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SENSITIVE DETERMINATION OF TYROSINE METABOLITES, *p*-HYDROXYPHENYLACETIC ACID, 4-HYDROXY-3-METHOXYPHENYLACETIC ACID AND 4-HYDROXY-3-METHOXYMANDELIC ACID, BY GAS CHROMATOGRAPHY—NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRY

APPLICATION TO A STABLE ISOTOPE-LABELLED TRACER EXPERIMENT TO INVESTIGATE THEIR METABOLISM IN MAN

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SUMMARY

A method has been established for studying the dynamic metabolism of tyrosine to its metabolites in humans using a deuterium-labelled amino acid. Phenylalanine-d_5 was administered orally to human subjects (5 mg/kg) and the levels of *p*-hydroxyphenylacetic acid-d_4, 4-hydroxy-3-methoxyphenylacetic acid-d_3, and 4-hydroxy-3-methoxymandelic acid-d_3 excreted into urine every hour were determined by gas chromatography—negative-ion chemical-ionization mass spectrometry. This method was also applied to some patients with depression and it was possible to detect a slight alteration in the excretion of some compounds compared with the control.

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INTRODUCTION

The correlations between affective disorders and the levels of neurotransmitters in the brain have been pointed out for many years and the levels of neurotransmitters or their metabolites in body fluid have been determined by different methods. However, in many cases, the changes were not large compared with experimental errors or individual differences, and no conclusive results have yet been obtained.

In view of this background, we thought that it would be more practicable for us, in terms of discovering the metabolic differences between subjects with affective disorders and the control, if we could determine the dynamic turnover rate of a metabolite rather than measuring the pool size of each metabolite.

So far, we have studied the in vivo metabolism of phenylalanine and tyrosine, which are precursors of catecholamines, using deuterium-labelled phenylalanine as a tracer to distinguish its metabolites from endogenous metabolites. We found that the turnover rate of tyrosine is profoundly suppressed in depression [1]. This observation implies that some metabolic alterations of catecholamines or other tyrosine metabolites might be present in depression. Based on this assumption, we have focused our remarks on measuring the turnover of *p*-hydroxyphenylacetic acid (PHPA), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) and 4-hydroxy-3-methoxymandelic acid (vanilmandelic acid, VMA) to compare the flows of three different metabolic pathways simultaneously by means of a stable isotope-labelled tracer experiment.

It is desirable to reduce the amount of tracer so that the added tracer may not disturb the physiological equilibrium. Because of this limitation, it is essential to develop highly sensitive techniques to determine stable isotope-labelled compounds. Therefore, we have already established super-sensitive methods for the determination of PHPA, HVA and VMA by means of gas chromatography—negative-ion chemical-ionization mass spectrometry (GC—NICI-MS) [2].

This communication deals with the establishment of a method for a deuterium-labelled tracer experiment to study the metabolism of tyrosine to its metabolites in man. [2′,3′,4′,5′,6′-$^2H_5$]-L-Phenylalanine (Phe-d_5) was used as a tracer and the excretion of PHPA-d_4, HVA-d_3 or VAM-d_3 into urine was measured by GC—NICI-MS. This method was used to investigate metabolic alterations in subjects suffering from depression. As a result it was found that the flow to PHPA or HVA was slightly suppressed in some cases, although individual differences were clearly observed in depressive patients as well as in the control.

EXPERIMENTAL

Chemicals

Phe-d_5 used as a tracer was prepared according to Matthews et al. [3]. Its isotopic purity was 95% as a d_5 form.

p-Hydroxyphenylacetic acid and 3,4-dihydroxybenzaldehyde were

purchased from Wako Pure Chemicals (Osaka, Japan) and 3,4-dihydroxyphenylacetic acid from Aldrich (Milwaukee, WI, U.S.A.). Deuterium oxide, deuterochloric acid (37% in ^2H_2O) and acetic acid-d_4 were from Merck (Darmstadt, F.R.G.). Esterase (from porcine liver) was obtained from Sigma (St. Louis, MO, U.S.A.).

The other reagents and solvents used in this study were all analytical grade.

Syntheses of deuterium-labelled compounds used as internal standards

[2,2,2',3',5',6'-$^2H_6$] p-Hydroxyphenylacetic acid (PHPA-d_6). PHPA (50 mg) was dissolved in a mixture of acetic acid-d_4 (0.4 ml) and deuterium oxide (0.5 ml) and the solvent was evaporated in a stream of nitrogen at 90°C. This procedure was repeated twice to remove active protones as completely as possible. The resulting residue was dissolved in a mixture of acetic acid-d_4 (0.3 ml), deuterium oxide (0.3 ml) and deuterochloric acid (37% in ^2H_2O, 0.8 ml). The solution was sealed in a pyrex tube and heated in a titanium autoclave at 190°C for 5 h. The product was extracted with ethyl acetate and washed with water, and the organic layer was evaporated to dryness. The resulting residue was treated with deuterium-labelled acids once more, according to the procedure described above. The yield of the final product was 29 mg. It gave a single peak with high-performance liquid chromatography (HPLC) (the analytical conditions are described below). The isotopic purity was 99% as PHPA-d_6, measured by gas chromatography—mass spectrometry (GC—MS) after it was converted to the pentafluorobenzyltrimethylsilyl derivative as described below.

[2,2,2',5',6'-$^2H_5$] -3'-[$^2H_3$] methoxy-4'-hydroxyphenylacetic acid (HVA-d_8) and [2,2,2',5',6'-$^2H_5$] -3'-hydroxy-4'-[$^2H_3$] methoxyphenylacetic acid (iso-HVA-d_8). [2,2,2',5',6'-$^2H_5$] -3,4-dihydroxyphenylacetic acid (DOPAC-d_5) was prepared in the same manner as described for the preparation of PHPA-d_6. The crude product was dissolved with 3 *M* Tris—HCl buffer (pH 8.6) and then applied to an alumina column (Woelm Pharma). The column was washed with water and the washings discarded. Then it was eluted with 0.1 *M* hydrochloric acid. From the eluate, DOPAC-d_5 was extracted with ethyl acetate (yield 65%).

DOPAC-d_5 (120 mg) was dissolved in methanol (1 ml) and the solution was chilled in a ice-bath. Then sodium methoxide (2.1 *M* in methanol, 890 μl: 2.5 equiv.) was added to the solution and the reaction mixture was stirred for 20 min at 0°C. Methyliodide-d_3 (120 μl: 2.5 equiv.) was then added and the mixture was stirred for 1 h at 20°C. The reaction mixture was acidified with 0.1 *M* hydrochloric acid and extracted with ethyl acetate. After ethyl acetate had been removed, the extract was dissolved with 0.1 *M* phosphate buffer (pH 8.0) and esterase (90 U) was added. The mixture was incubated for 24 h at 37°C. Alumina (20 g) and 3 *M* Tris—HCl buffer (pH 8.6, 2 ml) were added, and the mixture was stirred using a vortex mixer. Then alumina was filtered off and washed with water. The filtrate and the washings were combined. From this solution, HVA-d_8 and iso-HVA-d_8 were purified by HPLC (yield of HVA-d_8 2.5 mg, iso-HVA-d_8 3.0 mg). The isotopic purity of both of these compounds was 91%.

[2',5',6'-$^2H_3$] -3'-[$^2H_3$] methoxy-4'-hydroxymandelic acid (VMA-d_6). 3,4-Dihydroxybenzaldehyde-d_3 was prepared in the same manner as the preparation of PHPA-d_6 except for the reaction conditions (the acid solution was heated at

150°C for 30 min). The extract from the reaction mixture with ethyl acetate was applied to a silica gel column (15 cm × 1.2 cm I.D.). After the column was washed with dichloromethane, 3,4-dihydroxybenzaldehyde-d_3 was eluted with ethyl acetate.

3,4-Dihydroxybenzaldehyde-d_3 was then O-methylated in the same manner as described for the preparation of HVA-d_8. The resulting mixture of vanillin-d_6 and isovanillin-d_6 was purified by silica-gel thin-layer chromatography (silica gel F_{254}, Merck, 2 mm × 20 cm × 20 cm) developed with dichloromethane—methanol—*n*-hexane (15:1:1). The yield was 52 mg.

Then, deuterated vanillin was converted to VMA [4]. Dioxane (0.6 ml), bromoform (120 μl) and the solution (1 ml) of potassium hydroxide (1.1 g) and lithium chloride (0.4 g) in deuterium oxide (4 ml) were added to the mixture of vanillin-d_6 and isovanillin-d_6. The solution was stirred at room temperature for 55 h. Then, deuterium oxide (15 ml) was added to the reaction mixture and the pH was adjusted to 9 with deuterochloric acid. The remaining vanillin was washed out with diethyl ether. The aqueous layer was acidified with deuterochloric acid (to pH 1), and the product was extracted with ethyl acetate. VMA-d_6 was purified by HPLC (yield 4 mg). The isotopic purity of VMA-d_6 was 98%.

Gas chromatography—negative-ion chemical-ionization mass spectrometry

A Finnigan 4000 GC—MS instrument equipped with a pulsed positive-ion negative-ion chemical-ionization accessory was used for the selective-ion monitoring in the present study. The column used was an OV-101 FS-WCOT capillary column (25 m × 0.25 mm I.D., Gasukuro Kogyo, Tokyo, Japan). Methane was used as the carrier gas and also served as the chemical-ionization reagent gas. The ion source pressure and electron energy were maintained at 0.15 Torr and 90 eV, respectively.

High-performance liquid chromatography

A JASCO Tri Rotar I high-performance liquid chromatograph was used for the purification of synthesized deuterium-labelled compounds and urinary samples. A 250 mm × 4 mm I.D. stainless-steel column packed with NS-Gel C_8 (5 μm, Sakata Shoukai, Tokyo, Japan) was used. It was covered with a column jacket at 50°C.

The separation was carried out using a reproducible gradient-elution technique [5]. The first solvent was 0.01 *M* acetic acid containing Na_2EDTA (10 mg/l), and the second solvent was 50% aqueous acetonitrile solution. The sample solution was injected from an autosampler (KSST-60, Kyowa Seimitsu) and the eluate was collected by means of a fraction collector under the time control using a sequence controller (Omron SCY-PO). The details of this apparatus and procedures were described in our previous paper [2].

Derivatization

To each sample, which was dried in vacuo, 100 μl of 5% pentafluorobenzyl bromide in acetone and 5 mg of a mixture of sodium sulphate and sodium carbonate (1:1) were added and sealed into a glass ampule. The mixture was

heated at 40°C for 1 h. Benzene (500 μl) was added and the supernatant was evaporated to dryness in a stream of nitrogen. Then, 100 μl of a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide and pyridine (2:1) were added to the residue and the solution was allowed to stand for more than 1 h at room temperature. A 1-μl aliquot of the resulting solution was subjected to GC—NICI-MS.

Tracer experiment

Phenylalanine-d_5 (5 mg/kg) was administered orally at 11.00 a.m. and urine was collected every hour. An aliquot of each urine containing 0.2 mg of creatinine was sampled and PHPA-d_6, HVA-d_8, iso-HVA-d_8 and VMA-d_6 (50 ng each) were added as internal standards. The acids were extracted with ethyl actate at pH 1, purified by HPLC as described above, and derivatized for GC—NICI-MS analysis.

Calculation

The quantities of labelled and non-labelled metabolites were calculated from their relative peak heights to that of a standard sample on mass fragmentography. Thus, the values obtained were corrected by considering the isotopic purity of the Phe-d_5 administered and the natural abundance of stable isotopes in each derivative.

RESULTS AND DISCUSSION

The precise determination of labelled components in a stable isotope-labelled tracer experiment can best be accomplished using a highly labelled compound as an internal standard for the component that is required to be determined. The reasons for this are: first, the peak derived from an internal standard does not have to overlap the peak from the tracer or endogenous metabolite on mass fragmentography; second, it behaves chemically in the same way as these substances; third, it gives a peak at nearly the same retention time (within the isotopic effect), which is helpful as a means of assigning the peak derived from the tracer on mass fragmentography. So we synthesized PHPA-d_6, HVA-d_8 and VMA-d_6 as internal standards for the determination of the tracer, i.e. PHPA-d_4, HVA-d_3 and VMA-d_3 derived from Phe-d_5.

Fig. 1 shows the synthetic pathways of the deuterium-labelled compounds for internal standards.

A method for the introduction of three deuteriums to the methyl position of HVA or VMA was developed whereby methyl iodide-d_3 was used as a source of deuterium. With this procedure, the 3,4-dihydroxyphenyl group was converted to the 3-[$^2H_3$]methoxy-4-hydroxyphenyl group and the 3-hydroxy-4-[$^2H_3$]methoxyphenyl group, in nearly equivalent proportions. This method was applied to the conversion from DOPAC-d_5 to HVA-d_8 and iso-HVA-d_8. These products were separated from each other by gas—liquid chromatography after derivatization.

For the synthesis of VAM-d_6 we tried, at first, to exchange the aromatic and benzyl hydrogens of VMA or 3,4-dihydroxymandelic acid to deuterium by acid catalysis according to the method reported by Karoum et al. [6]. However,

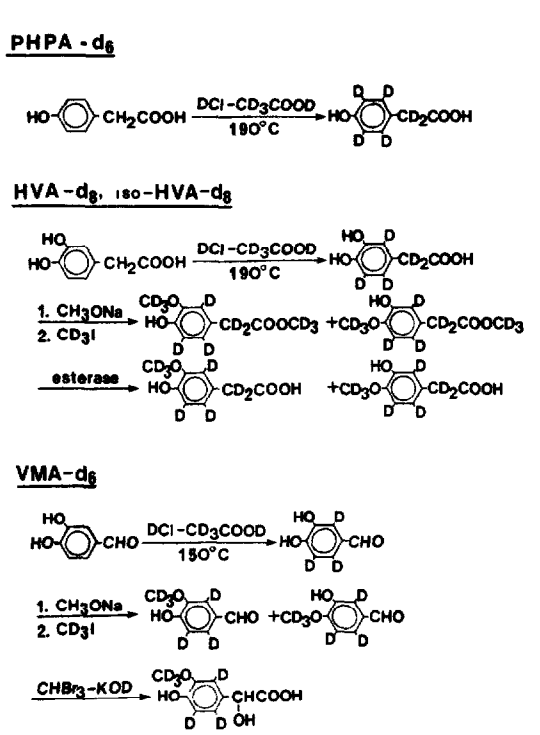

Fig. 1. Synthetic pathways of PHPA-d_6, HVA-d_8 and VMA-d_6, which were used as internal standards in the GC—NICI-MS determination of PHPA-d_4, HVA-d_3 and VMA-d_3, metabolized from Phe-d_5.

these materials were immediately decomposed, even at 120°C, under these strongly acidic conditions. A new method was therefore intended to synthesize deuterium-labelled VMA. First, 3,4-dihydroxybenzaldehyde-d_3 was prepared by means of a deuterium-hydrogen exchange reaction catalysed by strong deuterium-labelled acids. It was then converted to a mixture of vanillin-d_6 and isovanillin-d_6 by means of O-[$^2H_3$]methylation as described above. Finally, this mixture was treated with bromoform under alkaline conditions and VMA-d_6 was obtained. Interestingly, no iso-VMA-d_6 was produced. This phenomenon is due to the lability of isovanillin to alkali.

Each of the synthesized materials, PHPA-d_6, HVA-d_8, iso-HVA-d_8 and VMA-d_6, gave a single peak on HPLC and their isotopic purity was sufficient for use as internal stadards judged by mass fragmentography (data are shown in the experimental section).

In the previous study [2], we used PHPA-d_4 and HVA-d_5 as the internal standards for the determination of PHPA, HVA and VMA, respectively. For this study, all deuterium-labelled compounds of the target were prepared for us as internal standards so as to avoid the experimental errors due to recovery variation in the course of purification and derivatization. PHPA, HVA and VMA fractions prepared from urinary samples by ethyl acetate extraction and HPLC separation were collected and converted to each of the derivatives indicated in Fig. 2. The yields of these derivatives were all excellent. These

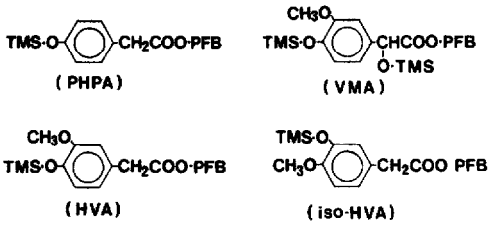

Fig. 2. Derivatives of PHPA, HVA and VMA for GC—NICI-MS analysis.

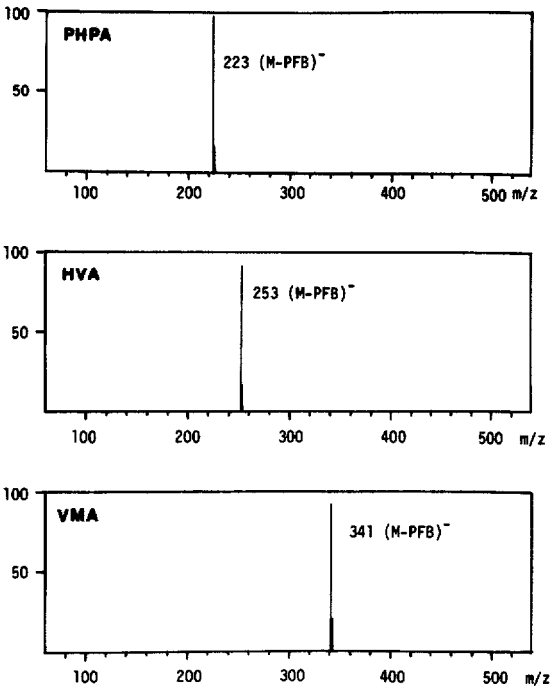

Fig. 3. Negative-ion chemical-ionization mass spectra of derivatives of PHPA, HVA and VMA.

derivatives produce the $(M - PFB)^-$ anion as efficiently and as predominantly as other pentafluorobenzyl (PFB) derivatives studied previously [7] in response to NICI-MS analysis (their mass spectra are shown in Fig. 3). These techniques make it possible to determine the super-sensitivity of the tracer. The reaction conditions of the derivatization are so mild that no exchange reactions between deuterium in the labelled compounds and hydrogen in the media have ever been detected during analysis.

The microanalytical methods established here were next applied to stable isotope-labelled tracer experiments using human subjects.

Fig. 4 shows the time courses of the tracer levels of PHPA-d_4, HVA-d_3 and VMA-d_3 excreted into the urine when Phe-d_5 (5 mg/kg) was administered orally to healthy subjects and patients with depression. Maximum excretion of PHPA-d_4, HVA-d_3 and VMA-d_3 emerged between 1 and 2 h, 2 and 4 h and 4 and 6 h,

respectively, and the difference between the control and the patients was not clearly observed. However, there appears to be some tendency for the levels of PHPA-d_4 and HVA-d_3 to decrease slightly in some patients. These preliminary results imply the possibility that the suppressed turnover of tyrosine oberved in depression causes some metabolic disorders of dopamine or tyramine (whose main metabolites are thought to be HVA and PHPA, respectively), although it is not clear at present whether these metabolic alterations really stem from the repressive condition or not. Studies on this are currently in progress.

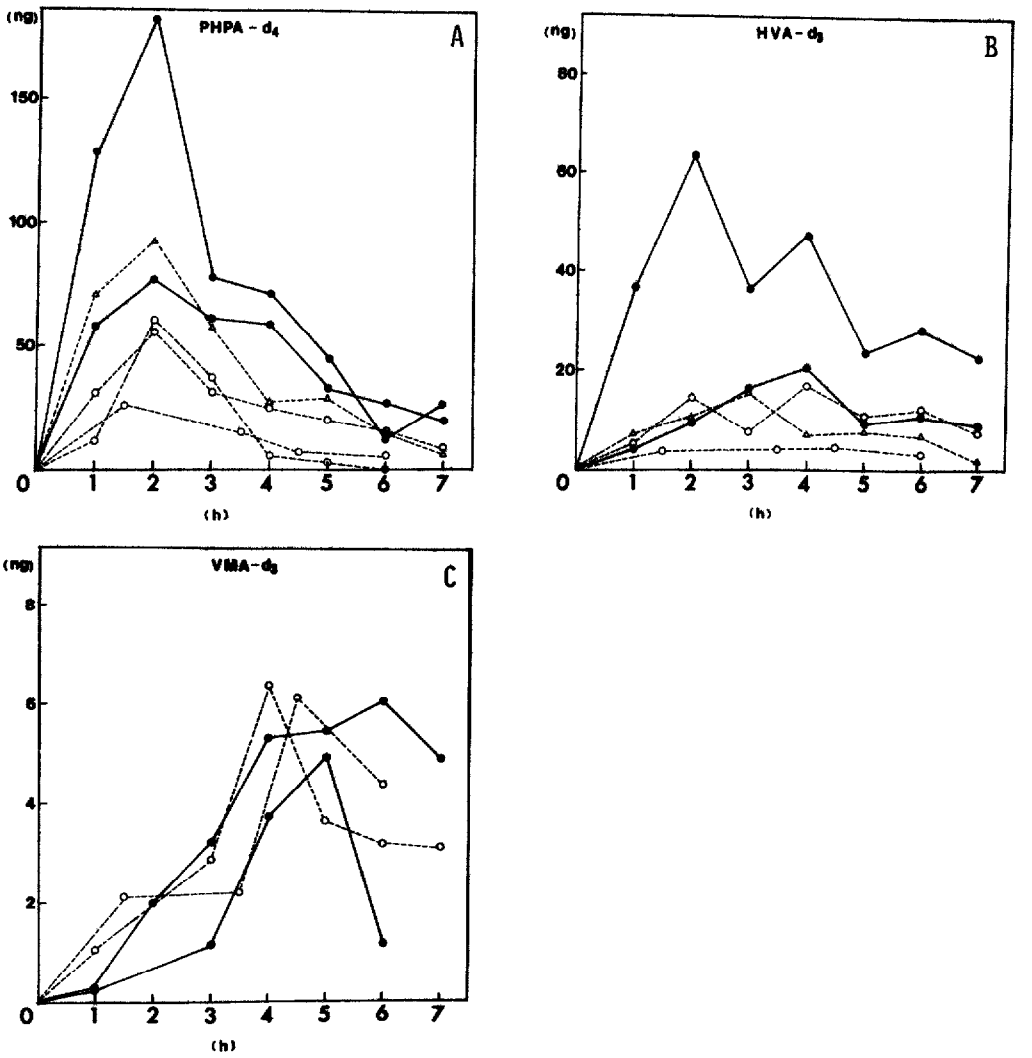

Fig. 4. Time courses of the tracer levels of (A) PHPA-d_4, (B) HVA-d_3 and (C) VMA-d_3, excreted into urine. The tracer levels are expressed as the weight of tracer every hour per 5 mg of Phe-d_5. •, Values of normal subjects; ○, values of patients with depression; △, values of patients on antidepressant therapy.

Fig. 5 illustrates a mass fragmentogram of a urinary sample monitored by the mass number of HVA. The results indicated the presence of iso-HVA in urine. This was ascertained by the observation that the peak assigned to iso-HVA emerged at the same time as the peak of the iso-HVA-d_8, which was added as an internal standard. The ratio of iso-HVA to HVA is in the range 1—3%. In contrast to the presence of iso-HVA, we could detect little iso-VMA. These results correspond well with the work reported by Muskiet et al. [8] (iso-HVA/HVA = 6%, iso-VMA/VMA = 0.7%).

CONCLUSION

A method for stable isotope-labelled tracer experiments from Phe-d_5 to its metabolites, PHPA-d_4, HVA-d_3 and VMA-d_3, in humans has been established using GC—NICI-MS.

This method was applied to a comparison between the control and depressive patients in terms of the metabolism of these components. As a result, a slight disorder of the PHPA or HVA metabolism was detected in subjects with depression. It appears that this method has the potential to contribute to the clinical study of other disorders in which metabolic changes of catecholamines or tyramine are suspected.

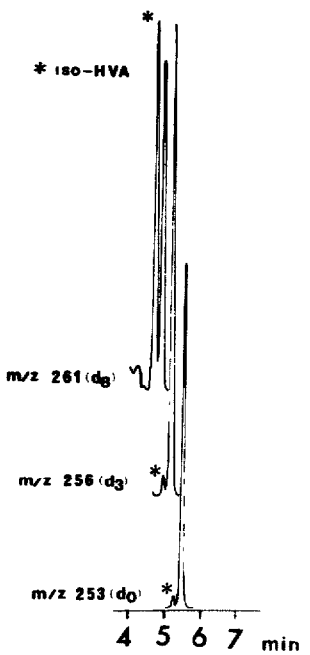

Fig. 5. Mass fragmentogram of HVA and iso-HVA derivatives in a urinary sample. The mass number of $(M - PFB)^-$ was monitored on NICI-MS using the selective-ion monitoring technique. Key: d_8 = internal standard; d_3 = tracer derived from Phe-d_5; d_0 = endogenous metabolite.

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