A Simple Fluorometric Determination of Vitamin C

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A simple and accurate method for determination of vitamin C (ascorbic acid (AsA) and dehydroascorbic acid (DHA)) using 4,5-dimethyl-*o*-phenylenediamine (DMPD) was investigated. It was found that DMPD is a useful fluorescent reagent. The reaction product of DMPD with DHA showed strong and stable fluorescence (Ex; 360 nm, Em; 440 nm). Fluorometric derivatives were extracted with isobutanol or *n*-butanol. Extraction with isobutanol was superior to that with *n*-butanol in terms of specificity, since fluorometric derivatives of keto acids were extracted with *n*-butanol, together with DHA. The fluorescence intensity of DMPD derivatives was absolutely stable in isobutanol for at least 24 h. The sensitivity of determination of vitamin C was improved by removing several non-fluorometric compounds coexisting in the samples. The derivative derived from AsA was easily separated from those of keto acids by an HPLC method. The determination of vitamin C in natural products was thus improved by extraction and the HPLC method.

Key words vitamin C; 4,5-dimethyl-*o*-phenylenediamine; HPLC; fluorometric determination; ascorbic acid; dehydroascorbic acid

2,4-Dinitrophenylhydrazine (DNPH) method[1—3] has been used to determine total vitamin C, that is, ascorbic acid (AsA, reduced form) plus dehydroascorbic acid (DHA, oxidized form) as described in "The Standard Methods of Analysis of Hygienic Chemists."[4] However, we consider that improvements must be made in regards to several points. The method lacks specificity because the degradation products of 2,3-diketogulonic acid (DGA), which have no vitamin C activity, react with DNPH. Additionally, highly concentrated sulfuric acid is used many times in the procedure. High performance liquid chromatography (HPLC), using a UV-detector and electrochemical detector (ECD) has been designed for determination of AsA,[5—12] but these methods are not good for determination of total vitamin C, and are also affected by contaminants. Since the oxidation reaction of AsA to DHA proceeds readily, it can be concluded that this reaction may be useful for determination of total vitamin C. The fluorometric determination of total vitamin C based on 2-glycerodihydroxyethyl-3-ketofuro[3,4-b]quinoxaline, which is the reaction product of *o*-phenylenediamine (OPD) and DHA, has given excellent results.[13] This method is rarely affected by DGA and is simple to use, but also has several disadvantages: 1) OPD reacts with many kinds of compounds such as keto acids and reductones[14,15] and 2) the fluorescent products show weak intensities. In order to remove these disadvantages for determination of vitamin C, we examined new fluorescent-derivatizing reagents. Herein we report a simple and accurate method for determination of total vitamin C using 4,5-dimethyl-*o*-phenylenediamine (DMPD).

Experimental

Chemicals 4-Methyl-*o*-phenylenediamine (MPD), DMPD, 4-chloro-*o*-phenylenediamine (CPD), *p*-nitro-*o*-phenylenediamine (NPD), 2,3-diaminopyridine (DAP), and 1,2-diaminoanthraquinone (DAA) were purchased from Tokyo Chemical Industry Co., LTD. (Tokyo, Japan). OPD and 3,4-diaminobenzoic acid (DBA) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). 2,3-Diaminonaphthalene (DAN) was obtained from Aldrich Chemical Co., Inc.. 4,5-Dimethoxy-1,2-diaminobenzene (DDB), L-AsA and quinine sulfate were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan).

Solutions a) Fluorescent derivative reagents; 10 mg Each of OPD, MPD, DDB and DAP were dissolved in 10 ml of 0.005 mol/l H_2SO_4, respectively. 10 mg Each of DMPD, DBA and CPD were also dissolved in 1 ml of EtOH, and 9 ml of 0.005 mol/l H_2SO_4 was added. b) 0.05 mol/l I_2 solution; 1.3 g of I_2 was dissolved in 10 ml of 40% KI, and 0.1 ml of diluted HCl (2.36 ml of concentrated HCl diluted to 10 ml with H_2O) was added. The solution was diluted with H_2O to 100 ml and stored in a refrigerator. The solution was diluted with H_2O to 0.005 mol/l concentration prior to use. c) 0.1 mol/l $Na_2S_2O_3$; 2.5 g of $Na_2S_2O_3$ and 0.02 g of Na_2CO_3 were dissolved in 100 ml of H_2O and stored in a refrigerator. The solution was diluted with H_2O to 0.01 mol/l concentration prior to use. d) AsA standard solution; 10 mg of L-AsA was dissolved in 100 ml of 3% phosphoric acid (meta)–8% CH_3COOH (1 : 1) solution and diluted to appropriate concentrations prior to use.

Sample Solutions for Quantitative Analysis Sample containing AsA was homogenized in 3% phosphoric acid (meta)–8% CH_3COOH (1 : 1) solution and filtered through a filter paper. The filtrate was diluted to a suitable concentration.

Reactions of AsA with Fluorescent Derivative Reagents One ml of a test solution or standard solution was mixed successively with 0.1 ml of 0.005 mol/l I_2, 0.1 ml of 0.01 mol/l $Na_2S_2O_3$, 0.5 ml of 1 mol/l NaOH and 0.1 ml of fluorescent derivative reagent. The solution was allowed to stand for 30 min at room temperature, protected from light, until reaction was complete. The fluorescence intensity of the reaction mixture was determined directly. In cases where extraction and separation were required, the reaction scale was doubled, and then extracted with 4 ml of solvent.

Determination of Total Vitamin C Content by the DNPH Method Total vitamin C was determined according to the standard methods of analysis for hygienic chemists.[4]

Apparatus a) HPLC: HPLC was performed with a Nihon-Bunko Model with a twinkle type pump and FP-110 fluorometric detector. HPLC conditions were as follows: column: Finepak SIL C18-10 (250 mm×4.6 mm), mobile phase: CH_3OH–0.1% phosphoric acid(meta) (7 : 3), flow rate: 1.0 ml/min, Injection volume: 10 μl, measured wavelength: Ex; 360 nm, Em; 440 nm. b) Spectrofluorometer: Hitachi Model F-3000.

Recovery of Added AsA Several μg of AsA per 1 ml of test sample was added to samples containing a known quantity of AsA. The total vitamin C in samples was determined by the prescribed methods.

Results and Discussion

Mutual Comparison of Fluorometric Derivative Reagents a) Fluorescence Spectra of *o*-Diamine Compounds and Their Reaction Products with AsA: A comparison of the fluorescence spectra of *o*-diamine compounds and their reaction products with AsA is summarized in Table 1. Table 1 indicates that these compounds, except for NPD, DAN and DAA, can be dissolved at a concentration of 1 mg/ml. The products from reaction of AsA (10 μg/ml) (previously oxidized to DHA) and six fluorometric derivative reagents

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Table 1. The Features of Fluorometric Reagents and Their Derivatives with AsA

Fluorometric reagent	Solubility[a] (1 mg/ml)	Fluorometric reagent alone			Fluorometric derivative		
		Ex (nm)	Em (nm)	Strength[b] (%)	Ex (nm)	Em (nm)	Strength[c] (%)
OPD	+	298	337	3.91	350	423	0.45
MPD	+	296	341	4.22	355	428	0.64
DMPD	EtOH	300	344	3.97	361	443	0.95
DDB	+	305	360	3.42	378	451	6.11
DBA	EtOH	326	426	0.35	360	430	0.46
CPD	EtOH	305	355	0.21	356	419	0.41
DAP	+	351	395	4.01	347	395	4.68
NPD, DAN, DAA	−						
Quinine(1 μg/ml)		350	448	100			100

a) One gram of fluorometric reagent was dissolved in 1 ml of 0.005 M H_2SO_4. +; soluble, EtOH;soluble when dissolved in one tenth volume of EtOH and then diluted to 1 mg/ml by 0.005 mol/l H_2SO_4, and −; partially soluble or insoluble. *b*) Measured at a concentration of 0.1 mg/ml and at the Ex and Em maxima of each reagent, and expressed as percentage of quinine–H_2SO_4 (1 μg/ml). *c*) AsA (10 μg/ml) was treated with fluorometric reagents as described in Experimental, and expressed as percentage of quinine–H_2SO_4 (1 μg/ml).

showed fluorescence. MPD, DMPD, DDB and DAP derivatives showed stronger fluorescence than that of the OPD derivative. Furthermore, these compounds, except the CPD and DAP derivatives, showed a maximum wavelength in the fluorescence spectra at 64—99 nm separation from the respective fluorometric derivative reagents. DMPD derivatives produced the best results for quantitative analysis.

b) Extractability of Fluorometric Derivatives with Organic Solvents: If fluorometric derivatives derived from vitamin C can be extracted with organic solvents, as in the cases of vitamin B_1 and B_2, the sensitivity of determination of vitamin C should be improved by removing several types of coexisting non-fluorometric compounds. We examined the partition coefficients of fluorometric derivatives of vitamin C between organic solvents (isobutanol, *n*-butanol, chloroform, ethylacetate, dichloromethane, ethylether, and *n*-hexane) and aqueous solution on the basis of fluorescence intensity. It became clear that the fluorometric derivatives derived from CPD, DMPD and MPD were extracted considerably with isobutanol and *n*-butanol. In particular, over 80% of the DMPD derivative was extracted with isobutanol and n-butanol. (data not shown) On the other hand, the fluorometric derivatives of OPD, DDB and DBA were hardly extracted with most solvents tested.

c) Stability of Fluorometric Derivatives: The stability and the temperature influence of fluorometric derivatives produced from the reaction of AsA (10 μg) (previously oxidized to DHA) and DMPD were examined. In the reaction mixture, the fluorescence intensity of the DMPD derivative was absolutely stable for 1 h at 30—50 °C though it decreased at 80 °C. In the extracting solvent (isobutanol), the fluorescence intensity of the derivatives from DMPD, CPD and OPD were absolutely stable for at least 24 h at room temperature (22 °C).

d) Reactivity of Keto Acids and Sugars with Fluorometric Derivative Reagents, and Their Extractability with Isobutanol: The reactivity of keto acids and sugars with four fluorometric derivative reagents (DMPD, OPD, CPD, DDB) was investigated under the same conditions for AsA. The molar amounts of keto acids used were the same as AsA (10 μg, 5.68×10^{-8} mol). The derivatives of ketoglutaric acid, oxalacetic acid and pyruvic acid in the reaction mixture showed 1.4—12.7% of the intensity of AsA. These values were decreased by extraction with isobutanol. The fluorometric derivative of AsA is extracted to the same extent with *n*-butanol and isobutanol. The extraction with isobutanol was superior to that with *n*-butanol in terms of specificity, since fluorometric derivatives of keto acids were extracted with *n*-butanol together with that of AsA, as can be seen with the DMPD derivative. All sugars tested have shown very little fluorescence under these conditions.

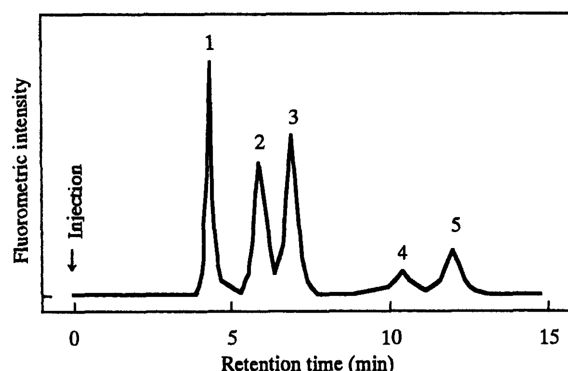

Fig. 1. HPLC Chromatogram of Fluorometric Derivatives from the Reaction of AsA and α-Keto Acids with DMPD

1. AsA, 2. α-ketoglutaric acid, 3. pyruvic acid, 4. oxalacetic acid, 5. α-ketovaleric acid, 6. α-ketoisocaproic acid.

e) HPLC Method for Separation of Fluorometric Derivatives of AsA and Keto Acids: The mixture of AsA (10 μg) (previously oxidized to DHA) and five keto acids (100 μg each) was reacted with DMPD, and the fluorometric derivatives were extracted with isobutanol, followed by HPLC separation. Figure 1 indicates that the DHA derivative was easily separated from those derived from keto acids.

f) Spectra of Fluorometric Derivatives from the Reaction of DHA and DMPD: The excitation and emission spectra of the fluorometric derivatives produced in the reaction of DHA and DMPD were measured as described in Experimental. Gentle-sloping excitation and emission spectra with maximum values at 361 and 443 nm, respectively were obtained (spectra not shown). Thus, we decided to utilize 360 nm for excitation and 440 nm for emission. On the other hand, fluorescence was not be observed when AsA was directly reacted with DMPD.

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Table 2. Comparison of Determination of Total Vitamin C in Natural Products and Recovery of Added AsA

	Method A (DMPD-Extr.)		Method B (DMPD-Extr.-HPLC)		Method C (DMPD-HPLC)		Method D (DNPH[a])	
	Found[b]	Recovery[c]	Found[b]	Recovery[c]	Found[b]	Recovery[c]	Found[b]	Recovery[c]
Chicken liver	18.7	102.5±2.3	16.6	102.2±0.9	16.9	101.0±0.6	22.7	96.5±6.9
Navel orange, rind	118.1	101.3±1.1	109.1	98.8±1.3	108.1	97.4±1.7	118.4	100.2±1.2
Orange, rind	160.0	99.3	160.0	99.3				
juice	45.0	102.0±6.8	45.0	102.0±6.8				
Lemon, rind	209.0	98.8±0.8	209.0	98.8±0.8				
juice	41.4	98.3±1.4	41.4	98.3±1.4				
Sausage	2.5	96.0	2.5	96.0				
Beef liver	21.8	109.0±2.8	21.8	109.0±2.8				

a) DNPH method described in the literature.[4] *b*) mg/100g (ml). *c*) mean±SD, %.

Determination of Total Vitamin C Content by DMPD Derivatization Since we demonstrated that DMPD is superior to OPD as a fluorometric derivative reagent in terms of fluorescence intensity and extractability, we examined the determination of total vitamin C content using the DMPD derivative.

a) Calibration Curve: One ml of AsA standard solutions were oxidized with 0.1 ml of 0.005 mol/l I_2, and 0.1 ml of 0.01 mol/l $Na_2S_2O_3$ was then added. The solution was adjusted to pH 4 with about 0.5 ml of 1 mol/l NaOH and reacted with 0.1 ml of 0.1% DMPD for 30 min at room temperature. The solution was extracted with 2 ml of isobutanol, and dried over anhydrous Na_2SO_4. The fluorescence intensity (Ex; 360 nm, Em; 440 nm) of extracts was proportional to the quantity of AsA. The final extracts were injected into the HPLC apparatus, and measurements suggested an adequate correlation (correlation coefficient: 1.000).

b) Recovery of Added AsA: Table 2 shows the results of total vitamin C and added AsA contents in chicken liver and navel orange rind by four methods. Thus, after the fluorometric derivative reaction with DMPD was completed, AsA contents in samples were determined by: 1) method A: extraction with isobutanol; 2) method B: HPLC separation after extraction with isobutanol; 3) method C: HPLC separation without extraction; 4) method D: DNPH method.[4] The recoveries of added AsA gave satisfactory results (almost 100%). Vitamin C content determined by method A agreed with that of method D, though the contents determined by both methods A and D were higher compared with the results determined by methods B and C. These results mean that fluorometric derivatives of keto acids were separated from that of AsA by extraction and HPLC.

c) Determination of Total Vitamin C Content in Natural Products: Table 2 also shows the results of determination of total vitamin C contents in natural products by the methods described above (methods A, B). Good values were obtained in each case. The values determined by method B were the same, or lower than those by method A. These results support the notion that fluorescent contaminants were removed by HPLC.

Finally, to summarize our studies, we have described a simple and accurate method for determination of vitamin C using DMPD. DMPD derivatives showed strong fluorescence and were absolutely stable. The sensitivity of determination of vitamin C was improved by extraction and an HPLC method.

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