Drug Development and Industrial Pharmacy, 23(10), 951-957 (1997)

RESEARCH PAPER

Kinetic and Biopharmaceutical Study of an Antiviral Agent in Aqueous Vehicle and Human Gastric Fluid

Andrew B. C. Yu,[1,*] Glenn Portmann,[2] and Daryl Simmons[3]

[1]*Department of Pharmaceutics, Albany College of Pharmacy, New Scotland Av., Albany, New York 12208*
[2]*Pharmaceutics Research Division, Sterling Winthrop, Inc., 1250 S. Collegeville Rd., Collegeville, Pennsylvania 19426*
[3]*Nycomed R. & D., Inc., 466 Devon Park Drive, P.O. Box 6630, Wayne, Pennsylvania 19087-8630*

ABSTRACT

WIN 54954 is an investigative antiviral compound that is insoluble in water and very unstable in acidic pH. Both factors can potentially limit in vivo absorption and availability. Degradation involves hydrolytic opening of the oxazoline ring, which results in loss of antiviral activities. The degradation product in acid is mostly an aminoethylester, WIN 55696, which further decomposes in basic medium into the hydroxyethylamide, WIN 55795. WIN 54954 decomposition in buffer solution is pH dependent and follows first-order kinetics. The stability half-life of WIN 54954 in 0.1 N hydrochloric acid is less than 1 hr at body temperature. A series of in vitro experiments were conducted to evaluate the stability of the drug in human gastric fluid (HGF) from volunteers. These studies demonstrate that the presence of digestive enzymes of HGF did not alter the nature of oxazoline ring opening substantially. The decomposition products identified were similar to products identified in in-vitro buffered solutions. The rate of decomposition is faster in genuine human gastric fluid than in 0.1 N HCl. Decomposition is also pH dependent and does not vary among individuals.

*To whom correspondence should be addressed.

INTRODUCTION

WIN 54954 is an oxazoline compound (Fig. 1) chemically related to Disoxoral, which was reported to be active against virus in several studies (1–5). WIN 54954 has improved antiviral activity over Disoxoral but is chemically less stable. The isoxazole group is common to both Disoxoral and WIN 54954, and is known to be stable at physiological conditions. The 2-methyl substituted oxazoline (pKa = 5.5) is unstable to acid but relatively stable at neutral or slightly basic pH (7). No related aromatic oxazolines were studied in gastric fluid. WIN 54954 is stable at physiological pH of the small intestine but requires acidic pH for solubilization. The products of WIN 54954 decomposition were identified and the mechanism elucidated.

Biopharmaceutical studies were initiated to study various factors that influence WIN 54954 stability, formulation, and availability. WIN 54954 decomposition was evaluated first in buffered solutions, then in simulated gastric fluid (SGF), (U.S. Pharmacopeia XIII-National Formulary XVII), and finally confirmed in gastric fluid from human volunteers (HGF). The present report provides details on analytical method development, strategies for overcoming instability, and a rationale for the development of these antivirals.

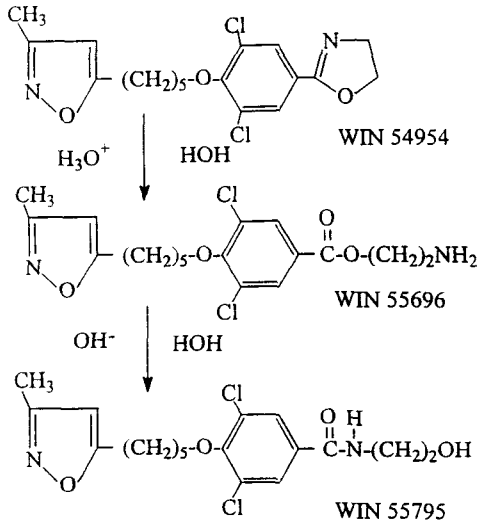

Figure 1. Degradation pathway for WIN 54954 in buffer solutions.

MATERIALS AND METHODS

HPLC Method

Mobile phase: methanol:acetonitrile:water:sodium acetate buffer, pH 5.45, 0.5 M : octanesulfonic acid (450:350:150:50:1)

Pump: 1.0 mL/min (Model 501, Waters Associates, Milford MA)

Injection: 50 µl (712 WISP, Waters Associates)

Guard column: RP-18 (New Guard 15 × 3.2 mm, 7 µm, Brownlee Labs Inc., Santa Clara, CA)

Analytical column: Whatman 10 ODS-3, 25 cm (Whatman Chemical Separations, Clifton, NJ)

Detection: UV, 254 nm, (PDA m991, Waters Associates)

Standard preparation: WIN 54954, 55696, and 55795 (Fig. 1) standard solutions were prepared in methanol. The concentration range, retention times, and statistical parameters of each set of standards are shown in Table 1.

Spectrophotometric Kinetic Study

A Bausch and Lomb Spectronic 2000 spectrophotometer was used to obtain spectral scan from 330 to 230 nm (Fig. 2). WIN 54954 solution samples from pH 1.1 to 5.2 were scanned at various times for kinetic determination. WIN 54954 has a lower absorption peak than the decomposition products at these pH values. The solutions were maintained at 37°C with a Distek constant-temperature bath. Calculations of the decomposition rate constants (k) were performed by linear regression using Cricket Graph.

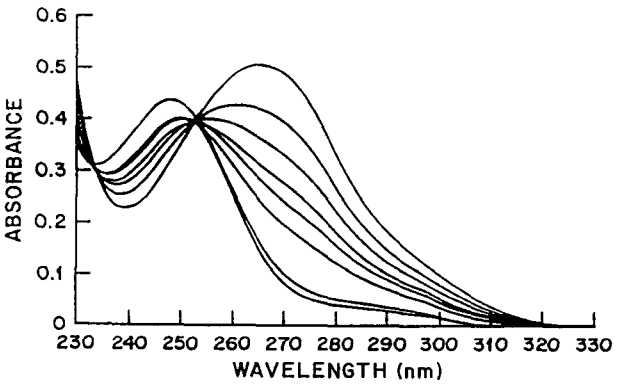

Figure 2. WIN 54954 absorbance vs. wavelength at different reaction times in 0.1 N HCl at 37°C.

Table 1

Standard Concentrations Versus Mean Peak Areas (n = 3) of Three WIN Compounds

Concentration (μg/ml)	WIN 54954 (Peak Area)	WIN 55795 (Peak Area)	WIN 55693 (Peak Area)
25.0	0.0380	-	0.0313
12.5	0.0195	0.0143	0.0157
5.0	0.0143	0.00561	0.00465
2.5	0.00927	0.00263	0.00228
0.25	-	0.000458	0.000301
Correlation Coefficient	1.00	1.00	0.997
Slope	1.25	1.14	1.28
Intercept	0.600	-0.030	-0.80
Peak retention	9.8 min	4.9 min	9.0 min

Solution Stability

WIN 54954 solution state stability was examined at various pH values, solvents, and temperatures to determine the rate of decomposition and to separate the degradation products formed. Aqueous buffered solutions of 15 to 20 μg/ml in 25% ethanol were maintained at 37°C and sampled at various times to determine the kinetics of WIN 54954 decomposition. The buffer solutions consisted of 0.2 M lactate (pH values 4.2 and 5.3), citrate (pH 6.6), and phosphate (pH values 7.2, 8.4, and 9.5). Solubilities of WIN 54954 were determined by HPLC after 24 hr of equilibrium at room temperature. Chromatographic assay allowed the drug to be separated from any decomposition product that may have formed during equilibration.

Stability in HGF and SGF

HGF was collected from the Sterling Pharmacology Study Unit at Albany Medical Center Hospital in accordance with the Volunteer Procedural Protocol. The fluid collection, preparation, and pH determination have been described previously (6,7). WIN 54954 solutions ranging from 0.5 to 1 mg/ml in gastric fluid were dispensed in 5.0-ml aliquots and maintained at 37°C with a Distek constant-temperature bath. The samples were taken periodically for up to 2 hr and assayed for WIN 54954 and its ring-opened decomposition products, identified as WIN 55696 and WIN 55795. WIN 55969, which also hydrolyzed in HGF, was further studied at the same temperature by adding WIN 55696 to HGF. Samples were drawn and assayed periodically in a similar manner.

RESULTS AND DISCUSSION

HPLC Assays

Good standard-curve linearity of peak areas versus concentration is shown for WIN 54954, and related degradation products WIN 55696 and WIN 55795 (Table 1). A typical HPLC chromatogram is shown in Fig. 3. Blank gastric fluid had no chromatographic interferences at the retention times for products. The parent compound WIN 54954 (8) was sufficiently resolved from the decomposition products. The retention time of each compound is listed in Table 1. The decomposition products include an aminoethylester (WIN 55696), and the hydroxyethylamide (WIN 55795).

Solubility

Aqueous solubilities of WIN 54954 are very low, ranging from less than 1 μg/ml (pH values 4.9 to 9.5) to about 50 μg/ml at pH 1.1 due to sale formation at the weakly basic oxazoline nitrogen. Several nonaqueous solvents were also studied because the low aqueous solubility may lead to a dissolution and absorption problem. The solubility is very high in ethanol, polyethylene glycol (PEG) 400, and other solvents (Table 2). Powder WIN 54954 is stable at 70°C, but clear melts were formed rapidly because the melting point is 42°C. Stability in alcohol and PEG is enhanced but a

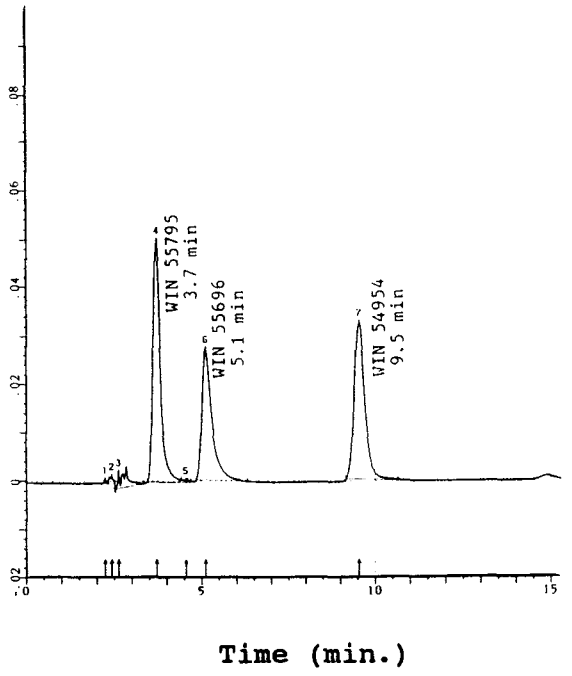

Figure 3. Chromatogram showing separation of WIN 54954 from decomposition products: WIN 54954-peak 3, WIN 55696-peak 2, and WIN 55795-peak 1.

hydroalcoholic solution is still unstable. A soft gelatin capsule prepared using a blend of PEG is stable during storage, although the stability of WIN 54954 in HGF still needs to be determined.

Oxazoline Ring-Opening Kinetics

The hydrolytic pathways were investigated by conducting a kinetic study of WIN 54954 at various pH levels and measuring the hydrolytic products. In order to keep the drug in solution throughout the entire pH range, 25% ethanol was used as a cosolvent. These solutions of WIN 54954 (15 or 20 μg/mL) in 25% ethanol were stored at 37°C and then assayed at various time intervals. The HPLC method was used for solutions with pH values of 6.5–9.5 and the spectrophotometric procedure was chosen for solutions with pH values of 1.1–5.2. Formation of only one hydrolytic product in acidic solutions, the aminoethylester (WIN 55696) (Figure 1), was shown by high-performance liquid chromatography (HPLC). This is illustrated by the changes in UV spectra shown in Figure 2. Because the peak absorbance at 266 nm for WIN 54954 decreases as a function of time, a new peak absorbance at 248 nm appears. The spectrum obtained after the reaction is complete (240 min or 16 half-lives) is identical to the spectrum obtained for a standard solution of WIN 55696. An isobestic point observed at 253.5 nm also indicates that only one product is formed in the reaction. The reaction kinetics can be determined by plotting the logarithm of the absorbance differences as a function of time at each respective peak wavelength as shown in Fig. 4. Similar rates were obtained for WIN 54954 decreases and WIN 55696 increases by linear regression analysis (half-lives of 14–16 min). Similar UV spectral changes with an isobestic point at 253.5 nm resulted after decomposition of solutions with apparent pH val-

Table 2

Solubilities of WIN 54954 in Various Solvents/Buffers

Solvent	WIN 54954 Concentration
0.1 N HCl	about 50 μg/ml
0.01 N HCl	about 30 μg/ml
Water (pH 4.9 to 9.5)	<1 μg/ml
25% ethanol in pH 7.1 buffer	38 μg/ml
50% ethanol in water	6.5 μg/ml
Absolute ethanol	>500 mg/ml
Propylene glycol	80 mg/ml
Polyethylene glycol 400	>200 mg/ml
Polyethylene glycol 300	>200 mg/ml
Corn oil	>200 mg/ml
Sesame seed oil	170 mg/ml

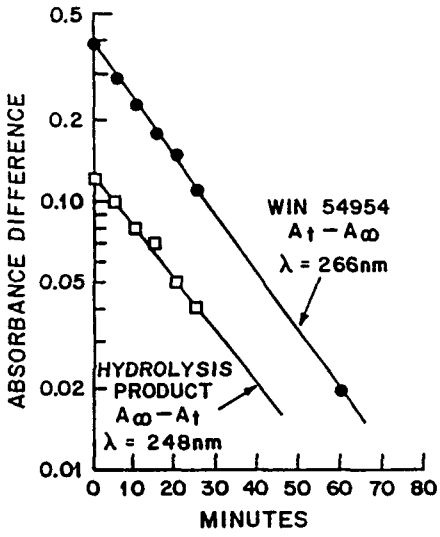

Figure 4. Log absorbance difference vs. time for WIN 54954 solution (15 μg/ml) and its reaction product in 0.1 N HCl at 37°C.

ues of 1.1–5.2. The first-order rate constants were determined by log linear regression at various pH values and plotted on Fig. 5. The correlation coefficients ranged from 0.96 to 0.99.

The sigmoidal range of the reaction rate versus pH curve is indicative of a mechanism involving reaction of the protonated and nonprotonated species, as shown in Fig. 6 and in the following expression:

$$\text{Reaction rate} = k_1[BH^+] + k_2[B]$$

The overall rate constant (k) for this mechanism (9) is expressed as follows, where K_a is the dissociation constant for the protonated WIN 54954:

$$K = \frac{k_1[H^+] + k_2K_a}{[H^+] + K_a}$$

Accurate measurement of the pKa for WIN 54954 was not possible due to its rapid hydrolysis in acidic solutions. The pKa is 4.7 for a related compound, 5-[5-[4-(4,5-dihydro-2-oxazolyl)phenoxy]pentyl]-3-methylisoxazole, which is more stable in acidic conditions. The theoretical curve in Fig. 5 was calculated based on a k_1 of 58 days^{-1} and a k_2 of 0.024

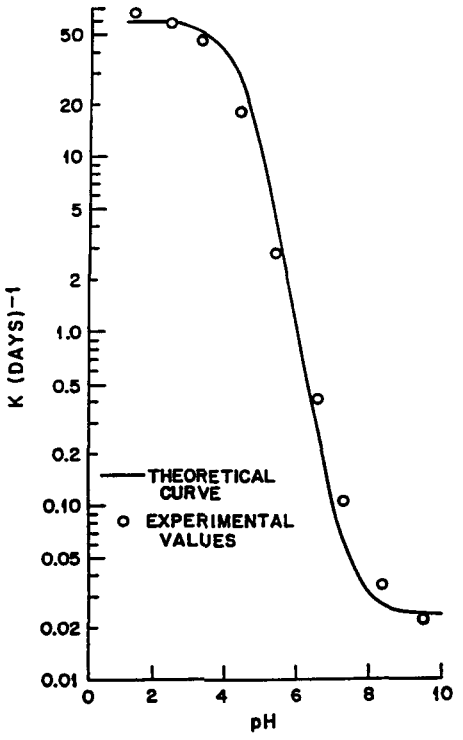

Figure 5. Log WIN 54954 hydrolysis rate constant (days^{-1}) vs. pH at 37°C.

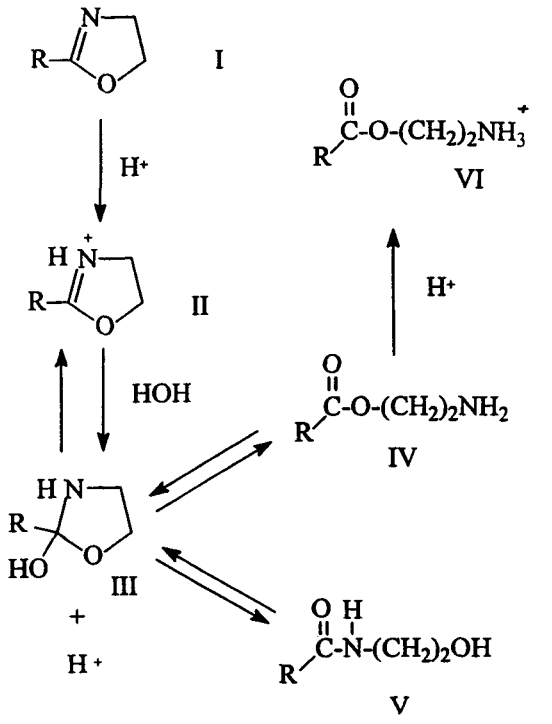

Figure 6. Degradation mechanism of an oxazoline compound.

days^{-1}, and a apparent K_a of 7.94 × 10^5 (pKa = 4.1). There is close agreement between the experimental values for K at different pH values and the shape of the theoretical curve, confirming that the proposed mechanism is consistent and appropriate, and in accordance with that proposed for 2-methyloxazoline (10) and in a review of oxazoline (11). The high reactivity of the protonated compound to water is evident by the greater than 2400-fold-higher rate compared to the basic form. The electron withdrawal property of the two chlorine groups in WIN 54954 accelerates the hydrolytic opening of the oxazoline ring. About a 75-fold enhancement is evident compared to previous results with the nonchlorinated phenyl compound WIN 51711 (8). A general reaction mechanism for oxazolines is shown in Fig. 6.

Stability in HGF and SGF

WIN 54954 decomposition and formation of products in HGF and SGF are shown in Figs. 7 and 8. WIN 54954 decomposition half-life is pH dependent in all three fluids (0.1 N HCl, SGF, and HGF) as shown in Table 3. The fluid pH ranged from 1.2 to 1.75. In most

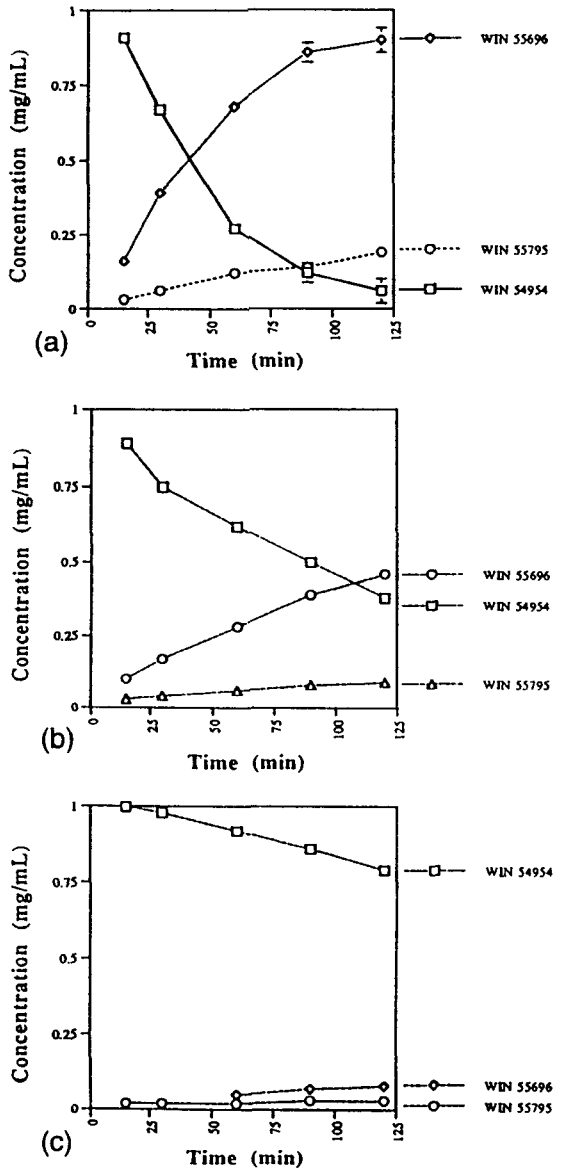

Figure 7. WIN 54954 decomposition in human gastric fluid at 37°C: (a) WIN 54954 decomposition in human gastric fluid at pH 1.4. (b) WIN 54954 decomposition in human gastric fluid at pH 1.8. (c) WIN 54954 decomposition in human gastric fluid at pH 3.0.

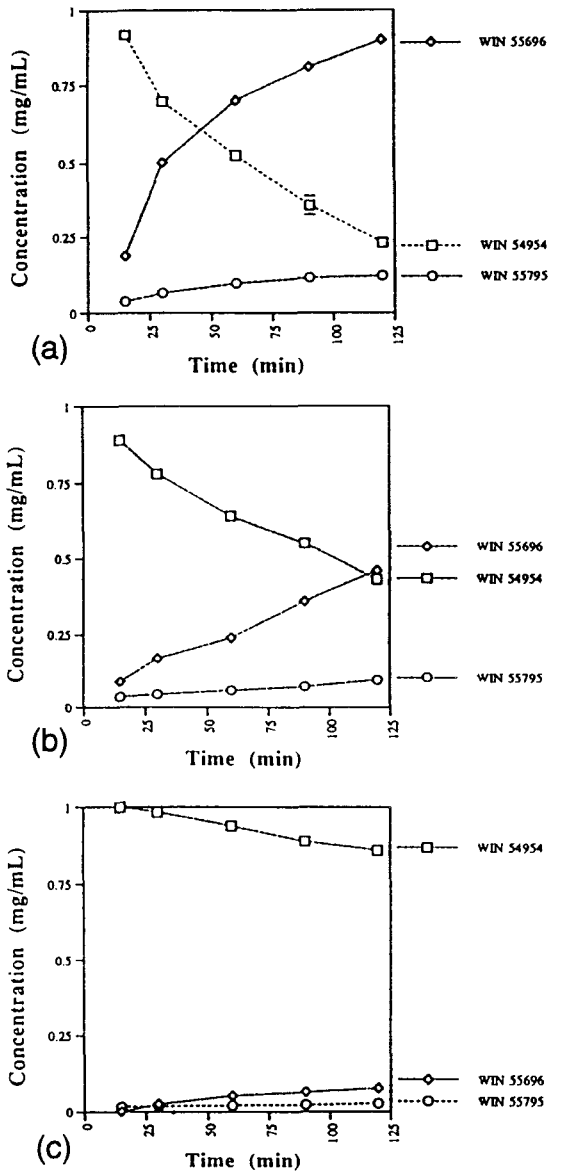

Figure 8. WIN 54954 decomposition in simulated gastric fluid at 37°C: (a) WIN 54954 decomposition in simulated gastric fluid at pH 1.4. (b) WIN 54954 decomposition in simulated gastric fluid at pH 1.8. (c) WIN 54954 decomposition in simulated gastric fluid at pH 3.0.

cases, the mean half-life was shorter in HGF or SGF compared to 0.1 N HCl. At pH 1.2, the half-life in SGF is 10 min, far faster than the half-life of 36 min in 0.1 N HCl. Also the rate of formation of the reaction products (WIN 55696 and 55795) are shown in Figs. 7 and 8. The main hydrolysis product, WIN 55696, is produced faster at lower pH values than the minor amounts of WIN 55795. At 2 hr, the sum of the three compounds amounts to 97% of theory.

SGF was shown to be a better predictor of hydrolysis potential in the human gastric environment than HCl. This may be due to an enhanced dissolution rate or in-

Table 3

WIN 54954 Hydrolysis Rate in Three Fluids at 37°C

	HGF (Range in Parenthesis)	SGF (Range in Parenthesis) $n = 3$	Hydrochloric Acid, 0.1 N (Range in Parenthesis) $n = 3$
pH	$t_{1/2}$ (min)	$t_{1/2}$ (min)	$t_{1/2}$ (min)
1.20	ND	10 (9–12)	36 (31–40)
1.40	24 $n = 1$	22 (20–25)	75 (66–86)
1.75	102 $n = 6$, (77–121)	84 (77–96)	127 (120–134)

crease in drug solubility due to the presence of peptides in the SGF and HGF. Solubility was previously shown to be higher in HGF for this drug (7). At higher pH values the amide product (WIN 55795) can also form from the ester compound (WIN 55696) (Fig. 1). Formation of the amide product at pH 10 is shown in Fig. 9. In the gastric fluid this reaction was not significant, since little WIN 55795 was formed.

CONCLUSION

Hydrolytic ring-opening reactions of WIN 54954 in solution state were evaluated using buffered solutions and HGF. Decomposition products formed in gastric fluid are predicted from in vitro experiments, but the rates of decomposition were faster in the presence of HGF. Physical factors of low solubility, low melting point, and instability in aqueous systems were major considerations in determining to formulate the product into a nonaqueous soft gelatin capsule for clinical trial.

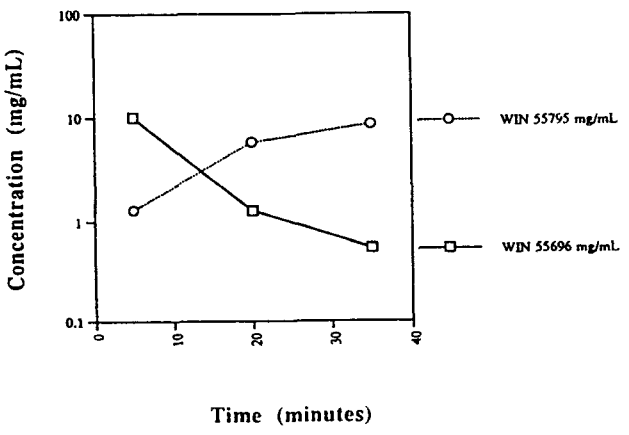

Figure 9. Hydrolysis rate of WIN 55696 and formation of WIN 55795 at pH 10.

REFERENCES

1. G. D. Diana, M. A. McKinlay, M. J. Otto, V. Akullian, and C. Oglesby, J. of Med. Chem. 28, 1906–1910, 1985.
2. G. D. Diana, M. J. Otto, and M. A. McKinlay, Pharmacol. Ther. 29, 287, 1985.
3. G. D. Diana, M. A. McKinlay, C. J. Brisson, E. S. Zalay, J. V. Miralles, and U. J. Salvador, J. of Med. Chem. 28, 748, 1985.
4. M. G. Rossman, et al., TIBS 12: 313, 1987.
5. J. Smith, et al., Science, 233, 1286, 1986.
6. U.S. Pharmacopeia XXIII-National Formulary XVIII, Rockville, MD 1995, p. 2053.
7. D. M. Simmons, Drug Devel. & Indust. Pharm., 19, 10, 1103–1112, 1993.
8. Andrew B. C. Yu and Glenn A Portmann, Drug Devel. & Indust. Pharm., 16, 13, 1971–1983, 1990.
9. K. A. Connors, G. L. Amidon, and V. J. Stella, *Chemical Stability of Pharmaceuticals*, 2nd ed., P48, Wiley Interscience, 1986.
10. T. C. Bruice and S. J. Benkovic, *Bioorganic Mechanism*, Vol. 2, Benjamin-Cummings, 1966.
11. J. A. Frump, Chem. Revs., 71, 494, 1971.