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Determination of methylimidazoleacetic acids in human urine by gas chromatography

SIR,—In man, about one half of injected labelled histamine is excreted in urine as 1-methylimidazole-4-acetic acid (1,4-MeImAA) (Schayer, 1959). Tham (1965, 1966) described a gas-liquid chromatographic method for the assay of this acid and its isomer 1-methylimidazole-5-acetic acid (1,5-MeImAA), in human urine. The MeImAAs are separated from urine by ion-exchange chromatography, converted to the methyl esters, extracted into chloroform from alkaline buffer and then analysed by gas-liquid chromatography. A method for estimating the excretion of these compounds by thin-layer chromatography has also been published (Granerus & Magnusson, 1965).

This department has used Tham's method for some time with columns of nominally the same chemical specification, but removal of neutral esters from the urine extracts, by chloroform extraction from acid buffer, was needed to eliminate interfering peaks (Fig. 1A) without substantially affecting the basic MeImAA methyl esters (Fig. 1B), and as an internal standard 1-benzylimidazole proved more convenient. In view of the current interest in histamine and its metabolism, the modified technique may interest others.

Urine samples (24 hr) collected in polythene bottles containing 10 ml 12 N hydrochloric acid are stored at 1° until analysis in batches of ten. Aliquots (50–100 ml) are adjusted to pH 8·8 with 10 N sodium hydroxide and filtered. Aliquots (40–80 ml) of the filtrates are run on 40 × 1·7 cm columns of Dowex

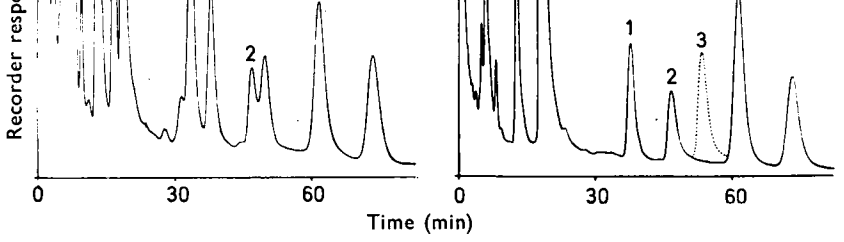

FIG. 1. A. Gas chromatogram of a urine sample purified on Dowex 1, esterified, and then extracted into chloroform from pH 8·0 buffer. Peak 1 corresponds to 1,4-methylimidazoleacetic acid, methyl ester and peak 2 corresponds to 1,5-methylimidazoleacetic acid, methyl ester.

B. Gas chromatogram of the same urine sample, prepared as for (A) above, but further purified by chloroform extraction at pH 4·0 before extraction at pH 8·0. Peaks 1 and 2 are as above; the dotted peak 3 shows the position of 1-benzylimidazole when added as internal standard.

1X8 (acetate form, washed with water until the effluents are pH $>5{\cdot}5$). Flow rates of 25 ml/hr are maintained with a 10-channel peristaltic pump (Watson-Marlow, Bucks) at the column outlets. After adsorption of the urine, the columns are washed with water (25 ml) and eluted with 0·5 M acetic acid. Fractions are collected at 60 min intervals in a collector (L.K.B. Instruments Ltd.) arranged so as to allow simultaneous collection of 10 eluates. The pH of the fractions falls slowly at first and then changes suddenly from 4–5 to <3. The fraction immediately preceding this point and the two fractions after it are combined and evaporated to dryness at 80° *in vacuo*. The dry residues are refluxed for 3 hr with $\approx$4 N hydrogen chloride in redistilled dry methanol (15 ml) under anhydrous conditions. The reaction mixtures are cooled (ice-bath), neutralized (2·5 g of solid sodium bicarbonate) and filtered through Hirsch funnels. The filtrates are evaporated to not less than 0·5 ml at 40° *in vacuo* and the residues dissolved in 3·0 M K_3PO_4/1·5 M citric acid buffer, pH 4·0 (9·0 ml) transferred to test tubes with one 1·0 ml rinse with water and shaken for 10 min with A.R. chloroform (25 ml). After centrifugation, the lower chloroform layers are removed by aspiration and discarded. This is repeated twice. The aqueous phases are made alkaline (pH 8·0) by addition of 3·0 M K_3PO_4 (7·0 ml) and shaken for 15 min with *redistilled* A.R. chloroform (25 ml). After centrifugation, the aqueous layers are removed by aspiration and the chloroform phases shaken for 2 min with anhydrous calcium sulphate ($\approx$1 g) and filtered into 25 ml measuring cylinders using Whatman No. 54 filter papers. Volume losses during filtration are taken into account when calculating the quantities of MeImAA methyl esters present. A solution of 1-benzylimidazole in chloroform (100 μg/ml) is added (0·5 ml) to each extract as an internal standard. The extracts are evaporated to about 2 ml in flasks and then to about 100 μl in conical test tubes, at 40° *in vacuo* (and excluding water vapour) on a rotary evaporator.

Extracts are analysed on a 13 ft (3·96 m) $\times$ 4 mm i.d. silanized glass column packed with 10% ethylene glycol adipate on 100–120 mesh Gas-Chrom Q coated with 1% polyvinylpyrrolidinone (Applied Science Laboratories, State College, Pennsylvania). We use a Pye 104 gas chromatograph with hydrogen flame ionization detector at 190°, a carrier gas (nitrogen) flow rate of 100 ml/min and amplifier attenuator setting 5×10^{-10} A.F.S. The instrument is calibrated as described by Tham (1966), except that 1-benzylimidazole is used as internal standard. 10 μl of extract is injected into the column packing without additional heating at the injection-point. Samples can be stored at 1° in test tubes with ground glass stoppers until analysis. Extracts have been re-assayed without change after storage for 16 weeks under these conditions.

Recovery of 1,4-MeImAA added to urine samples is 59·2% $\pm$ 1·5% (mean $\pm$ standard error of the mean, N = 18). The recovery of the 1,5-isomer has not been examined because of shortage of material, but it is assumed to be the same. Tham found this to be so for his technique. The weighted mean coefficient of variation for analysis of duplicate samples is 4% for 1,4-MeImAA and 8% for the 1,5-isomer. The lower recovery obtained with the method described, compared with Tham's, is due to loss of ester during the extraction with chloroform at pH 4·0, and the lower recovery obtained with the equilibrium extraction at pH 8·0, compared with the continuous extraction technique used by Tham.

This technique has been found more convenient in some respects than that of Tham. The pH 4·0 chloroform extraction is a complication and reduces the final recovery of MeImAA, but it does increase the specificity of the analysis.

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ADDENDUM—Since completion of this manuscript, it has been found that the pH 4·0 chloroform extraction step can be omitted if the Dowex 1 columns are eluted with 0·5M acetate buffer, pH 4·0. This gives an even cleaner gas chromatogram than Fig. 1B. Recovery and reproducibility have not yet been determined.

Effect of two non-steroidal anti-inflammatory agents on alkaline and acid phosphatases of inflamed tissue

SIR,—A large increase of phosphatase was found by histological methods to take place in inflamed tissue (Monis & Rutenburg, 1960; Georgiev & Bachvarova, 1962), but no biochemical data have been presented. Continuing our observations on naphthypramide [α-isopropyl-α-(2-dimethylaminoethyl)-1-naphthylacetamide], a new anti-inflammatory agent (Coppi & Bonardi, 1968), we report in this paper on its activity, compared to phenylbutazone, on alkaline and acid phosphatases of inflamed tissue.

Inflammation and treatments with anti-inflammatory drugs were as previously described (Coppi & Bonardi, 1968). The inflamed tissue from paw pads of animals, killed 48 hr after kaolin and 24 hr after carrageenan subplantar injection

TABLE 1. EFFECT OF NAPHTHYPRAMIDE AND PHENYLBUTAZONE ON ALKALINE AND ACID PHOSPHATASES OF INFLAMMED TISSUE OF RATS

Treatment	Dose of drug mg/kg (oral)	Alkaline phosphatase μg of P/mg N				Acid phosphatase μg of P/mg N			
		Rats No.	Mean ± s.e.	P*	P**	Rats No.	Mean ± s.e.	P*	P**
Kaolin oedema									
Normal control	—	12	71 ± 3	—	—	14	175 ± 10	—	—
Inflammation	—	16	202 ± 21	<0·001	—	9	309 ± 30	<0·001	—
Inflammation + naphthypramide	100 × 4	13	116 ± 5	<0·001	0·001 < P <0·01	8	246 ± 11	<0·001	>0·05
Inflammation + phenylbutazone	50 × 4	12	110 ± 6	<0·001	0·001 < P <0·01	8	218 ± 8	<0·001	0·01 < P <0·02
Carrageenan oedema									
Normal control	—	13	73 ± 4	—	—	9	168 ± 37	—	—
Inflammation	—	10	250 ± 16	<0·001	—	10	284 ± 28	0·02 < P <0·05	—
Inflammation + naphthypramide	80 × 3	10	173 ± 15	=0·02	<0·001	10	207 ± 12	>0·05	0·02 < P <0·05
Inflammation + phenylbutazone	40 × 3	10	134 ± 8	0·02 < P <0·05	<0·001	10	178 ± 15	>0·05	0·001 < P <0·01

* Statistical significance of difference between treated and normal controls.
** Statistical significance of difference between treated and inflamed controls.