Hydrolysis of Fatty Acid Esters of Acetaminophen in Buffered Pancreatic Lipase Systems I

CARL T. BAUGUESS ˣ, FARID SADIK, JULIAN H. FINCHER, and CHARLES W. HARTMAN †

Abstract □ A series of fatty acid esters of acetaminophen were prepared beginning with the acetate, the propionate, and all even-numbered fatty acids and going through the octadecanoate. The enzymatic hydrolysis of all derivatives was studied *in vitro* with varying amounts of lipase added to the hydrolysis mixtures. Under the conditions of the *in vitro* hydrolysis, it was observed that all derivatives were hydrolyzed more readily in an aqueous medium at pH 7.8. A positive relationship was seen between the hydrolysis rates and the concentration of lipase at this pH. There was a negative relationship between the chain length of the acyl moiety and the corresponding hydrolysis rates. The short chain esters were hydrolyzed at rates many times more rapid than the long chain esters. The intermediate chain-length esters, *p*-acetamidophenyl decanoate, *p*-acetamidophenyl laurate, and *p*-acetamidophenyl myristate, were hydrolyzed at intermediate time periods extending over 12 hr, approaching completion at 97.5, 87.5, and 80.5%, respectively, when 18 Wilson units of lipase was used in each milliliter of hydrolysis mixture. The longer chain esters, *p*-acetamidophenyl palmitate and *p*-acetamidophenyl stearate, were hydrolyzed to the extent of 16 and 8%, respectively, over 12 hr under the same *in vitro* conditions.

Keyphrases □ Acetaminophen—fatty acid esters, hydrolysis in buffered pancreatic lipase systems □ Esters, acetaminophen, fatty acid—hydrolysis in buffered pancreatic lipase systems □ Hydrolysis—fatty acid esters of acetaminophen, buffered pancreatic lipase systems □ Lipase—enzymatic hydrolysis of fatty acid esters of acetaminophen

The objective of this work was to investigate the feasibility of employing fatty acid ester derivatives of a drug for use as a substrate for GI enzymes to control the release of the free drug from its dosage form. A second objective was to determine if a combination of the esters and appropriate enzymes in a dosage form could be utilized to achieve a uniform and prolonged therapeutic effect from a single dosage unit of the drug without producing an excessive initial plasma concentration. Acetaminophen (*N*-acetyl-*p*-aminophenol, 4-hydroxyacetanilid) was selected as a model drug for this study.

The synthesis of *p*-acetamidophenol acetate, butyrate, hexanoate, and stearate and enzymatic hydrolysis in serum lipase as reported previously (1) provided the basis for the investigation. In the previous study, it was noted that the short chain esters of acetaminophen were hydrolyzed very rapidly while the stearate was hydrolyzed so slowly that no attempt was made to quantitate the results. In view of these findings, it was felt that a complete series of saturated fatty acid esters should be prepared and tested in pancreatic lipase for comparative purposes. Intermediate to the hexanoate and the stearate should be one or more esters that could be hydrolyzed slowly over 10–12-hr in the presence of GI enzymes.

Fletcher *et al.* (2) investigated the absorption of lincomycin and esters of lincomycin from rat intestines. They reported that the 7-butyrate and 7-propionate esters of lincomycin were absorbed more rapidly than the corresponding 2-esters. This difference was attributed to intestinal hydrolysis rates for the two positions on lincomycin in which the 7-esters were hydrolyzed more slowly than the 2-esters. Since both types of esters were absorbed to some extent intact, increased absorption of these soluble intact esters over the lincomycin base was explained on the basis of an increase in lipid solubility.

Glazko *et al.* (3, 4) found that the intact esters of chloramphenicol are poorly absorbed and gave indirect evidence that the rate of absorption of chloramphenicol depends upon the rate of enzymatic hydrolysis. The hydrolysis rates for these esters appeared to depend on particle size, chemical nature of the esters, and the ability of the compound to be wetted.

Contrary to previous reports, they found that some of the more water-soluble esters were hydrolyzed more slowly than water-insoluble esters such as the palmitate if the latter had a sufficiently large surface exposed to enzymatic attack. Suspensions containing very small particles of chloramphenicol palmitate produced excellent blood levels in dogs, while dry crystalline preparations were poorly absorbed. When the esters of chloramphenicol were not readily hydrolyzed *in vitro* by enzymes under optimum conditions, poor absorption resulted regardless of the physical form of the esters.

GI absorption of drugs in particulate form usually results after the drug is in the dissolved state (5). When dissolution is rate limiting, particle size is of great importance to the rate of transfer from the GI tract to ultimate sites of action. In a recent review article about particle size, it was reported that when the solubility of a drug is less than 0.1 mg/ml, the effect of particle size could be an important factor in consideration of physiological availability, while other workers contend a solubility up to 1 mg/ml may be a factor in drug availability (5). Fatty acid esters of acetaminophen are poorly soluble in water, and the GI absorption of acetaminophen should be limited by the amount of enzymes facilitating ester hydrolysis in the intestines and the available surface area in the dosage unit.

EXPERIMENTAL

Reagents—The following were used: acetaminophen[1], acetic anhydride[2], propionic anhydride[3], butyric anhydride[3], hexanoyl

[1] Eastman Organic Chemicals.
[2] J. T. Baker Chemical Co.
[3] Aldrich Chemical Co.

Table I—Acetaminophen Derivatives, Melting Points, Solubilities, and Average Particle Size

Derivative	Melting Point	Solubility, mg/ml	Particle Size, Average Diameter in Microns[a]
Acetaminophen	168–172°	20.00	—
Acetate	153–156°	0.32	30.0
Propionate	130–133°	0.32	11.5
Butyrate	140–143°	0.33	11.0
Hexanoate	107–109°	0.018	32.0
Octanoate	103–105°	0.010	8.7
Decanoate	107–109°	0.009	7.3
Laurate (dodecanoate)	111–113°	0.006	4.3
Myristate (tetradecanoate)	114–116°	0.006	2.7
Palmitate (hexadecanoate)	117–118°	0.005	2.7
Stearate (octadecanoate)	117–118°	0.005	2.1

[a] As determined by the Fisher subsieve sizer.

chloride[1], octanoyl chloride[1], decanoyl chloride[4], dodecanoyl chloride[1], tetradecanoyl chloride[1], hexadecanoyl chloride[1], octadecanoyl chloride[1], and lipase[5].

Instruments—The necessary equipment included a submersion rotator[6], a subsieve sizer[7], and a spectrophotometer[8].

Preparation of Acetaminophen Esters—A general synthetic procedure was followed in the preparation of the derivatives of acetaminophen (5) and involved the heating with stirring for 24 hr of the required acid anhydride or acyl halide with acetaminophen in anhydrous pyridine. The derivatives were precipitated from cold water, washed in a 5% solution of sodium carbonate, and recrystallized from *n*-propyl alcohol and water except in the case of *p*-acetamidophenyl propionate where methanol and water were used. The preparation of the esters of acetaminophen from the acid anhydrides using excess amounts of acetyl, propionyl, and butyryl anhydrides presented no problem in the isolation of the products.

Fatty acid chlorides and acetaminophen were used in approximately equimolar quantities in the synthesis of the larger analogs of this series of derivatives. The products were all white, crystalline powders except the acetate and stearate, which were off-white powders. The acetaminophen derivatives along with their melting points, solubilities, and average particle sizes are shown in Table I.

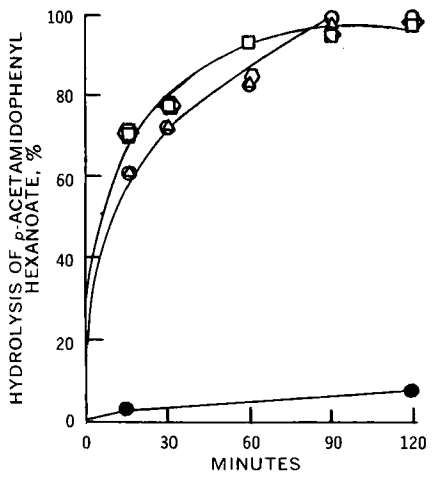

Figure 1—*Percent hydrolysis of* p-*acetamidophenyl hexanoate with varied amounts of lipase and calcium chloride in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 18 units/ml; □, 9 units/ml; △, 4.5 units/ml; ○, 2.25 units/ml; and ●, control, 0 units/ml.*

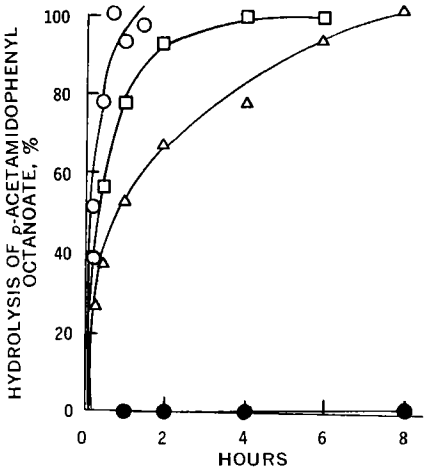

Figure 2—*Percent hydrolysis of* p-*acetamidophenyl octanoate with varied amounts of lipase and calcium chloride in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 9 units/ml; □, 4.5 units/ml; △, 2.25 units/ml; and ●, control, 0 units/ml.*

Solubilities—The solubilities of the acetaminophen derivatives were determined by placing an excess of each derivative in water at 25°. Test tubes containing the derivative and distilled water were agitated with the submersion rotator for 6 hr. The clear filtrates obtained by filtration through filter paper[9] were diluted successively until absorption could be determined spectrophotometrically. Dilutions were unnecessary with the larger esters because the saturated solutions beginning with the octanoate show very low absorbance.

The results of these determinations are recorded in Table I. The acetate, propionate, and butyrate derivatives, according to official description, were classified as "very slightly soluble," while the remaining derivatives were "insoluble" (7).

Particle Size—Prior to *in vitro* and *in vivo* testing, all derivatives were passed through a 120-mesh wire cloth sieve. Further classification of particle size was accomplished by the determination[7] of average particle diameters for each derivative.

Lipase Activity—Commercial preparations of pancreatic lipase were assayed for the hydrolytic activity with olive oil as the substrate by the method of Lazo-Wasem (8). All references to lipase activity in this study were based on the Wilson unit per milligram. The lipase used in the *in vitro* hydrolysis studies had an activity of 2.96 Wilson units (±0.069 *SE*).

Stability of Esters in Simulated Gastric Fluid—To establish the stability of the derivatives under simulated gastric conditions, two representative esters were selected: one short chain fatty acid derivative and one long chain fatty acid derivative. Separately, 100 μmoles of *p*-acetamidophenyl acetate and *p*-acetamidophenyl decanoate was digested in 10 ml of simulated gastric fluid USP (pH 1.35), maintained at 37°, agitated, and titrated with 0.0499 *N* sodium hydroxide solution on a recording pH meter for 4 hr. No appreciable hydrolysis in either sample (less than 5% hydrolysis) was noted.

Preparation of Hydrolysis Formulation—An accurately weighed sample of ester and enzyme was triturated in a cold mortar. Individual weighings were made from each batch to contain 100 μmoles of the ester, which was equivalent to 15.1 mg of acetaminophen. The required ester and enzyme were transferred to dry Pyrex test tubes (15-ml capacity). A 0.5-ml aliquot of a 0.2 *M* solution of calcium chloride was added to, and mixed with, the powder prior to the addition of 9.5 ml of a 0.1 *M* phosphate buffer solution, also containing 0.1 *M* sodium chloride. The pH of the buffer

[4] Matheson, Coleman & Bell Co.
[5] Reheis Chemical Co.
[6] Scientific Industries, Inc.
[7] Model 95, Fisher Scientific Co.
[8] Beckman DU model 2400, Beckman Instruments, Inc.
[9] Whatman No. 1.

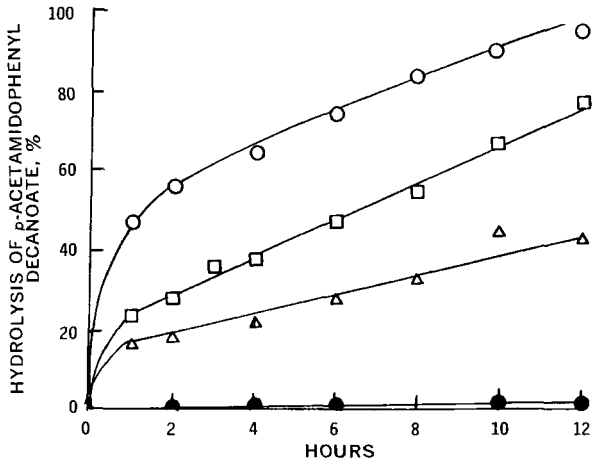

Figure 3—*Percent hydrolysis of* p-*acetamidophenyl decanoate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities):* ○, *18 units/ml;* □, *9 units/ml;* △, *4.5 units/ml; and* ●, *control, 0 units/ml.*

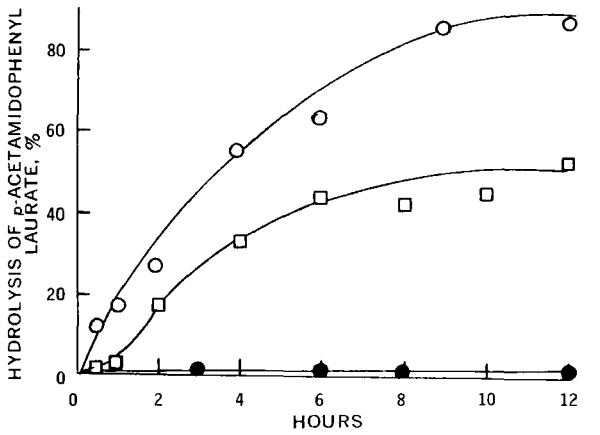

Figure 4—*Percent hydrolysis of* p-*acetamidophenyl laurate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities):* ○, *18 units/ml;* □, *9 units/ml; and* ●, *control, 0 units/ml.*

solution was adjusted to accommodate the addition of calcium chloride with a resultant pH of 7.8.

Each tube was stoppered and placed on a submersion rotator. The submersion rotator was placed in a constant-temperature bath maintained at 37° and rotated at a maximum speed of 30 cpm. At timed intervals, one or more of the tubes were removed from the bath and filtered through filter paper[9]. A 0.1-ml sample of the clear filtrate was diluted to 10 ml with distilled water, and the absorbance was determined against a reagent blank at 245 nm.

At 100% hydrolysis, each dilution contained 15.1 μg/ml of acetaminophen. The derivatives beginning with the hexanoate through the stearate were assayed by Procedure B (see *Analytical*), which takes advantage of a great difference in the solubility of acetaminophen as compared with the ester solubility and the dilution factor of 1:100 employed.

Analytical—The acetate, propionate, and butyrate derivatives are sufficiently soluble to require a different assay approach from the longer carbon derivatives. Acetaminophen absorbs more strongly at 280 nm than do the ester derivatives. The assay was accomplished by using an equimolar quantity of each derivative in the reagent blank containing an inactive enzyme, and the extent of hydrolysis was determined by the increase in absorbance as compared with a standard mixture of the ester and acetaminophen.

Procedure A: Determination of Acetaminophen in Presence of Acetaminophen Derivatives—Standard solutions containing 75.5 μg/ml (0.5 μmole/ml) of acetaminophen and 0.5 μmole/ml of the short chain esters (*p*-acetamidophenyl acetate, propionate, and butyrate equivalent to 75.5 μg/ml of acetaminophen) were prepared in the *in vitro* reagents. The esters of acetaminophen do not absorb strongly at 280 nm, but acetaminophen absorbs strongly at this wavelength.

In the determination of percent hydrolysis in the *in vitro* experiments, Procedure B was followed except that a 0.50-ml sample was used instead of a 0.10-ml sample and the dilutions were read at 280 nm against a reagent blank containing the ester.

Procedure B: Determination of Acetaminophen in In Vitro *Hydrolysis of Long Chain Acetaminophen Derivatives*—A 30.2-mg sample of acetaminophen, accurately weighed, was transferred to a 100-ml volumetric flask, and the flask was diluted to volume with 0.1 *M* phosphate buffer, pH 7.8. Aliquots of 1.0, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 ml of the standard solution were diluted to 1 ml with the phosphate buffer and diluted to 10.0 ml with distilled water. The absorbance of each sample against a reagent blank was measured at 245 nm.

RESULTS AND DISCUSSION

All melting points are consistent with those reported for *p*-acetamidophenyl acetate, butyrate, hexanoate, and stearate (1). The melting point for *p*-acetamidophenyl propionate prepared in this laboratory was slightly higher (about 4°) than that previously reported (9). The melting points for this series decrease from about 154° for the acetate to a minimum of 104° for the octanoate derivative and from the octanoate increase with fatty acid chain length to 118° for the stearate.

The melting point observed for the propionate derivative (132°) suggests an oscillating series similar to that reported for the fatty acid ester derivatives of salicylic acid, in which the fatty acid moieties with the even number of carbon atoms exhibited higher melting points in a series of compounds than the odd numbered in the same series (10).

Hofstee (10) recognized that an assay for crude lipase, using olive oil as the substrate, would not serve as a comparative standard for all substrates. The purpose of the assay here was to establish the quality of the various preparations and to standardize the activity in terms of Wilson units in the *in vitro* studies. The assay was especially useful in following the stability of the enzyme preparations during the investigation.

The release of acetaminophen in this study depends solely upon the hydrolysis of each derivative. The rate of hydrolysis of all derivatives was enhanced by the presence of pancreatic lipase as seen in the *in vitro* hydrolysis studies. Any loss of enzyme activity would seriously alter the outcome of an experiment, and esters of acetaminophen are hydrolyzed very slowly under gastric conditions.

The results obtained by Procedures A and B are illustrated in Figs. 1–7 except for *p*-acetamidophenyl acetate, *p*-acetamidophenyl propionate, and *p*-acetamidophenyl butyrate, which were hydrolyzed so rapidly as to be almost complete in 15 min or less.

p-Acetamidophenyl hexanoate was hydrolyzed more rapidly than the corresponding octanoate ester. Both esters were hydrolyzed less rapidly than the short chain esters under the same hy-

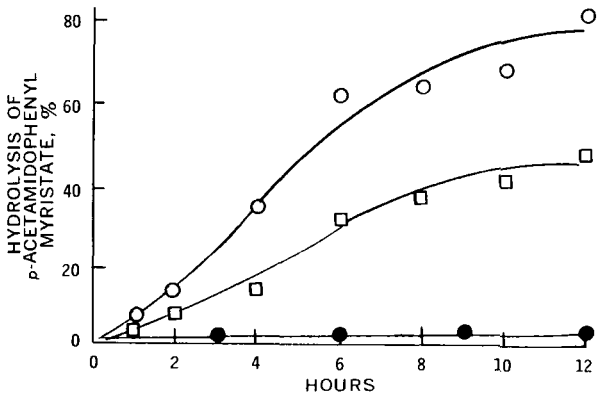

Figure 5—*Percent hydrolysis of* p-*acetamidophenyl myristate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities):* ○, *18 units/ml;* □, *9 units/ml; and* ●, *control, 0 units/ml.*

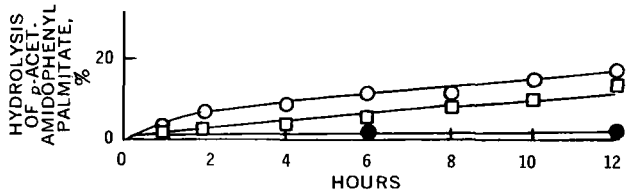

Figure 6—*Percent hydrolysis of* p-*acetamidophenyl palmitate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 18 units/ml; □, 9 units/ml; and ●, control, 0 units/ml.*

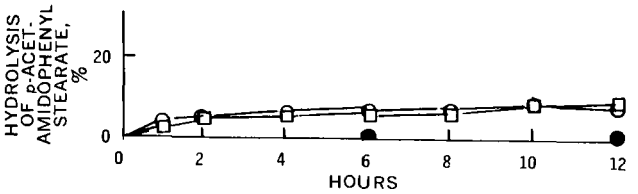

Figure 7—*Percent hydrolysis of* p-*acetamidophenyl stearate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 18 units/ml; □, 9 units/ml; and ●, control, 0 units/ml.*

drolysis conditions. None of the short chain esters through the octanoate ester appears to saturate the enzymes available in the hydrolysis system for any of the hydrolysis formulas shown in Figs. 1 and 2.

When the substrate concentration is in excess of the amount required to attain maximal velocity, the reaction proceeds at a zero-order rate; however, if the concentration is less than a saturation concentration, the rate of reaction becomes dependent upon the concentration of substrate and the reaction proceeds at a first-order rate (11). This appears to be the case with the short chain derivatives under these conditions.

In general, as the chain length in the fatty acid moiety was increased, a decrease in the hydrolysis rate was noted. The effects of varied lipase concentration are demonstrated in Figs. 3–5, showing the percent hydrolysis of *p*-acetamidophenyl decanoate, laurate, and myristate, respectively. A marked decrease in the rate of hydrolysis was noted for all three derivatives as the concentration of enzyme was decreased. The short chain esters were hydrolyzed rapidly at all enzyme concentrations. The long chain esters, *p*-acetamidophenyl palmitate and stearate, were hydrolyzed very slowly at the two enzyme concentrations studied.

Some difference was noted in Fig. 6 for *p*-acetamidophenyl palmitate but not to the same extent as observed for the derivatives of intermediate chain length. An insignificant difference was seen for *p*-acetamidophenyl stearate (Fig. 7). Both were hydrolyzed less than 10% in 12 hr. Since one purpose of the study was to find a derivative that would be hydrolyzed slowly and completely over a 12-hr period, it was apparent that the derivatives and some of the *in vitro* conditions illustrated in Figs. 3–5 for *p*-acetamidophenyl decanoate, laurate, and myristate met this specification.

Formulas of *p*-acetamidophenyl palmitate and stearate in Figs. 6 and 7 released acetaminophen at a rate lower than the therapeutic level. The short chain derivatives, *p*-acetamidophenyl acetate, propionate, and butyrate were hydrolyzed so rapidly that it was doubtful if the rate of hydrolysis would be a limiting step in their absorption from the GI tract if compared with acetaminophen.

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† Deceased.

ˣ To whom inquiries should be directed.