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DETERMINATION OF THE URONIC ACID COMPOSITION OF ALGINATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

GEOFFREY ANNISON*
School of Microbiology, The University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)
NORMAN W. H. CHEETHAM
School of Chemistry, The University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)
and
IAIN COUPERWHITE
School of Microbiology, The University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)
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SUMMARY

A high-performance liquid chromatographic (HPLC) method to determine the D-mannuronate/L-guluronate (M/G) ratio in alginates is described. Comparison between the HPLC procedure and a proton magnetic resonance method is made. The HPLC procedure can be used to determine the absolute M/G ratio in some sample types. In others it needs calibration against a method such as proton magnetic resonance.

INTRODUCTION

Alginic acid, a commercially important algal polysaccharide is a co-polymer of (1 → 4) linked α-L-guluronic acid and (1 → 4) linked β-D-mannuronic acid. The residues are arranged in irregular blocks along the linear chain[1]. Considerable geographical and species variations occur in the proportions of mannuronic and guluronic acids (M/G ratio) and this has a profound effect on the physical properties of the polymer, and hence its industrial applicability. A reliable and convenient means of determining the M/G ratio has been sought for many years. In the past methods have included total acid hydrolysis followed by ion-exchange chromatography[2], colourimetry[3], infra-red spectroscopy[4–7], polarimetry[8], gas chromatography[9] and enzymic degradation[10,11]. These methods are either too unreliable or tedious to perform. More recently ^{1}H and ^{13}C nuclear magnetic resonance (NMR) have been used to determine M/G ratios[12,1]. These techniques are both fast and accurate but due to the expensive and complex equipment required cannot be considered to be useful for routine alginate analysis.

The analysis of polyuronic acids by high-performance liquid chromatography

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(HPLC) has been described[13]. The iduronic acid/glucuronic acid (I/G) ratios of mixtures of chondroitin sulphate and dermatan sulphate were determined following a methanolysis procedure. The uronic acids were chromatographed as their methyl ester methyl glycosides. Correct I/G ratios, however, could only be determined by relating the results to standard preparations of known uronic acid composition.

In this paper the analysis of the methanolysis products of alginic acid by HPLC as a means of determining M/G ratios is described.

EXPERIMENTAL

The HPLC system consisted of the following Waters Assoc. (Milford, MA, U.S.A.) instruments: M6000 pump, U6K injector, R401 refractive index detector and a radial compression Z module. Quantitation was achieved with a Waters Assoc. 703 data module. The column was a Waters Dextropak plastic cartridge, 10 × 0.8 cm which for use was compressed in the Z module. Distilled water was used as eluent at a flow-rate of 2.5 ml/min. Methanolysed mannuronolactone (Sigma) was used as a standard. Injection volumes were 20 μl.

^{1}H NMR spectra were obtained with a Bruker CXP300 nuclear magnetic resonance spectrometer operating at 300 MHz. A sweep width of 3000 Hz was used with 8K data points and line broadening of 0.5 Hz. Spectra were recorded at 80°C. The NMR method was checked by using alginate fractions of known M/G ratio, kindly provided by Dr. B. Larsen, Institute of Marine Biochemistry, Trondheim, Norway.

Methanolic hydrochloric acid (1 *M*) was prepared by adding acetyl chloride to dry methanol.

The five alginate samples analysed (samples A–E) were all obtained from Reckitt and Colman Australia.

Preparation of alginate samples for HPLC analysis

Alginates were converted to their acid forms using the method of Haug[14].

Samples of alginic acid (40 mg) were partially hydrolysed in screw-top test tubes (4 ml 0.3 *M* hydrochloric acid, 100°C, 2 h with agitation). Partial hydrolysates were evaporated to dryness at room temperature and residues were methanolysed (8 ml, 1 *M* methanolic hydrochloric acid, 100°C, 24 h with agitation) in sealed glass ampoules. The reaction mixture was centrifuged (bench centrifuge). The supernatant was removed and neutralised with Amberlite IRA-400 (HCO_3^-) resin. The methanol was removed by evaporation (hot air oven, 50°C). The residues were resuspended in distilled water (250 μl), filtered (0.2-μm Millipore) and analysed by HPLC.

Preparation of samples for ^{1}H NMR analysis

The method of analysis of alginates by ^{1}H NMR was based on that of Grasdalen *et al.*[12].

A 2–3-mg amount of sample was dissolved in ^2H_2O (0.4 ml). The solutions were adjusted to p^{2}H 7 with NaO^{2}H. EDTA was added in order to complex any divalent cations.

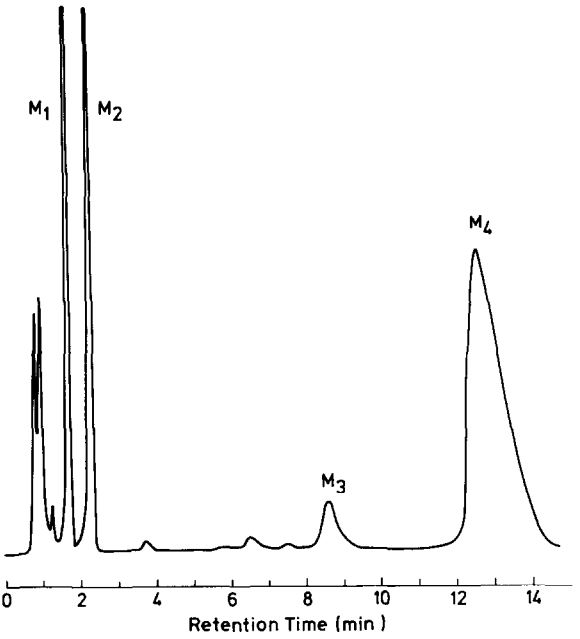

Fig. 1. HPLC of methanolysis products of D-mannuronolactone. Column: Dextropak. Flow-rate: 2.5 ml/min (water).

Preparation of mixtures of polymannuronic and polyguluronic acid

Sodium alginate (Sigma) was converted to the acid form. Fractions rich in guluronic acid (G fraction) and mannuronic acid (M fraction) were prepared using the method of Haug *et al.*[15]. Their uronic acid composition was determined by ^{1}H NMR. Samples of the two fractions and mixtures of differing proportions of each fraction were methanolysed as described previously, and analysed by HPLC.

Determination of M/G ratios

M/G ratios of samples were determined from NMR spectra by comparing the areas of resonance assigned to the M anomeric protons with those assigned to the G anomeric protons.

M/G ratios were determined from HPLC by comparing M peak areas (M_1, M_2 and M_4) with G peaks area (G_{1-4}).

RESULTS AND DISCUSSION

In the HPLC profile of the methanolysis products from D-mannuronolactone, four major peaks (M_{1-4}) are observed (Fig. 1). Earlier peaks in or near the void volume could be the methyl esters of mannuronic acid and free mannuronic acid[16]. The minor peaks may also be from M but are more likely from G as the mannuronolactone was derived from alginic acid. By analogy with methanolysis studies of glucuronic acid[16], M_1 and M_2 are expected to be the lactone glycosides. Similarly M_3

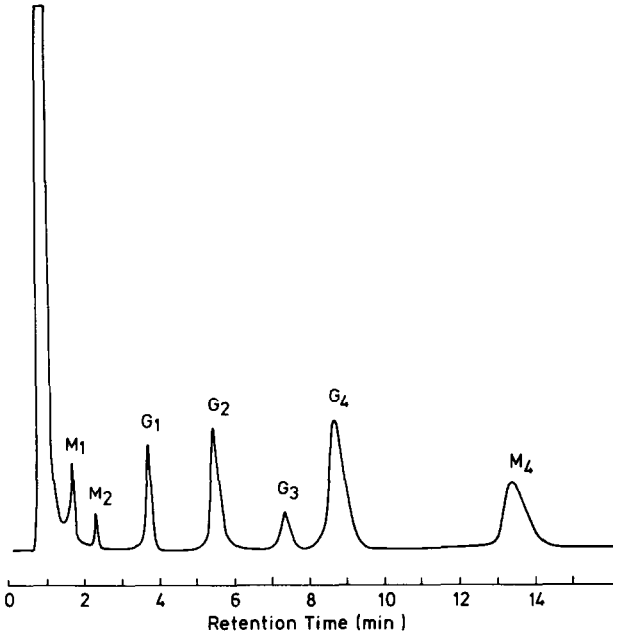

Fig. 2. HPLC of methanolysis products of alginate G fraction.

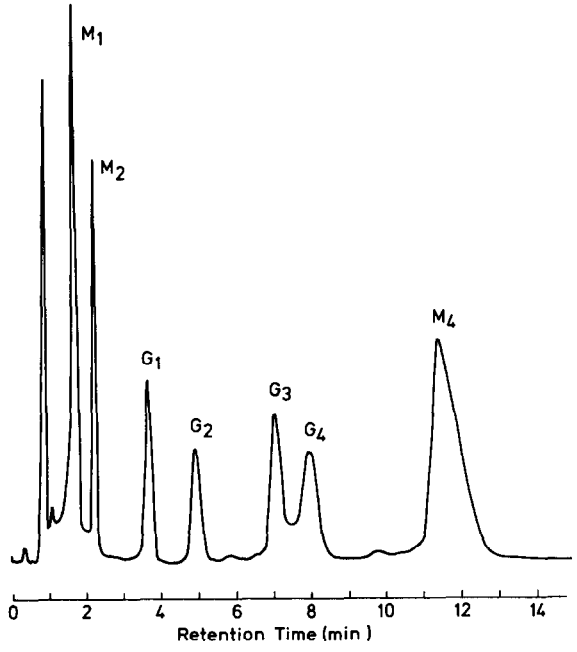

Fig. 3. HPLC of methanolysis products of partial hydrolysate of alginate sample C.

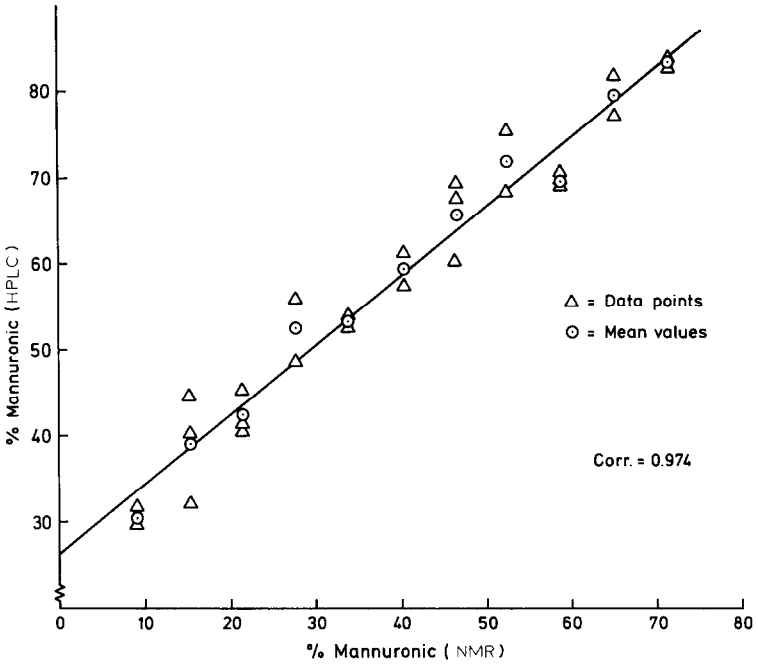

Fig. 4. Mannuronic acid content (%) of synthetic mixtures. HPLC method *vs.* NMR method.

and M_4 are likely to be the methyl ester methyl glycosides. These proposals have not yet been verified.

Fig. 2 shows the HPLC profile of the methanolised G fraction (91% G by NMR). Four peaks (G_{1-4}) were attributed to L-guluronic acid. They appear in the chromatograms of all samples containing G and are likely to be lactone glycosides and ester glycosides. The size of the M peaks reflects the relatively small amount of M in the G fraction. Peak M_3 is under the leading edge of peak G_4. This is suggested by the assymmetric shape of peak G_4. As peak M_3 was consistently about 1% of total M it was not allowed for in calculations.

The HPLC profile of the methanolysis products alginate sample C is shown in Fig. 3. The peaks previously assigned to M and G are clearly resolved except for M_3 as mentioned above.

Inspection of Figs. 1–3 reveals that the elution times vary somewhat. Total sample loading can affect these times. Samples high in carbohydrate (*e.g.* Fig. 3) lead to earlier emergence of peaks compared to ones with lighter loads. Standards were run regularly however to confirm peak identities.

M and G block fractions of known composition (determined by NMR) were combined to give mannuronic acid contents ranging from 10% to 70%. The uronic acid composition of these mixtures was also determined by HPLC. Although the HPLC method tends to overestimate the M content, especially at low levels of M (Fig. 4), a good correlation (0.974) is observed between it and the NMR procedure. A close correlation (0.893) is also observed in the analysis of natural alginates (Fig. 5). It is clear that the estimation of absolute M/G ratios using the HPLC method

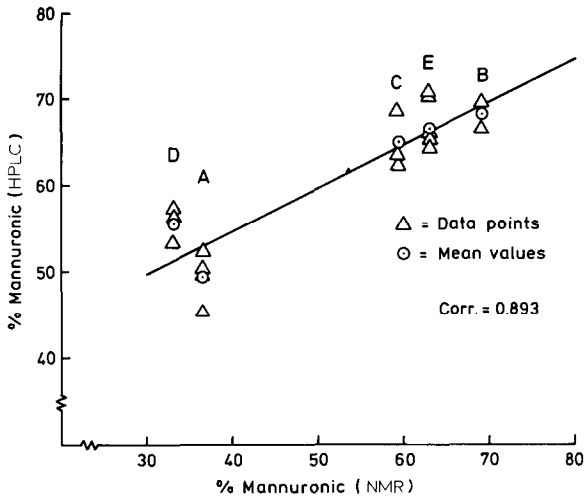

Fig. 5. Mannuronic acid content (%) of alginate samples A–E. HPLC method *vs.* NMR method.

would require a calibration against the NMR method. A similar conclusion was reached about an HPLC procedure which was used for the estimation of I/G ratios[13]. Without calibration, however, the HPLC method may still be used to differentiate readily between alginate samples. The accuracy of the HPLC method is best for high M alginates (Fig. 5) and could probably be used without further calibration for alginates of this type.

There appears to be two possible reasons for the overestimation of M values of some samples. Methanolysis is incomplete in that there is always considerable insoluble material remaining. It may be that the M residues *per se* are more susceptible to methanolysis than the G residues. This is the case for aqueous acid hydrolysis[17]. The results of the HPLC analysis of the synthetic mixtures of M and G fractions also support this suggestion. Considerable variation may occur in the size and arrangement of the M blocks, G blocks and MG blocks in alginates[1]. Samples A and D, the only low M alginates available have 36.5% M and 33.0% M respectively as determined by NMR. The corresponding HPLC values are 49.4% and 55.6% M. This discrepancy may be due to differences in the block structure. Thus the absolute amount of M as well as the block structure could be affecting the susceptibility of a particular sample to hydrolysis and subsequent methanolysis.

Although the present method does not yield absolute M/G ratios in all cases, it is considered that it could be valuable in industry for comparability purposes. Each laboratory could establish its own series of standard HPLC profiles. The method is relatively fast (1–2 days) compared with the full hydrolysis–ion-exchange procedure (6 days) of Haug and Larsen[2] and is simpler than several others.

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