ELSEVIER

Journal of Chromatography B, 655 (1994) 195–200

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of ascorbic acid in human plasma by high-performance liquid chromatography with electrochemical detection using a hydroxyapatite cartridge for precolumn deproteinization

Hiroshi Iwase*, Ichiro Ono

Central Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan

(First received October 1st, 1993; revised manuscript received February 7th, 1994)

Abstract

Ascorbic acid (AA) was determined in human plasma by high-performance liquid chromatography with electrochemical detection using a hydroxyapatite cartridge for plasma deproteinization. The proposed method is simple, rapid (deproteinization time, within 1 min; analysis time, *ca.* 15 min), sensitive [detection limit, *ca.* 240 ng/ml plasma (at a signal-to-noise ratio of 2:1)], highly selective and reproducible [relative standard deviation, *ca.* 2.8% ($n = 3$)]. The calibration graph for AA was linear in the range 0.1–10 ng per injection (20 μl). The recovery of AA was over 90% by the standard addition method.

1. Introduction

High-performance liquid chromatography (HPLC) with UV detection, electrochemical detection (ED) and fluorescence detection has proved to be a useful method for the determination of ascorbic acid (AA) in foods [1–6] and biological fluids [6–12]. Usually, the determination of AA in fluids has been performed by HPLC with UV detection at 245 or 254 nm [7,8] or ED set at 600–800 mV *versus* an Ag/AgCl reference electrode [6,8,9,11,12] after deproteinization of plasma (100–500 μl) with metaphosphoric acid (MA) or perchloric acid followed by centrifugation for 10 min or more or standing for 10 min or more than 30 min to ensure complete deproteinization.

AA is known to be unstable in aqueous solution [13]. Iriyama *et al.* [9] reported that human serum was prepared from blood just prior to analysis within 10 min. Yoshiura and Iriyama [12] reported that the total time from the sample preparation to the injection was within 30 min.

Measurement of unstable AA in biological fluids requires a simple and rapid deproteinization of the biological fluids and simple, rapid, highly selective, reproducible and sensitive microsample analysis.

In a previous paper [5], we reported the highly selective and sensitive determination of biologically and pharmacologically important AA and dehydroascorbic acid (DHAA) in juices by HPLC with ED set at <300 mV *versus* an Ag/

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00104-D

AgCl reference electrode using an internal standard. The rapid and mild reduction of DHAA to AA was carried out by addition of 10 m*M* sodium phosphate buffer (pH 6.8) containing L-cysteine (2.5 mg/ml). The preceding paper [14] reported a precolumn deproteinization method using a hydroxyapatite cartridge (PCPure cartridge, Moritex or Koken, Tokyo, Japan) for the determination of theophylline and diazepam in human plasma by HPLC with UV detection. By using a PCPure cartridge, human plasma was simply and rapidly deproteinized within 1 min without the need for centrifugation, a dilution method or an injection method [14]. The injection method was simple and convenient in comparison with the dilution method, because no dilution of samples prior to passing through the cartridge was required. A simple and rapid injection method was used for the unstable AA.

This paper deals with the choice of the eluent, elution of AA and an internal standard (α-methyl-L-dopa) from a PCPure cartridge and the stability of AA in the protein-free eluate. The validity of the determination of AA in human plasma by HPLC with ED set at <300 mV *versus* an Ag/AgCl reference electrode using L-cysteine as a precolumn reductant was checked. Further, this paper also reports a comparison of analytical data for AA using the proposed method and a published method.

2. Experimental

2.1. Reagents and materials

AA was purchased from Tokyo Kasei (Tokyo, Japan), L-cysteine from Ajinomoto (Tokyo, Japan) and α-methyl-L-dopa from Nacalai Tesque (Kyoto, Japan). Other reagents were of analytical reagent grade. PCPure cartridges were obtained from Moritex or Koken. The cartridges were used as received.

2.2. Preparation of standard AA solution

A standard aqueous AA solution (10 μg/ml) was freshly prepared prior to use. The AA in this solution was stable at 5°C for 30 min and then the peak height decreased.

2.3. Preparation of internal standard (α-methyl-L-dopa)

An internal standard (α-methyl-L-dopa) [5] aqueous solution (125 μg/ml) was freshly prepared prior to use. α-Methyl-L-dopa in this solution was stable at 5°C for 24 h, then the peak height decreased.

2.4. Preparation of eluent (10 mM sodium phosphate buffer (pH 6.8) containing L-cysteine)

A 10 m*M* sodium phosphate buffer (pH 6.8) containing L-cysteine (2.5 mg/ml) was freshly prepared prior to use. This eluent was stable at 5°C for 3 days. After 3 days, quantitative reduction of DHAA to AA was not possible with this eluent [5].

2.5. Plasma collection

Blood was freshly collected in from healthy individuals heparinized tubes and plasma was obtained by centrifugation at 1700 *g* for 15 min at 5°C. The plasma obtained was then used immediately for the determination of AA.

2.6. Sample preparation for determination of AA

Aliquots of 20 μl of plasma and 10 μl of α-methyl-L-dopa were directly injected into a PCPure cartridge and then 10 m*M* sodium phosphate buffer (pH 6.8) containing L-cysteine was passed through. The protein-free eluate (elution of the first 800 μl) [14] was used as the test solution. An aliquot (20 μl) was injected into the chromatograph.

2.7. Apparatus and conditions

A Model 655 A-11 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Model Σ875 electrochemical

detector (Irica, Kyoto, Japan) was used. The applied potential was set at 300 mV *versus* an Ag/AgCl reference electrode. The samples were applied by a Rheodyne Model 7125 sample loop injector with an effective volume of 20 μl. HPLC was carried out on a 15 × 0.46 cm I.D. reversed-phase Inertsil ODS-2 (5-μm) column (GL Sciences, Tokyo, Japan) using 100 m*M* KH_2PO_4 (pH 3, adjusted with phosphoric acid)–1 m*M* ethylenediaminetetraacetic acid disodium salt (EDTA·2Na) as the mobile phase at a flow-rate of 0.6 ml/min at room temperature.

3. Results and discussion

3.1. Effect of eluent on stability of AA

Several eluents were examined for the elution of AA in human plasma from the PCPure cartridge. Both AA and α-methyl-L-dopa must be rapidly eluted and must remain stable as long as possible in the protein-free eluate.

The initial effort was focused on the effect of the eluent on the stability of AA in aqueous solution. The stability of AA in aqueous solution was evaluated by comparing the peak-height ratio of a standard of AA (200 ng), which corresponds to 20 μl of blood (AA level *ca.* 10 μg/ml) [6,8,12] diluted in each eluent (800 μl) and analysed periodically by HPLC with ED.

The variation of the AA level in several eluents [0.9% sodium chloride solution, deionized water, 10 m*M* sodium phosphate buffer (pH 6.8) and 10 m*M* sodium phosphate buffer (pH 6.8) containing 0.25% L-cysteine] were tested. As shown in Fig. 1, the AA content in 0.9% sodium chloride solution decreased with time at room temperature and was undetectable after 3 h, because AA is oxidized in the presence of chloride [13]. The AA content in deionized water also decreased at room temperature. AA in L-cysteine-free 10 m*M* sodium phosphate buffer (pH 6.8) was stable for 15 min and then the level decreased at room temperature. On the other hand, AA in 10 m*M* sodium phosphate buffer (pH 6.8) containing 0.25% L-cysteine was stable for *ca.* 1 h at room temperature and for 2 h at 5°C, and then the level decreased slightly. It was also found that α-methyl-L-dopa was stable for 24 h in this eluent at 5°C.

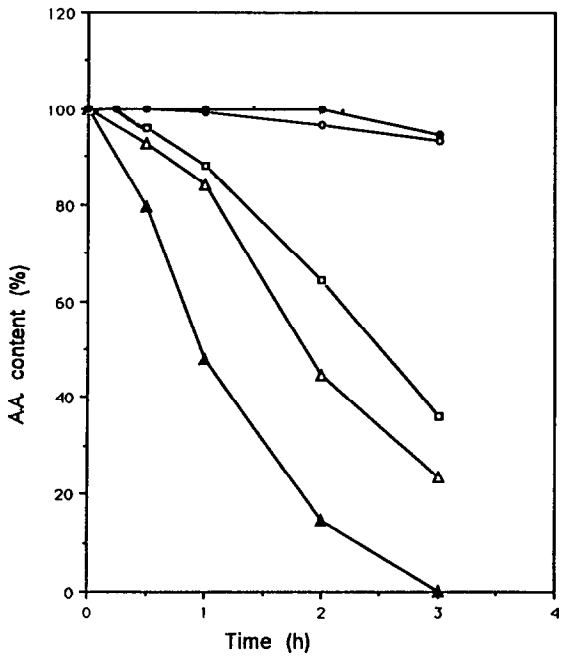

Fig. 1. Variation of AA content with time in aqueous solutions. Initial AA concentration = 200 ng in 800 μl. α-Methyl-L-dopa concentration = 1.25 μg in 800 μl. Solution: (▲) 0.9% sodium chloride (room temperature); (△) deionized water (room temperature); (□) 10 m*M* sodium phosphate buffer (pH 6.8) (room temperature); (●) 10 m*M* sodium phosphate buffer (pH 6.8) containing L-cysteine (5°C); (○) 10 m*M* sodium phosphate buffer (pH 6.8) containing L-cysteine (room temperature).

MA solution was used to precipitate protein and prevent oxidation of AA [9,11]. AA in MA solution was stable for 1 h at room temperature. However, the PCPure cartridge was unstable in acidic solution. Therefore, MA was not used as the eluent.

From the above results, 10 m*M* sodium phosphate buffer (pH 6.8) containing 0.25% L-cysteine was chosen as the eluent, because both AA and α-methyl-L-dopa were stable and protein-free eluate was obtained as described previously [14].

3.2. Elution of AA and α-methyl-L-dopa

The second effort was focused on the rapid elution of both AA and α-methyl-L-dopa from a PCPure cartridge with 10 m*M* sodium phosphate buffer (pH 6.8) containing 0.25% L-cysteine. Standard AA (200 ng) and α-methyl-L-dopa (1.25 μg) were injected into the cartridge followed by elution with the above eluent. Each fraction (300 μl) was analysed by HPLC with ED.

As can be seen in Fig. 2, both AA and α-methyl-L-dopa were eluted completely in both fractions 1 and 2 (600 μl) within 1 min. Protein-free eluate (elution of the first 800 μl) [14] was used in this study to ensure complete elution of both compounds. Accordingly, it was concluded that 10 m*M* sodium phosphate buffer (pH 6.8) containing 0.25% L-cysteine is a suitable eluent, because both the AA and α-methyl-L-dopa were eluted stably from the PCPure cartridge and protein-free eluate was obtained [14].

3.3. Chromatography

The next effort was focused on the determination of AA in human plasma by HPLC with ED set at <300 mV *versus* an Ag/AgCl reference electrode after deproteinization of human plasma using a PCPure cartridge.

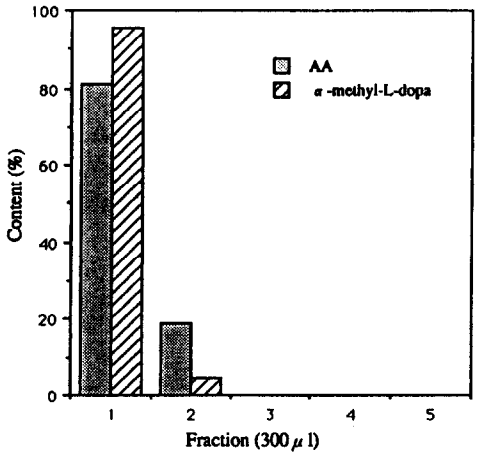

Fig. 2. Contents of AA and α-methyl-L-dopa in consecutive fractions (300 μl). Amount of AA = 200 ng; amount of α-methyl-L-dopa = 1.25 μg. Eluent: 10 m*M* sodium phosphate buffer (pH 6.8) containing 0.25% L-cysteine.

Usually, AA in foods and biomedical samples has been determined by HPLC with ED set at 600 mV or more *versus* an Ag/AgCl reference electrode [6,8,9,11,12] or by HPLC with UV detection at 245 or 254 nm [7,8]. A typical hydrodynamic voltammogram of AA, α-methyl-L-dopa, cysteine, tyrosine and tryptophan was illustrated in a previous paper [5].

When the determination of AA was performed by HPLC with ED set at ≥600 mV *versus* an Ag/AgCl reference electrode or by HPLC with UV detection at 245 or 254 nm, not only AA, but also cysteine, tyrosine and tryptophan were detected, as described previously [4,5]. It took about 60 min after the retention time of AA to elute the other compounds completely. On the other hand, the chromatography for AA can be highly selective with rapid detection (analysis time *ca.* 15 min) in the presence of other compounds by ED set at 300 mV *versus* an Ag/AgCl reference electrode. A typical chromatogram of AA (AA concentration *ca.* 145 ng in 800 μl of sample solution; AA concentration at the limit of detection *ca.* 4.8 ng in 800 μl of sample solution, at a signal-to-noise ratio of 2:1) in human plasma is shown in Fig. 3.

Usually, 100–500 μl of human plasma or serum have been deproteinized with MA followed by centrifugation for 10 min or more or standing for 10 min or more than 30 min [8,9,11]. Thus, the published methods require larger amounts of sample and more time. In this study, only 20 μl of plasma were deproteinized without the need for centrifugation and an even smaller volume of plasma can be he used for the determination of AA.

3.4. Stability of AA

The variation with time of the content of AA stored at 5°C and room temperature after deproteinization of human plasma by the proposed method was examined. AA was stable for 30 min under both conditions. The peak-height ratio of AA to α-methyl-L-dopa was constant for 30 min, then decreased. When the above sample was

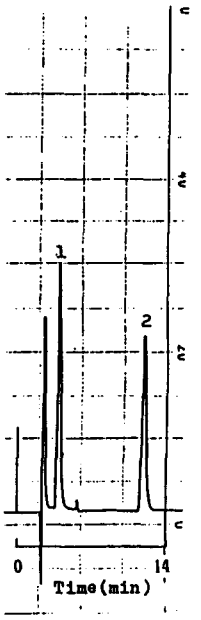

Fig. 3. Chromatogram of AA in human plasma obtained by HPLC with ED set at 300 mV *versus* an Ag/AgCl reference electrode. Amount of AA injected, 3.63 ng in 20 μl (AA concentration $\approx$ 145 ng per 800 μl of sample solution). HPLC was carried out on a 15 × 0.46 cm I.D. column of Inertsil ODS-2 (5 μm) using 100 m*M* KH_2PO_4 (pH 3, adjusted with phosphoric acid) 1 m*M* EDTA·2Na as the mobile phase at a flow-rate of 0.6 ml/min under ambient conditions. Peaks: 1 = AA; 2 = α-methyl-L-dopa.

stored for 24 h at 5 and −20°C, the AA content was *ca.* 80 and 98%, respectively (Table 1). On the other hand, when human plasma was stored for 24 h at −20°C without preparation by the proposed method, the AA content was about 21% (Table 1). This confirms that AA was stable in the presence of L-cysteine.

Accordingly, the determination of AA should be carried out within 30 min without storage. It may be considered that the determination of AA is possible when L-cysteine is added to blood just prior to plasma preparation.

Table 1
Comparison of AA content in samples stored at 5 and −20°C for 24 h after depproteinization by the proposed method

Temperature (°C)	Sample	AA content (%)
5	Protein-free plasma	80.1
−20	Protein-free plasma	98.2
−20	Plasma	21.2

3.5. Determination of AA

The calibration graph for AA was constructed by plotting the peak-height ratio of AA to the internal standard against the amount of AA. Satisfactory linearity was obtained in the range 0.1–10 ng on-column ($y = 0.342x - 0.023$, where y = peak-height ratio and x = amount of AA in ng).

Known amounts of AA and DHAA were added to human plasma and the overall recoveries were determined by the standard addition method. As indicated in Table 2, the recoveries of AA and DHAA were >90% and *ca.* 80%, respectively.

Table 3 gives the results for AA concentration in plasma samples from four human males. The relative standard deviation (R.S.D) was *ca.* 2.8% ($n = 3$) with no addition of AA.

A comparison was made of the results for AA in plasma samples from three human males using the proposed method and a published method [9]. The data in Table 4 indicate that the AA contents determined by the two methods were almost identical.

DHAA in MA solution and L-cysteine-free eluent was not detected. In L-cysteine-containing eluent, DHAA was reduced to AA [5].

Using the L-cysteine eluent, the content was not increased. As DHAA was determined as the difference between the total AA after DHAA reduction and the AA content of the original sample, these results confirm that DHAA was not detected in freshly prepared human plasma within 1 min by the proposed method.

Behrens and Madere [6] reported that the level of DHAA in rat plasma appear to be high (32.5% of the total vitamin C) in comparison with those in human and guinea pig plasma, in which the values did not exceed 10%. However, Liau *et al.* [8] reported that the use of homocysteine to reduce DHAA to AA did not lead to an increase in total AA. This aspect being studied further.

Table 2
Recoveries of AA and DHAA added to human plasma

Compound	Added (μg/ml)	Found (μg/ml)	Recovery ($n = 3$) (%)
AA	0	7.18	–
	5.0	11.76	91.6
	10.0	16.86	96.8
	20.0	27.45	101.4
DHAA	0	0	–
	10.0	8.11	81.1

Table 3
Results for the determination of AA in plasma samples from four human males.

Sample	Concentration (mean ± S.D., $n = 3$) (μg/ml)
A	7.73 ± 0.22
B	9.17 ± 0.23
C	7.95 ± 0.22
D	8.88 ± 0.25

Table 4
Comparison of results for AA obtained using the proposed method and a published method [9]

Method	AA ($n = 3$) (μG/ml)		
	A	B	C
Proposed method			
L-Cysteine eluent	7.73	9.17	7.95
L-Cysteine-free eluent	7.75	9.15	8.02
Published method			
MA [9]	7.68	9.18	7.98

4. Conclusion

The use of a commercially available PCPure cartridge for the simple and rapid deproteinization of human plasma and the use of 10 m*M* sodium phosphate buffer (pH 6.8) containing 0.25% L-cysteine as the eluent seems to be very useful for the determination of unstable AA in human plasma by HPLC with ED. The proposed method is satisfactory with respect to selectivity, simplicity and rapidity in comparison with published methods [6,8,9,11,12]. This method established here seems to be applicable to the routine determination of AA in human plasma in clinical chemistry. The proposed method is simple, rapid (deproteinization time of human plasma, within 1 min; analysis time for AA, *ca.* 15 min), sensitive (detection limit, *ca.* 240 ng ml plasma at a signal-to-noise ratio of 2:1), reproducible [R.S.D. *ca.* 2.8% (n = 3)] and highly selective without the need for clean-up and with recoveries of over 90% for AA.

5. References

[1] D.B. Dennison, T.G. Brawley and G.L.K. Hunter, *J. Agric. Food Chem.*, 29 (1981) 927.
[2] W.A. Behrens and R. Madere, *J. Liq. Chromatogr.*, 15 (1992) 753.
[3] W.D. Grahham and D. Annette, *J. Chromatogr.*, 594 (1992) 187.
[4] H. Iwase, *J. Chromatogr.*, 505 (1992) 277.
[5] H. Iwase and I. Ono, *J. Chromatogr.*, 654 (1993) 215.
[6] W.A. Behrens and R. Madere, *Anal. Biochem.*, 165 (1987) 102.
[7] R.R. Howard, T. Peterson and P.R. Kastl, *J. Chromatogr.*, 414 (1987) 434.
[8] L.S. Liau, B.L. Lee, A.L. New and C.N. Ong, *J. Chromatogr.*, 612 (1993) 63.
[9] K. Iriyama, M. Yoshiura, T. Iwamoto and Y. Ozaki, *Anal. Biochem.*, 141 (1984) 238.
[10] M. Yoshiura, T. Iwamoto and K. Iriyama, *Jikeiika Med. J.*, 32 (1985) 21.
[11] M.A. Kotnink, J.H. Skala, H.E. Sauberrlich and S.T. Omaye, *J. Liq. Chromatogr.*, 8 (1985) 31.
[12] M. Yoshiura and K. Iriyama, *J. Liq. Chromatogr.*, 9 (1986) 177.
[13] A.P. De Leenheer, W.E. Lambert and H.J. Nelis, *Modern Chromatographic Analysis of Vitamins*, Marcel Dekker, New York, 2nd ed., 1992, p. 235.
[14] H. Iwase, K. Gondo, T. Koike and I. Ono, *J. Chromatogr.*, 655 (1994) 73.