Journal of Chromatography, 265 (1983) 335–341
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,926

Note

Determination of neutral, amino and N-acetyl amino sugars as alditol benzoates by liquid–solid chromatography

RYUICHI OSHIMA* and JU KUMANOTANI
Institute of Industrial Science, University of Tokyo, 7-22-1, Roppongi, Minatoku, Tokyo 106 (Japan)
(Received April 13th, 1983)

Constituent monosaccharides of polysaccharides and glycoconjugates have been analyzed by gas–liquid chromatography (GLC) as their trimethylsilylated methylglycosides after methanolysis or as their alditol acetates after hydrolysis[1]. The former derivatives give complicated chromatograms due to the presence of anomers of methylglycosides. Only one peak is obtained for each of the latter derivatives[2] and excellent separations are attainable[3,4]. However, amino sugars and their N-acetylated substances cannot be discriminated by this procedure. A recent paper described a GLC–mass spectrometric (MS) method for solving this problem[5].

Benzoates of saccharides are suitable for LC analyses because of their high absorptivity in the UV region (λ_{max} = 240 and 275 nm). Lehrfeld[6] first reported the separation of perbenzoates of intact carbohydrates by liquid–solid chromatography (LSC). Piconde levels of benzoates of oligosaccharides have since been analyzed by LC[7].

We now demonstrate that neutral and amino sugars as alditol benzoates can simultaneously be analyzed by LSC, and also that amino sugars and N-acetylated substances are readily distinguished.

EXPERIMENTAL

Materials

Most sugar samples, benzoyl chloride, benzoic anhydride and 4-dimethylaminopyridine were commercial samples. 4-O-Methyl-D-glucose was a gift from Professor A. Ishizu. Pyridine was dried over sodium hydroxide.

Instruments

The liquid chromatograph comprised a Milton Roy minipump (SF-0396), a damper (Type DM; Umetani Seiki Co., Osaka, Japan), a syringe-loading sample injection valve (7125, Rheodyne) with a 20-μl loop and a gradient elution assembly (Toyo Soda, Tokyo, Japan). Effluent was monitored by a UV detector (UVIDEC 100II; JASCO, Tokyo, Japan) at 275 nm. Stainless-steel columns (150 × 4.5 and 150 × 8 mm) were packed with 3-μm silica gel (Develosil 60-3; Nomura Chemicals, Setocity, Aichi, Japan).

0021-9673/83/$03.00

Derivatization

A mixture of aqueous solutions of sugars (10 mg, 0.2 ml) and $NaBH_4$ (50 mg, 0.3 ml) was allowed to stand for 2 h at 40°C, acidified to pH 2.5–3 by adding 4 *M* hydrochloric acid, evaporated and coevaporated with methanol (1 ml), and dried at 50°C *in vacuo* for several hours. Dry pyridine (2 ml), benzoic anhydride (0.2 ml) and 4-dimethylaminopyrine (*ca.* 100 mg) were added to the residue and the mixture was stirred at 50°C for 2 h in a stoppered flask; methanol (1 ml) was then added. After evaporation and vacuum distillation at 60°C, the residue was partitioned between chloroform and water (2 ml each), and the organic layer was evaporated and subjected to gel permeation chromatography (TSK gel, G2000HG, 60 × 2.2 cm × 2; chloroform) to remove remaining methyl benzoate.

RESULTS AND DISCUSSION

Derivatization

Alditol benzoates of neutral monosaccharides were obtained using either benzoyl chloride or benzoic anhydride with 4-dimethylaminopyridine (catalyst) in py-

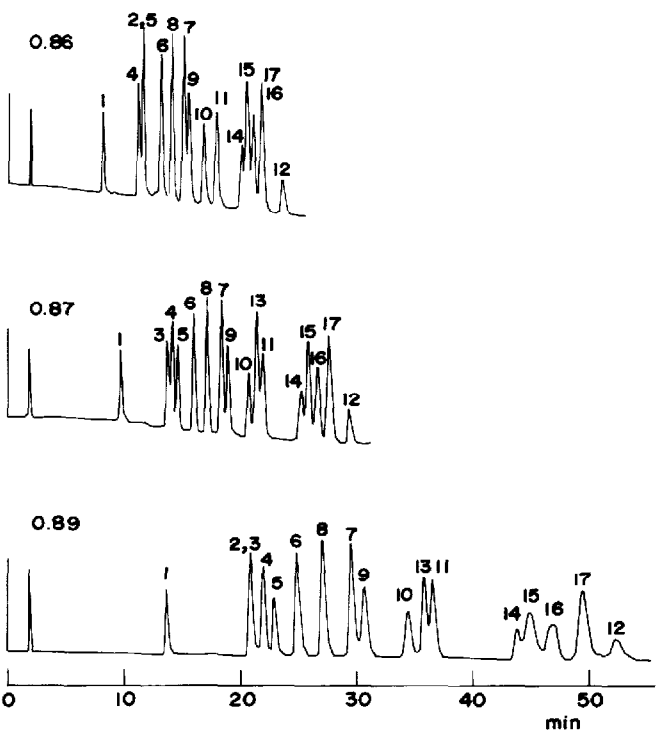


Fig. 1. Chromatograms of alditol benzoates using ternary eluents with varying *n*-hexane content as indicated on each elution curve, the ratio [dichloromethane]/([dichloromethane + [dioxane]) being constant at 0.87. Conditions: column, Develosil 60-3 (3 μm), 150 × 4.5 mm; detection, 275 nm, 0.16 a.u.f.s.; flow-rate, 1.25 ml/min. Peaks are alditol benzoates of: 1 = digitoxose; 2 = 2-deoxyribose; 3 = 6-deoxyglucose; 4 = rhamnose; 5 = fucose; 6 = ribose; 7 = xylose; 8 = arabinose; 9 = 2-deoxyglucose; 10 = 2-deoxygalactose; 11 = 3-O-methylglucose; 12 = 4-O-methylglucose; 13 = allose; 14 = altrose; 15 = glucose; 16 = mannose; 17 = galactose.

ridine, after reduction of sugars with $NaBH_4$. This was also the case for N-acetyl amino sugars. However, when bezoylated with benzoyl chloride, amino sugars gave two peaks in the chromatograms. Benzoylation of amino sugars by benzoic anhydride with 4-dimethylaminopyridine in pyridine gave a single peak in the chromatogram.

Separation

Since it was difficult to separate hexitol benzoates from each other by reversed-phase LC, all efforts were made to achieve separation by LSC on a silica gel column.

Since the presence of amide functions, either acetamide or benzamide, was found to increase the capacity factors of substances, gradient elution was required for simultaneous separation of alditol benzoates of neutral and amino sugars. An elution system comprising solvents with weak absorptivities in the UV region was preferred.

When *n*-hexane–ethanol mixtures were employed as eluent, alditol benzoates exhibited very small capacity factors even at ethanol contents as low as 1 %. We then made use of the ternary mobile phase, *n*-hexane–dioxane–dichloromethane, expecting to obtain good selectivity[8] as a result of variation of solvent composition. At a fixed

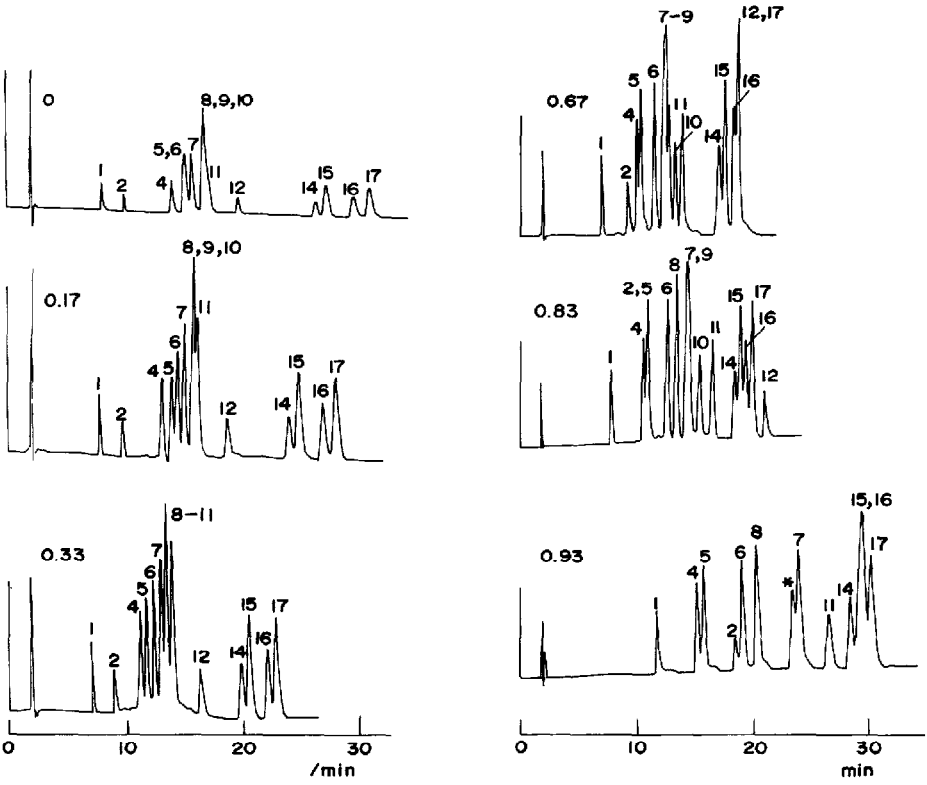


Fig. 2. Chromatograms of alditol benzoates using ternary eluents with a fixed *n*-hexane content (0.85), the ratio [dichloromethane]/([dichloromethane] + [dioxanel]) being varied as indicated on each elution curve. Other chromatographic conditions and peak numbers as in Fig. 1. A peak marked with an asterisk was not identified.

dioxane–dichloromethane ratio of 2:1 the elution order of alditol benzoates of neutral sugars was independent of the *n*-hexane content (Fig. 1). When the *n*-hexane content was fixed at 85%, the elution order varied with the ratio of dioxane and dichloromethane (Fig. 2). Fig. 3 illustrates the dependence of solute retention time on the dioxane–dichloromethane ratio.

The elution behaviours of benzoates of representative 2-amino-2-deoxyhexitols and 2-acetamido-2-deoxyhexitols were examined at a fixed *n*-hexane concentration varying the dioxane–dichloromethane ratio (Figs. 4 and 5). It is seen that the elution order was independent of the dioxane–dichloromethane ratio.

Based on the results of Figs. 3–5, simultaneous separation of benzoates of neutral and amino sugars was achieved using the ternary eluent with a dioxane–dichloromethane ratio of 2:1 (Fig. 6). Linear gradient elution enabled us to determine common neutral and amino sugars, but 2-deoxyhexoses cannot be discriminated. However, inspection of the data in Figs. 3–5 may yield an adequate solvent composition for separation of sugar mixtures under consideration.

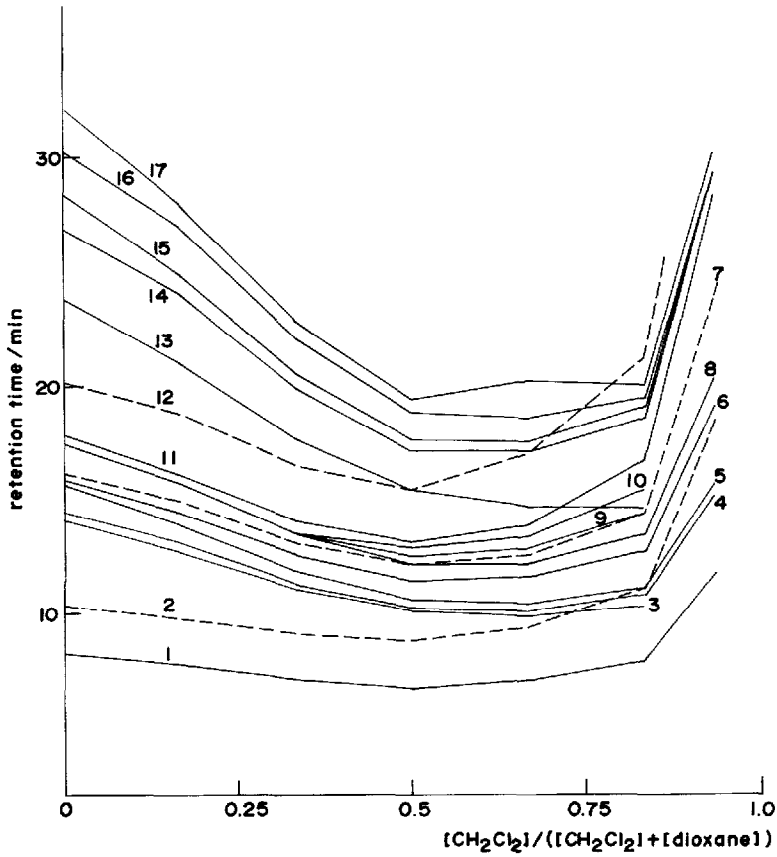
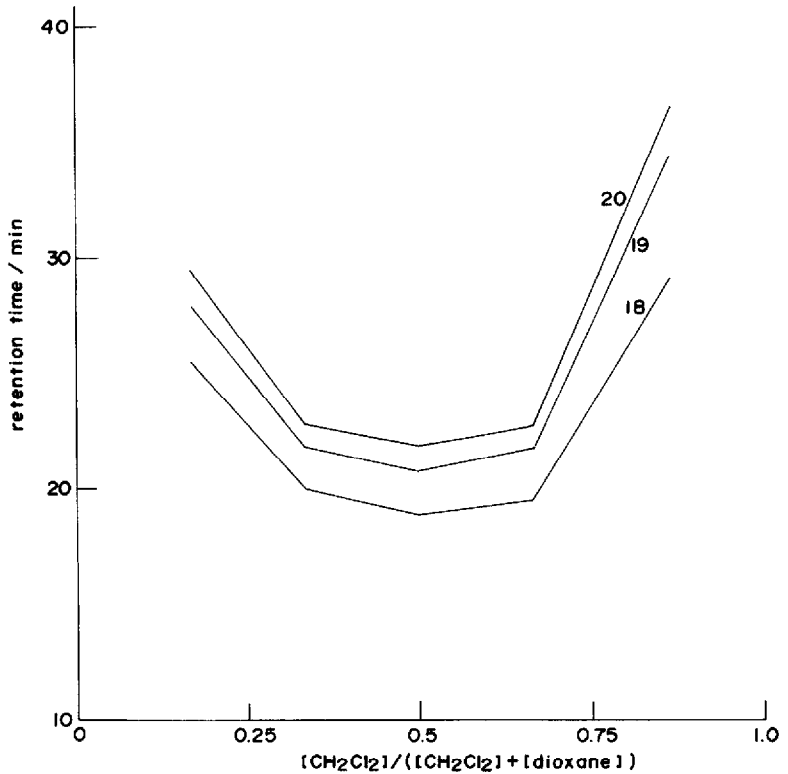


Fig. 3. Retention time of alditol benzoates of neutral monosaccharides using ternary eluents with varying [dichloromethane]/([dichloromethane] + [dioxanel]) the *n*-hexane content being fixed at 0.85. Chromatographic conditions and peak numbers on curves as in Fig. 1.



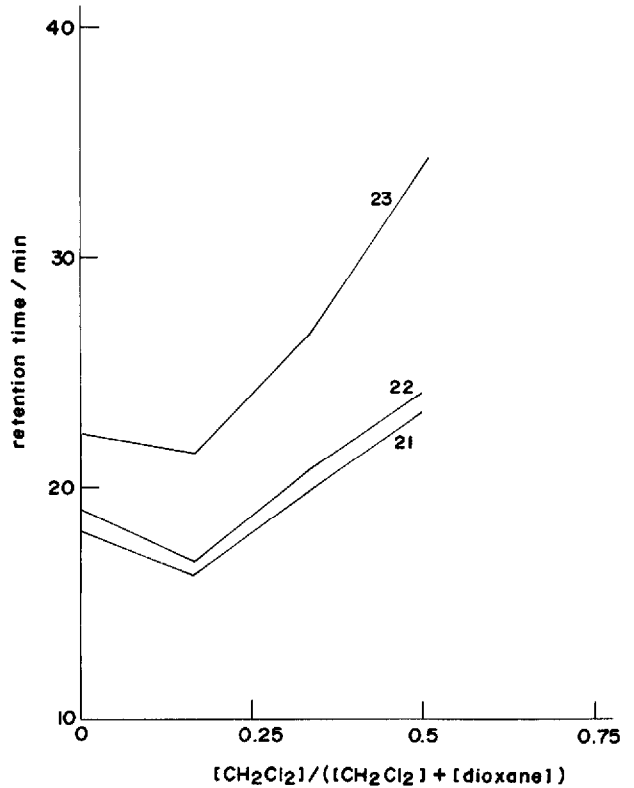


Fig 4. Retention time of alditol benzoates of 2-amino-2-deoxyhexose using eluents with varying [dichloromethane]/([dichloromethane] + [dioxane]) ratios, the *n*-hexane content being fixed at 0.80. Chromatographic conditions as in Fig. 1. Numbers on curves represent alditol benzoates of: 18 = glucosamine; 19 = mannosamine; 20 = galactosamine.

Fig. 5. Retention time of alditol benzoates of 2-acetamido-2-deoxyhexoses using ternary eluents with varying [dichloromethane]/([dichloromethane] + [dioxane]), the *n*-hexane content being fixed at 0.73. Chromatographic conditions are same as in Fig. 1. Numbers on curves represent alditol benzoates of: 21 = N-acetylmannosamine; 22 = N-acetylglucosamine; 23 = N-acetylgalactosamine.

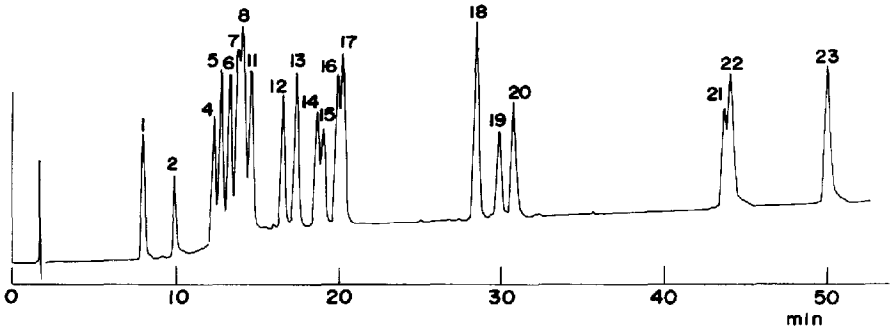


Fig. 6. Chromatogram of alditol benzoates with linear gradient elution using the ternary eluent, *n*-hexane–dioxane–dichloromethane (110:10:5 to 20:10:5 in 80 min). Other chromatographic conditions and peak numbers as in Figs. 1, 4 and 5.

The efficiency of this LSC separation is inferior to that of the fused-silica capillary GLC separation of alditol acetates[4]. Nonetheless, it is advantageous that this system can easily distinguish amino-sugars and N-acetylated analogues, and offers a tool for preparative purposes.

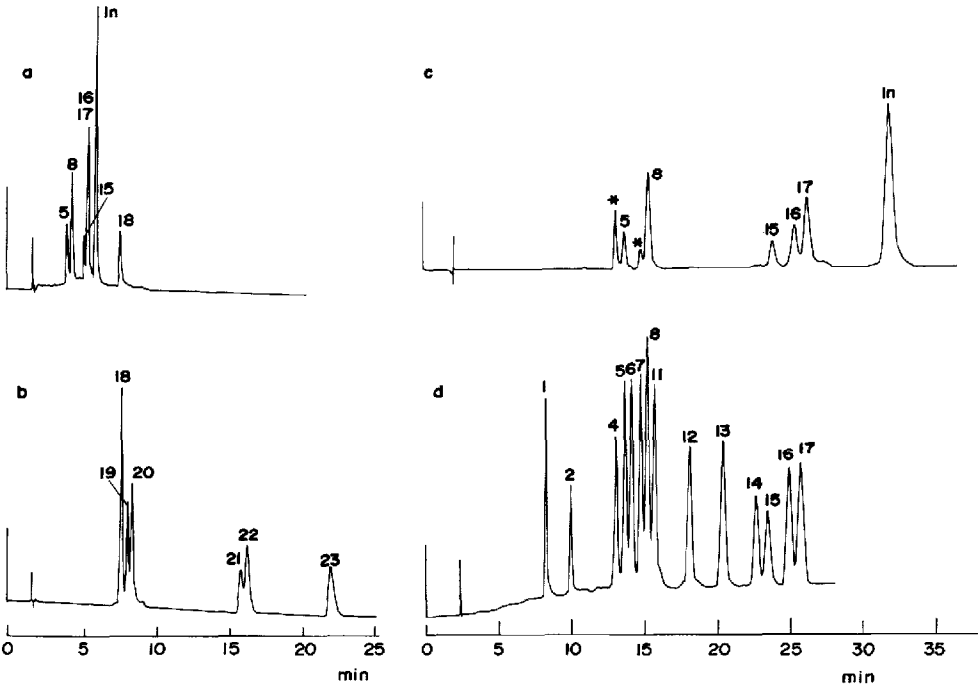


Fig. 7. Chromatograms of alditol benzoates from component sugars in the water-insoluble glycoprotein separated from the lac tree (*Rhus vernicifera*) (a and c). Elution curves b and d are for those of standard mixtures. Conditions for curves a and b: column, Develosil 60-3, 150 × 4.5 mm; eluent, *n*-hexane–dioxane–dichloromethane (40:10:5); flow-rate, 1.25 ml/min; detection, 275 nm, 0.16 a.u.f.s. Conditions for curves c and d: column, Develosil 60-3, 150 × 8 mm; eluent, *n*-hexane–dioxane–dichloromethane (150:20:5); detection, 275 nm, 0.08 a.u.f.s.; flow-rate, 2.5 ml/min. Peak numbers as in Figs. 1, 5 and 6; those marked with an asterisk were not identified; In = benzoate of innositol as internal standard.

The present procedure was applied to sugar analysis of the water-insoluble glycoprotein separated from the sap of lac tree (*Rhus vernicifera*). After hydrolysis of the glycoprotein (*ca.* 1 mg) with 2 *M* trifluoroacetic acid for 6 h at 100°C, the hydrolyzate was reduced with $NaBH_4$, benzoylated with 10% benzoic anhydride and 5% 4-dimethylaminopyridine in dry pyridine for 2 h at 65°C and analyzed by LSC to give the chromatograms of Fig. 7a and c; isocratic elution was employed because of the reproducibility of retention time. By comparison with chromatograms of standards (Fig. 7b and d), fucose, arabinose, glucose, mannose, galactose and glucosamine were identified, in good agreement with the results of GLC analysis of the hydrolyzate in the form of the alditol acetates. N-Acetyl amino sugars may be N-deacetylated in the course of hydrolysis.

ACKNOWLEDGEMENT

We express our sincere gratitude to Professor A. Ishizu for a supply of 4-O-methyl-D-glucose.

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