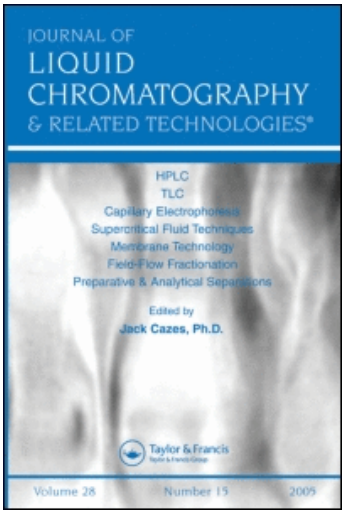

Journal of Liquid Chromatography & Related Technologies

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

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To cite this Article Baker, John K. , Kapeghian, John and Verlangieri, Anthony(1983) 'Determination of Ascorbic Acid and Dehydroascorbic Acid in Blood Plasma Samples', Journal of Liquid Chromatography & Related Technologies, 6: 7, 1319 — 1332

To link to this Article: DOI: 10.1080/01483918308080001

URL: http://dx.doi.org/10.1080/01483918308080001

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1319-1332 (1983)

DETERMINATION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID IN BLOOD PLASMA SAMPLES

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ABSTRACT

Following the stabilization of the plasma samples with $HClO_4$ and EDTA, the samples could be directly analyzed by HPLC using electrochemical detection and reversed-phase columns. The accuracy and precision of the method was evaluated using plasma samples spiked with ascorbic acid (10 μg/ml) and the results were also compared to the classical colorimetric procedure. Dehydroascorbic (5 μg/ml) was determined in plasma samples using UV detection following derivatization at room temperature for 45 minutes with o-phenylenediamine.

INTRODUCTION

In normal human adults the plasma concentration of ascorbic acid ranges from 4 to 14 μg/ml (1) while the reported values for dehydroascorbic acid range from 0.6 to 2.0 μg/ml (2,3). The most widely used method for the analysis of ascorbic acid in plasma is based on the colorimetric reaction with 2,6-dichlorophenolindophenol (1). "Total ascorbic acid" in plasma has been measured by dinitrophenylhydrazine derivatization of the dehydroascorbic acid initially present and the ascorbic acid oxidized to dehydroascorbic acid (1,2).

Ascorbic acid has been determined in animal tissue samples in the 200 to 3,000 μg/gm range using HPLC analysis with UV detection at 254 min (4). In the present study, it was also confirmed that HPLC analysis

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with UV detection was satisfactory for the high ascorbic acid concentrations found in tissue samples, however, it was not satisfactory for the 4 to 14 μg/ml range found in plasma samples. HPLC analysis with electrochemical detection has been used for the analysis ascorbic acid in marine invertebrates in the 4 to 30 μg/g range (5) and this detection technique was utilized in the present study.

Dehydroascorbic acid could theoretically be detected using electrochemical methods in the reductive mode but the diffusion current is only a small fraction of the expected value (6). While this manuscript was in preparation, a HPLC method for dehydroascorbic acid in fruit juice (100 μg/ml range) using derivatization with o-phenylenediamine was published (7). This highly conjugated derivative formed in aqueous solution at room temperature within 30 minutes and it was moderately stable (8). In the present study, the o-phenylenediamine derivatization procedure has been modified for the analysis of dehydroascorbic acid in plasma in the 0 to 5 μg/ml range.

EXPERIMENTAL

Determination of Ascorbic Acid by HPLC:

Approximately 3 ml of whole blood was collected in a tube containing 10 mg EDTA and immediately centrifuged for 5 minutes. Then 500 μl of the plasma was transferred to a fresh sample tube containing 25 μl of concentrated (70%) perchloric acid, agitated using a vortex mixer, then centrifuged to yield a clear, nearly colorless layer. The total time from sample collection to stabilization with perchloric acid was generally less than 10 minutes.

A calibration sample of 10 μg/ml ascorbic acid was prepared fresh daily using ascorbic acid (reagent grade, Fisher Scientific Co.) with 0.1 mg/ml Na_2EDTA and 0.1 N $HClO_4$ as the diluent. Unstablized solutions

of ascorbic acid in this concentration have a half-life of only 1-2 hours and were not satisfactory for calibration purposes. A calibration solution of 10 μg/ml of uric acid was also freshly prepared.

A 5.0 μl sample of the stabilized plasma sample or calibration sample was injected into the HPLC system using a U6K injector (Water's Assoc.). A C-18 guard column and a 3.9 mm x 30 mm μ-Bondapak C-18 analytical column was used with a mobile phase (1.5 ml/min) prepared with 0.2 mg/ml Na_2EDTA, 0.001 M N-octylamine, and 0.1M sodium acetate. The pH of the mobile phase was adjusted to 5.0 with glacial acetic acid before use.

A glassy carbon electrode at +0.60 volts (100 nA range) was used with an electrometer (Model LC-3A, Bioanalytical Systems, Inc.) and a strip chart recorder or a recording integrator (Model 3390A, Hewlett Packard). If the calibration sample was less than 25% of a full scale response, the electrode surface was polished (every 3-4 wks. typical).

Determination of Dehydroascorbic Acid:

Stock solution of 1.0 mg/ml of dehydroascorbic acid (ICN nutritional Biochemicals) were prepared using distilled water and these standard solutions were discarded if not utilized within 4 hours. Calibration samples of 5 μg/ml dehydroascorbic acid were prepared immediately before use because of the poor stability of the dilute solutions.

In a typical determination, 3 ml of whole blood was collected in a tube containing 10 mg EDTA, centrifuged, and then 500 μl of the plasma (or 5 μg/ml standard) was transferred to a fresh tube containing 50 μl of 0.5 mg/ml o-phenylenediamine. The total time between collection to the blood sample and the addition of the o-phenylenediamine was typically less than 7 minutes.

Following a mininum 45 min. incubation period, a 10 µl sample was subjected to HPLC analysis. A µ-Bondapak C-18 column was used with a mobile phase (1.5 ml/min) comprised of 6.6 g K_2HPO_4, 8.4 g of KH_2PO_4, 400 ml CH_3OH and 3.6 liters of water. Dual UV detectors set at 254 nm and 280 nm using the 0.02 AUFS range (Model 440, Waters Assoc.) were used for the detection of the dehydroascorbic acid derivative. The identity of the peak was verified by a comparison of retention time and the absorbance ratio observed with the two UV detectors.

Colorimetric Determination of Ascorbic Acid:

As an additional tool in establishing the validity of the HPLC method, the most commonly used colorimetric procedure (1) was also utilized for the analysis of some of the plasma samples. In this procedure, the blood was collected and the plasma was stablized using exactly the same procedure as used for the HPLC determination. A 500 µl sample of the stabilized plasma, 300 µl of a citrate buffer (0.22 g/ml trisodium citrate adj. to pH 4.15 with glacial acetic acid), and 1.0 ml of 2,6-dichlorophenolindophenol (0.1 mg/ml in water) were mixed directly in a spectrometer sample cell. After exactly 30 sec., the absorbance (A_1) of the sample at 520 nm was measured. Approximately 2 mg of ascorbic acid crystals were added to sample and the absorbance reading (A_2) of the mixture was measured again. The difference in the reading (A_1-A_2) was taken to be proportional to the ascorbic acid content and the procedure was calibrated using the same procedure.

RESULTS AND DISCUSSION

Using the ion-pair HPLC system with electrochemical detection, the normal physiological levels of ascorbic and uric acids present in human plasma could be detected easily (Fig. 1). Using the 100 nA range (the least sensitive range on the unit) the ascorbic acid peak appeared as a

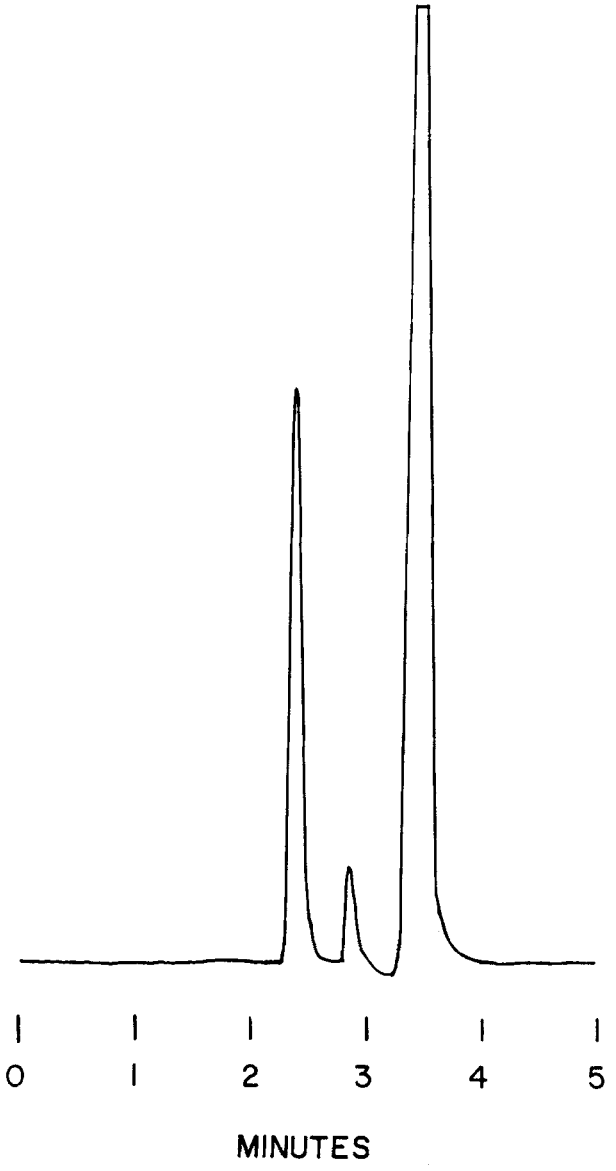

FIGURE 1: Chromatogram of Human Plasma Containing Uric Acid (t_R=3.4 min) and 10 μg/ml Ascorbic Acid (t_R=2.3 min.)

nearly full scale deflection at 2.30 min while the solvent peak at 1.3 minutes was just barely discernible. In the examination of fresh plasma samples from man, guinea pig, and monkey; it was found that the size of the ascorbic acid peak was of the same order of magnitude for the three species but there were marked variations in the size of the uric acid peak between species (man > guinea pig >> monkey).

To determine the accuracy and precision of the HPLC method, human plasma that had been depleted of ascorbic acid was spiked with 10.0 µg/ml of ascorbic acid, then the pooled specimen was divided into five samples for HPLC analysis and five samples for the classical colorimetric method. The results of this comparison (Tab. 1) showed that the HPLC method gave accurate results, however, the standard deviation of the series of measurements was higher than the standard deviation observed for the colorimetric method. If greater precision were needed, a fixed-loop injector could be substituted for the open-loop style injector to improve the precision of the volume of sample delivered to the column.

TABLE 1

Comparison of the Standard Colorimetric and HPLC Methods for the Determination of Ascorbic Acid (Spiked at 10.0 µg/ml) in Human Plasma

Plasma Sample	Colorimetric Method	HPLC Method
1	11.53 µg/ml	11.38 µg/ml
2	11.59 µg/ml	10.47 µg/ml
3	10.92 µg/ml	9.77 µg/ml
4	10.97 µg/ml	9.17 µg/ml
5	11.08 µg/ml	9.29 µg/ml
average	11.22 µg/ml	10.02 µg/ml
standard dev.	±0.32 µg/ml	±0.92 µg/ml
plasma blank	1.79 µg/ml	0.26 µg/ml

The analysis of the spiked plasma samples with the colorimetric method gave results that were 12% higher than the true value. The plasma blank also gave a fairly high false background value (1.79 µg/ml) which probably arose from redox reactions with other plasma constitutents with the indophenol dye. Though the absorbance reading for the plasma blank was taken 30 sec after the addition of the indophenol dye, the absorbance at 520 nm by the dye continued to fall at a fairly rapid rate.

In the early stages of this investigation, a number of different methods for stabilizing ascorbic acid were investigated. Metaphosphoric acid worked well for simple solutions or tissue samples of ascorbic acid, but it was found not to be useful for the stabilization of plasma samples. Trichloroacetic acid was also investigated, but it did not give a clear solution after precipitation of the plasma proteins. The combinations of EDTA and $HClO_4$ was found to be the most useful for the stabilization of ascorbic acid and precipitation of plasma proteins. Using this method, samples could be stored at room temperature for 6 hr. with no loss, 24 hr. at 5° with 14% loss, 7 days at -70° with no loss.

There are frequent references to rapid decomposition of ascorbic acid in plasma samples (1,9), but there are few kinetic studies using the more selective analytical procedures. A large sample of monkey plasma was collected and stored at room temperature. At various time periods, a portion of the plasma was stabilized and the ascorbic acid content was determined using the HPLC procedure. The results of this experiment (Fig. 2) showed the ascorbic acid was lost at a high rate ($t_{\frac{1}{2}}$ = 0.55 hr.) and the loss was much faster than would occur in a simple solution. The same monkey plasma sample was stored at 5° for 7 days, returned to room temperature, spiked with ascorbic acid, then

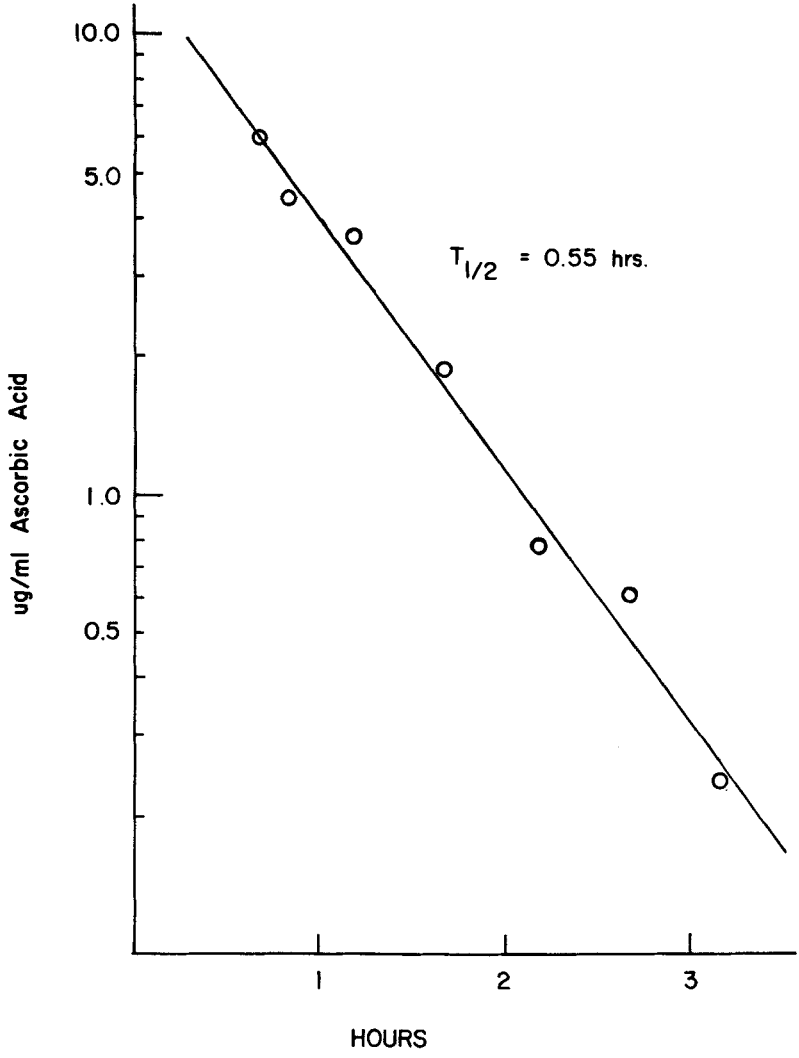

FIGURE 2: Degradation of Ascorbic Acid in a Fresh, Unstabilized Monkey Plasma Sample Held at Room Temperature.

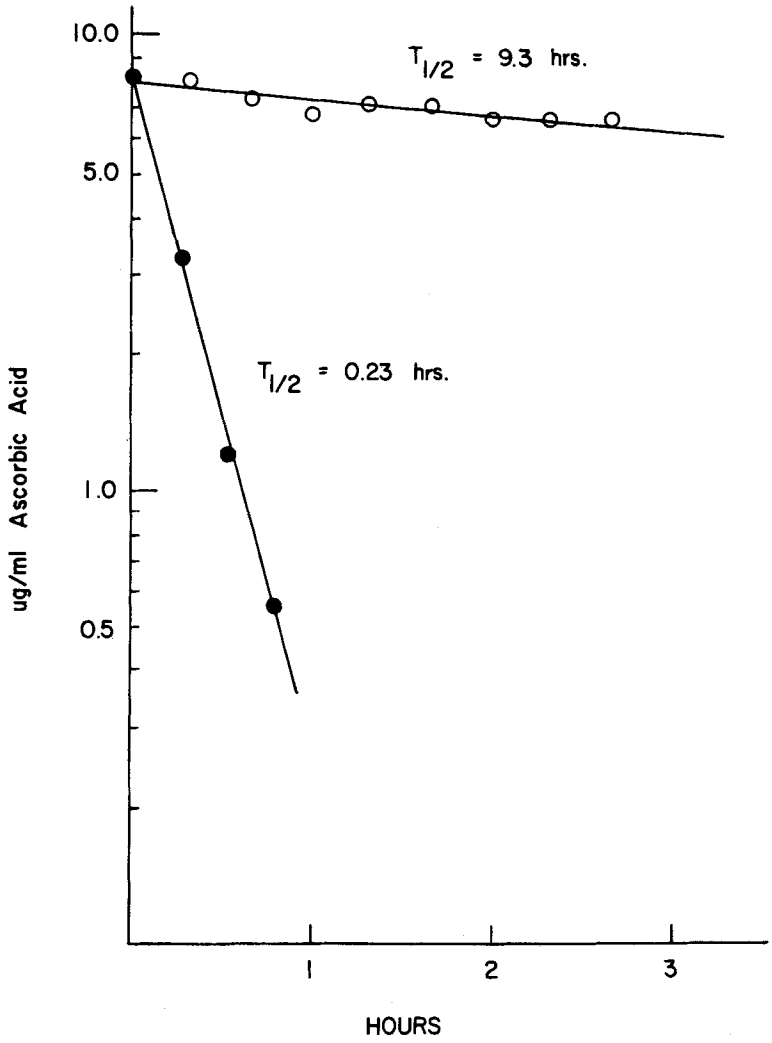

FIGURE 3: Degradation of Ascorbic Acid in Unstabilized Human Plasma Samples Held at Room Temperature. Filled circles-fresh plasma. Open circles-aged plasma.

assayed at various time periods. In this aged plasma sample, the ascorbic acid appeared to be much more stable ($t_{\frac{1}{2}}$ = 0.95 hr) compared to the fresh sample.

Similar results were also obtained using human plasma (Fig. 3). In freshly collected human plasma, the shortest half-life observed was 0.23 hours. In human plasma that had been stored at 5° for several weeks, then returned to room temperature, the half-life of ascorbic acid ($t_{\frac{1}{2}}$ = 9.3 hr) was considerably longer. Previous literature reports (1,9) have indicated that there was a 5-8% loss per hour, but the age of the plasma samples used for the evaluation was not indicated. The present study indicated that the stability of ascorbic acid in freshly drawn, hemolysis-free plasma samples was much poorer than previously estimated. Thus if one desires to have an accurate estimate of the ascorbic acid plasma levels of a given subject, it is essential that the total time between collection of the whole blood and the addition of the $HClO_4$ stabilizer be less than 10 minutes.

The method that was developed for the HPLC analysis of dehydroascorbic acid utilized a reaction with o-phenylenediamine (Fig. 4). Though this reaction has been studied for over 50 years, there are some indications that the structure shown for the product in Figure 4 may not be correct (10). However, under the conditions used for the derivatization of dehydroascorbic acid in the present study, only one product was detected in the model systems or with the plasma samples. The reaction occurs rapidly at room temperature in neutral aqueous solutions or in plasma samples. The derivative appeared as a peak at 8.7 min, was well separated from the excess o-phenylenediamine reagent at 5.5 min, and could be detected at the 5 μg/ml level in human plasma (Fig. 5). A derivatized human plasma blank did not show any other peak at that retention time. As an additional tool in the identification of

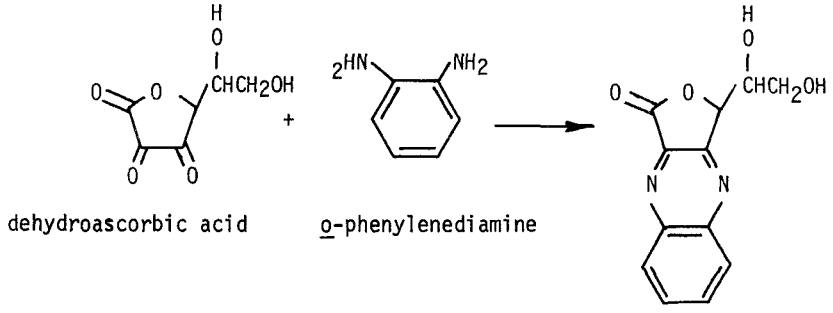

FIGURE 4: Derivatization Reaction for Dehydroascorbic Acid Showing Tentative Structure of Reaction Product.

the dehydroascorbic acid derivative peak, dual 254 nm and 280 nm detectors were used. With this system, the ratio of the detector response (A_{254}/A_{280} = 1.33) for the peak was very reproducible from week to week and the ratio was markedly different from other peaks naturally occurring in the plasma samples.

To establish the accuracy and precision of the method, human plasma was spiked with 5.0 µg/ml dehydroascorbic acid and divided into 5 specimens for analysis. The results of this analysis (Tab. 2) indicated that the precision of the method was satisfactory with a ±4.0% relative standard deviation. However, the average value for the five determinations (4.19 µg/ml) was found to be 16% lower than the expected

TABLE 2

Determination of Dehydroascorbic Acid (Spiked at 5.0 µg/ml) in Human Plasma

Plasma Sample	Dehydroascorbic Acid
1	4.34 µg/ml
2	4.40 µg/ml
3	4.07 µg/ml
4	4.11 µg/ml
5	4.07 µg/ml
average	4.19 µg/ml
standard dev.	±0.17 µg/ml (± 4.0%)

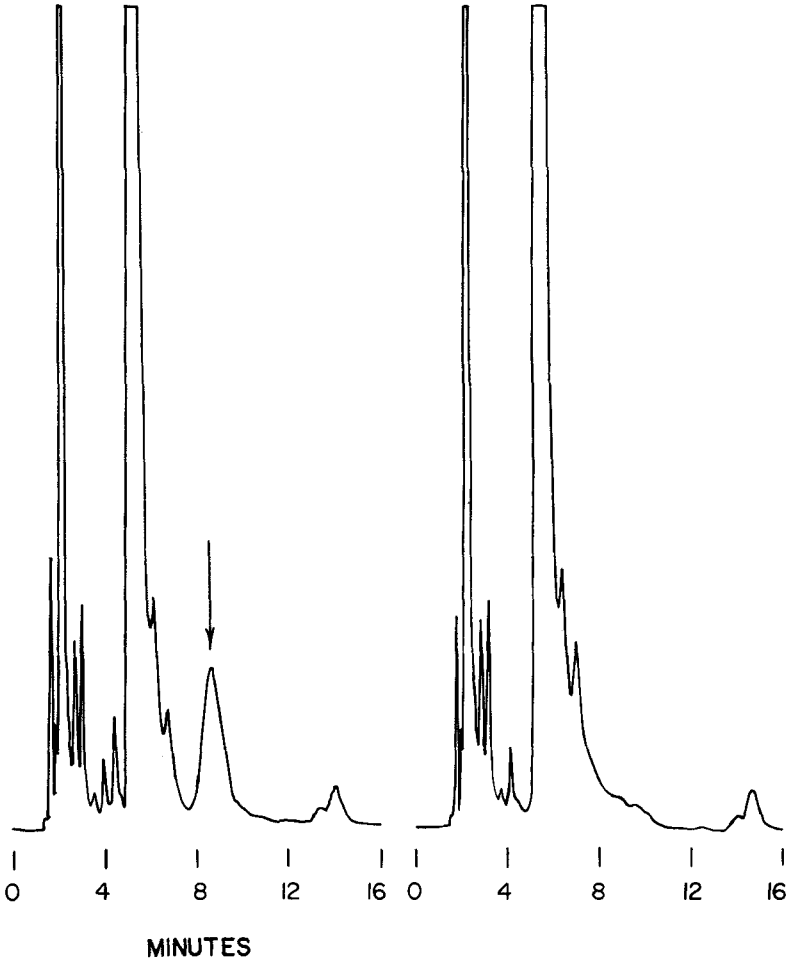

FIGURE 5: Chromatograms of Human Plasma containing Dehydroascorbic Acid Derivatized with o-Phenylenediamine. Left-sample spiked with 5.0 μg/ml dehydroascorbic acid, arrow indicated position of derivative. Right-derivatized plasma blank. Excess o-phenylenediamine appears at 5.5 min. in both chromatograms.

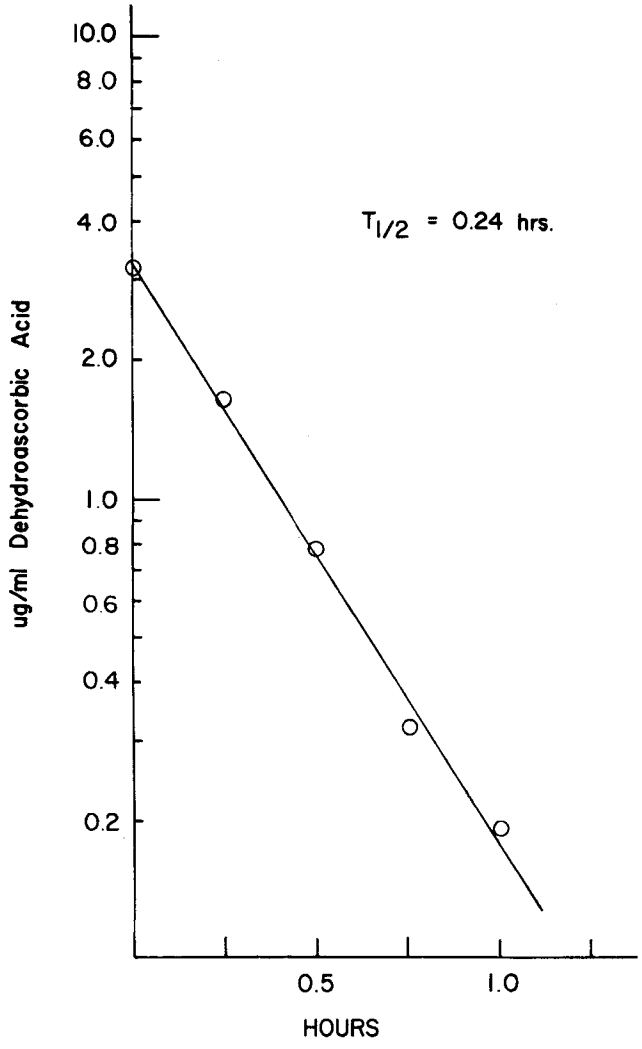

FIGURE 6: Degradation of Dehydroascorbic Acid in Unstabilized Human Plasma Held at Room Temperature.

value. Though the samples were handled quickly, it would appear that a significant portion of the added dehydroascorbic acid decomposed before the derivatization reagent was added.

To determine the stability of dehydroascorbic acid in the sample, human plasma was spiked with the material at room temperature, and samples were assayed over a one hour period. The results of this experiment (Fig. 6) showed that dehydroascorbic acid had a very short half-life ($t_{\frac{1}{2}}$ = 0.24 hr) under these conditions. With a half-life of only 15 minutes, it was essential to keep the time between the collection of the sample and the assay to a minimum.

ACKNOWLEDGEMENT

This work was supported in part by the Coronary Heart Disease Research Project of the American Health Assistance Foundation, Washington, D.C., and in part by the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi.

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