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STABILITY OF [(*N*-PIPERIDINE)METHYLENE]DAUNORUBICIN HYDROCHLORIDE AND [(*N*-PYRROLIDINE)METHYLENE]DAUNORUBICIN HYDROCHLORIDE IN SOLID STATE

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Abstract: The influence of temperature and relative air humidity on the stability of two novel derivatives of daunorubicin: [(*N*-piperidine)methylene]daunorubicin hydrochloride and [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride was investigated. The process of degradation was studied by using high-performance liquid chromatography with ultraviolet (UV) detection. The kinetic and thermodynamic parameters of degradation were calculated.

Keywords: [(*N*-piperidine)methylene]daunorubicin hydrochloride, [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride, stability in solid state, kinetic and thermodynamic parameters

Anthracycline antibiotics are compounds of a glycoside structure, containing sugar and aglycone moieties (Fig. 1). The sugar is usually daunozamine, an aminosugar of the hexoses group. The aglycone moiety consists of four six-carbon rings, where rings B and D are aromatic and ring C is quinone. Ring A contains a small substituent with the carbonyl group. Anthracyclines are a group of anticancer drugs with an established position in the treatment of malignant neoplasms. The mechanism of action of these antibiotics involves:

- direct intercalation of DNA, resulting in the biosynthesis of macromolecular stoppage,
- induction of oxidative stress inside cells by generating free radicals,
- combining with DNA and its alkylation,
- cross-linking of DNA strands,
- interrupting the DNA-helicase activity,
- direct impact on cell membranes and interruption of their activity,
- induction of DNA damage and apoptosis with topoisomerase II activity stoppage.

Each of these processes is closely connected with the structure of anthracyclines. The compact inner arrangement of the aglycone rings is reflected by their flat spatial structure. That is especially typical of rings B, C and D. This is crucially important for the intercalation of DNA by anthracyclines. The flat structure of the rings allows them to penetrate between the two DNA strands and react with the bases. Anthracyclines mainly react with cytosine and guanine. The remaining parts of the anthracycline molecule, the sugar and the cyclohexane ring A, do not penetrate the DNA strands but stay on the outside of the double helix. Their role is to stabilize the newly created DNA-anthracycline complex. That happens thanks to the electrostatic interaction between the antibiotic molecule and the outside parts of the nucleotides or through cross-linking between the sugar moiety and the nucleotides. According to the latest research, it is the part of the anthracycline molecule, which does not penetrate DNA, that has the most significant role in DNA intercalation.

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The main disadvantage of anthracyclines is their general toxicity, especially cardiotoxicity. Therefore, while looking for new derivatives, it is important to maintain the antitumor activity of the base drug and to ensure lower general toxicity. The complex structure of anthracyclines allows many modifications, such as the introduction of a new substituent, the modification of the existing ones or the development of selective stereoisomers of given compounds. Notably, chemical modifications can include both the aglycone and the sugar moiety. Many novel anthracycline derivatives modified in the sugar moiety have been synthesized. It was proven that a group of 3'-formamide-substituted daunorubicin derivatives show a much lower tendency to produce free radicals. Consequently, their similar antitumor activity to that of daunorubicin is combined with lower toxicity to the healthy cells of the body (1–3).

[(*N*-piperidine)methylene]daunorubicin hydrochloride (PIP) and [(*N*-pyrrolidine)-methylene]daunorubicin hydrochloride (PYR) were obtained by the replacement of the primary amino group at C-3' of the daunosamine moiety in daunorubicin hydrochloride by an amidine substituent containing a piperidine ring or a pyrrolidine ring, respectively (Fig. 1) (3).

Previous studies proved that daunorubicin hydrochloride and its new derivatives are vulnerable to degradation in the solid state (4, 5) in aqueous (6–9) and in intravenous (10) solutions.

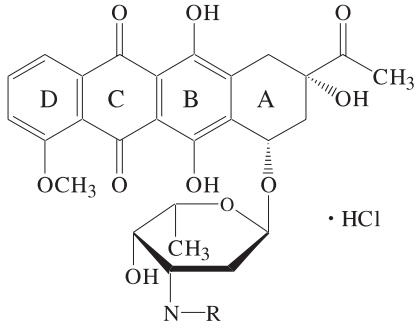
In the solid state, the degradation of daunorubicin hydrochloride (DAU) and its amidine derivative [(N-morpholine)metylene]daunorubicin hydrochloride (MOR) (Fig. 1) at an increased temperature and relative air humidity (RH > 50%) was a first-order autocatalytic reaction relative to DAU or MOR concentration (4, 5).

In aqueous solutions, in the pH range 0.45–13.08, the degradation of daunorubicin hydrochloride and its derivatives is a pseudo-first order reaction (6–9). For the daunorubicin hydrochloride amidine derivatives the reactions of protonated molecules catalyzed by hydrogen ions occurred at a similar rate but significant differences in the degradation rate were observed in spontaneous hydrolysis under the influence of water. In the pH range from 0.5 to 13.1, daunorubicin hydrochloride was more stable than its amidine derivatives and demonstrated the greatest stability in the pH range from 4 to 6. Its amidine derivatives are the most stable at pH ≈ 3. At pH < 4 DAU degraded to aglycone daunorubicinone and amino sugar daunosamine. Under more stressful conditions and in an alkali environment aglycone is degraded to more simple structures (11).

The photodegradation of the daunorubicin hydrochloride derivatives and epirubicin in solution is a pseudo-first-order reaction, which depends on substrate concentration (9, 12). The products of photodegradation at a wavelength of 365 nm were red and colorless at 510 nm.

All of the previous studies demonstrated that the differences in the chemical structures of the daunorubicin hydrochloride derivatives did not influence their stability or the kinetic mechanism of their degradation.

The aim of this study was to determine the effect of temperature at RH ≈ 76.4% on [(*N*-piperidine)methylene]daunorubicin hydrochloride (PIP) and [(*N*-pyrrolidine)methylene]-daunorubicin hydrochloride (PYR) and to evaluate their stability at 373 K in dry air (0% RH).



Derivative	R
DAU	H_2
PYR	=CH–N
PIP	=CH–N
MOR	=CH–N O

Figure 1. The chemical structure of daunorubicin hydrochloride (DAU), [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride (PYR), [(*N*-piperidine)methylene]daunorubicin hydrochloride (PIP) and [(*N*-morpholine)methylene]daunorubicin hydrochloride (MOR)

EXPERIMENTAL

Chemicals, reagents and solutions

[(*N*-piperidine)methylene]daunorubicin hydrochloride (PIP) and [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride (PYR) were obtained from the Institute of Biotechnology and Antibiotics in Warszawa. Quinidine hydrochloride was used as an internal standard. All other chemical substances and solvents were the products of Sigma and were of analytical or high-performance liquid chromatographic grade. High-quality pure water was prepared by using a Millipore purification system (Exil SA 67120, Millipore, Molsheim, France).

Instrumentation

The chromatographic apparatus consisted of an LC-61 isocratic pump, an SPD-6AV UV-Vis detector set at 254 nm (Shimadzu) and Rheodyne Berkeley 7120 injector with a 25 µL loop. Separations were performed on a LiChrospher 100-RP 18 column (250 × 4 mm; 5 µm particle size; Merck).

Chromatographic conditions

Chromatographic separations and quantitative analysis were performed by using an HPLC method (13, 14).

The mobile phase consisted of a mixture of acetonitrile and water (50 : 50, v/v) with the addition of 2.88 g/L sodium lauryl sulfate and 1.4 mL/L phosphoric acid (V) (1.42 g/mL). The flow rate was 1.5 mL/min. The internal standard was a solution of quinidine hydrochloride (0.100 g/mL). All chromatographic procedures were conducted at ambient temperature.

Conditions of kinetic studies

For the experiments, 0.005 g samples of PIP and PYR were weighed into 5 mL vials. The samples of the substances tested for the influence of temperature in a humid environment were inserted in desiccators containing saturated solutions of sodium chloride (≈ 76.4% RH). These samples were placed in heat chambers set to the desired temperatures: 343, 353, 363 and 373 K.

To evaluate the stability of PIP and PYR in dry air, the vials containing 0.005 g of these substances were immersed in sand bath placed in the heat chambers at 373 K.

Each batch to be studied comprised 10–15 samples. At specific time intervals, determined by the rate of degradation, the vials were removed, cooled to room temperature and the contents dissolved in a mixture of acetonitrile and water (1 : 1 v/v). The resultant solutions were quantitatively transferred into volumetric flasks and completed to a total volume of 10.0 mL with the same mixture of solvents. To 1.0 mL of the resultant solution (after filtration) 1.0 mL of the internal standard solution was added. Samples (25 µL) were injected onto the column.

Calculations

The rates constant of a first-order reaction were calculated from:

$$\ln c = \ln c_0 - k_{obs} \times t \qquad \text{(equation 1)}$$

where c_0 and c are concentrations at time t = 0 and t, respectively, and k_{obs} is the observed rate constant of degradation, while the rates constant of a first-order autocatalytic reaction relative to the substrate concentration were calculated from:

$$\ln c_t/(c_0 - c_t) = -k_{obs} \times t + g \qquad \text{(equation 2)}$$

where c_0 and c_t are substrate concentrations at t_0 and t; $c_0 - c_t$ are product concentrations at time t; g is a constant related to the induction time and k_{obs} is the observed rate constant of degradation (15).

Thermodynamic parameters (E_a, activation energy; $\Delta H^{\neq}$, enthalpy; $\Delta S^{\neq}$, entropy) were calculated from:

$$E_a = -a \times R \qquad \text{(equation 3)}$$

$$\Delta H^{\neq} = E_a - T \times R \qquad \text{(equation 4)}$$

$$\Delta S^{\neq} = R \times (\ln A - \ln (k_B \times T/h)) \qquad \text{(equation 5)}$$

where: k_B = Boltzmann's constant (1.3807×10^{-23} J × K^{-1}); h = Planck's constant (6.626×10^{-34} J × s); R = universal gas constant (8.314 $K^{-1} \times mol^{-1}$), T = temperature [K]; a = slope of the Arrhenius relationship; A = frequency coefficient where: (ln A = b) (15).

Statistical parameters of the respective equations were calculated using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Kinetics of degradation of PIP and PYR

The degradation of PIP at an increased temperature and ≈ 76% RH was a first-order reaction relative to the substrate concentration (Fig. 2) and the rate constants were calculated from equation 1.

The degradation of PYR in the same environment was a first-order autocatalytic reaction relative to the substrate concentration (Fig. 3) and the rate constants of this reaction were calculated from equation 2.

The semilogarithmic plots $c_t/(c_0 - c_t) = f(t)$ were straight lines and their slopes corresponded to the rate constants of the reaction ($-k_{obs}$).

The degradation of PIP at an increased temperature and 0% RH was a first-order autocatalytic

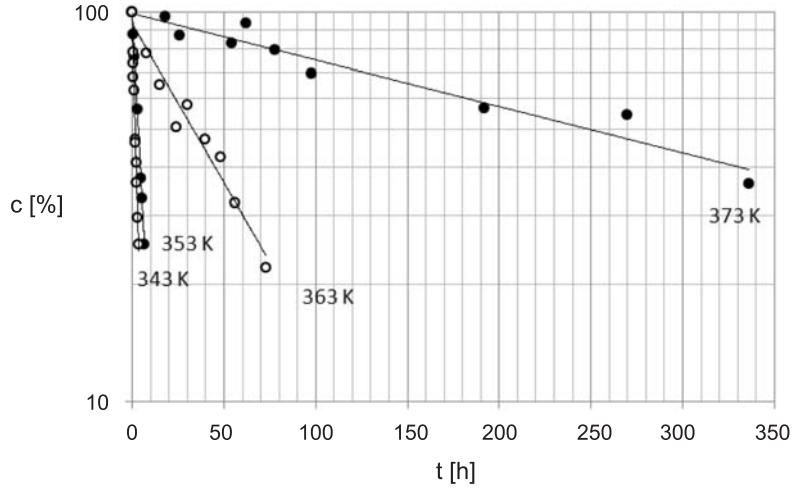


Figure 2. Semilogarithmic plots of ln c[%] = f(t) for the degradation of [(*N*-piperidine)methylene]daunorubicin hydrochloride (PIP) at RH ≈76.4%

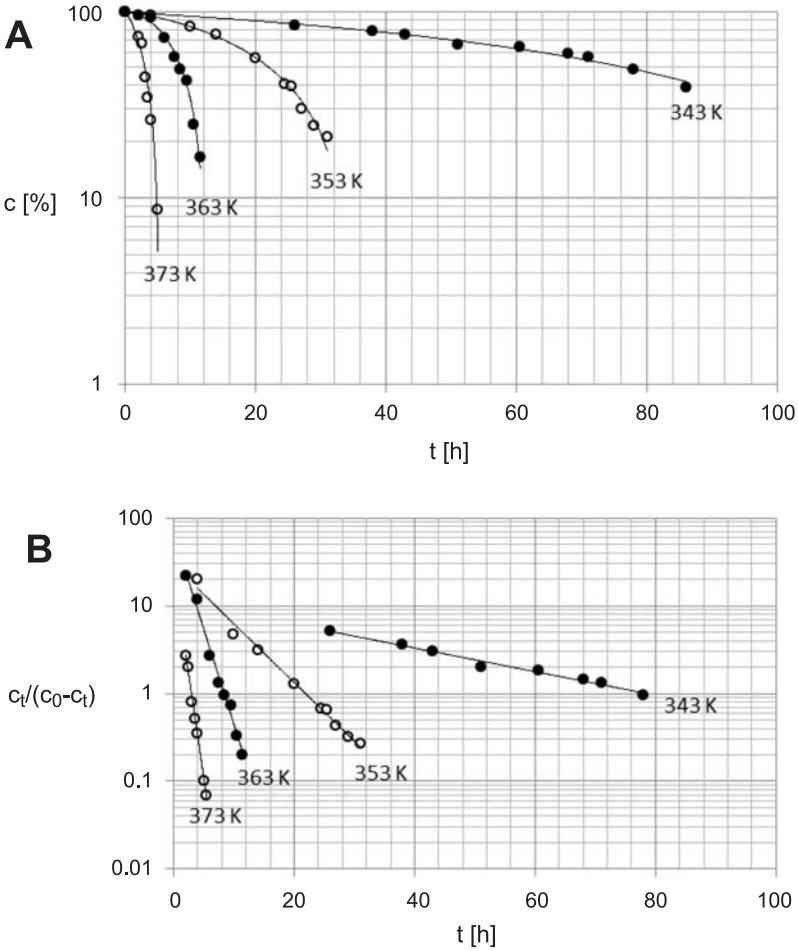


Figure 3. Semilogarithmic plots of ln c[%] = f(t) (**A**) and ln $c_t/(c_0 - c_t)$ = f(t) (**B**) for the degradation of [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride (PYR) at RH ≈76.4%

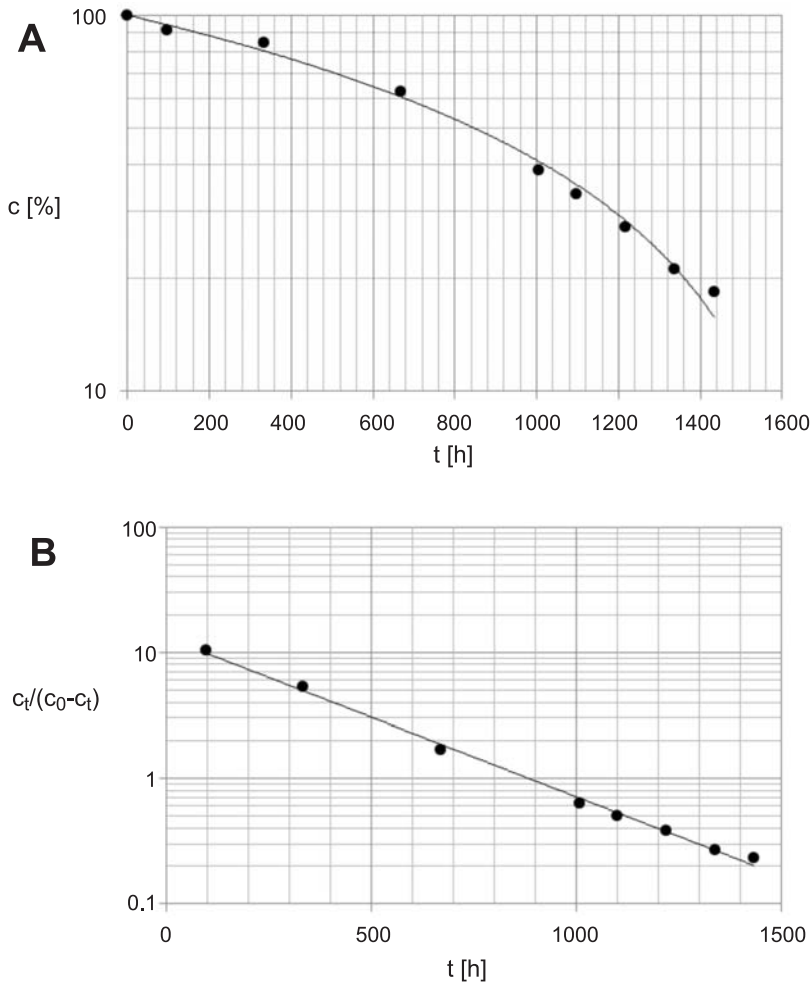


Figure 4. Semilogarithmic plots of ln c[%] = f(t) (**A**) and ln $c_t/(c_0 - c_t)$ = f(t) (**B**) for the degradation of [(*N*-piperidine)methylene]daunorubicin hydrochloride (PIP) at 373 K and RH = 0%

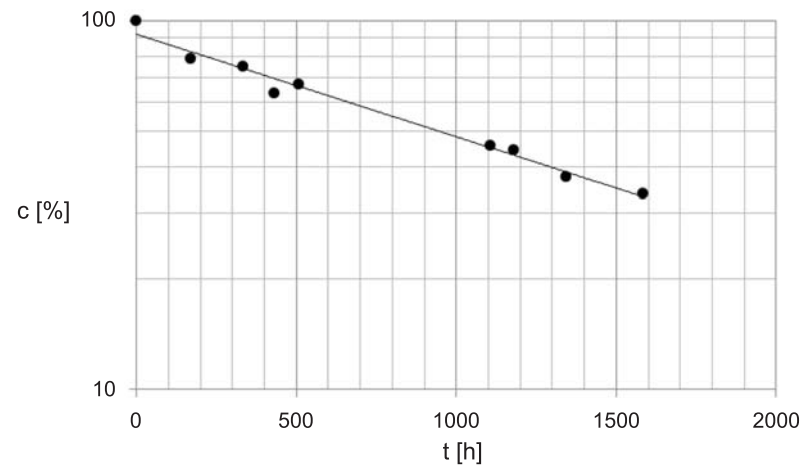


Figure 5. Semilogarithmic plot of ln c[%] = f(t) for the degradation of [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride (PYR) at 373 K and RH = 0%

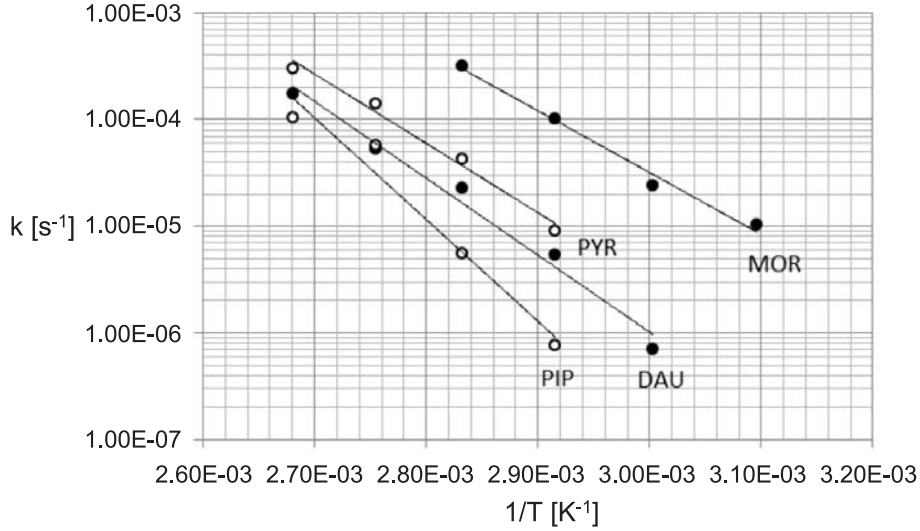


Figure 6. Semilogarithmic plot of ln k[s^{-1}] = f(1/T) [K^{-1}] for the degradation of daunorubicin hydrochloride (DAU) and its amidine derivatives [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride (PYR) [(N-piperidine)methylene]daunorubicin hydrochloride (PIP) and [(N-morpholine)methylene]daunorubicin hydrochloride (MOR) at RH ≈ 76.4%

Table 1. Kinetic and thermodynamic parameters of the degradation of daunorubicin hydrochloride (DAU) and its amidine derivatives [(*N*-pyrrolidine)methylene]daunorubicin hydrochloride (PYR) [(N-piperidine)methylene]daunorubicin hydrochloride (PIP) and [(N-morpholine)methylene]daunorubicin hydrochloride (MOR) in solid state at constant relative air humidity (RH = 76.4%).

T [K]	$(k_i \pm \Delta k) \times 10^5$ (s^{-1})	Statistical evaluation of ln $k_i = f(T^{-1})$	Thermodynamic parameters
[(*N*-piperidine)methylene]daunorubicin hydrochloride (PIP)			
343	0.0762 ± 0.015	a = -21950 ± 13232 S_a = 3075 b = 50.1 ± 37.0 S_b = 8.60 r = - 0.981	E_a = 182.5 ± 110.0 [kJ mol^{-1}] $\Delta H^{\neq}$ = 180.0 ± 110.0 [kJ mol^{-1}]* $\Delta S^{\neq}$ = 171.5 ± 62.8 [JK^{-1} mol^{-1}]*
353	0.564 ± 0.062		
363	5.68 ± 0.51		
373	10.4 ± 0.9		
[(*N*-pyrrolidine)methylene]daunorubicin hydrochloride (PYR)			
343	0.910 ± 0.113	a = -14647 ± 4145 S_a = 963.5 b = 31.3 ± 11.6 S_b = 2.69 r = - 0.996	E_a = 121.8 ± 34.5 [kJ mol^{-1}] $\Delta H^{\neq}$ = 119.3 ± 34.5 [kJ mol^{-1}]* $\Delta S^{\neq}$ = 15.3 ± 148.5 [JK^{-1} mol^{-1})]*
353	4.27 ± 0.39		
363	13.9 ± 1.5		
373	29.9 ± 3.1		
[(*N*-morpholine)methylene]daunorubicin hydrochloride (MOR) (10)			
323	1.03 ± 0.23	*a* = -13316 ± 4639 S_a = 1078 *b* = 29.6 ± 13.7 S_b = 3.19 r = 0.9935	E_a = 110 ± 39 [kJ mol^{-1}] $\Delta H^{\neq}$ = 108 ± 39 [kJ mol^{-1}]* $\Delta S^{\neq}$ = 1 ± 130 [JK^{-1} mol^{-1}]*
333	2.37 ± 0.25		
343	10.1 ± 0.7		
363	31.6 ± 0.9		
Daunorubicin hydrochloride (DAU) (4)			
333	0.071 ± 0.008	*a* = -16581 ± 3972 S_a = 1248 *b* = 35.94 ± 11.3 S_b = 3.5 r = 0.9916	E_a = 138 ± 33 [kJ mol^{-1}] $\Delta H^{\neq}$ = 135 ± 33 [kJ mol^{-1}]* $\Delta S^{\neq}$ = -149 ± 203 [JK^{-1} mol^{-1}]*
343	0.53 ± 0.07		
353	2.26 ± 0.38		
363	5.25 ± 1.95		
373	17.5 ± 0.2		

E_a = activation energy; $\Delta H^{\neq}$ = enthalpy; $\Delta S^{\neq}$ = entropy were calculated from equations 3, 4 and 5, respectively. * calculated for 298 K.

reaction relative to the substrate concentration (Fig. 4), whereas the degradation of PYR in dry air was a first-order reaction relative to the substrate concentration (Fig. 5).

For the interpretation of the straight line plots $\ln c_t/(c_0 - c_t) = f(t)$ and $\ln c_t = f(t)$ such statistical parameters as slope (a), error range of slope (Δa) , intercept (b), error range of intercept (Δb), standard deviations s_a, s_b, s_y and the coefficient of linear correlation (r) were calculated by using the least squares method. The values of Δa and Δb were obtained for f = n – 2 degrees of freedom, with $\alpha = 0.05$. Statistical parameters of the respective equations were calculated using Microsoft Excel 2010.

The values of reaction rate constants k_{obs} were used to calculate the Arrhenius relationship in order to interpret the influence of the temperature on the reaction rate at ≈ 76.4% RH. The energy, enthalpy and entropy of activation for 298 K were calculated based on the parameters of the slope $\ln k_i = f(T^{-1})$ (Table 1). The influence of temperature on the stability of PIP and PYR was described as:

$$\ln k_{PIP} = (2.19 \pm 1.32)10^4 T^{-1} - (50.12 \pm 37.03)$$

$$\ln k_{PYR} = (1.47 \pm 0.41)10^4 T^{-1} - (31.32 \pm 11.63)$$

The slope *a* expresses the effect of temperature on the stability of PIP and PYR in the solid state (Table 1). After comparing the plots $\ln k = f(1/T)$ a parallelism test proved that the influence of temperature on the rates degradation of PIP, PIR, MOR (5) and DAU (4) did not show any statistically significant differences. Because the kinetic mechanisms of degradation of DAU and its derivatives differed, the values $t_{0.5}$ were used to compare their stability, which could be ordered as follows: DAU > PIP > PIR > MOR.

Although the energy of activation of PIP, PYR, DAU and MOR obtained at an increased temperature did not show statistically significant differences, the stability of daunorubicin hydrochloride and its three derivatives were compared as follows: PIP > DAU > PYR > MOR.

The rate constants of PIP and PYR degradation at 373 K and 76.4% RH were $(1.04 \pm 0.09)10^{-4}$ s^{-1} and $(2.99 \pm 0.31)10^{-4}$ s^{-1}, respectively, while at 373 K and 0% RH they were $(8.08 \pm 0.48)10^{-7}$ s^{-1} and $(1.78 \pm 0.21)10^{-7}$ s^{-1}, respectively, which demonstrated that increased relative air humidity determined their degradation.

CONCLUSIONS

The degradation of PIP was a first-order reaction relative to the substrate concentration at an increased temperature and relative air humidity, whereas at an increased temperature and 0% RH it was a first-order autocatalytic reaction relative to the substrate concentration. The degradation of PYR, DAU and MOR was a first-order autocatalytic reaction relative to the substrate concentration at increased temperature and ≈76.4% RH and it was a first-order reaction relative to the substrate concentration in dry air.

The study demonstrated that the kinetic mechanism of the degradation of the derivatives of daunorubicin hydrochloride depends on storage conditions. The influence of relative air humidity on the stability of the studied substances indicated that relative air humidity determines the rate and mechanism of their degradation. The stability of daunorubicin hydrochloride and its amidine derivatives is similar in the solid state. It is otherwise known that the amidine derivatives of DAU have superior pharmacological properties, especially by demonstrating lower cardiotoxicity compared to their parent compound. Of the derivatives studied in this work, the greatest antiproliferative activity was shown by MOR; however, this derivative exhibits the lowest stability in solid state (5) and in aqueous solutions (8).

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