here, it is time-consuming and may suffer from incomplete derivatisation and high backgrounds arising from hydrolysis of excess reagent and impurities [20]. It is furthermore completely incompatible with ascorbate analysis, as the pH adjustments required for derivatisation will cause spontaneous oxidation and degradation of L-AA and L-DHA, respectively. Similar comments apply to the HPCE-analysis of *N*-ethylmaleimide-blocked thiols [21], and several other approaches involving derivatisation, e.g., during pre-derivatisation for HPCE with 1,1'-[ethenylidenebis(sulfonyl)]bisbenzene, [22], or with 2,3-naphthalenedicarboxyaldehyde [23].

Other published procedures for GSH or thiol analysis in which there is no sample derivatisation [24,25], are incompatible with the acidic, high ionic strength extraction solvents used in this study. Still other methods require electrochemical detection which is not a widely available commercial detection system for HPCE [26–28], although there are numerous publications detailing the HPLC analysis of thiols with electrochemical detection [29–34]. We are aware of only one instance in which the simultaneous analysis of L-AA and GSH has been reported [5], but in this method additional separate handling is required for GSSG (NADPH-dependent reduction with glutathione reductase) and L-DHA (DTT reduction) measurement.

It is vital when dealing with reactive constituents such as L-AA and GSH to quench the equilibrium between the reduced and oxidised components during extraction and to prevent further oxidation to L-DHA and GSSG. As discussed in Refs. [35–37], 3% MPA is the most efficient extraction and stabilisation solvent for L-AA analysis and, as noted by others, acid quenching involving MPA is the most rapid means of inactivating γ-glutathione-*S*-transferases and fixing the GSH/GSSG equilibrium during extraction [38,39]. In addition, the use of an acid such as MPA efficiently precipitates proteins which would otherwise interfere in the subsequent analyses. Work in our laboratory has shown that both L-AA and GSH are essentially stable for several hours in this solvent at 4°C (unpublished data). The use of perchloric acid by comparison has been reported to produce massive GSH oxidation in erythrocyte extracts [40], and the use of trichloroacetic acid causes a 30% drop in the yields of ascorbate [8,35,41,42]. For these reasons we concentrated on developing an HPCE method which could accommodate the use of 3% MPA/1 mmol/l EDTA as extraction solvent. The work presented in this paper demonstrates that it is possible to obtain good resolution between L-AA/L-DHA and GSH/GSSG with this extraction solvent and that the method can be applied to the determination of the antioxidant status of *Arabidopsis thaliana* leaf tissue, *A. thaliana* cell suspension culture and *Nicotiana tabacum* (data not shown). While detection limits are not as low as described in some other publications, this can possibly be improved by the use of more sophisticated detection systems and increasing the quantity of tissue extracted.

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Pathology, Huddinge University Hospital, Sweden. Samples were collected in test tubes without any preservatives and, before transport to the laboratory, one volume of 50% (v/v) aqueous ethanol was added to the samples, as previously described [13].

2.2. Sample treatment

A 200-μl volume of absolute ethanol was added to a 40-μl sample and the precipitation was completed by keeping the mixture at 4°C for 30 min. The precipitate was recovered by centrifugation in a Beckman microfuge (11 000 *g* for 5 min) and the HA and galactosaminoglycans were digested by chondroitinases ABC and AC (0.1 unit/ml of each enzyme) in a 25 m*M* Tris–HCl (100 μl) solution that was buffered at pH 7.5, for 90 min at 37°C [14–16]. Removal of the non-degraded heparan sulphate and proteins/glycoproteins was performed following ultrafiltration of the digestion mixture by centrifugation (11 000 *g* for 10 min) on a Centricon 3 membrane (cut-off 3000 Da). A 5–10 μl portion of the filtrate was then taken for direct determination of HA-derived disaccharides by HPCE. In parallel, aliquots of 20 μl from the filtrate were analysed by HPLC [13].

2.3. Analysis of HA by HPCE

Capillary electrophoresis was performed using a Beckman HPCE instrument (P/AGE system 5510), equipped with a diode array detector with a window of 800×100 μm, set at 232 nm. The analysis of HA was carried out using an uncoated fused-silica capillary tube (75 μm I.D., 55 cm total length and 50 cm from the injection point to the detector) at 25°C. Before each run, the capillary tube was washed with 0.1 *M* NaOH for 1 min and then with the operating buffer for 4 min. The samples to be analysed were injected automatically by using the pressure injection mode in which the sample was pressurised for 10 s. The injection volume can be estimated by the Poiseuille equation, as proposed by the manufacturer (Beckman), giving an estimated volume of 6 nl per second of injection time. For optimal separation, the electrophoresis was performed under previously described conditions [26], using 15 m*M* sodium phosphate, pH 3.00, as the operating buffer, at 20 kV and by reversing the electrodes so that the disaccharides would migrate from the negative (cathode) to the positive (anode) electrode. Peak areas were recorded and evaluated using the Beckman software system Gold V810. Quantitation of the Δ-disaccharide content in samples was performed using precisely known amounts of Δdi-nonS_{HA} dissolved in chondroitinase digestion buffer to give working standard solutions of 0.1, 1.0, 10, 50 and 100 ng/l and 0.5, 10, 25, 50 and 100 μg/l. Before use, the operating buffer was degassed by vacuum filtration through a 0.2-μm membrane filter, followed by agitation in an ultrasonic bath.

3. Results and discussion

3.1. HPCE determination of HA

The determination of HA in effusions was based on the analysis of Δdi-nonS_{HA} produced by digestion with chondroitinases ABC and AC. Since these enzymes cleave the galactosaminoglycans (CS/DS), the separation between Δdi-nonS_{CS} and Δdi-nonS_{HA} is essential for the determination of HA in clinical samples. As shown in Fig. 2, using reversed-polarity HPCE and 15 m*M* phosphate operating buffer, pH 3.00, the separation between Δdi-nonS_{HA} and Δdi-nonS_{CS} was complete within 14 min. The migration of these disaccharides as double peaks is due to the presence of α- and β-anomeric forms, which, however, do not interfere with the separation. Quantification was based on the area of both anomeric peaks. Sulphated Δ-disaccharides (mono- and oversulphated ones), produced by the degradation of galactosaminoglycans by chondroitinases, did not interfere with the HA analysis, since they migrated much earlier (3–7 min), as evaluated by the migration of the various commercially available standard sulphated Δ-disaccharides. The higher electrophoretic mobility of sulphated disaccharides is also in accordance with their higher charge density.

The sensitivity and the linearity of the method were tested by measuring the peak areas of standard Δdi-nonS_{HA} at various concentrations. The peak areas were found to be linearly related to the amount of the injected disaccharide, up to 10 μg/l, i.e., the entire interval tested. The detection limit for Δdi-

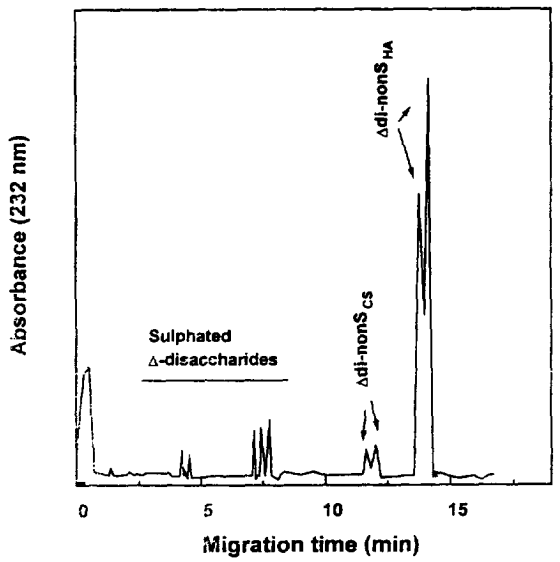

Fig. 2. HPCE profile of HA- and galactosaminoglycan-derived Δ-disaccharides obtained by chondroitinase digestion of a pleural effusion from a patient with mesothelioma. HPCE was performed using a 15 m*M* phosphate buffer, pH 3.00, with reversed polarity at 20 kV.

$nonS_{HA}$ (molar extinction coefficient of 5500 M^{-1} cm^{-1}), estimated as twice the baseline noise, which was as small as $1 \cdot 10^{-5}$ AU, corresponds to approximately 100 ng/l (250 pmol/l). By performing three injections within a 95% confidence interval and using an injection interval of 10 s, as little as 500 fg of HA is required. The loading, however, of the injector with an aliquot of approximately 5 μl would give a similar 95% accuracy when the sample contains approximately 100–250 ng/l. This sensitivity is also considerably higher on a concentration basis than that of the previously reported HPLC procedure [13].

3.2. Applications to the analysis of HA in effusions

In this study, three patients with pleural MM and five cases with effusions from other causes were analysed by HPCE and the values compared to those obtained by the standard HPLC procedure. As shown in Table 1, the two analytical techniques gave virtually the same results. The determined amounts of HA-derived glucuronic acid (GlcA) in patients with mesothelioma by HPCE indicated that the HPCE analysis is in accordance with the HA-derived GlcA cut-off value of 75 μg/ml (100% specificity and 56% sensitivity), as previously proposed for HPLC by Nurminen et al. [13].

Table 1
Hyaluronan content in effusions determined by HPCE and HPLC[a]

	HPCE	HPLC
Mesothelioma patients	45	46
	89	90
	323	318
Non-mesothelioma patients	1.6	1.7
	2.9	2.8
	9.3	9.3
	9.3	9.5
	16.8	18.6

[a] Results are expressed as the amount of HA-derived GlcA in μg/ml effusion and are the average of three experiments in triplicate. Variations in the amounts of disaccharides measured were less than 5% in all cases.

The obtained results indicate that HPCE can be used as an alternative to HPLC for the analysis of hyaluronan in effusions. The sensitivity obtained by this HPCE procedure is sufficiently high [26] to allow the analysis of HA in serum as well. The analysis of a large series of samples is, however, necessary before the procedure can be recommended for routine diagnostic use.

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