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Determination of plasma N^{τ}-methylhistamine in vivo by isotope dilution using benchtop gas chromatography–mass spectrometry

Edward E. Tredget[a,*], Takashi Iwashina[a], Paul G. Scott[b], Aziz Ghahary[a]

[a]*Department of Surgery, University of Alberta, Edmonton, Alb. T6G 2B7, Canada*
[b]*Department of Biochemistry, University of Alberta, Edmonton, Alb. T6G 2B7, Canada*

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Abstract

A practical sensitive and specific method for determination of the stable metabolite of histamine, N^{τ}-methylhistamine, in human plasma using benchtop gas chromatography–stable isotope dilution mass spectrometry has been developed. N^{τ}-Methylhistamine, a principal metabolite of histamine in humans, was extracted and purified from human plasma using a two-step procedure with Sep-Pak silica cartridges. Quantitation of N^{τ}-methylhistamine was made possible by the synthesis of N^{τ}-[$^2H_3$]methylhistamine used as an internal standard. Derivatization with pentafluoropropionyl anhydride of extracts of human plasma yielded the bis-pentafluoropropionyl derivative of N^{τ}-methylhistamine for measurement using selected ion monitoring of the m/z 417/420 ion pair after electron impact on a benchtop gas chromatography–mass spectrometry (GC–MS). By improvements in the plasma extraction technique, inclusion of a synthetic internal standard and the development of a sensitive and stable derivative of the histamine metabolite, N^{τ}-methylhistamine was found to be significantly elevated in the plasma of patients with the dermal fibroproliferative disorder, hypertrophic scarring as compared to age-matched normal volunteers (98.5±29.5 pg/ml, $n=9$, versus 43.3±16.5 pg/ml, $n=8$, $p<0.05$). As such, this method affords a sensitive, specific and practical approach to measurement of histamine metabolites in plasma and other biological fluids.

Keywords: N^{τ}-Methylhistamine

1. Introduction

Histamine is a biogenic amine formed by pyridoxal phosphate-dependent decarboxylation of the amino acid, histidine in mast cells, eosinophils and keratinocytes and has many important biological functions in the skin and other organs. In wound healing, histamine is released from mast cells by many stimuli including growth factors, such as IL-3 and IL-4 [1–5]. Histamine stimulates fibroblast proliferation and collagen synthesis and has been implicated in the pathogenesis of fibrotic disorders of the skin, including keloids and hypertrophic scarring, as well as systemic mastocytosis and other dermatologic disorders [6,7]. In systemic mastocytosis, pathologic increases of histamine have been measured in plasma and other biologic fluids, where improvements in clinical status of the patient have been associated with reductions in histamine concentrations [3,4]. Such patients often suffer uncontrollable pruritis and

*Corresponding author.

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wound discomfort despite long term administration of antihistamine medications [1]. Thus, modern therapies directed toward these disorders would benefit from accurate and sensitive analysis of histamine and its metabolites.

In vivo, histamine is catabolized via conversion to N^{τ}-methylhistamine by histamine N-methyltransferase before subsequent deamination to N^{τ}-methylimidazole acetic acid by monoamine oxidase [8–10]. Alternatively, by a second pathway, histamine is deaminated to imidazole acetic acid by oxidative deamination and conjugated to ribose as described by Schayer and Karjala [9]. Although the predominance of either metabolic pathway appears to be species specific, in man, rapid methylation is the principal degradative mechanism of histamine which results in a very short systemic half-life (approximately 102 s) and low basal levels less than 0.4 ng/ml [11]. Significant basal urinary excretion of N^{τ}-methylimidazole acetic acid limits the usefulness of this metabolite in the assessment of endogenous histamine production [13]. Thus, measurement of N^{τ}-methylhistamine in biological fluids such as plasma and urine has been proposed as a more reliable measure of endogenous mast cell degranulation because it is more stable and less influenced by sex, age and dietary factors [12–14].

Many methods for measuring N^{τ}-methylhistamine have been described including thin-layer chromatography (TLC), radioimmunoassays (RIA) [15], high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS). Unfortunately, TLC is semiquantitative and recently developed radioimmunoassays although convenient and simple, often are confounded by other serum proteins yielding spuriously elevated results. Using HPLC, histamine and its metabolites have been detected by fluorescence or electrochemical detector [16], but this method lacks sensitivity and specificity as compared to gas chromatography–stable isotope dilution mass spectrometry methods which have improved reliability and accuracy for analysis of N^{τ}-methylhistamine in biological fluids [17–21]. However, low recovery of N^{τ}-methylhistamine from biological samples after extraction of plasma samples has required GC–MS measurement using a negative ion chemical ionization (NICI) detector for improved sensitivity [21,22]. By refinement of the extraction methods of N^{τ}-methylhistamine [23], inclusion of a synthetic internal standard, N^{τ}-[$^{2}H_{3}$]methylhistamine, and development of the bis-pentafluoropropionyl (PFP) derivative of N^{τ}-methylhistamine, detection of stable metabolites of histamine in the plasma has become practical and efficient using benchtop gas chromatography–electron impact mass spectrometry as described herein. This approach has facilitated the assessment of clinical approaches for treating thermally injured patients suffering intense pruritus associated with hypertrophic scarring associated with systemic elevation of histamine and its metabolites.

2. Experimental

2.1. Materials

N^{τ}-Methylhistamine dihydrochloride and N^{π}-methylhistamine dihydrochloride were obtained from Sigma (St. Louis, MO, USA), pentafluoropropionic anhydride (PFPA) was from Aldrich (Milwaukee, WI, USA). N^{τ}-[$^{3}H_{3}$]Methylhistamine was synthesized in our laboratory as described elsewhere [23]. Radioactivity determination of tritium labeled methylhistamine was performed in ACS (Aqueous Counting Scintillant, Amersham Canada, Oakville, Canada) using a Beckman LS 6000 IC liquid scintillation spectrometer. All chemicals and solvents used were of analytical grade quality. Sep-Pak Vac silica cartridges 1 cc (100 mg) were purchased from Waters (Milford, MA, USA). NMR spectra were determined on a Bruker AM 300 spectrometer, using C[^{1}H]Cl_{3} as solvent with $Me_{4}Si$ as internal standard. The high resolution mass spectra were determined on an AE150 mass spectrometer.

2.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS was performed with a Hewlett-Packard (Palo Alto, CA, USA) 5890 gas chromatograph and HP5970 mass selective detector controlled by a HP5970 Chem station. The column employed was a 15 m×0.25 mm I.D., DB-1 fused-silica capillary

column, 0.25-μm film thickness (J&W Scientific, Folson, CA, USA). The flow-rate of carrier gas (ultra-high purity helium) was 41 ml/min. Chromatographic conditions were as follows: initial temperature, 50°C; initial hold time, 0.5 min; 20°C/min from 50 to 170°C, 50°C/min from 175 to 250°C; injector temperature, 250°C; transfer line temperature, 280°C. Electron impact ionization (70 eV) was used with the selected ion monitoring (SIM) mode, detecting ions specific for the bis-pentafluoropropionyl (PFP) derivative of N^{τ}-methylhistamine (m/z 417), and the bis-PFP derivative of N^{τ}-[$^{3}H_{3}$]methylhistamine (m/z 420) as internal standard. Peak integration was performed by using the software settings of the Hewlett-Packard MS Chem station, where the peak width was 0.020 arbitrary mass spectrometry units and the threshold was 7 arbitrary mass spectrometry units. Dwell time in SIM was 100 ms and the cycles per second was 3.7. Calibration curves were constructed through a range of N^{τ}-methylhistamine (0 pg to 75.0 pg/μl) by dilutions of a standard in 0.01 *M* HCl solution which was not extracted but evaporated to dryness and derivatized as described. The expressed value for the peak area ratio was obtained by dividing the N^{τ}-methylhistamine peak area by the peak area of the internal standard. Samples were diluted into the linear range of the calibration curve where necessary.

2.3. Blood collection and plasma preparation

All subjects were restricted from histamine-rich foods for 24 h prior to blood sampling in the mid-afternoon. Whole venous blood samples (5 ml) were collected by venipuncture using 10-ml vacutainers containing 15 mg of EDTA (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA) from thermally injured patients suffering from hypertrophic scarring involving more than 5% of the total body surface area and age-matched normal healthy volunteers. Blood samples were immediately stored on ice until the plasma was prepared within 30 min after the collection by centrifugation in a cooled centrifuge (4°C) for 110 min at 1000 *g*. Plasma was transferred to polypropylene tubes with polypropylene pipettes and stored at −80°C until analyzed.

2.4. Extraction of N^{τ}-methylhistamine from plasma

As described previously, 0.5 ml of plasma in a polypropylene tube was added to 10 μl of internal standard solution (920 pg/μl of N^{τ}-[$^{3}H_{3}$]methylhistamine) and 1.0 ml of borate buffer solution (pH 9.0). The solution was mixed and then applied to a Sep-Pak silica cartridge (100 mg). The cartridge was washed with water and eluted with acidic methanol solution. The eluates were combined and evaporated to dryness under vacuum. The residue was dissolved in 20% MeOH in $CHCl_3$. The solution was applied to a Sep-Pak silica cartridge (100 mg), washed with 20% MeOH in $CHCl_3$ and eluted with ammonium basic MeOH–$CHCl_3$ solution. The eluates were combined and evaporated to dryness under vacuum. The residue was used for derivatization.

2.5. Recovery of N^{τ}-methylhistamine

To assess recovery of N^{τ}-methylhistamine from column chromatography, radioactive N-methylhistamine (0.63 μCi, mixture of the about 2:1 ratio N^{τ}-[$^{3}H_{3}$]methylhistamine and N^{π}-[$^{3}H_{3}$]methylhistamine) and 1.0 ml of borate buffer solution (pH 9.0), was added to normal human plasma (0.5 ml) and urine samples (0.5 ml) in polypropylene tubes. The radioactive N-methylhistamine was purified with the extraction procedures described above except that the combined eluates in each step were evaporated under a stream of nitrogen gas. ^{3}H-radioactivity of the residue was measured by liquid scintillation counting.

2.6. Preparation of pentafluoropropionyl (PFP) derivative of N^{τ}-methylhistamine

A 200-μl volume of ethyl acetate, 40 μl of pyridine and 100 μl of PFP anhydride were added to the plasma extract in a screw-capped glass tube. The mixture was heated at 60°C for 40 min before under a stream of nitrogen gas at room temperature. A 0.5-ml volume of 0.5 mol/l Tris-buffer solution (pH 7.0) and *n*-hexane (2×1.5 ml) was added to the residue. The mixture was shaken with a vortex mixer for 1 min and centrifuged. *n*-Hexane layers were combined and evaporated to dryness with a Speed

Vac concentrator. The residue was dissolved in 5–15 μl of ethyl acetate and analyzed by GC–MS.

2.7. Synthesis of N^{τ}-[$^2H_3$]methylhistamine

N^{α}-Acetylhistamine (3 g, 19.6 mmol) was dissolved in 35 ml of 10% NaOH solution. To the solution was added dimethyl-[$^2H_6$] sulphate (2 ml, 21.1 mmol) on an ice bath and then stirred for 5 min. A further 35 ml of 10% NaOH solution and 2 ml of dimethyl-[$^2H_6$] sulphate were added and the mixture stirred as before. The reaction mixture was heated for 30 min at 105°C, cooled, saturated with sodium chloride, and extracted with chloroform (3×80 ml). The combined chloroform layers were dried over anhydrous sodium sulphate and evaporated under vacuum to give 1.94 g of crude N^{α}-acetyl-N^{τ}-[$^2H_3$]methylhistamine as an oil. N^{α}-Acetyl-N^{τ}-[$^2H_3$]methylhistamine was dissolved in 20 ml of a 6 *M* HCl solution and deacetylated at 105°C for 2 h. The solution was cooled to room temperature, made strongly alkaline, and saturated with sodium chloride. The aqueous solution was extracted with chloroform (3×40 ml) and the combined chloroform layers were dried over anhydrous sodium sulphate, treated with 0.5 ml of HCl-saturated ethanol and evaporated under vacuum to give 1.4 g of N^{τ}-[$^2H_3$]methylhistamine-2HCl (oil) (methyl-C[$^2H_3$], 99.7%).

2.8. Synthesis of bis-PFP derivative of N^{τ}-methylhistamine

A 200-μl volume of ethyl acetate, 40 μl of pyridine and 100 μl of pentafluoropropionic anhydride was added to 2 mg of N^{τ}-methylhistamine in a screw-capped glass tube. The mixture was heated at 60°C for 40 min and evaporated to dryness under a stream of nitrogen gas. The residue was dissolved into 1.5 ml of Tris-buffer (pH 7.0) and extracted three times with 3.0 ml of *n*-hexane. The combined *n*-hexane layers were evaporated to dryness under vacuum. The residue was submitted to NMR spectrometry and high resolution mass spectrometry.

2.9. Lower limit of detection

The lower limit of N^{τ}-methylhistamine standard injected during GC–MS analysis which could be distinguished above background by a signal-to-noise ratio of at least 5:1 was determined. This amount of N^{τ}-methylhistamine was extrapolated to a plasma concentration by correcting for potential losses during extraction.

2.10. Precision and accuracy

The precision with which N^{τ}-methylhistamine could be measured was determined by analyzing on the same day five aliquots of the same plasma obtained from a patient with a 90% total body surface area burn. Five 0.5-ml aliquots of the plasma to which 10 μl (920 pg/μl) internal standard were added were extracted and assayed as described and the coefficient of variation was calculated as a measure of intra-assay variation.

Accuracy of the assay was determined by adding a known amount of N^{τ}-methylhistamine to five aliquots of the same plasma along with the 10 μl internal standard solution and by comparing the calculated N^{τ}-methylhistamine concentration of the set of plasma samples to a second set to which only the internal standard was added. To five 0.5-ml plasma aliquots, 183 pg of authentic N^{τ}-methylhistamine and 10 μl of internal standard were added and to another five 0.5-ml plasma aliquots, only 10 μl of the internal standard solution was added. The samples were then extracted and analyzed. By subtracting the calculated N^{τ}-methylhistamine concentration of the second set from the first set, the accuracy of measuring 366 pg/ml was determined and was expressed as a mean percentage change from 366 pg/ml. The inter-assay variability was determined by analyzing the N^{τ}-methylhistamine concentration in five plasma aliquots of 0.5 ml which were extracted and assayed on five different days.

3. Results and discussion

The PFP derivative of N^{τ}-methylhistamine was chosen for GC–MS and was made by reaction of N^{τ}-methylhistamine with PFP anhydride in the presence of pyridine. For the authentic compound, the PFP derivative gave a single peak with a retention time of 6.47 min with a molecular ion of *m/z* 417 of relative intensity 46.3% in the mass spectrum of the total ion chromatogram (Fig. 1). The molecular ion

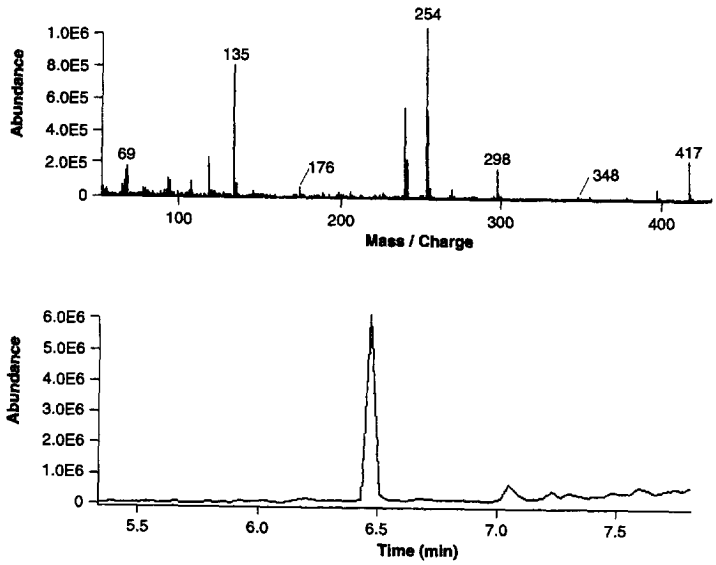

Fig. 1. GC–MS analysis of bis PFP derivative of authentic N^{τ}-methylhistamine. (Top) Mass spectrum of the peak at 6.47 min, (bottom) total ion chromatography.

of the PFP derivative and its fragmentation pattern were also confirmed by high resolution mass spectrometry using direct probe insertion (Table 1). Using NMR spectrometry, the signal of only one singlet appeared at δ 7.08 ppm in the spectrum of the derivative, suggestive of a low field signal associated with a proton on the 5-position on the imidazole ring and reaction at the 2-position by the electrophilic fluoroacyl group. This chemical structure of the bis-PFP derivative of N^{τ}-methylhistamine has been reported previously by the reaction with perfluoroacetic anhydrides under basic reaction conditions [17,18].

The internal standard, N^{τ}-[$^2H_3$]methylhistamine was synthesized by a modification of the method of Keyzer et al [18]. N-Methylation of nitrogen on the imidazole ring was carried out with dimethyl-[$^2H_6$] sulphate at 105°C for 30 min under strong basic conditions. The final crude trideuterated N^{τ}-methylhistamine dihydrochloride was obtained at a yield of 38.4% with the C[$^2H_3$] group at 99.7% purity, and a molecular ion of m/z 420 by GC–MS. The mass spectrum and total ion chromatogram of the bis PFP derivative of N^{τ}-[$^2H_3$]methylhistamine are shown in Fig. 2. As illustrated, a second peak was due to the isomer, N^{π}-methylhistamine which had a retention time of 6.89 min and was easily separated from N^{τ}-methylhistamine by capillary GC–MS, as described previously by Keyzer et al. [18]. This allowed the principal product, N^{τ}-[$^2H_3$]methylhistamine, to be used directly as a internal standard for quantitation by isotope dilution

Table 1
Molecular ion and main fragment ions of N^{α}-pentafluoropropionyl 2-pentafluoropropionyl N^{τ}-methylhistamine by electron impact high resolution mass spectrometry

m/z	Measured	Calculated	Relative intensity (%)
M^+ (molecular ion)	417.05309	417.05350	30.0
$[M-F]^+$	398.05397	398.05511	4.9
$[M-C_2F_5]^+$	298.06143	298.06149	14.5
$[M-C_2F_5CONH_2]^+$	254.04877	254.04900	100.0
$[M-C_2F_5CONHCH_2]^+$	241.04013	241.04002	51.8
$C_7H_7N_2O^+$	135.05893	135.05583	91.3
$C_5H_6N_2^+$	94.05325	94.05310	10.5

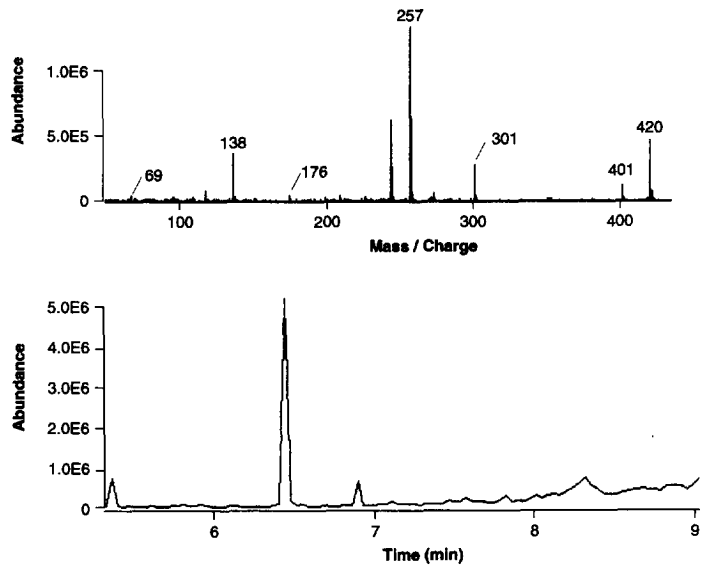

Fig. 2. GC–MS analysis of bis PFP derivative of synthesized N^{τ}-[$^2H_3$] methylhistamine. (Top) Mass spectrum of the peak at 6.44 min, (bottom) total ion chromatogram. The peak at 6.89 min in (b) was bis PFP derivative of N^{π}-[$^2H_3$]methylhistamine. (The peak showed a molecular ion m/z 420).

analysis using selected ion monitoring of the ion pair m/z 417/420. As illustrated in Fig. 3, selectivity and specificity of the analysis was thus possible by selected ion monitoring of authentic N^{τ}-methylhistamine (m/z 417) and its internal standard N^{τ}-[$^2H_3$]methylhistamine (m/z 420).

Standard solutions were prepared by dilution of solutions of N^{τ}-methylhistamine into the range of 0 to 75 pg/μl in a 0.01 *M* HCl solution. Samples were prepared in triplicate before 10 μl of N^{τ}-[$^2H_3$]methyhistamine (920 pg/μl) was added as a internal standard and the sample solutions were evaporated, derivatized, but not extracted before being analyzed by GC–MS. The calibration curve was linear in the range 0–75 pg ($y = -1.576 \cdot 10^{-2} + 3.873 \cdot 10^{-2}x$, $R^2 = 0.999$) (Fig. 4). Using this approach, the detection limit for N^{τ}-methylhistamine in a 0.01 *M* HCl solution was 2.8 pg actual on the column amount with a signal-to-noise ratio of 5:1. Assuming an average recovery of 90% for N^{τ}-methylhistamine [23] and injection of one fifth of the sample, these values correspond to a minimum measurable level of approximately 12–25 pg/ml for N^{τ}-methylhistamine in plasma. This level of sensitivity approaches that reported previously by Payne et al. using GC–NICI-MS techniques [22]. The internal standard was stable in 0.01 *M* HCl solution at 4°C over a number of months. Extracted plasma samples were stored dry at −10°C until derivatization and prompt analysis in groups of 5–6.

The inter-assay coefficient of variation was calculated from five aliquots of plasma which were extracted and analyzed for N^{τ}-methylhistamine on the same day and resulted in a mean±S.E. of 112.06±4.27 pg/ml. The coefficient of variation was 7.6%. When a known amount of N^{τ}-methylhistamine, 366 pg/ml, was added to five plasma aliquots and the endogenous N^{τ}-methylhistamine concentration was subtracted from the concentration obtained from the plasma samples, a net measurement of 353.42±28.56 pg/ml was obtained. Therefore, the accuracy with which 366 pg/ml of N^{τ}-methylhistamine can be measured was estimated to be 96.4% suggesting that little absorption of N^{τ}-methylhistamine occurs under these conditions. The intra-assay variability for N^{τ}-methylhistamine was 7.2% and the inter-assay variability was 13.7%.

Illustrated in Fig. 5 is typical GC–MS chromatogram of the bis-PFP derivatives of N^{τ}-methylhistamine (m/z 417) and N^{τ}-[$^2H_3$]methylhistamine (m/z

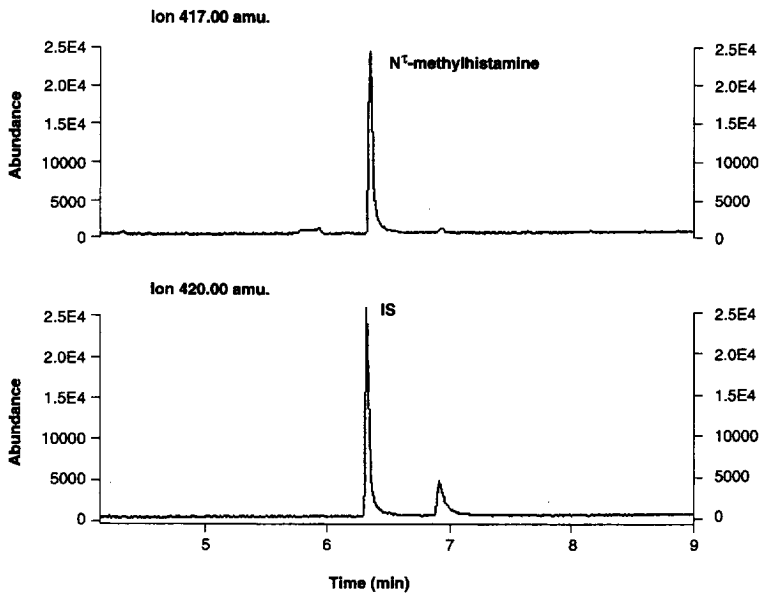

Fig. 3. GC–MS analysis of the mixture of bis PFP derivatives of authentic N$^{\tau}$-methylhistamine and synthesized N$^{\tau}$-[$^{2}H_3$]methylhistamine (internal standard). (Top) Selected ion monitoring of the m/z 417, (bottom) selected ion monitoring the m/z 420. The second peak in (b) was bis PFP derivative of N$^{\pi}$-[$^{2}H_3$]methylhistamine. The N$^{\tau}$-methylhistamine/N$^{\tau}$-[$^{2}H_3$]methylhistamine (internal standard) ratio (D_0/D_3) was 0.30 and the N$^{\tau}$-methylhistamine concentration was 9.20 ng/ml.

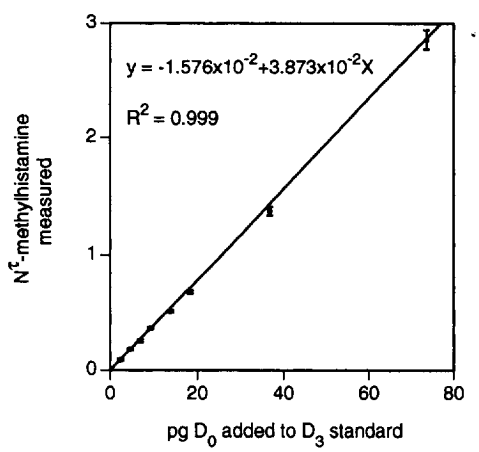

Fig. 4. Standard curve for N$^{\tau}$-methylhistamine. The ordinate represents the measured N$^{\tau}$-methylhistamine/N$^{\tau}$-[$^{2}H_3$]methylhistamine (internal standard) ratio (D_0/D_3). On the abscissa is increasing amounts of D_0-N$^{\tau}$-methylhistamine standard added to D_3-N$^{\tau}$-methylhistamine. The curve covers a range of 0–75.0 pg/μl N$^{\tau}$-methylhistamine in 0.01 *M* HCl and displays linearity with a high correlation coefficient of 0.999.

420) obtained by selected ion monitoring in the plasma of a burn patient with severe wound pruritus secondary to the dermal fibroproliferative disorder, hypertrophic scarring. Using this method, nine patients with hypertrophic scarring over greater than 5% of their body surface area demonstrated a greater than two fold elevation of plasma N$^{\tau}$-methylhistamine as compared to eight age-matched healthy human volunteers (98.5±29.5 pg/ml vs. 43.3±16.4 pg/ml, $p<0.05$ by unpaired *t*-test) (Fig. 6).

N$^{\tau}$-Methylhistamine in biological fluids has been determined with electron impact [17–19], chemical ionization [20] and electron capture negative ion chemical ionization mass spectrometry [21,22]. Prior to using these methods, N$^{\tau}$-methylhistamine has been purified from plasma using either column chromatography procedures or by solvent extraction [18–20]. Unfortunately, we have found the recovery of radioactive N$^{\tau}$-methylhistamine from human urine by chloroform extraction to be as low as 26.3±2.6% to 21.8±3.0%, for *n*-butanol–chloroform (1:4, v/v) extraction [23]. After using similar approaches to blood analysis, the peak of N$^{\tau}$-methylhistamine by selected ion monitoring after electron impact ioniza-

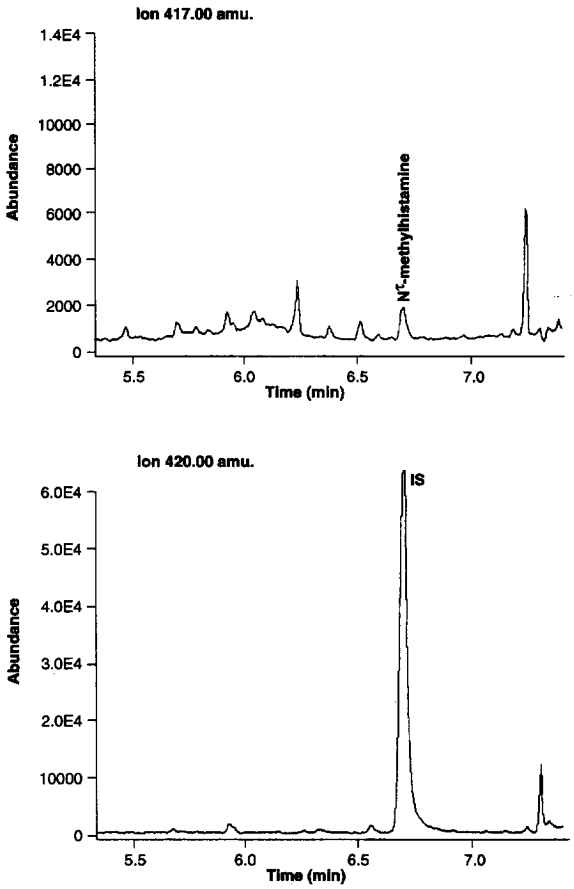

Fig. 5. Ion chromatography of N$^{\tau}$-methylhistamine (m/z 417) and internal standard (m/z 420) in plasma of burn patients with hypertrophic scarring. The ratio of N$^{\tau}$-methylhistamine/N$^{\tau}$-[$^{2}H_{3}$]methylhistamine (internal standard) (D_0/D_3) was 0.13 and the concentration was 225 pg/ml.

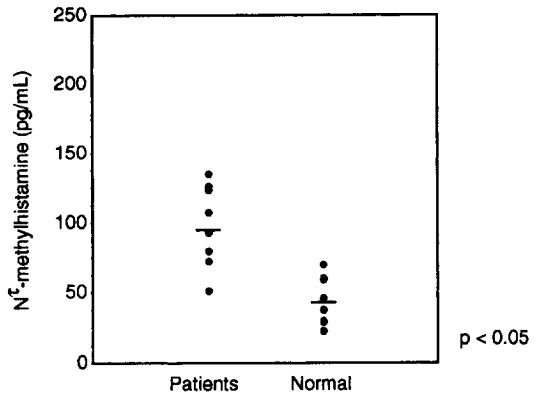

Fig. 6. Plasma levels of N$^{\tau}$-methylhistamine in normal and in burn patients with hypertrophic scarring.

tion on GC–MS has been of relatively low concentration and often interrupted by the interfering peaks arising from uncharacterized components of plasma and serum. By synthesis of a radioactive internal standard of N$^{\tau}$-methylhistamine, improvements in extraction methods using a two step purification procedure with Sep-Pak silica cartridges have increased the efficiency to at least 82.5±0.3% [23]. In addition, the plasma residue obtained through this approach was free of interfering peaks after derivatization of N$^{\tau}$-methylhistamine and the internal standard, enabling their measurement using selected ion monitoring.

The use of electron capture negative ion chemical ionization mass spectrometry for the analysis of N$^{\tau}$-methylhistamine offers the advantage of somewhat increased sensitivity afforded by much less fragmentation of the molecular ion [21]. Unfortunately, the high instrument cost and its limited availability restricts its widespread use. However, improvements in the purification and extraction of N$^{\tau}$-methylhistamine from plasma renders benchtop mass spectrometry practical and versatile. In addition, similar approaches to other biologic fluids in addition to plasma, will facilitate investigation and assessment of therapeutic modalities in the treatment of wound healing disorders such as hypertrophic scarring, as well as other conditions in which histamine metabolism is disordered including nephritis [19], asthma [24], anaphylaxis [20] systemic mastocytosis [5], polycythaemia vera with severe pruritus, and with urticaria [25].

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