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Aqueous Stability of SB 210661: Kinetics and Primary Degradation Mechanisms of an *N*-Hydroxyurea-containing 5-Lipoxygenase Inhibitor

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Abstract

SB 210661, (*S*)-*N*-hydroxy-*N*-[2,3-dihydro-6-(2,6-difluorophenylmethoxy)-3-benzofuranyl]urea, is a potent and selective inhibitor of 5-lipoxygenase. Its aqueous stability was primarily evaluated to support development of analytical methods and formulations. The results also add to the growing database on the stability of *N*-hydroxyurea compounds.

Comparison of the stability of SB 210661 with that of two other *N*-hydroxyurea-containing compounds, zileuton and Abbott-79175, supported a common primary degradative pathway at pH > 5 and different degradative pathways at pH < 5. The pathway at pH > 5 is consistent with the hydrolysis of the *N*-hydroxyurea group, whereas for SB 210661, the pathway at pH < 5 is consistent with specific acid-catalysed nucleophilic displacement of the *N*-hydroxyurea group by water.

Leukotrienes have been implicated in the pathogenesis of several inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, asthma, and allergic rhinitis (Chabot-Fletcher et al 1995). A key step in the formation of leukotrienes from arachidonic acid is mediated by the enzyme, 5-lipoxygenase (Rouzer & Samuelsson 1985). 5-Lipoxygenase is responsible for converting arachidonic acid to 5-hydroperoxyeicosatetraenoic acid, a reactive intermediate, that is converted to epoxide leukotriene A_4 (LTA_4) (Rouzer et al 1986). LTA_4 is the pivotal intermediate from which all other leukotrienes are formed. Direct inhibition of 5-lipoxygenase has been achieved with chemically diverse compounds including benzofurans, hydroxamates, indazolidinones and *N*-hydroxyurea derivatives (Chung 1995).

SB 210661 (Figure 1) is a novel bicyclic *N*-hydroxyurea 5-lipoxygenase inhibitor. The selectivity and potency of the 5-lipoxygenase inhibitory activity of the defluorinated analogue of SB 210661 (i.e. SB 202235) has been reported by Chabot-Fletcher et al (1995) and Adams et al (1996).

Previous reports have detailed the pH-dependent kinetics and mechanisms of degradation of the *N*-hydroxyurea-containing 5-lipoxygenase inhibitors, zileuton (Alvarez & Slade 1992; Chang et al 1995) and Abbott-79175 (Trivedi & Fort 1995). Structures of both molecules are shown in Figure 2. This report will add to the growing database on the stability of these analogues, as a result of evaluation of the stability of SB 210661. Similarities and differences among these compounds will be highlighted and discussed.

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Materials and Methods

Materials

SB 210661 and its hydroxylamine derivative 2,3-dihydro-*N*-hydroxy-6-(2,6-difluorophenylmethoxy)-3-benzofuranamine, were synthesized by the Chemical Development Department at SmithKline Beecham. Two lots of SB 210661 (AJK-19080-146 and LMT-19212-57) were used in this study. The purity of both lots, as determined by high-performance liquid chromatography (HPLC), was greater than 99·4%, and the purity of the hydroxylamine derivative (Lot #LL-19179-185) as determined by HPLC was greater than 95%. Purified water was from a Milli-Q system (Millipore, Milford, MA), methanol and acetonitrile were HPLC grade and other chemicals were ACS reagent grade.

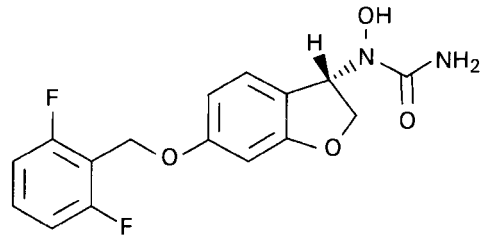
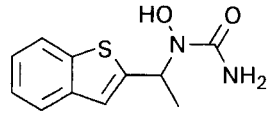
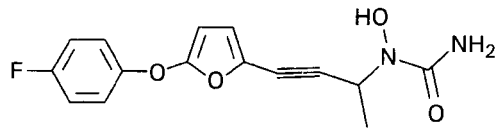


Figure 1. Chemical structure of SB 210661 ((*S*)-*N*-hydroxy-*N*-[2,3-dihydro-6-(2,6-difluorophenylmethoxy)-3-benzofuranyl]-urea; $C_{16}H_{14}F_2N_2O_4$).



zileuton



Abbott-79175

Figure 2. Chemical structures of zileuton and Abbott-79175.

HPLC assay

SB 210661 and its primary degradation products were monitored by HPLC analysis performed with a Shimadzu (Columbia, MD) SIL-6A system comprising gradient pumping system, column oven, system controller, autosampler with 100-μL sample loop, and variable-wavelength UV detector operating at 226 nm. Separations were performed on a 4·6 mm × 25 cm, 5 μm particle size Cosmosil 5C18-AR column (Phenomenex, Torrance, CA) maintained at 40°C. The injection volume was 15 μL and the mobile phase was an acetonitrile–water gradient; the flow rate was 1 mL min^{-1}. During analysis the amount of acetonitrile in the mobile phase was linearly increased from 34% to 90% in 15 min, held at 90% acetonitrile for 5 min, then re-equilibrated at 34% acetonitrile for 10 min before the next injection. SB 210661 was initially quantitated by comparison with a standard solution the concentration of which was in the linear range of the detector response. The SB 210661 peak and the two primary degradation product peaks were clearly resolved from secondary degradation products (Figure 3).

pH Measurement

Solution pH values were measured with an Orion (Boston, MA) Model 920 pH meter calibrated daily before use by use of two buffers (Baxter Diagnostics, Deerfield, IL) whose pH values encompassed those of the samples (except $0 < pH < 2$).

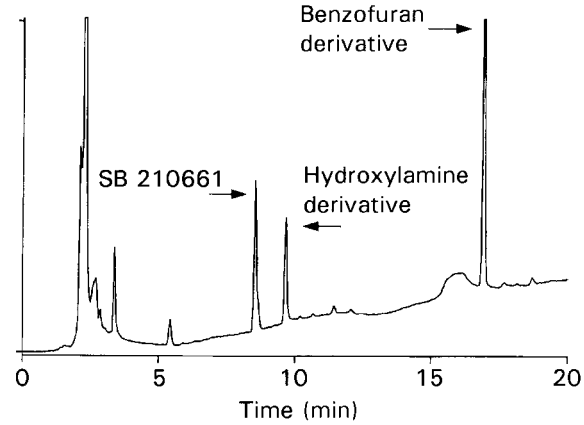


Figure 3. Chromatogram of a sample containing SB 210661 and the two primary degradants to show their relative retention using the described HPLC method. The sample was prepared by adding the hydroxylamine derivative to a degraded solution of SB 210661 (pH 2; 25°C for 5 h).

pK_a Determination

The pK_a of SB 210661 was determined spectrophotometrically. Thirteen buffers, ranging in pH between 8 and 12, of ionic strength 0·15 M, were prepared by mixing a 50 mM phosphate buffer (pH 8·0, ionic strength 0·15 M) with 0·01 M NaOH, (ionic strength 0·15 M). Samples (1 mL) of a solution (14·75 μg mL^{-1}) of SB 210661 in methanol were diluted to 50 mL with each buffer. The absorbance of these solutions at 220, 225, 230, and 240 nm was measured by means of an HP8452A UV spectrophotometer (Hewlett-Packard, Palo Alto, CA). A single measurement was made for each solution, resulting in a total run time of approximately 1 h. The absorbances were used in standard calculations, described by Albert & Serjeant (1971), to obtain the apparent pK_a which was uncorrected for activity coefficients.

Kinetics experiments

To study the effect of pH on the solution stability of SB 210661, samples of SB 210661 in various solvent systems (Table 1) were prepared at 10 μg mL^{-1} by adding 100 μL of a 5 mg mL^{-1} methanolic solution to 50-mL volumetric flasks containing each solution. Samples with pH values ranging from 3·0 to 12·0 were transferred to ampoules; these were flame-sealed and placed in a storage chamber at 25 ± 0·5°C. For samples with pH values ≤ 2·0, the volumetric flasks were placed in a temperature-controlled water bath set at 25 ± 0·5°C.

The effect of buffer concentration on the rate of degradation of SB 210661 was studied using acetate buffers of pH 4·2, 4·6 and 5·1. Buffer solutions were prepared at 25 mM, 50 mM, 75 mM and 100 mM while keeping the ionic strength at 0·15 M.

Table 1. Effect of pH on the degradation rate constants for SB 210661 at 25°C and ionic strength 0·15 M.

pH	pH-Maintaining species	Degradation rate constant (h^{-1})	Standard deviation of rate constant	r^2 Value of first-order plot
1·0	HCl-NaCl	$2·06 \times 10^{-1}$	$8·17 \times 10^{-4}$	0·9999
2·0	HCl-NaCl	$2·08 \times 10^{-2}$	$1·73 \times 10^{-4}$	0·9995
3·0	HCl-NaCl	$2·26 \times 10^{-3}$	$1·90 \times 10^{-5}$	0·9998
4·2	Acetate	$3·29 \times 10^{-4}$	$2·65 \times 10^{-6}$	0·9996
4·6	Acetate	$2·25 \times 10^{-4}$	$2·85 \times 10^{-6}$	0·9990
5·1	Acetate	$1·88 \times 10^{-4}$	$2·56 \times 10^{-6}$	0·9989
6·0	Phosphate	$1·72 \times 10^{-4}$	$4·25 \times 10^{-6}$	0·9969
8·0	Phosphate	$2·25 \times 10^{-4}$	$6·17 \times 10^{-6}$	0·9956
11·9	NaOH-NaCl	$6·42 \times 10^{-4}$	$8·79 \times 10^{-6}$	0·9989
12·8	NaOH-NaCl	$7·71 \times 10^{-4}$	$2·68 \times 10^{-5}$	0·9940

At pH 6·0 and 8·0, the pH was maintained with 50 mM phosphate buffers.

Samples were analysed initially and at time intervals appropriate for monitoring the progress of degradation. Samples in pH 6·0 and 8·0 buffers were assayed neat whereas all other samples were diluted with 500 mM pH 7·4 phosphate buffer before HPLC analysis. This was done to slow the rate of degradation to a point where analysis time was insignificant. A sample chromatogram showing the order of elution of SB 210661 and the two primary degradation products is shown in Figure 3. SB 210661 elutes at 8·9 min, the hydroxylamine derivative at 9·8 min, and the benzofuran derivative at 17·1 min.

The initial concentration of each sample was verified by use of an external standard prepared at the target concentration (10 $\mu g\,mL^{-1}$). Degradation was monitored by recording the peak area of SB 210661 as a percentage of the initial area. The natural log of this value was plotted against time then regression analysis was performed. The slope of the regression line defined the rate constant at each pH (Table 1). The log of the degradation rate was plotted against pH to obtain a pH-rate constant profile (Figure 4).

Extraction of comparative data for zileuton and Abbott-79175

The pH–k_{obs} profile for zileuton was constructed using acid- and water-catalysed rate constants predicted for 25°C (Alvarez & Slade 1992). The comparable profile for Abbott-79175 was generated using the Arrhenius relationship and the temperature-dependence of the observed rate constants reported by Trivedi & Fort (1995). The associated rate constants were calculated using equation 2 and assuming a pK_a value of 10·5, consistent with the spectrophotometrically generated values of 10·46 and 10·51 for SB 210661 and zileuton (Alvarez & Slade 1992), respectively.

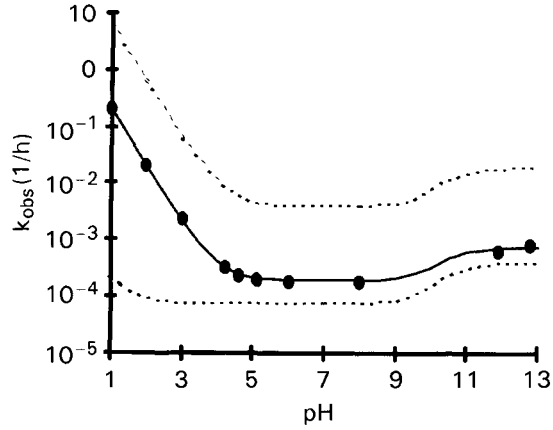


Figure 4. Plots of the observed rate constant against pH for Abbott-79175 (top), SB 210661 (middle), and zileuton (bottom) at 25°C and $\mu = 0·15$ M.

Reactivity of the hydroxylamine derivative

The stability of the hydroxylamine derivative (the final product in Figure 5) was studied at pH 2·0. A sample was prepared by adding a methanolic solution (5 $mg\,mL^{-1}$; 200 μL) to hydrochloric acid (0·01 M; ionic strength 0·15 M; 100 mL). Samples were analysed and the degradation rate determined as already described.

Results and Discussion

Examination of the pH–k_{obs} profile

Under all the conditions studied the degradation of SB 210661 was observed to be a pseudo-first-order process (see r^2 values in Table 1). The reactions were typically followed for one to three half-lives, for a minimum of seven samples. The acetate buffers used to maintain pH between 4·2 and 5·1 had no apparent catalytic effect on the degradation rate. Buffer effects were not studied at pH 6·0 and 8·0 because previous studies (Alvarez & Slade 1992) have shown that buffer catalysis is modest at elevated temperatures and even less significant at lower temperatures. For example, in carbonate

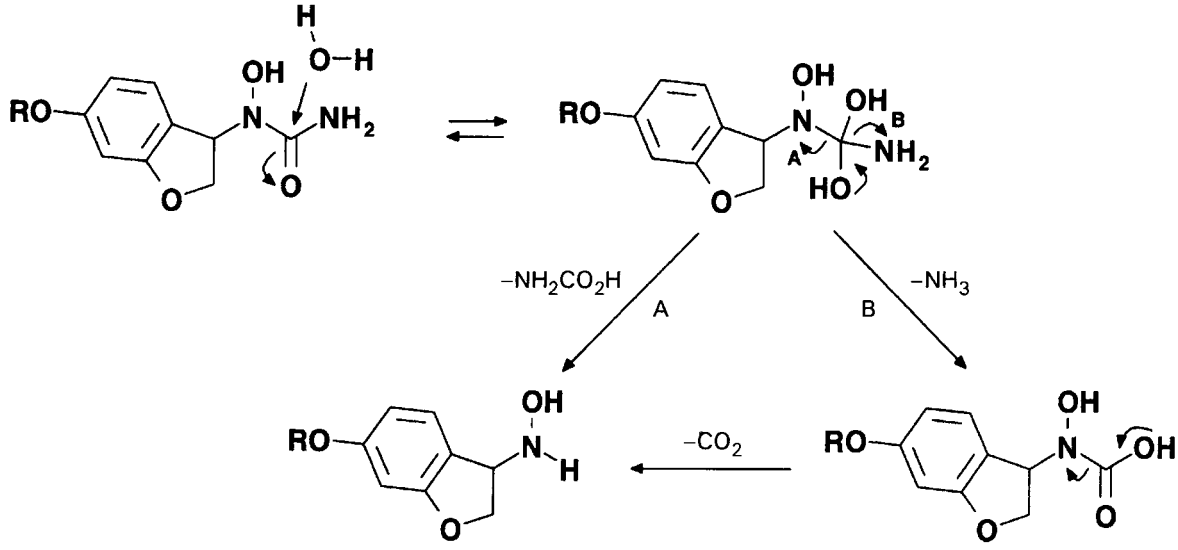


Figure 5. Postulated mechanism for the hydrolysis of SB 210661 at pH > 5.

buffer of pH 10·03, the buffer-dependent rate constant for zileuton was 0·38 and 0·06 $M^{-1} h^{-1}$ at 82 and 61°C, respectively.

The dependence of the observed degradation rate constant, k_{obs}, on pH at 25°C and ionic strength 0·15 M is shown in Table 1 and Figure 4. Figure 4 also depicts the extrapolated pH-rate constant profiles for zileuton and Abbott-79175. Over the pH range investigated, k_{obs} can be described for each compound by the relationships:

$$k_{obs} = k_H a_{H+} f_{HA} + k_0 f_{HA} + k'_0 f_{A-} \quad (1)$$

or

$$k_{obs} = (k_H[a_{H+}]^2 + k_0 a_{H+} + k'_0 K_a)/(a_{H+} + K_a) \quad (2)$$

where k_H is the rate constant for the specific acid-catalysed degradation of the free acid species; k_0 and k'_0 are the rate constants for the water-catalysed hydrolysis of the free acid and anionic species, respectively; a_{H+} is the hydrogen-ion activity; K_a is the ionization constant; and f_{HA} and f_{A-} are the fractions of the given compound present in the free acid and anionic forms, respectively. The theoretical profiles in Figure 4 were constructed with equation 2 and the kinetic and thermodynamic parameters listed in Table 2.

Mechanisms of degradation

Comparison of the pH-dependent chemical reactivities of SB 210661, zileuton, and Abbott-79175 at 25°C reveals that the shapes of the three profiles are comparable in the pH region dominated by the k_0 and k'_0 terms (i.e., pH > 5), implying the involvement of a common degradative mechanism and hence implicating the reactivity of the common functionality, the *N*-hydroxyurea group. In contrast, the shapes of the profiles in the pH region dominated by the k_H term (i.e. pH < 5) are similar for SB

Table 2. Comparison of the degradative rate constants and pK_a values at 25°C for SB 210661, Abbott-79175 and zileuton.

	SB 210661	Abbott-79175*	Zileuton†
Rate constant for specific acid-catalysed degradation of the free acid species	2·06 (±0·00)	60·6 (±0·8)	$1·63 \times 10^{-3}$
Rate constant for water-catalysed hydrolysis of the free acid	$1·93 (\pm 0·15) \times 10^{-4}$	$4·05 (\pm 3·58) \times 10^{-3}$	$7·66 \times 10^{-5}$
Rate constant for water-catalysed hydrolysis of the anionic species	$7·29 (\pm 0·29) \times 10^{-4}$	$1·98 (\pm 0·66) \times 10^{-2}$	$4·18 \times 10^{-4}$
pK_a	10·46	—	10·51

* The rate constants were determined by curve-fitting the pH-dependent rate constants; these were extrapolated to 25°C by use of the Arrhenius relationship, from the work by Trivedi & Fort (1995) assuming a pK_a of 10·5 at 25°C. † The rate constants and pK_a value were determined by Alvarez & Slade (1992).

210661 and Abbott-79175 but different for zileuton. This supports the mechanistic involvement of a group other than the *N*-hydroxyurea group for at least one of the compounds.

The primary degradation reaction for all three compounds at pH > 5 is hydrolysis of the group that all three have in common, the *N*-hydroxyurea group. Two kinetically equivalent pathways can account for the *N*-hydroxylamine product. The first step of both pathways involves nucleophilic attack by water at the carbonyl carbon to form the corresponding tetrahedral intermediate. As shown in Figure 5, the tetrahedral intermediate can degrade via two pathways, decomposition to form either the *N*-hydroxylamine product or the *N*-carboxylic acid intermediate. As described previously by Alvarez & Slade (1992), the *N*-carboxylic acid would be a short-lived intermediate which would rapidly decompose to form the *N*-hydroxylamine derivative. The considerably faster rate of degradation of Abbott-79175, compared with zileuton, has been attributed to differences between the inductive effects of the substituents attached to the *N*-hydroxyurea group (Trivedi & Fort 1995). Using a similar argument, this suggests that the inductive effects arising from the substituents attached to the *N*-hydroxyurea group of SB 210661 more closely approximate the effects seen with zileuton where the corresponding rate constants differ by a factor of 2·5 (i.e. the k_0 term) and a factor of 1·7 (i.e. the k'_0 term).

The primary degradation reaction at pH < 5 differs for the three compounds. For zileuton, hydrolysis of the *N*-hydroxyurea group, to form the *N*-hydroxylamine derivative (Alvarez & Slade 1992), is still the primary degradation reaction, whereas for Abbott-79175, hydrolysis of the furan group (Trivedi & Fort 1995) is the primary degradation reaction. For SB 210661, HPLC-electrospray ionization mass spectrometric analysis of degraded samples suggests that the primary degradation product formed under acidic conditions is the benzofuran derivative. An authentic sample, obtained from the Synthetic Chemistry Department of SmithKline Beecham, was added to a degraded sample and analysed by HPLC to confirm the identity. A mechanism consistent with formation of the benzofuran derivative is shown in Figure 6. It involves nucleophilic attack of water at the substituted ring carbon, leading to expulsion of the *N*-hydroxyurea group and to formation of the corresponding 3-hydroxybenzofuran derivative. Because this derivative is not detected during the course of the degradation reaction, it would be a reactive intermediate which rapidly dehydrates to form the corresponding benzofuran derivative. The acid-catalysed dehydration of several other 3-hydroxy-substituted benzofurans has been well documented (Bisagni et al 1975; Ghosh et al 1989; Kraus et al 1990; Teo et al 1992).

Additionally, a similar dehydration reaction has been postulated to occur with zileuton under very

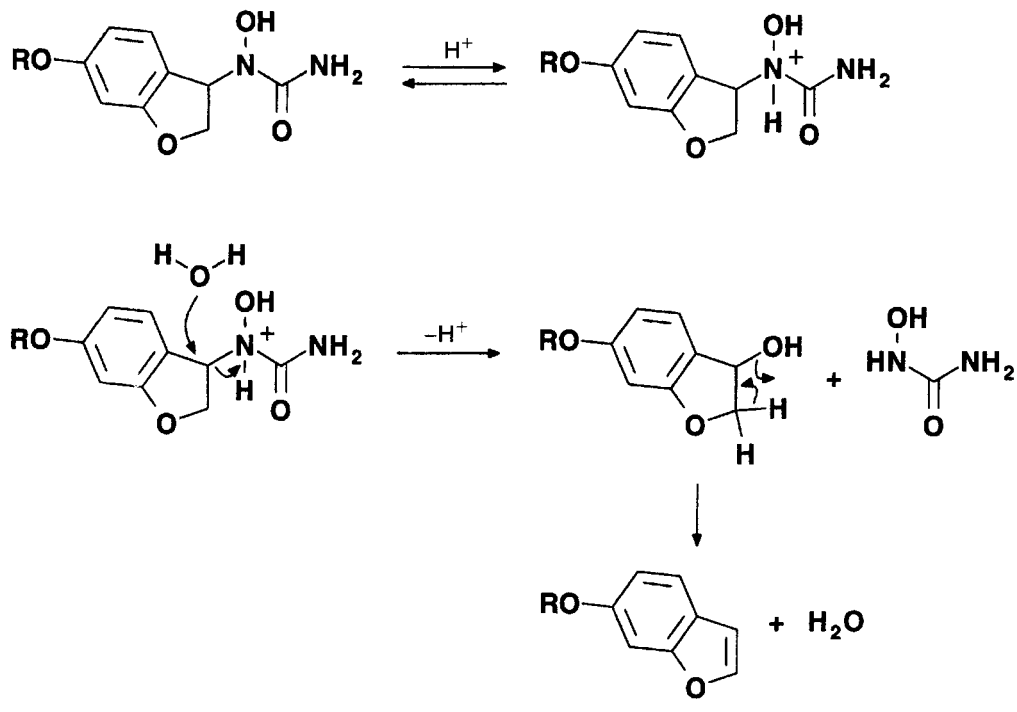


Figure 6. Postulated mechanism for the degradation of SB 210661 at pH < 5.

acidic conditions (pH < 2). However, unlike SB 210661, the hydroxylated species is detectable for zileuton. The greater reactivity of the hydroxylated species of SB 210661 might be related to the energetically favourable aromatic nature of the benzofuran product (Dean & Sargent 1984).

Another mechanistic distinction between the two compounds is that for zileuton the hydroxylated species appears to form from the hydroxylamine derivative, whereas for SB 210661 it does not. The hydroxylamine derivative of SB 210661 is stable at pH 2 over the time frame when SB 210661 is completely converted to the benzofuran derivative.

Conclusions

The aqueous stability of SB 210661 was investigated to support the development of a pharmaceutical formulation. The results were evaluated and compared with those of two other 5-lipoxygenase inhibitors, zileuton and Abbott-79175. The three compounds degrade in the same way at pH > 5, by hydrolysis of the *N*-hydroxyurea group. However, under acidic conditions all the compounds degrade differently. For zileuton, the reaction still involves *N*-hydroxyurea hydrolysis whereas for SB 210661 and Abbott-79175 the reactions involve more chemically labile groups.

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