Alkyl esters of 6-methoxy-2-naphthylacetic acid as potential prodrugs – Synthesis, physicochemical properties, chemical stability and enzymatic hydrolysis

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Various alkyl esters of 6-methoxy-2-naphthylacetic acid (6-MNA), the active metabolite of nabumetone (1), have been synthesized with the objective of obtaining the prodrugs that would release the active drug only. To assess their prodrug potential, these esters are evaluated for solubility, partition coefficient and capacity factors. The correlation between the partition coefficients, capacity factors and ester chain lengths are established. The hydrolysis of these prodrugs has been studied at various temperatures and pseudo first order rate constants and half lives in aqueous buffer, human plasma and liver homogenate were determined. The hydrolysis of the representative methyl ester has been studied in various buffers of pH range 1.2 to 9. Second order rate constants for specific acid and specific base catalyzed hydrolysis and first order rate constant for spontaneous hydrolysis are determined. The esters show maximum stability between pH 4-5 and are easily cleavable in liver homogenate.

Nabumetone (1) is a recently developed NSAID with proven efficacy and safety. It is a nonacidic prodrug and therefore possesses the potential to avoid NSAID-mediated direct gastric damage while maintaining its efficacy as an anti-inflammatory agent via peripheral action of its active metabolite, 6-methoxy-2-naphthylacetic acid, 6-MNA (2). However, nabumetone is not converted into 6-MNA quantitatively because many interrelated metabolic pathways occur simultaneously forming a number of other metabolites due to its oxidation, O-demethylation, reduction.

These observations suggest that development of a prodrug which could release only 6-MNA will be a step in the development of a safer nonsteroidal anti-inflammatory drug (NSAID) with improved efficacy. In an attempt to make therapy safer and less complex, we reported the synthesis of a number of glycolamide esters of 6-MNA as potential prodrugs and studied their physicochemical properties, chemical stability and enzymatic hydrolysis. These studies further prompted us to prepare alkyl esters of 6-MNA and evaluation of their prodrug potential. In this paper we report the synthesis, physicochemical properties, chemical stability and enzymatic hydrolysis of these alkyl esters of 6-MNA.

Results and Discussion

Alkyl esters of 6-MNA (2a-2f) were synthesized by refluxing 6-MNA and appropriate alcohol in the presence of small amount of sulphuric acid. The structures of these derivatives were determined from their elemental analysis and spectral data reported in the experimental section.

Melting point, capacity factor and lipophilicity of prodrugs

The significance of melting points lies not only in controlling ease of handling, but also in controlling solubility. In simple alkyl esters of 6-MNA, by increasing the length of the alkyl chain from CH₂ to (CH₂)₄CH₃, the melting points decreased consistently. This is explained by the generalizations that if a moiety being homologated is capable of efficient packing or strong intermolecular interactions, adding a methyl group will interfere with the packing and/or interactions and will thus lower the melting point. Adding additional methylene group will continue to interfere with the efficient crystal formation until the chain is long enough to cause the molecules to pack in a hydrocarbon like crystal. If the substitution is by two isomeric hydrocarbons, the compact and rigid isomer will have higher melting point and this is evident from the observation that 2d and 2f have higher melting points than 2c and 2e.

It is generally recognized that solubility and lipophilicity play an important role in governing the overall biological performance of drugs. For orally administered drugs, it has been mentioned that drugs having
octanol-water partition coefficient of 100 or more (log P ≥ 2) are well absorbed provided they have a minimum solubility of 10 μg/mL.\(^6\) To assess this potential, the solubilities of the ester prodrugs 2a-2f were determined in 0.05 M phosphate buffer at pH 7.4 (25°C). The melting point, solubility, capacity factor (k') and the log partition coefficient (log P) have been listed in Table 1.

Figure 1 depicts the plot of log P for esters (2a-2f) as a function of the number of added carbon atoms (N) on the ester moiety. Excellent linearity is observed between the two parameters, which is represented by the relationship:

\[
\text{Log } P = 0.546 N + 2.525 \quad r = 0.999 \quad n = 4 \quad \ldots \ (1)
\]

The methylene group contribution obtained from the slope of the log P against ester chain length is 0.546 which is comparable to 0.527, a hydrophobic fragment constant for methylene group mentioned for octanol/water system.\(^9\)

\[
\text{Figure 2 depicts the plot of logarithm of the capacity factors (log k') as a function of the number of carbon atoms on the ester moiety. The relationship is explained by the following equation:}
\]

\[
\text{Log } k' = 0.0915N - 0.0605 \quad r = 0.999 \quad n = 4 \quad \ldots \ (2)
\]

The slope of this line, i.e., methylene group contribution, calculated for the chromatographic system is different from the value calculated from Eqn.1 because the free energy of transfer of one methylene

\[
\begin{align*}
\text{Table 1} & \quad \text{Melting points, yields, solubility, capacity factors and partition coefficients of the various esters of 6-MNA} \\
\hline
\text{Sl. No.} & \text{R} & \text{m.p.} & \text{Yield} & \text{Solubility}^a & \text{Capacity}^b & \text{Log } P^c \\
\hline
2 & \text{H} & 74-75 & 87 & 14.3 & 0.1630 & 3.04 \\
2a & \text{CH}_3 & 55-56 & 93 & 13.1 & 0.2678 & 3.60 \\
2b & \text{CH}_3\text{CH}_3 & 51-52 & 93 & 4.2 & 0.3979 & 4.14 \\
2c & \text{CH}_3\text{CH}_2\text{CH}_3 & 71 & 82 & 2.8 & 0.3674 & 4.00 \\
2d & \text{CH}_2\text{CH}(\text{CH}_3)_2 & 40-41 & 79 & 2.1 & 0.4961 & 4.75 \\
2e & \text{CH}_2\text{CH}(\text{CH}_3)_2 & 45-46 & 76 & 2.0 & 0.4861 & 4.71 \\
2f & \text{CH}_2\text{CH}(\text{CH}_3)_2 & & & & & \\
\hline
\end{align*}
\]

a. Solubility in phosphate buffer of pH 7.4
b. Capacity factor in mobile phase acetonitrile/phosphate buffer of pH 4.0 (70:30)
c. Log partition coefficient between octanol and phosphate buffer of pH 7.4 (25°C)
group from acetonitrile/aqueous mobile phase to bonded octadecyl silane phase would be different from the energy required to transfer such a group from pure water to octanol.

Combining the log P data with the log $k'$ data (Table 1), it is observed that good correlation exists between log P and log $k'$ as shown in Figure 3 and explained by Eqn (3).

$$\log P=6.001 \log k'+2.87 \quad r=0.998 \quad n=6 \quad ... (3)$$

The solubility and lipophilicity data (Table 1) show that ester derivatives 2a and 2b possess optimum physicochemical properties (solubility and partition coefficient) required for oral absorption i.e. $\log P \geq 2$ and solubility $>10 \mu g/mL$.

Chemical stability of esters of 6-MNA (2a-2f)

The kinetics of hydrolytic breakdown of the esters 2a-2f was studied in phosphate buffer of pH 7.4 at elevated temperatures. Under the experimental conditions used, the degradation of all the esters displayed strict first-order kinetics for several half lives. All the esters got converted to 6-MNA quantitatively as shown in the Figure 4. For methyl ester 2a, typical first order plots for the degradation at different temperature are shown in Figure 5.

The Arrhenius equations explaining the hydrolysis of esters, (2a-2f) are as under:

$$\log k_a=-5199.48/T+13.94 \quad n=3 \quad r=0.999 \quad ... (4)$$

$$\log k_b=-5096.83/T+13.30 \quad n=3 \quad r=1.000 \quad ... (5)$$

$$\log k_c=-4957.89/T+12.87 \quad n=3 \quad r=0.999 \quad ... (6)$$

$$\log k_d=-4868.90/T+12.06 \quad n=3 \quad r=0.999 \quad ... (7)$$

$$\log k_e=-4728.31/T+12.12 \quad n=3 \quad r=0.999 \quad ... (8)$$

$$\log k_f=-4773.01/T+12.12 \quad n=3 \quad r=0.998 \quad ... (9)$$

The values of the rate constants at 37°C for esters (2a-2f) calculated from the Arrhenius equations and half lives ($t_{1/2}$) at 37°C are listed in Table II.

The effect of pH upon hydrolysis of the methyl ester of 6-MNA (2a) was examined over a pH range of 1.2-9.0 at 70°C. As seen from Figure 6, pH rate profile for 2a is U-shaped, indicating the occurrence of specific acid and base catalyzed as well as spontaneous or water catalyzed reaction, which is given by the following rate expression:

$$K=k_0 + k_{H^+}a_{H^+} + k_{OH^-}a_{OH^-} \quad ... (10)$$

where $a_{H^+}$ and $a_{OH^-}$ refer to the hydrogen ion and hydroxide ion activity, respectively. The $a_{OH^-}$ was calculated from the measured pH at 70°C according to the formula:

$$\log a_{OH^-} = pH - 12.80 \quad ... (11)$$

Values of the second order rate constants for the specific acid ($k_{H^+}$) and specific base ($k_{OH^-}$) catalyzed hydrolysis were determined from the straight line portions of the pH-rate profiles at low and high pH values after adjusting the slope to $-1$ to $+1$ by iteration respectively. The value of first order rate constant for spontaneous hydrolysis $k_0$ (h$^{-1}$) of 2a was obtained on the basis of Eqn. 10 and found to be $5.3 \times 10^{-1}$. The ester 2a showed maximum stability at pH 4.8.
Enzymatic hydrolysis

In order to be useful as prodrugs of 6-MNA, the ester derivatives should be readily converted to the parent drug in vivo. Simple aliphatic and aromatic esters are often not biotable in vivo to ensure a sufficiently high rate and extent of prodrug conversion. For example, much reduced anti-inflammatory activity observed for methyl or ethyl esters of naproxen, fenbufen and indomethacin, relative to free acids may be ascribed to the resistance of the esters to be hydrolyzed in vivo. It is evident from the data that the rates of hydrolysis of simple alkyl ester in 80 % human plasma were slower, the half lives being 862 s for 2a, 4940 s for 2b and 1047 s for 2c (Table II). These results prompted us to carry out additional studies in liver homogenate to assess the prodrug potential of these ester derivatives. It has been reported that enalapril is not hydrolyzed by human plasma and the conversion of this ethyl ester to the active enalaprilic acid takes place predominantly in liver. The concentration of carboxysterases is 15000 times more in liver than in plasma. Based on these observations, we studied the hydrolysis of these ester derivatives of 6-MNA in rat liver homogenate. At a concentration of $5 \times 10^{-6}$ to $1 \times 10^{-3}$

Table II — Data for the rate of hydrolysis of various ester prodrugs of 6-MNA in 0.05M phosphate buffer, 80% human plasma and 0.2% liver homogenate at 37°C

<table>
<thead>
<tr>
<th>SL. No.</th>
<th>Buffer (%)</th>
<th>First-order rate constant (k)</th>
<th>Half-lives (t1/2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>80%human plasma (s⁻¹)</td>
<td>0.2% liver (s⁻¹)</td>
</tr>
<tr>
<td>2a</td>
<td>1.49×10⁻³</td>
<td>8.039×10⁻⁴</td>
<td>7.992×10⁻³</td>
</tr>
<tr>
<td>2b</td>
<td>0.71×10⁻³</td>
<td>1.043×10⁻⁴</td>
<td>2.546×10⁻³</td>
</tr>
<tr>
<td>2c</td>
<td>0.77×10⁻³</td>
<td>6.618×10⁻⁴</td>
<td>5.808×10⁻³</td>
</tr>
<tr>
<td>2d</td>
<td>0.22×10⁻³</td>
<td>--</td>
<td>5.34×10⁻²</td>
</tr>
<tr>
<td>2e</td>
<td>0.74×10⁻³</td>
<td>--</td>
<td>5.09×10⁻²</td>
</tr>
<tr>
<td>2f</td>
<td>0.54×10⁻³</td>
<td>--</td>
<td>3.27×10⁻²</td>
</tr>
</tbody>
</table>

-- Calculated for 37°C from Arrhenius equations as discussed in text; -- Not performed.

Figure 6 — The pH-rate profile for the hydrolysis of methyl ester of 6-MNA (2a) in aqueous buffer ($\mu = 0.5$) at 70°C

Figure 7 — Plots showing first-order kinetics of hydrolysis of the ester prodrugs of 6-MNA in 2e, 2b and 2a

Conclusions

The correlations between the log P-ester chain length, log $k'$-ester chain length and log P-log $k'$ are linear and their relationship would very likely allow extrapolation for compounds containing more carbon atoms. The methylene group contribution calculated from this series is 0.546, which is very close to 0.527 as reported earlier. The esters (2a-2f) can be detected and quantitated using appropriate mixtures of acetonitrile and 0.02M phosphate buffer of pH 4.0 even in the presence of hydrolytic degradation product. 6-MNA allowing kinetic studies to be carried out easily.
The esters (2a-2f) showed hydrolytic degradation by strict first order kinetics and all of these got converted to 6-MNA quantitatively. As concluded from pH-rate profile of 2a, these ester prodrugs show occurrence of specific acid and base catalyzed, as well as spontaneous or water catalyzed reactions. The maximum stability of 2a was observed at pH 4.8.

Although the esters (2a-2c) showed good first order kinetics in human plasma, their t_{1/2} were high and it was not readily convertible to its parent compound 6-MNA as compared to hydrolysis in liver homogenate. The rapid cleavage of alkyl ester prodrugs (2a-2f) to 6-MNA in liver homogenate (0.2% w/v) makes these esters suitable prodrug candidate for further evaluation. Compounds 2a and 2b possess optimum physicochemical properties required for oral absorption and may be selected for further studies. These prodrugs may thus possess the potential to avoid 6-MNA mediated direct gastric damage while maintaining their efficacy via the systemic action of the active metabolite 6-MNA.

Experimental Section

**Materials and Methods:** High-performance liquid chromatography (HPLC) was done with a Waters apparatus consisting of two pumps M501 controlled by automated gradient controller 680, a Waters 484 tunable absorbance detector and a 20 μL Rheodyne loop injection valve. Readings of pH were carried out on a Control Dynamics pH meter. Melting points were determined on a liquid paraffin bath in open capillaries and are uncorrected. Microanalysis was performed on Perkin-Elmer 2400 CHN elemental analyzer. ^1^H NMR and ^13^C-NMR spectra were obtained using a Varian EM-390 instrument. Mass spectra were obtained from Micromass 7070F by Vg (England) and for IR, spectra Perkin-Elmer 882 infrared spectrometer was used.

**Preparation of 6-methoxy-2-naphthylacetic acid**

2. 6-Methoxy-2-naphthylacetic acid was prepared starting from 2-naphthol by methylation,^16^ acylation,^15^ followed by Willgerodt-Kindler reaction^18^ and hydrolysis. The pure acid was obtained by recrystallization from methanol.

**Preparation of alkyl esters of 6-MNA (2a-2f).** 6-MNA (2) (1.62g, 7.5 mmole) was dissolved in appropriate dried alcohol (50mL) and sulphuric acid (0.5 mL) was added and the reaction mixture was refluxed for 8 hr. The solvent was removed in vacuo, the residue was diluted with water (50mL) and extracted with ethyl acetate (3×50mL). The combined ethyl acetate extract was washed successively with 1% sodium bicarbonate solution (2×50mL), water (3×50mL) and dried over magnesium sulphate. The solvent was removed under vacuum and the residue on recrystallisation from ethyl acetate-hexane afforded the title compounds.

**Methyl 6-methoxy-2-naphthylacetate** 2a: Yield 1.5g (85.5%); mp 74-75°C (Found: C, 72.73; H, 6.16. Requires C, 73.02; H, 6.13) IR (KBr): 1733 (C=O), 1264 (C-O-C), 1202, 1158, 1130 (C-O), 1027 (C-O-C), 850 and 815 (C-H); ^1^H NMR (CDCl₃): δ 7.5 (6H, m, ArH) 3.95 (3H, s, OCH₃), 3.75 (2H, s, ArCH₂), 3.7 (3H, s, -COOCH₃); ^1^C NMR (CDCl₃): δ 41.03 (ArCH₃), 51.78 (COOCH₃), 55.01 (OCH₃), 105.47 (C-5), 118.80 (C-7), 126.90, 125.79, 127.66 and 128.96 (C-1, C-3, C-4 and C-8), 128.84, 128.96 and 133.45 (C-2, C-9 and C-10), 157.48 (C-6), 171.96 (COO); MS (m/z): 230 (M'^+',49%), 171 (100%), 128 (24%).

**Ethyl 6-methoxy-2-naphthylacetate** 2b: Yield 1.7g (93%); mp 55-56°C (Found: C, 73.28; H, 6.20. Requires C, 73.75; H, 6.60); IR (KBr): 1736 (C=O), 1267 (C-O-C), 1202, 1150 (C-O), 1029 (C-O-C), 852 and 815 (C-H); ^1^H NMR (CDCl₃): δ 7.5 (6H, m, ArH), 4.2 (2H, q, J = 7Hz, COOCH₂CH₃), 3.9 (3H, s, OCH₃), 3.75 (2H, s, ArCH₂), 1.25 (3H, t, J = 7Hz, COOCH₂CH₃); ^1^C NMR (CDCl₃): δ 14.40 (COOCH₂CH₃), 41.21 (ArCH₃), 55.03 (OCH₃), 60.66 (COOCH₂CH₃), 105.48 (C-5), 118.77 (C-7), 126.87, 127.58, 127.70 and 129.00 (C-1, C-3, C-4 and C-8), 128.79, 129.17 and 133.44 (C-2, C-9 and C-10), 157.46 (C-6), 171.55 (COO); MS (m/z): 244 (M'^+', 48%), 171 (100%), 128 (19%).

**Propyl 6-methoxy-2-naphthylacetate** 2c: Yield 1.8g (93%); mp 51-52°C (Found: C, 73.90; H, 7.23. Requires C, 74.39; H, 7.02); IR (KBr): 1735 (C=O), 1267 (C-O-C), 1206, 1151 (C-O), 1029 (C-O-C), 850 and 814 (C-H); ^1^H NMR (CDCl₃): δ 7.5 (6H, m, ArH), 4.1 (2H, t, J = 7Hz, COOCH₂CH₂CH₃), 3.9 (3H, s, OCH₃), 3.75 (2H, s, ArCH₂), 1.6 (2H, m, COOCH₂CH₂CH₃), 0.9 (3H, t, J=7Hz, COOCH₂CH₂CH₃); ^1^C NMR (CDCl₃): δ 10.33 (COOCH₂CH₂CH₃), 21.96 (COOCH₂CH₂CH₃), 41.41 (ArCH₃), 55.21 (OCH₃), 66.44 (COOCH₂CH₂CH₃), 105.63 (C-5), 118.97 (C-7), 127.01, 127.73, 127.87 and 129.13 (C-1, C-3, C-4 and C-8), 128.95, 128.96 and 133.59 (C-2, C-9 and C-10), 157.61 (C-6), 171.82 (COO); MS (m/z): 258 (M'^+',48%), 171 (100%), 128 (17%).

**Isopropyl 6-methoxy-2-naphthylacetate** 2d: Yield 1.6g (82%); mp 71°C (Found: C, 74.39; H, 7.02. Requires C, 74.39; H, 7.02). IR (KBr): 1729
I, 55.21 (CDCI\textsubscript{3}): (6H, m, ArH), 5.1 (1H, sept, J = 7Hz). COOCH(CH\textsubscript{3})\textsubscript{2}, 3.9 (3H, s, OCH\textsubscript{3}), 3.7 (2H, s, ArCH\textsubscript{2}), 1.25 (6H, d, J = 7Hz). COOCH(CH\textsubscript{3})\textsubscript{2}, 41.41 (ArCH\textsubscript{2}), 55.21 (OCH\textsubscript{3}), 68.18 (COOCH(CH\textsubscript{3})\textsubscript{2}), 105.63 (C-5), 118.89 (C-7), 126.97, 127.68, 127.85 and 128.95 (C-1, C-3, C-4 and C-8), 129.16, 129.49 and 133.56 (C-2, C-9 and C-10), 157.58 (C-6), 171.82 (COO); MS (m/z): 258 (M\textsuperscript{+},39%), 171 (100%), 128 (19%).

\textbf{n-Butyl 6-methoxy-2-naphthylacetate 2e:} Yield 1.7g (79%); mp 40-41\degree C (Found: C, 74.78; H, 7.44. Requires C, 74.97; H, 7.4; IR (KBr):1734 (C=O), 1267 (C-O-C), 1252, 1155 (C-O), 1031 (C-O-C), 849 and 811 (C-H); \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \delta 7.5 (6H, m, ArH), 4.1 (2H, t, J = 7Hz). COOCH(CH\textsubscript{3})\textsubscript{2}, 3.95 (3H, s, OCH\textsubscript{3}), 3.8 (2H, s, ArCH\textsubscript{2}), 1.2-1.6 (4H, m. COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 0.9 (3H, t, J = 7Hz). COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}; \textsuperscript{13}C NMR (CDCl\textsubscript{3}): \delta 13.65 (COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 19.89 (COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 30.62 (COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 41.37 (ArCH\textsubscript{2}), 55.14 (OCH\textsubscript{3}), 66.70 (COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 105.61 (C-5), 118.92 (C-7), 127.00, 127.73, 127.86 and 129.12 (C-1, C-3, C-4 and C-8), 128.95, 129.35 and 133.60 (C-2, C-9 and C-10), 157.61 (C-6), 171.78 (COO); MS (m/z): 272 (M\textsuperscript{+},43%), 171 (100%), 128 (24%).

\textbf{Isobutyl 6-methoxy-2-naphthylacetate 2f:} Yield 1.6g (76%); mp 45-46\degree C (Found: C, 74.40; H, 7.17. Requires C, 74.97; H, 7.40; IR (KBr): 1729 (C=O), 1267 (C-O-C), 1209, 1142 (C-O), 1028 (C-O-C), 852 and 813 (C-O), 1031 (C-O-C), 849 and 811 (C-H); \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \delta 7.5 (6H, m, ArH), 3.9 (2H, d, J = 7Hz). COOCH(CH\textsubscript{3})\textsubscript{2}, 3.95 (3H, s, OCH\textsubscript{3}), 3.75 (2H, s, ArCH\textsubscript{2}), 1.9 (1H, m. COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 0.9 (6H, d, J=7Hz). COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}; \textsuperscript{13}C NMR (CDCl\textsubscript{3}): \delta 19.01 (COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 27.71 (COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 41.42 (ArCH\textsubscript{2}), 55.17 (OCH\textsubscript{3}), 70.9 (COOCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 105.63 (C-5), 118.92 (C-7), 126.99, 127.74, 127.89 and 129.12 (C-1, C-3, C-4 and C-8), 128.94, 129.36 and 133.58 (C-2, C-9 and C-10), 157.60 (C-6), 171.74 (COO); MS (m/z): 272 (M\textsuperscript{+},43%), 171 (100%), 128 (20%).

\textbf{Analysis of 6-MNA and esters (2a-2f) by HPLC}

A reversed phase HPLC procedure was used for the quantitative determination of 6-MNA and the esters (2a-2f). A Bondapack C18, 125A, 10\micro m (3.9 x 300 mm) column was eluted with a mobile phase consisting of acetonitrile/0.02 M phosphate buffer of pH 4.0. The flow rate was 1.0 mL min\textsuperscript{-1} and the column effluent was monitored at 254 nm. Under these conditions the retention times of the esters were in the range of 4-6.5 min. It was assured that in each case adequate separation of the ester from the hydrolysis product, 6-MNA (2), was achieved. Quantification of the compounds was done from standard plots made from the standards chromatographed under the same conditions.

\textbf{Determination of solubility}

The solubility of the esters (2a-2f) in 0.05 M phosphate buffer of pH 7.4 was determined at 25\degree C by adding excess amount of the esters to the buffer in screw capped test tubes. The mixtures were placed in an ultrasonic bath for 10 min and then shaken gently in a water bath for 20 hr. It was ensured that saturation equilibrium was established. After filtration the filtrate was analyzed by HPLC.

\textbf{Determination of partition-coefficient}

The partition-coefficients of the esters (2a-2f) were determined in octanol-buffer system at 25\degree C. The aqueous phase was 0.05 M phosphate buffer of pH 7.4. The buffer solution and octanol were mutually saturated at 25\degree C before use. The traditional shake flask method was used for determining different concentrations by HPLC to afford rapid evaluation and better reliability.\textsuperscript{19,20,21} The compounds were dissolved in 2 mL octanol in a 10 mL screw capped tube. After addition of 2 mL buffer, the phases were mixed on a cyclo mixer for 15 min and then shaken in a water bath for 8 hr. The tubes were centrifuged at 3000 rpm for 30 min. One mL of the octanol layer was removed, diluted, 20\microL of this was injected and the peak area measured. The ratio of the peak area multiplied by the dilution factor gives the partition-coefficient.

The lipophilicity of the derivatives was also evaluated by means of reversed phase chromatography.\textsuperscript{22} In this method the capacity factor (k') of a solute is taken as a measure for the relative lipophilicity and is calculated as:

\[ k' = (t_R - t_0) / t_0 \]  

where \( t_0 \) is the retention time of the solute and \( t_R \) is the elution time of the solvent.

\textbf{Chemical hydrolysis}

The hydrolysis rate constants were determined at near physiological conditions, i.e. pH 7.4 at elevated temperatures ranging from 60-90\degree C, depending on the rate determined by preliminary studies.
The activation energy of the reaction was calculated from the rate constants at elevated temperature using Arrhenius Eqn.

\[ K = A e^{-E_{a}/RT} \]  

...(13)

From energy of activation, rate constants at 37°C were calculated. The hydrolysis of 2a was also investigated as a function of pH in various buffers in order to gain pharmaceutically important information. The buffers used were hydrochloric acid, acetate, phosphate and carbonate. A constant ionic strength (μ) of 0.5 was maintained by adding calculated amount of potassium chloride.

The reactions were initiated by adding 100 μL of the stock solution of the ester derivatives in acetonitrile to 20 mL of preheated plasma solutions in screw capped test tubes, the final concentrations of the compounds being \( 4 \times 10^{-6} - 1 \times 10^{-5} \) M. The solutions were kept in water bath at constant temperatures and at appropriate intervals, samples were taken and chromatographed. In cases where rate of hydrolysis was found to be very slow in preliminary studies, the solutions of the esters were filled in ampoules and kept in water bath at constant temperatures. Pseudo-first order rate constants for the degradation of these esters were determined from the slope of the linear plots of the logarithms of residual ester against time.

### Enzymatic hydrolysis

#### Hydrolysis on human plasma

The hydrolysis of the ester prodrugs of 6-MNA (2a), having solubility of more than 5 μg/mL, was studied in 80% human plasma at pH 7.4. The reaction was initiated by adding 20-50 μL of a stock solution of the ester in acetonitrile to 2-5 mL of preheated plasma solution, the final concentration of the compounds being \( 4.24 \times 10^{-3} \) to \( 1 \times 10^{-1} \) M. The solution was kept in water bath at 37°C and at appropriate time intervals samples of 100 to 250 μL were withdrawn and added to 1000-5000 μL of cold acetonitrile or methanol in order to deproteinise the solution. After immediate mixing and centrifugation for 5 min at 7000 rpm, 20 μL of the clear supernatant was analyzed by HPLC for the remaining ester prodrug. The values of rate constants (k) and half-lives (t½) were calculated from the slopes of linear plots of logarithms of remaining prodrug versus time using linear regression.

#### Hydrolysis in liver homogenate

The hydrolysis of simple alkyl esters (2a-2f) was also studied in 0.2% liver homogenate at 37°C. Liver homogenate was prepared by homogenizing the section of rat livers in tissue homogenizer containing a glass body and Teflon pestle at 0-5°C. The homogenate was diluted with 0.05 M phosphate buffer (pH 7.4) to make a 10% w/v liver homogenate and centrifuged at 10,000 rpm at 4°C. The supernatant was diluted and used for the experiments. The reactions were studied in a way similar to that described under human plasma hydrolysis studies.

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### References