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Kinetics of hydrolysis of meclofenoxate hydrochloride in human plasma

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The kinetics of hydrolysis of meclofenoxate hydrochloride in human plasma have been compared with those of clofibrate. The hydrolysis rate in fractionated plasma was determined in the presence and absence of a plasma esterase inhibitor, tetraethyl pyrophosphate. The kinetic data indicated that clofibrate decomposed only by esterase-induced hydrolysis, which was inhibited by binding of clofibrate to plasma proteins. In contrast to clofibrate, meclofenoxate decomposed rapidly in human plasma via spontaneous hydrolysis as well as esterase-induced hydrolysis. The spontaneous hydrolysis appeared to be inhibited by some components present in the esterase fraction isolated from plasma, while no significant inhibition of the hydrolysis by protein binding was observed.

Investigation of drug decomposition in plasma is necessary to obtain reliable data on the pharmacokinetics and fate of drugs in-vivo, but the literature on the kinetics of drug decomposition in plasma is small (Altmayer & Garrett 1983).

Meclofenoxate hydrochloride [2-(dimethylamino)-ethyl(4-chlorophenoxy)acetate hydrochloride] is a psychostimulant, which is considered to penetrate into the brain as the intact ester (Miyazaki et al 1971, 1976). It is known to be hydrolysed rapidly to *p*-chlorophenoxyacetic acid and dimethylaminoethanol in aqueous solution at physiological pH (Grabowska 1970; Yamana et al 1972), which suggests that rapid hydrolysis of meclofenoxate would occur in the circulation, however, the hydrolysis in plasma has not been studied kinetically. The present study was designed to evaluate the hydrolysis of meclofenoxate in plasma and to clarify the effects of various components in plasma on the hydrolysis rate. The kinetics of hydrolysis of clofibrate, which is known to bind largely to plasma protein, were also compared with those of meclofenoxate.

Materials and methods

Meclofenoxate hydrochloride was supplied by Dainippon Pharmaceutical Co. Ltd, Osaka, Japan. Fresh frozen human plasma was obtained from Kokuritsu Shoni Hospital (Tokyo, Japan); it had been prepared by addition of 28 mL of CPD solution (citric acid 17·0 mM, sodium phosphate 8·2, sodium citrate 102, glucose 129) to 200 mL of fresh plasma. Phosphate-buffered solution, pH 7·5, contained the same concentrations of citrate, phosphate and glucose as the fresh frozen plasma. Egg phosphatidylcholine was purchased from Sigma Chemical Co. (St Louis, USA). All other chemicals were of reagent grade.

Fractionation of human plasma. A 10 mL aliquot of the human plasma was applied to a Cellulofine GCL-2000 m column (26 mm × 100 cm) (Seikagaku Kogyo Co., Tokyo, Japan), and eluted with the phosphate-buffered solution at a rate of 0·4 mL min^{-1} and fractions of 3 mL each were collected. Column fractions were assayed for proteins by measuring the absorption at 280 nm, and were also assayed for esterase activity by means of Cholinesterase B-Test (Wako Pure Chemical Ind. Ltd, Osaka, Japan) with benzoylcholine as a substrate.

Kinetic measurement. The kinetic study of hydrolysis of meclofenoxate was carried out at 25 °C (the rapid hydrolysis in plasma could not be followed at 37 °C). Meclofenoxate was dissolved to make 2×10^{-3} M in the human plasma diluted with the phosphate-buffered solution and in the fractions of human plasma having different levels of esterase activity, obtained by gel-filtration. A 1 mL aliquot of the sample solution was taken at appropriate intervals so as to determine the apparent zero-order rate constant at the initial stage of the hydrolysis. The initial hydrolysis rate was estimated on the basis of the linear increase in the amount of hydrolysed products formed as a function of time. The kinetics of hydrolysis of clofibrate (1×10^{-4} M) was similarly studied at 37 °C.

The kinetic study on the spontaneous hydrolysis was carried out in three fractions of human plasma obtained by gel-filtration: a fraction having the strongest esterase activity (esterase fraction), a fraction containing most of the plasma albumin (representing the strongest absorption at 280 nm) (albumin fraction), and a fraction not containing any plasma components (a fraction eluted before the first peak of absorption at 280 nm). In order to inhibit the esterase-induced hydrolysis, tetraethyl pyrophosphate was added to each medium to give 95·7 μg mL^{-1}. Then, meclofenoxate was dissolved to make 0·5 to 2 mM in each fraction.

The extent of protein binding of meclofenoxate was preliminarily determined in the buffered plasma (the plasma diluted 1·5-fold with the phosphate-buffered solution), the esterase fraction, and the albumin fraction, after addition of tetraethyl pyrophosphate

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(95·7 μg mL^{-1}), with a Micropartition system MPS-1 (Amicon, Lexington, USA). Immediately after meclofenoxate was dissolved in each solution to make 1 mM at 25 °C, the solution was transferred to a Micropartition cell and centrifuged at 3000 rev min^{-1} at 4 °C. The concentration of *p*-chlorophenoxyacetic acid in the filtrate and that in the solution before centrifugation were determined after 10 min standing, when meclofenoxate was completely hydrolysed to *p*-chlorophenoxyacetic acid. Approximate extent of protein binding was estimated by comparing the concentrations.

Preparation of liposomes. Small unilamellar liposomes of neutral charge were prepared from egg phosphatidylcholine alone by a sonication procedure as described by Szoka & Papahadjopoulos (1980). The phospholipid was dissolved in chloroform in a 10 mL pear-shaped flask, and the chloroform was evaporated, leaving a thin film on the bottom of the flask. The flask was transferred to a desiccator and placed under vacuum for 3 h to remove remaining chloroform. The dried lipid was suspended in the phosphate-buffered solution and ultrasonically irradiated with a probe-type sonicator (Model UR-200P, Tomy Seiko Co., Tokyo, Japan) at 20 kHz and 4 °C under nitrogen for 1 h. Titanium fragments released from the sonication probe and undispersed phospholipid were removed by centrifugation at 100 000*g* for 30 min at 4 °C. To obtain positively and negatively charged liposomes, stearylamine and diacetylphosphate were added to give 17% of the total lipid, respectively. The kinetic study to clarify the effect of liposomes on the hydrolysis rate of meclofenoxate was carried out in the phosphate-buffered solution with 2 mM lipid.

Determination of meclofenoxate, clofibrate and their hydrolysed products by HPLC. Meclofenoxate and the hydrolysed product were determined in the following manner. A 1 mL aliquot of the sample solution was added to 4 mL of acetonitrile containing 0·7 mM diethylphthalate as an internal standard with vigorous shaking. Five μL of the solution was injected into a chromatograph (model 655, Hitachi Ltd, Tokyo, Japan) equipped with a multiple wavelength detector and a TSK 410 column (150 × 4 mm) (Toyo Soda Kogyo Corp., Tokyo, Japan). The mobile phase consisted of 50 mM phosphate buffer (adjusted to pH 2·5) and methanol (1:1), which was delivered at a rate of 1 mL min^{-1}. The column eluate was monitored at 280 nm. Clofibrate was determined by the same method, except that the mobile phase consisted of phosphate buffer (pH 2·5) and acetonitrile (4:6); the internal standard was diisopropylphthalate, and the column eluate was monitored at 225 nm.

Results

Since an increase in the pH value of human plasma was observed in the course of kinetic measurement because of elimination of carbon dioxide, hydrolysis of meclofenoxate was followed in the human plasma buffered with a relatively high concentration of phosphate-buffered solution. Initial rates of hydrolysis of meclofenoxate were determined in the buffered plasma prepared from five different batches of human plasma having different levels of esterase activity, and they are plotted against the esterase activity in Fig. 1. The hydrolysis of meclofenoxate was also followed in the fractions of human plasma obtained by gel-filtration using the phosphate-buffered solution as an eluent. Fig. 2 shows the gel-filtration pattern of human plasma, illustrating the total amount of protein and the esterase activity determined using benzoylcholine as a substrate. Initial hydrolysis rates determined in the esterase fraction are also plotted against the esterase activity in Fig. 1. A linear relationship was observed between the initial rate and the esterase activity, with a y-intercept representing spontaneous hydrolysis of meclofenoxate, independent of the esterase. Hydrolysis in the esterase fraction appeared to be faster than that in the buffered plasma with the same level of esterase activity, though the difference was small.

The hydrolysis rate of clofibrate, which is known to bind to plasma proteins, was also measured in the buffered plasma and the esterase fractions of plasma, having different levels of esterase activity. The results are shown in Fig. 3. The hydrolysis rate of clofibrate in the buffered plasma showed a large scatter regardless of the esterase activity, though a linear relationship that passed through the origin was observed between the rate in the esterase fraction and the esterase activity.

The exact extent of protein binding of meclofenoxate cannot be determined because it is impossible to inhibit the spontaneous hydrolysis of meclofenoxate during the measurement. The extents of protein binding in the buffered plasma and in the esterase and albumin fractions isolated from plasma were approximately

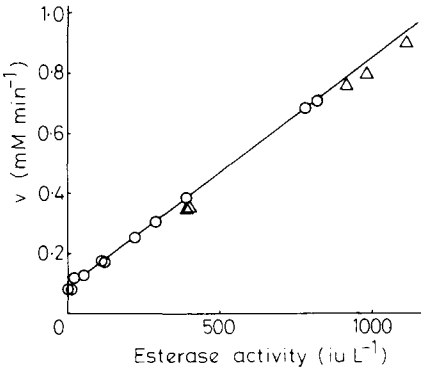

FIG. 1. Initial rates of hydrolysis of meclofenoxate in the buffered plasma (△) and the esterase fraction (○), as a function of the esterase activity (25 °C).

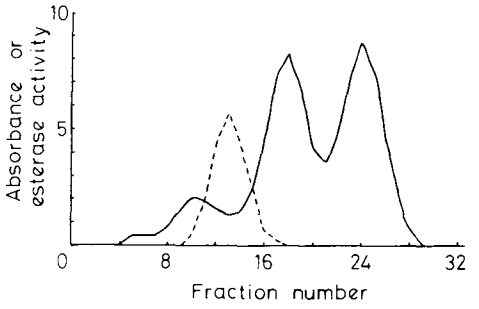

FIG. 2. Gel-filtration pattern of human plasma. Elution patterns of protein (——) and esterase activity (– – –).

determined by a micropartition method. The concentrations of unbound meclofenoxate in the buffered plasma and in the albumin fraction were found to be less than that in the phosphate-buffered solution (80 and 90% of that in the phosphate-buffered solution, respectively), but no reduction in the concentration of unbound meclofenoxate was observed in the esterase fraction.

The spontaneous hydrolysis of meclofenoxate in the fractions isolated from plasma (the esterase fraction, the albumin fraction and the fraction without any plasma protein (the phosphate-buffered solution)) was followed after addition of tetraethyl pyrophosphate to inhibit the esterase-induced hydrolysis. Fig. 4 shows the plots of the initial rate of spontaneous hydrolysis as a function of the initial concentration of meclofenoxate. A linear relationship between the initial rate and the initial concentration was observed for the spontaneous hydrolysis in the phosphate-buffered solution and in the esterase fraction, and the apparent first-order rate constants were estimated from the slopes to be 0·0625 and 0·0452 min^{-1}, respectively. On the other hand, the initial rate of the spontaneous hydrolysis in the albumin fraction was found to be significantly less than that in the phosphate-buffered solution.

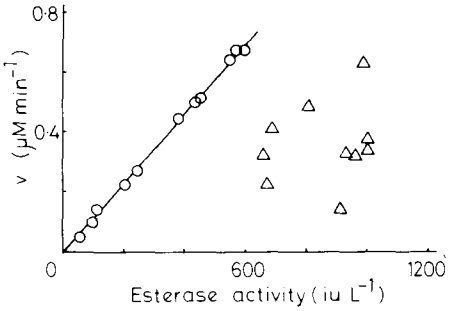

FIG. 3. Initial rates of hydrolysis of clofibrate in the buffered plasma (△) and the esterase fraction (○), as a function of the esterase activity (37 °C).

The effect of liposomes on the spontaneous hydrolysis rate of meclofenoxate was studied by comparing the initial rate measured in the presence of liposomes with that in the phosphate-buffered solution. Inhibition of the spontaneous hydrolysis of meclofenoxate was observed when variously charged unilamellar liposomes were added. In the presence of the same concentration of lipid as meclofenoxate, neutral liposomes, and negatively and positively charged liposomes reduced the spontaneous hydrolysis rate of meclofenoxate to 51, 50 and 79% of that in the absence of liposomes, respectively.

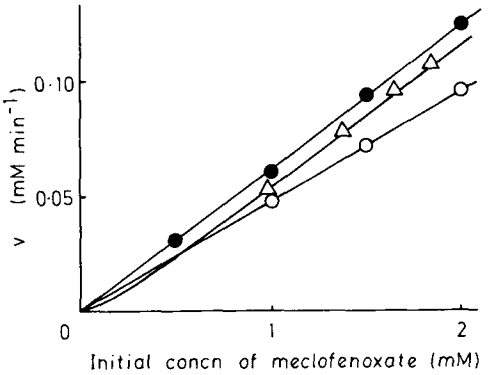

FIG. 4. Initial rates of hydrolysis of meclofenoxate in the phosphate-buffered solution (●), in the albumin fraction (△) and in the esterase fraction (○), as a function of initial concentration. All values represent mean ± s.d. of five measurements.

Discussion

There seem to be many factors in plasma affecting the hydrolysis, to which meclofenoxate is subjected before it penetrates into the brain as the intact ester. Protein binding could be a major factor governing the hydrolysis rate of drugs in plasma, as reported for the hydrolysis of clofibrate (Altmayer & Garrett 1983). The present kinetic study, in which hydrolysis of meclofenoxate in plasma was compared with that of clofibrate, represents a better insight into the fate of meclofenoxate in-vivo. The initial hydrolysis rate of clofibrate in the buffered plasma was found not to be correlated linearly with the esterase activity, while the initial rate in the esterase fraction isolated from plasma increased in proportion to the esterase activity (Fig. 3). This suggests that clofibrate is hydrolysed in plasma only by plasma esterase and that the hydrolysis rate of clofibrate in plasma is remarkably enhanced by removing the fraction which contains large amounts of plasma proteins, to which clofibrate binds. In contrast with the hydrolysis of clofibrate, meclofenoxate was found to decompose in plasma by spontaneous and enzyme-induced hydrolysis,

as indicated by a linear relationship with a certain value of y-intercept observed between the initial rate and the esterase activity (Fig. 1). The small difference between the hydrolysis rate of meclofenoxate in the buffered plasma and that in the esterase fraction suggests a small effect of protein binding of the drug on the hydrolysis rate in the concentration range studied. In a preliminary study on protein binding of meclofenoxate, a low level of protein binding was observed in the buffered plasma and in the albumin fraction of plasma, and not in the esterase fraction. This suggests that the low level of protein binding of meclofenoxate in plasma can be mainly ascribed to the albumin fraction, and that protein binding in the esterase fraction is negligible.

Based on the results described above, meclofenoxate seems to decompose in human plasma in the following manner:

$$\text{Meclofenoxate–Esterase} \xrightarrow{k_{cat}} p\text{-chlorophenoxyacetic acid} + \text{dimethylaminoethanol}$$

$$\text{Meclofenoxate–Esterase} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{Meclofenoxate} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Meclofenoxate–Protein}$$

$$\text{Meclofenoxate} \xrightarrow{k_0} p\text{-chlorophenoxyacetic acid} + \text{dimethylaminoethanol}$$

If the initial concentration of meclofenoxate is much larger than that of esterase, as is the case in the present experiment, the initial hydrolysis rate, v can be written as follows:

$$v = \frac{k_{cat}[E_0][M]}{K_m + [M]} + k_0[M] \quad (1)$$

where [M] and $[E_0]$ are the concentrations of unbound meclofenoxate, and esterase, respectively, and $K_m = (k_{-1} + k_{cat})/k_1$. Since protein binding is negligible in the esterase fraction, [M] in equation 1 can be considered to be equal to the initial concentration of meclofenoxate, $[M_0]$. On the basis of the relationship between the initial rate observed in the esterase fraction and the esterase activity, shown in Fig. 1, k_{cat}, K_m and k_0 were estimated to be $0{\cdot}928 \times 10^{-3}$ min^{-1}, $0{\cdot}416 \times 10^{-3}$ and $0{\cdot}0433$ min^{-1}, respectively, by the least-squared regression analysis (Yamaoka et al 1981). The estimate of k_0, the rate constant of spontaneous hydrolysis, coincided with the k_0 value estimated for the esterase fraction on the basis of the dependence of the initial rate determined in the presence of a plasma esterase inhibitor on the initial concentration of meclofenoxate ($0{\cdot}0452$ min^{-1}) (Fig. 4), and it was significantly smaller than the k_0 value estimated for the spontaneous hydrolysis in the phosphate-buffered solution ($0{\cdot}0625$ min^{-1}) (Fig. 4). The decrease of the rate constant of spontaneous hydrolysis in the esterase fraction in comparison with that in the phosphate-buffered solution cannot be interpreted in terms of protein binding, because protein binding is negligible in the esterase fraction as described above. The inhibition of the spontaneous hydrolysis may be ascribed to some plasma components present in the esterase fraction. For example, lipophilic components could affect the hydrolysis rate (Yotsuyanagi & Ikeda 1980; D'Silva & Notari 1982). This may be supported by the observation that the spontaneous hydrolysis rate of meclofenoxate was reduced by addition of variously charged unilamellar liposomes.

On the other hand, the observation that the initial rate of the spontaneous hydrolysis in the albumin fraction was less than that in the phosphate-buffered solution (Fig. 4) can be interpreted by assuming albumin binding of meclofenoxate. The initial rate observed in the albumin fraction could be fitted by a curve simulated on the assumption that the concentration of meclofenoxate was decreased by albumin binding (assuming that the albumin concentration is $0{\cdot}15 \times 10^{-1}$ M, the dissociation constant is $0{\cdot}001$ M, and k_0 is $0{\cdot}0625$ min^{-1}).

In conclusion, meclofenoxate was found to decompose rapidly in human plasma via spontaneous hydrolysis as well as esterase-induced hydrolysis. The hydrolysis was not significantly inhibited by protein binding in the concentration range studied, but the spontaneous hydrolysis appeared to be inhibited by some components in human plasma. The present in-vitro kinetic data, obtained at 25 °C, suggests a rapid hydrolysis of meclofenoxate in-vivo (37 °C). It is suggested that a significant amount of meclofenoxate that enters the circulation could be hydrolysed before it penetrates into the brain.

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