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Short communication

Determination of muramic acid in organic dust by gas chromatography–mass spectrometry

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Abstract

A method is described for the quantitation of muramic acid, a marker of bacterial peptidoglycan, in organic dust. House dust samples were hydrolysed in hydrochloric acid and then extracted with hexane to remove hydrophobic compounds. The aqueous phase was evaporated, heated in a silylation reagent to form trimethylsilyl derivatives, and analysed by gas chromatography–mass spectrometry. The muramic acid derivative gave two peaks upon injection into the gas chromatograph–mass spectrometer. Injection of 10 pg of the derivative gave a signal-to-noise ratio of 17 for the dominating peak when using selected ion monitoring in the electron impact mode, and a linear calibration curve was achieved upon analysis of samples containing 5–1500 ng of muramic acid. In a house dust sample, 40 ng of muramic acid was found per mg of dust; the coefficient of variation was 8.2% ($n = 6$, 1.2 mg of dust analysed). The described method is rapid and simple to apply, and should therefore become widely used for measuring peptidoglycan in many types of environmental samples, including organic dust.

1. Introduction

Inhalation of air-borne microorganisms is suspected to have a wide variety of clinical effects, but little is known about the microbial structures responsible for the development of the individual symptoms. One major reason for this lack of knowledge is that the methods used to measure the air-borne microorganisms have, in many instances, been insufficient. For example, culture-based methods detect only viable organisms, and microscopy methods have limited specificity. Biological assays based on the *Limulus* amoebocyte lysate test respond to almost all Gram-negative bacterial endotoxins (lipopolysaccharides) and also to some fungi [i.e. those containing (1-3)-β-glucan], but the specificity of these assays has been questioned [1–3]. Alternatively, determination of 3-hydroxy fatty acids and ergosterol by gas chromatography–mass spectrometry (GC–MS) can be applied for the characterization and quantification of endotoxins [3–6] and fungal biomass [7,8] in organic dust; these analytes may serve as specific chemical markers of larger, bioactive microbial structures [9].

Non-viable Gram-positive bacteria are not usually considered to be a health hazard upon inhalation, although peptidoglycan (PG) —the

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major structure of eubacterial cell envelopes—exhibits a toxicity panorama which is similar to that of endotoxin [10], and the levels of PG are usually much higher in Gram-positive than in Gram-negative bacteria [11]. Muramic acid (MuAc), an amino sugar, has been suggested for use as a chemical marker for GC–MS determination of PG in organic dust. Two derivatives of MuAc have been applied for this purpose: (1) the alditol acetate derivative, and (2) the trifluoroacetyl derivative. The former derivative is chemically stable and produces a single chromatographic peak for each sugar analyzed but is time-consuming and relatively complicated to prepare [11,12]; the latter derivative, on the other hand, is easily prepared and exhibits high detection sensitivity, but can lead to rapid deterioration of the GC column and requires access to negative ion-chemical ionization (NICI) GC–MS [9,13]. The present report describes the development of an alternative GC–MS method, based on the electron impact (EI) mode, for use in determining muramic acid in organic dust. The method is both convenient and rapid and allows detection in the low picogram range.

2. Experimental

2.1. Chemicals

Hydrochloric acid (HCl, 37%) and bis-(trimethylsilyl)trifluoroacetamide (BSTFA, 98%) were purchased from Janssen Chimica (Geel, Belgium), hexane (99%) from Labscan (Dublin, Ireland), muramic acid (MuAc) from Sigma (St. Louis, MO, USA), 3-hydroxytridecanoic acid (3-OH 13:0) from Larodan Lipids AB (Malmö, Sweden), and pyridine (p.a.) from Merck (Darmstadt, Germany). All chemicals were used without further purification.

2.2. Bacteria and dust samples

The studied bacteria included *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*, all isolated from clinical specimens and identified according to routine diagnostic methods. The bacteria were cultivated on agar plates overnight at 37°C, washed with saline, and freeze-dried before GC–MS analysis. Dust samples collected from a vacuum-cleaner used in a private home were used as models in the experiments.

2.3. Preparation of samples for GC–MS analysis

Samples of bacterial cells (typically 0.5–1 mg) or house dust (typically 2–5 mg) were heated overnight at 100°C in 1.5 ml 4 *M* aqueous HCl in 10-ml test tubes equipped with Teflon-lined screw caps. The hydrolysates were then extracted with 1 ml of hexane and centrifuged, and the phases were subsequently separated. The upper phase was either discarded or saved for analysis of 3-hydroxylated fatty acids as described elsewhere [4]. A 1.2-ml aliquot of the lower (aqueous) phase was transferred to a separate test tube and evaporated to dryness under a stream of nitrogen at 60°C, and then the internal standard (50 ng of 3-OH 13:0 acid methyl ester) was added. Samples were again evaporated and dried under vacuum in a desiccator for 30 min. Trimethylsilyl (TMS) derivatization was accomplished by adding 50 μl of BSTFA and 10 μl of pyridine to the test tube and heating at 80°C for 30 min. After reaction, 100 μl of hexane was added and the sample was mixed and then transferred to a conical vial. Thereafter, the sample was washed with 20 μl of water (pH 7.5), using a Pasteur pipette, and then centrifuged (2 min, 2500 *g*). The hexane layer was transferred to another conical vial containing 5 μl of pyridine and 20 μl of BSTFA, and again heated at 80°C for 15 min. Finally, the samples (1-μl aliquots) were analyzed by GC–MS, either immediately or after storage at 4°C.

2.4. GC–MS

A VG Trio-1S GC–MS system (Manchester, UK) was used. The gas chromatograph was a Hewlett-Packard Model 5890 (Avondale, PA, USA) equipped with a fused-silica capillary column (25 m $\times$ 0.25 mm I.D) containing cross-linked DB-5 (film thickness 0.25 μm) as the

stationary phase. Injections were made using a Hewlett-Packard Model 7673 autosampler in the splitless mode. Helium was used as the carrier gas, at a constant inlet pressure of 7 kPa, and the temperature of the column was programmed from 120 to 260°C at a rate of 20°C/min. The injector and the interface (between GC and MS) were kept at 260°C. TMS derivatives were analysed in the EI mode using an ion source temperature of 220°C; ionization was performed at 70 eV.

2.5. Conditions for derivatization and hydrolysis

TMS derivatization conditions were evaluated by heating 1-μg aliquots of free MuAc standard and 50 ng of 3-OH 13:0 methyl ester (internal standard) in 50 μl of BSTFA and 10 μl of pyridine for 15, 30, and 120 min at 80°C, and for 15 min at 60, 80 and 100°C (samples in duplicate). In two additional samples, silylation was allowed to occur overnight at room temperature. After reaction, 50 μl of hexane was added to each sample; the preparations were then directly subjected to GC–MS analysis. The ratios of the areas of peaks representing TMS-derivatized MuAc (m/z 185) to the areas of the peaks of the internal standard (m/z 301) were calculated.

Separate amounts of dried *E. coli* cells (1-mg aliquots, duplicate samples) were heated in 1 ml of 4 *M* HCl at 100°C for 1, 2, 5 and 18 h, respectively, to study the release of MuAc from bacterial peptidoglycan during hydrolysis. The hydrolysates were extracted with hexane and a portion of the aqueous phase was transferred to a separate test tube, evaporated, supplemented with the internal standard (50 ng of 3-OH 13:0 methyl ester), derivatized, and analyzed by GC–MS as described above.

2.6. Quantification, detection sensitivity, and reproducibility

To construct a calibration curve, portions of free MuAc standard, i.e. 5, 50, 100, 500, 1000 and 1500 ng, were placed in separate test tubes (duplicate samples). The samples were heated in 4 *M* HCl and then subjected to the same preparation procedure as the bacterial and dust samples (see above). The curve was constructed by plotting the ratios of the areas of peak m/z 185 (representing TMS-derivatized MuAc) to the areas of m/z 301 (internal standard) against the amounts of MuAc added.

The lowest detectable amount of the MuAc–TMS derivative injected into the GC–MS was determined after direct silylation of MuAc standard followed by serial dilutions.

The reproducibility was evaluated by hydrolysing a dust sample (12 mg) with 1.5 ml of 4 *M* HCl overnight. After centrifugation, six 150-μl aliquots of the hydrolysate, each corresponding to 1.2 mg of dust, were distributed in 6 test tubes; then 1 ml of water and 1 ml of hexane were added to each tube. After shaking, the hexane layer was separated and discarded. The lower aqueous layer was evaporated to dryness under a stream of nitrogen, thereafter the internal standard (50 ng of 3-OH 13:0 methyl ester) was added, and the samples were dried, TMS-derivatized, and analysed for MuAc as described above for bacterial and dust samples.

3. Results

3.1. Analysis of muramic acid standard

The MuAc–TMS derivative appeared as two chromatographically well-separated peaks. Both peaks gave mass spectra that were characterized by a strong ion of m/z 185 ($M-426$) that probably originated from the cleavage of two $O(CH_3)_3Si$ groups, one $CH_3CHCOO(CH_3)_3Si$ group and one $CH_2O(CH_3)_3Si$ group. Predominant ions in the low mass region were found at m/z 73 (cleavage of an $(CH_3)_3Si$ group) and at m/z 147 (cleavage of two vicinal TMS groups), and in the high mass region at m/z 506 [probably due to loss of an $O(CH_3)_3Si$ group and an oxygen from the $CH_3CHCOO(CH_3)_3$ Si group] (Fig. 1).

The calibration curve was linear within the studied range (5–1500 ng of MuAc in samples prepared for analysis) and followed the equation $y = 0.05 + 0.004x$, $R = 0.995$ (x = nanograms of

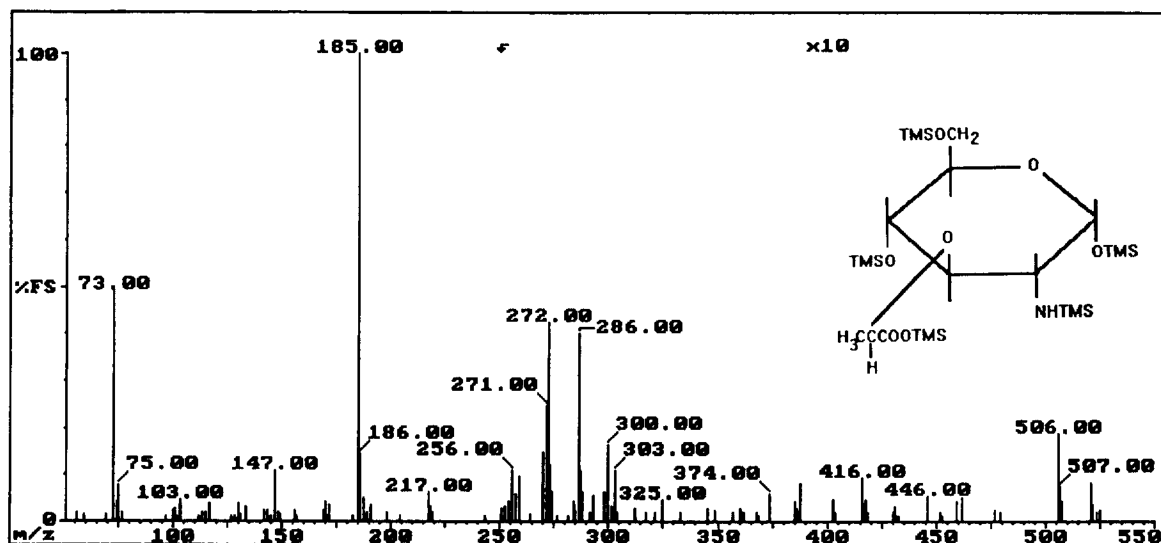

Fig 1. Mass spectrum of the trimethylsilyl derivative of muramic acid.

muramic acid; y = area ratios of ions 185/301) (non-weighted linear regression). An injection of 10 pg of MuAc derivative gave a signal-to-noise ratio of 17 for the dominating peak upon selected ion monitoring (SIM, m/z 185) (Fig. 2), and analysis of 5 ng of the MuAc standard after the entire sample preparation procedure gave a signal-to-noise ratio of 40.

Silylation of the free MuAc standard at 80°C for 15, 30 or 120 min or at 100°C for 15 min gave virtually identical yields of the TMS derivative (the area ratios of m/z 185 to m/z 301 was 4.4 in all cases). Overnight reaction at room temperature gave the lowest yield (area ratio 0.9), whereas 15 min silylation at 60°C gave an area ratio of 1.8. No degradation of the MuAc–TMS derivative was observed after storage of final preparations at 4°C for at least 20 days in the silylation reagent (data not shown).

3.2. Bacterial and dust samples

An amount of 40 ng of MuAc per mg of dust was found in a studied house-dust sample. The coefficient of variation was 8.2% (1.2 mg of dust analyzed, $n = 6$, Fig. 2).

Hydrolysis of bacterial cells in 4 M HCl for 1 h at 100°C was found to be adequate for releasing muramic acid; longer hydrolysis time did not improve the yield (data not shown). The amounts of MuAc detected per mg (dry weight) of *E. coli*, *Ps. aeruginosa*, and *P. mirabilis* were 6.3, 1.4, and 9.5 μg, respectively. A result from the analysis of *P. mirabilis* is shown as an example (Fig. 2). A new GC column of the same type used to analyse the MuAc standard and the dust samples was installed prior to the analysis of the bacterial cultures, which explains the differences in retention times.

4. Discussion

Inhalation of air-borne microorganisms has been shown to cause various clinical symptoms, and a connection with the development of asthma and allergy is suspected. Clearly, specific and sensitive measurement methods are required for firm correlation of the different microbial structures inhaled to individual symptoms. GC–MS analysis of organic dust allows accurate determination of specific microbial monomeric constituents, so-called chemical markers, thereby making it possible to characterize the microbial composition in a studied sample [9].

The two peaks of the TMS derivative of MuAc

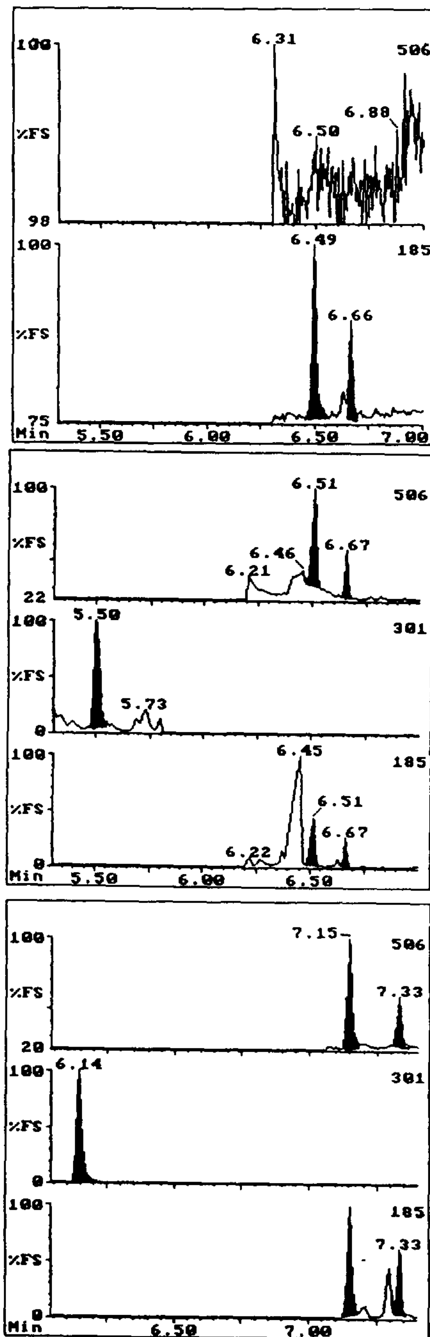

Fig. 2. Selected ion monitoring detection of the trimethylsilyl derivative of 10 pg of muramic acid standard (upper), muramic acid in 12 μg of house dust (center), and muramic acid in 200 ng of *Proteus mirabilis* (lower) (injected amounts). The muramic acid derivative was monitored at m/z 185 and 506; the 3-hydroxytridecanoic acid derivative was monitored at m/z 301.

probably represent different anomers formed due to the asymmetric centre at C_1, in analogy with α- and β-forms of glucopyranose produced when glucose is dissolved in water [14]. The ion m/z 185 is likely to constitute a very stable entity due to the formation of a strong hydrogen bond between the hydrogen in the amino group (linked to C_2) and the oxygen (linked to C_3); this bond may also prevent the second hydrogen in the amino group from being substituted by a TMS group. Preparations were extracted in hexane–water after TMS derivatization in order to remove traces of HCl that might bind strongly to the amino group in, for example, amino sugars and amino acids present in the samples, or to the added pyridine; the hexane phase was transferred to a new test tube and re-heated in the silylation mixture. This purification step was carried out to reduce the risk of introducing HCl, formed by decomposition of ammonium chloride salts in the heated injector, into the GC–MS system.

The method described here offers nearly the same high level of detection sensitivity for MuAc as for the trifluoroacetyl derivative analysed by NICI-GC–MS [13]. Heating samples for 1 h appeared to quantitatively release MuAc from PG, and TMS derivatization was complete after 15 min. Thus, the described method is considerably more rapid than previous methods used for the determination of muramic acid in organic dust [11,12]. The long hydrolysis time (18 h) and the use of 3-OH 13:0 as an internal standard were employed to demonstrate the possibility of combined determination of MuAc and 3-hydroxy fatty acids (markers of endotoxin) in the same sample. After hydrolysis, the hexane phase (containing 3-hydroxy fatty acids) and the aqueous phase (containing muramic acid) can be subjected to GC–MS analysis, in both cases using 3-OH 13:0 as an internal standard. Initially, we used methylglucamine as an internal standard as described by others [12], but found later that the TMS derivative of the compound was unstable (data not shown).

Our results show that MuAc in organic dust can be determined by GC–MS just as simply as 3-hydroxy fatty acids [4] and ergosterol [7]. The

fact that conventional EI-GC–MS is applicable for all these markers means that less complicated and expensive instrumentation can be applied. These methods should therefore be useful in the characterization of the microbiological composition of environmental samples, including airborne organic dust.

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