Journal of Chromatography, 370 (1986) 103–112
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 983

QUANTIFICATION OF 2-KETO-3-DEOXYOCTONATE IN (LIPO)POLYSACCHARIDES BY METHANOLYTIC RELEASE, TRIFLUOROACETYLATION AND CAPILLARY GAS CHROMATOGRAPHY

KLAUS BRYN* and ERIK JANTZEN
Statens Institutt for Folkehelse, Geitmyrsvn. 75, 0462 Oslo 4 (Norway)
(First received October 8th, 1985; revised manuscript received July 28th, 1986)

SUMMARY

Several conditions of acidic anhydrous methanolysis were examined to optimize the release and minimize the degradation of unphosphorylated 2-keto-3-deoxy-D-*manno*-octonic acid (KDO) from bacterial lipopolysaccharides and polysaccharides. The reaction was monitored by capillary gas chromatography after derivatization by trifluoroacetic anhydride. The best results were obtained by use of 2 *M* hydrochloric acid at 60°C for 2 h. Under these conditions a single KDO component appeared, and KDO was quantitatively released from all model compounds except when glycosidically linked to hexosamines. For quantitative cleavage of this linkage a reaction time of 6 h was required at 60°C, giving rise to 5–10% of secondary KDO products. The KDO detection limit was about 250 pmol (50 ng) and the molar response was the same as for glucose. The KDO derivative gave a mass spectrometric fragmentation pattern consistent with a pyranosidic methylketoside methyl ester structure. Differentiation of KDO linkage types could be obtained by determination of the rates of KDO release by mild methanolysis.

INTRODUCTION

Lipopolysaccharides (LPS) of Gram-negative bacteria consist of a polysaccharide and a lipid A part, usually linked together by 2-keto-3-deoxy-D-*manno*-octonic acid (KDO)[1]. In some capsular polysaccharides (PS) KDO is a major constituent, *e.g.*, in K12[2] and K13[3] of *Escherichia coli*, and in the group-specific PS of *Neisseria meningitidis* 29e[4].

Quantification of the KDO content in bacterial PS or LPS is usually performed by spectrophotometric methods involving a hydrolysis step, as in the most commonly used thiobarbituric acid assay[5–7]. These methods suffer from several limitations. Major difficulties are due to the lability of KDO under the acidic conditions required for its release[8] and to interferences from other deoxy sugars that frequently occur in Gram-negative bacteria[5], *e.g.*, neuraminic acid[9]. Further, substitution in the 4- or 5-position of KDO may give the chromophore in low yield[10].

When the release of KDO is performed by hydrochloric acid in methanol in-

0021-9673/86/$03.50

stead of by acidic hydrolysis, degradation apparently can be reduced[11,12]. Thus, the KDO content of LPS can be determined with reasonable accuracy by methanolysis followed by gas chromatography (GC), although a complex KDO peak pattern is observed[11]. We wanted to overcome this difficulty by establishing more optimal conditions for the release of KDO. In this work we studied the appearance of KDO products during methanolysis at lower temperatures for various periods of time. For most ketosidic and glycosidic linkages complete release and negligible degradation were obtained at 60°C for 2 h, and by use of defined conditions we could discriminate between several types of KDO linkages simultaneously present in the same polymer.

EXPERIMENTAL

Materials

Solvents of analytical-reagent grade (Rathburn Chemicals, Walkerburn, U.K.) were used without further purification. Methanolic hydrochloric acid was prepared by bubbling dry hydrogen chloride gas (Messer Griesheim, Düsseldorf, F.R.G.) into methanol until saturation and dilution to 2 *M* with methanol.

α-Methyl mannoside and *meso*-inositol were obtained from Koch-Light (Colnbrook, U.K.), methyl *n*-heptadecanoate from Sigma (St. Louis, MO, U.S.A.) and C^2H_3OH of Uvasol grade, trifluoroacetic (TFA) anhydride and acetonitrile from Merck (Darmstadt, F.R.G.). Synthetic 3-hydroxymyristic acid (3-OH-14:0) and myristoxymyristic acid was a gift from Ernst Th. Rietschel (Forschungsinstitut Borstel, F.R.G.), synthetic KDO (ammonium salt) from Chris Galanos [Max-Planck-Institut für Immunbiologie (MPI), Freiburg, F.R.G.], authentic L-glycero-D-*manno*-heptose and rough LPS (Rd and Re) of *Salmonella minnesota* from Otto Lüderitz (MPI) and *Escherichia coli* polysaccharides K12, K13 and K82 from Klaus Jann (MPI). LPS of *N. meningitides* was from Carl Frasch (Office of Biologics, FDA, MD, U.S.A.).

Smooth LPS of *Yersinia enterocolitica* O3 and of several salmonellae were prepared in our laboratory by the hot aqueous phenol procedure[13]. Capsular polysaccharide of *N. meningitidis* group 29e was obtained by either hot or cold buffered[14] phenol extraction. Both LPS and polysaccharide were purified by ultracentrifugation twice at 105 000 *g* for 4 h. An extract containing LPS and the polyneuraminic acid PS of serogroup C of *N. meningitidis* was obtained by the hot phenol method.

Methanolysis and trifluoroacetylation

Lyophilized aliquots of polysaccharides and LPS (10–100 μg) were methanolysed (2 *M* hydrochloric acid; 0.1–1 ml) in PTFE-lined screw-capped 2-ml vials. After flushing with nitrogen, the vials were heated as indicated. In kinetic experiments methanolysis was stopped by chilling (−50°C with acetone–dry-ice) and by diluting three-fold with cold methanol. Hydrochloric acid and methanol were removed with nitrogen and the residue was converted into the TFA derivatives[11]. Before injection into the gas chromatograph the TFA anhydride concentration was decreased to 10%.

Gas chromatography and mass spectrometry (MS)

GC analyses were carried out on a fused-silica capillary column (25 m × 0.2 mm I.D.) with SE-30 methylsilicone as the stationary phase (Hewlett-Packard, Avondale, PA, U.S.A.), operated in the splitless mode with helium as carrier gas and

programmed from 90°C (2 min) to 270°C at 8°C min^{-1}. Several samples were also chromatographed on a more polar methylsilicone column (5% phenyl, SE-54) under similar conditions.

GC–MS was carried out with a Hewlett-Packard 5992A apparatus equipped with a glass capillary column (25 m × 0.5 mm I.D.) containing CP-Sil 5 (methylsilicone) (Chrompack, Middelburg, The Netherlands) with helium as carrier gas. Mass selection was performed at 70 eV at 170°C, with scanning of masses from 50 to 700. Additional analyses were performed on a Finnigan 4023 instrument at the Centre for Industrial Research, Oslo.

Primary identification of the GC peaks was made by comparison of retention times with those of methanolysed reference substances[11]. The identities were confirmed by MS and interpretation of the fragmentation patterns was verified by additional MS analyses after methanolysis in C^2H_3OH.

Gas chromatographic quantification of KDO and other components

meso-Inositol, α-methyl mannoside and methyl *n*-heptadecanoate were used as internal standards, added after lyophilization of the samples. Relative molar response factors were determined by methanolysis of polymers of known structures (Tables I and II). The LPS marker 3-hydroxytetradecanoic acid (as the TFA derivative of the methyl ester) was given a response value of 1.00[11].

Spectrophotometric determination of KDO

The thiobarbituric acid assay was applied as described by Brade *et al.*[15], with hydrolysis in 1 *M* hydrochloric acid for 4 h in sealed ampoules, followed by periodate treatment. The calibration graph was based on the ammonium salt of KDO, hydrolysed in four different amounts (32–96 μg), each hydrolysate being processed further in duplicate.

RESULTS

Release of KDO

Various conditions of methanolysis were examined in order to optimize the yield of liberated KDO. Different KDO-containing polymers with known structures were methanolysed (2 *M* hydrochloric acid) at 37, 60 and 85°C for various periods of time, and the reaction products were analysed as TFA derivatives by capillary GC.

When the *E. coli* polysaccharides K12 (Rha-Rha-KDO)n and K13 (Rib-KDO)n[2,3] were methanolysed at 85°C, a time-dependent change in the GC peak pattern was apparent. KDO appeared primarily as a single peak (A in Fig. 1) which slowly decreased, accompanied by the appearance of a new major (heterogeneous) peak (B) and several smaller peaks. The sum of peak areas A and B rapidly rose to a maximum and then fell to *ca.* 70% of the maximum after 12–16 h. At this stage the ratio of the areas of peaks A and B was *ca.* 1.2:1. This characteristic chromatographic development was reasonably reproducible and largely independent of the nature of the KDO-containing polymer, as indicated earlier in studies on LPS[11]. We have now observed this peak pattern for a large number of LPS and polysaccharide preparations. However, owing to its complexity (Fig. 1B) the conditions were clearly not optimal for more accurate KDO determinations.

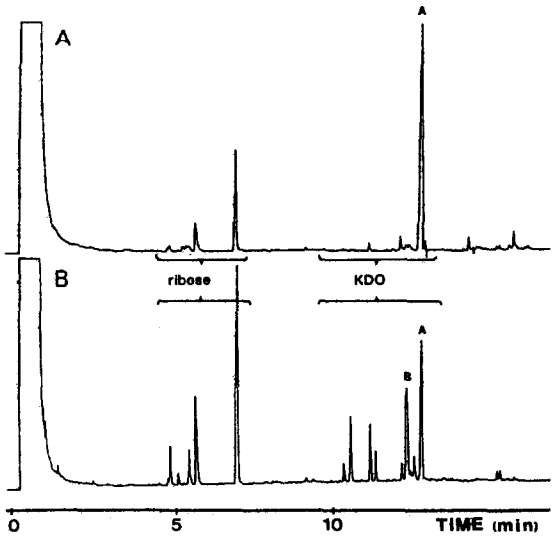

Fig. 1. Gas chromatography of methanolysed *E. coli* K13 polysaccharide (Rib/1-7KDO)n on a 25 m OV-1 fused-silica capillary column. Temperature programmed from 90 to 270°C at 8°C/min. Splitless injection. (A) 2 *M* HCl, 60°C, 2 h; (B) 2 *M* HCl, 85°C, 18 h. Peak A, TFA-derivatized methylketoside methyl ester of KDO; peak B, TFA-derivatized degradation product.

On lowering the temperature to 60°C only a single KDO peak (peak A, Fig. 1) was initially recorded, and the two polysaccharides K12 and K13 were completely cleaved in about 1 h (Table I). After 2 h peak B constituted about 5%, increasing to 5–10% after 6 h and 10–15% after 8 h. For *Salmonella* LPS a more complex development appeared during methanolysis. Less than 10 min were sufficient for complete release of KDO from Re LPS, containing solely a KDO disaccharide unit attached to lipid A[16]. For Rd LPS, with a terminal heptose linked to the KDO cluster[16], a biphasic release of KDO was observed (Fig. 2). About 60% was split off in 10 min, and about 90 min were required for completeness of the reaction, indicating a relatively slow cleavage of the heptose–KDO bond.

The use of a temperature of 37°C for methanolysis of K12 and K13 drastically decreased the rate of KDO release (Table I), and only KDO peak A was detected. At this low methanolysis temperature intermittently high amounts of oligosaccharides were formed; for K13 as much as 38% appeared as disaccharides after 15–20 min, which may be of interest for the isolation of KDO-containing oligosaccharides.

Release rates of KDO from various types of linkages

The biphasic methanolytic release of KDO from *Salmonella* Rd LPS (Fig. 2) allowed the selective quantitation of KDO moieties that were glycosidically or ketosidically linked. As seen in Table I, the half-times for release of KDO varied considerably with the type of linkage involved. The following order of increasing stability towards methanolysis was found: ketosidic < ribofuranosidic < rhamnosidic < heptosidic < hexosaminidic. With the exception of the last linkage, all were cleaved

TABLE I

STABILITY OF SOME COMMON (LIPO)POLYSACCHARIDE LINKAGES TOWARDS METHANOLYSIS

Samples were methanolysed (2 *M* hydrochloric acid) at 37, 60 and 85°C and aliquots were analysed by GC as indicated in the text.

*Linkage**	*Parent structure*	*Time required for 50% release (min)*		
		37°C	*60°C*	*85°C*
KDO2–6GlcN	*Salmonella* LPS; Re; Rd	10	≪5	–
KDO2–4KDO	*Salmonella* LPS; Re; Rd	10	≪5	–
Hep1–5KDO	*Salmonella* LPS Rd	400	30	≪10
Rib*f*1–7KDO	*E. coli* K-13 polysaccharide	40	–	–
Rha1–5KDO	*E. coli* K-12 polysaccharide	120	15	–
GalN1–7KDO	*N. meningitidis* polysaccharide 29e	–	70	–
Abq1–3Man	*Salmonella abortus-equi* LPS	<5	–	–
Man1–4Rha	*Salmonella abortus-equi* LPS	75	–	–
Gal1–4Glc	Lactose; fucosyllactose	75	15	–
Fuc1–2Gal	Fucosyllactose	–	<5	–
GlcN–(3-OH-C_{14}) (amide)	*Salmonella* LPS	–	⩾960	360
GlcN–(3-OH-C_{14}) (ester)	*Salmonella* LPS	–	45	15
(3OH-C_{14})–C_{16} (acyloxy)	*Salmonella* LPS	35	5	–
(3-OH-C_{14})–(2-OH-C_{14}) (acyloxy)	*Salmonella* LPS	35	5	–

* Abbreviations: KDO, 2-keto-3-deoxy-D-*manno*-octonic acid; GlcN, D-glucosamine; Hep, L-glycero-D-*manno*-heptose; Rib*f*, D-ribofuranose; Rha, L-rhamnose; Abq, D-abequose; Man, D-mannose; Gal, D-galactose; Glc, D-glucose; Fuc, L-fucose; 3-OH-C_{14}, 3-hydroxymyristic acid; 2-OH-C_{14}, 2-hydroxymyristic acid; C_{16}, palmitic acid. –, Not determined.

quantitatively (>95%) within 2 h at 60°C. The galactosamine–KDO linkage required 6 h at 60°C or 2 h at 85°C for complete cleavage.

For comparison, Table I also shows the stabilities of some linkages not involving KDO, but frequently occurring in LPS or polysaccharides. A very rapid release of 3,6-dideoxyhexoses was apparent (abequose and tyvelose behaved similarly). Amide linkages showed high stability. There was a marked difference between fatty acids ester-linked to glucosamine and those with an acyloxy linkage to the 3-hydroxy group of amide-bound fatty acids. Hence three types of fatty acid linkage may be discriminated by methanolysis.

Molar response

The molar response of KDO and some other sugars (after methanolysis at 60°C for 2 h) was established by the use of structurally well defined polymeric saccharides such as the two *E. coli* polysaccharides K12 and K13. The first of these (Rha-Rha-KDO)n in combination with the disaccharide rutinose (Rha-Glc) revealed a KDO molar response of 0.44. This value was also obtained by use of K13 (Rib-KDO)n in combination with D-ribose. By coincidence this value is the same as for

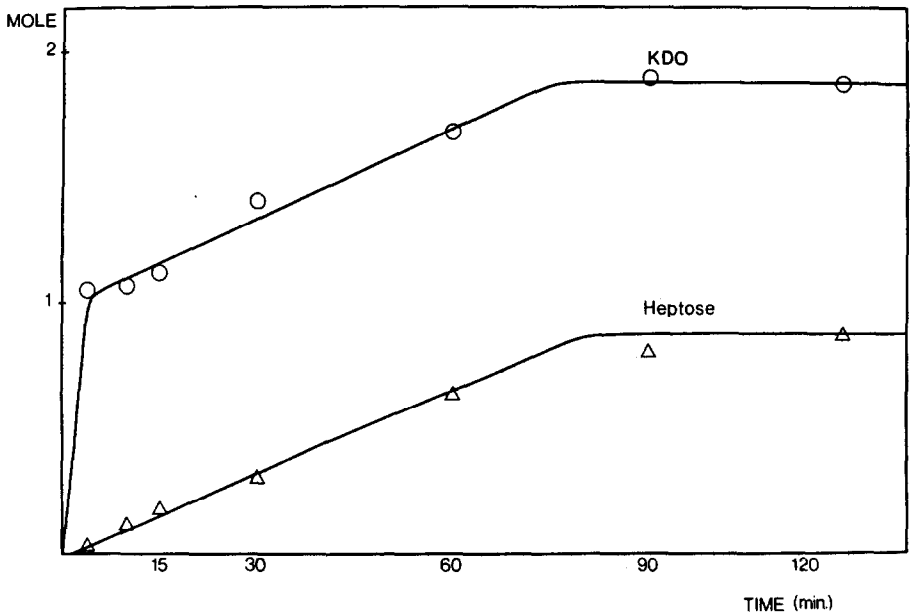

Fig. 2. Release of KDO and L-glycero-D-*manno*-heptose from *Salmonella* Rd LPS at different times of methanolysis (2 *M* HCl, 60°C). Aliquots were withdrawn at the indicated intervals and treated as described in the text.

glucose (Table II), and slightly lower than reported earlier[11]. The detection limit for KDO was about 250 pmol (50 ng) when analysed on a well used GC column.

GC–MS analysis of KDO methanolysis products

The mass spectrum of the primary and main methanolysis product formed under mild conditions (peak A, Fig. 1) is shown in Fig. 3. The fragmentation is consistent with a per(trifluoroacetylated) O-methylketoside of methyl 3-deoxyoctu-

TABLE II

MOLAR RESPONSE FACTORS OF KDO IN RELATION TO SOME OTHER SUGARS

Samples were methanolysed (2 *M* hydrochloric acid; 60°C for 2–6 h); see text and Table I for experimental details and abbreviations. Response of glucose was set as 0.44 (ref. 11).

Sugar	*Parent structure*	*Molar response*
D-Glucose	–	0.44
D-Galactose	Fucosyllactose (Fucl-2Gall-4Glc)	0.44
L-Fucose	Fucosyllactose	0.38
L-Rhamnose	Rutinose (Rhal-6Glc)	0.38
D-Ribose	D-Ribose	0.32
KDO	*E. coli* polysaccharide K12; (-3Rhal-2Rhal-5KDO2-)n	0.44
	E. coli polysaccharide K13; (-3Rib*f*1-7KDO2-)n	0.44

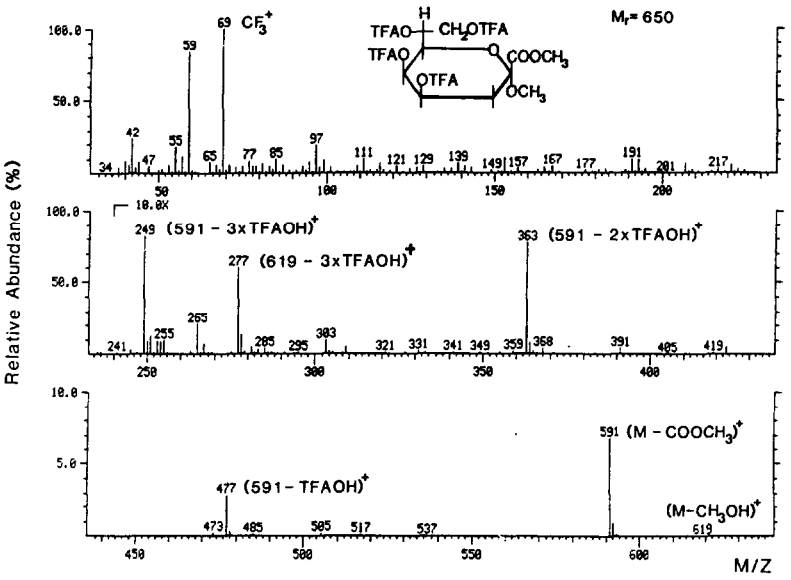

Fig. 3. Electron-impact mass spectrum with suggested fragment composition of TFA-derivatized methylketoside methyl ester of KDO (peak A in Fig. 1).

losonate in a pyranose ring. No molecular ion (*m/z* 650) was seen, but minor fragments of *m/z* 631 ($M^+ - F$) and *m/z* 619 ($M^+ - CH_3OH$) were occasionally observed. A prominent fragment was *m/z* 591 (loss of $COOCH_3$), and the series *m/z* 477, 363 and 249 formed by additional loss of one to three TFAOH groups. Such sequential loss of TFAOH or TFAO appears to be typical of acetylated sugars[17,18]. MS of the secondary peak B was complicated by its heterogeneity. However, constant spectra were obtained throughout the peak, indicating a mixture of chemically closely related substances. These appear to be entirely different from the primary (peak A) compound, as several distinct fragment ions of peak A were completely missing from the mass spectra of peak B. A prominent fragment (*m/z* 59) arising from the carboxyl methyl ester group was present in the spectra of both peaks A and B, indicating an absence of a lactone ring. Hence an anhydro structure is suggested for the conversion product (peak B) of KDO after methanolysis at 85°C.

Comparison of the GC method and spectrophotometry for determination of KDO

The KDO contents of LPS from *N. meningitidis* and of the capsular PS K82, (Rha-Rha-KDO)n from *E. coli* were determined both by the GC procedure (methanolysis in 2 *M* hydrochloric acid at 60°C for 2 h) and by the thiobarbituric acid assay. From Table III it appears that the values obtained by GC vary slightly less, especially when a new column is used. Apparently the amounts of KDO found in the LPS by GC (15.7%) is in good agreement with structural data (see Table III), assuming 3 mol of KDO per mole of LPS. In comparison, the corresponding value obtained by spectrophotometry probably is too high owing to a higher rate of degradation of the standard monomeric KDO than for the KDO bound in LPS (see Discussion). For both methods low KDO values were obtained for polysaccharide

TABLE III

KDO IN (LIPO)POLYSACCHARIDES DETERMINED BY SPECTROPHOTOMETRY AND METHANOLYSIS–GAS CHROMATOGRAPHY

The spectrophotometric method used was the thiobarbituric acid assay, with hydrolysis in 1 *M* hydrochloric acid at 100°C for 4 h[15]. For GC the methanolysis was performed in 2 *M* hydrochloric acid at 60°C for 2 h, followed by trifluoroacetylation and GC (see Fig. 1 and text).

Material	*Method*	*Samples (n)*	*Mean content of KDO (%)*	*S.D.**
LPS of strain M986	GC	5	15.7	0.6
*N. meningitidis***	Spectrophotometry	5 × 2	23.3	1.2
	Calculated***	–	17.0	–
E. coli poly-	GC; old column	5	31.3	1.8
saccharide K-82	GC; new column	5	33.2	1.0
	Spectrophotometry	3 × 2	38.7	2.7
	Spectrophotometry	5 × 2	33.5	2.3

* S.D., standard deviation. For the GC method values were determined from a single GC profile of separately methanolysed samples; for the spectrophotometric method each hydrolysate was processed in duplicate and the means used for calculation of standard deviations.

** Quantification of LPS in the samples was based on the 3-OH-14:0 fatty acid determined by GC[11], and the calculation was based on 6 mol of fatty acids per mol and the published structure of *N. meningitidis* oligosaccharide type L3[19,20].

*** The content of KDO in LPS of *N. meningitidis* is unknown. Assuming 3 mol of KDO per mole of LPS, as for enterobacteria[16], a KDO value of 17% was calculated.

K82 (theoretical value 43%). This may be due to an unknown content of water or other impurities, as the accuracy of the KDO value recorded by GC was supported by the rhamnose:KDO ratio of 2.0:0.93 (2:1 expected).

One important advantage of the GC method for KDO determinations, the lack of interference from other deoxy sugars, is illustrated in Fig. 4. In a phenol–water extract of *N. meningitidis* of serogroup C, with both LPS and the sialic acid-containing group PS, KDO and neuraminic acid could be simultaneously determined, the latter sugar being only partly N-deacetylated under these mild conditions.

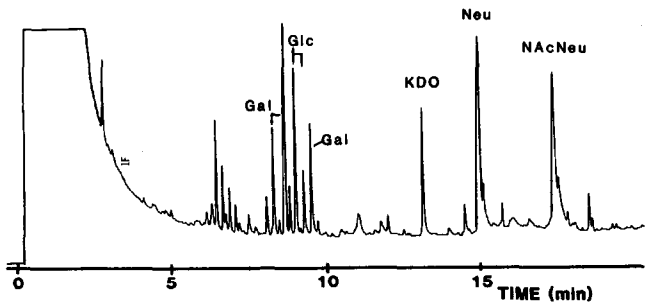

Fig. 4. Gas chromatography of a phenol–water extract of *N. meningitidis* serogroup C, containing LPS and capsular polysaccharide, after methanolysis in 2 *M* HCl at 60°C for 2 h and trifluoroacetylation. Conditions for GC as in Fig. 1. Abbreviations: Gal, galactose; Glc, glucose; Neu, neuraminic acid; NAcNeu, N-acetylneuraminic acid.

DISCUSSION

Release of KDO from (lipo)polysaccharides by acidic hydrolysis may cause a major conversion of the labile sugar to lactone[21,22] or anhydro ring forms[23]. Because as much as 70% of KDO may be converted into products that do not contribute to the colour yield in the thiobarbituric acid assay[15], careful correction for the degradation caused by hydrolysis is required by use of authentic KDO. Brade *et al.*[15] recommended that the conditions for hydrolytic release of KDO should be optimized for each polymer to be analysed.

When hydrolysis is replaced with methanolysis for the release of sugars, these are stabilized as methyl glycosides and ketosides[1,24,25]. However, KDO degradation was not avoided during methanolysis in 2 *M* hydrochloric acid for 18 h at 85°C, our conditions for LPS composite analysis[11]. The major degradation product was probably an anhydro structure rather than a lactone, as judged by the MS analysis. When the release of KDO from LPS or PS was performed by 2 *M* hydrochloric acid in methanol at 60°C, the degradation was almost completely prevented. At this temperature KDO was released quantitatively from most linkages after 2 h (Table I). KDO appeared as a single structure (peak A, Fig. 1) identified by GC–MS as the methyl ester of the methylketoside. Although the glycosidic linkage between KDO and hexosamine required 6 h for quantitative cleavage, the method compares well in speed and simplicity with another GC method for the determination of KDO[12], requiring reaction times of 2 days. Hence acidic methanolysis at 60°C followed by GC of TFA derivatives appears to be a good approach for KDO determination.

Degradation of KDO might also be avoided by lowering the acid strength, but we found no obvious advantages with methanolysis in 0.1–1 *M* hydrochloric acid (data not shown). During methanolysis, loss of hydrochloric acid due to reaction with methanol may occur[26] and we prefer to use 2 *M* hydrochloric acid to ensure a sufficient concentration for consistent cleavage of the more resistant KDO linkages.

The methanolysis–GC method presented shows slightly less variability than the spectrophotometric determination of KDO (Table III). Although the exact amount of KDO in the *N. meningitidis* LPS is unknown, the GC value (15.7%) comes very close to what could be calculated (17%) assuming 3 mol of KDO per mol of LPS. The higher KDO value (23.3%) found by spectrophotometry can probably be explained by a faster degradation of monomeric KDO used to establish the calibration graph, and released from ketosidic linkages, than for the heptosyl-linked KDO in LPS. The latter KDO moiety is released considerably more slowly than ketosidically linked KDO[15], and hence may have been better protected from degradation during hydrolysis. For the methanolysis–GC procedure such biphasic KDO release presents no analytical problem, as degradation of KDO does not occur. The sensitivity (about 250 pmol) is comparable to that obtained by spectrophotometry[15]. A considerably higher sensitivity can probably be obtained by using an electron-capture detector to take advantage of the many fluorine atoms in TFA derivatives, or by utilizing mass spectrometric detection (selected ion monitoring) with m/z 591 as a diagnostic ion.

Phosphate esters of KDO may occur in LPS[16], and the phosphate linkages are resistant towards methanolysis and all commonly used conditions of aqueous hydrolysis. Analysis of phosphate-substituted KDO by liquid chromatography may be

an attractive alternative as localization of the phosphate group may also be achieved[27].

The advantage of the methanolysis–GC procedure for KDO determination in cases of an abundance of deoxy sugars that would interfere in spectrophotometric tests is illustrated in Fig. 4, where KDO from LPS and neuraminic acid from capsular PS could be determined simultaneously. Hence our procedure may have certain potential in the determination of KDO in meningococcal capsular PS vaccines of serogroups B, C, Y and W135. We have determined KDO without difficulty in the presence of more than a 100-fold excess of neuraminic acid in such preparations[28,29].

REFERENCES

1 C. Galanos, O. Lüderitz, E. T. Rietschel and O. Westphal, in T. W. Goodwin (Editor), *International Review of Biochemistry, Vol. 14, Biochemistry of Lipids II*, University Park Press, Baltimore, 1977, p. 239.
2 M. A. Schmidt and K. Jann, *Eur. J. Biochem.*, 131 (1983) 509.
3 W. F. Vann and K. Jann, *Infect. Immun.*, 25 (1979) 85.
4 A. K. Bhattacharjee, H. J. Jennings and C. P. Kenny, *Biochemistry*, 17 (1978) 645.
5 V. S. Waravdekar and L. D. Saslaw, *J. Biol. Chem.*, 234 (1959) 1945.
6 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971.
7 A. Weissbach and J. Hurwitz, *J. Biol. Chem.*, 234 (1959) 705.
8 C. S. Hershberger, M. Davis and S. B. Binkley, *J. Biol. Chem.*, 243 (1968) 1585.
9 S. S. Kuwahara and M. C. Snetting, *Anal. Biochem.*, 100 (1979) 118.
10 D. Charon and L. Szabo, *Carbohydr. Res.*, 34 (1974) 271.
11 K. Bryn and E. Jantzen, *J. Chromatogr.*, 240 (1982) 405.
12 J. H. Banoub, D. H. Shaw and F. Michon, *Carbohydr. Res.*, 123 (1983) 117.
13 O. Westphal, O. Lüderitz and F. Bister, *Z. Naturforsch., Teil B*, 7 (1952) 148.
14 WHO Expert Committee on Biological Standardization, *27th Report, Technical Report Series*, No. 594, WHO, Geneva, 1976, 50.
15 H. Brade, C. Galanos and O. Lüderitz, *Eur. J. Biochem.*, 131 (1983) 195.
16 O. Lüderitz, O. Westphal, A. M. Staub and H. Nikaido, in G. Weinbaum, S. Kadis and S. J. Ajl (Editors), *Microbial Toxins*, Vol. IV, Academic Press, New York, 1971, p. 145.
17 W. A. König, H. Bauer, W. Voelter and E. Bayer, *Chem. Ber.*, 106 (1973) 1905.
18 D. Charon and L. Szabo, *J. Chem. Soc., Perkin Trans. 1*, (1979) 2369.
19 H. J. Jennings, A. K. Bhattacharjee, L. Kenne, C. P. Kenny and G. Calver, *Can. J. Biochem.*, 58 (1980) 128.
20 H. J. Jennings, K. G. Johnson and L. Kenne, *Carbohydr. Res.*, 121 (1983) 233.
21 L. D. Melton, E. R. Morris, D. A. Rees, D. Thom and S. M. Bociek, *Carbohydr. Res.*, 81 (1980) 295.
22 D. Charon and L. Szabo, *J. Chem. Soc., Perkin Trans. 1*, (1973) 1175.
23 W. A. Volk, N. L. Salomonsky and D. Hunt, *J. Biol. Chem.*, 247 (1972) 3881.
24 R. E. Chambers and J. R. Clamp, *Biochem. J.*, 125 (1971) 1009.
25 J. P. Zanetta, W. C. Breckenridge and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291.
26 Y. Kishimito and N. S. Radin, *J. Lipid Res.*, 6 (1965) 435.
27 A. K. Shukla, R. Schauer, F. M. Unger, U. Zähringer, T. E. Rietschel and H. Brade, *Carbohydr. Res.*, 140 (1985) 1.
28 K. Bryn, L. O. Frøholm, E. Holten and K. Bøvre, *NIPH Annals (Oslo)*, 6 (1983) 91.
29 K. Bryn and L. O. Frøholm, to be published.