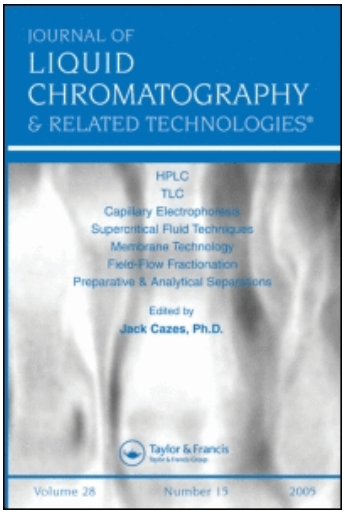

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Ascorbic Acid in Blood Plasma or Serum and in Seminal Plasma Using a Simplified Sample Preparation and High-Performance Liquid Chromatography Coupled with Uv Detection

Marina Patriarca[a]; Antonio Menditto[a]; Gino Morisi[a]
[a] Laboratorio di Biochimica Clinica Istituto, Roma, Italy

To cite this Article Patriarca, Marina , Menditto, Antonio and Morisi, Gino(1991) 'Determination of Ascorbic Acid in Blood Plasma or Serum and in Seminal Plasma Using a Simplified Sample Preparation and High-Performance Liquid Chromatography Coupled with Uv Detection', Journal of Liquid Chromatography & Related Technologies, 14: 2, 297 — 312
To link to this Article: DOI: 10.1080/01483919108049616
URL: http://dx.doi.org/10.1080/01483919108049616

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(2), 297-312 (1991)

DETERMINATION OF ASCORBIC ACID IN BLOOD PLASMA OR SERUM AND IN SEMINAL PLASMA USING A SIMPLIFIED SAMPLE PREPARATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH UV DETECTION

MARINA PATRIARCA, ANTONIO MENDITTO, AND GINO MORISI

Laboratorio di Biochimica Clinica
Istituto Superiore di Sanita'
Viale Regina Elena 299
00161 Roma - Italy

ABSTRACT

A simplified method of sample preparation and high-performance liquid chromatography procedure using UV detection is described for the determination of ascorbic acid (AA) in blood plasma or serum and seminal plasma. Within two hours from collection samples are treated with dithioerythritol (DTE) and then stored under Argon at -80°C. Prior to analysis, protein precipitation is initiated with the addition of cold methanol. AA elution is carried out on a C_{18} reverse phase column using dodecyltrimethylammonium bromide as an ion-pairing agent. The detection is accomplished by measuring ultraviolet absorption at 265 nm. The analysis time for sample is 10.5 min, the retention time of AA being 9.7 min. Within- and between-day coefficients of variation are 2.9% and 4.9% for blood serum, 1.0 % and 2.3 % for seminal plasma. Mean analytical recovery of 102.5 ± 3.7% was found analyzing a serum pool after addition of a standard amount of AA. AA levels are stable for at least 43 days under the described storage conditions.

Detection limit (3x sd) was of 1.8 ng/injection. Mean concentration of blood serum AA levels, measured in 165 male subjects, aged 56-76 years, was 8.9 ± 4.0 mg/L (50.5 ± 22.7 μmol/l). Seminal plasma AA levels observed in 14 adult subjects, aged 23-35 years, ranged from 25.8 to 232.4 mg/l (146.5 to 1319.6 μmol/l), with a mean concentration of 114.4 ± 69.0 mg/l (649.6 ± 391.8 μmol/l).

INTRODUCTION

Ascorbic acid (AA) is an essential cofactor for optimal activation of enzymes involved in various hydroxylation reactions (1), and plays a key role in protecting living organisms from the dangerous action of free radicals (FRs) (2, 3). Although the two described functions may appear very different, in both cases the same biochemical mechanism is involved, AA acting as a powerful reducing agent and providing readily available electron equivalents. In this process, AA oxidizes and becomes a radical itself (semidehydroascorbate radical, ascorbate free radical, AFR), but, unlike other FRs, AFR is relatively unreactive and decays by disproportionation to AA and dehydroascorbic acid (DHAA) (2 AFR $\longrightarrow$ AA + DHAA), thus interrupting the propagation of free radical chain reactions (4).

From a pathophysiological point of view having FRs being proposed as risk factors for ischemic heart disease (IDH), a putative protecting role has been suggested for AA (5, 6). Apart from the antioxidant hypothesis, other mechanisms of AA protection against IHD are conceivable as well. AA, among other biochemical pathways, is involved in the biosynthesis of collagen (1), in the synthesis of carnitine from lysine (1) and in the transformation of cholesterol into bile acids (7).

To a different extent, many experimental and clinical evidences provide direct demonstration of the importance of the antioxidant activity of AA in the pathophysiology

of reproduction (8-10). In spermatozoa, FRs induce peroxidation of plasma membrane lipids and lead to loss of structural integrity, decreases motility, viability and metabolic activity ultimately leading to cell death (11-13). In addition, as previously mentioned, AA is involved in the biosynthesis of carnitine, a biochemical marker of epydidymal integrity.

Several methods have been described for AA detection in biological fluids and tissues (14). The commonly used spectrophotometric determination, following various colorimetric reactions based on the oxidation of AA, has been shown to be poorly specific and positively affected by the presence of other reducing substances. The enzymatic method with ascorbic acid oxidase is highly selective but, due to being rather complicate and time consuming, is not suitable for routine analysis.

Rapid, reliable, and sensitive AA determinations have been performed both using ion exchange and ion paired reverse phase HPLC (15-23), mostly coupled with electrochemical detection (ECD). Under appropriate conditions, unattended operations can be carried out, saving operator time without affecting reliability of results. Nevertheless, ECD response may be time dependent, thus requiring standards to be run frequently (22). On the contrary, only a few Authors (16, 21) used UV detection because of its relatively low sensitivity compared to ECD. In addition, most AA preserving and stabilizing substances - i.e. metaphosphoric acid (MPA), trichloroacetic acid and diethylendiamminotetraacetic acid -showing intense UV absorption, elute very close to AA and their extraction (21) is time consuming and may lead to loss of accuracy. Moreover, MPA has been reported to cause major problems when matched with paired ion reverse phase HPLC (17).

The aim of this investigation was to develop a simple, sensitive and reliable HPLC method, coupled with UV detection, for AA determination in small sample volumes

(100-300 µl) of blood plasma or serum and seminal plasma, using a simple pretreatment procedure.

MATERIALS AND METHODS

Reagents

Deionized water (18 MΩ /cm), obtained with a Milli-Q purification system (Millipore, Bedford, MA, USA), was used for all reagent and standard solutions.
AA standard solutions were prepared from L(+)ascorbic acid (99.7%, Merck, Darmstadt, FRG). Uric acid (UA, 99.7 %, SRM 913) was from National Institute of Standards and Technology (Gaithersburg, MD, USA).
Ascorbate oxidase (133 U/mg) was purchased from United States Biochemical Corporation (Cleveland, OH, USA), and uricase (0.22 U per vial) from BDH Chemicals Ltd., (Poole, UK). 1,4-dithioerythritol (DTE, > 99%, Fluka Chemie, AG Bucks, Switzerland) was used for preservation of AA. HPLC grade methanol was from J.T.Baker, (Deventer, The Netherlands).
All other chemicals were of the highest grade commercially available.

Standard and Working Solutions

A stock standard solution containing 100 mg/l AA and 1 g/l DTE in water was prepared by weighing, subdivided into aliquots and stored at -80°C.
Working standards - containing 1.0, 2.5 and 5.0 mg/l AA (when blood serum or plasma samples were analyzed), or 5, 15 and 30 mg/l (during seminal plasma sample analysis) - were prepared daily by appropriately diluting the stock solution.

Pure methanol and methanol containing 1 g/l DTE (DTE-methanol) were stored at -20°C and used to initiate protein precipitation in blood components (plasma or serum) and seminal plasma.

Sample Collection and Storage

Venous blood samples were obtained after an overnight fast from 165 male subjects between the ages of 55 and 75 years participating in a survey of IHD. For plasma collection plastic tubes containing Li-heparin (32331 Sarstedt, Nunbrecht, West Germany) were used, whereas serum samples were obtained allowing blood to clot in glass tubes. Within two hours from collection, heparinized and non-heparinized blood samples were centrifuged at 1550 x g for 10 minutes and 300 µl of plasma or serum were transferred to polystyrene tubes, added to 60 µl of a 9 g/l DTE water solution (final concentration 1.5 g/l) then vortexed for 15 seconds, and stored at -80°C under Argon until the day of the analysis.

Seminal fluids were obtained into sterile plastic containers from 14 apparently healthy volunteers, aged 23-35 years, attending an out-patient infertility clinic. After collection, semen specimens were allowed to liquefy at room temperature for 30 minutes and then centrifuged at 400 x g for 15 minutes. Each seminal plasma sample was divided in two aliquots and DTE (final concentration 10 g/l) was added to one of them. Routinely, both of these aliquots were assayed within 1 h from collection, and, for further analyses, the DTE-containing aliquot was also stored at -80°C under Argon.

Sample Analysis

Blood components (serum or plasma) were diluted threefold with DTE-methanol. DTE-treated and DTE-

untreated seminal plasma aliquots were diluted tenfold with DTE-methanol and pure methanol, respectively. All samples were vortexed for 20 seconds and centrifuged at 10000 x g for 2 minutes. Each supernate was passed through a 0.45 μm disposable filter (Supelco Inc., Bellefonte, PA, USA) and kept at -20°C until a 20 μl aliquot was injected onto the HPLC system.

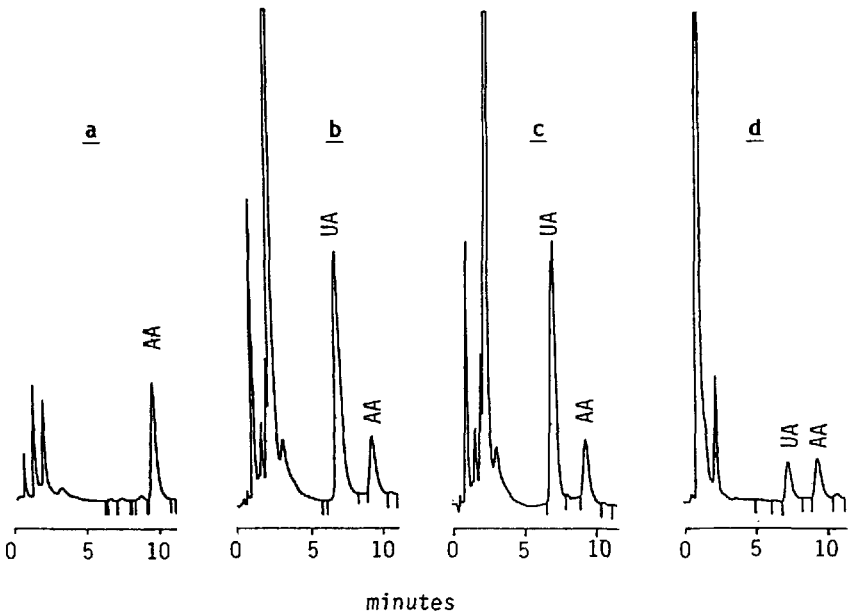

FIGURE 1: Representative chromatograms for: (a) aqueous solution 2.5 mg/l AA (14.2 μmol/l); (b) blood serum 4.5 mg/l (25.6 μmol/l) AA ; (c) blood plasma 4.5 mg/l (25.6 μmol/l) AA and (d) seminal plasma 26.7 mg/l (151.6 μmol/l) AA. For chromatograms (a), (b) and (c) the detector sensitivity was set at 0.001 AUFS, whereas for chromatogram (d) the AUFS was 0.005.

HPLC System

We used a Perkin-Elmer Series 4 liquid chromatograph (Perkin Elmer, Norwalk, CT, USA), a Rheodine 7125 injector (Rainin Instruments, Woburn, MA, USA) with a fixed 20 µl loop, a Supelguard LC18 precolumn (20 x 4.6 mm) and Supelcosyl LC18 Column (150 x 4.6 mm), both packed with 5 um ODS materials (Supelco Inc., Bellefonte, PA, USA), a LC 95 UV/visible double beam, variable wavelength spectrophotometric detector and a LCI-100 computing integrator (Perkin-Elmer).
The mobil phase was 1 mM ammonium formate, 7 mM dodecyltrimethylammonium bromide, and 40% methanol, taken to pH 5.9 with hydrochloric acid. It was filtered through a 0.45 µm nylon filter (MSI, Westboro, MA, USA) and degassed with a helium sparging system. Elution was isocratic at a flow rate of 0.9 ml/min at room temperature. The detector sensitivity was set at 0.001 or 0.005 AUFS during blood components (plasma and serum) or seminal plasma assays, respectively.

RESULTS

Identity and Specificity

Peak identity was proven both by the correspondence of standard solution and sample retention times (9.7 min.) and the coelution of added and endogenous AA.
Addition of ascorbate oxidase to the body fluids examined, followed by incubation for 2 h at room temperature, led to complete loss of the AA peak, showing the absence of coeluting substances.
Representative chromatograms for (a) aqueous solution, (b) blood serum, (c) blood plasma and (d) seminal plasma are shown in Fig. 1.

Resolution from the preceeding peak (Fig.1), identified as uric acid by means of comparison of retention time with a standard solution, coelution with added UA and the disappearance of the peak after treatment of samples with uricase, was 1.6.

Analytical Performance

For blood sample analysis, linear calibration curves between 0 and 5 mg/l (28.4 μmol/l) were obtained, whereas, during seminal plasma analysis, linearity was tested between 0 and 30 mg/l (170.3 μmol/l).
The detection limit, calculated as 3x standard deviation obtained from 10 replicate measurements of a serum sample, containing 0.8 mg/l AA (4.5 μmol/l), resulted in 1.8 ng/injection (10 pmol/injection), in relation to 0.3 mg/l (1.7 μmol/l) in the undiluted sample.
Within-day imprecision for blood serum was evaluated by analyzing in duplicate 27 samples, and the between-day imprecision by measuring AA levels in 27 samples on two different days. The pooled standard deviation (SD_p) was calculated in both cases with the following formula:

$$SD_p = \sqrt{\frac{\sum^{n}_{i=1} (x_{i1} - x_{i2})^2}{2n}}$$

where x_{i1} and x_{i2} are the first and second measurement on the i_{th} sample and n is the number of samples.
The obtained SDs_p and relative mean coefficients of variation (CV_m) are reported in Table 1.
Within- and between-day imprecision were 2.1% and 5.0% for blood plasma analysis (n=10) and 1.0% and 2.3% for determination of AA in seminal plasma (n=14). In order to evaluate analytical recovery, a human serum pool, after an overnight incubation at 40° C resulting in a

TABLE 1
Within- and Between-day Imprecision for Blood Serum

	n	Mean mg/l	SD_p mg/l	Range mg/l	CV_m %
Within-day variation	27	9.7	0.29	2.6 - 13.8	3.0
Between-day variation	27	9.2	0.44	2.0 - 18.6	4.9

complete loss of endogenous AA, was spiked with known amounts of AA to the final concentrations of 3.0 mg/l (17.0 μmol/l) and 10.0 mg/l (56.8 μmol/l). Mean analytical recovery, obtained from 12 measurements, was 93.6 ± 5.9% for the 3.0 mg/l pool and 102.5 ± 3.6% for the 10.0 mg/l pool.

Stability of AA

To assess the reliability of the described storage conditions, two human serum pools were divided into aliquots and analyzed approximately once a week for 43 days of storage. In the two pools, containing 10.5 and 11.0 mg/l AA, respectively, the recorded loss of AA was negligible or nil and the CVs (4.1% and 4.5%) were as expected relative to the observed between-day imprecision.

In order to verify if any loss of AA occured in serum samples after treatment with DTE-methanol, 27 samples were assayed immediately after protein precipitation, stored at -20°C and assayed again 72 hours later. For all samples virtually no detectable decrease of AA concentration was observed. Mean AA levels were 9.2 ± 4.9 mg/l (52.2 ± 27.8 μmol/l) at time 0 and 9.1 ± 4.9

mg/l (51.7 ± 27.8 μmol/l) after 72 h, and the CV_m was 3.0%.

Blood Serum AA Levels

Serum AA levels were evaluated from 165 subjects, which were divided, according to smoking habit, into two subgroups, smokers and nonsmokers. The obtained results are reported in Table 2. Statistical analysis of data, by means of the ANOVA test did not point out any significant difference between serum AA levels of smokers and nonsmokers (F ratio = 1.71, $p > 0.05$).

Blood Plasma versus Blood Serum

Plasma and serum AA levels have been evaluated from 27 subjects, analyzing both plasma and serum obtained from the same blood sample. Mean blood plasma and serum AA concentrations were 9.6 ± 4.2 mg/l (54.8 ± 23.8 μmol/l) and 9.2 ± 4.2 mg/l (52.3 ± 23.8 μmol/l), respectively.

Using the ANOVA test no significant difference was found between plasma and serum AA levels (F ratio = 0.143, $p = 0.71$). The CV_m was 6.5 %.

TABLE 2
Serum AA Levels (mg/l) in Male Adults, Smokers and Nonsmokers

	Total	Smokers	Nonsmokers
Subjects	165	55	110
Age (mean ± SD)	65 ± 5	65 ± 5	65 ± 5
Age (range)	56-76	56-76	56-76
AA, (mean ± SD)	8.9 ± 4.0	8.4 ± 4.8	9.2 ± 3.5
AA, (range)	1.3-18.7	1.3-18.7	1.8-17.9

Seminal Plasma AA Levels

The mean AA concentration observed in seminal plasma obtained from 14 subjects was 114.4 ± 69.0 mg/l (649.6 ± 391.8 μmol/l), ranging from 25.8 mg/l to 232.4 mg/l (146.5-1319.6 μmol/l). In twelve cases we measured AA levels both in the presence and absence of the reducing agent DTE, in order to measure AA and DHAA differentially . Mean values in DTE-treated and DTE-untreated samples were 122.5 ± 71.7 mg/l (695.5 ± 407.1 μmol/l) and 122.3 ± 71.4 mg/l (694.4 ± 405.4 μmol/l) , respectivly. No significant difference was found between the two groups ($p = 0.942$) using ANOVA test and, the observed CV_m (1.1%) did not differ from within-day imprecision (1.0 %). The data demonstrates that DHAA, if present, accounts for only 1% of total vitamin C content.

DISCUSSION

In our method sample pretreatment procedure has been extremely simplified and permits UV detection in the absence of any extraction procedure.

AA is eluted in a relatively short time and well resolved from UA, that could also be quantified in the same chromatogram, although with reduced sensitivity, because the chosen wavelength does not coincide with the absorbance maximum for UA (286 nm).

The method, unlike colorimetric procedures, is unaffected by the presence of other reducing substances, and the absence of interferences has been proven by the complete loss of the peak at 9.7 minutes after treatment with ascorbate oxidase.

The observed detection limit, 0.3 mg/l in the undiluted sample, is well below the lowest AA concentration measured in blood serum (1.3 mg/l), thus allowing us to discern even AA severe depletion.

Both within- and between-day imprecision compares favourably with data obtained by other Authors, using HPLC coupled with UV detection (16, 21), and makes our procedure suitable not only for nutritional assessment, but also for research purposes.

Mean analytical recovery obtained for both low and high AA levels, provides evidence of good accuracy, within the range of experimental imprecision.

Owing to rapid degradation of AA (15), sample collection and storage conditions have been carefully investigated in order to assure correspondence between measured and actual AA concentrations in body fluids.

Okamura (24) described a complete reduction of DHAA in blood plasma samples by 2.50 mmol/l dithiothreitol optimal pH range is between 6.5 and 8.0. Hernanz (21) reported that DTE 2 mmol/l prevents AA oxidation in blood serum extracts. In our experience, the DTE concentration required to stabilize AA levels in blood serum or plasma was 1.5 g/l (10 mmol/l). In seminal plasma, AA was protected from oxidation only in the presence of 10 g/l (65 mmol/l) DTE.

Nevertheless, in the long term, AA degradation still occurrs. Infact we found decreased AA levels in serum samples stored at -80°C, after three days from DTE addition (data not shown), whereas, when O_2 was removed (bubbling Argon) before storing samples at low temperature, AA levels were stable for at least 43 days.

Reliability of the described conditons for AA preservation, have been confirmed by the absence of the Electron Spin Resonance (ESR) signal, related to AFR in treated blood serum samples. Inasmuch as AFR is the

intermediate stage in the formation of DHAA, its absence provides evidence that no degradation of AA is occurring.

The mean AA level observed in our 165 adult and aging male subjects agrees with data reported by others in studies carried out in USA (25, 26) and Japan (27, 28), whereas they are noticeably higher than those reported for elderly Italian subjects in two recent investigations (29, 30).

To our knowledge, it is the first time that AA levels in seminal plasma are quantified by means of HPLC coupled with UV detection. Our data on AA seminal plasma levels confirm those reported by other Authors (3). In seminal plasma samples examined, DHAA represented not more than 1% of total vitamin C content.

REFERENCES

1. Levine M., New Concepts in the Biology and Biochemistry of Ascorbic Acid. N Engl J Med 314, 892-902, 1986.

2. Anderson R., Lukey P.T., A Biological Role for Ascorbate in the Selective Neutralization of Extracellular Phagocyte-Derived Oxidants. Ann NY Acad Sci 498: 229-246, 1987.

3. Anderson R., Theron A.J., Physiological Potential of Ascorbate, β-Carotene and α-Tocopherol Individually and in Combination in the Prevention of Tissue Damage, Carcinogenesis and Immune Dysfunction Mediated by Phagocyte Derived Reactive Oxidants. World Rev Nutr Diet 62: 27-58, 1990.

4. Halliwell B. and Gutteridge J.M.C., Free Radicals in Biology and Medicine, Second Edition, Clarendon Press, Oxford, 1989.

5. Duthie G.G., Wahle W.J., James W.P.T, Oxidants, Antioxidants and Cardiovascular Disease. Nutr Res Rev 2, 51-62, 1989.

6. Gey K.F., Stahelin H.B., Puska P., Evans A., Relationship of Plasma Levels of Vitamin C to Mortality from Ischemic Heart Disease. Ann N Y Acad Sci 498, 110-123, 1987.

7. Ginter E., Cholesterol: Vitamin C Controls its Transformation to Bile Acids. Science 179, 702-704, 1973.

8. Chinoy N. J., Buch-nee R. P., Mehta R.R., Seethalakshmi L., Sharma J.D. and Chinoy M.R., Effects of Vitamin C Deficiency on Physiology of Male Riproductive Organs of Guinea Pigs. Int J Fertil 31, 232-239, 1986.

9. Harris W.A., Harden T.E. and Dawson E.B., Apparent Effects of Ascorbic Acid Medication on Semen Metal Levels. Fertil Steril 32, 455-459, 1979.

10. Dawson E.B., Harris W.A. Powell L.C., Relationship between Ascorbic Acid and Male Fertility. World Rev Nutr Diet 62, 1-26, 1990.

11. Jones R. and Mann T., Lipid Peroxidation in Spermatozoa. Proc R Soc Lond 184, 103-113, 1973.

12. Jones R. and Mann T., Lipid Peroxidation in Spermatozoa, Formation, Role of Plasmalogen and Physiological Significance. Proc R Soc Lond 193, 317-324, 1976.

13. Jones R., Mann T. Sherins R., Peroxidative Breakdown of Phospholipids in Human Spermatozoa, Spermicidal Properties of Fatty Acids Peroxides and Protective Action of Seminal Plasma. Fertil Steril 31, 531-536, 1979.

14. Pachla L.A., Reynolds D.L. Kissinger P.T., Analytical Methods for Determininig Ascorbic Acid in Biological Samples, Food Products and Pharmaceuticals. J Assoc Off Anal Chem 68, 1-12, 1985.

15. Baker J.K., Kapeghian J., Verlangieri A., Determination of Ascorbic Acid and Dehydroascorbic Acid in Blood Plasma Samples. J Liq Chromatogr 6, 1319-1332, 1983.

16. Parviainen M.T., Nyyssonen K., Penttila I. M., Seppanen K., Rauramaa R., Salonen J.T.S., Gref C. G.: A Method for Routine Assay of Plasma Ascorbic Acid Using High-Performance Liquid Chromatography. J Liq Chromatogr 9, 2185-2187, 1986.

17. Kutnink A.M., Hawkes W.C., Schaus E.E., Omaye S.T., An Internal Standard Method for the Unattended High-Performance Liquid Chromatographic Analysis of Ascorbic Acid in Blood Components. Anal Biochem 166, 424-430, 1987.

18. Howard R.R., Peterson T., Kastl P.R., High-Performance Liquid Chromatographic Determination of Ascorbic Acid in Human Tears. J Chromatogr 414, 434-439, 1987.

19. Lopez-Anaya A., Mayersohn M., Ascorbic and Dehydro-Ascorbic Acid Simoultaneously Quantified in Biological Fluids by Liquid Chromatography with Fluorescence Detection and Comparison with a Colorimetric Assay. Clin Chem 33, 1874-1878, 1987.

20. Omaye S.T., Schaus E.E., Kutnink M.A., Hawkes W.C.: Measurement of Vitamin C in Blood Components by High-Performance Liquid Chromatography. Ann N Y Acad Sci 498, 389-401, 1987.

21. Hernanz A, High-Performance Liquid Chromatographic Determination of Ascorbic Acid in Serum Using Paired-Ion Chromatography and UV Spectrophotometric Detection. J Clin Chem Clin Biochem 26, 459-461, 1988.

22. Margolis S.A., Davis T.P., Stabilization of Ascorbic Acid in Human Plasma, and its Liquid-Chromatographic Measurement. Clin Chem 34, 2217-2223, 1988.

23. Nagy E., Degrell I., Determination of Ascorbic Acid and Dehydroascorbic Acid in Plasma and Cerebrospinal Fluid by Liquid Chromatography with Electrochemical Detection. J Chromatogr 497, 276-281, 1989.

24. Okamura M., An Improved Method for Determination of L-Ascorbic Acid and L-Dehydroascorbic Acid in Blood Plasma. Clin Chim Acta 103, 259-268, 1980.

25. Garry P.J., Goodwin J.S., Hunt W.C., Gilbert B.A., Nutritional Status in a Healthy Elderly Population: Vitamin C. Am J Clin Nutr 36, 332-339, 1982.

26. Schectman G., Byrd J.C., Gruchow H.W., The Influence of Smoking on Vitamin C Status in Adults. Am J Public Health 79, 158-162, 1989.

27. Murata A., Shiraishi I., Fukuzaki K., Kitahara T., Harada Y., Lower Levels of Vitamin C in Plasma and Urine of Japanese Male Smokers. Internat J Vit Nutr Res 59, 184-189, 1989.

28. Itoh R., Yamada K., Oka J., Echizen H., Murakami K., Sex as Factor in Levels of Serum Ascorbic Acid in a Healthy Elderly Population. Internat J Vit Nutr Res 59, 365-372, 1989.

29. Fidanza F., Brubacher G., Simonetti M.S., Mariani Cucchia L., Nutritional Status of the Elderly. Internat J Vit Nutr Res 54, 355-359, 1984.

30. Porrini M., Simonetti P., Ciappellano S., Testolin G., Vitamin A, E and C Nutriture of Elderly People in North Italy. Internat J Vit Nutr Res 57, 349-355, 1987.