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Analysis of N^{α}-methylhistamine by gas chromatography–mass spectrometry

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Abstract

A gas chromatography–electron capture mass spectrometry assay has been developed for the histamine H_3 receptor agonist, N^{α}-methylhistamine (N^{α}-MH). The assay is linear from 50 pg–10 ng, with a limit of detection of 50 pg/ml for gastric juice and plasma, and 50 pg/sample for bacteria (10^7–10^8 CFU) and gastric tissue (5–10 mg wet weight). The limits of quantification are 100 pg/ml for gastric juice (%RSD=1.4) and plasma (%RSD=9.4), and 100 pg/sample for bacteria (%RSD=3.9) and tissue (%RSD=5.8). N^{α}-MH was not present in human plasma, but low levels (1.4 ng/ml and 0.4 ng/ml) were detected in two samples of human gastric juice obtained from patients infected with *Helicobacter pylori*.
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1. Introduction

Gastric infection with the Gram negative microaerophile, *Helicobacter pylori,* predisposes to duodenal ulcers and gastric cancer [1]. Infection with the bacterium is most common in less developed countries and in lower socioeconomic groups [2,3]. The mechanisms by which *H. pylori* can cause gastrointestinal disease are of considerable interest. In particular, infection is associated with an alteration of gastric acid production. Recent work has indicated that N^{α}-methylhistamine (N^{α}-MH) may be involved in the pathogenesis of helicobacter-associated disease. N^{α}-MH is a histamine H_3-receptor agonist [4,5] and, as H_3-receptors have been shown to be present in human gastric mucosa, it was suggested by Courillon-Mallet et al. that N^{α}-MH has the potential to alter the normal regulation of acid secretion in the stomach and cause gastrointestinal disease [6]. This group, using a radioenzymatic assay, have shown that N^{α}-MH is present in gastric mucosal biopsies from helicobacter-infected patients, and given evidence that it is produced directly by *H. pylori* [6].

As part of a wider study to investigate the factors involved in the physiological control of gastric acid secretion and plasma gastrin in human subjects colonised by *Helicobacter pylori*, it was necessary to

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determine whether N^{α}-MH could be generated in these subjects in vivo, and if it were present in gastric secretions at sufficient concentration to exert a physiological effect on histamine receptors. The only method currently available to measure N^{α}-MH is a modification of a radioenzymic assay for histamine [6], and there is some doubt as to the specificity of this assay. Capillary column gas chromatography–electron capture mass spectrometry (GC–ECMS) offers a sensitive and specific method to measure a wide range of analytes in biological matrices. The technique is superior to classical mass spectrometric methods such as electron impact or positive ion chemical ionisation in that ionisation is more efficient (particularly when halogenated derivatising groups are incorporated) and fragmentation is normally minimal, with the majority of the ion current being carried by the molecular ion or a simple fragment. This results in picogramme sensitivity. The aim of this work was to develop a GC–ECMS assay capable of measuring N^{α}-MH in gastric juice, gastric tissue, plasma and bacterial cultures.

2. Experimental

2.1. Materials

N^{α}-Methylhistamine dihydrochloride was purchased from Calbiochem-Novabiochem Ltd (Nottingham, UK) and $[^{2}H_{4}]$histamine dihydrochloride was supplied by MSD Isotopes (Montreal, Canada). $[^{2}H_{3}]$Iodomethane (deuteromethyliodide), 3,5-*bis*-trifluoromethylbenzyl bromide, 3,5-*bis*trifluoromethylbenzoyl chloride, trifluoroacetic acid, acetonitrile (ACS grade), diisopropylethylamine and decane were obtained from Sigma-Aldrich Ltd (Poole, UK). Other organic solvents and hydrochloric acid were of 'Analar' grade. Diisopropylethylamine was passed through a column of basic alumina, and ethyl acetate was redistilled before use. 'Isolute' CBA solid-phase extraction columns were supplied by Jones Chromatography (Hengoed, UK). Water was of MilliQ grade. Samples of human gastric juice were kindly provided by Dr Julia Newton, School of Clinical Sciences, Medical School, Newcastle upon Tyne. Gastric tissue was obtained from rabbits.

2.2. Synthesis of N^{α}-trideuteromethyl-$\alpha,\alpha,\beta,\beta$-tetradeuterohistamine ($[^{2}H_{7}]N^{\alpha}$-MH)

$\alpha,\alpha,\beta,\beta$-Tetradeuterohistamine dihydrochloride in water (32 μmol in 240 μl) was treated with 128 μl sodium hydroxide solution (0.5 *M*). An aliquot of the aqueous solution (50 μl) containing the free base (4.35 μmol) was placed in a 1 ml glass ampoule together with ethanol (200 μl) and deuteromethyliodide (50 μl). The vial was immersed in an acetone/solid carbon dioxide bath until its contents were frozen, then flushed with nitrogen and flame-sealed. The vial was heated at 60°C for 30 min. The vial contents were evaporated under nitrogen and the residue dissolved in methanol (5.75 ml) to give a solution with a nominal concentration of 100 μg $[^{2}H_{7}]N^{\alpha}$-MH/ml. Samples of this stock solution were derivatised as described below and analysed by gas chromatography–electron capture mass spectrometry. By comparing the response of the di-3,5-*bis*trifluoromethylbenzyl derivative of $[^{2}H_{7}]N^{\alpha}$-MH with that obtained from an equivalent amount of N^{α}-MH, it was estimated that a yield of approximately 15% had been obtained for the deuteromethylation reaction. A serial dilution of the stock solution was made with methanol and standard solutions of $[^{2}H_{7}]N^{\alpha}$-MH were stored at −20°C until required.

2.3. Sample preparation

Four isolates of *H. pylori* from our laboratory were grown at 37°C on Columbia agar containing 7% horse blood (TCLS, Buckingham, UK) and 20 mg/l nalidixic acid, 2 mg/l amphotericin B, and 3 mg/l vancomycin. The plates were incubated for 48 h in a microaerophilic environment by using gas packs (Campypak, BBL, Maryland, USA). Bacteria were washed three times in phosphate buffered saline, and $\sim 10^{8}$ CFU resuspended in phosphate buffer (0.1 *M*, pH 7.4, 1 ml) in a 1.5 ml Eppendorf tube to which had been added 15 ng of $[^{2}H_{7}]N^{\alpha}$-MH. The suspension was acidified with concentrated hydrochloric acid (100 μl) to pH 1. The suspension was disrupted with an ultrasonic probe (3×30 s) at mid power under ice cooling. After centrifugation (Eppendorf centrifuge 5412, 15 min, 10 000 *g*), the clear supernatant was transferred to a screw-capped

glass vial (4 ml capacity) and washed with hexane (600 μl).

Samples of gastric tissue from four rabbits were collected and were subdivided into separate 10 mg aliquots. These were homogenised in 0.1 *M* HCl (3×100 μl) in a glass homogeniser containing 15 ng of $[^2H_7]N^{\alpha}$-MH. The homogenate was sonicated as outlined above and washed with hexane.

Gastric juice which had been stored at −20°C was allowed to thaw at room temperature. Samples were then centrifuged (15 min, 800 *g*) to remove any particulate matter that was present. An aliquot of gastric juice (1 ml) was placed in a glass vial containing 15 ng of $[^2H_7]N^{\alpha}$-MH, and acidified with hydrochloric acid (1 *M*, 100 μl). The vial contents were mixed and, after the pH had been checked to be ~1, washed with hexane (600 μl).

Blood was obtained by venapuncture and collected into heparinised tubes. Plasma was prepared by centrifugation (15 min, 800 *g*), and an aliquot (1 ml) was mixed in a glass tube with 100 μl of $[^2H_7]N^{\alpha}$-MH stock solution (15 ng). The tube was cooled on ice for 15 min and ice-cold methanol (4 ml) was added. The tube contents were vortex mixed and the glass tube was left to stand a further 5 min on ice. The mixture was then centrifuged (15 min, 800 *g*) and the clear supernatant transferred to a clean glass tube. The supernatant was evaporated to dryness under nitrogen and the residue extracted with hydrochloric acid (0.1 *M*, 2×500 μl). The combined acid extract was then washed with hexane (600 μl).

2.4. Solid phase extraction

The aqueous acidified solutions were taken to dryness overnight under vacuum at room temperature. Each residue was extracted with methanol (2×750 μl) and the methanol extract was evaporated to dryness under nitrogen. The extract was redissolved in 1.05 ml of dilute ammonia (~1 m*M*, adjusted by dilution to pH 8)–methanol (20:1, v/v) and the solution was applied to an ion-exchange Isolute CBA column (200 mg sorbent mass, 3 ml reservoir volume) which had been preconditioned with methanol (2×2 ml), phosphate buffer (10 m*M*, pH 7.4, 2 ml) and water (2 ml). The column was washed with pH 8 aqueous ammonia (2×2 ml), after which N^{α}-MH was eluted with 1% trifluoroacetic acid in water (2 ml). The eluate was taken to dryness overnight under vacuum and the residue was transferred to a glass vial (2 ml capacity) with methanol (2×750 μl). This solution was stored in methanol at −20°C.

2.5. Derivatisation procedure

Methanol present in sample extracts and standards was removed by evaporation under nitrogen and a 5% solution of 3,5-*bis*trifluoromethylbenzyl bromide in acetonitrile (80 μl) and diisopropylethylamine (20 μl) was added. The reaction mixture was left at room temperature overnight and then excess reagents removed under nitrogen. The residue was dissolved in hydrochloric acid (0.1 *M*, 200 μl) and washed twice with hexane (750 μl). Sodium carbonate solution (0.5 *M*, 200 μl) was added and the alkaline product extracted with ethyl acetate (2×750 μl).

The di-3,5-*bis*trifluoromethylbenzoyl derivative of N^{α}-MH was also prepared when the assay was under development. Samples were treated with 100 μl of 3.5-*bis*trifluoromethylbenzoyl chloride–diisopropylethylamine–ethyl acetate (1:1:100, v/v/v) for 60 min at room temperature. Excess reagents were removed under nitrogen, the derivative dissolved in 200 μl of saturated sodium bicarbonate solution and extracted into ethyl acetate (2×600 μl). For both derivatives, ethyl acetate was removed under nitrogen and the residues reconstituted in decane (20 μl). Aliquots of 2 μl were injected into the gas chromatograph–mass spectrometer.

2.6. Mass spectrometry

Gas chromatography–electron capture mass spectrometry (GC–ECMS) was carried out on a Finnigan MAT 4500 gas chromatograph quadrupole mass spectrometer (ThermoQuest Corporation, San Jose, CA, USA). The gas chromatograph was equipped with a 15 m×0.25 mm I.D. DB5 J&W fused-silica capillary column which was routed directly into the mass spectrometer ion source. Helium was used as carrier gas at a head pressure of 70 kPa. The gas chromatograph was fitted with a Grob-type capillary injector operated in the splitless mode and maintained at a temperature of 270°C. The gas chromato-

graph oven temperature was held at 150°C for 1 min, then raised to 270°C at 20°C min^{-1}. The mass spectrometer was operated in the electron capture negative ion mode with an electron energy of 100 eV. Ammonia gas was admitted to an indicated ion source pressure of 0.05 kPa and the indicated ion source temperature was maintained at 150°C. The mass spectrometer was set to monitor negative ions at *m/z* of 350.3 and 357.3 and data acquisition and reduction were performed by an INCOS data system.

Electrospray MS was carried out by flow injection analysis on a VG Quattro II mass spectrometer in the positive ion mode. Samples were introduced to the source in acetonitrile–water (20:80, v/v).

3. Results

N^{α}-MH was converted almost quantitatively into the di(3,5-*bis*trifluoromethylbenzoyl) (di-tFMBO) derivative following treatment with 3,5-*bis*trifluoromethylbenzoyl chloride (Fig. 1). The derivative chromatographs on GC as a single peak with a retention time of 5.6 min, and generates an intense electron capture mass spectrum with a molecular ion ($M^{-\cdot}$) at *m/z* 605.4. There is little fragmentation, with >95% of the ion current carried by the molecular ion. The naturally occurring histamine metabolite, 1-methylhistamine (1-MH, N^{τ}-MH) produced two di-tFMBO derivatives as previously reported [7]. Both isomers generated similar mass spectra ($M^{-\cdot}$ at *m/z* 605.4), and were well separated on GC from N^{α}-MH, eluting at 4.75 min and 6.37 min. The other ring methylated isomer, 3-methylhistamine (3-MH, N^{π}-MH) produced predominantly a mono derivative eluting at 4.68 min with an intense molecular ion ($M^{-\cdot}$) at *m/z* 365.3 and little fragmentation. A small amount of a di-derivative was also formed, eluting at 7.17 min with an $M^{-\cdot}$ ion at *m/z* 605.4.

Reaction of N^{α}-MH with 3,5-*bis*trifluoromethylbenzyl (tFMB) bromide resulted in the formation of a single di-tFMB derivative, eluting from GC at 5.1 min (Fig. 1). Electron capture ionisation gave a simple mass spectrum with essentially a single ion at *m/z* 350.3 corresponding to M-$tFMB^{-}$ (Fig. 2a). The deprotonated molecular ion ($M–H^{-}$) at *m/z* 576.4 represented <1% of the base peak. 1-MH and 3-MH also generated di-tFMB derivatives, but both were chromatographically distinct from N^{α}-MH, eluting at 4.7 min and 5.2 min respectively. Their mass spectra were markedly different from N^{α}-MH, with both isomers producing molecular ion species, and exhibiting marked fragmentation. 1-Methylhistamine generated an $M–H^{-}$ ion at *m/z* 576.4 with major fragment ions at *m/z* 349.3 ($M–H–tFMB^{-}$)

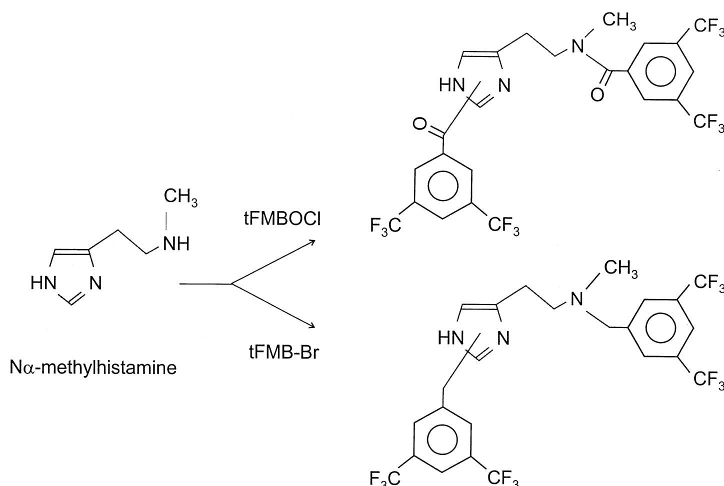

Fig. 1. N^{α}-MH is converted to di-tFMBO and di tFMB derivatives in good yield. In both cases, only a single di-derivative is formed. The positions of substitution of the fluorinated derivatives on the imidazole ring have not been determined.

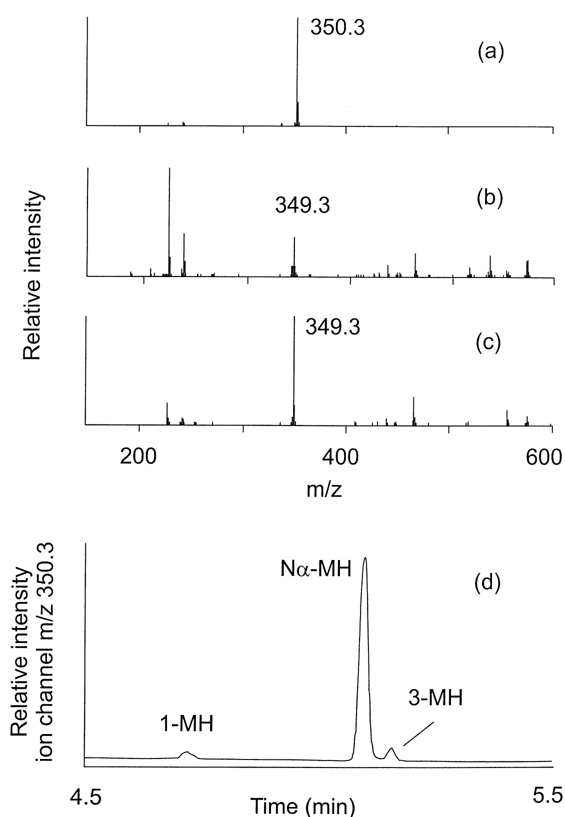

Fig. 2. The electron capture mass spectra of the di-(3,5-*bis*trifluoromethylbenzyl) derivatives of (a) N^{α}-methylhistamine, (b) 1-methylhistamine and (c) 3-methylhistamine. The m/z 350.3 selected ion profile for a mixture of the di-tFMB derivatives of N^{α}-MH (4 ng), 1-MH (80 ng) and 3-MH (80 ng) is shown in (d).

and 227.3 ($tFMB^-$), whereas 3-MH formed the molecular ion (m/z 577.4) with the base peak at m/z 349.3 ($M-H-tFMB^-$) (Fig. 2b, 2c). The response within the m/z 350.3 ion channel for N^{α}-MH was two hundred fold greater than for either of the other two isomers (Fig. 2d). Less than 5 pg of the di-tFMB and di-tFMBO derivatives of N^{α}-MH could be detected using selected ion monitoring with $S/N>10$ and both derivatives would therefore be suitable, in principle, for use in a GC–MS assay.

The internal standard, $[^2H_7]N^{\alpha}$-MH, was generated in a single step reaction by the deuteromethylation of $[^2H_4]$histamine (Fig. 3) and analysis of the reaction product by flow injection analysis positive ion electrospray mass spectrometry indicated that a mixture of unchanged histamine and mono-, di- and trideuteromethylated histamines had been obtained (with protonated molecular ions at m/z 116.1, 133.1, 150.1 and 167.2 respectively). The required product, $[^2H_7]N^{\alpha}$-MH, constituted about 15% of the reaction mixture and no further purification was undertaken. Following derivatisation, the di-tFMBO derivative of the deuterated internal standard eluted 1 s earlier than derivatised N^{α}-MH and generated an intense molecular ion at m/z 612.4. The m/z 612.4 ion chromatogram contained a number of interfering peaks which were found to arise from the diisopropylethylamine base. Elimination of the base, or replacement with triethylamine, lutidine or pyridine resulted in poor reaction yields, and so this derivative was abandoned. The di-tFMB derivative of $[^2H_7]N^{\alpha}$-MH also eluted 1 s before the corresponding derivative of N^{α}-MH, and generated an analogous mass spectrum with m/z 357.3 as base peak with less than 0.05% carryover into the m/z 350.3 ion channel (Fig. 3). No chromatographic interference from derivatising reagents was observed and this derivative was used for all further work. An unextracted standard curve was prepared with standards containing N^{α}-MH (0–10 ng) and $[^2H_7]N^{\alpha}$-MH (15 ng). Using selected ion monitoring of ions of m/z 350.3 and m/z 357.3, an eight point standard curve was obtained for the intensity ratio I_{350}/I_{357} plotted against amount of N^{α}-MH added, and this was linear with a negligible intercept ($y=0.0645x+0.001$, $r^2>0.999$). The inter assay precision based on ten standard curves was 14.4% for a 100 pg standard.

An extraction protocol was developed to handle gastric juice, plasma, gastric tissue and bacteria. The basis of the extraction was the use of a weak cation exchange resin, utilising a pH 8 loading step, where the positively charged analyte binds to the negatively charged sorbent. At pH 1, these ionic interactions are lost and N^{α}-MH is eluted from the resin. Using homogenised gastric tissue samples, recoveries through the extraction procedure were estimated initially with $[^{14}C]$-histamine at 80% ($n=2$), and later, by comparing the response of the deuterium labelled internal standard in the m/z 357.3 channel (in instrument units of ion current) for unextracted and extracted standards. The relative ion response reduced from 2.26 ± 0.14 to 1.66 ± 0.42 (mean±SD, $n=8$), corresponding to a recovery of 73%. Six samples of normal gastric juice and plasma were

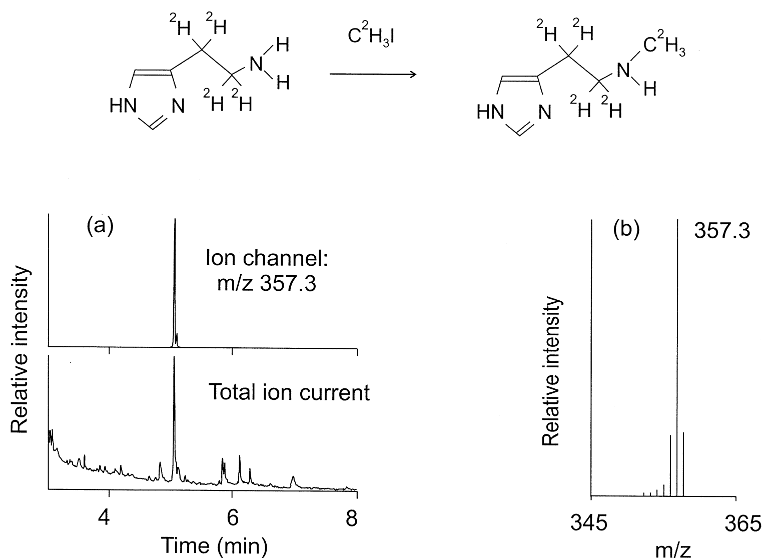

Fig. 3. $[^2H_7]N^{\alpha}$-methylhistamine was prepared by deuteromethylation of $[^2H_4]$-histamine. The di-tFMB derivative elutes at 5.1 min, and is the major species in the total ion current chromatogram. The m/z 357.3 selected ion profile is also shown (a). The base peak of the mass spectrum at m/z 357.3 is shown in (b). There is <0.05% contribution at m/z 350.3.

spiked with between 100 pg and 5 ng of N^{α}-MH and stored at −20°C for up to six months. There was no appreciable change in N^{α}-MH content on storage.

Extracted standard curves were produced from both gastric tissue homogenates and gastric juice. The y intercepts were <0.002, indicating that no endogenous N^{α}-MH was present, and there was no chromatographic interference. The curves were linear from 50 pg to 10 ng, and the slopes differed by <3% from the corresponding unextracted curve, which was then used for all future assays. The limit of quantification (LOQ) of N^{α}-MH was 100 pg for a 1 ml sample. The mean value was 100±1.4 pg (%RSD=1.4) for gastric juice and 90±8.5 pg (%RSD=9.4) for plasma ($n=6$). As the sample size of bacteria and tissue was variable (usually 10^7–10^8 CFU *H. pylori* and 5–10 mg gastric tissue, wet weight, containing 0.5–1 mg acid soluble protein), this limit was expressed in terms of pg/sample. The LOQ of N^{α}-MH in *H. pylori* and gastric tissue was also 100 pg/sample. At this level 100 pg samples gave mean values after extraction of 92±3.6 pg (%RSD=3.9) and 93±5.4pg (%RSD=5.8) respectively ($n=6$). Limits of detection of N^{α}-MH were set at 50 pg/sample (bacteria and tissue) and 50 pg/ml (plasma and gastric juice). At this level, the amine could be clearly differentiated from blank extracts with $S/N>10$.

Four strains of *H. pylori* were cultured for 48 h, and bacterial pellet extracts analysed for the presence of N^{α}-MH. There was no evidence for N^{α}-MH in any of the bacterial samples studied. In each case, the presence of the internal standard in the GC ion profile demonstrated that N^{α}-MH had not been lost during the extraction (Fig. 4a). Similarly, N^{α}-MH was below the limit of detection in three normal plasma samples. In contrast, N^{α}-MH was detected in two samples of gastric juice provided by Dr J Newton, University of Newcastle (at 1.4 ng/ml and 0.4 ng/ml) obtained from *H. pylori* positive patients (Fig. 4b) but was below the limit of detection in seven other samples. Again, the internal standard was present in each case.

4. Discussion

A GC–ECMS assay has been developed which is suitable for the analysis of N^{α}-MH in a wide variety of biological matrices ranging from plasma and gastric secretions, to tissue and bacterial homogenates. This involves a high yield extraction protocol

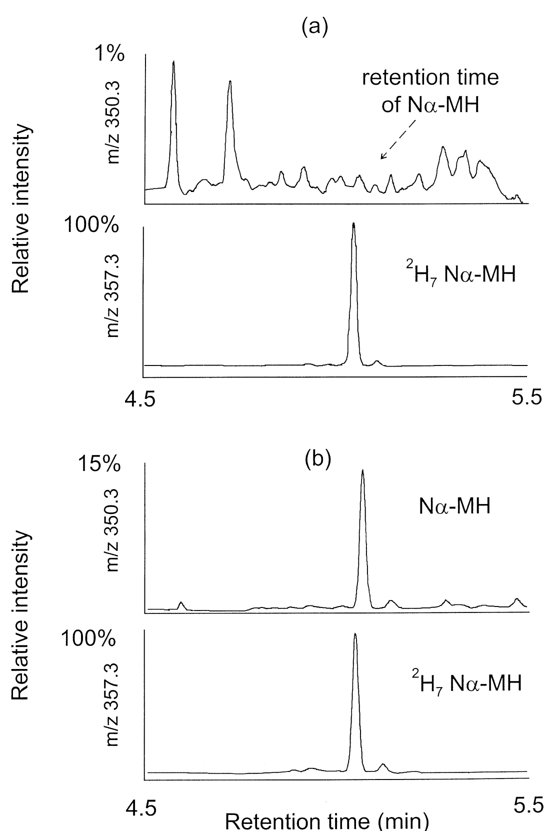

Fig. 4. Selected ion profiles for m/z 350.3 (N^{α}-MH) and m/z 357.3 (internal standard, [$^2H_7$]N^{α}-MH) of (a) an extract of *H. pylori* in which N^{α}-MH is undetectable, and (b) a sample of human gastric juice from a patient infected with *H. pylori* which is positive for N^{α}-MH.

centred on ion-exchange extraction, conversion to the di-tFMB derivative and capillary column GC–ECMS in the selected ion monitoring mode. An assay based on GC–ECMS has previously been applied to measure urinary excretion of the histamine metabolite 1-methylhistamine, using the di-tFMBO derivative [7]. In this study, although the di-tFMBO derivative of N^{α}-MH could be generated in good yield, by-products from the reaction led to interference in the selected ion channel of the internal standard, precluding its use. The related di-tFMB derivative, which has been used successfully for heterocyclic amine analysis [8], was applied to N^{α}-MH instead of the di-tFMBO derivative. This derivative possesses good chromatographic properties and, although it generates only a weak molecular ion species, the M–tFMB$^-$ fragment (m/z 350.3) carries the majority of the ion current and is suitable for monitoring N^{α}-MH. [$^2H_7$]N^{α}-MH was prepared as an internal standard. Although the reaction of histamine with methyl iodide results in only a 15% yield, purification was unnecessary as the other methylated derivatives did not interfere with the assay.

It, perhaps, seems retrograde to use GC techniques at a time when HPLC–mass spectrometric methods, particularly in the pharmaceutical industry, are the preferred mode of analysis. There are, however, occasions where GC–MS offers a number of benefits over HPLC–MS(MS), particularly for the analysis of low molecular weight, isomeric compounds. Even the requirement for derivatisation can be beneficial. In the case of N^{α}-MH, the electron capturing reagent only reacts with specific functional groups, leaving many impurities invisible to the mass spectrometer. Further, using the di-tFMB and di-tFMBO derivatives, N^{α}-MH can be readily differentiated on GC–ECMS from the other naturally occurring methylhistamines.

Both unextracted and extracted standard curves were prepared during the development of the assay, as comparison of the two curves shows whether there are coeluting impurities in the internal standard channel which would be revealed if the slopes of the curves are different. This comparison can also show the presence of any endogenous analyte (or a coeluting impurity) in the biological matrix which would be indicated by an increased intercept for the extracted standard curve. In this study, the unextracted and extracted curves were indistinguishable.

Using this specific assay, there was no evidence for N^{α}-MH in our cultures of *H. pylori*. The limit of detection of the assay was 50 pg/sample (~50 pg/ 10^8 CFU). This is in contrast to the results reported by Courillon-Mallet et al. [6] who found high levels of N^{α}-MH in three cultured *H. pylori* strains. This could arise from strain differences as *H. pylori* are a very heterogeneous population. N^{α}-MH was, however, present in two samples of gastric juice collected from infected patients demonstrating that this histamine receptor agonist is produced in humans in vivo. N^{α}-MH was not detected in a number of other gastric juice samples. Either N^{α}-MH is not produced in all infected patients, perhaps due to inhibition of

its biosynthetic pathway or lack of substrate, or there may be increased metabolism via further methylation or oxidation in these subjects.

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