Journal of Chromatography, 212 (1981) 65–73
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,824

SEPARATION OF REDUCED DISACCHARIDES DERIVED FROM GLYCOSAMINOGLYCANS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

GEORGE JIA-LONG LEE, DER-WU LIU, JOSEPH W. PAV and HOWARD TIECKELMANN*
Department of Chemistry, State University of New York at Buffalo, Buffalo, NY 14214 (U.S.A.)
(Received March 26th, 1981)

SUMMARY

A high-performance liquid chromatographic (HPLC) method for the analysis of reduced unsaturated disaccharides derived from enzymatic digestion followed by reduction with sodium borohydride of chondroitin sulfates, dermatan sulfate, heparan sulfate and heparin is described. This method is well suited for the HPLC analysis of glycosaminoglycans (GAGs) because the possibility of obtaining anomeric forms of unsaturated disaccharides is eliminated which provides a major advantage for quantitation. This procedure is more sensitive than existing HPLC methods for the determination of enzymatic degradation products from GAGs. In particular, the resolution of disaccharide products from heparan sulfate is improved after reduction. The applicability of this method for the determination of GAGs in biological samples is demonstrated.

INTRODUCTION

Glycosaminoglycans (GAGs) are members of a group of related heteropolysaccharides which can be degraded by specific enzymes to produce unsaturated disaccharides containing a reducing end. Two major groups of disaccharides have been reported: (1) disaccharides from chondroitin sulfate isomers after digestion with chondroitinase enzymes[1], (2) disaccharides from heparan sulfate and heparin after digestion with heparinase and/or heparitinase enzymes[2–4]. The assays of these disaccharide products provide useful tools for identification, structural analysis and quantification of GAGs[5,6]. High-performance liquid chromatography (HPLC) has been shown to be a successful technique for these assays. It is more sensitive, precise and faster than other chromatographic methods[7–11].

When HPLC is used for the quantitation of the unsaturated disaccharides from GAGs two peaks often have been observed for each disaccharide, which has been considered to be due to the presence of sugar anomeric forms[7,11]. Ototani *et al.*[10] also have assumed that anomers are present during the ion-pair reversed-phase HPLC of chondroitin sulfate disaccharides. These GAG disaccharides contain a reducing end which is the anomeric center in each molecule and therefore mixtures of α- and β-

0021-9673/81/0000–0000/$02.50

anomeric forms may be detected under appropriate chromatographic conditions. This phenomenon interferes with separations and has limited the quantification of these disaccharides.

In this report the effects of sodium borohydride reduction of sugar carbonyl to terminal hydroxymethyl groups are described. This approach does indeed eliminate anomeric forms and also increases the sensitivity and resolution in HPLC of enzymatic degradation products from GAG.

EXPERIMENTAL

Apparatus

The HPLC system employed in this work included a Model 6000A solvent delivery system, a U6K universal injector and a Model 440 UV detector (Waters Assoc., Milford, MA, U.S.A.). The recorder used was a Houston Instrument (Austin, TX, U.S.A.) OmniScribe A5211-5 dual-pen recorder. Peak heights, peak areas and retention times were measured by an on-line Model Supergrator-1 integrator (Columbia Scientific, Austin, TX, U.S.A.). A prepacked HPLC column, Partisil-10 PAC, 10 μm, 25 cm $\times$ 4.6 mm I.D. (Whatman, Clifton, NJ, U.S.A.) was employed.

Reagents and materials

Chondroitin 4-sulfate, the unsaturated disaccharides (ΔDi-OS, ΔDi-6S and ΔDi-4S)* from chondroitin sulfate isomers, chondroitinase ABC (E.C. 4.2.2.4) from *Proteus vulgaris* and chondroitinase AC (E.C. 4.2.2.5) from *Arthrobacter aurescens* were products of Seikagaku Kogyo, Tokyo, Japan and were purchased from Miles Labs. (Elkhart, IN, U.S.A.). Chondroitin 6-sulfate was purchased from Calbiochem (San Diego, CA, U.S.A.). The unsaturated disaccharides (ΔDi-HS_b-I, ΔDi-HS_b-II, ΔDi-HS_b-III and ΔDi-He_a-I)** from heparan sulfate and heparin were generous gifts from Dr. Alfred Linker (University of Utah, Salt Lake City, UT, U.S.A.). The urine sample from a patient with Maroteaux-Lamy disease was supplied by Dr. John T. Dulaney (University of Tennessee, Memphis, TN, U.S.A.).

Methanol and acetonitrile, HPLC grade, were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Sodium borohydride and sodium tetraborate were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Doubly distilled water passed through two mixed-bed ion-exchange cartridges was used in preparation of ammonium formate buffer for the HPLC mobile phase. The buffer was passed through a 0.45-μm filter prior to use.

Isolation of urinary GAGs and enzymatic procedure

The isolation of GAGs from 2 ml urine specimens and the enzymatic digestions

---

* ΔDi-OS = a non-sulfated disaccharide from chondroitin; ΔDi-6S = a 6-sulfated disaccharide from chondroitin 6-sulfate; ΔDi-4S = a 4-sulfated disaccharide from chondroitin 4-sulfate. For detailed structures and preparations, see ref. 1.

** ΔDi-HS_b-I = a non-sulfated disaccharide from heparan sulfate; ΔDi-HS_b-II = a disaccharide from heparan sulfate containing one sulfate group on the acetylglucosamine unit; ΔDi-HS_b-III = a disaccharide from heparan sulfate containing N-sulfated glucosamine; ΔDi-He_a-I = a trisulfated disaccharide from heparin. For detailed structures and preparations, see refs. 2 and 4.

of standard chondroitin sulfates and urinary GAGs by chondroitinases were carried out as described previously[8,12].

Chemical reduction

Standard unsaturated disaccharides (200 μg), and enzymatic degradation product mixtures obtained from 200 μg standard chondroitin sulfates or from 1 ml Maroteaux-Lamy urine were individually dissolved in 200 μl of 0.1 *M* sodium borate buffer (pH 8.2). A 2.5-mg amount of sodium borohydride in 100 μl of 0.1 *M* sodium borate (pH 8.2) was added to each solution in several steps. The vortex mixing was done with a Super-Mixer (Matheson Scientific) after each addition. After shaking gently at room temperature for 3 h, the solutions were refrigerated for 3 h and then neutralized to pH 5.0 with 0.5 *M* acetic acid to destroy excess sodium borohydride. Each solution was dried under a stream of nitrogen and the residue, dissolved in 90% aqueous methanol, was applied to the HPLC column.

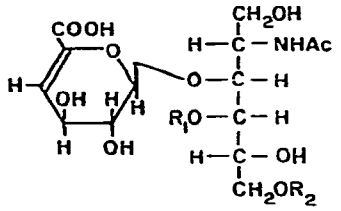
HPLC

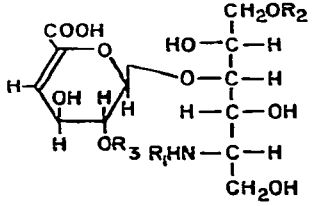
An aliquot from each reduced disaccharide product was injected and chromatographed at 1.5 ml/min with a ternary solvent system of acetonitrile–methanol–ammonium formate buffer on a Whatman Partisil-10 PAC column. The composition of the mobile phase was systematically varied in order to select the optimal conditions for separation.

The chromatography was carried out isocratically at room temperature. Details are given separately with each chromatogram.

RESULTS AND DISCUSSION

Sodium borohydride has been widely used for the derivatization of reducing sugars (neutral sugars[13,14], amino sugars[15,16] and sugar acids[17,18]) for gas chromatography. The reduction of the unsaturated disaccharides of GAGs using sodium borohydride was reported previously[4,19] and is assumed to reduce the carbonyl groups to give the structures shown in Fig. 1. Recently, in sequencing studies of





	R_1	R_2
1. r△Di—0S	H	H
2. r△Di—6S	H	SO_3H
3. r△Di—4S	SO_3H	H

	R_1	R_2	R_3
4. r△Di-HS_D-I	Ac	H	H
5. r△Di-HS_D-II	Ac	SO_3H	H
6. r△Di-HS_D-III	SO_3H	H	H
7. r△Di-He_A-I	SO_3H	SO_3H	SO_3H

Fig. 1. Structures of reduced disaccharides from GAGs.

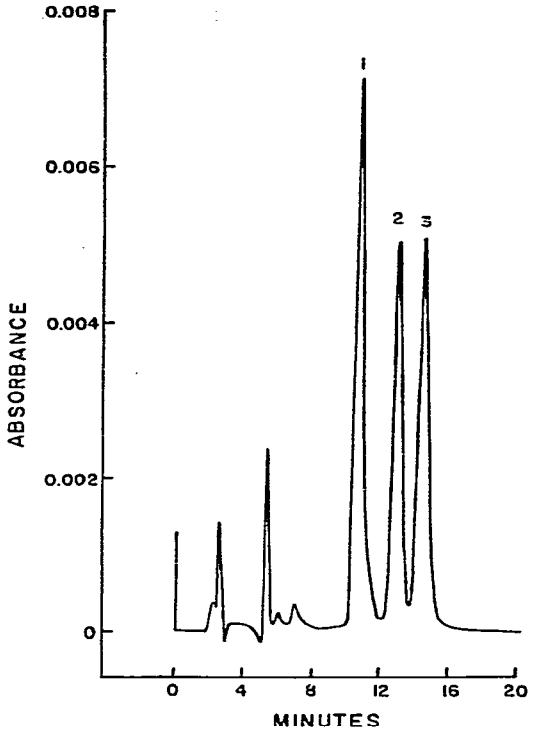


Fig. 2. HPLC of the reduced unsaturated disaccharides from chondroitin sulfate isomers. Peaks: 1 = rΔDi-OS; 2 = rΔDi-6S; 3 = rΔDi-4S. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Solvent system: acetonitrile–methanol–0.5 *M* ammonium formate, pH 6.0 (69:14:17). Flow-rate: 1.5 ml/min. Pressure: 600 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

chondroitin sulfates, sodium borohydride has been used to form reduced oligosaccharides and unsaturated disaccharides which were analyzed by HPLC on a Partisil-10 SAX anion-exchange column[20]. Compounds 1–7 are the reduced disaccharides from ΔDi-OS, ΔDi-6S, ΔDi-4S, ΔDi-HS_b-I, ΔDi-HS_b-II, ΔDi-HS_b-III, and ΔDi-He_a-I, respectively. A separation of the three reduced chondroitin sulfate disaccharides rΔDi-OS, rΔDi-6S and rΔDi-4S is shown in Fig. 2. The resolution of these three reduced compounds is similar to that of non-reduced disaccharides[8]. However, the efficiency in the chromatography of the reduced compounds is much higher as indicated in Table I which shows the comparison of the number of theoretical plates of reduced and non-reduced disaccharide peaks under a variety of compositions of mobile phases. The effective baseline separations for reduced and non-reduced disaccharides were achieved by mobile phases 1 and 2, respectively. It is clearly indicated that the number of theoretical plates of reduced disaccharide peaks is increased from two- to seven-fold when compared with non-reduced disaccharides depending on acetonitrile–methanol ratio and the molarity and pH of aqueous ammonium formate in the mobile phase.

Fig. 3 illustrates the HPLC separation of reduced non-sulfated and monosulfated disaccharides from heparan sulfate. Much better resolution was achieved in

TABLE I

THE DEPENDENCE OF THE NUMBER OF THEORETICAL PLATES (N) FOR THE SEPARATION OF REDUCED AND OF NON-REDUCED CHONDROITIN SULFATE DISACCHARIDE PEAKS ON ACETONITRILE–METHANOL RATIOS AND pH AND MOLARITY OF AMMONIUM FORMATE IN THE MOBILE PHASE

N was calculated by half peak height method, using $N = 5.54 (t_R/W_{\frac{1}{2}})^2$ where t_R = retention time and $W_{\frac{1}{2}}$ = peak width at half peak height. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Flow-rate: 1.5 ml/min.

Disaccharide	*N*			
	Acetonitrile–methanol–ammonium formate (molarity, pH) ratio			
	(1) 69:14:17 (0.5 M, pH 6.0)	*(2) 59:24:17 (0.5 M, pH 6.0)*	*(3) 69:14:17 (0.5 M, pH 5.0)*	*(4) 69:14:17 (0.8 M, pH 6.0)*
ΔDi-OS	572	646	504	441
ΔDi-6S	871	870	604	680
ΔDi-4S	820	854	653	695
rΔDi-OS	2861	1766	2681	3215
rΔDi-6S	2428	1930	2458	2930
rΔDi-4S	2459	1920	2506	2833

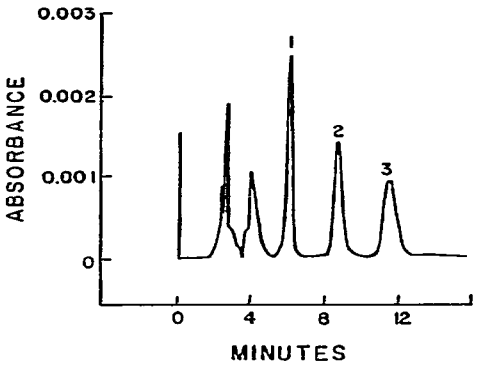


Fig. 3. HPLC of the reduced unsaturated disaccharides from heparan sulfate. Peaks: 1 = rΔDi-HS_b-I; 2 = rΔDi-HS_b-II; 3 = rΔDi-HS_b-III. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Solvent system: acetonitrile–methanol–0.5 M ammonium formate, pH 4.5 (60:20:20). Flow-rate: 1.5 ml/min. Pressure: 750 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

comparison with HPLC of non-reduced compounds[11]. Each reduced compound formed from chondroitin sulfates as well as heparan sulfate gave a single and much sharper peak under all chromatographic conditions. This is an important advantage for quantitation and supports the conclusion that anomeric forms were eliminated.

The retention behavior of these reduced disaccharides, as chromatographed on the cyano-amino bonded stationary phase, showed a strong and sensitive dependence on the ratio of acetonitrile and methanol content in the eluent. Table II shows the capacity ratios (k') of these reduced disaccharides determined as a function of the

TABLE II

THE DEPENDENCE OF CAPACITY RATIOS (k') OF THE REDUCED DISACCHARIDES FROM GAGs ON THE ACETONITRILE AND METHANOL CONTENT OF THE MOBILE PHASE

Values given are mean k' values determined from five separate chromatograms. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Flow-rate: 1.5 ml/min. UV detection at 254 nm, 0.01 a.u.f.s.

Reduced disaccharide from chondroitin sulfates	k'					
	Ratio of acetonitrile–methanol–0.5 M ammonium formate (pH 6.0)					
	0:83:17	*19:64:17*	*39:44:17*	*59:24:17*	*69:14:17*	*74:9:17*
rΔDi-OS	0.94	1.01	1.31	2.23	3.40	5.96
rΔDi-6S	7.43	4.56	3.35	3.30	4.27	7.12
rΔDi-4S	7.26	4.47	3.51	3.74	4.90	8.35
Reduced disaccharide from heparan sulfate	*Ratio of acetonitrile–methanol–0.5 M ammonium formate (pH 4.5)*					
	0:80:20	*20:60:20*	*40:40:20*	*60:20:20*	*65:15:20*	*75:5:20*
rΔDi-HS_b-I	0.99	1.01	1.11	1.56	1.95	6.90
rΔDi-HS_b-II	6.78	4.09	3.05	2.73	3.06	8.54
rΔDi-HS_b-III	10.85	7.09	4.90	3.93	4.24	11.05

concentration of acetonitrile and methanol of the mobile phase, while keeping the content of aqueous ammonium formate constant. The k' values of reduced sulfated disaccharides from both chondroitin sulfates and heparan sulfate first decrease with increasing acetonitrile content and decreasing methanol content, pass through a minimum, and then increase sharply. The k' values of reduced non-sulfated disaccharides (rΔDi-OS and rΔDi-HS_b-I) increase slightly in the region in which k' values of reduced sulfated disaccharides decrease and then increase sharply as did the reduced sulfated disaccharides. A reversed retention order of rΔDi-6S and rΔDi-4S was observed when these two organic solvent contents were varied. The k' values of all reduced disaccharides decrease with increasing aqueous ammonium formate content. After using a column for a period of time with a given mobile phase, the retention times of all disaccharides may decrease due to loss of column efficiency. An adjustment of mobile phase composition was necessary in order to maintain the optimal conditions for separation. The difference in retention behaviors of these compounds from column to column can also be overcome in the same manner. The reduced trisulfated disaccharide (rΔDi-He_a-I) derived from heparin can be quantitated using an ion-pair reversed-phase method as described for HPLC of the non-reduced trisulfated disaccharide[11].

In order to evaluate the linearity and the sensitivity of this procedure, the following experiment was performed. Duplicate aliquots containing 200 μg of the unsaturated disaccharides were prepared. One set of these aliquots was reduced with sodium borohydride and the other set was used without chemical modification. Both sets, reduced and non-reduced disaccharides, were chromatographed separately under the conditions which gave effective separations for each disaccharide isomer group. The peak height of each disaccharide was plotted against the amount of

compound injected. Excellent linear relationships exist for both sets of disaccharides as shown in Fig. 4. The results also indicate that the detection sensitivity is increased by a factor of at least 2 by employing sodium borohydride reduction.

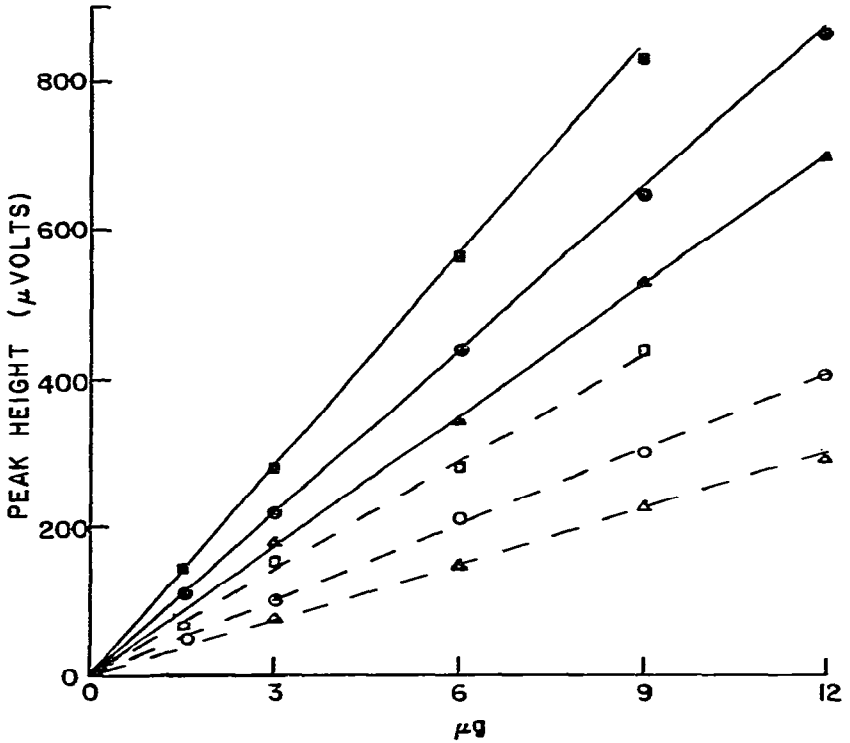


Fig. 4. The linearity of HPLC analysis for reduced disaccharides and the comparison of detection sensitivity between reduced products and original non-reduced compounds. Curves: ■, rΔDi-OS; ●, rΔDi-6S; ▲, rΔDi-4S; □, ΔDi-OS; ○, ΔDi-6S; △, ΔDi-4S.

The applicability of this reduction procedure to biological samples was demonstrated by the analysis of enzymatic degradation products of standard chondroitin sulfates and urinary GAGs from a patient with Maroteaux-Lamy disease (Fig. 5). The two top plots in Fig. 5 illustrate HPLC separations of reduced disaccharides obtained after the treatment of either chondroitin 4-sulfate (left) or chondroitin 6-sulfate (right) with chondroitinase ABC and borohydride reduction. As expected, rΔDi-4S and rΔDi-6S are the major products of chondroitin 4-sulfate and chondroitin 6-sulfate, respectively. The two bottom plots in Fig. 5 show separations of reduced disaccharides from Maroteaux-Lamy urinary GAGs after digestion with chondroitinase ABC (left) or AC (right) followed by borohydride reduction. The large difference in amounts of rΔDi-4S produced by the two different chondroitinases confirms the excretion of large amounts of dermatan sulfate[1], a characteristic of Maroteaux-Lamy disease[21]. Several additional impurity peaks appear in the chromatographs. However, they are eluted long before reduced disaccharide peaks and do not interfere with this quantification.

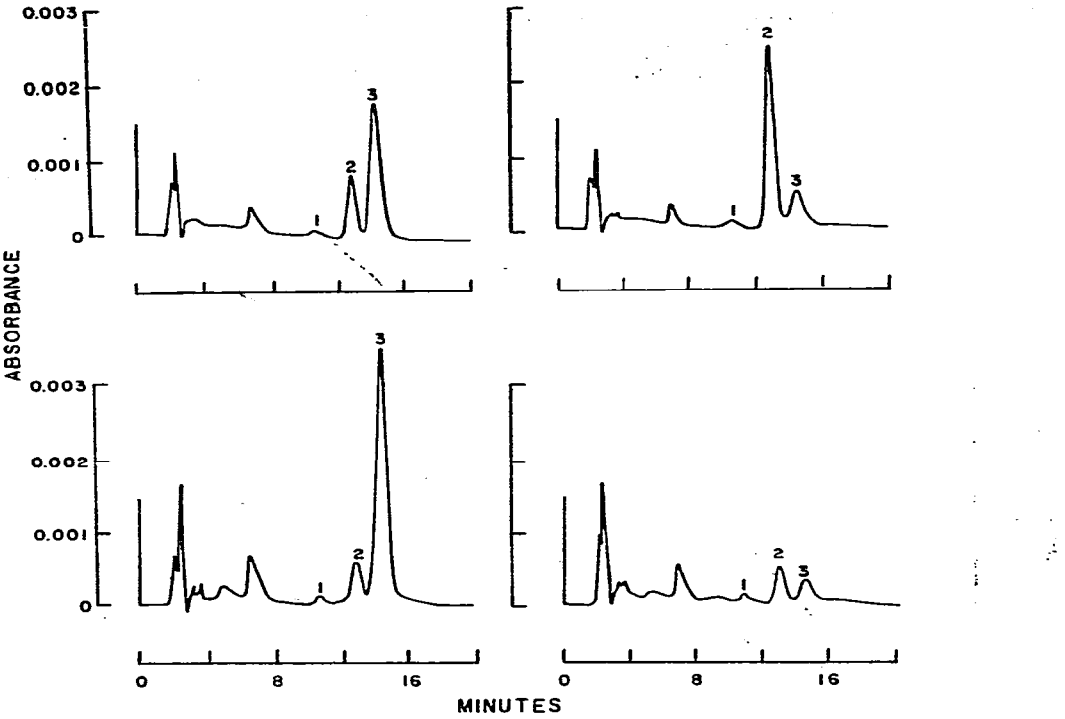


Fig. 5. HPLC of products of enzymatic degradation followed by sodium borohydride reduction of standard chondroitin sulfates and urinary GAGs: standard chondroitin 4-sulfate (top left) and chondroitin 6-sulfate (top right) degraded by chondroitinase ABC; GAGs from a Maroteaux-Lamy urine degraded by chondroitinase ABC (bottom left) and by chondroitinase AC (bottom right). Peaks: 1 = rΔDi-OS; 2 = rΔDi-6S; 3 = rΔDi-4S. The amount injected was 2.5% of the reaction products from 100 μg standard chondroitin sulfates or from GAG of 1 ml Maroteaux-Lamy urine. Chromatographic conditions as in Fig. 2.

In this laboratory the procedure described is a valuable addition to the existing methodology for the analyses of GAGs. It permits a facile analysis of lower levels of GAGs in biological samples when compared to previously described procedures involving enzymatic degradation.

REFERENCES

1 H. Saito, T. Yamagata and S. Suzuki, *J. Biol Chem.*, 243 (1968) 1536.
2 P. Hovingh and A. Linker, *Carbohyd. Res.*, 37 (1974) 181.
3 M. E. Silva, C. P. Dietrich and H. B. Nader, *Biochim. Biophys. Acta*, 437 (1976) 129.
4 P. Hovingh and A. Linker, *Biochem. J.*, 165 (1977) 287.
5 N. Taniguchi, S. Koizumi, K. Masaki and Y. Kobayashi, *Biochem. Med.*, 14 (1975) 241.
6 A. Linker and P. Hovingh, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 36 (1977) 43.
7 G. J.-L. Lee, J. E. Evans and H. Tieckelmann, *J Chromatogr.*, 146 (1978) 439.
8 G. J.-L. Lee and H. Tieckelmann, *Anal. Biochem.*, 94 (1979) 231.
9 A. Hjerpe, C. A. Antonopoulos and B. Engfeldt, *J. Chromatogr.*, 171 (1979) 339.
10 N. Ototani, N. Sato and Z. Yosizawa, *J. Biochem.*, 85 (1979) 1383.
11 G. J.-L. Lee and H. Tieckelmann, *J. Chromatogr.*, 195 (1980) 402.

12 G. J.-L. Lee, J. E. Evans, H. Tieckelmann, J. T. Dulaney and E. W. Naylor, *Clin. Chim. Acta*, 104 (1980) 65.
13 J. H. Kim, B. Shom, T.-H. Liao and J. G. Pierce, *Anal. Biochem.*, 20 (1967) 258.
14 W. F. Lehnhardt and R. J. Winzler, *J. Chromatogr.*, 34 (1968) 471.
15 Z. Tamura, T. Imanari and Y. Arakawa, *Chem. Pharm. Bull.*, 16 (1968) 1864.
16 L. J. Griggs, A. Post, E. R. White, J. A. Finkelstein, W. E. Moeckel, K. G. Holden, J. E. Zarembo and J. A. Weisbach, *Anal. Biochem.*, 43 (1971) 369.
17 F. Eisenberg, Jr., *Anal. Biochem.*, 60 (1974) 181.
18 M. B. Perry and R. K. Hulyalkar, *Can. J. Biochem.*, 43 (1965) 573.
19 A. Linker and P. Hovingh, *Biochemistry*, 11 (1972) 563.
20 S. R. Delaney, H. E. Conrad and J. H. Glaser, *Anal. Biochem.*, 108 (1980) 25.
21 A. Dorfman and R. Matalon, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 630.