GAS CHROMATOGRAPHIC ANALYSIS OF HISTAMINE METABOLITES IN URINE

QUANTITATIVE DETERMINATION OF RING METHYLATED IMIDAZOLE-ACETIC ACIDS IN HEALTHY MAN

RICHARD THAM
Department of Toxicology, Swedish Medical Research Council and Department of Pharmacology, Karolinska Institutet, Stockholm (Sweden)
(Received December 22nd, 1965)

One method of studying the endogenous production and release of histamine in man is to estimate the excretion of the amine and its metabolites in urine. Studies on urinary metabolites of subcutaneously administered ^{14}C-histamine have elucidated the catabolic pathways of the amine[1]. About 50 % of injected labelled histamine is excreted as 1-methylimidazole-4-acetic acid (1,4-MeImAA), about 30 % as imidazoleacetic acid, part of which is coupled with ribose, 5 % as 4-(2-aminoethyl)-1-methylimidazole (1,4-methylhistamine) and only about 2 % as unchanged histamine. These studies with labelled material unfortunately do not give any quantitative information about the endogenous production and liberation of histamine.

Several investigators have estimated the excretion of unchanged histamine in an attempt to study the endogenous liberation in physiological and pathological conditions (for a review see ref. 2). Since, however, most of the histamine is transformed into other products, this determination gives a poor indication of histamine production.

Imidazoleacetic acid can be estimated and it has been found to be a normal constituent of urine in man[3,4]. The urinary excretion of this acid is influenced by the histidine contents in food[4,5] and is thus not suitable for studies of the liberation and production of histamine. Methods for estimating the excretion of 1,4-methylhistamine have also been published[6,7].

In previous papers from our laboratory 1,4-MeImAA has been identified by means of gas chromatography as a normal constituent of human urine[8,9]. The metabolite has also been identified in urine with paper chromatographic methods[10]. Since 1,4-MeImAA, according to the isotopic experiments referred to above, is the main metabolite of histamine, it is very likely that the estimation of the excretion of this acid will give a good insight into the endogenous liberation and production of histamine. The present investigation deals with the 24 h excretion of 1,4-MeImAA in healthy human subjects.

In a previous investigation 1-methylimidazole-5-acetic acid (1,5-MeImAA) was shown to be a normal constituent of human urine[9]. The daily excretion of this isomer is also estimated in the present study in order to throw some light on its origin.

EXPERIMENTAL

Materials

For the materials used, see ref. 9.

Separation and esterification of imidazoleacetic acids in urine[9]

Urine was collected in bottles containing hydrochloric acid in amounts sufficient to bring the pH of the urine below 2. The urine was stored at —20° before analysis. An aliquot of urine corresponding to 40–100 mg of creatinine was concentrated *in vacuo* at 50° to 15–20 ml, the pH was brought to 8.8 by addition of 5 *N* NaOH and the volume adjusted to 25 ml. After centrifugation, the supernatant was applied on an ion exchange column, Dowex 1 × 10, 200–400 mesh, acetate form (prepared as in ref. 9), 400 mm × 16 mm i.d. The flow rate was held constant at 15 ml/h by a peristaltic pump at the outlet end of the column. The column was run at room temperature. After the urine had been adsorbed on the column, it was washed with 25 ml of water. Elution was then started with 0.5 *M* acetic acid, the effluent being collected in portions of 5 ml. The pH of the fractions dropped rather suddenly from 4–5 to less than 3. The three fractions immediately preceding this point and the seven consecutive fractions following it were combined in a 100 ml pear-shaped glass flask and freeze-dried. To the dry residue was added 15 ml of methanolic hydrogen chloride[9] and the flask was immediately fitted with a reflux condenser, dried at 150° before use and topped with a calcium chloride drying tube. The solution was then refluxed for 3 h at 90° in an oil bath. It was then cooled in an ice bath and neutralized by rapid addition of a 20% aqueous solution of sodium carbonate. It was evaporated *in vacuo* at 40° to dryness and the residue dissolved in 5 ml of phosphate buffer, *M*/15, pH 8.0. The solution was transferred to a continuous extraction apparatus, the glass flask was rinsed with another 5 ml of phosphate buffer, which was then added to the extraction tube. The solution was then extracted for 4 h with 40 ml of chloroform at 80°. The chloroform extract was collected in a 100 ml pear-shaped glass flask, 2.0 ml of an ethanolic solution of 1-methylimidazole-4-acetonitrile (0.1 mg/ml) was added as internal standard and the solution evaporated *in vacuo* at 40°. The residue was dissolved in 0.1–0.3 ml of methanol and stored at —20° prior to the gas chromatographic analysis.

Gas chromatography

Gas chromatographic analysis was performed with an F & M Model 400 apparatus equipped with a hydrogen flame ionization detection system. The columns consisted of 2.6 m or 3.4 m × 3.2 mm glass tubes, and contained 10% ethylene glycol adipate (EGA) on 100–120 mesh Gas Chrom P, silanized and coated with 1% polyvinylpyrrolidone. The preparation of the column, and the gas chromatographic conditions, were the same as desribed in a previous paper[9]. About 7 μl of the urine extract was injected with a Hamilton syringe. For quantitative determinations, the areas of the peaks representing 1-methylimidazole-4-acetonitrile, 1,4-MeImAA (methyl ester) and 1,5-MeImAA (methyl ester) were measured (height × width of the peak at half height). From the calibration curve (see below) the amount of methyl ester of 1,4-MeImAA and 1,5-MeImAA in the urine extract was determined. The corresponding amount of free acid was calculated, correction being made for losses

occurring during the procedure (the amount of methyl ester was multiplied by the factor (140 × 100)/(154 × 81) = 1.12; see Results and Discussion).

Synthesis of reference compounds

1-Methylimidazole-4-acetonitrile. This was synthesized according to PYMAN[11]. The picrate was converted to the free base by running through an ion exchanger, Dowex 1, converted to the OH^--form.

Methyl ester of 1-methylimidazole-4-acetic acid. 0.55 g of 1-methylimidazole-4-acetic acid hydrochloride was dissolved in a small amount of methanol and cooled to +4°. An ethereal solution of diazomethane[12,13] was added until the yellow colour persisted. After 15 min the excess of diazomethane was removed with a stream of nitrogen and the solution evaporated *in vacuo* at 40°. The residue was dissolved in a small amount of methanol and transferred to a microdestillation apparatus. The solvent was removed *in vacuo* and the residue then destilled at 0.05 mm Hg, bath temperature 105°. The destillate (0.15 g) consisted of a clear, oily, slightly yellow liquid.

Analysis, calculated for $C_7H_{10}N_2O_2$: C 54.5, H 6.54, N 18.17; found: C 53.6, H 7.10, N 18.01. The product proved to be gas chromatographically pure when run on two columns, EGA and F 60-Z[14]. The product also appeared as one distinct spot on thin-layer chromatograms (silica gel), developed with acetone–methanol–water (6:1:3) or in chloroform–methanol–acetic acid (12:6:1) and subsequently placed in a tank with iodine.

Methyl ester of 1-methylimidazole-5-acetic acid. This was prepared from 1-methylimidazole-5-acetic acid hydrochloride in the same way as described above, giving the ester as a clear, oily, slightly yellow liquid.

Analysis, calculated for $C_7H_{10}N_2O_2$: C 54.5, H 6.54, N 18.17; found: C 53.5, H 6.71, N 17.95. The product proved to be gas chromatographically pure when run on two different columns and appeared as one spot on thin-layer chromatograms as described above.

Preparation of calibration curve

To 2.0 ml portions of an ethanolic solution of 1-methylimidazole-4-acetonitrile (0.1 mg/ml) were added varying amounts of ethanolic solutions of the esters of 1,4-MeImAA and of 1,5-MeImAA. The solutions were evaporated *in vacuo* and the residue dissolved in 0.1–0.3 ml of methanol. This was then analyzed gas chromatographically as described above. The ratio of the areas of 1,4-MeImAA (methyl ester) and of 1,5-MeImAA (methyl ester) to that of 1-methylimidazole-4-acetonitrile was measured and a calibration curve constructed. A new calibration curve was prepared whenever a new solution of the internal standard was used or when a new gas chromatographic packing was used.

Estimation of creatinine in urine

This was done with a "Technicon Auto Analyzer"[15]. The method is a modification of the procedure of FOLIN AND WU[16].

RESULTS AND DISCUSSION

Reliability of the method

For quantitative gas chromatographic estimation of 1,4-MeImAA and 1,5-MeImAA it was found convenient to use an internal standard added to the urine extract[17,18]. This will correct for variations in concentration and volume of injected sample and for variations in instrumental response. The requirements for the internal standard are that it should separate completely on the gas chromatographic column from the methyl esters of 1,4-MeImAA and 1,5-MeImAA, but at the same time it should have a retention time within the same range as these compounds. Further it must not occur normally in the urine extract and must not be superimposed on other peaks in the chromatogram. Of several compounds, both imidazoles and others, which were tried as possible internal standards, 1-methylimidazole-4-acetonitrile was found to be the most suitable (Fig. 1). This compound gives a symmetrical peak on the EGA column at 175°. The retention time lies between those of the methyl esters of 1,4-MeImAA and 1,5-MeImAA. In order to get a complete separation from 1.5-MeImAA (methyl ester) it was necessary to use a longer column than that report-

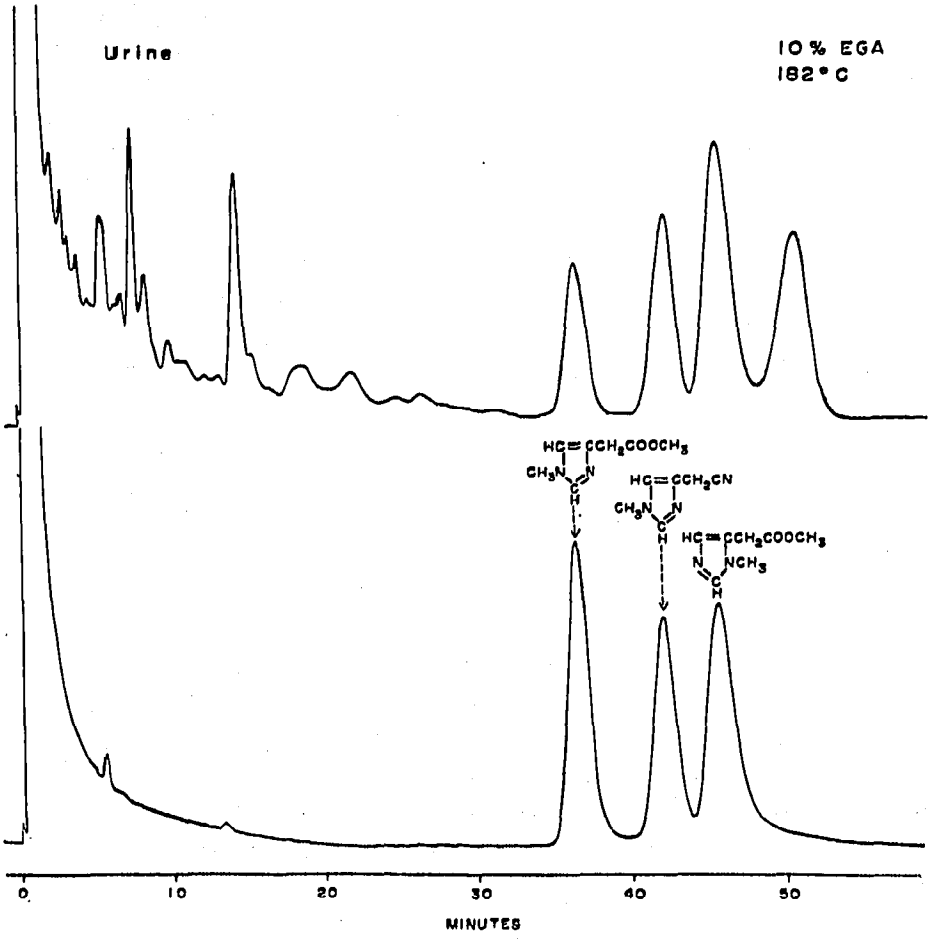

Fig. 1. Upper panel: gas chromatogram of a urine extract treated as described in the text. Internal standard (1-methylimidazole-4-acetonitrile) had been added. Lower panel: gas chromatogram of a mixture of the methyl esters of 1,4-MeImAA and 1,5-MeImAA and of the internal standard. Number of theoretical plates, calculated for 1-methylimidazole-4-acetonitrile: 3500.

ed in a previous publication[9]. 1-Methylimidazole-4-acetonitrile does not interfere with other peaks in the chromatogram, nor does it occur normally in urine extract.

The peaks of 1-methylimidazole-4-acetonitrile and of the methyl esters of 1,4-MeImAA and 1,5-MeImAA were symmetrical, and it was sufficient for quantitative determinations to calculate the product of the height and width of the peak at half height. A calibration curve was constructed as described above (Fig. 2). It is seen that the same amount of 1,4-MeImAA (methyl ester) and of 1,5-MeImAA (methyl ester) gave the same gas chromatographic response.

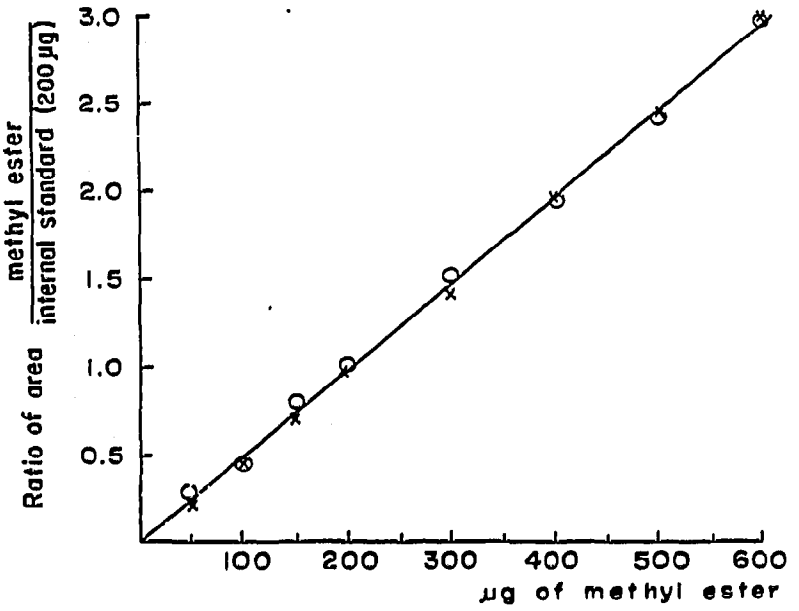

Fig. 2. Gas chromatographic calibration curve for the methyl esters of 1,4-MeImAA and 1,5-MeImAA (see text). (×) 1,4-MeImAA (methyl ester); (○) 1,5-MeImAA (methyl ester).

In order to estimate the losses during the ion exchange procedure, esterification and extraction, experiments were made with addition of known amounts of authentic 1,4-MeImAA and 1,5-MeImAA. In each experiment, two identical samples of urine were analyzed, to one of which a known amount of authentic 1,4-MeImAA and/or 1,5-MeImAA had been added. From the results of the analyses, the recovery of added acid was calculated (Tables I and II). The recovery of 1,4-MeImAA in 31 experiments was 81.6 ± 0.8 %. The recovery of 1,5-MeImAA in 12 experiments was 80.9 ± 1.8 %. No significant difference in the recovery, expressed as % of added authentic acid, was found between samples with a low and with a high content of the corresponding acids. Based upon these experiments, a value of 81 % recovery for both isomers was used throughout the investigation. This recovery is in good agreement with a previous investigation with labelled material[9], where labelled 1,4-MeImAA was added to a urine sample from which the imidazolic acids were subsequently separated and esterified almost exactly in the same way as in the present investigation. The recovery of added labelled acid was found to be about 80 %.

The reproducibility of the method was also investigated. From each of several different urines, 2–4 identical samples (corresponding to an adequate amount of creatinine) were analyzed in the usual way. The original amounts of 1,4-MeImAA and

TABLE I

RECOVERY OF 1,4-MeImAA

1,4-MeImAA found in urine sample without added authentic compound (μg)	*1,4-MeImAA found in urine sample with added authentic 1,4-MeImAA* (μg)	*Amount of added authentic 1,4-MeImAA* (μg)	*Recovery of added authentic 1,4-MeImAA* (%)
27	86	79	74.7
27	155	159	80.5
27	227	238	84.0
68	332	300	88.0
68	336	300	89.3
68	459	500	78.2
75	212	159	86.1
75	427	397	88.7
82	259	200	88.5
82	241	200	79.5
91	150	75	78.7
91	241	200	75.0
91	352	300	87.0
95	127	40	80.0
95	449	450	78.7
123	274	200	75.5
123	545	500	84.4
150	268	150	78.7
150	285	159	84.9
150	309	200	79.5
167	373	250	82.4
173	227	70	77.1
173	282	125	87.2
173	318	175	82.9
182	236	70	77.1
182	261	100	79.0
182	286	125	83.2
182	341	200	79.5
182	491	400	77.3
395	491	125	76.8
445	709	300	88.0
Mean (± standard error of the mean) 81.6 ± 0.8			

1.5-MeImAA in the samples were calculated, correction being made for losses occurring during separation and esterification. The standard deviation and the coefficient of variation were then calculated (Table III). The standard deviation was higher for 1,5-MeImAA than for 1,4-MeImAA. This probably depends on gas chromatographic factors, since in some gas chromatograms the peak corresponding to 1,5-MeImAA (methyl ester) was not completely separated from the following one, the identity of which is unknown. When this occurs, the estimation of the peak area is more uncertain. With some of the EGA-packings, the peak corresponding to 1,5-MeImAA (methyl ester) also showed a slight degree of tailing.

The 24 h excretion of 1,4-MeImAA and 1,5-MeImAA

The 24 h excretion of 1,4-MeImAA and 1,5-MeImAA by healthy individuals of either sex and of ages ranging from 19 to 65 years was determined (Table IV). Diets

TABLE II

RECOVERY OF 1,5-MeImAA

1,5-MeImAA found in urine sample without added authentic compound (μg)	*1,5-MeImAA found in urine sample with added authentic 1,5-MeImAA* (μg)	*Amount of added authentic 1,5-MeImAA* (μg)	*Recovery of added authentic 1,5-MeImAA* (%)
60	200	159	88.1
75	115	50	80.0
80	290	250	84.0
127	182	75	73.3
168	623	600	75.8
273	345	100	72.0
273	405	175	75.4
273	418	175	82.9
273	523	300	83.3
347	484	159	86.2
347	567	238	92.4
450	682	300	77.3
Mean ($\pm$ standard error of the mean) 80.9 $\pm$ 1.8			

TABLE III

ANALYSIS OF IDENTICAL SAMPLES

Experiment No.	*Amount of 1,4-MeImAA found in identical samples (values corrected for losses)* (μg)				*Standard deviation** s	*Coefficient of variation* $\frac{s \times 100}{M}$
1	79	92	92	81		
2	96	101	112	90		
3	162	162	148	146	8	4%
4	164	161	170	166		
5	197	177	184	194		
6	549	554				
	Amount of 1,5-MeImAA found in identical samples (values corrected for losses) (μg)					
1	28	43	44	38		
2	84	101	90			
3	106	103				
4	140	129				
5	171	134	151		16	9%
6	157	157				
7	207	179				
8	291	269				
9	392	354	403	352		

* s calculated according to ref. 35.

TABLE IV

EXCRETION OF 1,4-MeImAA AND 1,5-MeImAA BY HEALTHY SUBJECTS

Subject	*Age*	*24 h excretion of 1,4-MeImAA* (μg)	*μg of 1,4-MeImAA per mg of creatinine in urine*	*24 h excretion of 1,5-MeImAA* (μg)	*μg of 1,5-MeImAA per mg of creatinine in urine*
BH ♀	19	1210	1.5	700	0.9
BK ♀	19	1480	2.0	Not measurable*	
KE ♀	21	1120	1.2	Not measurable*	
MM ♀	22	3520	2.8	7580	6.1
LN ♀	22	2330	1.8	710	0.5
AL ♀	24	2420	2.2	1080	1.0
UT ♀	29	3920	2.0	4090	2.1
MH ♀	35	1570	1.6	4940	4.9
JG ♀	38	2360	1.8	1840	1.4
GN ♀	40	3550	2.8	7610	5.9
JK ♀	42	1390	1.0	3550	2.4
BP ♀	47	2100	1.2	3270	1.9
AB ♀	51	3100	2.4	2100	1.5
CH ♀	51	760	1.4	1390	2.5
IJ ♀	62	1460	1.1	560	0.4
EJ ♀	65	3030	1.5	3140	1.9
BF ♂	20	2060	1.7	9250	7.6
TH ♂	23	1560	1.7	11400	12.3
BP ♂	27	2220	1.9	2020	1.7
UA ♂	27	3020	1.7	2100	1.2
CH ♂	29	1790	1.9	Not measurable*	
KN ♂	30	4490	2.7	1110	0.7
RT ♂	30	4050	2.4	2430	1.4
TA ♂	30	1970	1.3	3720	2.5
JL ♂	34	4490	2.6	5610	3.3
RL ♂	35	4260	2.3	6730	3.6
BA ♂	38	3310	1.5	7520	3.5
EH ♂	42	4330	2.2	3080	1.6
GL ♂	44	4260	2.7	Not measurable*	
GK ♂	45	2020	2.0	1120	1.1
BH ♂	47	3610	1.6	1280	0.6
KP ♂	58	2200	1.5	1170	0.8
JA ♂	62	3100	1.4	8570	3.8
ES ♂	65	1930	1.5	810	0.6
Range		760–4490	1.0–2.8	0–11400	0–12.3
Mean ± standard error of the mean		2650 ± 190	1.9 ± 0.1	(3250)	(2.3)
Standard deviation		1100	0.5		

* "Not measurable" means amounts below 0.3 μg/mg of creatinine.

were uncontrolled. Since for various reasons collection of 24 h urine volume may be a rather inexact procedure, it was found convenient to correlate the excretion of the imidazolic acids to that of creatinine. This procedure is frequently used in studies of amino acid metabolism[19,20] and has also been used in gas chromatographic studies of phenolic acids in urine[21].

The mean 24 h excretion of 1,4-MeImAA by 34 healthy individuals was 2.65 mg

(0.76–4.49 mg; Table IV). The relation to the creatinine content of the urine was 1.9 μg (1.0–2.8 μg) per mg of creatinine. It is obvious that the excretion of 1,4-MeImAA by the examined subjects varied less when expressed in terms of creatinine ratios than when expressed in absolute amounts excreted per 24 h. No significant difference in the urinary excretion in men and women was observed, and the mean value was therefore calculated for both groups together.

GREEN *et al.* found that the 24 h excretion by healthy man of 1,4-methylhistamine, the metabolic precursor of 1,4-MeImAA, was 140–480 μg[6,7]. The molar ratio of the mean excretion of 1,4-methylhistamine to 1,4-MeImAA is thus about 1:8. This is in good agreement with experiments with labelled material. Upon injection of ^{14}C-histamine the ratio between excreted labelled 1,4-methylhistamine and 1,4-MeImAA is about 1:10[1,22].

The mean value of the daily excretion of unchanged histamine, reported by different investigators[4,7,23–29], varies from 9–49 μg. The molar ratio to the excretion of 1,4-MeImAA thus lies between 1:230 and 1:40. The ratio of ^{14}C-histamine to ^{14}C-1,4-MeImAA in urine upon injection of ^{14}C-histamine is about 1:17[1,22]. There are of course several possible explanations for this discrepancy; thus it might be possible that besides histamine, there are other precursors to 1,4-methylhistamine and 1,4-MeImAA. Another explanation may be that the excretion pattern of exogenous histamine is not the same as that of histamine from endogenous sources[30].

The 24 h excretion of 1,5-MeImAA was found to be very variable. The excretion by 34 healthy individuals varied from scarcely detectable amounts to 11.40 mg per 24 h (12.3 μg/mg of creatinine). The high degree of variation indicates that this compound may be of dietary origin. Other experiments on individuals fed parenterally give further evidence for this surmise[31]. It is unlikely that the excretion of 1,5-MeImAA reflects the endogenous liberation of histamine. One possible precursor of 1,5-MeImAA is α-amino-β-(1-methyl-5-imidazole)propionic acid (1-methylhistidine). This compound is a normal constituent of human urine[32,33] and has long been known to be a component of the dipeptide anserine[34]. It is notable that also the isomeric α-amino-β-(1-methyl-4-imidazole)propionic acid (3-methylhistidine) is excreted in human urine[33].

ACKNOWLEDGEMENTS

I thank Dr. R. BLOMSTRAND, Department of Clinical Chemistry, Serafimerlasarettet, for laboratory facilities and assistance placed at my disposal.

This research was supported by the Swedish Medical Research Council, Project No. 40X-677-91, and Reservationsanslaget, Karolinska Institutet.

SUMMARY

A gas chromatographic method for the identification of 1-methylimidazole-4-acetic acid and 1-methylimidazole-5-acetic acid in urine, previously described, has been modified to allow the quantitative estimation of the 24 h excretion of the acids by man. The mean excretion of the two acids by healthy individuals was determined. The excretion of 1-methylimidazole-4-acetic acid, expressed in terms of creatinine ratios, varied within a rather narrow range (1.0–2.8 μg/mg of creatinine). It seems

very likely that the estimation of this acid will give valuable information regarding the endogenous liberation and production of histamine. The daily excretion of the isomer, 1-methylimidazole-5-acetic acid was found to be very variable. The metabolic origin of this compound is discussed.

REFERENCES

1 R. W. SCHAYER AND J. A. D. COOPER, *J. Appl. Physiol.*, 9 (1956) 481.
2 H. DUNÉR AND B. PERNOW, *Acta Med. Scand.*, 168 (1960) 307.
3 A. HANSON, *Naturwiss.*, 44 (1957) 470.
4 H. DUNÉR, S. O. LILJEDAHL AND B. PERNOW, *Acta Physiol. Scand.*, 51 (1961) 41.
5 D. D. BROWN, O. L. SILVA, P. B. MCDONALD, S. H. SNYDER AND M. W. KIES, *J. Biol. Chem.*, 235 (1960) 154.
6 J. P. GREEN, D. H. FRAM AND N. KASE, *Nature*, 204 (1964) 1165.
7 D. H. FRAM AND J. P. GREEN, *J. Biol. Chem.*, 240 (1965) 2036.
8 R. THAM, *Life Sci.*, 4 (1965) 293.
9 R. THAM AND B. HOLMSTEDT, *J. Chromatog.*, 19 (1965) 286.
10 J. W. KERR, *Brit. Med. J.*, (1964 II) 606.
11 F. L. PYMAN, *J. Chem. Soc.*, 99 (1911) 2172.
12 A. F. MCKAY, *J. Am. Chem. Soc.*, 70 (1948) 1974.
13 C. M. WILLIAMS, *Anal. Biochem.*, 4 (1962) 423.
14 B. HOLMSTEDT, W. J. A. VANDENHEUVEL, W. L. GARDINER AND E. C. HORNING, *Anal. Biochem.*, 8 (1964) 151.
15 *Technicon Auto Analyzer, Methodology N 11 a*, Technicon Laboratories, Research Park, Chauncey, N.Y., 1963.
16 O. FOLIN AND H. WU, *J. Biol. Chem.*, 38 (1919) 81.
17 E. BROCHMANN-HANSSEN, *J. Pharm. Sci.*, 51 (1962) 1017.
18 W. J. A. VANDENHEUVEL AND E. C. HORNING, in H. A. SZYMANSKI (Editor), *Biomedical Applications of Gas Chromatography*, Plenum Press, New York, 1964, p. 89.
19 D. I. FOWLER, P. M. NORTON, M. W. CHEUNG AND E. L. PRATT, *Arch. Biochem. Biophys.*, 68 (1957) 452.
20 A. HUNTER, *Clin. Chim. Acta*, 12 (1965) 2.
21 C. M. WILLIAMS AND C. C. SWEELEY, in H. A. SZYMANSKI (Editor), *Biomedical Applications of Gas Chromatography*, Plenum Press, New York, 1964, p. 225.
22 K. NILSSON, S.-E. LINDELL, R. W. SCHAYER AND H. WESTLING, *Clin. Sci.*, 18 (1959) 313.
23 M. ROBERTS AND H. M. ADAM, *Brit. J. Pharmacol.*, 5 (1950) 526.
24 H. M. ADAM, R. B. HUNTER AND T. W. G. KINNEAR, *Quart. J. Exptl. Physiol.*, 36 (1950) 49.
25 R. G. MITCHELL, H. R. BUTT AND C. F. CODE, *J. Clin. Invest.*, 33 (1954) 1199.
26 H. DUNÉR AND B. PERNOW, *Scand. J. Clin. Lab. Invest.*, 8 (1956) 296.
27 J. A. OATES, E. MARSH AND A. SJOERDSMA, *Clin. Chim. Acta*, 7 (1962) 488.
28 H. T. GRAHAM, *Pharmacologist*, 5 (1963) 274.
29 G. N. BEALL, *Int. Arch. Allergy*, 26 (1965) 1.
30 A. V. FURANO AND J. P. GREEN, *Nature*, 199 (1963) 380.
31 R. THAM, to be published.
32 J. M. SEARLE, AND R. G. WESTALL, *Biochem. J.*, 48 (1951) PI.
33 H. H. TALLAN, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 206 (1954) 825.
34 V. DU VIGNEAUD AND O. K. BEHRENS, *Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol.*, 41 (1939) 917.
35 W. DIXON AND F. MASSEY, *Introduction to Statistical Analysis*, 2nd Ed., McGraw-Hill, New York, 1957.