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Synthesis, Biological Evaluation and QSPR Studies of Amino Acid Conjugates of Cinmetacin

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Abstract: Synthesis and biological evaluation of amino acid conjugates of cinmetacin was carried out to improve some pharmacokinetic properties and to minimize some undesirable side effects (especially ulcerogenic effect). Dissolution studies and hydrolysis studies on simulated intestinal fluid (pH 7.4) follow the first order kinetics. The quantitative structure property relationship studies reveals that rate of hydrolysis of the compounds is inversely related to partition coefficient values. The study of acute and chronic anti-inflammatory and ulcerogenic activity gave statistically significant results and it concluded that the compounds minimize the gastric side effects of cinmetacin remarkably.

Key words: Cinmetacin, QSPR, NSAIDS, anti-inflammatory, ulcerogenic

INTRODUCTION

Cinmetacin is a synthetic non-steroidal anti-inflammatory agent that belongs to the class of heteroaryl acetic acid derivatives. The anti-inflammatory activity of cinmetacin is attributed to the inhibition of the enzyme prostaglandin synthetase. In addition to anti-inflammatory activity, cinmetacin also possesses analgesic and antipyretic properties. It is indicated in the management of pain and inflammatory in conditions such as rheumatoid arthritis, ankylosing spondylitis and acute gout (Furst and Munster, 2001). Unfortunately, the clinical use of cinmetacin is seriously limited owing to adverse effects such as gastric ulceration and haemorrhage, the most frequent of all adverse effects pertaining to administration of NSAIDS (Rang *et al*., 1999). It is probably caused by a combination of local irritations produced by the free carboxylic group of NSAIDS and by local inhibition of cytoprotective action of prostaglandins on gastric mucosa (Shanbhag *et al*., 1992). One of the recent approaches for circumventing such therapeutic problems is the concept of drug derivatization or prodrug approach (Hyo-Kyung and Gordon, 2000) that incorporates targeting and metabolic considerations in to the drug design process. The prodrug approach, a chemical approach using reversible derivatives, is often useful in the optimization of the clinical application of a drug and improving the therapeutic properties of a wide variety of drugs through development of prodrugs or soft drugs. In conjunction with the aforementioned, the present study examines the applicability of produg approach to minimize the GIT adverse effects and improve the physiochemical properties of cinmetacin.

Amino acid conjugates of NSAIDS have been proved useful as prodrugs at many instances (Dhaneshwar and Chaturvedi, 1994; Eric *et al*., 1994; Persico *et al*., 1988). For example, glycine amides of ketoprofen and several other well-known NSAIDs are significantly less irritating to gastric mucosa, while their anti-inflammatory activities were comparable to their parent drugs (Dhaneshwar and Chaturvedi, 1994). Experimental studies by Okabe *et al*. (1974 and 1976) suggested that several amino

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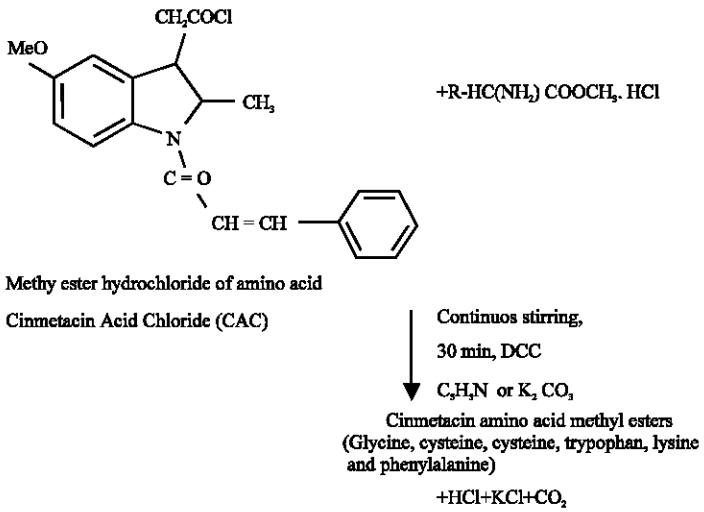

Fig. 1: Synthesis of cinmetacin amino acid conjugates. Acylation of amino acid methyl ester hydrochloride with cinmetacin acid chloride

acids are quite potent inhibitors for aspirin induced mucosa injury. Furthermore, amino acids are non-toxic and valuable dietary supplements. Related to the foregoing, the present study describes the synthesis, physicochemical properties and hydrolytic studies of amino acid conjugates of cinmetacin for potential use as prodrugs with improved therapeutic profile.

Chemistry

Amino acid conjugates of the cinmetacin were synthesized by conversion of acid group of drug to acid chloride using phosphorous pentachloride with continuous stirring at 40-45°C for 5 min. Alcoholic thionyl chloride and amino acid (Mann and Saunders, 1986, March, 2003) were refluxed for 6-14 h at 60-70°C with continuous stirring on a magnetic stirrer by Ronald's method to obtain methyl ester hydrochloride of amino acid. Acid chloride of drug and methyl ester hydrochloride of amino acid formed an amide bond between the drug and the amino acid with continuous stirring for 6 h in ice cooled basic solution (Silverstein *et al*., 1998) (Fig. 1).

MATERIALS AND METHODS

Materials

Synthetic grade chemicals were used for the synthesis of amino acid conjugates and analytical grade chemicals were used for analytical works.

Methodology for Synthesis of Compounds

Synthesis of Cinmetacin Conjugate of Amino Acids

Ice cooled, 10% w/v potassium carbonate solution (150 mL) or pyridine 3 mL (to dissolve pyridine 20 mL benzene) was taken in a 250 mL beaker and CDME (1.647 g) or TME (2.673 g) or CME (2.104 g) or CGME (2.45 g) or CLME (2.25 g) or CPME (1.4 g) was added to it. The reaction mixture was stirred for 30 min at room temperature. The reaction temperature was then reduced and maintained at 10°C. Cinmetacin acid chloride (5 g) was added, in small portions with continuous stirring. The reaction mixture was stirred for 4-6 h (for CGME, CLME and CPME, the reaction mixture was refluxed for 25-40 h at 60-70°C). The compound, so obtained was washed with 0.5% cold potassium carbonate and then recrystallized from methanol.

Dissolution Study

Dissolution studies were performed on USP XXI dissolution rate test apparatus using simulated gastric fluid (50 mL of potassium chloride (0.2 M) and hydrochloride at pH 1.2) and simulated intestinal fluid (pH 7.4). Conjugated derivatives remain as such in simulated gastric fluid up to 2 h, but the synthesized compounds attain a peak concentration after 60 min in simulated intestinal fluid. The hydrolysis studies were done in simulated intestinal fluid (pH 7.4) (USP, 2003). The amount of drug released and the remaining amount of product was estimated quantitatively by reverse phase HPLC (model Shimadzu SPD-6AV) using RP5C-18 column and methanol: phosphate buffer (70:30) as mobile phase with 1 ml/min flow rate and UV 290 nm detector.

Screening of Biological Activity

Anti-inflammatory Activity (Acute)

The suspension of test compounds was prepared in distilled water using 2% gum acacia and in all cases; control received the same quantity of gum acacia. Anti-inflammatory activity was evaluated by carrageenin-induced rat paw oedema method of Winter *et al.* (1962). Six albino rats of either sex weighing about 100-150 g, were randomly distributed in control as well as in experimental group. At 0 h, the test compounds were administered orally and after half an hour, carrageenin (0.05 mL, 1%) was injected in to the planter tissue of paw using 26 guage needle. The paw oedema was measured, before and at regular intervals of 1 h, for four hours after the injection of carrageenin (Winter *et al*., 1962).

Cotton Pellet Implantation Method: (Chronic)

The effect of drugs on chronic inflammation was studied by employing CPIM. The pellets of surgical cotton wool weighing 100 mg were sterilized in an autoclave at 15 lb pressure at 121°C for 30 min. These sterilized pellets were aseptically implanted subcutaneously in both the groins of each rat, under ether anesthesia. Drugs were administered once daily for seven days. The rats were sacrificed on the eighth day and the pellets were taken out. After removal of subcutaneous fat, hair and all the extraneous tissues, the pellets were dried overnight at 60°C in hot air oven and weighed. The amount of granulation was compared with that of control. The percent inhibition of granulation or percent anti-inflammatory activity was calculated by the following formula .

$$\text{Percent anti-inflammatory activity} = (1-(Vt/Vc))*100 \quad (1)$$

Vt =Mean weight of granulation tissue in drug treated group

Vc = Mean weight of granulation tissue in controlled group

Ulcerogenic Index

Six albino rats of either sex weighing about 100-150 g were randomly distributed in test as well as standard group. They were starved for 18 h. Drugs were administered orally once daily for seven days. The controls were fed only 2% gum acacia suspension. The rats were sacrificed on the eighth day and stomachs were removed and fixed in 10% formalin. Each stomach was clamped with hemostats at the oesophagal and pyloric ends and inflated with 8-10 mL of air introduced using a syringe fitted with a 26 gauge needle. After 2-5 min, the stomachs were opened along the greater curvature and number of lesions was examined by means of 2×2 binocular magnifier. The lesions (sheding of epithelium, petechial hemorrhages, one or two small ulcers, many ulcers and perforated ulcer) were considered to be positive for ulcerogenic response (Shrivastava *et al*., 2003a,b). The ulcerogenic index was found out by:

$$\text{Ulcerogenic index} = \text{number of ulcers} + \text{Ulcer Score} + \%\ \text{Incidence/Number of Animals.} \quad (2)$$

Quantitative Structure Property Relationship Study (QSPR)

Our attempt was to synthesize compounds with practically equivalent biological activity as that of the parent compound, i.e., prodrug approach. It was subjected to QSPR analysis by relating the determined physicochemical constants, i.e., partition coefficient and rate of hydrolysis. QSAR Easy software was used to perform the Multiple Regression Analysis (linear and non linear) (Hansch *et al*., 1990; Gupta and Kapoor, 1995).

RESULTS

Amino acid conjugates of cinmetacin were synthesized in cold basic solution and the yield obtained varied from 65-80%. The physiochemical properties were calculated and are presented in Table 1. The structure of the synthesized compounds were confirmed by elemental analysis, IR and mass spectrometry analysis (Leffler and Grunwald, 1963). The results obtained for nitrogen estimation (elemental analysis) and molecular weight determination coincided with the calculated values of the compounds (Table 1). The IR spectra obtained from Perkin-Elmer IR Spectrophotometer (Model 841) using KBr pellets showed some significant peaks, which are specific for the amino acid conjugates. Jeol Mass Spectrometer (Model D-300) was used to confirm the molecular weight of the synthesized compounds (Table 1). The solubility in benzene was performed and the concentration of the synthesized compounds was estimated by spectrophotometric method (Shimadzu 160A). It varied between 0.0632-0.2582 as compared to their parent drug 0.9635 mg ml^{-1}. Partition coefficient of the cinmetacin and its amino acid conjugates was determined in octanol/simulated intestinal fluid [(SIF) pH 7.4] system (Table 1).

The half-life of the synthesized conjugates in simulated intestinal fluid and the hydrolysis constant values are given in Table 2. The correlation between hydrolysis constant (k) of the compounds and partition coefficient was determined by QSPR analysis. The relationship obtained through multiple linear and non-linear regression methods are as follows.

Table 1: Physical constants and physico-chemical characteristics of the synthesized compounds

Comp. code	Molecular formula	Molecular weight		M.P. (°C)*	Partition coefficient	Hydrolysis constant (K) in phosphate buffer (pH 7.4)	$T_{1/2} = 0.693/K$
		Calcd.	Found**				
Control	$C_{21}H_{19}NO_4$	349.39	349	165°C	21.2540	--	--
CCME[b]	$C_{25}H_{26}N_2O_5S$	466.55	466	65°C	2.555	0.017	40.76
CCDME[c]	$C_{50}H_{26}N_4O_{10}S_2$	931.09	931	200°C	1.412	0.022	31.50
CTME[d]	$C_{33}H_{40}N_3O_5$	548.59	548	67°C	7.520	0.028	34.75
CGME[e]	$C_{24}H_{24}O_5N_2$	420.39	420	97°C	14.3379	0.014	49.500
CLME[f]	$C_{28}H_{34}O_5N_3$	491.39	491	115°C	9.8957	0.016	43.310
CPME[g]	$C_{31}H_{32}O_6N_2$	510.39	510	145°C	5.2368	0.021	33.000

* Melting point Uncorrected. ** Determined by mass Spectrophotometry. a- Cinmetacin, b- Cinmetacin Cystein methyl ester, c- Cinmetacin Cystine Dimethyl ester, d- Cinmetacin Tryptophan methyl ester, e- Cinmetacin Glycine methyl ester, f- Cinmetacin Lysine methyl ester, g- Cinmetacin Phenyl alanine methyl ester

Table 2: Anti-inflammatory activity (Chronic and Acute) of amino acid conjugates of Cinmetacin

Comp. code	Oral dose (mg kg)$^{-1}$	Percent anti-inflammatory activity (Chronic)	Oral dose (mg kg)$^{-1}$	Percent of anti-inflammatory activity (acute)			
				1 h	2 h	3 h	4 h
CN	20.00	61.03	20.00	38.90	54.50	67.80	66.67
CCME	106.83	56.45	26.71	27.78	45.45	60.71	60.00
CCDME	107.08	59.65	26.54	22.22	45.45	64.29	63.33
CTME	126.18	62.19	31.40	33.33	50.00	67.86	66.67
CGME	ND[a]	ND	24.06	50.64	49.94	49.39	49.03
CLME	ND	ND	28.12	51.6	51.11	50.32	49.10
CPME	ND	ND	29.21	53.7	51.57	50.72	49.32

a: Not determined

Table 3: Ulcerogenic index of amino acid conjugates of cinmetacin on gastric mucosa

Comp. code	Oral dose (mg kg)$^{-1}$	No. of ulcer	Ulcer score	Percent incident (%)	Ulcer index
CN	80.00	9.25	4.82	100.00	30.74
CCME	106.87	3.12	2.23	50.00	13.68
CCDME	107.08	3.02	1.00	16.67	6.80
CTME	126.18	2.62	2.25	33.33	10.43
CGME	96.25	0.75	1.05	31.23	7.005
CLME	112.51	0.89	0.9	33.33	7.345
CPME	116.88	1.26	1.75	51.00	11.51

Linear eq.

$$\text{Log K} = (-0.365455) \log P + (-1.434934). \quad (1)$$
$$n = 6, r = 0.974, R^2 = 0.948, \text{F-test} = 18.351, \text{t-test} = 4.284, \text{SD } 0.039.$$

Non-linear eq.

$$\text{Log K} = (-0.485044) \log P + (0.077895) \log P^2 + (-1.39453). \quad (2)$$
$$n = 6, R = 0.975, R^2 = 0.950, \text{F-test} = 6.368, \text{t-test} = 2.524, \text{SD} = 0.055.$$

According to Hansch analysis, the partition coefficient is negatively correlated with the rate of hydrolysis in linear and non-linear method.

The anti inflammatory activity of the synthesized compound was determined by Carregeenin induced rat hind paw oedema method reported by Winter *et al.* (1962) (Formula 1) for acute and chronic inflammation, respectively (Table 2). Ulcerogenic index was calculated by method described by Robert and Janson (2001) (Formula 2) using albino rat (Table 3).

DISCUSSION

The results obtained from different analytical methods provide the structural conformity of the cinmetacin amino acid conjugate. Nitrogen estimation by micro analytical techniques showed that the percentage of nitrogen in amino acid conjugate differ from the parent cinmetacin, which has one nitrogen atom with the percentage of 3.96%. In CCDME, 6.01% nitrogen was found experimentally give the conclusion of four nitrogen atoms are present in the molecule when compared with the CTME, which showed 7.65% (three nitrogen atoms are present). CGME and CLME have five nitrogen atoms. These results revealed that the amino acid conjugates have more number of nitrogen atoms than cinmetacin.

IR spectrophotometric analysis revealed that the strong N-H stretching of amides at 3402-3417 cm^{-1} and C (=O) NH carbonyl stretching at 1560-1550 cm^{-1} appeared in all the conjugates showed that product is formed. Absence of O-H stretching and C = O stretching bands in acid group confirms the amide linkage has formed in the conjugate. In cinmetacin cystinate methyl ester, the band at 2560 cm^{-1} showed the S-H stretching due to cystine amino acid.

In cinmetacin peak at 349 is due to the parent peak (M^+). The m/z of 420, 491, 510, 466, 931 and 548 in renders information about the molecular weight (M^+) of cinmetacin amino acid conjugates. Fragmentation pattern from the mass spectrometer also confirm the C = O (NH) has formed because only amino acid conjugates given the fragments with R-C = O (NH) pattern. Increase in the molecular weight of the conjugates with respect to the parent drug confirms the expected compound has formed.

Partition coefficient of the cinmetacin and its amino acid conjugates in octanol/simulated intestinal fluid [(SIF) pH 7.4] system showed that glycine, lysine, phenylalanine, cysteine, cystine and tryptophan conjugates of cinmetacin showed that the decrease in their partition coefficient value as

compared with the parent drug. It means the conjugates can ionize in the intestine. It also confirmed by the hydrolysis and dissolution studies of the conjugates in simulated gastric fluid (pH 7.4), which suggest that the conjugate is not ionized in the stomach and is only dissolved in the intestine. This result attained the object through reduce the gastric irritation and the possibility of the ulcer formation by the inhibition of cytoprotective prostaglandin in the gastric mucosa is reduced. The time for the release of drug from the conjugate and amino acid present in the conjugate heals and reduce the ulcers present in the stomach due to parent drug.

The inverse relationship between partition coefficient and hydrolysis constant obtained from the QSPR studies reveals that the rate of hydrolysis is faster in intestine as the compound has lower partition coefficient. It means, as the compound gets hydrolyzed faster, it is absorbed faster since it is less lipophilic. Thus compound would not exert gastrointestinal side effects and can enter the intestine without ionization in stomach.

Anti-inflammatory screening of parent and conjugated drug for 3 h showed that the amino acid release the drug substantially at the intestine and is comparable with the parent drug. Amino acid conjugates showed reduced ulcerogenic index as compared with cinmetacin, which has an ulcer index of 30.74. Cinmetacin produced red haemorrhage, erosion and 1 to 4 score range ulcers in all the rats. This result suggests that the CCDME, CGME and CLME have low ulcer index comparable with the other conjugates. This ulcer index suggests that the amino acid conjugates retain the anti-inflammatory activity of the parent compound at the same time it reduces the ulcer index of the parent compound and the drugs are released in the intestine. The amino acid released from the conjugate having the healing effect of the mucosal membrane, which ultimately reduce the ulcer index. The results of anti-inflammatory and ulcerogenic activity were statistically significant. Thus it is quite natural to presume that one of the serious side effects of nonsteroidal anti-inflammatory drugs that are ulcerogenic activity has been successfully overcome in the synthesized amino acid conjugates of cinmetacin.

From the study, it is concluded that the finding of the work attain the object by reducing the ulcer index with retention of anti-inflammatory activity of the parent drug. The conjugates undergo hydrolysis and dissolution in the intestinal fluid because of reduction in the partition coefficient. The QSPR studies also confirm that the decrease in partition coefficient, increase the hydrolysis rate of amino acid conjugate in intestine. The amino acid conjugated with the highly gastric ionizable carboxylic group of cinmetacin, reduces the partition coefficient and bypasses to intestine and dissociate in the intestine, hence the ulcer formation is reduced. This study may extend to design and improve the pharmacokinetic properties of cinmetacin for target delivery.

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