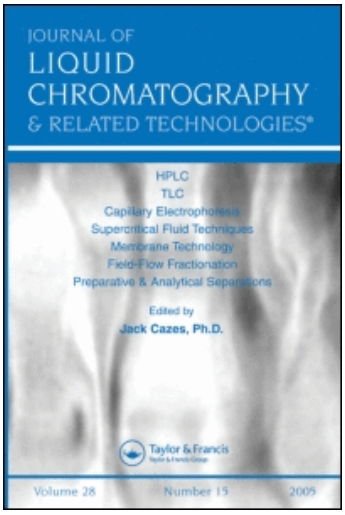

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Method for Routine Assay of Plasma Ascorbic Acid Using High-Performance Liquid Chromatography

Markku T. Parviainen[a]; Kristiina Nyyssönen[a]; Ilkka M. Penttilä[a]; Kari Seppänen[b]; Rainier Rauramaa[b]; Jukka T. S. Salonen[c]; Carl-G. Gref[d]

[a] Department of Clinical Chemistry Kuopio, University Central Hospital, Kuopio, Finland [b] Kuopio Research Institute of Exercise Medicine, Kuopio, Finland [c] Research Institute of Public Health Kuopio University, Kuopio, Finland [d] National Public Health Institute Helsinki, Finland

To cite this Article Parviainen, Markku T. , Nyyssönen, Kristiina , Penttilä, Ilkka M. , Seppänen, Kari , Rauramaa, Rainier , Salonen, Jukka T. S. and Gref, Carl-G.(1986) 'A Method for Routine Assay of Plasma Ascorbic Acid Using High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 9: 10, 2185 — 2197

To link to this Article: DOI: 10.1080/01483918608074142

URL: http://dx.doi.org/10.1080/01483918608074142

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(10), 2185-2197 (1986)

A METHOD FOR ROUTINE ASSAY OF PLASMA ASCORBIC ACID USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Markku T. Parviainen[1]*, Kristiina Nyyssönen[1], Ilkka M. Penttilä[1], Kari Seppänen[2], Rainier Rauramaa[2], Jukka T. S. Salonen[3], and Carl-G. Gref[4]

[1]*Department of Clinical Chemistry*
Kuopio University Central Hospital
SF-70210 Kuopio, Finland
[2]*Kuopio Research Institute of Exercise Medicine*
Kuopio, Finland
[3]*Research Institute of Public Health*
Kuopio University
Kuopio, Finland
[4]*National Public Health Institute*
Helsinki, Finland

ABSTRACT

A description is given of a specific and simple liquid chromatographic method for the determination of vitamin C (ascorbic acid) in protein-free plasma. The analysis time per sample is only 4 min, the retention time of ascorbic acid being 2.4 min. A detection limit of 0.3 mg/l (1.7 μmol/l) was achieved using the routine attenuation of the detector. The day-to-day coefficient of variation is less than 7.2 % at a physiological level and the within-day variation is less than 4.7 %. The method recovered 101 % (mean) of ascorbic acid supplemented to plasma. Mean plasma ascorbic acid concentration was 11.1 mg/l (63 μmol/l) in healthy adult volunteers, whereas considerably lower values were found in a group of randomly selected 54-year-old-men.

*) To whom all correspondence should be addressed

 0148-3919/86/0910-2185$3.50/0

INTRODUCTION

Ascorbic acid (vitamin C) possesses tremendous biological and pharmacological significance. Ascorbic acid acts as a unique reducing agent for the prolyl hydroxylase catalyzed synthesis of hydroxyproline in collagen (1). Overt vitamin C deficiency with distinct clinical and radiological signs, known as scurvy, has become rare in developed countries although mild vitamin C deficiency is encountered occasionally (2). Daily consumption of large quantities of ascorbic acid is claimed to have many beneficial effects against illnesses such as the common cold (3).

Several methods have been developed for assay of ascorbic acid in various biological samples or in plant materials, many of these methods having serious drawbacks. Commonly used method of dye titration with 2,6-dicholorophenol-indophenol (4) or the ketone derivatization method with 2,4-dinitrophenylhydrazine (5) are non-specific for ascorbic acid and in addition are laborious and time-consuming. Similar problems are also encountered with other recently proposed methods based on modifications of the reducing capacity of ascorbic acid (6, 7). The enzymatic mathod (8) with ascorbic acid oxidase coupled to the colour reaction or fluorometric method (9) seem to be more specific than the aforementioned methods, but are still rather complicated and are less suitable for routine determination of ascorbic acid in plasma.

High-performance liquid chromatography (HPLC) is a powerful tool for vitamin analyses. HPLC has been used for assaying the ascorbic acid content in foods, including fruits and vegetables (10, 11, 12, 13), and in beverages (14). Some of these methods (10, 13) are also capable of resolving and quantifying dehydroascorbic acid, an oxidized form of ascorbic acid found in minute quantities in foods. Doner and Hicks (15) were the first to separate ascorbic acid and its oxidation products plus erythorbic acid and its oxidation products

simultaneously using a weak anion exchange amino column. Ascorbic acid, however, was the only component in orange juice and urine that they were able to demonstrate. Their assay involved both refractive index and UV detection. Rose and Nahrwold (16) proposed that ascorbic acid and dehydroascorbic acid be analyzed simulataneously using an amino column plus UV detection with two wave-lengths. In quinea pig plasma only ascorbic acid levels were detected (11 ± 1 mg/l). Identification and quantification of ascorbic acid in extrates of human lymphocytes was described by Liebes et al. (17), who used a strong anion exchange column. Tsao and Salimi (18) used a reverse-phase ODS column and electrochemical detection for ascorbic acid and isoascorbic acid, which is biologically a relatively weak analog of ascorbic acid, from brain samples. Fluorometric determination of total vitamin C in whole blood with precolumn derivatization was recently described by Speek and his collaborators (19). Ascorbic acid was first oxidized enzymatically to dehydro-ascorbic acid, which was changed to a quinoxaline derivative analyzed using a reverse-phase column. Reference values of vitamin C in whole blood of healthy Dutch adults ranged from 20 to 80 μmol/l (3.5-14.1 mg/l) with a mean value of 54 μmol/l (9.5 mg/l). Other recently described HPLC methods include measurement of uric acid, ascorbic acid and some related metabolites in biological fluids using anion-exchange HPLC with UV detection (20). Two very recent works utilized reversed-phase HPLC combined with electrochemical detection of body fluid metabolites (21, 22).

In the present study, weak anion exchange HPLC with UV detection was used to develop a rapid, fully automatizable, reliable and sensitive method for analysis of levels of ascorbic acid in human plasma. Sample preparation is simple and involves only dilution of the plasma sample to 5 % metaphosphoric acid, centrifugation, and direct quantification.

MATERIALS AND METHODS

Reagents

The stock ascorbic acid (Sigma) standard 1000 mg/l was freshly prepared in water and further diluted to the final standard concentration (routinely 10 mg/l). The working standard was handled the same as the samples.

A 5 % metaphosphoric acid solution was prepared by washing the metaphosphoric acid crystals (Fluka) before the final solution was dissolved and filtered. The solution was stored at $+4^{\circ}$ C for one week.

Sample preparation

Venous blood was obtained from subjects who had fasted 12 h. The sample was drawn (at 7-9 AM) using Venoject VT-050 HL heparin tubes. The plasma was separated and handled within 30 minutes by centrifuging (1000 x g) at $+20^{\circ}$ C for 10 min. The plasma samples were stabilized in duplicate by adding 0.5 ml of plasma to 4.5 ml of 5 % metaphosphoric acid, followed by careful vortexing. The plasma proteins are precipitated and ascorbic acid is simultaneously stabilized. The stabilized samples were stored at -80° C for not more than one month if not analyzed immediately. Prior to analysis the sample tubes were centrifuged, and the clear supernatant thus obtained was injected directly into the liquid chromatographic column. The working standards were also diluted 0.5 ml + 4.5 ml in 5 % metaphosphoric acid.

Experimental HPLC

HPLC was performed using commercially available components. Samples were analyzed isocratically using either an Altex 110A pump and Schoeffel variable UV

detector SF 770 set to 254 nm, a Varian Vista 5500 Liquid Chromatograph and UV-200 variable UV detector set to 254 nm or Perkin-Elmer Series 10 Liquid Chromatograph and LC-15B UV detector at 254 nm. These chromatographs were equipped with a Rheodyne Model 7125 injection valve with a 20 µl fixed sample loop. For recording we used either a Perkin-Elmer Model 56 or Model R100 recorder. A fully automated ascorbic acid analysis was performed using Perkin-Elmer Series 4 Liquid Chromatograph plus LC-terminal, and P-E ISS-100 Autosampler, LC-95 UV/visible Spectrophotometer Detector and LCI-100 Laboratory Computing Integrator.

A Resolution stainless-steel column (150 x 4.6 mm I.D.) home-packed with Resolution-NH_2 3 µm (equals to Spherisorb® S3NH_2 bulk packing), was initially made available by Mr. Sandy Fuchs, Kaukomarkkinat Oy (Espoo, Finland). Commercially available amino columns (e.g. Spherisorb®, µBondapak®) probably are applicable, but the method we describe is subject for alterations when these are to be used. The eluent consisted of a 25 % solution of 2.5 mM potassium dihydrogen phosphate in acetonitrile (Merck, chromatography grade). A flow-rate of 1.2 or 1.5 ml/min (column back-pressure 1500-2000 psi, 10-15 MPa) was used. Ascorbic acid was quantified by comparing the peak heights of the samples or controls with those given by known concentrations of standards.

RESULTS AND DISCUSSION

The linearity of the detector response to ascorbic acid was established by using this calibrator from 0 to 100 mg/l. The standard was handled in the same way as the samples.

Ascorbic acid was separated from the standards and plasma samples as illustrated in Figures 1 and 2. A metaphosphoric acid blank (Fig. 1) showed only two minor reagent peaks at 1.2 and 3.6 min. These are readily differentiated

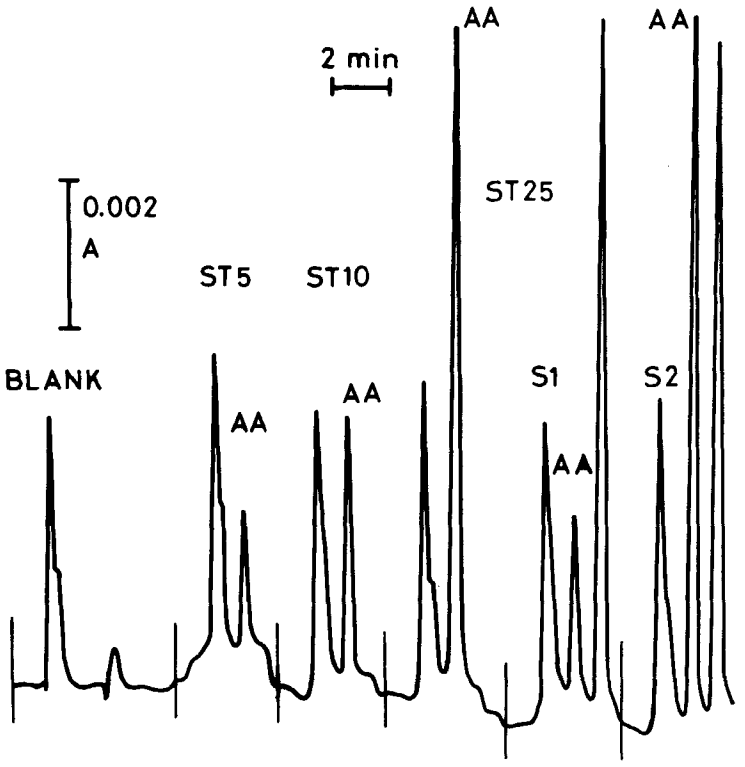

Figure 1.
Separation of ascorbic acid from plasma.
Blank: 5 % metaphosphoric acid injection;
5: ascorbic acid sandard 5 mg/l;
10: ascorbic acid standard 10 mg/l;
25: ascorbic acid standard 25 mg/l;
S1: a normal plasma sample;
S2: same as in S1 but ascorbic acid (20 mg/l) was added.
Retention time of ascorbic acid (AA): 2.4 min.

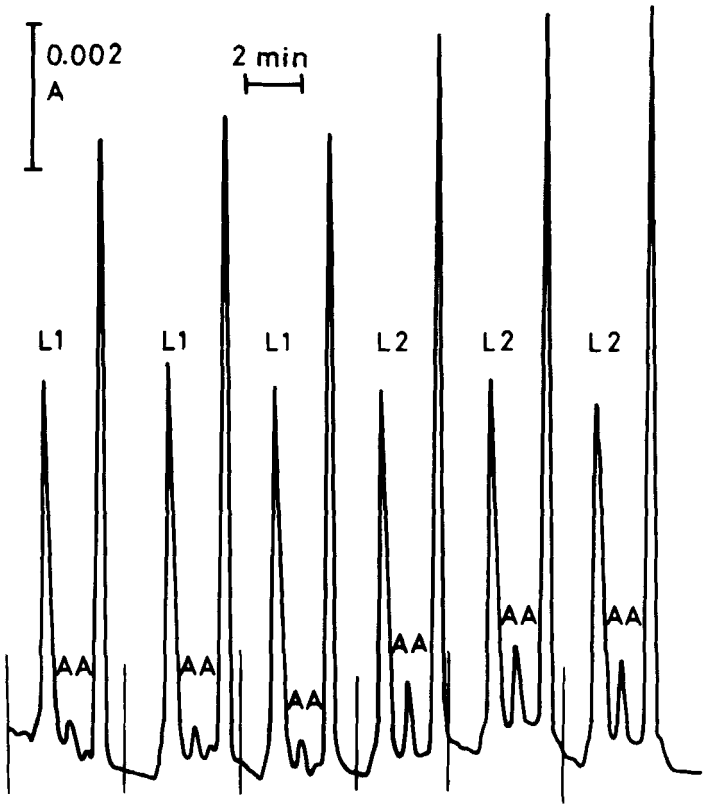

Figure 2.
Two plasma samples containing low concentrations of ascorbic acid were injected in triplicate (marked as L1 and L2, respectively).
Retention time of ascorbic acid (AA): 2.4 min.

from the ascorbic acid peak at 2.4 min both in standards and in plasma samples (Fig. 1). An unknown peak was observed in all plasma samples eluting at 3.2 min, and not possessing any interference. Repeated injections of two plasma samples containing very low ascorbic acid concentrations are shown in Figure 2. The ascorbic acid concentration in sample L1 was only 1.6 mg/l indicating a true vitamin C depletion, and the concentration in sample L2 was 3.5 mg/l indicating a

mild deprivation. The peak heights in repeated injections were essentially the same (Fig. 2).

Protein in the samples is precipitated with 5 % metaphosphoric acid, which gives a clear protein-free supernatant, and in addition, also stabilizes vitamin C (9, 16). Stabilized plasma samples can be stored at -80° C up to four weeks without any appreciable loss of ascorbic acid (data not shown).

The absolute recovery of ascorbic acid from plasma was measured by analysing plasma supplemented with 10 mg/l or 20 mg/l of ascorbic acid before deproteinization. The recovery (n=10) was 101 ± 8 % (mean ± SD), compared with the standard made up in 5 % metaphosphoric acid. No internal standard is thus needed in the ascorbic acid analysis.

Precision was determined in the series by repeated analysis of plasma samples containing 6.7 and 26.8 mg/l of ascorbic acid. The coefficients of variation was 4.7 % (n=15) and 2.4 % (n=14). The precision evaluated from day to day with these samples was 7.2 % (n=12) and 3.1 % (n=13) respectively.

A detection limit of 0.3 mg/l was achieved using the routine detector attenuation of about 0.016 absorbance units of the full scale. This seems to be well enough also for the detection of a grave ascorbic acid depletion.

Plasma ascorbic acid concentrations were measured from 19 adult volunteers of which 8 were females and 11 were males. The plasma ascorbic acid concentrations were 11.1 ± 2.4 mg/l (mean ± SD, 63 ± 14 μmol/l) with a range of 6.1 - 16.5 mg/l (35 - 94 μmol/l). In addition the plasma ascorbic acid levels were determined from a randomly selected group of 54-year-old men living in the city and rural area of Kuopio (n=155). The total range of ascorbic acid was 0.7 - 22.0 mg/l (4 - 125 μmol/l) with a mean value of 7.7 ± 4.4 mg/l (± SD, 44 ± 25 μmol/l). Eleven of these men (7.1 %) had ascorbic acid levels below 2.0 mg/l (11 umol/l), which is regarded as ascorbic acid depletion (23). In addition, 27 of the remaining 144 men (17.5 % of total 155) had an ascorbic acid level

between 2.1 - 4.0 mg/l (12 - 23 μmol/l) and were thus suspected of having mild vitamin C deprivation. According to the recent study of Speek et al. (19), a vitamin C level under 20 μmol/l (3.5 mg/l) would be regarded as below normal. Moreover, our results for plasma ascorbic acid in adult volunteers can be superimposed on those of Speek et al. for vitamin C in whole blood (ascorbic acid plus dehydroascorbic acid). Results obtained by conventional chemical methods (9, 23, 24) are also compared to those obtained by the present HPLC method (Table I). In Table I is shown a comparison of plasma vitamin C assays obtained by our proposed method and by the method of Brubacher and Vuilleumier (9). Revealing correlation is 0.9335 between these two methods. Plasma vitamin C assays using the method of Brubacher and Vuilleumier (9) were done by one of us in the National Public Health Institute in Helsinki. The comparison between the methods was done by analysing 27 randomly selected plasma samples obtained from the group of 54-year old men (n=155).

In addition to test the reliability of our new assay for vitamin C, the effect of large oral doses of ascorbic acid on the levels of vitamin C in plasma were tested in a short trial. Eight healthy adult volunteers received either 500 mg or 1 g ascorbic acid daily for one week. In the four subjects who received 1 g, the basal level (mean 8.7 mg/l) rose 84 % after one week compared with only 11 % in the four subjects who received 500 mg (mean basal level 11.6 mg/l). One week after supplementation ceased, plasma ascorbic acid was still elevated 54 % in the 1 g supplement group, and fell to the initial level after an additional week. The initial level was reached one week after cessation of the 500 mg supplement. According to Yung et al. (25), only about 30 % of the ingested ascorbic acid in tablet form is absorbed, and the ascorbic acid half-life is only hours (about 4 hours) when the body stores are saturated. In our test, 1 g of ascorbic acid gave a longer saturation of the body stores than did 500 mg. Further studies, however, are needed to investigate the kinetics of ascorbic acid.

Table 1. Plasma vitamin C assays by our method compared with the method of Brubacher and Vuilleumier (1974).

Brubacher and Vuilleumier (x)	HPLC (y)	Brubacher and Vuilleumier (x)	HPLC (y)
mg/l		mg/l	
1.9	2.3	7.1	7.6
4.8	6.7	11.9	12.4
8.6	11.5	2.9	4.5
4.9	5.2	9.9	7.4
6.5	6.7	1.6	1.5
4.1	3.8	11.3	10.4
4.1	3.9	7.5	7.1
5.2	5.2	2.1	1.8
5.9	5.1	5.8	5.9
9.6	13.2	10.3	10.5
10.8	11.3	4.5	5.4
3.0	2.7	1.5	1.7
8.6	10.9	6.1	8.5
2.2	2.4		

Linear regression equation: $y(HPLC) = 1.035x + 0.265$
$r = 0.9335$, $n = 27$
$x = 6.03$ mg/l; $SD_x = 3.21$ mg/l
$y = 6.50$ mg/l; $SD_y = 3.56$ mg/l
paired t-test: n.s.

Further studies of the correlation of ascorbic acid levels to other biochemical indices in plasma and to health habits are now in progress.

In conclusion, we have described a fast and reliable method for analysis of ascorbic acid in human plasma. Our method resembles to that described by Rose and Nahrwold (16) but, however, it became evident during the course of this study that their method is not readily applicable to clinical use. Plasma samples stabilized with 5 % metaphosphoric acid can be stored at -80° C at least one month without noticeable deterioration of ascorbic acid. Moreover, measurements of plasma

ascorbic acid is recommended since the levels found in whole blood (23) are similar, and also because plasma itself seems to be the primary carrier of ascorbic acid to the tissues (26), thus giving a reliable assessment of the overall vitamin C status.

ACKNOWLEDGEMENTS

We thank Drs. Tarja Mononen, M.D. and Ilkka Mononen, M.D. for inspiring discussion.

REFERENCES

1. Tuderman L, Myllylä R, Kivirikko K I: Mechanism of the prolyl hydroxylase reaction. 1. Role of co-substrates. Eur J Biochem 80, 341-348, 1977.

2. Vartiainen E, Puska P, Huttunen J, Karppanen H, Gey F: Vitamiinit ja sepelvaltimotauti (in Finnish). Suomen Lääkärilehti 38, 3146-3150, 1983.

3. Pauling L: Vitamin C. The Common Cold and the Flu. W H Freeman and Company, San Francisco, 1976.

4. Sauberlich H, Goad W C, Skala J H, Waring P P: Procedure for mechanized (continous-flow) measurement of serum ascorbic acid (vitamin C). Clin Chem 22, 105-110, 1976.

5. Roe J H, Kuether C A: The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenyl-hydrazine derivative of dehydroascorbic acid. J Biol Chem 147, 399-407, 1943.

6. Jagota S K, Dani H M: A new colorimetric technique for the estimation of vitamin C using Folin phenol reagent. Anal Biochem 127, 178-182, 1982.

7. McGown E L, Rusnak M G, Lewis C M, Tillotson J A: Tissue ascorbic acid analysis using ferrozine compared with the diphenylhydrazine method. Anal Biochem 119, 55-61, 1982.

8. Samyn W: "True" ascorbic acid in plasma and serum: investigation of a new method. Clin Chim Acta 133, 111-117, 1983.

9. Brubacher G, Vuilleumier J P: In Clinical Biochemistry. Principles and Methods (Eds. Curtius HCh and Roth M). W De Gruyter, Berlin, 1974, p. 992.

10. Wimalasiri P, Wills R B H: Simultaneous analysis of ascorbic acid and dehydroascorbic acid in fruit and vegetables by high-performance liquid chromatography. J Chromatogr 256, 368-371, 1983.

11. Ashoor S H, Monte W C, Welty J: Liquid chromatographic determination of ascorbic acid in foods. J Assoc Off Anal Chem 67, 78-80, 1984.

12. Shaw P E, Wilson C W: Ascorbic acid content of some tropical fruit products determinated by high-performance liquid chromatography. J Agric Food Chem 30, 394-396, 1982.

13. Wills R P H, Wimalasiri P, Greenfield H: Liquid chromatography, microfluorometry and dye-titration determination of vitamin C in fresh fruit and vegetables. J Assoc Off Anal Chem 66, 1377-1379, 1983.

14. Dennison D B, Brawley T G, Hunter G L K: Rapid high-performace liquid chromatographic determination of ascorbic acid and combined dehydroascorbic acid in beverages. J Agric Food Chem 29, 927-929, 1981.

15. Doner L W, Hicks K B: High-performance chromatographic separation of ascorbic acid, erythorbic acid, dehydroascorbic acid, dehydroerythorbic acid, diketogulonic acid and diketogluconic acid. Anal Biochem 115: 225-230, 1981

16. Rose R C; Nahrwold D L: Quantitative analysis of ascorbic acid and dehydroascorbic acid by high-performance liquid chromatography. Anal Biochem 114, 140-145, 1981.

17. Liebes L F, Kuo S, Krieqel R, Pelle E, Silber R: Identification and quantification of ascorbic acid in extracts of human lymphocytes by high-performance liquid chromatography. Anal Biochem 118, 53-57, 1981.

18. Tsao C S, Salimi S L: Differential determination of L-ascorbic acid and D-isoascorbic acid by reversed-phase high-performance liquid chromatography with electrochemical determination. J Chromatogr 245, 355-358, 1982.

19. Speek A J, Schrijver J, Schreurs W H P: Fluorometric determination of total vitamin C in whole blood by high-performance liquid chromatography with pre-column derivatization. J Chromatogr 305, 53-60, 1984.

20. Hatch L L, Sevanian A: Measurement of uric acid, ascorbic acid, and related metabolites in biological fluids. Anal Biochem 138, 324-328, 1984.

21. Kutnink M A, Skala J H, Sauberlich H E, Omaye S T: Simultaneous determination of ascorbic acid, isoascorbic acid (erythorbic acid) and uric acid in human plasma by high-performance liquid chromatography with amperometric detection. J Liq Chromatogr 8, 31-46, 1985.

22. Iriyama K, Yoshiura M, Iwamoto T: Electrochemical detection of ascorbic acid in the eluate from reversed-phase high-performance liquid chromatography with newly developed rigid-type porous polymer packing (polymetaacrylate gels). J Liq Chromatogr 8, 333-344, 1985.

23. Garry P J, Goodwin J S, Hunt W C, Gilbert R A: Nutritional status in a healthy elderly population: vitamin C. Am J Clin Nutr 36, 332-339, 1982.

24. Burr M L, Bates C J, Milbank J E, Yarnell J W G: The relationship between plasma ascorbate and lipid concentrations in fasting men. Hum Nutr Clin Nutr 36C, 135-139, 1982.

25. Yung S, Mayersohn M, Robinson J B: Ascorbic acid absorption in humans: a comparison among several dosage forms. J Pharm Sci 71, 282-285, 1982.

26. Evans R M, Currie L, Cambell A: The distribution of ascorbic acid between various cellular components of blood in normal individuals, and its relation to the plasma concentration. Br J Nutr 47, 473-482, 1982.