Adaptation of the Phosphotungstate Method to Determine Reduced and Oxidized Vitamin C in Blood Plasma

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The phosphotungstate reagent (PTR) was used for quantitative spectrophotometric determination of physiological forms of vitamin C in blood plasma. An immediate action of PTR on the first half of the tested samples allowed to determine reduced vitamin C concentrations (I) at 700 nm. 10 mM dithiothreitol added to the second half of the samples reduced oxidized vitamin C in it – hence the total amount of this vitamin was reduced with a concentration (II) determined as above (remains of dithiothreitol were removed with *N*-ethylmaleimide). The difference of results (II) and (I) gave the concentration of oxidized vitamin C. The method is characterised by fault-less analytical parameters: correlation coefficients of analytical curves > 0.99, recovery factor 100.5%, variation coefficients intra- and inter-serial < 3% and < 5%, respectively, detection limit 0.05 μM. The simplicity of the method enables an easy control of the ratio of oxidized and reduced vitamin C concentrations in blood plasma – the biomarker of the level of oxidative damage to cells.

Key words: Vitamin C – Reduced and Oxidized, Blood, Phosphotungstate Method

Introduction

Vitamin C in blood plasma is found in two physiological forms: reduced (AA) and oxidized (DAA) – their sum being total vitamin C (TVC). Both forms establish an oxido-reduction system AA/DAA which enables effective deactivation of reactive oxygen species (ROS) by AA (Halliwell, 1991; Oliveira *et al.*, 2003; Rose and Bode, 1993; Rutkowski and Grzegorczyk, 1999).

The content of both forms of vitamin C in blood plasma of healthy subjects is constant: AA 95–99%, DAA 1–5%. But, if AA is used up in deactivation of ROS, its contribution to TVC significantly decreases along with a slight increase in DAA content (Rumsey and Levine, 1998; Schorah *et al.*, 1996; Sies and Stahl, 1995). As a result there is a change of the concentration ratio of the vitamin C forms in blood plasma proportional to the intensity of this process. The mentioned ratio has been accepted as one of the biomarkers for the level of oxidative damage to cells (Lykkesfeldt *et al.*, 1995, 1997; Rose and Bode, 1993; Schorah *et al.*, 1996).

While carrying out studies on oxygen metabolism disturbances during precancerous and neoplastic conditions of the alimentary tract we have decided to control this biomarker in our patients. The choice of the determination method of both forms of vitamin C in plasma that would be appropriate for a hospital laboratory was a difficult problem. Therefore, the adaptation of our own phosphotungstate method (Rutkowski and Grzegorczyk, 1998), presented here and intended to analyse AA, was elaborated.

Material and Methods

Chemicals and equipment

AA, DTT, NEM, all of "Sigma Ultra" purity grade (Sigma, St. Louis, USA), and oxalic acid *p.a.* (POCh, Gliwice, Poland) were used for the development of the determination method. In control testing bovine serum was also used (Bio-Wet, Lublin, Poland) as well as analytically pure: DAA (Merck, Darmstadt, Germany), cysteine, homocysteine, reduced glutathione, 2-mercaptoethanol (all from Sigma), and PO (POCh, Gliwice, Poland). Distilled water from a quartz distillation apparatus was used in order to avoid any influence from heavy metal ions which are introduced to water by metal distillers. The absorbance measurements were performed on a Lambda 14-P (Perkin-Elmer, Überlingen, Germany) with the use of glass semi-microcuvettes of 1 ml capacity.

Abbreviations: AA, ascorbic acid; C. C., correlation coefficient; C. V., variation coefficient; DAA, dehydroascorbic acid; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PO, potassium oxalate; PTR, phosphotungstate reagent; R. F., recovery factor; ROS, reactive oxygen species; TVC, total vitamin C.

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Solutions

The determinations were performed with the use of PTR prepared according to the previously described manner (Rutkowski and Grzegorczyk, 1998). In order to obtain it a suspension of 150 g of sodium tungstate and 60 g of anhydrous sodium monohydrogen phosphate in 240 ml of water was prepared, which was heated to dissolve the ingredients; afterwards 145 ml of 3.7 M sulfuric acid (VI) was added. The solution was mildly heated for 2 h under reflux, and after cooling down to ambient temperature the pH was brought to 1.0 by a dropwise adding of concentrated sulfuric acid (VI) with stirring. The standard AA solution concentration was 56.8 μM and a solvent for it was 50 mM oxalic acid. Other solutions used for the performance of determinations were 10 mM DTT and 40 mM NEM.

Standard solutions of AA and DAA used for obtaining analytical curves had the concentrations: 14.2; 28.4; 56.8; 85.2; 113.6 μM and were prepared with bovine serum as a solvent with the addition of 2 mg/ml PO. Sulfoorganic reducers other than DTT for control tests, containing only one -SH group (Table I), were used as 20 mM solutions.

Developed procedure for two forms of vitamin C in plasma determination

Venous blood freshly collected on PO as an anticoagulant was used for the investigations. After centrifugation of blood cells (650 × *g*, 10 min) a 1.5 ml sample of plasma was subjected to actions described below, for which ready test tubes marked "A" (AA), "T" (TVC) and "S" (standard sample) were prepared.

"A" tubes with 750 μl of the tested plasma, "T" tubes with 750 μl of the tested plasma and 250 μl of DTT solution (stirred), and "S" tubes with 750 μl of standard solution AA were measured; they were left at ambient temperature for 20 min. The following substances were added to the test tubes and stirred: to "T" – 250 μl of NEM solution; to "A" and "S" – 500 μl of a fresh mixture of DTT and NEM solutions 1:1 (v/v) each. To all of the tubes 750 μl of PTR was added, the tube content was stirred and left at ambient temperature for 30 min, afterwards tubes "A" and "T" were centrifuged at 7000 × *g* for 10 min. The absorbance of supernatants from tubes "A" and "T" and of the liquid from tube "S" was measured at 700 nm against a 1:1 mixture (v/v) of PTR and 50 mM oxalic acid as a reference sample.

Concentrations (μmol/l) of AA and TVC were calculated from the equations:

$$\text{(I)} \quad c\,(\text{AA}) = \frac{A_A}{A_S} \cdot c(\text{s}) \qquad \text{(II)} \quad c\,(\text{TVC}) = \frac{A_T}{A_S} \cdot c(\text{s})$$

where A_A and A_T are the absorbance values for AA and TVC respectively, A_S is the absorbance value of the standard sample, and c(s) the concentration of the standard solution.

Then, the concentration of DAA was calculated as the difference of concentrations (II) and (I):

$$c\,(\text{DAA}) = c\,(\text{TVC}) - c\,(\text{AA})$$

The obtained results allowed us to calculate the concentrations ratio for both forms of vitamin C in the tested plasma.

Results

The usage of PTR for the determination of AA formed *in situ* through a reduction of DAA in tested plasma required the control of its analytical usefulness in conditions presented here. PTR reacts selectively with AA but it is true for those applications where analysed plasma does not contain any additional reagents (Rutkowski and Grzegorczyk, 1998) – this is fulfilled in the first half of tested samples. Yet for the second half of these samples PTR is added to plasma already containing DTT and NEM introduced previously, so a possibility of side reactions disturbing the determinations must have been taken into account. Moreover, DAA reduction time with DTT had to be controlled and compared to the action of this reductant with other compounds similar to it.

Control of PTR usefulness for analysis of two forms of vitamin C in blood plasma

In order to check whether PTR is useful for the determination of AA created from DAA under the influence of DTT two series of standard solutions of the same bovine serum made at first were used. The I series were solutions of AA, and the II series were TVC solutions obtained by blending equal volumes of AA and DAA standard solutions of the same concentrations just before the investigations. All the solutions were submitted to the elaborated analytical procedure (see "Materials

and Methods") treating solutions of the I series as samples with "A" symbols, and solutions of the II series as samples with "T" symbols (preliminary adopted time of DTT action was 10 min). Based on the measured absorbance values analytical curves for AA and TVC were drawn, their correlation coefficients calculated and the numeral values of the measurement points from both curves were compared.

Results of the tests showed that both obtained analytical curves were linear within the whole range of studied concentrations and crossed the 0 point (Fig. 1 – first two lower curves), their C. C. being > 0.99. The values of measurement points for the TVC curve were twice compared to the respective points of the AA curve.

Analytical curves were, however, only slightly inclined and this suggests a partial decomposition of both unstable forms of vitamin C in standard solutions, despite the fact that they were prepared just before the tests. New solutions were prepared with the addition of PO (2 mg/ml) that acts as an anticoagulant for the blood analysed for AA with the phosphotungstate method and stabilises vitamin C; later these actions for obtaining analytical curves were repeated. A large, but not uniform increase in inclination was found: the increase in curve angle for TVC was too small and this evidenced a not complete transformation of DAA to AA within the preliminary adopted time of DTT action.

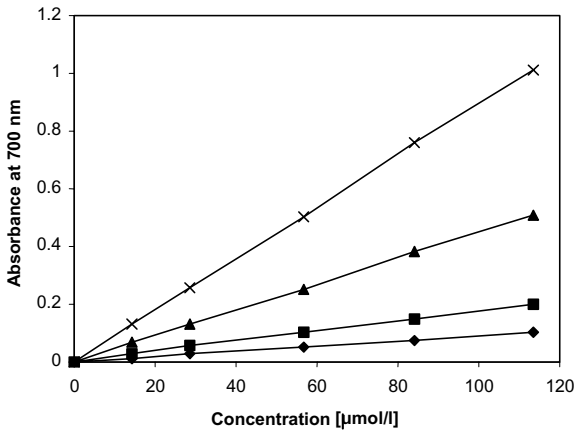

Fig. 1. Analytical curves for AA and TVC standard solutions in bovine serum, submitted to the analytical procedure presented in the text, before and after optimisation of DTT action time. The first two lower curves were obtained before optimisation of DTT action time (adopted time 10 min); measurement points are for the following solutions: –◆– AA, –■– TVC. The two upper curves were obtained for an optimised time of DTT action (20 min) with addition of PO (2 mg/ml) to standard solutions; measurement points are for following solutions: –▲– AA, –×– TVC.

Optimisation of DTT action time

A standard solution of 56.8 μM DAA with the addition of PO was submitted to the developed analytical procedure (see "Materials and Methods") at 20 °C according to the actions for samples marked "T", with DTT action time equal 5, 10, 15, 20, 25 and 30 min. Basing on the measured absorbance values an optimal curve was drawn (Fig. 2) which shows that a quantitative effect of transition took place after 20 min. Using this optimum time of exposure to DTT, analytical curves were performed (as above) for the standard solutions containing additions of PO. Regularity of inclination increase was found – the absorbance values for TVC were double the values for AA. Both curves crossed the 0 point, fulfilled the Lambert-Beer law within the whole range of the studied concentrations (Fig. 1 – two upper curves), and their C. C. were > 0.99. Detection limits of AA and DAA were both 0.05 μM.

Proper precision of the method modified this way was approved by the repeatability results examined during subsequent 5 d when concentrations of AA and DAA were determined in a randomly picked plasma sample stored at – 80 °C.

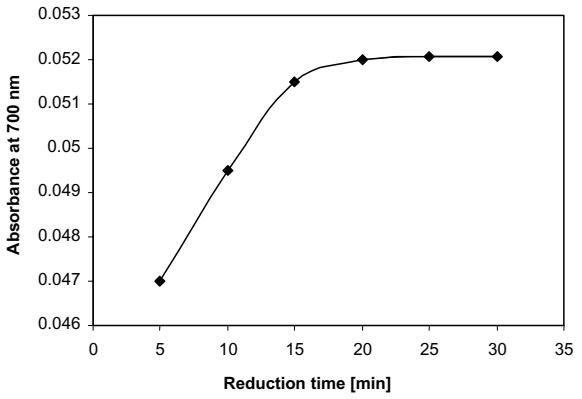

Fig. 2. Optimisation curve of DAA reduction time (56.8 μM solution in bovine serum with addition of 2 mg/ml PO) to AA by DTT (10 mM solution) at 20 °C.

Everyday average concentrations (n = 10) of both forms of vitamin C were used for the calculation of intra-serial C. V. which had the values < 3%. The inter-serial C. V. calculated based on everyday average concentrations (n = 5) was < 5%.

Comparison of activity of various reductants with -SH groups

Effectiveness of DAA transition to AA performed by DTT was compared to other easily accessible reductants containing -SH groups, such as: cysteine, homocysteine, reduced glutathione and 2-mercaptoethanol. The aim of this comparison was to assess the level of their usefulness for this method and perhaps to find a reductant better than DTT or equally efficient like DTT that could serve as a possible substitute. For tests of each of those compounds a new series of standard DAA solutions with PO addition was used, and the time of reduction was 20 min (optimal value for DTT). Standard solutions were submitted to the developed analytical procedure according to actions for samples marked "T", using subsequent tested reductants instead of DTT. Based on the measured absorbance values analytical curves were drawn for the efficiency of subsequent reductants (Fig. 3).

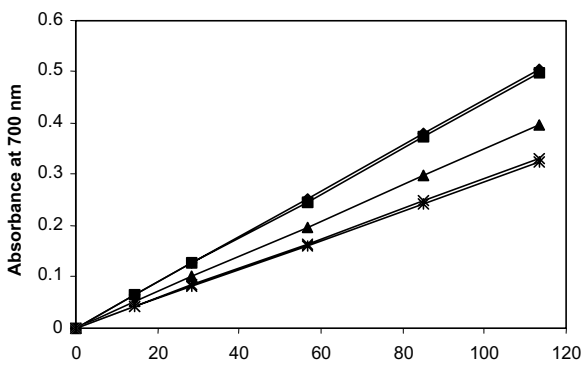

Fig. 3. Comparative analytical curves for DAA standard solutions (in bovine serum with the addition of 2 mg/ml PO) submitted to actions according to the analytical procedure presented in the text for a sample marked "T", with use of various reductants containing -SH groups (action time of every one of them was 20 min). Used reductants: –◆– DTT (10 mM solution), –■– homocysteine, –▲–glutathione reduced, –×– cysteine, –*– 2-mercaptoethanol (four last were used as 20 mM solutions, for their molecules include only one -SH group).

Table I. Recovery factor values for DAA to AA transformation performed by various reductants with -SH groups. A 56.8 μM solution of DAA (in bovine serum with addition of 2 mg/ml PO) was used for tests. The solution underwent actions according to the analytical procedure presented in the text just as samples marked "T"; reductants solutions had concentrations given in caption to Fig. 3.

Reductant	Recovery factor values after the time of reductant action	
	10 min	20 min
DTT	102.4%	100.5%
Homocysteine	97.9%	98.7%
Glutathione reduced	59.1%	78.8%
Cysteine	63.2%	65.6%
2-Mercapto-ethanol	45.6%	64.3%

Comparison of the obtained curves showed significant differences in reductants' ability for quantitative transition of DAA to AA in the used reaction environment.

The test was made more precisely by calculating recovery factors of transition from DAA to AA for a 56.8 μM standard solution of DAA, obtained during usage of each of the tested reductants. In order to do it a determined concentration of formed AA that was established under the influence of each reductant was divided by the prepared concentration of the initial DAA solution and results were expressed in percents. The obtained recovery factor values (average from three tests) are shown in Table I.

The results revealed that none of the tested compounds was a better reductant than DTT. Only homocysteine was equally efficient, yet other reductants proved to be ineffective for use. It must be said, however, that homocysteine has to be used at a concentration twice as big as the concentration of DTT, for the molecule has only one -SH group. Since molar masses of both compounds are almost similar (154.2 g/mol for DTT and 135.2 g/mol for homocysteine) homocysteine consumption would be almost double comparing to DTT consumption. It must be also said that the homocysteine price doubles that of DTT (for the same quantity). So, DTT usage is four times cheaper than homocysteine for similar analytic effects.

Table II. Results of clinical analyses of vitamin C forms in blood plasma obtained with the developed method and values of ratios of determined concentrations. The way of expressing concentrations of vitamin C forms ratio proposed by the authors is marked as a grey field.

	Diagnostic groups	TVC [μmol/l]	AA [μmol/l]	DAA [μmol/l]	AA:DAA	DAA:AA
I.	Healthy persons (*n = 15*)	55.7 (48.6–62.4)	54.3 (47.1–60.3)	1.1 (0.3–1.7)	49.4 *	0.02 *
II.	Chronic gastritis with focal dysplasia (*n = 15*)	21.5 (15.0–34.6)	18.9 (13.4–31.1)	2.4 (1.5–3.8)	7.9	0.13
III.	Gastric cancer (*n = 15*)	16.8 (10.9–28.2)	14.1 (7.8–20.6)	3.0 (1.8–4.5)	4.7	0.21

n: number of persons examined.
* Performing Student's t-test on the calculated ratios of average concentrations of vitamin C forms showed significant statistically differences between group I and groups II, III and IV ($p < 0.01$).

Example results of clinical analysis obtained with the developed method

The propriety of analytical parameters of the developed method makes it suitable for routine determinations in clinical needs. Alternative methods known from literature are therefore not applicable as they are not sufficiently selective (Deutsch and Weeks, 1965; Gero and Candido, 1969), too time consuming and not sufficiently specific (Okamura, 1980; Roe, 1954) or the cost of reagents (Moeslinger *et al.*, 1995) or equipment (*i.e.* Levine *et al.*, 1999; Lykkesfeldt *et al.*, 1995) required are too high.

The obtained results are shown in Table II appointing average values of determined concentrations (with ranges for each group given in brackets). They are in full agreement with data published in medical literature (Gail *et al.*, 2001; Khanzode *et al.*, 2003; Oliveira *et al.*, 2003; Schorah *et al.*, 1996; You *et al.*, 2000). The above results enable assessing a diagnostically significant biomarker in patients' blood: the obtained values of concentrations ratios of vitamin C forms are given in Table II. The results of the study show that it is more rational to calculate DAA:AA ratios than inverted ratios suggested by literature (Lykkesfeldt *et al.*, 1995, 1997; Schorah *et al.*, 1996), as they increase with intensification of pathological changes.

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Deutsch M. J. and Weeks C. E. (1965), Microfluorometric assay for vitamin C. J. Assoc. Off. Agr. Chem. **48**, 1248–1253.

Gail M. H., Brown L. M., and You W. C. (2001), Chemoprevention of gastric dysplasia: randomized trial of antioxidant supplements and anti-*Helicobacter pylori* therapy. J. Natl. Cancer Inst. **93**, 559–560.

Gero E. and Candido A. (1969), Une technique chimique du dosage de l'acide ascorbique total par une reaction d'oxido-reduction. J. Int. Vitaminol. **39**, 252–255.

Halliwell B. (1991), Reactive oxygen species in living systems: source, biochemistry and role in human disease. Am. J. Med. **91** (suppl. 3C), 14–22.

Khanzode S. S., Khanzode S. D., and Dakhale G. N. (2003), Serum and plasma concentration of oxidants and antioxidants in patients of *Helicobacter pylori* gastritis and its correlation with gastric cancer. Cancer Lett. **195**, 27–31.

Levine M., Wang Y., and Rumsey S. C. (1999), Analysis of ascorbic acid and dehydroascorbic acid in biological samples. Methods Enzymol. **299**, 65–76.

Lykkesfeldt J., Loft S., and Poulsen H. E. (1995), Determination of ascorbic acid and dehydroascorbic acid in plasma by high-performance liquid chromatography with coulometric detection: are they reliable biomarkers of oxidative stress. Anal. Biochem. **229**, 329–335.

Lykkesfeldt J., Loft S., Nielsen J. B., and Poulsen H. E. (1997), Ascorbic acid and dehydroascorbic acid as biomarkers of oxidative stress caused by smoking. Am. J. Clin. Nutr. **65**, 959–963.

Moeslinger T., Brunner M., Volf I., and Spieckerman P. G. (1995), Spectrophotometric determination of ascorbic acid and dehydroascorbic acid. Clin. Chem. **41**, 1177–1181.

Okamura M. (1980), An improved method for determination of L-ascorbic and L-dehydroascorbic acid in blood plasma. Clin. Chim. Acta **103**, 259–268.

Oliveira C. P., Kassab P., Lopasso F. P., Souza H. P., Janiszewski M., Laurindo F. R., Iriya K., and Laudanna A. A. (2003), Protective effect of ascorbic acid in experimental gastric cancer: reduction of oxidative stress. World J. Gastroenterol. **9**, 446–448.

Roe J. H. (1954), Chemical determination of ascorbic acid, dehydroascorbic acid and diketogulonic acid. Meth. Biochem. Anal. **1**, 115–139.

Rose R. C., and Bode A. M. (1993), Biology of free radical scavengers: an evaluation of ascorbate. FASEB J. **7**, 1135–1142.

Rumsey S. C., and Levine M. (1998), Absorption, transport and disposition of ascorbic acid in humans. J. Nutr. Biochem. **9**, 116–130.

Rutkowski M. and Grzegorczyk K. (1998), Colorimetric determination of vitamin C concentration in blood plasma with phosphotungstic reagent – a modification of Kyaw method. Diagn. Lab. **34**, 511–520.

Rutkowski M. and Grzegorczyk K. (1999), Vitamins with antioxidant action – a general characterisation. Part III: Vitamin C. Farm. Pol. **55**, 74–79.

Schorah C. J., Downing C., Piripitsi A., Gallivan L., Al-Hazaa A. H., Sanderson M. J., and Bodenham A. (1996), Total vitamin C, ascorbic acid, and dehydroascorbic acid concentrations in plasma of critically ill patients. Am. J. Clin. Nutr. **63**, 760–765.

Sies H. and Stahl W. (1995), Vitamins E and C, β-carotene, and other carotenoids as antioxidants. Am. J. Clin. Nutr. **62**, 1315S–1321S.

You W. C., Zhang L., Gail M. H., Chang Y. S., Liu W. D., Ma J. L., Li J. Y., Jin M. L., Hu Y. R., Yang C. S., Blaser M. J., Correa P., Blot W. J., Fraumeni J. F., and Xu G. W. (2000), Gastric dysplasia and gastric cancer: *Helicobacter pylori*, serum vitamin C, and other risk factors. J. Natl. Cancer Inst. **92**, 1607–1612.

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