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RESEARCH PAPERS

Preformulation Development of a Human Leukocyte Elastase Inhibitor for Oral Dosage

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ABSTRACT

WIN 64733 solubility and partition coefficients were determined with a precise and accurate HPLC method. Solubility decreased with increasing pH (140 mg/mL at pH 2.0, 86 mg/mL at pH 3.8, and 48 mg/mL at pH 5.2) but equilibrated within 30 hours. In purified water the maximum concentration was 102 mg/mL with pH 3.6. The maximum solubility in hydroxypropyl-beta-cyclodextrin (HP-β-CD) is only 43 mg/mL. However, unlike the water solution, the cyclodextrin preparation did not precipitate when added to 0.1 N HCl or 0.9% NaCl. The precipitated chloride salt of WIN 64733 is amorphous. Excess compound that was wetted, but not dissolved, formed liquid crystals.

In human gastric fluid (HGF), WIN 64733 did not decompose in the solid or liquid state for 2 hours, or precipitate from solution despite NaCl and HCl in the HGF. WIN 64733 solid is stable to heat (50°C), light (1000 ft-candles), humidity (75% RH), and heat plus humidity for 6 weeks. Solutions of WIN 64733 are unstable, with first-order decomposition rates that are light independent at pH's 2.0 to 6.6. Decomposition was more rapid at higher pH's and increased temperature. This occurred by hydrolysis of the ester linkage forming an N-hydroxymethyl fragment that subsequently decomposed to saccharin and formaldehyde, verified by the Hantzsch reaction.

Since solubility is high in water and HGF, dissolution in the gastric environment would favor absorption of an oral dose.

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INTRODUCTION

WIN 64733 is a potent and selective inhibitor of human leukocyte elastase and is targeted for the therapy of chronic obstructive pulmonary diseases including bronchitis, emphysema, and other inflammatory pulmonary diseases such as cystic fibrosis. It is a development compound for formulation in an oral delivery system. A preformulation study was conducted to determine certain physical and chemical properties of WIN 64733 that are useful in the design and formulation of a stable and orally bioavailable delivery system. The solubilities in water and human gastric fluid (HGF) in addition to the decomposition mechanism make this an interesting preformulation profile. The effect of hydroxypropyl-beta-cyclodextrin (HP-β-CD) on both its stability in the presence of chlorides and its aqueous solubility provides insight to formulation strategies with cyclodextrin. Cyclodextrins are commonly used to overcome aqueous solubility problems of lipophilic drugs (1–3). HP-β-CD is not to be regarded simply as an excipient for solubility enhancement since other physicochemical properties can be affected, such as stability (1,3,4,5), reduction of unwanted side effects (6), prevention of crystal growth (7), and reduction of irritation caused by injected drugs (8,9). HP-β-CD was investigated here as a stabilizing agent for WIN 64733 in the presence of chlorides, since a liquid oral dose was considered.

MATERIALS AND METHODS

WIN 64733 (as the HCl salt) and purified water were obtained from Sterling Winthrop Pharmaceuticals Research Division. HPLC grade methanol, isopropanol, 190 Ethanol, acetonitrile, and ammonium acetate were purchased from J. T. Baker Inc., Phillipsburg, NJ. Octanol, formaldehyde, and acetylacetone were supplied by Sigma Chemical Co. (St. Louis, MO). Glacial acetic acid was purchased from Mallinckrodt Specialty Chemicals Inc., Paris, KY, and hydroxypropyl-beta-cyclodextrin (HP-β-CD) from American Maize Products Company (Hammond, IN). The Sterling Pharmacology Study Unit at Albany Medical Center (Albany, NY) supplied the human gastric fluid from paid healthy volunteers as per the protocol previously reported (10).

HPLC Method

The HPLC system consisted of the following Waters equipment (Waters Chromatography, Milford, MA): model 510 pump, model 710B WISP, model 991 photodiode array detector with version 6.22a software, and a model 5200 printer plotter. A Whatman 10 ODS-3 (250 × 4.6 mm, Whatman, Inc., Clifton, NJ) analytical column was used with a mobile phase of 65% acetonitrile and 35% pH 4.0 ammonium acetate buffer (0.1 M), delivered at 1 mL/min. The peak areas were integrated at 280 nm.

Solubility

WIN 64733 was added to 4 mL glass vials with Teflon®-lined screw caps, containing 3 mL each of one of the following solvents: isopropanol, methanol, octanol, water, and 0.1 M sodium acetate buffers at pH's 2, 4, and 6.

The capped vials were rotated on a Glas-Col model RD350 rotating mixer (Terra Haute, IN) for 48 hours. Sample aliquots were taken at timed intervals and filtered through 0.2 mm membrane microfuge filters (Rainin Instrument Co. Inc., Woburn, MA), using a Beckman microfuge (Beckman Instruments Inc., Palo Alto, CA). Portions of the clear supernatant were quantitatively diluted in acetonitrile for HPLC analysis.

Supersaturated solutions were prepared in screw-cap vials by placing WIN 64733 in the buffers in an ultrasonic bath for an hour. The preparations were allowed to cool and recrystallize overnight, then filtered with 0.2 mm microfuge filter tubes and analyzed by HPLC to compare with the solubilities determined by continuous mixing. The remaining solid material was examined microscopically with a Leitz Laborlux Pol microscope (Upstate Technical, Syracuse, NY) at 630×, for crystal characteristics.

Solutions of WIN 64733 in water and 28% HP-β-CD were added to 0.9% NaCl and 0.1 N HCl to test the drug solubility in chloride ion solutions for comparison with the values obtained in actual HGF.

The pH's of the buffered and water solutions were determined with a Beckman F71 pH meter.

Solid-State Stability

WIN 64733 in 4 dram screw-cap glass vials were placed in 37°C and 50°C ovens and in the light cabinet (1000 ft-candles, combined fluorescent and incandescent). A control was maintained in the lab cupboard protected from light at room temperature and all samples were mixed with a spatula for uniformity prior to sampling, and portions were quantitatively dissolved in acetonitrile for HPLC analysis.

Solution Stability

1. Decomposition and pH Stability

Solutions of WIN 64733 were prepared at approximately 20 μg/mL in water and 0.1 M acetate buffers at pH's 2, 4, and 6.6. Aliquots of the solutions were transferred to 4 mL glass vials with Teflon®-lined screw caps. Sample vials of each pH were heat stressed at 37°C and 50°C, and subjected to light at room temperature for 6 weeks. A control at each pH was maintained at room temperature and protected from light in the lab cupboard.

2. Formaldehyde Assay

A colorimetric assay (2) was used to measure formaldehyde produced during stability testing of WIN 64733.

Solution A consisted of 2 M ammonium acetate, 0.05 M acetic acid, and 0.02 M acetylacetone in purified water.

a. Standard Preparation

A formaldehyde standard stock solution in water containing 80.6 μg/mL was further diluted, to prepare working standards of the concentrations 0.81, 1.61, 3.22, 4.84, and 6.45 μg/mL. The formaldehyde standards were mixed 1:1 with Solution A.

b. Sample Preparation

The control and 50°C samples from the 5-week stability station were assayed: 1.5 mL of Solution A were mixed with 0.5 mL each of sample and 1 mL of water. An assay blank was prepared with water and the appropriate amounts of the reagents. After 6 hours of incubation at room temperature, the samples and standards were read at 412 nm on a Hewlett Packard 845VA Diode Array spectrophotometer (San Fernando, CA).

Characterization in Human Gastric Fluid

Solid WIN 64733 was added to three samples of HGF at 37°C, in screw-cap glass vials, to determine solubility and stability in the gastric environment for a 2-hour period since this is the maximum residence time expected. The mixtures were examined microscopically and the pH of each sample was determined with a Beckman F70 pH meter. Decomposition was quenched by quantitatively diluting the entire vial contents with acetonitrile, and the remaining drug (solid/solution) was assayed by HPLC.

Solutions of WIN 64733 in water were added to portions of the same HGF samples to achieve drug concentrations of 10–11 mg/mL. The solutions were assayed initially and at 2 hours. Quantitative dilution with acetonitrile was used to quench decomposition reactions prior to HPLC analysis.

RESULTS AND DISCUSSION

HPLC Method

Peak areas were integrated at 280 nm. WIN 64733 eluted at 9.9 minutes with a tailing factor (t) of 1.2, and reproducibility (%RSD) of 0.3%, which were within the USP XXII limits [t less than 2 and %RSD not more than 4 (11)]. Resolution was not calculated since no other compounds eluted near the WIN 64733 peak.

Aqueous Solubility: Water, HP-β-CD, and Human Gastric Fluid

Solubility equilibrium was reached within 30 hours (Table 1). No decomposition peaks were seen in the chromatograms within the 48-hour testing period. The results obtained in water and buffers, with the mixing and recrystallization methods, were similar, and solubility was linearly dependent on pH ($r^2 = 0.982$). Sonication and recrystallization from supersaturated water solutions was more rapid than mixing for 30 hours and resulted in the formation of amorphic particles less than 1 mm in diameter.

Because of the high aqueous solubility of WIN 64733, HP-β-CD was tested primarily as a stabilizing

Table 1

Equilibrium Solubility of WIN 64733 and Solution pH's After 48 Hours on a Laboratory Rotator

Solvent	Concentrations (mg/mL)		pH
	Recrystallization	Mixing	
Water	101	102	2.8
5% HP-β-CD		33	3.1
28% HP-β-CD		43	2.9
50% HP-β-CD		64	2.8
Buffers			
pH 1.9	140	140	
pH 3.8	95	86	
pH 5.2	48	48	
Octanol		1.7	

agent for WIN 64733 solutions in the presence of chlorides. Aqueous solutions, at drug concentrations of 1–40 mg/mL, precipitated immediately as amorphic particles, when added to 0.1 N HCl or 0.9% NaCl solutions due to the common ion effect (13,14). At 40 mg/mL in 28% HP-β-CD, no precipitate formed over a 16-hour period when WIN 64733 was added to 0.9% NaCl or 0.1 N HCl solutions and no decomposition occurred.

An interesting but unexpected observation was that cyclodextrin decreased WIN 64733 aqueous solubility (Table 1). As the cyclodextrin concentration was increased, the WIN 64733 solubility increased linearly (r^2 = 0.977), but was always less than in pure water. This may be due to HP-β-CD changing the solvation characteristics of water. Although HP-β-CD decreased aqueous solubility, it protected drug solutions from precipitation in the presence of chlorides. WIN 64733 has a high aqueous solubility, and may bind with the free OH groups of HP-β-CD, shielding the pyrrolidine ring, which is the site of protonation and therefore where the chloride counter ion would reside. Experiments to better understand the nature of the inclusion complex with WIN 64733 are ongoing.

HGF chlorides, present as HCl, and NaCl, did not retard dissolution of WIN 64733 when HP-β-CD was not present. Drug solubilization was higher in HGF (about 10 mg/mL) than in water (1.4 mg/mL) for the same 2-hour time period due to intrinsic gastric fluid components (Table 2). Biological fluids have been shown to increase the aqueous solubility of various compounds (10,15).

Undissolved solids from mixed samples formed liquid crystals, shown by microscopy as the maltese crosses that were seen under cross-polarized light at 630× (Figure 1). This phenomenon occurred in water, in cyclodextrin solutions, and in HGF.

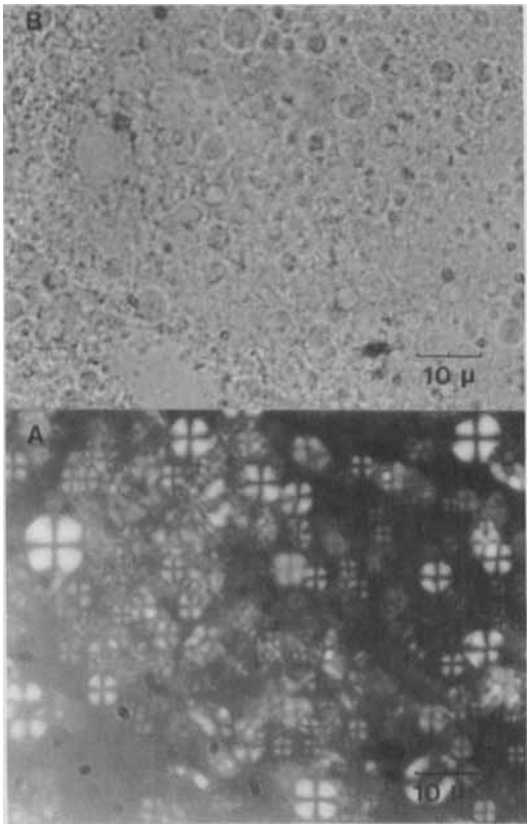

Figure 1. Liquid crystals of WIN 64733 in water, viewed at 630×: (A) identified by maltese crosses under cross-polarized light, and (B) shown as seen under ordinary light.

Characterization in Human Gastric Fluid

The HGF solubility results were obtained by HPLC with approximately 80 mg of WIN 64733 suspended in human gastric fluid for up to 2 hours at various pH's (Table 2). No decomposition peaks were seen in the HPLC chromatograms. The variation in solubility concentration is due in part to the short 2-hour mixing time, which was not long enough to achieve equilibrated solubility, and to the individual gastric fluid composition. Individual gastrointestinal fluids were previously shown to have different effects of the solubility of lipophilic compounds (12). A pH effect on solubility did not occur here and was not expected because of the HGF solutes and the short duration, but 2 hours is the maximum expected gastric residence time for oral dosages.

WIN 64733 solutions did not decompose in HGF (Table 2). The drug concentration remained unchanged for 2 hours at 37°C and no chloride precipitation was seen. These results indicate that WIN 64733 dissolution would begin in the stomach and significant decomposition would not occur. This is favorable for absorption of an oral dose.

Table 2

WIN 64733 Solubility and Solution Stability After 2 Hours in Human Gastric Fluid

Fluid pH	Solubility (mg/mL)	Percent WIN 64733 Remaining
1.6	11.0	99.0
2.5	9.7	99.4
6.2	10.9	98.9

Solid-State Stability

WIN 64733 was unaffected by heat (37°C and 50°C) and light over a 6-week stress period. There were no decomposition product peaks on any of the chromatograms, and both samples assayed at 100% at all temperatures. Similarly, moisture in the 75% relative humidity chambers did not chemically affect the compound.

Solution Stability

1. Kinetics of Decomposition

WIN 64733, in solution, showed first-order decomposition that was unaffected by light. At pH 2, the control and light samples were too stable to calculate breakdown values from 6 weeks of testing (Table 3). All drug solutions decomposed faster at elevated temperature and higher pH (Figure 2a, b, c, and d). At 50°C, the degradation was biphasic with a rapid initial drop in drug concentration in pH 6.6 buffer and in water (Figure 2d).

Table 3

Rate Constants k' (day^{-1}) and Half Lives $t_{1/2}$(days), Based on 6 Weeks of Data

Condition	Sample	k'(day^{-1})	$t_{1/2}$(days)
Control	pH 2.0	95% of the drug remaining	
Control	pH 4.0	0.0086	81
Control	pH 6.6	0.0217	32
Control	Water	0.0173	40
Light	pH 2.0	94% of the drug remaining	
Light	pH 4.0	0.0096	73
Light	pH 6.6	0.0318	22
Light	Water	0.0119	58
37°C	pH 2.0	0.0113	61
37°C	pH 4.0	0.0550	13
37°C	pH 6.6	0.290	2.4
37°C	Water	0.065	11
50°C	pH 2.0	0.0381	18
50°C	pH 4.0	0.123	5.7
50°C	pH 6.6	0.25	2.8
50°C	Water	0.18	3.8

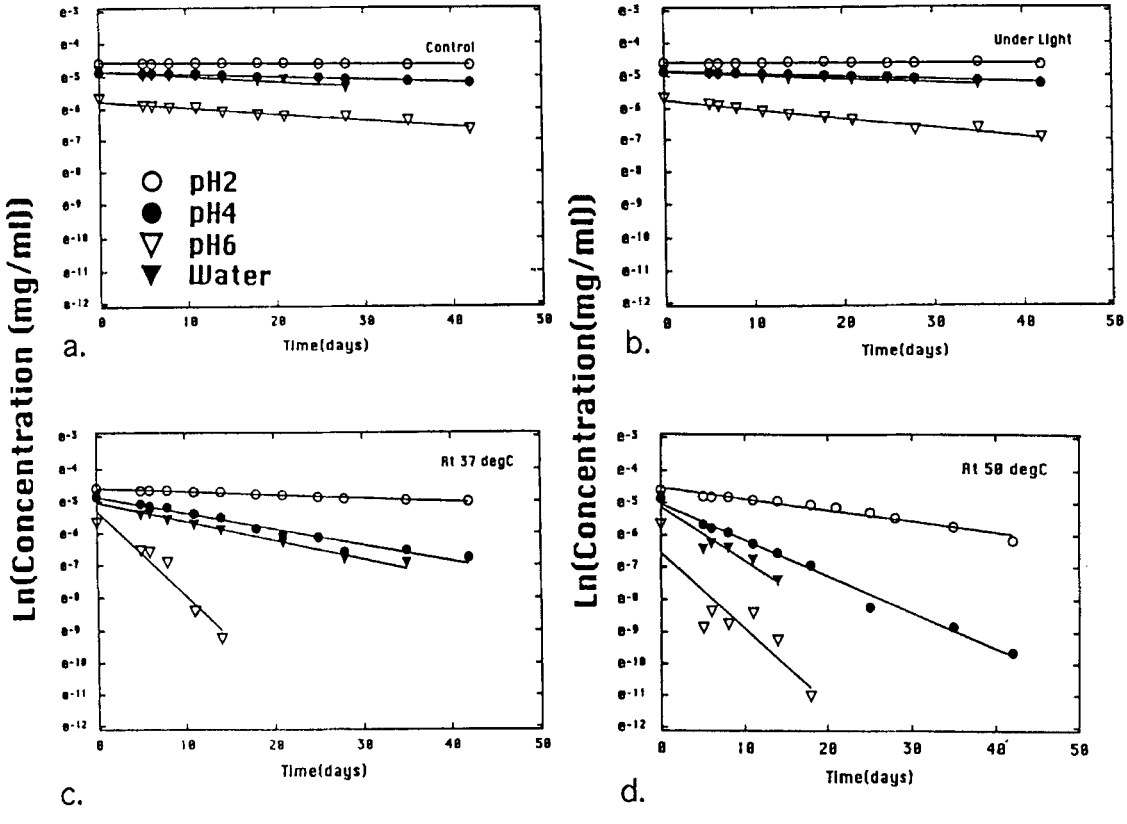

Figure 2. WIN 64733 concentration versus time (days): (a) in the dark at room temperature, (b) exposed to light at room temperature, (c) at 37°C, and (d) at 50°C.

The k' and $t_{1/2}$ values are based on a best-fit line. All other conditions resulted in first-order decomposition.

2. pH/Rate Decomposition Profile

The values were calculated using a general form for a pH-dependent rate equation, $y = mx + b$ where, y = log k (degradation), m = slope, x = pH, and b = y-intercept (Table 4).

The slope can provide insight to the reaction mechanism. For example, in simple ester hydrolysis, one may find "bell-shaped" pH/rate profile with slopes of -1, 0, and +1. The portion of the profile with slope -1 implies the presence of a single mechanism involving specific acid catalysis, where H_3O+ functions as the proton donor. Where the slope equals 0, the major degradation pathway is due to spontaneous hydrolysis, i.e., water acts as the nucleophile and there is no net flux of protons in the transition state. The portion of the profile with slope +1 involves loss of a proton to hydroxide ion or direct attack of the hydroxide ion in the transition state.

If the slopes for the decomposition of WIN 64733 were closer to +1, specific-base catalysis might be involved in the rate-limiting step. The observation of decreased solubility with increasing pH may make the apparent rate (at higher pH) seem lower, due to solubility becoming partially rate limiting. In this case, the similarity of the slopes (ranging from 0.16 to 0.38) suggests a common decomposition pathway for this set of conditions.

3. Formaldehyde Assay

Absorbance at 420 nm was proportional to the intensity of the yellow color that developed in the presence of formaldehyde, and linear with its concentration in the standard series (r^2 = 1.000).

Table 4

pH/Rate Profile Data Determined by the Linear Equation $y = mx + b$

Condition	y-Intercept	Slope	r^2
37°C	-2.66	0.352	1.0
50°C (overall)	-1.79	0.204	0.98
50°C (initial)	-2.08	0.311	0.99
50°C (final)	-1.66	0.156	0.88
Light	-3.67	0.376	0.97
Control	-3.24	0.27	0.98

Table 5

Formaldehyde Concentrations at 50°C, Formed at pH's 2.0, 4.0, and 6.0

Station pH	Formaldehyde Concentration (mg/mL)
2.0	1.62
4.0	2.16
6.0	0.93
Water	1.74

The control samples exhibited absorbance, equivalent to the blank. The 50°C samples formed formaldehyde during decomposition (Table 5).

Acetylacetone and formaldehyde (from WIN 64733 decomposition) form diacetyldihydrolutidine in the presence of excess ammonium salt (11). The overall reaction is quantitative at pH 5.5-6.5, and a proposed reaction mechanism is shown in Figure 3.

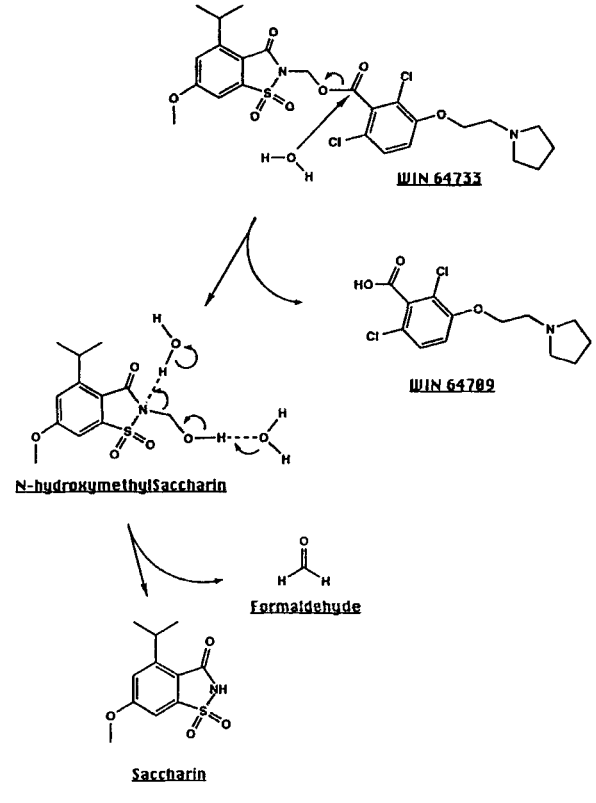

Figure 3. Proposed mechanism for formaldehyde release during degradation of WIN 64733, as determined by the Hantzsch reaction.

CONCLUSIONS

WIN 64733 is a stable solid, resistant to the stresses of moisture, light, and heat in combination, or individually. The aqueous and human gastric fluid solubilities imply good dissolution of the drug in vivo. Because of the lack of decomposition in gastric fluid, no enteric protection would be required for a solid dosage. In fact, the drug solubilities in the acid solutions indicate that dissolution would begin in the stomach and would not be retarded by the chloride content. This is favorable for absorption.

At pH's higher than 4, WIN 64733 is unstable in solution and susceptible to light-independent hydrolysis. The decomposition rate in solution is increased by heat and higher pH. A stable solution would be difficult to formulate at a tolerable pH. Aqueous solutions of the drug precipitate in the presence of chlorides (0.1 N HCl and 0.9% NaCl). In contrast, WIN 64733 dissolution in human gastric fluid is 10-fold that in water, and aqueous solutions do not precipitate in human gastric fluid. WIN 64733 in HP-β-CD solutions is unaffected by chlorides but the aqueous solubility is reduced by HP-β-CD. A stable lyophilized preparation with cyclodextrin might be possible for IV administration. The formulation would be reconstituted with normal saline just before use.

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