International Journal of Pharmaceutics, 40 (1987) 249–255
Elsevier

IJP 01362

N-(Acyloxyalkoxycarbonyl) derivatives as potential prodrugs of amines. II. Esterase-catalysed release of parent amines from model prodrugs

U.S. Gogate [1] and A.J. Repta [1,2]

[1] *Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045 (U.S.A.)*
and [2] *INTERX Research Corp., Merck and Co., Lawrence, KS 66046 (U.S.A.)*

(Received 6 April 1987)
(Modified version received 18 June 1987)
(Accepted 19 June 1987)

Key words: Amine prodrug, Acyloxyalkoxycarbamate; Plasma esterase; Enzyme catalysis

Summary

N-(Acetoxyethoxycarbonyl) derivatives of primary amines released a major fraction of the parent amine in the desired free form in plasma but a significant fraction of the undesired *N*-acetylated parent amine was also produced. The fraction of the parent amines released from the carbamate derivatives of the primary amines was greater in human plasma than in pH 7.4 buffer. In human plasma, the *N*-(acetoxyethoxycarbonyl) derivative of a secondary amine released the parent amine in a quantitative manner at a rate higher than that observed in pH 7.4 buffer. Experimental results suggested that the observed catalysis of the release of the parent amines from *N*-(acetoxyethoxycarbonyl) derivatives of primary and secondary amines was due to participation by plasma esterases. The data suggested that *N*-(acetoxyethoxycarbonyl) derivatives are well suited for use as prodrugs of secondary amines. Their utility as prodrugs of primary amines is more problematic and cannot be predicted prior to in-vivo studies for the individual compound.

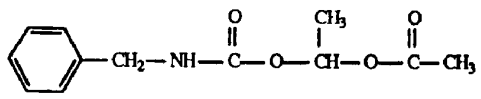
Introduction

The rationale for the use of *N*-(acyloxyalkoxycarbonyl) derivatives as prodrugs of amines has been discussed previously (Gogate et al., 1987; Gogate (1987)). It was shown that *N*-(acetoxyethoxycarbonyl) derivatives (I–IV, Table 1) of primary and secondary amines are likely to yield prodrug candidates with sufficient stability for formulation as solids or as liquids. In order for these compounds to be useful as prodrugs, they should revert rapidly and quantitatively to the parent amines in the human body. To assess the suitability of these derivatives as prodrugs, information regarding probable release of the parent amines from the model prodrugs was needed. The carboxylesterases present in the human body would be expected to cleave these acetoxyethoxycarbonylamine compounds, I–IV, by hydrolysis of the ester portion as proposed earlier (Gogate et al., 1987). In the present studies, recovered human

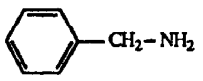
Correspondence: A.J. Repta, Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045, U.S.A.

TABLE 1

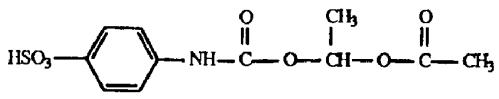
Structures of the 4 model prodrugs studied and their parent amines



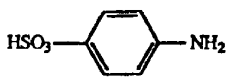
N-(Acetoxyethoxycarbonyl)benzylamine (I)



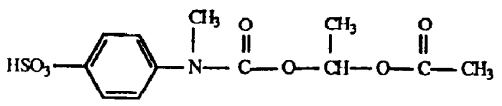
Benzylamine



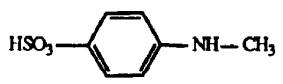
4-(N-(Acetoxyethoxycarbonyl)amino)benzenesulfonic acid (II)



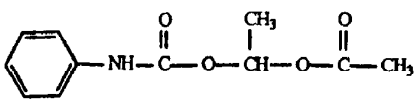
4-Aminobenzenesulfonic acid



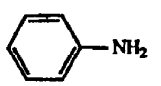
4-(N-(Acetoxyethoxycarbonyl)-N-methyl)benzenesulfonic acid (III)



4-Methylaminobenzenesulfonic acid



N-(Acetoxyethoxycarbonyl)aniline (IV)



Aniline

plasma was used as a representative source of esterase activity present in the body. Two shortcomings of using plasma were recognised. Esterase activity in other tissues will differ in both magnitude and specificity from that in plasma (Casida et al., 1966). Also, the total metabolic pathways available in the body will not be restricted to those present in plasma. In spite of these shortcomings, human plasma represents a convenient and often-used (Patel, 1980; Nielson, 1986) biological fluid for preliminarily assessing the possible fate of the acetoxyethoxycarbonyl derivatives of amines in a biological medium. Various experiments were performed to obtain information about the kinetics, mechanism and products of degradation of model prodrugs I–IV in human plasma. The methodology and results of these experiments and their in-vivo implications are presented in this paper.

Materials and Methods

Materials

Recovered human plasma was obtained from the Community Blood Center of Kansas City, MO. Di-isopropylfluorophosphate (DFP) was obtained from Aldrich Chemical Co. Porcine liver esterase was obtained from Sigma Chemical Co., (E 3128). The model prodrugs (I–IV) were prepared as described previously (Gogate et al., 1987). The high performance liquid chromatographic (HPLC) system and analytical column used for analysis of various compounds in human plasma were also identical to those described earlier (Gogate et al., 1987) with the modification that an additional ODS-Hypersil column (5 cm × 4.6 mm (i.d.)) was also used as a guard column to protect the analytical column during plasma studies.

Methods

Kinetics of degradation in plasma

The kinetics of degradation of compounds I–IV in recovered human plasma at $37 \pm 0.1°C$ were studied by analyzing the solutions with HPLC. The mobile phases used in analyzing various compounds are listed in Table 2 along with the compounds analyzed and their retention volumes. All analyses were done at ambient temperature. A mobile phase flow rate of 1 ml/min was used and the column effluent was monitored at 254 nm. The quantitation of compounds was achieved by measuring the peak heights in relation to those of standard solutions chromatographed under the same conditions. In a typical degradation experiment an accurately weighed sample was placed in a 10-ml volumetric flask. Acetonitrile (1 ml) was added to dissolve the sample. A sufficient amount of distilled water was added to bring the volume to 10 ml. An aliquot (1 ml) from this solution was transferred to a second 10-ml volumetric flask and a sufficient quantity of recovered human plasma (equilibrated at 37°C) was added to bring the volume to 10 ml. This plasma solution was then placed in a thermostated waterbath maintained at 37°C. Unless otherwise indicated, the initial concentration of the compound under study was 10–100 μM. The loss of the compound in the plasma solution was followed by withdrawing samples from the solution at various time points, subjecting them to the sample preparation steps given below and analysing by HPLC. The apparent first-order rate constants for degradation were obtained by linear regression analysis of natural log of concentration vs time (correlation coefficient > 0.97).

The effect of the presence of DFP on the degradation of III in plasma was also studied. The procedure for the experiment was identical to that mentioned above with the exception that a specified amount of DFP was added to the plasma solution at the beginning of the experiment.

Sample preparation for plasma studies. A plasma sample (0.5 ml) was diluted with an equal volume of methanol to precipitate plasma proteins. Independent studies demonstrated that addition of methanol terminated the enzymatic degradation. After vortex mixing and centrifugation at 750 × g for 5 min the clear supernatant liquid was directly injected and analysed by HPLC.

TABLE 2

Mobile phases used for studying the degradation of various compounds in human plasma

Mobile phase	Compounds analysed (retention volumes in ml)
35% CH_3CN : water [a]	I (11.0)
CH_3CN : acetate buffer (0.1 M, pH 4.6) (45 : 100) (0.05% THABr) [b]	II (9.0)
CH_3CN : phosphate buffer (0.1 M, pH 4.6) (48 : 100) (0.05% THABr)	III (6.0)
CH_3CN : acetate buffer (0.1 M, pH 4.6) (30 : 100) (0.05% THABr)	sulfanilic acid (7.1), *N*-acetylsulfanilic acid (8.7) *N*-methylsulfanilic acid (5.0)
40% CH_3CN : water [a]	IV (9.5)
10% CH_3CN : water [a]	aniline (11.5), acetanilide (15.0)

[a] Organic solvent brought to volume with water.
[b] THABr, tetrahexylammonium bromide.

Product analysis

Following the completion of the hydrolysis reaction, the plasma solutions were analysed for the amounts of the various degradation products generated. Analytical procedures used were identical to those reported earlier (Gogate et al., 1987).

Kinetics of degradation in the presence of porcine liver esterase

The initial rates of degradation of III were studied as a function of its initial concentration in the presence of porcine liver esterase. These studies were carried out at $37 \pm 0.1°C$ in Sorenson's isotonic phosphate buffer, pH 7.4. The procedure used for this set of experiments was identical to that used during kinetic studies, as reported in a previous communication (Gogate et al., 1987). The concentrations of porcine liver esterase used were 0.1 and 0.2 units/ml in both sets of experiments.

Results and Discussion

Degradation of compounds I–IV in human plasma

Compounds I–IV exhibited apparent first-order degradation kinetics in plasma indicating that the initial concentration of the particular compound under study was well below saturation level for plasma enzymes. The apparent first-order rate constants for degradation of all 4 compounds in plasma at 37 °C are listed in Table 3. Following completion of the hydrolysis reaction, the plasma solutions were analysed for the amounts of the various degradation products generated. It was anticipated that the products of degradation of I–IV containing the benzene nucleus would be detected by the UV detector ($\lambda = 254$ nm) that monitored the HPLC effluent. For compounds I, II and IV two detectable degradation products were observed at $\lambda = 254$ nm, whereas only one such detectable degradation product was observed for III. The respective parent amine and the *N*-acetylated parent amine were the detectable degradation products for compounds I, II and IV, whereas the parent amine (i.e. *N*-methylsulfanilic acid) was the only detectable degradation product for III in plasma. The percents of the degradation products formed from the 4 compounds in plasma are also listed in Table 3. The rate constants for degradation of the compounds I–IV in pH 7.4 buffer at 37 °C and the percents of the products formed (Gogate et al, 1987) are also listed in Table 3 for the purpose of comparison.

One or more components present in human plasma appeared to be catalyzing degradation of *N*-(acetoxyethoxycarbonyl)amine compounds as suggested by the observation that rate constants for degradation of all the 4 compounds, I–IV, in plasma were greater than those in pH 7.4 buffer at 37 °C (Table 3). The detectable degradation products of compounds I, II and IV in plasma were the same as those observed in pH 7.4 buffer, i.e., the parent amines and *N*-acetylated parent amines. Previously the generation of the parent amine from these compounds at pH 7.4 was accounted for by ester hydrolysis and the formation of *N*-acetylated parent amine was accounted for by an intramolecular acyl transfer reaction (Gogate et al., 1987). The same two general reactions can also account for formation of parent amines and *N*-acetylated parent amines from I, II and IV in plasma. The relative amounts of the two degradation products in plasma were different from those in pH 7.4 buffer. The respective parent amine was the major degradation product for each of the compounds I, II and IV in plasma, whereas the *N*-acetylated parent amine was the major degradation product in pH 7.4 buffer. Thus, it appears that catalysis of ester hydrolysis occurs in plasma at the expense of intramolecular acyl transfer reaction. As was observed in pH 7.4 buffer, *N*-methylsulfanilic acid was the only detectable de-

TABLE 3

Rate constants of degradation and percent products formed from compounds I–IV in pH 7.4 buffer [a] *and in human plasma at 37 °C*

Compound	Medium	k_{obs} (h^{-1})	% Amine formed	% *N*-Acetylated amine formed
I	pH 7.4 buffer	0.198 ± 0.012	2.2 ± 0.3	97.8 ± 0.5
	human plasma	0.387 ± 0.014	–	[b]
II	pH 7.4 buffer	0.070 ± 0.002	10.0 ± 1.9	90.0 ± 2.0
	human plasma	0.188 ± 0.011	65.4 ± 3.7	34.6 ± 3.7
III	pH 7.4 buffer	$(7.1 \pm 0.3) \times 10^{-3}$	100.0 ± 1.8	0
	human plasma	0.112 ± 0.012	100.0 ± 2.2	0
IV	pH 7.4 buffer	0.203 ± 0.009	2.5 ± 0.2	97.5 ± 0.4
	human plasma	0.609 ± 0.012	85.9 ± 4.0	14.1 ± 4.0

[a] From Gogate et al. (1987).

[b] The parent amine was the major degradation product.

gradation product of III in plasma. Since III is incapable of undergoing the intramolecular acyl transfer reaction, it degrades in plasma solely by ester hydrolysis which is catalysed by plasma components.

Esterase participation in the hydrolysis of N-(acetoxyethoxycarbonyl) derivatives of amines

N-(Acyloxyalkoxycarbonyl) derivatives of the type studied here were suggested (Alexander, 1984) as prodrugs of amines with the anticipation that esterases present in human body will catalyze release of the parent amines from such prodrugs. The results described clearly indicate that components of human plasma catalyze the release of the parent amines from these types of prodrugs. Various experiments were performed to assess whether the observed catalysis of the release of the parent amines in plasma could be attributed to plasma esterases. The results of those experiments and their implications are described below. Of the modified carbamates under investigation, III was chosen in preference to I, II and IV for studying enzyme catalysis, because the potentially interfering intramolecular acyl transfer reaction does not occur in hydrolysis of III in buffers or in plasma. Accordingly, the enzyme catalysis of the ester hydrolysis reaction could be studied unambiguously, if III was used as a substrate.

Effect of the substrate concentration on initial rates of degradation of III in plasma

This set of experiments was performed to examine the saturable nature of the catalysis of hydrolysis of III by human plasma. Initial rates of degradation of III were studied in human plasma as a function of its initial concentration at 37 °C. These initial rates were corrected for non-enzymic hydrolysis by subtracting from them the initial rates of degradation of III in pH 7.4 buffer under similar conditions. The data obtained clearly demonstrated the saturable nature of the plasma component-catalyzed hydrolysis of III. A Lineweaver–Burk (1934) plot (1/initial rate of degradation vs 1/initial concentration) of the data for the degradation of III in recovered human plasma at 37 °C is shown in Fig. 1 along with the

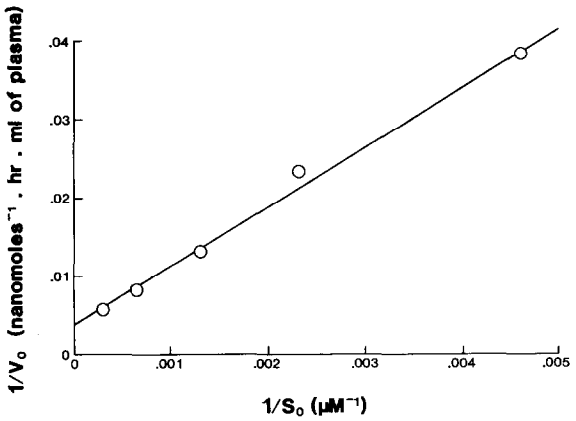


Fig. 1. Lineweaver–Burk plot for the initial rates of degradation of III in human plasma. The values of V_{max} was calculated (by linear regression analysis, $r = 0.996$) to be 255 nmol/h/ml plasma and $K_m = 1920$ μM.

calculated values of V_{max} and K_m. The Lineweaver–Burk plot in Fig. 1 appears to be linear ($r > 0.99$ for linear regression analysis). These data suggest that the catalytic hydrolysis of III observed in human plasma is a saturable process and hence is enzyme mediated.

Hydrolysis of III in the presence of porcine liver esterase

This set of experiments was performed to dem-

TABLE 4

Initial rates for degradation of III in pH 7.4 Sorenson buffer at 37 °C in the presence of porcine liver esterase

Porcine liver esterase concentration (U/ml)	Initial concentration of III (μM)	Initial rates of degradation (nmol/h/ml medium ± S.D.)
0.1	20.3	1.5 ± 0.1
	40.6	2.6 ± 0.4
	86.6	3.5 ± 0.8
	216.3	7.3 ± 1.2
	573.8	7.6 ± 1.3
0.2	20.4	3.4 ± 0.4
	40.7	6.2 ± 0.6
	65.3	8.0 ± 0.4
	147.9	11.5 ± 1.5
	347.9	12.2 ± 1.5

onstrate that compound III is a substrate for porcine liver esterase. The initial rates of degradation of III in the presence of porcine liver esterase were studied as a function of the initial concentration of III. These initial rates were corrected for non-enzymic degradation by subtracting from them the initial rates of degradation of III under similar pH and temperature conditions, but in the absence of enzyme. The corrected initial rates of degradation are listed in Table 4 as a function of porcine liver esterase concentration and the initial concentration of III. Lineweaver–Burk plots (1934) for those two experiments are shown in Fig. 2 along with the calculated values of V_{max} and K_m. The plots for these studies at two different porcine liver esterase concentrations are linear (Fig. 2), suggesting a presence of saturable catalytic process by porcine liver esterase. The increase in the value of V_{max} with an increase in porcine liver esterase concentration clearly demonstrates that the porcine liver esterase enzyme catalyzes the hydrolysis of the compound III.

Enzyme inactivation by DFP

The effects of the presence of DFP on the initial rates of hydrolysis of III in recovered human plasma and in pH 7.4 Sorenson buffer in the presence of porcine liver esterase were studied at 37 °C. DFP is a known inhibitor of porcine liver esterase (Augusteyn, 1969) and of esterases present in human plasma (Tsujita, 1983). Therefore, if the catalysis of hydrolysis of III by plasma and by porcine liver esterase is inhibited by addition of DFP, it would support the supposition that hydrolysis of compound III is catalysed by the esterases present in human plasma and porcine liver. For the enzyme inhibition studies in plasma, the initial concentration of III and DFP were 400 μM and 2 mM, respectively. The results of these experiments are summarised in Table 5. For enzyme-inhibition studies in pH 7.4 Sorenson buffer, the initial concentration of III used was 80 μM, porcine liver esterase concentration used was 0.2 U/ml and DFP concentration used was 1 mM.

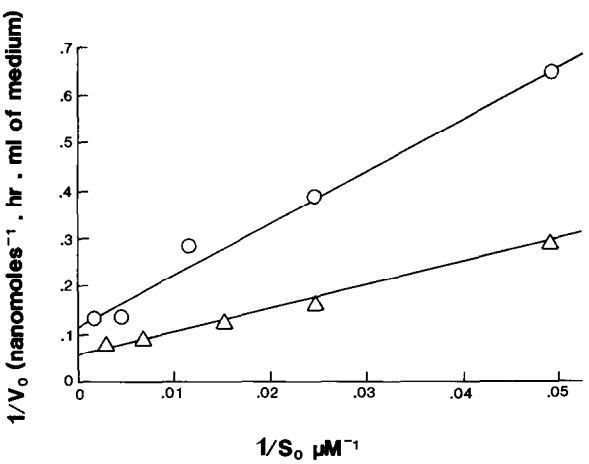


Fig. 2. Lineweaver–Burk plots for the initial rates of degradation of III in the presence of porcine liver esterase. Porcine liver esterase concentration and the values of V_{max} and K_M for the two experiments were, respectively: 0.1 U/ml (○), 1 nmol/h/ml medium, 84 μM, and 0.2 U/ml (△), 2 nmol/h/ml medium, 87 μM. Values of V_{max} and K_M were calculated by linear regression analysis. In both cases $r > 0.99$.

TABLE 5

The initial rates for degradation of III at 37 °C in buffer and in human plasma with and without DFP

Medium	DFP concentration (mM)	Initial rates of degradation [a] (nmol/h/ml medium)
pH 7.4		
Sorenson buffer	0	2.8
Human plasma	0	34.0
Human plasma	2	5.5

The initial concentration of III = 400 μM.
[a] Average of two determinations.

TABLE 6

The initial rates of degradation of III at 37 °C in Sorenson buffer (pH 7.4) with and without porcine liver esterase and DFP

Expt. no.	DFP concentration (mM)	Porcine liver esterase concentration (U/ml)	Initial rates of degradation [a] (nmol/h/ml medium)
1	0	0	0.5
2	0	0.2	8.5
3	1	0.2	1.2

Initial concentration of III = 80 μM.
[a] Average of two determinations.

The results of these experiments are summarized in Table 6. From the results reported in Tables 5 and 6, it is apparent that the catalysis of hydrolysis of III by human plasma and by porcine liver esterase is inhibited by the addition of DFP. These results clearly demonstrate that compound III (and probably related compounds) are substrates for esterases present in plasma.

Summary and Conclusions

From the results of the plasma studies described above, it is apparent that plasma components do enhance the rate and extent of release of the parent amine from the respective *N*-(acetoxyethoxycarbonyl) derivatives. Results of the various related experiments indicate that this observed catalysis of release of parent amines is primarily the result of esterase activity. In the case of prodrug derivatives of the primary amines (such as I, II and IV) quantitative release of the parent amine does not occur, but the major fraction of parent amine is released in the desired free form with the corresponding *N*-acetylated amine being the minor undesired side product. Esterase activity in various body tissues (especially liver) has been shown to be greater than that in plasma (Casida et al., 1966). If, in the whole body, the same two degradation mechanisms hold and the intramolecular route is uncatalysed, the fraction of free parent amine released might be expected to increase to nearly 100%. However, such extrapolation may not be justified because of high substrate specificity involved with enzyme activity. Thus, while promising, the utility of acyloxyalkoxycarbonyl derivatives as prodrugs of primary amines cannot be assessed with high confidence without conducting in-vivo studies in human or other target species with the specific compound of interest. However, the prospects of using modified carbamates as prodrugs of secondary amines appear very promising since the potentially interfering intramolecular acyl transfer reaction is not possible for those compounds. The results suggest that the modified carbamates will release the parent secondary amines rapidly and in quantitative yields in-vivo.

Acknowledgements

This work was supported by grants from the Parenteral Drug Association Foundation and INTERX Research Corporation, Merck Sharp and Dohme Research Laboratories, Merck and Co, Inc.

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