Gas chromatography–mass spectrometry assay for determination of N^{τ}-methylhistamine concentration in canine urine specimens and fecal extracts

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Objective—To develop and validate a gas chromatography–mass spectrometry (GC-MS) method for determination of N^{τ}-methylhistamine (NMH) concentration in canine urine and fecal extracts and to assess urinary NMH concentrations in dogs with mast cell neoplasia and fecal NMH concentrations in dogs with protein-losing enteropathy.

Sample Population—Urine specimens were collected from 6 healthy dogs and 7 dogs with mast cell neoplasia. Fecal extracts were obtained from fecal specimens of 28 dogs with various severities of protein-losing enteropathy, as indicated by fecal concentration of α_1-proteinase inhibitor.

Procedures—NMH was extracted directly from urine, and fecal specimens were first extracted into 5 volumes of PBSS containing 1% newborn calf serum. N^{τ}-methylhistamine in specimens was quantified via stable isotope dilution GC-MS. The assay was validated via determination of percentage recovery of known amounts of NMH and interassay coefficients of variation. Urinary excretion of NMH was evaluated by means of NMH-to-creatinine concentration ratios.

Results—Recovery of NMH in urine and fecal extracts averaged 104.6% and 104.5%, respectively. Interassay coefficients of variation ranged from 5.4% to 11.7% in urine and 12.6% to 18.1% in fecal extracts. Urinary NMH excretion was significantly increased in dogs with mast cell neoplasia, compared with that in healthy dogs. No correlation was detected between severity of protein-losing enteropathy and fecal NMH concentration.

Conclusions and Clinical Relevance—This method provided a sensitive, reproducible means of measuring NMH in canine urine and fecal extracts. High urinary NMH-to-creatinine concentration ratios in dogs with mast cell neoplasia are consistent with increased histamine release in this disease. (*Am J Vet Res* 2009;70:167–171)

Histamine, a potent mediator of many physical manifestations of inflammation, is stored in granules within mast cells. Mast cells are ubiquitous in the body but exist in particularly high numbers in the skin and gastrointestinal tract.[1,2] In circulation, histamine has a short half-life. Following its release from mast cells, the compound is, in part, converted to NMH via the action of histamine methyltransferase and is subsequently oxidized by monoamine oxidase.[3] N^{τ}-methylhistamine is considered a stable metabolite of histamine, and measurement of NMH concentration has been proposed as a method of assessing histamine release in vivo.[4]

Abbreviations

GC-MS	Gas chromatography–mass spectrometry
NMH	N^{τ}-methylhistamine
PFP	Pentafluoropropionyl anhydride

Diagnosis of gastrointestinal disease is often hampered by the inaccessibility of the gastrointestinal tract to diagnostic specimen collection. For this reason, the development of minimally invasive markers of gastrointestinal disease is an area of active interest in human and comparative gastroenterology. Mast cell activity and systemic release of histamine have been investigated in human patients with inflammatory bowel disease, and disease activity reportedly correlates with urinary or fecal NMH excretion.[5,6]

Measurement of NMH concentration in plasma samples by use of inexpensive bench-top GC-MS equipment has been described by others.[3] The purpose of the study reported here was to modify this method for use with canine urine specimens and fecal extracts, allowing minimally invasive assessment of mast cell activity and histamine release in mast cell–mediated disease in dogs. Specifically, we sought to develop and validate

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a GC-MS method for determination of NMH concentration in canine urine and fecal extracts and to assess urinary NMH concentration in dogs with mast cell neoplasia and fecal NMH concentrations in dogs with protein-losing enteropathy.

Materials and Methods

Specimen collection—Urine specimens were obtained via midstream catch during natural voiding from pet dogs (n = 6; control dogs) owned by students and staff of the Texas A&M University College of Veterinary Medicine and Biomedical Sciences. Physical examinations were performed to ensure all dogs were apparently healthy. Dogs with pruritic skin disease or recent exposure to a glucocorticoid drug were excluded. Each urine specimen was divided into aliquots and stored at –80°C until use. Urine specimens were also obtained from client-owned pet dogs (7) with mast cell neoplasia via midstream catch, cystocentesis, or indwelling urinary catheter at the discretion of the attending clinician and as appropriate to the clinical management of the dog. These urine specimens were excess or remainder specimens destined for disposal and were not specifically obtained from the dogs for the purpose of the study.

Fecal extracts (n = 28) were selected from specimens submitted to our clinical services laboratory for measurement of the concentration of fecal α_1-proteinase inhibitor. Extracts were selected on the basis of reported concentrations of fecal α_1-proteinase inhibitor to provide a range from low concentrations (< 5 µg/g of feces, which is considered the upper reference limit in dogs), to excessive concentrations (> 100 µg/g of feces, which is considered characteristic of protein-losing enteropathy). The fecal specimens used in the study were excess or remainder specimens destined for disposal and were not specifically obtained from dogs for the purpose of the study. Specimens (approx 1 g of wet weight) were stored at –20°C until processing. Urine specimen collection from the 6 aforementioned healthy control dogs was conducted following a protocol for animal use and care that was approved by the Clinical Research Review Committee at the College of Veterinary Medicine and Biomedical Sciences.

Reagents—A stock solution containing 5,000 pg of trideuterated NMH[a]/µL was prepared in analytic-grade laboratory water that had been purified by reverse osmosis filtration and ion-exchange filtration. Standard solutions containing various concentrations of nondeuterated NMH[b] ranging from 0 to 5,000 pg/µL were prepared by serial dilution with analytic-grade laboratory water.

GC-MS—Gas chromatography–mass spectrometry was carried out with a gas chromatograph[c] interfaced to a mass spectrometer[d] operating in electron impact mode. Separation was achieved by means of a 30-m column[e] with an inner diameter of 0.25 mm and film thickness of 0.25 µm, with helium as a carrier gas. The gas chromatograph was operated in a constant flow mode at 2.0 mL/min, with a splitless mode for 30 seconds at injection, and the remainder of the separation was carried out with a 15:1 split-flow ratio. The temperature program used was essentially that as described by others,[3] with the exception that a lower transfer line temperature (250°C) and a lower carrier gas flow rate were used. The mass spectrometer was operated in electron impact ionization mode (ionization potential, 70 eV) with selected ion monitoring at masses specific for the bis-PFP derivative of NMH and the trideuterated bis-PFP derivative of trideuterated NMH (mass-to-charge ratio, 417 and 420, respectively). Dwell times for each ion were 100 milliseconds with 5 cycles/s. Peak area integration was calculated with the default settings of software for the GC-MS system. Retention times were confirmed daily by GC-MS evaluation of a specimen of pure, nondeuterated NMH that was evaporated to dryness and derivatized directly.

Extraction and derivatization of NMH from canine urine—N^{τ}-methylhistamine was extracted from canine urine specimens (0.5 to 1 mL) essentially as described elsewhere.[3] Briefly, a known quantity of trideuterated NMH (50 pg) was added to each specimen. Specimens were diluted with an equal volume of borate buffer (pH, 9.0) and mixed for 30 seconds on a standard laboratory vortex machine, then applied to a silica cartridge[f] under light vacuum. The cartridge was washed twice with 1 mL of ultrapure water, then eluted with three 1-mL fractions of 0.1nM HCl in methanol[g] into borosilicate glass test tubes. The fractions were combined and evaporated under a stream of nitrogen.

After drying, the walls of the test tubes were rinsed twice with 150 µL of 20% methanol in chloroform,[g] for a total volume of 300 µL. The specimens were then redissolved in the methanol and chloroform solution and applied to a second silica cartridge under light vacuum. The cartridge was washed with 150 µL of 20% methanol in chloroform, then eluted into a clean borosilicate test tube with 4 aliquots of a mixture of methanol, chloroform, and ammonium hydroxide (ratio of 25:25:1 vol/vol). The eluates were evaporated to dryness, and bis-PFP derivatives were generated by reaction with pyridine[b] (40 µL) and PFP[g] anhydride (100 µL) mixed with 200 µL of ethyl acetate.[b] The test tubes were covered with paraffin laboratory film and heated at 64°C for 40 minutes, then evaporated to dryness under a stream of nitrogen.

The dried derivatives were cleaned via solvent partitioning. Derivatives were dissolved in 500 µL of 0.5M Tris buffer, then 1.5 mL of hexane[g] was added to each tube. The specimens were mixed on a vortex machine for 1 minute, then the solvents were allowed to separate. The hexane layers were carefully collected and transferred to a 4-mL glass vial, then a second aliquot of 1.5 mL of hexane was added to each extract. Solvent portioning was repeated, and the hexane extracts were combined in the 4-mL vial and evaporated to dryness under a stream of nitrogen, then resuspended in 50 µL of ethyl acetate. One microliter of the final extract was injected into the GC-MS analyzer.

Extraction and derivatization of NMH from canine fecal specimens—Thawed fecal specimens were initially extracted with 5 volumes of PBSS containing 1% newborn calf serum[b] as described elsewhere.[7] Specimens were shaken for 20 minutes on a standard labo-

ratory orbital platform, then centrifuged at 3,000 × *g* for 5 minutes. The supernatants were filtered, 5 µL was removed for use in an α_1-proteinase inhibitor ELISA as described,[7] and the remainder was stored at –80°C until processed further. Prior to extraction of NMH from fecal extracts, 50 pg of trideutrated NMH was added to 500 µL of each specimen. Subsequent processing of the fecal extract specimen, derivatization, and GC-MS analysis were exactly as described for the urine specimens.

Evaluation of recovery and reproducibility of NMH measurements—Pure NMH in aqueous solution was added in known quantity to 3 urine specimens and 3 fecal extract specimens at 5 different concentrations prior to extraction and derivatization. The reproducibility of the method was assessed by calculating the coefficients of variation for results for the urine specimens and fecal extracts, each of which was separately assayed 10 times.

Evaluation of urinary NMH-to-creatinine concentration ratios—Urine creatinine concentration was determined at a commercial laboratory by means of an automated dry chemistry analyzer system. The urine NMH-to-creatinine concentration ratio was determined by division of the NMH concentration (pg/µL) by urine creatinine concentration (mg/dL). Median urinary ratios of NMH concentration to creatinine concentration were compared between the 6 healthy controls and dogs with mast cell neoplasia by use of the Mann-Whitney test.

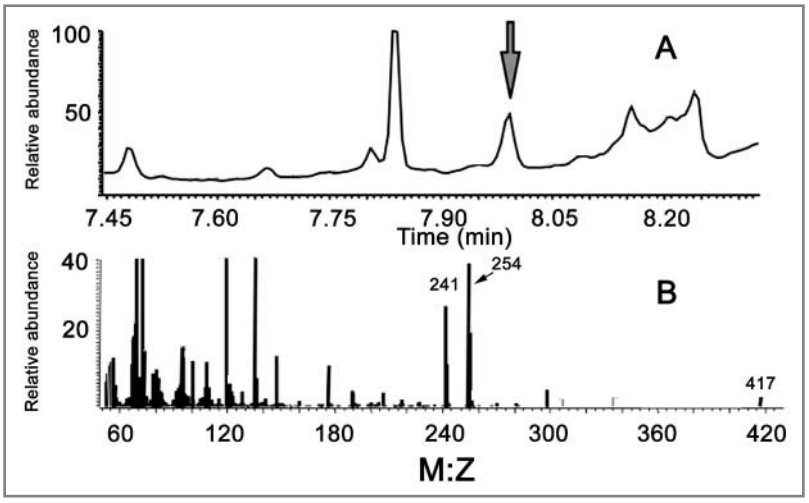

Figure 1—Representative full-scan chromatogram (mass-to-charge ratio [M:Z] range, 50 to 440) indicating the elution of endogenous NMH from the GC-MS at 7.99 minutes (arrow) after extraction from a canine urine specimen (A) and electron impact ionization fragment pattern of the indicated peak (B). The peak indicated in panel A represents a fragmentation pattern consistent with that of NMH, with a parent ion at an M:Z of 417. The specimen represented in the chromatogram contained no trideuterated NMH.

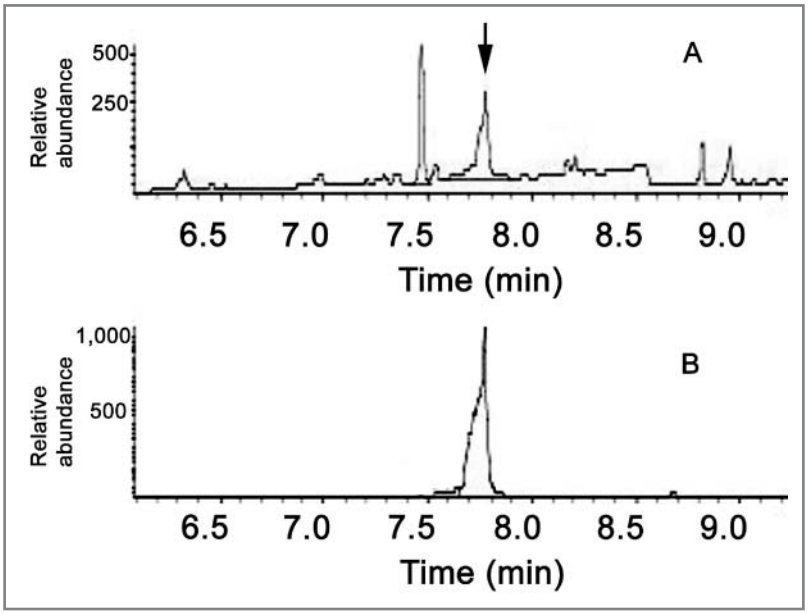

Figure 2—Representative selected ion monitoring trace chromatograms of the result of elution of NMH from a canine fecal extract. Undeuterated methylhistamine is represented by the peak in the M:Z 417 chromatogram (A; arrow), whereas the trideuterated NMH internal standard is represented by the peak in the M:Z 420 chromatogram (B). *See* Figure 1 for remainder of key.

Results

Detection of NMH—The bis-pentafluoropropionyl derivative of NMH was eluted from the gas chromatograph at 7.99 minutes. N^{τ}-methylhistamine was readily detected in canine urine and fecal specimens to which NMH had not been intentionally added (**Figures 1 and 2**). The NMH peak was clearly resolved from other peaks. Standard curves, generated by addition of pure NMH to a constant quantity of trideuterated NMH in aqueous solution before extraction and derivatization, revealed good linearity of results from 0 to 5,000 pg/µL (linear regression equation, $y = 0.2999x + 32.407$; $R^2 = 0.998$; **Figure 3**). The lower limit of detection of the assay, based on a signal-to-noise ratio of > 5, was approximately 20 pg of NMH/µL of urine or fecal extract.

Recovery and reproducibility of NMH measurements—Recovery of NMH from the 3 urine specimens to which it had been added ranged from 81.01% to 143.47%, with a mean recovery across all specimens of 104.6%. The recovery of added NMH from the fecal extracts ranged from 88.63% to 114.98%, with mean recovery across all specimens of 104.18%. The coefficients of variation for the urine specimens ranged from 5.4% to 11.7%, whereas the coefficients of variation for the fecal extracts ranged from 12.6% to 18.1%.

Urinary NMH-to-creatinine concentration ratios in healthy dogs and dogs with mast cell neoplasia—Dogs with mast cell neoplasia had significantly greater NMH-to-creatinine concentration ratios than did the healthy control

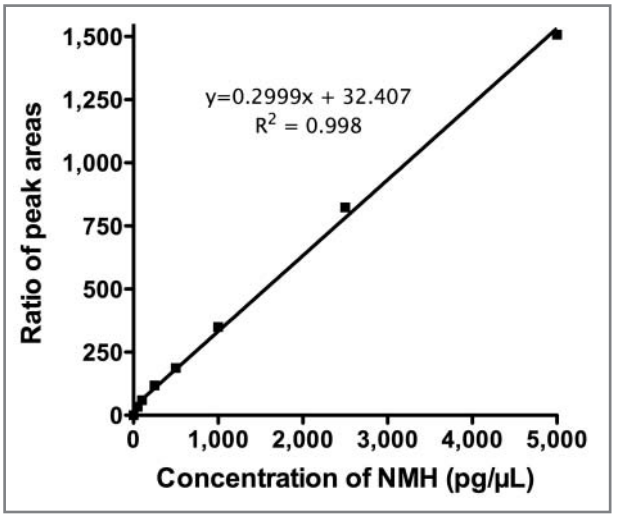

Figure 3—Ratios of peak areas of M:Z 417:420 for standard curves generated via gas chromatographic evaluation of pure NMH added to a constant quantity of trideuterated NMH in aqueous solution at 7.99 minutes. Linear regression analysis (equation provided in graph) revealed results were strongly linear through a wide range of NMH concentrations (0 to 5,000 pg/µL).

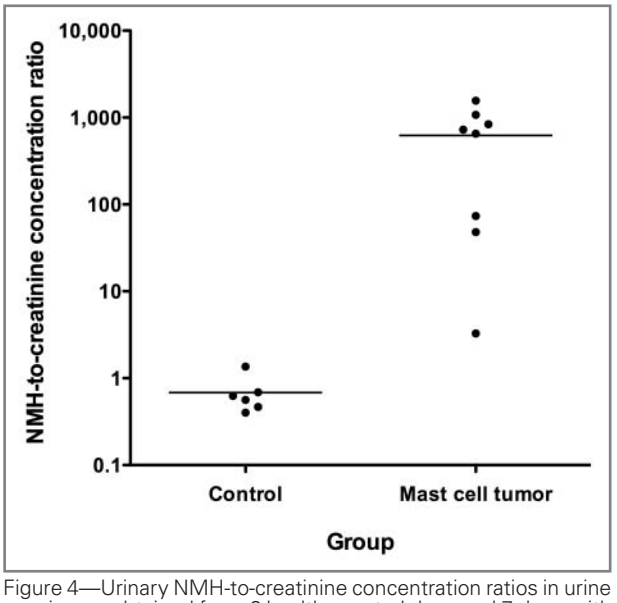

Figure 4—Urinary NMH-to-creatinine concentration ratios in urine specimens obtained from 6 healthy control dogs and 7 dogs with mast cell neoplasia. Notice that the scale of the y-axis is logarithmic (log_{10}). Horizontal bar represents the median value.

dogs (Mann-Whitney test; $P < 0.001$; **Figure 4**). The dogs with mast cell neoplasia had a median NMH-to-creatinine concentration ratio of 687.6 (range, 3.27 to 1,566; n = 7), whereas the healthy control dogs had a median value of 0.59 (range, 0.40 to 1.36; n = 6). The lowest value for the NMH-to-creatinine concentration ratio among dogs with mast cell neoplasia was 2.4 times as great as the highest value detected among the healthy control dogs.

NMH concentration in fecal extracts from healthy dogs and dogs with protein-losing enteropathies— There was no correlation between fecal concentration of α_1-proteinase inhibitor and fecal concentration of NMH (Spearman $\rho = -0.008$; $P = 0.97$). The concentrations of NMH detected in fecal extracts ranged widely, from undetectable to 1,959 µg/g of feces.

Discussion

The study reported here involved an extension of a method[3] for determination of NMH by means of conventional bench-top equipment for GC-MS. Findings indicated that NMH can be extracted from canine fecal specimens with a rapid and low-cost procedure and without interfering with determination of the concentration of α_1-proteinase inhibitor in a fecal specimen. The method used had acceptable linearity, reproducibility, and quantitative recovery and is therefore suitable for use in additional investigations of histamine release in gastrointestinal disease.

N^{τ}-methylhistamine concentrations were measured in urine specimens from dogs with mast cell neoplasia and compared with values in specimens from healthy control dogs. To account for variations in glomerular filtration rate and urine concentration among these specimens, the measured NMH concentrations were normalized by calculation of the NMH-to-creatinine concentration ratio.[8,9] The finding of high urinary NMH-to-creatinine concentration ratios in dogs with mast cell neoplasia was consistent with the increase in release and metabolism of histamine associated with this disease. The relationship, if any, between histologic or biological grade of mast cell neoplasia in dogs and this urinary ratio is an area of active interest.

The concentration of fecal α_1-proteinase inhibitor is considered an indicator for gastrointestinal protein loss because α_1-proteinase inhibitor has a molecular mass similar to that of albumin; however, in contrast to albumin, α_1-proteinase inhibitor is able to withstand gastrointestinal degradation by virtue of its proteinase inhibitory activity.[7] Mast cell accumulation in the gastrointestinal mucosa and histamine release into the gastrointestinal tract reportedly occur in human patients with inflammatory bowel disease,[5] whereas in domestic dogs, dietary intolerance and histamine release may play a role in the development of protein-losing enteropathy in Soft Coated Wheaten Terriers.[10] In the study reported here, fecal NMH concentration was determined in canine fecal extracts selected from submissions to a laboratory for determination of fecal concentration of α_1-proteinase inhibitor. Specimens were deliberately selected to cover a range of α_1-proteinase inhibitor concentrations, from not detectable to excessive. The NMH concentrations detected in fecal extracts from healthy dogs in our study ranged widely, suggesting that gastrointestinal histamine release in healthy dogs is variable. Because specimens were selected to evaluate a broad range of fecal concentrations of α_1-proteinase inhibitor, and we did not select specimens on the basis of specific breed or diagnosis of dogs, it remains possible that significant differences in fecal NMH concentrations would be detected in more carefully defined groups of dogs.

a. CDN Isotopes, Pointe Claire, QC, Canada.
b. Sigma Chemical Co, St Louis, Mo.
c. Carlo Erba GC 8000^{TOP}, Thermo Corp, Waltham, Mass.
d. ThermoFinnigan Voyager, Thermo Corp, Waltham, Mass.
e. J&W DB-1ms column, Agilent Corp, Palo Alto, Calif.
f. Sep-Pak cartridge, Waters, Milford, Mass.
g. VWR International, West Chester, Pa.

References

1. Yu LC, Perdue MH. Role of mast cells in intestinal mucosal function: studies in models of hypersensitivity and stress. *Immunol Rev* 2001;179:61–73.
2. Stenton GR, Vliagoftis H, Befus AD. Role of intestinal mast cells in modulating gastrointestinal pathophysiology. *Ann Allergy Asthma Immunol* 1998;81:1–11.
3. Tredget EE, Iwashina T, Scott PG, et al. Determination of plasma N^{τ}-methylhistamine in vivo by isotope dilution using benchtop gas chromatography-mass spectrometry. *J Chromatogr B Biomed Sci Appl* 1997;694:1–9.
4. Keyzer JJ, Breukelman H, Wolthers BG, et al. Measurement of N^{τ}-methylhistamine concentrations in plasma and urine as a parameter for histamine release during anaphylactoid reactions. *Agents Actions* 1985;16:76–79.
5. Bischoff SC, Grabowsky J, Manns MP. Quantification of inflammatory mediators in stool samples of patients with inflammatory bowel disorders and controls. *Dig Dis Sci* 1997;42:394–403.
6. He SH. Key role of mast cells and their major secretory products in inflammatory bowel disease. *World J Gastroenterol* 2004;10:309–318.
7. Melgarejo T, Williams DA, Asem EK. Enzyme-linked immunosorbent assay for canine α_1-protease inhibitor (Erratum published in *Am J Vet Res* 1998;59:524). *Am J Vet Res* 1998;59:127–130.
8. Stout L, Gerspach JM, Levy SM, et al. Dimethyl sulfoxide does not trigger urine histamine release in interstitial cystitis. *Urology* 1995;46:653–656.
9. Yun SK, Laub DJ, Weese DL, et al. Stimulated release of urine histamine in interstitial cystitis. *J Urol* 1992;148:1145–1148.
10. Vaden SL, Hammerberg B, Davenport DJ, et al. Food hypersensitivity reactions in Soft Coated Wheaten Terriers with protein-losing enteropathy or protein-losing nephropathy or both: gastroscopic food sensitivity testing, dietary provocation, and fecal immunoglobulin E. *J Vet Intern Med* 2000;14:60–67.