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Liquid chromatographic measurement of L-ascorbic acid and D-ascorbic acid in biological samples

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Abstract

D- and L-Ascorbic acids have been separated using liquid chromatography (LC) on a polymer-coated silica-based NH_2 column and the L-isomer has been quantified in human serum, rat serum, rat lung, rat lung perfusate, infant formula (SRM 1846) and mixed food sample (SRM 2383). The D-isomer was observed only in trace amounts in the mixed food sample. The results demonstrate that ascorbic acid was stable on the column and completely recovered from supplemented samples of human serum and that this method of analysis is accurate, precise and has broad application exhibiting no dependence on the nature of the matrices evaluated herein.

Keywords: Ascorbic acid; Vitamins

1. Introduction

Ascorbic acid (AA) is a six-carbon carbohydrate that exists in two stereoisomeric forms: an L-isomer and a D-isomer. The D-isomer is also known as isoascorbic acid (IAA) or erythorbic acid. The use of IAA as an antioxidant to preserve foods has led to the development of methods to separate and quantify both IAA and AA in prepared foods and in plasma or serum. There are three basic liquid chromatographic (LC) methods for separating AA and IAA: (a) reversed-phase ion pair separation on octadecyl silane (ODS) columns [1–5], (b) reversed-phase separation on styrene-divinylbenzene columns [6–8] and (c) ion-exchange separation on aminopropyl silane columns [9–14]. (Although the aminopropyl silane column [11] separated the stereoisomers, the AA underwent on-column oxidation that could not be completely eliminated.) The detection methods are (a) direct amperometric detection, (b) ultraviolet detection or (c) fluorescence detection of the *o*-phenylenediamine post-column derivative [8,9]. The advantages and disadvantages of the use of the various detection systems and analytical procedures have been extensively reviewed [15]. We have selected the amperometric method because in our hands it is stable, and is more selective and sensitive than the ultraviolet method [16].

The AA isomers may be reversibly oxidized to their respective dehydro isomers (DHAA and DHIAA). The oxidized forms of AA and IAA have been measured by post-column detection of the *o*-

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phenylenediamine derivatives [8,9] or by reducing them with dithiothreitol (DTT) and measuring the oxidized forms by calculating the difference between the reduced and total amounts of AA and IAA [10,16,17].

The purpose of the present study is to report a method using amperometric detection that completely resolves AA from IAA, uric acid and DTT; to document the capability of this method; to quantify the amounts of AA and DHAA and to demonstrate that the method is not matrix dependent and that little or no IAA is present in a variety of biological materials including human plasma, infant formula, mixed food diet, rat lungs, serum and the perfusate of isolated rat lungs.

2. Experimental

2.1. Materials

AA, IAA and DTT were purchased from Sigma (St. Louis, MO, USA). The AA, examined by proton magnetic resonance, contained <0.1% impurities, including those related to AA or the degradation products of AA. The acetonitrile was HPLC grade. Human serum was purchased from Interstate Blood Bank (Memphis, TN, USA). It was free of anticoagulants and tested negative immunologically for the hepatitis B and HIV viruses. The serum was stored at −70°C until used.

Rat lungs from 350–400 g Sprague–Dawley rats were isolated and were perfused in a recirculating system at 15 ml/min with a bicarbonate-buffered salt solution containing 50 g/l Ficoll (Pharmacia Biotech, Piscataway, NJ, USA). The perfusate effluxed the left atrium into a reservoir and was recirculated in a temperature controlled system at 37°C for 60 min. Rat serum was collected when the lungs were isolated. Aqueous metaphosphoric acid (MPA), 100 g/l, was added to the rat serum, the lung perfusate and the homogenized lung tissue samples to a final MPA concentration of 50 g/l to precipitate the proteins and prevent the oxidation of AA to DHAA. The samples were stored at −70°C.

AA was measured in two food items: infant formula, standard reference material (SRM) 1846 and a mixed food sample, candidate SRM 2383, Vitamins and Carotenoids in Food (NIST Standard Reference Materials Program, Gaithersburg, MD, USA). The infant formula was a dry powder which was supplemented with stabilized exogenous vitamins and prepared and packaged by Analytical Research Systems (Indianapolis, IN, USA) in 30-g lots and vacuum packed in Mylar pouches. The mixed food sample, consisting of a pureed mixture of meat, vegetables, fruit juice and pasta, was prepared by Gerber Products (Fremont, MI, USA) in heat-sterilized vacuum-packed 4-ounce bottles and was not supplemented with exogenous AA. The mixed food samples were divided into three groups that were stored at either room temperature, 4°C, or −70°C. After five months of storage the AA and DHAA were measured in representative samples from each mixed food sample group.

2.2. Method of analysis

The samples were analyzed by LC on a 250×4.6 mm Capcell Pak NH_2 column (Shiseido, Tokyo, Japan). The column was equilibrated at 40°C at a flow-rate of 1 ml/min with a solvent composed of 0.680 g monobasic potassium phosphate, 200 ml water, 800 ml acetonitrile and 7.5 ml concentrated phosphoric acid. It is important to note that this separation is very dependent on the solvent composition and slight variations cause significant degradation in the resolution of the AA isomers. The analysis was done isocratically using a Varian Model 5560 chromatograph, a Model 8085 autosampler (Varian Associates, Sunnyvale, CA, USA) and a Model 400 electrochemical detector (EG&G, Princeton Applied Research, Princeton, NJ, USA). The electrochemical detector was set at 700 mv, which is slightly above the point at which the asymptotic response curve reaches a plateau. The chromatograms were integrated with Maxima 800 software (Waters, Milford, MA, USA). Some serum samples were also analyzed on a styrene divinylbenzene, fast acid analysis column (Bio-Rad, Hercules, CA, USA) as previously described [16,17].

2.3. Preparation of supplemented serum samples

Supplemented serum was prepared gravimetrically. Two sets of samples were prepared using two

different batches of serum: (a) Lots 178, 179 and 180 and (b) Lot 179B. A weighed amount of a MPA (50 g/l) solution of AA was added to a weighed amount of serum to which was then added an equal volume of 100 g/l of aqueous MPA (which was also weighed). Aliquots (1 ml) of the resulting constantly stirred suspension were distributed into washed 2-ml cryules (Wheaton, Millville, NJ, USA) using a positive-displacement automatic pipette. The vials were sealed and stored at −70°C where they were stored until analysis. The process of sample preparation was completed within 3 h thus minimizing the time that oxidation of the AA could occur. The final concentrations of the supplemented AA in the serum samples are summarized in Table 1.

2.4. Determination of AA and DHAA

AA and DHAA were extracted from the serum samples and analyzed using external standards according to the method previously described [16]. The rat serum and lung perfusate samples were assayed by the same procedure as that used for human serum. The rat lung samples were similarly assayed after ten-fold dilution.

The infant formula samples were prepared for analysis by weighing all liquids and solids to the nearest mg on a calibrated top-loading balance. To assess the homogeneity of the material, two samples were prepared by weighing approximately 5 g of sample into each of two 125-ml bottles. Next, 40 ml of aqueous solution containing monobasic potassium phosphate (0.05 mol/l) and DTT (1 g/l) were weighed into the bottle and the material was mixed and allowed to dissolve. After all samples had dissolved, 10 ml of aqueous MPA (500 g/l) and 20 ml of acetonitrile were sequentially added and weighed. The samples were mixed and then centrifuged at 1000 *g* for 15 min at 5°C. The upper layer contained insoluble material. Four ml were removed from the clear lower layer for further analysis by LC. Each sample, in random order, was diluted with the extraction solvent with a positive-displacement automatic pipette. The first set of samples was diluted 50 fold (0.100 ml to 4.90 ml of solvent) and the second and third sets were diluted 75 fold (0.100 ml to 7.400 ml of solvent). The diluted samples were mixed, transferred to autosampler vials and analyzed on a Capcell Pak NH_2 column in a random order.

Each food sample was analyzed twice, the first time for AA and IAA and the second time for total AA (AA+DHAA) and total IAA (IAA+DHIAA), by suspending 1 g of sample in 5 ml water and vortex-mixing it for 15 s. To the first sample 1 ml of aqueous MPA (400 g/l) was added, the suspension was vortex-mixed for 15 s, then 2 ml of acetonitrile was added and the suspension was vortex-mixed again for 15 s. After centrifuging the suspension (1000 *g*, 30 min, 5°C) the clear phase was transferred to 1.8-ml vials for LC analysis. The second sample was treated identically except that after the material was suspended in water, 1 ml of 0.5 mol/l of dibasic potassium phosphate containing 100 g/l of DTT was added to reduce the DHAA and the DHIAA. The

Table 1
Recovery of supplemented AA from human serum

Sample I.D.	AA (mean±S.D.) (μmol/l)	AA+DHAA[a] (mean±S.D.) (μmol/l)	Supplemented AA (μmol/l)
Serum 1	5.1±1.1 (4)[b]	11.5±0.2 (4)	
178	10.2±0.4 (4)	27.0±0.5 (4)	15.8[c]
179	12.1±3.9 (10)	43.5±1.5 (10)	32.1[c]
180	15.1±0.4 (4)	42.7±0.3 (4)	31.6[c]
Serum 2	18.9±6.5 (4)	52.2±1.6 (4)	
179B	67.2±26.4 (5)	154.4±1.1 (10)	101.3[d]

[a] This value was obtained by reducing the DHAA to AA with DTT prior to analysis.
[b] The value in parentheses is the number of measurements made.
[c] This value represents the amount of AA physically added into serum 1 and is comparable to the difference between the measured total AA in this sample and that in serum 1.
[d] This value represents the amount of AA physically added into serum 2 and is comparable to the difference between the measured total AA in this sample and that in serum 2.

sample was vortex-mixed for 15 s, kept at room temperature for 30 min and 1 ml of aqueous MPA (400 g/l) was added. The second sample was then treated the same as the first sample.

3. Results

Fig. 1 illustrates the baseline resolution of a mixture of standards of AA, IAA, MPA and uric acid (UA) on the Capcell Pak NH_2 column. Both DTT and MPA elute very near the solvent front and do not interfere with the quantification of the analytes of interest, IAA and AA. It is important to note that the AA isomers are well resolved from each other. UA, which also gives an electrochemical response, is also completely resolved from the AA isomers. The calibration curve for AA is linear over at least the range of 1.4 to 14 μmol/l ($y=9.96\cdot10^{-8}x-1.47\cdot10^{-4}$, $R^2=0.9986$, $n=12$), the concentration range over which the extracted samples were measured, and the intercept was <0.2% of the AA peak areas of the extracts of the serum samples.

3.1. Determination of AA in human serum

Table 1 summarizes the results of the assay by the current method for AA and AA+DHAA in the four lots of serum samples supplemented with AA as well as the unsupplemented serum samples. It is apparent that each lot of serum contains a different amount of AA and DHAA. During the process of supplementation some of the added AA was oxidized to DHAA, which is indicated by the low levels of AA in the absence of DTT as compared to the total amount of AA added to each lot. However, all of the supplemented AA is accounted for if the amount of supplemented AA is added to the amount of AA+DHAA in the unsupplemented serum for each lot. The relative standard deviation of the total AA is between 0.7 and 3.5%. However, the R.S.D. for the AA only (without DTT reduction of DHAA) is large and variable as compared with that of the total AA.

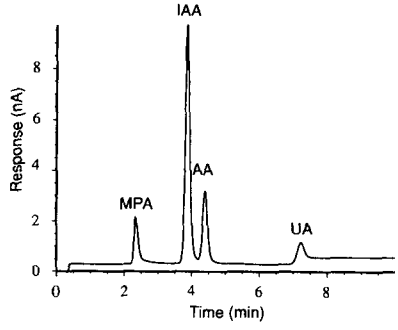

Fig. 1. Chromatogram of AA, IAA, UA and MPA Standards on a Capcell Pak NH_2 Column. Chromatographic conditions are described in Experimental Section 2.2.

Fig. 2 illustrates the relative levels of AA and total AA (AA+DHAA) obtained for two different lots of serum that were supplemented with different amounts of AA. These chromatograms indicate that one of the serum samples (Lot 180, chromatograms 1 and 2, panels A and B) contained an unknown material (U1) with the same retention time as IAA. Lot 179B did not contain this material (chromatograms 3 and 4, Panel A). This material did not react with AA oxidase, which oxidizes both AA and IAA to their dehydro forms; it was unstable upon storage at −70°C for 2 months; and it could not be regenerated by reduction with DTT. All of the DHAA that had been formed during the preparation of the samples or during storage was converted to AA upon treatment with DTT (Table 1), both immediately after preparation and after 22 months storage at −70°C.

Samples 179 and 180 were analyzed by an alternative method on the fast acid analysis column [16,17]. This column exhibits a different order of elution of the four major components, MPA, AA, UA and DTT, yet the total AA content is indistinguishable from that obtained using the Capcell Pak NH_2 column (Table 2) suggesting that few if any interfering substances co-elute with the AA.

3.2. Determination of AA in infant formula

Fig. 3 illustrates a typical chromatogram of an extract of SRM 1846 (powdered infant formula). The sample was treated with DTT to measure the total AA. In addition to the MPA and DTT only AA and UA were detected. There was no evidence of IAA in any of these samples. Two samples from each of twelve randomly selected packets were extracted and the total AA in each extract was measured in duplicate. The total AA content of this material was

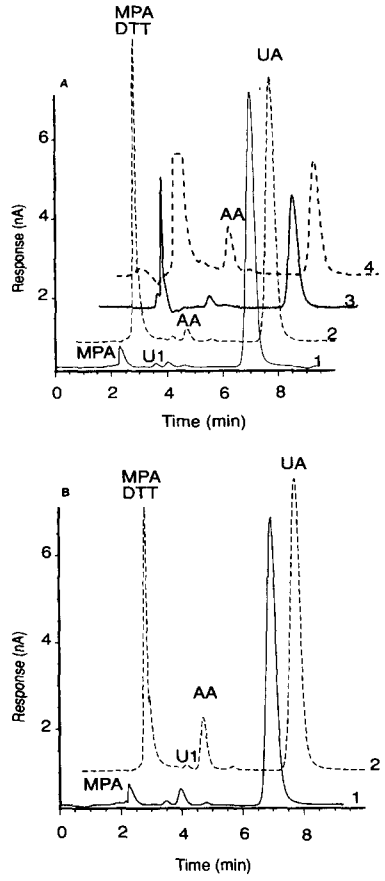

Fig. 2. Chromatograms of AA in human serum. The chromatograms of the unsupplemented samples in panel A illustrate the AA in Lot 180 (curve 1); the total AA in Lot 180 after the DHAA was reduced with DTT (curve 2); the AA in Lot 179B (curve 3), and the total AA in Lot 179B after the DHAA was reduced with DTT (curve 4). The samples chromatographed in panel B were supplemented with AA (31.6 μmol/l of AA) and the chromatograms represent; the AA in Lot 180 supplemented (curve 1) and the AA in Lot 180 supplemented and after the DHAA was reduced with DTT (curve 2).

1.22±0.06 (S.D.) mg/g of powder. The S.D. included a between packet relative standard deviation (R.S.D.) of 1.8%, a between sample R.S.D. of 1.6% and a measurement error R.S.D. of 1.1%. The between-day and between-packet variance were both statistically significant, but the between-sample variance was not. The between-packet variance was in excess of the between-sample variance. The measurement error (R.S.D. of 1.1%) indicates the precision of this method. As the result of the analysis of this SRM by Analytical Research Systems Corp. was 1.15 mg AA/g of powder, the difference (0.07) between the NIST measured value and the stated values is insignificant.

Table 2
Comparison of measurement of AA in human serum on two different types of column

Lot No.	Total AA (mean±S.D.) (μmol/l)[a]	
	Capcell Pak NH_2	Bio-Rad fast acid analysis
179	43.5±1.5 (10)[b]	43.0±0.3 (4)
180	42.7±0.3 (4)	43.2±1.7 (4)

[a] This value equals the sum of the AA+DHAA obtained by incubation with DTT.
[b] The values in parentheses are the number of measurements made.

3.3. Determination of AA in a mixed food sample

Fig. 4 illustrates a series of chromatograms of extracts of the candidate SRM 2383 (food reference material). There is a small peak in each of the chromatograms that has a retention time similar to that for IAA. However, it represents less than 3% of the AA+DHAA content and suggests that if IAA is present it is only a minor component. Both the AA and total AA were measured in this heat-sterilized material that was stored at several different temperatures [room temperature (~22°C), 4°C and −70°C] for 5 months to assess the effect of temperature on AA degradation. The results are summarized in Table 3. They indicate that 78% of the AA degraded

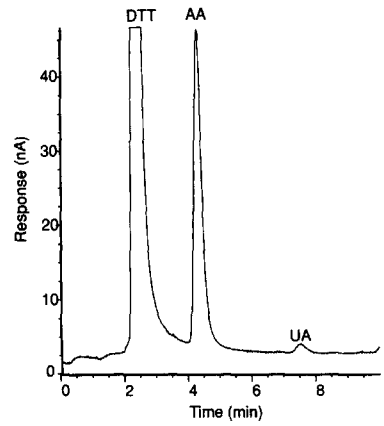

Fig. 3. Chromatogram of AA in SRM 1846 Infant Formula.

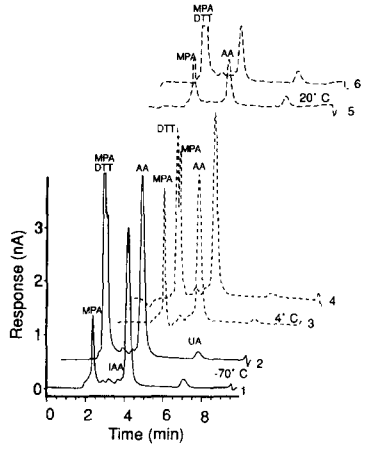

Fig. 4. Chromatograms of AA in a mixed food sample, candidate SRM 2383 stored at three different temperatures. Curves 1, 3 and 5 represent the AA present before DTT reduction and curves 2, 4 and 6 represent the total AA after reduction of the DHAA with DTT after storage at −70, 4 and 20°C, respectively.

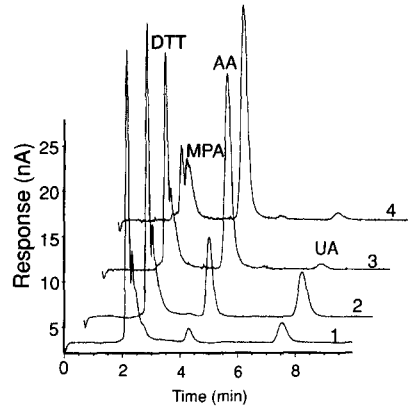

Fig. 5. Chromatograms of the total AA in rat tissues. All samples were reduced with DTT. Curve 1 represents the total AA in lung perfusate; curve 2, in serum; curve 3, in the supernatant fluid from a 20% homogenate of lung and curve 4, in the 20% homogenate of lung.

beyond DHAA at room temperature (~22°C) within 5 months. At 4°C and −70°C the total AA content changed very little, but at 4°C 15% more of the AA was oxidized to DHAA than at −70°C. Less than 5% of the total AA was degraded beyond DHAA at 4°C. The DHAA measured in the samples stored at −70°C probably represents the initial concentration of DHAA at the time of sample preparation since very little oxidation of AA has been observed in other biological samples stored at −70°C for six months or less [15,17,18]. These results suggest that AA is not very stable in this food sample at 20°C and that the instability can be monitored by measuring both the AA and the total AA.

Table 3
Effect of storage conditions on the stability of AA in mixed food material

Storage temperature (°C)	Concentration (mean±S.D., n=8) (μmol/l)	
	AA	AA+DHAA[a]
~22[b]	50±7	58±3
4	163±9	214±9
−70	191±25	226±4

[a] Samples were reduced with DTT before being assayed.
[b] Room temperature.

3.4. Analysis of AA in rat tissue

Fig. 5 illustrates a series of chromatograms of DTT reduced extracts of rat serum, rat lung homogenate and the rat lung perfusate from the isolated rat lung preparation. In all chromatograms, except for the peaks at the solvent front, only AA and UA were detected. Both the rat serum and the rat lung contained measurable amounts of AA, 54.4±2.5 μmol/l in the rat serum and 0.51±0.06 mmol/l in the 20% rat lung homogenate. Fig. 6 illustrates that AA is released into the rat lung perfusate and the amount of AA increases as the perfusion continues over 60 min. Furthermore, the majority of the total AA is in the reduced form indicating that either the AA is not rapidly oxidized, or that perfusion through the lungs, or material(s) secreted or cells released into the perfusate keep the AA in the reduced state. The release of UA into the perfusate paralleled that of AA.

4. Discussion

A chromatographic method must be robust if it is to be a useful technique. That is, the analyte must be stable during the analytical process; both the extraction and chromatographic procedures must be

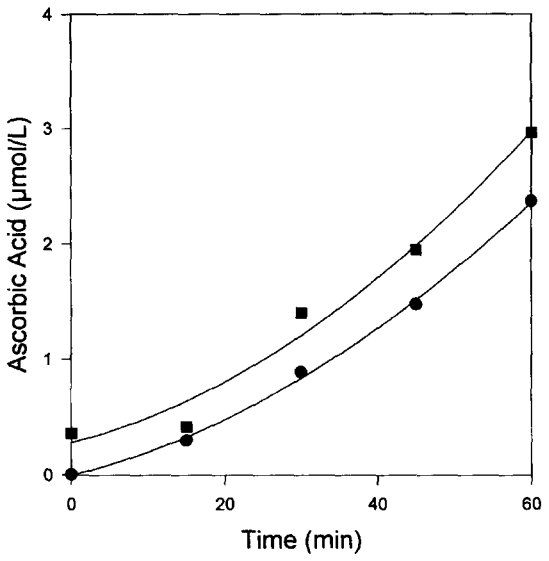

Fig. 6. Time course of the release of AA and DHAA into the perfusate of an isolated rat lung. (■) Total AA; (●) AA before reduction of DHAA with DTT.

reproducible; the results should be accurate and the uncertainty should be small; the detector should have a high degree of selectivity for the analytes of interest; the signal for each analyte of interest should be free from significant interferences; and the method should be free from matrix effects.

The separation of AA isomers by LC on silica based aminopropyl columns [11,12] is well documented; however, AA is not stable on this type of column [11]. The development of the polymer-coated silica-based NH_2 (Capcell Pak NH_2) column offered an opportunity to reevaluate the separation of AA isomers on an amine column without oxidation of the AA. In the present study, using this column, the AA is stable during the chromatography process as indicated by a linear standard curve that has a very small positive intercept. This small intercept is approximately 0.2% of the lowest concentration of the AA standard (1.4 μmol/l) and is well below the AA content measured in the sample extracts evaluated in this study. Furthermore the reagents, MPA and DTT, elute at the solvent front and UA elutes well after the AA and IAA. The elution position of the DTT is the same as that observed on the aminopropyl column [11], and the elution position of both the DTT and the UA are much more favorable on the NH_2 column than on the fast acid analysis column used in earlier studies [16–18]. The advantage of the new chromatographic method presented in this study is that it permits the assay of the total amounts of AA and IAA after DTT reduction of the oxidized forms.

The extraction and chromatography of the AA from infant formula and human serum is both reproducible and accurate. The analysis of a series of supplemented serum samples (Table 1) is characterized by small R.S.D. values (3.5%) indicating that the extraction of the total AA and chromatography are very reproducible. The recovery of essentially all of the supplemented AA, (the difference between the AA in the DTT treated unsupplemented serum and the AA in the DTT treated supplemented samples) is clearly demonstrated in Table 1. After 22 months of storage the AA content remained essentially the same giving further support to the accuracy, stability and reproducibility of both the method of storage and the chromatography. The variability in the DHAA content appears to be matrix dependent [25].

The selectivity and advantages of using an electrochemical detector to measure AA and related compounds are well established [15,16]. However, there were several small peaks detected in the chromatograms of one of the unsupplemented serum samples. The peak with the retention time of AA was destroyed completely by AA oxidase. None of the remaining peaks were modified by this enzyme nor were they regenerated by treatment with DTT after they were destroyed during storage for 22 months, Thus there appears to be a small amount of material that has the same retention time as IAA but does not possess the properties of IAA since the material is not oxidized by AA oxidase or reduced by DTT (compare chromatograms 1 and 3 in Fig. 2A). Since not all sera contain materials that co-elute with IAA, it will be necessary to evaluate each serum sample for IAA after it has been completely reduced and for co-eluting materials after the IAA has been completely oxidized. This applies not only to human serum but to all matrices regardless of which analytical method is used.

A robust method should also be uninfluenced by the sample-matrix composition and diverse concentrations of the analyte of interest. Although it is

impossible to demonstrate that a method is independent of all matrix effects, the method in the present study has been applied to the measurement of AA in a wide variety of materials. In all cases, the AA chromatograms showed a well defined symmetrical peak with the same retention time. Only in the case of the mixed food reference material were impurities observed that were not fully resolved from the AA peak and these were very small, constituting less than 3% of the AA in the material that was stored at −70°C. The extraction of the total AA appears to be essentially 100% both from the serum (Table 1) [16,17] and from the infant formula. Thus, we have demonstrated the utility of this method in the measurement of AA, IAA and DHAA in human serum, infant formula (SRM 1846), a mixed food sample (SRM 2383), several rat tissues and rat lung perfusate.

The results on the food reference material clearly illustrates the instability of AA in some matrices (Table 3). In this mixed food sample, which was vacuum packed and heat sterilized, 75% of the AA degraded beyond DHAA within 5 months of storage at room temperature. Even at 4°C, 25% of the AA oxidized in that period of time. Only at −70°C did the AA remain stable for the entire storage period. This pattern of stability is consistent with our studies on the stability of AA in human serum [16,17].

The measurement of AA in the rat tissues (serum and lung) and the isolated lung perfusate clearly illustrates the general applicability of this method to a broad variety of biological samples. These studies were undertaken to demonstrate that AA is a major antioxidant [22,23] released into the perfusate of isolated rat lungs. The concentration of AA continues to increase during the one-hour perfusion period. The DHAA content appears to remain relatively constant as the AA content increases. A concomitant increase in the release of uric acid, a second major free radical scavenger [19–21], was also observed. The rise in the AA content is consistent with its antioxidant activity. The relatively constant level of DHAA is consistent with the possible uptake and reduction of the DHAA from the perfusate by the lung cells and the subsequent release of AA back into the perfusate [24]. Thus, the lack of complete stoichiometry between the AA and the reduced ferricytochrome *c* noted by Schapira [22] may simply reflect the regeneration of AA by the cells of the isolated perfused lungs or cells released into the perfusate. This is also consistent with the observation in whole blood samples of an equilibrium between the plasma AA and DHAA at a ratio of approximately 9:1 [18].

The extraction process described in this study provides a procedure for successfully extracting total AA from a variety of biological materials. Furthermore, in the case of human serum and powdered infant formula this extraction process has been shown to be quantitative. It has the advantage of being done in a single vessel without dilutions or transfers and of measuring the reduced and total content of both L- and D-AA isomers by a single method.

Thus, the method described above has the following advantages within the limitations of this study: (a) it is applicable to a variety of matrices that includes both foods and animal tissues, (b) it permits the simultaneous measurement of AA and IAA with the caveat that one must be aware of the need to test for the sporadic occurrence co-eluting substances, (c) it also allows for the measurement of the oxidized forms of AA and IAA, (d) it utilizes a simple extraction procedure that does not require sample transfer and (e) it is both accurate and precise for total AA as demonstrated in Table 1 and as previously demonstrated [17,25].

5. Note

Certain commercial equipment, instruments and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the equipment or material is necessarily the best for the purpose.

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