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Separation and determination of reduced vitamin C in polymerized hemoglobin-based oxygen carriers of the human placenta

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Abstract

The molybdenum blue method was used to determine the content of reduced vitamin C (Vc) in a solution of polymerized hemoglobin-based oxygen carriers (HBOCs) of the human placenta. The conditions of absorption wavelength, HCl addition, and reaction time, were investigated. The results of validation experiments showed that under the optimized conditions, a standard curve was confirmed with good linearity of 0.9985, for the Vc amount ranging from 0–200 μg. The values for relative standard deviation (*RSD*) of the precision and repeatability were both below 5%. Vc recovery was in the range of 97–102%. The conclusion could be made that a reduction in Vc content could be tested effectively by the molybdenum blue method.

Keywords: hemoglobin-based oxygen carriers, molybdenum blue method, spectrometry, Vitamin C

Introduction

Hemoglobin-based oxygen carriers (HBOCs) have been paid much attention during the past decades due to their potential applications in blood substitutes and oxygen therapeutics (Sakai et al. 2013, Silverman and Weiskopf 2009). To reduce the side effects such as vasoconstriction and renal toxicity, HBOCs were prepared through hemoglobin polymerization or modification to form hemoglobin (Hb) complexes with higher molecular weight. Due to the absence of enzymatic protective systems in red blood cells, HBOCs are subjected to oxidation during the spontaneous and uncontrolled oxidation process of ferrous Hb (Fe^{2+}), leading to the formation of methemoglobin (MetHb) and free radicals (Chang 2010), which was considered to be one of main reasons for the adverse reactions of HBOCs in clinical trials (Linberg et al. 1998). It was believed that only HBOCs with a MetHb content less than 10% can effectively deliver oxygen to hypoxic tissues (Buehler et al. 2010). Thus, to prevent the formation of MetHb and free radicals, certain antioxidants were administered for attenuation of oxidative reactions.

As a typical non-enzymatic antioxidant with lower molecular weight in human plasma, Vc attracts much attention because of its powerful antioxidative properties (Harrington et al. 2010, Dorman et al. 2002). The results of our previous research work showed that polymerized human placental HBOCs could be protected against antioxidative stress by Vc, indicating its potential applications in the development of HBOCs (Chen et al. 2013). During the pharmaceutical development and production process, it is necessary to ensure the safe dose of any additives. Therefore, building a method for determination of the Vc content has a significant role in the development of HBOCs.

Until now, Vc content was usually tested through the fluorescence method (Matsuoka et al. 2012), phenanthroline monohydrate method (Zenki et al. 2004), and iodimetry method (Paixao et al. 2006), which were all used for non-protein samples. As derivatives of hemoglobin, it was necessary to isolate the polymerized hemoglobin from solutions before the detection of Vc. In this work, as solutions of polymerized human placental hemoglobin were being chosen as samples, the HBOCs were isolated through the precipitation method in solutions using HCl at certain concentrations. Then, the Vc content of the supernatant was tested through the molybdenum blue method, which is considered to be fast, effective and low-cost.

Materials and methods

Chemicals and reagents

Human placental blood was provided by Sichuan New Life Stem Cell Polytron Technologies Inc. (Chengdu, China). The standard Vc was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The other chemicals used were of analytical grade. The water used in this research was obtained from the RO/BIO Water System (18.2 MΩ cm, PALL Corporation, USA).

Instruments

UV/Visible Spectrophotometer (Ultrospec 6300™, Amersham Biosciences, America); Experimental pH meter (FE20, Mettler-Toledo Instruments, China); Electronic Balance (AL204, Mettler-Toledo Instruments, China); Blood Cell Analyzer (BC-3200, Mindray, China); Electric Thermostatic

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Water Bath (DK-8AD, Hengyi Technology, China); RO/BIO Water System (Cascada™, PALL, America).

Experimental methods

Preparation of HBOCs

The polymerized human placental hemoglobin was prepared as described previously (Li et al. 2006).

Preparation of the coloring reaction solution

1. Oxalic Acid-Ethylene Diamine Tetraacetic Acid (OA-EDTA) solution

The OA-EDTA solution was prepared as follows: 6.3000 g OA (with crystal water) and 0.0584 g EDTA were dissolved in pure water and introduced into a 1000 ml flask. The OA-EDTA solution obtained contained 0.05 mol/L OA and 0.2 mmol/L EDTA.

2. Metaphosphoric Acid-Acetic Acid (MA-AA) solution

The MA-AA solution was prepared by introducing 15.0000 g MA and 40 ml AA into a 500 ml volumetric flask and diluted with pure water to 500 ml. The solution could be obtained after filtration.

3. Ammonium Molybdate (AM) solution

The AM solution, at a concentration of 0.037 mol/L, was prepared by dissolving 25.0000 g AM with pure water, in a 500 ml volumetric flask.

Pretreatment of polymerized human placental HBOC solution

A certain amount of hydrochloric acid solution with the concentration of 12 mol/L, 2.0 ml of HBOC solution, and 2.0 ml of OA-EDTA solution, were introduced into a centrifuge tube, and then the mixed solution was diluted to a final volume of 8 ml. After being agitated for 1 min and centrifuged at 7500 rpm for 15 min, the polymerized hemoglobin was precipitated, and the supernatant could be used as the sample solution for Vc detection.

Standard curve of Vc

In each Brown volumetric flask with a volume of 10 ml, the coloring reaction solutions were added, including 3 ml of OA-EDTA solution, 1 ml of MA-AA solution, 2 ml of ammonium molybdate solution, and certain amounts of HCl, followed by the addition of 10, 40, 80, 120, 160, and 200 µl respectively of the standard Vc solution, with a concentration of 1 mg/ml (equal to 10, 40, 80, 120, 160, and 200 µg of Vc quantity). After the coloring reaction solution was diluted with pure water to the required volume and the color reaction was completed, the absorbance of the coloring solutions at certain wavelengths were tested, with pure water as the reference. The standard curve could be obtained by the linear relationship between the absorbance value and the amounts of Vc added.

Detection and calculation of Vc in sample solutions

Sample solutions containing unknown amounts of Vc were added to the coloring reaction solution in a flask, to trigger the color reaction. After the reaction was completed, the absorbance of the resultant color solution was tested, with pure water as the reference. Each sample was tested three times, for calculation of the average value.

The content of Vc in the sample solutions after pretreatment could be calculated from the absorbance value by a standard curve, and then the reduced Vc amounts in the HBOC solutions could be calculated as below:

$$C_{\mathrm{Vc}}\,(\mathrm{mg/ml}) = \frac{m \times V_2}{1000 \times V_1 \times V_3}$$

m: Vc content in coloring reaction solution in the volumetric flask (µg)

V_1: Volume of sample solution added into coloring reaction solution after pretreatment (ml)

V_2: Total volume of sample solution after pretreatment (ml)

V_3: Volume of HBOC solution for pretreatment (ml)

Results

Effects of HCl concentration on the precipitation of HBOCs

Two milliliters of HBOC solution was added into OA-EDTA solutions with HCl concentrations of 0.50, 0.75, 1.00, 1.25, 1.50 and 2.00 mol/L, to investigate the effect of HCl concentration on the precipitation of HBOCs. After centrifugation, the absorbance of supernatants at the wavelength of 652 nm (A_{652}) was tested, and a lower A_{652} value indicated more effective precipitation. The results of the precipitation experiment are shown in Figure 1. It can be seen that the A_{652} decreased with the increase in HCl concentration, which indicates that the OA-EDTA solution with higher HCl concentration shows more effective precipitation. When the concentration of HCl increased to above 1.00 mol/L, the A_{652} values of the supernatants were constant, and showed nearly no significant differences compared to the reference, indicating the complete precipitation of HBOCs. Therefore, the concentration of HCl should be above 1.00 mol/L, to ensure the complete precipitation of HBOCs.

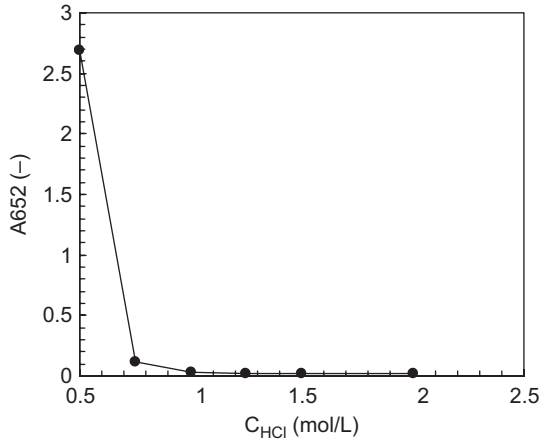

Figure 1. A_{652} of the supernatants with different HCl concentrations, after centrifugation.

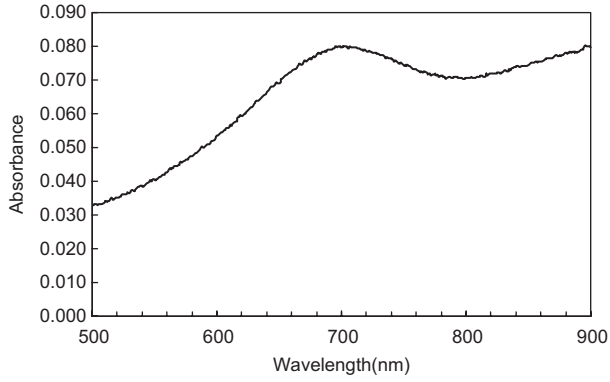

Figure 2. The absorbance curve of the coloring solution between 500 and 900 nm.

Optimization of color reactions

Maximum absorption wavelength

Fifty microliters of Vc solution with a concentration of 0.1% (w/v) were added into a coloring reaction solution; after completion of the reaction, the absorbance values of the coloring reaction solution were measured at different wavelengths in the range of 500–900 nm, and the results are shown in Figure 2. From the spectrum curve, an absorption peak can be detected in the range of 650–760 nm, and the maximum absorption is seen to be located at 710 nm, which was chosen as the test wavelength in this study.

Additional amount of HCl

Fifty microliters of Vc solution, with a concentration of 0.1% (w/v) were added into coloring reaction solutions, with different amounts of HCl addition at 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 ml, respectively. After completion of the reaction, the absorbance of the reaction solutions at the wavelength of 710 nm (A_{710}) was tested, and the results are shown in Figure 3. It can be seen that when smaller amounts of HCl were added, ($\leq$ 0.5 ml), the A_{710} values increased with the increase in the amount of HCl added. When the amounts of HCl added were above 0.5 ml, the A_{710} values gradually decreased, to remain constant with further increase in the amount of HCl added, within the range of experimental study. Therefore, it can be concluded that A_{710} can remain stable when the amount of HCL added ranges from 1.00 to 2.00 ml.

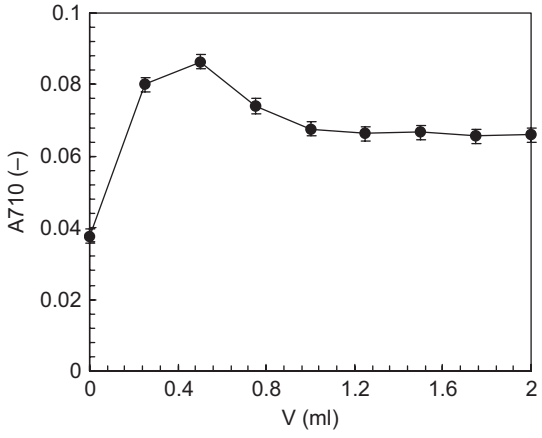

Figure 3. Effects of amounts of HCl added, on the value of A_{710}.

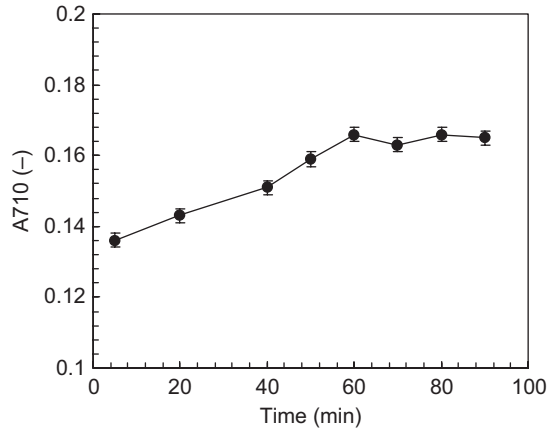

Figure 4. Effects of time on the coloring reaction.

Colorimetric reaction time

A quantity of thirty µl of Vc solution, with the concentration of 0.1% (w/v), was added into the coloring reaction solutions, and the A_{710} values were tested at certain time intervals. The data in Figure 4 show that the A_{710} values gradually increased during the early reaction time. After the reaction time of 60 min, the A_{710} values remained constant, indicating that the coloring reaction was nearly complete. Thus, it is reasonable to believe that the A_{710} value can be tested 60 min after the start of the coloring reaction.

Method validation

Standard curve

Based on the optimization of the conditions of the coloring reaction, the standard curve could be obtained, and the data are shown in Figure 5. The results of the standard curve suggest that with the addition of Vc ranging from 0 to 200 µg, the linear coefficient of the standard curve is 0.9985, indicating a good linearity between the A_{710} and the amounts of Vc added.

Sensitivity

In sensitivity experiments, different amounts of Vc solution at a concentration of 0.01% (w/v), were added to the coloring reaction solutions. Each sample was tested three times, and the data are shown in Table I. It can be seen that the

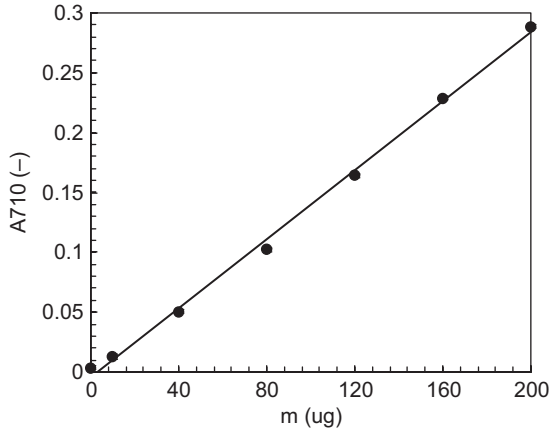

Figure 5. The standard curve of Vc testing.

Table I. Results of sensitivity experiments.

Vc quantity(μg)	0	2	4	6	8	10	12
A_{710}-1	0.005	0.007	0.008	0.008	0.008	0.013	0.017
A_{710}-2	0.007	0.005	0.006	0.007	0.009	0.013	0.014
A_{710}-3	0.004	0.009	0.007	0.007	0.007	0.011	0.015
A_{710}-av	0.005	0.007	0.007	0.007	0.008	0.012	0.015

A_{710} value with the addition of 12 μg of Vc, was about 3 times more than that on addition of 0 μg Vc, indicating the detection limitation of 12 μg.

Repeatability and precision

The repeatability and precision were evaluated using the relative standard deviations (*RSD*). Three samples with different Vc concentrations were prepared and then analyzed by the established method. Each sample was tested in six replicates and the data are listed in Tables II and III. The RSD values were 3.24%, 3.76% and 4.54% for repeatability, and 3.26%, 2.30% and 2.81% for precision, indicating good repeatability and precision of the colorimetric method developed.

Recovery

The recovery experiments were carried out through the decrement method, in which a certain amount of Vc was added into the reaction solution for reference, before the addition of a spiked amount of Vc. The detected amount of standard Vc addition could be obtained by subtracting the reference amount from that of the total Vc. In this experiment, three levels of standard samples were tested. The data of results, in Table IV, show that the average recoveries were 101.81%, 97.93% and 97.02% respectively, indicating good accuracy of the colorimetric method established.

Discussion

Metaphosphoric acid and phosphomolybdate can interact with reduced Vc in acidic conditions, leading to the formation of the molybdenum blue complex. The amounts of molybdenum blue complex obtained are proportional to the reduced amounts of Vc in the reaction. Based on this principle, the reduced Vc content could be detected through the molybdenum colorimetric method. In this work, the quantities of the metaphosphoric acid and ammonium molybdate were in excess, relative to that of reduced Vc, which ensured the completion of coloring reaction. For the accuracy of testing, it is necessary to add the OA-EDTA solution into the coloring reaction solution during the process of addition, to avoid the interferential effect of free metal cations by breaking the reaction between the reduced Vc and the oxidation cations, especially by the free Fe^{3+} cations.

Due to the dark pigment color of the HBOCs, the molybdenum colorimetric method cannot be used directly to detect the Vc content in the HBOC solution. In this work, the HBOCs were isolated through precipitation in acidic solution, to eliminate the interference of HBOCs in Vc detection. In previous studies, sulfuric acid was usually used as the acidic medium in the colorimetric reaction solution (Sayed Elnenaey et al. 1979, Eldawy et al. 1975). However, the system of conversion of HBOCs usually contained Ca^{2+} cations, which were subjected to precipitation by the sulfate radicals. Therefore, hydrochloric acid was used as the acidic medium for the HBOC precipitation and coloring reaction in this experiment, because of the negative effects of Cl^{-} on the reaction. Due to the controversial reports about the maximum absorption (Sayed Elnenaey et al. 1979, Eldawy et al. 1975, Tiwari 2010) and the changes in the acidic medium, it is necessary to optimize the reaction conditions, including the maximum absorption wavelength, HCl addition, and reaction time. In this work, the effects of temperature on the reaction were not investigated, because detailed elucidation has been provided in previous work (Tiwari 2010).

Vc is a kind of efficient natural antioxidant, and can react with a variety components in the HBOC solution, including free Fe^{3+}, free oxygen, Hb-O_2, and MetHb. These reactions lead to the rapid variation in Vc content, which the recovery experiments should take into consideration. In this work, the decrement method was used, based on the assumption of nearly equivalent consumption of Vc by the reference and samples during the testing. The detected amounts of standard Vc addition could be obtained by subtracting the reference amount from that of total Vc. Consequently, the decrement method was proved to be suitable for Vc detection in HBOCs by the recovery experiments, yielding results in the range of 97–102%.

HBOCs are usually preserved in the solution system containing additives such as glucose, Ringer's solution, saline, and hydroxyethyl starch, which prove to bear a negative effect on the molybdenum blue method (Sayed Elnenaey et al. 1979). In addition, the reagents used in this work are easy to obtain and the operations during the testing are simple, suggesting the simplicity and convenience of the testing process in the molybdenum blue method.

Table II. Results of repeatability experiments.

	A_{710}-1	A_{710}-2	A_{710}-3	A_{710}-4	A_{710}-5	A_{710}-6	*RSD*值
Sample A	0.212	0.201	0.214	0.200	0.205	0.200	3.24%
Sample B	0.112	0.118	0.107	0.116	0.114	0.110	3.76%
Sample C	0.025	0.024	0.025	0.027	0.026	0.025	4.54%

Table III. Results of precision experiments.

	A_{710}-1	A_{710}-2	A_{710}-3	A_{710}-4	A_{710}-5	A_{710}-6	*RSD*值
Sample A	0.218	0.223	0.217	0.207	0.209	0.225	3.26%
Sample B	0.160	0.159	0.155	0.159	0.164	0.166	2.30%
Sample C	0.035	0.034	0.034	0.034	0.036	0.036	2.81%

Table IV. Results of recovery experiments.

Samples	1	2	3	References
Spiked amount of Vc addition(mg/ml)	1.00	0.50	0.20	0.12
Detected amount of total Vc addition(mg/ml)	1.14	0.61	0.31	
Detected amount of standard Vc addition (mg/ml)	1.02	0.49	0.19	
Recovery	101.81%	97.93%	97.02%	

Average of 3 experiments.

Conclusion

In this work, the molybdenum blue method was used to detect the Vc content in the solution of HBOCs, and the conditions of the colorimetric reaction were optimized. The results show that HBOCs could be isolated effectively by the OA-EDTA solution with HCl as the acidic medium, which has no interferential effects on the reaction. The RSD of the data from method validation were below 5%, and the recoveries were between 97% and 102%. In conclusion, the molybdenum blue method can detect the Vc content effectively in the solution of HBOCs, and thus has potential application in development of HBOCs due to its convenient, low-cost, and simple operation.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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