Carbohydrate Research, 30 (1973) 143–154

THE TRIMETHYLSILYLATION REACTIONS OF HEXOSAMINES, AND GAS-CHROMATOGRAPHIC SEPARATION OF THE DERIVATIVES

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(Received December 22nd, 1972; accepted in revised form, April 6th, 1973)

ABSTRACT

Several methods for trimethylsilylation were applied to the 2-amino-2-deoxy-hexoses, and the resulting derivatives were characterized by gas–liquid chromatography as to retention index, response factor, and degree of substitution on the amino group. Conditions were found for replacing none, one, or both of the amino protons with trimethylsilyl groups. The average response-factor for the trimethylsilyl derivatives was found to be 6.27 g of docosane per mole of hexosamine, or 1.13 mg of docosane per mg of hexosamine. One of the methods investigated was applied to the determination of hexosamines in acid hydrolyzates of glycosaminoglycans. The 2-acetamido-2-deoxy derivatives were also studied, but the several derivatives have not yet been identified.

INTRODUCTION

Because of the widespread occurrence of hexosamines with other carbohydrates in glycosaminoglycans, glycoproteins, and glycolipids, methods for their separation have been sought. The separation of trimethylsilyl (TMS) derivatives of carbohydrates by gas–liquid chromatography (g.l.c.) for both qualitative and quantitative analyses has found widespread application, but, until recently, the only method used for producing these derivatives was that originated by Sweeley *et al.*[1], namely, treatment with hexamethyldisilazane (HMDS) and chlorotrimethylsilane (TMCS) in pyridine (2:1:10 by volume). Although the hexosamines exist in Nature primarily as the 2-acetamido-2-deoxy derivatives, the conditions required for their liberation by hydrolysis invariably remove the acetyl groups, to afford the amine hydrochloride. However, the results reported in the literature for TMS derivatives of the hydrochlorides have been quite variable. Thus, Perry[2] could not observe elution of any derivative, and Sweeley and Walker[3] observed more peaks than would be predicted on the basis of two anomers. Because of these and other difficulties, most investigators have *N*-acetylated the hexosamine hydrochlorides prior to[1–5] or concurrently with[6] silylation.

The derivative afforded by application of this method to 2-amino-2-deoxy-D-galactose was shown to have *O*-TMS groups only[7]. Hence, Perry's failure[2] to

observe any derivative can be attributed to irreversible adsorption of the highly polar free amino group, as he, unlike subsequent investigators, did not utilize a highly deactivated support. Recently, it was shown that addition of the powerful silyl donor *N,O*-bis(trimethylsilyl)acetamide (BSA) to the usual reaction-mixture [2:1:10 (*v/v*) HMDS–TMCS–pyridine] caused substitution of a single TMS group on the amino group[8]. Another method, involving silylation with HMDS in *N,N*-dimethylformamide (DMF), has been claimed to be specific for hexosamine hydrochlorides[9], but the derivative has not been characterized.

Most studies have employed pyridine as the solvent, despite the fact that reaction of catecholamines with BSA, which causes both amino protons to be replaced by TMS groups, is much faster in acetonitrile than in pyridine[10]. Furthermore, BSA catalyzed by 1% of TMCS is a more powerful silyl donor than BSA alone[10] (used unsuccessfully by Kärkkäinen and Vihko[8]). Consequently, a systematic study, using conditions previously employed for other amino compounds, has now been made of the reactions of the biologically important 2-amino-2-deoxy- and 2-acetamido-2-deoxy-hexoses. Conditions were found for quantitatively silylating the hydrochlorides, to afford derivatives having no, one, or two TMS groups on the amino nitrogen atom.

EXPERIMENTAL

Materials. — The silylating reagents, namely, chlorotrimethylsilane (TMCS), hexamethyldisilazane (HMDS), *N,O*-bis(trimethylsilyl)acetamide (BSA), and *N*-(trimethylsilyl)imidazole (TSIM) were purchased from Pierce Chemical Co. (P.O. Box 117, Rockford, Illinois 61105) in septum-capped bottles, and were used without purification. Pyridine and *N,N*-dimethylformamide (DMF), reagent grade, were obtained from Fisher Scientific Co. (690 Miami Circle, N. E., Atlanta, Georgia 30324); the former was dehydrated with, and stored over, Davison Molecular Sieves (Davison Chemical Co., Baltimore, Maryland 21203). Acetonitrile (ACS) containing 0.1% of water was obtained from Sargent–Welch (Birmingham, Alabama). Hydrocarbons were obtained from Applied Science Laboratories (P.O. Box 440, State College, Pennsylvania 16801). The 2-amino-2-deoxy-D-galactose hydrochloride (Grade I) and 2-amino-2-deoxy-D-glucose hydrochloride were obtained from Sigma Chemical Corp. (P.O. Box 14508, St. Louis, Missouri 63178). Glycosaminoglycans were kindly presented by Dr. J. A. Cifonelli; they were of the highest purity available.

The conditions of use of the various silylation reagents are summarized in Table I. All mixtures were prepared fresh daily. Dried samples were placed in small vials, 1.0 ml of silylating reagent was added, the internal standard, 10 µl of docosane solution (40 mg/ml of heptane), was added, and the vial was capped with a septum cap. Liquid samples were added to the vial and lyophilized prior to silylation.

Gas–liquid chromatography. — G.l.c. analyses were performed on a Hewlett–Packard 7610 High Efficiency Chromatograph equipped with a 7671 Automatic Sampler and a 3373 B Integrator (Hewlett–Packard Company, P.O. Box 28234,

TABLE I

REAGENTS AND CONDITIONS EMPLOYED FOR THE PER(TRIMETHYLSILYL)ATION OF HEXOSAMINES

Method	*Reagents and relative volumes*	*Reaction conditions*	*References*
Ia	HMDS–TMCS–pyridine (2:1:10)	r.t., 20 min	1, 3
Ia′	above, but reagents newly received	r.t., 20 min	
Ib	Ia+BSA (1 vol)	r.t., 20 min	8
IIa	BSA–pyridine (1:4)	injected immediately	
IIb	BSA–TMCS–pyridine (100:1:400)	injected immediately	
III	HMDS–DMF (1:5)	105°, 3 min	9
IV	TSIM–pyridine (1:4)	60°, 20 min	12
Va	BSA–TMCS–MeCN (100:1:400)	60°, 10 min	
Vb	Same as Va	60°, 5 h	10
VI	BSTFA–TMCS–pyridine (100:1:400)	60°, 3 h	

Atlanta, Georgia 30328), and a flame-ionization detector. The glass column (4 ft × 1/8 in.) was packed with 3.8% of UCW-98 silicone on 100/120 Diatoport S (provided pre-packed by Hewlett–Packard). This column has an efficiency of 0.8 mm, expressed as HETP, for the per(trimethylsilyl)ated carbohydrates. Helium (99.999% purity) was employed as the carrier gas. The column was operated at its temperature of optimal efficiency (160°), with the injector and detectors maintained at 200°. The carrier gas was passed through at the optimal gas-velocity (50 ml/min). Peaks were detected with the flame-ionization detector, with an attenuation level of 10^3 used on the electrometer. The usual injection volume was 3 μl. The usual internal standard was docosane ($C_{22}H_{46}$) (40 mg/ml of heptane), and 10 μl of this solution was added to samples after the addition of the silylating agents. For the determination of retention indices, a solution of hexadecane ($C_{16}H_{34}$; 100 μg/ml), octadecane ($C_{18}H_{38}$; 200 μg/ml), eicosane ($C_{20}H_{42}$; 300 μg/ml), and docosane ($C_{22}H_{46}$; 400 μg/ml) was prepared in heptane, and 1 μl was co-injected with the sample.

In studies made to determine the response factor, stock solutions of the respective hexosamines (10 mg/ml of distilled water) were prepared, and allowed to mutarotate for at least 2 days at room temperature prior to use. Aliquots of the samples were transferred to 1-ml glass vials, to give respectively 50, 100, 250, 1,000, 2,000, and 5,000 μg of the hexosamine hydrochloride per vial; the samples were lyophilized, and the residues were silylated with 1 ml of the silylating reagent by one of the methods already described.

Analysis of glycosaminoglycans. — Samples (1 mg) of sodium chondroitin 4-sulfate, dermatan sulfate, hyaluronate, and calcium heparan sulfate were hydrolyzed in 0.3 ml of 1M hydrochloric acid for 3 h (ref. 11) or 0.3 ml of 6M hydrochloric acid for 16 h (ref. 9) at 105° in heavy-walled, conical vials sealed with Teflon-faced caps (Pierce Chemical Co., "ReactiVials", 1 ml size). The samples were lyophilized, and the residues silylated with 0.2 ml of reagent according to method IIb (see Table I). The volume of internal standard added was 2 μl (80 μg).

RESULTS AND DISCUSSION

The g.l.c. analysis of derivatized, free (reducing) sugars has always presented certain problems, one of which is the multiplicity of peaks that may be observed. Thus, for a suitable derivative (that is, one produced by a method of derivatization that does not introduce additional peaks), it is theoretically possible to obtain a peak for the derivative of each anomer of both the pyranose and furanose forms, in addition to that of the acyclic, free carbonyl form. Even though this formation is not usually observed, and only two peaks result from a mutarotated sample, the presentation of retention data for carbohydrates can still be complex.

As the retention time is a highly irreproducible characteristic that depends upon several instrumental variables, retention data are usually presented relative to some internal standard, but, unfortunately, most workers in the carbohydrate field have chosen different standards. The normal alkanes have been used in the present work as standard solutes in terms of whose retentions the relative retentions of other solutes can be expressed; this is usually recorded in the form of the retention index, I, of Kovats[12]. A series of normal alkanes, in this case, $C_{16}H_{34}$, $C_{18}H_{38}$, $C_{20}H_{42}$, and $C_{22}H_{46}$, are chromatographed under the conditions being studied, and a plot is made of log t_R (where t_R is the adjusted retention-time) *versus* 100× the number of carbon atoms. As this graph is linear, the retention index of any compound can be found by taking the log of its retention time and finding the corresponding value on the abscissa. The retention indices observed for the hexosamines under study, together with the relative sizes of the peaks and the response factors relative to docosane, are summarized in Table II. Results are also expressed as retentions relative to the C_{22} hydrocarbon standard.

In order to assign peaks to various anomers, the pure, crystalline compounds were also studied. The response factors were measured as follows. For a mass-sensitive detector (such as the flame-ionization detector), it may be shown[13] that

$$W_i = W_j A_{ij} S_j / S_i = W_j A_{ij} / S_{ij},$$

where W is the weight of the respective compound in the mixture, A_{ij} is the area of i relative to the area of j, and S is the absolute mass-sensitivity of the detector. In practice, compound j is taken as the standard compound, so that, from a plot of W_i *versus* A_{ij}, it is possible to determine the relative response factor, S_{ij}. This relationship is independent both of the total volume of the sample and the volume of sample injected. For the present purposes, it was desirable to express results in terms of micromoles of hexosamine, N_i, in the sample; therefore, a plot was made of N_i *versus* A_{ij}. If more than one peak resulted, the total area of all of the peaks was used. A least-squares method[14] was applied to the data to obtain values of $S'_{ij} = S_{ij} M_i$, where M_i is the molecular weight of the hexosamine.

For the hexosamine hydrochlorides, three distrinct derivatives were produced for each anomeric form. Thus, for 2-amino-2-deoxy-D-glucose, pairs of peaks having retention indices of 1917 and 1973, 1978 and 2030, or 2009 and 2063 were observed,

whereas, for 2-amino-2-deoxy-D-galactose, pairs of peaks were observed having indices of 1885 and 1944, 1946 and 2022, or 1975 and 2055. For both hexosamines, the first pair of peaks corresponds to the derivatives previously characterized as having no TMS substitution on the amino group[7,8]. The second pair of derivatives produced by methods Ib, II, and, to a lesser extent VI, has previously been shown by gas chromatography–mass spectrometry to contain a single TMS substituent on the amino group. The third pair of derivatives, then, must correspond to the fully substituted form, *i.e.*, that in which both amino protons have been replaced by TMS groups. This conclusion is verified by the longer retention-time on the nonpolar, liquid phase UC-W98 (which implies a greater molecular weight) and by the reaction conditions needed for formation. Interestingly, production of the per(trimethylsilyl) derivative from the catecholamines[10] required heating for 5 h at 60°, whereas the per(trimethylsilyl) derivatives are produced from the hexosamine hydrochlorides by heating for only 10 min.

The reaction mixture employed in method Ia is, perhaps, the single most popular silylating reagent in use today, as it has a high reactivity toward a variety of functional groups. The combination is many times more reactive than either component alone, and this property has been explained[15] as being due to mutual catalysis, *i.e.* the reaction of HMDS, a base, is acid-catalyzed, whereas the reaction of TMCS, an acid, is base-catalyzed. The pyridine used as solvent also plays a complex role, and *inter alia*, is an acid acceptor that drives the reaction toward completion. Despite its high reactivity toward hydroxyl groups of all kinds, the mixture is not particularly reactive in replacing hydrogen atoms of amino or amide groups. It should be noted that, when all of the reagents are fresh (Method Ia′), some penta-TMS derivative is formed; this is the most probable explanation of the "extra" peaks reported by Sweeley and Walker[3].

BSA, a relatively new TMS donor, has received only very limited use in the carbohydrate field, despite its finding considerable application in steroid chemistry, particularly with TMCS (1% v/v) as a catalyst[16,17]. As previously mentioned, BSA was added to the mixture[8] used in method I to produce the *N*-TMS derivative. A similar silyl donor, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [25% (*v/v*) in *N,N*-dimethylacetamide], has been used to silylate hexosamines[18], but the derivatives resulting were not characterized. In our hands, BSTFA in pyridine (20% *v/v*) afforded the *N*-TMS derivative only, in 85% yield, even with TMCS as the catalyst and with prolonged heating. Interestingly, the use of BSA, alone, has been reported to lead to a variety of products. In a study of hexoses, Pierce[15] reported that a number of peaks were produced, whose relative areas were time-dependent; this was attributed to anomerization, but, as will be seen later, was probably due to the presence of partially silylated material. Of more interest is the report[8] of a similar phenomenon when hexosamine hydrochlorides were treated with BSA in pyridine, and, although the penta-TMS derivative preponderated, multiple peaks resulted. These results contrast with those now reported; by using BSA alone in pyridine, the hydrochlorides were found to be completely converted into the penta-TMS derivative.

TABLE II

RETENTION AND RESPONSE DATA FOR TRIMETHYLSILYL DERIVATIVES OF 2-AMINO- AND 2-ACETAMIDO-2-DEOXYHEXOSES

Hexosamine	*Silylation method*	*Relative areas for retention indices observed* (%)						*Response data*	
								Intercept	S'_{ij}[a]
		1917 (0.301)[b]	1973 (0.389)	1978 (0.414)	2030 (0.518)	2009 (0.439)	2063 (0.557)		
2-Amino-2-deoxy-D-glucopyranose·HCl, mutarotated	Ia	54	46	—	—	—	—	0.06 ±0.08	6.15 ±0.17
	Ia′	34	22	36	8	—	—	—	—
	Ib	—	—	56	44	—	—	—	—
	IIa	—	—	60	40	—	—	0.02 ±0.01	6.55 ±0.03
	IIb[c]	—	—	58	42	—	—	0.05 ±0.03	5.90 ±0.17
	III	56	44	—	—	—	—	0.14 ±0.14	5.23 ±0.17
	IV	64	36	—	—	—	—	—	—
	Va,b	—	—	—	—	70	30	0.02 ±0.02	6.37 ±0.02
	VI	10	5	55	30	—	—	—	—
crystalline	Ia	97	—	3	—	—	—	—	—
	Ia′	58	—	42	—	—	—	—	—
	IIb	—	—	100	—	—	—	—	—
	Va	—	—	—	—	100	—	—	—
		1885 (0.262)	1944 (0.341)	1946 (0.342)	2022 (0.510)	1975 (0.382)	2055 (0.538)		
2-Amino-2-deoxy-D-galactopyranose·HCl, mutarotated	Ia	45	55	—	—	—	—	0.02 ±0.10	6.33 ±0.55
	Ia′	51	41	[c]	8	—	—	—	—
	Ib	—	—	64	36	—	—	—	—
	IIa	—	—	69	31	—	—	0.04 ±0.02	7.17 ±0.10
	IIb[c]	—	—	68	32	—	—	0.07 ±0.03	6.12 ±0.15
	III	65	35	—	—	—	—	0.19 ±0.06	7.50 ±0.45

TABLE II (*continued*)

Hexosamine	*Silylation method*	*Relative areas for retention indices observed (%)*						*Response data*	
								Intercept	S'_{ij}[a]
	IV	12	88	—	—	—	—	—	—
	Va,b	—	—	—	—	62	38	0.03 ±0.02	6.28 ±0.09
	VI	8	4	50	38	—	—	—	—
crystalline	Ia	—	99	—	1	—	—	—	—
	IIb	—	2	—	98	—	—	—	—
	Va	—	—	—	—	—	100	—	—
			2076 (0.591)	2118 (0.714)	2146 (0.803)	2190 (0.997)			
2-Acetamido-2-deoxy-	Ia		—	—	100	—		0.05 ±0.03	6.00 ±0.10
D-glucopyranose,	IIa		5–20	25–45	40–60	10–20		—[d]	—[d]
mutarotated	IIb		—	12	80	—		0.07 ±0.03	6.37 ±0.17
	IV		—	6	94	—		—	—
	Va,b		—	43	57	—		0.09 ±0.04	6.38 ±0.20
crystalline	Va,b		—	54	46	—		—	—
			2093 (0.631)	2099 (0.647)	2122 (0.720)				
2-Acetamido-2-deoxy-	Ia		11	—	89			0.10 ±0.04	5.70 ±0.15
D-galactopyranose,	IIb		—	43	57			0.12 ±0.06	6.88 ±0.18
mutarotated	IV		13	—	87			—	—
	Va,b		—	42	58			0.09 ±0.04	6.31 ±0.08
crystalline	Va,b		—	8	93			—	—

[a]Units are mg of hydrocarbon per mole of hexosamine. [b]Retention index and retention relative to docosane in parentheses. [c]Unresolved from the $I = 1944$ peak. [d]One peak, unresolved from the C_{22} internal standard.

It should be noted that the amine hydrochlorides provide their own acid catalyst in the form of hydrogen chloride. However, the pyridine employed by Kärkkäinen and Vihko[8] was distilled from, and stored over, potassium hydroxide, whereas that employed in this study was not; it is possible that a small amount of potassium hydroxide dissolves in the pyridine, and neutralizes the hydrogen chloride, thus removing the acid catalyst. Dissolved potassium hydroxide may also be responsible for the puzzling report of lability of the N–$SiMe_3$ bond published[8] by these authors. In the present investigation, it was found that the derivatives produced by any of the methods listed in Table I are completely stable (as determined by constancy of area) for at least 24 h, and that some (Methods IIb, IV, and V) are stable for at least 48 h. The extensive precautions taken by most investigators to maintain anhydrous conditions are surprising, in view not only of the fact that several workers have reported that trimethylsilylations proceed in high yield under aqueous conditions[15], but also, that small amounts of water actually exert a powerful catalytic effect, at least for BSA in pyridine or acetonitrile[10].

The presence of the endogenous hydrochloric acid as the acid catalyst is undoubtedly responsible for the success of the reaction with HMDS in DMF, as Radhakrishnamurthy *et al.*[9] reported that carbohydrates other than the hexosamine hydrochlorides are unreactive under these conditions. The effect of the hydrogen chloride may be seen by comparing the results afforded by BSA, alone, with the hexosamine hydrochlorides and the 2-acetamido-2-deoxyhexoses. If a sample of α-D-galactopyranose is silylated by Method Ia or IIb, a single peak having $I = 1922$ results, but silylation by Method IIa (BSA alone) affords three peaks, having $I = 1890$, 1922, and 1965, with relative areas that vary with time. This result was attributed by Pierce[15] to anomerization catalyzed by BSA, as the relative proportions vary with time, but this explanation cannot be correct, because addition of 1% of TMCS to the sample causes disappearance of the peaks having $I = 1890$ and 1965 in less than two minutes, and the response factor for the remaining peak is now identical to that of the peak given by methods Ia or IIb. The most satisfactory explanation is that, in the absence of an acid catalyst, the replacement of the final proton is slow.

Chromatography of the TMS derivatives of the crystalline hexosamines allows assignments to be made for their α and β anomers. 2-Amino-2-deoxy-D-glucose hydrochloride usually occurs as the α anomer[19], whereas the β anomer is the more common form of 2-amino-2-deoxy-D-galactose hydrochloride[20]. Consequently, the following assignments can be made for 2-amino-2-deoxy-D-glucose. The peaks at $I = 1917$, 1978, and 2009 are due to the α-pyranose anomer, whereas those at $I = 1973$, 2030, and 2063 are due to the β-pyranose anomer. The number of TMS substituents on the amino group is, respectively for each pair of anomers, none, one, and two. For 2-amino-2-deoxy-D-galactose, the α-pyranose forms are eluted at $I = 1885$, 1946, and 1975, whereas the corresponding β-pyranose forms are eluted at $I = 1944$, 2022, and 2055.

These data confirm the rule proposed by Sweeley *et al.*[1] that, for hexopyranoses in both the *CI* and the *IC* conformation, the slower-moving anomer is that having the

bond to the anomeric hydroxyl group in the equatorial position. For both of the hexosamines studied, this is the α anomer, as confirmed by our data for the tetrakis-, pentakis-, and hexakis-(trimethylsilyl)ated derivatives.

The *N*-acetyl derivatives of these hexosamines were also studied, and some differences in behavior from that of the hexosamine hydrochlorides were noted. The 2-acetamido-2-deoxy sugars afford no endogenous, acid catalyst, and a large difference in reactivity was noted between BSA alone and BSA containing 1% of TMCS. With BSA alone, the ratios of the peak sizes were extremely variable, although the total area varied much less. At least two different derivatives are produced in various proportions by all of the methods employed, and the problem of multiple peaks was encountered with all of these silylating methods, including that using TSIM, which, presumably, silylates hydroxyl groups only. Only 2-acetamido-2-deoxy-D-glucose affords a single peak, and then only when per(trimethylsilyl)ated by method Ia. The most probable explanation is that the faster-moving component is formed by silylation of the amido group. Without some additional resonance-stabilization by the substituting group, secondary amides (which are, in any event, rather unreactive), tend to undergo *N*-silylation, although substitution on the carbonyl group is possible[12]. As silylation of the amide group interferes with its ability to form hydrogen bonds, it was to be expected that this type of substitution would lower the boiling point and, hence, decrease the retention time.

Although the anomers of the faster-moving derivative of 2-acetamido-2-deoxy-D-galactose appear to be resolved, resolution does not occur with the slower-moving derivative, nor with any of the derivatives of 2-acetamido-2-deoxy-D-glucose. Because of the poor resolution, both of the anomeric forms and of the two derivatives, afforded by the column employed, no attempt was made to inter-relate the peaks with the respective anomeric forms.

The response factors (relative to docosane), obtained by plotting the total area of all of the hexosamine peaks *versus* the number of moles of hexosamine in the sample, are also summarized in Table II. The data were adequately represented as linear functions and, in most cases, the intercept appeared not statistically significant. The only exception was the product obtained by method III, for which the intercept was significantly different from zero. A small curvature at low concentrations was also noted. This method also produced a variety of very minor peaks (not included in the area calculations) which, although insignificant at the higher concentrations of hexosamine (greater than 100 μg/ml), constituted a significant proportion at levels less than this.

If the results are averaged for the methods that produce only a single type of derivative, an average of 6.27 ±0.08 mg of hydrocarbon per mole of hexosamine (1.13 ±0.02 mg of hydrocarbon per mg of hexosamine) is obtained. This value can be used as the response factor for all of the hexosamines. It should be noted that the presence of two additional trimethylsilyl groups per molecule would not affect the response factor appreciably, as the difference in the proportion of carbon contained is small.

Because of its sensitivity and the resolution it provides, g.l.c. is an ideal method for analyzing glycosaminoglycan hydrolyzates for released monosaccharides. Table III summarizes the results of analyzing hydrolyzates obtained under comparatively mild,

TABLE III

ANALYSIS OF HEXOSAMINES IN HYDROLYZATES OF GLYCOSAMINOGLYCANS

Sample	*Conditions of hydrolysis*	*Relative areas for observed retention indices* (%)			*Recovery of total theoretical hexosamine*[d]
		1945[a]	*1978*[b]	*2022+2030*[c]	
Hyaluronate	3 h, 1M	tr.	65	35	33
	16 h, 6M	tr.	71	29	108
Dermatan sulfate	3 h, 1M	61	2	37	92
	16 h, 6M	68	2	30	64
	16 h, 6M	71	2	29	81
Chondroitin 4-sulfate	3 h, 1M	65	tr.	35	27
	16 h, 6M	67	tr.	33	96
Heparan sulfate	3 h, 1M	tr.	70	30	7.5
	16 h, 6M	tr.	70	30	96

[a]Derivative of 2-amino-2-deoxy-α-D-galactopyranose; tr. = trace. [b]Derivative of 2-amino-2-deoxy-α-D-glucopyranose. [c]Unresolved derivatives of β anomers. [d]Calculated from chemically determined, percent composition.

as well as more vigorous, conditions. The problem of mutarotation appears to offer fewer problems than might have been anticipated, particularly in view of the poor resolution of the derivatives of the β anomers. The fact that the relative ratios of the α and β anomers are relatively independent of the conditions of hydrolysis indicates that the areas due to the α anomers may be used to quantitate the individual hexosamines when both are present in a mixture. The differences in susceptibility of the various glycosaminoglycans to hydrolysis can be an important source of error. This is particularly true of measurements of the relative amounts of heparan sulfate and dermatan sulfate in the urine of patients having mucopolysaccharidoses, in that the conditions necessary for completely hydrolyzing heparan sulfate result in considerable decomposition of the dermatan sulfate. The higher lability of the dermatan sulfate can, however, be exploited for its identification and quantitation by subjecting the sample to hydrolysis for 3 h in 1M hydrochloric acid. Not only is the release of hexosamine virtually quantitative, but a large peak due to iduronic acid is also identifiable. Use of small vials having Teflon-faced caps is important to obtaining the hexosamines in high yield; if 5-ml vials are used instead for hydrolysis of 3-mg samples dissolved in 1 ml of M hydrochloric acid, the yields are lessened by 25–75%.

The problems of anomers and of the distribution of the anomeric forms are worthy of some further mention. In the absence of any changes induced by the silylating agent, the percentages given in Table II should reflect the equilibrium distribution of the two anomeric forms, as these were obtained from aqueous solutions

at mutarotational equilibrium. There are, however, some differences evident in the data in Table II. Furthermore, there is usually some variability for a given silylating agent; this variability is, almost exclusively, a function of the time that elapses between lyophilization and silylation. Immediately upon removal of the water, the hexosamines form an amorphous layer on the bottom of the vial, but, with time, the thermodynamically stable anomer begins to crystallize out. Significant changes can occur overnight, and, after 5 days, the less-stable anomer has almost completely disappeared. This circumstance can be exploited to produce a single peak for each sugar.

Each of the trimethylsilyl derivatives has its own particular advantages. The tetrakis(trimethylsilyl) derivatives of the β anomers are resolvable on the customary 4-foot column, and this is a definite advantage for quantitative analysis. Although this derivative from the hydrochlorides can be produced somewhat specifically by method III, the presence of other carbohydrates results in broad, diffuse peaks that interfere with the amino sugar peaks[9]. Unfortunately, the peaks due to tetrakis-(trimethylsilyl) derivatives of the α anomers are not resolved from the peaks due to galactose. The pentakis- and hexakis-(trimethylsilyl) derivatives are well separated from other carbohydrates but, unfortunately, the β anomeric derivatives are not resolved from each other by this short column, and thus, the utility of these derivatives for quantitative analysis in mixtures is limited, unless the ratio of the two anomers is constant. The separation from other carbohydrates suggests, however, that either method IIb or Va may prove advantageous in the assay of glycoproteins and glycolipids.

ACKNOWLEDGMENTS

The author thanks Mrs. Hope Stinger for her excellent technical assistance, Dr. Julian Menter for his helpful advice, and Dr. J. N. Thompson for his aid in the preparation of the manuscript of this article. This work was supported, in part, by the National Institute of General Medical Sciences, National Institutes of Health Grant 1PO1-GM 18252-02.

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