Synthesis, biological evaluation and kinetic studies of glyceride prodrugs of diclofenac

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The prodrugs (glyceride derivatives) 3a and 3b of diclofenac were prepared by reacting 1, 2, 3-trihydroxy propane-1,3-dipalmitate/stearate with the acid chloride of diclofenac as potential prodrugs to reduce the gastrointestinal toxicity associated with them. These prodrugs were evaluated for their ulcerogenicity, anti-inflammatory and analgesic activity. It was found that the prodrugs were significantly less irritating to the gastric mucosa as indicated by severity index of 0.86, 0.78 compared to 1.6 of diclofenac. The prodrugs 3a and 3b showed better anti-inflammatory and analgesic activity than the parent drug. The hydrolysis of prodrugs 3a and 3b were studied at pH 3, 4, 5 and 7.4. The HPLC analysis showed that the prodrugs were resistant to hydrolysis at pH 3, 4 and 5 indicating that they did not hydrolyze in acidic environment, whereas at pH 7.4 the prodrugs readily released the parent drug in significant quantities. The plasma levels of diclofenac were also analyzed by HPLC in rats after single oral dose of the prodrugs. The results indicated that the parent drugs were readily released. The concentration of diclofenac during the study was found higher in animals treated with prodrugs 3a and 3b compared with animals treated with diclofenac. The concentration of diclofenac was found to be 38.59, 33.6 and 30.36 µg/ml in animals treated with prodrugs 3a, 3b and diclofenac respectively. In conclusion, all these studies indicated that the glyceride prodrugs of diclofenac might be considered as potential biotabile prodrugs of diclofenac.

Keywords: Anti-inflammatory activity, Analgesic activity, Ulcerogenicity, Prodrugs, Diclofenac, HPLC.

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In recent years, considerable attention has been paid on the design of prodrugs in order to improve the delivery characteristics and decrease the side effects of a variety of the NSAIDs1. A number of prodrugs have been synthesized with an aim to decrease the side effects related to the intrinsic mechanism responsible for their activity e.g. gastric irritation produced by NSAIDs. The gastric irritation/ulceration associated with NSAIDs is from mild to life threatening. The condition is manifested due to the local action of free carboxylic group and inhibition of production of cytoprotective prostaglandins5,6. Temporarily masking the acidic group of NSAIDs has resulted in reduced gastrointestinal toxicity due to less direct contact with the stomach mucosa. Cioli et al7, have studied the gastric ulceration produced by NSAIDs after oral and intravenous administration of the drugs and reported that after intravenous administration significantly fewer ulcers are produced compared with oral administration of the same drug. To a great extent derivatization of the carboxylic function of the NSAIDs produces prodrug forms with adequate stability at the acidic pH of the stomach, thus preventing local irritation on stomach mucosa and also capable of releasing the parent drug spontaneously or enzymatically in the system following their absorption5. The aim of our study has been to mask the free carboxylic acid group of diclofenac temporarily by synthesizing their glyceride prodrugs, which can pass the stomach as such and increase their absorption pertaining to the absorption of natural triglycerides. Triglycerides being the major constituents of the dietary fat, their absorption involves simple hydrolysis mainly by pancreatic lipase to monoglycerides and free fatty acids. These prodrugs, therefore, do not involve risk of unwanted effects after the prodrug is hydrolyzed. The synthesized compounds were evaluated for their ulcerogenic, anti-inflammatory and analgesic effects. The in vitro and in vivo kinetics of the prodrugs was also studied. The prodrugs 3a and 3b were obtained by condensing the acid chloride of diclofenac 2, prepared by the standard procedure8 in presence of dry pyridine with 1, 2, 3-trihydroxy propane-1,3-dipalmitate/stearate8 (Scheme 1).
Materials and Methods

Materials—Except dihydroxy acetone, which was purchased from E. Merck KGaA, Germany, all other reagents were obtained from E. Merck (India) Ltd. All the solvents in these studies were dried and distilled before use. Wistar rats of either sex weighing between 150-200 g and Swiss albino mice of either sex weighing between 25-30 g were procured from Animal House, Jamia Hamdard, New Delhi. TLC of the synthesized compounds was carried out in petroleum ether:ethyl acetate (5:1) solvent system. The TLC spots were located by exposing to iodine vapours. Melting points were uncorrected and recorded in liquid paraffin bath using open-end capillaries. 1H NMR spectra were recorded on a Bruker spectrospin Avance DPX200, 300 MHz in CDCl3. Mass spectra were recorded on a JEOL SX102/DA-6000 Mass spectrometer and FT-IR spectra were recorded on Perkin-Elmer Spectrometer. HPLC analysis was done on a Shimadzu Model LC-10ATVP (Japan) system containing a quaternary pump, UV detector and c18 reverse phase column. Dissolution was carried out by SR8 Plus dissolution test apparatus, Hanson Research, USA. Samples were filtered with 0.45 µm millipore filter and eluted with 73% phosphate buffer (45 mM, pH 7) and 27% acetonitrile: tetrahydrofuran (7:3 v/v) at 1.1 ml/min. The effluent was monitored at 273 nm by UV detector, Shimadzu Model SCL-JOA VP, Japan.

Preparation of glyceride prodrug (3a)—It was prepared by standard procedure8,9 (Scheme 1). After stirring 1 and 2 in pyridine for 40 hr at room temperature, the contents were diluted with water and extracted with CH2Cl2. The CH2Cl2 extracts on evaporating to dryness gave a solid mass which was crystallized from petroleum ether; yield: 68%; mp: 135°-37°C; Rp: 0.71; elemental analysis (%): calcd for: C29Hn17O6NCl2, C: 69.58, H: 9.11, N: 1.65; found: C: 69.66, H: 9.26, N: 1.52. 1H NMR (δ ppm): 0.88 (t, 6H, 2x CH3), 1.25 (m, 24x CH2), 1.63 (m, 4H, 2x CH2 β to CO), 2.37 (m, 4H, 2x CH2 α to CO), 3.78 (s, 2H, CH2 of benzyl), 4.16 (m, 5H, 2xCH2 + C-H of glycerol), 6.41 (s, 1H, NH, O=C exchange), 7.03 (m, 1H, H-5), 7.18 (m, 1H, H-4′), 7.35 (dd, J=2,8Hz, 1H, H-3), 7.41 (dd, J=2,8Hz, 1H, H-6), 7.50 (m, 2H, H-3′, 5′), 7.54 (m, 1H, H-4). IR (KBrl/mm, cm⁻¹): 3460.4 (N-H), 2920.3, 2853.6 (C-H), 1730.6 (C=O), 1612.0 (aromatic), 1170.3 (C-O-O), 786.4, 738.6.

Preparation of glyceride (3b)—It was prepared following the same procedure as for 3a except that 1,3-di stearyl glyceride was used; yield: 72%; mp: 169-71°C; Rp: 0.71; elemental analysis (%): calcd for: C33H35O6NCl2, C: 70.58, H: 9.43, N: 1.55; found: C: 70.66, H: 9.62, N: 1.69. 1H NMR (δ ppm): 0.87 (t, 6H, 2xCH3), 1.25 (m, 28x CH2), 1.62 (m, 4H, 2x CH2 β to CO), 2.37 (m, 4H, 2x CH2 α to CO), 3.78 (s, 2H, 

Scheme 1—Steps involved in the synthesis of prodrugs 3a and 3b
**3a, n=14, palmityl, (M+ 845*, 847*, 849*, analyzing for C49H77O6N2Cl,)
**

**3b, n=16, stearyl, (M+ 901*, 903*, 905*, analyzing for C53H85O6N2Cl)**

(Molecular ion peaks not observed)

**CH2 of benzyl), 4.18 (m, 5H, 2xCH2 + C-H of glycerol), 6.42 (s, 1H, NH, D2O exchange), 7.03 (m, 1H, H-5), 7.18 (m, 1H, H-4'), 7.32 (dd, J=2, 8Hz, 1H, H-3), 7.43 (dd, J=2, 8Hz, 1H, H-6), 7.5 (m, 2H, H-3', 5'), 7.52 (m, 1H, H-4), 1R (KBr/Vmax cm-1): 3466.7 (NH), 2922.6, 2853.9 (CH), 1739.7 (C=O), 1616.0 (aromatic), 1178.7 (C-O-O), 790.8, 740.6.

A further support for the successful synthesis of the prodrugs 3a and 3b was obtained by mass spectral analysis, which showed the diagnostic peaks (Fig-1).

**Anti-inflammatory activity**—The anti-inflammatory activity was evaluated using carrageenan induced paw edema in rat by the Winter et al. method20. Wistar rats (150-200 g) were divided into 4 groups of 6 animals each. Group I served as a control group without using the drug, group II received diclofenac 20 mgkg-1, group III and IV received prodrugs 3a and 3b respectively in doses molecularly equivalent to diclofenac. A stock solution of 4 mg/ml, 18.47 mg/ml and 19.57 mg/ml was prepared as a homogeneous suspension in aqueous solution of sodium CMC (0.5% w/v) and each animal received 0.75-1.0 ml orally of the respective drugs. Thirty min after administration of drugs, each rat received a subplantar injection of 0.1 ml of 1% carrageenan solution in its left hind paw. The measurement of the hind paw volume was done by using a Ugo Basile Plethysmometer before any treatment (V0) and at 2 and 3 hr (V1) after the administration of the drugs. All the results are expressed as mean ± SEM. Statistical evaluation was performed using analysis of variance followed by t-test for sub group comparison (level of significance is P< 0.001). The percentage inhibition was calculated from equation 1.
Inhibition (%) \( = \frac{[V_1 - V_o]_{\text{control}} - (V_1 - V_o)_{\text{treated}}}{[V_1 - V_o]_{\text{control}}} \times 100 \) \( \ldots (1) \)

**Analgesic activity**—Analgesic activity was carried out by using acetic acid induced writhing (Seigmund et al. 1957) method in Swiss albino mice (25-30 g) of either sex. A 1% v/v solution of acetic acid was used as writhing inducing agent. Test compounds were administered orally 2 hr prior to acetic acid injection. Number of writhings for 10 min in control and test compounds were counted and compared. Analgesic activity was measured as per cent decrease in writhing in comparison to control. Mice were divided into 4 groups of 6 animals each. Group 1 served as a control group without using the drug while group II received diclofenac 20 mg/kg, group III and IV received prodrugs 3a and 3b in doses molecularly equivalent to the diclofenac. A stock solution of 1, 3.69 and 3.91 mg/ml was prepared as a homogenous suspension in aqueous solution of sodium CMC (0.5%/w/v) and each animal received 0.5-0.6 ml orally of the respective drugs. Acetic acid was administered intraperitoneally 1 ml/100g body weight of the animal. All the results are expressed as mean ± S.E.M. Statistical evaluation was performed using analysis of variance followed by t-test for sub group comparison (level of significance is \( P < 0.0001 \)). The percentage protection was calculated from equation 2.

\[
\text{Protection} \% = 100 - \left[ \frac{\text{no. of writhing in test}}{\text{no. of writhing in control}} \times 100 \right] \quad \ldots (2)
\]

**Ulcerogenicity**—Gastrointestinal toxicity was determined by the method of Cioli et al. The studies were carried out on healthy Wistar rats (150-200 g), procured from animal house, Jamia Hamdard, N. Delhi. The activity was carried out at a dose of 50 mg/kg. Group I served as control and received vehicle only. Group II received diclofenac whereas group III and IV received test compounds 3a and 3b in doses molecularly equivalent to the parent drug. Drugs were administered as a homogenous suspension in aqueous solution of sodium CMC (0.5%/w/v). The animals were fasted for 8 hr prior to a single dose of each of the standard and test compound and sacrificed 17 hr later during which period food and water remained available. The gastric mucosa of the rats was examined by means of binocular magnifier (4x). The mean score of each treated group minus the mean score of the control group was considered the ‘severity index’ of gastric damage. The results are summarized in Table 1.

**In vitro pharmacokinetic studies**—In vitro hydrolysis kinetics of prodrugs 3a and 3b was carried out by HPLC method. Buffer solutions of different pH 3, 4, 5 and 7.4 were prepared. The mixtures were equilibrated at 37 °C for 1 hr and 100 mg of each sample was added to them. The mixtures were agitated by an overhead stirrer. At selected time intervals 15, 30, 45, 60, 75, 90, 105 and 120 min, 0.1 ml of the mixture was withdrawn, diluted up to 10 ml with mobile phase and 20 ml of this solution was injected for analysis. At pH 7.4 samples were withdrawn at 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hr. Calibration curve for diclofenac was prepared in the concentration range of 1-100 μg/ml. The samples were eluted by using 73% phosphate buffer (45 mM, pH 7) and 27% acetonitrile: tetrahydrofuran (7:3 v/v) at a flow rate of 1.10 ml/min and the effluent was monitored at 273 nm.

**In vivo hydrolysis studies**—In vivo studies on prodrugs 3a and 3b were carried out in rats by HPLC. Rats were divided into 3 groups of 6 animals each. Group I received diclofenac orally 20 mg/kg, while groups II and III received 3a (57.09 mg/kg) and 3b (60.87 mg/kg) a dose molecularly equivalent to diclofenac. The animals were fasted overnight prior to experiments. Blood samples were collected at different time intervals 0.5, 1, 2, 4, 6 and 8 hr. Plasma was separated by centrifugation at 4000 rpm and refrigerated (– 20°C) till analysis. Calibration curve of diclofenac was prepared in rat plasma in concentration range of 1-40 μg/ml. In a 5 ml centrifuge tube 0.05 ml of plasma was taken and to it was added 1.5 ml acetonitrile and vortexed for 2 min. The precipitated proteins were separated by centrifugation at 4000 rpm for 15 min. Separation was achieved on a c18 reverse phase column and detected with UV detector. Mobile phase used was 73% phosphate buffer (45 mM, pH 7) and 27% acetonitrile: tetrahydrofuran (7:3 v/v) run at a flow rate of 1.10 ml/min and the effluent was monitored at 273 nm. The injection volume was 20 μl.

The experimental protocol was approved by the “Jamia Hamdard Animal Ethics Committee”. The care of the animals was as per the "Guidelines for the Use of Animals in Scientific Research" prepared by the Indian National Science Academy, New Delhi.
Table 1—Comparative chart of diclofenac, 3a and 3b with respect to their dose and biological activity.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diclofenac</th>
<th>3a</th>
<th>3b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>20</td>
<td>57.09*</td>
<td>60.87*</td>
</tr>
<tr>
<td>Anti-inflammatory activity</td>
<td></td>
<td>54.20</td>
<td>67.28*</td>
</tr>
<tr>
<td>2hr</td>
<td></td>
<td></td>
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<tr>
<td>3hr</td>
<td></td>
<td>79.80*</td>
<td>82.69*</td>
</tr>
<tr>
<td>Analgesic activity</td>
<td></td>
<td>60**</td>
<td>64**</td>
</tr>
<tr>
<td>Ulcerogenicity (%) of animals with ulcers</td>
<td>80</td>
<td>38</td>
<td>33</td>
</tr>
<tr>
<td>Severity index#</td>
<td>1.6</td>
<td>0.86</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* The dose quantities are molar equivalent of diclofenac
** P < 0.001 compared to control, *** P < 0.0001 compared to control
# 'Severity index' mean score of treated group minus mean score of control group

Results and Discussion

Anti-inflammatory activity was carried out by the method of Winter et al. on rat paw \(^1\). Percentage reduction in paw edema at 2\(^{nd}\) and 3\(^{rd}\) hr in comparison to the control is given in Table 1. The compounds showed good anti-inflammatory activity with a percent inhibition of 67.28, 68.22 at 2\(^{nd}\) and 82.69, 80.76 at 3\(^{rd}\) hr compared to 54.20 and 79.80 by diclofenac at 2\(^{nd}\) and 3\(^{rd}\) hr respectively. Analgesic activity was carried out by the method of Seigmund et al. \(^1\). The decrease in number of writhings expressed as percentage protection by test compounds with reference to the control is given in (Table 1). Prodrug 3a showed 64% protection, while 3b and diclofenac gave 59.59% and 60% respectively. The synthesized prodrugs were tested for gastrointestinal side effects by the method of Cioli et al. \(^1\). The prodrugs were significantly less ulcerogenic than the parent drugs, a severity index of 0.86 and 0.78 compared to 1.6 with diclofenac was observed (Table 1). Hydrolysis kinetics of the synthesized glyceride prodrugs 3a and 3b was studied in aqueous phosphate buffer solution at pH 7.4. Under the experimental conditions the target compounds hydrolyzed to release the parent drug (Fig. 2). A graph of log concentration of residual drug vs time was plotted for 3a and 3b, a straight line was obtained suggesting pseudo first order rate kinetics. The rate constant (k\(_{obs}\)) and the corresponding half-lives (t\(_{1/2}\)) for the respective prodrugs were calculated from the linear regression equation correlating the log concentration of the residual produg vs time. The slope of the curve is 5\(\times\)10\(^{-4}\) and k\(_{obs}\) was found to be 8.6\(\times\)10\(^{-4}\) with t\(_{1/2}\) of 37.25 hr for diclofenac. To examine the degradation of glyceride prodrugs in pH as

Fig. 2—Hydrolysis profile of prodrugs of diclofenac 3a and 3b at pH 7.4

of stomach, pH 3, 4 and 5 were selected, because the mean fasting stomach pH of adult is approximately 2 and increases to 4-5 following ingestion of food \(^2\). NSAIDs are not recommended to be taken in fasting state; consequently pH 3, 4 and 5 were selected to mimic the appropriate clinical range. An assay time of 2 hr was selected, after that stomach emptying would normally be effectively complete. The findings are shown in (Fig. 3). The hydrolysis at pH 3, 4, and 5 was insignificant, whereas the drug was readily released at pH 7.4 indicating that the prodrugs were resistant to acidic environment as desired but would release the parent drug in the system. The release of parent drug from the prodrugs was also studied in rats \(^3\). A comparison of the plasma concentration vs time curves of diclofenac indicated that the esters were rapidly hydrolyzed to the parent drug. At all times, the concentration of diclofenac in the animals treated with
3a and 3b were relatively higher in comparison to the animals receiving diclofenac. Furthermore, 8 hr after administration of the prodrugs 3a and 3b, the concentration of diclofenac was approximately 2 times higher compared with the parent drug treated animals, indicating a sustained release. C\textsubscript{max} of diclofenac in case of animals treated with prodrugs 3a and 3b was 38.59 and 33.6 μg/ml compared to 30.36 μg/ml in the animals treated with diclofenac (Fig. 4). In vivo evaluation study indicated that the prodrugs were readily hydrolyzed to release the active drug and also improved bioavailability and hence the glyceride prodrugs of diclofenac might be considered as potential biolabile prodrugs of diclofenac.

**Conclusion**

The glyceride derivatives of diclofenac were successfully synthesized and evaluated for gastrointestinal toxicity, anti-inflammatory activity, analgesic activity, ulcerogeneity and hydrolysis profile. The prodrugs were considerably less ulcerogenic compared to parent drug indicating that the gastrointestinal toxicity due to direct contact of the free carboxylic function reduced with good anti-inflammatory and analgesic activity. The prodrugs were resistant to hydrolysis at pH 3, 4 and 5 further supporting the finding that the prodrugs were resistant to acidic environment, whereas the parent drug was quantitatively released at pH 7.4. The plasma concentration of diclofenac in prodrugs treated animals was constantly high compared to the animals treated with pure diclofenac, the concentration was 2 time high even after 8 hr of drug administration suggesting that the drug was readily released and for a longer duration of time. It can be concluded that the glyceride prodrugs may be used as non-ulcerogenic prodrug for NSAIDs.

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**References**


