Serum-Catalyzed Hydrolysis of Metronidazole Amino Acid Esters

MOO J. CHO[x] AND LYNN C. HAYNES

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Abstract □ Glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), and lysine (Lys) esters of metronidazole were synthesized using dicyclohexylcarbodiimide (DCC) coupling or a mixed-anhydride route, using *tert*-butyloxycarbonyl (*tert*-Boc) amino acids. Human serum-catalyzed hydrolysis of these esters at 37°C give half-lives varying from 4.5 min for the Phe ester to 96 h for the Ile ester. Also determined was the pH–rate profile for hydrolysis in aqueous buffers at 25°C. A linear relationship was observed between the logarithmic value of the hydrolysis rate constant in serum and that of the OH^--catalyzed hydrolysis of cationic esters. This finding may indicate that the esters studied are "equally" poor substrates for binding to the enzymes in serum and, thus, the difference observed in the serum-catalyzed hydrolysis rate is solely derived from the chemical lability of an ester bond. Interestingly, the extent of chemical activation observed in the buffer system appears to be amplified in the serum-catalyzed hydrolysis.

Metronidazole [2-methyl-5-nitroimidazole-1-ethanol], the drug of choice for the treatment of certain anaerobic infections, possesses a low intrinsic solubility (~10 mg/mL at 25°C). To overcome this solubility problem in developing a parenteral dosage form, the phosphate ester was previously proposed as a water-soluble prodrug.[1] As a part of our continuing interest in the subject, we have explored the possible use of amino acid esters of metronidazole and report the relationship between the rate of hydrolysis of these esters in serum and buffered solutions. After the present study was completed, two publications[2,3] described similar studies on metronidazole esters of mainly unnaturally occurring amino acids.

Experimental Section

N,N-Dimethylaminopyridine (DMAP), trifluoroacetic acid (TFA), and the *tert*-butyloxycarboxyl (*tert*-Boc) derivatives of glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), and phenylalanine (Phe) were purchased from Sigma; the bis-Boc derivative of lysine (Lys) was from Bachem. Isobutyl chloroformate and dicyclohexylcarbodiimide (DCC) were from Aldrich. Dimethyloctylamine (DMOA) used in the HPLC mobile phases, from the Ethyl Corp., was used as received. The preparative silica gel LC columns used were Lobar Size B or C from Merck. The anionic-exchange resin used was Dowex 2-×8. The following HPLC components were used: Altex model 110A pump; Rheodyne model 7000 constant-volume injector; C_{18} (10-μm) column from Waters Associates coupled with an RP_{18} guard column from Rainin; Dupont model 837 variable-wavelength detector; Hewlett-Packard model 3380A integrator. A Varian model T-60 NMR spectrometer and a Perkin-Elmer model 621 IR spectrophotometer were used.

General Method for Synthesis—All amino acid esters of metronidazole, except the Ile ester, were synthesized by means of the DCC coupling of *tert*-Boc amino acids and metronidazole in the presence of a catalytic amount of DMAP. The stoichiometry used was generally 1:3:3:0.05 for metronidazole:*tert*-Boc amino acid:DCC:DMAP. For example, in 40 mL of dry pyridine, 6.19 g of DCC and 5.07 g of *tert*-Boc-Ala were dissolved and allowed to stand at room temperature for 15–30 min. The dicyclohexylurea was removed by filtration, and then the filtrate was added in a dropwise manner to 40 mL of a pyridine solution containing 1.72 g of metronidazole and 61 mg of DMAP. The mixture was allowed to stand for 60 min at room temperature. The solvent was then removed under reduced pressure at 50°C, and the residue was taken up in ethyl acetate. The latter was successively washed with 100 mL of 0.1 M formate buffer at pH 4.0, 100 mL of 0.1 M phosphate buffer at pH 7.6, and water. The organic phase was dried over Na_2SO_4, and the solvent was removed under reduced pressure at 50°C. In some cases, *tert*-Boc amino acid esters of metronidazole were purified at this stage from the mixture by means of silica gel column chromatography. The *tert*-Boc group was subsequently removed with 25% TFA in dry methylene chloride for 60 min at room temperature. In the case of the *tert*-Boc-Val ester, it was treated with 75% TFA overnight. After the solvent and excess TFA were removed under reduced pressure, the resulting TFA salts of metronidazole amino acid esters were converted to their chloride salts by means of anionic-exchange column chromatography. If required, the HCl salts were further purified by recrystallization from isopropyl alcohol. The Ninhydrin test[4] was used in detecting free amino groups on TLC plates throughout the study.

A mixed-anhydride route was used in the synthesis of the Ile ester. To a tetrahydrofuran (THF) solution of *tert*-Boc-Ile and *N*-methylmorpholine, isobutyl chloroformate was added in a dropwise manner under a nitrogen atmosphere. The precipitate was removed by filtration, and then a mixture of metronidazole and DMAP in THF was added. The stoichiometry adopted was 1:1:1:1:0.05 for metronidazole : *tert*-Boc-Ile : *N*-methylmorpholine : isobutyl/chloroformate: DMAP. The reaction was allowed to proceed at room temperature for 60 min. The subsequent workup procedure was identical with that used in the synthesis of the other esters.

In all cases, the final product was characterized by mp, elemental analysis, NMR, and IR. The overall yield for analytical samples was in the range of 10%. No attempts were made to measure the minute extent of racemization which might have occurred during the synthesis. The mobile phases for TLC were: (A) methylene chloride:isopropyl alcohol:methanol:28.4% NH_4OH (80:15:4:1); (B) *sec*-butyl alcohol:pyridine:acetic acid:H_2O (37.5:25:7.5:30).

Glycine Ester—R_f 0.72 (A); mp, 187–189°C; ^1H NMR (CD_3OD): δ 2.55 (s, 3, CH_3Ar), 3.9 (s, 1, CH), 4.7 (m, 4, CH_2CH_2), and 7.9 ppm (s, 1, ArH).

Anal.—Calc. for $C_8H_{13}ClN_4O_4$: C, 36.30; H, 4.95; Cl, 13.40; N, 21.17. Found: C, 36.02; H, 5.08; Cl, 13.64; N, 20.98.

Alanine Ester—R_f 0.53 (A); mp, 140–142°C; ^1H NMR (CD_3OD): δ 1.4 (d, 3, CH_3), 2.6 (s, 3, CH_3Ar), 4.2 (m, 1, CH), 4.8 (m, 4, CH_2CH_2), and 8.0 ppm (s, 1, ArH).

Anal.—Calc. for $C_{11}H_{14}ClN_4O_4$: C, 38.89; H, 5.43; Cl, 12,75; N, 20.15. Found: C, 38.94; H, 5.55; Cl, 12.82; N, 20.32.

Phenylalanine Ester—R_f 0.77 (B); mp, ~70°C; ^1H NMR (CD_3OD): δ 2.6 (s, 3, CH_3Ar), 3.4 (m, 2, CH_2Ar), 4.3 (m, 1, CH), 4.8 (m, 4, CH_2CH_2), 7.2 (s, 5, ArH), and 8.2 ppm (s, 1, ArH); IR (KBr): 2900 (br, NH_3) and 1750 cm^{-1} (CO_2R).

Lysine Ester—R_f 0.58 (B); mp, ~70°C; ^1H NMR (D_2O): δ 1.8 (m, 6, CH_2 in R), 2.6 (s, 3, CH_3Ar), 3.0 (t, 2, CH_2NH_2), 4.1 (m, 1, CH), 4.8 (m, 4, CH_2Ar), and 8.1 ppm (s, 1, ArH); IR (KBr): 2950 (br, NH_3) and 1750 cm^{-1} (CO_2R).

Valine Ester—R_f 0.73 (A); mp, ~70°C; ^1H NMR (CD_3OD): δ 1.2 (d, 6, CH_3 in R), 2.2 (m, 1, CH), 2.6 (s, 3, CH_3Ar), 4.0 (d, 1, HCO), 4.8 (m, 4, CH_2CH_2Ar), and 8.1 ppm (s, 1, ArH).

Anal.—Calc. for $C_{11}H_{19}ClN_4O_4$: C, 43.07; H, 6.24; Cl, 11.56; N, 18.26. Found: C, 41.90; H, 6.22; Cl, 11.46; N, 18.03.

Leucine Ester—R_f 0.83 (A); mp, 140–143°C (dec.); ^1H NMR (CD_3OD): δ 1.0 (d, CH_3 in R), 2.8 (m, 2, CH_2 in R), 2.9 (s, 3, CH_3Ar), 4.2 (t, 1, HCO), 4.8 (m, 4, CH_2CH_2Ar), and 8.4 ppm (s, 1, ArH).

Anal.—Calc. for $C_{12}H_{21}ClN_4O_4$: C, 44.93; H, 6.60; Cl, 11.05; N, 17.47. Found: C, 45.13; H, 6.78; Cl, 11.07; N, 17.43.

Isoleucine Ester—R_f 0.77 (A); mp, 197–199°C; ^1H NMR (CD_3OD): δ 1.2 (m, 9, R), 2.1 (s, 1, not assigned), 2.8 (s, 3, CH_3Ar), 4.1 (m, 1, HCO), 4.8 (m, 4, CH_2CH_2Ar), and 8.5 ppm (s, 1, ArH, assignment tentative).

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Anal.—Calc. for $C_{12}H_{20}ClN_4O_4$: C, 45.07, H, 6.30; Cl, 11.09; N, 17.52. Found: C, 44.76; H, 6.61; Cl, 10.90; N, 17.46.

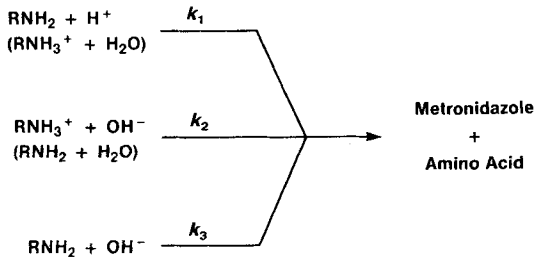
Hydrolysis in Human Serum and Buffers—After the blood from human volunteers was allowed to coagulate at room temperature for 60 min, the whole blood was centrifuged at 1000×*g* for 20 min at 4°C. The resulting serum was decanted into fractions of ~10 mL and stored at −10°C until used. Hydrolysis was initiated by adding an aqueous solution of an ester to a given volume of serum which had been previously equilibrated at 37°C. In all cases, the serum was diluted <1%. The initial substrate concentration was ~1 mg/mL except in the case of the Val and Ile esters, where 0.5 mg/mL was used. At a given time, 0.5 mL of serum was withdrawn and added to 2.0 mL of methanol. The solution was vortexed and centrifuged. The supernatant was diluted five times (usually 1.0 mL to 5.0 mL) with an appropriate HPLC mobile phase. Using a constant-volume injector, the above sample was injected directly onto a reversed-phase column. The mobile phases used were: for the Gly and Ala esters, 10% methanol:90% 0.02 M citrate buffer:0.02 M DMOA at a final pH of 6.0; for the Val ester, 15% methanol:85% 0.02 M citrate buffer:0.02 M DMOA at a final pH of 6.0; for the Leu ester, 25% methanol:75% 0.02 M citrate buffer:0.02 M DMOA at a final pH of 5.5; for the Ile ester, 50% methanol:50% 0.1 M phosphate buffer:0.005% sodium 1-decanesulfonate at a final pH of 6.7; for the Phe ester, 25% methanol:75% 0.02 M citrate buffer:0.02 M DMOA at a final pH of 5.0; for the Lys ester, 0.02 M citrate buffer without methanol:0.02 M DMOA at a final pH of 6.0. The ester peaks generally eluted at $\kappa' = 2.0–4.0$ at P = 1500–2500 psi. The eluant was monitored at λ = 317 nm.

Hydrolysis in aqueous buffers was monitored at 25 ± 3°C. The final ionic strength *(I)* was, in all cases, adjusted to 0.10 M: 0.01 M from buffer components and 0.09 M from NaCl. The buffers used were acetate, succinate, phosphate, Tris, carbonate, and butylamine. For pH values close to 2 and 12, HCl and NaOH were used, respectively. The remaining metronidazole ester was determined by means of reversed-phase HPLC using the aforementioned HPLC conditions. In general, the hydrolysis was monitored for at least two half-lives ($t_{1/2}$) and followed first-order kinetics in all cases. The hydrolysis rate constant (k_{obs}) was calculated from $k_{obs} \cdot t_{1/2} = \ln 2$.

Results and Discussion

The ability to predict the in vivo conversion rate of a prodrug to its parent compound is highly desirable in various pharmacokinetic models.[5–7] One objective of the present study is to correlate the serum-catalyzed hydrolysis to some structural parameters of the pro-moiety. The former can serve as a sufficient, if not a necessary, condition for the in vivo conversion, particularly for parenterally administered prodrugs.

In human serum, all seven esters studied hydrolyzed at 37°C, following a first-order kinetic process at least over a period of two half-lives ($t_{1/2}$) at an initial ester concentration between 0.5 and 1.0 mg/mL. In terms of $t_{1/2}$, the hydrolytic lability varied significantly: 22.0 min for Gly, 6.49 for Ala, 313 for Val, 21.0 for Leu, 5730 for Ile, 4.50 for Phe, and 9.63 for Lys esters of metronidazole. Logarithmic values of the rate constant (k_s) are listed in Table I. It is interesting to note that the $t_{1/2}$ for the Gly ester reported in this study, 22 min, is much smaller than the 41 min observed in 80% human serum.[2]



Scheme I

In buffered solutions, the pH–rate profile (Fig. 1) appears to satisfy the three microscopic reaction pathways shown in Scheme I, in which RNH_2 and RNH_3^+ represent neutral and protonated species of an ester, respectively. Kinetically equivalent reactions are also listed inside parentheses. Scheme I leads to the following expression of k_{obs}:

$$k_{obs} = \frac{K_a}{K_a + (H^+)} \left[k_1 (H^+) + \frac{k_2 \cdot K_w}{K_a} + k_3 (OH^-)\right] \quad (1)$$

The values of k_{obs} are obtained at I = 0.10 M, only 0.01 M of which was derived from the buffer components. The highest buffer concentration used was on the order of 0.02 M for the acetate buffer. The potential general acid/base or nucleophilic catalysis due to the buffer components was ignored in the present study.

The pH dependence of k_{obs} was subjected to the simplex least-squares method of curve fitting[8] to eq. 1, resulting in the values of pK_a, k_1, k_2, and k_3 listed in Table I. The foregoing analysis of the rate–pH profile ignored the possible inductive effects of the protonation of the imidazole nitrogen. Its pK_a was previously reported to be 2.6.[1] Similarly, the effect due to protonation of the ϵ-amino group of the Lys ester is ignored; its pK_a is 10.8.[9] Close examination of the rate–pH profile revealed no indication of these effects in either case, perhaps due to their distance from the ester moiety.

If serum is to be considered as a pooled enzyme system, the substrate binding to various enzymes present in the serum as well as chemical reactivity of an ester bond would be important to consider. In the former, such physical properties as the Hansch π value,[10,11] Wolfenden's so-called hydration potential,[12] and the hydrophobicity scale for amino acids developed by Bull[13,14] would be important determinants. In the latter, one of the three microscopic rate constants, Taft's steric parameter (E_s),[10] Charton's steric parameter (ν),[15] or

Table I—Serum-Catalyzed Hydrolysis of Amino Acid Esters of Metronidazole and Physiocochemical Properties of the Amino Acid Side Chain

Ester	-log k_s[a]	pK_a	Binding			Reactivity[e]				
			π[b]	H[c]	ΔG[d]	ν[f]	E_s[b]	k_1	k_2	k_3
Gly	3.28	7.20[g]	0	2.39	0.81	0	0	7.27	158	1.07
Ala	2.75	7.18	0.56	1.94	0.61	0.52	−1.24	3.41	436	7.34
Val	4.43	7.18	1.53	1.99	−0.75	0.76	−1.71	11.8	74.5	0.729
Leu	3.26	7.08	2.09	2.28	−1.65	0.98	−2.17	1.73	361	2.23
Ile	4.70	7.51	2.04	2.15	−1.45	1.02	−2.37	10.1	39.1	0.489
Phe	2.59	6.51	2.01	−0.76	−1.52	0.70	−1.62	0.457	467	2.02
Lys	2.92	6.86[h]	−1.94	−9.52	0.46	0.70	−1.63	1.60	498	7.13

[a] Serum-catalyzed hydrolysis rate constant (s^{-1}) at 37°C. [b] From ref. 10. [c] From ref. 12. [d] From ref. 13. [e] See Scheme I for the definition of k_i ($s^{-1}M^{-1}$). [f] From ref. 15. [g] Obtained by rapid titration. [h] Rapid titration resulted in 7.04.

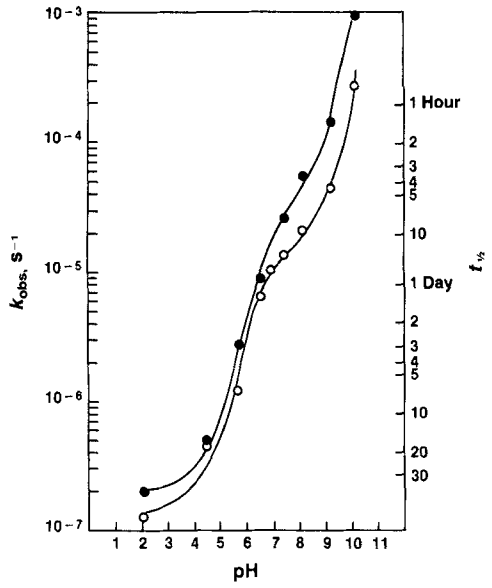


Figure 1—*Dependence on pH of the observed rate constant (k_{obs}) and half-life ($t_{1/2}$) for the hydrolysis of Phe (○) and Lys (●) esters of metronidazole at I = 0.10 M and 25 °C.*

some other chemical properties would be important. Due to the limited information, Taft's polar substituent parameter σ^* is excluded from the present consideration. These five parameters are listed in Table I.

Of all regression analyses, with one of those five parameters or with two parameters each representing either the binding or the chemical lability, a most significant correlation was discovered between k_s and k_2 or k_3 (Fig. 2). With k_2, for example, $p < 0.001$ testing H_0:slope = 0, and:

$$\log k_s = 1.80 \log k_2 - 7.58 \quad (R^2 = 0.92) \quad (2)$$

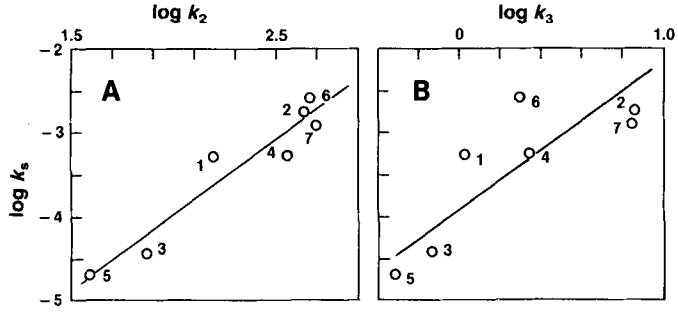


Figure 2—*Relationships between serum-catalyzed hydrolysis at 37 °C (k_s) and the second-order rate constant (k_2) for the OH^--catalyzed hydrolysis of the cationic species at 25 °C (A) and that (k_3) for the OH^--catalyzed hydrolysis of the neutral species (B). Key: (1) Gly; (2) Ala; (3) Val; (4) Leu; (5) Ile; (6) Phe; (7) Lys esters of metronidazole.*

As defined in Scheme I, k_2 and k_3 are the second-order rate constants for the OH^--catalyzed hydrolysis of the cationic and neutral species of the esters, respectively. Numerous mechanistic studies indicate that the enzymatic hydrolysis of ester substrates occurs by a double displacement reaction. When enzymes are not saturated with the substrate, the relevant "chemical" reaction is the enzyme acylation step. It was, therefore, predicted that the acyl substituent effects on nucleophilic attack by the enzyme should be similar to those exerted in the OH^--catalyzed hydrolysis of esters.[16] The present study thus appears to support the prediction.

In both Fig. 2A and B, the least-squares analysis of linearity results in a slope in the range of 1.80. If it is assumed that the total concentration of all relevant enzymes is constant throughout the present study, then k_2 can be considered as a second-order rate constant. That the slope is much greater than unity indicates a higher sensitivity to substituent effects of serum-catalyzed hydrolysis than hydrolysis in the buffers. However, a quantitative interpretation of this amplification of chemical activation is not possible in the present study since k_2 and k_3 were determined at 25°C, whereas k_s was determined at 37°C.

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