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SENSITIVE DETERMINATION OF DEUTERATED AND NON-DEUTERATED INDOLE-3-ACETIC ACID AND 5-HYDROXYINDOLE-3-ACETIC ACID BY COMBINED CAPILLARY GAS CHROMATOGRAPHY–NEGATIVE-ION CHEMICAL IONIZATION MASS SPECTROMETRY

TOKISHI HAYASHI*, HIROSHI NARUSE and FUMIO MATSUDA

National Centre of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi-Machi, Kodaira, Tokyo 187 (Japan)

and

YOSHIO IIDA

Department of Engineering, Seikei University, Kichijoji-Kita-Machi, Musashino, Tokyo 180 (Japan)

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SUMMARY

Sensitive methods for the determination of deuterated and non-deuterated indole-3-acetic acid and 5-hydroxyindole-3-acetic acid by combined capillary gas chromatography–negative-ion chemical ionization mass spectrometry were developed. Indole-3-acetic and 5-hydroxyindole-3-acetic acids were converted into pentafluorobenzyl and trifluoroacetylmethyl derivatives, respectively, after pre-purification by high-performance liquid chromatography. These derivatives were separated by gas chromatography and determined by selected ion monitoring. In the determinations, indole-3-acetic-2,2,2′,4′,5′,6′,7′-d_7 acid and 5-hydroxyindole-3-acetic-3,3-d_2 acid were used as internal standards. The methods developed in this work were used for the determination of deuterated and non-deuterated indole-3-acetic acid and 5-hydroxyindole-3-acetic acid in human urine samples collected before and after administration of L-tryptophan-3,3-d_2.

INTRODUCTION

It is well known that a large part of tryptophan is metabolized in vivo through a route involving formylkynurenine, kynurenine, etc., and the remaining small part is metabolized through serotonin (5-HT) and tryptamine (TA). The rela-

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tionship between their metabolism and several psychiatric diseases such as autism and depressive syndromes is gaining increasing attention. In addition, a great deal of research relating to the levels of indoleamines [1–8] has been conducted. However, no conclusive results have yet been obtained, because the difference between normal and abnormal levels might not be so large when compared with individual variations. To investigate such delicate metabolic alterations, it seemed necessary to develop a new method. These results prompted us to start a study concerning the development of a tracer method for tryptophan (Trp) metabolism using a stable isotope-labelled compound. In a previous paper [9], sensitive methods for the determination of deuterated and non-deuterated Trp, TA and 5-HT by combined capillary gas chromatography–negative-ion chemical ionization mass spectrometry (GC–NICI-MS) were reported.

This paper describes the development of a highly sensitive and reliable method for the determination of deuterated and non-deuterated indole-3-acetic acid (IAA) and 5-hydroxyindole-3-acetic acid (5-HIAA) in body fluids using GC–NICI-MS. Tracer experiments were also carried out using L-tryptophan-3,3-d_2 (Trp-d_2) as the tracer.

EXPERIMENTAL

Apparatus

A Finnigan 4000 GC–MS system equipped with a pulsed positive-ion, negative-ion accessory (Finnigan, CA, U.S.A.) was used. Sample injections were performed with a solvent cut injector (Gaskuro Kogyo, Tokyo, Japan). GC separations were carried out with an OV-101 coated fused-silica capillary column (25 m × 0.25 mm I.D.). The end of the capillary column was connected directly to the mass spectrometer. Methane was used as the GC carrier gas and chemical ionization reagent gas. The capillary column head pressure was held at 58.8 Pa, which gave a flow-rate of methane of 1.0 ml/min. The ion-source pressure, the ion-source temperature, the electron energy and the emission current were maintained at 20 Pa, 250°C, 90 eV and 300 μA, respectively.

The high-performance liquid chromatographic (HPLC) system used for the pre-purification of the sample was constructed from a one-chamber gradient programmer, a Model KHP-010 high-pressure pump, a KSST-60 auto-sample injector (Kyowa Seimitsu, Tokyo, Japan), a UVIDEC-100 II UV detector (Japan Spectroscopic, Tokyo, Japan), a constant-temperature circulator and electromagnetic valves, as shown in Fig. 1.

The one-chamber gradient programmer used in this work was described in a previous paper [10]. This system was automatically controlled with a Stysmac PO programmable controller (Omron, Tokyo, Japan) and HPLC separation was carried out with a stainless-steel column (250 mm × 4 mm I.D.) packed with NS-Gel C_8 (5 μm) (Sakata, Tokyo, Japan). The separation conditions are given in the legend to Fig. 2.

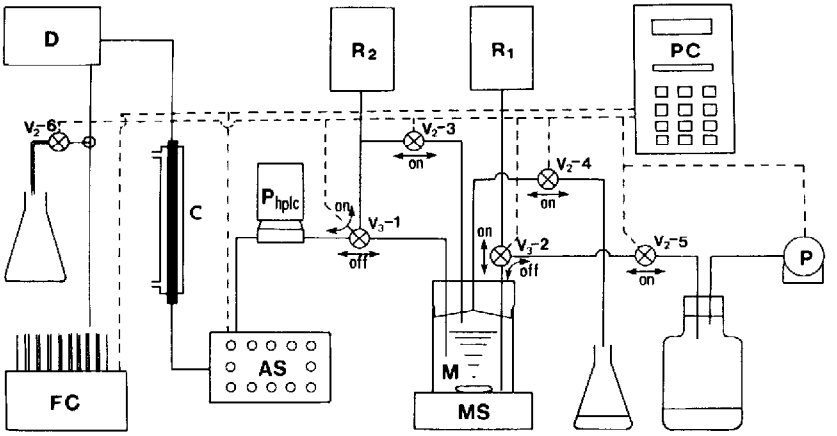

Fig. 1. Schematic diagram of the automated HPLC system used for the pre-purification of IAA and 5-HIAA. R_1 = reservoir for solvent 1; R_2 = reservoir for solvent 2; PC = programmable controller; P_{hplc} = high-pressure pump for mobile phase; P = vacuum pump; V = electromagnetic valve; M = mixer (20 ml); MS = magnetic stirrer; AS = auto-sample injector; C = column; D = UV detector; FC = fraction collector.

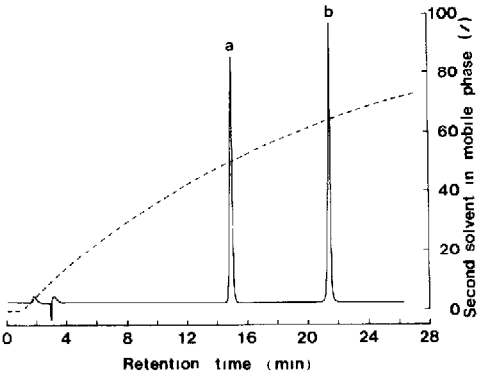

Fig. 2. High-performance liquid chromatogram of a standard mixture of (a) 5-HIAA and (b) IAA. Column, NS-Gel C_8 (5 μm) (250 mm × 4 mm I.D.); column temperature, 50°C; mobile phase, first solvent 0.01 *M* acetic acid (10 mg/l EDTA); second solvent 50% aqueous acetonitrile; flow-rate, 1.0 ml/min; detector, UV (280 nm)

Reagents

Analytical-reagent grade chemicals were used. Trifluoroacetic anhydride (TFAA) and pentafluorobenzyl bromide (PFB-Br) were purchased from Merck Japan (Tokyo, Japan), IAA and 5-HIAA from Wako (Osaka, Japan) and indole-3-acetic-2′,2,2,4′,5′,6′,7′-d_7 acid (IAA-d_7) and 5-hydroxyindole-3-acetic-2,2-d_2 acid (5-HIAA-d_2) from Merck Frosst Canada (Montreal, Canada). Standard stock solutions of these acids were prepared by dissolving them in ethanol containing 0.1% mercaptoethanol so that the concentration of each acid was 400 μg/

ml. These solutions were stored at −20°C and diluted with ethanol just before use. A solution of diazomethane in diethyl ether was prepared from nitrosomethylurea by the usual method.

Administration of Trp-d_2

Trp-d_2 (10 mg/kg) was administered orally to a healthy male, aged 42 years, at 11.00 a.m. and blood samples (heparinized) were obtained from a forearm vein before and after dosing at intervals, together with urine collection. The blood samples were centrifuged at 10 000 *g* for 15 min in a refrigerated centrifuge. Plasma samples obtained were stored at −20°C until analysed. After measurement of the volume, aliquots of the urine samples were stored at −20°C until analysed.

Procedure for the determination of deuterated and non-deuterated IAA and 5-HIAA

Two 1.0-ml urine samples were placed in test-tubes and 100 μl of internal standard solution containing 200 ng of IAA-d_7 and 50 ng of 5-HIAA-d_2 were added to one of them. Both of these samples were treated as follows: after addition of 300 mg of sodium chloride and 0.1 ml of 2 *M* hydrochloric acid, the acids were extracted with 6 ml of ethyl acetate by shaking vigorously for 5 min. The ethyl acetate layer was dried over sodium sulphate and evaporated to dryness. The residue was dissolved in 50 μl of ethanol and diluted with 300 μl of water. A 300-μl volume of the resulting solution was injected into the HPLC system. The eluate relating to IAA or 5-HIAA was fractionated and the acids were extracted with 6 ml of ethyl acetate in a similar manner to the extraction from the urine sample. The ethyl acetate was evaporated to dryness after being dried over sodium sulphate, then each residue was dissolved in a few drops of methanol and transferred to a glass ampoule for derivatization. For the determination of IAA, only the eluate obtained from the sample to which the internal standard solution had been added was treated.

After removal of the solvent, the derivatization for GC–NICI-MS was carried out in the following manner. The residue containing 5-HIAA was dissolved in 50 μl of methanol and 0.5 ml of the diazomethane solution was added to it. After a few minutes at room temperature, the excess of reagent and solvent were removed in a stream of nitrogen. Then, 50 μl of a mixture of TFAA and acetonitrile (1:1) was added to the residue and 1 μl of the solution obtained was used for the GC–NICI-MS analysis. The residue containing IAA was mixed with 100 μl of 2.5% PFB-Br solution in acetone and 2–3 mg of a mixture of sodium carbonate and sodium sulphate (1:1) at room temperature for 10 min to convert IAA into the PFB derivative. The reaction mixture was diluted with 0.3 ml of *n*-hexane and the supernatant was transferred into another ampoule. After evaporation of the solvent and excess of reagent, the ampoule was placed in a vacuum drying apparatus at room temperature for 30 min to remove the excess of reagent as completely as possible. The resulting residue was dissolved in 50 μl of ethyl acetate and 1 μl of the solution was used for GC–NICI-MS analysis.

Procedures for the determination of deuterated and non-deuterated plasma Trp, urinary 5-HT and urinary TA

GC–NICI-MS analysis of these compounds was carried out according to the methods reported previously [9].

RESULTS AND DISCUSSION

It has been reported [11] that NICI-MS could provide 100–1000 times greater sensitivity than that available by means of the positive-ion mode for certain compounds and that the electron affinity inherent in the sample structure would be the most important factor. Effective derivatizations for the highly sensitive detection of many biologically important compounds for GC–NICI-MS were discussed in previous papers [12,13]. From these data, the derivatives shown in Figs. 3 and 4 were selected for the highly sensitive determination of IAA and 5-HIAA by GC–NICI-MS. Figs. 3 and 4 show the methane NICI mass spectra of these

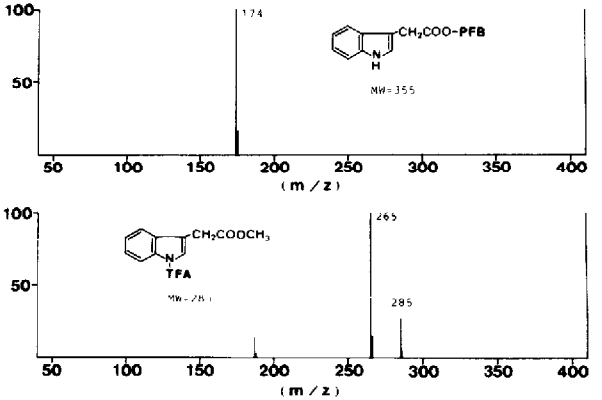

Fig. 3. Methane NICI mass spectra of PFB and TFA methyl derivatives of IAA.

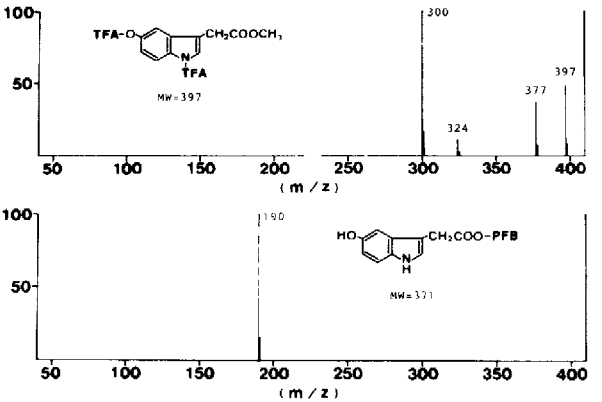

Fig. 4. Methane NICI mass spectra of TFA methyl and PFB derivatives of 5-HIAA.

derivatives. With IAA, both the PFB and the TFA methyl derivatives showed good volatility and high sensitivity in GC–NICI-MS analysis. The NICI mass spectrum of the PFB derivative consists of a single fragment anion, which was assigned to $(M-PFB)^-$, and in that of the TFA methyl derivative three fragment signals were observed at m/z 187, 265 and 285, which could be assigned to $(M-H-TFA)^-$, $(M-HF)^-$ and M^-, respectively. It is very important to understand the origin of the hydrogen in HF which is eliminated at the formation of the most dominant fragment peak, $(M-HF)^-$, in the work using deuterated compounds as a tracer and an internal standard. Investigations using some deuterated compounds suggested that hydrogen was eliminated as HF from the methylene in the side-chain. Therefore, this derivative may not be useful if IAA with deuterated methylene is used as an internal standard or a tracer.

Both derivatives of 5-HIAA also showed good ionization efficiency in the methane NICI mode and gave useful dominant peaks, $(M-TFA)^-$ and $(M-PFB)^-$. The PFB derivative, however, showed less volatility than the TFA methyl derivative which was employed in this work.

Fig. 5 shows selected ion current profiles which were generated by only 200 fg of the PFB derivative of IAA and 400 fg of the TFA methyl derivative of 5-HIAA.

A stable isotope-labelled compound is usually used as an internal standard in GC–MS because of the close similarity of its chemical and physical properties to those of the non-labelled compound. The use of a stable isotope-labelled compound also makes it unnecessary to correct for losses through different kinds of analytical treatments of the sample. In this work, IAA-d_7 and 5-HIAA-d_2 were used as internal standards for the determination of IAA and 5-HIAA. The determination of deuterated and non-deuterated 5-HIAA in a urine sample collected before and after oral administration of Trp-d_2 was carried out by the double iso-

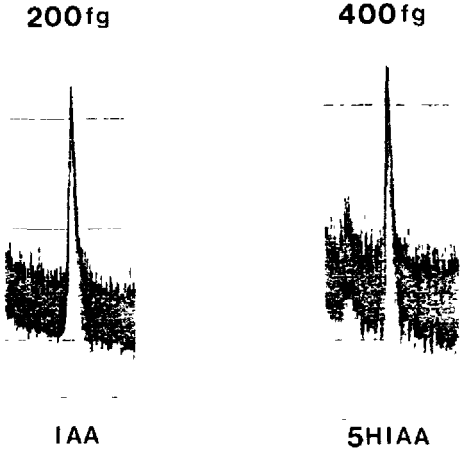

Fig. 5. Selected ion responses obtained by monitoring ions at m/z 174 and 300 for IAA and 5-HIAA.

tope dilution method, because the deuterated 5-HIAA metabolized from Trp-d_2 is 5-HIAA-d_2.

Next, the conditions for the derivatization of IAA and 5-HIAA were investigated. The effect of the reaction time at room temperature on the yield of the PFB derivative of IAA was investigated. The yield of the PFB derivative reached a plateau after a reaction time of ca. 10 min. When the effect of methylation with diazomethane on the yield of the TFA methyl derivative of 5-HIAA was studied, the maximum yield was observed after ca. 1 min and gradually decreased. It is well known that the reactivity of a carboxyl group with diazomethane is higher than that of a phenol group. The decrease in the yield might be due to methylation of the phenol group. Trifluoroacetylation of the methyl derivative of 5-HIAA was completed under the conditions described under Experimental.

In selected ion monitoring using the deuterium-labelled compound as an internal standard, the internal standard must not suffer from the D–H exchange reaction. Therefore, we investigated whether or not the internal standards, IAA-d_7 and 5-HIAA-d_2, suffer from this exchange in the derivatization. We observed no detectable D–H exchange reaction under the conditions employed for both internal standards.

In tracer experiments, administration of large amounts of a labelled compound might not permit a metabolic investigation under physiological conditions. Therefore, highly sensitive methods are necessary for the determination of much smaller amounts of labelled compound and its metabolites than those of endogenous compounds. Generally, pre-purification is very important for the determination of trace amounts of compounds in complex mixtures such as biological fluids.

In this work, HPLC was used for the clean-up of IAA and 5-HIAA in a human urine sample. Fig. 6 shows the chromatogram obtained on pre-purification with UV detection (280 nm). The shadowed part around 14 min is the elution area of

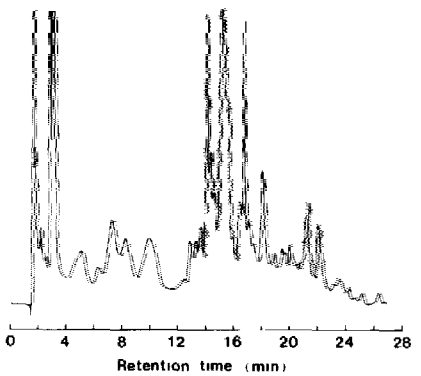

Fig. 6. High-performance liquid chromatogram obtained for the pre-purification of IAA and 5-HIAA in a urine sample.

5-HIAA and that around 21 min corresponds to IAA. This system could be run automatically and the reproducibilities of the retention times of both 5-HIAA and IAA were within 0.4%. Also, neither internal standard suffered from D–H exchange in the pre-purification treatment.

Calibration graphs for the determination of deuterated and non-deuterated IAA were prepared in the following manner. The peak-height ratio of IAA-d_0 to that of IAA-d_7 (I.S.) was plotted on the ordinate after correction for the signal due to the isotopic peak, and the amount (nmol) of IAA-d_0 on the abscissa. The graph was linear and passed through the origin in the concentration range from at least 5 pmol to 2 nmol of IAA-d_0.

The calibration graph for IAA-d_2 was prepared in the following manner. When the calibration graph for IAA-d_0 was expressed as $y=ax$, that for IAA-d_2 was expressed as $y=(K_2/K_0)ax$, where K_2 and K_0 are the ratios of the ion abundances at m/z 176 and 174 to the total abundance of the isotopic ions corresponding to $(M-PFB)^-$ for the derivatives of IAA-d_2 and IAA-d_0 samples, respectively. The value of K_2 was calculated from the relative isotopic intensities for IAA-d_0 and the percentage of deuterated species of Trp-d_2, assuming that the isotopic peak-height ratios corresponding to $(M-PFB)^-$ of each species are analogous and that each species was equally metabolized to IAA in vivo.

The calibration graph for 5-HIAA-d_0 was prepared in a similar manner to that for IAA-d_0 and the graph for 5-HIAA-d_2 in a similar manner to that for IAA-d_2.

The levels of deuterated and non-deuterated 5-HIAA in sample solution were calculated as follows:

$$\text{5-HIAA-}d_0\ (\text{nmol/ml of sample}) = \frac{SP_0}{SP_1 \cdot C_0 \cdot SV}$$

$$\text{5-HIAA-}d_2\ (\text{nmol/ml of sample}) = \frac{SP_2}{SP_1 \cdot C_2 \cdot SV}$$

where SP_0, SP_2 and SP_1 are the peak intensities at m/z 300 derived from 5-HIAA-d_0, that at m/z 302 derived from 5-HIAA-d_2 and that at m/z 302 derived from the internal standard in the sample to which the internal standard solution was added, C_0 and C_2 are the slopes of the calibration graphs for 5-HIAA-d_0 and 5-HIAA-d_2, respectively, and SV is the sample volume (ml). SP_i, SP_2 and SP_0 can be calculated as follows:

$$SP_i = \frac{NDD2 \cdot ADD0 - NDD0 \cdot ADD2}{NDD2 \cdot KI - NDD0}$$

$$SP_2 = \frac{ADD2 - ADD0 \cdot K0 - SP_1(1 - K0 \cdot KI)}{1 - K0 \cdot KI}$$

$$SP_0 = ADD0 - SP_2 \cdot K2 - SP_i \cdot KI$$

where $NDD0$ and $NDD2$ are the peak heights at m/z 300 and 302 of the sample

TABLE I

PLASMA LEVELS OF DEUTERATED AND NON-DEUTERATED Trp IN A HEALTHY MALE (42 YEARS) AFTER THE ORAL ADMINISTRATION OF Trp-d_2 (10 mg/kg)

Time (min)	Plasma level (nmol/ml)	
	Trp-d_2	Trp-d_0
0	0	55.3
15	44.2	57.4
30	83.6	59.4
45	74.4	58.2
60	85 4	70.8
90	61.6	55.8
120	57 1	63.8
180	37.5	62.8
240	32 6	64.2
360	9.2	60.3

TABLE II

URINARY LEVELS OF DEUTERATED AND NON-DEUTERATED 5-HT, TA, 5-HIAA AND IAA IN A HEALTHY MALE (42 YEARS) AFTER THE ORAL ADMINISTRATION OF Trp-d_2 (10 mg/kg)

Time (min)	Urinary level (nmol/ml)			
	5-HT-d_2 (5-HT-d_0)	TA-d_2 (TA-d_0)	5-HIAA-d_2 (5-HIAA-d_0)	IAA-d_2 (IAA-d_0)
0–60	0.352 (0.642)	0.152 (0.236)	0.376 (7.08)	0.628 (15.05)
60–120	0.243 (0.411)	0.242 (0.282)	0.441 (3.09)	1.512 (7.08)
120–180	0.102 (0.304)	0.099 (0.226)	0.424 (4.51)	1.325 (6.40)
180–240	0.058 (0.337)	0.060 (0.263)	0.393 (5.59)	2.122 (12.38)
240–300	0.050 (0.330)	0.026 (0.222)	0.341 (5.39)	1.459 (10.53)
300–360	0.035 (0.305)	0.014 (0.237)	0.315 (5.36)	1.168 (9.58)

without addition of internal standard; *ADD*0 and *ADD*2 are those of the sample with addition of internal standard, *K*0 is the ratio of the height of the peak at m/z 302 to that at m/z 300 of 5-HIAA-d_0; *K*I is the ratio of the height of the peak at m/z 300 to that at m/z 302 of the internal standard; and *K*2 is the ratio of the height of the peak at m/z 300 to that at m/z 302 of 5-HIAA-d_2, which were cal-

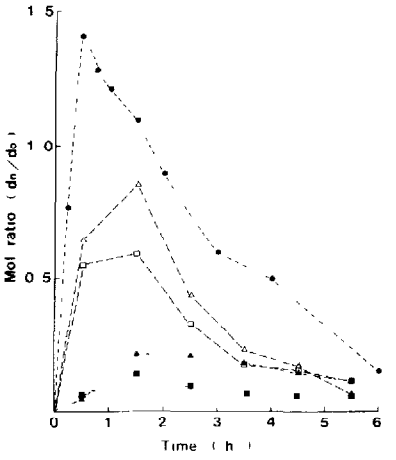

Fig. 7. Time course of the molar ratios of plasma levels of Trp-d_2, and urinary levels of TA-d_2, 5-HT-d_2, IAA-d_2 and 5-HIAA-d_2 to the non-deuterated compounds after oral administration of Trp-d_2 (10 mg/kg) to a healthy male. ●, Plasma Trp; △, urinary TA; □, urinary 5-HT; ▲, urinary IAA; ■, urinary 5-HIAA.

culated from the relative isotopic peak intensities for 5-HIAA and the percentage of deuterated species of Trp-d_2 used as a tracer.

Tables I and II give the plasma levels of deuterated and non-deuterated Trp and the urinary levels of deuterated and non-deuterated 5-HT, TA, 5-HIAA and IAA in a healthy male aged 42 years after the oral administration of Trp-d_2 (10 mg/kg). Fig. 7 shows the time course of the ratios of plasma Trp-d_2 to Trp-d_0, urinary 5-HT-d_2 to 5-HT-d_0, urinary TA-d_2 to TA-d_0, urinary 5-HIAA-d_2 to 5-HIAA-d_0 and urinary IAA-d_2 to IAA-d_0.

As described above, the amounts of deuterated and non-deuterated 5-HIAA and IAA excreted into the urine after oral administration of Trp-d_2 could be monitored by the present method. Some investigations concerning in vivo tryptophan metabolism in several psychiatric diseases such as depression and infantile autism are now being carried out using the present method together with the methods reported previously [9]. The results will be discussed in subsequent papers.

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