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# SEPARATION OF EIGHT EPIMERIC 2-AMINOALDOHEXOSES AS AMINOHEXITOL ACETATES BY GAS CHROMATOGRAPHY AND ANALYSIS OF BACTERIAL HETEROGLYCANS CONTAINING RARE 2-AMINOALDOHEXOSES AND 2-AMINOURONIC ACIDS

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## SUMMARY

A method has been developed for the separation of all eight of the possible 2-aminohexitols as alditol acetates derived from 2-aminoaldohexoses by gas chromatography on a capillary column wall coated with Poly A-103 (25 m × 0.25 mm I.D.). For the depolymerization of bacterial polysaccharides containing amino sugars or aminouronic acids, we used methanolysis, which resulted in transglycosylated amino sugar methylglycosides and aminouronic acid methylglycoside methyl esters without appreciable destruction. The latter compounds were converted into amino sugar methylglycosides by reduction with sodium borohydride. This step allows differentiation between amino sugars and aminouronic acids.

The usefulness of the method is demonstrated on cell wall polysaccharides of *Shigella sonnei*, *Vibrio parahaemolyticus*, *Micrococcus lysodeikticus* and *Methanobacterium thermoautotrophicum* containing rare aminouronic acids.

## INTRODUCTION

In recent years, numerous papers have been published on polysaccharides containing amino sugars or aminouronic acids rarely occurring or even hitherto unknown in nature. For the analysis of amino sugars, gas chromatography is a widely accepted method, although most of the procedures published are restricted to the separation of glucosamine, galactosamine and mannosamine. Perry and Webb[1] reported the systematic resolution of all eight of the possible epimeric 2-aminoaldohexoses as alditol acetates on neopentyl glycol sebacate polyester phase. This method resulted in poor resolution in our hands. On the other hand, we found that the use of a polyamide stationary phase, Poly A-103, introduced by Niedermeier and Tomana[2], is very promising for the analysis of 2-aminohexitolacetates.

Further difficulties can be expected in preparing monomeric amino sugars and aminouronic acids by hydrolysis. The strong glycosidic bonds withstand the hydrolytic conditions normally used for neutral polysaccharides and more drastic treatment, *e.g.*, with 4–6 *M* mineral acid at elevated temperature, leads to considerable decomposition, especially for aminouronic acids.

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To overcome these problems, we used methanolysis instead of hydrolysis, which resulted in transglycosylated amino sugar methylglycosides or transglycosylated-transesterified aminouronic acid methylglycoside methyl esters without appreciable destruction[3]. The latter compounds could be easily converted into amino sugar methylglycosides by reduction with sodium borohydride. This step allows a clear differentiation between amino sugars and aminouronic acids[4,5].

This paper gives a detailed description of the method and reports on its use in the analysis of bacterial heteropolysaccharides.

## EXPERIMENTAL

### *Materials*

2-Amino-2-deoxy-D-glucose (D-glucosamine), 2-amino-2-deoxy-D-galactose (D-galactosamine) and 2-amino-2-deoxy-D-mannose (D-mannosamine) were obtained from Sigma (St. Louis, MO, U.S.A.). 2-Amino-2-deoxy-D-allose (D-allosamine), 2-amino-2-deoxy-D-altrose (D-altrosamine), 2-amino-2-deoxy-D-gulose (D-gulosamine), 2-amino-2-deoxy-D-idose (D-idosamine) and 2-amino-2-deoxy-D-talose (D-talosamine) were synthesized by the method of Perry and co-workers[6–8] in our laboratory.

*Shigella sonnei* phase I O-polysaccharide was prepared as described previously[5]. K-15 antigen of *Vibrio parahaemolyticus* A 55 (05:K 15)[9] and cell wall polysaccharide of *Micrococcus lysodeikticus* containing 2-amino-guluronic acid and 2-aminomannuronic acid were kindly supplied by Dr. M. Torii (Research Institute for Microbial Diseases, Osaka, Japan). Cell walls of *Methanobacterium thermoautotrophicum* containing, in addition glucosamine and galactosamine, 2-aminotaluronic acid[10] was a gift from Dr. O. Kandler (Botanisches Institut der Universität München, Munich, F.R.G.).

### *Preparation of samples for gas chromatography*

*A. Methanolysis.* Samples of 2 4 mg of polysaccharides containing amino sugars and aminouronic acids were thoroughly dried in a vacuum desiccator over phosphorus pentoxide at 65°C. Methanolysis was performed with 1 *M* hydrochloric acid prepared in anhydrous methanol (0.5 ml), according to Chambers and Clamp[3], for 8 h at 80°C. The acid was then neutralized with solid silver carbonate and the methanolysate containing methylglycosides and methylglycoside methyl esters was reacetylated overnight in the same tube with acetic anhydride (0.05 ml) at room temperature. Silver salts were removed by centrifugation and washed with anhydrous methanol (2 × 1 ml). The supernatants were collected and evaporated to dryness *in vacuo*. Traces of acetic acid were removed from the residue by repeated dissolution in methanol and evaporation to dryness.

*B. Reduction of methylglycoside methyl esters to amino sugar methylglycosides.* The residue was dissolved in water (0.5 ml) and was treated with sodium borohydride (10 mg in 0.5 ml of water) overnight at room temperature. Sodium ions were removed using Amberlite CG-120 ($H^+$) resin. Samples were evaporated to dryness *in vacuo* and boric acid was distilled off as methyl borate by repeated addition of methanol (five times).

*C. Hydrolysis.* The residue which contained amino sugar methylglycosides was hydrolysed with 4 *M* hydrochloric acid (0.2 ml) for 2 h at 100°C. The solution was then evaporated to dryness under reduced pressure.

*D. Reduction and peracetylation of amino sugars.* The hydrolysate was redissolved in water (0.3 ml) and the sugars were reduced with 10 mg of sodium borohydride in 0.5 ml of water (2 h at room temperature). The reduction was stopped with 0.2 ml of concentrated acetic acid and the solution was evaporated to dryness. Borate ions were removed by repeated evaporation (at least five times) from 1.5 ml of methanol and 0.05 ml of acetic acid. The samples were stored *in vacuo* over sodium hydroxide pellets. Peracetylation was carried out in a mixture of dry pyridine and acetic anhydride (1:1) (1 ml) at 100°C for 2 h. The reaction mixture was diluted with 2 ml of water and evaporated twice under reduced pressure. The aminoalditol acetates that resulted were redissolved in chloroform and used for gas chromatography.

*Gas chromatography*

Analyses were carried out on a Varian Aerograph 2740 instrument equipped with a type 86820 inlet splitter (Hamilton, Bonaduz, Switzerland) and a type BFU-

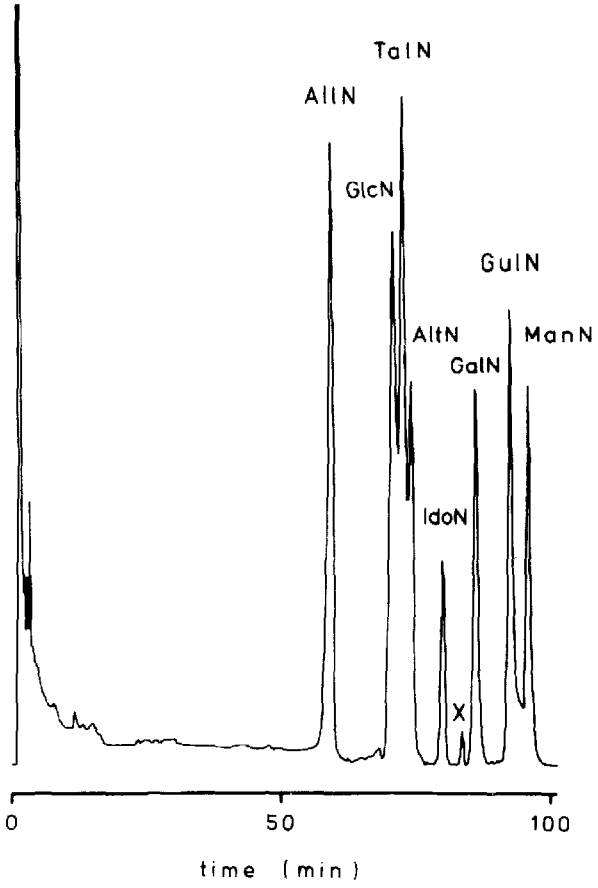


Fig. 1. Capillary gas chromatography of a standard mixture of 2-aminohexitol acetates. Conditions of sample preparation and chromatography are given in the text. For retention times and relative retention times see Table I. AllN, allosaminitol; GlcN, glucosaminitol; TalN, talosaminitol; AltN, altrosaminitol; IdoN, idosaminitol; GalN, galactosaminitol; GulN, gulosaminitol; ManN, mannosaminitol; X, artefact.

101 flow control unit (Chrompack, Middelburg, The Netherlands). For the analysis, a wall-coated glass capillary column was used (25 m long × 0.25 mm I.D.). The column was coated with Poly A-103 (Applied Science Labs, College Station, OH, U.S.A.) and operated under the following conditions: carrier gas (nitrogen) flow-rate, 2.5 ml/min; splitting ratio, 1:5; make-up gas flow-rate, 27.5 ml/min; flame-ionization detector using hydrogen at 30 ml/min and air at 300 ml/min; injector temperature, 250°C; detector temperature, 250°C; column temperature, 175°C isothermal for 50 min, then increased at 0.5°C/min to 210°C.

## RESULTS AND DISCUSSION

### *Separation of standard mixture of amino sugars*

A 1–2-mg amount of standard amino sugars was converted into aminohexitol acetates as described in section *D* under Experimental. In a previous experiment, we attempted to separate aminoalditol acetates on a conventional support-coated column (6 ft. × 1/4 in. I.D. 1% Poly A-103 on Gas-Chrom Q, 100–200 mesh) at 195°C (isothermal). Altrosaminitol, glucosaminitol and talosaminitol could not be resolved under these conditions. A standard mixture without talosamine gave a fairly well resolved shoulder of altrosaminitol on the glucosaminitol peak.

Fig. 1 shows the separation of eight aminoalditol acetates on a capillary column. The peaks are clearly separated and allow identification of the components from their relative retention times (Table I). Between the peaks of idosaminitol and galactosaminitol, a peak was observed that we assume represents an artefact.

TABLE I

CAPILLARY GAS CHROMATOGRAPHIC RETENTION TIMES OF 2-AMINOHEXITOL ACETATES DERIVED FROM STANDARD 2-AMINOALDOHEXOSES AND IDENTIFICATION OF AMINO SUGARS IN SUGAR COMPOSITION ANALYSES OF BACTERIAL HETEROGLYCANS

| *Substances* | *Derivative of* | *Retention time (min)* | *Relative retention time* | *Identified aminohexitol* |
|---|---|---|---|---|
| Standard sugars | Allosaminitol | 59.40 | 0.83 | |
| | Glucosaminitol | 71.50 | 1.00 | |
| | Talosaminitol | 73.32 | 1.03 | |
| | Altrosaminitol | 74.90 | 1.05 | |
| | Idosaminitol | 80.90 | 1.13 | |
| | Galactosaminitol | 87.06 | 1.22 | |
| | Gulosaminitol | 93.51 | 1.31 | |
| | Mannosaminitol | 96.97 | 1.36 | |
| Bacterial heteroglycans | *V. parahaemolyticus* K-15 antigen | 93.70 | 1.31 | Gulosaminitol |
| | | 97.40 | 1.36 | Mannosaminitol |
| | *S. sonnei* lipopolysaccharide | 75.52 | 1.06 | Altrosaminitol |
| | *M. thermoautotrophicum* cell wall | 72.92 | 1.02 | Talosaminitol |
| | | 89.52 | 1.25 | Galactosaminitol |
| | *M. lysodeikticus* polysaccharide | 96.54 | 1.35 | Mannosaminitol |

*Detection of amino sugars and aminouronic acids of bacterial heteroglycans*

Alditol acetates of neutral sugars appear on the isothermal part of the chromatogram after about 50 min. We did not try to separate neutral sugar components considering the poor resolving power of Poly A-103, especially for the most common

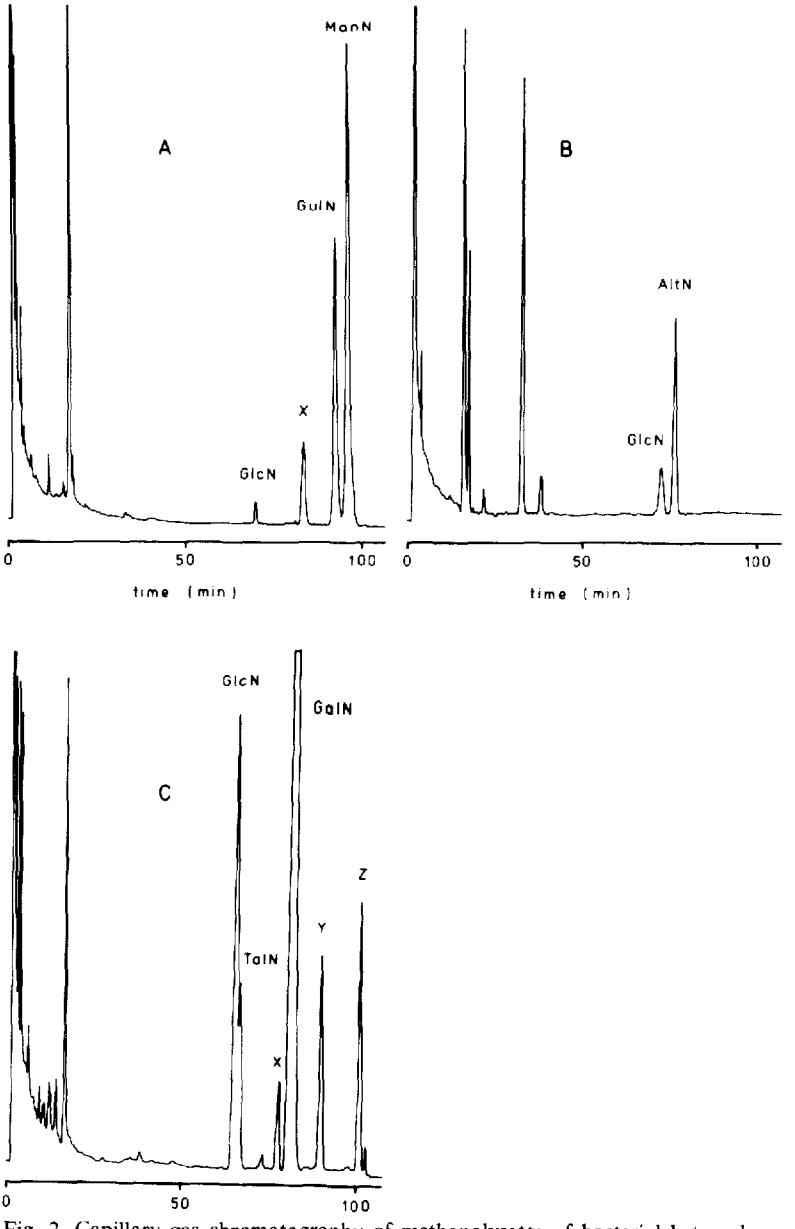


Fig. 2. Capillary gas chromatography of methanolysates of bacterial heteroglycans. For experimental details, see text. A, K-15 antigen of *Vibrio parahaemolyticus* A55 (05:K15); B, lipopolysaccharide of *Shigella sonnei* phase I; C, cell wall of *Methanobacterium thermoautotrophicum*. See Fig. 1 for peak identification. Y and Z: unidentified peaks.

hexoses galactose and glucose, as reported by Niedermeier and Tomana[2]. However, analysis of these compounds with appropriate column packings used for neutral alditol acetates is possible using the same samples[11]. Similarly, no attempt was made to evaluate the results quantitatively because of the complex of derivatization procedure, the steps of which may influence the recovery of each of the components differently.

Fig. 2A and B show chromatograms of purified K-15 antigen and lipopolysaccharide of *Shigella sonnei* after derivatization as described under Experimental. In K-15 antigen of *Vibrio parahaemolyticus* gulosaminuronic acid and mannosaminuronic acid were detected[9]. *Shigella sonnei* phase I lipopolysaccharide contains, in addition to glucosamine, L-altrosaminuronic acid[5]. Both aminouronic acids and aminoaldohexoses appear on these chromatograms as aminohexitol acetate peaks. The efficiency of the method is demonstrated especially by the chromatogram for the crude sample of cell wall of *Methanobacterium thermoautotrophicum*[10] in which, in addition to a large amount of glucosaminitol and galactosaminitol, the talosaminitol peak derived from talosaminuronic acid can be readily distinguished (Fig. 2C). On this chromatogram two further unidentified peaks were observed that do not correspond to any of the known amino sugars. The relative retention times of aminoalditol acetates established on these chromatograms are given in Table I.

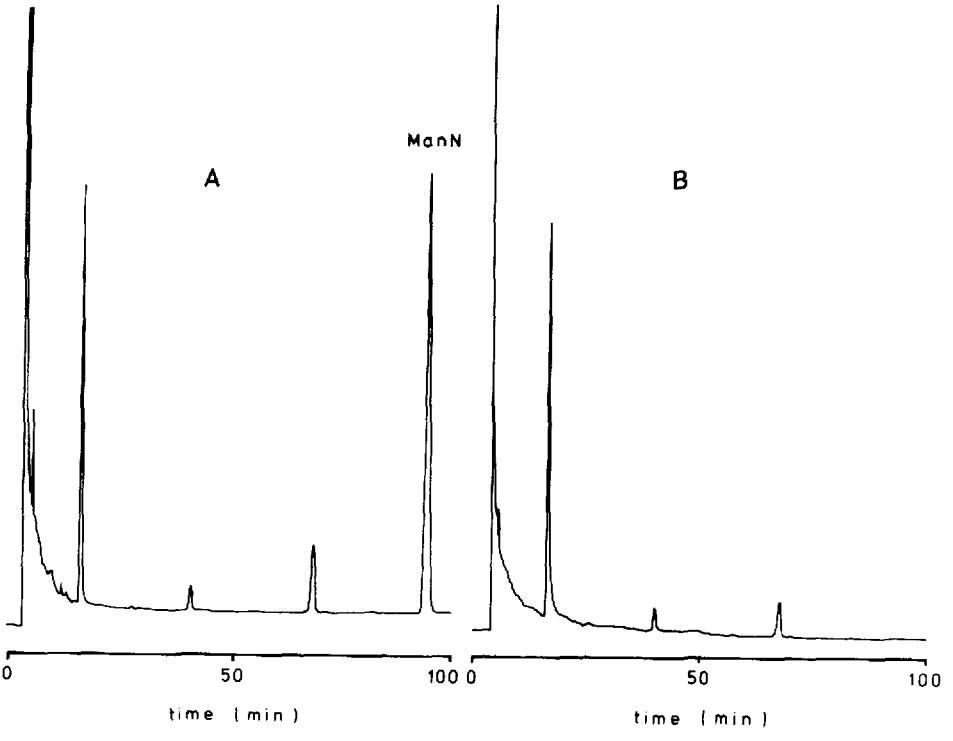


Fig. 3. Capillary gas chromatography of the methanolysate of *Micrococcus lysodeikticus* cell wall polysaccharide (A) with reduction of aminouronic acid methyl esters as described in section *B* under Experimental and (B) without reduction. Conditions of sample preparation and chromatography are given in the text. See Fig. 1 for peak identification.

*Differentiation between amino sugars and aminouronic acids*

To distinguish amino sugars and aminouronic acids, samples after methanolysis were divided into two parts. One part was treated as described in sections *B*, *C* and *D* under Experimental. The other part, omitting step *B*, was immediately hydrolysed (*C*), reduced and peracetylated (*D*). When aminouronic acid was present in the methanolysate, the last procedure resulted in non-volatile peracetylated 5-aminoaldonic acid, and therefore no peak appeared on the chromatogram.

Fig. 3 shows the chromatogram of the methanolysate of *Micrococcus lysodeikticus* cell wall polysaccharide containing mannosaminuronic acid as the sole amino sugar[12], with (A) and without (B) reduction of methyl esters, indicating that the mannosaminitol peak of the chromatogram in Fig. 3A was derived from mannosaminuronic acid.

This method is also suitable for the rapid screening of heteroglycans of unknown structure prepared from microbial and other sources containing 2-amino sugars or 2-aminouronic acids hitherto unknown in nature.

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