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ION CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF SULFATE IN COMPLEX CARBOHYDRATES

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SUMMARY

A procedure is described for the quantitation of sulfate in complex carbohydrates. Samples are taken to dryness in the presence of a small amount of sodium hydroxide and the sulfate is liberated by pyrolysis. Sulfate is then quantitated by single-column ion chromatography eliminating the interference by ions such as phosphate, chloride, sodium and calcium which frequently occurs with colorimetric methods. The reproducibility of the method is approximately 10%. This approach cannot be directly applied to glycoproteins because a portion of the sulfur in sulfur-containing amino acids appears to be converted to sulfate. However, this method can be used to measure sulfate in oligosaccharides of glycoproteins if the protein components are removed prior to analysis.

INTRODUCTION

A variety of biologically important substances including the proteoglycans[1–3] and certain glycoproteins[4–7] contain sulfate covalently attached to carbohydrate. Colorimetric methods have been described to measure sulfate in these complex carbohydrates after liberation by acid hydrolysis[4,8–10] or pyrolysis[7,11]. We and others have observed (unpublished observations) that it is extremely difficult to obtain reproducible and reliable sulfate quantitation using colorimetric methods. Recently, methods for the quantitation of sulfate by ion chromatography have become available[12–15]. In this communication we report a method for the quantitation of sulfate in complex carbohydrates which employs single-column ion chromatography.

EXPERIMENTAL

Reagents

Glc-6S (glucose-6-sulfate), Chond-4S (chondroitin-4-sulfate), KHP (potassium hydrogen phthalate), glucuronolactone, amino acids and the albumin preparations

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were obtained from Sigma (St. Louis, MO, U.S.A.). ^{4}Di-OS [2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose], ^{4}Di-4S [2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose], ^{4}Di-6S [2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose] and Chond-6S (chondroitin-6-sulfate) were obtained from Miles (Naperville, IL, U.S.A.). PHA (phthalic acid) and certified 0.05 *M* sulfuric acid were obtained from Fisher (Philadelphia, PA, U.S.A.).

Ion chromatography

The ion chromatograph consisted of a Beckman 110A HPLC pump equipped with a 110-40 pulse dampner, a 210 injector equipped with a 0.1-ml sample loop (Beckman Scientific, Palo Alto, CA, U.S.A.), a Wescan Anion Guard Column (No. 269-003), Standard Analytical Anion Column (No. 269-001), 213A Conductivity Detector and strip chart recorder (Wescan Instruments, Santa Clara, CA, U.S.A.). KHP solutions (routinely 6 m*M*) were prepared in water which had been deionized and then glass distilled. Methanol (1%, v/v) was added to retard microbial growth. The pH of KHP solutions was adjusted with 1 *M* potassium hydroxide or 6 m*M* PHA. All buffers were filtered through a 0.45-μm filter and thoroughly degassed before use. Other KHP eluents were prepared in a similar manner. A flow-rate of 2.0 ml/min was routinely used. Sulfate standards were prepared by diluting 0.05 *M* sulfuric acid in water or KHP. The conductivity detector was utilized at the most sensitive setting. Under these conditions, the strip chart recorder gave a deflection of approximately 3 mm for each nmol sulfate and the lower limit of detectability was approximately 1 nmol. All glassware was thoroughly washed, rinsed with distilled water, dilute nitric acid and with distilled water again. For each set of unknowns a standard curve was prepared by injecting various amounts of sulfate. Water blanks and sulfate standards were also routinely carried through the pyrolysis procedure and analyzed for sulfate to assess potential contamination and procedural losses, respectively.

Pyrolytic liberation and quantitation of sulfate

Pyrolysis was essentially performed as described by Silvestri *et al.*[11]. Samples containing between 5 and 25 nmol sulfate were dissolved in water. Aliquots were pipeted into 75 × 10 mm borosilicate culture tubes; 0.1 ml of 0.01 *M* sodium hydroxide was added per ml solution. The samples were then taken to dryness in a Savant SpeedVac Centrifuge (Savant Instruments, Hicksville, NY, U.S.A.) and heated in a Fisher burner for 6 s unless otherwise noted. The pyrolyzed samples were redissolved in KHP and aliquots were injected into the ion chromatograph.

The free sulfate in monosaccharide, disaccharide, chondroitin sulfate and amino acid solutions was quantitated by direct injection into the ion chromatograph. To determine the free sulfate in albumin preparations, solutions of albumin were treated with an equal volume of acetonitrile and then incubated 15 min at 4°C. The precipitate was removed by centrifugation at 12800 *g* for 15 min. Sulfate in the supernatant was then determined. Any free sulfate in solution was subtracted from the value obtained after pyrolysis.

GlcUA

GlcUA (glucuronic acid) was quantitated by the method of Bitter and Muir[16] using glucuronolactone as the standard.

RESULTS

Optimum conditions for sulfate quantitation

Initially the elution buffer recommended by the column manufacturer (4 m*M* KHP, pH 4.5) was evaluated. Samples dissolved in other buffers, those containing buffer anions as well as those whose pH was different from that of the elution buffer frequently exhibited a phthalate-system peak[15] (see Fig. 1) which eluted sufficiently close to sulfate to interfere with its quantitation. KHP eluants in the pH range of 4.0 to 4.5 had this problem. The phthalate-system peak results from the interaction of hydrogen ions and/or buffers in particular samples with the KHP eluent (Wescan Instruments, personal communication). The system peak could be eliminated by preparing standards from sodium sulfate or by preparing them from sulfuric acid in KHP adjusted to the pH of the elution buffer. To avoid this problem, we chose

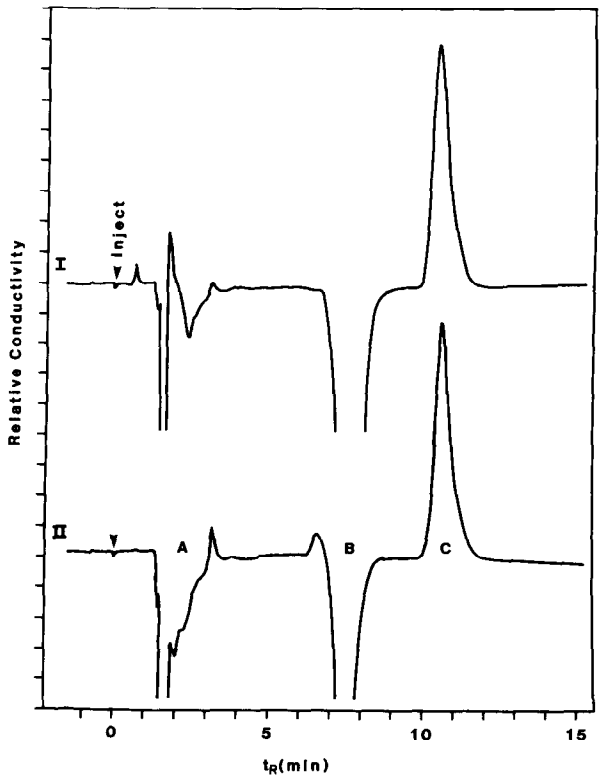

Fig. 1. Typical sulfate elution profiles obtained using single-column ion chromatography. Column: Wescan Anion Guard and Standard Anion; Eluent: 6 m*M* KHP, pH 3.9, 2 ml/min; sample: 25 nmol sulfate in KHP (I) or Water (II); detector: Wescan 213A conductivity detector. Peaks: A = sample solvent front; B = phthalate-system peak; C = sulfate.

conditions where the system and the sulfate peaks were clearly resolved. KHP elution buffers with pH's > 4.5 or < 3.9 separated sulfate from the phthalate-system peak (Table I). However, the silica-based column utilized may undergo accelerated deterioration with eluents above pH 4.5. An eluant pH of 3.9 was found to be optimal even though sulfate is relatively strongly retained[12,13] (Table I). Increasing the ionic strength of the eluant to 6 m*M* slightly decreased the retention times (t_R) of all ions. Thus, 6 m*M* KHP, pH 3.9, was selected as the eluant. Under these conditions sulfate is adequately resolved from the phthalate-system peak (Fig. 1) and samples can be injected at approximately 15 min intervals. No other components eluted after sulfate even when the elution was continued through 30 min. The anions routinely encountered in biological samples eluted earlier and did not interfere with the quantitation of sulfate even if present in fairly high concentrations.

TABLE I

RETENTION TIMES (t_R) FOR COMMON ANIONS IN VARIOUS KHP ELUENTS DURING SINGLE-COLUMN ION CHROMATOGRAPHY

Anion or peak	*t_R (min) at a flow-rate of 2.0 ml/min with*			
	6 mM KHP pH 3.9	*4 mM KHP pH 3.9*	*4 mM KHP pH 4.7*	*4 mM KHP pH 5.0*
Pseudopeak*	1.5	1.5	1.5	1.5
Cl^-	3.0	3.5	2.5	2.5
NO_2^-	3.2	3.8	2.9	2.8
NO_3^-	3.6	4.5	3.1	3.1
HCO_3^-	–**	–	–	3.1***
I^-	6.5	6.5	4.2	4.4
$H_2PO_4^-$	–	–	5.7***	4.2***
SO_4^{2-}	10.5	15.0	6.1	5.7
HSO_3^-	10.5	15.0	6.1	5.5
$S_2O_3^{2-}$	12.3	ND§	ND	ND
Phthalate-system§§	7.5/6.5	11.8/9.9	8.2/7.3	8.8/8.1

* Due to cations in and the solvent of the injected sample.

** Where no t_R is given, no discernible peak was observed when 50 nmol was injected.

*** A broad peak with conductivity lower than the eluant was observed.

§ Not determined.

§§ The phthalate-system peak was positive, negative or biphasic depending on the pH difference between the injected sample and the eluent buffer. The first t_R denotes the primary phthalate-system peak while the second t_R denotes the secondary phthalate-system peak when biphasic. The secondary peak was not always present but was consistently observed when phosphate or bicarbonate was injected.

Sulfite, even when prepared in an excess of dithiothreitol, consistently co-eluted with sulfate but yielded considerably lower conductivity per unit mass (3% for sodium sulfite; 24% for sodium hydrogen sulfite). It is possible that the sulfite preparations utilized were slightly contaminated with sulfate, that some sulfite was oxidized to sulfate in solution or that these anions essentially co-elute[12,13]. No other sulfite peaks were observed.

A standard curve was prepared with each set of unknowns. The peak height was directly proportional to the amount of sulfate injected (Fig. 2). Measuring peak

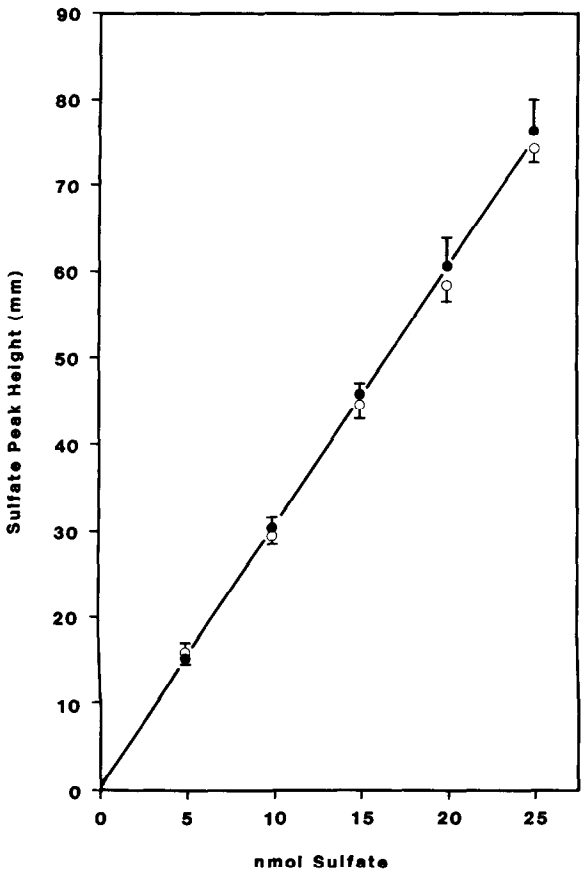

Fig. 2. Typical calibration curves for sulfate standards prepared in (○), water, $y = 0.84 + 2.92x$, $r = 0.994$; or (●), KHP, $y = 0.65 + 3.04x$, $r = 0.991$.

areas, determined by multiplying peak height with peak width at one-half peak height, yielded no improvement in precision. Standard curves carried through the pyrolysis procedure yielded equivalent standard curves to those prepared by direct injection.

Optimum pyrolysis conditions

Silvestri *et al.*[11] suggested that sodium hydroxide be added to complex carbohydrates prior to pyrolysis in order to obtain quantitative recovery of sulfate. We observed that recovery of sulfate from samples heated in a Fisher burner for 6 s was not quantitative unless a small amount of sodium hydroxide was dried down with the sample prior to heating. For example, omitting sodium hydroxide from Glc-6S resulted in sulfate levels 42% lower than those observed when sodium hydroxide was added. The stability of inorganic sulfate was further evaluated by heating samples at 550°C for 15 min in the presence and absence of sodium hydroxide. Under both neutral and acidic conditions, the recovery of sulfate was slightly less than quantitative (Table II).

TABLE II

STABILITY OF INORGANIC SULFATE HEATED AT 550°C FOR 15 min

Sulfate	*Additions*	*Heated*	*Sulfate peak height (mm)*	*Recovery (%)*
20 nmol	none	no	63.7 ± 0.3*	100
20 nmol**	none	no	66.2 ± 3.6	104 ± 6
20 nmol	none	yes	58.5 ± 2.6	92 ± 4
20 nmol	0.8 μequiv. sodium hydroxide	yes	61.7 ± 0.8	97 ± 1
20 nmol	0.8 μequiv. hydrochloric acid***	yes	53.7 ± 1.5	84 ± 2
20 nmol	0.8 μequiv. hydrochloric acid***	no	61.3 ± 3.5	96 ± 5

* Mean ± S.D., $n = 3$ except as noted below.

** These samples represent sulfate prepared in water and analyzed by direct injection into the ion chromatograph ($n = 6$). All heated samples as well as the 20 nmol standards to which hydrochloric acid was added were prepared from this solution.

*** Hydrochloric acid was partially removed by the addition and evaporation of methanol three times[7].

As observed by Silvestri *et al.*[11] pyrolysis for 6 s in a Fisher burner appears to be optimal (Fig. 3). Shorter times resulted in incomplete destruction of the samples while longer times appear to result in the loss of sulfate. Thus, 6 s was routinely utilized. However, pyrolysis for approximately 20 s was required when the sulfate associated with albumin was analyzed because shorter times did not result in complete destruction of the samples.

With each set of unknowns, water blanks and sulfate standards were carried through the pyrolysis procedure and analyzed for sulfate to assess potential contamination and procedural losses. Water blanks typically contained non-detectable amounts of sulfate (0.39 ± 0.76 nmol) and recovery of sulfate was quantitative (100 ± 8%).

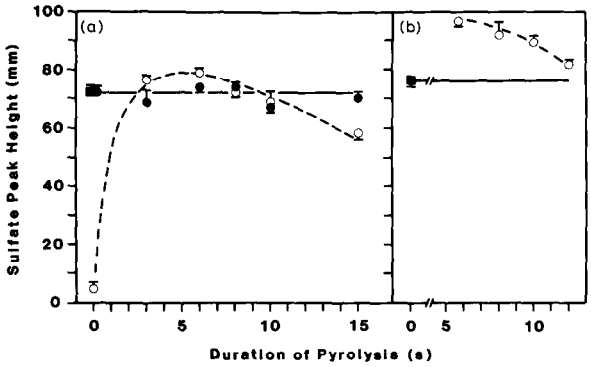

Fig. 3. Effect of varying the duration of pyrolysis on the sulfate quantitated from (a) Glc-6S, ■ = 20 nmol sulfate, not pyrolyzed; ● = 20 nmol sulfate, pyrolyzed; ○ = 20 nmol Glc-6S, pyrolysed and (b) Chond-4S, ■ = 20 nmol sulfate; ○ = 16 μg Chond-4S, pyrolyzed. Pyrolysis of Chond-4S for less than 6 s resulted in incomplete destruction of the samples. Each point illustrates the mean ± S.D. of triplicate observations. Note that a duration of 6 s appears to be optimal.

TABLE III

SULFATE IN COMPLEX CARBOHYDRATES AS QUANTITATED BY SINGLE-COLUMN ION CHROMATOGRAPHY AFTER PYROLYSIS

	Molar ratio: sulfate to glucose or GlcUA
Glc-6S	0.92 ± 0.07*
^{4}Di-OS	0.01 ± 0.02
^{4}Di-4S	1.16 ± 0.13
^{4}Di-6S	1.30 ± 0.10
Chond-4S	0.97 ± 0.06
Chond-6S	1.46 ± 0.03

* Mean ± S.D., $n = 6$. Each compound was assayed at two dose levels in triplicate. GlcUA was determined by the method of Bitter and Muir[16].

Sulfate in complex carbohydrates

The sulfate in a series of model complex carbohydrates was determined (Table III). The values obtained agree reasonably well with those theoretically expected as well as estimates provided by the suppliers which were obtained with alternate methods[1,11]. The reproducibility of the method was evaluated by assaying sulfate on Glc-6S on three separate occasions. In addition to the molar ratio noted above, sulfate to glucose molar ratios of 1.09 ± 0.15 and 0.99 ± 0.04 were also observed. These data suggested that sulfate on complex carbohydrates could be quantitated with an intra-assay coefficient of variation of approximately 10% and an inter-assay coefficient of variation of approximately 8.5%. The overall mean molar ratio observed in the three determinations was 1.00.

Potential for interference by sulfur-containing amino acids

The above results suggest that sulfate in complex carbohydrates can be quantitated by this method. In order to determine if the method could potentially be applied to glycoproteins, the conversion of sulfur in sulfur-containing amino acids and proteins was evaluated. Pyrolysis of methionine, cysteine, cystine and cysteic

TABLE IV

CONVERSION OF THE SULFUR IN THE SULFUR-CONTAINING AMINO ACIDS TO SULFATE DURING PYROLYSIS

Amino acid	*Molar ratio: sulfate/amino acid*	
	Without sodium hydroxide	*With sodium hydroxide**
Methionine	0.085 ± 0.010 (4)**	0.825 ± 0.188 (3)
Cysteine	0.074 ± 0.018 (4)	1.005 ± 0.071 (3)
Cystine	0.055 ± 0.017 (4)	1.097 ± 0.255 (3)
Cysteic acid	0.126 ± 0.025 (4)	0.835 ± 0.093 (3)

* 0.1 μequiv. sodium hydroxide per 25 nmol acid was added to these samples.

** Mean ± S.D. (*n*).

TABLE V

CONVERSION OF SULFUR IN ALBUMIN TO SULFATE DURING PYROLYSIS FOR 20 s[1]

The following assumptions were used in the calculations: (a) molecular weights of 66000 and (b) both BSA and RSA have 34 half-cystine, 1 cysteine and 4 (BSA) or 6 (RSA) methionine residues per molecule[18]. Each sample received 0.1 μequiv. sodium hydroxide per nmol albumin prior to pyrolysis.

Compound	*Amount assayed*	*Molar ratio*	
		Sulfate/albumin	*Sulfate/sulfur*
BSA	1 nmol	8.27 ± 0.12*	0.212 ± 0.003
BSA	2 nmol	3.60 ± 0.01	0.092 ± 0.003
RSA	1 nmol	5.35 ± 2.05	0.130 ± 0.050
RSA	2 nmol	3.23 ± 0.31	0.079 ± 0.007

* Mean ± S.D., $n = 3$ except for RSA at 1 nmol where $n = 2$.

acid under basic conditions appeared to convert a high percentage of the sulfur to sulfate (Table IV). The sulfate generated during the pyrolysis of bovine serum albumin (BSA) and rat serum albumin (RSA) was also evaluated because these proteins contain large numbers of sulfur-containing amino acids. Although the conversion of sulfur was less than quantitative, it appeared that a significant percentage was oxidized to sulfate (or possibly sulfite) during pyrolysis (Table V). Thus, this method is not directly applicable to glycoproteins unless the oligosaccharides are separated from the protein before analysis.

DISCUSSION

In our early studies we attempted to quantitate sulfate in complex carbohydrates by colorimetric methods[4,8–10]. However, we found those methods to be tedious and unreliable. Thus, we evaluated ion chromatography[12–15] as a possible alternative and found it to be reproducible. Knowing that the method was going to be ultimately applied to biological samples, we sought elution conditions which would resolve sulfate from other anions and interfering substances which might be present in biological samples. A KHP elution buffer with a pH of 3.9 was found to fulfill these requirements. Increasing the concentration of KHP from 4 to 6 m*M* at pH 3.9 decreased the retention times of all anions and thus enabled samples to be injected at shorter intervals. Under the conditions described, samples can be injected every 15 min. Furthermore, ions such as phosphate, chloride, sodium and calcium which affect sulfate quantitation by colorimetric methods[10,11] can be present in the samples and do not interfere with the ion chromatography unless present in high concentrations.

Silvestri *et al.*[11] and others[7] have used the term "pyrolysis" to denote the destruction of biological samples by heat. In the context of mass spectrometry, pyrolysis usually denotes the destruction of molecules by heat in a nitrogen atmosphere to prevent oxidation. As applied herein, pyrolysis is performed in room air which presumably provides sufficient oxygen to eliminate carbon as oxygen containing gases. Although inorganic sulfate appears to be stable during short periods of heating, recovery of sulfate from biological samples (which may generate acids during their

destruction) or from samples heated under acidic conditions, is less than quantitative. Perhaps some of the sulfate is lost (evaporated) as sulfuric acid and/or decomposed into sulfur-containing gases under acidic conditions. Thus, pyrolysis must be performed under basic conditions to prevent the loss of sulfate. Application of these procedures to a series of model complex carbohydrates which contain sulfate yielded reasonable, reliable and reproducible quantitation of sulfate.

During the course of these studies, it was observed that pyrolysis of proteins and sulfur-containing amino acids under basic conditions appears to result in the generation of sulfate and/or sulfite. Thus, this method cannot be applied directly to glycoproteins. In more recent experiments, the sulfate associated with certain glycoproteins was liberated by hydrolysis with hydrochloric acid[10,11] or methanolysis[17]. After the hydrochloric acid was removed by repeated evaporation with water or methanol, the amino acids and proteins were removed by passage over a AG 50W-X8 (H^+) cation-exchange column and the sulfate was quantitated by ion chromatography[19].

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