International Journal of Pharmaceutics, 99 (1993) 73–78

IJP 03285

Degradation kinetics of the antitumour drug nimustine (ACNU) in aqueous solution

O.A.G.J. Van der Houwen [a], J.H. Beijnen [b], A. Bult [a], O. Bekers [c], R.M.J. Goossen [a] and W.J.M. Underberg [a]

[a] *Department of Pharmaceutical Analysis and Toxicology, Faculty of Pharmacy, State University of Utrecht, P.O. Box 80.082, 3508 TB Utrecht (The Netherlands),* [b] *Slotervaart Hospital / Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam (The Netherlands) and* [c] *Leyenburg Hospital, Leyweg 275, 2545 CH 's-Gravenhage (The Netherlands)*

(Received 1 March 1993)
(Accepted 15 April 1993)

Key words: Degradation kinetics; Nimustine; ACNU; Nitrosourea; Degradation mechanism

Summary

The degradation kinetics of the nitrosourea derivative nimustine has been investigated over the H_0/pH region −1 to 11 at 25°C. The stability tests were performed using a stability-indicating high-performance liquid chromatographic assay. The first degradation product of nimustine is 3,4-dihydro-7-methylpyrimido[4,5-α]pyrimidine-2-(1H)one. Maximum stability of nimustine is around pH 3. At higher and lower pH values the degradation rate increases proportionally with [OH^-] and [H^+], respectively. From the log k_{obs}-pH plot a pK_a value of 6.24 could be derived. The influences of ionic strength and buffer ions were investigated at pH 5 and 9 but showed no effect on the degradation rate. Temperature effects were studied at pH 1.0, 5.0 and 9.0. The degradation mechanism of nimustine is discussed.

Introduction

Nimustine (3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride; ACNU; NSC-245382) (Fig. 1) is a nitrosourea derivative. Other representatives of this class of anticancer compounds are lomustine, semustine and carmustine (Fig. 1). The nitrosoureas exert their antitumour effect through alkylation of nucleic acids and carbamoylation of proteins. Phase II clinical trials with nimustine have demonstrated activity in lung cancer, colorectal cancer, gastric cancer, chronic lymphatic leukemia, Hodgkin and non-Hodgkin lymphoma, melanoma and sarcoma (Saito 1980). The treatment results in brain tumors and brain metastases are at least interesting (Saijo and Niitani, 1980; Joss et al., 1986). In the treatment of small-cell carcinoma of the lung, nimustine reduced the rate of brain metastasis and prolonged the survival of patients (Saijo and Niitani, 1980). Nimustine also has a strong cytocidal effect

Correspondence to: J.H. Beijnen, Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands.

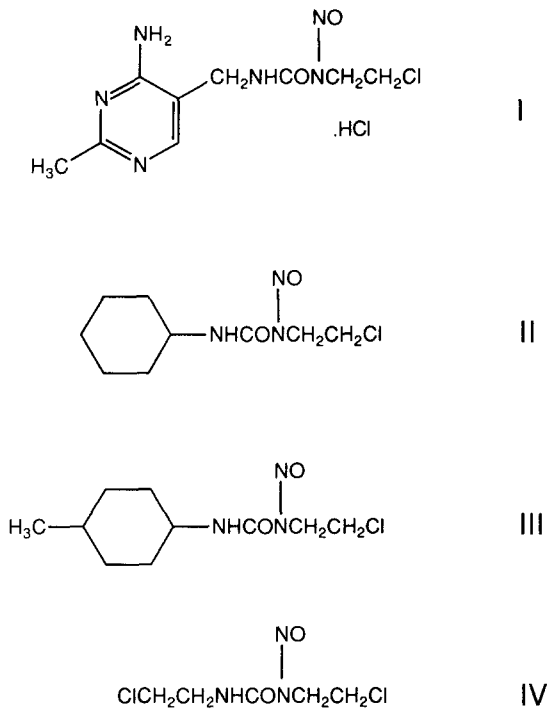

Fig. 1. Chemical structures of nitorosurea anticancer drugs. I, nimustine hydrochloride; II, lomustine; III, semustine; IV, carmustine.

against gliomas and has been tested against malignant leptomeningeal tumors after intrathecal administration in rats (Arita et al., 1988). Parenteral administration of the nitrosoureas is hampered by their poor water solubility. On the other hand, nimustine, formulated as a hydrochloride salt, has high water solubility (1 g dissolves in 11 ml of water) and can be administered intravenously. The nitrosoureas are very labile in aqueous media. Bosanquet (1985) reviewed the literature on the chemical stability of this class of cytotoxic compounds. The degradation of the nitrosoureas has been investigated extensively, however, stability data on nimustine are scanty except for some fragmentary information (Bosanquet, 1985). The objective of this study was to extend insight into the degradation kinetics of nimustine and the influences of external factors (pH, ionic strength, buffers, temperature) on these processes.

Experimental

Chemicals

Nimustine hydrochloride (ACNU 50®) originated from ASTA Pharma AG (Frankfurt, Germany). All other chemicals were of analytical grade and deionized water was used throughout.

Buffer solutions

For the kinetic studies the following solutions and buffers were used: H_0/pH −1 to 3, perchloric acid; pH 3–6, acetate; pH 6–9, phosphate; pH 9–11, carbonate. The buffer concentrations were 0.01 M except for the experiments where the influence of the buffer concentration was tested. The pH values between 1 and 11 were measured at 25°C with a Beckman Gel-filled combined glass-reference electrode and an E512 pH meter (Metrohm, Herisau, Switzerland). Extension of the acidity scale below 1 was accomplished with the Hammett acidity function (Bates, 1973). A constant ionic strength of 0.3 was maintained by addition of an appropriate amount of sodium chloride, except for the solutions where the concentration of protons exceeded 0.3 M and for the experiments where the influence of the ionic strength was investigated.

Kinetic measurements

The kinetic studies were conducted at 25 ± 0.2°C. The reactions were initiated by adding 30 μl of a stock solution of nimustine (2 mg/ml) to 3.0 ml pre-heated buffer solution to achieve an initial drug concentration of 20 μg/ml (6.5×10^{-5} M). The reaction solutions were kept in a thermostatically controlled water-bath, protected from light. At regular time intervals 10 μl samples were withdrawn and immediately injected into the HPLC system. All experiments were performed in duplicate.

Apparatus and analytical procedures

The reaction solutions were analyzed by high-performance liquid chromatography (HPLC) and ultraviolet (UV) detection. The HPLC equipment consisted of a U6K injection device, a Model M6000 solvent delivery system (both from Waters Associates, Milford, MA, U.S.A.) and a Spec-

troflow 773 UV absorbance detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) or a PU 4021 Multichannel UV detector (Pye Unicam Ltd, Cambridge, U.K.). The detector operated at 274 nm. The detector signal was recorded by an SP 4270 integrator (Spectra Physics, Santa Clara, CA, U.S.A.). The stainless-steel analytical column (125 × 4 mm internal diameter) was packed with Lichrosorb RP-8 material (particle size: 5 μm) (Merck, Darmstadt, Germany). The eluent comprised 250 ml methanol and 750 ml of 0.05 M phosphate buffer; the pH of this mixture was adjusted to 5.5 with phosphoric acid 85%. The flow rate was 1.0 ml/min. Quantitation of undegraded nimustine was based on peak area measurements.

Results and Discussion

Analytical procedures

The UV spectrum of nimustine in water displays a maximum at 242 nm ($\epsilon = 13\,800$ M^{-1} cm^{-1}) and a minimum at 213 nm ($\epsilon = 6700$ M^{-1} cm^{-1}). During degradation in aqueous media the absorbance at 242 nm decreases and a maximum around 280 nm emerges. These spectral changes allow the degradation to be followed spectrophotometrically. However, HPLC was preferred as this technique also provides direct information on the degradation products. Typical HPLC chromatograms, demonstrating the separation between nimustine and its degradation product, are depicted in Fig. 2. A similar HPLC system has been used for the determination of nimustine in blood and brain tissues (Tatsuhara et al., 1990). The stability-indicating capability of the HPLC assay was investigated using a multichannel photo-diode UV detector connected with the HPLC system. On-line UV spectra of the nimustine peak were recorded at different stages ($t = 0$, $t = t_{1/2}$, $t = 4t_{1/2}$) of the degradation process. There were no qualitative differences between these spectra, indicating that the nimustine peak does not co-elute with a degradation product with a different UV spectrum. In the H_0/pH region -1 to 11 the only detectable degradation product of nimustine is 3,4-dihydro-7-methylpyrimido[4,5-α]pyrimidine-2-(1H)one (DMPP) (Fig. 3). When the conversion of nimustine into DMPP is followed spectrophotometrically an isosbestic point at 274 nm is found. The molar absorptivities of both compounds are, thus, identical at 274 nm. This wavelength was chosen for HPLC detection since this detection wavelength allows simple establishment of the mass balance between nimustine and its degradation product(s). The peak area of nimustine at $t = 0$ and the sum of peak areas of residual nimustine and DMPP were equal during degradation over at least four half-lives. This mass balance indicates that nimustine degrades into only one product (DMPP). Deviations

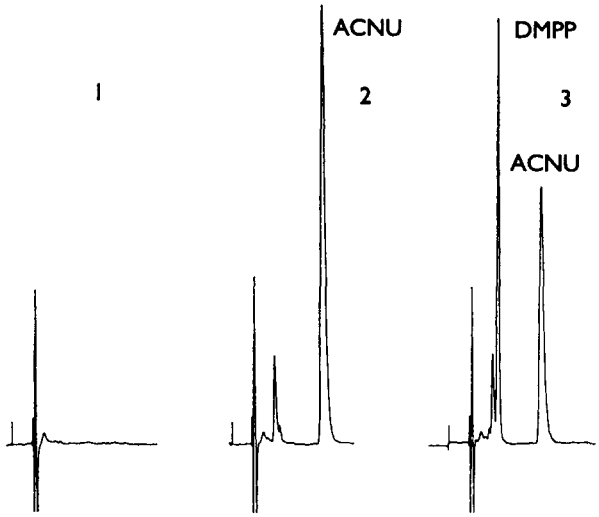

Fig. 2. HPLC chromatograms of nimustine solutions at pH 9 at various stages of degradation: (1) blank solution; (2) $t = 0$; (3) $t = t_{1/2}$. The retention time of nimustine (ACNU) is 5.5 min and that of DMPP is 2.5 min.

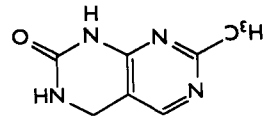

Fig. 3. Chemical structure of DMPP (3,4-dihydro-7-methylpyrimido[4,5-α]pyrimidine-2-(1H)one).

from this pattern were found at pH ≤ 1 which paralleled the emergence of other degradation products.

Degradation products

In the pH region 1–11 the degradation of nimustine involves the conversion into the cyclized pyrimidopyrimidine DMPP with a retention time of 2.5 min (Figs 2 and 3). The identity of the degradation product as DMPP was determined by comparison of the elution volumes of this compound and DMPP (Nakamura et al., 1977; Nishigaki et al., 1985a). At pH values < 1, extra peaks with retention times of 7 and 10 min and additional peaks in the solvent front appeared in the chromatograms. Under these conditions DMPP is not stable and consecutive degradation reactions occur with a significant rate.

Degradation kinetics

Order of reaction Under the experimental conditions, the degradation reactions of nimustine in buffers exhibited a pseudo-first order kinetic behaviour. This is indicated by the linearity of plots of the natural logarithm of residual nimustine concentration vs time over at least four half-lives. Neither the order of the reaction nor the degradation rate is influenced by the initial drug concentration (tested over the range: 2–67 μg/ml) which is in agreement with a first order kinetic model.

Standard deviation in k_{obs} The standard deviation (SD) in the observed (pseudo) first order rate constants, k_{obs}, was determinated by replicate measurements ($n = 19$) at pH 9.0 (0.01 M carbonate buffer; $\mu = 0.3$). The value of $k_{obs} \pm$ SD was $6.34 \pm 0.41 \times 10^{-4}$ s^{-1}. The relative standard deviation is 6.5%. All other rate constants were measured in duplicate and the results are the mean values.

Influence of ionic strength The influence of ionic strength, μ, on the degradation kinetics was measured in 0.01 M carbonate buffers (pH 9), 0.01 M acetate buffers (pH 5.0) and perchloric acid solutions (pH 1) with different amounts of sodium chloride added with μ between 0.1 and 1.0. The results of the tests have been enumerated in Table 1 and demonstrate that the ionic strength has no effect or only a marginal influence on the decomposition rate of nimustine.

TABLE 1

k_{obs} values (in s^{-1}) for the degradation of nimustine in 0.01 M buffers [a] as a function of ionic strength (μ) (temperature, 25°C)

pH	μ	k_{obs}	pH	μ	k_{obs}
1.0	1.0	1.1×10^{-5}	9.0	1.0	7.0×10^{-4}
	0.7	1.0×10^{-5}		0.7	7.0×10^{-4}
	0.5	9.2×10^{-6}		0.5	6.9×10^{-4}
	0.3	1.1×10^{-5}		0.3	6.3×10^{-4}
	0.1	7.3×10^{-6}		0.1	6.7×10^{-4}
5.0	1.0	6.5×10^{-6}			
	0.7	6.2×10^{-6}			
	0.5	5.8×10^{-6}			
	0.3	6.3×10^{-6}			
	0.1	5.8×10^{-6}			

[a] pH 1.0, perchloric acid solution; pH 5.0, acetate buffer; pH 9.0, carbonate buffer

Influence of buffers The effects of buffer concentration were tested at pH 5 and 9. The results are given in Table 2. No significant influence on the degradation rate was observed.

Influence of pH In Fig. 4 the pH-rate profile for nimustine at 25°C is given. As buffers and ionic strength have no significant catalytic effect on the degradation rate the profile does not need to be corrected for these parameters. The profile was recorded in 0.01 M buffer solutions with $\mu = 0.3$. The pH values of the buffers did not change during degradation. The pH-rate profile is characterized by linear segments at pH < 0 and pH > 9 where the degradation rate is linearly

TABLE 2

k_{obs} values (in s^{-1}) for the degradation of nimustine in various buffer [a] solutions ($\mu = 0.3$; temperature, 25°C)

pH	[Buffer]	k_{obs}	pH	[Buffer]	k_{obs}
5.0	0.2	6.3×10^{-6}	9.0	0.2	6.8×10^{-4}
	0.1	6.3×10^{-6}		0.1	6.7×10^{-4}
	0.05	6.1×10^{-6}		0.05	6.6×10^{-4}
	0.01	6.3×10^{-6}		0.01	6.3×10^{-4}

[a] pH 5.0, acetate buffer; pH 9.0, carbonate buffer.

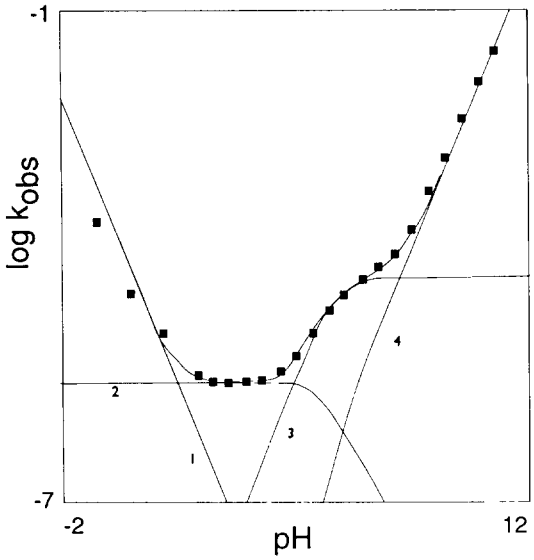

Fig. 4. Log k_{obs}-pH profile for the degradation of nimustine (temperature, 25°C; 0.01 M buffers; $\mu = 0.3$; k_{obs} in s^{-1}). (1) Proton catalyzed degradation of protonated nimustine; (2) proton catalyzed degradation of neutral nimustine and solvent-catalyzed degradation of protonated nimustine; (3) solvent catalyzed degradation of neutral nimustine and hydroxyl-catalyzed degradation of protonated nimustine; (4) hydroxyl catalyzed-degradation of neutral nimustine. The closed squares are experimental values and the plot is the fitted graph from Eqn 1 and the values listed in Table 3.

related to $[H^+]$ and $[OH^-]$, respectively. At pH < 0 the slope of the linear part of the profile is -0.7 and at pH > 9, 0.9. The latter value is near unity indicating specific hydroxyl catalysis in this pH region. At H_0 0 and H_0 -1 the observed degradation rate is lower than the calculated model values as can be seen in Fig. 4. The deviations in this strongly acidic medium may be due to significant differences between proton activities and concentration, water activity and, possibly, differences in solvatation of the nimustine ions. The inflection point around pH 7 indicates that the compound is involved in a protolytic equilibrium in this region. The pH-rate profile was, therefore, modelled using the general equation describing a compound existing in solution as a neutral or protonated, positively charged species which are subject to proton, water and hydroxyl catalyzed decomposition reactions (Van der Houwen et al., 1988):

$$k_{obs} = \left(M_1[H^+] + M_2 + M_3[H^+]^{-1} + M_4[H^+]^{-2}\right) \times \left(1 + K_1[H^+]^{-1}\right)^{-1} \quad (1)$$

where the macro reaction constants (M_1–M_4) are defined:

$$M_1 = k_{1,H} \quad (2)$$

$$M_2 = k_{0,H} \cdot K_a + k_{1,S} \quad (3)$$

$$M_3 = k_{0,S} \cdot K_a + k_{1,OH} \cdot K_w \quad (4)$$

$$M_4 = k_{0,OH} \cdot K_a \cdot K_w \quad (5)$$

with K_a as the acid dissociation constant and the autoprotolysis constant of water K_w. The subscripts 1 and 0 of the micro reaction constants (k) correspond to a positively charged and neutral nimustine molecule, respectively; the subscripts H, S and OH relate these nimustine species to the proton, solvent and hydroxyl catalyzed reactions. Macro and micro reaction constants and the acid dissociation constant K_a have been listed in Table 3. The micro reaction constants $k_{0,H}$, $k_{1,S}$ and $k_{0,S}$, $k_{1,OH}$, which are combined in the single macro reaction constant M_2 and M_3, respectively, are kinetically indistinguishable. It is, therefore, impossible to determine the individual contribution of these constants to the pH profile (Van der Houwen et al., 1988). On the other hand, the micro reaction constants $k_{1,H}$ and $k_{0,OH}$

TABLE 3

Macro and micro reaction constants for the degradation of nimustine and the acid dissociation constant at 25°C

M_1	7.7×10^{-5} M^{-1} s^{-1}	$k_{1,H}$ 7.7×10^{-5} M^{-1} s^{-1}
M_2	2.7×10^{-6} s^{-1}	$k_{0,OH}$ 45.2 M^{-1} s^{-1}
M_3	3.3×10^{-11} M s^{-1}	K_a 5.75×10^{-7} (pK_a 6.24)
M_4	2.6×10^{-19} M^2 s^{-1}	

can be calculated from Eqns 2 and 5. The contributions of the individual macro reaction constants to k_{obs} are shown in Fig. 4. It is clear from these plots that M_1 and M_4 largely determine the extremities of the profile while M_2 and M_3 dominate in the pH region 2–6. From Fig. 4 it can also be concluded that a good correlation exists between the experimental values and the fitted curve.

The kinetically derived pK_a value of nimustine is 6.24 (25°C) and can, apparently, be attributed to the dissociation of the conjugated acid of the primary amino function attached to the pyrimidine ring. Theoretically, the other nitrogen atoms in the nimustine molecule have protophilic properties but protonation probably occurs only at pH values beyond the region of this study or at the pH extremities studied here.

Influence of temperature The effect of temperature on the degradation rate of nimustine was determined in 0.01 M carbonate buffer (pH 9.0), 0.01 M acetate buffer (pH 5.0) and in perchloric acid solution (pH 1) over the range 5–75°C with $\mu = 0.3$. The Arrhenius equation is obeyed. From the slope and the intercept of a plot of ln k_{obs} vs the reciprocal of absolute temperature, the activation energy ($\Delta H^{\neq}$) and frequency factor (A) were calculated. The results are: pH 9.0, $\Delta H^{\neq} = 118$ kJ mol^{-1}, $A = 2.8 \times 10^{17}$ s^{-1}; pH 5.0, $\Delta H^{\neq} = 105$ kJ mol^{-1}, $A = 1.4 \times 10^{13}$ s^{-1}; pH 1.0, $\Delta H^{\neq} = 90$ kJ mol^{-1}, $A = 5.7 \times 10^{10}$ s^{-1}.

Degradation mechanism

Degradation of nimustine in the pH region 1–11 leads to the formation of only one product, DMPP. Nishigaki et al. (1985b) proposed that the hydrolysis of nimustine in aqueous solutions proceeds through the formation of an isocyanate intermediate after chloroethyldiazohydroxide is split off the nimustine side chain. An intramolecular cyclization of the isocyanate intermediate follows instantaneously with the formation of DMPP. Due to the loss of the alkylating moiety in DMPP the compound no longer has any cytotoxic activity (Nishigaki et al., 1985b). This reaction can be utilized when nimustine solutions must be inactivated before disposal but, of course, should be prevented during the preparation and storage of, e.g., infusion fluids with the drug.

References

Arita, N., Ushio, Y., Hayakawa, T., Nagatani, M., Huang, T.-Y., Izumoto, S. and Mogami, H., Intrathecal ACNU – a new therapeutic approach against malignant leptomeningeal tumors. *J. Neuro-Oncol.*, 6 (1988) 221–226.

Bates, R.G., *Determination of pH; Theory and Practice,* Wiley, New York, 1973, pp. 165–169.

Bosanquet, A.G., Stability of solutions of antineoplastic agents during preparation and storage for in vitro assays. General considerations, the nitrosoureas and alkylating agents. *Cancer Chemother. Pharmacol.*, 15 (1985) 83–95.

Joss, R.A., Siegenthaler, P., Ludwig, C., Alberto, P., Castiglione, M.M. and Cavalli, F., Phase II trial of nimustine (ACNU; 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride) in patients with small cell carcinoma of the lung after failure on combination chemotherapy. *Invest. New Drugs,* 4 (1986) 175–179.

Nakamura, K., Asami, M., Kawada, K. and Sasahara, K., Quantitative determination of ACNU (3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride), a new water-soluble anti-tumor nitrososurea, in biological fluids and tissues of patients by high-performance liquid chromatography: I. Analytical method and pharmacokinetics. *Ann. Rep. Sankyo Res. Lab.,* 29 (1977) 66–74.

Nishigaki, T., Nakamura, K., Kinoshita, T., Kuwano, H. and Tanaka, M., Identification of major metabolites of 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride. *J. Pharmacobio. Dyn.,* 8 (1985a) 401–408.

Nishigaki, T., Nakamura, K.-I. and Tanaka, M., In vitro metabolism of ACNU 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride, a water soluble antitumor nitrosourea. *J. Pharmacobio. Dyn.,* 8 (1985b) 409–416.

Saijo, N. and Niitani, H., Experimental and clinical effect of acnu in japan, with emphasis on small-cell carcinoma of the lung. *Cancer Chemother. Pharmacol.,* 4 (1980) 165–171.

Saito, T. Continuation of phase II study of 1-(4-amino-2-methylpyrimidine-5-yl)-methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU). *Recent Res. Cancer Res.,* 70 (1980) 91–106.

Tatsuhara, T., Tabuchi, F., Yamane, M. and Huori, T., Rapid and simple method for the determination of nimustine hydrochloride in human blood and brain by high-performance liquid chromatography. *J. Chromatogr.,* 526 (1990) 507–514.

Van der Houwen, O.A.G.J., Beijnen, J.H., Bult, A. and Underberg, W.J.M., A general approach to the interpretation of pH degradation profiles. *Int. J. Pharm.,* 45 (1988) 181–188.