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High-throughput analysis of Vitamin C in human plasma with the use of HPLC with monolithic column and UV-detection

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Abstract

Vitamin C plays a central role in the body. One of its important functions is its role as an antioxidant, and accurate measurements are important for interpretations of this role. However, its reactive nature and instability complicates the assessment, especially in biological samples. A high-throughput chromatographic method using monolithic column and UV-detection was developed for the assessment of plasma ascorbic acid and total ascorbic acid. The method showed excellent analytical sensitivity, specificity, precision, recovery and linearity during the validation study. The method was used for the assessment of ascorbic acid and total ascorbic acid during several clinical studies.

Keywords: Vitamin C; Ascorbic acid; Dehydroascorbic acid; Plasma; Monolithic column

1. Introduction

Vitamin C is one of the most ubiquitous vitamins ever discovered and plays a paramount role as an antioxidant and a free radical scavenger, able to moderate the oxidative stress effects of various diseases [1]. Humans, guinea pigs, and some fruit-eating bats must satisfy their Vitamin C requirements through the diet, due to the lack of the last enzyme on the Vitamin C synthesis pathway, L-gulono-lactone oxidase [2].

Vitamin C is widely sold and used as a preventive and therapeutic agent for the common cold, and doses as high as 8 g per day has been shown to have small but significant effects on the duration but not on the number of incidences in limited groups of people [3,4]. However, the daily recommended intake of Vitamin C supplementation are within the range of 30–200 mg [5,6] and although Vitamin C is a powerful antioxidant it may also function as a prooxidant at higher dosages [6,7].

The role as a powerful antioxidant and cofunction in enzymatic processes emphasize the necessity of accurate methods for the assessment of Vitamin C in biological samples.

At biological pH the main form of Vitamin C is ascorbate (AA), which is rapidly oxidized to dehydroascorbate (DHAA) and depleted in extra cellular biological fluid [1]. Oxidation in plasma is influenced by sample concentration, temperature, light, pH, dissolved oxygen, solvent, ionic strength, and the presence of ferric ions or oxidizing enzymes [8–11]. DHAA is even more unstable and may undergo irre-

Abbreviations: AA, ascorbic acid; DHAA, dehydroascorbic acid; 2,3-DKG, 2,3-diketogulonate; TAA, total ascorbic acid; HPLC, high performance liquid chromatography; UV, ultraviolet; MS, mass spectrometry; ECD, electrochemical detection; FLD, fluorescence detector; NaH_2PO_4, sodium dihydrogen phosphate; Na_2EDTA, disodium ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate buffered saline; BSA, bovine serum albumin; TCEP, tris[2-carboxyethyl]phosphine hydrochloride; MPA, meta-phosphoric acid; SRM, standard reference material; NIST, The National Institute of Standards and Technology; VWD, variable wavelength detector; DAD, diode array detector; TCA, trichloroacetic acid; PCA, perchloric acid; DTT, dithiothreitol; RP, reversed phase; VIS, visible

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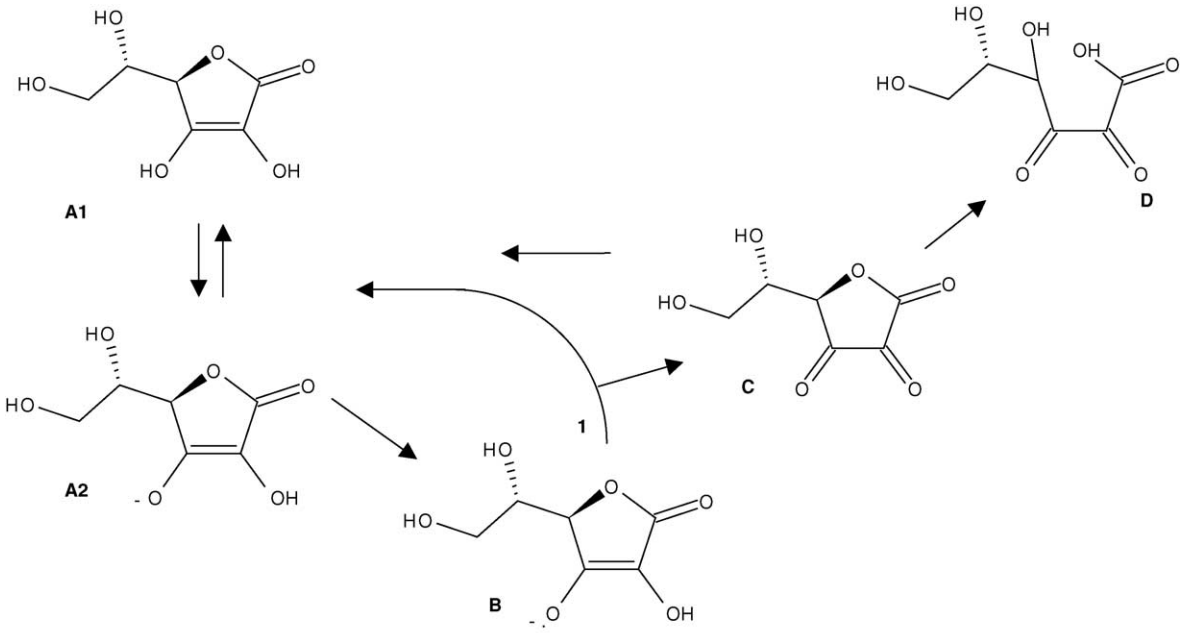

Fig. 1. Reaction scheme for the oxidation of Vitamin C. The main form of Vitamin C, ascorbic acid, AA (A1) (reduced form), is found in its ionic form, ascorbate, (A2), at biological conditions. When oxidized, the ascorbyl free radical, AFR (B), is formed. The AFR undergo a spontaneous dismutation reaction (1) where one molecule of AA and one molecule of dehydroascorbic acid, DHAA (C) (oxidized form) are formed from two molecules of AFR. At biological conditions DHAA will rapidly and irreversible undergo hydrolysis to 2,3-diketogulonate, 2,3-DKG (D).

versible hydrolysis to 2,3-diketogulonate (2,3-DKG) under biological conditions [12–14] (Fig. 1). The significance of AA and DHAA as an antioxidant couple in biological systems are well established [2,15,16] and in healthy adults, the level of DHAA are normally below 2% of that of AA [17]. In oxidative stress related conditions, however, elevated levels of DHAA as compared to total AA (TAA) has been reported [17–19].

Numerous assays for AA and DHAA have been published, and they can be divided into three categories—enzymatic, spectrophotometric and chromatographic assays [20]. Enzymatic and spectrophotometric assays is often influenced by interferences leading to overestimation of AA in biological samples [21–24], and the necessity of modern high performance liquid chromatographic (HPLC) methods for the determination of Vitamin C in biological samples have been thoroughly reviewed [20,25,26].

AA can very easily be detected with the most commonly employed HPLC detectors as ultraviolet (UV), mass spectrometry (MS) and electrochemical detector (ECD) systems, whereas the response of UV and ECD detectors to DHAA is low. The most common approach for detection of DHAA has been pre or post column derivatization and subsequent detection by UV or fluorescence (FLD) [27,28], or pre or post column reduction to AA, and detection by UV or ECD [29,30].

Since the detection properties of DHAA by most common HPLC detectors is poor and the compound is unstable under most conditions of analysis, transformation to a more stable compounds before analysis is desirable. The reduction of DHAA to AA prior to analysis and the use of reduction agents and subtraction methods has been reported as essential for accurate AA and DHAA determination in biological samples [25,31].

Traditionally the run time of a HPLC method for the assessment of Vitamin C in biological samples exceeds 10 min [32,33]. Analysis time down to 4 min has recently been reported with the use of subtraction for the determination of AA and TAA in plant material [34].

In a clinical setting, the number of samples is often large and it is essential to reduce the run time, considering both the limited stability of the analytes and that AA and TAA are determined by two injections in the same sample. Monolithic HPLC columns allow high-throughput analysis due to the reduced column backpressure and as a consequence, the possibility of using high flow rates (∼7 mL/min) with little loss of efficiency. These columns have been used in the field of high throughput analysis of drugs and metabolites, chiral separations, analysis of pollutants and food-relevant compounds, as well as in bioanalytical separations such as in proteomics [35].

In this study we have developed a rapid, accurate, robust and specific chromatographic method for high-throughput assessment of AA and TAA in biological samples based on the use of monolithic columns. Delay between sample preparation and analysis were minimized, allowing processing of 100 samples in less than 3.5 h. The stability of prepared samples stored in the HPLC autosampler compartment with and

without temperature controlling were studied. The method was validated and used for high-throughput analysis of samples in several clinical studies.

2. Materials and methods

2.1. Chemicals, reagents and solutions

L-AA, L-DHAA, D-AA, sodium dihydrogen phosphate (NaH_2PO_4) dodecyltrimethyl ammonium chloride, disodium ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (Na_2EDTA), phosphate buffered saline (PBS) bovine serum albumin (BSA) tris[2-carboxyethyl]phosphine hydrochloride (TCEP) meta-phosphoric acid (MPA) and trizmabuffer were obtained from Sigma–Aldrich (Oslo, Norway). HPLC-grade acetonitrile and the Chromolith Performance HPLC column and guard column kit were obtained from Merck (Oslo, Norway). Standard reference material (SRM) of AA and TAA in human serum was obtained from The National Institute of Standards and Technology (NIST) (Gaithersburg, USA). All reagents and solutions were prepared in Milli-Q water, which was made from a Millipore ultra-pure water system (Millipore Corp., USA). Heparin, EDTA and citrate vacutainer tubes were purchased from BD Vacutainer (Franklin Lakes, USA). Stabilyte vacutainer tubes were obtained from Biopool AB (Umeå, Sweden).

2.2. Preparation of standards and solutions

The aqueous part of the mobile phase consisted of 2.5 mmol/L NaH_2PO_4, 2.5 mmol/L dodecyltrimethyl ammonium chloride and 1.25 mmol/L Na_2EDTA in milli-Q water. 10% MPA (w/v) was prepared by dissolving 10 g MPA dissolved in milli-Q water to a final volume of 100 mL. The reduction reagent solution contained 2.3 mmol/L TCEP in 800 mmol/L trizmabuffer (pH 9.0).

Validation of the method for the determination of AA in human plasma was performed using spiked solutions of AA in AA depleted human heparin plasma. Human plasma was AA depleted by leaving it on the bench for 96 h. Plasma was spiked to 50, 100 and 200 μmol/L using a freshly prepared 10 mmol/L L-AA stock solution prepared in 1% BSA in PBS.

2.3. Sample preparation

Whole blood was sampled in heparin vacutainer tubes from healthy, adult volunteers. Plasma was generated by centrifugation at 2800 × *g* in 10 min at +4 °C. One part plasma was thoroughly mixed with one part cold, freshly prepared 10% MPA in an eppendorf tube, using a vortexmixer for 30 s. The tube was immediately frozen to −70 °C until further handled and analysed.

At the time of analysis, the samples were thawed at room temperature, before wortexmixing and centrifugation at 3500 × *g* in 15 min at +4 °C. Of the clear supernatant, 100 μL was used for direct determination of AA, and 100 μL for determination of TAA, as described below.

2.3.1. Direct determination of AA

The clear supernatant (100 μL) was diluted with 400 μL of the aqueous part of the mobile phase in an autosampler vial. The sample was centrifuged at 3500 × *g* in 10 min at +4 °C and 5 μL was used for chromatographic analysis.

2.3.2. Determination of TAA/reduction of DHAA

The clear supernatant (100 μL) was subjected to reduction with the addition of 50 μL 2.3 mmol/L TCEP in 800 mmol/L trizmabuffer (pH 9.0). After wortexmixing, the sample was left light protected in room temperature for 7 min for complete reduction of DHAA, before 350 μL of the aqueous part of the mobile phase was added. The sample was centrifuged at 3500 × *g* in 10 min at +4 °C and 5 μL was used for chromatographic analysis.

2.3.3. Concentration of DHAA

The concentration of DHAA is determined by subtracting the concentration of AA from the TAA concentration.

2.4. Chromatographic method

An Hp 1100 series LC apparatus were used for chromatographic analysis. All data were handled by ChemStation 6.0. The mobile phase was prepared by addition of 2% acetonitrile to the aqueous part, prepared as described above. The flow rate was 6.0 mL/min. For separation of AA from other plasma constituents, a Chromolith Performance RP18-e, 4.6 mm × 100 mm column was used, with a Chromolith Performance RP18-e, 4.6 mm × 10 mm guard column. The injection volume used was 5 μL. A variable wavelength UV detector (VWD) was used at 264 nm.

2.5. Validation of the chromatographic method

The chromatographic method was validated with emphasis on specificity, linearity within the expected concentration range, accuracy, precision, recovery, detection limits and robustness.

The specificity of the method was verified by co-elution of authentic standards and by evaluation of the spectra of the peak of interest, using a diode-array detector (DAD). The linearity of the method was investigated with the use of a 3-point calibration curves within and exceeding the expected concentration range, generated on a daily basis from freshly prepared standard solutions, prepared in AA depleted heparin plasma. The accuracy of the method was verified with the use of a standardized reference material for ascorbic acid in serum, provided by NIST (SRM970) [36]. The precision of the method was investigated by calculation of the precision on 3 concentration levels using freshly prepared standard solutions in AA depleted human heparin plasma within and exceeding the expected concentration range. The detection

and quantification levels were established with the use of a signal to noise ratio of 3 and 10, respectively. The robustness was investigated with the use of different columns ($n = 2$) and pH variations in the mobile phase.

3. Results and discussion

3.1. Sample preparation

Preservation and stabilization in the sample preparation step to prevent AA oxidation and DHAA hydrolysis in biological samples is critical to obtain accurate assay results. As both forms are subjected to rapid degradation, no delay must take place between sampling and preservation.

The use of different vacutainer tubes as EDTA, citrate and heparin gains different values for AA and DHAA. In accordance to reported data and the WHO recommendations for laboratory investigations, heparin tubes are used for assessment of Vitamin C in human plasma [37]. Both AA oxidation and DHAA hydrolysis are further prevented to some extent by acidification. Acids as trichloroacetic acid (TCA), perchloric acid (PCA) and MPA have been used for stabilization of Vitamin C in biological samples. However, MPA have been shown to be more efficient, considering both stabilization of AA and DHAA and precipitation of sample proteins [9].

Several reduction agents have been employed for the reduction of DHAA in biological samples. Commonly used are TCEP and dithiothreitol (DTT). With the use of both reduction agents, the pH has to be elevated to above 8 for an efficient reduction. At this pH DHAA is rapidly hydrolyzed to 2,3-DKG and it is essential that the reduction step is limited with respect to the time interval. TCEP reduce DHAA to AA with higher efficiency at room temperature, as compared to DTT [31], and is less likely to interfere as it is not detected in the chromatographic system.

For the assessment of Vitamin C in human plasma, whole blood collected in heparin tubes were processed for plasma generation immediately after sampling, and the obtained plasma were acidified and frozen without further delay. Samples were stored at −70 °C for up to 3 months before analysis. At the time of analysis, samples were thawed, and immediately prepared as described under materials and methods for the determination of AA and TAA.

3.2. Use of internal standard

Very few published method for the determination of AA and TAA in biological samples uses an internal standard. Several compounds could have been considered for the use as an internal standard for the assessment of Vitamin C in biological samples. Hydroquinone has been employed as an internal standard for the determination of ascorbic acid in aqueous humor and plasma [38]. The isomeric form of ascorbic acid, isoascorbic acid, has also been employed for the determination of Vitamin C in plasma [39]. Both these compounds are well suited chromatographic; they are structure-like and are well separated from ascorbic acid.

There are several reason why the use of these internal standards in Vitamin C analysis is troublesome. One explanation could be that both compounds may be oxidized during storage and sample preparation, and as they are added to the samples. With the use of reduction agent, quantification will be problematic. The isomeric form isoascorbic acid may also be present in biological samples.

Due to the use of reduction agents and subtraction method, it was not possible to find a suitable internal standard during this study.

3.3. Chromatographic method

A representative chromatogram of AA in human plasma is shown in Fig. 2.

Due to its polar nature and small molecular weight, AA has little retention in regular reversed phase (RP) chromatographic conditions. Although chromatographic methods have been reported for the determination of AA without the use of ion-pair reagent [34], we chose to use dodecyltrimethyl ammonium chloride as an ion-pair reagent in the mobile phase to obtained increased retention. For chelating of trace metals, Na_2EDTA was added to the mobile phase, and NaH_2PO_4 was used for pH control. With the use of this mobile phase composition, the retention time of AA was 0.7 min with a flow rate of 6 mL/min. The retention time was highly reproducible with CV < 1%. The identity of the peak was verified by co-elution with an authentic standard, prepared in 1% BSA in PBS. Further identification was done using a DAD for spectra analysis. Due to flow limitation in the DAD cell, the flow was reduced to 3 mL/min and AA eluted

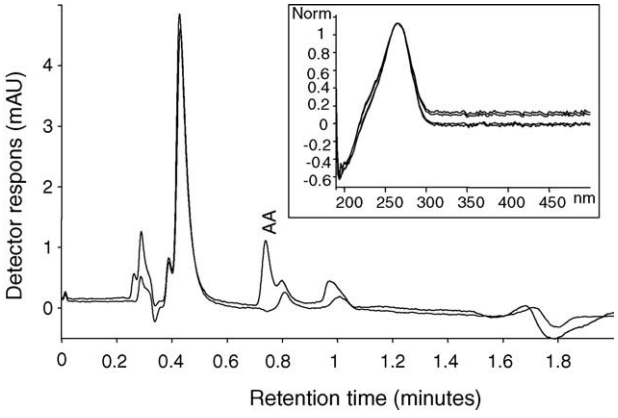

Fig. 2. Chromatograms of ascorbic acid, AA (whole line), in human plasma and ascorbate oxidase treated human plasma (dotted line). Samples were prepared as described in materials and methods. Ascorbate oxidase treated samples were handled as described elsewhere [46]. The mobile phase consisted of 2% acetonitrile in 2.5 mmol/L NaH_2PO_4, 2.5 mmol/L dodecyltrimethyl ammonium chloride and 1.25 mmol/L Na_2EDTA, and the flow rate were 6 mL/min. The column was a Chromolith Performance RP-18E, 4.6 mm × 100 mm, and the UV detector was set at 264 nm. The separation was performed at room temperature.

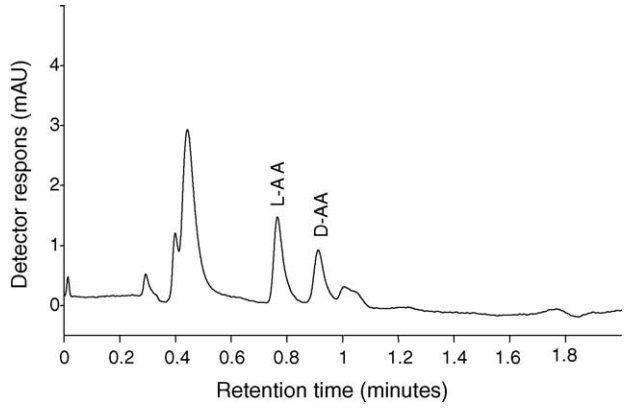

Fig. 3. Chromatogram of the separation of L-AA and D-AA in standard solutions containing 50 μmol/L L-AA and 30 μmol/L D-AA. Standard solutions was prepared as described in materials and methods. The mobile phase consisted of 2% acetonitrile in 2.5 mmol/L NaH_2PO_4, 2.5 mmol/L dodecyltrimethyl ammonium chloride and 1.25 mmol/L Na_2EDTA, and the flow rate were 6 mL/min. The column was a Chromolith Performance RP-18E, 4.6 mm × 100 mm, and the UV detector was set at 264 nm. The separation was performed at room temperature.

at 1.2 min. The UV–vis (VIS) spectra of the AA peak are inserted in Fig. 2. The identity of the AA peak was confirmed by treating human plasma samples with ascorbate oxidase, as described elsewhere [46]. Fig. 2 shows chromatograms of both human plasma and ascorbate oxidase treated human plasma, and demonstrate that the peak exclusively contains L-AA. As Fig. 2 demonstrates, a possible interference is present in the tail of the AA peak in human plasma samples. As this peak only contributed to the peak area and not the peak height, this potential problem was solved with the use of peak height for quantification of AA.

Since D-AA may be present in Vitamin C enriched foodstuff, it may also be absorbed in the same manner as L-AA, and consequently found in human plasma samples. Since the biological function of D-AA may differ from that of L-AA, it is essential that these isomeric compounds are well separated in the chromatographic system. Fig. 3 shows a chromatogram of a standard solution containing 50 μmol/L L-AA and 30 μmol/L D-AA. The compounds are clearly separated and may be quantified individually.

The use of non-volatile ion pair reagents in the mobile phase excluded the use of MS as detector system. Both ECD ad UV can be employed for the detection of AA in biological samples. As both ECD and UV perform equally well in Vitamin C analysis, it is natural to choose UV due to its improved robustness and simplicity.

The AA peak area and added concentration of AA to AA-depleted heparin plasma displayed linear relationship for a 3-point calibration curves over the selected concentration range, 50–200 μmol/L. Calibration curves were generated on a daily basis. The slopes of the calibration curves were consistent. r^2 were <0.999, with coefficients of variations below 1%. Linearity and day-to-day variation are shown in Table 1.

The accuracy of the method was verified with the use of a standardized reference material for AA in acidified serum, provided by NIST (SRM970). For reference material level I (14.8 μmol/L) deviation was 7% and for reference material level II (57.6 μmol/L) deviation was 5%, as compared to AA standards prepared in AA-depleted heparin plasma. The values were within the expected SD for the reference material.

The CV for the precision of the method was 4.2–4.5% and 4.3–4.6% for within-day ($n = 5$) and between-day ($n = 5$) estimations, respectively. Estimations were based on measured concentrations of AA in AA-depleted heparin plasma spiked with known concentrations of AA. Precision and recovery data are shown in Table 2.

The detection limit of AA in human plasma was 1.5 μmol/L, and the limit of quantification was 4.95 μmol/L. The method was robust with the use of different Chromolith Performance columns ($n = 2$, from different batches) and a pH range from 4.5 to 4.9 in the mobile phase.

Validity of accurate AA and DHAA measurements in clinical plasma samples are highly dependent on the availability of a method of demonstrated precision and accuracy for measurement of each analyte. The presented HPLC method for AA and TAA determinations in biological material with the use of subtraction method [40,31] showed excellent analytical sensitivity, specificity, precision, recovery and linearity,

Table 1
Linearity and day-to-day variation ($n = 5$)

	Slope[a] mean ($n = 5$)	CV (%) (slope)	r mean ($n = 5$)	CV (%) (r)
Within-day	$y = 0.0534x + 0.006$	5.2	0.9995	0.04
Between-day	$y = 0.0516x - 0.0638$	6.3	0.9986	0.02

[a] y = peak area, x = concentration.

Table 2
Reproducibility and recovery

Compound	Added to plasma (μmol/L)	Mean measured ($n = 10$)	CV (%)		Recovery, (%) ($n = 10$)
			Within-day ($n = 5$)	Between-day ($n = 5$)	
AA	50	45.7	4.5	4.6	91.3
	100	90.8	4.2	4.3	90.8
	200	189.6	4.3	4.5	94.8

Table 3
Mean values for AA and TAA in different studies

Study no.	*n*	Parameters investigated	Population	Age	AA, baseline mean (μmol/L)	DHAA, baseline mean (μmol/L)
1	41	AA/TAA at time intervals ($n = 4$) after intervention	Healthy adults	29 ± 10	63.7 ± 18.9	4.1 ± 3.6
2	60	AA/TAA at time intervals ($n = 2$) after intervention	Adults; smokers, elevated blood pressure or high cholesterol	55 ± 9	47.3 ± 15.4	5.6 ± 4.3
3	20	AA/TAA at time intervals ($n = 3$) after intervention	Premature children	5 weeks	42.1 ± 33.1	5.8 ± 4.2

and there was sufficient separation of AA from other plasma constituents. The identity of the AA peak was confirmed by a UV–vis spectrum and the AA peak showed purity above 99%. A relation between the observed value (calibration curve) and a known value (NIST) were established, and found to be satisfactory. Retention time for ascorbic acid was 0.7 min, but to avoid interferences from unknown peaks in human plasma, an injection interval of 2 min was used. This allowed throughput of 30 samples per hour. Though the chromatographic conditions (mobile phase, column and detection) differ little compared to earlier methods [27,41], the use of a monolithic column is a significant improvement. The reduced backpressure obtained with the use of a monolithic column, and as a consequence the ability to use a higher flow rate allows a higher throughput of samples, as compared to assays where conventional HPLC columns is used [45].

Prepared heparin plasma samples, as described above, showed little degradation of AA when stored in the HPLC autosampler at +4 °C for up to 4 h, as shown in Fig. 4. However, when the autosampler temperature control was turned off and samples were left at room temperature, rapid degradation of AA was observed even at short time intervals (Fig. 4).

These data emphasise the necessity of a high-throughput method for accurate determination of AA and TAA in biological samples, as AA is subjected to rapid degradation even in acidified prepared samples at short time intervals when left at room temperature, or at longer time intervals at +4 °C.

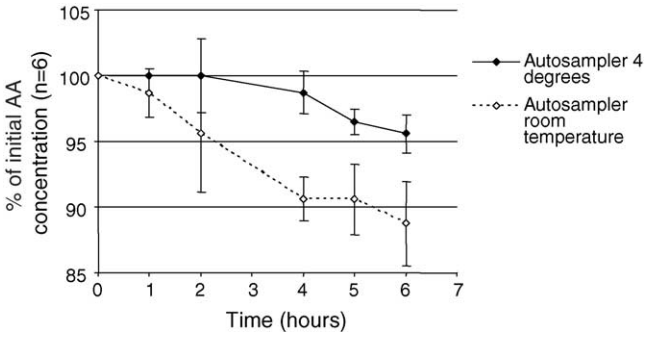

Fig. 4. Degradation of AA in prepared plasma samples stored in autosampler at room temperature and +4 °C. AA levels were measured in plasma after acidification and further sample preparation as described in materials and methods. Values are presented as median percentage of initial AA peak area at time intervals in hours. Data series bars represent standard deviations.

The reported method with a time interval of 1.7 min between injections allows the run of 100 samples, 50 for AA and 50 for TAA, within 3 h and 30 min. Within this time interval a degradation of less then 1.5% is observed, if sample are kept at +4 °C in the autosampler compartment. If kept at room temperature, a degradation of nearly 10% is observed in the same time interval.

The degradation of AA and TAA in samples could potentially be corrected for with the use of an appropriate calibrator that ideally would show similar degradation of AA. However, since AA is degraded to different extend between individuals this correction can never be achieved in an accurate manner.

3.4. Application

The high-throughput method was used for the assessment of plasma AA and DHAA in 3 studies conducted in collaboration with other clinical institutions. All studies were approved by the local ethical committee. The analytical method showed excellent analytical performances during the studies.

Mean values of AA and DHAA are summarized in Table 3. In study 1, healthy adults ($n = 41$) at age 29 ± 10 participated. The mean plasma AA was 63.7 ± 18.9 μmol/L. Plasma DHAA was 4.1 ± 3.6 μmol/L, and mean DHAA to TAA ratio were 0.06.

In study 2, adults ($n = 60$) at the age of 55 ± 9 participated. The inclusion criteria were smoking, elevated blood pressure or high cholesterol. Mean plasma AA was 47.3 ± 15.4 μmol/L and mean plasma DHAA was 5.6 ± 4.3 μmol/L, mean ratio of DHAA to TAA was 0.11.

In study 3, AA and DHAA were measured in premature children at the age of 5 weeks. Mean plasma AA was 42.1 ± 33.1 μmol/L and mean plasma DHAA was 5.8 ± 4.2 μmol/L. Ratio of DHAA to TAA was 0.12.

In those subjects that presumably were subjected to elevated global oxidative stress, that is participants in studies 2 and 3, elevated DHAA levels and depleted AA were detected, as compared to study 1, where healthy individuals where participating. These finding are in accordance to previously reported levels of AA and DHAA in healthy individuals

and in individuals subjected to oxidative stress conditions [18,42–45].

4. Conclusion

The presented high-throughput HPLC method for AA and TAA determinations in biological material with the use of subtraction method showed excellent analytical sensitivity, specificity, precision, recovery and linearity, and AA was separated from other plasma constituents. The method has been employed for the assessment of Vitamin C in several clinical studies (Table 3), as well as in several other studies.

The method may also be applied to other biological material if attention is paid to immediate sample acidification and reduced temperature during storage. In addition, the identity of the AA peak should be confirmed from its UV-spectrum in addition to retention time. We have with success quantified Vitamin C with the presented method in several tissues from mice and also in infant formula (data not shown).

The method is in use in our laboratory on a daily basis and in total several thousand samples have been analyzed.

The highly unstable nature of Vitamin C in prepared samples, the difficulty of finding an appropriate internal standard to overcome this problem in combination with a large number of samples in a clinical setting, precludes accurate and precise assessment of Vitamin C in biological samples. An ultra high-throughput method as presented here allows analysis of a large number of samples, while still maintaining high accuracy and precision.

The presented method has proven it self as robust, and the analysis time of 1.7 min allows a throughput of 100 samples in less than 3.5 h.

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