Journal of Chromatography B, 879 (2011) 2834–2839

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short Communication

The determination of ascorbic acid and uric acid in human seminal plasma using an HPLC with UV detection

Roman Kanďár[a,*], Petra Drábková[a], Radek Hampl[b]

[a] Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Studentska 573, 532 10 Pardubice, Czech Republic
[b] Sanus, In Vitro Fertilization Clinic, Pardubice, Czech Republic

ARTICLE INFO

Article history:
Received 20 June 2011
Accepted 8 August 2011
Available online 16 August 2011

Keywords:
Ascorbic acid
Uric acid
Dithiothreitol
High-performance liquid chromatography with ultraviolet detection
Protein precipitants
Human seminal plasma

ABSTRACT

Oxidative stress has been proposed as one of the potential causes for infertility in men. Ascorbic acid and uric acid play important role in protection of spermatozoa against free radicals. A method for the simultaneous determination of ascorbic acid and uric acid in human seminal plasma using HPLC with UV detection and investigation their clinical significance as antioxidants protecting male germ cells against oxidative damage are described. Semen samples were obtained from consecutive male partners of couples presenting for a fertility evaluation. After liquefaction, the samples were centrifuged and the supernatants were diluted with dithiothreitol solution and after a filtration injected onto an analytical column. For the separation, a reverse-phase column MAG 1, 250 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm, was used. The mixture of ethanol and 25 mmol/L sodium dihydrogenphosphate (2.5:97.5, v/v), pH 4.70 was used as a mobile phase. Analytical performance of this method is satisfactory for both ascorbic acid and uric acid: the intra-assay and inter-assay coefficients of variation were below 10%. Quantitative recoveries from spiked seminal plasma were between 92.1 and 102.1%. We have found no significant differences in both ascorbic acid and uric acid concentration between the smokers and non-smokers (351.0 ± 237.9 μmol/L and 323.7 ± 99.5 μmol/L vs. 444.8 ± 245.5 μmol/L and 316.6 ± 108.9 μmol/L, $p > 0.05$). This assay is a simple and reproducible HPLC method for the simultaneous measurement of ascorbic acid and uric acid in human seminal plasma.

1. Introduction

Oxidative stress induced by reactive oxygen (ROS) and nitrogen (RNS) species has been proposed as one of the potential causes for infertility in men [1–3]. Spermatozoa are susceptible to free radical-induced damage because their cytoplasmic membrane contains large percentage of polyunsaturated fatty acids [4,5]. Furthermore spermatozoa cytoplasm disposes of low activity of antioxidant enzymes [6]. On the other hand relatively high concentration of low-molecular weight antioxidants (ascorbic acid, uric acid, glutathione, taurine, hypotaurine, α-tocopherol, β-carotene, and coenzyme Q_{10}) plays maybe the most important role in protection of spermatozoa against ROS [6–10].

Numerous methods have been described for the analysis of ascorbic acid (AA) and uric acid (UA) in various biological samples. Methods for determination of AA include the reducing properties of the 1,2-enediol group that lead to absorbance changes in indicator dyes (2,6-dichlorophenol-indolphenol) [11,12], the ketone derivatization method with 2,4-dinitrophenylhydrazine [13] or o-phenylenediamine [14], an enzymatic method with ascorbic acid oxidase [15] and high-performance liquid chromatography (HPLC) methods with UV–vis [16,17], fluorimetric [18,19], electrochemical [20,21], and MS detection [22], eventually gas chromatography (GC) [23,24] or high-performance capillary electrophoresis (HPCE) [25,26]. For the measuring of serum or urine UA is widely used an uricase method [27] whereas a direct method quantifies the decrease of UV absorbance at 293 nm (absorption maximum of UA) and indirect method quantifies the amount of hydrogen peroxide formed by uricase action. Enzymatic methods can be affected by the presence of different interfering compounds. Hence the separation techniques as HPLC with UV [28], electrochemical [29] and MS detection [30,31], GC [32] and HPCE [33] are preferred. Numerous HPLC methods have been developed for the simultaneous determination of AA and UA in serum, plasma, urine and tissues [34,35], but practically no against human seminal plasma.

Oxidation of antioxidants during sample storage and preparation is the major problem at their measurement [36,37]. Therefore sample preparation is essential for accurate analysis of AA and UA. While UA is relative stable, AA is easily oxidized to dehydroascorbic acid (DHAA). Oxidation of AA and DHAA hydrolysis is influenced by temperature, light, pH, dissolved oxygen, solvent, ionic strength,

* Corresponding author. Tel.: +420 466037714; fax: +420 466037068.
E-mail address: roman.kandar@upce.cz (R. Kanďár).

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doi:10.1016/j.jchromb.2011.08.007

and the presence of oxidizing enzymes or some divalent cations [14].

Some authors point out that HPLC-UV method for the simultaneous determination of AA and UA in biological samples has low sensitivity and selectivity required the additional sample preparation [31]. The aim of this study was to develop and validate HPLC-UV method with a rapid and simple sample preparation for the routine simultaneous determination of AA and UA in human seminal plasma.

2. Experimental

2.1. Reagents and chemicals

Ascorbic acid, uric acid, hydrochloric acid, metaphosphoric acid, ortho-phosphoric acid, perchloric acid, trichloroacetic acid, oxalic acid, sulfosalicylic acid, sodium dihydrogenphosphate, sodium hydrogenphosphate, sodium acetate, ammonium acetate, dithiothreitol, dithioerythritol, sodium borohydride, and sodium hydrosulfite were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC-gradient grade methanol, ethanol and acetonitrile were from Merck KgaA (Darmstadt, Germany). For the quantification of UA, lyophilized calibrators were used (0, 708 and 1439 μmol/L; Lot 8JD178, 8JD278, and 8JD378) from Dade Behring (Newark, DE, USA), lyophilized serum UA controls chemTRAK® (Lot TLM10081 and TLM10082) were from MAS® Controls (Passau, Germany). All the other chemicals were of analytical grade.

2.2. Instrumentation

Chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-10ADvp solvent delivery system, SIL-10ADvp autosampler, CTO-10ASvp column oven, SPD-10Avp variable wavelength spectrophotometric detector and SCL-10Avp system controller. Data were collected digitally with Clarity chromatography software (DataApex, Prague, Czech Republic).

2.3. Subject and samples

Semen samples were obtained from consecutive male partners ($n = 100$, 29 smokers in the age 18–47 years, mean age 31 years and 71 non-smokers in the age 19–52 years, mean age 32 years) of couples presenting for a fertility evaluation at the Sanus, In Vitro Fertilization Clinic, Pardubice, Czech Republic. The diagnosis of infertility for these patients was formulated according to the World Health Organization (WHO) guidelines (1999). None of the participants had a serious or chronic disease and took any medications and vitamins on the day of semen collection. A written informed consent was obtained from all participants before starting the protocol and the Institutional Review Board of the Sanus, In Vitro Fertilization Clinic of Pardubice, Czech Republic, approved the study.

2.4. Semen samples collection

Semen samples were collected into sterile plastic containers by masturbation after a period of sexual abstinence of 2–3 days. After 30 min of liquefaction at room temperature, the raw semen specimens were divided into two portions, the first to estimate the sperm concentration, sperm morphology and sperm motility, the second to be centrifuged (3500 × *g*, 10 min, and room temperature). The seminal plasma was kept at −80 °C for the determination of AA and UA.

2.5. Sample preparation

Reducing agent (1 mmol/L dithiothreitol [DTT]) was carefully added (1.0 mL) to seminal plasma or mixed standards (20 μL). After incubation (4 °C, 10 min) and centrifugation (33 000 × *g*, 10 min, 4 °C), supernatants were filtered through a nylon filter (pore size 0.20 μm, 4 mm diameter, Supelco, Bellefonte, PA, USA), transferred into 1.0-mL amber vials and purged with nitrogen for 10 s (Linde Gas, Prague, Czech Republic).

2.6. Chromatography method

Chromatography of AA and UA was accomplished using an isocratic elution on a MAG 1, 250 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm analytical column fitted a MAP, 20 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm guard column (Labio a.s., Prague, Czech Republic) and a PEEK pre-column filter, and pore size 0.5 μm (Supelco, Bellefonte, PA, USA) at 25 °C. The mobile phase consisted of 2.5% ethanol in 25 mmol/L sodium dihydrogenphosphate (v/v), pH 4.70 ± 0.05. The flow rate was kept constant at 0.5 mL/min. The optimum response of AA was observed when wavelength was set at 265 nm, while UA was monitored at 292 nm. The amount of AA and UA was quantified from the corresponding peak area using Clarity chromatography software (DataApex, Prague, Czech Republic). The concentration of AA and UA in the samples was determined from the calibration curve.

2.7. Statistical analysis

The data are presented as mean values ± S.D. Differences between the smokers and non-smokers were analyzed with the use of the Student's *t*-test and analysis of correlation was carried out using Spearman rank order correlation (software QCexpert, Trilobyte, Pardubice, Czech Republic). A $p < 0.05$ value was considered statistically significant.

3. Results and discussion

3.1. The effectiveness of various protein precipitants and reducing agents, ascorbic acid stability

An oxidation of AA during a sample preparation is major problem at its measurement. Many of the protein precipitants are acids, which not only precipitate proteins but also prevent hydrolysis of the lactone ring and inhibit oxidation. Commonly used acids are metaphosphoric acid (MPA) and trichloroacetic acid (TCA). Organic solvents have been used instead of acids. We have investigated stability of AA and UA in semen and seminal plasma samples immediately after semen sample collection. Semen sample was stored at room temperature and aliquots were analyzed at 5-min time intervals for 60 min. Levels of AA were practically stable for at least 30 min and UA levels were stable for at least 60 min (Fig. 1A). The results suggest that during 30-min semen liquefaction at room temperature is both AA and UA stable. On the other hand seminal plasma was stored at either 4 °C or −20 °C and aliquots were analyzed at 30-min time intervals for 10 h. Levels of AA were stable only in seminal plasma stored at −20 °C while UA levels were stable both at 4 °C and −20 °C (Fig. 1B).

We have tested number of protein precipitants with regard to stability and recovery. Cold protein precipitant (10% MPA, 1.0 mol/L perchloric acid, 10% TCA, 10% sulfosalicylic acid, mixture of 10% TCA and 10 mmol/L oxalic acid, acetonitrile (ACN) with 0.1 mol/L hydrochloric acid, ACN with 0.1 mol/L acetic acid, methanol with 0.1 mol/L hydrochloric acid, ethanol with 0.1 mol/L hydrochloric acid) was carefully added (400 μL) to seminal plasma or mixed

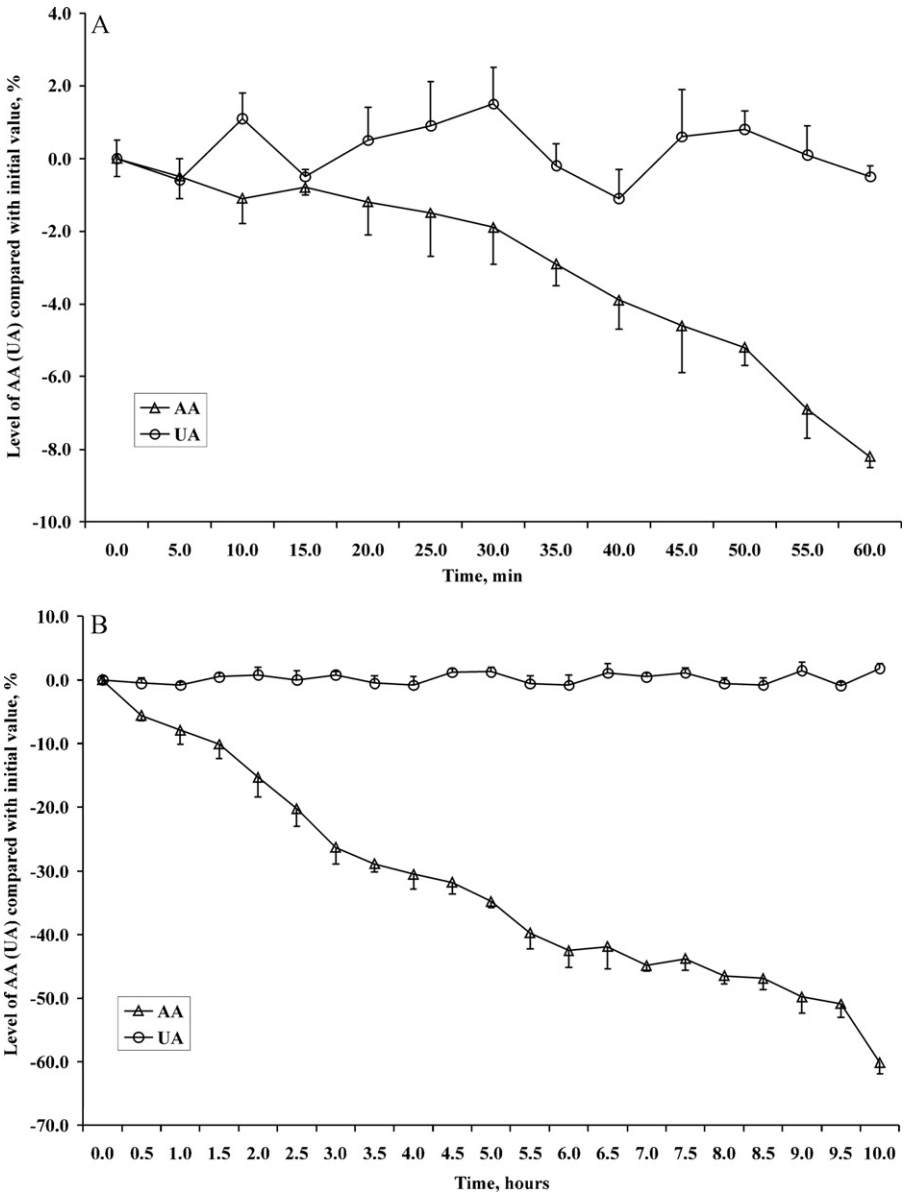

Fig. 1. Stability of AA and UA in semen sample during storage (semen liquefaction) at room temperature for 60 min (A) and stability of AA and UA in seminal plasma sample during storage at 4 °C for 10 h (B). Mean ± S.E.M. (standard error of the mean) of triplicate assays is recorded.

standards (200 μL). After incubation (4 °C, 10 min) and centrifugation (22 000 × *g*, 10 min, 4 °C), supernatants (100 μL) were diluted with DTT solution with different concentration (900 μL), and filtered through a nylon filter (pore size 0.20 μm, 4 mm diameter), transferred into 1.0-mL amber vials and purged with nitrogen for 10 s. The only MPA as a protein precipitant led to satisfactory recoveries and stability. However, all of tested protein precipitants interfered with HPLC analysis. One of the few HPLC methods for the determination of seminal plasma AA is that by Colagar and Merzony [3]. They used methanol as a protein precipitant, however, they did not test AA stability. Our results point out that AA is not stable in samples precipitated with organic solvents.

The relevant step of sample preparation for accurate analysis of AA is the addition of an antioxidant or a reducing agent. We have tested number of reducing agents (DTT, dithioerythritol, sodium borohydride, and sodium hydrosulfite) with different concentrations. The best results were obtained for a solution of DTT (1 mL of 1 mmol/L DTT to 20 μL of seminal plasma sample). Ascorbic acid was stable in seminal plasma samples treated with DTT more than 10 h at 4 °C (cooled autosampler).

3.2. High-performance liquid chromatographic assay of ascorbic acid and uric acid

Ascorbic acid and UA were separated on a reverse-phase column using an isocratic system of ethanol and sodium dihydrogenphosphate. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of AA and UA as well as pooled seminal plasma were used for study of the mobile phase composition. Several mobile phases (namely different buffers containing ethanol) were assayed. We prefer the using of an eco-friendly mobile phase, because the organic solvents such as ACN and methanol are considered as significant pollutants. Optimization of the separation was obtained after studying the effect of sodium dihydrogenphosphate concentration (from 5.0 to 100.0 mmol/L) and ethanol concentration. The retention behavior was studied in dependence of pH value of the mobile phase

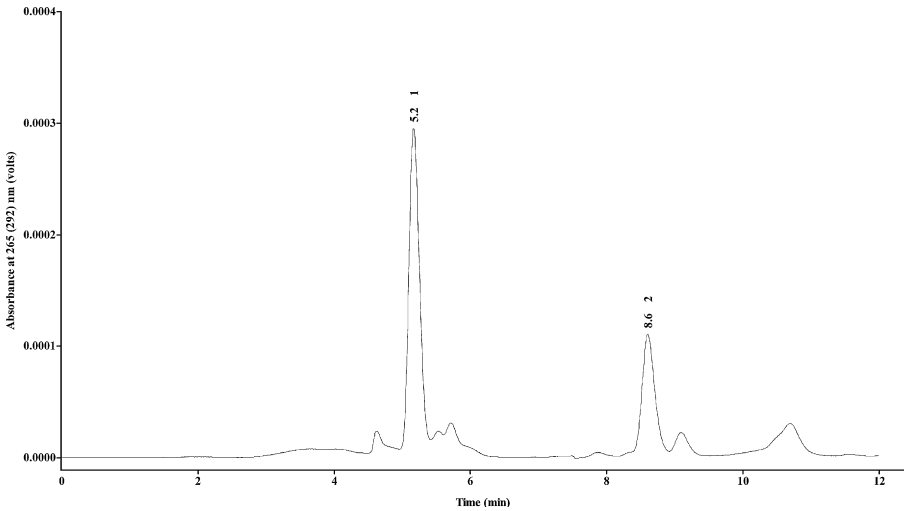

Fig. 2. An HPLC chromatogram of AA (582.0 μmol/L) and UA (228.1 μmol/L) in human seminal plasma. Peaks: (1) AA and (2) UA. HPLC conditions: an isocratic elution (mobile phase: 2.5% ethanol in 25 mmol/L sodium dihydrogenphosphate, pH 4.70), the stationary phase was an analytical column MAG 1, 250 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm fitted a MAP, 20 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm guard column and a PEEK pre-column filter, pore size 0.5 μm, the flow rate was kept constant at 0.5 mL/min, separation ran at 25 °C and AA was monitored at 265 nm, while UA at 292 nm.

Table 1
Precision of AA and UA in human seminal plasma.

	AA		UA	
	Mean ± S.D., μmol/L	CV, %	Mean ± S.D., μmol/L	CV, %
(A) Precision (within-day)				
10	68.3 ± 2.6	3.8	198.7 ± 6.2	3.1
10	342.1 ± 9.9	2.9	483.5 ± 10.6	2.2
(B) Precision (between day)				
10	346.3 ± 30.1	8.7	472.5 ± 37.3	7.9

in the range 4.0–6.5. The optimal pH 4.7 was chosen for the best separation and detection of AA and UA. Column temperature was changed from 20 to 45 °C. The mobile phase conditions leading to the best separation were: 2.5% ethanol in 25 mmol/L sodium dihydrogenphosphate, pH 4.70 ± 0.05. Optimal temperature interval was from 20 to 25 °C. The criteria were the resolution, stability of the absorbance, and the analysis speed. Pursuant to records, we can establish that the presented method is highly robust. Two different columns, both of C18 type, were assayed in the research: a Discovery (Supelco, Bellefonte, PA, USA) and a MAG 1, Labiospher. The MAG 1, Labiosper column was selected for further experiments. Two lengths of selected column (150 and 250 mm) were assayed and that of 250 mm length yielded the best resolution with acceptable retention times for both AA and UA in human seminal plasma. The 250 mm column provided a proper separation between tested analytes and interferences. An HPLC chromatogram of AA and UA in human seminal plasma is shown in Fig. 2.

Precision of AA and UA analysis for seminal plasma samples are shown in Table 1. To determine the within-day precision, the seminal plasma samples were analyzed ten times in the same day under the same conditions. Similarly, results on the between-day precision were obtained on the same seminal plasma samples, which were analyzed in 10 different days. The coefficients of variation were below 10%. The spike recoveries, obtained after the dilution with 1.0 mmol/L DTT, ranged between 92.1 and 102.1% for AA and 93.2 and 101.3% for UA (Fig. 3). The calibration curve (11-point for a determination of analytical parameters and 7-point for routine analysis) was linear in the whole tested range: 10.0–2000.0 μmol/L of AA and 5.0–1000.0 μmol/L of UA. The regression lines obtained from the combination of 10 standard curves were $y = 5.606x - 19.87$ μmol/L for AA and $y = 8.211x - 24.15$ μmol/L for UA. The mean slope, intercept and correlation coefficient (R) for the calibration curves were 5.606 (95% confidence interval, 5.373–5.872), 3.5 μmol/L (2.0–5.4 μmol/L), and 0.9998 for AA, 8.211 (95% confidence interval 7.325–9.145), 2.9 μmol/L (1.7–4.0 μmol/L), and 0.9997 for UA. The lowest concentration that can be quantified with acceptable accuracy and precision was 10.0 μmol/L (2.0 pmol/inject) for AA and 5.0 μmol/L (1.0 pmol/inject) for UA. Furthermore, limits of detection for AA and UA, defined as signal-to-noise (S/N) ratio of 3:1, were 2.0 μmol/L (0.4 pmol/inject) and 1.5 μmol/L (0.3 pmol/inject), respectively (detector sensitivity: 0.001 absorbance units full scale, AUFS).

3.3. The determination of ascorbic acid and uric acid in human seminal plasma

Levels of AA and UA in seminal plasma as well as examined sperm parameters in the patients are shown in Table 2. We found no significant differences in all follow-up parameters between non-

Table 2
Comparison of selected sperm parameters quality, AA and UA in the smoker and non-smoker men.

	Non-smoker men ($n = 71$)	Smoker men ($n = 29$)	p^*
Age (years)	32 ± 5	31 ± 6	0.103
AA (μmol/L)	444.8 ± 245.4	351.9 ± 237.9	0.098
UA (μmol/L)	316.6 ± 108.9	323.7 ± 99.5	0.738
Volume (mL)	3.1 ± 1.3	2.8 ± 1.1	0.415
Sperm count ($\times 10^6$/mL)	42.1 ± 36.9	50.6 ± 51.4	0.519
Total sperm ($\times 10^6$)	116.3 ± 92.5	155.0 ± 174.1	0.799
Normal morphology (%)	10.9 ± 7.4	12.3 ± 8.1	0.540
Vitality (%)	72 ± 18	73 ± 19	0.577

* Mann–Whitney rank sum test.

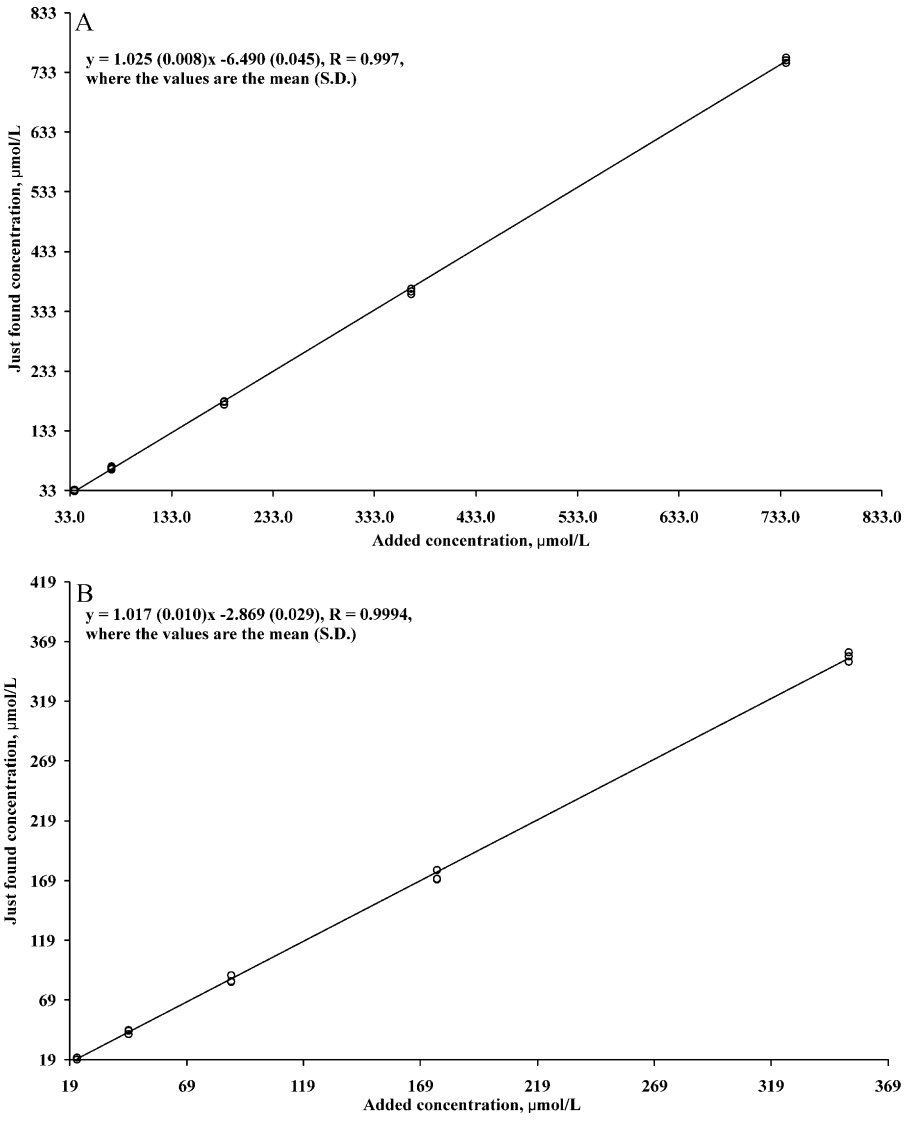

Fig. 3. Recovery experiment: AA (A) and UA (B). Values of triplicate assays are recorded. Slopes correspond to the mean recovery 97.6% (S.D. = 3.7%) for AA and 97.9% (S.D. = 2.9%) for UA.

smokers and smokers. We observed significant correlation between AA concentration and age ($R = -0.373$, $p = 0.0462$), between AA concentration and normal morphology of spermatozoa ($R = 0.385$, $p = 0.0391$), and between UA concentration and spermatozoa vitality ($R = 0.378$, $p = 0.0431$) in smokers. Kul'Krauchas et al. [38] state that a smoking in men is associated with about 20–40% decline of serum AA levels and an increasing of spermatozoa abnormalities. Dawson et al. [39] recommend a supplementation of AA to smokers with a view to improved spermatozoa quality. Mostafa et al. [10] found significantly decreased AA levels in smokers versus non-smokers while Colagar and Marzony [3] found no significant differences just like us. The values of AA and UA in human seminal plasma differ between laboratories and AA levels range at wide interval. We assume that this wide interval is due to differences in eating habits of individuals.

This method was developed for the simultaneous determination of AA and UA as important antioxidants in seminal plasma of male partners of couples presenting for a fertility evaluation at the Sanus, In Vitro Fertilization Clinic of Pardubice. For the measurement of antioxidants in biological materials is desirable to have a single, reliable and inexpensive method. Our method fully satisfied these conditions; a sample preparation is simple and significantly prevents AA oxidation and degradation, the analytical parameters are sufficient. Presented method is cheap (about 2500$/1000 analyses; i.e. HPLC instrument, analytical column, mobile phase, filters, and chemicals for sample preparation costs) and sample preparation includes only a dilution with DTT solution and a filtration. Within 2 years we have carried out more than 1000 analyses and have found out, that lifetime of the analytical column is more than 1000 injects. Therefore we can establish that the method is inexpensive and suitable for clinical trials.

Acknowledgements

The Ministry of Education (Grants SGFChT07/2011 and MSMT 0021627502) supported this work.

The authors thank staffs of Sanus, In Vitro Fertilization Clinic in Pardubice for the seminal plasma samples.

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