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DETERMINATION OF 3-DEOXY-D-*manno*-OCTULOSONIC ACID (KDO), *N*-ACETYLNEURAMINIC ACID, AND THEIR DERIVATIVES BY ION-EXCHANGE LIQUID CHROMATOGRAPHY

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A liquid chromatography (1.6 MPa) system for the analysis of 3-deoxy-D-*manno*-2-octulosonic acid (KDO), *N*-acetylneuraminic acid (Neu5Ac), methyl α- and β-glycosides of Neu5Ac and KDO, α-heptosyl-(1→5)-KDO, various sialyllactoses, α-KDO-(2→4)-KDO, α-KDO-(2→4)-KDO methyl α-glycoside, β-KDO-(2→4)-KDO methyl β-glycoside, D-glucuronic acid, D-glucurono-3,6-lactone, and D-galacturonic acid has been developed. Separation was achieved within 10 and 30 min by the use of a small column filled with a strongly basic, anion-exchange resin, Aminex A-29, and 0.75 or 10mM sodium sulfate solutions as mobile phases. This method allowed the determination of KDO and sialic acids in amounts of 100 ng (0.5 nmol) and 200 pg (0.6 pmol), respectively.

INTRODUCTION

2-Keto-3-deoxy-D-*manno*-octulosonic acid (KDO) is known as a constituent of the inner-core of lipopolysaccharides, the endotoxins of gram-negative bacteria[1]. It has also been detected in some acidic capsular polysaccharides of *Escherichia coli* and *Neisseria meningitidis* along with sialic acids[2,3].

KDO is usually determined by the thiobarbituric acid (TBA) assay, after acid-catalyzed hydrolysis of KDO-containing biopolymers[4,5]. This acidic hydrolysis causes several degradation products that have been partially characterized[6] with respect to their reactivity with TBA. The main disadvantage of the determination with TBA is that all 3-deoxyaldulosonic acids, including sialic acids, give[7,8] a chromophore having an absorption maximum at 549 nm. L-Fucose, 2-deoxyribose, and some unsaturated fatty acids yield a similar chromophore. The presence of these substances interferes with the exact determination of KDO. To overcome

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these problems, we adapted an l.c. system, developed for the analysis of various sialic acids[9], for the analysis of KDO and its derivatives. It allows the detection of these substances also in the presence of sialic acids.

RESULTS AND DISCUSSION

The retention times of KDO, sialic acids, related glycoside compounds, and some acidic monosaccharides on a column of Aminex A-29 in l.c. are given in Tables I and II. A 0.75mM aqueous sodium sulfate solution was used as eluent (Table I), except for substances containing a KDO-disaccharide, for which the ionic strength was raised up to 10mM to obtain shorter retention times (Table II). To

TABLE I

RETENTION TIMES OF VARIOUS ACIDIC MONOSACCHARIDES AND SOME OLIGOSACCHARIDE DERIVATIVES ON ANION-EXCHANGE LIQUID CHROMATOGRAPHY USING 0.75mM SODIUM SULFATE AS MOBILE PHASE[a]

Compounds	*Retention times (s)*
α-*N*-Acetylneuraminyl-(2→6)-*N*-acetyllactosamine	115
α-*N*-Acetylneuraminyl-(2→6)-lactose	135
α-*N*-Acetylneuraminyl-(2→3)-lactose	175
β-*N*-Acetylneuraminyl-(2→6)-*N*-acetyllactosamine	179
N-Acetylneuraminic acid	360
N-Glycolylneuraminic acid	480
N-Acetylneuraminic acid methyl β-glycoside	267
N-Acetylneuraminic acid methyl α-glycoside	441
KDO	535
α-Heptosyl-(1→5)-KDO	395
KDO methyl α-glycoside	392
KDO methyl β-glycoside	502
D-Glucurono-3,6-lactone	90
D-Glucuronic acid	687, 818[b]
D-Galacturonic acid	605

[a]For further conditions, see Experimental section. [b]The occurrence of two peaks may be due to the presence of α and β anomers.

TABLE II

RETENTION TIMES OF KDO DISACCHARIDES AND RELATED COMPOUNDS ON ANION-EXCHANGE LIQUID CHROMATOGRAPHY USING 10mM SODIUM SULFATE AS MOBILE PHASE[a]

Compounds	*Retention times (s)*
KDO methyl α-glycoside	139
KDO methyl β-glycoside	171
α-KDO-(2→4)-KDO	1032
α-KDO-(2→4)-KDO methyl α-glycoside	664
β-KDO-(2→4)-KDO methyl β-glycoside	1477

[a]For further conditions, see Experimental section.

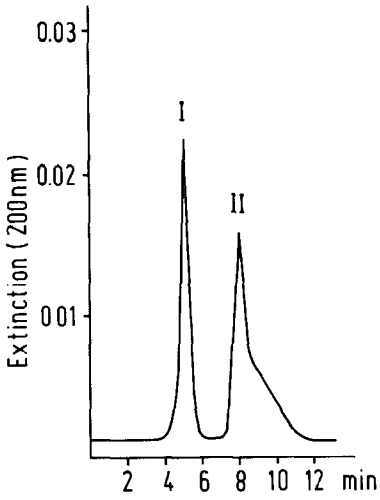

Fig. 1. Profile of elution from anion-exchange l.c. of Neu5Ac (I) and KDO (II) using 0.75mM sodium sulfate as mobile phase. The flow rate was 0.5 mL/min at 1.5 MPa. Eluted substances were monitored at 200 nm.

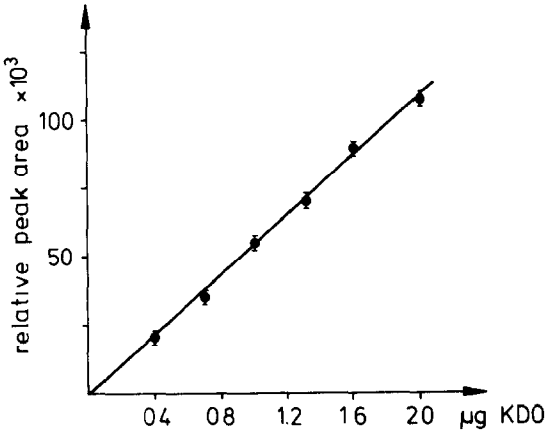

Fig. 2. Calibration curve for the ammonium salt of KDO on anion-exchange l.c. using 0.75mM sodium sulfate as mobile phase. The flow rate was 0.5 mL/min at 1.5 MPa. KDO was monitored at 200 nm and 0.001 a.u.f.s.

illustrate the extent of the separation of KDO and Neu5Ac, the elution profile is shown in Fig. 1.

The minimum amount of KDO that could be determined by l.c. was 0.5 nmol (Fig. 2). Thus, the determination of KDO by this method is 2 to 3 times more sensitive than by the TBA assay. The corresponding minimum value obtained by l.c. for Neu5Ac is about 1 ng (~3 pmol) (Fig. 3). At a signal-to-noise ratio of 3:1, even 200 pg (0.6 pmol) of sialic acid could be identified. This represents an ~100–500-fold higher sensitivity when compared with the TBA assay[8]. The greater sen-

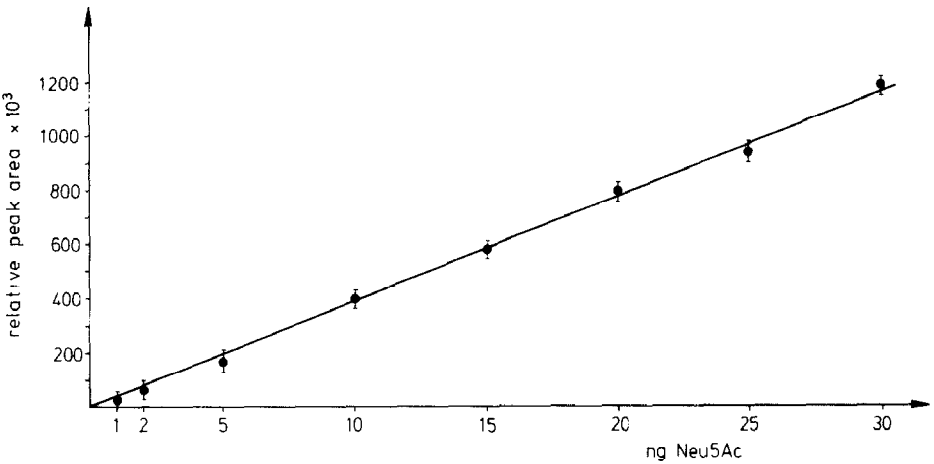

Fig. 3. Calibration curve for *N*-acetylneuraminic acid (Neu5Ac) on anion-exchange l.c. using 0.75mM sodium sulfate as mobile phase. The flow rate was 0.5 mL/min at 1.5 MPa. Neu5Ac was monitored at 200 nm and 0.001 a.u.f.s.

sitivity of the determination of sialic acids by l.c. is due to the absorbance of *N*-acyl groups at 200 nm.

The absorbance peak observed at 200 nm is not specific for sugars, and any impurity absorbing at this wavelength and being eluted in the same range as KDO or sialic acid may cause a wrong interpretation. To increase the specificity of the analysis, sialic acid samples were incubated with *N*-acetylneuraminate lyase (EC 4.1.3.3) which cleaves sialic acid into pyruvic acid and 2-acylamino-2-deoxy-D-mannose. The disappearence of the sialic acid peak after incubation with the enzyme gave a specific test for sialic acids[10]. It would be possible to use KDO-aldolase[7] (EC 4.1.2.23) in a similar way for a specific test of KDO; this enzyme, however, is not commercially available.

The quantitative determination of KDO, sialic acid, and related compounds described herein has several advantages over methods previously described[7,8]. It requires small amounts of substances and, in most cases, does not require the intensive purification steps that are necessary for the analysis of these substances by the TBA reagent, and especially by g.l.c., g.l.c.–m.s., and n.m.r. spectroscopy[7,11]. These purification steps are tedious and may lead to loss of material or of substituents (*e.g.*, *O*-acetyl groups) on the sugars.

The presence of KDO together with Neu5Ac has been reported for various strains of gram-negative bacteria[9]. KDO may be quantitatively estimated in the presence of Neu5Ac by determining the sialic acid content with the orcinol–Fe^{3+}–HCl reagent[8]. As KDO does not yield a chromophore similar to that given by sialic acid with orcinol, the difference between the values from the TBA and orcinol determinations gives the amount of KDO. However, if sialic acids other than Neu5Ac are present, this determination may lead to wrong values for KDO, as the molar extinction coefficients, in the TBA assay, for over 20 differently *N,O*-acylated sialic acids occurring in Nature are distributed over a wide range (between ε

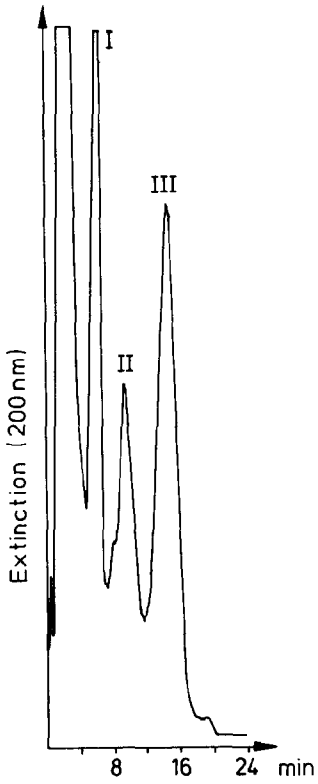

Fig. 4. Profile of elution from anion-exchange l.c. of KDO and some of its derivatives isolated by acid hydrolysis from *Salmonella minnesota* R-form LPS. L.c. conditions are the same as described in legend to Fig. 1: (I) α-Heptosyl-(1→5)-KDO, (II) KDO, and (III) unidentified KDO derivative.

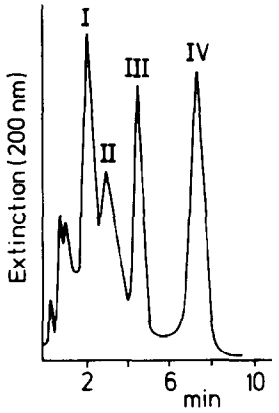

Fig. 5. Profile of elution of the methyl α- and β-glycosides of Neu5Ac, and α and β anomers of sialyllactose by l.c. The conditions are the same as described in the legend to Fig. 1: (I) α-*N*-Acetylneuraminyl-(2→6)-*N*-acetyllactosamine, (II) β-*N*-acetylneuraminyl-(2→6)-*N*-acetyllactosamine, (III) *N*-acetylneuraminic acid methyl β-glycoside, and (IV) *N*-acetylneuraminic acid methyl α-glycoside.

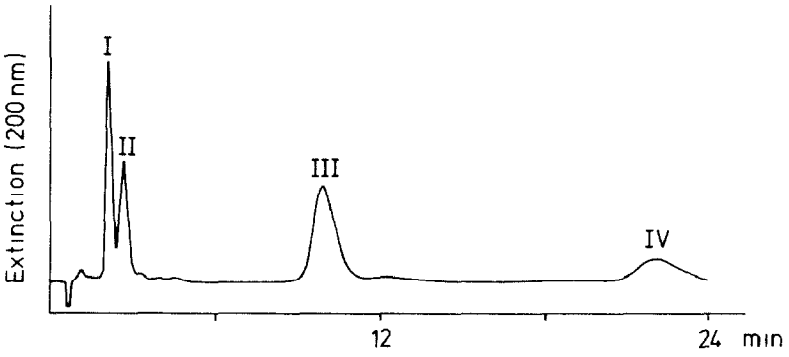

Fig. 6 Profile of elution by l.c. using 10mM sodium sulfate as mobile phase of: (I) KDO methyl α-glycoside, (II) KDO methyl β-glycoside, (III) α-KDO-(2→4)-KDO methyl α-glycoside, and (IV) β-KDO-(2→4)-KDO methyl β-glycoside. The other conditions were the same as described in the legend to Fig. 1.

70 000 and 3 500)[11,12], whereas the extinction coefficients given by the orcinol reaction for these sialic acids[11] are almost all the same. Furthermore, the unsaturated 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (Neu2en5Ac) gives a positive reaction with orcinol, but a negative with TBA. These difficulties in the analysis of a natural mixture of KDO and sialic acids may be overcome by l.c., as illustrated in Fig. 1.

Fig. 4 shows a separation profile of KDO and its derivatives that were isolated from *Salmonella minnesota* R-form LPS after acid hydrolysis. The fractions from l.c. were collected and identified by the TBA assay and high-voltage paper electrophoresis in the presence of reference substances. In some cases, the substances purified by high-voltage paper electrophoresis showed, apart from the main substances, additional peaks in l.c., whereas the KDO derivatives purified by l.c. showed a single spot in paper electrophoresis.

The present method was also applied to the study of synthetic derivatives of KDO and Neu5Ac, such as methyl α- and β-glycosides (Table I, Figs. 5 and 6). In the case of the methyl glycosides of Neu5Ac, the β anomer was eluted faster than the α anomer, but, in the case of sialyllactose, the opposite elution sequence of the anomers was observed (Fig. 5). In contrast to Neu5Ac, the α anomer of the methyl glycosides of KDO was eluted before the β anomer (Fig. 6). This separation by l.c. will be helpful for the control of the synthesis of, and the study of enzymic action on, different anomers.

For the separation of (KDO→KDO) disaccharides and some derivatives, a higher concentration of sodium sulfate (10 mM) was required. In this system, the synthetic methyl α- and β-glycosides and the naturally occurring [α-KDO-(2→4)-KDO] disaccharide could also be separated (Table I, Fig. 6). However, the separation of the substances reported in Table I was not as efficient at this higher sulfate concentration than in 0.75mM sodium sulfate.

In conclusion, a fast and sensitive method for the determination of KDO and

sialic acids by l.c. has been developed. This method will be helpful in the study of metabolic reactions of KDO and sialic acids[10,13] and in the analysis of natural products.

EXPERIMENTAL

Materials. — The ammonium salts of KDO, KDO methyl α- and β-glycoside, α-KDO-(2→4)-KDO methyl α-glycoside, and β-KDO-(2→4)-KDO methyl-β-glycoside were prepared as described previously[14]. α-Heptosyl-(1→5)-KDO and α-KDO-(2→4)-KDO disaccharides were prepared from *Salmonella minnesota* R-form LPS by mild acid hydrolysis, followed by preparative high-voltage paper electrophoresis[15].

N-Acetyl- and *N*-glycolyl-neuraminic acid were prepared from porcine submandibular-gland glycoproteins[16]. α-*N*-Acetylneuraminyl-(2→6)-*N*-acetyllactosamine, and α-*N*-acetylneuraminyl-(2→3)- or -(2→6)-lactose were isolated from bovine colostrum[17]. The methyl β-glycoside of Neu5Ac was prepared as described previously[18]. The methyl α-glycoside of Neu5Ac and β-*N*-acetylneuraminyl-(2→6)-*N*-acetyllactosamine were kindly provided by Prof. Paulsen. D-Glucurono-3,6-lactone, D-glucuronic acid, and D-galacturonic acid were obtained from Merck (Darmstadt). *Clostridium perfringens N*-acetylneuraminate lyase (EC 4.1.3.3) was obtained from Sigma (Munich).

Liquid chromatography. — L.c. was performed with a Spectra–Physics apparatus SP 8000 (see ref. 9), equipped with a small stainless-steel column (40 × 4.6 mm) filled with Aminex A-29 ion-exchange resin (Bio-Rad, Munich). Sialic acids, KDO, and related substances were eluted isocratically with 0.75mM Na_2SO_4 at a flow rate of 0.5 mL/min at 1.6 MPa. KDO disaccharides and their methyl α- and β-glycosides were eluted under the same conditions, however, with 10mM Na_2SO_4. The eluted substances were monitored at 200 nm (Photometer SF 773, Kratos, Karlsruhe), the detector sensitivity being between 0.05 and 0.001 a.u.f.s., and the attenuation varying between 5 and 40 mV.

Analysis of sialic acids and KDO after l.c. — Sialic acids eluted from the l.c. column were further analyzed with a micro adaptation of the HIO_4–thiobarbituric acid and orcinol–Fe^{3+}–HCl assays[8]. *N*-Acetyl- and *N*-glycolyl-sialic acids were also identified by incubation with *N*-acetylneuraminate lyase, which cleaves them into pyruvic acid and 2-acylamino-2-deoxy-D-mannoses[10]. KDO was identified by the HIO_4–thiobarbituric acid test[5]. Furthermore, KDO and its derivatives were identified by high-voltage paper electrophoresis in the presence of reference substances[15].

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