Journal of Chromatography, 152 (1978) 405–411
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 10,701

PERIODATE OXIDATION ANALYSIS OF CARBOHYDRATES

VII*. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CONJUGATED ALDEHYDES IN PRODUCTS OF PERIODATE OXIDATION OF CARBOHYDRATES BY DUAL-WAVELENGTH DETECTION OF THEIR 2,4-DINITROPHENYLHYDRAZONES

SUSUMU HONDA and KAZUAKI KAKEHI
Faculty of Pharmaceutical Sciences, Kinki University, Kowakae, Higashi-osaka (Japan)
(Received September 20th, 1977)

SUMMARY

Conjugated aldehydes in the products of the periodate oxidation of glycosides were converted quantitatively into their 2,4-dinitrophenylhydrazones by treatment of the products with 2,4-dinitrophenylhydrazine hydrochloride in 1,2-dimethoxyethane, and the hydrazones formed were separated by high-performance liquid chromatography. The hydrazones were then determined accurately by application to a column of LiChrospher SI-100, development with a chloroform–isooctane–methanol system by gradient elution and peak detection at 352 and 435 nm with two internal standards (L-rhamnose 2,4-dinitrophenylhydrazone and D-xylose 2,4-dinitrophenylosazone, respectively). The possible use of this procedure for the elucidation of the structures of carbohydrates is suggested.

INTRODUCTION

When a glycoside is oxidized with periodate, a dialdehyde compound is formed, which consists of various aldehydes linked through hemialdal and hemiacetal bondings. Determination of the aldehydic composition of this dialdehyde compound offers more useful information on interglycosidic linkages than that provided by measurement of periodate consumption. Recently we reported a simple method for the simultaneous determination of component aldehydes by thin-layer or column chromatographic separation and subsequent spectrophotometric measurement of their 2,4-dinitrophenylhydrazones[1,2]. This paper describes an improvement of this analysis by using high-performance liquid chromatography (HPLC).

---

* For Part VI, see ref. 2.

EXPERIMENTAL

Materials

A reagent-grade sample of 2,4-dinitrophenylhydrazine hydrochloride was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). All specimens of aldehydes were of the highest grade commercially available. The solvents used were of spectroscopic grade. L-Rhamnose 2,4-dinitrophenylhydrazone and D-xylose 2,4-dinitrophenylosazone (internal standards) were prepared according to the literature[3]. The procedure for the preparation and the physical constants of authentic 2,4-dinitrophenylhydrazones of other aldehydes have been reported previously[1]. Model dialdehydes, I and II, were also prepared as described previously[1]. The conditions for the periodate oxidation of glycosides were as described in Part VI[2].

Apparatus

HPLC was performed on a Hitachi 635 instrument, equipped with a variable-wavelength effluent monitor and a gradient apparatus. UV spectra were measured on a Shimadzu UV-210 spectrophotometer with a 1-cm quartz cell. Proton magnetic resonance (PMR) spectra were recorded in dimethyl sulphoxide-d_6 by using a Hitachi R-20A instrument (60 MHz), and expressed on a δ-scale.

Procedure for simultaneous determination of conjugated aldehydes

The procedure for conversion of conjugated aldehydes into their hydrazones was essentially the same as in Part VI[2]. Dissolve a sample of a dialdehyde compound or a deionized oxidation product (*ca.* 0.5 μmole) in a saturated solution (200 μl) of 2,4-dinitrophenylhydrazine hydrochloride in 1,2-dimethoxyethane, and keep the resultant solution for 3 h at 25°. Add a 1,2-dimethoxyethane solution (400 μl) of internal standards containing L-rhamnose 2,4-dinitrophenylhydrazone and D-xylose 2,4-dinitrophenylosazone (0.5 μmole of each), and analyze the hydrazones by injecting 1 μl of the reaction solution into the HPLC column [50 cm × 0.21 cm I.D., LiChrospher SI-100 (Merck, Darmstadt, G.F.R.)]. The flow-rate was 0.5 ml/min. A gradient solvent system was prepared by adding a 10% chloroform solution of methanol continuously to a 5% chloroform solution (5.0 ml) of isooctane at a flow-rate of 0.5 ml/min, keeping the volume of the solution in the reservoir constant. The hydrazones of hydroxyaldehydes and the bishydrazone of glyoxal were determined at 352 nm (0.32 a.u.f.s.) and 435 nm (1.28 a.u.f.s.), respectively, by the peak-weight method.

Isolation and structural studies of isomeric hydrazones of D*-glyceraldehyde*

D-Glyceraldehyde was dissolved in a saturated solution of an equivalent amount of 2,4-dinitrophenylhydrazine hydrochloride in ethanol and the solution was allowed to stand for 5 h. The crystalline product separated was collected and recrystallized twice from ethanol (mp. 174–175°); λ_{max} = 353 nm (ε = 19,300; ethanol). *m/e*: 270 (molecular ion), 239 (base peak), 193, 183, 167. PMR data: 3.55 (2 proton-multiplet, $-CH_2OH$ at $C_{3'}$), 4.20 (1 proton-multiplet, $-CH(OH)-$ at $C_{2'}$), 4–5 (2 proton-multiplet, 2 × OH), 7.88 (1 proton-doublet, aromatic proton at C_6), 7.90 (1 proton-doublet, $-CH{=}N-$ at $C_{1'}$, $J_{1',2'}$ = 5.9 Hz), 8.35 (1 proton-quartet, aromatic proton at C_5, $J_{5,6}$ = 9.1 Hz), 8.82 (1 proton-doublet, aromatic proton at C_3, $J_{3,5}$ = 2.3

Hz), 11.37 (1 proton-singlet, –N*H*–). Analysis: $C_9H_{10}N_4O_6$ requires C 40.00%, H 3.73%, N 20.74%; found, C 39.77%, H 3.73%, N 20.25%.

The mother liquor was concentrated to an amorphous solid. The solid was dissolved in a small volume of 1,2-dimethoxyethane and the solution was applied to thin-layer plates of silica gel (Wakogel B-5) and developed with chloroform–methanol (10:1, v/v). The upper zones (R_F 0.37) of the minor component were scraped off and extracted with chloroform–methanol (5:1, v/v). The extract was evaporated to dryness and the residue was crystallized and recrystallized five times from ethanol (m.p. 138–141°); λ_{max} = 453 nm (ε = 19,100; ethanol). *m/e*: 270 (molecular ion), 239 (base peak), 193, 183, 167. PMR data: 3.61 (2 proton-multiplet, –CH_2OH at $C_{3'}$), 3.3–4.2 (2 proton-multiplet, 2 × O*H*), 4.50 (1 proton-multiplet, –C*H*(OH)– at $C_{2'}$), 7.00 (1 proton-doublet, –C*H*=N– at $C_{1'}$, $J_{1',2'}$ = 3.5 Hz), 7.82 (1 proton-doublet, aromatic proton at C_6), 8.31 (1 proton-quartet, aromatic proton at C_5, $J_{5,6}$ = 10.2 Hz), 8.82 1 proton-doublet, aromatic proton at C_3, $J_{3,5}$ = 3.0 Hz), 13.0 (1 proton-singlet, –N*H*–). Ananlysis: $C_9H_{10}N_4O_6$ requires C 40.00%, H 3.73%, N 20.74%; found, C 39.88%, H 3.85%, N 20.45%.

RESULTS AND DISCUSSION

Suitable conditions foı•the separation of 2,4-dinitrophenylhydrazones were established by trying various column packings and solvents. The best results was obtained when LiChrospher SI-100 was used and the column was developed with a chloroform–isooctane–methanol by gradient elution. Fig. 1 shows the separation of

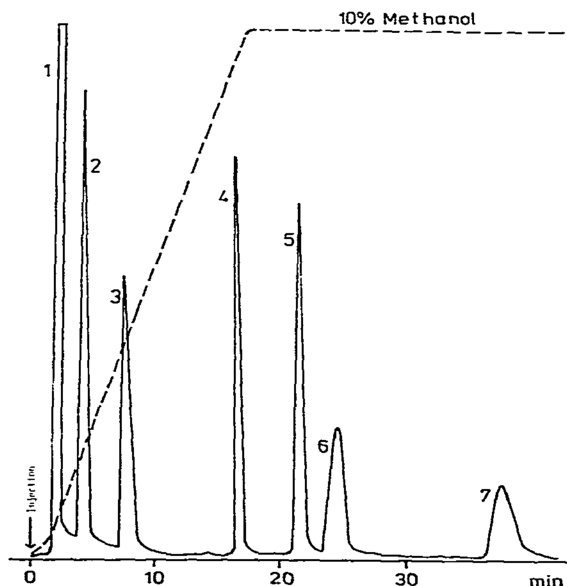

Fig. 1. Separation of an artificial mixture of equimolar 2,4-dinitrophenylhydrazones of various aldehydes. The mixture was chromatographed immediately after dissolution in 1,2-dimethoxyethane and peaks were detected at 352 nm. The broken line indicates the concentration of methanol in the eluate. Peaks: 1 = glyoxal bis-DNP; 2 = the reagent; 3 = glycolaldehyde DNP; 4 = D-glyceraldehyde DNP; 5 = D-erythrose DNP; 6 = D-arabinose DNP; 7 = D-glucose DNP.

an artificial mixture of hydrazones of aldehydes possibly formed from various glycosides of oligo- and polysaccharides. The hydrazones separated were detected at 352 nm. All of the hydrazones of hydroxyaldehydes gave their absorption maxima around this wavelength. Glyoxal was an exceptional aldehyde and formed the bishydrazone. Owing to its low polarity, the bishydrazone eluted at the void volume of the column and its peak was partly superimposed on that of the hydrazine reagent. Therefore, the determination of glyoxal becomes inaccurate, especially if a large excess of the reagent is used.

The UV spectra of the hydrazine reagent, the bishydrazone of glyoxal and the hydrazone of D-glyceraldehyde, a typical hydroxyaldehyde, are shown in Fig. 2. The absorption maximum of the bishydrazone of glyoxal was observed at 435 nm, shifted bathochromically by *ca.* 85 nm from those of the other two compounds (352 nm). Its molar absorptivity was *ca.* 40,000, which is 2.5 times larger than those of the reagent and the hydrazones of hydroxyaldehydes. It is noticeable that the absorption of the reagent at 435 nm was very low, so that the overlap of the bishydrazone of glyoxal and the reagent was negligible when the eluate was detected at this wavelength, provided that too great an excess of the reagent was not used. This wavelength was, however, unsuitable for the detection of the hydrazones of other aldehydes, as their intensities were too weak. Consequently, it is preferable to determine hydrazones with a dual-wavelength system, the hydrazones of hydroxyaldehydes at 352 nm and the bishydrazone of glyoxal at 435 nm, with two internal standards: L-rhamnose 2,4-dinitrophenylhydrazone for the former and D-xylose 2,4-dinitrophenylosazone for the latter. The molar response factors of hydrazones relative to the internal standards are given in Table I.

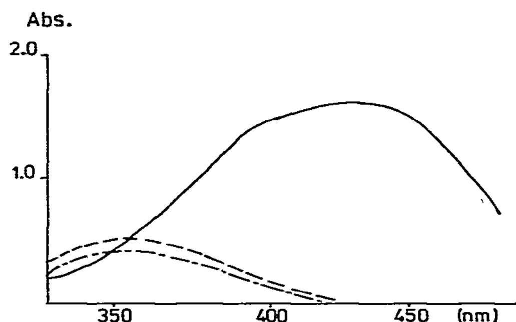

Fig. 2. Comparison of the UV adsorption spectra of the hydrazine reagent (-·-·-), the bishydrazone of glyoxal (———) and the hydrazone of D-glyceraldehyde (— — —).

Fig. 3 shows the chromatogram obtained for model dialdehyde I, which was prepared by periodate oxidation of methyl α-D-glucopyranoside and contains glyoxal and D-glyceraldehyde in equimolar proportions. A slightly faster eluting small peak (4′) was observed in the D-glyceraldehyde region, which was also detected when free D-glyceraldehyde was subjected to a similar analytical treatment. This small peak could be eliminated by repeated recrystallization of the product obtained from free D-glyceraldehyde. However, when a 1,2-dimethoxyethane solution of the purified product was allowed to stand, the small peak appeared again and its intensity in-

TABLE I

RELATIVE MOLAR RESPONSE OF THE HYDRAZONES

Hydrazone	*Relative molar response*	
	352 nm	*435 nm*
Glyoxal bis-DNP	—	1.26
Glycolaldehyde DNP	1.32	—
D-Glyceraldehyde DNP	1.47	—
D-Erythrose DNP	1.24	—
D-Arabinose DNP	0.98	—
D-Glucose DNP	0.77	—
L-Rhamnose DNP (Internal standard)	1	—
D-Xylose bis-DNP (Internal standard)	—	1

creased gradually to give an equilibrium state. The compound that gave the small peak was isolated by preparative thin-layer chromatography and purified by repeated recrystallization, but the spectral behaviour of the purified product was almost the same as that of the compound which gave the large peak (4). This evidence, together with the analytical data, suggests that both compounds were isomers of hydrazones of D-glyceraldehyde. The azomethine proton at $C_{1'}$ of the major isomer resonated at a lower field of 7.90 ppm with a larger coupling constant of 5.9 Hz in comparison with those of the minor isomer which appeared at 7.00 ppm with a coupling constant of 3.5 Hz. These PMR data indicate that the major and minor compounds are *syn*- and *anti*-isomers, respectively, analogous to aldoxime isomers[4]. Therefore, the sum of the weights of both peaks should be used for the determination of D-glyceraldehyde. Such

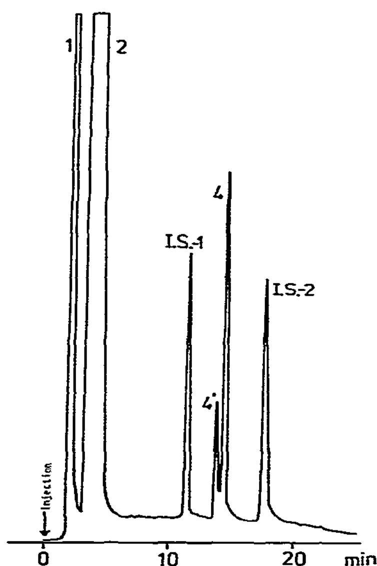

Fig. 3. Analysis of the component aldehydes in dialdehyde I. Internal standards: I.S.-1 = D-xylose DNP; I.S.-2 = L-rhamnose DNP; 4 = D-glyceraldehyde DNP (*syn*-isomer); 4' = D-glyceraldehyde DNP (*anti*-isomer). Assignment of other peaks as in Fig. 1.

TABLE II

DETERMINATION OF THE COMPONENT ALDEHYDES IN DIALDEHYDES I AND II

Dialdehyde	*Amount of sample (μmole)*	*Component aldehyde*			
		Glyoxal (μmole)		*D-Glyceraldehyde (μmole)*	
		Theoretical	*Found*	*Theoretical*	*Found*
I	0.20	0.20	0.21	0.20	0.19
	0.50	0.50	0.52	0.50	0.48
	1.00	1.00	1.00	1.00	0.96
II	0.20	0.20	0.20	0.20	0.20
	0.50	0.50	0.52	0.50	0.49
	1.00	1.00	1.03	1.00	0.97

TABLE III

ANALYSIS OF CONJUGATED ALDEHYDES IN PRODUCTS OF PERIODATE OXIDATION OF METHYL GLYCOSIDES

Glycoside	*Period of reaction (h)*	*Molar ratio to glyoxal*[a]	
		Glycolaldehyde	*D-Glyceraldehyde*
Methyl β-L-arabinopyranoside	24	1.00 (1)	0.00 (0)
Methyl β-D-xylopyranoside	24	0.96 (1)	0.00 (0)
Methyl α-D-glucopyranoside	24	0.00 (0)	0.93 (1)
Methyl β-gentiobioside	48	0.00 (0)	0.94 (1)

[a] Numbers in parentheses are theoretical values.

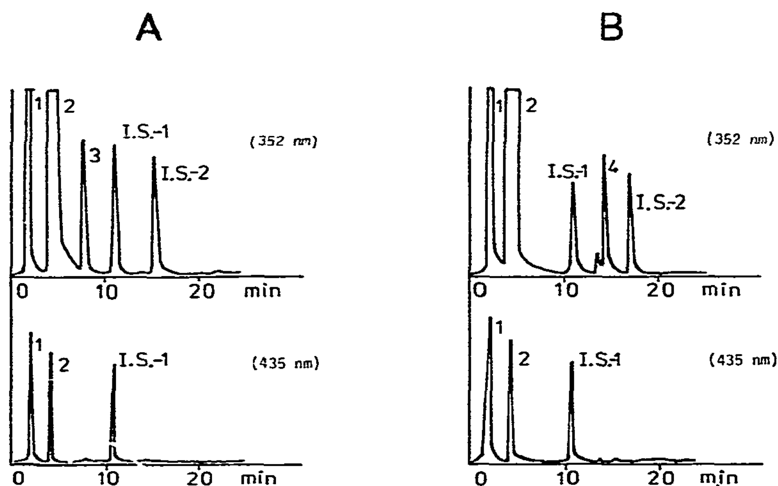

Fig. 4. Analysis of the conjugated aldehydes in products of periodate oxidation of (A) methyl β-D-xylopyranoside and (B) methyl β-gentiobioside. Peak assignment as in Figs. 1 and 3.

peak splitting was not observed for the other hydrazones. Analysis of dialdehyde II, prepared by oxidation of methyl β-D-glucopyranoside gave similar results.

Table II shows the accuracy of the determination of the component aldehydes in the isomeric dialdehydes I and II. In either instance, both glyoxal and D-glyceraldehyde were determined accurately.

On the basis of these results, several glycosides of monosaccharides were oxidized with periodate and the conjugated aldehydes in the products were determined. Table III gives the molar ratios of hydroxyaldehydes to glyoxal. An example of the oxidation of an oligosaccharide that has a 1,6-linkage (methyl β-gentiobioside) was also studied. The molar ratio of D-glyceraldehyde to glyoxal was also in good agreement with the theoretical value, suggesting the possible utility of this HPLC method for the determination of interglycosidic linkages of oligo- and polysaccharides. The chromatograms for methyl β-D-xylopyranoside and methyl β-gentiobioside are shown in Fig. 4.

REFERENCES

1 S. Honda, K. Kahehi and K. Takiura, *Anal. Chim. Acta*, 77 (1975) 125.
2 S. Honda, K. Kahehi and K. Takiura, *Carbohyd. Res.*, 47 (1976) 213.
3 C. Neuberg and E. Strauss, *Arch. Biochem.*, 11 (1946) 457.
4 P. Finch and Z. Merchant, *J. Chem. Soc., Perkin I*, (1975) 1682.