The Effect of Selected Antioxidants on the Kinetics of Changes in the Stability of an HTK Solution: A Technical Note

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INTRODUCTION

There have been numerous reports on the effectiveness of HTK (histidine-tryptophane–α-ketoglutaric acid) solutions in transplantology.[1,2] However, there have been no reports concerning its long-term stability. The stability of an HTK solution is very important, for both technological and economic reasons.

For this reason, the study aimed at determining the stability of an HTK solution using the changes of the histidine content at elevated temperatures as the basis for calculations. Histidine is the main active substance and with the highest content (198 mM). Histidine and histidine hydrochloride make a very strong buffer system. At pH values ranging from 6.0 to 6.8 the amino acid shows a much higher buffer capacity than other commonly used buffers. It also slows down the process of cell acidifying.[1,2] The study aimed at tracing the kinetics of the changes in the stability of the amino acid as affected by various antioxidants (vitamin C, cysteine, fumaric acid) in a concentration of 0.3 mM. The antioxidants and their concentrations were selected on the basis of earlier experiments.

MATERIALS AND METHODS

Composition of the HTK solution

The composition of the HTK solution (mM) was histidine, 198; mannitol, 30; tryptophane, 2; α-ketogluatric acid, 1; Na^+, 15; K^+, 10; Mg^{2+}, 4; Ca^{2+}, 0.015; and Cl^-, 50. The pH value was 7.20 and the osmolarity approximately 310 mOsm.[1]

Four solutions were prepared: (1) HTK, (2) HTK + cysteine, (3) HTK + vitamin C, and (4) HTK + fumaric acid. The solutions were prepared with the use of "pure for analysis" substances, certified (Sigma-Aldrich, Poznan, Poland).

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Antioxidants

The antioxidants were cysteine hydrochloride (Sigma Chemical), vitamin C (F.Z. "Pliva," Cracow, Poland, and fumaric acid (F.Z.N.P. "Biochefa," Sosnowiec, Poland).

Preparing the HTK solutions

An antioxidant (0.3 mM) was dissolved in 70 mL of water pro injection. Afterward, the remaining components of the solutions were added from the smallest to the largest amount. After they were completely dissolved, the pH values of the solutions were brought to 7.30 to 7.40 with 1 M solution of NaOH and the solutions were filled with water pro inj to 1000 mL. The solutions thus obtained were filtered at 50 mL/min through a sterilizing membrane filter of cellulose acetate "Sartopure GF 2," with a pore diameter of 0.22 μm. After being filtered, the solutions were poured into sterile bottles, of 100 mL each, and closed with sterile rubber caps. A unit pack contained 100 mL of solution. The bottles were made of transparent glass, I hydrolytic class. The hydrolytic resistance of the inner surface corresponded to the class HC 1.

The solutions were made according to the adopted good manufacturing practice (GMP) rules, at a site with laminar airflow.

Preparing a histidine standard curve

The histidine content was determined by the Pauli method (imidasole system).[3] In a basic environment, solutions containing imidazole compounds, including histidine, become red as a result of a diazo reaction with sulphanilic acid. Eight minutes after the diazo reaction, the extinction of the colored solution is measured at a wavelength of $\lambda = 530$ nm.

An amount of 1 mL of the working solution of histidine was put into test tubes containing solutions of 5, 10, 20, 30, 35, 40, 50, and 60 μg/mL; 2 mL of sulphanilic acid, 1 mL 5% solution of sodium nitrite, and 3 mL 10% solution of sodium carbonate was added to each tube. The solutions were stirred and the absorbances of the samples were measured after 8 minutes against a blind sample as a reference.

The standard curve of the relationship between the absorbance and the histidine concentration (0.71, 1.43, 2.86, 4.29, 5.00, 5.71, 7.14, and 8.57 μg/mL) is shown in Figure 1.

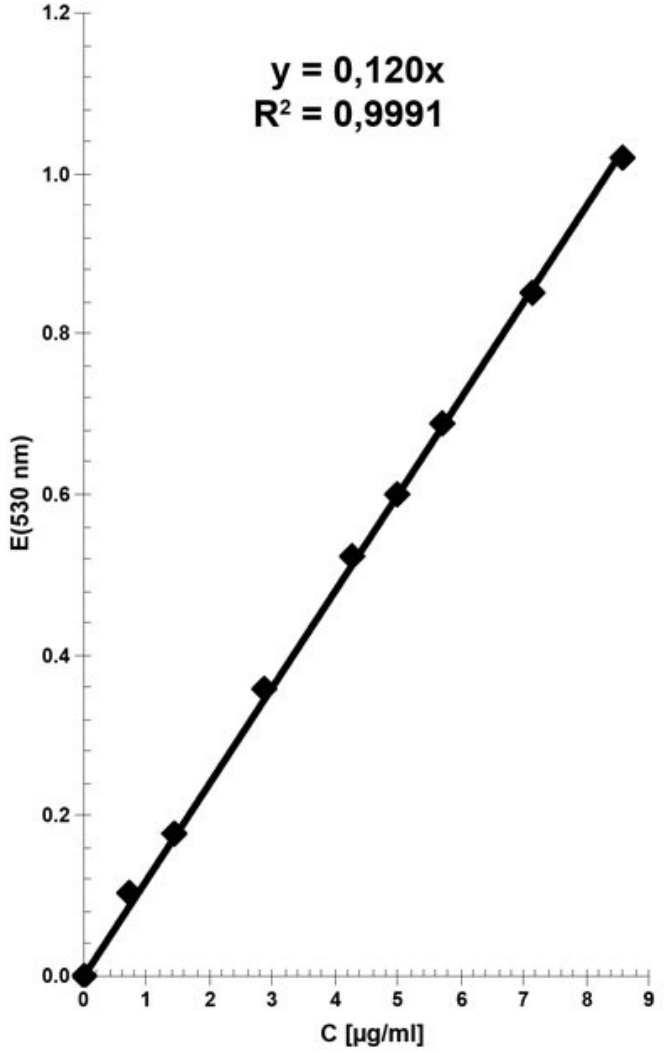

Figure 1. Standard curve showing the relationship between histidine concentration and absorbance.

The relationship between histidine concentration was described by a linear function: y = 0.120x. The value of the regression coefficient (R^2) was close to [1.0] (R^2 = 0.9991); the linear regression error – 0.000753. The precision of the analytic method (s = 0.63%) was calculated from the standard deviation (SD) value, relative standard deviation (Sr) and the confidence interval of the mean value (x + dx). The molar absorption coefficient (the method sensitivity) equaled 2.27×10^4 ($1 \times mol^{-1} \times cm^{-1}$].[4] The results were presented as the mean value for 5 samples.

The absorbance of the samples was measured in 1-cm thick glass cuvettes, with a UV-VIS "CE 3021" spectrophotometer with accessories (Cecil Instruments, Cambridge, England). The photometric accuracy of the apparatus equaled ± 0.005 A.

Testing the stability of HTK solutions

The stability of the HTK solutions was tested using an accelerated aging test.[5,6] The determination of the solution stability was based on the kinetics of the rate of change of histidine concentration at elevated temperatures. Before the tests, the absolute seal of the bottles was tested by submerging them in 1% aqueous solution of methylene blue. The experiment was conducted at 2 temperatures differing by 10°C: 50°C ± 0.2 (323 K) and 60°C ± 0.2 (333 K). The temperatures were selected based on the rate of histidine decomposition, which was determined in an experiment conducted earlier. There were 15 unit packs taken for the experiment. During the experiment conducted at 60°C, samples were taken at intervals from 0 to 70 hours. During the experiment conducted at 50°C, samples were taken at intervals from 0 to 180 hours. Immediately after being taken out of the ultrathermostat (UTU 4, filled with water), the samples were cooled down by immersing them in ice water to inhibit the decomposition process. The first sample was not taken out until its temperature equaled that of the thermostat. The histidine content was determined in the samples. The histidine content in the first sample was taken as the initial value (100%) for time t = 0. The experiment was conducted until the degree of histidine decomposition had reached 50%.

Determining the order of the reaction of histidine decomposition

The reaction order was determined by the graphic method, based on the histidine concentration in the samples after the accelerated aging test was conducted. The logarithm of histidine concentration (lg C%) was plotted against the time and temperature of solutions. The significance of adjustment of the line to the experimental data was described by the regression coefficient (R^2).

Determining the parameters of histidine decomposition rate

The obtained results provided grounds for calculating the following: histidine decomposition reaction rate constants (k) for 60°C (333 K) and 50°C (323 K); activation energy (E); frequency coefficient (lg A); histidine decomposition reaction rate constant at the temperature of the solution storage – 5°C (278 K), and the storage time (stability – t90%). Stability is the time in which the histidine content decreases to 90%.

The reaction rate constants (k) were calculated for 60°C (333 K) and for 50°C (323 K) from the following formula:

$$k = 2,303 \cdot lg\ [C_o \div (C_o - C_x)] \div t\,[min^{-1}], \qquad (1)$$

where Co is the initial concentration of histidine at time t = 0 and Cx is the histidine concentration after a certain time.

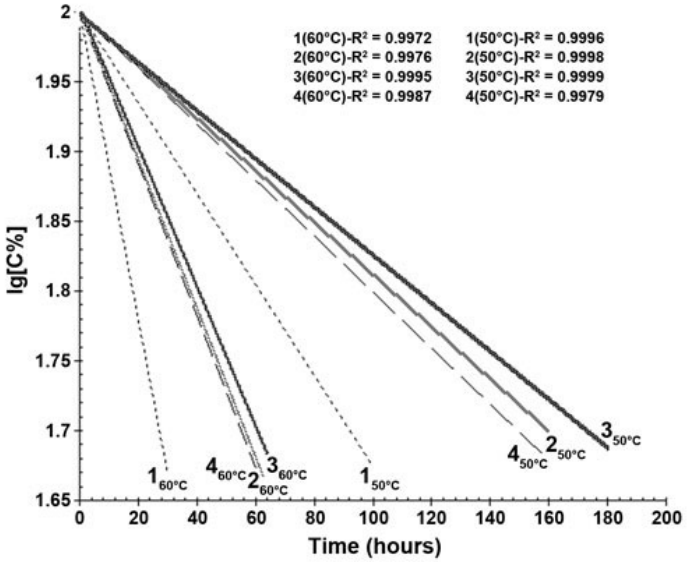

Figure 2. The relationhip between the logarithm of histidine concentration and the time and temperature of heating.

The energy of activation (E) and logarithm of the frequency coefficient (A) was calculated from the transformed Arrhenius' formula:

$$lg\ A = lg\ k + E \div (2,303 \cdot R \cdot T), \qquad (2)$$

where A is a constant value called the frequency coefficient; E is the energy of activation (cal × mol^{-1}); R is the gas constant = 1.987 (cal × mol^{-1} × K^{-1}]; and T is the absolute temperature (K). This formula was also used for calculating the histidine decomposition reaction rate constant at 5°C (278 K). The decrease in histidine concentration to 90% was calculated from the following formula:

$$t_{10\%} = \frac{0.104}{k}[days] \qquad (3)$$

Calculations

The results were shown as the mean value from 3 results (x); the SD was also calculated. The statistical analysis was conducted for the level of significance of *P* less than .05.

Results and Discussion

HTK solutions were prepared with such physical, chemical, and microbiological properties as are required of solutions for infusion.[5] The solutions were colorless, transparent, with no solid particles, sterile, and nonpyrogenic.

When solutions containing histidine are stored, the process of the compound's oxidation takes place. The reaction consists mainly of addition of the free radical to the aromatic ring. This is a relatively quick reaction as the resulting free radical adducts are stabilized by the resonance action of the aromatic ring.[7] After the accelerated aging test, the histidine concentration was observed to have decreased in each solution. The relationship between the logarithm of histidine solution (lg C%) on the one side and time temperature of heating on the other is shown in Figure 2. The significance of adjusting the line to the experimental data was described by the regression coefficient (R^2).

The lines that go through the point of intersection of the X and Y axes indicate that the decomposition of histidine is a reaction of the first order. At 50°C and 60°C the reaction rate is the highest in the HTK solution with no antioxidant. The slowest decomposition was observed in the solution with vitamin C. At 60°C the rate of histidine reaction was similar in the solution with fumaric acid and in that containing cysteine. At 50°C it was observed that the presence of fumaric acid made the histidine decomposition a little faster than that which took place in the solution with cysteine. Table 1 shows the parameters describing the kinetics of histidine decomposition.

The rate of decomposition reaction in the solution containing vitamin C equaled 0.58 × 10^{-4} (min^{-1}) at 50°C. This value was 3 times lower than at 60°C. The histidine decomposition in the solution with vitamin C was also about twice as slow as that which took place in the solution without an antioxidant at both temperatures. The heating of histidine results in the creation of a diketopiperazine derivative: 2,5-dimethyl-β-(4-imidazolil)-diketopiperazine.[8]

The energy of activation (E) and the frequency coefficient (lg A) were taken to calculate the rate of histidine decomposition at 5°C, which ranged from 0.162 × 10^{-6} to 0.3010^{-6} (min^{-1}). The decomposition of the amino acid in

Table 1. The Parameters of the Kinetics of Histidine Decomposition Rate in HTK Solutions

Solutions	Decomposition Rate Constant (k), min^{-1}		Activation energy (E), cal × mol^{-1}	lg (A)	k (5°C), min^{-1}	t10%, d
	60°C	50°C				
HTK	3.57 × 10^{-4}	1.16 × 10^{-4}	23.945	12.27	0.2810^{-6}	260
HTK + cysteine	1.910^{-4}	0.68 × 10^{-4}	21.980	10.71	0.275 × 10^{-6}	265
HTK + vitamin C	1.74 × 10^{-4}	0.58 × 10^{-4}	23.453	11.64	0.162 × 10^{-6}	450
HTK + fumaric acid	1.86 × 10^{-4}	0.68 × 10^{-4}	21.440	10.33	0.3010^{-6}	242

the solution containing vitamin C was slower by 42.3% than in the solution without an antioxidant. Fumaric acid accelerated the decomposition of histidine by approximately 7%. Cysteine did not significantly affect the rate of histidine decomposition. Cysteine is oxidized by giving off a hydrogen atom from the thiol group (SH) and the reaction results in the creation of cystine.

The study shows that vitamin C is the most effective antioxidant. At 5°C the HTK solution with vitamin C was stable for 450 days, that with cysteine for 265 days, and that with fumaric acid for 242 days. The solution without an antioxidant was stable for 260 days. An addition of 0.3 mM vitamin C increased the stability of the HTK solution by the factor close to 2. A relatively small addition of vitamin C does not significantly affect the effectiveness of the solution. In an HTK solution, vitamin C probably acts as a hydrophilic antioxidant, which protects the aqueous environment of a cell and prevents oxidative damage to DNA.[8,9] When oxidized, vitamin C yields dehydroascorbic acid, which is subsequently hydrolyzed to 2,3-diketogulonic acid, which is then oxidized to oxalic acid.

Summary and Conclusions

The effect of selected antioxidants (vitamin C, cysteine, fumaric acid) on the stability of a 0.3-mM solution of HTK has been determined. The stability of the HTK solution was determined using the changes in histidine content at elevated temperatures. The rate of the amino acid decomposition in this solution was 42.3% lower than in a solution without an antioxidant.

Vitamin C is the most effective antioxidant. An HTK solution stored at +5°C is stable for 450 days with vitamin C added to it, for 265 days with cysteine, for 242 days with fumaric acid, and for 260 days with no antioxidant.

Acknowledgments

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