*Journal of Chromatography*, 487 (1989) 1–7
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4487

# GAS CHROMATOGRAPHIC DETERMINATION OF (PHOSPHORYLATED) 2-KETO-3-DEOXYOCTONIC ACID, HEPTOSES AND GLUCOSAMINE IN BACTERIAL LIPOPOLYSACCHARIDES AFTER TREATMENT WITH HYDROFLUORIC ACID, METHANOLYSIS AND TRIFLUOROACETYLATION

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(First received May 16th, 1988; revised manuscript received September 2nd, 1988)

## SUMMARY

Quantification of phosphorylated sugar constituents of lipopolysaccharides has been performed by the following sequence: dephosphorylation by treatment with hydrofluoric acid, cleavage to monomeric constituents by methanolysis and analysis of the released sugars by capillary gas chromatography. Lipopolysaccharides of *Salmonella minnesota* $Rd_1P^+$, *Bordetella pertussis* NIH 114 and *Vibrio cholerae*, NAG and 95R strains, were used as model substances. Comparison of the chromatographic data obtained from hydrofluoric acid-treated and untreated lipopolysaccharide preparations indicated that all lipopolysaccharides examined contained one moiety of glucosamine bound to phosphate in a stable linkage. 2-Keto-3-deoxyoctonic acid appeared phosphorylated to a variable extent. Lipopolysaccharides of the two *V cholerae* strains contained one moiety of fully phosphorylated 2-keto-3-deoxyoctonic acid, whereas in that of *S minnesota* $Rd_1P^+$ only one of the three moieties was phosphorylated. Lipopolysaccharide of *B pertussis* had one moiety of 2-keto-3-deoxyoctonic acid, ca. 70% phosphorylated. All four of the preparations examined contained L-glycero-D-*manno*-heptose in amounts varying from 2.6 to 5.2 moieties. In the lipopolysaccharides of *B pertussis* and strain 95R of *V cholerae* this sugar was unphosphorylated, whereas the two remaining strains contained one phosphorylated moiety of this sugar. Phosphorylated lipopolysaccharide constituents can be analysed by this approach on a 50–100 $\mu g$ scale.

0378-4347/89/$03.50 

## INTRODUCTION

Lipopolysaccharides (LPS; endotoxins) are constituents of the outer membrane of all genuine Gram-negative bacteria [1,2]. Owing to the wide spectrum of pathophysiological effects of these unique substances in the host during infection, LPS have been subjected to intensive studies for several decades [3,4]. Furthermore, there is an increasing interest in LPS constituents for use as chemical markers in bacterial systematics [5].

For compositional analysis of LPS, a gas chromatographic (GC) technique has been published [6,7]. This method is based on methanolysis of the macromolecules into monosaccharides (as methyl glycosides) and fatty acids (as methyl esters). After derivatization of hydroxyl and amino groups with trifluoroacetic anhydride (TFAA), a simultaneous GC analysis of sugars and fatty acids is performed. However, phosphorylated sugar moieties frequently occurring in LPS are not detected by this technique, i.e. phosphates of 2-keto-3-deoxyoctonic acid (KDO), L-glycero-D-*manno*-heptose (LD-Hep), D-glycero-D-*manno*-heptose (DD-Hep) and 2-deoxy-2-amino-D-glucose (glucosamine, GlcN) [6,7]. Generally, quantification of phosphate-linked sugars in complex material is an analytical difficulty that can be overcome by removal of the phosphate group. This can be done enzymically [8,9] or, as in the present study, by a chemical method.

It is well known that concentrated aqueous hydrofluoric acid at low temperatures acts highly specifically as a dephosphorylating agent, leaving glycosidic and other linkages uncleaved [10–14]. Prehm et al. [15] took advantage of this in structural studies of LPS from *Escherichia coli*, and recently Caroff et al. [16,17] found a considerable increase in colorimetric response for KDO when LPS of *Bordetella pertussis* and *Vibrio cholerae* were treated with hydrofluoric acid.

Application of a GC system for quantification of the dephosphorylated sugars may have advantages over colorimetry related to the separation of the products from the complex and often interfering "background" of other compounds. Also, several dephosphorylated sugars may be determined simultaneously, and their identity can be verified using mass spectrometry (MS).

## EXPERIMENTAL

### *Materials*

All solvents were of analytical-reagent grade (Rathburn Chemicals, Walkerburn, U.K.) and 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. Hydrofluoric acid, 48% (w/w), p.a. grade, was from Merck (Darmstadt, F.R.G.) and TFAA, p.a. grade, from Aldrich Chemie (Steinheim, F.R.G.). "Rough" LPS of *Salmonella minnesota* $Rd_1P^+$ was a gift from Otto Lüderitz (Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.). LPS from *Neisseria meningitidis* and *B. pertussis* were extracted by the hot aqueous phenol procedure [18] followed by extraction with 90% phenol–chloroform–hexane (2:5:8, v/v) (PCP-extraction, ref. 19), dialysis and ultracentrifugation twice at 105 000 *g* for

4 h. LPS from two strains of *V. cholerae* (non-agglutinating H11 NAG and rough 95R strains) were gifts from Kevin W. Broady (University of New South Wales, Kensington, Australia).

*Hydrolysis by hydrofluoric acid*

Lyophilized aliquots of LPS (50–100 $\mu$g) were mixed with 0.3 ml of aqueous 48% (w/w) hydrofluoric acid in closed polyethylene tubes and kept at 4°C for 72 h. Then 0.3 ml of water was added, and after flushing with nitrogen for 2 h, 0.6 ml of water was added and the samples were freeze-dried to remove traces of hydrofluoric acid. Residues were dissolved in water, transferred to PTFE-lined screw-capped glass vials of 1.1 ml capacity and lyophilized.

*Methanolysis and trifluoroacetylation*

LPS preparations, untreated as well as dephosphorylated, were suspended in 0.3 ml of 2 *M* hydrochloridic acid in methanol, vortex-mixed and briefly flushed with nitrogen. Methanolysis was performed both at 85°C for 18 h [6] and at 60°C for 2 h [7]. After removal of methanol and hydrochloric acid by nitrogen the samples were derivatized with TFAA [6]. The reagents were removed under nitrogen, and the residue was redissolved in 10% TFAA (v/v) in acetonitrile prior to GC injection.

*Gas chromatography–mass spectrometry*

The GC analyses were carried out on two different instruments, a Hewlett-Packard (HP) 5710 (Avondale, PA, U.S.A.), equipped with an HP 18740 B capillary column control, and a Dani 6500-HR (Monza, Italy). Both instruments were equipped with flame-ionization detectors and fused-silica capillary columns (25 m×0.2 mm I.D.) (SGE, Ringwood, Victoria, Australia) with cross-linked SE-30 as stationary phase. Helium, at a flow-rate of 1.5 ml/min, served as carrier gas. Injections were made in the splitless mode in the HP 5710 gas chromatograph with the split valve open after 1 min, and the injector temperature at 250°C. The Dani 6500-HR gas chromatograph was equipped with a temperature-programmed vaporizer operating from 70 to 250°C in splitless mode. The temperature of the detectors was 300°C, and the temperatures of the columns were programmed (starting 2 min after injection) from 90 to 280°C at 8°C/min. Peak areas and retention times were recorded on a Perkin-Elmer LCI-100 recorder (Norwalk, CT, U.S.A.) and an HP Model 3390A integrator, respectively.

GC peaks were identified both by retention time data from our LPS data files and by MS. GC–MS was performed with a Varian 3300 GC (Walnut Creek, CA, U.S.A.), equipped with a fused-silica column (25 m×0.2 mm I.D.) (HP) with cross-linked SE-30 as stationary phase. The chromatograph was connected to the MS instrument (Finnigan Ion-Trap 700; San Jose, CA, U.S.A.) via a heated (250°C) fused-silica transfer line.

Response factors, determined relative to 3-hydroxymyristic acid (3-OH-14:0) [as the trifluoroacetyl (TFA) derivatized methyl ester], were used for quantification of the carbohydrates [6,7].

RESULTS

*Conditions for hydrofluoric acid hydrolysis*

To establish the time required for complete hydrofluoric acid dephosphorylation, the yields of GlcN, LD-Hep and KDO obtained from *N. meningitidis* LPS were determined at intervals by methanolysis at 85 °C for 18 h. A constant level of GlcN was obtained after 24 h, whereas 48 h or more were needed for maximum recovery of LD-Hep (data not shown). A reaction time of 72 h at 4 °C was subsequently used as standard.

*Analysis of phosphorylated LPS constituents*

LPS of two *V. cholerae* strains, of *B. pertussis* and of *S. minnesota* were analysed both before and after hydrofluoric acid treatment. As an example, the corresponding chromatographic profiles of LPS of *V. cholerae* 95R, methanolysed at 60 °C, which is optimal for KDO analysis [7], is shown in Fig. 1. Evidently, and as previously described [8], all KDO in this particular LPS is phosphorylated. KDO appeared as a significant peak only after treatment with hydrofluoric acid (Fig. 1). On identification by GC–MS the KDO peak showed the characteristic fragment of $m/z$ 591 ($[M-COOCH_3]^+$) and also $m/z$ 477 and $m/z$ 363, corresponding to additional losses of one and two TFAOH groups, respectively [7].

The other GC peaks labelled in Fig. 1 were identified by GC–MS after meth-

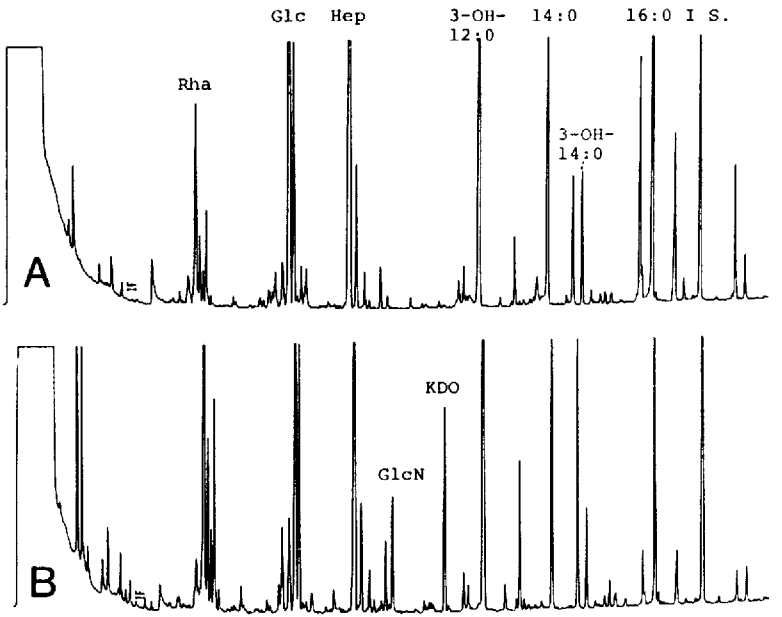


Fig. 1. Gas chromatograms of LPS of *Vibrio cholerae* (strain 95R) after methanolysis (2 *M* hydrochloric acid, 60 °C, 2 h) and TFA derivatization, without (A) and with (B) primary dephosphorylation by treatment with hydrofluoric acid (48%, w/w, in water, 4 °C, 72 h). For chromatographic conditions and experimental details see text. Peaks: I.S. = internal standard (heptadecanoic acid methyl ester); Rha = rhamnose; Glc = glucose; Hep = L-glycero-D-*manno*-heptose; GlcN = glucosamine; KDO = 2-keto-3-deoxyoctonic acid; 3-OH-12.0 = 3-hydroxylauric acid; 14:0 = myristic acid; 3-OH-14.0 = 3-hydroxymyristic acid; 16 0 = palmitic acid.

TABLE I

PHOSPHORYLATED LPS CONSTITUENTS DETERMINED BEFORE AND AFTER TREATMENT WITH HYDROFLUORIC ACID

Samples (100 μg, analysed in duplicates), both native and dephosphorylated [hydrolysed in 48% aqueous hydrofluoric acid (HF) at 4°C for 72 h], were methanolysed (2 *M* hydrochloric acid in methanol at 85°C) for 18 h (heptoses and GlcN) and at 60°C for 2 h (KDO), followed by trifluoroacetylation and GC. Molar ratios relative to 3-OH-14 0 were calculated on the basis of GC data

| Compound | *V cholerae* H11 NAG | | *V cholerae* 95R | | *S minnesota* $Rd_1P^+$ | | *B pertussis* NIH 114 | |
|---|---|---|---|---|---|---|---|---|
| | −HF | +HF | −HF | +HF | −HF | +HF | −HF | +HF |
| DD-Hep | 2.7 | 6.2 | −* | −* | −* | −* | −* | −* |
| LD-Hep | 4.0 | 5.2 | 4.8 | 4.6 | 2.0 | 2.9 | 1.2** | 1.3** |
| GlcN | 1.4 | 2.4 | 1.5 | 2.5 | 1.0 | 2.0 | 1.7** | 2.5** |
| KDO | <0.1 | 1.1 | 0.1 | 0.8 | 1 8 | 2.6 | 0.3 | 0.8 |
| 3-OH-14 0 (amide-linked) | 2.0 | 2.0 | 2.0 | 2.0 | 2 0 | 2.0 | 2.0 | 2.0 |

*Not detected

**Values low due to especially stable glucosaminisidic linkages.

anolysis either at 60°C for 2 h or at 85°C for 18 h. The mild conditions cleave the amide linkage between GlcN and 3-OH-14:0 to only a small degree [7], and hence only small amounts of these constituents were apparent in the figure. Several unidentified peaks in Fig. 1 are believed to be oligosaccharides not cleaved by methanolysis at 60°C, as they were absent after methanolysis at 85°C.

The molar yields of DD-Hep, LD-Hep,GlcN and KDO from the four LPS preparations with and without hydrofluoric acid treatment are given in Table I. All data are given relative to the two amide-linked 3-OH-14:0 moieties. These were determined after differential methanolyses at 60 and 85°C. (The amounts of 3-OH-14:0 have been corrected for 25% residual uncleaved amide linkages recently established by using 4 *M* hydrochloric acid at 100°C for 18 h.)

For all four LPS preparations roughly one additional moiety of GlcN became apparent after treatment with hydrofluoric acid, which can be related to the 4′-phosphorylated GlcN moiety of lipid A.

The LD-Hep moieties in LPS of *V. cholerae* H11 NAG and of *S. minnesota* are shown in Table I to be partially phosphorylated, as is also the case of DD-Hep in the former LPS. LD-Hep of *V. cholerae* 95R and *B. pertussis* LPS, however, are apparently not phosphorylated.

All LPS preparations examined contained phosphorylated KDO. In agreement with previous studies [8,16], the LPS of the two *V. cholerae* strains showed only tra amounts of unphosphorylated KDO, and this study indicates the presence of one moiety of phosphorylated KDO. On the other hand, the *S. minnesota* LPS seems to contain two moieties of KDO without and one with phosphate, whereas the *B. pertussis* LPS apparently contained one moiety of KDO, which was predominantly phosphorylated.

## DISCUSSION

In structural studies of LPS the quantitative determination of KDO is difficult, especially when the labile sugar is phosphorylated. Presence of a phosphate group

in the 5-position apparently prevents the formation of chromophoric products in the most commonly used colorimetric tests [8,16]. In GC analysis of KDO after methanolysis [6,7] phosphorylation of KDO similarly presents a problem, since phosphate is not removed from KDO under the methanolysis conditions employed, nor from heptoses or GlcN, leaving these sugar moieties undetected. Strong hydrolytic conditions as a primary dephosphorylation step cannot easily be applied since KDO is acid-labile. As a result several LPS have been reported to be completely devoid of KDO [17]. For other LPS, KDO is at the best only semiquantitatively determined. In one case even fructose has been suggested to replace KDO as a ketosidic link to lipid A [20].

Aqueous hydrofluoric acid cleaves chemical bonds with different specificity depending on concentration and temperature [10]. At 4°C, the concentrated hydrofluoric acid cleaves sugar–phosphate esters selectively, leaving glycosidic linkages largely intact [10–14]. Caroff et al. [16] found increased colorimetric yields of KDO in a thiobarbituric acid assay after hydrofluoric acid treatment of LPS of *B. pertussis* and of *V. cholerae*, which had earlier been reported to contain phosphorylated KDO [8,21].

In most established LPS structures, GlcN occurs in a diphosphorylated disaccharide, the "backbone" of lipid A, where the phosphate group in the 4′-position forms a stable linkage [22]. All four LPS examined showed at least one additional moiety of GlcN after treatment with hydrofluoric acid (Table I), indicating complete dephosphorylation of the 4′-GlcN of lipid A.

The increased yields of DD-Hep and LD-Hep following hydrofluoric acid treatment indicate that these constituents are partially phosphorylated in LPS of *V. cholerae* (NAG strain). DD-Hep is presumed to be a biosynthetic precursor of LD-Hep in LPS of some bacteria [23]. Also in LPS of *S. minnesota* $Rd_1P^+$, LD-Hep appears to be partially phosphorylated. The three moieties of LD-Hep found in this LPS conflict with the previously reported value of two [24]. In contrast, LPS of *B. pertussis* and of *V. cholerae* 95R apparently contain only unphosphorylated LD-Hep.

Quantification of hydrofluoric acid-dephosphorylated KDO appears relatively uncomplicated after methanolysis at 60°C, as for unphosphorylated KDO in untreated LPS [7]. However, after exposure to hydrofluoric acid, the proportion of KDO appearing as the main KDO peak (Fig. 1B) decreased from 95 to 90%, as judged from MS analysis (data not shown). This effect became more evident when methanolysis was performed at 85°C, as the degradation of KDO was greater and more pronounced than normal. Hence methanolysis at the higher temperature should be avoided for quantification of KDO in LPS treated with hydrofluoric acid.

In this work we have demonstrated that the combined use of hydrofluoric acid treatment, methanolysis and GC overcomes inherent problems in the analysis of phosphorylated LPS constituents. It would be of interest to explore in detail the potential of the procedure for the analysis of LPS, especially since it can be used in a micro-scale e.g. for subfractions obtained by electrophoretic separations or other fractionation techniques.

ACKNOWLEDGEMENTS

We thank Drs. O. Lüderitz and K.W. Broady for the gifts of lipopolysaccharides used in this investigation. This work was supported by the Swedish Work Environmental Fund. A travel grant (A.S.) from the Nordic Research Courses is greatly appreciated.

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