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Gas Chromatographic Determination of Urinary Indole-3-acetic Acid

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Gas chromatographic analysis of urinary indole-3-acetic acid as the trifluoroacetyl derivative of its methyl ester was carried out. A 10 ml portion of urine sample, acidified and saturated with sodium chloride, was extracted with benzene. After methylation with diazomethane in ether, indole-3-acetic acid was converted to the trifluoroacetyl derivative. Indole-3-butyric acid was used as an internal standard. This method was applied to human urine samples and the results showed the presence of 1.5—3.5 mg/day or 0.31—2.50 μg/mg creatinine of indole-3-acetic acid in normal urine, while the excretion of indole-3-acetic acid was higher in leukemia, gastric cancer, and phenylketonuria.

Keywords——indole-3-acetic acid; gas chromatography; urinary component; trifluoroacetylation; clinical chemistry

Indole-3-acetic acid (IAA) is formed as one of the metabolites of tryptophan and the presence of IAA in human urine has been verified by many investigators. An elevated excretion of IAA has been reported in the urine of patients with a hereditary syndrome such as phenylketonuria and Hartnup disease.[2]

To date, gas chromatography of IAA has been reported by several workers.[3-5] Determination of IAA by gas chromatography requires a prior preparation of suitable volatile derivatives. Grunwald, *et al.*[3] converted eleven indole acids into their respective methyl esters using diazomethane. Brook, *et al.*[4] demonstrated that diazomethylation accompanied by trifluoroacetylation offers a suitable derivative for an electron capture analysis. Recently, Seeley and Powell[5] reported a gas chromatographic analysis of the plant hormone IAA in which they also showed that the trifluoroacetyl derivative of IAA methyl ester possesses excellent chromatographic properties which permits quantitative and qualitative analyses in the nanogram to picogram level, employing an electron capture detector. This latter method has been applied to an analysis of apple seed hormones. We are not aware, however, of any report dealing with a gas chromatographic determination of urinary IAA. The present paper describes a procedure for the measurement of urinary IAA, in which it was separated by gas chromatography as the trifluoroacetyl derivative of IAA methyl ester. Flame ionization detection was sensitive enough to quantitate urinary IAA. The retention time and the gas chromatographic–mass spectrometric pattern of the experimental peak obtained from a human urine sample agreed completely with those of authentic IAA.

Experimental

Apparatus and Conditions——Gas chromatography was carried out with a Shimadzu Model GC-4APF gas chromatograph equipped with a flame ionization detector (FID). The column (glass, 200×0.3 cm int. diam.) was packed with Gas-Chrom Q (100—120 mesh) coated with 1.5% SE-30.

1) Location: *a*) *Mitahora Higashi, Gifu 502, Japan*; *b*) *Misasagi Higashiyama-ku, Kyoto 607, Japan.*
2) H. Weissbach, W. King, A. Sjoerdsma, and S. Udenfriend, *J. Biol. Chem.*, **234**, 81 (1959).
3) C. Grunwald, M. Vendrell, and B.B. Strowe, *Anal. Biochem.*, **20**, 484 (1967).
4) J.L. Brook, R.H. Biggs, P.A. St. John, and D.S. Anthony, *Anal. Biochem.*, **18**, 453 (1967).
5) S.D. Seeley and L.E. Powell, *Anal. Biochem.*, **58**, 39 (1974).

Mass spectra were obtained with a Hitachi Model RMU-6E MS coupled to the gas chromatograph. The operating conditions are the same as those described in a previous paper.[6]

Standard Procedure——NaCl (3.5 g) was added to 10 ml of urine sample, in a 50 ml centrifuge tube, acidified with 1 ml of 1 N HCl and the mixture was extracted with three 10 ml portions of benzene. Low-speed centrifugation (Tominaga centrifugal machine VE-KU) for about 5 min was required to separate the two phases. The combined benzene extracts were concentrated to about 5 ml by evaporation under a reduced pressure in a 10 ml pear-shaped flask. To this solution was added 0.5 ml of AcOEt solution of 2-mercaptoethanol (0.4 ml/50 ml) which acts as a stabilizer, 1 ml of 0.02% indole-3-butyric acid in AcOEt as an internal standard and about 1 ml of freshly prepared CH_2N_2 in ether. The mixture was allowed to stand in ice for 30 min, after which the solvent was evaporated to dryness under a reduced pressure. The residue was dissolved immediately in 2 drops of AcOEt. Trifluoroacetylation was carried out at a cool temperature with 10 drops of $(CF_3CO)_2O$ and the mixture was allowed to stand overnight. An aliquot (1 μl) of this solution was injected into the gas chromatograph. Quantitation was carried out using a working curve obtained from the control urines spiked with known amounts of IAA.

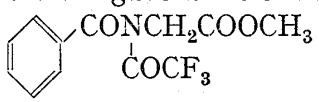
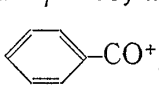
Results and Discussion

In a study designed to eliminate interference from co-existing substances in the urinary IAA extract, two extracting solvents were tested. Ethyl acetate, although it was the best for the extraction of IAA from aqueous solution, extracted undesirable foreign substances, as seen on the gas chromatographic record (see Fig. 1A), when applied to urine. Benzene was found to be a suitable solvent for purification to reduce mixing of interfering substances and, although it extracted IAA in a lower yield than ethyl acetate, the yield was improved when the urine was saturated with sodium chloride at a pH ranging from 1.0 to 5.0. In this case IAA was found to be extracted with a recovery of nearly 100% (see Fig. 1B).

The optimal condition for the trifluoroacetylation of IAA methyl ester was then examined. Treatment of a small amount of IAA methyl ester in 2 drops of ethyl acetate with trifluoroacetic anhydride (10 drops) produced two peaks on the gas chromatogram. Reaction for 4 hr, however, resulted in complete derivatization and a single peak. Indole-3-butyric acid was used as an internal standard, which was expected to give a suitable retention time and show a similar behavior as IAA in its derivatization and chromatographic separation, because of its similar structure. IAA was apt to decompose during evaporation and derivatization which resulted in varied measurement values. When 2-mercaptoethanol was used as a stabilizer, the calibration curve was linear and passed through the origin up to 100 μg/10 ml concentration range of IAA. To check the precision of this method, five 10 ml portions of an identical urine sample spiked with 40 μg of IAA were determined by this over-all procedure and the mean recovery was 95.1% (S.D.±3.1).

The GC–MS spectrum of trifluoroacetylated IAA methyl ester is given in Fig. 2. A molecular ion was observed at *m/e* 285 and the peak at *m/e* 225 corresponds to the loss of $COOCH_3$. Other prominent fragments are reasonably illustrated in Fig. 2.

The retention time and the GC–MS pattern of apparent urinary IAA agreed completely with those of authentic IAA. From these results, the derivative formed was concluded to be N-trifluoroacetyl IAA methyl ester.

When ethyl acetate was used for extraction of urinary IAA, a large disturbing peak Y sometimes appeared near the retention time of trifluoroacetylated IAA methyl ester, as shown in Fig. 1A and, when a larger volume of urine sample was treated by the same technique, a marked increase of peak Y was observed accompanied by a marked decrease of peak X. In order to explain this phenomenon, the structure of peak X was examined by gas chromatography–mass spectrometry, and its spectrum gave a molecular ion *m/e* 289, and a base peak 105. They seem to correspond to  and  , respectively.

6) M. Naruse, K. Hirano, S. Kawai, T. Ohno, Y. Masada, and K. Hashimoto, *J. Chromatogr.*, **82**, 331 (1973).

Table I. Determination of Free IAA in Human Urine

Sample[a]	Sex[b]	Age (yrs)	IAA value
A	m	22	1.7 IAA (mg)/day
B	f	27	1.5
C	m	24	2.8
D	m	22	3.5
E	m	23	0.61 IAA (μg)/creatinine (mg)
F	m	23	0.49
G	m	30	0.31
H	m	48	2.50
I	f	42	0.48
J	f	22	1.74
K	f	22	0.32
L	f	22	0.85
M	m	68	7.66
N	m	18	14.04
O	m	70	5.06
P	m	10	12.47

a) A—L: normal subjects; M, N: leukemia; O: gastric cancer; P: phenylketonuria.
b) m: male, f: female.

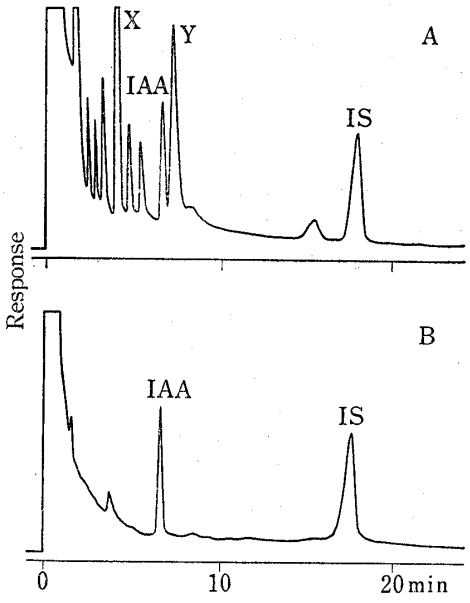


Fig. 1. Gas Chromatography of Urinary IAA as the Trifluoroacetyl Methyl Ester

(A) extracted with ethyl acetate.
(B) extracted with benzene.
Is (internal standard): indole-3-butyric acid.
Conditions: 1.5% SE-30 on Gas-Chrom Q (100—120 mesh), glass column (2.0 m×3 mm int. diam.), 140°, FID.

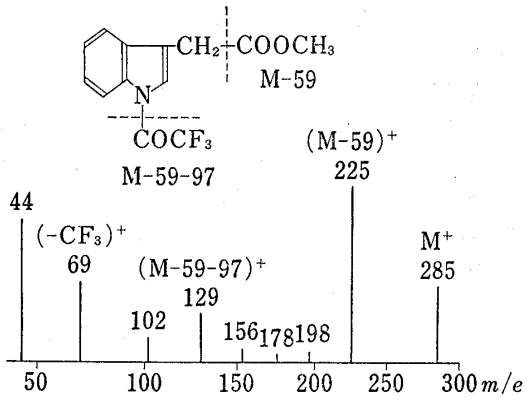


Fig. 2. GC–MS Spectrum of Trifluoroacetate Methyl Ester of IAA

Further identification was obtained by the fact that the retention time of peak X agreed completely with that of trifluoroacetylated methyl ester of authentic hippuric acid. The large, disturbing peak Y therefore is considered to be due to incomplete derivatization of hippuric acid.

These techniques were applied to the determination of IAA in human urine and its results are given in Table I. The results for samples A—D show the presence of approximately 1.5 to 3.5 mg/day of IAA in normal human urine. It is difficult to ensure complete urine collection over a 24 hr period. The results for sample E—P are therefore expressed as a ratio with respect to the urinary creatinine. Creatinine in each urine sample was determined by the alkaline picrate method. In four patients with leukemia, gastric cancer, or phenylketonuria, excretion of IAA was found to be higher.

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