Adaptation of the Phosphotungstate Method for the Determination of Vitamin C Contents in Animal and Human Tissues

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The usefulness of phosphotungstate reagent for vitamin C determination in tissue homogenates has been confirmed. An optimal homogenization medium was selected: 1.8 M solution of HPO_3 in 1.3 M CH_3COOH. With this medium the analytical curve (at 700 nm) demonstrated the right linearity, correlation and recovery coefficients were appropriately high (0.999 and 99.8%) and the values of intraserial and interserial variation coefficient were low (< 5% and < 10%, respectively). It makes this method sensitive, easily repeatable, and useful for vitamin C determination in animal and human tissues, including neoplastic ones.

Introduction

In natural and medical studies there is sometimes need for determination in tissues of contents of vitamin C – an important hydrophilic antioxidant (Yew, 1983; Mezzetti *et al.*, 1995; Henning *et al.*, 1997; El Bassiouni *et al.*, 1998; Fournier *et al.*, 2000). However, it is sensitive to oxygen, increased temperature, low and high pH and heavy metals ions (Bode, 1997; Rutkowski and Grzegorczyk, 1999). Its determination in tissues is confronted with many difficulties and descriptions of determinations, related even to the same types of tissues differ significantly as regards the composition of media used for their homogenization or the employed analytical methods (Omaye *et al.*, 1979; Carr *et al.*, 1983; Washko *et al.*, 1992; Oliveira and Watson, 2001). Data on vitamin C contents in human tissues in case of various diseases are therefore rarely found in literature.

In our study on the role of vitamin C in colonic oncogenesis, vitamin C was determined in the removed tumours with the use of our own phosphotungstate method (Rutkowski and Grzegorczyk, 1998) originally designed for the determination of this vitamin in plasma.

Abbreviations: A, absorbance; AA, ascorbic acid; AcOH, acetic acid; C. C., correlation coefficient; C. V., variation coefficient; M_{AA}, molar mass of AA; MPA, metaphosphoric acid; PTR, phosphotungstate reagent; R. F., recovery factor.

Material and Methods

Chemicals

High purity AA ($C_6H_8O_6$) "ACS Reagent" (Sigma, St. Louis, U. S. A.) and 40–44% MPA (HPO_3)(Riedel de Haën, Seelze, Germany) were used in the studies. Other reagents were of analytical purity.

Solutions

In the I and II stage of the studies homogenization media were used of composition as in Table II. To prepare the medium for human tissue homogenization, about 36 g of MPA were dissolved in about 60 ml 1.3 M AcOH (CH_3COOH). The solution was filtered through a paper filter into the 100 ml volumetric flask and filled up with 1.3 M AcOH. The medium is durable for 2 weeks when stored at + 4 °C. Vitamin C was determined with PTR made according to an earlier description (Rutkowski and Grzegorczyk, 1998) from sodium

tungstate ($Na_2WO_4 \cdot 2H_2O$), sodium hydrophosphate (Na_2HPO_4) and 3.7 M sulfuric acid (H_2SO_4). Vitamin C standard solution of 56.8 μmol/l concentration was obtained from AA using ten times diluted homogenizing medium as a solvent in the given stage of studies (the medium selected for human tissue was diluted with 1.3 M AcOH).

Tissues used for the investigation

During I and II stage of the study animal muscular tissue was homogenized with the addition of AA (inner pattern) given below. It played the role of a model of human tissue. In optimal conditions (stage III) surgically obtained colonic adenocarcinomas as well as samples of healthy part of this colon were homogenized. The obtained samples were stored at – 80 °C and subjected to homogenization and vitamin C determination within a month.

Homogenization of tissues

Samples of animal tissue (4 g of each) with added 11.36 μmol (2 mg) AA were homogenized as the human tissue but in 4 times larger volumetric scale applying successive investigated homogenization media. 25% Homogenates (v/w) were obtained.

As for the human tissue homogenate, the procedure was the following: the tissue was defreezed, dried with filter paper, chopped and a sample of 1 g was weighted (with accuracy of 0.001 g), then homogenized for 2–3 min with in a glass homogenizer in 2.5 ml of the earlier described medium, cooled with water and ice. The homogenate was transferred quantitatively into a volumetric flask of 25 ml capacity, filled up with 1.3 M AcOH and after stirring it was transferred into centrifugation tube which was kept for 20 min at + 4 °C to isolate lipid layer and then stirred at + 4 °C (1500 × *g*, 15 min) and the supernatant was filtered through the filter paper.

Diluting of homogenates to the analytical curves

Out of the homogenates made in I and II stage of the study 25 ml of each sample were left and the rest was diluted 2-, 4- and 8-fold with the medium appropriate for the given homogenate. A series of standard homogenates made with particular media with added AA concentrations: 14.2; 28.4; 56.8 and 113.6 μmol/l were obtained in this way.

Procedure of vitamin C determination

1.5 ml of filtered supernatant was placed into a centrifugation tube, 1.5 ml of PTR was added with stirring and after 30 min the fluid from above the sediment called "tested sample" was centrifuged (3500 × *g*, 10 min). Absorbance A_{700} of this sample and of standard vitamin C solution subjected to the same procedure (without centrifugation) was measured at 700 nm, using as the reference, the mixture 1:1 (v/v) of PTR and the solvent applied to prepare the standard solution.

Calculations

Initially, the vitamin C concentration in tissue homogenate was calculated according to the formula:

$$c_{\text{vitC}}\ [\mu\text{mol/l}] = \frac{A_{test.}}{A_{stand.}} \cdot c_{\text{stand.}} \qquad (1)$$

where: $c_{vit.C}$ -concentration of vitamin C in homogenate, $A_{test.}$-absorbance of the tested sample, $A_{stand.}$-absorbance of standard solution, $c_{stand.}$-concentration of standard solution. The obtained result was used to calculate vitamin C contents in the investigated tissue according to the formula:

$$\text{contents of vitamin C}\ [\mu\text{g/g}] = \frac{c_{vit.C}}{m} \cdot 4.4 \qquad (2)$$

where: *m*-weight mass of the investigated tissue [g], 4.4-coefficient resulting from the mathematical relation between constant elements of the calculations explained below.

Since 1 mg AA = 5.68 μmol (M_{AA} = 176.12 g/mol), then dividing $c_{vit.C}$ by 5.68 μmol/l changes into mg/l, which divided by 40 (quotient of definition volume 1000 ml for molar concentration and the volume of 25 ml homogenates) gives the amount of vitamin in 25 ml of tissue homogenate of mass *m*. Dividing the result by *m*, the contents of vitamin C in the investigated tissue is obtained in mg/g, which multiplied by 1000 changes into μg/g. A sequence of operations results from that: $c_{vit.C} \cdot 1000/5.68 \cdot 40 \cdot m$, in which the quantity $1000/5.68 \cdot 40$ is constant and equals 4.4, being the coefficient in formula (2).

Results

The study on the application of our own phosphotungstate method (Rutkowski and Grzegorczyk, 1998) for vitamin C determination in tissues consisted of three stages. In stage I the usefulness of PTR in such analyses was investigated. Obtaining positive results allowed to select, in stage II, the composition of homogenization medium ensuring the transfer of total vitamin C from tissues to homogenates and maintain its stability in them. In stage III the vitamin was determined according to the method worked out in human tissues samples.

PTR usefulness in vitamin C determination in homogenates

A series of standard vitamin C solutions (described in "Diluting of homogenates…") was prepared in water homogenate of animal muscular tissue with the addition of AA (acc. to "Homogenization of tissues"). The use of water as homogenization medium was meant to eliminate possible PTR reaction with components from other media. The solutions were subjected to procedures acc. to "Procedure of vitamin C determination" (during A_{700} measurements the participation of endogenous vitamin C was subtracted), and on the basis of mean A_{700} values from 3 measurements an analytical curve was drawn (Fig. 1-II). Its character enabled recognizing PTR to be useful in vitamin C determination in homogenates. The curve was linear and passed through point 0 of A,c coordinate system, and the value of C. C. = 0.994 proved a high accuracy of the determination.

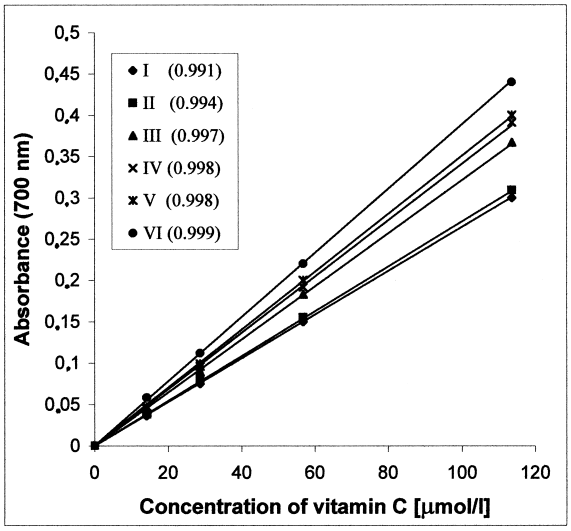

Fig. 1. Analytical curves for vitamin C determinations in tissue homogenates performed in the selected homogenizing media.
The composition of individual homogenizing media I–VI was specified in Table I. Numbers in brackets are C. C. values of particular curves. Values of A determine means from 3 measurements of samples subjected to analytical procedure described in "Materials and Methods" (the participation of endogenous vitamin C was subtracted).

Selection of optimal homogenization medium

The study comprised MPA solutions given in Table I and, for comparison, 0.15 M solution of NaCl. According to the accepted procedure, in each of these media the same animal muscular tissue samples were homogenized with the addition of AA, and diluting the homogenates with the media used for their preparation, earlier described series of standard solutions were prepared. Subjecting them to analytical procedures the same as above, on the basis of mean A_{700} values from 3 measurements analytical curves related to particular investigated media were drawn (Fig. 1), and C. C. values were calculated.

Table I. Determined and standard A values and R. F. coefficients of tissue homogenates of vitamin C concentration 56.8 μmol/l prepared in homogenizing media used in investigations.

Type of homogenizing medium	$A_{deter.}$	$A_{stand.}$	R. F. [%]
I: 0.15 M NaCl	0.1544	0.1588	97.2
II: water	0.1561	0.1599	97.6
III: 1.8 M MPA	0.1871	0.1884	99.3
IV: 0.4M MPA in 5.0 M AcOH	0.2004	0.2016	99.4
V: 0.4 M MPA in 1.3 M AcOH	0.1982	0.1974	100.4
VI: 1.8 M MPA in 1.3 M AcOH	0.2198	0.2201	99.8

Measurements of A (700 nm) were carried out in the samples subjected to analytical procedure described in "Material and Methods" (the participation of endogenous vitamin C was subtracted). $A_{deter.}$ values (means from 3 determinations) refer to homogenates of about 4 g of animal muscular tissue with the addition of 2 mg of AA of dilution corresponding to the mentioned concentration. $A_{stand.}$ values (means from 3 determinations) refer to standard solutions of AA of the concentration 56.8 μmol/l prepared in successive media used for tissue homogenizing. R. F. coefficients were calculated as proportions $A_{deter.}$: $A_{stand.}$ for each medium.

R. F. value obtained with the use of each medium was calculated taking into consideration the concentration of 56.8 μmol/l – the centre of the investigated concentration range AA. For this purpose in all media AA solutions of this concentration were made and subjected to the accepted, analytical procedure and the mean values A_{700} were calculated from 3 measurements determined as $A_{\text{stand.}}$. Taking into consideration the obtained earlier mean values A_{700} for the investigated media, corresponding with the homogenates dilutions of vitamin C concentration of C 56.8 μmol/l – here determined as $A_{\text{deter.}}$, R. F. [%] value was calculated for each medium as the ratio $A_{\text{deter.}}$: $A_{\text{stand.}}$ (Table I).

The best results were obtained for 1.8 M of MPA solution in 1.3 M AcOH: the largest inclination angle of the analytical curve, C. C. = 0.999, R. F. = 99.9%. Repeatability was investigated using homogenate prepared in this medium of vitamin C concentration 56.8 μmol/l stored for 5 days at – 80 °C. Intraserial C. V. calculated on the basis of mean concentration (n = 10) determined every day was < 5%. Every day means (n = 5) were used to calculate interserial C. V., which was < 10%.

Vitamin C determination in human tissue homogenates

Twenty samples were investigated in which the vitamin C concentration (μg/g of tissue) was the following:

a) colonic adenocarcinoma (n = 10)
   contents of vitamin $C_{\text{min.}}$ = 77; contents of vitamin $C_{\text{max.}}$ = 107; contents of vitamin C_{mean} = 90.8 ± 3.72
b) healthy (normal) colon (n = 10)
   contents of vitamin $C_{\text{min.}}$ = 32; contents of vitamin $C_{\text{max.}}$ = 55; contents of vitamin C_{mean} = 44.5 ± 2.36

A higher content of vitamin C in neoplastic tissue in comparison with healthy tissue corresponds with the literature data (Krasner and Dymock, 1974; Anthony and Schorah, 1982).

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