative ulcerogenic index of the synthesized prodrug is shown as bar diagram in Fig. 4.

Characterization of the prodrugs establishes that the prodrugs had been formed in pure form and conforms to expected properties. Spectroscopic data are suggestive of the formation of desired prodrugs. Biopharmaceutical studies revealed that the prodrugs have lesser protein binding and better absorption but slow onset of action. After prodrugs administration all the prodrugs shown lesser anti-inflammatory activity in the initial phase, but after some time the activity was increased gradually. After 6 h of administration, prodrugs showed nearly equal effect in comparison to standard drug. This might be due to various factors including hydrolysis rate, dissolution rate, etc. Similar pattern of results was obtained in analgesic activity, which might be due to same factor involved in the anti-inflammatory activity. Ulcerogenic index of the prodrug was found much lesser in comparison to standard drug. The minimized side effects obtained in the prodrug might be due to inhibition of direct contact of carboxyl group of the drug to the gastric mucosa, which is mainly responsible for the damage. It is also due to negligible dissolution as well as hydrolysis in acidic buffer (pH 1.2).

On the basis of the results, it is concluded that prodrug approach can be successfully applied in attaining the goal of minimized gastrointestinal toxicity without loss of desired anti-inflammatory and analgesic activities of the drug. The good pharmacological response indicates that the absorption of the prodrugs might be regulated by some other means like presence of amino acid transport system besides dissolution.

Out of all the synthesized compounds, prodrug, viz. AR-11, AR-19 and AR-20 showed excellent pharmacological response and encouraging hydrolysis rate both in SIF and in 80% human plasma. On contrary prodrugs with increased aliphatic side chain length showed enhanced partition coefficient but diminished dissolution and hydrolysis rates. Such prodrugs can be considered for sustained release purpose.

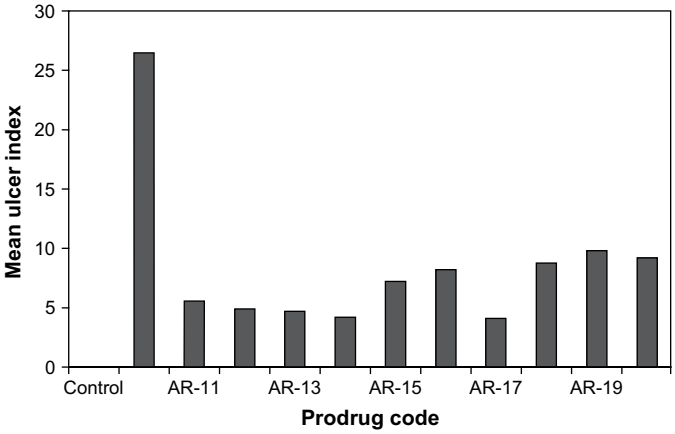


Fig. 4. Ulcerogenic indices of ketorolac and its amide prodrugs.

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