Journal of Chromatography, 315 (1984) 451–456
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,245

Note

Reversed-phase high-performance liquid chromatography of hydrazones of 3-deoxy-D-*manno*-oct-2-ulosonic acid and neuraminic acids

P. A. McNICHOLAS*, M. BATLEY and J. W. REDMOND
School of Chemistry, Macquarie University, North Ryde, N.S.W. 2113 (Australia)
(Received September 17th, 1984)

Analyses of neuraminic acids and 3-deoxy-D-*manno*-oct-2-ulosonic acid (KDO) are usually carried out colorimetrically[1,2]. The thiobarbituric acid test, which has recently been improved[3], is subject to interference from deoxy sugars[4], while the diphenylamine method[2] cannot be used in the presence of large amounts of other carbohydrates. The semicarbazide assay is insensitive and the conditions used in the recognised procedure do not provide reproducible derivatization[5]. Neuraminic acids have been estimated using gas chromatography (GC) and gas chromatography–mass spectroscopy of trimethylsilyl derivatives[6] and by ion-exchange high-performance liquid chromatography (HPLC) using either short-wavelength ultraviolet (UV) detection or post-column derivatization[7,8]. Similarly KDO has been studied using GC of peracetylated methyl glycoside methyl ester[9,10], trimethylsilyl[11] and trifluoroacetyl[12] derivatives but only one HPLC study appears to have been carried out[13].

As part of a program of study of the lipopolysaccharides (LPS) of Gram-negative bacteria we require a reliable method for analysis of KDO. We now report a simple, sensitive analysis of neuraminic acids and KDO by reversed-phase HPLC of their *p*-nitrophenylhydrazones.

EXPERIMENTAL

Materials

HPLC solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); N-acetyl- and N-glycolylneuraminic acid, oxaloacetic acid, 2-keto-D-gluconic acid, and pyruvic acid from Sigma (St. Louis, MO, U.S.A.); 2-phenoxyethanol from Ajax (Sydney, Australia); sodium borohydride from BDH (Port Fairy, Australia); sodium cyanoborohydride from Aldrich (Milwaukee, WI, U.S.A.). KDO was synthesised by the method of Ghalamor and Heath[14] and *p*-nitrophenylhydrazine from BDH (Poole, U.K.) was recrystallised from ethanol.

Equipment

Two LDC Constametric pumps (Models I and IIG) with a dynamic gradient mixer were operated in conjunction with an LDC Gradient Master. A Rheodyne Model 7120 syringe-loading injector was used with a 20-μl loop and detection was by means of an LDC UVIII monitor operating at 254 nm. A Brownlee guard column

0021-9673/84/$03.00 © 1984 Elsevier Science Publishers B.V.

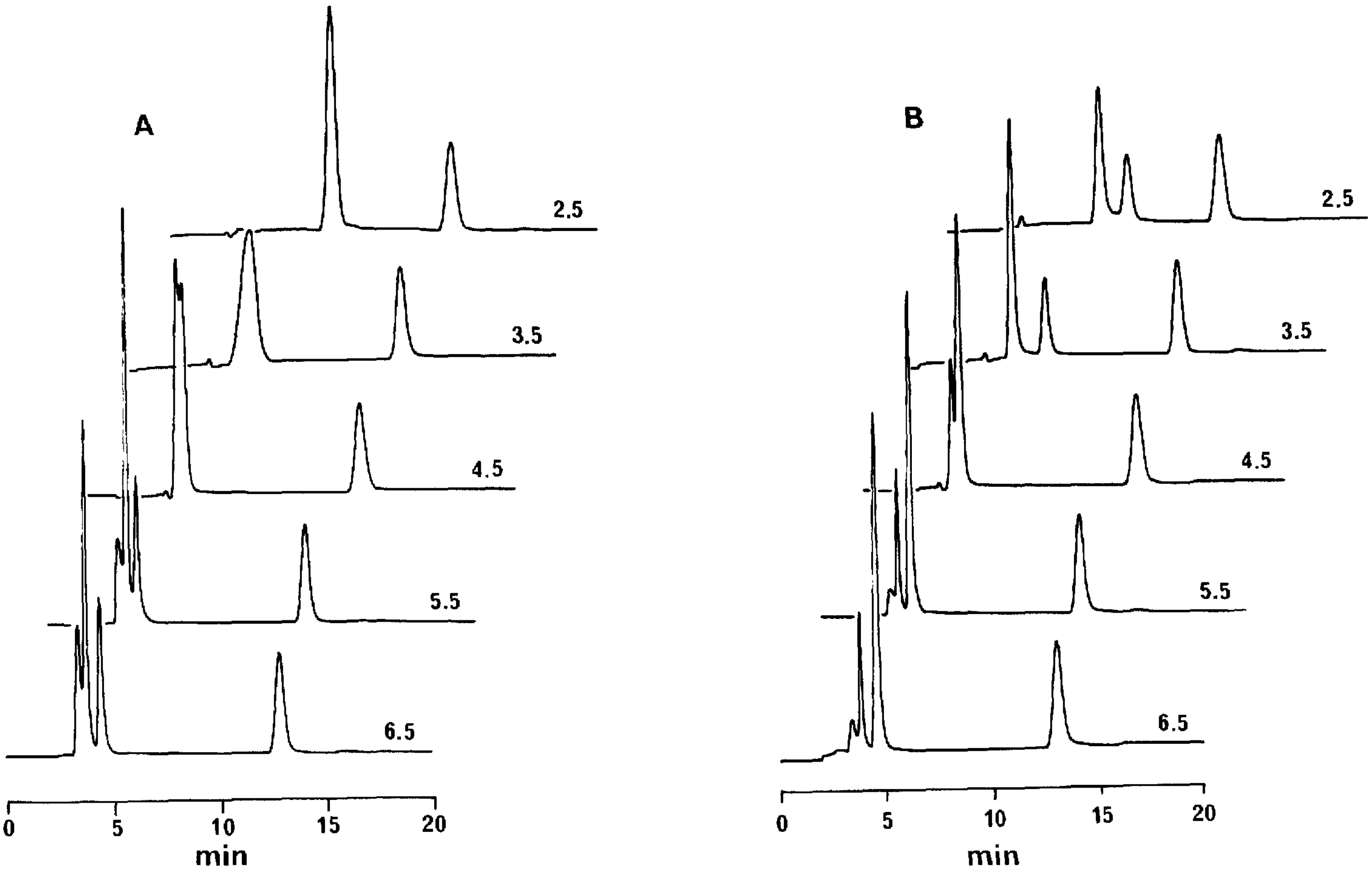

Fig. 1. Separation of keto acids by HPLC. Elution conditions: pre-column and column, RP-8 (10 μm); mobile phase, acetonitrile–50 m*M* potassium phosphate buffer (20:80) at specified pH; flow-rate, 1 ml/min. A, KDO; B, N-acetylneuraminic acid.

(40 × 4.6 mm I.D.) and a Chrompack Stock column (250 × 4.6 mm I.D.) were used, each packed with LiChrosorb RP-8 (10 μm). The eluting solvent was acetonitrile–50 m*M* potassium phosphate buffer and the flow-rate was 1 ml/min. The data were digitized and stored as previously described[15].

Derivatisation

Aliquots (0.100 ml) containing 5–100 μg of KDO (ammonium salt) or the neuraminic acid to be tested were carefully placed into the bottoms of a series of fusion tubes, followed by 100 m*M* potassium phosphate buffer (pH 4.5) (0.200 ml). Aliquots (0.100 ml) of *p*-nitrophenylhydrazine solution [0.5% (w/v) in methanol] were then added. The contents of the tubes were mixed and the tubes centrifuged briefly before sealing and heating in a water bath at 60°C for 45 min.

The tubes were then cooled and opened, ethyl acetate (1 ml) was added to each and the mixtures agitated well on a vortex mixer. After brief centrifugation to give clear separation of the layers, the organic layers were removed and rejected. The ethyl acetate extraction was repeated, and then an aliquot (0.100 ml) of the aqueous layer was removed from each tube, mixed with a solution of 2-phenoxyethanol [0.1% (v/v) in water, 0.025 ml]. The mixture was blown gently under a stream of nitrogen to remove traces of ethyl acetate and used directly for HPLC analysis. A full (20-μl) loop was always injected.

Attempted reduction of hydrazone derivatives

To a solution of the KDO derivative (0.400 ml), representing approximately 40 μg KDO, was added sodium cyanoborohydride or sodium borohydride (2 mg). The mixture was kept at 37°C for up to 3.5 h and then used directly for HPLC.

RESULTS AND DISCUSSION

The chemistry of saccharide hydrazones has been extensively reviewed[16]. Hydrazones are formed most rapidly at pH 4 to 5 in the presence of large concentrations of phosphate buffer[16]. Weakly basic hydrazines are better suited for this condensation since they are less likely to form salts under the acidic conditions of the reaction. Pilot experiments indicated that derivatization with *p*-nitrophenylhydrazine for 45 min at pH 4.5 and 60°C is optimal.

Our first HPLC studies of the *p*-nitrophenylhydrazones employed a LiChrosorb NH_2 column using dilute potassium hydrogen phosphate as the eluent. While the initial chromatographic performance was excellent, the column quickly deteriorated to the point where it was unusable. Subsequent experiments were therefore carried out using reversed-phase separation.

Any chromatographic procedure employing hydrazone derivatives is complicated by the ill-defined nature of their structure. Saccharide hydrazones can form either a Schiff-base or a cyclic hydrazino structure[16]. The Schiff-base structure can give rise to a mixture of geometric isomers, while the cyclic hydrazino class can exhibit both anomeric and ring size isomerism. These many structural possibilities are expected to lead to complicated chromatograms.

Elution of the KDO derivative at pH 6.5 gave three peaks (Fig. 1a) which could not arise from geometric isomerism alone. Isolation of these components and

re-chromatography showed them to be in equilibrium. If two of the components were geometric isomers then reduction of the carbon-nitrogen double bond, with sodium borohydride or sodium cyanoborohydride[17], would convert them into a single new peak. This did not occur and it is concluded that these three peaks represent derivatives of the cyclic hydrazino form. Their properties are therefore expected to be those of a glycosylamine rather than a hydrazone. Consistent with this, the derivatives hydrolyse slowly at room temperature and pH 4.5, with a half life of approximately 16 h[15,18]. Furthermore it is known[19] that there is a tendency toward a greater percentage of cyclic forms as the basicity of the hydrazine is decreased.

The chromatographic behaviour of derivatives of KDO and neuraminic acids is further complicated by the presence of a free carboxylic acid. In fact the derivatives are considerably more acidic than the parent keto acids[20], and require suppression of their ionization for satisfactory chromatography in a reversed-phase system.

Elution of the KDO derivative at varying pH values (Fig. 1a) shows that a single peak can be obtained at pH 3.5, while at pH 2.5 greater retention and sharper peaks are obtained. Elution with acetonitrile–50 mM phosphate buffer (pH 2.5) (20:80) was adopted as the standard procedure for the analysis of KDO. This procedure is similar to that of Buslig[21] for 2-keto dicarboxylic acids. The pH-dependence of elution of the N-acetylneuraminic acid derivatives is somewhat different (Fig. 1b). The retention time of the minor component is particularly sensitive to change in pH between 4.5 and 3.5, indicating that it is less acidic than the major component. Although a single peak can be obtained within this range elution was routinely carried out at pH 2.5. Under these conditions it was possible to separate N-glycolyl- from N-acetylneuraminic acid (Fig. 2).

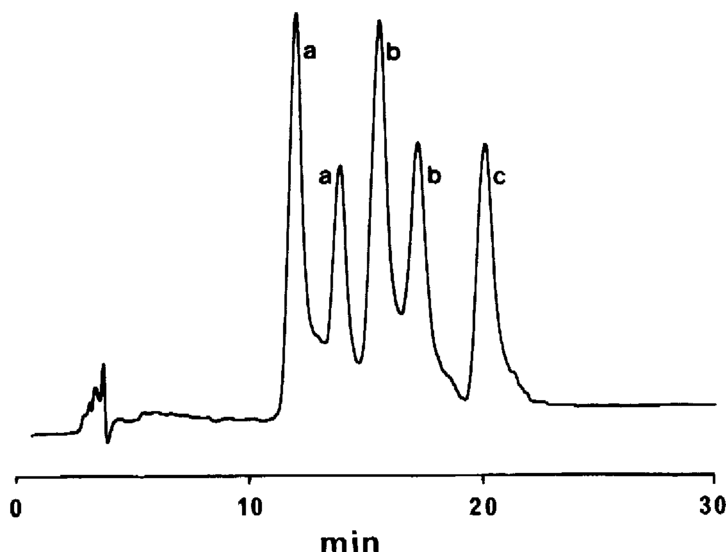

Fig. 2. Separation of neuraminic acids by HPLC of their p-nitrophenylhydrazones. Elution conditions: pre-column and column, RP-8 (10 μm); mobile phase, acetonitrile–50 mM phosphate buffer (pH 2.5) (15:85); flow-rate, 1 ml/min. Peaks: a = N-glycolylneuraminic acid; b = N-acetylneuraminic acid; c = 2-phenoxyethanol.

Several 2-keto acids were investigated for use as internal standards. Pyruvic acid is unsatisfactory because its *p*-nitrophenylhydrazone is soluble in ethyl acetate, the solvent used to remove the derivatizing reagent. The derivative of oxaloacetic acid has a very long retention time and that of 2-keto-D-gluconic acid exhibits a poor peak shape under the chromatographic conditions used. 2-Phenoxyethanol was chosen as the internal standard but, to avoid partial loss on ethyl acetate extraction, it was added after derivatization. Quantitation of KDO over a ten-fold range gave a linear plot with a precision of ±1.0% (Fig. 3) with better than ±0.5% reproducibility. Linearity with N-acetylneuraminic acid, based on the total area of the two peaks, is better than ±2%.

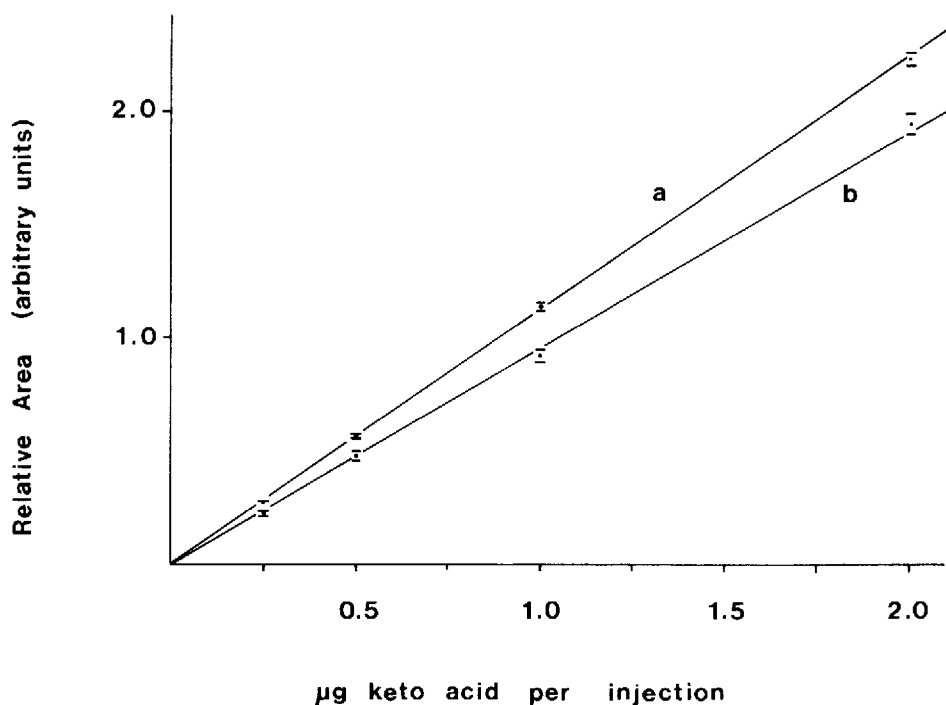

Fig. 3. Calibration plot for *p*-nitrophenylhydrazone derivatives of keto acids. Elution conditions: precolumn and column, RP-8 (10 μm); mobile phase, acetonitrile–50 m*M* phosphate buffer (pH 2.5) (20:80); flow-rate 1 ml/min. Calibration lines: a = KDO; b = N-acetylneuraminic acid.

The standard procedure has not been optimized for sensitivity. Typically, however, *p*-nitrophenylhydrazone equivalent to 2 μg of KDO gives a major peak with full-scale response on a detector setting of 0.032 a.u.f.s. at 254 nm. Detection at 397 nm would permit a four-fold improvement in sensitivity. As an analysis of KDO and neuraminic acids our method has the advantages of simplicity, speed and the use of standard equipment.

REFERENCES

1 W. Dröge, V. Lehmann, O. Lüderitz and O. Westphal, *Eur. J. Biochem.*, 14 (1970) 175.
2 R. Chaby, S. R. Sarfati and L. Szabo, *Anal. Biochem.*, 58 (1974) 123.
3 Y. D. Karkhanis, J. Y. Zeltner, J. J. Jackson and D. J. Carlo, *Anal. Biochem.*, 85 (1978) 595.
4 M. A. Cynkin and G. Ashwell, *Nature (London)*, 186 (1960) 155.
5 M. Batley, J. W. Redmond and P. A. McNicholas, in preparation.
6 Y. Sugarawa, M. Iwamori, J. Portoukalian and Y. Nagai, *Anal. Biochem.*, 132 (1983) 147.
7 A. K. Shukla, N. Scholz, E. H. Reimerdes and R. Schauer, *Anal. Biochem.*, 123 (1982) 78.
8 A. K. Shukla and R. Schauer, *J. Chromatogr.*, 244 (1982) 81.
9 D. Charon and L. Szabo, *J. Chem. Soc. Perkin Trans. I*, (1979) 2369.
10 J. H. Banoub, D. H. Shaw and F. Michon, *Carbohyd. Res.*, 123 (1983) 117.
11 E. Jantzen, K. Bryn and K. Bovre, *Acta. Pathol. Microbiol. Scand. Sect. B: Microbiol.*, 84 (1976) 177.
12 K. Bryn and E. Jantzen, *J. Chromatogr.*, 240 (1982) 405.
13 R. C. Seid, Jr., H. Schneider, S. Bondarew and R. A. Boykins, *Anal. Biochem.*, 124 (1982) 320.
14 M. A. Ghalambor and E. C. Heath, *Biochem. Biophys. Res. Commun.*, 11 (1963) 288.
15 M. Batley, J. W. Redmond and A. Tseng, *J. Chromatogr.*, 253 (1982) 124.
16 L. Mester and H. S. El Khadem, in W. Pigman, D. Horton and J. D. Wander (Editors), *The Carbohydrates Chemistry and Biochemistry*, Vol. 1B, Academic Press, New York, 2nd ed., 1980, pp. 929–988.
17 C. F. Lane, *Synthesis*, (1975) 135.
18 B. Capon and B. E. Connett, *J. Chem. Soc.*, (1965) 4497.
19 S. L. Zhdanov and A. A. Potehkin, *Zh. Org. Khim.*, 13 (1977) 469; *C.A.*, 86 (1977) 190370s.
20 H. Terada, T. Hayashi, S. Kawai and T. Ohno, *J. Chromatogr.*, 130 (1977) 281.
21 B. S. Buslig, *J. Chromatogr.*, 247 (1982) 193.