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A method for the simultaneous determinations of ascorbic acid and dehydroascorbic acid in human plasma using RHPLC-UV.

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Abstract

A method combining high performance liquid chromatography HPLC with UV-Visdetection in order to determine ascorbic acid and dehydroascorbic acid in human plasma was developed. Ascorbic acid was extracted from human plasma and then stabilized using 10% metaphosphoric acid. The analysis was done using a symmetry C18 column and mobile phase consisted of 100 mMpotassiumdihydrogenphosphate (KH_2PO_4) and 10 mMHexadecyltrimethylammonium bromide (HTAB). The flow rate was kept constant at 1 mL/min. The concentration of dehydroascorbic acid was calculated by subtracting the initial ascorbic acid concentration from the total ascorbic acid concentration after reduction by dithiothreitol(DTT) reagent. The calibration curve was linear within the tested range of 1-100µg/mL for ascorbic acid. After administration of 4000 mg of vitamin C tablets to healthy Iraqi volunteers, this method was successfully applied to a human pharmacokinetic study of both ascorbic acid and its oxidation product.

Keywords: HPLC, Method validation, Ascorbic acid, Dehydroascorbic acid.

INTRODUCTION

Ascorbic acid is one of the organic acids which is soluble in water and known widely as Vitamin C as the main sources of this compound are the fruits and vegetables (1,2). Ascorbic acid is considered an important vitamin that engages in many physiological activities in living organisms. It is involved in the synthesis of collagen in connective tissue that is responsible for wound healing and fracture repair (3). Ascorbic acid has essential functions for human body as it acts as antioxidant and plays important role in metabolisms and synthesis of adrenaline (4,5). Moreover, ascorbic acid is used widely for preventing and treating scurvy and common cold. Also, dehydroascorbic acid (DHAA) is an important compound because of its role in normal cellular homeostasis (6). The ratio of dehydroascorbic acid (DHAA) to ascorbic acid (AA) concentrations is known as a marker of oxidative stress (7). The accurate detection of AA is very important but it is not easy because the AA is unstable in the presence of moisture, air, heat, light, oxygen and it easily decomposes to biologically inactive compounds.

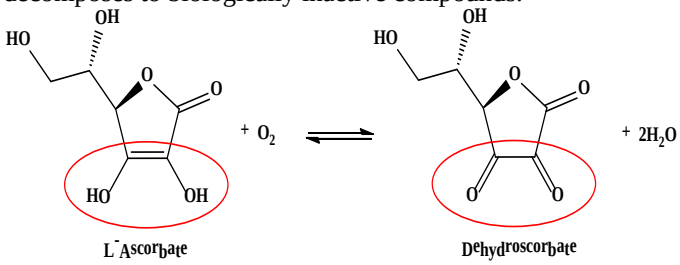

Fig1. Schematic representation of ascorbic acid and dehydroascorbic acid.

Additional advantage includes considering the ascorbic acid as one of the strongest reducing materials and radical scavengers as it acts as a primary defense against aqueous radicals in the blood. (8, 9). Ascorbic acid has a great importance in various physiological and biochemicalfields of crops (10,11,12). The average concentration of vitamin C in human blood is 50-100 µM, 95 % of it is ascorbic acid and the remaining is dehydroascorbic acid as shown in fig.1 (13).

Therefore, the determination of ascorbic acid concentration is of great interest and can be achieved by various methods. Many of them are based on traditional methods such as spectrophotometry colorimetry (14, 15), potentiometry and enzymatic methods (16, 17). Also, AA can be detected using a high performance liquid chromatography paired with ultraviolet detector system at the wavelengths 265 nm or 240 nm. DHAA can be measured directly using highly sensitive HPLC electrochemical detector ECD (18). The assessment of DHAA concentration is based on the difference between AA and total AA concentrations in human plasma (19). After the reduction of DHAA to AA, the total AA is estimated then the DHAA concentration is calculated from the difference between total AA and free AA, which was present in the sample (18, 20). AA is easy to detect using amperometric and coulometric systems because of its high electroactivity. However, it is difficult to detect DHAA using electrochemistry because of its low electroactivity and has weak absorbance in the UV. Therefore, DHAA is reduced to AA with dithiothreitol (DTT) and then electrochemical detection is carried out to calculate the difference of AA concentrations before and after the reduction of DHAA (19, 21).

The purpose of this study was to develop and validate HPLC method for the simultaneous determinations of AA and DHAA in human plasma with simple and rapid sample preparation.

Chemicals and reagents

L-ascorbic acid, D-isoascorbic acid, potassium dihydrogenphosphate, dithiothreitolreagent (DTT), hexadecyltrimethyl ammonium bromide (HTAB)were purchased from Sigma-Aldrich and used without further purification.Metaphosphoric acid, methanol, waterwere purchased from Fisher Scientific. The solutions were prepared using water purified with Milli-pore system(resistivity ≥18 MΩ cm.)

HPLC apparatus and conditions

The higher performance liquid chromatography HPLC was composed of a 9012 pump, a C18 column, and a prostar 330 PDA detector, all from Varian.Chromatographic conditions were studied to achieve the appropriate system suitability before performing the validation assay.The mobile phase was 100 mMKH_2PO_4 and10 mMHTAB. The mobile phase was pumped isocraticallyat a flow rate of 1 mL/min.

Standard preparation

Ascorbic acid and isoascorbic acid were dissolved in 50% methanol to prepare stock solution of 1 mg /mL. Standards of 10, 25,50,100,250,500,1000 of AA were prepared by diluting the working solutions of AA with 50% methanol. As described in study by Karlsen et al(22) human plasma was depleted of AA by leaving it for four days on the bench. The human plasma calibration curve was prepared at different concentration 1, 10, 60, 80, and 100.The ascorbic acid in blood is unstable because it rapidly degrades to DHAA therefore metaphosphoric acid was added as an efficient stabilizer and protein precipitator for analysis of AA (23). After the samples were vortexed for 1 min , samples were centrifuged at 12000 rpm for 12 min at 4^0C. The supernatants were collected in injection vials, and then for the analysis 10 µL were injected into an HPLC system.

Determination of total ascorbic acid

The blank plasma was reduced by adding 50 microliter of 5mM DTT at pH 7.7 and 50 microliter of IS. The reaction was for 20 min at room temperature in the dark. After that, 400 microliter of metaphosphoric acid was added to each sample as an efficient stabilizer and protein precipitator. Then the samples were vortexed for 1 min, and centrifuged at 12000 rpm for 12 min at 4^0C. The supernatants were collected in injection vials, and then for the analysis 10 microliter was injected into an HPLC system.

Method validation

This method was validated using spiked solutions of ascorbic acid in AA-depleted plasma. The parameters: limit of quantification (LOQ), limit of detection (LOD), accuracy, precision,linearity, and recovery were studied. The LOD and LOQ weredetermined byserial dilutions of known concentration solutions to obtain signal/noise ratios of 3:1for LOD and 10:1 for LOQ. The linearity was checked using a calibration curve with eight different concentration. Calibration curves were constructed by plotting the response ratios versus the concentrations. The accuracy was studied by the replicated analysis of samples that contain known amount of the analyte.The precision was investigated by injection repeatability and analysis repeatability of spiked plasma samples.The deviation of the mean serves as accuracy measurement. The recovery was determined as the ratio of the peak area for the extracted samples to that of direct injection of equivalent concentration. The intra- day precision and accuracy were investigated within one day by analyzing of five replicate samples. The inter-day precision and accuracy were investigated using five separate days in identical concentrations.

Applications in pharmacokinetic studies

In this study, we included healthy male volunteers aged between 50-20yearsold. Both urinalysis and blood chemistry results were required. Volunteers with evidence of drug abuse, illness, or alcoholism were not enrolled in the study.After administration of 4000 mg of vitamin C tablet to five healthy volunteers, study of the pharmacokinetics of Ascorbic acid and dehydroascorbic acidwasachieved. Blood samples were taken from each volunteer. Blood samples were collected into heparinized tubes at pre dosing and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6 h post dosing. Next plasma was separated immediately using centrifuge at 2000 rpm for 15 min. Then the plasma was stored frozen at -80 ^{0}C until analysis.

RESULTS

Sample preparation

Plasma samples were stored at different temperature (-70,-4, 25^{0}C) for 60 min. As shown in fig.2, the highest relative recovery was achieved after 10 min at -70 ^{0}C. However, result at -4,25^{0}C showed the instability of ascorbic acid in plasma over time. Therefore, plasma was storage at -70 ^{0}Cthen the sample preparation of AA in plasmawas done. It is important to add a stabilizer like HPO_3 to avoid the oxidation of AA in plasma samples.Dithiothreitol (DTT)was used as a reducing agent. DTT with different concentration has been employed 5mM, 10 mM, and 20 mM.We used 5mM of DTT for all analyses because the same total concentrations of AA were determined for every DTT concentration. We calculated the amount of DHAA from the different between the total AA and the reduced AA.

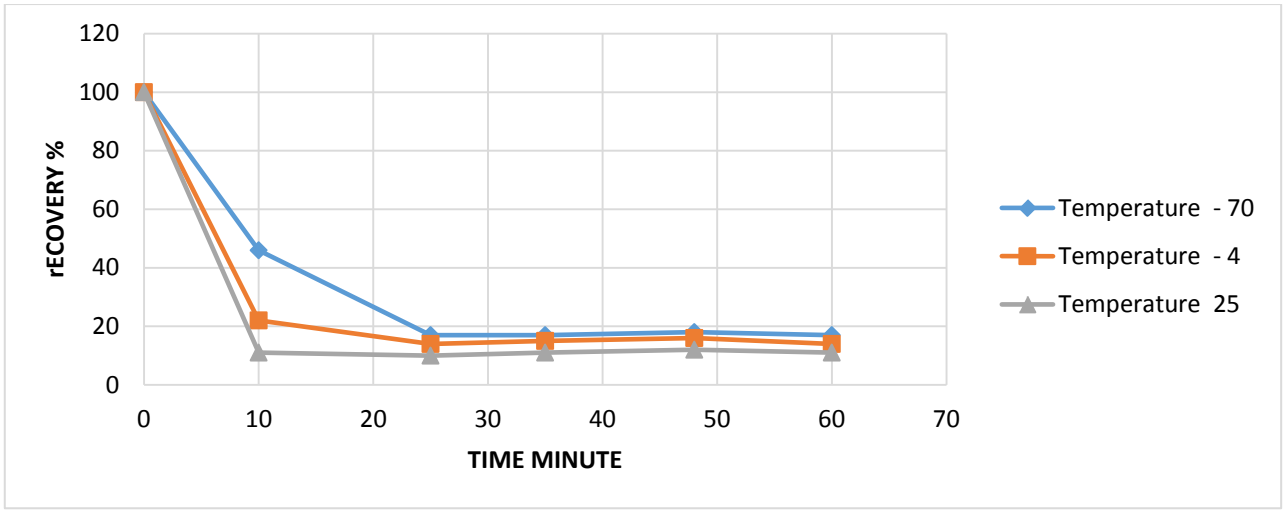

Fig 2. Recovery of ascorbic acid contents in standard solutions stored at different temperature: -70, -4, and 25 ^{0}C.

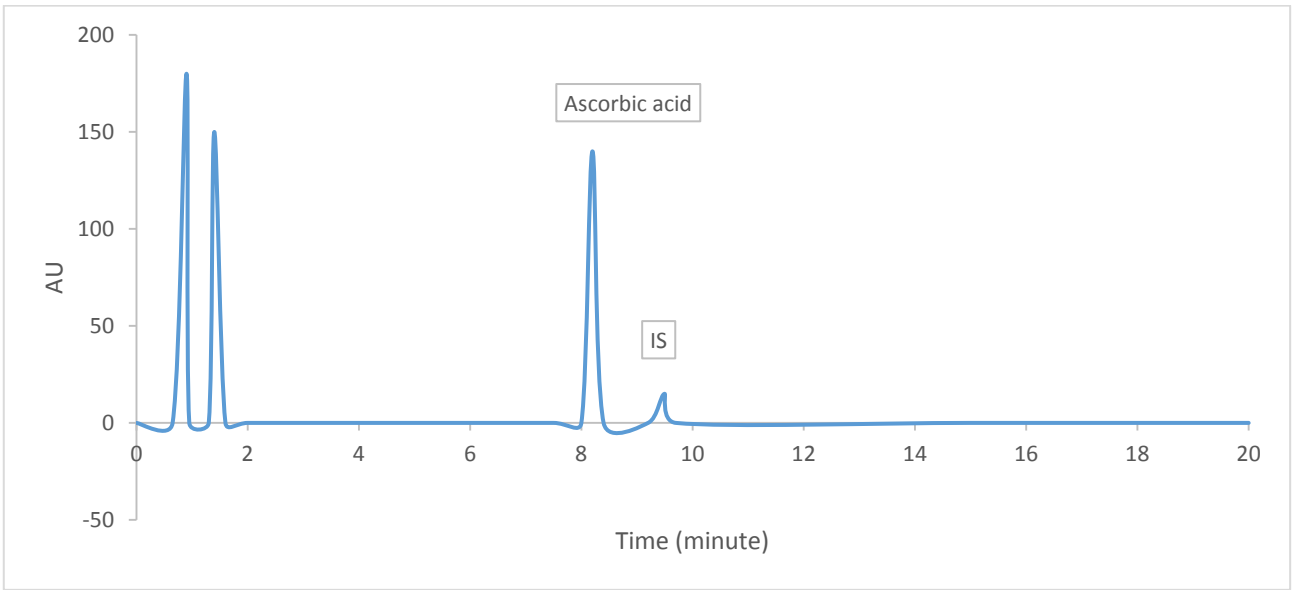

Fig 3.Chromatogram represent ascorbic acid and internal standards in human plasma.

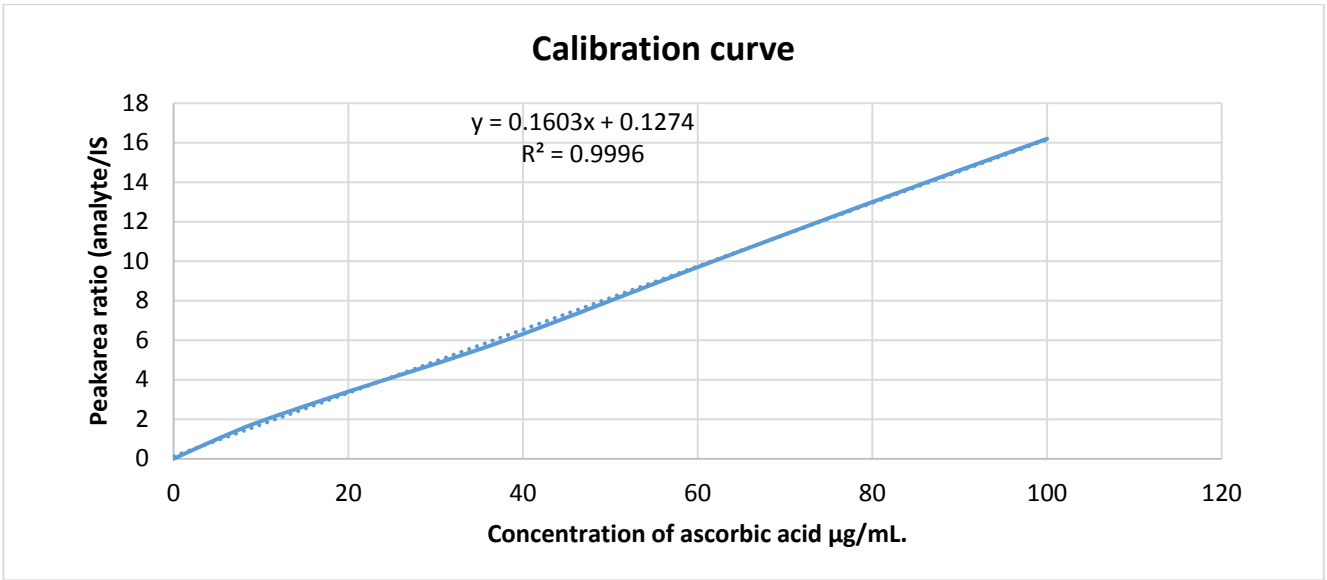

Fig 4. Calibration curve of ascorbic acid.

Chromatographic separation

As shown in Figure 3 representative HPLC chromatogram obtained from a human plasma sample. The identification of corresponding peaks was performed by comparing the retention time of the sample with the retention time of the separately run pure standards using the same analytical conditions.

Linearity

The calibration curve of the blank and eight standards ranging between 1-100µg/mL, the correlation coefficient (r^2) was 0.999 and this indicates excellent linearity as shown in Figure 4.

Accuracyand precision

Four concentrations (1, 10, 60, 80 µg/mL) of the quality control samples were used to determine accuracy and precision.Precisions within ±13% and accuracies within 82–113% were established. The accuracy and precision of intra-day measurements of the analytical method were 82.12–102.13% and 2.23–12.92%, respectively as shown in Table 1. The accuracy and precision of inter-day measurements were 84.37–101.31% and 1.37–9.26%, respectively as shown in Table 2.

Intra-day	Conc 1 µg/mL	Conc 10 µg/mL	Conc 60 µg/mL	Conc 80 µg/mL
Mean	0.82	9.42	61.28	78.36
CV %	12.92	6.74	3.46	2.23
Accuracy %	82.12	94.23	102.13	97.95

Table 1. Intra-day precision and accuracy of ascorbic acid

Inter-day	Conc 1 µg/mL	Conc 10 µg/mL	Conc 60 µg/mL	Conc 80 µg/mL
Mean	0.84	9.23	60.79	78.48
CV %	9.26	5.43	2.60	1.37
Accuracy %	84.37	92.39	101.31	98.1

Table 2. Inter-day precision and accuracy of ascorbic acid.

Recovery

The recovery of AA and internal standard were 97.52-114.29% and 99.91%, respectively as shown in table 3. Ascorbic acid recovery tests were performed for three quality control concentrations.

Concentration µg/mL	Recovery Intra-day	Recovery Inter-day
1	104.32±2.87	97.52±4.71
10	112.65±5.46	108.27±9.18
60	114.29±6.82	113.63±10.82
80	103.48±4.23	101.96±4.84

Table 3. intra – and inter-day recovery of ascorbic acid.

DHAA Concentration

The concentration of DHAA was calculatedfrom the different between the total AA and the reduced AA as shown in table 4. The results show that it is possible to utilize the described HPLC-UV method to determine the amount of AA and DHAA accurately. The analysis of one sample (including the sample preparation) requires Less than 15 minutes.

Free Ascorbic acid µg/mL	Total ascorbic acid µg/mL	Dehydroascorbic acid
AA 50 µg/mL	AA 50 µg/mL + 5 mM DTT	Total AA – free AA
34.52 µg/mL	42.76 µg/mL	8.24 µg/mL

Table 4. Concentration of dehydroascorbic acid.

Pharmacokinetic study

After administration of 4000 mg of ascorbic acid to five healthy Iraqi volunteers average age of 24 years, average weight of 74.6 kg, this method has been successfully applied to a pharmacokinetic study of ascorbic acid and its oxidant. The mean concentration time profile for ascorbic acid is shown in Fig 5.The mean concentration time profile for dehydroascorbic acid is shown in Fig 6.

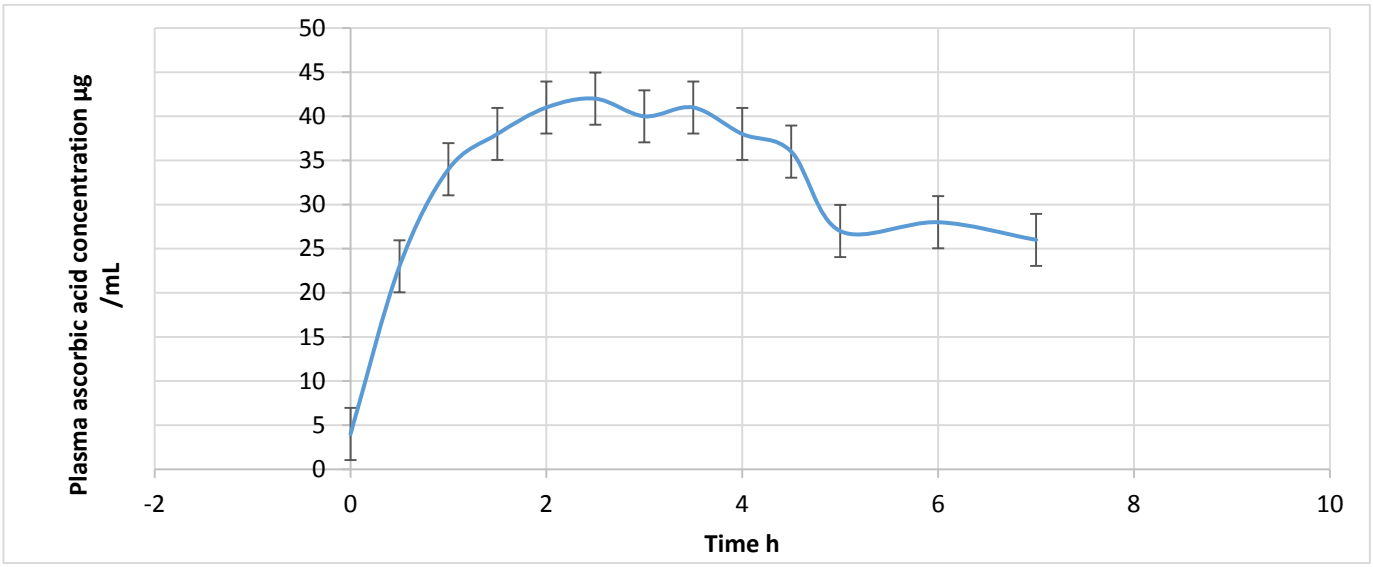

Fig 5. Mean concentration- time profile of ascorbic acid.

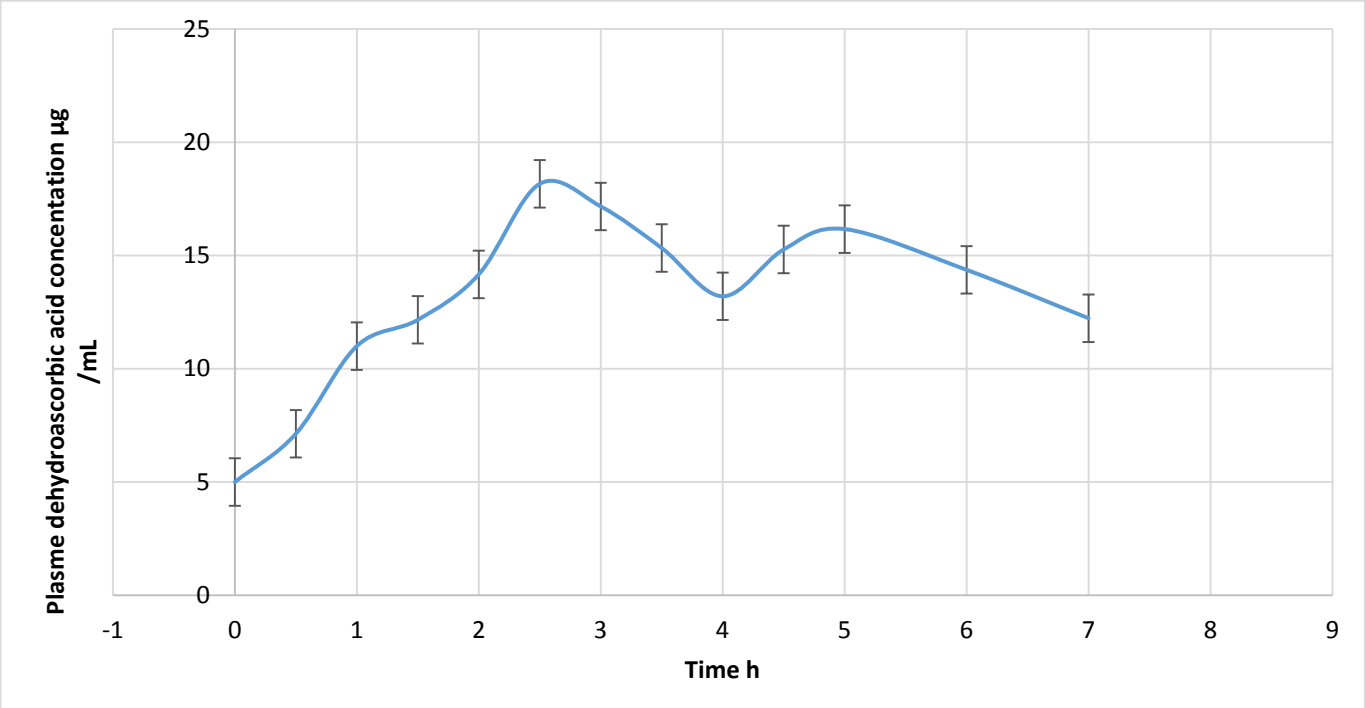

Fig 6. Mean concentration time profile of dehydroascorbic acid.

DISCUSSION

A validated, sensitive and rapid HPLC- UV method was successfully developed for the simultaneous determination of AA and DHAA levels in human plasma. The method provides a accurate, rapid, and reproducible for the determination of AA with great retention time lees than 10 min. The LOQ for AA was 1 µg/mL. Good performance was obtained using reversed-phase column. A simple and cheap preparation stepwasneeded. This validated method was successfully applied to pharmacokinetic study of AA and DHAA in healthy subjects after oral administration of vitamin C.

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