DETERMINATION OF TOTAL L-ASCORBIC ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN HUMAN PLASMA

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Abstract - The total vitamin C content in human plasma is widely accepted as an indicator of the tissue status of vitamin C. A liquid chromatography method with ultraviolet detector (264 nm) for measuring ascorbic acid in human plasma was developed. A C_{18} reversed-phase column and cetrimide as an ion-pairing agent was employed. Ascorbic acid (AA) was measured after reducing L-dehydroascorbic acid to L-ascorbic acid with dithiothreitol. The stability of the ascorbic acid in plasma, metaphosphoric acid and trichloroacetic acid was also evaluated. The analytical parameters, including linearity (1-60 μg/ml), accuracy (98.98%), repeatability (2.8%) and reproducibility (7.2%), showed that the method is reliable for measuring the total vitamin C content in plasma.

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Key Words: L-ascorbic acid, total vitamin C, HPLC

INTRODUCTION

L-Ascorbic acid has been proposed as having cancer-chemo-preventive activity (1,2). Vitamin C is widely claimed to have a protective role in the oxidative damage and degenerative diseases (3,4,5). The rapidly increasing interest in vitamin C intake and status indices has urged the need to offer a valid method for determination of L-ascorbic acid. Of these indices, plasma vitamin C concentration is the most widely preferred mainly because it is sensitive both to variations in the intake and body stores of the vitamin (6). The total vitamin C content in blood plasma and leucocytes is widely accepted as an indicator of the tissue status of vitamin C (7). The total vitamin C of a sample is the sum of L-ascorbic acid and its oxidized form, L-dehydroascorbic acid (8). The biological activities of these two are similar (7).

L-ascorbic acid in plasma or serum has been measured most often by colorimetric methods based on the oxidation-reduction properties of L-ascorbic acid or the formation of chromogenic derivatives, such as hydrazone (9).

The preferred method for determination of L-ascorbic acid is the reduction of 2,6-dichlorophenolindophenol (10,11).

Accurate measurement of ascorbic acid in biological samples (such as plasma) depends on a reliable method for stabilizing this material in the matrix of interest and the availability of a reliable assay for this analyte. High-performance liquid chromatography (HPLC) methods have been proved suitable for determining ascorbic acid in physiological fluids and tissues (8,12,13). In this study vitamin C is determined in human blood plasma by HPLC using a UV detector. Also a simple method for sample preparation is proposed.

MATERIALS AND METHODS

Reagents

L-Ascorbic acid, dithiothreitol (DTT), trichloroacetic acid (TCA), metaphosphoric acid (MPA), methanol, cetrimide, 4'-hydroxyacetanilide and sodium dihydrogen phosphate were from Merck (Darmstadt, Germany). Double-distilled deionized water was used throughout.

Standard preparation

Stock solution of L-ascorbic acid (125 μg/ml) with dithiothreitol (5mM) was prepared in water, which appeared stable for weeks in dark at 4°C. Stock solution of L-ascorbic acid was used for preparation of working standards (1,2,5,10,20,35,40,50,55 and 60 μg/ml) and these working standards were further diluted by water. Internal standard solution of 4'-hydroxyacetanilide (4 μg/ml) with dithiothreitol (5mM) was prepared in metaphosphoric acid 10%.

Sample preparation

Venous blood samples were drawn from the forearm vein of volunteers (25-35 years), and were transferred to tubes with heparin to obtain plasma. Plasma was separated by centrifugation at 3500 g for 5 min at 4°C. In a 2ml PTFE-capped centrifuge tube, 200 μl of plasma was mixed with 200 μl of internal standard solution. The precipitated proteins were removed by centrifugation at 18000 g for 5 min at 4°C and the supernatant was filtered through a 0.22 μm filter (Sartorius, Goettingen, Germany). Finally, a 5 μl aliquot of the clear phase was injected directly onto the HPLC column.

Liquid chromatography

The HPLC system included a manual injector (Rheodyne, California, USA), a HPLC pump (Maxi-star K-1000, Knauer, Berlin, Germany), a C_{18} reversed-phase column (Nova-Pak C_{18} 4 μm 250 × 4.6 mm, Waters, Milford, USA), a spectrophotometer UV-Visible detector (K2500, Knauer, Berlin, Germany) and a computer software (Eurochrom 2000 Ver. 1.6, Knauer, Berlin, Germany) as integrator.

The mobile phase consisted of phosphate buffer (50 mM, pH = 4.5) with 2.5 mM cetrimide, filtered through a 0.22 μm filter (Sartorius, Goettingen, Germany). The flow rate was 1 ml/min, and the analysis was carried out at room temperature. The UV detector was set at 264 nm and 0.001 AUFS. 4′-hydroxyacetanilide was used as the internal standard.

Standard calibration

A similar procedure to that of the sample preparation was used substituting 200 μl of diluted standard solution for plasma sample and then calibration curve were drawn by plotting peak area ratios against L-ascorbic acid concentrations.

RESULTS

Chromatography conditions

Preliminary experiments showed that phosphate was superior to other buffers and electrolytes (sulfate, acetate, etc).

Experiments were carried out with cetrimide, as an ion-pairing agent.

The first variable to be studied in detail was the effect of pH on the peak separation of ascorbic acid, 4′-hydroxyacetanilide (internal standard) and plasma components on chromatogram.

In this study it was noticed that at pH 4.5, resolution was excellent. The next variable to be evaluated was the concentration of ion-pair. The best results were obtained with 2.5 mM cetrimide (7).

L-ascorbic acid can be determined by its ultraviolet absorbance at 245 nm, but Hegenauer and Saltman reported that measurement at 260 nm could avoid some of the interferences (14) and we noticed that determination at 264 nm was much more suitable.

The chromatogram of vitamin C in plasma is shown in figure 1.

Table 1. Some of method validation data, as recovery, precision and sensitivity.

Recovery			
Sample (n=5) (μg/ml)	Spike added (μg/ml)	The spiked sample(n=3) (μg/ml)	Recovery%
9.61 ± 0.13	2	11.57 ± 0.07	99.58
	5	14.43 ± 0.42	98.16
	10	19.53 ± 0.38	99.2
			Mean = 98.98

Precision (RSD%)		
9.6 μg/ml (Sample)	Repeatability (n=10)	Internal reproducibility (n=5)
	2.8%	7.2%
5 μg/ml (Standard)	3%	3%
20 μg/ml (Standard)	1.8%	2.8%

Sensitivity and linearity range			
Calibration range (n=14)	R^2	Slope [RAUC/(μg/ml)]	Intercept (RAUC)*
0-60 μg/ml	0.996	0.0323	0.07895

* RAUC: Relative area under the curve

Sample preparation

The samples were deproteinized by methanol, trichloroacetic acid 10% and metaphosphoric acid 10% (15). Metaphosphoric acid 10% was chosen because it could precipitate proteins and prevent the oxidation of L-ascorbic acid (16,17,18).

Another advantage was the similarity of metaphosphoric acid to the mobile phase because it hydrolyzed to phosphate (19).

Due to L-ascorbic acid instability, dithiothreitol was added to samples. Dithiothreitol prevents the oxidation of L-ascorbic acid and converts dehydroascorbic acid to L-ascorbic acid (20). The DTT-stabilized samples are not suitable for colorimetric assays that involve either an oxidation or reduction step. On the other hand the MPA-stabilized samples are compatible with most colorimetric and liquid chromatographic methods (21).

We stabilized samples by DTT and MPA because

both of them are suitable for liquid chromatographic methods. Therefore, the determined concentration is the total ascorbic acid.

Linearity and analytical range

The correlation between the peak area ratios and the L-ascorbic acid concentrations was evaluated over the range 0-60 μg/ml and was found to be linear ($y = 0.07895 + 0.0323x$; $R^2 = 0.996$; $n=14$). A standard addition curve for plasma (9.6 (g/ml) with added amounts of 2, 5 and 10 μg ascorbic acid was plotted, which showed no significant difference with the calibration curve of the standard solution.

Accuracy

The accuracy of the method was verified by means of recovery assay. This was accomplished by an analyzing standard solution and spiked (enriched) plasma. The analytical recovery was 98.98% for total ascorbic acid.

Precision

The repeatability of the method was calculated by using the measured data of a single day. In contrast internal reproducibility of the method was calculated by using the measured data of 5 successive days. Both values were expressed by relative standard deviation. The precision data are shown in Table 1.

Sensitivity

Sensitivity of the method was expressed by angular coefficient (slope) of the calibration curve. The method validation (22) data are shown in Table 1.

Limit of detection and limit of determination

Limit of detection was 0.2 μg/ml, which was calculated from a signal to noise ratio of 3.

Limit of determination was 0.4 μg/ml, which was calculated from a signal to noise ratio of 6 (23).

Stability

Due to the instability of L-ascorbic acid, it should be determined immediately after obtaining the samples, but the equipment required is not available in most laboratories. This has necessitated forwarding the samples to other institutions, which is inconvenient and expensive and may result in inaccurate estimates of vitamin C content (due to the instability of the compound in transit and sample preparation). Therefore, it is useful to have an estimation of the stability of the stored samples. In our study the stability of L-ascorbic acid in plasma, trichloroacetic acid 5%, metaphosphoric acid 5% with dithiothreitol 2.5mM were studied at 4° C and -28° C.

The obtained results are summarized in Table 2.

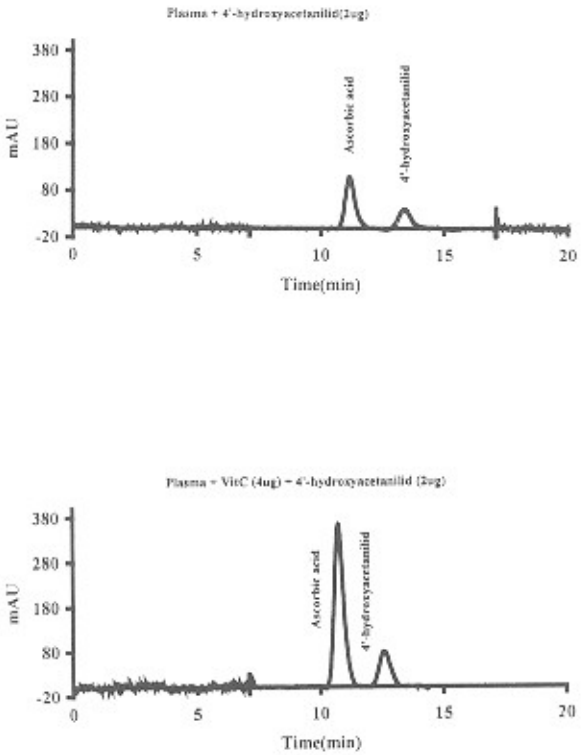

Fig 1. A typical ascorbic acid and internal standard chromatogram in plasma sample and spiked sample.

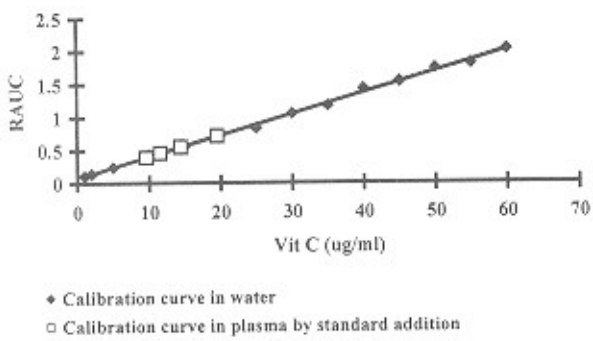

Fig 2. Ascorbic acid stability data in plasma, TCA, MPA and MPA with DTT at 4°C and -28°C.

Table 2. Ascorbic acid stability data in plasma, TCA, MPA and MPA with DTT at 4°C and -28°C.

Total ascorbic acid (μg/ml)								
	Stored at 4°C				Stored at -28°C			
Hours	Spiked Plasma	TCA 5%	MPA 5%	MPA 5% & DTT 2.5mM	Spiked Plasma	TCA 5%	MPA 5%	MPA 5% & DTT 2.5 mM
0	59.75	60.00	60.00	60.02	59.75	60.00	60.10	60.00
2	55.11	55.42	58.90	59.10	59.13	59.44	61.27	59.80
4	50.78	51.39	60.10	61.05	56.96	57.57	60.04	60.10
6	48.92	49.23	59.20	60.00	54.49	54.80	58.07	58.90
8	42.11	42.11	60.05	58.09	53.25	52.94	59.80	60.05
10	34.70	35.93	-	58.60	-	-	57.90	60.00
21	-	-	59.00	-	43.97	43.97	-	-
23	19.53	19.22	59.91	58.50	42.73	43.04	59.60	58.06
25	17.37	17.68	60.03	59.91	-	-	60.80	59.10
Days								
2	9.66	10.09	58.90	60.10	26.63	30.11	60.20	59.90
4	N.D.*	N.D.	59.36	60.02	5.20	7.12	60.00	60.25
6	-	-	60.13	59.66	N.D.	N.D.	59.48	61.06
8	-	-	59.90	60.00	-	-	59.77	59.80

* N.D.: Not detected

\- Not determined

DISCUSSION

The data show that ascorbic acid content in plasma and trichloroacetic acid solution decreased very fast at 4°C and -28°C, whereas the ascorbic acid was stable in metaphosphoric acid 5% and metaphosphoric acid 5% with dithiothreitol 2.5 mM.

Margolis and Duewer (1996) demonstrated that total ascorbic acid in properly prepared human plasma was stable at -70°C for at least 6 years when preserved with dithiothreitol. Also they showed that total ascorbic acid in human plasma or serum preserved with metaphosphoric acid (MPA) degrades slowly, at the rate of no more than 1% per year (21).

Therefore, samples should be determined immediately or when it is not possible to analyze the samples immediately, they should be stabilized by metaphosphoric acid and dithiothreitol and kept at 4°C or lower until analysis. In conclusion the method validation parameters show that the method is very sensitive, accurate, reliable and adequate for measuring the total vitamin C content in human plasma, and it can be performed on a relatively small blood sample, and without the need for sophisticated sample preparation procedures.

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