Systematic Study on the Chemical Stability of the Prodrug Antitumor Agent Carzelesin (U-80,244)

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Abstract □ The chemical stability of the novel anticancer agent carzelesin in aqueous buffer/acetonitrile (1:1, v/v) mixtures has been investigated utilizing a stability-indicating reversed-phase high-performance liquid chromatographic assay. The degradation kinetics of carzelesin has been studied as a function of pH, buffer composition, ionic strength, and temperature. Degradation of carzelesin follows (pseudo-) first-order kinetics. A pH–rate profile, using rate constants extrapolated to zero buffer concentration, was constructed demonstrating that carzelesin is most stable in the pH region 1–4. The degradation rate of carzelesin was not significantly affected by buffer components and by the ionic strength. In addition to the formation of the degradation products U-76,-073, U-76,074, and aniline in alkaline medium and in acetate buffer solution, another degradation product was formed in acetate buffer solution. In perchloric acid buffer solution (pH* < 3), U-76,073 and U-76,-074 could not be detected as degradation products.

Introduction

Carzelesin (U-80,244; NSC 619029; Figure 1) is a synthetically derived cyclopropylpyrroloindole (CPI) analog of the highly potent, alkylating, antitumor agent antibiotic CC-1065.[1] The CPI analogs are DNA minor-groove binders containing a cyclopropyl group, which mediates N^3-adenine covalent adduct formation in a sequence-selective fashion with no intercalation.[1] It has been speculated that these CPI derivatives selectively inhibit the binding of regulatory proteins to DNA, thereby modifying the transcription of specific genes that are important for neoplastic cell growth.[2] Carzelesin showed activity against a broad panel of human tumor xenografts in preclinical *in vivo* studies in mice[1] and *in vitro* in a number of gynecologic cancer cell lines.[3] Carzelesin was designed to be an inactive prodrug, requiring chemical or enzymatic activation to the DNA-reactive form.[1] Activation of carzelesin requires two steps, *i.e.*, (a) hydrolysis of the carbanilate substituent to form U-76,073 followed by (b) ring closure of the chloromethyl group to form the cyclopropyl-containing DNA-reactive U-76,074 (Figure 1).

Recently, a study[4] has been conducted dealing with the characterization and pharmaceutical formulation of carzelesin according to the "Guidelines for the formulation of investigational cytotoxic drugs", which were drawn up by the Joint Formulation Working Party of the EORTC/CRC/NCI.[5] A stable parenteral formulation of carzelesin in a polyethylene glycol 400 (PEG 400)/absolute ethanol/Tween 80 (6:3:1, v/v/v; PET formulation) solution was developed,[4] which is now being tested in phase I clinical trials.[6] During the manufacturing process of clinical batches of carzelesin in the PET formulation, observations were made indicating that the stability of the drug in this formulation was dependent upon the interbatch variability of the excipients used in the vehicle. Accelerated stability studies (at 40 °C) of carzelesin in the PET formulation showed that the variable stability characteristics were caused mainly by the pH differences between batches of PEG 400.[7] This stimulated us to investigate systematically the chemical stability of carzelesin in solutions, which can aid to further optimize the pharmaceutical formulation, and to get more insight into the degradation mechanism.

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Experimental Section

Materials—*Chemicals*—Carzelesin, U-76,073, and U-76,074 were synthesized by The Upjohn Company (Kalamazoo, MI)[8,9] and provided through the New Drug Development Office (NDDO) of the EORTC (Amsterdam, The Netherlands). Aniline was obtained from Aldrich Chemical Co. Ltd. (Axel, The Netherlands). All other chemicals used were of analytical grade, and deionized water was used throughout.

Buffer Solutions—Due to the poor aqueous solubility of carzelesin, the kinetic studies were performed in water/acetonitrile mixtures (1:1, v/v). The aqueous components were 0.02 or 0.03 M buffer solutions, except in the experiments where the effects of buffer concentration were studied. The following buffers were used: $0 \le$ pH < 3, perchloric acid; $3 \le$ pH < 6, acetate; $6 \le$ pH < 9, phosphate; and pH ≥ 9, carbonate. The pH values of the aqueous buffer solutions were measured using an Ingold Semimicro combination pH electrode and a Model 654 pH meter (Metrohm AG, Herisau, Switzerland), which had been calibrated on fully aqueous standard solutions. The pH values of the buffer solutions were adjusted with either 0.1 M perchloric acid solution or 0.1 M sodium hydroxide solution to the appropriate pH values. Then, acetonitrile was added to the buffer solutions and the apparent pH values (called pH*) of these mixtures (except for pH* 0, which was calculated), at the study temperature, were measured using the same electrode system.

A constant ionic strength (μ) of 0.3 was maintained for each solution by addition of the appropriate amount of sodium chloride, except in experiments where the effect of the ionic strength on the degradation of carzelesin was investigated and where the OH^- and H^+ concentrations were higher than 0.3 M.

Degradation Kinetics—*Kinetic Measurements*—The kinetic studies were carried out over at least 3 half-lives in the dark (in order to prevent possible photolytic degradation), and the temperature of the study was 25 °C in the pH* range 6−10, 60 °C in the pH* range 1.5−5.5, and 70 °C at pH* 0. The reaction was initiated by addition of 100 μL of a solution of carzelesin in absolute ethanol (1 mg/mL) to 5.0 mL of preheated buffer/acetonitrile solution (1:1, v/v) to give an initial concentration of approximately 20 μg/mL (2.7×10^{-5} M). The reaction solutions were kept in screw-capped brown glass vials in a thermostatically controlled water bath. At appropriate time intervals 200-μL samples were withdrawn and directly analyzed for undegraded carzelesin and the degradation products U-76,073 and U-76,074, by a stability-indicating high-performance liquid chromatography (HPLC) assay. All kinetic studies were performed in duplicate.

Influence of Temperature—The effect of temperature on the degradation of carzelesin was studied at pH* 1.5 (0.1 M perchloric

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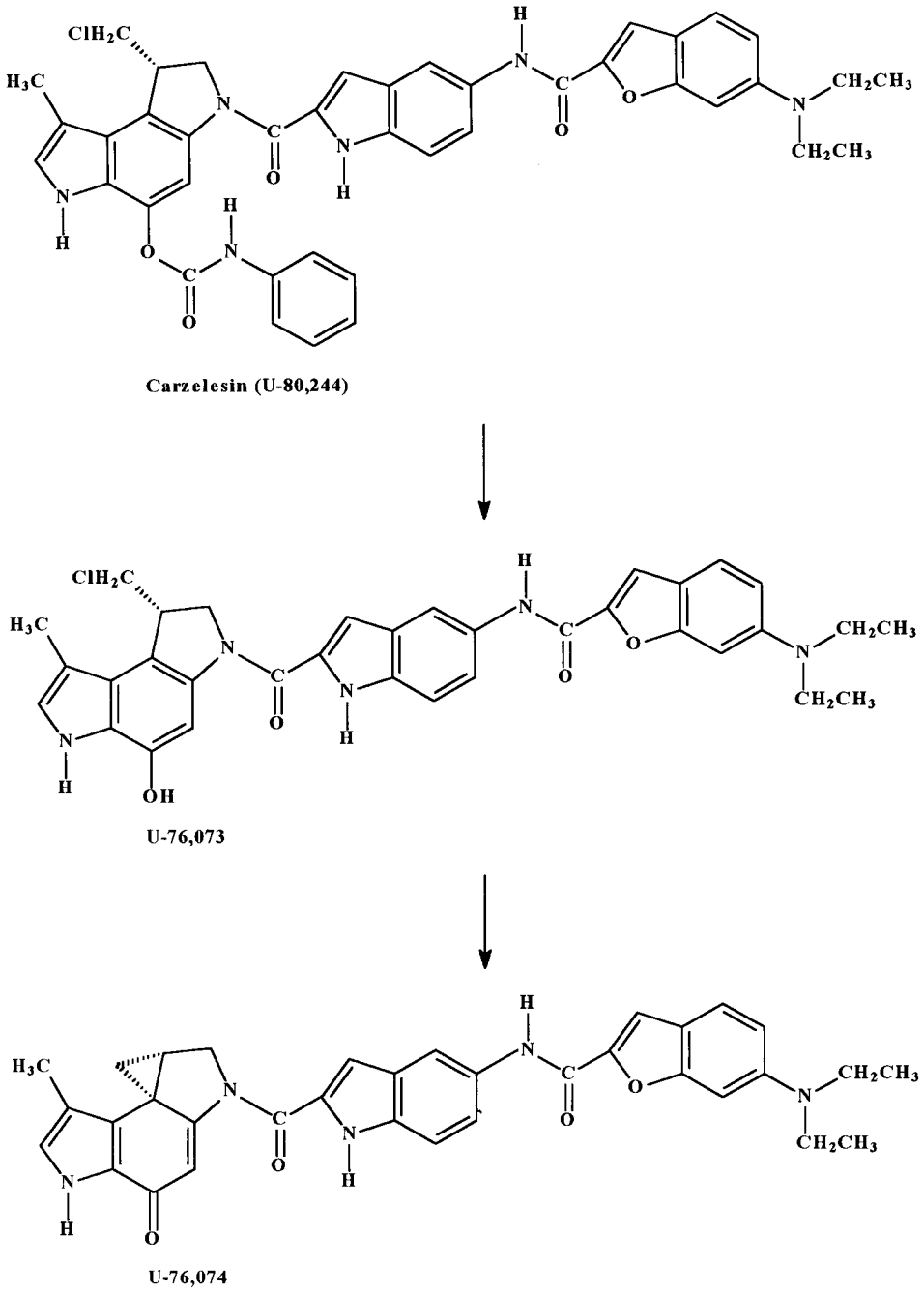

Figure 1—Chemical structures of carzelesin and the major degradation products U-76,073 and U-76,074.

acid solution; μ = 0.3) in the temperature range 60–80 °C and at pH* 7.2 (0.02 M phosphate buffer; μ = 0.3) between 25 and 45 °C.

Analytical Procedures—*HPLC*—The liquid chromatographic system consisted of a Model 510 HPLC pump, a Model 441 absorbance detector (both from Millipore Waters Chromatography, Milford, MA), a Model ISS-100 autosampler (Perkin-Elmer, Uberlingen, Germany), and a Model SP 4270 integrator (Thermo Separation Products (TSP), San Jose, CA). Ultraviolet detection at 254 nm was used. A μ Bondapak C-18 analytical column (300 × 3.9 mm i.d.; particle size 10 μm; Millipore Waters Chromatography) was used. The mobile phase consisted of 2 mM phosphate buffer pH 6.5/acetonitrile (25:65, v/v). The flow rate was 1.0 mL/min, and the injection volume was 20 μL. Chromatography was performed at room temperature. Quantitation of carzelesin, U-76,073, and U-76,074 was based on peak area measurements using a Model SP 4270 integrator (TSP). Chromatographic data processing was performed with the WINner/286 data system (TSP).

Calibration curves of carzelesin and the degradation products in the mobile phase were linear (r > 0.990) in the concentration range of interest.

Ultraviolet/Visible (UV/Vis) Spectrophotometry—UV/vis absorption spectra of carzelesin and degradation mixtures were recorded with a Model GBC 918 UV/vis spectrophotometer (GBC Scientific Equipment Ltd., Victoria, Australia). The 1-cm quartz cells were kept in a thermostatically controlled cell compartment at 25 or 40 °C. Degradation reactions were initiated by addition of 40 μL of a solution of carzelesin in absolute ethanol (1 mg/mL) to 3.0 mL of preheated buffer/acetonitrile solution (1:1, v/v) to give an initial concentration of approximately 13 μg/mL (1.8×10^{-5} M).

In addition, UV/vis absorbance spectra of reference standards of the degradation products were recorded for identification purposes.

Identification of Degradation Products—For the confirmation of the identity of the degradation products, U-76,073 and U-76,074, and for recording spectra of other degradation products, a Model Waters 996

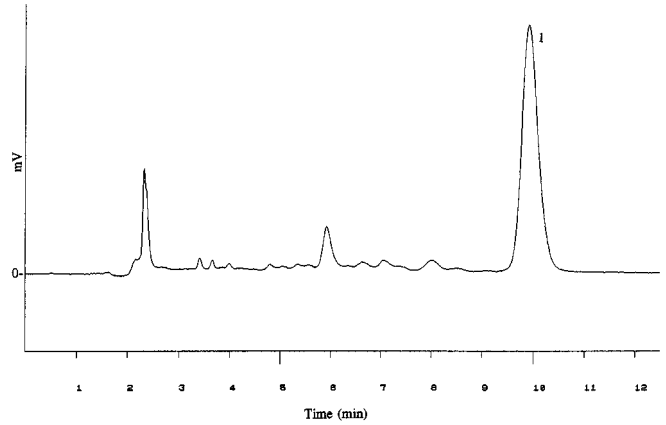

Figure 2—HPLC chromatogram of partly degraded ($t = t_{1/2}$) carzelesin in acidic medium (0.1 M perchloric acid buffer solution; pH* 1.5; $\mu = 0.3$). Peak 1: carzelesin.

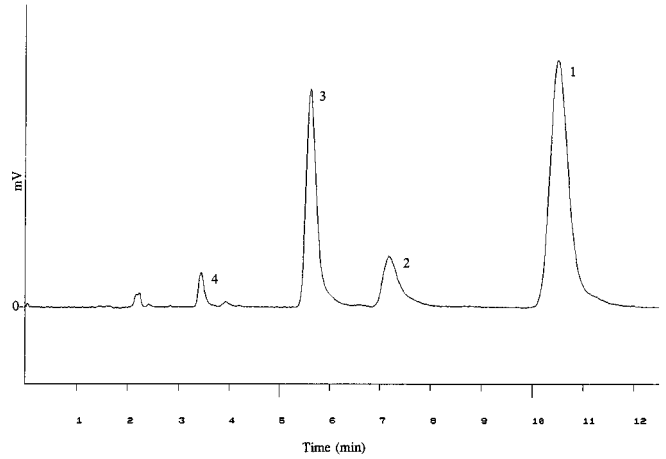

Figure 3—HPLC chromatogram of partly degraded ($t = t_{1/2}$) carzelesin in alkaline medium (0.03 M phosphate buffer; pH* 7.2; $\mu = 0.3$). Peak 1: carzelesin. Peak 2: U-76,073. Peak 3: U-76,074. Peak 4: aniline.

photodiode array (PDA) detector (Millipore Waters Chromatography) was used on-line with the HPLC system.

Results and Discussion

Analytical Procedures—Carzelesin is poorly soluble in water (1−2 μg/mL). Furthermore, carzelesin, dissolved in aqueous solution, tends to adsorb to container materials. This problem could be solved by performing the degradation studies in aqueous buffer/acetonitrile mixtures. The optimal buffer/acetonitrile ratio, in terms of carzelesin solubility, adsorption prevention, and buffer capacity requirements, was 1:1 (v/v).

UV/vis spectrophotometry measurements showed similar spectra for carzelesin and its degradation products, thus indicating that this is not an adequate technique to study the degradation reactions. A stability-indicating HPLC method was, therefore, used to resolve the degradation products from the parent carzelesin. Typical HPLC chromatograms of the degradation mixtures of carzelesin in acidic and alkaline media are depicted in Figures 2 and 3, respectively.

Degradation Kinetics—*Order of Reactions*—The degradation of carzelesin in acidic as well as alkaline media displays (pseudo-) first-order kinetics over several half-lives. The observed (pseudo-) first-order rate constant (k_{obs}) for the overall degradation rate was calculated by linear regression analysis of a plot of the natural logarithm of residual carzelesin concentration versus time (eq 1),

$$\ln[\text{carzelesin}]_t = \ln[\text{carzelesin}]_0 - k_{obs}t \qquad (1)$$

where $[\text{carzelesin}]_t$ and $[\text{carzelesin}]_0$ are the concentration of carzelesin at time t and the initial concentration, respectively. Figure 4 shows first-order plots of carzelesin at several pH* values. Figure 5 shows a typical degradation curve of carzelesin and the subsequent appearance curves of the degradation products U-76,073 and U-76,074. After hydrolysis of the carbanilate ring moiety, carzelesin was converted into U-76,-073, which was then, after ring closure, transformed into U-76,074. The formation of U-76,074 was characterized by a lag time, which is in accordance with the proposed mechanism where U-76,073 is formed first before subsequent transformation into U-76,074 can take place. The mass balance of carzelesin and its degradation products adds up to approximately 90%. Since the amounts of U-76,073 and U-76,-074 formed were calculated relative to the amount of carzelesin initially present at $t = 0$, the missing 10% can be explained by a slight decrease in molar absorptivities at the detection wavelength (254 nm). These observations have also been confirmed by UV/vis spectrophotometric experiments.

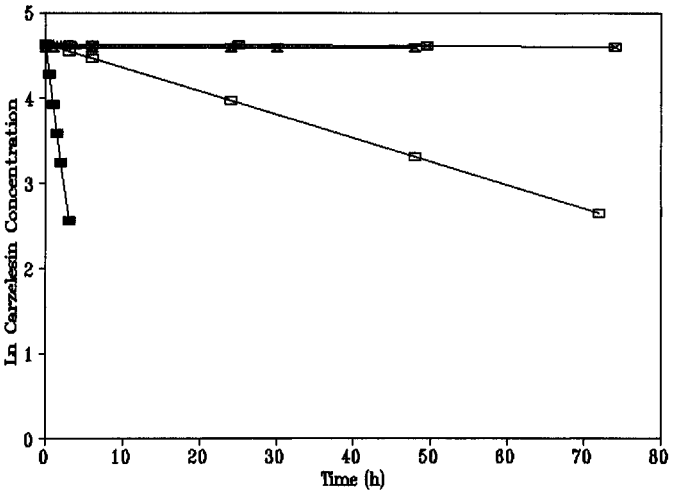

Figure 4—First-order plots of carzelesin at pH* 0.0 (hourglass symbol), 4.6 (⊠), 5.2 (▲), 7.7 (□), and 9.8 (■), respectively.

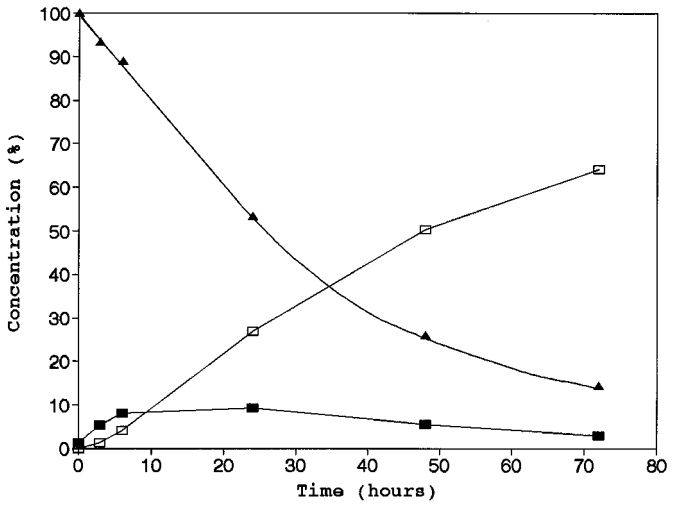

Figure 5—Typical degradation curve of carzelesin (▲) and appearance curves of U-76,073 (■) and U-76,074 (□) (concentrations have been calculated as percent of the initial carzelesin peak area at $t = 0$) at pH* 7.2 (0.03 M phosphate buffer; $\mu = 0.3$).

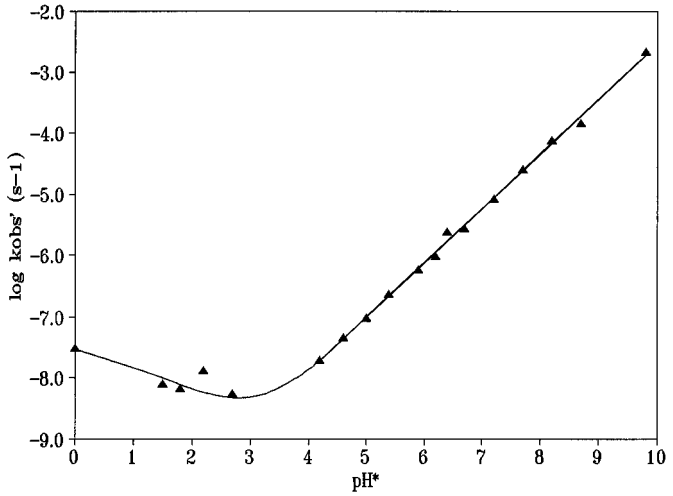

Figure 6—pH*–rate profile of carzelesin at 25 °C.

Standard Deviation in k_{obs}—The standard deviation (SD) of the overall rate constant, k_{obs}, was determined for the HPLC assay. These statistical experiments were performed at pH* 6.4 (0.03 M acetate buffer solution; $\mu = 0.3$) and 60 °C and at pH* 8.2 (0.03 M phosphate buffer solution; $\mu = 0.3$) and 25 °C, respectively. The value of k_{obs} (±SD) at pH* 6.4 was $1.5 \times 10^{-6} \pm 7.3 \times 10^{-8}$ s^{-1} ($n = 6$), and the relative SD is equal to 4.8%. The value of k_{obs} (±SD) at pH* 8.2 was $7.3 \times 10^{-5} \pm 2.2 \times 10^{-6}$ s^{-1} ($n = 4$), and the relative SD is equal to 3.0%.

Influence of pH—From previous stability experiments of carzelesin in the pharmaceutical formulation, it was concluded that the degradation of carzelesin is strongly influenced by pH.[7] Buffering is especially important for carzelesin because the ring closure step produces hydrochloric acid in addition to U-76,074. The overall rate constant (k_{obs}) for the degradation of carzelesin in buffer solutions is defined as

$$k_{obs} = k_0 + k_H[H^+] + k_{OH}[OH^-] + k_{buffer}[buffer] \quad (2)$$

where k_0 is the (pseudo-) first-order rate constant for the degradation in solvent only and k_H and k_{OH} are the second-order rate constants for proton- and hydroxyl-catalyzed degradation, respectively. The term k_{buffer}[buffer] represents the sum of the second-order rate constants for the degradation catalyzed by each of the buffer components multiplied by its concentration. For each pH the (pseudo-) first-order rate constant for [buffer] = 0, $k_{obs}' = k_0 + k_H[H^+] + k_{OH}[OH^-]$, was calculated as the intercept from the linear part of the plot of k_{obs} vs [buffer] at a fixed pH.

As the degradation of carzelesin at low pH and 25 °C was relatively slow, the kinetic experiments at low pH were performed at 60 °C in the pH* range 1.5−5.5 and at 70 °C at pH* 0. The effect of temperature on the degradation of carzelesin was studied at pH* 1.5 in the temperature range 60−80 °C and at pH* 7.2 in the temperature range 25−45 °C, respectively. The Arrhenius relationship, $\ln k_{obs} = \ln A - E_a/RT$, between the natural logarithm of the k_{obs} and the reciprocal of the absolute temperature holds. At pH* 1.5, ln A is equal to 26.49 (0.098) s^{-1} and E_a to 111.8 (6.75) kJ mol^{-1}, and at pH* 7.2, ln A equals 40.97 (0.037) s^{-1} and E_a is equal to 130.7 (2.08) kJ mol^{-1}. Since the activation energy was determined to be constant over the temperature range, these parameters were used to extrapolate k_{obs}' values obtained at 60 °C to k_{obs}' values at 25 °C for the construction of the pH*−rate profile at 25 °C (Figure 6). The profile shows distinct regions with a pH independent region between approximately 1 and 4, with a predominantly hydroxyl catalyzed part at higher pH values. Overall the degradation of carzelesin is catalyzed mainly by hydroxyl ions, which constitutes the major part of the profile. At pH* > 5, the pH*−rate profile has a slope of +0.9; this is close to unity and indicates that specific hydroxyl ion catalysis occurs.

With the use of eq 3,

$$k_{obs}' = k_0 + k_H[H^+] + k_{OH}[OH^-] \quad (3)$$

the specific rate constants for the degradation of carzelesin were calculated. The values of the specific rate constants k_H, k_0, and k_{OH} were 2.41×10^{-8} M^{-1} s^{-1}, 7.62×10^{-7} s^{-1}, and 3.50×10^{1} M^{-1} s^{-1}, respectively. The pH at which the minimum occurs in the pH−rate profile was determined

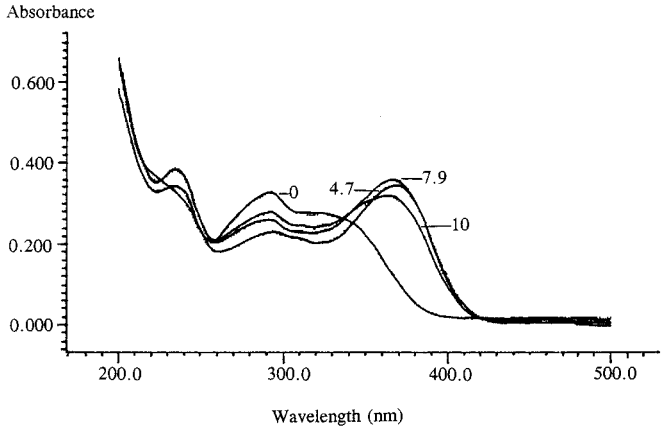

Figure 7—UV/vis spectra of carzelesin in 0.01 M buffer solutions at pH* 0, 4.7, 7.9, and 10, recorded at $t = 0$.

Table 1–Influence of Acetate, Phosphate, and Carbonate Buffer Concentration and pH on k_{obs} of the Degradation Reactions of Carzelesin at 25 °C

pH[a]	pH*[b]	[Buffer], M	k_{obs}, s^{-1}	pH[a]	pH*[b]	[Buffer], M	k_{obs}, s^{-1}
0.0	0.0	1.0	5.0×10^{-8}	5.4	6.2	0.01	1.0×10^{-6}
1.3	1.5	0.1	3.5×10^{-9}			0.03	8.1×10^{-7}
1.7	1.8	0.03	3.0×10^{-9}			0.05	6.9×10^{-7}
2.1	2.2	0.01	5.8×10^{-9}	5.9	6.7	0.01	2.7×10^{-6}
2.5	2.7	0.003	2.5×10^{-9}			0.03	2.4×10^{-6}
3.2	3.2	0.005	2.3×10^{-8}			0.05	2.3×10^{-6}
		0.01	2.2×10^{-8}	6.4	7.2	0.0025	9.0×10^{-6}
		0.03	1.9×10^{-8}			0.005	8.1×10^{-6}
		0.05	1.8×10^{-8}			0.01	7.8×10^{-6}
3.5	4.6	0.005	5.1×10^{-8}			0.02	7.8×10^{-6}
		0.01	4.4×10^{-8}			0.03	7.6×10^{-6}
		0.03	3.8×10^{-8}			0.04	7.3×10^{-6}
		0.05	3.7×10^{-8}			0.05	7.4×10^{-6}
3.9	5.0	0.005	9.9×10^{-8}	7.0	7.7	0.01	2.5×10^{-5}
		0.01	8.2×10^{-8}			0.03	2.8×10^{-5}
		0.03	7.2×10^{-8}			0.05	2.6×10^{-5}
		0.05	6.7×10^{-8}	7.5	8.2	0.005	6.1×10^{-5}
4.2	5.4	0.005	2.4×10^{-7}			0.01	7.8×10^{-5}
		0.01	2.1×10^{-7}			0.03	8.3×10^{-5}
		0.03	2.0×10^{-7}			0.05	8.3×10^{-5}
		0.05	1.9×10^{-7}	8.0	8.7	0.01	1.3×10^{-4}
4.7	5.9	0.005	5.6×10^{-7}			0.03	1.9×10^{-4}
		0.01	5.8×10^{-7}			0.05	2.2×10^{-4}
		0.03	5.6×10^{-7}	8.5	9.8	0.005	3.7×10^{-4}
		0.05	5.5×10^{-7}			0.01	1.1×10^{-3}
5.2	6.4	0.005	2.6×10^{-6}			0.03	2.7×10^{-3}
		0.01	2.2×10^{-6}			0.05	3.1×10^{-3}
		0.03	1.6×10^{-6}				
		0.05	1.6×10^{-6}				

[a] pH measured in the aqueous buffer solution. [b] pH* is the pH in the buffer/acetonitrile (1:1, v/v) mixture.

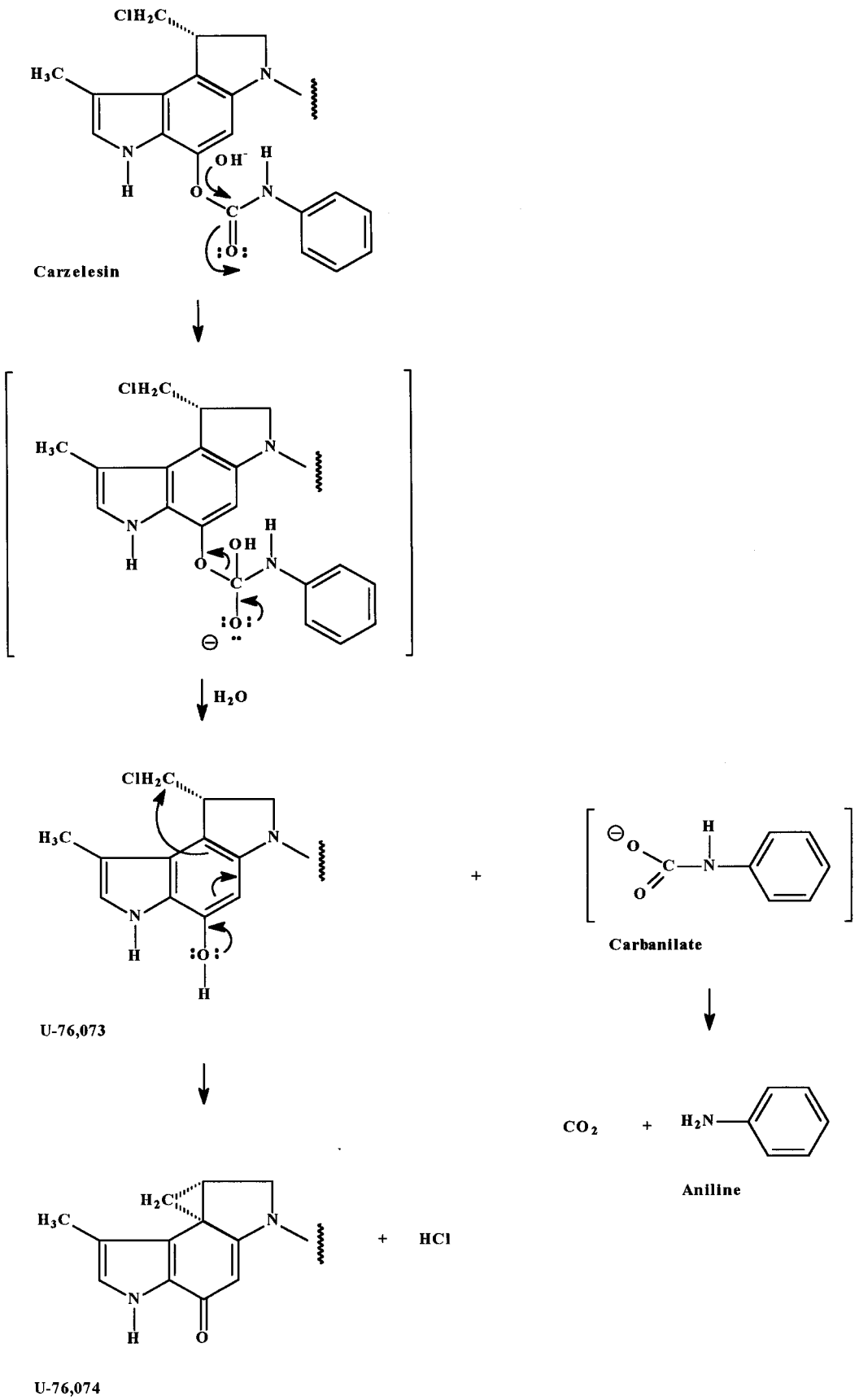

Figure 8—Proposed overall degradation scheme of carzelesin in alkaline medium.

graphically to be pH 2–3. The pH*–rate profile does not show inflections that could be attributed to acid–base equilibria of the compound. UV/vis spectra of carzelesin recorded in four buffer/acetonitrile solutions at pH* 0, 4.7, 7.9, and 10.0, however, showed that the spectrum at pH* 0 was significantly different from the other three spectra (Figure 7). Carzelesin

probably becomes protonated at this extremely low pH value. This might also explain the relatively slow degradation rate in this acidic pH region at pH* < 2. Protonated carzelesin will be positively charged and, therefore, less susceptible to proton-catalyzed degradation.

It is clear that the stability of carzelesin is extremely pH dependent with maximal stability around pH 3 (Figure 6). Degradation mainly involves hydroxyl-catalyzed reactions where $k_{OH} >> k_H$. In an earlier study it was found that the pH values of PEG 400 obtained from different suppliers were variable and had a major impact on the stability of the pharmaceutical formulation. It is advisable to adjust the pH of the PET formulation to around 3 in order to guarantee maximal drug stability. Shortly before intravenous infusion the PET formulation is diluted with 5% dextrose infusion solution, which increases the pH within physiologically accepted limits.[4]

Influence of Buffers—Experiments were performed where k_{obs} was measured at varying buffer concentrations, while pH, ionic strength ($\mu = 0.3$) and temperature (25 or 60 °C) were kept constant. Table 1 shows the influence of buffer ions on the degradation of carzelesin. All four different types of buffers appear to have a small, however, not significant effect on the degradation rate of carzelesin.

Influence of Ionic Strength—The influence of ionic strength on the degradation of carzelesin was investigated by adding various amounts of sodium chloride to buffer solutions of fixed pH, pH* 1.5 (0.1 M perchloric acid buffer solution) and pH* 7.2 (0.02 M phosphate buffer solution). The influence of ionic strength on the degradation of carzelesin at pH* 7.2 is negligible. However, at pH* 1.5 the ionic strength seems to have a stabilizing effect on the degradation rate of carzelesin. The higher the ionic strength, the lower the degradation rate of carzelesin.

Degradation Mechanism—Figures 2 and 3 show HPLC chromatograms of degradation products of carzelesin in acidic (perchloric acid buffer solution) and alkaline media, respectively. It appears that different degradation products are formed in the two regions of the pH*–rate profile. Figure 8 shows the proposed degradation mechanism of carzelesin in alkaline medium. Carzelesin degrades, in the presence of OH^- ions, mainly into U-76,073 and a carbanilate ion. U-76,-073 is subsequently converted into U-76,074 and hydrochloric acid. Carbanilate is unstable and decomposes into aniline and CO_2. The peak with a retention time of 3.6 min in Figure 3 is aniline. All three degradation products, U-76,073, U-76,-074, and aniline, were confirmed by photodiode array detection. The retention times in the HPLC chromatograms and UV/vis spectra were compared with those of reference standard solutions and were found to be identical.

In acidic medium (perchloric acid buffer solution) many peaks are formed. The intensity, however, is limited, and none of the peaks coeluted with U-76,073 and U-76,074. The stability of U-76,073 in a perchloric buffer solution (pH* 2.2; 60 °C) was then investigated, and it was found that the degradation half-life of U-76,073 was approximately 4 h, while the half-life of carzelesin was 130 h at this pH (60 °C). In perchloric acid buffer solutions, carzelesin may be converted into U-76,073; however, due to the short degradation half-life of U-76,073, this will not be detected. Several carzelesin degradation products formed at pH* 2.2 had retention times identical to that of the U-76,073 degradation products generated under these conditions.

The degradation profile of carzelesin in acetate buffer solutions ($3 \leq$ pH < 6) is different from that in perchloric acid solution at identical pH. In acetate buffer solutions, carzelesin degrades into U-76,073, which is subsequently converted into U-76,074. In addition, a peak with a retention time of 4.4 min is seen in the HPLC chromatogram, with a peak area of up to 40% of the amount of carzelesin present at $t = 0$. The on-line recorded UV/vis spectrum of this product was identical to that of U-76,073. Experiments to isolate and to elucidate the chemical structure of this intermediate degradation product are ongoing.

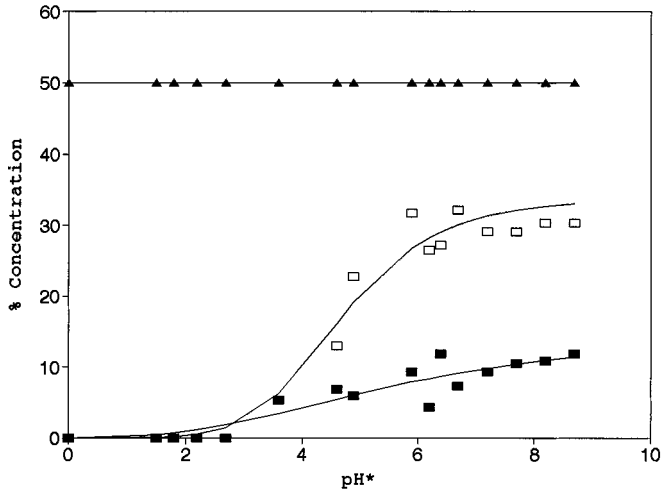

Figure 9—Quantification of the degradation products U-76,073 (■) and U-76,-074 (□) (calculated as percent of the initial carzelesin peak area at $t = 0$), at 50% degradation of carzelesin (▲), versus the pH*.

In order to determine the influence of pH on the amount of formed degradation products, at each pH* the amount of formed degradation products was quantitated as a function of 50% degradation of carzelesin. The results are displayed in Figure 9. The higher the pH*, the higher the amount of identified degradation products formed (calculated as the percentage of carzelesin present at $t = 0$). In the pH* range 6–10, the maximum amounts of U-76,073 and U-76,074 formed were constant and equal to 10 and 30%, respectively.

Conclusions

Degradation of carzelesin follows (pseudo-) first-order kinetics. The pH*–rate profile of carzelesin is determined mainly by specific hydroxyl catalysis. The degradation rate and mechanism of carzelesin degradation are strongly pH-dependent. Carzelesin is most stable in the pH* region 1–4.

The degradation rate of carzelesin is not significantly affected by buffer components and by the ionic strength at pH* 7.2. At pH* 1.5, however, the ionic strength seems to have a stabilizing effect on the degradation of carzelesin.

In addition to the formation of the degradation products U-76,073, U-76,074, and aniline in alkaline medium and in acetate buffer solution, another degradation product (t_R 4.4 min) was formed in acetate buffer solution. In perchloric acid buffer solution (pH* < 3), U-76,073 and U-76,074 were not detected. The degradation mechanism is not completely understood yet.

References and Notes

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