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ANALYSIS OF LIPOPOLYSACCHARIDES BY METHANOLYSIS, TRIFLUOROACETYLATION, AND GAS CHROMATOGRAPHY ON A FUSED-SILICA CAPILLARY COLUMN

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SUMMARY

A gas chromatographic method for simultaneous analysis of fatty acids and sugars of lipopolysaccharides (LPS) has been developed. The sample (1 mg or less) is methanolyzed at 85°C overnight in 2 M HCl in methanol. The released methyl esters and methyl glycosides are trifluoroacetylated and chromatographed on a methylsilicone-impregnated fused-silica capillary column. This column resolves all ordinary LPS sugars and fatty acids, and quantitative analysis is possible, including 2-keto-3-deoxyoctanoic acid (KDO), glucosamine and heptoses. 3,6-Dideoxyhexoses show some thermal degradation at 85°C during methanolysis, but this can be overcome by lowering the temperature to 37°C. For KDO the higher temperature similarly causes some degradation, but a reproducible response factor was found. The method appears to be useful for analysis of purified LPS as well as a means for monitoring for LPS content during purification of bacterial antigens of different kinds.

INTRODUCTION

Bacterial lipopolysaccharides (LPS, endotoxins) are encountered in almost all Gram-negative bacteria, and play an important role in bacterial infections. This group of highly complex structures have therefore been extensively studied; they have been shown to evoke a wide variety of biological responses in the host and are reported to affect almost all organ systems[1,2].

The most studied LPS are isolated from strains of *Salmonella*. The general structure is composed of three chemically different regions[3]. A lipophilic moiety (lipid A) consists of a disaccharide of glucosamine with one acid-labile and one acid-stable phosphate group, and hydroxylated and non-hydroxylated fatty acids in ester and amide linkages. Lipid A is ketosidically linked to a core polysaccharide consisting of 2-keto-3-deoxyoctanoic acid (KDO) and L-glycero-D-mannoheptose, both partly phosphorylated, together with several hexoses and hexosamines. Some LPS (rough, R) only contain lipid A and a biosynthetically completed or uncompleted core polysaccharide, but most LPS are of S type (smooth), and these have an additional region (O-chain) that is a polymerized oligosaccharide.

Owing to the complexity of LPS, with a variety of constituents and types of linkage, several different methods are commonly utilized for compositional analysis. These comprise separate colorimetric assays of KDO, hexosamine, heptose and neutral sugars, and thin-layer chromatography (TLC) or gas chromatography (GC) of sugars and fatty acids. It was therefore of interest to develop a rapid and simple method where most constituents could be quantitated simultaneously. Previously[4,5] we have described a GC technique for bacterial identification which was based on methanolyzed and trifluoroacetylated bacterial cells. In this communication we report a similar analytical approach for determination of LPS constituents, including the use of a fused-silica capillary column.

EXPERIMENTAL

Chemicals

Solvents of analytical grade were distilled before use. Methanolic HCl (2 M) was obtained by bubbling dry HCl gas (Fluka, Buchs, Switzerland) into dry methanol until saturation, and subsequent dilution. Most LPS preparations and the N-(3-hydroxymyristyl)glucosamine standard were gifts from O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, G.F.R. (*Salmonella abortus equi*; *S. typhi*; *S. minnesota*, Ra to Re). A LPS preparation from *Actinobacillus actinomycetemcomitans* was received from J. Jonsen and I. Olsen, University of Oslo, Norway. LPS of *Neisseria elongata* and *Yersinia enterocolitica* were our own preparations. All LPS samples were extracted and purified according to Westphal et al.[6] by the hot phenol procedure, followed by dialysis and ultracentrifugation. The K-13 polysaccharide of *Escherichia coli*[7] was obtained from K. Jann. Max-Planck-Institut für Immunbiologie. Type I polysaccharide of *Klebsiella*[8] was a gift from J. Eriksen, University of Oslo.

Fatty acid methyl ester standards were from Applied Science Labs. (State College, PA, U.S.A.). Ethanolamine, monosaccharides, disaccharides, mannan, and cerebroside were from Sigma (St. Louis, MO, U.S.A.).

Methanolysis and trifluoroacetylation

LPS preparations (0.1–1 mg) were suspended in 2 M HCl in methanol (1 ml) in teflon-lined screw-capped vials and kept at 85°C for 18 h[4,5]. Methanolysates were concentrated to dryness by nitrogen or on a rotary evaporator at room temperature. Trifluoroacetyl (TFA) derivatives were then formed by adding 50 μl of 50% trifluoroacetic anhydride (Merck, Darmstadt, G.F.R.) in acetonitrile (Merck) and heating to boiling for ca. 2 min. After 10 min at room temperature, the reaction mixture was diluted to 10% trifluoroacetic anhydride and injected onto the gas chromatograph. Derivatized samples could be stored for several months at −20°C in sealed capillaries.

Gas chromatography

The GC analyses were carried out on a Hewlett-Packard 5710 chromatograph equipped with a flame ionization detector and a Hewlett-Packard 18740B capillary column control. The fused-silica capillary column (25 m × 0.2 mm I.D., methylsilicone (SE-30) stationary phase, Hewlett-Packard, Avondale, PA, U.S.A.) was operated in splitless mode and with a carrier gas (helium) flow-rate of 1.5 ml min^{-1}.

Column oven temperature was held for 4 min at 90°C and then programmed at 8°C min^{-1} to 250°C. Peak areas and retention times were recorded by a Hewlett-Packard 3390A recorder-integrator.

Peak identification

Generally the constituents were identified by comparison of retention times with those of methanolyzed standards. In addition, the identities were confirmed by mass spectrometry (MS) using a Hewlett-Packard 5992A gas chromatograph–mass spectrometer instrument equipped with a glass capillary column (25 m × 0.5 mm, CP Sil 5 (methyl silicone), Chrompack, Middelburg, The Netherlands). The ionization conditions used were 70 eV at 170°C.

Heptose was identified by GC and MS of two mutant R form LPS that contained (Rd) or was devoid (Re) of heptose, as reported earlier[9]. Several specific fragments were recorded, e.g. m/e 591 (M − TFAO), 531 (M − TFAO and CH$_3$OCHO), 445 (M − CH$_3$O and 2 TFAO), and also the general fragment of m/e 157 (CH$_3$OCHOTFA).

KDO peaks were recognized by analysis of methanolysates of Escherichia coli polysaccharide K-13, a ribose-KDO polymer[7]. The same KDO peaks were also recorded for methanolysates of Salmonella LPS. Verification of identity was performed by MS, where characteristic fragments of M − 31 (loss of CH$_3$O) and M − 59 (loss of COOCH$_3$) were observed (to be published).

RESULTS AND DISCUSSION

Methanolysis

The fatty acids of LPS are usually liberated by saponification using aqueous methanol and NaOH or KOH, followed by methyl ester formation and GC. Analysis of the various monosaccharide constituents is more problematic, and several different assays are required for reliable identification and quantitation. For example, at least three different hydrolytic conditions are commonly utilized to avoid degradation of the most labile sugars and to accomplish complete cleavage of all glycosidic linkages of an oligosaccharide chain.

Methanolic HCl is usually considered to be a mild and effective reagent for cleaving oligosaccharide linkages[10,11]. Sugar–phosphate linkages are normally not cleaved and hence phosphorylated LPS constituents are not represented in the chromatograms. Otherwise, it is our experience that most glycosidic linkages are quantitatively broken and that most commonly occurring sugars are stable at the conditions used. Dideoxy sugars represent an exception in being partly degraded. A quantitative GC analysis of these fragile sugars requires methanolysis at a more moderate temperature (unpublished).

KDO, another common and fragile LPS constituent, can be determined by the presented method. However, a certain percentage of this sugar commonly occurs in phosphorylated form, and only the unphosphorylated moieties will be detected. During methanolysis of KDO at 85°C, an extensive conversion between different ring-forms takes place. After completed reaction, the two most abundant peaks (Fig. 1) constitute 70% of total KDO with a low coefficient of variation (below 5%), and these peaks can apparently be used for quantitation of unphosphorylated KDO.
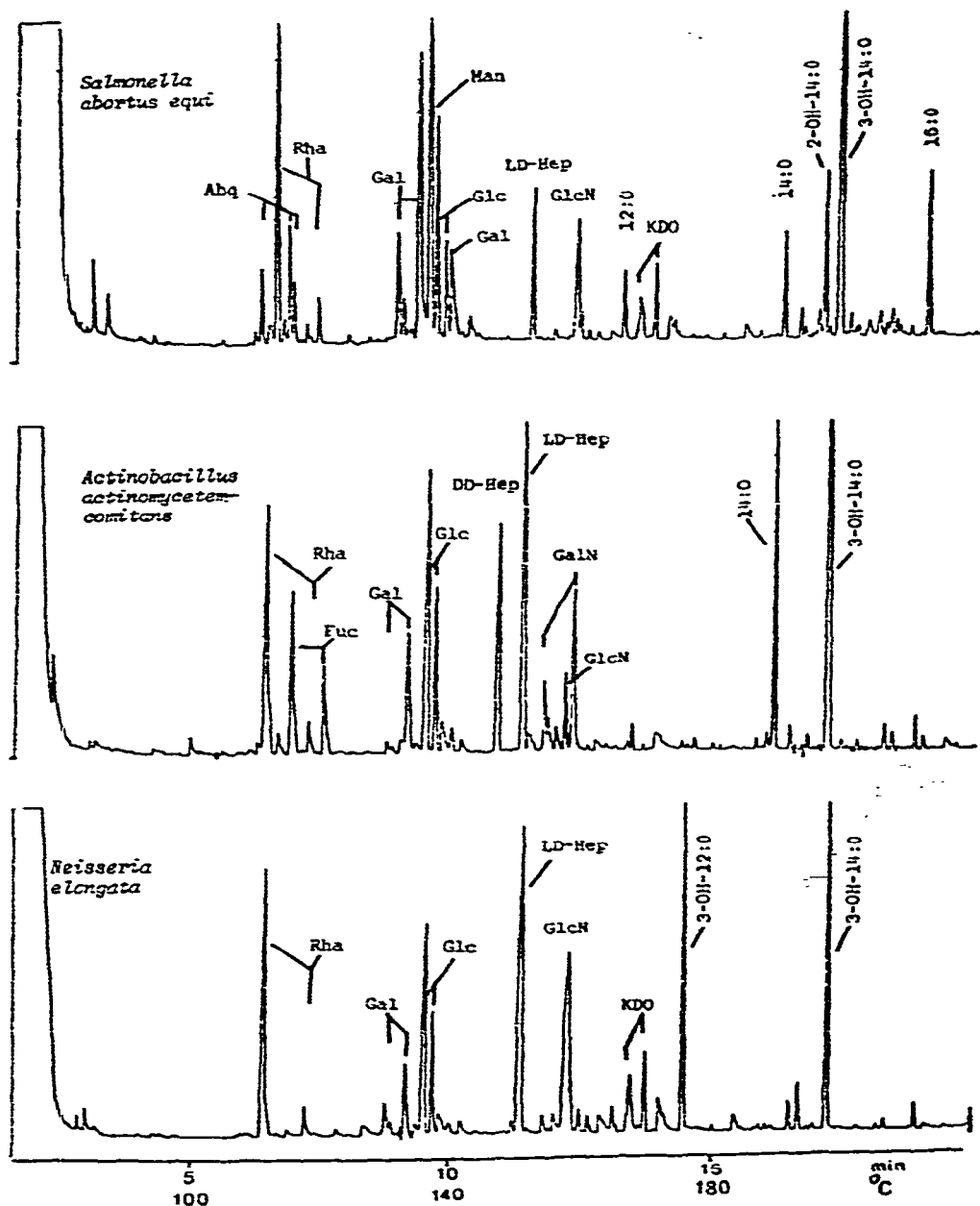
Fig. 1. Chromatograms of three LPS preparations after methanolysis and trifluoroacetylation. Conditions of sample preparation and chromatography are given in the text. Abbreviations: Abq, abequose; Rha, rhamnose; Fuc, fucose; Gal, galactose; Man, mannose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GlcN, 2-deoxy-2-aminoglucose; KDO, 2-keto-3-deoxy-octanoate; 12:0, dodecanoate; 3-OH-12:0, 3-hydroxydodecanoate; 14:0, tetradecanoate; 3-OH-14:0, 3-hydroxytetradecanoate; 16:0, hexadecanoate.

The fact that several peaks are obtained for each monosaccharide makes the chromatograms of many LPS rather complex. When a capillary column is used, the peaks are normally well resolved (Fig. 1), and the specific peak pattern of a sugar is a useful aid for its identification. The ratio of the various ring-forms was found to be relatively stable under the conditions used (Table I) and in agreement with previous reports[10,12]. The quantitation of a certain sugar may thus be based on one or more selected peaks.

TABLE I

GAS CHROMATOGRAPHIC DATA OF SOME COMMONLY OCCURRING LPS MONOSAC-CHARIDES AS TRIFLUOROACETYLATED METHYLGLYCOSIDES*

Component	Retention times (min)** and peak distribution (%)				Source
Ethanolamine	3.08				Synthetic
	100				
Abequose***	6.14	6.66	6.76		
	45	23	32		LPS of S. abortus equi
Tyvelose***	5.43	6.39	6.52		
	70	15	15		LPS of S. typhi
Rhamnose	6.40	7.22			
	88	12			LPS of S. abortus equi
	86	14			LPS of N. elongata
Fucose	5.00	6.67	6.92	7.51	
	6	8	55	30	LPS of A. actinomycetemcomitans
	10	18	51	22	Klebsiella type I polysaccharide
Ribose	6.89	7.15	7.24	8.31	
	8·	13	16	63	LPS of S. minnesota Ra §
	12	6	16	66	E. coli K-13 polysaccharide
Glucose	8.87	9.47	9.62		
	1	72	27		LPS of S. abortus equi
	7	66	27		LPS of S. typhi
	3	69	28		Lactose
	—	73	27		Cellobiose
	—	75	25		Cerebroside
Galactose	8.74	9.14	9.67		
	13	60	27		LPS of S. abortus equi
	13	62	25		LPS of S. typhi
	9	64	28		Lactose
Mannose	9.35	9.98			
	91	9			LPS of S. abortus equi
	95	5			Mannan
L-Glycero-D-mannoheptose	11.36				
	100				LPS of S. abortus equi
	100				LPS of N. elongata
D-Glycero-D-mannoheptose	10.89				
	> 80				LPS of A. actinomycetemcomitans
	> 80				LPS of Y. enterocolitica
Glucosamine	12.22				
	100				LPS of S. minnesota Ra and Re
	100				N-Acetylglucosamine §§
	100				N-(3-OH-14:0)-glucosamine

TABLE I *(continued)*

Component	Retention times (min)[*] and peak distribution (%)			Source
Galactosamine		*11.72*	*12.36*	
		28	72	N-Acetylgalactosamine[‡‡]
2-Keto-3-	*12.26*	*13.38*	*13.71*	
deoxyoctanoate	17	32	39	LPS of *S. abortus equi*
(KDO)[‡‡‡]	17	30	41	LPS of *S. typhi*
	18	37	29	LPS of *S. minnesota* Ra
	16	28	39	LPS of *S. minnesota* Re
	14	39	34	LPS of *N. elongata*
Neuraminic acid		*15.53*		
		100		N-Acetylneuraminic acid

* Conditions of methanolysis were 2 M HCl in methanol, 85°C, 18 h. Trifluoroacetylation was performed by heating to boiling with 50% trifluoroacetic anhydride in acetonitrile. Column: fused silica (25 m × 0.2 mm I.D.) coated with SE-30. Carrier gas (He) flow-rate 1.5 ml min^{-1}. Temperature program: 90°C for 4 min, 8° min^{-1} to 250°C.

** Retention times (italicized) given are normalized relative to a $C_{12:0}$ retention time of 13.10 min.

*** Abequose and tyvelose are partially degraded during methanolysis at standard conditions. Recorded figures for distribution are as obtained by mild methanolysis (2 M HCl, 37°C, 90 min).

‡ "Leaky" Ra mutant (O. Lüderitz, private communication) originating from a ribose-containing T1 strain[19].

‡‡ Free-N-unacylated hexosamines give a distinctly different pattern.

‡‡‡ Peak distribution of KDO varies with conditions of methanolysis, and two minor additional peaks are commonly found.

Derivatization

Quantitation of monosaccharides by GC is often performed as alditol acetates[13,14] or as silylated methylglycosides[15,16]. Zanetta *et al.*[10], and others[12,17], have amply demonstrated the applicability of trifluoroacetylated (TFA-derivatized) methylglycosides for GC of sugars from complex glyco-conjugates. In spite of these reports such derivatives are apparently still considered to be unstable and unsuitable in practical use[18].

As illustrated in Fig. 1, the trifluoroacetate derivatives of the monosaccharides are very volatile, and all except the peaks of KDO elute before dodecanoate ($C_{12:0}$). Accordingly, when the retention times (Tables I and II) and the individual response factors (Table III) are known, a considerable number of LPS constituents liberated by methanolysis can be determined simultaneously in a rapid and simple manner. Some loss of the more volatile fatty acid methyl esters (*e.g.* $C_{12:0}$) may occur during the evaporation step for removal of the methanolic HCl. Quantitative yields of these esters therefore require separate analysis including extraction into hexane[5].

Molar response

Molar response factors have been determined relative to 3-hydroxymyristic acid (TFA-derivatized), a widely occurring LPS constituent (Table III). A direct measure of the response ratio between fatty acids and monosaccharides was obtained by methanolysis and GC of synthetic N-(3-hydroxy-myristyl)-glucosamine, which shows structural similarity to lipid A. The relative response of the individual fatty acids was determined from standards. Similarly the molar response values of the

TABLE II

RETENTION TIMES OF COMMON LPS FATTY ACIDS AS (O-TRIFLUOROACETYLATED) METHYL ESTERS

Conditions as in Table I.

Chain length	Retention time (min)		
	Unhydroxylated	2-Hydroxy	3-Hydroxy
C_{10}	9.53	—	11.34
C_{12}	13.10	14.17	14.46
C_{13}	—	—	15.91
C_{14}	16.20	16.98	17.25
C_{15}	17.62	—	18.55
C_{16}	18.98	19.55	19.74
C_{18}	21.45	21.91	22.05

TABLE III

COMPOSITIONAL ANALYSIS OF THREE LIPOPOLYSACCHARIDES

See Table I, Fig. 1 and text for experimental details.

Component	Molar response*	Molar ratio			
		S. abortus equi		N. elongata	A. actino-mycetemcomitans
		GC value	Calc. from ref. 21		
Abequose	0.1**	10	11.9	—	—
Rhamnose	0.38	12.0	12.0	4.2	5.1
Fucose	0.38	—	—	—	6.9
Mannose	0.42	12.2	12.0	—	—
Galactose	0.40	13.1	12.6	1.7	3.7
Glucose	0.44	4.1	2.8	4.4	7.8
Glucosamine	0.44	1.5	3.0	5.2	1.1
Galactosamine	0.44	—	—	—	4.3
LD-Glyceromannoheptose	0.55	1.1	3.0	4.8	5.0
DD-Glyceromannoheptose	0.55	—	—	—	3.1
KDO	0.46	2.7	3.6	4.7	—
$C_{12:0}$	0.87	0.7	—	—	—
$C_{14:0}$	1.00	0.5	—	—	2.0
$C_{16:0}$	1.12	0.4	—	0.2	—
3-OH-$C_{12:0}$	0.88	—	—	2.0	—
2-OH-$C_{14:0}$	1.00	0.4	—	—	—
3-OH-$C_{14:0}$	1.00	3.8	—	2.1	6.0

* Relative to 3-OH-$C_{14:0}$ (TFA-derivatized).
** Response value inaccurate owing to degradation, see text.

monosaccharides were calculated on the basis of methanolysis and GC of various disaccharides, LPS preparations, polysaccharides and cerebroside (Table I). Molar response factors of heptose and KDO were determined by analysis of a series of nineteen preparations of S and R forms of *Salmonella* LPS of known composition. All these LPS preparations were found to exhibit KDO and 3-hydroxymyristic acid in a constant ratio, corresponding to approximately 3 moles per polysaccharide chain.

The relative amount of heptose showed dissimilarities among the nineteen *Salmonella* LPS preparations examined, presumably owing to variation in the degree of phosphorylation. However, in all cases the number of heptose moieties recorded was in accordance with the expected number of unphosphorylated heptose moieties in LPS[20].

Applications

The chromatograms of three LPS preparations are shown in Fig. 1. Generally, all peaks are well resolved and simultaneous determinations could be performed of individual hexoses, deoxy- and dideoxy-hexoses, heptoses, hexosamines and KDO, as well as of fatty acids. With a few exceptions, the quantitative data obtained (Table III) show good agreement with values expected for *S. abortus equi*[20,21]. A low yield of glucosamine was obtained (1.5 mole compared with 2 moles expected). *Salmonella* LPS has one glucosamine in the polysaccharide chain, and two phosphorylated moieties in the lipid A part, one of which is phosphorylated in the 1-position, and hence acid-labile. The reason for the low yield of glucosamine is not known, but it was observed also in S form LPS of *S. typhi*, in contrast to all R form LPS tested.

Previous analysis of *A. actinomycetemcomitans* LPS[22] detected two unidentified sugars. One of these, as indicated in Fig. 1, had chromatographic properties identical with D-glycero-D-mannoheptose, occurring also in LPS of *Yersinia enterocolitica*[23]. The ratio between the two heptoses in *A. actinomycetemcomitans* is 3 to 2, in accordance with the data of Kiley and Holt[22]. No KDO could be observed by GC of this LPS, which may indicate that all KDO moieties, if present, are phosphorylated. For LPS of *N. elongata*, the third example in Fig. 1, no analytical data have been published.

In addition to its usefulness in structural elucidations, this simple GC method is valuable for monitoring during purification of LPS. It may presumably also be of advantage for detection of LPS in bacterial vaccines and other bacteriological materials.

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