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Research Article

Influence of the Selected Antioxidants on the Stability of the Celsior Solution Used for Perfusion and Organ Preservation Purposes

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Abstract. The purpose of the following research was to improve the original Celsior solution in order to obtain a higher degree of stability and effectiveness. The solution was modified by the addition of selected antioxidants such as vitamin C, cysteine, and fumaric acid in the following concentrations: 0.1, 0.3, and 0.5 mmol/l. The solution's stability was estimated using an accelerated stability test based on changes in histidine concentrations in the solution using Pauly's method for determining concentrations. Elevated temperatures, the factor accelerating substances' decomposition reaction rate, were used in the tests. The research was conducted at four temperatures at intervals of 10°C: 60±0.2°C, 70±0.2°C, 80±0.2°C, and 90±0.2°C. It was stated that the studied substances' decomposition occurred in accordance with the equation for first-order reactions. The function of the logarithmic concentration (log%C) over time was revealed to be rectilinear. This dependence was used to determine the kinetics of decomposition reaction rate parameters (the rate constant of decomposition k, activation energy E_a, and frequency factor A). On the basis of these parameters, the stability of the modified solution was estimated at +5°C. The results obtained show that the proposed antioxidants have a significant effect on lengthening the Celsior solution's stability. The best results were reached when combining two antioxidants: vitamin C and cysteine in 0.5 mmol/l concentrations. As a result, the Celsior solution's stability was lengthened from 22 to 299 days, which is 13.5 times. Vitamin C at a concentration of 0.5 mmol/l increased the solution's stability by 5.2 times (t_{90}=115 days), cysteine at a concentration of 0.5 mmol/l caused a 4.4 times stability increase (t_{90}=96 days), and fumaric acid at a concentration of 0.5 mmol/l extended the stability by 2.1 times (t_{90}=48 days) in relation to the original solution.

KEY WORDS: accelerated stability test; antioxidants; ascorbic acid; Celsior; cysteine; fumaric acid.

INTRODUCTION

The Celsior solution is one of the standard solutions used in perfusion and organ preservation purposes. It has primarily been utilized in cardioplegia. The solution enables heart storage for a period of 4–6 h. It can be used to preserve the heart using the simple hypothermia method exclusively. The advantages of this method include fast cardiac standstill and a decrease in cardiac enzyme activity. Celsior is an extracellular solution with a low potassium concentration and a high concentration of sodium. Its composition enables the reduction of growth in the concentration of calcium ions in cardiac muscles, prevents cellular ederma and intracellular acidosis, as well as protects against the negative effects of free radicals. Glutamic acid prevents increases in Ca^{2+} concentrations in cells. Lactobionate and mannitol exhibit anti-edermal properties, whereas histidine prevents the acidification of the environment. Apart from histidine, glutathione also acts as an eliminator of free radicals. The tests conducted show that Celsior improves the functioning of the right atrium of the transplanted heart in a significant manner, restoring heart action with a potentially lower risk of rejection. The conducted tests indicate that the solution may also be used to store the liver, kidneys, and lungs (1–11).

The reperfusion period (the restoration of blood flow to tissues and organs) leads to oxygen deficiency in cells and, consequently, to irreversible changes in the transplanted organ. In the course of this process, the transformation of xanthic dehydrogenase into xanthic oxidase takes place. Electrons, which have been previously transported by NAD, will be transported by oxygen using oxidase, which is associated with the formation of free radicals (12). Antioxidants are often added to preservative solutions in order to reduce damage resulting from anoxia and reperfusion (13). The antioxidizing properties of vitamin C, fumaric acid, and cysteine were used in the test for histidine stabilization. Histidine–histidine hydrochloride forms a strong buffer system. This amino acid shows great buffer capacity when compared to other buffers used in solutions. It is also a substance of relatively significant stability. The energy of activation value is approximately 20,000 cal/mol (4).

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Glutathione (GSH) is one of the most important cellular antioxidants. Together with its oxidized forms (glutathione disulfide, GSSG), glutathione peroxidase and glutathione reductase, it eliminates both free and lipid radicals, protecting cellular membranes, DNA, and proteins against oxidative stress and xenobiotics (14,15). It acts as a hydrosulfide buffer and is indispensable to the maintenance of correct thiolic redox potential by means of sustaining hydrosulfide groups (SH) of proteins in reduced form. Reduced glutathione is necessary to maintain the proper structure of erythrocytes and to keep hemoglobin in non-oxidized form. Cells with an insufficient volume of glutathione undergo hemolysis. During the period of organ storage and reperfusion, a decrease in the concentration of reduced GSH and an increase in the volume of its reduced form (GSSG) in cells have been observed (16,17); therefore, its supplementation is very significant. The introduction of glutathione as a component of the solution contributes to a significant increase in manufacturing costs, which is additionally accompanied by the issue of low cellular absorption of glutathione (17). It was also proven that low concentrations of glutathione in the transplanted organs engender an immunosuppressive effect (14). The replacement of glutathione by cysteine may be a good solution. This results from the fact that the basic substrates of GSH synthesis are three amino acids: L-glutamic acid, L-cysteine, and glycine (14). The antioxidizing properties of cysteine are related to the opportunity of complexing metal ions and, therefore, protecting against hazardous Fenton reactions. It also has a radioprotective effect and protects lymphocytes against chromosome aberrations (17). In high concentrations, vitamin C is also a powerful antioxidant. It is classified as a water-soluble antioxidant. Vitamin C forms a strong oxidizing and reducing system: L-ascorbic acid/dehydro-L-ascorbic acid with the potential of enabling the reduction of inactive forms of oxygen. It neutralizes hydroxylic, oxyalcohol, superoxide, and nitric radicals (18–27). The application of fumaric acid in cardoplegia improves myocardial contractility and decreases the concentration of lactate dehydrogenase, which is correlated to increases in the number of dead cells at higher levels of activity. Its protective impact is related to the transformation of fumaric acid into succinic acid via a dehydrogenation reaction in Krebs cycle (28). It has also been shown to positively impact the restoration of proper function in immature myocytes (29).

The introduction of antioxidants as solution components may significantly reduce adverse changes in stored organs. Taking into consideration patients' waiting time, it is also recommended that the manufactured perfusion and preservation solutions are of the longest possible stability and, therefore, of greatest effectiveness. The introduction of antioxidants into the solutions may significantly increase their stability, thereby contributing to a reduction in manufacturing costs.

The aim of the study was to determine the influence of selected antioxidants on the stability of the Celsior solution. The accelerated stability test, enabling the rapid determination of solution stability, as well as the evaluation of stabilizers applied at different concentrations, was used to analyze stability. This method is based on quantitative dependence between the reaction rate and temperature, which can be evaluated using the laws of chemical reaction kinetics.

MATERIALS AND METHODS

The following were used for test purposes: the Celsior solution [prepared in accordance with the original composition of SangStat Medical Corporation USA (30)] with the following composition: histidine, 30 mmol/l; mannitol, 60 mmol/l; lactobionic acid, 80 mmol/l; glutamic acid, 20 mmol/l; reduced glutathione, 3 mmol/l; Na^+, 100 mmol/l; K^+, 15 mmol/l; Mg^{2+}, 13 mmol/l; Ca^{2+}, 0.25 mmol/l; Cl^-, 41.25 mmol/l; solution pH 7.20–7.40; osmolarity 320–360 mOsm/l. All reagents in the original solution, as well as the applied antioxidants (vitamin C, cysteine hydrochloride, and fumaric acid), were purchased from Sigma (St. Luis, MO, USA). All other chemicals were of analytical grade.

The solution was subsequently modified by replacing glutathione with the selected antioxidants, ascorbic acid, cysteine, and fumaric acid, in the following concentrations: 0.1, 0.3, and 0.5 mmol/l, respectively. The solution was prepared in such a manner that the components, in the amounts from least to greatest, have been dissolved in approximately 700 ml of water pro-injection. pH values of 7.20–7.40 in the prepared solution were achieved by adding 1 M solution of NaOH and adding 1,000 ml of water pro-injection. The solutions were then filtered using a "Sartopure GF 2" sterilizing infiltration trench made of cellulose acetate with pore sizes of 0.22 μm at a rate of 50 ml/min. In the next stage, the solution was poured into sterilized 100-ml glass bottles and closed with sterilized gum stoppers equipped with caps. The bottles were made of first-class transparent hydrolytic glass. The hydrolytic resistance of internal surfaces is of HC 1 class. The solutions were prepared in compliance with the GMP principles in a laminar airflow cabinet.

The modified solutions were prepared analogically, with a single difference consisting in the addition of an antioxidant at a relevant concentration at the beginning and adding the remaining components after the said antioxidant dissolves.

The physical and chemical properties of the prepared solutions were analyzed. Measurements of pH values were made using a microcomputer pH meter of CP-315 model, with combined electrode, manufactured by Elmetron (Poland). The accuracy of measurements amounted to ±0.01 pH. Density measurements were made on the basis of determining the masses of three solution samples of identical capacity. The viscosity of the tested solutions was determined using a method based on Stokes' law, the so-called falling ball method. A rotatory Höppler viscosimeter, model VEB MLW Prüfgeräte-Werk (Germany), was used for test purposes. The light refraction coefficient was measured using Abby's refractometer, RL1 type, manufactured by PZO (Poland). The measurement range, n_d, amounted to 1.3–1.7. The accuracy of the refraction coefficient measurement was in the range of 1.3–1.42, amounting to ±0.0004, whereas in the 1.42–1.7 range, it amounted to ±0.0002. Buffer capacity was determined by the number of HCl hydrogen ion moles indispensable to change the pH value of 1 ml of solution by 1 unit. Osmolarity measurements were made using a 800 cl osmometer manufactured by the Trident Med Company (Poland). The measurement accuracy of the osmometer amounted to ±1 mOsm/kg H_2O (±0.4%). Solution color was

determined for each series before and after an accelerated senescence test. The color was determined in daylight, in glass test tubes placed on a white matted background. The solution was deemed "colorless" if no change of color compared to a test tube containing distilled water, used as a control liquid, was observed.

Determination of Histidine Content in Celsior Solution Using Pauly's Method

An analysis of histidine in the course of processing chemical reactions during the accelerated stability test was carried out using Pauly's spectrophotometric method (31). Test tubes were filled with 1 ml of histidine working solution at concentrations of 5, 10, 20, 30, 35, 40, 50, and 60 μg/ml and then filled with 2 ml of sulfonic acid and 1 ml of 5% sodium nitrite solution; the solution was mixed and 3 ml of 10% Na_2CO_3 was subsequently added. The absorption of the resulting azo complex was measured using λ=530-nm wavelength with a blind trial as reference. The calibration curve of the dependence between absorption and the concentration of histidine, defined by the equation $y=0.120x$, was then produced. The correlation coefficient amounted to R^2= 0.999, whereas the linear regression error equaled 0.000753. The accuracy of the measurement of 0.63% was positively evaluated on the basis of standard deviation values, the relative standard deviation, as well as the confidence interval for the average value. Method sensitivity amounted to 2.27×10^4 l mol^{-1} cm^{-1}. The measurements were made in glass cuvettes of 1 -cm thickness using a "CECIL 3021" UV–vis spectrophotometer manufactured by the Chemist Instruments Company (Poland). The spectrophotometer's photometric accuracy amounted to ±0.005 A.

Substances used in the determination of histidine were of analytical grade and derived from Sigma and PPH "POCh" S.A. Gliwice (Poland).

Solution Stability Analysis Using Accelerated Stability Test

Solution stability was determined on the basis of the kinetics of the rate of histidine content change at raised temperatures (32–34). The examinations were preceded by a unitary packaging tightness test by immersing them in a 1% water solution of methylene blue. Five unitary packages containing the relevant liquid were placed in a ultrathermostat (U7; Remontom, Poland) filled with water (the degree to which a constant temperature was maintained amounted ±0.1°C). The test was conducted at four temperatures at intervals of 10°C, i.e., 60±0.2°C (333 K), 70±0.2°C (343 K), 80±0.2°C (353 K), and 90±0.2°C (363 K). The temperature range was determined on the basis of initially conducted tests of the rate of histidine degradation. Sampling was started at the moment of temperature equalization of the tested solution with an ultrathermostat temperature (time t=0, assumed content of determined substance is 100%). The samples taken were cooled in ice water directly after being removed in order to prevent further degradation. The tests were continued until more than 50% of the degradation of the determined substance was obtained.

Determination of Order of Histidine Degradation

The reaction order was defined on the basis of the results of analytically determining the histidine concentration in the sampled solutions after the accelerated stability test. It was stated that the degradation of the examined substances was compliant with the first-order reactions equation. Graphs of the concentration logarithm (log%C) over time are rectilinear. The above dependence was used to calculate the rate constant.

Determination of Kinetic Parameters of Degradation Rate

Rate constants (k) were calculated for the first-order reaction at four different temperatures using the following equation (32,33):

$$k = 2.303 \times \lg\left[C_0/(C_0 - C_x)\right]/t \quad \left[\min^{-1}\right] \qquad (1)$$

in which C_0 is the output content of the degraded substance determined over time t=0 (100%) and C_x is the content of determined substance after a specific period of time.

The results obtained for each temperature were presented as an average of five results (k). Furthermore, the standard deviation was calculated (SD).

Table I. Reaction Rate Constants for Histidine Degradation in Solutions at Various Temperatures

Solution	k 10^{-4} (min^{-1})			
	60 (°C)	70 (°C)	80 (°C)	90 (°C)
Celsior	1.860±0.016	3.320±0.017	5.840±0.018	9.990±0.012
+0.1 (mmol/l) vit. C	1.430±0.018	2.660±0.014	4.790±0.011	8.330±0.026
+0.3 (mmol/l) vit. C	1.060±0.013	2.100±0.010	4.004±0.016	7.470±0.014
+0.5 (mmol/l) vit. C	0.850±0.009	1.730±0.010	3.420±0.015	6.530±0.017
+0.1 [mmol/l] cysteine	1.520±0.023	2.840±0.017	5.080±0.014	8.730±0.026
+0.3 (mmol/l) cysteine	1.180±0.013	2.320±0.016	4.390±0.016	8.190±0.015
+0.5 (mmol/l) cysteine	0.920±0.010	1.870±0.013	3.650±0.011	6.840±0.014
+0.5 (mmol/l) vit. C +0.5 (mmol/l) cysteine	0.450±0.006	0.970±0.006	2.010±0.016	3.990±0.017
+0.1 (mmol/l) fumaric acid	1.730±0.021	3.130±0.022	5.450±0.014	9.450±0.027
+0.3 (mmol/l) fumaric acid	1.480±0.022	2.750±0.022	4.970±0.021	8.770±0.020
+0.5 (mmol/l) fumaric acid	1.310±0.016	2.480±0.020	4.620±0.018	8.320±0.020

On the basis of the obtained results (k), the graphs of ln$k$ dependence *versus* reciprocal absolute temperature were made. It was stated that the dependence $\ln k = f(1/T)$ is a linear function, allowing for the assumption that in the said cases, the Arrhenius equation in logarithm form was complied with:

$$\ln k = -\frac{E_a}{R} \times \frac{1}{T} + \ln A. \tag{2}$$

The results obtained confirm the thesis that within the temperature range analyzed, the activation energy (E_a) and frequency coefficient (A) may be considered as constant values.

For linear dependence $\ln k = f(1/T)$, the straight gradient coefficient (a) and intersection point with ordinate axis (b) was calculated using the linear regression method, as well as, based on it, the activation energy (E_a) and frequency coefficient (A).

$$\ln k = a \times \frac{1}{T} + b = -\frac{E_a}{R} \times \frac{1}{T} + \ln A \tag{3}$$

$$a = -\frac{E_a}{R} = \frac{n \times \sum_{i=1}^{n} \frac{1}{T_i} \times \ln k_i - \sum_{i=1}^{n} \frac{1}{T_i} \times \sum_{i=1}^{n} \ln k_i}{n \times \sum_{i=1}^{n} \left(\frac{1}{T_i}\right)^2 - \left(\sum_{i=1}^{n} \frac{1}{T_i}\right)^2} \tag{4}$$

$$b = \ln A = \frac{n \times \sum_{i=1}^{n} \ln k_i \times \sum_{i=1}^{n} \left(\frac{1}{T_i}\right)^2 - \sum_{i=1}^{n} \frac{1}{T_i} \times \sum_{i=1}^{n} \frac{1}{T_i} \times \ln k_i}{n \times \sum_{i=1}^{n} \left(\frac{1}{T_i}\right)^2 - \left(\sum_{i=1}^{n} \frac{1}{T_i}\right)^2} \tag{5}$$

$$\Delta a = \sqrt{\frac{n}{n-2} \times \frac{\sum_{i=1}^{n} (\ln k_i)^2 - a \times \sum_{i=1}^{n} \frac{1}{T_i} \times \ln k_i - b \times \sum_{i=1}^{n} \frac{1}{T_i}}{n \times \sum_{i=1}^{n} \left(\frac{1}{T_i}\right)^2 - \left(\sum_{i=1}^{n} \frac{1}{T_i}\right)^2}} \tag{6}$$

$$\Delta b = \Delta a \times \sqrt{\frac{1}{n} \sum_{i=1}^{n} \left(\frac{1}{T_i}\right)^2}. \tag{7}$$

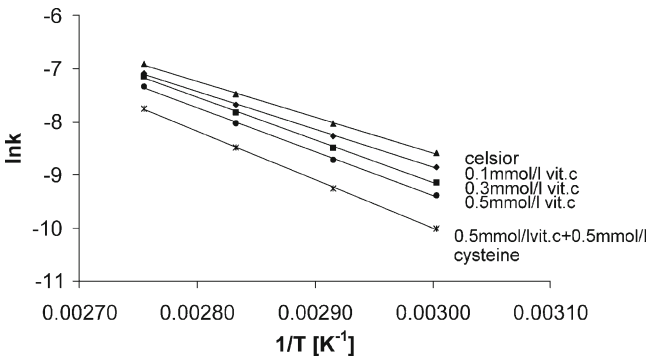

Fig. 1. Arrhenius plot for the first-order rate constant of histidine degradation in Celsior solution and in Celsior solution modified by adding vitamin C over the temperature range of 60–90°C

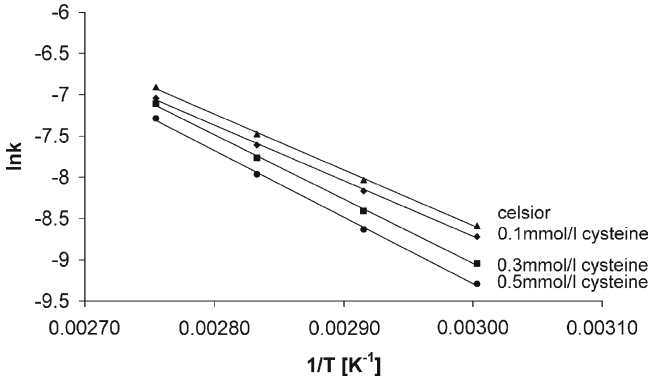

Fig. 2. Arrhenius plot for the first-order rate constant of histidine degradation in Celsior solution and in Celsior solution modified by adding cysteine over the temperature range of 60–90°C

Reaction rate constant at a temperature of 5°C was calculated using the determined E_a and A values:

$$\ln k_{278} = -\frac{E_a}{R} \times \frac{1}{T} + \ln A. \tag{8}$$

Time, in which 10% of the examined substance will be degraded at a temperature of 5°C, was calculated according to the following equation:

$$t_{90} = 0.1053/k_{278}. \tag{9}$$

Because the calculated values are a function of the other measured values, error t_{90} was determined using the total differential method.

$$\Delta f(x_1, x_2, .., x_n) = \sum_{i=1}^{n} \left|\frac{\delta f(x_i)}{\delta x_i}\right| \Delta x_i \tag{10}$$

$$\Delta t_{90} = \left|\frac{\delta t_{90}}{\delta k}\right| \Delta k = \left|-\frac{0.1053}{k^2}\right| \Delta k = \frac{0.1053}{k^2} \times \left(\left|\frac{\delta k}{\delta a}\right| \Delta a + \left|\frac{\delta k}{\delta b}\right| \Delta b\right) \tag{11}$$

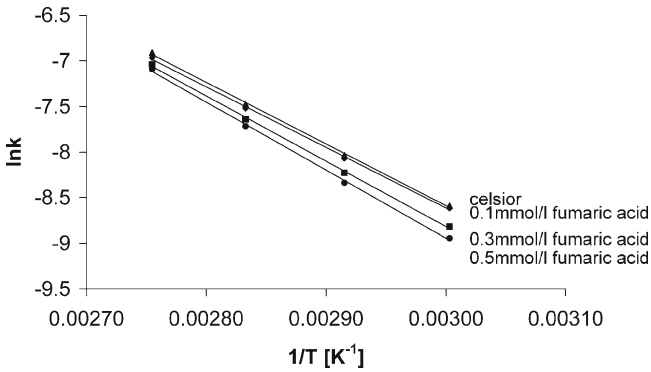

Fig. 3. Arrhenius plot for the first-order rate constant of histidine degradation in Celsior solution and in Celsior solution modified by adding fumaric acid over the temperature range of 60–90°C

Table II. Activation Energy (E_a) and Frequency Coefficient (A) Determined for Solutions

Solution	E_a (kJ mol^{-1})	ln$A$
Celsior	56.30±0.16	11.70±0.05
+0.1 (mmol/l) vit. C	59.10±0.14	12.50±0.05
+0.3 (mmol/l) vit. C	65.60±0.14	14.50±0.05
+0.5 (mmol/l) vit. C	68.40±0.14	15.30±0.05
+0.1 (mmol/l) cysteine	58.50±0.18	12.30±0.06
+0.3 (mmol/l) cysteine	65.00±0.17	14.40±0.06
+0.5 (mmol/l) cysteine	67.10±0.13	15.00±0.05
+0.5 (mmol/l) vit. C + 0.5 (mmol/l) cysteine	73.00±0.16	16.40±0.06
+0.1 (mmol/l) fumaric acid	56.80±0.20	11.90±0.07
+0.3 (mmol/l) fumaric acid	59.80±0.17	12.80±0.06
+0.5 (mmol/l) fumaric acid	62.10±0.16	13.50±0.06

$$\Delta t_{90} = \frac{0.1053}{k^2} \cdot \left(\frac{1}{T} \times e^{a \times \frac{1}{T} + b} \times \Delta a + e^{a \times \frac{1}{T} + b} \times \Delta b \right) = \frac{0.1053}{k} \times \left(\frac{1}{T} \times \Delta a + \Delta b \right). \quad (12)$$

RESULTS AND DISCUSSION

Based on the analyses conducted using Pauly's method, both the decrease in the histidine content of the Celsior solution and the order of the reaction were determined. Initial concentrations of histidine and reducing compounds in the examined solutions were assumed to be 100%, whereas subsequent concentrations were expressed as a percentage of the initial value. On the basis of the obtained results, the graphs of the logarithm of concentration dependence (log% C) of non-degraded substances over time were made. Transformations at all of the examined temperatures are congruent with the first-order reaction equations. Table I presents the calculated degradation rate constants (k) for first-order reactions at four different temperatures. The obtained results for each temperature were presented as an average of five results (k) together with the standard deviation. The chemical reaction rate resulting from the van't Hoff equation (proportion of chemical reaction rate after temperature change and rate of the same reaction before temperature change) increases for the Celsior solution, ranging between 1.71 and 2.15. It was also stated that the temperature coefficient, changing together with the temperature, has greater values at lower temperature ranges than at higher temperature ranges and grows together with the increase in the concentration of antioxidants.

The determined reaction rate constants have enabled settling of their dependence on temperature. Graphic presentation of this dependence in the form of ln$k$=f(1/T) is presented in Figs. 1, 2, and 3. The observed linear dependence between the reaction rate constant and temperature is determined by the Arrhenius equation. Deviations from the linear relation of the ln$k$ to 1/T function occurred in many studies (34). Its image is a straight $-E_a/RT$ gradient, which intersects the ordinate axis at point ln$A$ where 1/T=0. For linear dependence ln$k$=f(1/T), the straight gradient coefficient (a) and intersection point with the ordinate axis (b) were calculated using the linear regression method, in addition to, based on it, the activation energy (E_a) and frequency coefficient (A). The determined E_a and ln$A$ values are presented in Table II. Activation energy values express the stability of the examined solutions. The highest values of this parameter are exemplified by the Celsior solution modified by adding vitamin C and cysteine at concentrations of 0.5 mmol/l. The resulting value of the activation energy amounts to 73 kJ mol^{-1}. The lowest activation energy value (56.3 kJ mol^{-1}) was obtained by adding cysteine at a

Table III. The Stability of Celsior Solution and Modified Solution at 20°C and 5°C

Solution	20 (°C)		5 (°C)	
	k 10^{-6} (min^{-1})	t_{90} (days)	k 10^{-6} (min^{-1})	t_{90} (days)
Celsior	11.55±1.38	6.3±0.8	3.30±0.41	22.0±2.7
+0.1 (mmol/l) vit. C	7.74±0.81	9.5±1.0	2.10±0.23	35.0±3.8
+0.3 (mmol/l) vit. C	4.17±0.44	17.6±1.9	0.98±0.11	75.0±8.1
+0.5 (mmol/l) vit. C	2.90±0.30	25.2±2.6	0.64±0.07	114.7±12.2
+0.1 (mmol/l) cysteine	8.54±1.16	8.6±1.2	2.30±0.33	31.3±4.4
+0.3 (mmol/l) cysteine	4.75±0.60	15.4±1.9	1.10±0.15	65.0±8.4
+0.5 (mmol/l) cysteine	3.36±0.33	21.7±2.1	0.76±0.08	96.2±9.8
+0.5 (mmol/l) vit. C + 0.5 (mmol/l) cysteine	1.23±0.15	59.3±7.3	0.25±0.03	298.5±37.7
+0.1 (mmol/l) fumaric acid	10.43±1.54	7.0±1.0	3.00±0.45	24.7±3.8
+0.3 (mmol/l) fumaric acid	7.71±1.02	9.5±1.2	2.10±0.28	35.7±4.8
+0.5 (mmol/l) fumaric acid	6.06±0.73	12.1±1.5	1.50±0.19	47.8±5.9

Table IV. Physical and Chemical Properties of the Prepared Solutions after the Accelerated Stability Test at 90°C

Solution	Stability test	pH	Density (g/ml)	Light refraction coefficient	Osmolarity (mOsm/l)	Buffer capacity (mol/l)	Viscosity (Pa s)	solution color
Celsior	Before test	7.30	1.013	1.3417	333	0.019	1.112	Colorless
	After test	6.98	1.000	1.3420	332	0.019	1.102	Yellow strain
+0.1 (mmol/l) vit. C	Before test	7.30	1.020	1.3420	339	0.019	1.095	Colorless
	After test	6.98	0.993	1.3419	337	0.021	1.079	Yellow strain
+0.3 (mmol/l) vit. C	Before test	7.20	1.013	1.3429	337	0.023	1.068	Colorless
	After test	6.84	0.993	1.3420	333	0.021	1.062	Yellow strain
+0.5 (mmol/l) vit. C	Before test	7.20	1.013	1.3417	333	0.022	1.102	Colorless
	After test	6.77	0.993	1.3420	332	0.025	1.101	Yellow strain
+0.1 (mmol/l) cysteine	Before test	7.40	1.013	1.3420	341	0.021	1.201	Colorless
	After test	7.33	1.013	1.3415	326	0.022	1.157	Yellow strain
+0.3 (mmol/l) cysteine	Before test	7.22	1.066	1.3415	342	0.023	1.286	Colorless
	After test	7.03	1.013	1.3415	338	0.021	1.090	Yellow strain
+0.5 (mmol/l) cysteine	Before test	7.20	1.027	1.3415	340	0.021	1.112	Colorless
	After test	6.61	1.013	1.3415	337	0.021	1.067	Yellow strain
+0.1 (mmol/l) fumaric acid	Before test	7.20	1.020	1.3415	340	0.022	1.106	Colorless
	After test	6.82	1.020	1.3415	329	0.022	1.043	Yellow strain
+0.3 (mmol/l) fumaric acid	Before test	7.20	1.020	1.3415x	332x	0.021	1.128	Colorless
	After test	6.84	1.020	1.3415	329	0.019	1.073	Yellow strain
+0.5 (mmol/l) fumaric acid	Before test	7.20	1.013	1.3415	332	0.021	1.179	Colorless
	After test	6.77	1.006	1.3411	325	0.023	1.096	Yellow strain
+0.5 (mmol/l) vit. C +0.5 (mmol/l) cysteine	Before test	7.40	1.013	1.3420	341	0.021	1.201	Colorless
	After test	7.33	1.013	1.3415	326	0.022	1.157	Yellow strain

concentration of 0.1 mmol/l to the Celsior solution. The calculated values are contained in the 50–90 kJ mol^{-1} range, which reflects the average for most medicinal products.

The presence of constant E_a and A values in the examined temperature scope allows for the determination of reaction rate constants at the storage temperature of the tested solutions, as well as the determination of t_{90} degradation times. The calculated values have been presented in Table III. The chemical stability of an active substance constitutes the core element of tests of the stability of medicinal products. However, it is still important for the medicinal product's physical and chemical properties to remain unchanged. Table IV contains the physical and chemical properties of the Celsior solution as well as the properties of modified solutions following accelerated stability tests at a temperature of 90°C. At this temperature, the greatest changes in the analyzed parameters are to be assumed. pH, density, buffer capacity, osmolarity, light refraction coefficient, and viscosity values were examined. These values did not change significantly after conducting the accelerated stability test. The yellow strain color observed after the test may be a result of the Maillard reaction (35) taking place between the first-order amine groups (cysteine, histidine) and reducing sugars (emerging in liquids via hydrolysis). The change in the solutions' color proves the lowered effectiveness of such solutions.

Having analyzed the data presented in Tables I, II, and III, it may be stated that the addition of an antioxidant has a significant impact on the reaction rate constant. The lowest k value at a storage temperature of 5°C for the Celsior solution and its modified versions was observed in the original solution with the addition of vitamin C and cysteine at 0.5 mmol/l ($k=0.25\times10^{-6}$ min^{-1}) concentrations. The determinant of the solution's stability at a given temperature is given by the t_{90} value. Having analyzed the data from Table III, it may be stated that a combination of two optimal antioxidants, ascorbic acid and cysteine (at 0.5 mmol/l concentrations), resulted in extending the Celsior solution's stability from 22 to 299 days, provided that the physical and chemical parameters are not changed significantly (pH 7.33, osmolarity 326 mOsm/l). The remaining antioxidants also extended the solution's stability (Table III). Vitamin C at a concentration of 0.5 mmol/l increased stability by 5.2 times (t_{90}=115 days). Cysteine at a concentration of 0.5 mmol/l resulted in increasing the stability by 4.4 times (t_{90}= 96 days), whereas fumaric acid at a concentration of 0.5 mmol/l increased stability by 2.1 times (t_{90}=48 days) compared to the original solution. The antioxidizing properties of the selected antioxidants decrease in the following order: vitamin C > cysteine > fumaric acid. This dependence results from the redox potential of reactions present in the solution during the storage period.

Schemes of the chemical reactions present during the course of the tests have been defined. Histidine in the Celsior solution may be present in two tautomeric forms. Therefore, we may assume the presence of a tautomerization reaction. Its structure contains an imidazole ring which participates in four types of reactions: as an electrophile, giving and receiving electron pairs; it may also additionally provide a single electron or react chemically with two chemical compounds at the same time. This property arises from molecular orbital theory and is related to the presence of two nitrogen atoms (secondary pyrrole and tertiary pyridine) of different physical and chemical potentials (35). In the examined solutions, histidine is most likely subject to decarboxylation reactions, transforming into histamine [β-(4-imidazol)-ethylamine] (36,37).

Vitamin C is the most efficient antioxidant. It is capable of reacting with metal cations contained in liquids, generating monodent, and dissolving complex compounds of relatively low stability. The lactone ring and side chain are engaged in the complexion process. They initially dissociate the protons from the lactone ring at the O-3 position and, consequently at the O-2 position, which is related to the presence of C(2) OH…OC(1) hydrogen bonding, hindering dissociation of the OH(2) group proton. As a consequence, the following compounds are formed: $K(HAs)\cdot2H_2O$, $Na(HAs)\cdot2H_2O$, $Mg(HAs)_2\cdot4H_2O$, and $Ca(HAs)_2\cdot4H_2O$. The complexation reaction has a stabilizing effect on the examined solutions, as lower concentrations of cations prevent precipitation. Cysteine and histidine exhibit similar properties and form soluble complex compounds with K^+, Na^+, Mg^{2+}, and Ca^{2+} ions (38,39).

We were unable to find information in the available literature on antioxidants' effects on organ perfusion and the preservation of solution stability. The conducted tests on HTK solution stability, which depend on the applied antioxidants (40), confirm the hypothesis that antioxidants have a significant impact on extending the stability of solutions for perfusion and organ preservation purposes. An HTK solution stored at +5°C is stable for 260 days with no antioxidants, 450°days with vitamin C, 265°days with cysteine, and 242° days with fumaric acid (40).

CONCLUSIONS

Antioxidants have a significant impact on the stability of solutions used for perfusion and organ preservation. The conducted laboratory tests prove that a combination of two optimal antioxidants, ascorbic acid and cysteine (0.5 mmol/l concentrations), resulted in an increase in the Celsior solution's stability from 22 to 299 days. It is our belief that a modified Celsior solution manufactured under industrial conditions will exhibit greater stability.

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