

COMPARISON OF THREE DIFFERENT SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF VITAMIN C LEVELS IN HUMAN BODY FLUIDS

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ABSTRACT

In this investigation three different spectrophotometric methods have been compared to estimate ascorbic acid contents of various human body fluids. For this purpose twenty blood and urine samples were collected from middle aged adult subjects and preserved by the recommended methods. The 1st batch was analyzed by three methods for the ascorbic acid contents and the same methods were applied to the samples with added ascorbic acid in the 2nd batch of blood and in urine samples 3rd batch, taken from the same subjects. The results obtained have showed that all three test methods were valuable and applicable equally for blood and urine samples. Among these, the modified method of Roe and Keuther as described by Nino and Shah (1986) has been found the best in this regard. The percentage recovery from blood samples with added ascorbic acid was excellent with this method. For determining ascorbic acid in urine samples, above method again showed considerably better results. It is, therefore, conceivable that from the view point of sensitivity and specificity, the method modified has shown promising results for its application in human biological fluids.

Key Words: Ascorbic acid estimation, comparison of methods, human blood and urine

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INTRODUCTION

Ascorbic acid contains ene-diols which are oxidized by oxidizing agents and this gives basis for its determination (Anonymous, 2000; Basu and Dickerson, 1996; McCornick, 1997; McCornick, 1999). Numerous methods have been reported (Esteve *et al.*, 1997), for the determination of vitamin C but most of the methods are tedious, lengthy, time consuming and not free from interferences (Iqbal and Yaqub, 1980). The determination of vitamin C is of great clinical importance for evaluating its deficiency in various diseases (Bender and Mayes, 2003). Blood and urine examinations are usually taken as an index for checking the adequacy of this vitamin in body (Zilva and Pannal, 1985). Various acids are used for extraction of ascorbic acid, including acetic, trichloroacetic, meta-phosphoric and oxalic acids. The later two acids not only stabilize vitamin C, by reducing the pH of the medium, but also form complexes with metal ions e.g. copper, preventing the catalytic oxidation of this vitamin.

The methods in vogue are not very many but those available are un-satisfactory due to various reasons. A colorimetric method developed by Sarwar *et al* (1995) was based upon turbidometric measurement of fine orange colored suspension produced by the reaction of ascorbic acid with selenium dioxide. The method is considered useful for the test solutions and pharmaceutical preparations. Regarding the storage stability whole blood contents remains stable for about 3 hours if the specimen is refrigerated immediately. Vitamin C is stable for 2 weeks if the plasma specimen is promptly diluted with meta-phosphoric acid and stored at

-20°C. Trichloro-acetic acid can also be used in place of meta-phosphoric acid to prepare a protein free filtrate of plasma (Nino and Shah, 1986).

Another method (Wilson and Guilan, 1969) involves titration with 2, 6-dichlorophenol indo-phenol in an acid medium. Ascorbic acid reduces the indicator to a colorless form. The change in color is measured spectrometrically. A protein free filtrate is necessary which is prepared from fresh blood or plasma by deproteinization with meta-phosphoric or trichloro-acetic acid. The filtrate is titrated directly with the indicator reagent. The blue colored indicator becomes pink in acidic solution. The titration is continued till a persisting faint pink color is obtained. The method is suitable for determination of ascorbic acid in whole blood. A slightly modified method (Nelson and Somers, 1945; 1944; Meclink *et al.*, 1945) is based on the addition of an excess of the dye and measurement of the un-reacted portion subsequently. In this method determination of ascorbic acid is done by photometric measurement. Although this titration method is rapid and convenient, some difficulty is encountered in accessing end point because the indicator color is pale and tends to fade away. Other methods have been developed in which excess of a colored indicator is added to protein free filtrate and to standard solutions. Henry's modification (Henry, 1964) measures only the reduced ascorbic acid and not dehydro-ascorbic acid. Therefore, this method is not suitable for urine specimen since urine contains a sizeable fraction of dehydro-ascorbic acid also. Metal ions such as cuprous, stannous and ferrous interfere with the manual of the indicator dye method. In another method (a modification of Roe and Keuther 1943), method was narrated by Nino and Shah, 1986 dictated that ascorbic acid is oxidized to dehydro ascorbic acid either by cupric sulphate or 2, 6-dichlorophenol indophenol. The dehydro ascorbic acid in a strong acid medium, reacts with 2, 4-dinitrophenyl hydrazine to give dinitrophenyl hydrazone (a red color) which is measured spectrometrically. This procedure however does not distinguish between biologically active ascorbic acid, dehydro ascorbic acid, and the biologically inactive diketogulonic acid forms. Another big hazard is that the reagent can be auto-oxidizable if kept longer. Thiourea is added to dinitrophenyl hydrazine reagent, to prevent its oxidation by interfering substances. Similarly, in another method (Bessey, 1944), the reaction of ascorbic acid with diazotized 4-methoxy-2-nitroaniline in an acid medium, followed by addition of an alkali, yields an intense blue color and is utilized for ascorbic acid assays.

Among other methods of ascorbic acid estimation in different biological samples, Roe (1954) who used 2,4-dinitrophenyl hydrazine and Wollish *et al* (1954) have used 4-methoxy 2-nitroaniline. While some others have used 3,4 dinitrobenzoic acid and/or chloroauric acid as coloring agents. Among oxidation methods, 2,6-dichloro-phenol indophenol dye method was found to be satisfactory. However, Barakat *et al* (1955) found N-bromosuccamide as a selective and desirable oxidizing agent that brings about complete oxidation of ascorbic acid. This method is also selective and relatively better for the estimation of ascorbic acid. Therefore, in the present study we have compared three of the above mentioned methods for determination of ascorbic acid in biological i.e., blood and urine samples.

MATERIALS AND METHODS

Human urine samples were collected from 20 healthy, young adult subjects and were stored immediately at -4°C for determination of ascorbic acid. To avoid oxidation, the urine samples were preserved by adding 2 ml of glacial acetic acid. Similarly, fresh blood samples were collected from the same subjects and were immediately stored at -4°C in bottles containing 3% sodium citrate. The remaining blood and urine samples were stored at -20°C in a refrigerator for future record.

The vitamin C contents of blood and urine samples were determined in triplicate by applying three different methods given below. The results were compared by applying appropriate statistical test (Mohammad, 1991).

Method I (Iqbal and Yaqub, 1980):

Reagents: Standard ascorbic acid aqueous solution 0.08%, having added 2ml glacial acetic acid per 100 ml volume as a preservative, selenium dioxide solution 2%, carboxyl methyl cellulose (CMC) solution 2%, trichlor acetic acid (TCA) solution 20%. The deproteinization of blood was done with 20% TCA solution.

Procedure: Added 1,2,3,4 & 5 ml of blood drop-wise separately in 5 different centrifuge tubes with continuous shaking. Then 5ml of 20% trichlor acetic acid were stirred to obtain a fine suspension, allowed to stand for 5 minutes and centrifuged, showed clear supernatant solution which was filtered. For ascorbic acid estimation, 1.0ml of above filtrate was taken in 25 ml measuring flask separately. Ten ml of 2% CMC and 2ml of 2% selenium dioxide solution was added in each flask until an orange colored suspension developed. The volume in flask was made to the mark with distilled water & absorbance was read at 380 nm.

The same procedure was repeated with urine samples and absorbance was taken again at 380 nm. A separate experiment was conducted by adding known amount of ascorbic acid in blood, which was not previously deproteinized. The blood containing added ascorbic acid was deproteinized with 20% TCA as described previously. All the procedures were repeated thrice and the mean values were taken for comparison. Ascorbic acid added in urine samples was also estimated by the same method.

Method II (Barakat *et al.*, 1955):

The determination of ascorbic acid using N-Bromosuccinamide (NBS) was based on the following reaction:

Ascorbic acid + N-bromosuccinamide \square Dehydro ascorbic acid + Succinamide + Hydrogen Bromide

Reagents: KI 4%, bovine serum 0.1% (NBS), TCA 20%, soluble starch 1%, acetic acid 3% and ascorbic acid 0.1%

For standardization took 5 ml of 0.1% ascorbic acid, add 5 ml of KI and 2 ml of acetic acid. Then added 1 drop of soluble starch solution, titrated against 0.1% BS solution till light pink color was produced.

Procedure: In this method five ml of blood was added drop wise while shaking, to 5 ml

of 20% trichlor acetic acid in a 25 ml centrifuge tube and stirred to obtain a fine suspension. The suspension was allowed to stand for 5 minutes and then centrifuged. The clear supernatant solution was filtered and 5 ml of the filtrate was titrated immediately with 0.001 percent solution of N-Bromosuccinamide. For the urine samples the process of deproteinization was omitted and the samples were titrated with 0.1% NBS solution.

Method III (Nino and Shah, 1986):

For determination of plasma ascorbic acid by 2,4-dinitrophenyl hydrazine (Roe and Keuther⁽¹⁷⁾; Roe⁽²³⁾, Henry⁽¹³⁾). A slightly modification was done as described by Nino and Shah⁽¹¹⁾. The chemical reaction is as follows:

Ascorbic acid + Copper sulphate $\square \square$ Dehydro ascorbic acid.

Dehydro ascorbic acid + DNPH \square Bis 2,4 Dinitrophenyl hydrazone-ascorbate.

The ascorbic acid was converted to dehydro ascorbic acid by shaking with cupric sulphate solution and then coupled with 2,4- dinitrophenyl hydrazine in the presence of thiourea as a mild reducing agent. Sulphuric acid then converted dinitrophenyl hydrazone into a red colored compound, which was assayed calorimetrically. The thiourea is added to prevent oxidation of dinitrophenyl hydrazine reagent by interfering substances.

Reagents: Meta phosphoric acid 6%, sulphuric acid 4.5 molar, sulphuric acid 12 molar and 2,4-dinitrophenyl hydrazine, thiourea 5% solution, cupric sulphate 0.6% solution.

Dinitrophenyl hydrazine thiourea copper sulphate solution (DTCS): Five ml of thiourea and 5ml of copper sulphate add into 100 ml of DNPH solution, mix and store at 4C for a maximum period of 1 week. Standard solution 50 mg% in 6% meta phosphoric acid, always prepared fresh. Intermediate standard solution 5mg %. For working standard, took 0.5, 1, 2, 4, 6,10, 5.0 and 20.0 ml of IS made volume upto 25 ml by meta phosphoric acid giving final concentrations of 0.1, 0.4, 0.8, 1.2, 2.0, 3.0 and 4.0 mg per 100 ml.

Took 2.0 ml of freshly prepared meta-phosphoric acid in test tubes add 0.5 ml of sample or standard. Mixed and centrifuged at 2500 rpm for 15 minutes. Filtered and took supernatant for blank, sample and standard. Added 0.4 ml of DTCS in each and waited for 3 hrs at 37 °C. Added 2 ml of 12 molar sulphuric acid in each and read the absorbance at 520 nm against the reagent blank.

RESULTS AND DISCUSSION

The above mentioned three methods were applied to all the blood and urine samples. The results were compared with similar other samples from same subjects with added 1.0% ascorbic acid. The results have been recorded in Tables 1 and 2 for comparison between the above 3 methods.

Table 1: Comparison of ascorbic acid levels determined by three different methods in human actual blood and urine samples.

Actual Blood Samples Concentrations					Actual Urine Samples Concentrations				
Vol. Used (ml)	Actual Conc. (mg/dl)	Iqbal and Yaqub (1980)	Barakat et al (1955)	Nino and Shah (1986)	Vol. Used (ml)	Actual Conc. (mg/dl)	Iqbal and Yaqub (1980)	Barakat et al (1955)	Nino and Shah (1986)
1	0.8	0.78	0.79	0.79	5	1.5	1.34	1.36	1.46
2	1.6	1.52	1.55	1.56	10	3.0	2.88	2.90	2.92
3	2.4	2.30	2.32	2.36	15	4.5	4.38	4.40	4.45
4	3.2	3.12	3.15	3.16	20	6.0	5.90	5.92	5.95
5	4.0	3.90	3.92	3.96	25	7.5	7.30	7.34	7.42

Table 2: Comparison of Ascorbic acid added (AAA) samples with added ascorbic acid (5 mg/dl) determined by different methods in Human blood and urine samples

Added Blood Samples Concentrations					Added Urine Samples Concentrations				
Vol. Used (ml)	Actual Conc. (mg/dl)	Iqbal and Yaqub (1980)	Barakat et al (1955)	Nino and Shah (1986)	Vol. Used (ml)	Actual Conc. (mg/dl)	Iqbal and Yaqub (1980)	Barakat et al (1955)	Nino and Shah (1986)
1	4.0	3.90	3.95	3.95	5	7.50	6.70	6.80	7.30
2	8.0	7.60	7.75	7.80	10	15.0	14.44	14.5	14.60
3	12.0	11.5	11.6	11.8	15	22.5	21.9	22.0	22.25
4	16.0	15.6	15.75	15.8	20	30.0	29.5	29.6	29.75
5	20.0	19.5	19.6	19.8	25	37.5	36.5	36.7	37.1

The numerous titrimetric, colorimetric, polarographic and spectrophotometric methods have been developed for the determination of vitamin C. The method generally used, either do not take care of possible interference or involve a very lengthy and time-consuming procedure for the removal of interferences, making the specificity doubtful (Varlay and Govenlock 1981). The main purpose of the present study was, to evolve a reasonably good method for the determination of vitamin C in urine and blood. The method should be simple, specific and rapid to give better results and of course reproduce-

ability also. Present study was undertaken to overcome such difficulties. In this study, the recovery of the added compound from urine and blood was about 98-100 %. It was observed that the modified method of Roe and Keuther (1943) as cited by Nino and Shah (1986) were advantageous. The reducing agents like glucose, fructose, meta-bisulphite, cysteine, sulphite and substances like citric acid, tartaric acid, sodium benzoate and meta-phosphoric acid do not interfere in the ascorbic acid determination. The above spectrophotometric method seems quite specific, quick, accurate and convenient.

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