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DETERMINATION OF N^{τ}-METHYLHISTAMINE IN URINE BY GAS CHROMATOGRAPHY USING NITROGEN—PHOSPHORUS DETECTION

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SUMMARY

The determination of N^{τ}-methylhistamine in urine, using gas chromatography with nitrogen—phosphorus detection and the homologue N^{τ}-ethylhistamine as internal standard, is described. A comparison between the present method and a previously described stable isotope dilution mass fragmentographic method resulted in a regression line of $Y = 0.023 + 0.944X$ μmol/l with a correlation coefficient of 0.996.

The 24-h excretions of 35 normal adults on a free diet ranged from 0.4 to 1.8 μmol. Patients with mastocytosis, chronic myelocytic leukemia, anaphylactic reactions and a patient after bronchial provocation showed above normal values.

INTRODUCTION

Histamine, which is synthesized and stored by mast cells and basophils, is an important mediator of immediate hypersensitivity reactions. In man, the major metabolic pathway for the rapid degradation of histamine includes N-methylation, catalyzed by histamine methyltransferase (EC 2.1.1.8), leading to N^{τ}-methylhistamine. (For the nomenclature of substituted imidazoles see ref. 1.) About 70% of subcutaneously injected histamine is primarily metabolized by this methylation pathway, followed by subsequent deamination and dehydrogenation to N^{τ}-methylimidazoleacetic acid [2]. Both N^{τ}-methylhistamine and N^{τ}-methylimidazoleacetic acid are excreted in the urine.

In order to study the role of histamine during various pathological pro-

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cesses, several methods for the determination of histamine and histamine metabolites in body fluids have been described [3—6]. Notably those investigations in which the determination of histamine in plasma have been used, are hampered by the rapid metabolic degradation of histamine, resulting in large fluctuation in concentration within a short period of time [7]. As most studies have the aim to determine an increased production or liberation rate of histamine, it seems more practical to determine the major metabolites of histamine in the urine. In this respect the determination of N^{τ}-methylhistamine in urine has proved to be a useful parameter for the diagnosis and the follow-up of various pathological states in which histamine is involved, including mastocytosis, anaphylactic reactions, bronchial obstructive reactions, chronic myelocytic leukemia and bacterial overgrowth [3, 8].

The methods of determination of N^{τ}-methylhistamine described until now have been based on thin-layer chromatographic separation followed by fluorometric estimation of its dinitrofluorobenzene derivative [9], high-performance liquid chromatographic separation with fluorometric detection of dansylated N^{τ}-methylhistamine [10], and gas chromatographic separation in combination with mass fragmentographic quantitation of a perfluoroacylated derivative [3, 11]. These methods are fairly time-consuming, or need expensive apparatus like a mass spectrometer.

In this paper we report an assay of N^{τ}-methylhistamine in urine by gas chromatography with a nitrogen—phosphorus selective detector, which is more adequate for use in routine clinical laboratories. The results of the present method were compared to those obtained by the previously described determination of N^{τ}-methylhistamine, using stable isotope dilution mass fragmentography [3].

MATERIALS

N^{τ}-Methylhistamine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; diethylsulphate was from Fluka A.G., Buchs, Switzerland; heptafluorobutyric anhydride was from Pierce Chemical Co., Rockford, IL, U.S.A.; all other organic solvents and reagents were of analytical grade from Merck, Darmstadt, G.F.R.

METHODS

Synthesis of N^{τ}-ethylhistamine

The internal standard N^{τ}-ethylhistamine was synthesized analogously to the preparation of N^{τ}-methylhistamine-d_3 [3], using diethylsulphate instead of dimethylsulphate-d_6.

Preparation of standards

A standard solution, containing 20 μmol/l N^{τ}-methylhistamine in 0.01 mol/l HCl was prepared and stored at 4°C. From the internal standard a stock solution was prepared containing 50 μmol/l N^{τ}-ethylhistamine in 0.01 mol/l HCl. Various amounts of the standard solution, corresponding to 0, 1, 2, 4, 6 and 8 nmol of N^{τ}-methylhistamine, were pipetted into 15-ml glass stop-

pered tubes. After the addition of 100 μmol (5 nmol) of the internal standard solution, the mixtures were evaporated to dryness at 120°C under a stream of air. All pipetting was done with Oxford adjustable push-button pipettes with polypropylene disposable tips.

Extraction of urine samples

To 4 ml of urine, 0.1 ml of 6 mol/l HCl and 100 μl of the internal standard solution were added. The solution was mixed and the volume reduced to about 0.5 ml under a stream of air at 120°C. After cooling, 0.25 ml of a 10 mol/l NaOH solution and 0.3 g of NaCl were added. The solution was shaken, and extracted twice with 4 ml of chloroform by vortexing for 5 min and subsequent centrifugation. The combined chloroform layers were dried over anhydrous sodium sulphate. After the addition of 0.1 ml of acetic acid, the solution was evaporated to dryness at 40°C under a stream of nitrogen.

Derivatization

To the dry sample (standard or extract of urine) in a 15 ml glass stoppered tube, 0.1 ml of heptafluorobutyric anhydride and 0.1 ml of acetonitrile were added. The tubes were capped and incubated overnight at room temperature.

Isolation of the derivatives

The solutions were evaporated to dryness at room temperature under a stream of nitrogen, and the residues dissolved in 1 ml of 0.75 mol/l sodium phosphate buffer pH 7.0. The derivatives were extracted into 4 ml of ethyl acetate by vortexing for 2 min. After centrifugation the aqueous layers were discarded. To the ethyl acetate 1 ml of 0.1 mol/l HCl solution was added, and the derivatives containing an imidazole moiety back-extracted into the aqueous layer. The ethyl acetate layers were discarded, and the aqueous layers adjusted to pH 7.0 by the addition of 1 ml of 0.75 mol/l sodium phosphate buffer pH 7.0. Four millilitres of ethyl acetate were added, and after vortexing and centrifugation, the aqueous layers discarded.

The ethyl acetate layers were dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen at room temperature. The residues were dissolved in 100 μl of ethyl acetate, and 2-μl aliquots injected into the gas chromatograph.

Gas chromatography

Gas chromatography with nitrogen—phosphorus selective detection was performed with a Hewlett-Packard 5880 A gas chromatograph, equipped with a Model 7672 A autosampler and a 25 m × 0.22 mm I.D. SP-2100 coated, Carbowax-deactivated, fused silica column (Hewlett-Packard). The gas chromatographic conditions were as follows: injector temperature 260°C; oven temperature starting at 160°C for 6 min, then programmed to 220°C at 20°C/min, followed by 4 min at 220°C; detector temperature 300°C. The carrier gas was helium, split ratio 1:15 and the flow-rate 0.45 ml/min. The quartz insert of the injector was filled with a plug of glass wool and deactivated by rinsing with a solution of 0.05% (w/v) Carbowax 20M in chloroform and drying at 150°C.

Mass fragmentographic determination of N^{τ}-methylhistamine

The determinations of N^{τ}-methylhistamine by isotope dilution mass fragmentography were performed as described previously [3] with the following minor modifications: a Finnigan MAT 44-S mass spectrometer equipped with a Varian 3700 gas chromatograph was used under the conditions, split ratio 1:5, ionizing energy 70 eV, ion source temperature 200°C. The standard Finnigan MAT interface (open split coupling) was used.

RESULTS AND DISCUSSION

Methodology

The problems associated with the gas chromatographic analysis of imidazole compounds have been discussed previously [3, 4]. Adsorption, resulting in peak tailing and memory effects can be overcome either by introducing an acyl group into the imidazole ring, or by using a chromatographic system deactivated by Carbowax 20M. In the isotope dilution mass fragmentographic determination of N^{τ}-methylhistamine [3], the first solution was selected. The reaction of N^{τ}-methylhistamine with heptafluorobutyric anhydride (HFBA) in the presence of pyridine results in bis-HFB-N^{τ}-methylhistamine [3]. While investigating the use of this derivatization for the gas chromatographic analysis of N^{τ}-methylhistamine with the homologue N^{τ}-ethylhistamine as internal standard, it was found that this reaction often gave rise to varying yields for each compound. It was decided therefore to use the mono-HFB derivatives, which are quantitatively formed at room temperature. These derivatives, in combination with a Carbowax 20M deactivated insert and a Carbowax deactivated fused-silica capillary column, gave symmetrical gas chromatographic peaks, both of N^{τ}-methylhistamine-HFB and of N^{τ}-ethylhistamine-HFB.

Using a simple chloroform extraction from NaCl-saturated, alkalinized urine, many amines are extracted and subsequently derivatized to their HFB derivatives. When such a crude derivatized extract is further purified by adding 1 ml of phosphate buffer pH 7.0 and extraction with 4 ml of ethyl acetate, a chromatogram as shown in Fig. 1a is obtained. Indeed, many peaks are observed, making the determination of N^{τ}-methylhistamine with N^{τ}-ethylhistamine as internal standard troublesome.

In the same way as described for the determination of N^{τ}-methylimidazoleacetic acid, it was thought it would be possible to exploit the typical pK value of the imidazole ring for a direct clean-up of the crude derivatized extract [4]. In this method apolar interfering impurities were separated from the protonated imidazole-ring-containing compounds, by their selective extractability in an organic solvent at pH 1.0. However, such a clean-up procedure of the HFB derivatives at pH 1.0, using ethyl acetate as organic solvent, resulted in an almost quantitative extraction of N^{τ}-methylhistamine-HFB and the internal standard into the organic phase. In studying this phenomenon we found that ion-pair extraction of protonated substituted histamines and the heptafluorobutyrate anion occurred. To prevent the formation of this ion pair we investigated two post-derivatization clean-up methods. In the first method a methylation step for heptafluorobutyric acid was introduced.

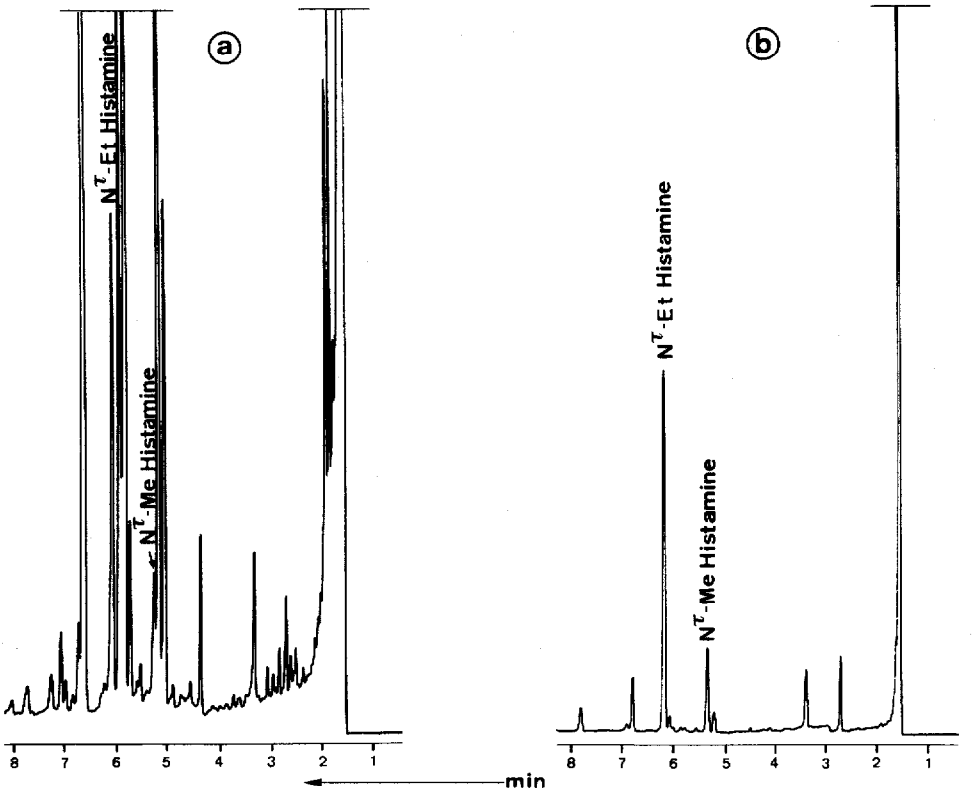

Fig. 1. Gas chromatograms of a urine of a normal person without a clean-up procedure after derivatization (a), and with an additional clean-up procedure after derivatization (b). The peak of N$^{\tau}$-methylhistamine corresponds to an amount of about 1.3 pmol on column.

To the derivatized and evaporated extract, 1 ml of 1 mol/l HCl in methanol was added and the mixture heated at 60°C for 1 h. The solution was evaporated to dryness at room temperature under a stream of nitrogen. In the second method an extraction of the derivatives with ethyl acetate and buffer pH 7.0 was included, prior to the extraction at pH 1.0. In this preextraction the heptafluorobutyrate anion will remain in the aqueous layer. Both methods resulted in a considerably clean-up, with a high recovery of N$^{\tau}$-methylhistamine-HFB and the internal standard. For routine analysis we prefer the second method in which three extraction steps at pH 7.0, pH 1.0 and pH 7.0 are used. A chromatogram of the same urine as shown in Fig. 1a, processed by the procedure described above, is shown in Fig. 1b. The overall recovery of N$^{\tau}$-methylhistamine and N$^{\tau}$-ethylhistamine amounted to 60%. Using this procedure, in combination with capillary gas chromatography and nitrogen—phosphorus detection, a specific and sensitive assay was obtained, without interference from extraneous compounds.

The calibration graph for the determination of N$^{\tau}$-methylhistamine in urine was linear in the range investigated (0—8 nmol). An example of the typical data for a regression line is $Y = 0.197X - 0.001$ (Y = amount of N$^{\tau}$-methylhistamine, X = peak height ratio), $r = 0.999$. When it was expected or found

that the concentration of N^{τ}-methylhistamine in urine was more than 2 μmol/l, 1 ml or less of this urine was taken instead of 4 ml in the extraction procedure.

The within-day reproducibility of this method was investigated by analyzing 20 aliquots of a pool urine. A concentration of 0.809 ± 0.028 μmol/l (mean ± 1 S.D.) was found, corresponding to a coefficient of variation of 3.5%. For the day-to-day variation these data were 0.801 ± 0.037 μmol/l (n = 10), coefficient of variation 4.7%. As a quality control in each series, two samples of a pool urine were incorporated, to one of which 4 nmol of N^{τ}-methylhistamine were added. When a difference greater than 2 S.D. between the value of this pool and the mean value, or a relative recovery of the spiked pool beyond 90—110% was found, the series was re-analyzed.

Correlation of the gas chromatographic and mass fragmentographic methods

To investigate the correlation between the gas chromatographic determination of N^{τ}-methylhistamine and the stable isotope dilution mass fragmentographic method previously described [3], 25 urine samples in the normal range (0.1—1.5 μmol/l) were analyzed by both methods. Fig. 2 shows the results of this correlation study. The regression line was calculated by the Deming method, as discussed by Wakkers et al. [12]. The correlation was found to be satisfactory, indicating that the present method is a good alternative to the mass fragmentographic determination.

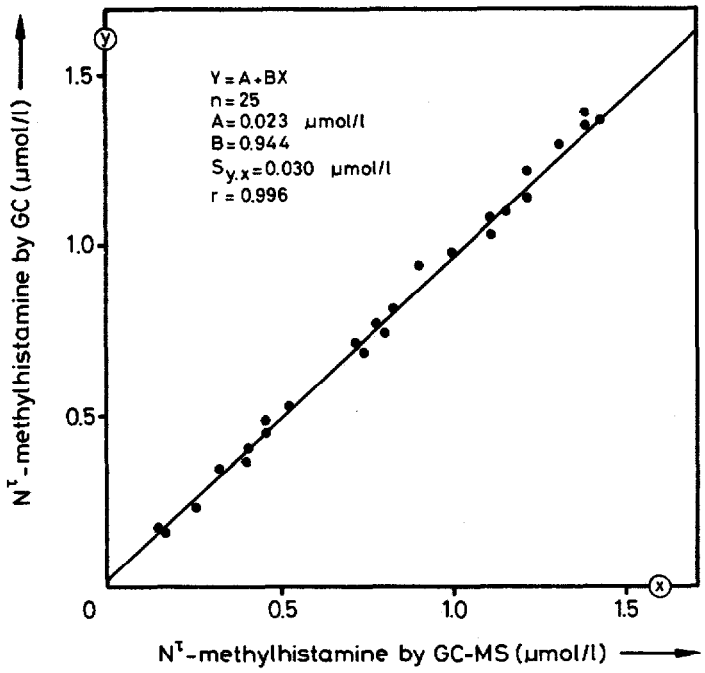

Fig. 2. Correlation between urinary N^{τ}-methylhistamine values obtained by a mass fragmentographic method (GC—MS) and the present gas chromatographic method (GC).

Urinary excretions of N^{τ}-methylhistamine

Urines were kept refrigerated during the collection period, and frozen at −20°C until assayed. Urines sent from elsewhere were collected in 6 mol/l HCl (10 ml/l urine). The N^{τ}-methylhistamine in urine stored at −20°C remains stable for at least six months. When non-preserved urines were kept at room temperature for several days, we observed in some cases a considerable increase in the N^{τ}-methylhistamine concentrations. This phenomenon was attributed to bacterial growth.

Since histamine may be present in the diet or may be synthesized from histidine by microorganisms in the lumen of the alimentary tract, Granerus [13] suggested that the investigations of histamine metabolism in man should be performed under standardized dietary conditions. To study the dietary influence in the N^{τ}-methylhistamine excretion, the 24-h excretions were determined in a group of eight normal persons, on a free diet and on a standardized diet. The standard diet, which was used the day before and on the day of the urine collection, contained 55 g of protein, and no bacterially processed foodstuffs like cheese or sauerkraut. The mean 24-h excretion of N^{τ}-methylhistamine on the free diet amounted to 1.27 μmol, range 0.85—1.57 μmol. Under the dietary conditions described above the values were 1.03 μmol and range 0.74—1.33 μmol. The difference between the excretion with and without a prescribed diet shows that, depending on the nature of the histamine investigation, it may be preferable to use dietary conditions.

In order to permit the measurement of N^{τ}-methylhistamine in urines collected over short time periods, N^{τ}-methylhistamine excretion may be expressed in relation to urinary creatinine. Table I shows the N^{τ}-methylhistamine excretions of some patients with an increased production or liberation rate of histamine. All patients were on a free diet. The normal range is based on

TABLE I

EXCRETION VALUES OF N^{τ}-METHYLHISTAMINE DURING VARIOUS PATHOLOGICAL STATES

Diagnosis	Specimen (h)	Urinary N^{τ}-methylhistamine	
		μmol per 24 h	μmol/mol creatinine
Systemic mastocytosis	24	29.40	5160
Systemic mastocytosis	24	16.10	1456
Systemic mastocytosis	24	8.92	610
Chronic myelocytic leukemia	24	103.0	10360
Chronic myelocytic leukemia	24	9.10	855
Chronic myelocytic leukemia	24	7.32	814
Anaphylactic reaction to:			
Acetylsalicylic acid	2		520
Glafenine	3		382
Iodamide	1		423
Iodamide	1		368
Iodamide	1		274
Normal range		0.4—1.8	40—160

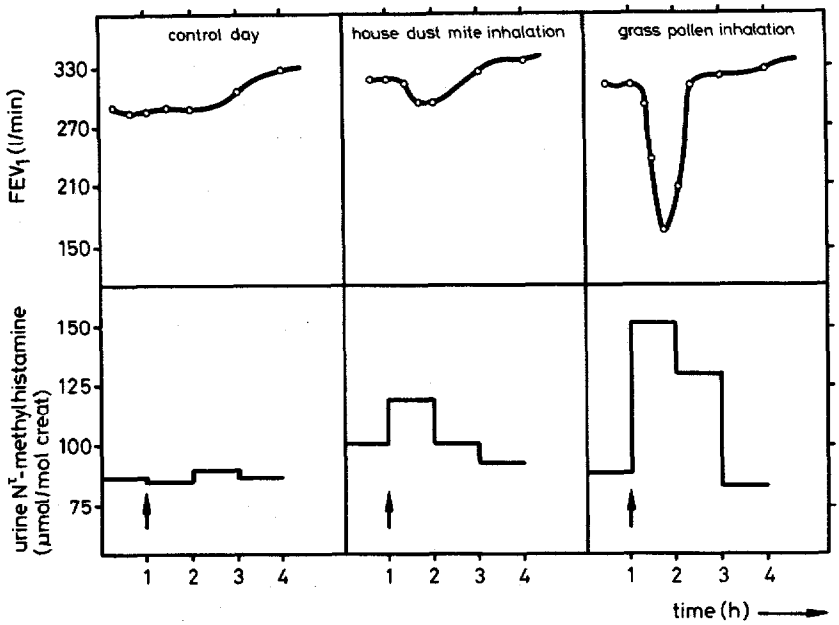

Fig. 3. The relationship between lung function (FEV_1) and urinary N^{τ}-methylhistamine excretion under different allergen challenges in the same patient. The arrows indicate the start of the 10-min allergen inhalation.

the determination of the excretions of 35 healthy adults on a free diet. The urines of the patients with an anaphylactic reaction were collected over a period directly after the onset of the reaction. The excretion of N^{τ}-methylhistamine of an asthmatic patient, on a control day and after bronchial provocation reactions, in relation to a parameter for the lung function, the forced expiratory volume during 1 sec (FEV_1), is shown in Fig. 3. The urines were collected in 1-h fractions. The association between the N^{τ}-methylhistamine excretion and the FEV_1 value was in agreement with the experiences of Löwhagen et al. [8].

In summary, it can be concluded that the described method permits the accurate determination of N^{τ}-methylhistamine in urine for clinical-chemical purposes, without the need of expensive gas chromatographic—mass spectrometric equipment. Although the extraction and clean-up procedures are somewhat more laborious than those of the formerly described mass fragmentographic procedure [3], in our hands it was possible to analyse up to 40 urine samples within one day by one technician, making use of an autosampler.

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