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Stability Study of Anticancer Agent N-[(Hexahydroazepin-1-yl)methyl]daunorubicin in Aqueous Solutions Using HPLC Method

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A high performance liquid chromatographic method with UV detection (254 nm), which has been applied for the determination of N-[(hexahydroazepin-1-yl)methyl]daunorubicin (HMD), is described. The samples of solution were chromatographed on LiChrospher RP 18 e column (125 mm × 4 mm, particle size 5 mm), using an eluent composed of: 9 volumes of acetonitrile, 1 volume of methanol and 10 volumes of a solution containing 2.88 g L^{-1} of sodium lauryl sulfate and 1.6 mL L^{-1} of phosphoric(V) acid. The method was validated with respect to selectivity, linearity and precision. The method was found appropriate for kinetic studies of the stability of N-[(hexahydroazepin-1-yl)methyl]daunorubicin (HMD) in aqueous solutions in the range pH 0.43-13.71. The pH-rate profile indicated (a) hydrolysis of protonated molecules of HMD depending on hydrogen ions and (b) spontaneous hydrolysis under the influence of water depending on the substrate charge. General acid-base hydrolysis of HMD was observed in phosphate (pH 1.89-3.10) and acetate (pH 4.01-5.65) buffers. The ionic strength effect and thermodynamic parameters of the reaction were determined.

Key Words: HPLC, [(N-[(Hexahydroazepin-1-yl)methyl]daunorubicin, Stability, log k-Profile, Thermodynamic parameters.

INTRODUCTION

The anthracyclines are very effective anticancer drugs but their clinical use is limited by their toxic effect on healthy tissue (cardiomyopathy) and the drug resistance of tumor cells. Chemical modification of a molecule can be one of the ways to reduce its carditoxicity and to overcome the resistance of cancer cells to the drug. During *in vitro* and *in vivo* studies N-[(hexahydroazepin-1-yl)methyl]daunorubicin (HMD) has demonstrated cytotoxic action comparable to that of daunorubicin and less cardiotoxicity than daunorubicin[1-3]. The stability of daunorubicin[4-6], [(N-pyrrolidine)metylene] daunorubicin[7] and [(N-morpholine)methylene]daunorubicin[8] have already been studied.

The aim of our study is to determine general and/or specific acid-base hydrolysis of HMD at pH 0.43-13.71 at 333, 323, 313 and 303 K. In order to determine the observed rate constants an isocratic HPLC method was used.

EXPERIMENTAL

(N-[(Hexahydroazepin-1-yl)methyl]daunorubicin hydrochloride (HMD) was prepared in the Department of Modified Antibiotics, Institute of Biotechnology and Antibiotics in Warsaw, Poland. N-[(Hexahydroazepin-1-yl)methyl]daunorubicin is a reddish powder, freely soluble in water and methanol. All chemicals (Sigma-Aldrich) and all solvents (POCH) were of analytical or HPLC grade.

Chromatographic conditions: Chromatographic separation and quantitative analysis were performed by using an HPLC method[7,8]. The analytical system consisted of an LC-6A pump (Shimadzu), a UV-vis (SPD-6AV) detector (Shimadzu), a Rheodyne with a 50 µL loop. A LiChrospher RP 18 e column (125 mm × 4 mm, particle size 5 µm, Merck, Germany) was used as the stationary phase. The mobile phase consisted of a mixture of 9 volumes of acetonitrile, 1 volume of methanol and 10 volumes of a solution containing 2.88 g L^{-1} of sodium lauryl sulfate and 1.6 mL L^{-1} of phosphoric(V) acid. The flow rate was 1.5 mL min^{-1}. The detection wavelength was 254 nm. The internal standard was a solution of quinine papaverine hydrochloride at a concentration of 50 µg mL^{-1}. The study was performed at ambient temperature. Although the used method has been evaluated and validated for determination of the four derivatives of daunorubicin in aqueous solutions[9]. The selectivity was examined for non-degraded and degraded samples at increased temperature.

Kinetic measurements

During preparation and kinetic studies all solutions of HMD were protected from light: The stability of HMD in

aqueous solutions was studied by using the stress degradation test. The degradation of HMD was studied at 303, 313, 323 and 333 K in hydrochloric acid (pH 0.43-1.39), phosphate buffers (pH 1.89-3.10 and 6.13-7.50), acetate buffer (pH 4.01-5.65), borate buffer (pH 7.70-9.75) and sodium hydroxide (pH 11.64-13.71). The pH values of the solutions and buffer standards were used to calibrate the pH-meter were measured at reaction temperatures. To indicate that buffer components catalyze the degradation of HMD at four concentration (0.4, 0.3, 0.2 and 0.1 M) of each buffer was made. The activity coefficients f_{HCl} and f_{NaOH} were obtained from the literature or calculated by interpolation of literature data[10]. The ionic strength (μ) of all solutions except solutions used to determine the influence of ionic strength was adjusted to 0.50 mol L^{-1} with a solution of sodium chloride (4 mol L^{-1}). Solutions of the desired pH and ionic strength of 0.50 mol L^{-1} were heated to the required temperatures and than a sample of HMD was added. The initial concentration of HMD was 0.2 mg mL^{-1}. At specified time intervals, determined by the rate of degradation, samples of the solutions (0.50 mL) were collected and instantly cooled in a mixture of ice and water. Samples with pH above 7.5 were neutralized by using HCl solutions at concentrations ensuring that their pH was *ca.* 2. To each such sample 0.5 mL of the internal standard solution was added. 50 μL samples of the so obtained solutions were injected onto the column. Microsoft Excel 2000 was used for the calculation of regression parameters.

RESULTS AND DISCUSSION

The method was selective for HMD in the presence of its degradation products and the internal standard (Fig. 1). In the chromatograms taken over a period of 0-12 min, the following peaks emerged: HMD, corresponding to the [(N-[(hexahydroazepin-1-yl)methyl]daunorubicin, with retention time 10.5 min; IS, corresponding to the internal standard, with retention time 2.9 min and P, corresponding to the products of HMD degradation, with retention times 0.9, 4.3, 6.2 and 8.1 min.

Observed rate constants: The degradation of HMD as a result of hydrolysis was a pseudo-first-order reaction described by the following equation:

$$\ln (A_{HMD}/A_{IS})_t = \ln (A_{HMD}/A_{IS})_0 - k_{obs}t \quad (1)$$

where: A_{HMD} and A_{IS} are the areas of the peaks of HMD and the internal standard, at time t = 0 and t, respectively. The slopes of semilogarythmic plots $\ln (A_{HMD}/A_{IS}) = f(t)$ with a negative sign were equal to the observed rate constants of the pseudo-first-order reaction of HMD degradation ($-k_{obs}$).

Buffer catalysis: To verify that k_{obs} determined at different concentration of buffer were statistically significant the parallelism test was used. In hydrochloric acid (pH 0.43-1.39), phosphate buffer (6.13-7.50), borate buffer (pH 7.70-9.75) and sodium hydroxide (pH 11.64-13.71) only the specific acid-base hydrolysis was observed ($k_{obs} = k_{pH}$). At constant pH, ionic strength and temperature, in the presence of excess buffer, the observed rate constant of degradation of HMD depend on the total concentration of phosphate (pH 1.89-3.10) and acetate (pH 4.01-5.65) buffers. The first-order rate constants k_{pH} under the conditions of general acid-base catalysis were calculated from the following equation:

$$k_{obs} = k_{pH} + k_B[B]_T$$

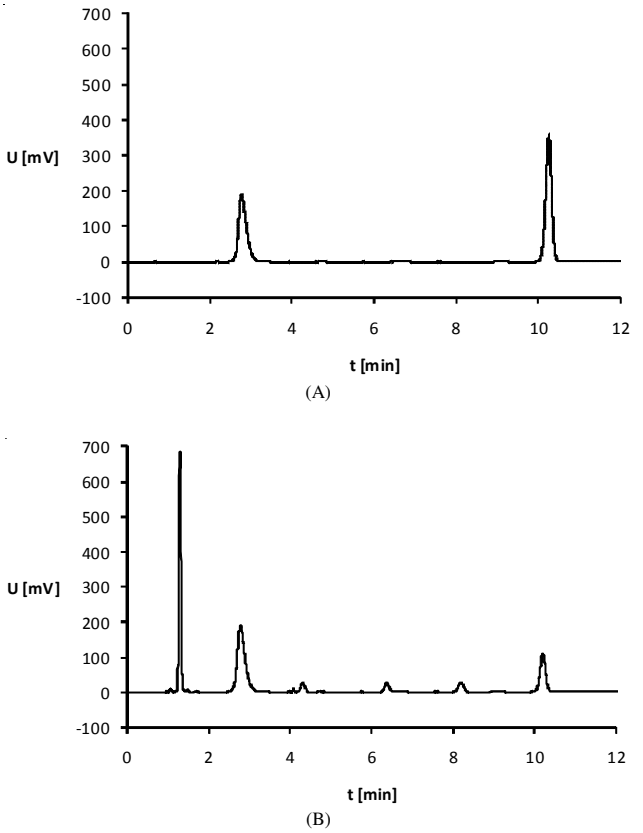

Fig. 1. HPLC chromatograms of HMD, its degradation products (P_1, P_2, P_3, P_4) and the internal standard (IS): (A) at t = 0 min; (B) at t = 50 min after degradation in borate buffer (pH = 8.85) at 333 K

where: k_{pH} = rate constant at zero buffer concentration, k_B = catalytic effect of the buffer, $[B]_T$ = total buffer concentration.

The plots $k_{obs} = f([B]_T)$ obtained for the phosphate (pH 1.89-3.10) and acetate (pH 4.01-5.65) buffers were linear and their slopes equaled k_B whereas the values of k_{obs} for $[B]_T = 0$ equaled k_{pH}.

pH-Rate profile: The values of k_{pH} were used to calculate the relationship $\log k_{pH} = f(pH)$. The semi-logarythmic relationship $k_{pH} = f(pH)$ indicates that in aqueous solutions of HMD at pH 0.43-13.71 the following reactions occur:

Hydrolysis of the protonated molecules of HMD catalyzed by hydrogen ions (k_1)

$$HMD^+ + H^+ \xrightarrow{H_2O} \text{Products}, k_1$$

Spontaneous hydrolysis of HMD under the influence of water, depending on the substrate charge

$$HMD^{+-} \xrightarrow{H_2O} \text{Products}, k_2$$

$$HMD \xrightarrow{H_2O} \text{Products}, k_3$$

$$HMD^- \xrightarrow{H_2O} \text{Products}, k_4$$

The total reaction rate is equal to the sum of the partial reaction rates:

$$k_{pH} = k_1 \times a_{H^+} \times f_1 + k_2 \times f_2 + k_3 \times f_3 + k_4 \times f_4 \quad (2)$$

where: a_{H^+} = hydrogen ion activity, f_1 - f_4 are the fractions of protonated molecules, zwitter ions, unprotonated molecules and monoanions of HMD. The values f_1 - f_4 were calculated using the values of pK_a of HMD that were:

$$303\ K: pK_{a_1} = 6,4;\ pK_{a_2} = 7,7;\ pK_{a_3} = 11,6$$

$$313\ K: pK_{a_1} = 6,3;\ pK_{a_2} = 7,7;\ pK_{a_3} = 11,4$$

$$323\ K: pK_{a_1} = 6,2;\ pK_{a_2} = 7,7;\ pK_{a_3} = 11,5$$

$$333\ K: pK_{a_1} = 6,5;\ pK_{a_2} = 7,7;\ pK_{a_3} = 10,5$$

The catalytic rate constants k_1 were calculated from the equation $k_{pH} = k_1 \times a_{H^+} f_1$. The plots $k_{pH} = f(a_{H^+})$ were linear, with a positive slope equal to k_1.

The catalytic rate constants k_4 were calculated as the mean values of k_{pH} at pH above 12. The catalytic rate constants k_2 were calculated from the dependence $k'_{pH} = f(f_2)$ $k'_{pH} = k_{pH} - (k_1 \times a_{H^+} \times f_1 + k_4 \times f_4)$ at the pH range 4.16-6.22, where $f_2 \rightarrow 1$.

The values of k'_{pH} for $f_2 = 1$ were equal to k_2. The catalytic rate constants k_3 were calculated as the mean values of k''_{pH} ($k''_{pH} = k_{pH} - (k_1 a_H + f_1 + k_2 f_2 + k_4 f_4)$ at the pH range 6.87-9.43, where $f_3 \rightarrow 1$.

The consistency between theoretical profile calculated from eqn. 2 and the experimental results indicates that the equation is correct (Fig. 2).

Influence of temperature: The catalytic rate constants were used to calculate the slope (a) of the plots $\ln k_i = f(1/T)$ and the values of ln A (A-frequency coefficient) for the partial reactions ($\ln k_i = \ln A - a(1/T)$; the Arrhenius relationship). These values were used to determine the energy of activation, enthalpy and entropy (Table-1).

The lowest energy of activation was observed in the reaction

$$HMD^- \xrightarrow{H_2O} \text{Products}, k_4$$

The entropy of the reactions of the unprotonated molecules and monoanions of HMD under the influence of water was negative, which may indicate the bimolecular character of these reactions.

Influence of ionic strength: In hydrochloric acid (0.10 mol L^{-1}, 333 K) rate constant increase linearly with increasing ionic strength. It confirms reaction between protonated molecules in this pH range.

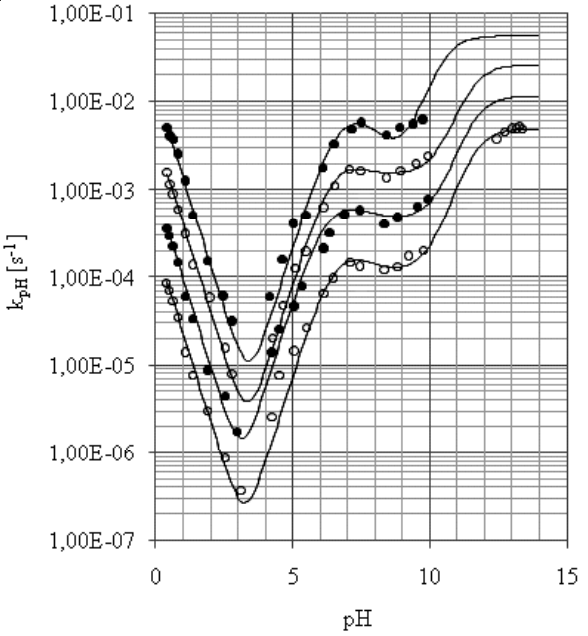

Fig. 2. pH-Rate profile for the degradation of HMD at 303, 313, 323 and 333 K. The points determined experimentally and the lines calculated from eqn. 2

In sodium hydroxide solution (0.05 mol L^{-1}, 293 K) none influences of the ionic strength could be observed. It confirms spontaneous hydrolysis of HMD under the influence of water in this pH range.

Conclusion

The hydrolysis of HMD involves hydrolysis of the protonated molecules of HMD depending on H^+ and spontaneous hydrolysis of the zwitter ions, unprotonated molecules and monoanions of HMD under the influence of water. A catalytic effect is demonstrated by the components of phosphate (pH 1.89-3.10) and acetate (pH 4.01-5.65) buffers. The components of phosphate (6.13-7.50) and borate (pH 7.70-9.75) buffers do not effect the reaction rate. N-[(Hexahydroazepin-1-yl)methyl]daunorubicin (HMD) is the most stable at pH 2-4.

TABLE-1
KINETIC AND THERMODYNAMIC PARAMETERS FOR THE HYDROLYSIS OF HMD IN AQUEOUS SOLUTIONS

Catalytic rate constants	Temperature (K)	k_i	Statistical evaluation $\ln k_i = f(1/T)$	Thermodynamic parameters
k_1 ($mol^{-1}\ s^{-1}$)	303	2.36×10^{-4}	r = -0.9999 a = -13871 ± 724 b = 37.4 ± 2.3	E_a = 115.3 ± 6.0 (kJ mol^{-1}) $\Delta H^{\neq}$ = 112.9 ± 8.5 (kJ mol^{-1}) $\Delta S^{\neq}$ = 66.3 ± 225.9 (J K^{-1} mol^{-1})
	313	1.00×10^{-3}		
	323	4.21×10^{-3}		
	333	1.43×10^{-2}		
k_2 (s^{-1})	303	1.94×10^{-4}	r = -0.9999 a = -12064 ± 793 b = 31.3 ± 2.5	E_a = 100.3 ± 6.6 (mol^{-1}) $\Delta H^{\neq}$ = 97.8 ± 9.1 (kJ mol^{-1}) $\Delta S^{\neq}$ =15.0 ± 224.1 (J K^{-1} mol^{-1})
	313	6.89×10^{-4}		
	323	2.16×10^{-3}		
	333	7.16×10^{-3}		
k_3 (s^{-1})	303	2.12×10^{-4}	r = -0.9906 a = -8616 ± 3619 b = 20.0 ± 11.4	E_a = 86.48 ± 46.13 (kJ mol^{-1}) $\Delta H^{\neq}$ = 84.0 ± 48.1 (kJ mol^{-1}) $\Delta S^{\neq}$ = 33.3 ± 99.6 (J K^{-1} mol^{-1})
	313	4.74×10^{-4}		
	333	1.53×10^{-3}		
	343	2.46×10^{-3}		
k_4 (s^{-1})	303	4.92×10^{-3}	r = -0.9999 a = -8194 ± 13 b = 21.73 ± 0.04	E_a = 67.94 ± 0.46 (kJ mol^{-1}) $\Delta H^{\neq}$ = 65.4 ± 2.9 (kJ mol^{-1}) $\Delta S^{\neq}$ = 64.8 ± 243.5 (J K^{-1} mol^{-1})
	313	*1.17×10^{-2}		
	323	*2.62×10^{-2}		
	333	*5.63×10^{-2}		

$\Delta H^{\neq}$ and $\Delta S^{\neq}$ were calculated for 298 K, *Value extrapolated, $E_a = -aR$ (J mol^{-1}); $\Delta H^{\neq} = E_a - RT$ (J mol^{-1}); $\Delta S^{\neq} = R(\ln A - \ln (k_B T)/h$ (J K^{-1} mol^{-1}), where: k_B = Boltzmann constant ($1.3807\ 10^{-23}$ J K^{-1}); h = Planck constant ($6.626\ 10^{-34}$ Js); R = universal gas constant (8.314 J K^{-1} mol^{-1}); T = temperature in K; a = vectorial coefficient of the Arrhenius relationship; A = frequency coefficient.

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