〈Original Article〉

Development of the enzymatic assay for whole blood choline using an automated biochemical analyzer

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Summary Background: The measurement of whole blood choline is useful for the diagnosis of acute coronary syndrome, but its routine measurement method has not yet been established. We developed an enzymatic assay for whole blood choline that is not affected by interfering substances in whole blood.

Method: For the measurement of choline, choline oxidase and DA-67 as a highly sensitive coloring reagent were used. To avoid the influences of interfering substances in whole blood, potassium iodate, hydroxylamine hydrochloride, and hexadecyltrimethylammonium bromide were added to the reagent.

Results: Within-run reproducibility tests (n = 20) showed a coefficient of variation of 0.37-2.30%. Choline oxidase did not react on endogenous analogues in samples, showing high specificity.

Conclusion: This method requires only 10 minutes, allowing rapid, direct measurement of whole blood choline. This method can be used for diagnosis and outcome prediction in patients with acute coronary syndrome (ACS).

Key words: Acute coronary syndrome (ACS), Whole blood choline, Enzymatic assay, Automated analyzer

1. Introduction

Acute coronary syndrome (ACS) is a general term for ischemic heart diseases that commonly show thrombosis associated with rupture of a coronary artery plaque. The biochemical markers used for the diagnosis of this syndrome are myocardial troponin T and troponin Ⅰ. Since ACS often shows atypical symptoms, risk evaluation before the onset of ACS is often inadequate, and the establishment of a new marker is indispensable[1].

In recent years, as a diagnostic parameter of ACS that is independent of age, sex, history of myocardial infarction, coronary risk factors, and electrocardio-

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grams, the whole blood and plasma choline levels have been reported to be useful[2]. Cell infiltration and the activation of macrophage metabolism around unstable plaques, increases in phospholipase D activity and platelet reactivity, and the fragility of unstable coronary plaques are reflected by changes in the choline concentration in blood cells. Therefore, choline in both plasma and whole blood is involved in tissue ischemia. In particular, the whole blood choline level was reported to be more important than the plasma choline level for the early detection of high-risk patients for unstable coronary plaques and outcome prediction[2]. The cut-off value of choline for adverse major cardiac events including myocardial infarction, unstable angina pectoris, and cardiac failure is 28.2 μ mol/L in whole blood and 25.0 μ mol/L in plasma. The sensitivity and specificity of the whole blood choline level for high-risk unstable angina were reported to be 86% each[3]. In addition, Möckel et al.[4] reported that the combination of whole blood choline, the N-terminal pro-B-Type natriuretic peptide (NT-proBNP), and lipoprotein-associated phospholipase A2 (Lp-PLA2) is optimal for early risk-stratification for ACS.

However, the previously reported method for measuring whole blood choline uses high-performance liquid chromatography-mass spectrometry, which cannot be performed in ordinary laboratories. In addition, since whole blood pretreatment requires more than 1 hour, this conventional method is not appropriate for ACS that should be promptly treated. To establish a rapid, direct, and inexpensive measurement method for whole blood choline, we developed an enzymatic assay that does not require whole blood pretreatment such as deproteinization and can be performed using an automated biochemical analyzer.

2. Materials and methods

2.1. Measurement principles

The measurement principles are shown in Fig. 1. Two molecules of hydrogen peroxide are produced from 1 molecule of choline using choline oxidase (COD). In the presence of peroxidase (POD), DA-67 as a highly sensitive leuco dye is oxidized with hydrogen peroxide, and the absorbance of the occurred methylene blue was measured at 660 nm. To avoid interfering substances in whole blood, potassium iodate, hydroxylamine hydrochloride, and hexadecyltrimethylammonium bromide as reducing agents were added to the reagent.

2.2. Measurement apparatus and reagents

Measurement apparatus: For analysis, the Hitachi 7170 automated analyzer (Hitachi High Technologies, Tokyo, Japan) was used.

$$\text{Choline} + O_2 \xrightarrow{\text{COD (EC 1.1.3.17)}} \text{Betaine aldehyde} + H_2O_2$$

$$\text{Betaine aldehyde} + O_2 \xrightarrow{\text{COD (EC 1.1.3.17)}} \text{Betaine} + H_2O_2$$

$$2H_2O_2 + \text{DA-67} \xrightarrow{\text{POD (EC 1.11.1.7)}} \text{Methylene blue (660nm)}$$

Fig. 1 Measurement principles for whole blood choline.
From one molecule of choline, two molecules of hydrogen peroxide are occurred by the action of COD. Hydrogen peroxide oxidizes DA-67 in the presence of POD, resulting in the formation of methylene blue. The choline concentration is calculated by measuring the absorbance of methylene blue at 660 nm.

Table 1 Measurement conditions

	Contents	Volume (μL)	Reaction time
Specimen	whole blood (EDTA-2K)	17	
Pre-diluent	0.75 mmol/L Hydroxylamine Hydrochloride	85	
Sample	Pre-diluent ＋Specimen	20	
R-1	pH 4.0, 0.01 mol/L Acetate buffer 0.1% TritonX-100 100 mmol/L Potassium iodate 0.05% Hexadecyltrimethylammonium Bromide	180	1 minute and 25 seconds
R-2	pH7.5, 0.20 mol/L Phosphate buffer 0.082 mmol/L DA-67 10 mmol/L Sodium thiosulfate 0.16 mmol/L Orange G	90	3 minutes and 35 seconds
R-3	pH 7.5, 0.07 mol/L Phosphate buffer 77.5 U/mL Peroxidase 60 U/mL Choline oxidase	20	1 minute and 2 seconds
Colorimetry	Measurement using a 2-point end assay (sub/main wavelength = 800 nm/660 nm)		1 minute and 2 seconds

Reagents: The reagents used included COD (EC 1.1.3.17) (Asahi Kasei Pharma, Tokyo, Japan), POD (EC 1.11.1.7) (Oriental Yeast, Tokyo, Japan), TritonX-100 (Sigma-Aldrich Japan, Tokyo, Japan), and Orange G (Kanto Chemical, Tokyo, Japan). The other reagents used were of analytic grade (Wako Pure Chemical Industries, Osaka, Japan). This assay system consists of a pre-diluent and 3 reagents (Table 1). The diluent contains 0.75 mmol/L hydroxylamine hydrochloride. The first reagent (R-1) contains 0.01 mol/L acetate buffer (pH 4.0, 25℃), 100 mmol/L potassium iodate, and 0.1% TritonX-100, and 0.05% hexadecyltrimethylammonium bromide. The second reagent (R-2) contains 0.20 mol/L phosphate buffer (pH 7.5, 25℃), 0.082 mmol/L DA-67, 10 mmol/L sodium thiosulfate, and 0.16 mmol/L Orange G. The third reagent (R-3) contains 0.07 mol/L phosphate buffer (pH 7.5, 25℃), 77.5 U/mL POD, and 60 U/mL COD. As the standard solution, choline chloride was dissolved in purified water to obtain a concentration of 100 μmol/L, and stored under refrigeration.

Measurement samples: For measurement, whole venous blood samples were collected using blood collection tubes containing EDTA-2K and stored in a refrigerator until measurement.

2.3. Measurement conditions

The 2-point end method and pre-dilution mode of the Hitachi 7170 automated analyzer were selected. The measurement temperature was 37℃. Each blood sample (17 μL) was mixed with 85 μL of the pre-diluent and used as a sample for analysis. Of the sample, 20 μL was dispensed into the cells, and 180 μL R-1 was added. After 1 minute and 25 seconds, 90 μL R-2 was added. After 3 minutes and 35 seconds, 20 μL R-3 was added. After 1 minute and 2 seconds, colorimetric analysis was performed at a main wavelength of 660 nm and sub wavelength of 800 nm (Table 1).

2.4. Evaluation methods

2.4.1. Determination of reagent compositions

Hemolysis methods: To measure choline contained in blood cells after hemolysis in whole blood, TritonX-100 as a surfactant was selected. TritonX-100 solution (0.05-0.3%) was produced, and 180 μL of this solution was mixed with 3.5 μL of whole blood, and the presence or absence of hemolysis was determined under a microscope.

Study of the concentrations of coloring and

enzymatic reagents: To evaluate the influences of the hemoglobin spectrum, 350 μL of the hemolyzed blood obtained after freezing whole blood was mixed with 1.8 mL of 0.05 mol/L phosphate buffer (pH 7.5, 25℃), and absorption spectra at 350-800 nm were measured using a spectrophotometer. The coloring reagent which can avoid the influences of the hemoglobin spectrum was selected. The optimal concentration of COD was calculated based on the reference[5].

Avoidance of the influences of reducing substances in whole blood: In whole blood, there are various reducing, interfering substances, causing negative errors in the oxidative coloring of choline-derived hydrogen peroxide. To avoid the influences of reducing substances, 3 reagents (potassium iodate, hydroxylamine hydrochloride, and hexadecyltrimethylammonium bromide) were evaluated. R-1 at a potassium iodate concentration of 0-200 mmol/L, a pre-diluent at a hydroxylamine hydrochloride concentration of 0-1.75 mmol/L, and R-1 at a hexadecyltrimethylammonium bromide concentration of 0-0.05% were prepared, and the optimal concentrations achieving the maximum avoidance of the influences of reducing substances were determined. The avoidance of the influences of reducing substances was evaluated using the recovery rate after the addition of 50 μmol/L choline solution to whole blood. The recovery rate was calculated as follows:
$(A - B)/(C - D) \times 100\%$

A: 1 volume of 500 μmol/L choline solution + 9 volumes of a sample from a healthy subject

B: 1 volume of physiological saline + 9 volumes of a sample from a healthy subject

C: 1 volume of 500 μmol/L choline solution + 9 volumes of physiological saline

D: 1 volume of physiological saline + 9 volumes of physiological saline

2.4.2. Basic quantitative characteristics evaluated using an automated biochemical analyzer

Using reagents determined by various evaluations of the reagent composition, the following tests were performed using a Hitachi 7170 automated analyzer.

Reproducibility tests: Measurement of within-run reproducibility was performed using choline solutions at 2 concentrations (10 and 100 μmol/L) and whole blood from healthy subjects 20 times each. Measurement of between-day reproducibility was performed using choline solutions at 2 concentrations (10 and 100 μmol/L) every day for 20 days.

Specificity tests: Among components containing nitrogen, sugars, and amino acids in the blood, ethanolamine, serine, alanine, creatinine, and carnitine were selected as blood components with a chemical structure resembling that of choline. The solutions of these choline analogues and choline (50 μmol/L) were prepared, and measurement was performed. The reactivity of each choline analogue as a percentage of that of choline solution was calculated.

Linearity tests: Serial dilutions of 100 μmol/L choline were prepared, and the choline concentration was determined in duplicate.

Dilution linearity: Using whole blood samples from healthy subjects and 500 μmol/L choline solution, A (1 volume of physiological saline + 9 volumes of a sample from healthy subjects) and B (1 volume of 500 μmol/L choline solution + 9 volumes of a sample from healthy subjects) were prepared. Subsequently, serial dilutions of whole blood samples were prepared by changing the mixture ratio of A to B, and the choline concentration in each dilution was determined.

Minimum limit of detection: Serial dilutions (0-20 μmol/L) of choline solution were prepared. Choline in each dilution was measured in triplicate, and the minimum limit of detection was obtained.

Influences of interfering substances in whole blood: As interfering substances in whole blood, ascorbic acid, albumin, reduced glutathione, and uric acid were selected. Solutions of each substance (20, 5000, 100, and 8 mg/dL) were prepared, and choline was added to obtain a final concentration of 50 μmol/L. The reactivity of solution containing each interfering substance was calculated as a percentage of that of 50 μmol/L choline solution.

Storage stability of samples: To confirm the storage stability of samples, samples from healthy subjects were stored at 25℃ (room temperature), 5℃

(refrigerator), and -20℃ (freezer), and measurement was performed for 7 days from immediately after blood collection.

2.4.3. Assay of choline in whole blood of healthy volunteers

Whole blood samples were obtained from 29 healthy volunteers, consisting of 13 males and 16 females aged 20-25 years (mean, 21 years), with informed consent of the subjects, and choline was measured.

3. Results

3.1. Determination of the reagent compositions

Hemolysis methods: Complete hemolysis was confirmed in ≥ 0.1% TritonX-100 dilutions.

Study of the concentrations of coloring and enzymatic reagents: To avoid the influences of the hemoglobin spectrum of the hemolyzed blood (Fig. 2), DA-67, with an absorption maximum at 666 nm, was selected as a coloring reagent. DA-67 has a molar absorption coefficient of 9×10^4 L/mol · cm, allowing highly sensitive measurement, but is unstable in the solution state, causing spontaneous coloring. For the stabilization of the coloring reagent, 10 mmol/L sodium thiosulfate and 0.16 mmol/L Orange G were added. Based on the Km value[5] of COD for choline (1.2×10^{-3} mol/L), the enzyme concentration at which COD's enzymatic reaction terminates within 1.3 minutes was estimated to be 60 U/mL of R-3 reagent.

Avoidance of the influences of reducing substances in whole blood: The recovery rate in the absence of potassium iodate, hydroxylamine hydrochloride, and hexadecyltrimethylammonium bromide was 24.5%. In the presence of potassium iodate alone, the condition associated with favorable avoidance of the influences of reducing substances was a potassium iodate concentration ≥ 100 mmol/L, and the recovery rate improved to about 50%. In the presence of both 100 mmol/L potassium iodate and hydroxylamine hydrochloride, the recovery rate was 80.3% at a hydroxylamine hydrochloride concentration of 0.75 mmol/L. We speculated that the hemoglobin protein oxidized by the oxidant causes poor recovery. Therefore, hexadecyltrimethylammonium bromide as a cationic surfactant was added for further improvement of the recovery rate. As a result, after the addition of 100 mmol/L potassium iodate, 0.75 mmol/L hydroxylamine hydrochloride, and 0.05% hexade-

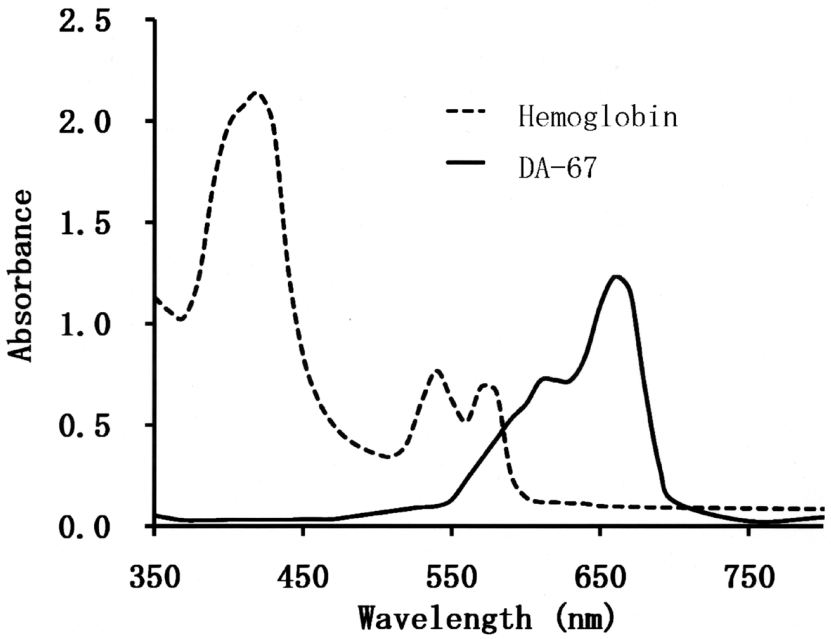

Fig. 2 Absorption spectra of hemoglobin and DA-67.
DA-67 is a coloring reagent with an absorption maximum at 666 nm.

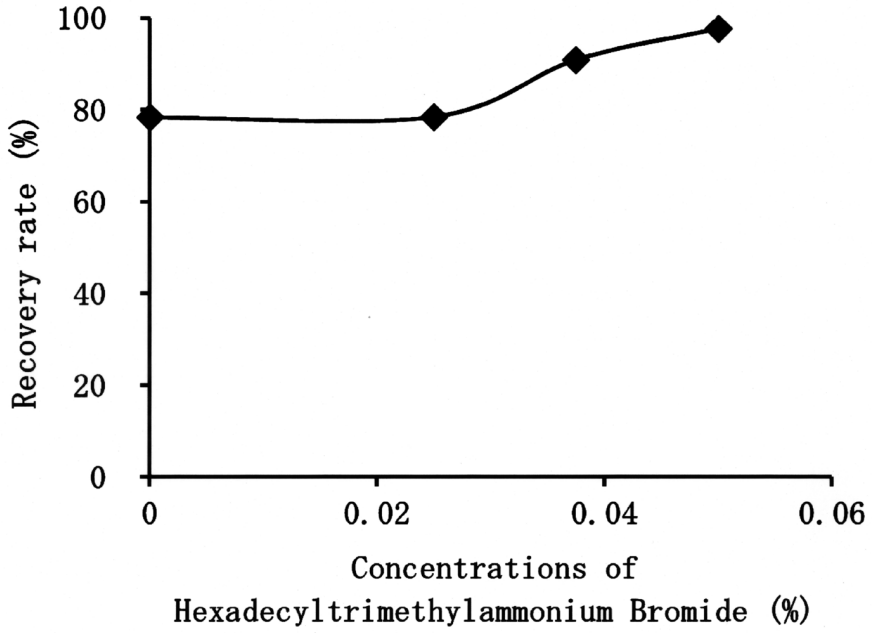

Fig. 3 R-1 solutions differing in the hexadecyltrimethylammonium bromide concentration (0-0.05%) were prepared. The graph shows the whole blood choline recovery rate using R-1 containing 100 mmol/L potassium iodate and pre-diluent containing 0.75 mmol/L hydroxylamine hydrochloride. After the addition of 0.05% hexadecyltrimethylammonium bromide, the recovery rate improved to 97.6%.

Table 2 Within-run and between-day reproducibilities

Sample	Within-run (n=20)		Between-day (n=20)	
	S1	S2	S1	S2
Mean (μ mol/L)	8.2	99.7	8.2	99.7
SD (μ mol/L)	0.19	0.37	0.20	0.43
CV (%)	2.30	0.37	2.43	0.43

cyltrimethylammonium bromide, the recovery rate was 97.6% (Fig. 3).

3.2. Basic quantitative characteristics evaluated using an automated biochemical analyzer

Reproducibility tests: Table 2 shows the within-run reproducibility (n = 20) as well as between-day reproducibility (n = 20) for choline solutions. For 10 and 100 μ mol/L choline solutions, the within-run reproducibility values were 2.30 and 0.37%, respectively, and the between-day values were 2.43 and 0.43%, respectively. Within-run reproducibility tests using whole blood samples from a healthy subject (n = 20) showed a mean value of 21.2 μ mol/L and coefficient of variation of 0.59%.

Specificity tests: Each choline analogue showed negligible reactivity, and the specificity of COD for choline as a substrate was high (Table 3).

Table 3 Specificity of choline oxidase

Substrate (50 μ mol/L)	Reactivity (%)
Choline	100.0
Ethanolamine	3.9
Creatinine	0.0
Carnitine	0.0
Aranine	0.0
Serine	0.0

Linearity tests: Favorable linearity was obtained in the choline concentration range of 0-100 μ mol/L. Dilution linearity: Serial choline dilutions containing whole blood components also yielded favorable

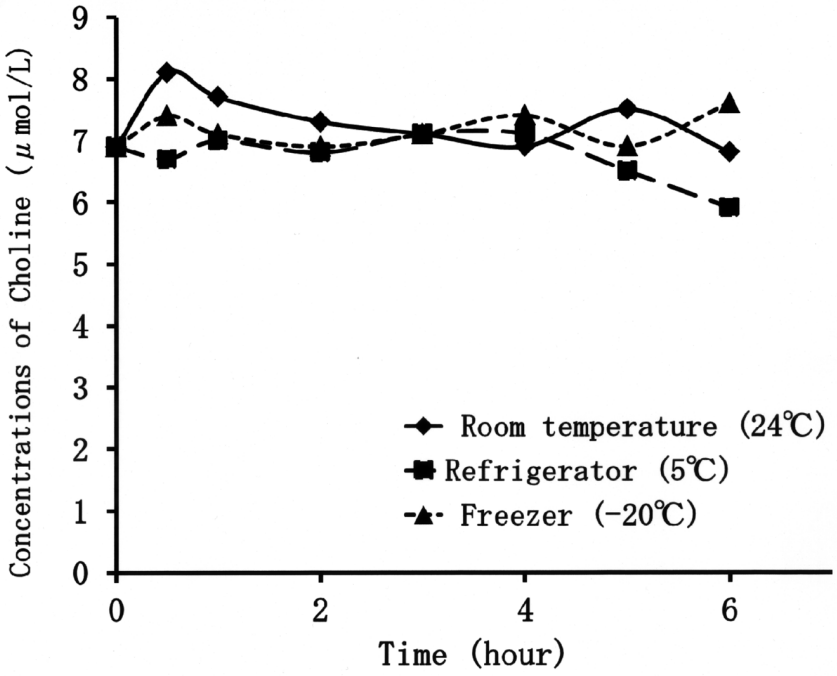

Fig. 4 Changes in the choline concentration until 6 hours after blood collection when whole blood samples from healthy subjects were stored at room temperature (25℃), in a refrigerator (5℃), or in a freezer (-20℃). The choline concentration was relatively stable (changes within ± 1.2 μ mol/L) under each storage condition.

linearity.

Minimum limit of detection: The choline concentration not overlapping with ± 3 SD for 0 μ mol/L was 2 μ mol/L, and, therefore, the minimum limit of detection was 2 μ mol/L.

Influences of interfering substances in whole blood: The rate of interference (%) by interfering substances in whole blood was 0.5-0.6%, showing their negligible influences on the color reaction of choline.

Storage stability of samples: Under each storage condition, samples were relatively stable (changes in the choline concentration, within ± 1.2 μ mol/L) until 6 hours after blood collection (Fig. 4). Until 7 days after blood collection, the choline concentration gradually increased, and this tendency was particularly marked in samples stored at room temperature (25℃).

3.3. Whole blood concentration of choline

In 3 subjects showing a choline concentration ≥ 25 μ mol/L, macroscopic hemolysis was observed. In 26 subjects without hemolysis, the mean value was 8.23 μ mol/L, and the standard deviation was 4.06 μ mol/L. The mean ± 2 SD interval in these samples was 0.1-16.3 μ mol/L.

4. Discussion

At present, most reagents for tests performed employing automated analyzers are used for serum or urine. There are only a few reagents used for whole blood or blood cells. This is because interfering substances such as reduced glutathione or hemoglobin, which affect measurement, are present at a higher concentration in whole blood than in serum, and accurate measurement is difficult. When whole blood components are measured, the influences of interfering substances are reduced by dilution or deproteinization before measurement. However, such a procedure is time-consuming and inappropriate for examination for ACS requiring early diagnosis and treatment. We previously developed a measurement method for whole blood choline by deproteinization with trichloroacetic acid[6-8], but this method required 25

minutes.

In this study, there were two major problems involved in the measurement of whole blood choline using an automated biochemical analyzer. One was interference in absorption by hemoglobin pigments, and the other was interference by various reducing substances that are present in whole blood components. To overcome these problems, we developed an enzymatic assay for choline in whole blood after repeated evaluation.

Concerning the influences of hemoglobin pigments on absorbance, the spectrum of hemoglobin pigments may cause positive or negative errors, depending on the measurement wavelength. As shown in the absorption spectrum of hemoglobin in Fig. 2, a strong red spectrum of hemoglobin is present at wavelengths shorter than 600 nm. DA-67 as a coloring reagent with an absorption maximum at 660 nm can markedly reduce the influences of hemoglobin. In addition, although the choline concentration in whole blood is low (i.e., < 25 μ mol/L), DA-67 has a high molar absorption coefficient, allowing highly sensitive measurement. Therefore, we considered DA-67 to be an optimal coloring reagent in this method.

Concerning reducing substances, in measurement methods using oxidation-reduction reactions, oxidizing or reducing substances in blood cause measurement errors. Among substances causing errors, reduced glutathione, hemoglobin, bilirubin, and ascorbic acid as reducing substances have marked influences, causing negative errors. Various reducing substrates are used for POD as the final detection enzyme of hydrogen peroxide. Therefore, inhibition due to competition with the coloring reagent occurs, causing negative errors. In addition, discoloration due to reduction after oxidation coloring occurs, causing unstable color development.

As pretreatment of interfering reducing substances before the measurement of choline, negative errors were avoided utilizing the oxidation ability of potassium iodate. Potassium iodate remaining after pretreatment oxidizes DA-67 as a coloring reagent, causing positive errors. Therefore, residual potassium iodate after pretreatment should be eliminated. Potassium iodate exhibits oxidation activity in the acid range (pH 4.0-6.0) and loses it in the neutral pH range. Therefore, after the addition of R-2, the buffer was adjusted to obtain a pH of 7.5 for the elimination of the oxidation activity of potassium iodate. The addition of potassium iodate to R-1 increased the recovery rate from 24.5 to 50%. However, the effects of potassium iodate alone were inadequate.

Subsequently, to further increase the effects of potassium iodate to avoid interfering substances, various substances as oxidation accelerators were evaluated. Potassium iodate mixed with very small amounts of reducing substances showed enhanced oxidation activity. At the initiation of the study, R-1 in which potassium iodate coexists with hydroxylamine hydrochloride was evaluated. However, the mixed reagent, in which an oxidizing agent coexists with a reducing agent, showed a gradual decrease in oxidation activity, and was extremely unstable. To overcome this problem, the pre-dilution mode of the automated analyzer was used. The reducing agent was added to sample dilutions, and the oxidizing agent was separately used as R-1. As a result, the reagent could be stored in the stable state, and the elimination of low molecular weight interfering substances became possible. Due to the use of dilutions containing hydroxylamine hydrochloride, the recovery rate improved to 80.3%, which was still inadequate.

We speculated that high molecular weight hemoglobin among interfering, reducing substances in whole blood most markedly affects the measurement system due to its highest concentration. Hemoglobin oxidized by R-1 is considered to oxidize DA-67, resulting in color development. Considering that hemoglobin is negatively charged at a pH of 7.5, we formed ionic bonds with hexadecyltrimethylammonium bromide as a cationic surfactant, and wrapped hemoglobin with the surfactant membrane in order to avoid the influences of hemoglobin by avoiding contact between DA-67 and hemoglobin. Due to the addition of hexadecyltrimethylammonium bromide, the recovery rate improved to 97.6%.

Basic quantitative characteristics using an automated biochemical analyzer showed favorable results regarding reproducibility, linearity, and

minimum limit of detection. Since this method allows quantitative measurement even at low concentrations, accurate measurement of whole blood choline, which was reported as a marker for ACS by Danne et al.[9] may be possible. Concerning the specificity of COD, no reaction with choline analogues in whole blood was observed, suggesting that this method allows the specific measurement of choline. In addition, there were negligible influences of interfering substances in whole blood, and serial dilutions containing whole blood components yielded favorable linearity. Therefore, the influences of interfering substances in whole blood can be adequately avoided using this method.

Concerning storage stability, whole blood samples were relatively stable for 6 hours from immediately after their collection at each temperature, which was consistent with the results reported by Yue et al.[10]. In the blood collection tube, phospholipase D acts on phospholipids in whole blood, increasing the choline concentration. Calcium ions as an activator for phospholipase D may have been inactivated by the chelating action of EDTA as an anticoagulant, resulting in the inhibition of an increase in the choline concentration. The increase in the choline concentration until 7 days after the blood collection day may have been because phospholipase D and other enzymes could not be completely inactivated by EDTA, and a small amount of choline in phospholipids was hydrolyzed over a long period. Although measurement immediately after blood collection is desirable, when it is impossible, storage under refrigeration is recommended.

Danne et al.[2] reported a mean choline level of 15.8 μ mol/L and a standard deviation of 9.5 μ mol/L in healthy subjects. The mean $\pm$ 2 SD interval of the whole-blood choline level obtained in this study was similar to that reported by Danne et al., confirming the validity of this method. In addition, evaluation of the quantitative characteristics of this method showed favorable results, suggesting that choline in whole blood could be measured in a short time (measurement time, 10 minutes) without pretreatment.

However, further studies are necessary to evaluate the correlation between HPLC-MS and this enzymatic assay and confirm the accuracy of this assay.

5. Conclusion

To eliminate interfering substances in whole blood, a whole-blood component assay not requiring deproteinization was established by combining potassium iodate, hydroxylamine hydrochloride, and hexadecyltrimethylammonium bromide. This method enabled specific and direct measurement of choline in whole blood using an automated biochemical analyzer. Compared with the conventional method requiring more than 1 hour, this method requires only about 10 minutes, and can also be performed in clinical laboratories without special equipment. This method of eliminate interfering substances also allows the measurement of other items in blood by changing the types of enzyme. Therefore, this measurement system is applicable to other whole blood components and blood cell components that are clinically useful, and its further application in the future is expected.

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Declaration of conflicting interests

None.

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Contributorship

All the authors have read the manuscript and have approved this submission.

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