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Determination of main low molecular weight antioxidants in urinary bladder wall using HPLC with electrochemical detector

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Abstract

The aim of the present work was to develop validated HPLC method using electrochemical detector for simultaneous detection of low molecular weight antioxidants (LMWA) in urinary bladder. Furthermore, the method was applied to study the distribution of LMWA in urinary bladder wall. The ascorbic acid (AA), glutathione in reduced (GSH) and oxidized (GSSG) form and uric acid (UA) were resolved by isocratic elution from C_{18} reversed-phase column. The bladder tissue sample preparation involved extraction with *meta*-phosphoric acid solution for LMWA stabilization. The AA, GSH and UA tissue peak was identified by different approaches. The obtained method validation parameters were in acceptable range: intra-day precision (<4.4%), intra-day accuracy (<8.4%), inter-day precision (<9.4%) and inter-day accuracy (<15.6%). Additionally, the method provided good linearity ($r^2 > 0.99$) and recoveries (98.9–112.6%). The distribution of LMWA in urinary bladder was determined by measuring their concentration in bladder wall layers: urothelium, lamina propria, muscularis and serosa. The validated method was able to quantify the reduced form of all three LMWA in all four bladder wall layers. The LMWA concentrations were decreasing from urothelium to serosa except of UA. The developed HPLC method with electrochemical detection of LMWA is simple, fast and can be used for simultaneous quantification of LMWA in tissues, which contain low concentrations of antioxidants.

Keywords: Low molecular weight antioxidants; HPLC; Electrochemical detector; Urinary bladder wall

1. Introduction

Antioxidants present one of the main defence systems against reactive oxygen and nitrogen intermediates. Low molecular weight antioxidants (LMWA) scavenge reactive intermediates in different metabolic processes by transforming them into less reactive species, and therefore, prevent the oxidative damage of biological structures. The capacity of LMWA is normally not exceeded, however, if there is an overproduction of reactive intermediates or if the capacity of LMWA and other antioxidants is too low, oxidative damage of biological structures occurs. Among the water-soluble LMWA ascorbic acid (AA),

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glutathione (GSH) and uric acid (UA) are the most important antioxidants. Their antioxidant role in different conditions is well established and described in details elsewhere (Becker et al., 1991; Rose and Bode, 1993; Halliwell and Gutteridge, 1999; Halliwell, 2001; Waring et al., 2001).

Oxidative damage of the urinary bladder wall has been associated with numerous disorders. Reactive intermediates, especially nitric oxide have been lately suggested to be involved in bladder inflammation, ischemia/reperfusion secondary to overdistension injury or bladder outlet obstruction in benign prostatic hyperplasia, carcinomas and urolithiasis (Lundberg et al., 1996; Grases et al., 1998; Olsson et al., 1998; Cohen et al., 2000; Lin et al., 2000; Parekh et al., 2001). Additionally, in the animal models of urolithiasis and sepsis, the decrease in ascorbic acid and glutathione bladder tissue concentrations in comparison with control group was observed (Paskaloğlu et al., 2004). However, bladder impairment due to the oxidative damage can be overcome by the application of different antioxidants in order to improve the antioxidant capacity of the endogenous antioxidants (Grases et al., 1998; Lin et al., 2000; Malini et al., 2000; Parekh et al., 2001; Paskaloglu et al., 2004). The determination of antioxidant status in the urinary bladder would, therefore, provide crucial data for rational targeting of antioxidant strategies in treatment of mentioned bladder disorders.

Various analytical methods for the determination of water-soluble LMWA in biological samples have been described; among them high-performance liquid chromatography (HPLC) coupled with the electrochemical detector (ECD) has many advantages, especially high selectivity and low limit of quantification. ECD with coulometric detection provides sensitivity and specificity for electroactive substances in the low picogram range. At the appropriate voltage setting of the electrodes, 100% of the substance will react when passing through the detector cell. Additionally, using the ECD composed of coulometric electrode array possible interferences of the co-eluted electroactive substances can be avoided, and therefore, the selectivity of detector is improved. The analytical method for water-soluble LMWA determination by HLPC with ECD allows simultaneous determination of the AA, GSH and UA (Rose and Bode, 1995), reduced and oxidized glutathione (Harvey et al., 1989; Smith et al., 1995; Lakritz et al., 1997) or ascorbic (Levine et al., 1999; Lykkesfeldt, 2000) or UA only (Inoue et al., 2003). The possibility to quantitatively determine antioxidant in its reduced and oxidized form is especially valuable, since their ratio can be used as an important indicator of oxidative stress.

The aim of the present work was to obtain simple, rapid and validated HPLC method for determination of water-soluble LMWA in urinary bladder tissue using electrochemical detection. The method development was directed toward simultaneous determination of AA, UA, and glutathione in reduced (GSH) and oxidized (GSSG) form. Additionally, the extraction procedure was optimise to obtain stable LMWA after their extraction from the bladder tissue. Finally, the obtained method was applied to determine water-soluble LMWA distribution in urinary bladder wall. Such information is of particular interest when determining the adaptation ability and vulnerability of biological system to frequent occurrence of oxidative stress.

2. Materials and methods

2.1. Materials

Water for all applications was obtained from a PURELAB classic purification system (Elga, UK) and its resistance was equal or higher than 18.2 MΩ. All used compounds were at least of analytical grade purity. L-Ascorbic acid, reduced and oxidized glutathione, ascorbate oxidase and *N*-ethylmaleimide were purchased from Sigma (USA), while disodium-EDTA, *ortho*- and *meta*-phosphoric acid, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck (Germany). Uric acid was obtained from Calibochem (Germany).

2.2. Chromatographic system

The HPLC system Agilent series 1100 consisted of the following units: isocratic pump, column oven operating at 25 °C, autosampler and an ESA CoulArray electrochemical detector model 5600 containing array of four cells set at 300, 600, 700 and 800 mV. Working and reference electrodes used in electrochemical detector were porous graphite and palladium, respectively. The units were connected to personal computer and their control was achieved by using the HP-ChemStation for LC software except of CoulArray

detector that was controlled by use of CoulArray for Windows software. For chromatographic separation Hypersil C_{18} column (particle size 5 μm; 250 mm × 4 mm; ThermoHypersil) that was connected to precolumn of the same type was applied. The isocratic mobile phase used by Rose and Bode (1995), was modified and consisted of 50 mM potassium phosphate. The pH of resulting solution was adjusted to 3 with *ortho*-phosphoric acid. The mobile phase was filtered through a 0.22 μm filter under vacuum and ultrasonically degassed before use. The flow rate of the mobile phase was 0.8 ml/min and the volume of injected samples was 20 μl.

2.3. Standard solutions

Stock solutions of AA, GSH and GSSG in concentrations 1 mg/ml were prepared by dissolving compounds in the mobile phase with 2 mM EDTA. The low water solubility of uric acid was overcome by preparing its stock solution in 10 mM phosphate buffer pH 9. Standards for HPLC measurements were prepared daily by appropriate dilutions of stock solutions with mobile phase containing 1 mM EDTA. The AA, GSH and UA standards were ranging from 0.5 to 50 μM, 0.8 to 80 μM and 0.05 to 5 μM, respectively.

2.4. Tissue sample preparation

2.4.1. Tissue isolation

Fresh pig urinary bladders, which were obtained at the local slaughterhouse, were immediately rinsed and during the tissue isolation kept in ice-cooled and carbogen (95% O_2, 5% CO_2) saturated phosphate buffer saline pH 7.4. The dorsal part of the bladder corpus was isolated and cut to small cylindrical pieces with serosal and mucosal surface of approximate diameter, i.e., 8 mm. The tissue pieces were blotted dry, laid between two parallel stainless plates and snap frozen in liquid nitrogen. Frozen tissue pieces had parallel mucosal and serosal bladder surfaces and were subsequently used for the validation of the HPLC method. For the determination of antioxidants distribution in the urinary bladder wall, the cylindrical pieces of the bladder wall were divided into two parts, one contained the mucosal and the other the serosal layer. The mucosal and serosal tissue pieces were processed in the same way as described above. The frozen bladder tissue pieces were kept not more than two weeks at −70 °C before being analyzed.

For HPLC method validation the tissue samples used for LMWA extraction contained the whole bladder wall. The frozen bladder pieces were sectioned by cryotome (CM 1850, Leica, Germany) into 20-μm thick sections perpendicular to mucosal layer and sections were placed into pre-weighted plastic microcentrifuge tubes.

The number of animals used in experiments is equivalent to the number of measurements indicated in the tables since each analysed tissue sample was obtained from only one animal and analysed only once.

For the determination of antioxidants distribution in the bladder wall their concentrations were determined in each layer of the bladder wall: urothelium, lamina propria, muscularis and serosa. In the preliminary experiments the thickness of the bladder wall layers was estimated microscopically (Bogataj et al., 2003). On the basis of estimated thickness the appropriate interval for tissue sectioning was chosen. Sections from mucosal tissue pieces starting at mucosal side from 0 to 100 μm, 100 to 200 μm and 2000 to 2100 μm in depth represented urothelium, lamina propria and muscularis sample, respectively. Tissue pieces were sectioned into 20-μm thick sections parallel to mucosal or serosal surface and sections were placed into pre-weighted plastic microcentrifuge tubes. The appropriate quantity of tissue (∼20 mg) for analysis was obtained by combining sections from selected layer from all tissue pieces of the same urinary bladder. The serosal sample was obtained by the same procedure. Serosal tissue pieces of the same urinary bladder were sectioned from serosal side and sections from 0 to 200 μm were combined.

2.4.2. Tissue LMWA extraction

Ice-cold extraction solutions (50 mM *ortho*-phosphoric acid with 1 mM EDTA, 500 mM *ortho*-phosphoric acid with 2 mM EDTA or 5% *meta*-phosphoric acid with 2 mM EDTA) were added to the weighed tissue samples placed in the microcentrifuge tubes to obtain the concentration of 0.05 mg of the wet tissue per μl of the extraction solution. These different extraction solutions were tested in order to prevent degradation of antioxidants upon their extraction from the tissue. Samples were homogenized for 1 min on ice and centrifuged at 20,000 rpm and 5 °C 15 min. Supernatants obtained by tissue

extraction with *ortho*-phosphoric acid were additionally diluted in the ratio of 1:1 (v/v) with mobile phase containing 1 mM EDTA and in case of extraction with *meta*-phosphoric acid supernatants were diluted in the same ratio with 0.625 M potassium phosphate containing 1 mM EDTA. Diluted supernatants were centrifuged for 2 min under the same conditions as before and immediately analyzed.

2.5. HPLC method validation

Accuracy, inter- and intra-day precision and limit of quantification were determined for standards of AA, GSH and UA (Guidance for Industry, 2001). Fresh six to eight standard solutions each containing AA, GSH and UA were prepared daily from the stock solutions (see Section 2.3 Standard solutions) and analysed in five different days. Calibration curves were prepared by plotting the peak areas against standard concentrations and evaluated by linear regression analysis. Additionally, as described above standard solutions at different concentration levels were prepared, analysed and used as quality control standard samples for calculation of intra- and inter-day precision and accuracy. The precision was calculated and presented as the coefficient of variation (C.V.). The accuracy was calculated by back calculation of quality control samples concentrations from the calibration curves, compared to its theoretical concentration and expressed as relative error (R.E.). The limit of quantification was determined in separate experiments where the concentration range of standards was broadened for all three antioxidants to range from 0.01 to 50 μM. The limit of quantification was defined as the standard concentration resulting in the lowest measurable peak area with acceptable precision (C.V. < 20%) and accuracy (80% < Accuracy < 120%) (Guidance for Industry, FDA, 2001). The acceptable R.E. for accuracy at the limit of quantification should, therefore, not exceed 20%.

The efficiency of extraction procedure using *meta*-phosphoric acid was determined. The tissue samples were spiked with known amount of AA, GSH and UA before homogenisation. Blank and spiked samples were extracted and analysed as described above. The concentrations of antioxidants were calculated using calibration curves obtained on the same day and recoveries were defined as ratio of recovered concentration of antioxidant versus its expected concentration.

Additionally, LMWA peak identification in supernatants was performed by three different approaches. First, the peak identity of AA, GSH and UA in supernatants was proved by comparison of retention times from standard solutions and supernatants. Second, the identity of tissue AA and GSH was confirmed by addition of specific reagents. The LMWA were extracted from the tissue using 50 mM phosphate buffer pH 7.0, and to supernatants ascorbate oxidase (EC 1.10.3.3) or *N*-ethylmaleimide were added to final concentration 0.13 U/μl or 10 mM, respectively. The samples were incubated at room temperature for 15 min, diluted with 5% *meta*-phosphoric acid containing 2 mM EDTA and analyzed. Third, the voltamograms for AA and GSH were constructed from standard solutions and supernatants. The identity of AA and GSH in supernatants was proved by comparison of their voltamograms with those obtained from standards.

3. Results and discussion

The literature information regarding the concentrations of LMWA in the urinary bladder wall are very poor regardless of numerous studies demonstrating existence of increased oxidative stress in different pathological conditions of the urinary bladder. The available data of LMWA in the urinary bladder support the conclusion that these concentrations are low in comparison with organs like liver where concentrations are high, and therefore, analytically easier to quantify (Mohandas et al., 1984). Moreover, oxidative stress is not uniformly expressed in the urinary bladder wall layers. Specially, the mucosa, which is directly exposed to toxic substances derived from urine, is very likely more frequently exposed to the oxidative stress. Additionally, the metabolism, which is an important source of the reactive intermediates, is more active in mucosa than in other bladder layers (Hypolite et al., 1993; Levin et al., 1996). Therefore, the analytical method for the determination of LMWA in the urinary bladder wall should have low limit of quantification in order to be able to measure their levels in very small tissue portions.

The initially selected mobile and stationary phases were adopted from the Rose and bode (1995) and good separation of all four antioxidants was obtained. As the high ionic strength of the mobile phase (200 mM)

could be problematic for long term analysis we decided to determine the influence of mobile phase on antioxidants chromatographic separation. The ionic strength in the tested range (200–50 mM) did not influence the separation of AA, GSH and UA, therefore, for further analysis 50 mM phosphate buffer with pH 3.0 and Hypersil C_{18} stationary phase were chosen. The separation of antioxidants in standard mixture (10 μM of AA, GSH, GSSG and UA) and in urinary bladder tissue sample is presented on Fig. 1A and B, respectively. The AA, GSH and UA were eluted in less than 8 min while the retention time of GSSG was approximately

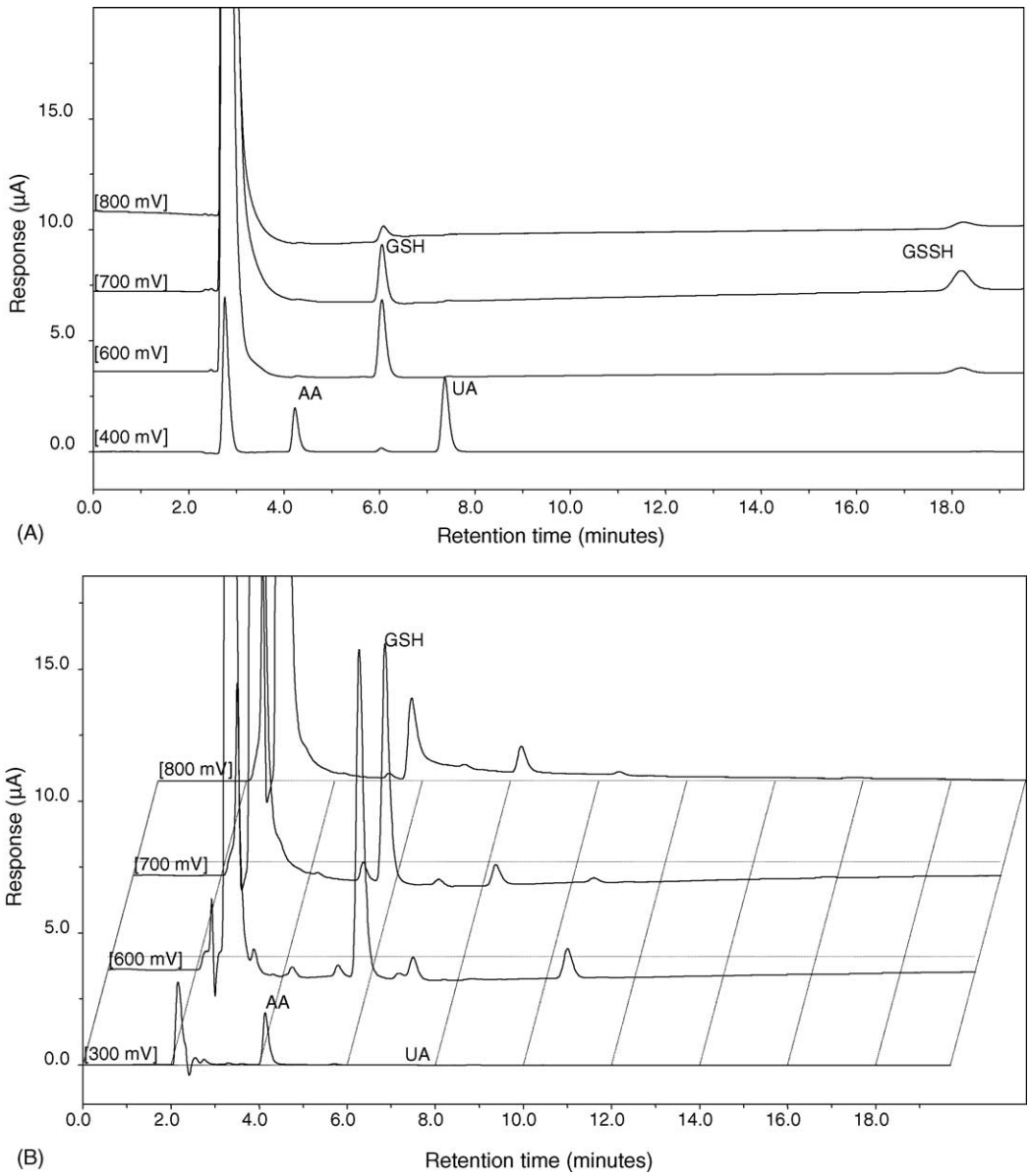

Fig. 1. Chromatograms of antioxidants in standard mixture (A) and in urinary bladder tissue sample (B) at selected cells potentials. The concentrations of AA, GSH, UA and GSSG in standard mixture were 10 μM, chromatographic conditions are described under Section 2.

18 min (Fig. 1A). The peak of UA in tissue sample is very small and not visible under the chosen magnitude of response scale (Fig. 1B), nevertheless the software, which we used for automatic determination of peak response, was able to determine UA response. The peak of GSSG was not detectable neither in this particular urinary bladder sample (Fig. 1B) nor in all further urinary bladder samples studied in the present work; therefore, the validation of the method did not include determination of GSSG's precision, accuracy and limit of quantification. It is very likely that the concentration of GSSG in urinary bladder sample was below the detection limit. This assumption is supported by literature data indicating physiological ratio of reduced and oxidized form of glutathione even as high as 150:1 (Lakritz et al., 1997). However, as can be seen from Fig. 2, the GSSG standard gave maximal response between 700 an 800 mV, therefore, response at 3rd or 4th detector cell could be used for GSSG quantification.

Optimal potentials for LMWA detection were determined by injecting 10 μM antioxidants standard mixture at selected cells potentials within the range −200 to 800 mV. The cumulative response of antioxidants at defined potential is shown on Fig. 2. On the basis of these results the cells potentials were set at 300, 600, 700 and 800 mV (potential versus reference electrode) and response at 300 mV was chosen for AA and UA quantification and 700 mV for GSH quantification.

The antioxidants peak identification was performed by three different approaches. First, retention times of LMWA in standard solutions and in bladder tissue samples differed for less than 4.6%. Second, identity of tissue AA was determined with ascorbate oxidase, which oxidized AA to electro-non-active dehydroascorbate, while tissue GSH was identified with *N*-ethylmaleimide which is specific thiols alkylator. In each case the peaks of AA and GSH in tissue samples treated with ascorbate oxidase or *N*-ethylmaleimide were removed as can be seen on chromatograms on Fig. 3. Third, voltamograms of AA and GSH obtained from standard solutions and bladder tissue samples are comparable (Fig. 2), therefore, the identity of tissue AA and GSH is additionally demonstrated.

Results of HPLC method validation studies are presented in Table 1. Good precision and accuracy were found. Intra-day precision ranged from 0.6 to 4.4%, while the inter-day precision ranged from 3.0 to 9.4% for all antioxidants. The accuracy expressed as

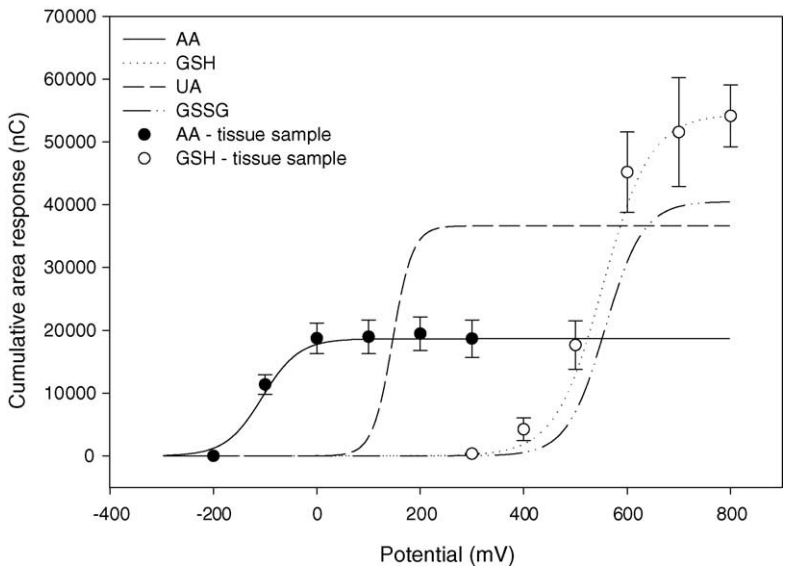

Fig. 2. Voltamograms for LMWA. The cumulative area response of the LMWA standards and tissue samples against potential. The lines present ($n = 3$) the fit of sigmoid function to average values of experimentally determined antioxidants standards responses. The experimentally obtained responses of the tissue AA or GSH were first relatively expressed against maximal response of tissue AA or GSH and afterwards multiplied with maximal response of AA or GSH standard, respectively and their average values and standard deviations are presented (points; $n = 3$). This calculation enable the acquisition of comparable values of standards and tissue antioxidants responses.

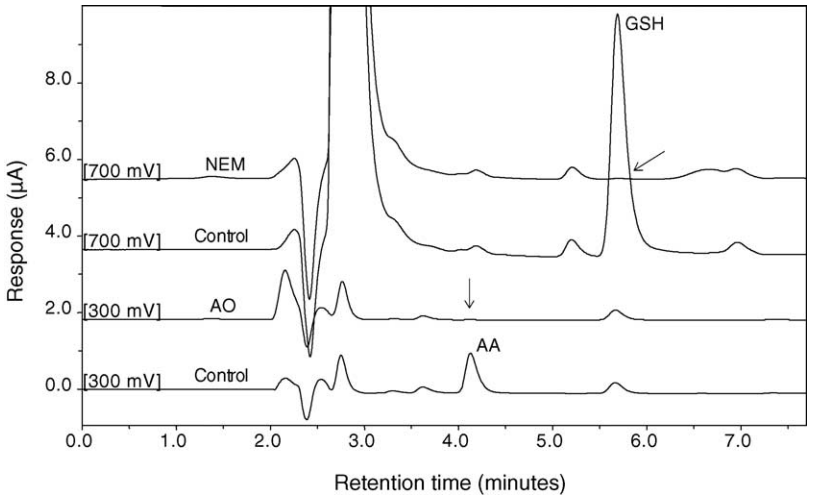

Fig. 3. The identification of AA and GSH peak in bladder tissue sample. Chromatograms obtained at 300 and 700 mV of the control and of the tissue bladder sample incubated with ascorbate oxidase (AO) or *N*-ethylmaleimide (NEM) are shown. The concentration of ascorbate oxidase and *N*-ethylmaleimide in tissue samples were 0.13 U/μl and 10 mM, respectively. The arrows indicate the missing peaks of AA and GSH.

relative error was in both studies below 12.9% except for the lowest concentration of GSH in inter-day study. Calibration curves in the studied concentration range were linear and correlation coefficient was always higher than 0.996. The limits of quantification of AA, GSH and UA were at 500, 50 and 10 nM concentration, respectively. The obtained antioxidants limits of quantification are comparable with those reported in the literature regardless that different authors use different definitions of these parameters what could influence on the calculated values. The reported limits of detection for AA are very variable and range from 100 to 1 nM (Ødum, 1990; Lykkesfeldt et al., 1995; Iwase, 2000), for GSH limits of detection (Smith et al., 1995) and quantification (Lakritz et al., 1997) are 20 nM and ∼40 nM, respectively, while for UA the reported limits of detection (Czauderna and Kowalczyk, 1997) and quantification (Inoue et al., 2003) are ∼400 nM and 20 nM, respectively. In preliminary extraction experiments different *ortho*- and *meta*-phosphoric acid solutions were used for extraction, however, only the *meta*-phosphoric acid solution prevented the oxidation of ascorbic acid. The AA and other antioxidants in bladder tissue samples extracted with *meta*-phosphoric

Table 1
Inter- and intra-day precision and accuracy of antioxidant standards at given concentrations

AA				GSH				UA			
QC (μM)	BCC (μM)	C.V. (%)	R.E. (%)	QC (μM)	BCC (μM)	C.V. (%)	R.E. (%)	QC (μM)	BCC (μM)	C.V. (%)	R.E. (%)
Intra-day precision and accuracy ($n = 3$)											
1	1.1 ± 0.02	1.5	8.4	0.1	0.1 ± 0.003	2.5	4.3	0.05	0.05 ± 0.002	4.4	3.1
10	9.4 ± 0.2	2.7	6.1	10	10.2 ± 0.1	0.6	1.6	1	1.0 ± 0.015	1.5	2.1
Inter-day precision and accuracy ($n = 5$)											
1	1.1 ± 0.05	4.4	12.9	0.8	0.9 ± 0.06	6.1	15.6	0.05	0.1 ± 0.005	9.4	2.6
10	9.5 ± 0.6	6.2	5.3	8	8.0 ± 0.3	4.1	0.5	0.5	0.5 ± 0.02	4.2	2.4
50	51.1 ± 2.5	5.0	2.3	48	48.7 ± 1.5	3.1	1.4	1	1.0 ± 0.02	3.0	2.0

Accuracy and precision were calculated as described in Section 2. QC, quality control standard sample; BCC, back calculated concentration of the sample; C.V., coefficient of variation and R.E., relative error.

Table 2
Recovery of urinary bladder LMWA extracted with *meta*-phosphoric acid

Spiked concentration (μM)			Recovery% ± S.D. ($n = 3$)		
AA	GSH	UA	AA	GSH	UA
34	15	0.42	109.3 ± 7.1	101.5 ± 1.2	101.8 ± 11.3
170	750	2.1	112.6 ± 1.6	102.1 ± 4.1	98.9 ± 2.3

Table 3
The distribution of LMWA in urinary bladder wall layers[a]

Urinary bladder wall layer	AA[b] (nmol/mg tissue wet weight)	GSH[b] (nmol/mg tissue wet weight)	UA[b] (nmol/mg tissue wet weight)
Urothelium	0.75 ± 0.11	2.7 ± 0.71	3.3E-03 ± 1.8E-03
Lamina propria	0.71 ± 0.12	1.3 ± 0.32	3.3E-03 ± 1.6E-03
Muscularis	0.35 ± 0.06	1.1 ± 0.28	2.1E-03 ± 3.4E-04
Serosa	0.18 ± 0.03	0.5 ± 0.13	2.6E-03 ± 1.3E-03

Mean LMWA concentrations and their standard deviations in particular bladder wall layer are shown. Experiments were performed on samples obtained from five different urinary bladders.

[a] $n = 5$.

[b] Average ± S.D.

acid were stable for 1 h after the extraction. Therefore, the recovery test was performed with *meta*-phosphoric acid as extraction medium and extracted antioxidants were immediately analyzed. The recoveries of LMWA in bladder tissue sample spiked with the known amount of LMWA are shown in Table 2. Good recoveries were obtained with values around 100%.

The method was applied to study the LMWA distribution in urinary bladder wall. The urinary bladder wall is composed of four tissue layers beginning with thin urothelium, lamina propria, muscularis and serosa. The isolated urinary bladder wall layers were analyzed and obtained concentrations are presented in Table 3. The concentrations of AA and GSH were decreasing from urothelium to serosa. On the other hand, the concentrations of UA were very variable for all studied layers. The obtained HPLC method provided new evidence of antioxidant distribution in urinary bladder wall layers and represents the appropriate method for indirect monitoring of the oxidative stress through LMWA concentrations even in tissues, which contain as low concentrations of LMWA as urinary bladder.

4. Conclusions

The HPLC method using electrochemical detector was developed for simultaneous determination of LMWA in urinary bladder tissue. The method involves antioxidants extraction and their stabilization with *meta*-phosphoric acid, as well as their separation using reversed phase chromatography and electrochemical detection. Different approaches for antioxidants peak identification in the bladder tissue samples were performed. Additionally, the method was applied to study the LMWA distribution in urinary bladder wall. We proved that the developed HPLC method was able to quantify simultaneously the reduced form of all three LMWA in all studied bladder wall layers. Moreover, the obtained method is simple, fast, and therefore, appropriate for simultaneous determination of LMWA concentrations even in tissues where their concentrations are low as in the urinary bladder wall. Finally, the experimental results demonstrated that concentrations of AA and GSH were decreasing from urothelium to serosa.

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