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SENSITIVE GAS—LIQUID CHROMATOGRAPHIC PROCEDURE FOR URINARY Nτ-METHYLIMIDAZOLE ACETIC ACID, AN INDEX OF HISTAMINE TURNOVER

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SUMMARY

A simple and specific gas—liquid chromatographic procedure, compatible with both nitrogen—phosphorous and electron-capture detection, and employing conventional packed columns, has been devised for urinary 1-methylimidazole-4-acetic acid (Nτ-MeImAA), an index of whole-body histamine turnover. Nτ-MeImAA is isolated by ion-exchange on Dowex 1 (CH_3COO^-), esterified by reaction with chloroethanol—boron trichloride and, depending upon detection employed, chromatographed on base-deactivated SP-2401 or SP-2250. [^{3}H]Nτ-MeImAA serves as internal recovery standard.

INTRODUCTION

In those species, including man, in which ring N-methylation is the predominant route for the catabolism of histamine [1—3], the major urinary product is 1-methylimidazole-4-acetic acid (Nτ-MeImAA). (For nomenclature of N-substituted imidazoles, see ref. 4.) Its positional isomer 1-methylimidazole-5-acetic acid (Nπ-MeIAA) also occurs in urine, but is probably of dietary origin. Nτ-MeImAA comprises a remarkably constant fraction of urinary radioactivity following a bolus of labeled histamine [2]. While the fate of exogenous histamine may not necessarily reflect that of the biogenic material, quantitative measurements of endogenous metabolites [5, 6] support the view that Nτ-MeImAA is the most valid of several available indices of histamine formation/release in man.

The advent of α-fluoromethylhistidine (α-FMH), a specific, irreversible inhibitor of histidine decarboxylase [7], provides a therapeutic approach to histamine-related disease states alternative or adjunctive to histamine receptor

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antagonism. In animals, direct measurement of tissue histamine demonstrates that α-FMH effects acute reductions in the nascent or rapidly turning-over pools of brain [8, 9] and stomach [8, 10], and upon chronic treatment, time-dependent decreases in the relatively inert mast-cell pools (unpublished). As a non-invasive methodology for assessing effects of α-FMH in man, urinary Nτ-MeImAA appears to be a feasible alternative.

A number of procedures for urinary Nτ-MeIAA have been described, all but the last are too cumbersome for routine application. Gas chromatographic methods with flame-ionization detection of Nτ- and Nπ-MeIAA's as methyl esters [6, 11] and ethyl esters [12] require large urine volumes and long run times. Photometric methods employing thin-layer chromatographic separations [13, 14] are semi-quantitative. The capillary gas chromatographic method with nitrogen detection of the isopropyl ester described by Keyzer et al. [15] is not, in our hands, adaptable to the more convenient and accessible packed columns.

The present report describes a procedure, suitable for routine analysis of urinary Nτ-MeImAA as its chloroethyl ester, using packed columns and either of two sensitive detection systems, nitrogen—phosphorous detection (NPD), or electron-capture detection (ECD).

EXPERIMENTAL

Reagents

Nτ-Methylimidazole hydrochloride was obtained from Calbiochem; [^{3}H]-histamine from New England Nuclear; AG1-X8, 100—200 mesh was purchased in the Cl^- form from Bio-Rad Labs. and successively converted through the OH^- and CH_3COO^- forms as per manufacturer's instructions; boron trichloride—2-chloroethanol reagent (10%, w/v) was purchased from Applied Science Labs.; and ethyl acetate, nanograde, from Mallinkrodt.

Preparation of [^{3}H] Nτ-methylimidazole acetic acid.

Each of three mice were injected intravenously with ca. $6 \cdot 10^7$ dpm of [^{3}H(G)] histamine, 8.5 mCi/μmol, and urine collected through 24 h. This was adjusted to pH 8 and chromatographed on AG1-X8 (CH_3COO^-) (see below), 58% of total count being recovered in the acetic acid eluate. This was evaporated to dryness and streaked on Whatman K6 silica gel plates, which were developed with *n*-butanol—toluene—methanol—5% ammonium hydroxide (2:1:1.3:1). The major band corresponding to [^{3}H] Nτ-MeImAA was located by autoradiography at R_F = 0.47 and eluted with methanol to yield a radiochemically pure stock solution ($12 \cdot 10^6$ dpm/ml) of indeterminate but high specific activity, such that spiking of urine samples with 20,000 dpm thereof introduces a negligible mass of the compound of interest.

Isolation and derivatization of MeImAA

All manipulations through injection into the gas chromatograph are carried out in a single 125 × 16 mm disposable culture tube. To a 2.0-ml aliquot of urine are added 20,000 dpm of internal standard, a drop of 0.1% thymol blue indicator, and 2 *M* ammonium hydroxide to pH 8—9. The sample is decanted onto a 2.0-ml bed of AG1-X8 (CH_3COO^-) in a Bio-Rad disposable poly-

propylene column (No. 731-1550), followed by a wash of ca. 10 ml of distilled water, the perchlorate and wash then being discarded. The columns are positioned onto the original tubes and eluted with 5 ml of 0.75 *M* acetic acid. The tubes are transferred to a Model 3-2200 Buchler Vortex evaporator fitted with the 56-place heating block at 80° C and evaporated to dryness in vacuo. To the dried residue is added 200 μl of the chloroethanol—boron trichloride reagent, the block covered, and temperature raised to maximum setting while maintaining slight negative pressure to insure sealing. After 60 min, agitation and full vacuum are applied to reduce the reaction mixture to dryness.

After cooling, the residue is dissolved in 1.0 ml of 0.2 *M* hydrochloric acid, which is twice washed by vortexing with 5-ml portions of ethyl acetate, made alkaline with 100 μl of 6 *M* ammonium hydroxide, and extracted with 5 ml of ethyl acetate. A 0.5-ml aliquot of the final extract is taken for scintillation counting, and a second aliquot promptly sealed in an injection vial for gas—liquid chromatography (GLC).

If the samples cannot be chromatographed within 24 h, the bulk of the final ethyl acetate extract is transferred to a clean tube, evaporated to dryness in vacuo, and reconstituted shortly before analysis in a volume of ethyl acetate that is one-fifth of the original. A corresponding reduction is made in the aliquot taken for counting and for injection into the gas chromatograph.

For construction of a standard curve, a pooled urine sample was constituted by mixing equal volumes of every fifth urine specimen, and 2.0-ml aliquots thereof spiked in duplicate with unlabeled Nτ-MeImAA at 0, 0.5, 1, 2, 3 and 5 μg/ml. These were thereafter treated in an identical manner with the unknown samples.

GLC conditions

An HP-5840A instrument fitted with a 36-place automatic sampler and both nitrogen—phosphorous and electron-capture detectors was employed. The following refers to use of NPD. For ECD, the same operating conditions apply except for column packing (SP-2250-DB), carrier gas (argon—methane, 95:5), and less stringent conditioning of the base-deactivated packing.

An 180 cm × 2 mm column of 3% SP-2401-DB on Supelcoport 100—120 mesh was conditioned at 250° C for three days with helium flow-rate at 30 ml/min, with periodic injections of 25 μl ethyl acetate, and a final injection of 20 μl Silyl-8®.

For analyses, helium flow-rate was at 20 ml/min, injector temperature 250° C, detector at 300° C, and oven temperature programmed for 5 min at 205° C then 5° C/min to 225° C, plus 3 min at the final temperature. Each sample was injected twice, with the automatic sampler adjusted to deliver 3 μl. Thus, a 24-h run will accommodate a standard curve series plus thirty unknown specimens, the preparation of which can readily be accomplished by one person in the course of a single working day.

RESULTS

In Fig. 1 are presented specimen chromatograms for a pure Nτ-MeImAA sample, a representative urine, and the same urine spiked with a known concen-

tration of Nτ-MeIAA. That the urine peak at retention time, t_R = 2.72 min represents Nτ-MeImAA is confirmed by superimposition of it and the reference compound in the spiked sample. By analogy to the respective retention times of the Nτ- and Nπ-isomers of MeImAA in other systems [11, 15], it might be assumed that the urine peak at t_R = 4.42 min represents Nπ-MeIAA. This is confirmed by results of the GLC—mass spectrometric (MS) analyses described below.

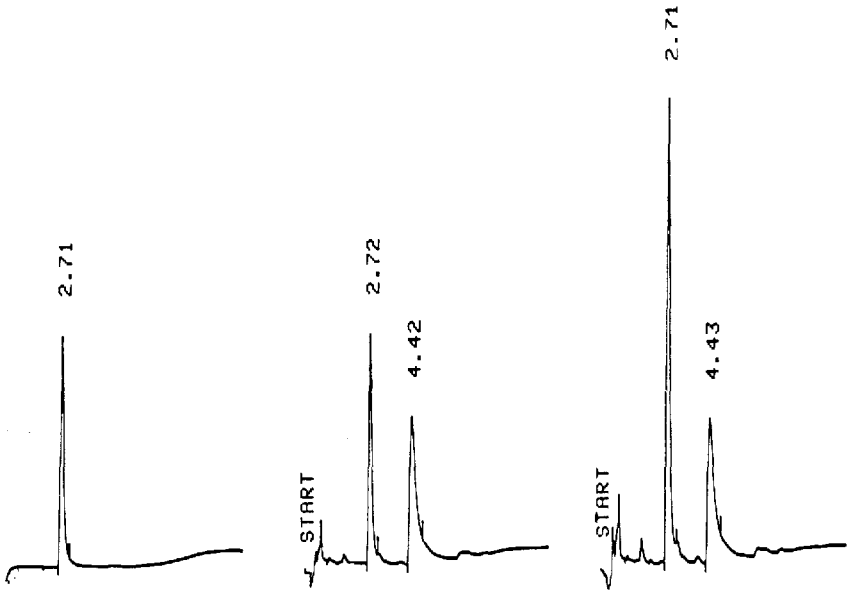

Fig. 1. Gas chromatograms (NPD) for, left to right: reference Nτ-MeIAA, 1.0 μg/ml in buffer; representative urine; and urine spiked with 1.0 μg/ml Nτ-MeIAA. Values above peaks denote retention times in minutes.

The same urine sample was analyzed by capillary GLC—MS employing an on-line HP-5710 gas chromatograph with a 25-m OV-17 fused silica capillary column, temperature programmed from 60°C to 230°C at 8°C/min, with helium as carrier gas at a flow-rate of 1 ml/min and interfaced to a VG—MM7035 mass spectrometer with dedicated 2035 data system. MS operating conditions were: ionization energy, 20 eV; accelerating voltage, 4 kV; and scan rate, 0.3 sec/decade.

A plot of total ionization current revealed surprisingly few peaks, major ones being centered at scan Nos. 1051 (22.4 min) and 1081 (22.8 min) (Fig. 2a). The mass chromatogram constructed for m/z = 202 (the molecular ion for either isomer of MeIAA) shows the same two peaks (Fig. 2b). Their respective mass spectra (Fig. 3a and b) show the same molecular ion at 202 with characteristic chlorine isotope cluster, and base peak at m/z = 95 without it, corresponding to removal of —CO · OCH_2CH_2Cl. The pronounced tailing of the t_R = 4.42 min peak in the gas chromatogram is also evident in the mass chromatogram peak at t_R = 22.8 min, lending further support to its identification as Nπ-MeIAA.

A typical standard curve for pooled urine spiked with authentic Nτ-MeIAA

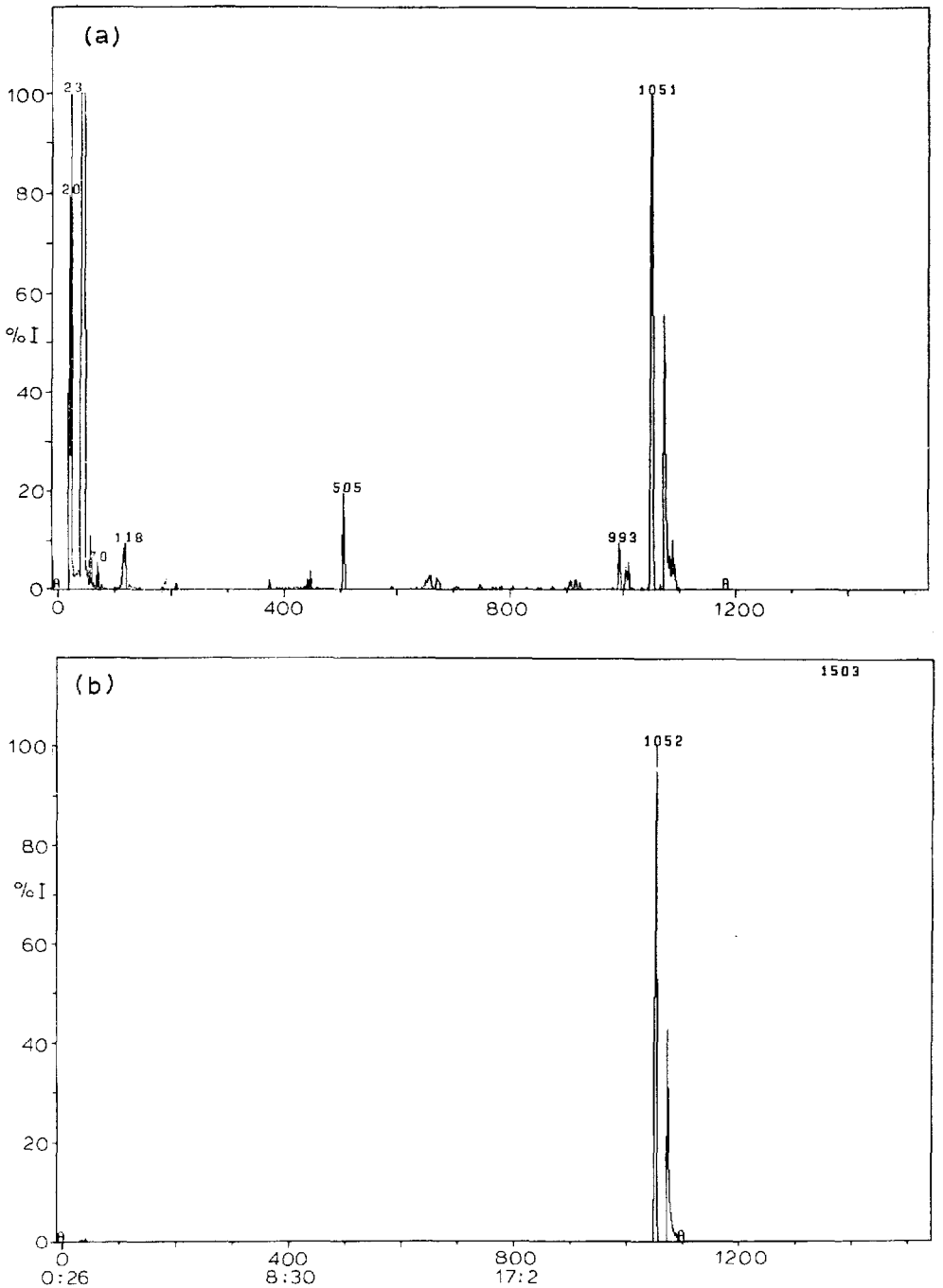

Fig. 2. Capillary gas chromatograms with MS detection for same urine as in Fig. 1. (a) total ionization current; (b) mass chromatogram constructed for molecular ion m/z = 202.

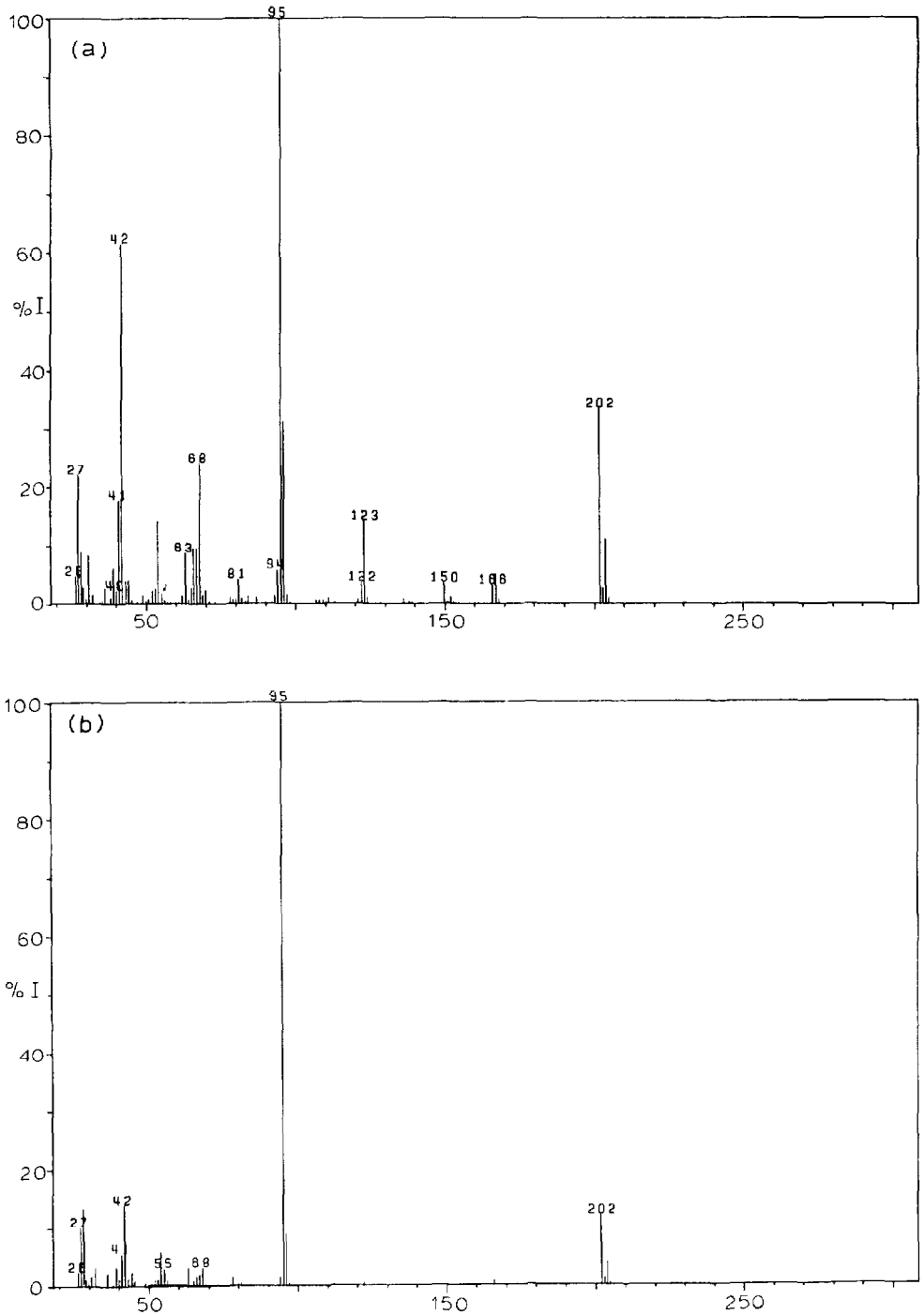

Fig. 3. Mass spectra for scans (a) No. 1052 (Nτ-MeImAA) and (b) No. 1081 (Nπ-ImAA).

(Fig. 4) demonstrates linearity between 2.87 μg/ml (the calculated endogenous level) and 7.87 μg/ml, the upper limit of which is in excess of all but one urine analyzed to date. For pure samples of Nτ-MeIAA in water, significant negative deviation from linearity occurs below 0.25 μg/ml.

Precision and reproducibility of the method were assessed by ten replicate analyses of two pooled urine samples on each of two occasions. For this purpose, as in routine use, standard curves were constructed from fresh dilutions of a frozen reference standard of Nτ-MeIAA (1 mg/ml), and detector sensitivity normalized with respect to fresh dilutions of a solution of Nτ-MeImAA chloroethyl ester, 830 μg/ml (by ^{14}C label). Intra-assay precision averaged 4.9%, and reproducibility between assays was < 1% (Table I), insuring the validity of storing of biological samples in the frozen state until assay.

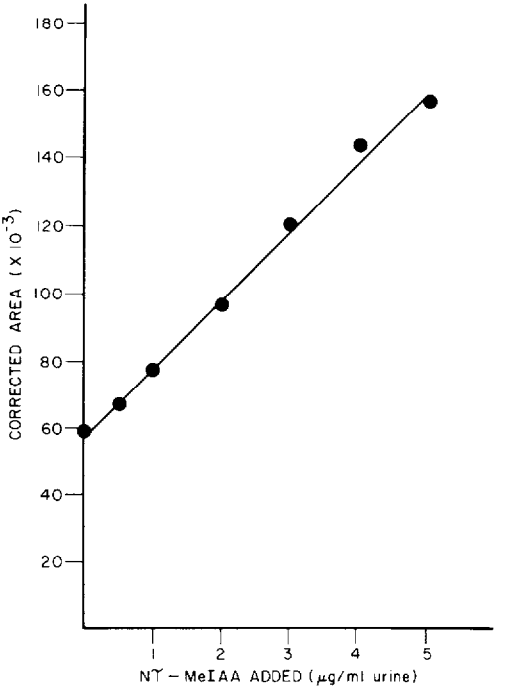

Fig. 4. Standard curve for Nτ-MeImAA added to pooled normal urine. Ordinate values are area at t_R = 2.7 min corrected for recovery of [^{3}H]Nτ-MeImAA[^{3}H].

TABLE I

PRECISION AND REPRODUCIBILITY OF ASSAY

Sample	Day	(μg/ml)			Relative S.D. (%)
		Range	Mean	S.D.	
1	3	1.69—1.95	1.83	0.10	5.5
	18	1.66—1.95	1.85	0.085	4.6
2	3	1.15—1.36	1.23	0.058	4.7
	18	1.17—1.34	1.25	0.055	4.6

TABLE II

DAILY URINARY EXCRETIONS OF N_{τ}-MeIAA AND ITS N_{π}-ISOMER IN NORMAL MALE SUBJECTS

Subject No.	Day	Urine volume (ml)	Creatinine (mg/24 h)	N_{τ}-MeImAA (μg/24h)	N_{τ}-MeImAA/ creatinine ($\times$ 1000)	N_{π}-MeImAA (μg/24 h)
1	1	1200	2724	2485	0.91	581
	7	990	1881	1926	1.02	nil
2	1	635	1829	1594	0.87	375
	7	1270	2718	3014	1.10	138
3	1	930	2348	1934	0.90	280
	7	1405	4468	2066	0.46	270
4	1	2180	2660	8590	3.23	735
	7	1630	2266	8095	3.57	nil
5	1	2265	2423	2537	1.05	nil
	7	2220	2775	2087	0.75	nil
6	1	1130	1808	5955	3.29	670
	7	1675	2260	8800	3.89	870
7	1	1062	2040	1485	0.73	87
	6	1835	2055	2605	1.26	691
	13	1815	2270	1580	0.70	154
	20	1950	1755	1365	0.78	263
	27	828	1610	1805	1.12	516
	34	910	2510	1765	0.70	235
8	1	985	1595	2445	1.53	3580
	6	1032	2350	16800	7.15	1090
	13	1440	2520	3010	1.19	4125
	20	530	1810	1675	0.92	1740
	27	832	2340	2413	1.03	2865
	34	2065	2975	3263	1.09	nil

The urines employed in development of this procedure were single voidings supplied on an ad libitum basis. In Table II are summarized 24-h excretion data for urines obtained in a controlled clinical environment from eight normal male volunteers; two subjects on six separate occasions, and six others on two occasions. Mean daily excretion of N_{τ}-MeImAA for all collections was 3720 $\pm$ 3590 μg/24 h, and excluding the one very atypical sample (subject No. 8, day 6), 3152 $\pm$ 2320 μg/24 h. Normalization of these data on the basis of corresponding creatinine excretion minimized intra-individual variations, but had no material effect upon inter-individual variation which, for this limited panel, is greater than the reported coefficients of variation imply for previous studies.

DISCUSSION

With the notable exception of the capillary GLC procedure recently described by Keyzer et al. [15], the present method offers obvious advantages

in terms of efficiency, sensitivity and precision, and with respect to the last, the relative convenience of using conventional packed columns. These, however, must be deactivated to minimize absorption to active sites typical of basic imidazoles. Each of the several base-deactivated packings available from Supelco was investigated, and SP-2401-DB and SP-2250-DB (compatible with NPD and ECD, respectively) were found to provide linear standard curves for Nτ-MeImAA to 0.25 μg/ml; i.e.: for an overall recovery of ca. 50% through the isolation and derivatization procedure, and an injection volume of 3 μl, 750 pg equivalents injected. By employing the pre-conditioning procedure specified above, no detrimental effects upon either NPD or ECD have been observed*.

The chromatograms in Fig. 1 are typical of those for some fifty urines examined to date with respect to the absence of significant peaks other than those of the compound of interest, and in most samples its Nπ-isomer. That this is not solely a function of detector selectivity is evident in the total ionization current chromatogram (Fig. 2) which unlike, for example, gas chromatograms obtained with flame-ionization detection (see refs. 6, 11, 12), contains no signals of intensity comparable to that of the compound of interest. This apparent enhancement in specificity of the isolation procedure is at the expense of recovery of the compound of interest, overall recoveries being low, but fairly reproducible, at 45 ± 9% for the [^{3}H] Nτ-MeImAA spike.

The present procedure yields values for urinary Nτ-MeImAA for normal man in substantial agreement with previously published ones [6, 12, 15]. Also consistent with prior reports is the high variability in excretion of its Nπ-isomer, both inter- and intra-individually. This variation, independent of Nτ-MeImAA or creatinine excretion lends further credence to the assumption that the Nπ-MeImAA is of dietary origin.

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*Base-deactivated packings are not recommended by manufacturer for use with NPD without extensive pre-conditioning.