Carbohydrate Research, 39 (1975) 168–176
© Elsevier Scientific Publishing Company, Amsterdam – Printed in Belgium

Note

A conductimetric method for the determination of sulphate and carboxyl groups in heparin and other mucopolysaccharides

BENITO CASU AND UGO GENNARO
Istituto Scientifico di Chimica e Biochimica "G. Ronzoni", 20133 Milano (Italy)
(Received April 25th, 1974; accepted for publication, June 13th, 1974)

In investigating the detailed structure and solution properties of heparins and chondroitins, the need had arisen for reliable, non-destructive methods for characterizing these polysaccharides. Among other information needed, the content of sulphate and uronic groups was essential in order to evaluate the charge distribution along the polyelectrolyte chain.

Most of the classical methods for the quantitative determination of sulphate and uronic groups of polysaccharides are either destructive or exceedingly time-consuming. Some methods are also unreliable, *e.g.*, the carbazole reaction for uronic acid groups[1], which gives consistently high values when applied to heparins and heparan sulphate samples.

Since sulphate and carboxyl groups have quite different acid dissociation constants[2], potentiometry and conductimetry should provide a simple approach to the determination of these groups in purified samples of mucopolysaccharides. A potentiometric procedure for determining the total acid content and the sulphate-to-carboxyl ratio of heparinic acids had been reported by Kuettner and Linderbaum[3]. Their method seems well suited for the former purpose, but the precision of the data for sulphate-to-carboxyl ratio was poor because of interference by the carboxyl groups in the sulphate titration. Moreover, chondroitin sulphates cannot be titrated potentiometrically in aqueous solution because of the very weak sulphate inflexion. The above-mentioned authors were able to circumvent this difficulty by using 70–80% *p*-dioxane as solvent, but the complete analysis requires two titrations, one in water for the total acid content, and one in water–*p*-dioxane for the sulphates. Since the sulphate inflexion point in the mixed-solvent titration curve is a function of the water–dioxane ratio and also of the purity of the dioxane, this second titration requires careful control of the experimental conditions.

Conductimetry apparently has not been applied to heparin and related mucopolysaccharides. It is known that conductimetric curves having sharp inflexion points are obtained for titrations of strong acids with strong bases, and *vice versa*[5].

In our experience, conductimetry can be conveniently used to titrate the sulphate groups in samples of dextran sulphate. On the contrary, conductimetry is not especially attractive for titrating weak acids such as uronic and polyuronic acids. Due to poor dissociation and buffering effects[5], the corresponding curves are "rounded off", and the evaluation of neutralization points is often uncertain. Nevertheless, the titration with a strong base of a mixture of a strong acid and a weak acid can usually be better performed by conductimetry than by potentiometry[6]. Although sulphated polyuronic acids such as heparin and chondroitin sulphate show polyelectrolyte behaviour[7] and cannot be regarded merely as "mixtures" of strong and weak acids (the sulphate and the carboxyl groups, respectively), it was expected that their conductimetric curves could afford a convenient method for rapid characterization.

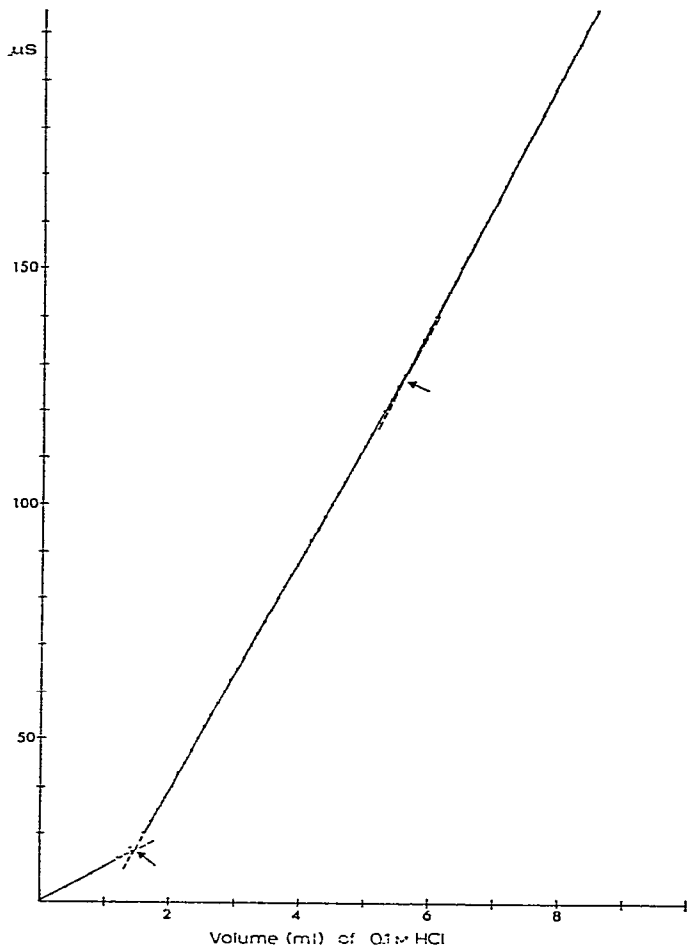

Fig. 1. Conductimetric titration curve of a sample of sodium heparinate (94 mg, dry basis) with standard acid. The first inflexion point corresponds to the complete displacement of Na^+ from carboxyl, and the second from sulphate.

The present Note describes the evaluation of conductimetry for the determination of the sulphate and carboxyl groups in heparins and chondroitins.

The conductimetric curves obtained by direct titration of the sodium salts of some mucopolysaccharides with standard acid were studied first. As shown in Fig. 1 for a sodium heparinate sample, the replacement of Na^+ ions (specific conductance $\lambda_+ = 50$) with protons ($\lambda_+ = 350$) and the introduction of Cl^- ions ($\lambda_- = 76$) causes an increase in the conductance of the solution. The slope of the curve (*i.e.*, the conductance change per unit volume of titrant) increases rather sharply when all of the counter-ions of the carboxyl groups (Na^+) have been replaced by protons. Such a slope change reflects the higher mobility of protons associated with the sulphate groups relative to those associated with the carboxyls. The replacement of all the Na^+ ions bound to the sulphate groups is indicated by a further increase in slope. Because of the excess of Na^+ and Cl^- ions, however, this second change is so slight as to be of little use for analytical purposes.

In Fig. 2, the conductance curves obtained by titrating the acid form of heparins and chondroitins with a strong base are shown; clearly, they are much more characteristic than those obtained by titration of the salt forms with acid. The conductance of the solutions, initially relatively high due mainly to the contribution of the mobile

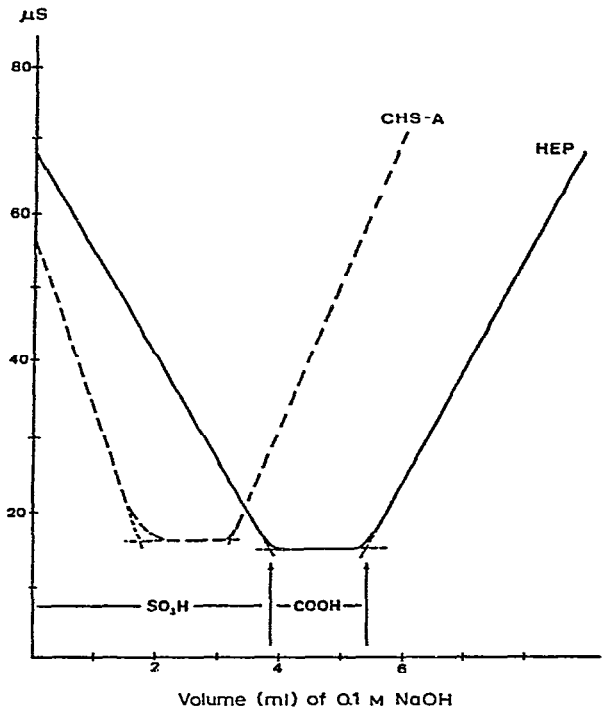

Fig. 2. Conductimetric titration curves of samples of heparinic acid (HEP, 88 mg of Na salt, dry basis) and chondroitin 4-sulphuric acid (CHS-A, 86 mg of Na salt, dry basis).

protons of the sulphate groups, decreases sharply as these protons are replaced by Na^+ ions. After all the SO_3H groups have been neutralised, however, the curves level out; in fact, the conductance barely changes during neutralisation of the carboxyls, giving characteristic plateau regions in the titration curves of both heparin and chondroitin. In the plateau region, the small contribution due to neutralisation of the largely undissociated carboxyl protons is compensated by the positive contribution due to the increasing concentration of Na^+ ions. After neutralisation of all the carboxyl groups, the conductance increases sharply again because of the added contribution of the mobile OH^- ions ($\lambda_- = 198$). The curves are rounded off near the equivalence points because of hydrolysis effects, the impact of which is different for different samples. Extrapolation of the three branches of the conductimetric curve gives two intersections, the first corresponding to the equivalence point of the sulphates, and the second to that of the carboxyls. The conductimetric titration of heparins and chondroitins in the acid form thus appears to provide a simple method for determining both the sulphate and the carboxyl groups.

This conductimetric approach to the analysis of mucopolysaccharides has been evaluated by using a number of heparin samples (including a desulphated heparin), and two chondroitins (4-sulphate and 6-sulphate). Since conversion of the samples from the original salt form into their acid form was effected with a cation-exchange resin (see Experimental), tests were made to ensure that contact with the resin did not affect the analytical data. Under the experimental conditions used, no loss of material or change in conductimetric data was observed. Na^+ analysis of effluents from the column (by atomic absorption) also suggested that no hold-up of heparins had occurred on the resin. Reproducibility tests, performed on ten separate samples of the same heparin, gave standard errors of $\pm 0.44\%$ for the sulphate and $\pm 2.95\%$ for carboxyl. Although, for convenience of handling, most experiments were performed with heparin or chondroitin samples of ~ 100 mg, substantially the same precision was achieved with 5-mg samples and a semimicro conductimetry cell.

Data for the SO_3^- and COO^- determinations are given in Table I, together with comparative data obtained by classical methods (oxidation–combustion for S analysis[8], and carbazole–borate for uronic carboxyl groups[9]). In general, the conductimetric data for the SO_3^- groups are somewhat higher (up to 5% on a relative basis) than those obtained from S analysis. This slight discrepancy seems more likely to be attributable to the oxidation–combustion analysis giving low values rather than conductimetry giving high values. In fact, several sets of S analyses by the oxidation–combustion method from different laboratories gave scattered data which, on the average, were lower than the more homogeneous set of data reported in Table I.

Conductimetric data for the COO^- groups of heparins are up to 20% lower than those obtained by the carbazole–borate method. Such a difference was not unexpected on account of previous reports on the unreliability of the carbazole method, even the borate modification[1]. Since factors affecting the intensity of the carbazole colour are not well understood, and are still a subject of investigation[10], no attempts were made to interpret the spread of the carbazole–borate data of Table I

TABLE I

SULPHATE AND CARBOXYL CONTENT OF HEPARINS AND CHONDROITINS, DETERMINED BY CONDUCTIMETRIC TITRATION OF THE CORRESPONDING ACID FORMS, COMPARED WITH DATA FROM CLASSICAL METHODS[a]

Sample	*Anticoagulant activity (USP units)*	SO_3^- (%)		COO^- (%)		SO_3^-/COO^- [c]
		Conductim.	*Ox./comb.*	*Conductim.*	*Carbazole-borate*	
Heparin, No. **1**	120	27.7	25.5	7.85	8.04	1.94
Heparin, No. **2**	100	29.0	27.0	7.87	8.87	2.02
Heparin, No. **3**	130	31.4	31.0	8.33	10.40	2.07
Heparin, No. **4**	150	31.6	30.6	8.53	10.00	2.04
Heparin, No. **5**	160	31.8	31.1	8.32	8.95	2.10
Heparin, No. **6**	175	33.1	32.6	8.62	8.64	2.11
Heparin, No. **7**	[b]	35.0	34.6	8.03	8.03	2.40
Desulphated heparin	[b]	18.6	16.7	10.00	10.13	1.02
Chondroitin 4-sulphate	[b]	16.0	15.7	8.17	8.31	1.08
Chondroitin 6-sulphate	[b]	16.1	15.5	8.80	9.10	0.98

[a]Data (expressed on dry-weight basis) are referred to the weight of the original sodium salts. [b]Data not obtained. [c]Molar ratio from conductimetric titrations.

over a 0 to +20% range relative to the conductimetric data. That surprisingly good correspondence between the conductimetric and colorimetric data was found for the two samples (6 and 7) having the highest degree of sulphation may be coincidental. For the chondroitin sulphate samples, the agreement between the conductimetric and colorimetric data is fairly good.

Although acid–base titration curves are by definition non-specific, conductimetry of the acid form of heparins and chondroitins provides quite typical curves that can be used, in conjunction with more specific methods, for the characterisation of these polysaccharides. In fact, the shape of the conductimetric curves of heparins differs from that of chondroitin (as an obvious consequence of the different sulphate-to-carboxyl ratio), and both curves differ from those of a mixture of equivalent amounts of a polysulphate (such as dextran sulphate) and a polyuronic acid (such as alginic acid), which usually show just one equivalence point corresponding to the total acid content of the mixtures.

No attempts were made to correlate specific conductance values of the solution (either at the beginning of the titration or at the equivalence points) with the chemical composition of the samples. It is conceivable that the absolute conductance values ("normalized" for the water content of the samples) are physico-chemical parameters associated with the density of the acid groups along the polyelectrolyte chain, as well as with the homogeneity of their distribution. The slope of the sulphate and carboxyl branches of the conductance titration curve should thus be affected by factors such as charge density, *O*-sulphate–*N*-sulphate ratio, *N*-acetyl content, and L-iduronic–D-glucuronic acid ratio. This aspect deserves further investigation with a large number of well-characterized samples.

The present conductimetric method provides a simple and rapid means for determining the absolute content both of sulphate and carboxyl groups in heparins and chondroitins, and perhaps in other acidic mucopolysaccharides. To determine the sulphate-to-carboxyl molar ratios only, there is no need to weigh the samples or to determine their weight loss on drying. Furthermore, if information is desired for the carboxyl groups only, a conductimetric titration of the sodium (or potassium) salts with standard acid is an even simpler procedure. As regards the *N*-sulphate groups, it appears that, under the present experimental conditions, the inflexion point for the titration of this grouping is virtually identical with that for the *O*-sulphate group and cannot be distinguished from it.

Since inorganic salts may interfere with the conductance measurements, the titration should be performed only on dialyzed samples or, preferably, after gel-filtration*. This is a drawback relative to more specific methods such as the titration *via* formation of paraffin-chain, quaternary ammonium complexes[11]. Non-purified samples should at least be checked for possible contamination by inorganic sulphates

*When working with non-desalted samples, it has to be kept in mind that NaCl, a common contaminant of commercial heparins, produces HCl, which, as a strong acid, interferes with the sulphate analysis.

or by sodium acetate. Both types of contaminant can be detected by i.r. spectrophotometry in aqueous solution[12]. Inorganic acetates can also be detected by p.m.r. spectroscopy[13]. It was observed that, although contamination with inorganic acetate caused the expected shift of the equivalence point for the carboxyls, it did not affect the inflexion point for the sulphate groups. The shift of carboxyl equivalence point is also accompanied by an increase in the slope of the carboxyl branch of the conductance curve, but in the presence of inorganic acetate it seems no longer possible to detect the original equivalence point for the uronic acid groups of the mucopolysaccharide.

EXPERIMENTAL

Materials and apparatus. — Heparin samples were from commercial sources (**1**–**4** from Syntex, Buenos Aires; **5** from Schuchardt, München; **6** from L.D.O., Milan; **7** from Upjohn, Kalamazoo). Samples **2**–**4** were obtained by purification of sample **1**, using Cetavlon[1]. The heparin samples were extracts from beef or hog intestinal mucosa, except for **7**, which was from beef lung. Samples **1**–**6** show a p.m.r. signal from *N*-acetyl, and can be classified as type-A heparins[13–15]. Sample **7**, with only a weak *N*-acetyl signal, was a type-B heparin. Approximate anticoagulant activities, indicated in Table I, were determined by the U.S.P. method. Desulphated heparin was prepared from sample **4** by treatment[16] with 0.04M HCl. Chondroitin sulphates were commercial samples (4-sulphate from Schuchardt, München, and 6-sulphate from Miles Laboratories, Elkhart, Ind.).

The sulphur content of all the samples was determined by the oxidation–combustion procedure[8]. The uronic acid content was determined by the carbazole–borate method using sodium D-glucuronate as a standard[9]. The loss of weight was determined by drying the samples to constant weight (~6 h) in a pistol dryer, at 66.7° (CCl_4) and 1 mmHg.

Before the conductimetric analyses, each sample was dialysed for 4 h against running, distilled water, in standard dialysis tubes (CRAMI, Milan); this duration was best for eliminating common inorganic salts and acetates, without producing any significant self-hydrolysis of heparin. The dialysed solutions were carefully brought to the original pH with NaOH, before recovering them by evaporation at ~40°. The conductimetric analyses were performed either on an automatic instrument (Metrohm Model E 365 B) or on a manual instrument (Derritron Model E 3924), using a standard cell (total volume 200 ml) or a semi-micro cell (30 ml). I.r. spectra were run on a Perkin–Elmer 337 grating instrument, and p.m.r. spectra on a Jeol-100 instrument.

Direct conductimetric titration of the carboxyl groups. — Samples (100 mg; or 5 mg using the semi-micro cell) of heparin or chondroitin (Na salt, undried) were dissolved in ~120 ml of distilled water (25 ml for semi-micro), and the solution was introduced into the conductimetric cell, adjusted to 150 ml (50 ml), and directly titrated with 0.1M HCl. The carboxyl content in the original mucopolysaccharide was

determined from the amount of standard acid corresponding to the first inflexion point, and expressed on a dry basis. The results were calculated as follows: COO^- % = A × 4.4 × 100/w(mg), where A = ml of 0.1M HCl corresponding to the first inflexion point; 4.4 = equivalent weight (mg) corresponding to 0.1 meq of COO^-; and w(mg) = dry weight of the sample.

Conductimetric titration of the sulphate and carboxyl groups in heparin and chondroitin acids. — Samples (100 mg) of heparin or chondroitin (Na salts, undried) were dissolved in ~30 ml of distilled water. The solution was passed through a column (15 × 150 mm) of Amberlite IR-120(H^+) resin until the effluent was neutral. The acid form of the mucopolysaccharide was eluted with water directly into the conductimetric cell, up to a volume of 150 ml. For small samples (5 mg), volumes were reduced 6-fold, and the measurements were made in a semi-microcell. The sulphate content in the original sample was determined from the first equivalence point, evaluated by extrapolation of the first two branches of the conductance titration curve, as shown in Fig. 2. The amount of standard base required to neutralize the carboxyl groups was determined by subtracting the amount required to neutralize the sulphate groups from the amount corresponding to the second inflexion point (total acid equivalent). The sulphate-to-carboxyl molar ratio was simply determined from the ratio of the amount of base required to neutralize the sulphate to that required to neutralize the carboxyl. The results were calculated as follows:

$$SO_3^- \% = \frac{A \times 8.0}{w(mg)} \times 100; \qquad COO^- \% = \frac{(B-A) \times 4.4}{w(mg)} \times 100;$$

and molar ratio $SO_3^-/COO^- = A(B-A)$; where A = ml of 0.1M NaOH corresponding to the first inflexion point; B = ml of 0.1M NaOH corresponding to the second inflexion point; 8.0 = equivalent weight (mg) corresponding to 0.1 meq of SO_3^-; 4.4 = equivalent weight (mg) corresponding to 0.1 meq of COO^-; and w(mg) = dry weight of the sample.

ACKNOWLEDGMENTS

The authors thank the N.A.T.O. Scientific Affairs Division for their generous support of this work, in the form of a joint grant (No 675) to Professor A. S. Perlin and B.C. They also thank Dr. G. G. Torri for the carbazole analyses, Mr. Pasta for sulphur analyses, Dr. I. Carletti for valuable technical help, and Professor A. S. Perlin for helpful discussions.

REFERENCES

1 L. Rodén, J. R. Baker, J. A. Cifonelli, and M. B. Mathews, *Methods Enzymol.*, 28 (1972) 73.
2 A. Albert and E. P. Serjeant, *The Determination of Ionization Constants*, Chapman and Hall, London 1971.
3 K. E. Kuettner and A. Linderbaum, *Science*, 144 (1964) 1228.
4 K. E. Kuettner and A. Linderbaum, *Biochim. Biophys. Acta*, 101 (1965) 223.

5 H. H. WILLARD, L. L. MERRITT, AND J. A. DEAN, *Instrumental Methods of Analysis*, Van Nostrand, New York, 1958, p. 568.
6 R. A. CHALMERS, *Aspects of Analytical Chemistry*, Oliver and Boyd, London, 1968, p. 111.
7 F. A. BETTELHEIM, in A. VEIS (Ed.), *Biological Polyelectrolytes*, Dekker, New York, 1970, p. 161.
8 B. A. LEWIS, F. SMITH, AND A. M. STEPHEN, *Methods Carbohyd. Chem.*, 5 (1965) 195.
9 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330.
10 J. TSE, M. S. THESIS, Department of Chemistry, McGill University, 1971.
11 J. E. SCOTT, *Methods Biochem. Anal.*, 8 (1960) 162.
12 B. CASU, A. S. PERLIN, AND G. SCOVENNA, unpublished data.
13 A. S. PERLIN, B. CASU, G. R. SANDERSON, AND L. F. JOHNSON, *Can. J. Chem.*, 48 (1970) 2260.
14 A. S. PERLIN, M. MAZUREK, L. B. JAQUES, AND L. W. KAVANAH, *Carbohyd. Res.*, 7 (1968) 369.
15 L. W. KAVANAH AND L. B. JAQUES, *Arzneimittel-Forsch.*, 23 (1973) 605.
16 I. DANISHEFSKY, *Methods Carbohyd. Chem.*, 5 (1965) 407.