Hydrolysis of 4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate by Esterolytic Enzymes from Various Sources

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Abstract □ Hydrolysis rates were determined for ATC in pH 7.4 phosphate buffer containing human plasma from a number of individuals; human plasma treated and stored in various ways; several Cohn fractions of human plasma; human plasma treated with various esterase inhibitors; and a number of commercially available enzymes. The variation among individual plasma samples was observed, as well as the way in which blood type, plasma concentration, lyophilization, freezing, thawing, storing the plasma at 25°, ethanol concentration, and substrate concentration influenced the catalytic potency of human plasma. Studies with the Cohn fractions and esterase inhibitors suggested that pseudocholinesterase was primarily responsible for the enzymatic activity of human plasma with respect to ATC hydrolysis. Some proteolytic enzymes were also found to be potent catalysts of ATC hydrolysis. It was concluded that, following oral administration, ATC would be exposed to many enzymes that are potent catalysts for its hydrolysis, both in the gastrointestinal tract and following absorption and distribution in body tissue.

Keyphrases □ 4-Acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC)—hydrolysis □ Enzymes, human plasma—ATC hydrolysis □ Pseudocholinesterase—ATC esterolytic hydrolysis

The authors' experience has indicated that acetaminophen and other drugs possessing the hydroxyl group may be converted to various carbonate esters and still retain the intrinsic therapeutic activity of the parent drug *in vivo*. Because of this, it is possible to prepare a series of compounds with approximately equivalent therapeutic activity on a molar basis, but with widely ranging differences in physical-chemical properties. These differences could be of importance to the pharmacist preparing variant forms of a drug because they might affect dissolution rate, physical form, and taste; they might also influence the absorption and stability of a drug, as well as the dosage form in which it can be employed.

The compound, 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC), is an interesting carbonate ester variant of acetaminophen. The hydrolysis rates of ATC in buffer solutions and in buffers containing plasma and intestinal fluid from rats and humans have been reported (1). It has also been established that the hydrolysis rates of carbonate and carboxylic acid esters of acetaminophen with a variety of structures are accelerated by the blood sera of humans and animals (2). Presumably, the catalysis produced by these body fluids is due to esterolytic enzymes; however, many proteolytic and other types of enzymes can also function as esterases. For this reason, it was of interest to determine what factors influence the *in vitro* catalytic potency of a crude enzyme system such as human plasma; and what relative catalytic potencies are obtained with various enzyme systems when ATC is the substrate.

Table I—Half-Lives for the Hydrolysis of ATC in 2% Human Plasma from Various Donors (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Sample No.	Blood Type	Half-Life (min.) (Average ± Mean Deviation)
12446	AB+	10.7
		12.0
		11.8 (av. 11.5 ± 0.5)
12449	O+	13.0
		10.2 (av. 11.6 ± 1.4)
12450	A+	19.5
		21.3 (av. 20.4 ± 0.9)
12448	B+	20.0
		17.1
		17.8 (av. 18.3 ± 1.1)
GMI	A−	15.0
S-12593	O+	14.3
S-12589	O+	21.5
S-12590	O+	19.0
LWD	O+	17.7
S-12591	A+	19.3
S-12594	A+	20.3
S-12592	A+	16.0
69255	B+	21.3
P-69334	B+	15.8
69338	B+	21.0
P-69254	B+	31.3
P-69332	B+	20.6
S	B+	25.8
		Av. 17.6 ± 4 min. (limits 10.2–31.3)

EXPERIMENTAL

Frozen citrated human blood plasma of various blood types from individual donors, and lyophilized Cohn fractions of human plasma were obtained.[1] Lyophilized whole human plasma was prepared from 110 ml. of Type O+ citrated human plasma. The other materials used were: human pseudocholinesterase,[2] horse pseudocholinesterase,[3] crystalline human serum albumin and the purified enzymes,[4] physostigmine sulfate and tetraethyl pyrophosphate (TEPP),[5] and sodium ethylenediaminetetraacetate (EDTA).[6]

Half-lives for the hydrolysis of ATC at 37° in 100% human plasma and in pH 7.4 phosphate buffer (0.1 *M*) containing 25, 50, and 75% human plasma or 7.5% lyophilized human plasma were determined by a chromatographic procedure previously described (1). All other half-lives were determined by a direct UV procedure previously described (1). All reactions followed apparent pseudo first-order kinetics.

RESULTS AND DISCUSSION

Half-lives for the hydrolysis of ATC in pH 7.4 phosphate buffer (0.1 *M*) containing 2% human plasma from 18 donors are shown in Table I. The half-lives varied from a minimum of 10.2 min. to a maximum of 31.3 min., with an average half-life of

[1] Through the Philadelphia Serum Exchange.
[2] Cutter Laboratories, Berkeley, Calif.
[3] Armour Pharmaceutical Co., Kankakee, Ill.
[4] Nutritional Biochemicals Corp., Clevelend, Ohio.
[5] K & K Laboratories, Jamaica, N. Y.
[6] Fisher Scientific Co., Fair Lawn, N. J.

Table II—Effect of Human Plasma Concentration on Hydrolysis Rate of ATC (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Plasma Concn., %		Half-Life, min.
Liquid		
2		19.5
4		13.9
6		11.2
25		6.1
50		4.2
75		3.4
100		2.9
Lyophilized (Liquid Equivalent, %)		
0.05	(0.667)	28
0.1	(1.33)	35
0.2	(2.67)	17.2
0.3	(4.00)	11.9
0.5	(6.67)	12.0
7.5	(100)	7.5

about 18 ± 4 min. There was considerable variation among individual samples of plasma with respect to their ability to accelerate the hydrolysis of ATC, but there appeared to be no correlation between blood type and catalytic potency. The results shown in Table I also indicate that, in repeated experiments with the same sample of plasma, a mean deviation of 5 to 10% in the measured half-life can be expected with the experimental technique employed.

Table II shows the effect of plasma concentration on the half-life of ATC at pH 7.4. As the concentration of either liquid or lyophilized human plasma was increased, the rate of hydrolysis of ATC increased (half-life decreased), but the increase was not directly proportional to the increase in plasma concentration with either material. Thus, one can expect ATC to be very rapidly hydrolyzed to free acetaminophen in blood plasma *in vivo*, but one would not necessarily expect it to be hydrolyzed 50 times faster in blood plasma *in vivo* than it is in 2% plasma solution *in vitro*. The half-life of ATC in a given concentration of lyophilized plasma was roughly equivalent to the half-life in an equivalent concentration of liquid plasma; this showed that lyophilization did not destroy or inhibit to any great extent the enzymes of human plasma that catalyze the hydrolysis of ATC.

To study the hydrolysis of ATC in plasma and the various enzyme systems using a direct UV procedure, it was necessary to dissolve the drug initially in a water-miscible solvent, to use a concentration of ATC in the final enzyme mixture which would optimize the UV analysis, and to freeze and thaw the plasma samples, perhaps several times, before use. These factors were investigated to see if they had a profound influence on the half-life of ATC in 2% human plasma in pH 7.4 phosphate buffer.

Table III—Effect of Ethanol, Substrate Concentrations, and Various Conditions of Plasma Storage on Hydrolysis Rate of ATC in 2% Human Plasma Solutions (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Effect of Ethanol Concn. (0.03 mg./ml. ATC)	
Ethanol Concn.	Half-Life, min.
0.75%	40
1	30
2	30
3	41
Effect of ATC Concn. (1% ethanol)	
ATC Concn., mg./ml.	Half-Life, min.
0.01	36
0.015	36
0.03	28
0.04	33.6

Effect of Conditions of Plasma Storage (1% ethanol, 0.03 mg./ml. ATC)

History	Half-Life, min.
1. Freshly drawn plasma	23
2. No. 1, frozen overnight, thawed rapidly[a]	22.1
3. No. 1, frozen overnight, thawed slowly[b]	24
4. No. 3, stored 19 hr. at 25°	29
5. No. 3, stored 43 hr. at 25°	25

[a] A 20-ml. vial was placed in 37° water until thawed. [b] A 20-ml. vial was placed in air at 25° until thawed.

Table IV—Catalytic Potency of Various Cohn Fractions of Human Plasma on the Hydrolysis of ATC (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Cohn Fraction	Concn., %	Half-Life, min.
I	0.1	—[a]
II	0.028	—[a]
III-0	0.027	56
III-1	0.035	—[a]
III-2	0.033	—[a]
III-3	0.033	—[a]
IV	0.1	10.6
IV-1	0.032	—[a]
IV-4	0.036	20.8
V	0.1	21.7
Crystalline human serum albumin	4	99

[a] Half-life over 240 min. No enzymatic activity.

The results are shown in Table III. Ethanol concentrations between 0.75 and 3% and ATC concentrations between 0.01 and 0.04 mg./ml. in the final enzyme mixture had no apparent effect on the hydrolysis rate; the half-lives were within experimental error. The half-lives produced by 2% solutions of human plasma that were freshly drawn, frozen and thawed rapidly, frozen and thawed slowly, or stored at 25° for 19 and 43 hr. were also within experimental error. These results suggest that low concentrations of ethanol and ATC had very little effect on the human plasma enzymes which hydrolyze ATC, and that storing plasma in small containers in a freezer and rapidly thawing it shortly before use can be expected to have little effect on the catalytic potency of this crude enzyme source with respect to hydrolysis of acetaminophen carbonate ester prodrugs.

Table IV shows the catalytic potency of several Cohn human plasma fractions (3) with respect to hydrolysis of ATC. The activity appears to be concentrated in the III-0, IV-4, and V fractions; the other fractions studied had no activity. Fraction V is composed mainly of albumin, which has been reported to be a catalyst in some esterolytic reactions (4). However, crystalline human albumin at relatively high concentrations (4%) showed practically no catalytic effect on the hydrolysis of ATC and it may be concluded that the esterolytic activity of Cohn fraction V shown in Table IV was not due to catalysis by albumin but by enzymes left in Fraction V by the Cohn fractionation procedure.

Cohn fractions III and IV are composed mainly of globulins and contain aromatic esterase (A-esterase) and pseudocholinesterase (C-esterase) which are reported to be the major esterolytic enzymes of human plasma (5). These enzymes are not separated in the Cohn fractionation procedure, however, and the experiments with the Cohn fractions were unable to distinguish which of these enzymes is responsible for the hydrolysis of ATC. Pseudocholinesterase has been shown to be identical to "procainesterase" (6), and it was of interest to know if this esterase is also involved in the hydrolysis of carbonate prodrug esters such as ATC. Pseudocholinesterases are inactivated by physostigmine and TEPP (7), but aromatic esterase is unaffected by these inhibitors. However, for aromatic esterase to exert its activity, calcium ions must be present in the medium (8). Table V

Table V—Effect of Calcium Ion and Cholinesterase Inhibitors on the Hydrolysis Rate of ATC in 2% Liquid Human Plasma (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Concn. of Ca^{++} or Inhibitor	Half-Life, min.
None	19
10^{-4} *M* $CaCl_2$	18
10^{-4} *M* EDTA	17
10^{-4} *M* Physostigmine sulfate	—[a]
10^{-4} *M* TEPP	—[a]

[a] Half-life over 240 min. No enzymatic activity.

Table VI—Half-Lives for Hydrolysis of ATC in Dilute Solutions of Purified Human Pseudocholinesterase (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Concn., %	Half-Life, min.
0.0025	9.2
0.005	5.8
0.01	1.1
0.02	0.63
0.03	0.32

shows that the esterolytic activity of 2% human plasma with respect to ATC hydrolysis was completely destroyed by $10^{-4}M$ physostigmine sulfate or $10^{-4}M$ TEPP, whereas the half-life for hydrolysis was unaffected by $10^{-4}M$ calcium chloride or $10^{-4}M$ EDTA. These results support the implication that human plasma pseudocholinesterase is primarily responsible for the esterolytic activity of human plasma with respect to ATC hydrolysis.

Human pseudocholinesterase is available commercially in a purified form, and Table VI shows half-lives for the hydrolysis of ATC in pH 7.4 phosphate buffer containing relatively low concentrations of this material. The results show that the reaction rate was roughly proportional to enzyme concentration in the 0.01 to 0.03% range. Half-lives of about 20 sec. were obtained for ATC in 0.03% solutions, confirming that human pseudocholinesterase is a very potent enzyme with respect to the hydrolysis of ATC. A comparison of the catalytic potency of human pseudocholinesterase with that of other commercially available enzymes (Table VII) shows that human and horse pseudocholinesterases were among the most potent catalysts, with human enzyme the most potent of all the enzymes studied.

Proteolytic enzymes, especially the chymotrypsins and trypsin, were also potent catalysts. As might be expected, pepsin and papain showed weak activity in this experiment; these enzymes are most active at more acidic pH's. It is also not surprising that acetylcholinesterase was only a very weak catalyst, since this enzyme is virtually specific for acetylcholine and has very little effect on most other esters (9).

The remaining enzymes listed in Table VII showed little or no activity under the conditions of this experiment. It would be difficult to explain why each enzyme behaved as it did, and explanations based on this brief study would have very little meaning. However, it can be concluded that, following oral administration, ATC will be exposed to many enzymes which are potent catalysts for its hydrolysis, particularly the proteolytic enzymes and pseudocholinesterase; it might also be surmised that other prodrug carbonate and carboxylate esters of acetaminophen, or other parent drugs which contain a hydroxyl group, may also be hydrolyzed by these enzymes.

SUMMARY

1. Individual specimens of human plasma varied in their catalytic potencies with respect to the hydrolysis of ATC, but there appeared to be no correlation between blood type and catalytic potency.

2. The hydrolysis rate of ATC in pH 7.4 phosphate buffer containing human plasma increased with increasing plasma concentration, but the increase was not proportional to the plasma concentration over the entire concentration range. Thus, the rate in 100% plasma was about seven times faster than the rate in 2% plasma.

3. Lyophilization of plasma, ethanol concentrations between 0.75% and 3%, ATC concentrations between 0.01 and 0.04 mg./ml., and freezing, thawing, or storing plasma at 25° for up to 43 hr. appeared to have very little effect on the catalytic potency of human plasma with respect to ATC hydrolysis.

4. The catalytic activity of human plasma appeared to be concentrated in the III-O, IV-4, and V Cohn fractions. The activity of Fraction V was probably due to contaminating enzymes from the other fractions, and inhibition studies with physostigmine and TEPP suggested that the activities of Fractions III-0 and IV-4 were due to pseudocholinesterase.

5. Human and horse pseudocholinesterases and the chymotrypsins were the most potent catalysts of ATC hydrolysis among the commercially available enzymes studied at pH 7.4. Thus, enzymes found in the gastrointestinal tract, liver, and blood are capable of rapidly hydrolyzing ATC and would probably hydrolyze other carbonate and carboxylic acid prodrug esters of acetaminophen as well. Consequently, free acetaminophen should be rapidly released following oral administration and dissolution of these types of compounds. It might also be expected that the pseudocholinesterases and the chymotrypsins would be active catalysts of carbonate and carboxylic acid ester prodrugs of other parent drugs which contain the hydroxyl group.

Table VII—Half-Lives for Hydrolysis of ATC in Dilute Solutions of Various Purified Enzymes (0.05%) (0.1 *M*, pH 7.4, Phosphate Buffer, 37°C.)

Enzyme	Half-Life, min.	Rate Relative to Buffer Alone
Human plasma cholinesterase (0.03%) (Cutter Labs.)	0.3	800
α-Chymotrypsin	0.65	370
β-Chymotrypsin	0.56	430
γ-Chymotrypsin	0.51	470
Δ-Chymotrypsin	0.53	450
Horse pseudocholinesterase (Armour Co.)	1.4	171
Acylase (hog kidney)	2.2	109
Trypsin (1–300)	3.0	80
Pancreatin	15	16
Peptidase	20	12
Malt Diastase	24	10
Pectin esterase	27	9
Maltase	38	6
Proteinase	66	4
Acetyl cholinesterase (bovine erythrocyte)	74	3
Erepsin	78	3
Lysozyme	99	2.3
Papain	124	2
Pepsin (1–20,000)	185	1.3
Penicillinase	> 240 (no activity)	—
Pepsin (blood group A free)	> 240 (no activity)	—
Carbonic anhydrase	> 240 (no activity)	—
Enterokinase	> 240 (no activity)	—
Lipase	> 240 (no activity)	—
Hog kidney extract	> 240 (no activity)	—
No enzyme	> 240 (no activity)	—

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