Carbohydrate Polymers **12** (1990) 307–314

A Suitable Method for a Rapid Estimation of Sulphated Positions in Polysaccharides of Pharmacological Interest

Jose Kovensky, Javier A. Covián & Alicia Fernandez Cirelli*

Departamento de Quimica Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellon II, Ciudad Universitaria, 1428 Buenos Aires, Argentina

(Received 25 April 1989; accepted 2 May 1989)

ABSTRACT

Most polysaccharides of pharmacological interest bear sulphate substituents. Methylation analysis is still a powerful method for structural elucidation. The butyllithium-methyl iodide procedure, previously reported for neutral glycans, has been applied to sulphated polysaccharides. Results obtained with heparin, heparan sulphates and synthetic derivatives of natural β-(1→3)-glucans show that it is a convenient method for the rapid estimation of sulphated positions.

INTRODUCTION

Sulphated polysaccharides are commonly used in the pharmacological field. Heparin is the anticoagulant most frequently employed in clinical therapies (Casu, 1985). The related glycosaminoglycan heparan sulphate was found to inhibit experimentally induced thrombosis in animals (Meuleman *et al.*, 1982). Several attempts were made to relate biological activity and structure, but it became difficult due to the structural variability of these complex polysaccharides. On the other hand, the addition of sulphate residues to neutral polysaccharides resulted in the generation of effective anti-HIV substances (Nakashima *et al.*, 1987). In fact, the sulphation of antitumour active β-(1→3)-glucans isolated from *Cyttaria harioti* Fischer led to products with mitogenic activity (Fernández Cirelli *et al.*, 1989). In previous studies in the laboratory with heparan sulphates (Kovensky & Fernández Cirelli, 1987) and sulphated

*Research Member of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

glucans (Fernández Cirelli *et al.*, 1987) the development of a suitable procedure to determine sulphated positions in the polymers became necessary. Methylation analysis is still one of the most successful and versatile methods for the structural determination of polysaccharides. The traditional Hakomori (1964) procedure is the preferred route to obtain fully methylated sugars, involving three steps: (a) methylsulphinyl carbanion generation; (b) alkoxide formation; (c) methyl iodide addition. A direct method using butyllithium for sugar alcoxide formation has been reported (Kvernheim, 1987). The methylation of several glucans was achieved in two steps instead of the three ones involved in the traditional method. In addition to the more simplified experimental procedure, shorter times of analysis have been employed, owing to the use of a stronger reagent. The extension of this method to the methylation of sulphated polysaccharides is reported in this paper.

EXPERIMENTAL

Materials

Heparin from bovine intestinal mucosa was kindly given by Sintex Argentina S.A. Heparan sulphate (HS) was obtained from heparin by-products (Sintex Argentina S.A.) by alkaline copper–ethanol precipitation (Roden *et al.*, 1972). L_1 is a fraction of heparan sulphate isolated from rat liver tissue (Kovensky & Fernández Cirelli, 1987). Glucans G and R were obtained by alkaline extraction from cell walls from *Cyttaria harioti* Fischer (Oliva *et al.*, 1986). Glucan G consists of a (1→3)-linked β-D-glucan backbone of which every second or third residue is substituted in the 6-position by a glucose unit. Glucan R has a more linear structure with branches at each 9 or 10 residue. Glucan L was obtained by periodate oxidation–Smith degradation of G; SG was obtained by sulphation of G with pyridinechlorosulphonic acid (Fernández Cirelli *et al.*, 1987).

Methylation of sulphated polysaccharides

The sulphated polysaccharides were converted into the corresponding pyridinium salts by treatment with Dowex-50 X-8 (H^+) followed by the addition of an excess of pyridine. The mixture was evaporated to dryness and further dried over P_2O_5. Each sample (10 mg) was dissolved in methylsulphoxide (2 ml) by stirring in an ultrasonic bath at room tem-

perature. Butyllithium (0·9 M in hexane; 2 ml) was added and the mixture was stirred for 2 h at 40°C. After cooling in an ice bath, methyl iodide (1·5 ml) was added. Stirring was continued at room temperature until a clear solution was obtained. The liquid was poured over one volume of distilled water and dyalized. The methylated polysaccharides were recovered by freeze-drying. Desulphation was accomplished by the method of Nagasawa *et al.* (1977) in 1 : 9 H_2O–Me_2SO (80°C 5 h).

Hydrolysis of desulphated glycosaminoglycans was performed with 0·25 M H_2SO_4 in acetic acid. After 18 h at 80°C an equal volume of water was added and the heating was continued for 5 h. The solution was deionized through a column of Biorad-AG 3X-4A (acetate form) resin, eluted with methanol and evaporated (Stellner *et al.*, 1973). Glucan depolymerization was achieved by dissolution in 72% H_2SO_4 (room temperature, 2 h). The solution was diluted with water to 12% and heated for 4 h at 90°C (Fernández Cirelli & Lederkremer, 1976).

Gas–liquid chromatography–mass spectrometry analysis

Gas–liquid chromatography was performed with a Hewlett Packard 5830 gas chromatograph equipped with a flame ionization detector on glass columns packed with: (a) 3% ECNSS-M on gas chrom Q (0·2 × 180 cm^2); (b) 2% OV-101 on Chromosorb W AW DMCS (60–80) (0·2 × 120 cm^2). Glucan hydrolysates were analysed using column (a) (injection temperature (T_i) 210°C, column temperature (T_c) 170°C, detector temperature (T_d) 210°C; flow 29 ml N_2/min); glycosaminoglycan hydrolysates were analysed using column (b) (T_i 250°C, T_c programmed from 120°C to 290°C, rate 6°C/min, T_d 250°C; flow 25 ml N_2/min). Gas–liquid chromatography–mass spectrometry (GLC–MS) was performed in a Varian 1440 Chromatograph coupled to a Varian MAT 166 data system in the same conditions (flow 28 ml He/min).

RESULTS AND DISCUSSION

Sulphated polysaccharides were converted into pyridinium salts prior to methylation in order to allow the dissolution of the samples in methyl sulphoxide. Homogenous reaction media lead to better results in methylation studies, at least with the Hakomori method (Barker *et al.*, 1984).

Methylation using butyllithium-methyl iodide was carried out on commercial heparin (8·5%S), heparan sulphate from bovine mucose (3·4%S), a high sulphated heparan sulphate from rat liver L_1 (6·4%S) and sul-

phated SG (8·9%S) and non-sulphated β-(1 → 3)-D-glucans (G. R. and L). Samples G, SG and heparin were also methylated by the Hakomori method for comparison.

The methylated polysaccharides were desulphated, hydrolysed and after reduction and acetylation were analysed by GLC–MS.

The GLC pattern (Table I) obtained for the methylation of the sulphated β-(1 → 3)-glucan SG indicated a preference for *O*-6 sulphation as can be expected taking into account the relative reactivities of the

TABLE 1
Gas–Liquid Chromatography of Partially Methylated Alditol Acetates of Glucans

Positions of methylation	*Molar proportion (%)*			
	G	*L*	*R*	*SG*
2,3,4,6	27·9	0	16·3	11·7
2,4,6	38·7	74·2	31·3	13·9
2,3,4	2·3	0	4·5	1·2
2,4	31·2	22·4	26·0	27·2
2	0	3·2	21·8	45·9

hydroxyl groups and previous results from spectroscopic analysis. In the ^{13}C-NMR spectrum of SG, no appreciable signal below 68·5 ppm attributable to *O*-unsubstituted *O*-6 could be observed. However, in the GLC pattern, peaks corresponding to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-sorbitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-sorbitol were detected. This result suggests partial removal of sulphate groups in the methylation conditions. A similar GLC pattern was obtained by the Hakomori procedure. The high proportion of monomethylated derivative could indicate *O*-4 sulphation. Partial methylation could be discarded since it was only observed for the non-sulphated glucan R and can be attributed to its insolubility in the reaction medium. Data for glucan L confirmed that Smith degradation was not successful for the elimination of all the oxidized side-chains, as was previously suggested by the ^{13}C-NMR spectrum (Fernández Cirelli *et al.*, 1987). Similar results were obtained by both methylation procedures for glucan G. It can therefore be concluded that the butyllithium method gives excellent results with neutral β-(1 → 3)-glucans when homogenous reaction media can be achieved, and although it is not suitable for a rigorous quantification of number and position of sulphate groups, it allows a rapid estimation of the sulphation pattern.

The results obtained for the methylation of glycosaminoglycans using butyllithium are in accordance with those obtained by the traditional Hakomori procedure, and showed that this method is suitable for structural analysis.

Glycosaminoglycans were not reduced prior to methylation since the main concern of this work was in the determination of the position of sulphate groups on glucosamine moieties. It is known that the strongly basic conditions of the Hakomori methylation lead to β-elimination of glycuronosyl residues. The same effect was observed when butyllithium was employed (Fig. 1, Table 2). Beta-elimination of uronic acid units results in a residue whose fragmentation pattern is shown in Scheme 1. Hence, the iduronic/glucuronic acid ratio cannot be quantified, unless previous reduction of carboxyl groups is performed.

From the mass spectra, it is possible to assign the peaks corresponding to β-eliminated uronic acids as arising from non-sulphated or 2-*O*-sulphated units. These peaks may be used to estimate the degree of sulphation of uronic acids. They would be of relative accuracy since part of these units could be lost through further degradations. The low release of sulphate, if any, is in the same order as that produced in Hakomori conditions.

The actual advantage of the procedure described lies in the estimation of the relative proportions of the different 2-amino-2-deoxy-D-glucopyranosyl residues, i.e., non-sulphated, 6-*O*-sulphated and 3,6-di-*O*-sulphated glucosamine. However, in some cases products of incomplete *N*-methylation could be observed (Fig. 1, Table 2), which can

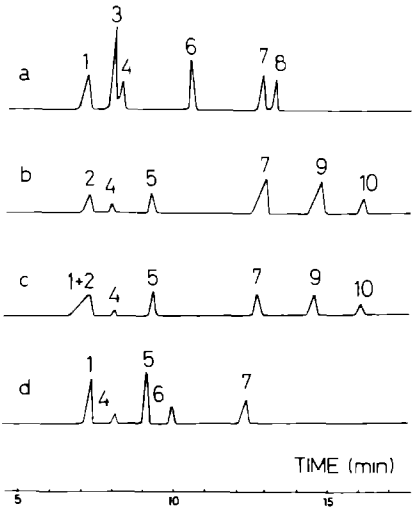
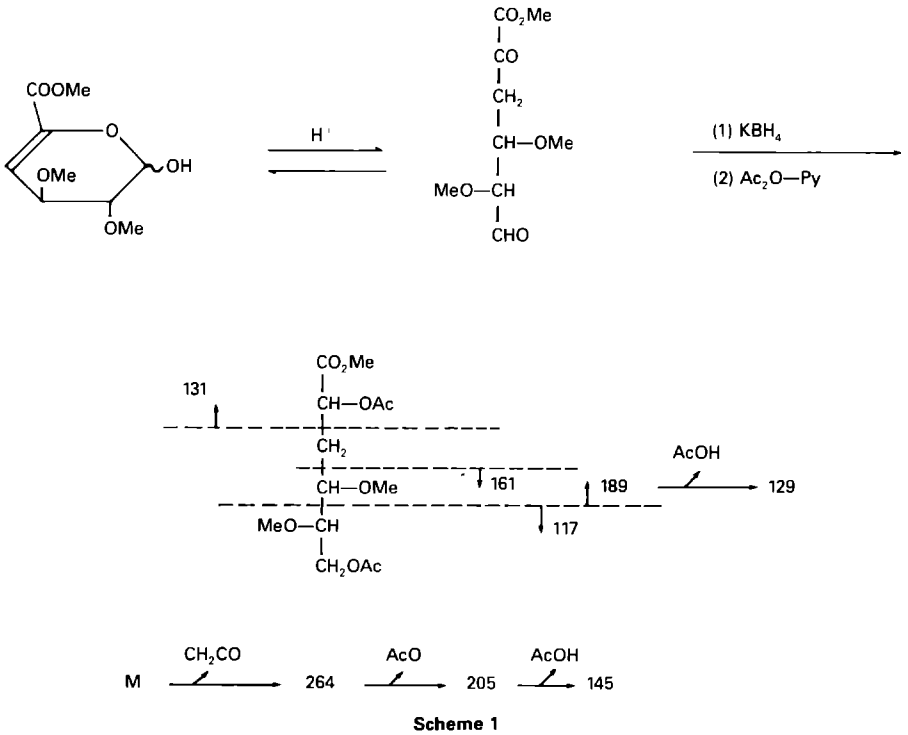


Fig. 1. Gas chromatography–mass spectrometry of methylated glycosaminoglycans. (a) HS (butyllithium procedure); (b) Heparin (Hakomori procedure); (c) Heparin (butyllithium procedure); (d) L_1 (butyllithium procedure).

TABLE 2
Gas–Liquid Chromatography–Mass Spectrometry of Glycosaminoglycans

Peak number	*Retention time (min)*	*Original unit*[a]	*Characteristic fragments*
1	7·3	Δ-4,5 UA (non-sulphated)	189, 161, 145, 131, 117
2	7·4	Δ-4,5 UA (2-*O*-sulphated)	189, 145, 131
3	8·1	Non-sulphated UA (NR)	175, 161, 117
4	8·3	Non-sulphated UA	233, 161, 117
5	9·2	2-*O*-sulphated UA	189, 145, 129
6	10·5	Non-sulphated hexosamine	158, 116, 233, 202, 129
7	12·8	6-*O*-sulphated hexosamine	158, 116, 261, 217, 202
8	13·2	Incomplete *N*-methylation of non-sulphated hexosamine	144, 84
9	14·7	3,6-di-*O*-sulphated hexosamine	158, 116, 289, 217, 145
10	16·1	Incomplete *N*-methylation of 6-*O*-sulphated hexosamine	144, 102

[a] UA = uronic acid; NR = non-reducing terminus.



Scheme 1

be attributed to an electrostatic repulsion between the attacking anionic species and those hexosamine residues that are *N*-sulphated in the original polysaccharide. Retention times of different units, and the main peaks of mass spectra that allowed their identification are shown in Table 2. Mass spectrometry of all the hexosamine residues showed fragments at m/z 158 and 116 (Stellner *et al.*, 1973). Identification of 1,4,5,6-tetra-*O*-acetyl-2-deoxy-3-*O*-methyl-(*N*-methyl-acetamido)-hexitol arising from 6-*O*-sulphated residues was made on the basis of fragments at m/z 261 and 217. The 3,6-di-*O*-sulphated glucosamine moieties lead to per-*O*-acetylated derivatives, which show fragments at m/z 289, 217 and 145, consistent with four consecutive carbon atoms bearing *O*-acetyl substituents. The greater advantage of the application of this method to glycosaminoglycans lies in its simplicity and shorter time of analysis to confirm or discard the presence of 3,6-di-*O*-sulphated glucosamine units. This residue is essential to account for the anticoagulant activity of heparin-like polysaccharides, because it confirms the active pentasaccharide sequence responsible for the specific binding to the protein inhibitor antithrombin III in plasma (Jacobsson *et al.*, 1986). The presence of this residue emerges clearly from the heparin GLC pattern (Fig. 1). It should be taken into account that the intensity of the 3,6-di-*O*-sulphated hexosamine is higher than expected, in both Hakomori and butyllithium procedures (Fig. 1. (b) and (c)). Nevertheless, this peak could not be observed in the GLC–MS pattern of heparan sulphates, indicating that this residue if present is in a very low proportion. This result is particularly meaningful for L_1, which displays a low anticoagulant activity in spite of its high degree of sulphation (Kovensky *et al.*, 1989, unpublished). Furthermore, this procedure may constitute a useful tool to correlate structural features and biological activity.

From the above results, it can be concluded that methylation analysis using butyllithium to generate the alkoxide anion appears to be an advisable method for the rapid estimation of sulphated positions.

ACKNOWLEDGMENTS

The authors are indebted to UBA (Universidad de Buenos Aires), CONICET (Consejo Nacional de Investigaciones Cientificas y Técnicas) and CIC (Comisión de Investigaciones Cientificas de la Provincia de Buenos Aires) for financial support. One of the authors (J.K.) also thanks CIC for a fellowship.

REFERENCES

Barker, S. A., Hurst, R. E., Settine, J., Fish, F. P. & Settine, R. L. (1984). *Carbohydr. Res.*, **125**, 291.
Casu, B. (1985). *Adv. Carbohydr. Chem. Biochem.*, **43**, 51.
Fernández Cirelli, A. & Lederkremer, R. M. (1976). *Carbohydr. Res.*, **48**, 217.
Fernández Cirelli, A., Oliva, E. M. & Covián, J. A. (1987). *An. Soc. Cientifica Argentina*, **217**, 13.
Fernández Cirelli, A., Covián, J. A., Ohno, N., Adachi, Y. & Yadomae, T. (1989). *Carbohydr. Res.*, **190**, 329.
Hakomori, S. (1964). *J. Biochem. (Tokyo)*, **55**, 205.
Jacobsson, K., Lindahl, U. & Horner, A. (1986). *Biochem. J.*, **240**, (3), 625.
Kovensky, J. & Fernández Cirelli, A. (1987). *An. Asoc. Quim. Argentina*, **75** (6), 563.
Kvernheim, A. L. (1987). *Acta Chem. Scand., Ser. B*, **41** (2), 150.
Meuleman, D. G., Hobbelen, P. M. J., Van Deden, G. & Moelker, H. C. T. (1982). *Thromb. Res.*, **27**, 353.
Nagasawa, K., Inoue, Y. & Kamata, T. (1977). *Carbohydr. Res.*, **58**, 47.
Nakashima, H., Osamu, Y., Tochikura, T., Yoshida, T., Mimura, T., Kido, Y., Motoki, Y., Kaneko, Y., Uryu, T. & Yamamoto, N. (1987). *Jpn. J. Cancer Res. (Gann)*, **78**, 1164.
Oliva, E. M., Fernandez Cirelli, A. & Lederkremer, R. M. (1986). *Exp. Mycol.*, **32**, 150.
Roden, L., Baker, J., Cifonelli, J. A. & Matthews, M. B. (1972). *Methods Enzymol.*, **28**, 73.
Stellner, K., Saito, H. & Hakomori, S. (1973). *Arch. Biochem. Biophys.*, **155**, 464.