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MODIFICATIONS IN THE ALDITOL ACETATE METHOD FOR ANALYSIS OF MURAMIC ACID AND OTHER NEUTRAL AND AMINO SUGARS BY CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY WITH SELECTED ION MONITORING

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SUMMARY

Two alditol acetate methods for the gas chromatographic (GC) analysis of neutral and amino sugars were compared. Following sodium borohydride reduction, one method uses methylimidazole as an acetylation catalyst without prior removal of water or borate salts and the other method uses sodium acetate after removal of borate and water. Depending on the acetylation conditions, muramic acid produced different derivatives. With methylimidazole, reliable derivatization of muramic acid was not possible, although other sugars derivatized reliably. With sodium acetate, all sugars tested were reproducibly derivatized. The utility of the sodium acetate method is shown by the trace GC–mass spectrometric analysis of muramic acid and rhamnose derived from bacterial peptidoglycan-polysaccharide complexes in mammalian tissue.

INTRODUCTION

The alditol acetate method for the gas chromatographic (GC) analysis of carbohydrates has been popular because a single peak is produced for each derivatized sugar and because the stability of the derivative permits sample storage for extended periods. However, the principal disadvantage of the alditol acetate method has been the large number of manual processing steps which make the procedure time consuming and tedious to perform[1–4]. There have been numerous attempts to develop alternative derivatives which are simpler to prepare, including trimethylsilyls[5], aldononitrile acetates[6,7], methyloxime acetates[8], and trifluoroacetates[9] with varying degrees of success.

In previous articles[10,11] we reported improvements in the alditol acetate

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method for analyzing mixtures of neutral and amino sugars. Modifications in the hydrolysis, neutralization, isolation, and derivatization steps have led to a considerable decrease in extraneous peaks as well as allowing the processing of multiple samples. The method has been used successfully along with fused-silica capillary columns and mass spectrometry (MS) for the characterization and identification of neutral and amino sugars including muramic acid in *Legionella* and other bacteria[12,13]. McGinnis[14] demonstrated that, using the catalyst methylimidazole, acetylation can proceed without removal of the borate generated during the reduction step, thus allowing for rapid and simple derivatization. Blakeney and co-workers[15,16] further developed an alditol acetate method based on the use of methylimidazole as a catalyst.

In the present study we describe an alditol acetate procedure that incorporates sample handling modifications with the use of methylimidazole as a catalyst[15], as well as improvements to our previously published sodium acetate catalyzed method[10]. Both procedures allow simplified processing of multiple samples and include extensive clean-up steps to reduce background interferences. During the development of these procedures we discovered some peculiarities about the alditol acetate derivatization reactions of muramic acid which make it incompatible with the methylimidazole catalyzed procedure. Our laboratories are particularly interested in muramic acid since it is a unique sugar found only in bacterial cell walls. We previously showed that this compound can be used as a chemical marker for bacterial cell walls in inflamed mammalian tissues[3]. We confirm this observation using the improved sodium acetate catalyzed alditol acetate procedure and demonstrate that rhamnose, a neutral sugar, can also be used as a chemical marker for bacterial components.

EXPERIMENTAL

Materials

L-Rhamnose, L-fucose, D-xylose, D-mannose, D-galactose, and D-glucose standards were obtained from Supelco (Bellefonte, PA, U.S.A.). 2-Deoxy-D-ribose, D-ribose, muramic acid, D-glucosamine hydrochloride, D-galactosamine hydrochloride, D-mannoheptulose, methylglucamine, and *E. coli* 0127:B8 lipopolysaccharide (LPS, phenol water extract) were obtained from Sigma (St. Louis, MO, U.S.A.). D-Arabinose was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.).

Glass-distilled acetic anhydride (Alltech, Deerfield, IL, U.S.A.), chloroform and methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were purchased. Reagent-grade glacial acetic acid (J. T. Baker, Phillipsburgh, NJ, U.S.A.) was glass-distilled prior to use. N,N-dioctylmethylamine was purchased from ICN (Plainview, NY, U.S.A.). Sodium borohydride and reagent-grade sulfuric acid were obtained J. T. Baker (Phillipsburgh, NJ, U.S.A.). N-methylimidazole was obtained from Sigma (St. Louis, MO, U.S.A.) and was glass-distilled prior to use. Ammonium hydroxide and hydrochloric acid (Ultrex grade) were purchased from J. T. Baker. Bond Elut hydrophobic extraction columns and Chem Elut hydrophilic columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). All glassware was washed with detergent, distilled water, 1 *M* hydrochloric acid, and chloroform.

Pre-derivatization sample processing

Typically, 1 mg of sample in 0.5 ml 1 *M* sulfuric acid was placed in a hydrolysis tube (Pierce, Rockford, IL, U.S.A.). Ten tubes were connected simultaneously to a vacuum manifold that was connected to both a nitrogen tank and a vacuum pump. This manifold configuration permitted each hydrolysis tube to be alternately evacuated and flushed with nitrogen to remove oxygen. The PTFE valves on the hydrolysis tubes were closed under vacuum and hydrolysis was performed in a Pierce Reacti-Therm heating module at 100°C for 3 h. After the tubes were cooled to room temperature, 10 μg (in 200 μl water) of internal standard (arabinose for neutral sugars and methylglucamine for amino sugars) and 2.5 ml of 20% N,N-dioctylmethylamine in chloroform were added. The mixtures were vigorously mixed on a Vortex mixer and allowed to settle into two layers on standing. A 1-ml Bond Elut column was prepared for each sample by rinsing with 2 ml methanol followed by 2 ml of distilled water. The upper, aqueous layer of each hydrolysis mixture was carefully pipetted onto a column. Each mixture was then pulled through the column by vacuum into a reaction vial fitted with a screw cap and a PTFE silicone liner (Pierce) and the column was washed with 1.0 ml of distilled water. The Vac-Elut system (Analytichem) permitted up to ten samples to be run simultaneously.

Methylimidazole catalyzed derivatization

The samples were dried overnight at 30°C under vacuum in a Vortex evaporator (Buchler, Fort Lee, NJ, U.S.A.) which allowed up to thirty samples to be processed simultaneously. The samples were redissolved in 250 μl water, and 60 μl of sodium borohydride solution (10 mg/ml, dissolved in N-methylimidazole) was added. The vials were incubated in a Reacti-Therm heating module at 37°C for 90 min. Excess sodium borohydride was destroyed by adding 20 μl glacial acetic acid to the sample. Volumes of 600 μl of acetic anhydride were added to each vial and the sample was heated at 37°C for 45 min in the Reacti-Therm heating module.

Sodium acetate catalyzed derivatization

After the hydrolysis, 50 μl of aqueous sodium borohydride (100 mg/ml) was pulled through the Bond Elut column along with the sample. The reduction was allowed to proceed overnight in a refrigerator. Excess sodium borohydride was destroyed by adding 2 ml of acetic acid–methanol (1:200, v/v) to the sample which was then evaporated to dryness in the Vortex evaporator at 60°C under vacuum. This step was repeated four additional times to ensure complete removal of the sodium borohydride. The vials were allowed to dry for 3 h after the last evaporation. After cooling to room temperature, 300 μl of acetic anhydride was added to each vial and the sample was heated at 100°C for 13–16 h in the Reacti-Therm heating module.

Post-derivatization sample processing

After acetylation, samples were cooled in an ice bath, and 0.80 ml of cold ammonium hydroxide (80%, v/v) was added. This addition was carried out slowly in order to minimize the heat generated during neutralization. Chloroform (1 ml) was added and the mixture was agitated and poured onto a Chem Elut column and eluted with 2 ml chloroform. To the eluent 0.6 ml of 1 *M* hydrochloric acid was added, followed by vortexing. The well agitated mixture was again poured onto a

fresh Chem Elut column and eluted with another 2 ml of chloroform. The chloroform solution was evaporated to dryness under vacuum. The sample was redissolved in about 40 μl of chloroform before analysis.

GC–MS

GC–MS analyses were carried out using a 5970 mass selective detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) interfaced via a capillary direct inlet to a HP-5890 GC equipped with a capillary inlet system and a 25 m × 0.20 mm I.D. OV-1701 fused-silica column that was prepared in our laboratory. Data analysis was carried out on a HP-59970 data station. Samples were injected with a split ratio of 20:1 and a column helium flow of 0.6 ml/min. The injection port temperature was 240°C and the GC–MS interface temperature was 270°C. The oven temperature was held at 140°C for 0.5 min and programmed at 25°C/min to 195°C, then programmed at 6°C/min to 250°C. The mass selective detector was routinely operated under "Autotune" conditions (standard ions 69, 219, and 502 of perfluorotributylamine, PFTBA). Five ions were monitored: m/z 84 (100 ms/cycle) for glucosamine and galactosamine, m/z 86 (50 ms) for methylglucamine, m/z 159 (25 ms) for deoxyribose, m/z 115 (100 ms) for all other neutral sugars, and m/z 168 (200 ms) for muramic acid.

Mass spectra containing molecular ions and high mass fragments required to identify muramicitol tetraacetate (MTA) and muramicitol pentaacetate (MPA) were obtained by tuning the instrument using the automated tuning software with ions 219, 414, and 502 of PFTBA. Spectra were acquired over the mass range 50–500 at a scan rate of 1.3 Hz.

RESULTS AND DISCUSSION

The objective of the studies reported here was the comparison of two derivatization methods for sugars, employing either methylimidazole or sodium acetate as an acetylation catalyst. The apparent advantage of methylimidazole was that removal of borate or water was unnecessary prior to acetylation[14–16]. Sample processing was thus greatly simplified and the method could be completed more rapidly. In our previous work with the alditol acetate method[10,11], improvements in sample handling and clean-up were introduced that permitted simultaneous analysis of multiple samples and trace analysis of sugars in biological matrices. A method combining the advantages of both procedures while minimizing any drawbacks would have considerable utility.

Instrumental considerations

In these studies, a fused-silica capillary column coated with OV-1701 was employed. This column provides baseline or better resolution for all components in 18 min. OV-1701 is also available as a bonded phase, providing greater temperature stability. Other polar columns such as SP-2330 also provide excellent resolution of alditol acetates[10]. We do not recommend the routine use of less polar columns as they provide inadequate resolution of some of the early eluting neutral sugars, although these may be resolved on a GC–MS using reconstructed ion profiles[13].

Selective GC detectors aid in the analysis of complex samples through elim-

ination of extraneous background peaks derived from the sample matrix. In addition, selective detectors are often more sensitive than the standard flame ionization detector. For example, Whenham[17] demonstrated the utility of the nitrogen–phosphorus detection (NPD) system in the determination of amino sugars as their alditol acetates. Pritchard and Niedermeier[9] have also shown enhanced sensitivity using an electron-capture detection (ECD) system with trifluoroacetate derivatives. The drawback of the NPD is that only amino sugars can be detected while neutral sugars give no signal; to achieve higher sensitivity with an ECD, a derivatization reaction that introduces a halogen into the compound of interest must be employed. A mass spectrometer in the selected ion monitoring (SIM) mode is a much more versatile and selective detector. Through appropriate selection of the monitored ions, all of the compounds of interest can be detected while the background is ignored. GC–MS with SIM is not limited to a particular class of compounds as is the NPD and does not require specific chemical derivatives as does the ECD.

Derivatization of muramic acid

Methylimidazole is an excellent catalyst for the acetylation of neutral and amino sugars without prior removal of water or borate. However, we found muramic acid (3-O-lactyl-glucosamine, Fig. 1A) did not produce a chromatographic peak in appreciable yield. Previously, we had noted that the alditol acetate of muramic acid is a lactam[3,13] and suspected that the presence of water might inhibit dehydration of the molecule to the lactam form. Muramicitol acetates in the free acid form would presumably be removed from the sample in the clean-up steps or would not pass through the GC column. Muramic acid exists naturally in the lactam form in bacterial endospores[18], and the aldononitrile acetate of muramic acid has been shown by IR spectrometry to be a lactam[19]. By including an extensive drying step between the reduction and acetylation steps (30°C overnight under vacuum) and performing the acetylation in the absence of water we were able to obtain two chromatographic peaks for muramic acid, as shown in Fig. 2B and C.

Because variations in the acetylation conditions produced differing ratios of the two muramic acid derivatives, incomplete acetylation was suspected. For example, acetylation at room temperature produced predominantly the later eluting derivative (Fig. 2B) while acetylation at 115°C produced the earlier eluting derivative (Fig. 2C). The mass spectra of the muramic acid derivatives are shown in Fig. 3. The spectrum of the earlier eluting derivative shows a strong ion at m/z 445 corresponding

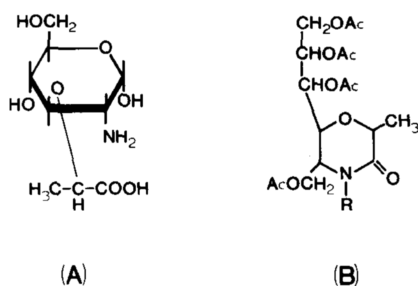

Fig. 1. The structures of (A) muramic acid and (B) the lactam forms of muramicitol acetate, where R = H for MTA and R = Ac for MPA.

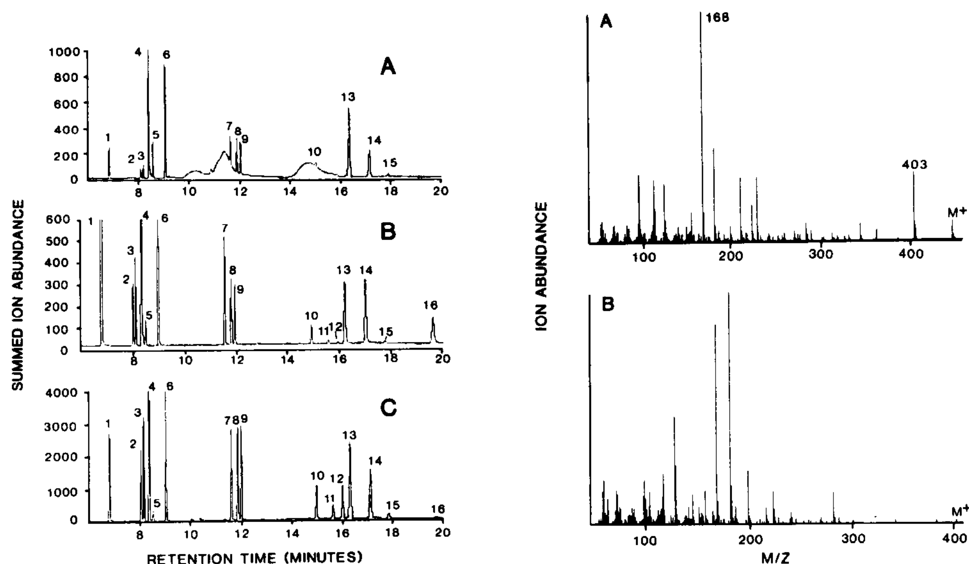

Fig. 2. Chromatograms of a mixture of sugars derivatized (A) in the presence of water, (B) in the absence of water at room temperature, and (C) in the absence of water at 115°C. Peak identification: 1 = deoxyribose, 2 = rhamnose, 3 = fucose, 4 = ribose, 5 = arabinose, 6 = xylose, 7 = mannose, 8 = galactose, 9 = glucose, 10 = L-glycero-D-mannoheptose or D-glycero-L-mannoheptose, 11 = D-glycero-D-mannoheptose, 12 = MPA, 13 = glucosamine, 14 = galactosamine, 15 = methylglucamine, 16 = MTA. The SIM parameters for chromatogram A are: m/z 115 monitored for 200 ms and m/z 159 for 200 ms from 3.0 to 15.7 min, and m/z 84 for 200 ms and m/z 86 from 15.7 to 20 min. The conditions for the other chromatograms are as noted in the Experimental section.

Fig. 3. Mass spectra of (A) MPA and (B) MTA.

to the molecular ion of the pentaacetate of the lactam form of muramicitol (Fig. 1B). The spectrum of the later eluting derivative contains a possible molecular ion at m/z 403, which corresponds to the tetraacetate in Fig. 1B. The pentaacetate has been acetylated at all possible functions whilst the tetraacetate has one unacetylated function. We suggest that this unacetylated function is the lactam nitrogen. The pentaacetate spectrum shows a very strong ion at m/z 403 (M − 42) indicating loss of ketene, but the corresponding M − 42 fragment is not apparent in the tetraacetate spectrum. If the primary source of ketene loss is acetylated hydroxyls, the lack of one O-acetyl group out of four should not totally eliminate this fragmentation pathway. On the other hand, if the ketene is lost from the single acetylated nitrogen, then lack of acetylation at this position would remove the M − 42 fragment from the spectrum. The presence of the secondary amide in the tetraacetate is to be expected because other amino sugars form secondary rather than tertiary amides[13]. A similar structure for the aldononitrile triacetate of muramic acid, based on a six membered lactam containing a secondary nitrogen, was postulated by Findlay *et al*[19].

Experiments varying the amount of methylimidazole, the acetylation time, and the acetylation temperature were performed to examine the effects of these variables on the formation of muramic acid derivatives. Although low temperatures favor MTA production, the reaction is not reproducible perhaps due to partial conversion

of MTA to MPA. The production of MPA was favored by high temperatures and/or long reaction times. Suitable reaction conditions for the reproducible formation of MPA were not found. At high temperatures or long reaction times, in the absence of water, glass distilled methylimidazole and glass distilled acetic anhydride undergo a browning reaction, a phenomenon we have previously seen with pyridine–acetic anhydride reactions[10]. This browning reaction may interfere with the reproducible production of MPA. In addition, tarry reaction products are deposited in the GC injection port, reducing the precision and accuracy of the analysis in the manner described by Grob and Bossard[20] and Grob and Neukom[21]. We conclude that the methylimidazole catalyzed alditol acetate method is unsuitable for the analysis of muramic acid.

Methylimidazole catalyzed derivatization of neutral and amino sugars

Despite the unsuitability of the methylimidazole method for the analysis of muramic acid, it may be useful for the analysis of other neutral and amino sugars. The modifications we have incorporated allow the simultaneous processing of multiple samples. The use of hydrolysis reaction tubes, a multi-sample manifold for sample evacuation, N,N-dioctylmethylamine for neutralization, a reduced pressure Vortex evaporator, disposable extraction columns with the multi-column Vac Elut system, and derivatization reaction vials and heating modules all contribute to decreasing the number of manual operations required and permitting the simultaneous analysis of multiple samples. These modifications have been described previously in greater detail[10] and can be adapted for use with the methylimidazole method.

The first step in the determination of the carbohydrate composition of a polysaccharide is the hydrolysis of the polymer to release the monomeric constituents. Sugars are notoriously unstable when heated to high temperatures in strong acid solutions. Any such degradation is undesirable because it results in erroneously low values for the sugars and may introduce additional peaks in the chromatogram. The use of sulfuric acid and the careful exclusion of air has been previously shown to minimize these effects[22]. The neutralization or removal of the acid may also cause degradation of the sugars; for example, if hydrochloric acid is employed and removed by evaporation, extraneous peaks may appear. Sulfuric acid is generally removed by neutralization with barium hydroxide; however, barium hydroxide neutralization is difficult to implement on a micro scale and sample may be lost by coprecipitation with barium sulfate. The use of N,N-dioctylmethylamine to neutralize sulfuric acid eliminates these problems[23]. Following the neutralization step, disposable hydrophobic columns are employed to remove lipids released by the hydrolysis as well as residual N,N-dioctylmethylamine. The internal standards, arabinose and methylglucamine, were chosen because they are not often present in bacterial cell walls and were added after the hydrolysis step and carried through the remaining steps[10]. The hydrolysis and hydrophobic column clean-up steps result in the sample containing an excess of water. Evaporation of water allows the entire sample to be derivatized, which is preferable for increased sensitivity in comparison to the derivatization of a small aliquot of the sample as suggested by others[15].

The next step in the chemical derivatization is a sodium borohydride reduction of the aldoses to alditols and subsequent acetylation. In the past, most workers have added sodium borohydride in aqueous solution[1,2,4,10]. Blakeney *et al.*[15] suggested

the addition of sodium borohydride in a dimethylsulfoxide (DMSO) solution, which is considerably more stable than an aqueous solution and facilitates storage and handling. More simply, sodium borohydride can be dissolved in methylimidazole to form a stable solution, eliminating the additional DMSO solvent from the reaction mixture.

It is possible to perform the acetylation reaction without removal of borate salts or water when methylimidazole is used as the catalyst. This results in the elimination of the multiple evaporation steps generally employed by other workers to remove borate[15].

Previous work[10,11] employed post-derivatization removal of residual hydrophilic components with chloroform–water extraction and hydrophilic columns. The present method removes not only salts, but also organic acids and bases initially present in the samples or resulting from the derivatization. Free acetic acid added to decompose the borohydride prior to acetylation and also generated from the acetylating reagent is removed along with other acidic contaminants by chloroform–base extractions and hydrophilic columns. The methylimidazole, added as a catalyst, and basic contaminants from the sample are removed by chloroform–acid extraction. Without these steps large quantities of methylimidazole and other contaminants may be present in the final solution for GC analysis.

Despite the use of clean-up steps, some extraneous peaks still appear in chromatograms. Some of the contaminant peaks elute early, primarily before the first carbohydrate peak, and appear to be derived from methylimidazole and its reaction products. Later eluting, very broad peaks appear only in samples acetylated at low temperature. These broad peaks do not appear in blank runs, and increase in size as the amount of sugar in the sample increases. These contaminants appear in the trace of ion 115, which is a characteristic fragment of sugar acetates and probably represent incomplete acetylation products of alditols. The presence of free hydroxyl groups could account for the broad peak profiles. Although these peaks clutter the chromatogram, they do not significantly interfere with the analysis.

The methylimidazole method as described in this report, with acetylation at 37°C in the presence of water, shows reduced sensitivity for rhamnose, fucose, and the heptoses (Fig. 2A). We have explored a large number of variations in reaction conditions, including reagent amounts, reaction temperature, reaction time, and presence or absence of water. For example, acetylating the sample for 4 h at 37°C in the absence of water increased the yields only slightly, although the broad background peaks were eliminated (Fig. 2B). The relative yields of rhamnose, fucose, and the heptoses were increased dramatically by increasing the acetylation temperature to 115°C in the absence of water (Fig. 2C). Unfortunately, these reaction conditions caused the methylimidazole–acetic anhydride browning reaction to create a significant amount of tarry product which was impossible to remove from the sample and which was deposited in the GC injection port and column. The use of extreme reaction conditions did not improve the analytical results sufficiently to compensate for this problem. We therefore recommend the acetylation be performed at 37°C for 45 min in the presence of water.

Linearity of methylimidazole method

In order to demonstrate the linearity of the methylimidazole method, a set of

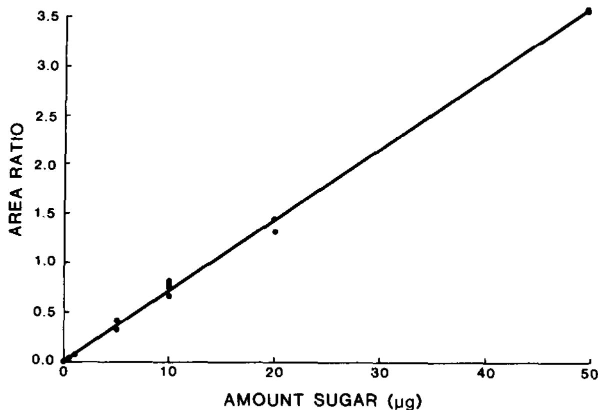

Fig. 4. Standard calibration plot for ribose. Peak area ratio of ribose to arabinose is plotted against amount of ribose in standard. The coefficient of determination is 0.999 and the estimated intercept is not significantly different from zero at the 95% level of confidence.

sugar standards, varying in amount from 0.5 to 50 μg, were derivatized in duplicate along with analytical blanks. The chromatographic peak area of each sugar was divided by the area of the internal standard, and plotted against the amount of sugar. Figs. 4 and 5 show examples of the calibration curves for neutral and amino sugars (ribose and glucosamine respectively). The curves were linear over the indicated range. The relative standard deviations for the nine sugars studied, based on four replicates at the 10-μg level, varied from 5 to 11%. As an example of the utility of this method, a sample of lipopolysaccharide (LPS) from *E. coli* 0127:B8 was analyzed. The composition was determined to be (by dry weight) 4.7% fucose, 1.5% ribose, 7.5% galactose, 2.2% glucose, 2.0% glucosamine, and 14% galactosamine. Although L-glycero-D-mannoheptose was found by the sodium acetate catalyzed procedure to be present in this sample in small quantity, it was below the limit of detection of this

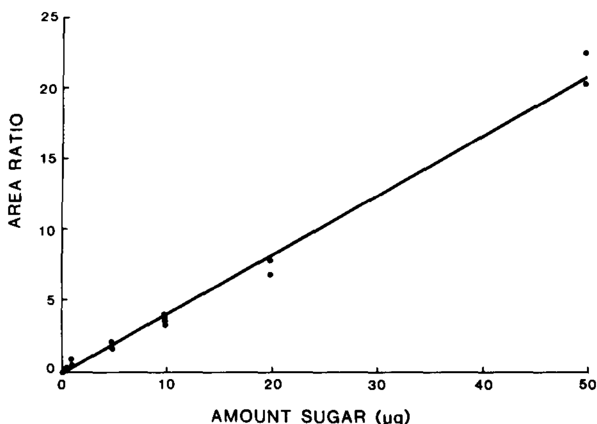

Fig. 5. Standard calibration plot for glucosamine. Peak area ratio of glucosamine to methylglucamine is plotted against amount of glucosamine in standard. The coefficient of determination is 0.991 and the estimated intercept is not significantly different from zero at the 95% level of confidence.

methylimidazole catalyzed method. The qualitative composition of *E. coli* 0127:B8 LPS has been previously described and agrees with the above results[24].

Trace analysis of chemical markers for bacteria in mammalian tissues

The sodium acetate catalyzed alditol acetate method[10,11], although more tedious to perform than the methylimidazole method, has considerably lower background and produces good yields of all neutral and amino sugars, including muramic acid. This is due to the use of extreme acetylation conditions which are possible because the catalyst, sodium acetate, does not react with acetic anhydride, the acetylating reagent. The completeness of acetylation is shown by the reproducible conversion of muramic acid into MPA. For trace analysis of carbohydrates in complex biological samples, it is essential that side reactions from the acetylating reagents are minimized, and good yields of the sugar of interest are obtained[3,13].

Previously, we have detected muramic acid in tissue samples using a complex alditol acetate procedure involving multiple sample preparation steps including thin-layer chromatography[3]. To illustrate the utility of the sodium acetate catalyzed al-

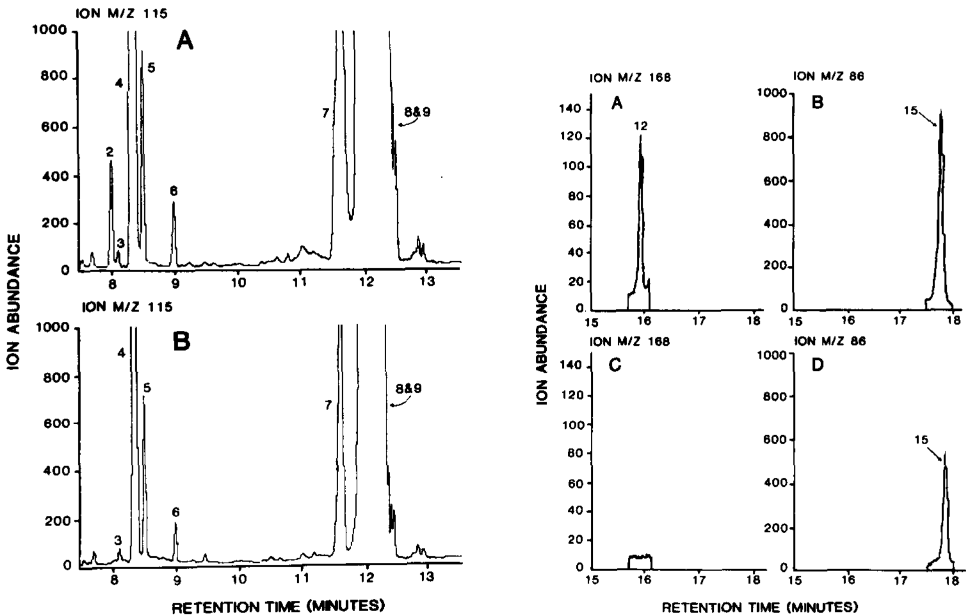

Fig. 6. Early portions of gas chromatograms of hydrolysates of 50 mg (wet weight) of liver samples four days post injection from (A) a rat intraperitoneally injected with streptococcal cell walls and (B) a control rat injected with phosphate buffered saline. Chromatograms clearly show rhamnose present in cell wall injected animals and absent in controls. In order to enhance sensitivity, ion m/z 115 was the only ion monitored during this portion of the chromatogram. For peak identification see Fig. 2.

Fig. 7. Late portions of chromatograms shown in Fig. 6 of hydrolysates of 50 mg of liver samples four days post injection from (A and B) a rat intraperitoneally injected with streptococcal cell walls and (B and C) a control rat injected with phosphate buffered saline. Chromatograms clearly show muramic acid present in cell wall injected animals and absent in controls. Ion m/z 168 (muramic acid) was monitored from 15.7 to 16.1 min (A and C) and ion m/z 86 (methylglucamine, the internal standard) was monitored from 17.5 to 18 min (B and D). For peak identification see Fig. 2.

ditol acetate method, analyses were performed on tissue samples from animals in which uveitis and polyarthritis had been elicited by the systemic administration of streptococcal cell wall fragments[3,25,26]. Control animals were injected with phosphate buffered saline alone. The animals were sacrificed four days post injection and samples of liver from experimental and control rats were analyzed for their carbohydrate composition. Amounts of 50 mg wet weight samples were hydrolyzed, derivatized, and analyzed by GC–MS. In order to increase sensitivity, only a single ion was monitored at each stage of the chromatographic analysis, m/z 115 (for neutral sugars) from 7.0 to 15.7 min, m/z 168 (for muramic acid) from 15.7 to 16.1 min, and m/z 86 (for methylglucamine, the internal standard) from 17.5 to 18.0 min. In the early portion of the chromatograms of normal livers, fucose, ribose, xylose, mannose, galactose, and glucose were all observed (Fig. 6B). Rhamnose was not detected. The chromatograms of livers from cell wall injected animals showed very similar profiles with the addition of an obvious rhamnose peak (Fig. 6A), corresponding to approximately 175 ng/mg wet weight of sample. The later portion of the chromatograms of normal animals showed only methylglucamine, the internal standard (Fig. 7C and D). Monitoring of an additional ion, m/z 84, allowed the detection of glucosamine and galactosamine (data not shown). Muramic acid was easily discernable in the chromatograms from livers of cell wall injected animals (Fig. 7A and B) at a level of *ca.* 36 ng/mg. These analyses were greatly facilitated by the use of the post-derivatization base and acid extractions described above which removed a large portion of the background generated by the liver samples and were simple to perform. Further trace analyses for bacterial components in mammalian tissues may provide new information on the fate of bacterial debris during the infectious process.

CONCLUSION

Muramic acid can produce two alditol acetate derivatives, both lactams, in the absence of water. The ratio of the amounts of these two derivatives, MTA and MPA, depends on the acetylation conditions. The more completely acetylated product, MPA, can be formed almost exclusively if vigorous acetylation conditions are employed.

We have compared two alternative procedures for producing volatile alditol acetate derivatives. The methylimidazole method is simple and rapid to perform; the sodium acetate method, while more tedious, has greater sensitivity and reduced background noise. For analyses where carbohydrates are present in high abundance and the sample matrix is of limited complexity, the methylimidazole method may be preferred for its speed and simplicity. For analyses where carbohydrates are present in low abundance or in complex matrices, the sodium acetate method is preferred for its greater sensitivity and reduced background. The sodium acetate method is also preferred for samples containing muramic acid.

The use of fused-silica capillary columns and GC–MS with SIM in this work has facilitated the selective and sensitive analysis of mixtures of neutral and amino sugars, some of which can be employed as chemical markers for bacteria. Further research in related areas might be profitably focused on simplifying sample handling, decreasing the time required for analysis, finding improved catalysts for acetylation, and further increasing the sensitivity and selectivity of the analytical method.

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