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Simultaneous high-performance capillary electrophoresis analysis of the reduced and oxidised forms of ascorbate and glutathione

Mark W. Davey, Guy Bauw, Marc Van Montagu*

Department of Genetics, Flanders Interuniversity Institute for Biotechnology, Universiteit Gent, K.L.Ledeganckstraat 35, B-9000 Ghent, Belgium

Abstract

We describe here a procedure for the simultaneous analysis of the oxidised and reduced forms of the major cellular hydrophillic antioxidants, ascorbic acid (vitamin C) and glutathione (γ-L-glutamyl-L-cysteinylglycine), by high-performance capillary electrophoresis. Separations are performed in uncoated fused-silica capillaries using 200 mmol/l borate pH 9.0, containing 20% (v/v) acetonitrile as the background electrolyte with fixed-wavelength UV absorbance detection at 185 nm. The influence of pH, organic solvent and other additives on the resolution of these compounds is described and we show that the optimised protocol is capable of simultaneously resolving other thiol components including, *N*-acetylcysteine and methyl-*S*-glutathione. The method is suitable for the analysis of these antioxidants in *Arabidopsis* and *Nicotiana* leaf tissue and is compatible with the use of the high ionic strength, acidic extraction solvents which are necessary to quench the redox equilibria of these labile components. ©1997 Elsevier Science B.V.

Keywords: Ascorbic acid; Glutathione; Dehydroascorbic acid; Glutathione disulphide

1. Introduction

The oxygenic environment of aerobic life inevitably leads to the production of a number of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), organic peroxides and radicals, as by-products of cellular metabolism. The presence of these ROS leads to the condition of oxidative stress, which if allowed to persist causes cell damage through lipid peroxidation and through alterations of protein and nucleic acid structure. Primary amongst the systems that exist to prevent or limit this oxidative damage are non-enzymatic antioxidants, such as glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine), ascorbate (L-AA, vitamin C) and α-tocopherol (vitamin E). In addition, there are also enzymatic defence mechanisms including superoxide dismutases (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase and other haemoprotein peroxidases.

The wide variety of cellular reactions in which L-AA and GSH are involved [1–4], as well as the importance of the ratio of oxidised/reduced forms as indicators of oxidative stress or pathological conditions, explains the great interest in the quantitative analysis of these compounds. However, to our knowledge there is only one publication dealing with the simultaneous determination of L-AA and GSH, by HPLC with electrochemical detection [5], and measurement of the oxidised forms (GSSG and L-DHA) with this method requires additional (separate) treatments and analyses. Rather, the majority of studies to date have employed completely different extraction and analytical methods for either L-AA/L-

*Corresponding author.

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DHA or GSH/GSSG analysis [6,7]. Clearly then, there are significant advantages to be gained in terms of labour and analysis times, with any system that is able to simultaneously quantify all of these compounds. The data we present in this article demonstrate that it is possible to obtain good resolution not only of the L-AA/L-DHA and GSH/GSSG pairs, but also of a number of other thiol compounds using a relatively simple background electrolyte (BGE) and uncoated fused-silica capillaries. We also show that the method is suitable for determining the antioxidant status of plant leaf tissue extracts, although difficulties remain in the quantitation of L-DHA levels because of the impurity of currently available commercial standards.

2. Experimental

2.1. Materials

Boric acid was purchased from Vel (Leuven, Belgium); 50% NaOH solution and D-isoAA were from J.T. Baker (Deventer, Netherlands); HPLC-grade methanol and HPLC-grade acetonitrile from Lab Scan (Dublin, Ireland); metaphosphoric acid (MPA), GSH, GSSG, L-AA, L-DHA, methyl-*S*-glutathione, *N*-acetyl cysteine and disodium EDTA from Aldrich Chemie (Brussels, Belgium). Water used was purified using a Millipore 'Nanopure' system. Polyimide-covered fused-silica capillaries (1 m long, 50 μm I.D., 393 μm O.D), cut according to requirements, and hydroxypropylmethylcellulose were obtained from Supelco (Sigma-Aldrich, Bornem, Belgium).

2.2. Instrumentation

All analyses were carried out on a Waters Quanta 4000 capillary electrophoresis system (Waters Associates, Milford, MA, USA), with fixed-wavelength UV absorbance detection at 185 nm or 254 nm. Injections were carried out hydrostatically by raising the inlet end of the capillary 10 cm relative to the outlet. Data was collected and analysed using a computer running the CE option of the Maxima 820 (version 3.3), chromatography and data analysis software (Waters Associates).

2.3. Electrophoretic conditions

Developmental work was carried out using a 40 cm to detector (47.5 cm total length)×50 μm I.D., uncoated fused-silica capillary. The analysis of biological extracts was carried out using 62.5 cm (70 cm total length)×50 μm I.D. Standard solutions were prepared in 3% metaphosphoric acid–1 mmol/l EDTA (MPA–EDTA, extraction solvent) and were injected hydrostatically for up to 50 s. The background electrolyte (BGE) consisted of 200 mmol/l boric acid adjusted to pH 9.0 with 50% NaOH solution and containing 20% (v/v) acetonitrile. This was prepared daily from a stock of 400 mmol/l borate pH 9.0, water and HPLC-grade acetonitrile. Separations were carried out under an applied voltage of +26 kV (47.5 cm total capillary length), which generated a current of around 85 μA, or at +30 kV (72.5 cm total capillary length) with a current of around 48 μA. Data were collected at a rate of 8 Hz, and the detector was set at a response time of 0.3 s. New capillaries were conditioned by purging under vacuum (17 mm Hg or 2200 Pa) for 15 min with 0.5 mmol/l LiOH, followed by water, and BGE at 24 kV for approximately 1 h. The regeneration of capillaries between successive analyses was carried out using a purge buffer, consisting of 100 mmol/l SDS, 100 mmol/l borate pH 9.0, 25% acetonitrile for 4 min and BGE for 6 min. The different buffer compositions examined in this work were prepared by diluting a stock of 400 mmol/l borate of the appropriate pH with nanopure water and the appropriate amount of additive (organic modifier, detergent, etc.), to give a final borate concentration of 200 mmol/l. A 0.01% solution of formamide was used as electroosmotic flow (EOF) marker. Changes in the migration behaviour of the standards were expressed as relative migration times (RMTs), i.e., migration time−migration time of the EOF.

2.4. Standard curves and detection limits

Standard solutions of GSH, GSSG, L-AA, D-isoAA, L-DHA, GS-Me and NAC were made up in ice-cold, helium-degassed MPA/EDTA at concentrations 1.00 mg/ml, and stored at 4°C for use on the same day. Serial dilutions of these stock solutions

were then made to estimate the minimum detection limits, set at approximately 3 times baseline noise.

2.5. Sample preparation

Aliquots of 50–200 mg of plant tissue were accurately weighed and snap frozen in liquid nitrogen. Samples were then crushed in liquid nitrogen using a pre-cooled pestle and mortar before addition of 0.4 ml extraction solvent (3% MPA/1 mmol/l EDTA). The sample with frozen buffer was thoroughly homogenised and allowed to stand on ice until the mixture thawed, whereupon it was transferred to a 2.0-ml Eppendorf tube with additional aliquots of extraction solvent. After centrifugation, at 14 000 rpm (20 800 *g*) for 7 min, the extraction volume was accurately weighed by transfer to a 2.0-ml Eppendorf tube. The pellet was resuspended in fresh extraction buffer and centrifuged as before. The supernatant was weighed again and combined with the first extract, and the total extract filtered through a disposable PVDF 0.22-μm filter and kept at 4°C in the dark prior to analysis. Samples were stored frozen at −20°C or −70°C.

3. Results and discussion

We have recently published a procedure for the analysis of L-AA in plant tissue based on extraction with 3% MPA/1 mmol/l EDTA and free solution capillary electrophoresis (FSCE) in 200 mmol/l borate buffer pH 9.0 [8], and we were interested in extending this work to allow the simultaneous quantitation of not only L-AA, but also L-DHA, GSH and GSSG, for the reasons discussed in the introduction. Experiments were carried out using the following mixture of pure standards dissolved in extraction buffer, 0.15 mg/ml L-AA, 0.1 mg/ml D-isoAA, 0.1 mg/ml GSH, 0.2 mg/ml GSSG and 0.2 mg/ml L-DHA, unless otherwise indicated.

3.1. Influence of organic modifiers on resolution

In 200 mmol/l pH 9.0, there was essentially no resolution of GSH and GSSG, although these components were well separated from L-AA and the slow-migrating L-DHA peaks. As summarised in Fig. 1A, the inclusion of up to 15% (v/v) methanol, significantly decreased the EOF, but had virtually no effect on the resolution of the compounds. By comparison, even at low concentrations, the addition of acetonitrile had a very dramatic effect, leading to baseline resolution of all compounds (including D-isoAA), at concentrations as low as 5% (v/v). These results are summarised in Fig. 1B and typical electropherograms are shown in Fig. 2.

The addition of tetrahydrofuran as organic modifier had a similar effect to acetonitrile, but its high UV absorbance at the detection wavelength leads to a loss of detection sensitivity and it is therefore unsuitable for use in this analysis.

It is interesting to speculate on the possible reasons for the different effects of methanol and acetonitrile on sample mobilities. Clearly, it cannot be simply a matter of hydrophobicity since even low concentrations of the less hydrophobic solvent (acetonitrile) significantly improved the resolution between the GSH/GSSG pair. Although methanol is known to decrease the EOF, probably by hydrogen bonding with free silanol groups on the capillary wall surface, and acetonitrile is known to increase the EOF [9], it is unclear how significant these effects will be at the high buffer concentrations used here. Indeed, increasing concentrations of acetonitrile had very little influence on the EOF (see Fig. 1). Rather, the major effect of the organic modifiers is likely to be on the suppression of the dissociation of the analytes. The differences between the two organic solvents in resolving the GSH/GSSG pair are therefore probably related to the fact that methanol primarily interacts with solutes as a proton acceptor, whereas acetonitrile forms mainly dipole interactions. As discussed in Section 3.3, resolution between GSH and GSSG is due to ionisation of the free sulphydryl of GSH and one explanation for the lack of resolution observed in the presence of methanol could be masking of this weakly acidic group through hydrogen-bond formation with methanol.

3.2. Effect of other additives

The inclusion of up to 100 mmol/l SDS in the 200 mmol/l borate pH 9.0/20% (v/v) acetonitrile BGE, caused a loss of resolution between GSH and GSSG (results not shown). Furthermore, as discussed by

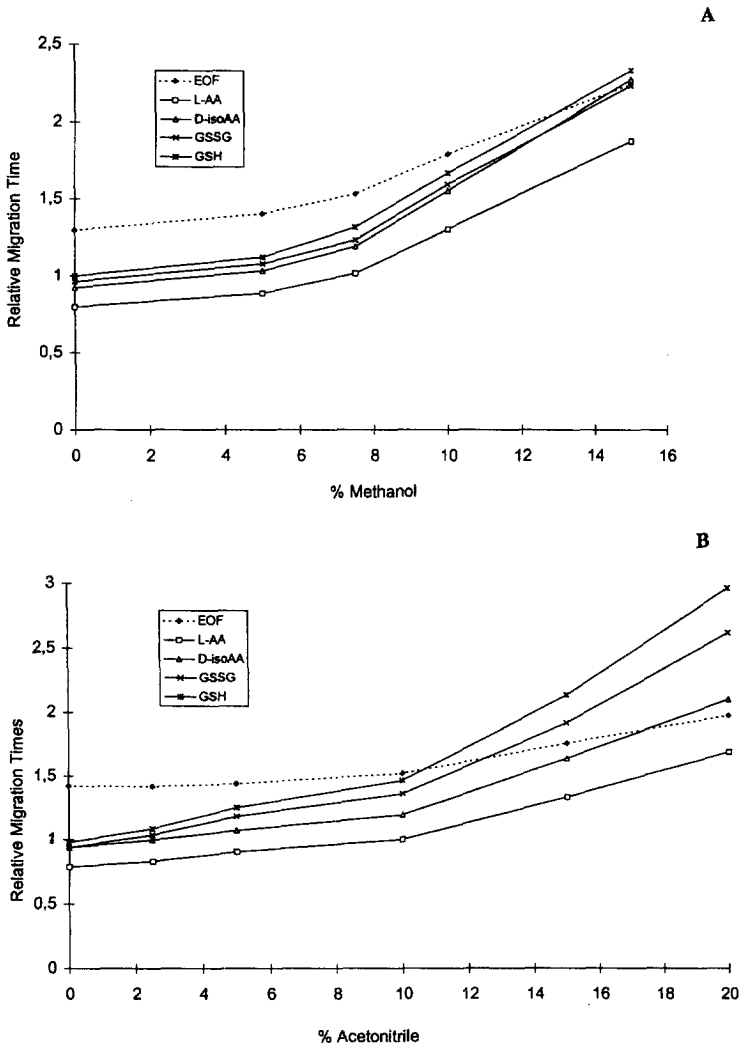

Fig. 1. Influence of proportion of organic modifier present in the BGE on the resolution of L-AA, D-isoAA, GSH and GSSG. Results expressed as RMTs (migration time of analyte−migration time EOF marker), except for EOF values which are expressed as absolute migration times. Analyses performed in a 40 cm (to detector)×50 μm fused-silica capillary at +26 kV (547 V/cm) and UV detection at 185 nm. Data collection rate of 8 Hz, with detector response time of 0.3 s rise. BGE consists of 200 mmol/l borate pH 9.0 with the indicated proportion of HPLC-grade methanol (A) or acetonitrile (B); 20-s hydrostatic injection.

others [10], the inclusion of SDS reduces the peak capacity of the system. The addition of 0.03% hydroxypropylmethylcellulose to modify wall interactions and reduce the evaporation of acetonitrile from the mobile phase did not improve resolution (results not shown).

3.3. Influence of BGE pH

The influence of the pH of the BGE on sample resolution is summarised in Fig. 3. The resolution between L-AA and D-isoAA gradually increases with the pH in a uniform manner over the range 8.25–9.5.

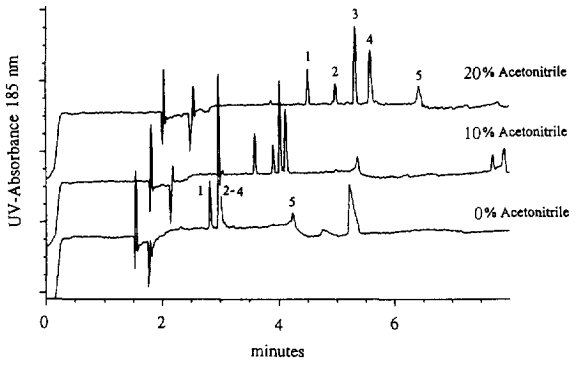

Fig. 2. Influence of the proportion of acetonitrile in 200 mmol/l borate pH 9.0, on the electropherograms obtained for a standard mixture of 0.15 mg/ml L-AA, 0.10 mg/ml D-isoAA, 0.1 mg/ml GSH, 0.2 mg/ml GSSG and 0.2 mg/ml L-DHA dissolved in 3% MPA–EDTA. Peaks labelled 1–5, respectively; 20-s hydrostatic injection. All other conditions as in Fig. 1.

As discussed previously [8], these changes represent the balance of the influence of pH on the EOF (through ionisation of the silanol groups of the capillary wall) and the shift in equilibrium of the borate–ascorbate complex (see also Refs. [11–13]). The gradual increase in RMTs (and resolution) of L-AA and D-isoAA at higher pH therefore results from the greater proportion of analyte that is complexed with borate. These anionic complexes migrate in the opposite direction to the EOF, with the result that their migration through the capillary is retarded. L-DHA was always found to migrate much more slowly than its reduced counterpart and was easily resolved from the other components, suggesting that it is able to interact strongly with boric acid.

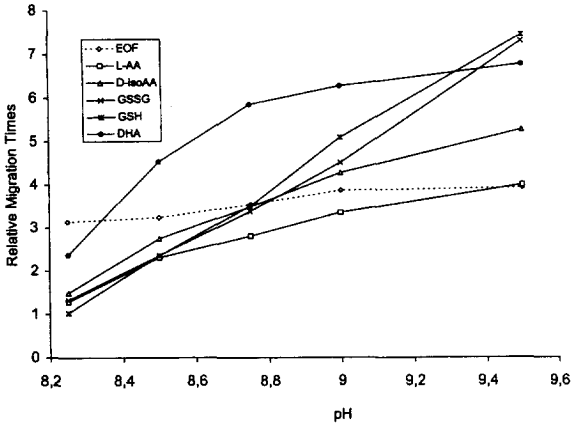

Fig. 3. Influence of pH of 200 mmol/l borate–20% (v/v) acetonitrile, on resolution of standards. All other conditions as in Fig. 1.

The pH of the BGE has a much more profound effect on the RMTs of GSH and GSSG relative to L-AA. This is probably due to changes in the ionisation of the terminal glutamate α-amino function (pK_a approximately 8.20, using the 'adjusted' ionisation constants reported in [14]). In other words, as the pH increases there is proportionally less of the amino function ionised, slowing the rate of cathodic migration and increasing RMTs. The dominant effect still remains one of anodic migration however as a result of the sum of the glycinyl α-carboxyl function and the glutamate terminal α-carboxyl group, both of which have pK_a values of around 3.20 [14]. Therefore within the pH range pH 8.25–9.5, GSH/GSSG will always be negatively charged and will tend to move against the EOF, which is still sufficiently strong to carry the analytes past the detector. The size of this net negative charge will increase slightly as the pH increases and as the ionisation of the terminal glutamate α-amino function decreases, explaining the increase in RMTs. The resolution observed between the GSH/GSSG pair, presumably arises from ionisation of the free sulphydryl group of GSH (pK_a approximately 10.3 [14]), which increases as the pH of the BGE increases. Therefore at higher pH values, GSH has a higher charge/mass ratio and migrates faster than GSSG towards the anode, and consequently has a longer RMT. As the pH decreases however, this effect becomes much less significant. As discussed earlier, methanol may mask this charge with the result that there was no resolution observed between GSH and GSSG.

The standardised conditions consisting of a BGE composition of 200 mmol/l borate pH 9.0–20% (v/v) acetonitrile at an applied voltage of +26 kV, also enabled simultaneous resolution of other thiol components including GSH-Me and NAC (Fig. 4). Although NAC tended to co-elute with D-isoAA (data not shown), either compound is potentially suitable for use as an internal standard as none of our

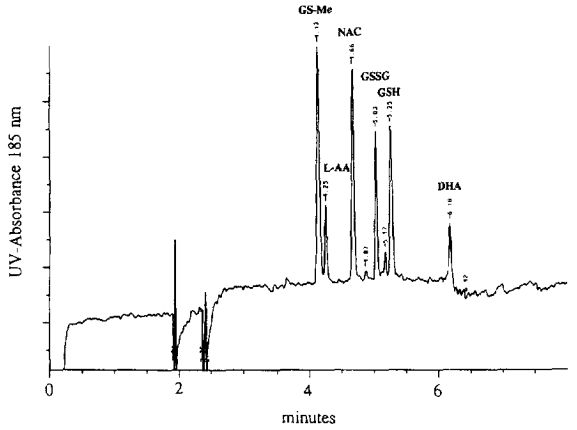

Fig. 4. Simultaneous resolution of 0.1 mg/ml L-AA, 0.2 mg/ml L-DHA, 0.1 mg/ml GSSG, 0.1 mg/ml GSH, 0.2 mg/ml Me-GSH and 0.1 mg/ml NAC. All other conditions as previously described in Fig. 1.

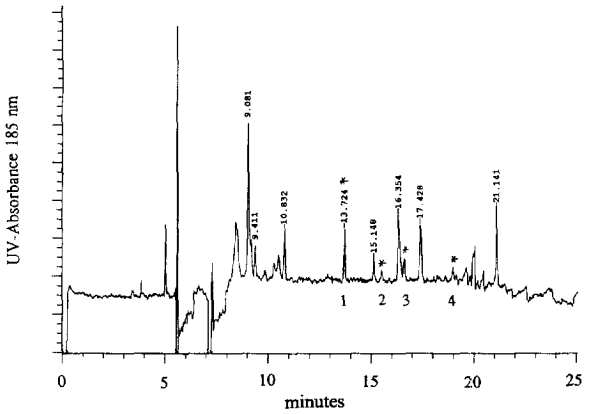

Fig. 5. Analysis of 3% MPA–EDTA extract of *Arabidopsis* leaf tissue. Extraction of 104 mg fresh weight leaf tissue, with a total of 900 μl extraction solvent. Analysis carried out in 62.5 cm×50 μm uncoated fused-silica capillary with UV absorbance detection at 185 nm. Data were collected at a rate of 8 Hz; 40-s hydrostatic injection; applied voltage, +30 kV (429 V/cm). Peaks with asterix labelled as follows: 1=L-AA; 2=GSSG; 3=GSH; and 4=L-DHA.

biological extracts contained detectable levels of NAC or D-isoAA.

3.4. Minimum detection limits

Detection limits, for the 50 μm capillary, set at approximately 3 times baseline noise were 0.005 mg/ml, or 28 μmol/l, for L-AA in extraction buffer (corresponding to a 40 s injection time). Linearity of detector response was observed between 0.01 and 0.5 mg/ml. For both GSH and GSSG, the minimum detection limits were in the region of 0.002 mg/ml (6.5 μmol/l). It was not possible to obtain accurate values for L-DHA because of apparent impurities in the commercially available standards.

3.5. Analysis of biological extracts

Extracts were prepared as described in Section 2.5, but the length of the capillary was increased from 47.5 to 72.5 cm total length. The increase in length was necessary to resolve several peaks migrating close to GSH/GSSG, the increased resolution being due to the higher efficiencies that result from the proportionally smaller injection plug. As can be seen in Fig. 5, these conditions produced good resolution in the region of elution of the antioxidants of interest and quantitation of L-AA and of GSH/GSSG is straightforward. As mentioned earlier, the lack of a pure L-DHA standard means that quantitation of this compound is difficult, particularly as we observed the formation of multiple degradation products with time. Under normal growth conditions, DHA and GSSG represent about 10–30% of the values of their respective reduced forms, and this can make detection difficult when the amount of tissue available is small. However, reduction of samples with DTT or with NADPH and glutathione reductase allows determination of the 'total L-AA' and 'total GSH' content of the sample, from which L-DHA/GSSG can be calculated by subtraction. Positive peak identification was made by spiking biological extracts with pure individual standards in extraction solvent.

4. Conclusions

A number of procedures for the estimation of thiol compounds by HPCE have been described in the literature. The classical approach used for the HPLC analysis of thiols involving pre-derivatisation with monobromobimane [15–17] has also been adapted to HPCE [18,19]. While monobromobimane derivatisation followed by HPLC-fluorescence detection is a more sensitive procedure than the method outlined

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Short communication

High-performance capillary electrophoretic analysis of hyaluronan in effusions from human malignant mesothelioma[1]

Nikos K. Karamanos[a], Anders Hjerpe[b,*]

[a]*Section of Organic Chemistry, Biochemistry and Natural Products, Department of Chemistry, University of Patras, 26110 Patras, Greece*

[b]*Department of Immunology, Microbiology, Pathology and Infectious Diseases, Division of Pathology, Huddinge University Hospital, Karolinska Institute F-42, S-141 86 Huddinge, Sweden*

Abstract

A procedure to quantify hyaluronan in effusions from human malignant mesothelioma using a highly sensitive and reproducible high-performance capillary electrophoresis (HPCE) method is presented. Following ethanol precipitation, hyaluronan and galactosaminoglycans were degraded to $\Delta^{4,5}$-disaccharides with a mixture of chondroitinases ABC and AC. Heparan sulphate and proteins/glycoproteins were separated by ultrafiltration on a Centricon 3 membrane, and hyaluronan-derived disaccharides were analysed by direct injection of the filtrate into a HPCE system. Determination of hyaluronan in effusions from five healthy individuals and three patients with mesothelioma gave values comparable to those found using the HPLC method. One of the advantages of the HPCE method as compared to HPLC is the low solvent consumption. The much lower detection limit (attomole level) of the HPCE method may also allow the analysis of hyaluronan content in serum. The contribution of HPCE in diagnosis of a neoplasm, such as human malignant mesothelioma, illustrates the great potential of this technique in the field of life sciences. ©1997 Elsevier Science B.V.

Keywords: Hyaluronan

1. Introduction

Human malignant mesothelioma (MM) is a neoplasm arising from mesodermally derived multipotential mesothelial cells. MM is mainly located in the large serosal cavities, such as pleura, peritoneum and pericardium. Due to the multidirectional differential capabilities of mesothelioma cells, MM shows a wide spectrum of histological patterns. Therefore, differential diagnosis from other tumours is often difficult (for an excellent book on mesothelial and mesothelioma cells see Ref. [1]). MMs produce substances that are usually present in the matrix of mesenchymal tissues [2–4]. Such a mesenchymal marker, found in extracellular matrix of most connective tissues, is hyaluronan (HA), which belongs to the glycosaminoglycan (GAG) family. HA is composed of the non-sulphated repeating disaccharide unit: →4D-glucuronic acid (β1→3) N-acetyl-glucosamine β1→ [5]. Patients with malignant mesothelioma often present effusions containing large amounts of HA. This secretion of excessive amounts of hyaluronan has been used for the diagnosis of MM [6–8]. Histochemical evaluation of MM using cationic dyes [9,10] is of limited useful-

*Corresponding author.

[1] With the present paper the authors wish to honour Professor Christos Antonopoulos on his 65th birthday for his significant contributions to the field of carbohydrate chemistry.

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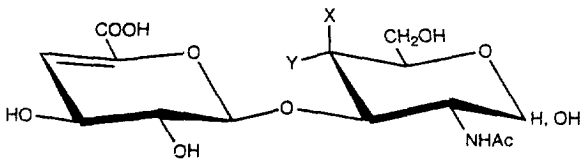
ness, due to the water solubility of HA during the staining procedure. Therefore, the determination of HA by chemical and/or enzymatic methods is more reliable. Among the methods described for HA analysis in effusions [11–13], the HPLC method reported by Hjerpe [11] and Nurminen et al. [13] takes an excellent position among the clinically useful methods, having simplicity, high specificity, accuracy and reproducibility.

HA and the galactosaminoglycans, chondroitin sulphate (CS) and dermatan sulphate (DS), are susceptible to digestion with chondroitinases ABC and AC, whereas other GAGs present in MM effusions, such as heparin and heparan sulphate are not degraded by these eliminases [5]. The disaccharide composition of HA and galactosaminoglycans is readily analysed following the digestion of the polysaccharides with chondroitinases ABC, AC and/or B, with or without simultaneous digestion with chondro-4- or 6-sulphatases [14–16]. The structure of the HÁ- and galactosaminoglycan-derived non-sulphated $\Delta^{4,5}$-disaccharides obtained after digestion with chondroitinases and chondrosulphatases is shown in Fig. 1.

These disaccharides have been analysed by HPLC [17–21] and capillary zone electrophoresis [22–24]. The HPLC methods have been widely and successfully used to analyse HA and galactosaminoglycan disaccharides in MM effusions, while the HA content of serum is often too low.

Capillary electrophoresis is a powerful technique which may offer an alternative method due to its high resolution, low sample and solvent consumption and high sensitivity. During the last decade, HPCE has been used for the analysis and structural characterisation of various types of glycoconjugates (for a review see Ref. [25]). Our group has recently described a reversed-polarity HPCE method for the analysis of HA- and galactosaminoglycan-derived Δ-disaccharides, using specific degradation of the polysaccharides with chondroitinases [26]. UV absorbance detection for these Δ-disaccharides at 232 nm enables the detection of attomole levels of these polysaccharide constituents [26].

In this paper, we describe a procedure by which the HA and galactosaminoglycan composition in effusions from human malignant mesothelioma can be analysed by an HPCE method of reversed polarity. The determination of HA in MM is performed within 14 min with high sensitivity and accuracy.



(1): X=H, Y=OH

(2): X=OH, Y=H

Fig. 1. Structure of the non-sulphated Δ-disaccharides of hyaluronan (1), and chondroitin sulphate and/or dermatan sulphate (2), produced by digestion with chondroitinases and chondrosulphatases.

2. Experimental

2.1. Chemicals and biological material

Standard preparations of Δdi-nonS_{HA}, 2-acetamido-2-deoxy-3-O - (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose, Δdi-nonS_{CS}, 2-acetamido-2-deoxy-3-O - (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose and the variously mono-, di- and trisulphated Δ-disaccharides were purchased from Seikagaku Kogyo (Tokyo, Japan). Chondroitinases AC and ABC were also obtained from Seikagaku. CSA from whale cartilage (grade I) and HA from human umbilical cord were obtained from Sigma (St. Louis, MO, USA). The non-sulfated CS (chondroitin) was prepared from squid skin as previously described by Karamanos et al. [27]. Centricon 3 membranes were obtained from Amicon (Beverly, MA, USA). All other chemicals used were of analytical-reagent grade. Membrane filters (0.2 μm) were purchased from Millipore (Waters, Milford, MA, USA).

The collection of biological material and the procedure followed was in accordance with the ethical standards of the Helsinki Declaration of 1975. The effusions were sent for HA analysis to the laboratory for Clinical Cytology at the Department of