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Chemical stability of ecomustine, a new antitumor agent in aqueous and biological media as assessed by high-performance liquid chromatography

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Summary. Ecomustine, or CY233 (NSC-609224), is a new water-soluble nitrosoureido sugar derived from acosamine. A high-performance liquid chromatographic assay (HPLC) developed to quantify the unchanged drug in aqueous solutions and biological specimens enabled us to study the chemical stability as a function of pH, light, and temperature. In buffered aqueous solutions, the kinetics of degradation of CY233 is a first-order process. The log *k*-pH profile demonstrated hydroxide ion-catalyzed solvolysis. The drug is most stable at pH 4, more stable than some other nitrosoureas in 5% glucose ($t_{1/2}$, 62–67 h) and in 0.9% isotonic saline ($t_{1/2}$, 25–37 h) solutions. Based on these findings, blood samples should be collected in cold tubes (4° C) containing citrate buffer (pH 4) and all manipulations should be protected from heat and light.

Introduction

The *N*-nitrosoureas (NUs) have been shown to increase the life span of mice bearing i.p. or intracerebrally implanted L1210 leukemia [32]. Chloroethylnitrosoureas (CENUs) such as BCNU, CCNU, and MeCCNU, were subsequently developed and have been shown to have considerable antitumor activity in a variety of experimental [29] and human malignancies [15].

The clinical efficacy of these lipophilic drugs that cross the blood-brain barrier [4, 33] has been limited by their delayed and cumulative hematological toxicity. Their chemistry has been extensively studied [19, 23]. A variety of structural modifications have been aimed at optimizing their activity against murine leukemia and/or at decreasing their hematological toxicity; hydrophilic NUs with sugar carriers, such as streptozocin [36], chlorozotocin [1], GCNU [30], CNUA [7], and RFCNU [22] show differences in human and animal therapeutic activity. These compounds possess an alkylating 2-chloroethyl group (except the methyl analog, streptozocin) as the active moiety and can cross-link nucleic acids and proteins [12, 25, 35]. The addition of a sugar carrier to the cytotoxic nitrosoureido moiety reduces bone marrow toxicity without significantly altering the antitumor activity [17].

Recently, Roger et al. [27] have synthesized several nitrosoureido derivatives of di- and trideoxy sugars on the premise that such compounds would be intermediate between water-soluble and water-insoluble CENUs. If activity and toxicity can be segregated on the basis of lipid solubility, we postulated that more potent and less toxic drugs should be produced by the creation of less lipophilic compounds [16]. Structural features such as the number of hydroxyl groups, the configuration at the anomeric center, and the absolute configuration of the sugar component may affect the antitumor activity of these new nitrosoureido sugars [28].

Ecomustine, methyl 3-[2-(chloroethyl)-3-nitrosoureido]-2,3-dideoxy-α-D-arabino-hexopyrannoside was the most active compound in vivo against L1210 leukemia, B16 melanocarcinoma, and Lewis lung carcinoma, with a very large therapeutic index. When treated with ecomustine at 20 mg/kg on day 1, >90% of the L1210 leukemia- and B16 melanocarcinoma-bearing mice survived for at least 60 days. The LD_{50} value for this compound was 42 mg/kg [28].

The antitumor activity of ecomustine is dose-dependent by all routes of administration (i.p., i.v., p.o.) [13]. It was only slightly active against the L1210 leukemia intracerebral form and the L1210/BCNU subline. This suggests that contrary to BCNU, ecomustine does not readily cross the blood-brain barrier. This compound is more effi-

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Abbreviations: *BCNU*, 1,3-bis(2-chloroethyl)-1-nitrosourea; *CCNU*, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; *MeCCNU*, 1-(2-chloroethyl)-3-methylcyclohexyl-1-nitrosourea; *GCNU*, 3-(tetraacetyl-glucopyranose-2-yl)-1-(2-chloroethyl)-1-nitrosourea; *CNUA*, 3-(3-(2-chloroethyl-3-nitrosoureido)-3-deoxy-D-allose; *RFCNU*, 1-(2-chloroethyl)-3-[1-(5′-paranitrobenzoyl-2′,3′-isopropylidene)-α,β-D-ribofuranosyl]-1-nitrosourea; *5-FU*, 5-fluorouracil

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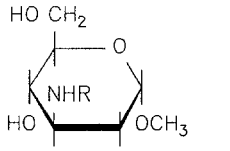

R = C(O)–N(NO)–CH_2CH_2Cl

Fig. 1. Structure of ecomustine

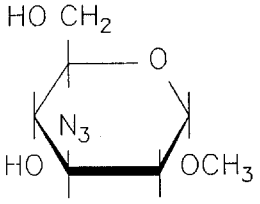

Fig. 2. Structure of the internal standard (azido)

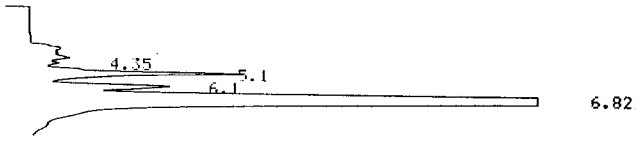

Fig. 3. High-performance liquid chromatogram of ecomustine, the internal standard (azido), and endogenous constituents in a blank plasma extract

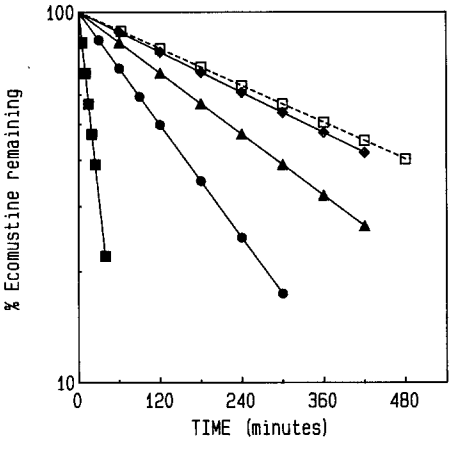

Fig. 4. Kinetics of degradation of ecomustine in buffer solutions at 37° C in daylight at pH 1.17 (▲), pH 2.6 (◆), pH 4.84 (□), pH 6 (●), and pH 6.7 (■)

cient than 5-FU against s.c. established murine colon 38 adenocarcinoma, known to be resistant to BCNU and to the major anticancer drugs [2]. A survey of the literature indicates that the antitumor and toxic actions of the CENUs are related to spontaneous decomposition to their alkylating and carbamoylating moieties in biological media [26, 36, 37].

In the present study, a high-performance liquid chromatographic (HPLC) assay was developed to determine the chemical stability of this new water-soluble nitrosoureido sugar and the influence of external factors such as pH, light, and temperature on the kinetics of the degradation process. A preliminary report on a portion of these results has been presented elsewhere [14].

Materials and methods

Chemicals. Ecomustine (Fig. 1) and the internal standard (azido, Fig. 2), a structural analog, were synthesized in the Therapeutic Chemistry Departement of the Choay Institute (Sanofi Recherche) [27].

Buffer solutions. The aqueous buffer solutions used in the kinetic studies are described in the literature [3] (Table 1). The pH values were measured at 20° C using a glass-reference electrode and a pH meter standardized at 20° C (Digital pH meter 646, Knick).

Apparatus and analytical procedures. HPLC analyses were carried out using a model 344 CRT-based gradient liquid chromatograph (Beckman Instruments) consisting of a 421 system controller, two 112 solvent-delivery modules, a 340 organizer (including the sample injection valve, the mixer, and the drain valve), and a microprocessor-controlled absorbance detector (model 165 variable-wavelength UV/visible detector).

The analytical column (30 cm × inside diameter, 3.9 mm) was packed with a reversed phase (Nucleosil C18, 10 µm). The detector wavelength was set at 230 nm. The mobile phase consisted of a mixture of methanol and water (40 : 60, v/v) and the flow rate was 1 ml/min. All drug analyses were run isocratically at pH 3.5–4. Quantitation of ecomustine was based on peak-area-ratio measurements using a C-R1B integrator (Shimadzu).

In all, 1 ml water (pH 3.2) or 1 ml cold (0–4° C) fresh human plasma sample was spiked with ecomustine (0.5–25 µg/ml) and internal standard (25 µg/ml). The mixture was then extracted twice with 2 ml cold (0–4° C) ethyl acetate, then centrifuged at 4° C for 2 min at 3,000 rpm. The organic phase was evaporated to dryness under a nitrogen stream, and the residue was taken up with 100 µl mobile phase and then injected onto the column. The calibration curve obtained was linear over a wide range of concentrations (0.5–25 µg/ml), with a correlation coefficient of 0.999 and a slope of 0.00105. The detection limit of pure material injected directly onto the chromatograph was 25 ng/ml, and the lower limit of sensitivity in plasma was 100 ng/ml, with a within-day coefficient of variation (CV) of 8.3%. The theoritical detection limit was 66 ng/ml, calculated from the equation.

$$DL = \text{Blank} + T(n, 95\%) \times Sc(\rightarrow 0),$$

where $T(n, 95\%)$ is the critical value of the distribution T with confidence intervals of 95%, n is the number of assays, and $Sc(\rightarrow 0)$ is the standard deviation of the lower concentration. The between-days reproducibility was 11.6%, with a precision of 7.1% within three repetition assays. Under these conditions, ecomustine and the internal standard had retention times in the range of 6.8–7.4 and 4.8–5.1 min, respectively (Fig. 3). The extraction efficiency of ecomustine and the internal standard from plasma was 78.2% and 62.8%, respectively.

Degradation kinetic studies. The stability of the drug in aqueous solutions was based on a previously published procedure of Wheeler et al. [37]. Buffer solutions at different pH were incubated in the presence of ecomustine (1 mg dissolved in 1 ml water) in a thermostatically controlled water bath at 37° C, protected from light. A 1-ml aliquot of the solution was removed at various intervals, then 100 ml internal standard (1 mg dissolved in 1 ml methanol) was added. The mixture was then extracted and immediately analyzed by HPLC as described above. The results are expressed as the percentage of initial drug concentration against time, and the half-lives were calculated from rate constants obtained from the nonlinear regression of amount-versus-time data in

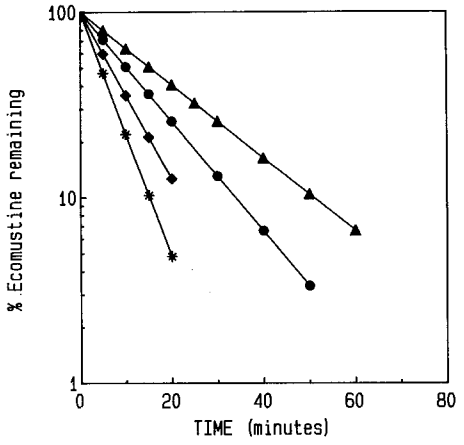

Fig. 5. Kinetics of degradation of ecomustine in buffer solutions at 37° C in daylight at pH 7.2 (▲), pH 7.4 (●), pH 8 (◆), and pH 8.7 (✳)

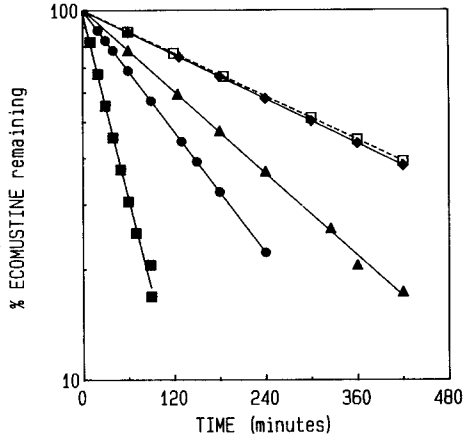

Fig. 6. Kinetics of degradation of ecomustine in buffer solutions at 37° C protected from light, at pH 1.32 (▲), pH 2.61 (◆), pH 4.86 (□), pH 6.1 (●), and pH 7.43 (■)

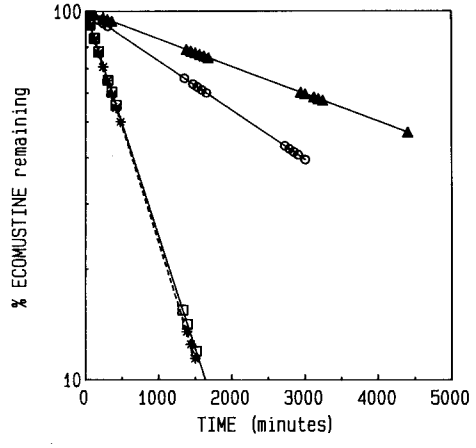

Fig. 7. Kinetics of degradation of ecomustine in buffer solutions at 37° C in daylight at pH 3.63 (□) and protected from light at pH 3.65 (✳), and at 20° C in 0.9% isotonic saline (○) and 5% glucose (▲) solutions

Table 1. Aqueous buffer solutions used in the degradation kinetic studies

pH	Buffer
1.17–1.32	KCl 0.2 M + HCl 0.2 *N*
2.6	Citrate 0.1 M + HCl 0.1 *N*
3.63	Citrate 0.1 M + HCl 0.1 *N*
4.84	Citrate 0.1 M + HCl 0.1 *N*
6.1	Phosphate 5×10^{-3}M
6.7	Phosphate 5×10^{-3}M
7.2	Phosphate 5×10^{-3}M
7.4	Phosphate 5×10^{-3}M
8	Phosphate 5×10^{-3}M
8.7	Phosphate 5×10^{-3}M

Table 2. Degradation rate constants and chemical half-lives of ecomustine as a function of pH at 37° C in daylight

pH	k (min^{-1})	log k	$t_{1/2}$ (min)
1.17	4.17×10^{-3}	–2.38	166
2.6	2.3×10^{-3}	–2.64	302
3.63	1.41×10^{-3}	–2.85	493
4.84	2.23×10^{-3}	–2.65	310
6	1×10^{-2}	–2	111
6.7	2×10^{-2}	–1.71	35.1
7.2	5×10^{-2}	–1.3	15.3
7.4	7×10^{-2}	–1.15	10.2
8	0.1	–1	6.7
8.7	0.15	–0.82	4.6

k, Degradation rate constants

Table 3. Degradation rate constants and chemical half-lives of ecomustine as a function of pH at 37° C, protected from light

pH	k (min^{-1})	log k	$t_{1/2}$ (min)
1.32	3.16×10^{-3}	–2.5	220
2.61	2.08×10^{-3}	–2.68	334
3.65	1.45×10^{-3}	–2.84	480
4.86	1.9×10^{-3}	–2.72	365
6.1	1×10^{-2}	–2	119
7.43	4×10^{-2}	–1.4	18.3

k, Degradation rate constants

Table 4. Chemical half-lives of ecomustine in plasma as a function of temperature

T (° C)	$1/T$ (K^{-1})	log k	$t_{1/2}$ (min)
4	3.61×10^{-3}	–3.3	1,359
24	3.36×10^{-3}	–2	72.9
37	3.23×10^{-3}	–1.3	13.2

k, Degradation rate constant

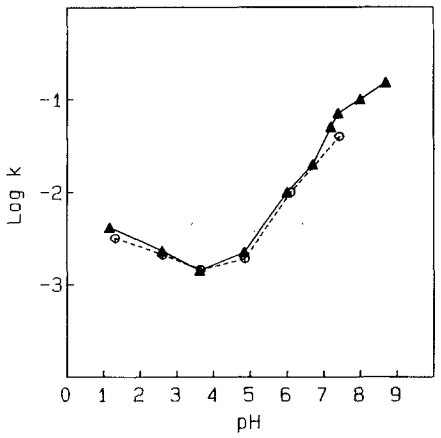

Fig. 8. Log k-pH profile for ecomustine in different buffers in daylight (▲) and protected from light (○)

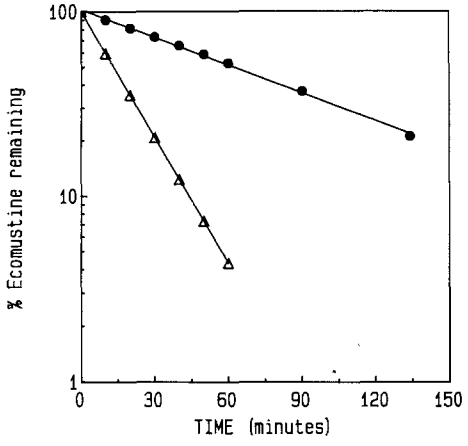

Fig. 9. Kinetics of degradation of ecomustine in plasma at 37° C (△) and at 24° C (●)

accordance with a first-order loss, i.e., $C = 100 * e^{-kt}$ (where C is the concentration of ecomustine at time t). The corresponding chemical half-life [10] is:

$$t_{1/2} = 1n2/k = 0.693/k.$$

The degradation of ecomustine at different pH values was also studied at 37° C in daylight.

Additional kinetic studies were done at various temperatures in plasma in 0.9% isotonic saline and 5% glucose solutions at room temperature (20° C), protected from light. The quantitative relation between specific reaction rates and temperature is the Arrhenius expression. Its logarithmic version is:

$$\log k = -\Delta Ha/2.303\,RT + \log P,$$

where T is the absolute temperature in degrees Kelvin and $R = 1.987$ [9].

Results

Effect of pH on the decomposition of ecomustine in aqueous solutions

Semilogarithmic plots (Figs. 4–7) of the percentage of initial drug concentration as a function of time at different

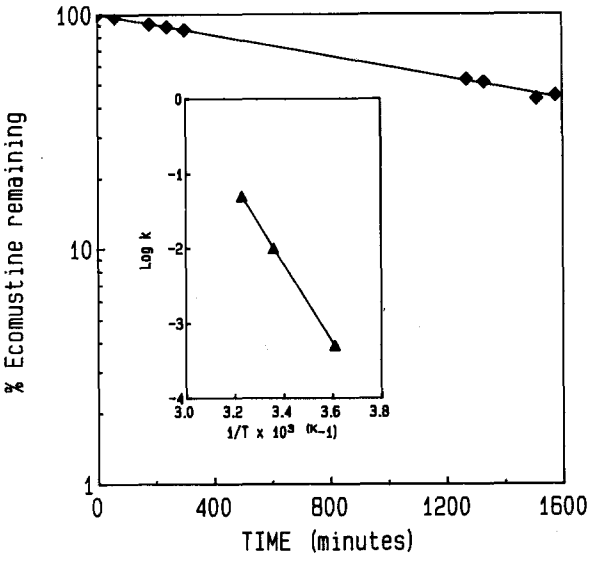

Fig. 10. Kinetics of degradation of ecomustine in plasma at 4° C (◆). *Insert:* Arrhenius plot

pH values were linear with rate constants (k in min^{-1}), indicating that the degradation follows apparent first-order kinetics. The apparent first-order rate constants (k, Tables 2–4) were obtained in accordance with $C = 100 \times e^{-kt}$.

Since a number of antitumor antibiotics have been shown to be sensitive to light [5], the effect of light on drug-degradation kinetics was also studied. The log k-pH profile for the solvolysis of ecomustine is given in Fig. 8, based on the data shown in Tables 2 and 3. The rate of ecomustine degradation was affected by both pH and light (at physiological pH). At pH 7.4 and 37° C, the degradation half-life under protection from light (18.3 min) was longer than that in daylight (10.2 min), with rate constants of 4×10^{-2} and 7×10^{-2}, respectively. Maximal stability was attained at ca. pH 4.

Effect of temperature on the decomposition of ecomustine in plasma

The influence of temperature on the degradation rate in pooled fresh human plasma (pH 7.5–7.9) was also studied. At 37° C, the half-life was 13.2 min, increasing to 72.9 min at 24° C and to 23 h at 4° C (Table 4; Figs. 9, 10). The Arrhenius plot of the data for the solvolysis of ecomustine is shown in the inset of Fig. 10. The pertinent ΔHa, the energy of activation of the reaction, and the log P, related to the entropy of the system, were 23.9 kcal·mol^{-1} and 15.6, respectively.

Decomposition of ecomustine in other media

At room temperature (20° C), the chemical half-life was between 25 and 37 h in 0.9% isotonic saline (pH 6.16–6.54) and between 62 and 67 h in 5% glucose (pH 5.38–5.78) solutions (Fig. 7).

Discussion

Prediction of the stability of drugs and pharmaceutical preparations depends on quantitative mathematical expressions that enable the calculation of rates of degradation by the substitution of the appropriate values for temperature, concentration, pressure, time, pH, oxygen content, light intensities, and wavelengths.

The importance of appropriate analytical methodology for drug decomposition has been extensively reviewed [6]. A specific high-performance liquid chromatographic (HPLC) assay with current widespread application [18, 21, 34] was developed for ecomustine in both aqueous solutions and biological fluids (sensitivity, 100 ng/ml). An azido analog was used as the internal standard (spectrophotometric detection, 230 nm). The HPLC methodology enables the separation of intact ecomustine from its degradation products and its quantification by UV detection.

The assay was used to determine the stability of ecomustine in aqueous solutions, to establish its log *k*-pH profiles in a dark environment and in daylight, and to evaluate the Arrhenius parameters from experiments carried out at different temperatures. It appears that ecomustine is sensitive to light at pH 7.4 (physiological pH). However, the effect of light is negligible between pH 3.5 and pH 6.5. Thus, it is not necessary to infuse ecomustine under protection from light since isotonic glucose solutions have pH values of 5.38–5.78.

Specific hydroxide-ion-catalyzed solvolysis is indicated by the fact that the slope of the log *k* vs pH plot between pH 5 and pH 8.7 approaches a value of +1. It has previously been demonstrated that neutral and alkaline solvolysis of *N*-nitrosoureas (NUs) is subject to spontaneous and specific hydroxide-ion catalysis [11]. Ecomustine has maximal stability at ca. pH 4. It is more stable than some other NUs in acidic media; therefore, it is suitable for oral administration. At pH 1.32, its half-life was longer (220 min) than that of RFCNU (22 min at pH 1.2) [20]. This could explain the improved antitumor activity of ecomustine against L1210 leukemia when it is given orally [13], since the drug can resist stomach acidity better than BCNU.

In plasma, the half-life of ecomustine at 37° C was also longer (13.2 min) than that of RFCNU (3.5 min) [20] but was shorter than that of CCNU in phosphate buffer at pH 7.2 (64 min) [24]. The half-life of BCNU in plasma is 45 min. The influence of temperature on the rate of decomposition of ecomustine in plasma confirms that it is best to refrigerate blood samples collected during pharmacokinetic studies in animals and to protect them from light both immediately and through analytical manipulations. The energy of activation (ΔHa, 23.9 kcal mol^{-1}) and the log P (15.6) obtained for ecomustine in the present study are in good agreement with those reported for other nitrosoureas [11, 20].

For the study of drug distribution in tissue and its disposition in biological media (urine, bile, faeces), the molecule was labeled with carbon 14 either on the chloroethyl moiety or on the urea carbonyl or methoxy group of the sugar residue [31]. Recent autoradiographic studies (in preparation) have shown that ecomustine does not cross the blood-brain barrier to any significant extent confirming our hypothesis based on the low antitumor activity of this compound against intracerebrally grafted L1210 leukemia in mice. The minimal radioactivity found in bone marrow is consistent with the limited myelotoxicity observed [8]. Results showed that a minor amount of radioactivity was excreted in the expired air ($^{14}CO_2$) after i.v. injection of [^{14}C-carbonyl]-ecomustine. This indicates that the potential isocyanate (in the usual decomposition scheme) is not readily hydrolysed to CO_2 and the corresponding amine but could rather undergo an internal cyclic reaction that could explain the low carbamoylating potential (as compared with that of BCNU) that has been observed in preliminary experiments.

The water solubility of ecomustine (30 g/l), its chemical stability profile in aqueous media, and its tissue distribution clearly emphasize the very original and promising position of ecomustine among the alkylating agents in general and more precisely among nitrosoureas.

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