Rapid and Specific Spectrophotometric and RP-HPLC Methods for the Determination of Ascorbic Acid in Fruits Juices and in Human Plasma

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Summary: Ascorbic acid (AA) is one of the most important water soluble vitamin in the human diet, present naturally in a wide range of foods, especially fruits and vegetables. The objective of this study was to develop a rapid, sensitive and specific method for the determination of ascorbic acid (vitamin C) from a variety of sources like fresh fruits or from human plasma using spectrophotometric technique or by RP-HPLC. Initially, estimation of vitamin C was carried out spectrophotometrically as UV spectroscopy is a trusted technique to monitor small quantities of drugs and vitamins. The assay was linear over the concentration range of 0.05-100 $\mu g/mL^{-1}$. In the second procedure, we attempted to separate and quantitate ascorbic acid from fruit juices as well as from human plasma by RP-HPLC with UV detection. This has been possible because of the diversity of columns and conditions of analysis available. Chromatographic separation was successfully achieved on a pre-packed Kromasil 100, C_{18} (5 μm 25 x 0.46) column using acetonitrile: water (60:40; v/v) as mobile phase at a flow rate of 0.75 mL^{-min} and effluent monitored at 265 nm. The assay was also linear over the concentration range of 0.05-100 $\mu g/mL^{-1}$, with recovery ranging from 99.0-100.0 % and intra and inter day CV <3 % when applied to the analysis of ascorbic acid from fruit juice available in Pakistan at the time of study. Grape fruit, malta, mosami, sweetlemon, fruiter, lemon, lime, custard apple, orange, lemon, guava and papaya juice were found to be very rich in as ascorbic acid, while chikoo, pear, apricot, peach, carrot and some other fruits were found to be poor sources of ascorbic acid.

Introduction

Ascorbic acid (AA) is an important anti-oxidant [1-3], that helps to protect against heart disease, stress and cancers. It is part of the cellular chemistry that provides energy; it is essential for sperm production, and for making the collagen protein involved in the building and health of cartilage, joints, skin and blood vessels. It has also been shown to be essential for the healing of wounds. It is a powerful antioxidant, immune-enhancing, naturally present in many foods especially fruits and vegetables [4-8].

Scurvy, the deficiency disease caused by lack of vitamin C is quite common in developing countries, which leads to slow healing of wound, anemia, epistaxis, dry and scaly skin, dyspnea, fatigue, easy bruises, splitting hair, gastrointestinal problems, arthritis and frequent infections [9, 10].

Several methods have been developed for the estimation of AA [11], high-performance liquid chromatography (HPLC), using a UV detector is currently the most commonly used technique for the analysis of ascorbic acid in food [12]. Few HPLC methods require other detectors like electrochemical [13, 14] or fluorimetric [15-17], due to low absorptivity of dehydroascorbic acid (DHA) in UV. Enzymatic methods using commercial test kits are also used for determination of AA levels [18-21].

The objective of this study was to develop easy, rapid, sensitive and specific method for the analysis of AA from variety of sources, e.g dosage formulation fruit juices and human serum. The methods were then applied to the analysis of AA contents of regularly consumed fruits available in Pakistani markets.

Result and Discussion

The proposed HPLC method does not require any reagent or sample preparation, it is simple and less time consuming. Further it is possible to analyze more sample concurrently than the enzymatic method. The ease of use of spectrophotometric or HPLC techniques make them an ideal quality control

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tool for the food and pharmaceutical industries. Furthermore, the enzymatic method does not determine the total vitamin C because of the reduction of dehydroascorbic acid (DHA) to ascorbic acid cannot be measured.

In reported methods, the mobile phase consisted of complicated buffers causing column corrosion. In our method, the mobile phase simply consisted of acetonitrile and water (60:40). Other ratios (10:90, 30:70, 20:80 v/v) were also tested for system suitability and optimization. The variation in the mobile phase leads to considerable changes in the chromatographic parameters, like peak symmetry and retention time. However, the ratio of 60:40 (v/v) yielded best results (Tables 1-4). A representative chromatogram of AA in water and serum is shown in Figs. 1 and 2, respectively.

Table-1: Estimation of vitamin C from fruit juices by UV Spectroscopy.

Fruits	Ascorbic acid (mg/100mL)	Fruits	Ascorbic acid (mg/100mL)
Apple	0.023	Mango	0.029
Apricot	0.036	Melon	0.025
Black current	0.071	Mosami	0.106
Black grapes	0.025	Musk melon	0.043
Carrot	0.029	Papaya	0.044
Coconut	0.051	Peach	0.044
Crabe apple	0.048	Pear	0.021
Custard apple	0.053	Persimmom	0.039
Fig	0.078	Pineapple	0.038
Fruiter	0.05	Plums	0.03
Grape fruit	0.081	Pomegranate	0.055
Grape green	0.023	Red cherries	0.162
Guava	0.105	Red grapes	0.03
Grewia	0.195	Sapodilla	0.025
Jambolan	0.03	Strawberry	0.042
Keeno	0.036	Sugar beet	0.015
Lemon	0.028	Sugarcane	0.026
Lime	0.039	Sweet lemon	0.106
Lychee	0.056	Tanarival	0.048
Malta	0.196	Water melon	0.042

The response for the detector was determined to be linear over the range 100-0.05 μg/mL, which was determined using standard calibration curve ($n = 6$). For inter and intra day validations AA solutions were analyzed at intervals of four hours a day four days. The correlation coefficient and intercept value were calculated in the corresponding statistical study ($p < 0.05$). Slope and intercept was obtained by regression equation ($y = a + bx$) and least square method which confirms linearity of the method. Correlation coefficients for ascorbic acid 0.999 were observed in both of experiments.

Table-2: Estimation of vitamin C from fruit juices by RP-HPLC.

Fruit juice	Conc (mg/dL)	Fruit juice	Conc (mg/dL)
Apple	0.01	Mango	0.0025
Apricot	0.02	Melon	0.0145
Black current	0.002	Mosami	0.2224
Black grapes	0.78	Musk melon	0.0010
Carrot	0.450	Papaya	0.3210
Coconut	0.123	Peach	3.2010
Crabe apple	0.251	Pear	4.2010
Custard apple	0.0005	Persimmon	5.2000
Fig	0.2323	Pineapple	0.0012
Fruiter	1.234	Plums	1.2330
Grape fruit	2.245	Pomegranate	2.2000
Grape green	2.025	Red cherries	6.6000
Guava	0.120	Red grapes	0.1200
Grewia	3.3412	Sapodilla	0.0001
Jambolan	0.123	Strawberry	4.4100
Keeno	1.023	Sugar beet	1.2000
Lemon	3.316	Sugarcane	6.3200
Lime	2.245	Sweet lemons	2.0100
Lychee	0.231	Tanarival	6.5820
Malta	8.842	Water melon	0.0012

Table-3: Calibration data, recovery, within-day precision and accuracy for ascorbic acid.

Concentration (μg/mL)	Mean found concentration (μg/mL)	Accuracy[a] (%)	Precision[b] RSD (%)
0.05	0.05	100.0	0.0100
0.09	0.09	100.0	0.0382
0.5	0.49	98.00	0.0665
0.9	0.90	100.0	0.0025
1	0.98	98.00	0.0477
5	4.98	99.60	0.0002
12.5	12.49	99.92	0.0002
25	25.00	100.0	0.0004
50	50.39	100.7	0.0045
100	100	100.0	0.0001

[a]Accuracy: found concentration expressed in % of the nominal concentration
[b]RSD relative standard deviation

Table-4: Calibration data, recovery, between -day precision and accuracy for ascorbic acid.

Concentration (μg/mL)	Mean found concentration (μg/mL)	Accuracy[a] (%)	Precision[b] RSD (%)
0.05	0.05	100.0	0.0100
0.09	0.09	100.0	0.0482
0.5	0.49	98.00	0.0675
0.9	0.90	100.0	0.0035
1	0.98	98.00	0.0477
5	4.98	99.60	0.0005
12.5	12.49	99.92	0.0003
25	25.00	100.0	0.0014
50	50.39	100.77	0.0055
100	100.0	100.0	0.0001

[a]Accuracy: found concentration expressed in % of the nominal concentration
[b]RSD: relative standard deviation

The accuracy and precision of the assay was measured by analyzing independently prepared solutions of ascorbic acid at two levels, repeatability and intermediate precision. Six-sample replicates were consecutively tested with the same and different equipments at concentration of 100-0.05 μg/mL.

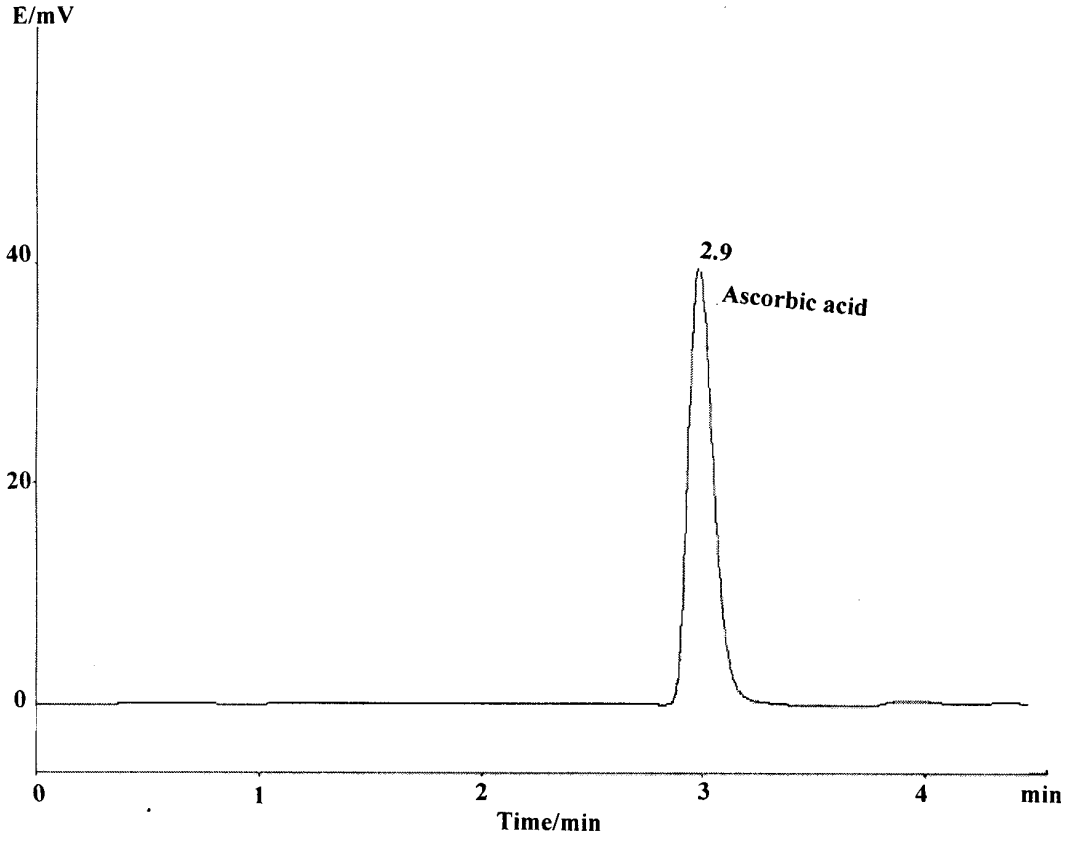

Fig. 1: Representative chromatograms of ascorbic acid.

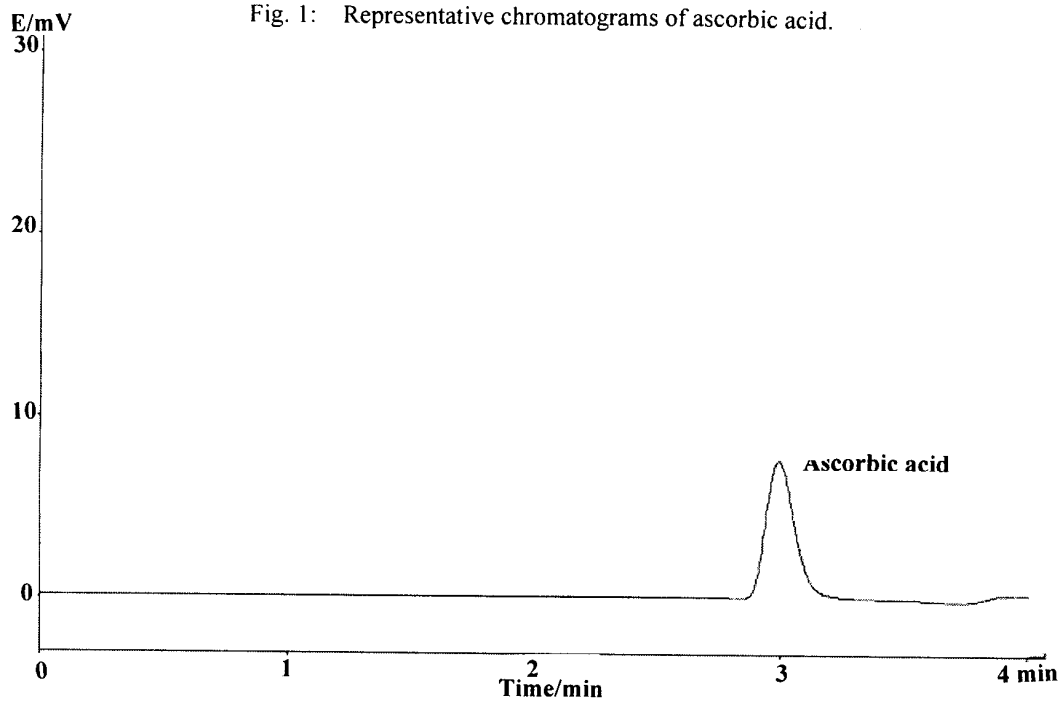

Fig. 2: Chromatogram of plasma sample containing ascorbic acid.

Ascorbic acid precision was assessed within-laboratories variations, within-day precision and between-day precision by using different equipments, analysts and days to analyze samples six times.

The AA under study is unstable and especially sensitive to light, heat and oxygen therefore, special precautions have to be taken during the experiments. However, the working solutions for spiking blank human plasma samples were freshly prepared from stock solutions, and used immediately.

The results in Tables 1-4 indicated that the percentage CV for the within-day ($n = 6$) and between-day ($n = 6$) are numerically all below 3.5 %. The obtained results indicated a good method precision with relative standard deviation < 3 %). The specificity of the chromatographic method was determined to ensure separation of ascorbic acid as shown in Figs. 1 and 2. This is evidenced by the lack of interfering peaks in the chromatograms containing respectively. Solutions of ascorbic acid was prepared and then injected to check for interference from only serum. The method demonstrated good resolution of ascorbic acid and was found to be free of interferences. The limit of detection (LOD 0.0001 mg/mL), and limit of quantification (LOQ 0.0003 mg/mL), of the both methods were, respectively.

Ruggedness of this method was evaluated in two different labs with two different instruments and analysts. The method did not show any notable deviation in results from acceptable limits.

The contents of AA in Pakistani fruits have been shown in Tables-5 and 6 according to which highest quantities of ascorbic acid were found in sugar beet (*Beta vulgaris*), sweet lemon (*Citrus limetta*), grewia (*Grewia asiatica*), mosambi (*Citrus sinensis*), red cherry (*Prunus serotina*), black current (*Ribes nigrum*), fig (*Ficus carica*), grapefruit (*Citrus paradisi*), guava (*Psidium guajava*), coconut (*Cocos nucifera*), fruiter (*Citrus aurantium*), lychee (*Litchi chinensis*), pomegranate (*Punica ganatum*), crabapple (*Malus floribunda*), papaya (*Carica papaya*), peach (*Prunus persica*), strawberry (*Fragaria ananassa*), keeno (*Citrus sinensis*) and water melon (*Citrullus lanatus*), while apple (*Malus domestica*), apricot (*Prunus persica*), carrot (*Daucus carota*), custard apple (*Annona reticulate*), grape green (*Vitis vinifera*), jambolan (*Syzygium cumini*), lemon (*Citrus lemon*), lime (*Citrus aurantium*),

Table-5: Within-day percent accuracy/ recovery of the method for plasma vitamin C analysis.

	<——Ascorbic acid(R.T = 2.9 minutes)——>		
Conc injected (μg.mL^{-1})	Found (mean[a])	% RSD	% accuracy
0.05	0.05	0.0112	100.99
0.09	0.09	0.0000	100.40
0.5	0.50	0.0000	99.96
0.9	0.89	0.0000	100.0
1	0.99	0.0000	99.60
5	4.98	0.0000	99.00
12.5	12.50	0.0003	98.89
25	24.99	0.0016	99.80
50	50.20	0.0000	100.0
100	100.99	0.0000	100.0

a =Mean found values represent "concentration mean" of six different samples for each concentration

Table-6: Between-day precision and accuracy of Ascorbic acid in human plasma.

Conc.	<—— concentration ——>						
Added (μg.mL^{-1})	Day 1	Day 2	Day 3	Day 4	Mean	% RSD recovered	% Accuracy
0.5	0.49	0.50	0.49	0.48	0.49	0.0161	98.60
0.9	0.90	0.89	0.99	0.98	0.99	0.0012	99.63
1	0.99	0.99	0.90	0.90	0.90	0.0159	98.67
5	4.99	4.98	4.99	4.99	4.99	0.0002	99.73
12.5	12.49	12.5	12.49	12.48	12.49	0.0003	99.95
25	25.0	24.99	25.0	24.80	24.95	0.0015	100.01
50	50.39	50.20	50.39	50.01	50.27	0.0018	100.59
100	100	100.99	100	100.0	100.25	0.0000	100.33

mango (*Mangifera indica*), melon (*Cucumis melo*), musk melon (*Cucumis melo*) pear (*Malus domestica*), persimmon (*Dispyros digyna*), pineapple (*Ananas comosus*), plums (*Prunus salicina*), red cherry (*Cornus officinalis*), sapodilla (*Achras sapota*), sugar cane (*Saccharum*) and tanarival (*Tamarindus indica*) contained small quantities of this vitamin.

To the best of our knowledge, this is the first study reporting AA contents of forty different fruit juices in Pakistan. These values are of fresh and mostly unpeeled fruits. It is well known that vitamin C is easily lost during processing and cooking. The losses during cooking alone have been estimated to be about 35 %. Therefore, only raw, uncooked and fresh fruits are good sources of vitamin C.

We compared our results with the existing data on vitamin C contents of fruits in USA. According to the United States Department (USDA, 1998), the high concentrations of vitamin C have been found to be in guava (183 mg/100 gm), strawberry (57 mg/100 gm), grape fruit (38 mg/100 gm), melon (18 mg/100 gm), pear (3.8 mg/100 gm), carrot (5.8 mg/100 gm) and lemon (52.9 mg/100 gm).

Our results also show high concentrations of ascorbic acid in grewia, mosami, red cherry, sugar

beet, sweet lemon, black current, fig, grape fruit, guava, coconut, fruiter, lychee, pomegranate, crabe apple, papaya, peach, strawberry, keeno and water melon. While there was remarkable concordance in the values of ascorbic acid in grape fruit, pear, verities of orange juice and melon juice from USA and Pakistan, difference too were noticed in contents of vitamin C in fruits from these two countries. These differences are most probably due to different regional varieties of fruits. It has been reported that the amount of vitamin could even vary between different samples of the same species.

Experimental

Materials Reagent and Chemicals

Fresh juices were bought from local market, the juices used in these studies were apple, apricot black currant, black grapes, carrot, coconut, crabe apple, custard apple, fig, fruiter, grape fruit, grape green, guava, keno, lemon, lychee, malta, mango, melon, mosami, muskmelon, papaya, peach, pear, persimmon, pineapple, plums, pomegranate, red cherries, red grapes, sapodilla, strawberry, sugar beet, sugar cane, sweet lemon, tanarivel and water melon. All reagents used were of analytical grade. An ascorbic acid reference standard was obtained from Merck Ltd. HPLC grade acetonitrile (Tedia, USA) and deionized filtered water was used to prepare a mobile phase.

Instrumentation and Analytical Conditions

Juice in each case was analyzed either by measuring the absorbance of aliquots at 265 nm on a UV/ Visible spectrophotometer, or by RP-HPLC method. The UV/Visible spectrophotometer (Shimadzu 1601) was coupled with a UV Pc loaded with UVPC 3.91 software, the chromatographic system Shimadzu comprised of LC-10AT VP, SPD-10AT VP, UV-Visible detector and Communication Bus Module integrator (102), Separations were performed on (Kromasil 100, C_{18} (5 μm 25 x 0.46) analytical reverse-phased column at ambient temperature. The samples were introduced through a Rheodyne injector valve with a 20 μL sample loop using mobile phase acetonitrile-water (60:40 % v/v), which was filtered daily using 0.45 μm membrane filter (Millipore, Germany), degassed in an ultrasonic bath, and pumped at a flow rate of 0.75ML^{-min} mL/minute using gradient pump system.

Estimation of Vitamin C from Fruit Juices by UV Spectrophotometer and RP-HPLC Technique

Fresh fruits of different varieties available in the market were obtained, each fruit was cut into two halves, squeezed by juice extractor centrifuged at 750 rev min^{-1} for 15 min and filtered the juice was diluted ten folds and allowed to stand on water bath at 37 °C for 30 minutes. These flasks were continuously shaken at 5 minutes interval for homogenous mixing after 30 minutes 5 mL sample was withdrawn and assayed, the samples were scanned in the region 190-400 nm and the maxima recorded at 265 nm.

Method for the Determination of Vitamin C by RP-HPLC

Preparation of Stock and Working Solutions

Stock solutions of ascorbic acid containing 100 µg/mL in deionized water were diluted to 100, 50, 25, 12.5, 5, 1, 0.9, 0.5, 0.09 and 0.05 mg/mL working standard solutions for the preparation of calibration curves. The stock solutions were stored at –20 °C.

Drug-plasma Solutions

The recovery of ascorbic acid from pooled human plasma was determined by the stated chromatographic conditions. Multiple blood samples (10 mL) of ten healthy non-smoker volunteers (age ranging from 22-25 years) not involved in any strenuous activity and not taking any medicaments were collected in evacuated glass tubes. The blood was then centrifuged at 3000 rpm for 10 minutes and plasma was separated and deprotinated by acetonitrile. The supernatant obtained was filtered through a 0.45 μ filter. Serum thus obtained was mixed in ratio of 1:1 AA solutions and stored at -20 °C pending drugs analysis.

Conclusion

The proposed spectrophotometric and HPLC method has been developed and validated and was found to be suitable for the determination of AA in raw materials, pharmaceutical formulation and fruits. The procedure employs a relatively simple clean-up procedure for sample preparation. The efficiency is enhanced by the use of only one isocratic chromatographic elution that separates and quantifies the AA. This method can be utilized in studies that

require monitoring of plasma vitamin C levels after oral administration of antioxidant supplements to human subjects.

The method has been successful in determining the AA concentration as low as 0.05 μg/mL. The presented results suggest that the proposed HPLC method is reliable, reproducible, and sensitive for determining AA. This chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method, including accuracy, linearity, recovery and precision data. It is a highly specific and precise analytical procedure confirmed by the statistical parameters and its chromatographic run time of 5 minute allows the analysis of a large number of samples in a short period of time. Therefore, this RP-HPLC method can be used as a routine sample analysis. However the UV result show the contents of ascorbic acid in Pakistani fruits, highest quantities of ascorbic acid were found in grewia, mosami, red cherry, sugar beet, sweet lemon, black current, fig, grape fruit, guava, coconut, fruiter, lychee, pomegranate, crabe apple, papaya, peach, strawberry, keeno and water melon. Apple appricot, carrot, custard apple, grape green, jambolan, lemon, lime, mango, melon, musk melon pear, persimmom, pineapple, plums, red cherry, sapodilla, sugar cane and tanarival contained small quantities of this vitamin.

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