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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Substituted aminoalcohol ester analogs of indomethacin with reduced toxic effects

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Abstract Synthesis and evaluation of five different *N,N*-disubstituted aminoethanol ester derivatives of indomethacin bearing structural resemblance to the aminoethanol ester class of anticholinergics are reported herein. The anticholinergic activity was incorporated into the intact esters to overcome the gastric toxicity of indomethacin, not only by blocking the acidic functionality but also by decreasing gastric secretions and motility. These derivatives exhibited *in vitro* stability in buffers of pH 2.0 and 7.4 for 6 h and were readily hydrolyzed by human plasma esterases to liberate the parent drug. All the derivatives were significantly less irritating to the gastric mucosa than the parent drug. Though only two esters showed antiinflammatory activity similar to that of the parent drug at equivalent dose levels, all the esters were equipotent to indomethacin in the mouse acetic acid–induced writhing assay for analgesic action. The present evaluation indicates that the combined pharmacological properties of these ester derivatives may prove useful for design and development of novel gastric sparing antiinflammatory molecules with potentially important therapeutic applications.

Keywords Antiinflammatory · Indomethacin · Ulceration

Introduction

The market for antiinflammatory drugs, squarely focused on selective cyclooxygenase-2 (cox-2) inhibitors during the past few years, no longer remains dominated by these drugs, following the alarming increase in their renal and cardiovascular adverse effects (Paul et al., 2004; Scheen, 2004). With the withdrawal of some cox-2 inhibitors, the demand for safer nonsteroidal antiinflammatory drugs (NSAIDs)

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still remains unmet. Chemical modification of the existing time-tested and clinically used drugs to overcome their side effects would be an economical and time-saving approach compared to new drug discovery, which is quite an expensive process.

The undesired gastrointestinal (GI) irritation principally limits the clinical utility of most of the conventional acidic NSAIDs. The main causes of NSAID-induced gastropathy are reduced mucosal cytoprotective prostaglandin (PG) levels, increased gastric acidity, and increased gastric motility. The increased gastric motility leads to a reduced mucosal blood flow, hypoxia, and destruction of the mucous bicarbonate barrier, which prevents back diffusion of pepsin and hydrogen ions from the lumen into the mucosal layer (Takeuchi, 1989). Moreover, microcirculation of gastroduodenal mucosa supplies energy and oxygen to mucosal cells, removes hydrogen ions and waste products, and transports bicarbonate to the surface of the gastric epithelium. Thus, the mucosal blood flow plays a very crucial role in supporting the defense mechanism of gastric mucosa (Akira and Tadashi, 1992). Local GI irritation via a direct contact effect due to the acidic carboxyl functionality is common to all nonselective cyclooxygenase inhibitor NSAIDs (Wallace and Cirino, 1994). To diminish the GI toxicity, many ester and amide prodrugs of NSAIDs have been reported in the literature (Ueda et al., 1991; Bonina et al., 2002). The inactive prodrugs temporarily block the acidic carboxyl group until their absorption and release the active parent drugs into the blood after absorption.

We thought of modifying the structure of NSAIDs to *N,N*-disubstituted aminoalcohol esters so that it would lead to the formation of anticholinergic molecules in the intact form, with a greatly reduced acidic character during their GI transit before absorption. The expected advantage of incorporation of anticholinergic activity was that the intact ester in stomach would inhibit gastric secretions and motility by its local anticholinergic action. Thus, contrary to the prior prodrug approach that relied on overcoming local GI irritation by simply blocking the acidic group of NSAIDs, the specially designed NSAID aminoalcohol esters would afford gastric protection by (1) masking the acidic carboxyl group temporarily until absorption, (2) inhibiting gastric acid secretion, and (3) decreasing the gastric motility to maintain the optimal mucosal blood flow, an important gastric mucosal defense factor (Akira and Tadashi, 1992). Hence, *N,N*-disubstituted aminoalcohol esters were specifically designed to possess a terminal tertiary ''nitrogen'' atom with an ethylene bridge between this terminal nitrogen and the carbonyl group of bulky ester, which are also the structural features of aminoalcohol ester class of anticholinergics (Latin and Fifer, 1981).

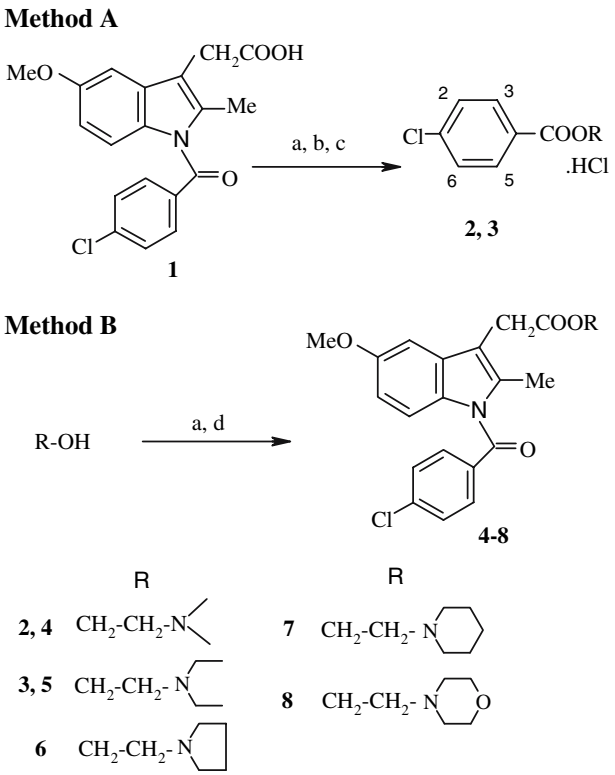
Previous studies from our laboratory have revealed that esterification of the carboxylic acid moiety, with different aminoalcohols, in NSAIDs such as flurbiprofen (Halen et al., 2006), naproxen (Halen et al., 2007), and diclofenac (Yadav et al., 2005) generates potent dual acting anticholinergic-antiinflammatory moieties with significantly reduced oral gastric toxicity. The promising results from these candidates prompted us to apply this potential approach to indomethacin (**1**), a potent indoleacetic acid class of antiinflammatory drug. We report here the synthesis and evaluation of potential esters of indomethacin that combine anticholinergic and antiinflammatory actions. Moreover, a methodology for

esterification of carboxyl compounds possessing other sensitive groups is discussed, ensuring a much broader synthetic utility.

Results and Discussion

Synthesis

Mahfouz et al. (1999) have reported that indomethacin esters can be obtained via the acid chloride formation followed by reaction with the respective aminoalcohols. However, our efforts to obtain the esters of indomethacin via the acid chloride yielded the unexpected esters of *p*-chlorobenzoic acid. The esters **2** and **3** so obtained, as per Scheme 1 (method A), were characterized as the hydrochloride salt forms. The infrared (IR) spectra showed strong ester (C=O) stretching bands at 1727–1739 cm^{-1} in their IR spectra and characteristic proton magnetic resonance (PMR) signals at δ 7.43–7.46 (d, 2H) for ring protons ortho to chloro and 7.97–8.01 (d, 2H) for ring protons ortho to keto ester moiety. From this observation, we conclude that in presence of thionyl chloride the parent acid undergoes degradation at the indole-amide linkage to liberate the *p*-chlorobenzoyl group, which results in the various undesired esters via the acid chloride. Interestingly, it was observed that even under basic conditions (in the presence of anhydrous potassium carbonate i.e., without being converted to acid chloride) indomethacin (**1**) reacted with the aminoalcohols to yield the undesired esters of *p*-chlorobenzoic acid. After failure to obtain the desired aminoalcohol esters by the reported acid chloride method, an easy access to the desired esters was finally made possible by using a two-step process (method B), that is (1) preparation of the *N,N*-disubstituted aminoethylchloride followed by (2) the reaction of indomethacin with the resulting chloro derivatives. The appropriate *N,N*-disubstituted aminoalcohol was reacted with thionyl chloride smoothly at room temperature to give the desired chloro derivative in good yields. The second step, i.e., reaction of resulting chloro derivative dissolved in chloroform, with indomethacin dissolved in aqueous bicarbonate solution, was carried out successfully in presence of a phase transfer catalyst. The simple work up involving the washing of the organic phase with ice-cold water, drying, and solvent removal yielded the oily esters **4–8**, which were found to be unstable on storage when converted to the hydrochloride salts. The basic esters were therefore characterized, preserved, and used as such for further studies. Features such as mild reaction conditions and simple workup with good yields broadens the scope of the present synthetic strategy for esterification of carboxylic compounds with groups sensitive to thionyl chloride. The present procedure offers a superior and cleaner method than the reported ones, involving use of dicyclohexylcarbodiimide as coupling agent (Holmberg and Hansen, 1979; Bonina et al., 1991; Abordo et al., 1998). All the esters were characterized by their spectral data.



Scheme 1 Reagents and reaction conditions: **(a)** $SOCl_2$; **(b)** R-OH, K_2CO_3; **(c)** dry HCl; **(d)** H_2O,$NaHCO_3$. **1,** Phase transfer catalyst

Hydrolysis studies

Chemical and enzymatic hydrolyses studies of the ester derivatives **4–8** were performed in aqueous buffer solutions (pH 2.0 and pH 7.4) and in human serum (80%), respectively. The corresponding half-lives in buffer solution and percent release of parent drug on enzymatic hydrolysis are given in Table 1. At pH 2.0, no noticeable hydrolysis was observed for all the derivatives until a period of 6 h. The observed acid stability of the ester derivatives fulfills the requirement for oral delivery system of NSAIDs, whereby the masking group should be acid stable to prevent the direct contact effects on the gastric mucosa. Apart from this, in the acidic pH of stomach, the polar protonated form (at the basic nitrogen) of these esters would resist absorption into the gastric cells owing to their decreased lipophilicity. This would prevent the intracellular entrapment of these compounds, and hence the local inhibition of the cytoprotective prostaglandin synthesis. It is well accepted that an easy absorption of the lipophilic form of NSAIDs at the gastric pH into the mucosal cells, followed by the entrapment due to the alkaline intracellular pH, is responsible for a more intense localized anti-PG action (Wallace and Cirino, 1994).

Table 1 Chemical and enzymatic hydrolysis of ester derivatives **4–8**

Compound	$t_{½}$(h) pH 7.4 buffer	% Release of indomethacin (in 80% human serum)		
		½ h	1 h	2 h
4	34	35.5	47.8	49.6
5	39	54.4	59.3	70.9
6	99	40.6	42.1	53.3
7	66	50.1	59.1	65.0
8	52	27.8	36.9	45.4

At pH 7.4, sufficiently long half-lives were observed for all the ester derivatives (Table 1). These data ensure the stability of the candidates throughout their GI transit followed by their absorption in the intact form to enter the circulation. The enzymatic susceptibility of all the compounds toward the human plasma esterases ensures a successful release of the parent acid after entering the circulation. Compound **6,** bearing a cyclic substituent at the N-terminus, exhibited the slowest rate of chemical hydrolysis and low percent release of parent acid whereas compound **5,** with an open chain diethyl linkage, showed an exactly opposite behavioral pattern toward chemical and enzymatic hydrolysis. Compound **5** exhibited not only a fast chemical hydrolysis but also the highest susceptibility toward serum esterases. Replacing the pyrrolidino substituent in **6** with the piperidino substituent (**7**) increases the susceptibility toward both types of hydrolysis; however, the rates are lower when compared to the diethylaminoethyl derivative **5**. Further, the higher percent release of parent acid in serum by compound **7** (piperidino derivative) in comparison to compound **8** shows that replacement of a $–CH_2–$ group with O, i.e., changing the polarity of the N-terminal substituent affects the binding of the esters to the esterase site. Hence, moving from an open chain to a cyclic substituent at the N-terminal of these esters has a substantial influence on their hydrolysis behavior.

The combined results of chemical and enzymatic hydrolyses studies reveal these esters to survive the GI conditions and enter the circulation as intact compounds, thereby successfully overcoming the local GI irritation. Besides this, all the esters have appeared to possess the chemical requirements (good aqueous stability and high enzymatic conversion) to be regarded as derivatives useful for oral administration. Further, the aminoethylalcohol moiety released on metabolism of the ester drug would be free of systemic toxicity and side effects owing to their rapid elimination as highly water soluble molecules.

Biological evaluation

All ester derivatives **4–8** exhibited anticholinergic activity with pA_2 values (Table 2) ranging from 4.92 to 5.19, though the activity was much weaker than that of the standard atropine sulfate ($pA_2 = 8.20$). The right parallel shift observed for the dose–response curve in the presence of the esters reveals them to be competitive

reversible inhibitors of acetylcholine at the smooth muscle receptors. Compounds **4** and **6** exhibited antiinflammatory activity comparable to that of the parent acid, and compounds **5, 7,** and **8** were less active than indomethacin at equimolar doses. A significant reduction was observed in the ulcerogenic potential of these compounds (**4–8**) when compared to their respective parent drugs (Table 2). Considering the stability of these derivatives in aqueous buffer solutions and the rapid enzymatic hydrolysis, it can be concluded that the observed ulcerogenicity may be due to conversion of the esters to indomethacin after absorption from the GI tract, which is responsible for the systemic inhibition of the prostaglandin synthesis. In acetic acid–induced writhing syndrome for evaluation of the peripheral analgesic activity, interesting results were observed. Except for **5**, all the other derivatives were equipotent to the parent drug in their analgesic action. In brief, in the series **4–8**, compound **4** was found to be the best among all the derivatives of indomethacin, with a significant reduction in gastric irritation while retaining the antiinflammatory and analgesic activity.

In conclusion, the present study indicates the tested esters represent potentially useful indomethacin derivatives for oral administration because they (1) are stable in aqueous solutions, (2) show anticholinergic activity in the gut, (3) are readily hydrolyzed in human plasma, (4) retain the antiinflammatory and analgesic activity of the parent acid, and (4) notably inhibit the GI irritation induced by indomethacin. There are reports on indomethacin esters and amides being cox-2 selective inhibitors (Kalgutkar et al., 2005; Khanna et al., 2006); hence, in addition, we expect that these esters, until they remain intact in the circulation, might afford gastric protection by having selectivity for cox-2 enzyme. These derivatives cannot be called prodrugs because unlike prodrugs they have a biological action (anticholinergic) before being hydrolyzed for release of the parent moiety. We believe that the present approach is a very rare example of drug design wherein a compound possesses a beneficial pharmacological action before entering the circulation and elicits the desired biological action on metabolism.

Table 2 Anticholinergic, antiinflammatory, analgesic activities and ulcerogenicity of indomethacin (**1**) and its derivatives **4–8**

Compound	pA_2	Edema volume (% inhibition)	Ulcer index	No. of writhings, % inhibition
Control	—	0.920 ± 0.06 (0.00)	0.000	36.60 ±1.44 (0.00)
Indomethacin		0.502 ± 0.04 (45.43)	0.547 ± 0.116*	21.20 ± 3.14 (42.08)
(Dose, mg/kg)		3.0	25.0	15.0
4	5.19	0.490 ± 0.05 (46.74)	0.149 ± 0.044*	22.00 ± 2.19 (39.89)
5	4.49	0.602 ± 0.12 (34.56*)	0.317 ± .058*	28.17 ± 1.58 (23.04*)
6	4.92	0.576 ± 0.03 (37.50)	0.341 ± 0.046*	23.83 ± 1.75 (37.16)
7	5.04	0.768 ± 0.06 (16.57*)	0.134 ± 0.042*	23.80 ± 2.52 (34.97)
8	5.16	0.708 ± 0.10 (23.09*)	0.259 ± 0.031*	23.60 ± 1.50 (35.52)

*$p < 0.05$ versus indomethacin. Results are expressed as means ± SEM

Experimental

Lambda (type IV) carrageenan and the aminoalcohols were purchased from Sigma-Aldrich (St. Louis, MO). Distilled water was used in the preparation of the buffer solutions. Anhydrous sodium sulfate was used as the drying agent. Melting points were taken in open capillaries and are uncorrected. The IR spectra were recorded on a Shimadzu-8300 FT-IR using a KBr disc. Absorbance was measured on a Shimadzu UV-1601 spectrophotometer. ^{1}H-NMR spectra were recorded on a 300 MHz instrument for solutions in $CDCl_3$.

Synthesis of *N,N*-disubstituted aminoethyl esters of indomethacin **(4–8)**

Method A. Indomethacin (**1**; 1.0 g, 2.79 mmol) was dissolved in dichloromethane by refluxing and then treated dropwise with thionyl chloride (0.4 ml, 5.37 mmol). The reaction mixture was stirred for 2 h at room temperature followed by the addition of the *N,N*-dimethylaminoethyl alcohol (1.5 ml, 0.015 mmol) and anhydrous potassium carbonate (1.0 g) with further stirring for 2 h at room temperature. The reaction mixture was diluted with chloroform, filtered, and the organic layer was washed with ice-cold water, dried, and the solvent removed to obtain an oily residue. The oily material was dissolved in dry isopropyl ether, and dry hydrogen chloride was passed to give a solid precipitate. The salt so obtained was filtered, crystallized, and identified as **2**. The above route was tried with the *N,N*-diethylaminoethyl alcohol and the compound so obtained was characterized to be the aminoalcohol ester **3** of *p*-chlorobenzoic acid.

2-Dimethylaminoethyl 4-chlorobenzoate hydrochloride (2). % Yield 57, mp 178–181°C, IR (cm^{-1}): 1731 (ester C=O stretching), ^{1}H-NMR: δ (ppm) ($CDCl_3$): 2.94 (s, 6H, $N(CH_3)_2$),3.47–3.50 (t, 2H, CH_2N), 4.82–4.85 (t, 2H, O–CH_2), 7.43–7.46 (d, 2H, Ar–3–CH and Ar–5–CH), 8.02–8.05 (d, 2H, Ar–2–CH and Ar–6–CH), $C_{11}H_{15}Cl_2NO$: Requires C, 53.2; H, 6.1; N, 5.6. Found C, 53.1; H, 6.3; N, 5.4%.

2-Diethylaminoethyl 4-chlorobenzoate hydrochloride (3). % Yield 55, mp 167–169°C, IR (cm^{-1}): 1727 (ester C=O stretching), ^{1}H-NMR: δ (ppm) ($CDCl_3$): 2.94 (s, 6H, $N(CH_3)_2$), 3.47–3.50 (t, 2H, CH_2N), 4.82–4.85 (t, 2H, O–CH_2), 7.43–7.46 (d, 2H, Ar–3–CH and Ar–5–CH), 8.02–8.05 (d, 2H, Ar–2–CH and Ar–6–CH), $C_{13}H_{19}Cl_2NO$: Requires C, 56.5; H, 6.9; N, 5.1. Found C, 56.1; H, 6.7; N, 5.3%.

Method B. *N,N*-Dimethylaminoethanol (1.0 ml, 0.01 mol) was reacted with thionyl chloride (1.5 ml, 0.02 mol) in dry chloroform (20 ml) under stirring at room temperature and refluxed for 15 min on a water bath. The reaction mixture was then cooled and ice-cold water (20 ml) was added to it. Sodium bicarbonate (3.5 g), potassium iodide (300 mg), indomethacin (1.5 g, 4.18 mmol), and tetraethylammonium bromide (PTC, phase transfer catalyst; 1.5 g) were added to the above solution and the reaction mixture was stirred for 15–18 h. The reaction mixture was monitored via thin-layer chromatography (TLC) for the absence of indomethacin in the organic layer, diluted further with chloroform (200 ml), and washed several times with ice-cold water. The chloroform layer was dried and removed completely to afford the oily product (**4**). The esters were preserved as oily bases without conversion into the corresponding hydrochloride salts, which were found to be

unstable. Compounds **5–8** (yield 60–73%) were prepared by this route (Scheme 1) and were characterized by their spectral data, purified by column chromatography, and purity checked by TLC. Different solvent systems were tried for crystallization but the semisolid oily ester bases could not be crystalized.

2-Dimethylaminoethyl 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetate (4). %Yield 62, UV (MeOH): λ_{max} 227 nm (log ε 4.40), IR (cm^{-1}): 1733 (ester C=O stretching), 1683 amide C=O, ^{1}H NMR: δ (ppm) ($CDCl_3$): 2.31 (s, 6H, $N(CH_3)_2$), 2.40 (s, 3H, $ArCH_3$), 2.63–2.69 (t, 3H, CH_2N), 3.65 (s, 2H, Ar–CH_2), 3.80 (s, 3H, Ar–OCH_3) , 4.24–4.26 (t, 2H, –OCH_2), 6.64–7.67(m,7H, ArH).

2-Diethylaminoethyl 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H* -indole-3-acetate (5). % Yield 67, UV (MeOH): λ_{max} 227 nm (log ε 4.48), IR (cm^{-1}): 1732 (ester C=O stretching), 1683(amide C=O stretching), ^{1}H-NMR: δ (ppm) ($CDCl_3$): 1.11 (t, 6H, $N(CH_2CH_3)_2$), 2.35 (s, 3H, Ar–CH_3), 2.73–2.85 (q, 4H, $N(CH_2CH_3)_2$), 2.98 (t, 2H, –CH_2N), 3.64 (s, 2H, Ar–CH_2), 3.78 (s, 3H, Ar–OCH_3), 4.21–4.26 (m, 2H, OCH_2), 6.63–7.66 (m,7H, ArH).

1-[2-(1-Pyrrolidino)]ethyl 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetate (6). % Yield 65, UV (MeOH): λ_{max} 227 nm (log ε 4.39), IR (cm^{-1}): 1733 (ester C=O stretching), 1683 (amide C=O stretching), ^{1}H-NMR: δ (ppm) ($CDCl_3$): 1.90–1.95 (m, 4H, $(CH_2)_2$), 2.33 (s, 3H, Ar–CH_3), 2.71–3.16 (m, 6H, $CH_2N(CH_2)_2$), 3.60 (s, 2H, Ar–CH_2), 3.81(s, 3H, Ar–OCH_3), 4.52–4.58 (t, 2H, OCH_2), 6.68–7.97(m,7H, ArH).

1-[2-(1-Piperidino)]ethyl 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetate (7). % Yield 73, UV (MeOH): λ_{max} 227 nm (log ε 4.29), IR (cm^{-1}): 1732 (ester C=O stretching), 1683 (amide C=O stretching), ^{1}H-NMR: δ (ppm) ($CDCl_3$): 1.25–1.68 (m, 6H, $(CH_2)_3$), 2.34 (s, 3H, Ar–CH_3), 2.55–2.93 (m, 6H, $CH_2N(CH_2)_2$), 3.60 (s,2H, Ar–CH_2), 3.79 (s, 3H, Ar–OCH_3), 4.48–4.58 (t, 2H, OCH_2), 6.65–7.97 (m,7H, ArH).

1-[2-(4-Morpholino)]ethyl 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetate (8). % Yield 60, UV (MeOH): λ_{max} 227 nm (log ε 4.40), IR (cm^{-1}): 1732 (ester C=O stretching), 1683 (amide C=O stretching), ^{1}H-NMR: δ (ppm) ($CDCl_3$): 2.40(s, 3H, Ar–CH_3), 2.43–2.75(m, 6H, $CH_2N(CH_2)_2$), 3.57–3.77 (m, 4H, CH_2OCH_2), 3.68 (s, 2H, Ar–CH_2), 3.83 (s, 3H, Ar–OCH_3), 4.24–4.27 (t, 2H, OCH_2), 6.64–7.67 (m, 7H, ArH).

Hydrolysis studies

Kinetics of hydrolysis of **4–8** in aqueous solutions. Reactions were initiated by maintaining a solution of the ester **4–8** (1 mg/ml), in pH 7.4 buffer and in pH 2.0 buffer at 37 ± 1°C. At definite time intervals until a period of 6 h, samples (1.0 ml) were withdrawn and transferred to a separating funnel containing buffer (pH 2.0, 9 ml). This acidified solution was extracted into solvent ether (3 × 5 ml). The pooled organic extract was removed completely, methanol (10 ml) was added to the residue, and absorbance was measured at 227 nm (λ_{max}) against a blank obtained by similar treatment. The rate constants, *k*, for hydrolysis of prodrugs were determined

by linear regression of log of residual ester versus time plots. Triplicate samples were analyzed and $t_{1/2}$ was calculated (Table 1) using the equation $t_{1/2} = 0.693/k$.

Kinetics of hydrolysis of **4–8** in pooled human serum. Pooled human serum (4.0 ml) was placed in a stoppered conical flask and maintained at 37 ± 1°C in a water bath. To this the derivative (**4**) solution (1.0 ml, 5 mg/ml in pH 7.4 phosphate buffer) was added, and at appropriate time intervals aliquots (0.5 ml) were withdrawn and transferred to a separating funnel containing trichloroacetic acid (1 ml, 10% wt/vol). Buffer solution (8.5 ml, pH 2.0) was added and this protein-precipitated solution was extracted with solvent ether (3 × 5 ml). Further treatment was as described under hydrolysis in aqueous solution. The percent release of the parent drug was calculated. Three determinations were performed to give average percent release of the parent drug (Table 1).

Biological evaluation

Indomethacin was administered orally as a suspension in carboxymethylcellulose (1% wt/vol). The derivatives **4–8** were administered orally at an equivalent dose in polyethylene glycol 400 (5% vol/vol) in distilled water. The protocol for the animal experiments performed was approved by the IAEC (Institutional Animal Ethics Committee) as registered under CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Government of India.

Anticholinergic activity. The anticholinergic activity was determined on isolated rat ileum (Winter et al., 1962; Halen et al., 2006a). Rats weighing 150–200 g were fasted overnight and housed singly; the abdomen was dissected and the ileum was placed in Tyrode solution at 37 ± 1°C with aeration. A 1.0–1.5 cm length of the tissue was mounted under tension (1*g*). The tissue was stabilized with washings of fresh Tyrode every 10 min and the dose–response curve (DRC) was recorded to obtain the maximum response for acetylcholine (ACh). The tissue was allowed to be in contact with the Tyrode containing the derivative 1(a–e) or atropine sulfate for half an hour and the DRC was repeated again for ACh. The percentage response was calculated to find the EC_{50} in the presence and absence of the antagonist (derivative or atropine). The pA_2 value was calculated via the following formula:

$pA_2 = -\log[M] + \log(x - 1)$ where, log[M] = molar conc. of antagonist and $x = EC_{50}$ found in presence of antagonist/EC_{50} found in the absence of antagonist.

Antiinflammatory activity. The antiinflammatory activity for the derivatives was determined via carrageenan-induced rat hind paw edema assay as described by Halen et al. (2006a) and Winter et al. (1962). Sprague–Dawley rats of either sex ($n = 6$, 150–200 g) were used. The carrageenan-induced rat hind paw edema assay was performed. The animals were fasted for 24 h with water *ad libitum*. Each animal was injected s.c. (0.1 ml, 1% wt/vol) with carrageenan suspension into the subplantar region of the left hind paw after 1 h of drug administration. The paw volume was measured immediately after injection and after 3 h using a plethysmometer (Ugo Basil). The control group received no drug. Indomethacin **(1)** was given at a dose of 3 mg/kg body weight and the derivatives were administered at dose equivalent to 3 mg/kg of indomethacin. Results (Table 2) are expressed as percentage inhibition of edema formation, calculated via the formula,

% inhibition of paw edema = $(1 - Ed_{drug}/Ed_{control}) \times 100$, where Ed_{drug} and $Ed_{control}$ are the edema volumes in drug-treated and control groups respectively.

Ulcerogenicity. Sprague–Dawley rats ($n = 6$, 150–200 g) of either sex were used to determine the ulcer indices (Parmar and Desai, 1993; Halen et al., 2006a). The rats were fasted for 36 h with water *ad libitum* before administration of drug solutions and for 4 h post-dosing. The control group received no drug. Indomethacin (25 mg/kg) was given orally and derivatives (**4–8**) were given at a dose equivalent to 25 mg/kg of indomethacin. The animals were killed, their stomach was dissected out, cut along the greater curvature, washed with normal saline, and the gastric mucosa was observed for the lesions via a 2×2 binocular magnifier and the results were expressed as ulcer index using the formula, Ulcer index = 10 (Au/Am), where, Am = total mucosal area, Au = Al + Ac + Ap, A_l = area of linear lesions ($l \times b$), Ac = area of circular lesions, and Ap = total no. of piteaches/5.

Analgesic activity. Analgesic activity of the derivatives (**4–8**) was assessed by the acetic acid–induced writhing assay in the mouse as described by Koster and Anderson (1959).The animals were divided via randomization in groups (each of six mice, 18–22 g) and were orally administered indomethacin (**1**; 15 mg/kg) or its ester derivatives **4–8** at dose equivalent to 15 mg/kg of indomethacin. After 1 h of drug administration, the writhing syndrome was elicited by the intraperitoneal injection of (10 ml/kg of body weight of 0.6% vol/vol in 0.9% saline) acetic acid and the number of writhes for each mouse was counted after 5 min of injection for a period of 20 min. The average number of writhes was determined in each group and the degree of analgesia was expressed as percentage inhibition calculated according to the formula: % inhibition of writhing = $(1 - T/S) \times 100$, where S and T are the number of writhes in the control and drug-treated group, respectively.

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