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Note

A comparison of some hydrolytic and gas chromatographic procedures used in methylation analysis of the carbohydrate units of glycopeptides

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In recent years, when employing methylation analysis in structural investigations of glycopeptides containing 2-acetamido-2-deoxyhexose residues, it has become standard practice to degrade the methylated glycopeptides by an acetolysis procedure[1] prior to determination of the released, partially methylated sugar residues as alditol acetates. The procedure was devised to protect the acetyl portion of the *N*-methylacetamido group that is quantitatively formed during methylation of amino sugar residues[1]. Such protection facilitates splitting of glycosidic linkages that become resistant to acid hydrolysis if the acetyl group is lost to give a positively charged methylaminohexosyl residue.

During methylation analysis of asparaginyl-carbohydrate glycopeptides by g.l.c. with glass-capillary columns, we have found that, whereas this hydrolytic procedure gives good recoveries of amino sugar derivatives and most of the hexitol derivatives, it gives low molar ratios for the triply substituted β-D-mannosyl residue found in the core of some glycopeptides of this type. The use of formolysis followed by hydrolysis[2] results in quantitative yields of all the methylated hexitol residues, but gives low and more variable yields of the methylated aminodeoxyhexitols. Therefore, in order to ensure accurate determination of all the methylated residues in such glycopeptides, it is necessary that both hydrolytic procedures are employed, or that correction factors are applied to compensate for losses by each method.

In the course of this work, we have also demonstrated that, for g.l.c. separations with glass-capillary columns, on-column injection[3] gives more reproducible results than do other injection procedures. This technique is of particular value for methylated aminodeoxy sugars, which often tend to give variable quantitative results.

For purposes of comparison, we analysed two glycopeptides (from ovalbumin and ovomucoid) known to contain triply substituted, β-D-mannosyl residues[4–6], and one (from fetuin) which did not[7]. After methylation, each product was subjected to acetolysis/hydrolysis[1] or formolysis/hydrolysis[2]. An on-column injection technique[3] was used for the introduction of the samples into a glass-capillary column wall-

TABLE I

COMPARISON OF METHYLATION ANALYSES OF GLYCOPEPTIDES USING (*A*) ACETOLYSIS AND (*B*) FORMOLYSIS PROCEDURES

Methylated derivative[a]	*Molar ratios*					
	Ovalbumin		*Ovomucoid*		*Fetuin*	
	(A)	(B)	(A)	(B)	(A)	(B)
2,3,4,6-Gal	—	—	1.0	1.0	1.0	1.0
2,3,4,6-Man	2.0	2.0	—	—	—	—
3,4,6-Man	1.0	1.1	1.1	1.0	2.0	2.1
2,4,6-Gal	—	—	—	—	2.8	2.7
2,3,4-Gal	—	—	—	—	2.2	2.5
3,6-Man	—	—	1.7	2.0	1.8	2.2
2,4-Man	0.9	0.9	—	—	1.9	1.9
2-Man	0.5	1.1	1.1	2.1	—	—
3-Man	—	—	1.1	1.1	—	—
3,4,6-GlcNAcMe	2.0	1.3	9.0	7.1	—	—
3,6-GlcNAcMe	1.9	1.2	4.6	3.3	8.5	5.3

[a]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, *etc*; 3,4,6-GlcNAcMe = 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucitol, *etc.*

coated with OV-1. A comparison of the results obtained is shown in Table I. To test the reproducibility of the methylation, a further eight samples of ovomucoid glycopeptide were methylated, and four were hydrolysed by each procedure. The variability in ratios was not significantly greater than for results from multiple injections of a single sample.

For the two glycopeptides which contained 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-D-mannitol, acetolysis gave molar ratios for this derivative which were about half those obtained when formolysis was employed. The other hexitol derivatives showed little difference in their ratios. Mass spectrometry was used to confirm that the peak corresponding to the 2-*O*-methylmannitol derivative obtained after formolysis contained no contaminant.

For all three glycopeptides, formolysis gave molar ratios for the amino sugar derivatives which were lower than those obtained by acetolysis. The formolysis samples gave small, subsidiary peaks which appeared immediately behind each of the peaks corresponding to the 3,4,6-tri-*O*-methyl- and the 3,6-di-*O*-methyl-*N*-methylacetamido derivatives. Mass spectrometry showed that these peaks represented the corresponding *N*-acetylacetamido derivatives. Other workers who have employed formolysis during methylation analysis of glycopeptides containing amino sugars have also identified *N*-acetylacetamido derivatives in addition to the *N*-methylacetamido products[8]. Only traces of the *N*-acetylacetamido derivatives were present in the acetolysis samples; even for the formolysis samples, the molar ratios of the *N*-acetylacetamido derivatives were much too small to account for the loss of amino sugar.

TABLE II

COMPARISON OF METHYLATION ANALYSIS RESULTS[a] OBTAINED WITH DIFFERENT INJECTION TECHNIQUES

Methylated derivative[b]	*Split injection*		*Splitless injection*		*On-column injection*	
	Molar ratio	*% rel. S.D. (n = 7)*	*Molar ratio*	*% rel. S.D. (n = 6)*	*Molar ratio*	*% rel. S.D. (n = 5)*
2,3,4,6-Gal	1.00		1.00		1.00	
3,4,6-Man	0.99	4.0	0.97	4.1	1.09	1.8
3,6-Man	1.60	10.0	1.52	9.9	1.97	1.0
2-Man	1.62	17.3	1.42	15.5	2.11	3.3
3-Man	0.77	18.2	0.77	50.7	1.05	1.9
3,4,6-GlcNAcMe	5.37	19.4	3.67	20.4	7.03	4.1
3,6-GlcNAcMe	2.33	26.6	1.51	31.8	3.23	4.3

[a]For ovomucoid glycopeptides hydrolysed by the formolysis procedure[2]. [b]For key, see Table I.

With capillary g.l.c., the injection technique used is another source of variability of ratios, particularly for such slow-running and sensitive compounds as amino sugars. A comparison was therefore made of results obtained by three injection techniques (Table II). Both split and non-split injections resulted in considerably lower and more variable molar ratios for the less-volatile derivatives in the samples than were obtained with on-column injection. Variability of results with split and non-split injection have been observed when determining triglycerides by capillary g.l.c.[9]. For accurate quantification of methylated sugars, therefore, on-column injection is recommended when using glass-capillary columns. Under these conditions, using an OV-1 column, much more reproducible results are obtained for amino sugar derivatives that have often been found difficult to quantify by g.l.c.

EXPERIMENTAL

Glycopeptides. — Exhaustive digestion with Pronase was employed to obtain glycopeptides from ovalbumin[4], asialo-ovomucoid[5], and fetuin[10]. Glycopeptide 4 was obtained from ovalbumin[4], and glycopeptide 1 from fetuin[10]. Glycopeptide 1 was a heterogeneous fraction from which a small amount of sialic acid had been removed.

Hydrolysis conditions. — The glycopeptides were methylated by the procedure of Stellner *et al.*[1], and half of the product was subjected to acetolysis/hydrolysis[1]. The other half was subjected to formolysis/hydrolysis as described by Lindberg[2], except that neutralisation was carried out with Amberlite IRA-400 (HCO_3^-) resin.

Chromatography. — The gas chromatograph was a Carlo Erba Fractovap 4160 model equipped with a flame-ionisation detector and Grob split/non-split injector as well as a non-vaporising, septum-less, on-column injector of the Grob type[3] with secondary cooling. For split injection, the column was maintained at 200° and

the splitting ratio was 40:1. For non-split injection, the column was cooled to 60° and, 30 sec after injection, the injector was vented and the temperature was raised rapidly to 200°. For on-column injection, the column was cooled to 60° during injection and then raised rapidly to 200° and maintained thereat. Separations were performed on glass-capillary columns (50 m × 0.3 mm) wall-coated with OV-1 (Phase Separations Ltd., Clwyd, U.K.).

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