Fluorimetric Determination of Ascorbic Acid in Vitamin C Tablets Using Methylene Blue

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In this study, a simple and sensitive fluorimetric method was described for the determination of Ascorbic Acid (AA). The procedure is based on the reaction between AA and Methylene Blue (MB). The fluorescence intensity of MB was measured at excitation and emission of 664 and 682 nm, respectively. MB concentration was decreased as a function of decreasing fluorescence intensity due to forming colorless form of MB (Leuco-MB) in the reaction between AA and MB. A linear relationship was obtained between the decreasing fluorescence intensity and the concentration of AA in the range of 3.0×10^{-7}—6.0×10^{-6} mol·l^{-1}. The detection limit was 2.5×10^{-7} mol·l^{-1}. The proposed method was applied successfully for the determination of AA in Vitamin C tablets.

Key words fluorimetric determination; ascorbic acid; methylene blue; Vitamin C tablets

Ascorbic acid (AA), also known as Vitamin C, is a water-soluble vitamin and needed for the growth and repair of tissues in all parts of the body. AA has vital importance in processes of oxidation and reduction in human organism participating of several metabolic reactions. AA has also been used for the prevention and treatment of the common cold, mental illness, infertility, cancer and AIDS.[1)] Due to the great importance of AA in human beings, the quantitative analysis of AA has gained increased significance in several areas of analytical chemistry such as pharmaceutical and food applications. It is also clinically important to determine its concentration in blood, urine and some tissues.

Many methods depend on the reaction of AA by means of suitable reagents, then the product of reaction or excess reagent monitored with optically (spectrophotometric,[2—4)] fluorimetric[5—8)] or chemiluminimetric[9)]), electrochemically (amperometric,[10,11)] voltammetric,[12,13)] potentiometric[14)] or conductometric[15)]) and chromatographically (High Pressure Liquid Chromatography[16,17)]). Spectrofluorimetric analysis is well known for its sensitivity and speed. Recently, fluorimetric determinations of AA have been developed based on the condensation reactions of AA with *o*-phenylenediamine[5)] and on the oxidation with mercury(II) of vitamin C to form quinoxaline derivate.[6)] The reaction products of these methods exhibit fluorescence. A simple, sensitive and selected fluorimetry for determination of AA is based on the reaction of AA in alkaline medium with 2,3-diaminonaphthalene without oxidant.[7)] The addition of beta-cyclodextrin (beta-CD) was greatly enhancing the fluorescence of the system. In another study,[8)] Thionine blue is reacted with AA in order to develop a simple, sensitive, selective and rapid fluorimetric method for determining AA. The product of this photochemical reaction, Leucothionine blue, is a highly florescent specimen, which is used for sensitive determination of AA in flow injection system.

In our study, Methylene Blue (MB), a member of thiazine dye groups like thionine blue, was used for the fluorimetric determination of AA. MB is widely used in a number of different areas *e.g.* a photo sensitizer to produce singlet oxygen in photodynamic therapy for the treatment of cancer. The highly colored oxidized form of MB can be reduced to the colorless leuco form Leuco-Methylene Blue (LMB), as shown in Fig. 1.[18)] The redox indicator properties of this couple have been used extensively in chemical analyses. The reaction between AA and MB was used for AA determination in pharmaceuticals or fruit juices by using spectrophotometry,[19)] amperometry[20,21)] or voltammetry.[22)]

In this study, the determination of AA is based on the measurement of decreasing of the fluorescence intensity of MB due to the reaction between AA and MB, where MB is reduced to colorless LMB and AA is oxidized to dehydroascorbic acid (DHAA). This method allows us to determine the amount of AA in the purified materials, specifically Vitamin C tablets.

Results and Discussion

Fluorescence Spectra of Methylene Blue The fluorescence bands of MB were obtained at 664 nm for excitation and 682 nm for emission peaks. Lee and Mills[18)] also obtained emission bands as 682 nm for MB and 452 nm for LMB. Although the reduced form of MB (LMB) exhibits a stronger relatively long-lived blue compared to that of its oxidized form, MB can be also used for the determination of AA. The emission peak of MB at 682 nm increased due to the increase of its concentration, which is shown in Fig. 2. A linear relationship between MB concentration and intensity was obtained over the concentration range of 1.0×10^{-7}—2.0×10^{-6} mol·l^{-1} MB ($y=49.082x+94.46$, $r^2=0.9969$). The excitation peak of MB at 664 nm also linearly increased depending onto the increase of its concentration.

The stability effect of fluorescence spectra of MB solutions is also examined in this study. Figure 3 shows the emis-

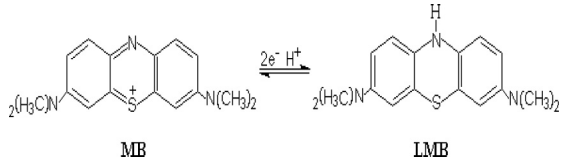

Fig. 1. The Redox Couple of Methylene Blue

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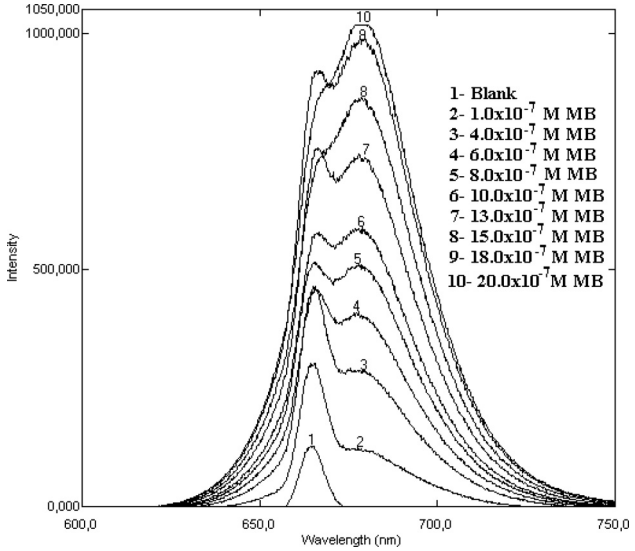

Fig. 2. Fluorescence Spectra of Methylene Blue (Emission Spectra λ_{em}=682 nm) Depending on Its Concentration

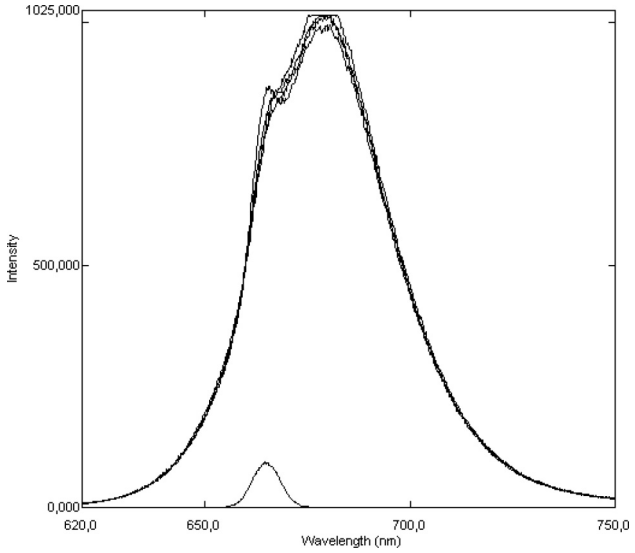

Fig. 3. The Fluorescence Spectra of 2.0×10^{-6} mol·l^{-1} Methylene Blue (λ_{em}=682 nm) Depending on Time

sion spectrum of 2.0×10^{-6} mol·l^{-1} MB as a function of time. Each spectrum was recorded at 1-min intervals. The spectrum was not changed with time, reflecting that the fluorescence spectrum of MB was highly stable with time.

The Effect of Ascorbic Acid on the Fluorescence of Methylene Blue In order to examine the effect of AA on the excitation spectrum of MB at 664 nm, 2.0×10^{-6} mol·l^{-1} MB solutions, each includes in the range of AA with different concentration, were prepared at N_2 atmosphere. Their spectra were recorded at 664 nm (Fig. 4). While the excitation intensity of 2.0×10^{-6} mol·l^{-1} MB was about 1000.0, above which intensity was decreased by increasing AA concentration in MB solutions. In the redox reaction between AA and MB (Eq. 1), AA was oxidized to DHAA, while MB was reduced to colorless LMB.

$$AA + MB_{(DeepBlue)} \leftrightarrow LMB_{Colorless} + DHAA \quad (1)$$

Thus, the amount as well as the fluorescence intensity of MB

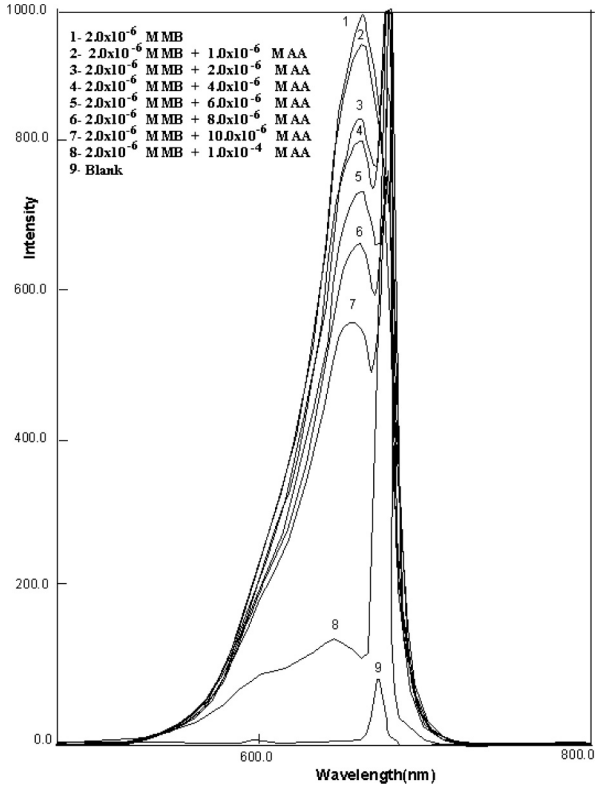

Fig. 4. The Fluorescence Spectra of 2.0×10^{-6} mol·l^{-1} Methylene Blue (Excitation Spectra λ_{ex}=664 nm) Depending on Ascorbic Acid Concentration

at 664 nm decreased depending the increase of AA concentration due to the formation of colorless LMB.

In a similar study,[8] thionine blue was reacted with AA under the light. The product of reaction between AA and thionine blue called Leucothionine blue was highly fluorescent characterized, which could be easily used for the sensitive determination of AA in a flow injection system. The overall reaction between AA and Thionine Blue is given by Eq. 2.

$$\underset{\text{(Blue Low Fluorescence)}}{\text{Thionine Blue}} + AA \xrightarrow{h\nu} \underset{\text{(Colorless Highly Fluorescence)}}{\text{Leuco-Thionine Blue}} + DHAA \quad (2)$$

A study by Leon and Catapano[23] has shown that indirect flow injection spectrophotometric determination of AA in Vitamin C tablets was accomplished by using the photochemical reduction of MB. In their study, they measured the decrease of MB absorbance at 662 nm depending on AA solutions with different concentration. However, in our study, we measured emission intensity of MB at 682 nm. Thus, the same mechanism can be given for the reaction between AA and MB.

Stable Test The effect of the reaction time on fluorescence intensity is also studied. 2.0×10^{-3} mmol·l^{-1} MB solutions, each including a solution of AA in the range of 5.0×10^{-7}—1.0×10^{-5} mol·l^{-1}, were recorded depending on time (Fig. 5). The results show that the fluorescence intensity of each solution remains stable for about 50—60 s at room temperature.

Calibration Curve and Detection Limit The calibration was made based on using working concentration of MB (2.0×10^{-3} mmol·l^{-1}). The results indicate that the fluorescence intensity of the system is a linear function of AA con-

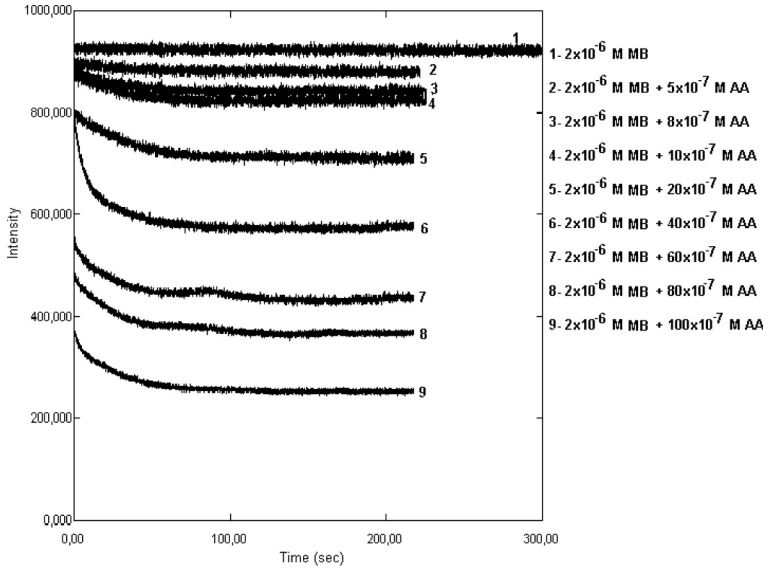

Fig. 5. The Fluorescence Intensity–Time Recordings Obtained with 2.0×10^{-6} mol·l^{-1} Methylene Blue Depending on Ascorbic Acid Solution with Different Concentration

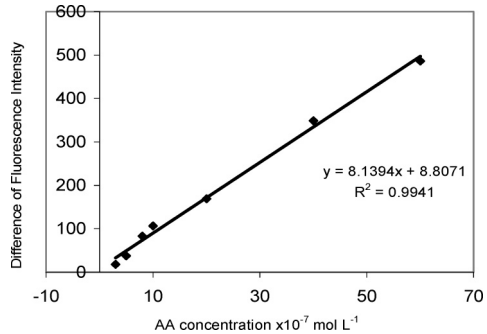

Fig. 6. The Calibration Plot over the Ascorbic Acid Concentration Range 3.0×10^{-7} to 6.0×10^{-6} mol·l^{-1}

centration in the range of 3.0×10^{-7}—6.0×10^{-6} M (Fig. 6). The regression coefficient was 0.9941 and detection limit was 2.52×10^{-7} mol·l^{-1}.

Compared to some methods, the proposed method has a good sensitivity and simplicity. For example, the sensitivity of this method is higher about ten fold than that of spectrophotmetric determination of AA by using MB.[19] The detection limit of another fluorimetric method, which is 1.3 μg·ml^{-1} (about 7.3×10^{-7} mol·l^{-1}), is lower than that of the proposed method. Although a similar method using thionine blue[8] is very rapid than this method, its concentration range (from 8.0×10^{-7} to 5.0×10^{-5} mol·l^{-1}) is lower than that of the proposed method (from 3.0×10^{-7} to 6.0×10^{-6} mol·l^{-1}). This can be explained that, although the lecothionine blue has highly fluorescent property, the decreasing of the fluorescence intensity of MB in the proposed method may be very sensitive. Results of our experiment were supported by this phenomenon. However, the sensitivity of other fluorimetric methods, *o*-phenylenediamine[5] and β-cyclodextrin[7] methods, is higher than that of the proposed method. The detection limits of these methods are 0.006 μg·ml^{-1} (about 3.4×10^{-8} mol·l^{-1}) and 8 ng·ml^{-1} (about 4.5×10^{-8} mol·l^{-1}), respectively. As a result, it can be concluded that this method is useful for rapid and sensitive determination of AA in vitamin C tablets.

Table 1. Tolerance towards Different Substances in the Determination of Ascorbic Acid[a)]

Additive type	Maximum tolerable concentration ratio
Glucose, fructose, alanin, saccharose, urea, acetat, benzoic acid	100
Citric acid	20

a) Ascorbic acid concentration 1.0×10^{-6} mol·l^{-1}

Table 2. Determination of Ascorbic Acid in Pharmaceutical Preparations

Sample	Amount of ascorbic acid (mg g^{-1})	
	Proposed method[a)]	Reference method
Redoxon (Roche)	205.203±9.20	218.02±1.14
Sandoz (Novartis)	129.87±7.72	145.63±2.02

a) Mean for five determinations.

Interference Study In order to assess the possible analytical applications of the fluorimetric method describe above, the effect of concomitant species on the determination of AA in real samples was studied by analyzing synthetic sample solution 1.0×10^{-6} mol·l^{-1} of AA and various excess amount of the common excipients were used in pharmaceutical preparations. Table 1 shows the assayed compounds. These compounds, with the exemption of urea, are present in most vitamin C tablets. Based on these results it might be concluded that there is no major interference by the presence of these species. The experimental results showed that the presence of hundred-fold excess of the all contaminant compounds and twenty-fold excess of the citric acid did not significantly influence affect the determination of AA upon the experimental conditions. So, it is possible to use this method for the direct determination of AA in the pharmaceuticals without separating the interfering materials.

Sample Determination The proposed method is used to determine AA in Vitamin C tablets and compared with standard triiodide procedure.[24] Table 2 lists the results, which

were the average of five replicate determinations, obtained on application of the proposed method. It can be seen that the results are in good agreement with that of the triiodide procedure.

Conclusion

The proposed method provides a simple and sensitive fluorimetric procedure by using MB for the determination of AA. While the reduced form of MB (LMB) was frequently used for fluorimetric determination of AA due to its strongly fluorescence property at 452 nm (for emission), the oxidized form of MB was only used for spectrophotometric determination of AA. Although MB has slightly fluorescence property in comparison to LMB, this study has shown that it could be used for fluorimetric determination of AA and the AA content of Vitamin C tablets (Sandoz and Redoxon). The decreased fluorescence intensity of MB is preferred; despite LMB is highly fluorescent property. It could be explained that the decrease of the fluorescence intensity of MB in the proposed method was more sensitive. In addition, the interferences at 452 nm can have fluorescence intensity.

Experimental

Reagents All chemicals were of analytical reagent grade and ultra pure water from Elga Maxima was used in all experiments.

Standard AA Solutions: A stock solution of AA (10^{-2} mol·l^{-1}) was prepared daily no more than 3 h prior to use by dissolving 44 mg of AA (Merck) in 25 ml deoxygenated ultra pure water. Working solutions of lower concentration were prepared by appropriate dilution with deoxygenated water. All solutions were kept in amber-colored bottles in the dark.

Stock Solution of Methylene Blue: MB (10^{-3} mol·l^{-1}) solution was prepared by dissolving the appropriate amount of the $MBCl \cdot xH_2O$ ($x=2$ or 3, Merck) in deoxygenated ultra pure water. Solutions of lower concentration were prepared by dilution with deoxygenated water.

Apparatus A RF 5301 Series Shimadzu Spectrofluorimeter was used to record spectra and carry out fluorescence measurements.

Preparation of Assay Solution Ten tablets of commercial sample (Sandoz and Redoxon) were powdered in an agate mortar. Later, an appropriate amount of the powder was weighed accurately and dissolved in 25 ml deoxygenated water. The final solution was centrifuged and diluted with cold water prior to analysis.

Procedure The fluorescence spectrum of MB was examined as depending on its concentration.

Ten milliliters 2.0×10^{-6} mol·l^{-1} MB solutions, each reacted with AA at different concentration, were prepared by deoxygenated water and their fluorescence intensities at 664 nm for excitation were recorded. Thus signal recorded are of decreasing fluorescence intensity owing to reduction of MB to LMB (colorless) with standard AA solutions.

An aliquot of dilute Vitamin C tablets samples were reacted by 10 ml 2.0×10^{-6} mol·l^{-1} MB solutions and decreasing of fluorescence intensities of these solutions were recorded at 682 nm for emission.

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