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Kinetics and mechanism of the enzymatic hydrolysis of aspirin phenylalanine ethyl ester

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Summary

The kinetics and the mechanism of the enzymatic hydrolysis of aspirin phenylalanine ethyl ester (I) were determined in the presence of α-chymotrypsin, carboxypeptidase A, butyryl cholinesterase (from horse serum) and in human plasma. In the presence of α-chymotrypsin or carboxypeptidase, I was found to hydrolyze to aspirin phenylalanine in accordance with Michaelis-Menten kinetics. In the presence of butyryl cholinesterase or human plasma, I was found to hydrolyze to salicyl phenylalanine ethyl ester. The data thus indicate that I does not cleave to aspirin in the presence of any of the enzymes above.

Introduction

It is known that oral administration of aspirin induces gastric irritation and bleeding because of local irritation of the gastric mucosal membrane by the very acidic aspirin particles (Leonard et al., 1970; Davison et al., 1966; Anderson, 1964). One possible approach to minimizing this side effect is masking the acidic carboxyl group of aspirin via prodrug formation. Upon administration, the neutral derivative would dissolve first, and then hydrolyze either in the GI tract or in the plasma, generating aspirin. Classical esterification of the carboxyl group results in non-irritating but insoluble species which do not revert to aspirin but rather to the

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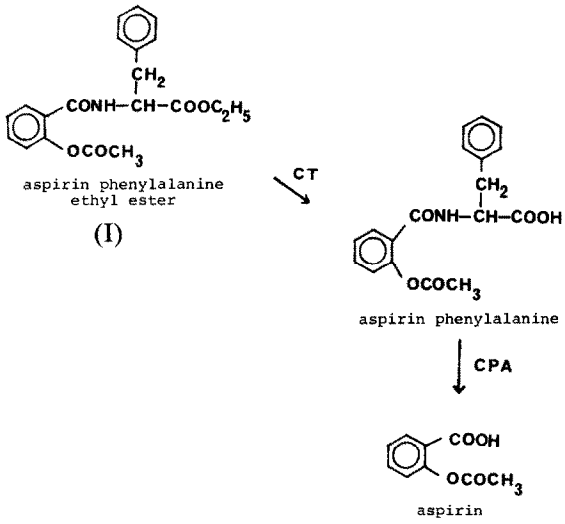
corresponding salicylate derivative (Pierre et al., 1968). However, esterification of the carboxyl moiety with protective groups that are equally labile to enzymatic hydrolysis as the acetyl group was found to generate aspirin in vivo. For example, the methylsulfinyl methyl ester of aspirin was found to generate aspirin and salicylic acid upon oral and intravenous administration to dogs (Loftsson et al., 1981). Thus, a prerequisite for any aspirin prodrug is that the masking group cleaves as fast as or much faster than the acetyl group.

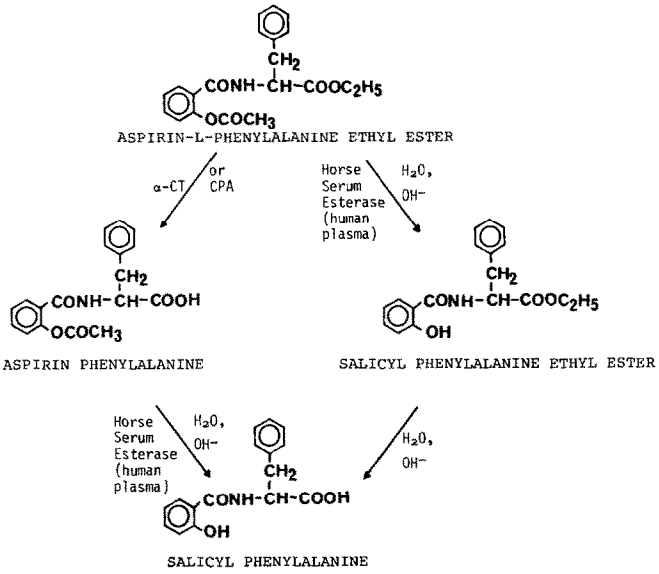
Recently, aspirin phenylalanine ethyl ester (I) was purported to be a prodrug for aspirin (Banerjee et al., 1981a, b and c). It was rationalized that, because of the specificity of certain enzymes such as α-chymotrypsin and carboxypeptidase A, I would first cleave to aspirin phenylalanine (II) and subsequently to aspirin by carboxypeptidase A (Scheme I). To support their hypothesis, the authors studied the rate of hydrolysis of I at pH values of 7.5 and 8 in the presence of α-chymotrypsin and separately determined the rate of hydrolysis of II at pH 8.5 in the presence of carboxypeptidase A. In their studies, the reactions were followed by measuring the consumption of sodium hydroxide using a pH-stat titration.

In a recent communication (Kawahara et al., 1983) employing a specific high-pressure liquid chromatographic (HPLC) assay and TLC assay, it was shown that compound I does not hydrolyze according to Scheme I but rather to its salicyl derivative according to Scheme II.

The purpose of this article is to describe, in detail, the kinetics and mechanism of hydrolysis of I and II in the presence of α-chymotrypsin and carboxypeptidase A. In addition, the hydrolysis of I and II in the presence of butyryl cholinesterase and the hydrolysis of I in the presence of human plasma is also reported.

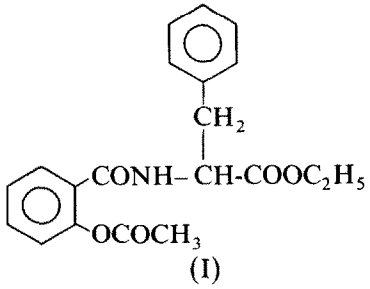


Scheme I.

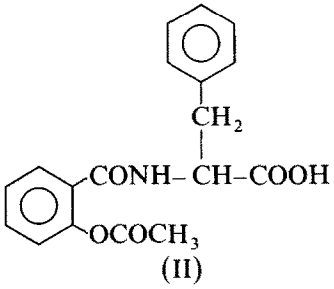


Scheme II.

The results of this study show that under any of these conditions, aspirin as not generated from I.



(I)



(II)

Experimental

Materials

Aspirin phenylalanine ethyl ester (I) and aspirin phenylalanine (II) were prepared according to the procedures previously described (Banerjee et al., 1981a; Kawahara et al., 1983). The N,N-dimethylaminoethyl ester of aspirin (III) was synthesized as the hydrochloride salt by treating a solution of 3 ml dimethylaminoethanol [1] in 50 ml dichloromethane with a solution of 9 g O-acetylsalicyloyl chloride in 50 ml

[1] Eastman Kodak Co., Rochester, NY.

dichloromethane and stirring the resulting mixture at room temperature for 4 h. The reaction mixture was then added to 600 ml of diethyl ether and stirred overnight. The crystalline product was recovered by filtration, recrystallized from methanol/ether and dried. The product (8 g) melts at 121°C and gave an NMR(^{1}H–) spectrum consistent with the structure of III. NMR($CDCl_3$): δ 12–13, broad, 0.8, –N–H; δ 7.0–8.2, m, 4, aromatic; δ 4.7–5.0, t, 2, –O–CH_2–; δ 3.4–3.8, t, 2, –CH_2–N; δ 2.9, s, 6, –N(CH_3); δ 2.4, s, 3, –OCOCH_3. α-Chymotrypsin and carboxypeptidase A were used as purchased (Eastman Kodak).

HPLC analysis

The HPLC conditions employed in these studies were previously reported (Kawahara et al., 1983).

Kinetics of hydrolysis of aspirin phenylalanine ethyl ester in the presence of α-chymotrypsin

Four stock solutions containing 7.04×10^{-3} M, 5.28×10^{-3} M, 3.52×10^{-3} M and 2.64×10^{-3} M of I in ethanol were prepared. A stock solution containing 4.62×10^{-7} M of α-chymotrypsin in 0.1 M phosphate buffer at pH 7.98 was also prepared. For the kinetics experiments, 0.8 ml of the solution of I was added to 2.0 ml of α-chymotrypsin solution and at appropriate time intervals, 0.25 ml aliquots of the reaction solution were withdrawn and mixed with 0.35 ml of 1 M phosphate buffer at pH 1.9 (to quench the reaction). Twenty-microliter aliquots were then injected into the HPLC column. The initial rate of hydrolysis at the various concentrations of I was determined from plots of change in concentration of I versus time.

Kinetics of hydrolysis of aspirin phenylalanine ethyl ester in the presence of carboxypeptidase A

Four stock solutions containing 7.04×10^{-3} M, 4.69×10^{-3} M, 3.52×10^{-3} M and 2.64×10^{-3} M of I in ethanol were prepared. A stock solution containing 1.21×10^{-5} M of carboxypeptidase A in 0.1 M phosphate buffer at pH 8.5 was also prepared. For the kinetics experiment, 0.5 ml of the solution of I was added to 2.5 ml of carboxypeptidase A solution, and at appropriate time intervals, 0.20-ml aliquots of the reaction mixture were sampled and mixed with 0.2 ml of 1.0 M phosphate buffer at pH 2.0 (to quench the reaction). Twenty-microliter aliquots were then analyzed by HPLC. The initial rate of hydrolysis at the various concentrations of I were determined as described above.

Kinetics of hydrolysis of aspirin phenylalanine ethyl ester, aspirin phenylalanine, N,N-dimethyl aminoethyl ester of aspirin and aspirin in the presence of butyryl cholinesterase: effect of enzyme concentration on the hydrolysis rate constants

Stock solutions containing 64.2, 32.1 and 16.0 units/ml of butyryl cholinesterase in 0.2 M phosphate buffer at pH 7.4 were prepared. Four stock solutions containing 4.28×10^{-3} M of I, II and N,N-dimethyl aminoethyl ester of aspirin (III) and 4.44×10^{-3} M of aspirin were prepared in dioxane. For the kinetic experiments, a

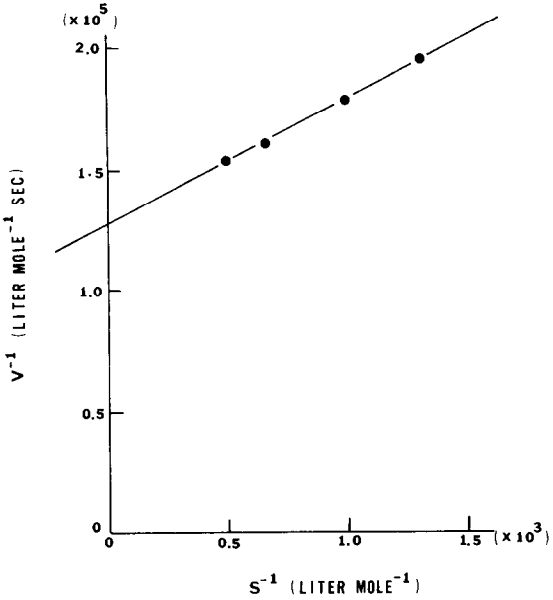


Fig. 1. Lineweaver-Burk plot for the hydrolysis of aspirin phenylalanine ethyl ester in the presence of 3.31×10^{-7} M α-chymotripsin at pH 7.98.

1.2-ml aliquot of the enzyme solution was placed in each of two 1-cm spectrophotometric cells. A 0.06 ml-aliquot of drug solution was added to the sample cell. The cell was shaken vigorously and the absorbance at 300 nm was measured at various time intervals. The observed first-order rate constants for the different enzyme concentrations were calculated from the slopes of semi-logarithmic plots of the absorbance values ($A_\infty - A_t$) versus time. The formation of the salicyl derivatives from I, II, III and aspirin was also confirmed by HPLC.

Hydrolysis of aspirin phenylalanine ethyl ester in the presence of human plasma

A solution containing 8.0×10^{-4} M of I in 0.1 M phosphate buffer at pH 7.4 was prepared. The hydrolysis of I in the presence of 10%, 40% and 100% plasma was then followed by HPLC.

Results and Discussion

The enzymatic hydrolysis of aspirin phenylalanine ethyl ester as shown in Scheme II was followed by using an HPLC method. This method was previously shown to separate I from all its hydrolytic products (Kawahara et al., 1983).

Figs. 1 and 2 show Lineweaver-Burk plots for the hydrolysis of I to II in the presence of α-chymotrypsin and carboxypeptidase A, respectively. It is apparent that the hydrolysis follows Michaelis-Menten kinetics. The V_{max}, K_m and k_{cat} values

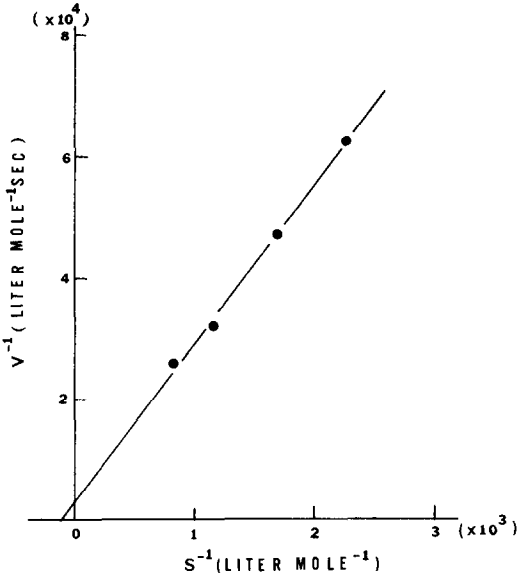


Fig. 2. Lineweaver-Burk plot for the hydrolysis of aspirin phenylalanine ethyl ester catalyzed by 1.0×10^{-5} M carboxypeptidase A at pH 8.5.

for the hydrolysis of I in the presence of α-chymotrypsin were calculated to be 7.47×10^{-6} mol · liter^{-1} · s^{-1}, 6.71×10^{-4} mol · liter^{-1} and 22.6 s^{-1}, respectively, which is in close agreement with those obtained by Banerjee and Amidon employing a pH-stat procedure (Banerjee and Amidon (1981b). The hydrolysis of I in the presence of carboxypeptidase was found to generate II and subsequently the salicyl derivative. The V_{max}, K_m and k_{cat} values for the hydrolysis of I to II in the presence of carboxypeptidase A were calculated to be 2.78×10^{-4} mol · liter^{-1} · s^{-1}, 7.15×10^{-3} mol · liter^{-1} and 27.8 s^{-1}, respectively. Thus, I cleaves to II in the presence of the two enzymes.

Non-enzymatic studies on the hydrolysis of I have also been performed under conditions that are identical to the enzymatic studies presented above except that carboxypeptidase was absent. These studies show the hydrolysis of I to have half-lives of 52.5 and 232.5 h at pH values of 8 and 7, respectively. These results are also in agreement with the findings of Banerjee and Amidon (1981a) that the half-life for hydrolysis of I at a pH of 7.5 is about 70 h. In the presence of as little as 1×10^{-5} M carboxypeptidase, however, 50% of the compound is hydrolyzed in approximately 10 min (Kawahara et al. (1983)).

In order to determine whether I generates aspirin in the presence of esterases, its hydrolysis in human plasma and by butyryl cholinesterase was determined. As shown in Fig. 3, the prodrug was found to cleave in human plasma generating the salicyl derivative (Scheme II). Similarly, in the presence of butyryl cholinesterase, prodrug I as well as compound II and aspirin were found to hydrolyze exclusively to their corresponding salicyl derivatives. The rate of hydrolysis of the acetyl group was

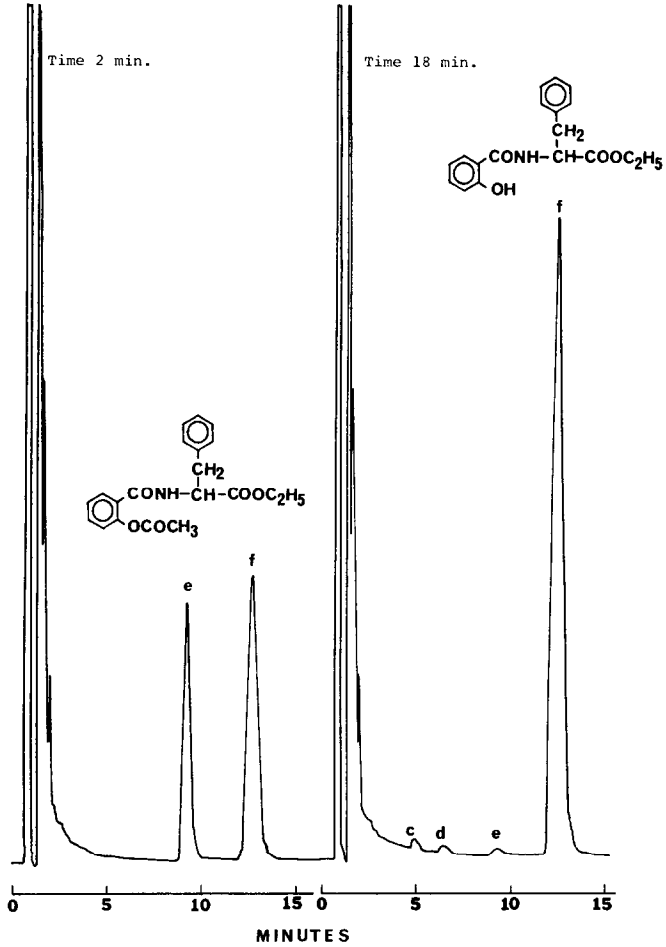


Fig. 3. HPLC chromatograms for the hydrolysis of I in human plasma. Key: (a) and (b) aspirin and salicylic acid (not shown), (c) aspirin phenylalanine, (d) salicyl phenylalanine, (e) I, and (f) salicyl phenylalanine ethyl ester.

first-order with respect to the compounds and dependent on enzyme concentration (Fig. 4). As is seen from the data of Fig. 4, the rate of hydrolysis is directly proportional to the concentration of enzyme and at any enzyme concentration there is a significant difference in the rate of hydrolysis of these compounds. Although these differences may reflect inherent differences in substrate specificities, they can be rationalized by electrostatic considerations. At pH values > 6 both II and aspirin are negatively charged, whereas I is neutral. Since the enzyme is also negatively charged above pH 6 (zwitterionic pK_a 5.8), its interaction with the negatively charged esters is less favorable than its interaction with I. In order to further verify that the observed differences in enzymatic hydrolyses were consistent with electrostatic considerations, the rate of hydrolysis by the same enzyme of a derivative

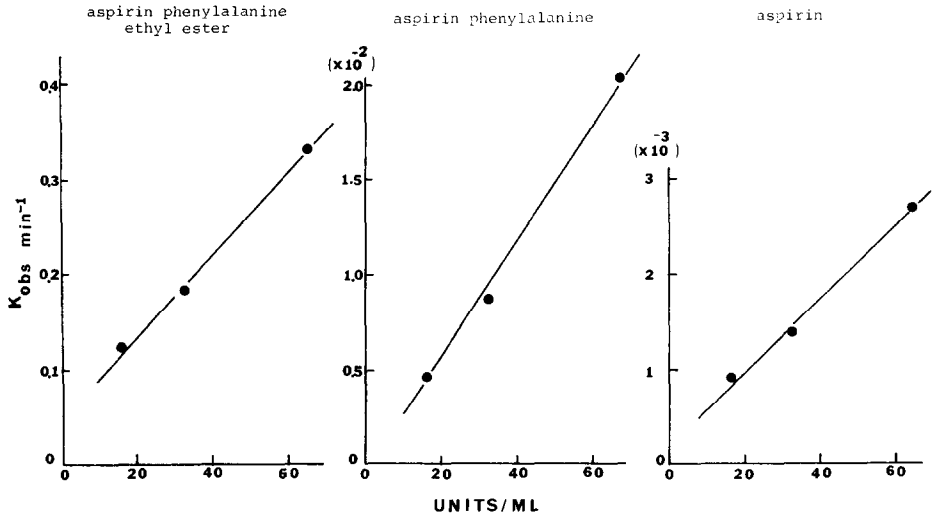
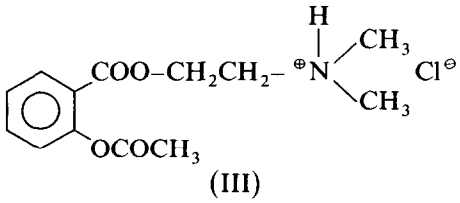


Fig. 4. Effect of cholinesterase butyryl concentration on the rate of salicyl derivative formation from aspirin phenylalanine ethyl ester, aspirin phenylalanine and aspirin.

positively charged at pH 6, N,N-dimethyl aminoethyl aspirin (III) (Garrett, 1958) was studied. The resulting data are shown in Table 1. It is apparent that the positively charged ester, III, is hydrolyzed much faster than the neutral or negatively charged esters. Such an enhancement can be rationalized by considering the favorable interaction between the positively charged substrate (pK_a 8.5) and the negatively charged enzyme. Such observations are also consistent with those reported earlier for the hydrolysis of salicylamide esters (Babhair and Hussain, 1983).



(III)

TABLE 1

HALF-LIVES FOR HYDROLYSIS OF COMPOUNDS I, II, III AND ASPIRIN IN THE PRESENCE OF 32 units/ml OF BUTYRYL CHOLINESTERASE AT pH 7.4 AND 37°C

Compound	$t_{1/2}$ (min)
I	3.8
II	78.1
III	0.04
Aspirin	498

The study above thus indicates that aspirin phenylalanine ethyl ester will not generate aspirin, regardless of the nature of the enzyme, but rather hydrolyzes eventually to the corresponding salicyl derivative. The study above also suggests that the enzymatic hydrolysis of esters can be varied significantly via changes in the electrostatic nature of the derivative and that such an approach may be used to control the release of the parent drug from its prodrugs.

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