A COMPARATIVE STUDY OF THE KINETICS OF CYTARABINE HYDROLYTIC DEAMINATION IN AQUEOUS SOLUTIONS

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Cytarabine (1-β-*D*-arabinofuranosylcytosine, I) is a drug belonging to the group of nucleoside antimetabolites [1]. At present, the drug is produced abroad both in dry (lyophilized) and liquid forms. The production of liquid forms is less labor-consuming and more economically effective. At the same time, it was reported [2 – 8] that cytarabine is subject to hydrolytic deamination in aqueous media with the formation of a therapeutically inactive 1-β-*D*-arabinosyluracil (II) and ammonia (Fig. 1).

Our task was to study the stability of cytarabine in aqueous solutions during a preset storage time. For this purpose, it was necessary to select a method that would be (i) capable of studying the kinetics and rate of the cytarabine hydrolytic deamination reaction and (ii) sufficiently simple and optimum from the standpoint of sensitivity and reproducibility.

Various methods were developed for determination of the content of I in medicinal preparations and for the study of hydrolytic deamination of this compound. These include TLC [9], RIA [10 – 13], mass spectrometry [14], gas chromatography in various modes, including those combined with other techniques [15, 16], UV spectrophotometry [17], extraction methods [18], and liquid chromatography at low [19] and high pressures [20 – 35].

The method of direct UV spectrophotometry [17] is based on the difference between the absorption spectra of I and its decomposition product II (having absorption peaks at 280 and 260 nm, respectively, in 0.1 N HCl). This technique is most simple and was widely used in the early investigations of the hydrolytic deamination reaction [2 – 4, 6 – 8].

In recent years, compounds I and II are more frequently determined by HPLC techniques offering some advantages over the other methods. Compounds belonging to arabinosyl nucleosides were predominantly studied by inversed-phase HPLC (IPHPLC) [20 – 22], although ion-exchange HPLC [23] and combined techniques can be used as well [27].

In application to nucleosides, IPHPLC can be used in several different modes [36], including chromatography under socratic conditions (most frequently used for the analysis of arabinosyl nucleosides) [20 – 22], gradient-elution chromatography, and ion-pair chromatography [29, 34, 35].

As is known [3], the maximum extinction coefficient of cytosine nucleosides is observed at low pH. Therefore, using these conditions we may reach a maximum sensitivity of spectrophotometric detection. At the same time, all the molecules of cytosine nucleosides studied under these conditions are ionized and, hence, exhibit weak adsorption on the immobile (nonpolar) phase that results in lower resolution. Thus, optimum possibilities for controlling the interaction of partitioned components with the immobile phase are provided by the method of ion-pair chromatography.

MATERIALS AND METHODS

Cytarabine. 99.4% purity; Pharmacopoeial Clause FS 42-2982 – 93; produced by the experimental plant at the Institute of Biological and Organic Chemistry, Academy of Sciences of the Republic of Belarus, Belarus.

UV spectrophotometric determination of compounds I and II [3, 4]. Using the optical density of a sample determined at 260 and 280 nm, the concentrations of compounds I and II are calculated by the following formulas:

$$C_{\mathrm{I}} = (0.927D_{280} - 0.389D_{260}) \times 10^{-4},$$

$$C_{\mathrm{II}} = (1.21D_{260} - 0.546D_{280}) \times 10^{-4},$$

where C_{I} and C_{II} are the concentrations of I and II in solution (mole/liter); D_{260} and D_{280} are the optical densities at 260 and 280 nm, respectively.

The initial solutions were diluted 2000 times with 0.1 M hydrochloric acid solution. The samples were measured to determine the optical densities at 260 and 280 nm, with reference to pure 0.1 M HCl solution. The measurements were performed in 10-mm-thick quartz cells on an SF-46 spectrophotometer (LOMO, Russia),

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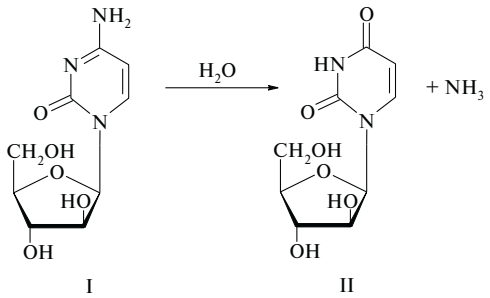

Fig. 1. Hydrolytic deamination of cytarabine (arabinosylcytosine).

IPHPLC determination of compounds I and II. The HPLC measurements were performed in a system equipped with a Model 64 high-pressure pump, Model 50 temperature programmer, variable wavelength monitor, and D-1000 injector with a 10 μl loop (all Knauer, Germany). The chromatograms were recorded with an LKB TZ-4620 line recorder (Czech Republic) and processed using a Chromatopac C-R3A integrator (Shimadzu, Japan). Sorbents and columns: Sepharon SGX C_{18} (5 μm), 150 × 4 mm; Econosil C_{18} 5U (5 μm), 250 × 4.6 mm; Hypersil ODS (6 μm), 250 × 3 mm. The HPLC analyses were performed at a flow rate of 1 ml/min. Mobile phase: trifluoroacetic acid (TFA) – water (1 : 99), pH 1.31. Prior to analysis, the system was ultrasonically outgassed at low vacuum in a Bandelin Sonorex TK52 setup (Knauer, Germany). The useful signals were detected at 260 nm.

Before sample application, the HPLC system was washed with the mobile phase for 20 min. The sample volume was 10 μl. The sample solutions were prepared by 20-fold dilution of the initial liquid preparation with distilled water.

The concentrations of I and II were determined using a column filled with Sepharon SGX C_{18}. The corresponding calibration plots were constructed [in peak area (mV × sec) versus concentration coordinates] for aqueous solutions of pure compounds I (concentration interval, 0 – 2 mg/ml) and II (0 – 0.16 mg/ml). The results of the calibration measurements were approximated using the least squares method by linear equations of the type

$$I = a_1 \times C,$$

where I is the integral peak intensity (mV × sec) and C is the component concentration in solution (mg/liter). Upon data processing, we obtained the following coefficients: $a_1 = 0.0248$ ($r^2 = 0.998$) for I and $a_1 = 0.0345$ ($r^2 = 0.999$) for II.

TABLE 1. The Parameters of Cytarabine Hydrolytic Deamination Kinetics Determined by Various Methods

Method	$k \times 10^{-3}$, h^{-1}	CV(%)	r^2
Loss of I (HPLC)	0.501	5.243	0.963
Loss of I (spectroscopy)	0.444	5.698	0.958
Formation of II (HPLC)	0.138	6.419	0.910
Loss of I (ammonia)	0.645	2.916	0.988

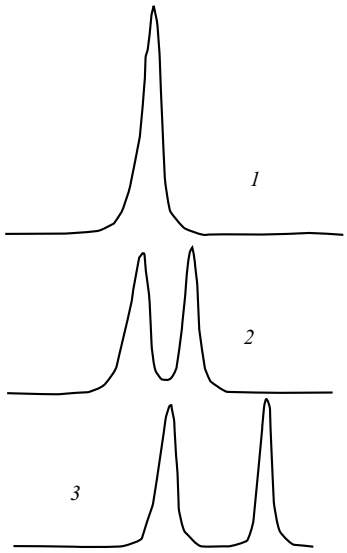

Fig. 2. Chromatograms of a mixture of compounds I and II on Sepharon SGX C_{18} (5 μm) sorbent eluted in a TFA – water system with various component ratios: (*1*) 0.25 : 100; (*2*) 0.5 : 100; (*3*) 1 : 100.

The sample concentrations were calculated by the formula

$$C = \frac{I \times 20}{a_1 \times 1000},$$

where C is the concentration of cytarabine (arabinosyluracil) in the sample (g/liter), I is the integral HPLC peak intensity (mV × sec), a_1 is the corresponding calibration coefficient, and 20 is the sample dilution factor.

Determination of ammonia. The content of ammonia was determined by a colorimetric method based on a reaction with the Nessler reagent. To 5 ml of a sample (diluted 50 – 200 times with distilled water) were added 0.25 ml of the Nessler reagent. The mixture was thoroughly stirred, allowed to stand for 10 min, and measured in the spectrophotometer at 400 nm against the blank solution. The corresponding calibration plot was constructed for ammonium sulfate (reagent grade) solutions.

The concentration of ammonia in the sample (mmole/liter) was calculated by the formula

$$C = a \times 0.298 \times D,$$

where D is the optical density at 400 nm, a is the sample dilution factor, and 0.298 is the coefficient determined for the calibration plot, which was linear in the 0.040 – 0.200 mmole/liter concentration interval.

Cytarabine decomposition kinetics. The kinetics of cytarabine decomposition (deamination) was studied in drug solution with a concentration of 20 g/liter and a pH adjusted to 7.0 ± 0.2 with 0.01 M NaOH solution. The sample solu-

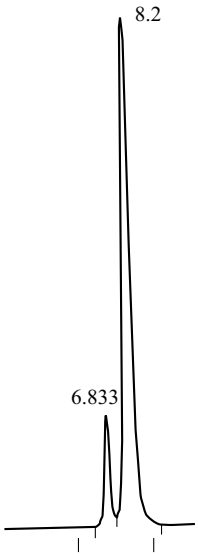

Fig. 3. A typical chromatogram of a cytarabine solution incubated at 70°C.

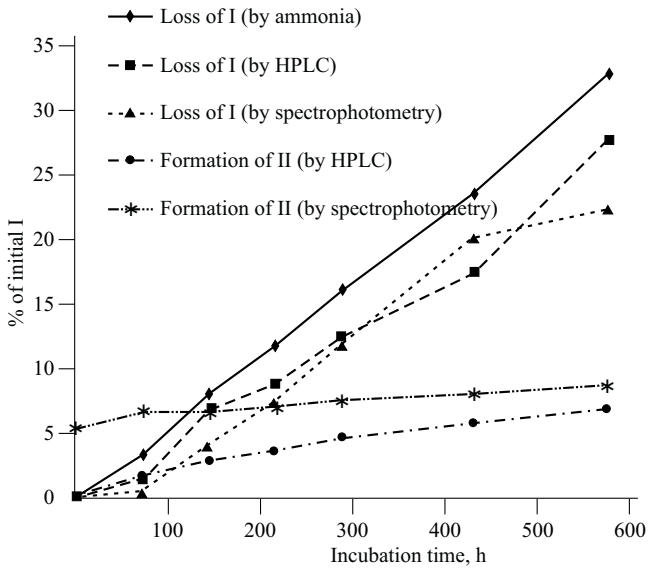

Fig. 4. Variation of the composition of an aqueous solution of cytarabine (I) with the time of incubation at 70°C by data of various methods.

tion was poured by 3-ml portions into 5-ml ampules. The ampules were sealed and incubated in a BT-120 thermostat (Czech Republic) at 70 ± 0.3°C. After incubation for a certain time, four samples were taken and analyzed for ammonia content.

RESULTS AND DISCUSSION

For comparative analysis and verification of data obtained by the direct spectrophotometric technique, we used the results of ion-pair inversed-phase HPLC measurements. The HPLC measurements were performed using a TFA – water system that is rather widely employed for ion-pair IPHPLC chemical analysis [36]. The samples were analyzed in a column filled with Sepharon SGX C_{18} sorbent. In preliminary experiments on HPLC of a I + II mixture separated using three eluents with different compositions, we selected a 1 : 99 TFA – water ratio (Figs. 2 and 3). Attempts at increasing the resolution by using different columns (with the same eluent) were unsuccessful.

According to the data reported in [2 – 8], the hydrolytic deamination of cytarabine in aqueous solutions leads to the formation of ammonia among other products (Fig. 1). We have used this fact for the first time for monitoring the reaction kinetics by measuring the ammonia concentration with the aid of the Nessler reagent.

Figure 4 shows variation of the composition of an aqueous solution of cytarabine (I) with increasing incubation time. Figure 5 presents data on the variation coefficients calculated for cytarabine hydrolytic deamination kinetics studied by various methods and plotted versus the amounts of determined substances.

The experimental data were used to calculate the rate constants for the reactions studied. According to the published data [2 – 4, 6, 8], we assumed that the hydrolytic deamination of I is a first-order reaction. The rate constants were calculated upon the least squares approximation of data by an equation of the type

$$\ln C = -k \times t,$$

where C is the current concentration of a given substance (expressed as a percentage of the initial value), t is the sample incubation time (h), and k is the reaction rate constant. The values of k calculated for all the methods employed are listed in Table 1 together with data on the relative standard errors CV (%) and correlation coefficients r^2 for the linearized experimental relationships.

As seen from the presented data, determination of the loss of compound I by the spectrophotometric method and HPLC leads to analogous results. The average variation coefficients determined for a large degree of decomposition of compound I amount to 20 and 12%, respectively. However, the error of determination exhibits a manifold increase at small decomposition levels (below 12 and 7% for the spectrophotometric method and HPLC, respectively) because the small value (the yield of deaminated I) is determined as a difference of two close values (initial and final cytarabine concentrations).

In the initial stage of cytarabine hydrolytic deamination, the reaction kinetics should be studied by determining the content of the reaction products. The HPLC technique is capable of determining the increments of II at sufficiently high precision (average variation coefficient is 7%) in the entire concentration range studied, beginning with virtually zero values. However, the rate of the formation of compound II is almost four times smaller than the rate of the loss of com-

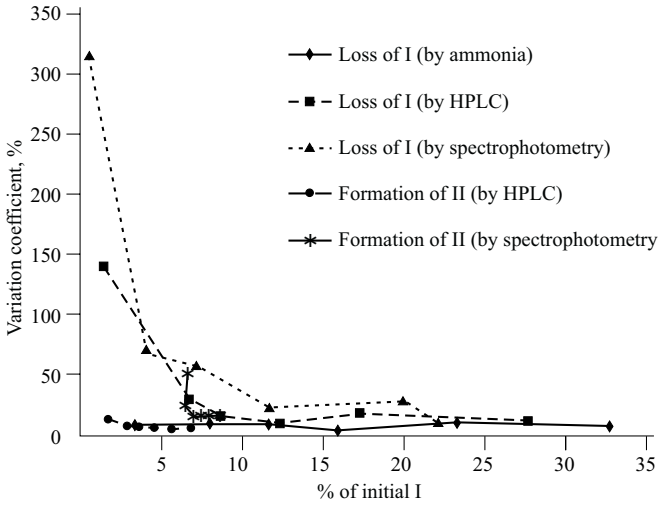

Fig. 5. Plots of the variation coefficient ($n = 4$) for cytarabine hydrolytic deamination kinetics versus the amounts of substances determined by various methods.

pound I. According to the published data [2, 4, 5], we may suggest that compound II exhibits subsequent conversion with the formation of optically inactive products. This is confirmed by the fact that the increment of II exhibits a tendency to slow down, whereas the cytarabine deamination proceeds linearly (or even slightly increases). For this reason, we may conclude that variation of the content of compound II does not adequately reflect the kinetics of the hydrolytic deamination of cytarabine.

The spectrophotometric determination of II in a mixture with I by the method described above also gives results that are rather inconsistent with the HPLC data.

However, the increment of ammonia – another product of hydrolytic deamination – is well correlated with the degree of cytarabine deamination. Determination of the hydrolytic deamination kinetics by measuring the ammonia increment is sufficiently precise (the variation coefficient not exceeding 7% in the entire range of concentrations), while being faster and simpler as compared to the HPLC procedure. However, it must be noted that study of cytarabine hydrolytic deamination kinetics by the "ammonia" technique leads to a somewhat (up to 30%) overstated estimate of the reaction rate. Additional ammonia production is probably also related to the conversion of compound II.

Thus, the proposed methods of evaluation of cytarabine stability in aqueous solutions, based on HPLC determination of compounds I and II and the colorimetric analysis of ammonia, can be used for drug stability monitoring in development of liquid medicinal forms of cytarabine.

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