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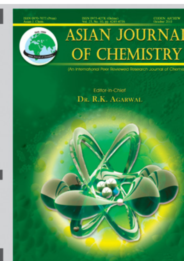

Synthesis, Characterization and Pharmacological Evaluation of Amino Acid Conjugates of Ketoprofen

N. DUBEY*, D.K. JAIN, U.S. BHADORIA and S. SINHA

College of Pharmacy, IPS Academy, Indore-452 012, India

*Corresponding author: Fax: +731 4041627; E-mail: nitindubeympharm@yahoo.com

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Ketoprofen is used for its antipyretic, analgesic and antiinflammatory properties by inhibiting cyclooxygenase-1 and cyclooxygenase-2 enzymes reversibly, which decreases production of proinflammatory prostaglandin precursors. Ketoprofen suffers from the general side effects of nonsteroidal antiinflammatory drugs, owing to presence of free carboxylic acid group. The study aimed to retard the adverse effects of gastrointestinal origin. Different conjugates of ketoprofen have been synthesized by amidation with methyl esters of amino acids namely, phenylalanine, lysine, arginine, glycine, cysteine, valine, glutamine, serine, proline and alanine. Synthesized conjugates were characterized and evaluated for analgesic and antiinflammatory activities.

Key Words: Ketoprofen, Amino acid conjugates, Analgesic, Antiinflammatory activity.

INTRODUCTION

Ketoprofen, one of non-steroidal antiinflammatory drugs, could not be used as up to its potential, because of its adverse reactions offered due to presence of free carboxylic acid group[1]. The non-steroidal antiinflammatory drugs are widely used for indications extending from inflammation and pain to cardiovascular and genitourinary diseases. Gastrointestinal side effects constitute the most frequent of all the adverse reactions of non-steroidal antiinflammatory drugs and often these reactions lead to gastrointestinal tract ulceration and hemorrhage[2,3]. Gastrointestinal mucossal injury produced by non-steroidal antiinflammatory drugs is generally believed to be caused by two different mechanisms. The first mechanism involves a local action composed of a direct contact while the other has indirect effect on the gastrointestinal mucosa. The direct contact effect can be attributed to a combination of a local irritation produced by acidic group of non-steroidal antiinflammatory drugs and local inhibition of prostaglandin synthesis in the gastrointestinal tract. The indirect effect can be attributed to combination of an ion trapping mechanism of non-steroidal antiinflammatory drugs from the lumen into the mucosa. The second mechanism is based on a generalized systemic action occurring after absor-ption, which can be demonstrated following intravenous dosing[4]. Recently, considerable attention has been focused in the develop-ment of bio-reversible derivatives, by temporarily masking the acidic group of non-steroidal antiinflammatory drugs, as a promising mean of reducing the gastrointestinal toxicity[5].

The purpose of this study is to mask the free acidic group by synthesizing its amino acid conjugates and to evaluate its analgesic and antiinflammatory activity.

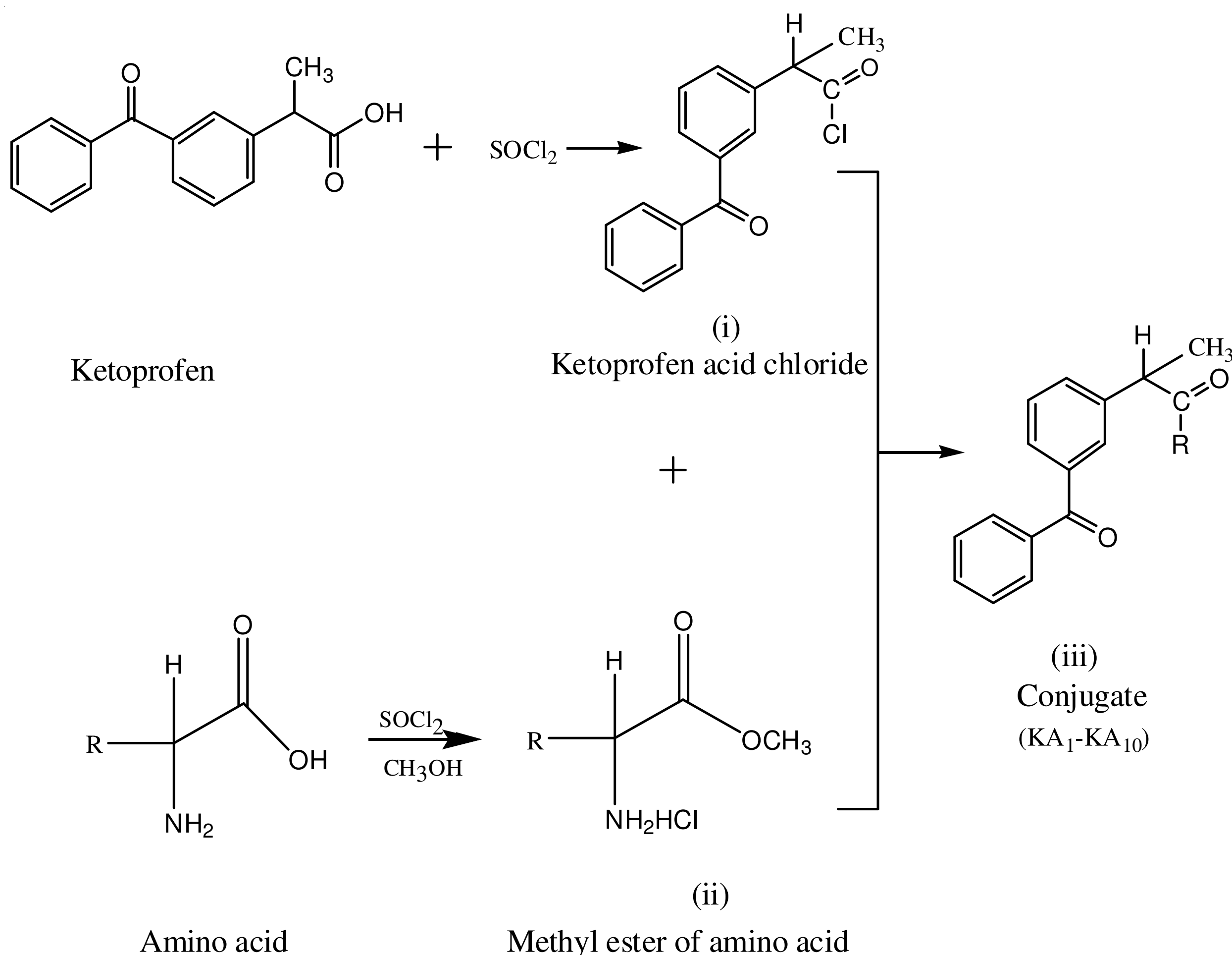
EXPERIMENTAL

All the chemicals used were of AR grade. Ketoprofen was obtained as a gift sample from Alkem Laboratories, Mumbai. The purity of the conjugates was ascertained by thin layer chromatography using iodine vapours as detecting agent. Melting points were determined by melting point apparatus (Biotecnics, 2304).

General procedure

Synthesis of ketoprofen acid chloride (i): 2-(3-Benzoylphenyl)propanoyl chloride; Ketoprofen (1.06 g, 0.005 M) was dissolved in minimum amount of chloroform and thionyl chloride (0.5 mL) was added slowly to it. The mixture was refluxed for 6-7 h at 60-70 ºC with continuous stirring on magnetic stirrer. The viscous liquid obtained was immediately poured on petri dish and dried to produce crude ketoprofen acid chloride (**Scheme-I**).

Synthesis of methyl ester hydrochlorides of amino acids (ii): Thionyl chloride (0.5 mL) was slowly added to methanol (22 mL) with cooling and amino acid (0.68 g, 0.005 M) was added to it. The mixture was refluxed for 6-7 h at 60-70 ºC



Scheme-I for the synthesis of amino acid conjugates of Ketoprofen

with continuous stirring on magnetic stirrer. The resulting solid product was collected, dried and re-crystallized from hot methanol followed by cooling. The purified crystals were collected and washed twice with ether: methanol mixture at 5:1 ratio followed by pure ether and dried to get pure amino acid methyl ester hydrochloride (**Scheme-I**).

Synthesis of conjugates of ketoprofen with amino acids (iii): Ice cold, aqueous sodium hydroxide solution (5 %, 5 mL) was taken in 250 mL beaker and methyl ester of amino acid (**ii**) (0.68 g, 0.005 M) was added to it. The reaction mixture was mechanically stirred for 0.5 h at room temperature, after which the beaker transferred to an ice bath kept on mechanical stirrer. Ketoprofen acid chloride (**i**) (1.36 g, 0.005 M) was added in small portions with continuous stirring for 6-7 h. The solid that separated out was filtered off. The crude prodrug was re-crystallized from methanol (**Scheme-I**).

Detection Method: The purity of the conjugates was ascertained by thin layer chromatography using mobile phase [chloroform:methanol:ammonia (5:2:3), water: *n*-propanol (5:5), water: *n*-propanol (3:7)] and iodine vapours as detecting agent. Melting points were determined by digital melting point apparatus (Biotecnics, 2304). ^{1}H NMR spectra were recorded on Jeol AL 300 FT-NMR using cadmium chloride as solvent. FT-IR spectra were recorded on Perkin Elmer 1600 (Japan) and mass spectra were recorded on Jeol SX-102 attached with 991 photodiode array detector (Japan).

Biological evaluation

***In-vitro* activity of synthesized conjugates**

a) Inhibition of protein denaturation: The reaction mixture (0.5 mL) consisted of 0.45 mL bovine serum albumin (5 % aqueous solution) and 0.05 mL of synthesized conjugates, pH was adjusted at 6.3 using a small amount of 1 N HCl. The samples were heated at 57 ºC for 3 min. After cooling the samples, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm. For control, 0.05 mL distilled water was used instead of conjugates, while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percent inhibition} = \frac{100 - (\text{O.D. of test} - \text{O.D. of product control})}{\text{O.D. of control}} \times 100$$

b) Effect on membrane stabilization: The reaction mixture was (4.5 mL) consisted of 2 mL hypotonic saline (0.25 % NaCl), 1 mL 0.15 M phosphate buffer (pH 7.4) and 1 mL solution of the synthesized conjugates in normal saline. 0.5 mL of 10 % rat RBC in normal saline was added. For control tests, 1 mL of isotonic saline was used instead of solution of the synthesized conjugates solution, while product control tests lacked red blood cells. The tubes were cooled under running tap water for 20 min. The mixtures were centrifuged and the absorbance of the supernatants read at 560 nm. Per cent membrane stabilizing activity was calculated as follows:

TABLE-1
STRUCTURE AND IUPAC NAME OF THE SYNTHESIZED CONJUGATES

Conjugate	Structure of conjugates	IUPAC name of conjugates
KA_1		Methyl 2-[2-(3-benzoylphenyl) Propanamido]-3-phenyl propanoate
KA_2		Methyl 6-amino-2-[2-(3-benzoyl phenyl)propanamido]hexanoate
KA_3		Methyl 2-[2-(3-benzoylphenyl)-propanamido]-5-guanidino pentanoate
KA_4		Methyl 3-[2-(3-benzoylphenyl) propanamido]-2-oxopropanoate
KA_5		Methyl 2-[2-(3-benzoylphenyl) propanamido]-3-mercapto propanoate
KA_6		Methyl 2-[2-(3-benzoylphenyl) propanamido]-3-methylbutanoate
KA_7		Methyl 2-amino-5-[2-(3-benzoyl phenyl)propanamido]-5-oxopentanoate
KA_8		Methyl 2-[2-(3-benzoylphenyl) propanamido]-3-hydroxy propanoate
KA_9		Methyl 4-[2-(3-benzoylphenyl) propanoyl]pyrrolidine-2-carboxylate
KA_{10}		Methyl 2-[2-(3-benzoylphenyl) propanamido]propanoate

TABLE-2
PHYSICAL CONSTANTS AND SPECTRAL DATA OF SYNTHESIZED CONJUGATES OF KETOPROFEN

Conjugate code	m.f.	m.w.	m.p. (ºC)	R_f value	IR spectra (cm^{-1})	Mass spectra	^1H NMR spectra
KA_1	$C_{26}H_{25}NO_4$	415	305-310	0.67	3296, 2980, 1708, 1651, 1508, 1371	415.14	7.31-7.70 (m, 6H, aromatic ring), 7.12 (d, 1H, CH in ring), 7.21 (d, 1H, CH in ring), 7.08 (s, 1H in ring), 3.29 (s, CH_2 in ring), 8.0 (NH), 4.8 (s, NH)
KA_2	$C_{23}H_{28}N_2O_4$	396	280-285	0.73	3853, 2879, 2733, 1697, 1651	395.6	7.31-7.70 (m, 7H, aromatic ring), 3.67 (d, 1H, CH in ring), 3.89(q, 2H, OCH_3), 1.52 (t, 2H, CH_2 in ring), 8 (NH), 4.42 (t, 2H, CH_2 in ring), 2 (NH)
KA_3	$C_{23}N_{28}N_4O_4$	424	300-305	0.69	3832, 2879, 2733, 1697, 1651	423.4	7.31-7.70 (m, 6H, aromatic ring), 3.89 (t, 2H, OCH_3 in ring), 1.52 (t, 2H, CH_2 in ring), 8 (NH)
KA_4	$C_{20}H_{19}NO_5$	353	310-315	0.81	3387, 3066, 2922, 1599, 1560, 1319, 1271	352.6	7.31-7.56 (m, 6H, aromatic ring), 3.89 (t, 2H, CH_2 in ring), 1.52 (t, 3H, OCH_3), 8 (NH), 3.67 (d, H, CH_2 in ring)
KA_5	$C_{20}H_{21}NO_4S$	371	330-335	0.83	3855, 2918, 2852, 1699, 1658, 1585 1585, 1491, 1408	370.4	7.31-7.70 (m, 6H, aromatic ring), 3.88 (t, 2H, CH_2 in ring), 1.54 (t, 3H, OCH_3), 7.8 (NH), 3.70 (d, H, CH_2 in ring)
KA_6	$C_{22}H_{25}NO_4$	367	330-335	0.79	3061, 2980, 2735, 1697, 1651, 1599	366.8	7.29-7.75 (m, 6H, aromatic ring), 7.16 (d, 1H, CH in ring), 7.25 (d, 1H, CH in ring), 7.1 (s, 1H in ring), 3.62 (s, CH_2 in ring), 8.2 (NH), 4.4 (s, NH)
KA_7	$C_{22}H_{24}N_2O_5$	396	340-345	0.84	3736, 2978, 2739, 1699, 1599, 1514	395.6	7.31-7.74 (m, 6H, aromatic ring), 7.14 (d, 1H, CH in ring), 7.28 (d, 1H, CH in ring), 7.18 (s, 1H in ring), 3.26 (s, CH_2 in ring), 7.8 (NH)
KA_8	$C_{21}H_{24}NO_5$	370	310-315	0.83	3454, 3061, 2733, 1697, 1597, 1446	370.14	7.26-7.80 (m, 6H, aromatic ring), 7.11 (d, 1H, CH in ring), 7.32 (d, 1H, CH in ring), 7.28 (s, 1H in ring), 3.67 (s, CH_2 in ring), 7.9 (NH), 4.5 (s, NH)
KA_9	$C_{22}H_{23}NO_4$	365	290-295	0.72	3483, 2978, 1699, 1656, 1597, 1446	365.14	7.32-7.79 (m, 6H, aromatic ring), 7.22 (d, 1H, CH in ring), 7.08 (s, 1H in ring), 3.22 (s, CH_2 in ring), 7.8 (NH), 3.9 (s, NH)
KA_{10}	$C_{20}H_{21}NO_4$	339	330-335	0.74	3508, 2729, 2640, 1705, 1651, 1575, 1419	339.16	7.33-7.72 (m, 6H, aromatic ring), 7.34 (d, 1H, CH in ring), 7.45 (d, 1H, CH in ring), 7.68 (s, 1H in ring), 3.62 (s, CH_2 in ring), 8.0 (NH), 4.72 (s, NH)

R_f^* value in solvent system: For KA_1, KA_3, KA_4, KA_6 – chloroform : methanol : ammonia (5:2:3); For KA_2, KA_5, KA_8 – water : *n*-propanol (5:5); For KA_7, KA_9, KA_{10} – water : *n*-propanol (3:7)

$$\% \text{ Stabilization} = \frac{100 - (\text{O.D. of test} - \text{O.D. of product control})}{\text{O.D. of control}} \times 100$$

***In vivo* activity**

a) Analgesic activity: The analgesic activity of the synthesized conjugates was determined by tail flick method using thermal stimulus. Wistar rats (100-200 g) were divided into twelve groups including standard and control, comprising of three rats each. The rats were placed in a holder through which the tail of the rat was protruded out. The current was adjusted so that more than 90 % rats flick the tail within range of 5-9 s. The drug/conjugate (dose of each conjugate was calculated equivalent to 20 mg/kg body weight) was administered orally in 1 % suspension of sodium carboxymethylcellulose. In all cases control received the same quantity of sodium carboxymethylcellulose.

$$\text{Per cent analgesia} = [1-(T_2/T_1)]100$$

where, T_1 was the reaction time in second before administration of drug/conjugate and T_2 was the reaction time in second after administration of drug/conjugate[6].

b) Antiinflammatory activity[7]: The antiinflammatory activity of synthesized conjugates was determined by hind paw edema method utilizing carrageenan as phlogistic agent in saline solution and was injected subcutaneously in the right hind paw of rats. (0.1 mL, 1 %). Wistar rats (100-200 g) were divided into 12 groups including standard and control, comprising of three rats each. The initial volume of right hind paw of albino rats was measured by vernier caliper, without administration of the drug/conjugate. The drug and its conjugates were suspended in saline solution and administered (20 mg/kg body weight of rat) orally 1 h before the carrageenan injection. The control group was treated with distilled water. The zone of inflammation was measured at 0.5, 1 and 2 h after carrageenan injection using vernier caliper.

RESULTS AND DISCUSSION

Conjugates of ketoprofen with 10 different amino acids were synthesized *i.e.* phenylalanine, lysine, arginine, glycine, cysteine, valine, glutamine, serine, proline and alanine. Structure of all the synthesized conjugates (Table-1) have been established on the basis of their physical characteristics data and their IR, ^1H NMR and mass spectral data[8] (Table-2). The synthesized conjugates showed the presence of amide and ester functional group along with the presence of aromatic and aliphatic ring which was also evident in the ^1H NMR spectra of the synthesized conjugates. They were evaluated for their *in vitro* and *in vivo* analgesic and antiinflammatory activity. They showed improved analgesic and antiinflammatory activity over the parent drug[9] (Table-3).

Significant protection in denaturation of proteins and membrane stabilization was shown by KA_1, KA_2, KA_3, KA_4, KA_5, KA_8 and KA_9 conjugate whereas KA_6, KA_7 and KA_{10} conjugate showed poor protection as compared to the standard ketoprofen (Table-3).

Conjugate KA_1, KA_4 and KA_6 showed potent analgesia from 84 to 80 %, conjugate KA_2, KA_8 and KA_9 showed good analgesia from 69 to 67 % and conjugate KA_3, KA_5, KA_7 and

TABLE-3
RESULT OF *IN-VITRO* AND *IN-VIVO* ACTIVITY OF SYNTHESIZED CONJUGATES AND KETOPROFEN

Conjugate Code	Inhibition of protein denaturation (%)	Membrane stabilization (%)	Analgesic activity (%)	Antiinflammatory activity (%)
KA_1	46.6	59.5	83.8	48.8
KA_2	53.3	46.8	67.7	50.4
KA_3	46.6	42.5	45.1	43.8
KA_4	53.3	70.2	80.6	33.3
KA_5	44.4	61.7	48.3	30.3
KA_6	31.3	25.5	83.8	28.4
KA_7	37.7	29.7	48.3	45.9
KA_8	44.4	53.1	67.7	36.2
KA_9	46.6	63.8	68.7	29.6
KA_{10}	24.4	34.4	45.1	33.1
Standard	42.2	40.4	54.8	30.2

KA_{10} showed lower analgesia from 49 to 45 % as compared to standard ketoprofen[10]. Conjugate KA_1, KA_2, KA_3, KA_4, KA_5, KA_7, KA_8 and KA_{10} showed good antiinflammatory activity whereas conjugate KA_6 and KA_9 showed lower antiinflammatory activity as compared to standard ketoprofen (Table-3).

Conclusion

On the basis of the results, it may be concluded that conjugate approach can be successfully applied in attaining the goal of minimized gastrointestinal toxicity without loss of desired analgesic and antiinflammatory activities of the drug.

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