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MEASUREMENT OF IMIDAZOLEACETIC ACID IN URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Imidazoleacetic acid (IAA), a histamine and histidine metabolite, was quantified in human urine by gas chromatography—mass spectrometry (GC—MS). The acid was separated by ion-exchange chromatography, derivatized as the *n*-butyl ester with boron trifluoride—butanol and the derivative extracted with chloroform. GC—MS analysis was carried out by selected-ion monitoring of ions *m/z* 81 and *m/z* 83 corresponding, respectively, to IAA and [^{15}N,^{15}N′]IAA used as internal standard. The mean IAA content in urine was about 8.02 nmol/mg of creatinine. The specificity of measurement was rigorously established by GC retention time, peak shape, ion abundance ratios, and recovery experiments. The method is capable of quantifying IAA in 0.05 ml of urine and in amounts as low as 0.20 nmol.

INTRODUCTION

Histamine (HA) is metabolized by mammals mainly by two enzymatic pathways [1—3], the relative importance of which varies with species and organs [4, 5]. In one pathway HA undergoes ring-methylation to form tele-methylhistamine (t-MH) which is oxidatively deaminated to tele-methylimidazoleacetic acid (t-MIAA). In the other pathway HA undergoes direct oxidative deamination to form imidazoleacetic acid (IAA). IAA can also be formed by metabolism of histidine [6].

Unlike t-MIAA, which has been identified and quantitatively measured in tissue and body fluids [7—17], IAA has not been extensively studied because of a lack of suitable methodology. IAA and its conjugate [18] have been identified and semiquantitatively measured by various techniques, mostly by thin-layer (TLC) and paper chromatography [19—29]. These techniques are tedious

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and usually lack accuracy and selectivity. While this paper was being prepared an enzymatic procedure [30, 31] was described reporting measurements of IAA in various rat tissues including brain and serum. We describe a simple, sensitive and selective gas chromatographic—mass spectrometric (GC—MS) method for the quantitative measurement of IAA. The method was used to measure the levels of free IAA in human urine.

EXPERIMENTAL

Materials

Imidazoleacetic acid hydrochloride and pyridylacetic acid (PAA) hydrochloride were bought from Calbiochem (La Jolla, CA, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively. Boron trifluoride–butanol (14%, w/v) was bought from Applied Science (State College, PA, U.S.A.). Analytical-grade anion-exchange resin AG 1-X4, 100—200 mesh, Cl^-, was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Standards were prepared in 0.01 *M* hydrochloric acid and stored at 4°C.

Synthesis of [^{15}N,$^{15}N'$]IAA · HCl

[^{15}N,$^{15}N'$]IAA was prepared by the hydrolysis of the corresponding [^{15}N,$^{15}N'$]imidazole-4-acetonitrile which was obtained by the oxidation of [^{15}N,$^{15}N'$]histidine · HCl according to the procedure of Bauer and Tabor [32]. Following reflux (3.5 h) of the nitrile in excess 2 *M* sodium hydroxide, the reaction mixture was acidified with concentrated hydrochloric acid. The solution was evaporated to dryness in vacuo on a rotary evaporator. The residue was dissolved in water and again evaporated; this treatment was repeated three more times to ensure complete removal of hydrochloric acid. The final dry residue was extracted twice with hot ethanol, filtered and cooled overnight at −20°C to obtain the product as a colorless crystalline material. The authenticity of the material was confirmed by nuclear magnetic resonance (NMR) and mass spectrometry.

^1H NMR (recorded on a Varian FT-20A instrument, in ppm downfield from TMS = 0) of the nitrile, in $(C^2H_3)_2CO$ solution: δ 7.77, dd, $J_{H-^{15}N_1}$ = 3.2 Hz, $J_{H-^{15}N_3}$ = 8.6 Hz; H_2, overlying $^{15}N_1$—H broad band; δ 7.27, br. d., $J_{H-^{15}N_1}$ = 4.7 Hz, H_5; δ 3.93, s, CH_2CN. (The spectrum of the crude nitrile also displayed a small (less than 0.54) unidentified doublet at δ 4.11, J = 2 Hz.) The electron-impact (EI) mass spectrum of the butyl ester of [^{15}N,$^{15}N'$]IAA exhibited the base peak at mass ion *m/z* 83 and a small molecular ion at *m/z* 184 (not shown).

Extraction and derivatization of IAA

To the urine sample (0.05 ml) was added [^{15}N,$^{15}N'$]IAA (200 ng) or PAA (100 ng) as internal standard, and the solution was adjusted to 2 ml with deionized water. In parallel, a standard curve was obtained by treating appropriate amounts of IAA with the internal standard. The sample was made alkaline by adding to each milliliter, 0.012 ml of 1 *M* sodium hydroxide and 0.1 ml of 50 m*M* Tris—acetate buffer (pH 9.0). The solution was subjected to

ion-exchange chromatography on columns (Isolab, polypropylene QS-Q; 1 cm I.D.) containing 2 ml of an ion-exchange resin (CH_3COO^-, analytical-grade Bio-Rad AG 1-X4 100—200 mesh), according to the method of Khandelwal et al. [12]. The column was washed with 4 ml of deionized water, and the acids were eluted by addition of 4 ml of 0.5 *M* acetic acid. To the eluate in 5-ml silanized screw-cap vials was added 0.1 ml of 0.01 *M* hydrochloric acid; the solution was taken to dryness at room temperature by vacuum centrifugation (Savant Instruments, Model SVC-100). The residue was rinsed with 0.2 ml of methanol and transferred to 1-ml silanized reaction vials and dried.

For derivatization, 0.1 ml of boron trifluoride—butanol was added to each vial. The mixture was vortexed and centrifuged. Samples were heated in a sand bath at 90°C for 90 min with intermittent mixing and then cooled on ice. Each vial then received 0.4 ml of toluene and 0.3 ml of unbuffered saturated Tris base, and the samples were vortexed and centrifuged for 1 min. After removal of the lower aqueous phase, 0.2 ml of water was added. The samples were again vortexed and centrifuged for 1 min and the aqueous phase was discarded. To each was added 0.1 ml of 0.1 *M* hydrochloric acid. Samples were vortexed and centrifuged for 1 min and the upper organic phase was aspirated. To the aqueous phase 0.05 ml each of chloroform and unbuffered saturated Tris base were added, and the mixture was vortexed and then centrifuged for 1 min. After removal of the upper aqueous phase, about 20 mg of anhydrous sodium sulfate were added to the lower chloroform layer.

Gas chromatography—mass spectrometry

GC—MS analysis was carried out on a 0.91 m × 2 mm silanized glass column containing a mixed phase of 1% Poly A-135 and 2% Silar 5CP on Gas-Chrom Q, 100—200 mesh with helium, flow-rate 30 ml/min, as carrier gas at 210°C. The major fragment ion of the derivative of IAA is *m/z* 81 (base peak), and the molecular ion is *m/z* 182. For quantification, *m/z* 81 of the derivative of endogenous or authentic IAA is monitored along with *m/z* 83 of the derivative of the internal standard, [^{15}N,^{15}N′]IAA.

EI (70 eV electron energy, 0.4 mA emission) and isobutane chemical ionization (CI) mass spectra were obtained on a Hewlett-Packard 5930A combined GC—MS system with a dual-ion source. A Hewlett-Packard 5933A data system linked to a Tektronix display terminal (Model 4012) equipped with a hard copy unit (Model 4610) was used to acquire, reduce, and process the data including the determination of peak areas of the selected-ion scans.

Urine samples

Samples of urine were collected from healthy male laboratory personnel in the morning and the creatinine content measured at 340 nm after the Jaffe alkaline picrate reaction [33].

RESULTS

Fig. 1 shows the EI (Fig. 1A) and isobutane CI (Fig. 1B) mass spectra of *n*-butyl ester of authentic IAA. The EI mass spectrum exhibited facile α-cleavage,

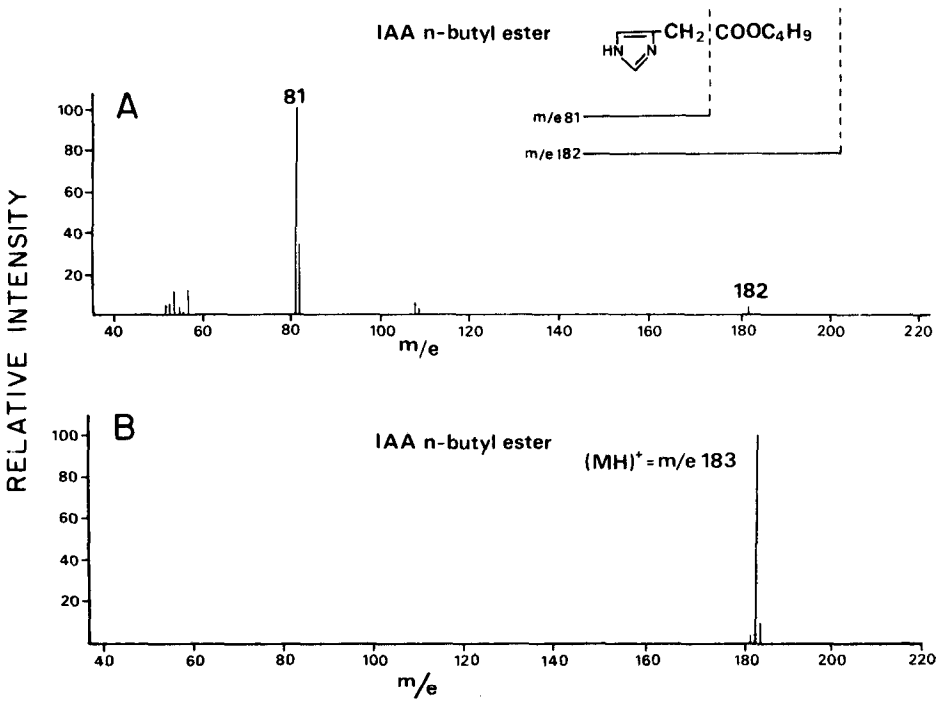

Fig. 1. EI (A), and CI (B) mass spectra of *n*-butyl esters of authentic IAA. For CI mass spectrum helium (0.6 Torr) was used as carrier gas, and isobutane (0.4 Torr), the reagent gas, was mixed at the end of GC column. The total-ion source pressure was kept at 1 Torr.

TABLE I

SELECTIVITY OF IAA MEASUREMENT IN URINE

The samples were extracted, derivatized and analyzed by GC—MS as described in the text.

Sample	t_R (min)	Mass ion abundance ratio (*m/z* 81/182)
Authentic IAA	8.2	11.73
Urine	8.2	11.20
Authentic IAA + urine extract	8.2	11.59

$[M—COOC_4H_9]^+$, to form a base peak at *m/z* 81 together with a small molecular ion at *m/z* 182. Isobutane CI mass spectrum showed an intense protonated molecular ion $[MH]^+$ *at m/z* 183. In the EI mass spectrum of $[^{15}N,^{15}N']$IAA butyl ester the base peak was shifted two mass units higher to *m/z* 83 whereas molecular ion was seen at *m/z* 184 (not shown).

Extent of esterification

The TLC on silica gel (diethyl ether—methanol—ammonium hydroxide, 50:50:1.5) of the IAA derivative showed no underivatized acid, indicating complete esterification. In addition, EI total-ion chromatography of IAA butyl ester showed only a single peak, confirming the formation of a single esterified product.

Selectivity of analysis

GC retention time, peak shape and mass ion abundance ratio (*m/z* 81/182) established the identity of the acid in urine. EI selected-ion monitoring (SIM) of urine extracts showed the same retention time (t_R) and nearly identical mass ion abundance ratios of authentic IAA; these results were obtained when standard and urine extracts were analyzed separately or by co-injection (Table I; Fig. 2).

Quantification of IAA

Standard curves of IAA carried through the entire assay procedure with two different internal standards, [^{15}N,^{15}N′]IAA and PAA, were linear on plotting the ratios of peak areas of acid esters (IAA/[^{15}N,^{15}N′]IAA, *m/z* 81/83 and IAA/PAA, *m/z* 81/93) against the amounts of IAA. The standard curve is linear from, at least, 25 to 250 ng. The suitability of [^{15}N,^{15}N′]IAA and PAA as internal standards was appraised by analysis of four different urine samples devoid of internal standard. No interfering peaks were seen in the urine samples at mass ions *m/z* 83 and *m/z* 93 at the t_R of [^{15}N,^{15}N′]IAA and PAA, respectively. The GC peaks of the esters of both endogenous IAA and [^{15}N,^{15}N′]IAA were fairly symmetrical and possessed a relatively short t_R (4.9 min) as shown in an EI-SIM scan of a urine sample (Fig. 3). The separate measurements of IAA in the same urine samples with two different internal standards were in good agreement and exhibited excellent correlation, r = 0.996, further confirming the selectivity of measurement. The IAA levels in four human urine samples ranged from 6.38 to 9.93 nmol/mg of creatinine [mean, 8.02; standard error of the mean (S.E.M.), 0.73].

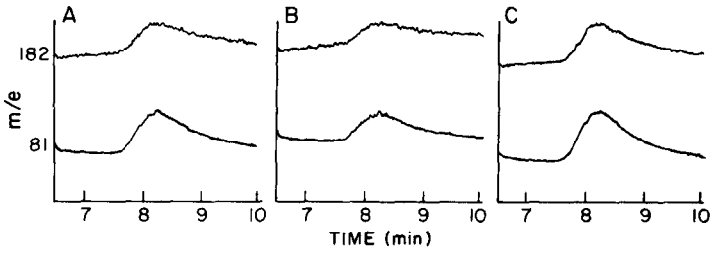

Fig. 2. EI-SIM of *n*-butyl esters of authentic IAA (A), urine extract (B), and co-injected authentic IAA and urine extract (C). GC—MS analysis was carried out on a 1.37-m long column.

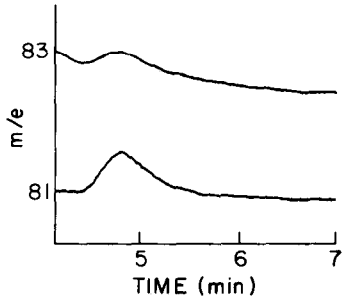

Fig. 3. EI-SIM scan of a urine extract analyzed as *n*-butyl ester. Mass ions *m/z* 81 and *m/z* 83 correspond respectively to endogenous IAA and [^{15}N,^{15}N′]IAA used as internal standard.

TABLE II

PRECISION OF THE METHOD FOR MEASURING IAA IN URINE (n = 2)

Sample	Ratio of peak areas m/z 81 (IAA)/m/z 83 ([^{15}N,^{15}N′]IAA)
1	0.128, 0.125
2	1.200, 1.270
3	0.250, 0.267

TABLE III

REPRODUCIBILITY OF GC—MS MEASUREMENTS IN URINE

Sample	Number of injections (n)	Ratio of peak areas m/z 81 (IAA)/m/z 83 ([^{15}N,^{15}N′]IAA)
1	4	0.47 ± 0.01 (S.E.M.)
2	2	0.45, 0.49
3	2	0.17, 0.20
4	2	0.26, 0.29

Recovery with the method

To one set of identical urine samples in triplicate (0.05 ml) were added 25 ng of IAA and internal standard. To the other set of aliquots of the same urine samples (0.05 ml) used to measure endogenous IAA, only the internal standard was added. All samples were subjected to the same extraction, derivatization and analysis procedure. To calculate recovery the average value of endogenous IAA was subtracted from the average value obtained from the spiked urine samples. The recovery of the 25 ng IAA added to urine was 26.35 ng (5% error).

Precision of the method

The precision of the entire assay procedure was assessed by analyzing three urine samples, randomly selected, each in duplicate. Table II shows that in these samples the ratio of the peak size, IAA/[^{15}N,^{15}N′]IAA, differed by 2—6% between the two aliquots.

Reproducibility of GC—MS measurements

The reproducibility of GC—MS measurements was excellent as assessed by repeated injections of the derivatized urine samples shown in Table III.

DISCUSSION

Although IAA has been previously identified and semi-quantitatively measured in human urine [19—29], there are few data on the endogenous levels of the free acid in urine. Recently, Imamura et al. [31] have reported levels of IAA in urine, serum and rat tissues by a radioenzymatic method. The method can measure amounts of IAA as low as 2 nmol. The GC—MS method described

here to quantify IAA (with either [^{15}N,^{15}N′]IAA or PAA as internal standard) is simple, sensitive, selective, and can measure a quantity at least as low as 0.2 nmol IAA. The related acids, t-MIAA, and its isomer, pros-methylimidazoleacetic acid (p-MIAA) and urocanic acid, a metabolite of histidine, also form *n*-butyl esters by our derivatization procedure [12].

Possessing similar chemical properties and the same GC characteristics as that of endogenous IAA, [^{15}N,^{15}N′]IAA serves as an ideal internal standard for quantitation. Blanks containing the same amount of [^{15}N,^{15}N′]IAA as used in the analysis of biological samples did not show any contribution to mass ion *m/z* 81 corresponding to IAA, showing that the internal standard does not interfere with the measurement of endogenous IAA. PAA, which is commercially available, works equally well as an internal standard. However, the isotopic IAA is preferred as an internal standard for measurement of low levels of IAA by GC—MS because it has chemical and GC characteristics similar to those of IAA.

The levels of free IAA measured in urine of four healthy subjects ranged from 6.38 to 9.93 nmol/mg of creatinine with an average excretion of 8.02 nmol/mg of creatinine.These values may be compared with others, with the assumption that the 24-h urine volume is 1200 ml and the 24-h creatinine excretion is 1.4 g [34]. Imamura et al. [31] found 6.6 nmol free IAA and 18.7 nmol conjugated IAA per mg creatinine. The sum of these values is in agreement with the findings of Mosebach et al. [20] who reported total urinary excretion of free and conjugated IAA to be 26 nmol/mg of creatinine.

Measurement of t-MIAA, the acid metabolite of HA formed by the methylating pathway, has been carried out in tissues and body fluids to assess histamine metabolism in disease [35—39] and after treatment with a drug [16]. Since IAA is a peripheral metabolite of HA in man, the availability of a reliable method to measure IAA should help to delineate the roles of HA and histidine in disease and in the action of drugs. There has been work done in this regard. Maslinski and Krajewska [40] have reported the effects on urinary excretion of endogenous IAA by guinea pigs adapted to histamine aerosols. According to Eliassen [41], treatment with aminoguanidine, an inhibitor of diamine oxidase (DAO), reduced total IAA from 70 to 4% in goat urine and 18 to 2% in pig urine. Similar reductions in free IAA (79%) and its conjugate levels (91%) have been recently reported in rat urine following aminoguanidine treatment [31]. In goat, oxidative deamination predominates whereas in pigs methylation is the dominant route of metabolism of histamine. In a study with radiolabeled material Granerus et al. [21] reported that treatment with aminoguanidine abolished excretion of radioactive IAA in human urine. In addition to its formation from histamine by direct oxidative deamination, IAA may be formed directly from histidine without histamine as intermediate. After histidine loading, as a means for early detection of megaloblastic anemia in pregnancy [22], it was observed that pregnant women excrete increased amounts of imidazole metabolites, including IAA. Levy et al. [23] reported that neonates with histidinemia excrete relatively less IAA than older children suffering with the same disease. Studies by Sharpless et al. [42] indicate that administration of L-3,4-dihydroxyphenylalanine and peripheral aromatic L-amino acid decarboxylase inhibitor (L-α-methyldopa hydrazine, MK 486) did

not appear to influence urinary excretion of IAA in patients with Parkinson's disease. Beaven et al. [43] have reported that small doses of salicylates increased urinary excretion of imidazoleacetic acid in man, rats and mice whereas the conjugated metabolite, ribosylimidazoleacetic acid, was decreased. In an investigation on protein—energy—malnutrition, Antener et al. [44] found unusually higher IAA in stool ultrafiltrates of these people. Further studies in man and laboratory animals will be facilitated by the method described here.

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