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FLUOROMETRIC DETERMINATION OF TOTAL VITAMIN C IN WHOLE BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PRE-COLUMN DERIVATIZATION

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SUMMARY

A reliable and semi-automated high-performance liquid chromatographic (HPLC) method is described for the determination of total vitamin C in whole blood. After deproteinization of whole blood and enzymatic oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid, the latter is condensed with *o*-phenylenediamine to its quinoxaline derivative. This derivative is separated on a reversed-phase HPLC column and detected fluorometrically. Total vitamin C in whole blood can be determined in concentrations as low as 0.2 μmol/l.

Special attention was paid to the stability of vitamin C in whole blood and of its quinoxaline derivative in the extract. Results of our investigations showed that total vitamin C in whole blood is stable for eight days at −20°C, provided ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and glutathione are immediately added to the blood sample. The quinoxaline derivative of vitamin C in the blood extract is stable for at least 24 h if stored in the dark at 4°C.

Routine vitamin C determinations can be carried out in a series of 100 samples within 48 h. The within-assay and between-assay coefficients of variation were 3.7% and 4.6%, respectively. The between-assay analytical recovery of *l*-ascorbic acid added to whole blood samples was 97.0 ± 7.0% (mean ± S.D.). Reference values of vitamin C in whole blood of normal healthy Dutch adults were found in the range 20—80 μmol/l.

INTRODUCTION

Vitamin C, a water-soluble vitamin, consists of *l*-ascorbic acid and its oxidized form dehydro-*l*-ascorbic acid. Both forms are equally biologically active [1]. Numerous methods have been described for the analysis of *l*-ascorbic acid and/or dehydro-*l*-ascorbic acid in various biological samples. These include the indicator-dye reduction method with dichlorophenolindophenol [2, 3], the ketone derivatization method with dinitrophenylhydrazine

[3—5], an enzymatic method with ascorbic acid oxidase [6] and high-performance liquid chromatographic (HPLC) methods with electrochemical [7—9] and UV detection [10—12].

However, the indicator-dye reduction and ketone derivatization methods are not very specific and have the drawback that blank values have to be determined by chemical interference in the colour-inducing reaction. The enzymatic method as described lacks sensitivity for the analysis of the low concentrations of vitamin C observed in whole blood and is difficult to perform in large-scale routine analysis. HPLC methods with electrochemical detection allow only the determination of *l*-ascorbic acid, while UV measurement suffers from the very low absorbance of dehydro-*l*-ascorbic acid. Keating and Haddad [12] described an HPLC method with UV detection for the analysis of *l*-ascorbic acid and dehydro-*l*-ascorbic acid in foodstuffs. They enhanced the absorbance of the latter by pre-column derivatization with *o*-phenylenediamine (OPDA) to 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ). However, their method has the drawback that *l*-ascorbic acid may be easily oxidized to dehydro-*l*-ascorbic acid during sample handling. This oxidation probably also occurs during deproteinization of blood when oxygen is released from oxyhaemoglobin [13]. Due to this oxidation and possible previous oxidation during blood collection, transport and storage, systematic errors may arise. Therefore, and since *l*-ascorbic acid and dehydro-*l*-ascorbic acid are equally biologically active, we developed a method for the analysis of total vitamin C in whole blood by incorporating an oxidation step in the procedure.

After enzymatic oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid, the latter, having insufficient UV absorbance, is condensed with OPDA to the highly fluorescent DFQ. This derivative is separated from interfering compounds on a reversed-phase HPLC column. Fluorometric detection then permits the determination of total vitamin C down to concentrations below those normally occurring in blood.

MATERIALS AND METHODS

Apparatus

HPLC was performed using a Gilson Model 302 constant-flow pump (Meyvis, Bergen op Zoom, The Netherlands), a Micromeritics 725 Autoinjector (CLI, Schijndel, The Netherlands), and a Kratos FS 950 Fluoromat fluorescence spectrophotometer (Kipp Analytica, Delft, The Netherlands) equipped with a mercury light source (type FSA 110), an excitation interference filter of 365 nm (type FSA 401) and an emission cut-off filter of 418 nm (type FSA 426).

A Knauer stainless-steel column (80 × 4.6 mm I.D.) was home-packed with ODS-Hypersil 3 μm (Shandon Southern Products, Astmoor, U.K., Cat. No. 580 × 24) by the balanced-density slurry technique using a Haskel pump type MCP 110 (Ammann Technik, Stuttgart, F.R.G.). Elution profiles were displayed on a Kipp BD-8 recorder (Kipp Analytica).

Reagents

EGTA—glutathione solution: A solution, containing 0.24 *M* ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 0.20 *M*

glutathione, was prepared by dissolving 1.5 g of glutathione (Sigma, St. Louis, MO, U.S.A.) in 25 ml of double-distilled water and adjusting the pH to 6.5 with 2 *M* sodium hydroxide. In this solution 2.25 g of EGTA (Sigma) was dissolved and the pH was adjusted to 6.5 with 10 *M* sodium hydroxide. The EGTA–glutathione solution was stable for at least two months if stored in the dark at 4°C.

Ascorbate oxidase spatulae were obtained from Boehringer (Mannheim, F.R.G.). *o*-Phenylenediamine (OPDA) was from Merck (Darmstadt, F.R.G.).

The HPLC mobile phase containing 0.08 *M* potassium dihydrogen phosphate and 20% (v/v) methanol, pH 7.8, was flushed with a stream of helium gas for 10 min before use.

l-Ascorbic acid stock standard solution contained about 350 mg/l *l*-ascorbic acid (Merck) in 1% (w/v) metaphosphoric acid. This solution was stored in the dark at 4°C and was stable for at least fourteen days. The *l*-ascorbic acid working standard solution was prepared daily by diluting 2 ml of the stock standard solution with water to 100 ml.

Blood extraction and derivatization procedures

Blood was collected from veins directly into 5-ml plastic tubes containing 0.1 ml of EGTA–glutathione solution. One millilitre of whole blood was slowly transferred with thorough mixing using a vortex mixer to a 10-ml plastic tube containing 4 ml of 0.3 *M* trichloroacetic acid (TCA). The tube was allowed to stand for about 20 min in the dark at 4°C, mixing once after about 10 min. Thereafter the tube was centrifuged at 2000 *g* and 4°C for 10 min. A 1.5-ml aliquot of the supernatant was transferred to a 5-ml plastic tube. After adding 0.2 ml of 4.5 *M* sodium acetate buffer pH 6.2 and an ascorbic acid oxidase spatula, the tube was placed in a waterbath at 37°C for 5 min, mixing once after about 2 min. After removal of the enzyme-carrying spatula 0.25 ml of a freshly prepared 0.1% (w/v) OPDA solution was added. After mixing, the tube was wrapped with aluminium foil to screen it from daylight and placed in a waterbath at 37°C for 30 min. Thereafter the tube was stored in the dark at 4°C for HPLC analysis within 24 h. The centrifuge tube, containing the pellet and a residual 2 ml of supernatant, was stored in the dark at −20°C for repeated derivatization and HPLC analysis, if required, within one week.

The working standard solution was derivatized by adding 1 ml to a 10-ml tube containing 4 ml of 0.3 *M* TCA and 1.2 ml of 4.5 *M* sodium acetate buffer pH 6.2. After adding an enzyme spatula, *l*-ascorbic acid was oxidized by heating in a water-bath at 37°C for 5 min. After wrapping the tube with aluminium foil, the resulting dehydro-*l*-ascorbic acid was condensed to DFQ by adding 0.5 ml of OPDA solution and heating in a water-bath at 37°C for 30 min. The tube was stored in the dark for HPLC analysis within 24 h.

Chromatographic conditions

HPLC analysis of DFQ was carried out by injecting 20 μl of the derivatized sample extract onto the ODS-Hypersil column. The column was eluted isocratically with the mobile phase at a flow-rate of 1.0 ml/min. The effluent was monitored with the fluorescence spectrophotometer. The recorder was set at 10 mV full scale. Duration of the chromatographic run was about 10 min per sample.

Calculation

The vitamin C concentration of the sample was calculated from peak heights with the working standard solution as the reference. In routine analysis this solution was run before each series of five samples. Assuming that whole blood contains 85% of aqueous phase [14] and taking into account the volumes of sample, added 0.3 *M* TCA, the supernatant aliquot and volumes of added buffer and reagent solution, the concentration of vitamin C in the original blood sample was calculated by multiplying the concentration of vitamin C found by a factor of $\left(\frac{0.85+4.0}{1.0}\right) \times \left(\frac{1.5+0.20+0.25}{1.5}\right) = 6.3$.

Selection of fluorometric parameters

Under the chromatographic conditions employed, DFQ had its excitation maximum at 355 nm and its emission maximum at 425 nm. The light source and filters for the fluorescence detector were chosen in accordance to these wavelengths. Both the excitation maximum and the emission maximum of DFQ proved to be pH-independent over the range 3.5—8.5. pH 7.8 was chosen as a result of an optimization procedure for best HPLC separation.

Recovery test

The recovery of *l*-ascorbic acid added to whole blood was determined by analysing 1-ml portions of whole blood to which 20 μl of the *l*-ascorbic acid stock standard solution had been added. The original vitamin C was raised in this way by 39.0 μmol of *l*-ascorbic acid per litre.

RESULTS

Characteristics of the HPLC procedure

Typical elution profiles of a derivatized blood extract and the working standard solution are shown in Fig. 1. In blank experiments only a solvent peak was recorded. The HPLC procedure afforded an excellent separation of DFQ from other extract components.

As far as has been investigated the fluorescence response was linear from concentrations corresponding to 0.3 μmol/l up to about 170 μmol/l in the whole blood sample. Assuming a signal-to-noise ratio of 3, the detection limit of the vitamin C assay described in this paper corresponds to a concentration of vitamin C in whole blood of 0.2 μmol/l.

Efficiency of the extraction procedure

To investigate the efficiency of the extraction of vitamin C by the procedure described under Materials and methods, 1.0-ml aliquots of a series of five different whole blood samples were deproteinized in the presence of ^3H_2O (24×10^3 dpm). After centrifugation and removal of the supernatant the residual pellet was extracted twice more with 4 ml of 0.3 *M* TCA. The three supernatants of each sample were analysed for vitamin C and ^3H_2O. It was found that after the first extraction 10.3 ± 1.3% (mean ± S.D.) of the total amount of vitamin C and 9.9 ± 0.4% (mean ± S.D.) of the total amount of

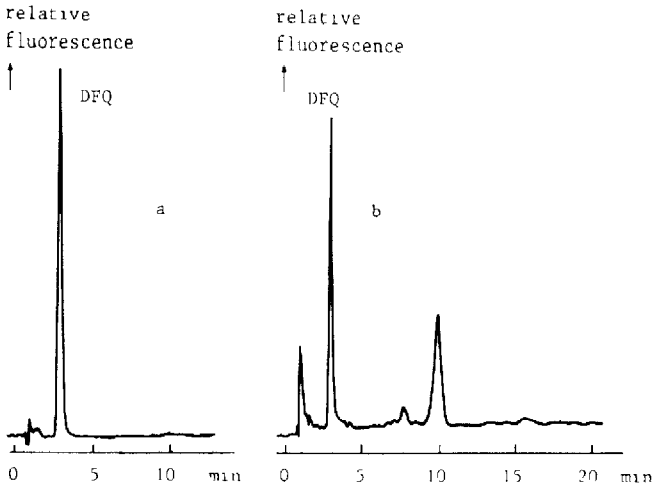


Fig. 1. Typical HPLC elution profiles of the working standard solution (a) and of a whole blood sample (b).

^3H_2O were still present in the pellet. From these results it was concluded that the efficiency of the extraction of vitamin C by the method described amounted to 100% for whole blood samples of 1 ml.

Derivatization

The minimum periods needed for complete oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid and for complete derivatization of the latter to DFQ were determined for whole blood samples as well as for the working standard solution. Portions (1 ml) of a pooled whole blood sample and of the working standard solution were analysed as described under Materials and methods, the reaction periods for the oxidation step being varied from 1 to 8 min in 1-min steps. Another eight 1-ml portions of the same pooled whole blood sample and of the working standard solution were analysed with reaction periods for the condensation reaction with OPDA ranging from 5 to 40 min in 5-min steps. All measurements were carried out in duplicate. The derivatized sample was injected onto the ODS-Hypersil column immediately after termination of the reaction period for the condensation reaction. For the working standard solution the minimum reaction periods to achieve maximum peak height of DFQ were 5 min and 25 min for the oxidation step and condensation reaction, respectively. For a whole blood sample these periods were 2 and 25 min, respectively. No decrease in peak height of DFQ was observed in tests with reaction periods of 8 min for oxidation and 40 min for derivatization as compared with the peak heights observed in tests with reaction periods of 5 and 25 min. The reaction periods of the assay were chosen in accordance with these findings.

Stability of DFQ

Derivatized extracts of a pooled whole blood sample were exposed to daylight at ambient temperature, or were stored in the dark at ambient

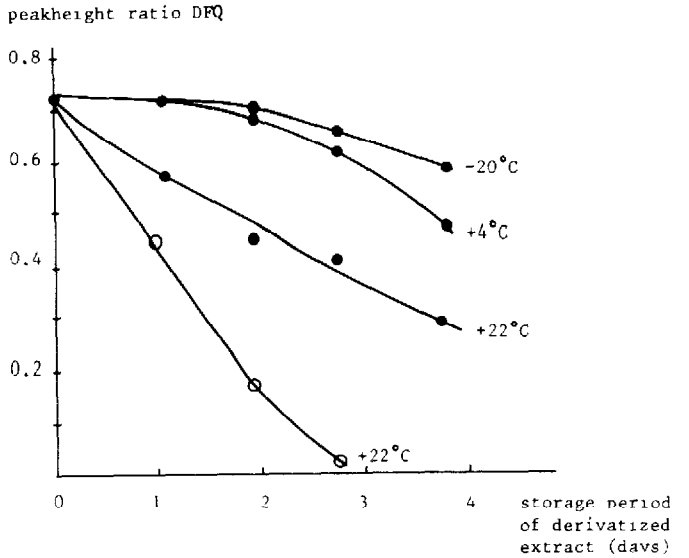
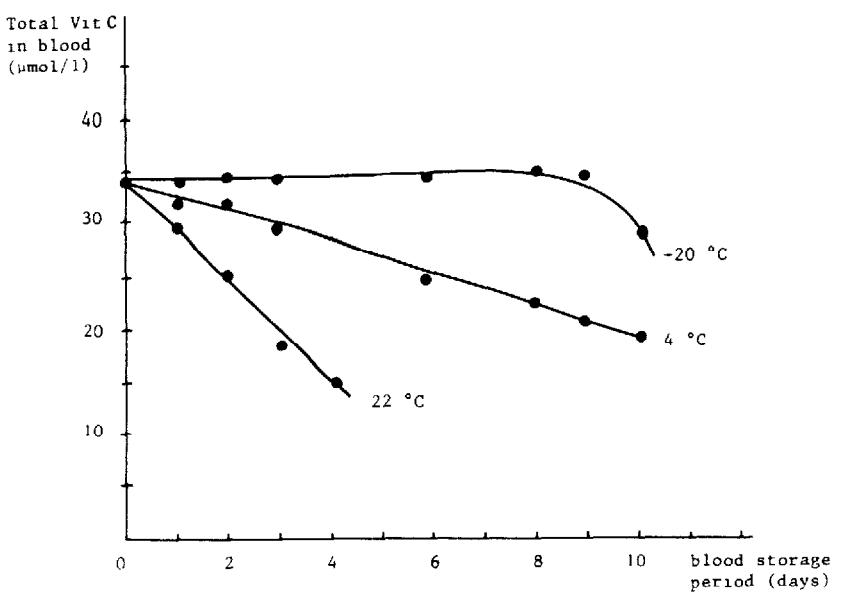


Fig. 2. Stability of DFQ from a whole blood sample under exposure to daylight (○) and in the dark (●). The ratio of the peak height of DFQ in the stored extract to that in a freshly prepared working standard solution is plotted against the storage period.

the extract by injecting 20 μl onto the ODS-Hypersil column. The peak height of DFQ was compared with the peak height of a freshly derivatized working standard solution. The results are shown in Fig. 2. As can be seen from this figure, DFQ deteriorates rapidly under daylight exposure whereas it is stable for at least one day if stored in the dark at 4° C or —20° C.

Stability of vitamin C in whole blood

In order to investigate the stability of vitamin C in whole blood after its collection in the EGTA—glutathione solution, 1-ml portions of a pooled whole



blood sample were stored in the dark at ambient temperature, 4° C and −20° C and were analysed during consecutive days. The results are shown in Fig. 3. From this figure it can be concluded that whole blood samples for vitamin C analysis may be stored in the dark at −20° C for a period of eight days without appreciable loss of vitamin C.

Precision and recovery

In order to test the within-assay and between-assay precisions of the method, several 1-ml portions of the same whole blood sample with and without the addition of 39.0 μmol/l *l*-ascorbic acid were stored in the dark at −20° C and analysed for vitamin C eight times in succession and also in a series of eight consecutive days. The results are given in Table I. The coefficient of variation (C.V.) of the between-assay is somewhat higher than the C.V. of the within-assay, although both are below 5%. Table I also shows the recovery of *l*-ascorbic acid added to whole blood (97–98%).

TABLE I

PRECISION OF THE METHOD FOR DETERMINATION OF TOTAL VITAMIN C

	Within-assay precision		Between-assay precision	
	Whole blood	Recovery test*	Whole blood	Recovery test*
n	8	8	8	8
Mean	39.8 μmol/l	97.7%	39.2 μmol/l	97.0%
S.D.	1.5 μmol/l	6.3%	1.8 μmol/l	7.0%
C.V.	3.7%		4.6%	

*Recovery tests were performed as described in Materials and methods.

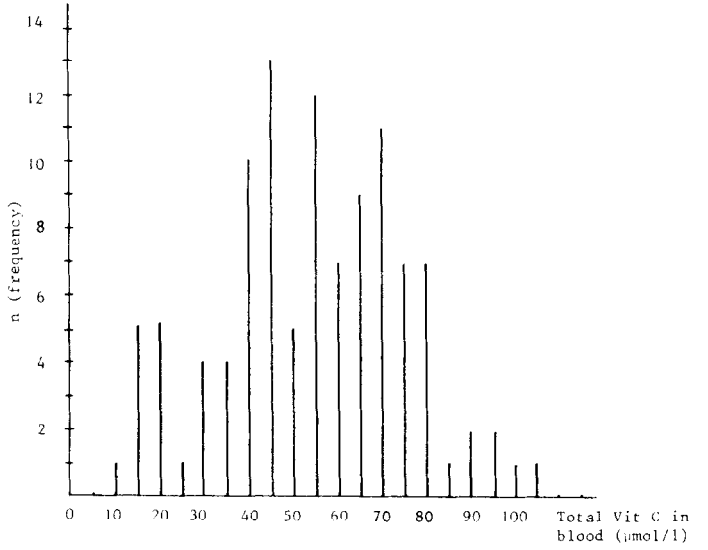


Fig. 4. Frequency distribution of the concentration of total vitamin C in whole blood samples of normal healthy Dutch adults (n = 108).

Establishing a reference range

From the analysis of vitamin C in whole blood samples of a group of 108 normal healthy Dutch adults (61 males and 47 females), a total range for vitamin C of 10—105 μmol/l was found with a mean value of 54 μmol/l. The frequency distribution of the vitamin C concentrations in these samples seems to be non-Gaussian (Fig. 4). For the assessment of a "normal" range, the distribution free method of Rümke and Bezemer [15] was used. By setting the limits of percentiles at 2.5% and 97.5% with a reliability of 95%, a reference range was obtained of 20—80 μmol/l. Accordingly, a concentration of vitamin C below 20 μmol/l would be regarded as below normal.

DISCUSSION

The procedure described in this paper provides a fast, sensitive and reliable method for the determination of total vitamin C in whole blood, i.e. the sum of the concentrations of *l*-ascorbic acid and dehydro-*l*-ascorbic acid. The method is suited for large-scale routine analysis. Precision and recovery are good. The linearity range and sensitivity permit the determination of total vitamin C in whole blood in concentrations far below and above normal. Systematic errors due to oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid during sample storage and handling are eliminated by incorporating this oxidation in the method. Blood samples containing EGTA and glutathione can be stored in the dark at −20°C for at least eight days without significant deterioration of vitamin C.

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