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SENSITIVE DETERMINATION OF DEUTERATED AND NON-DEUTERATED PHENYLALANINE AND TYROSINE IN HUMAN PLASMA BY COMBINED CAPILLARY GAS CHROMATOGRAPHY—NEGATIVE ION CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A combined capillary gas chromatography—negative ion chemical ionization mass spectrometric method for the determination of deuterated and non-deuterated phenylalanine and tyrosine in plasma was developed. Phenylalanine and tyrosine were converted to pentafluorobenzyl—trifluoroacetyl (PFB—TFA) and PFB—TFA—trimethylsilyl (TMS) derivatives, respectively, after prepurification with a Bio-Rad AG 50W-X2 cation-exchange column. These derivatives showed good gas chromatographic separation properties and provided intense $(M - PFB)^-$ ions. These ions were ideal for the specific and sensitive determination of deuterated and non-deuterated phenylalanine and tyrosine by selected ion monitoring assay. [2,2′,3,3,3′,4′,5′,6′-$^2H_8$] L-Phenylalanine and [2,2′,3,3,3′,5′,6′-$^2H_7$] L-tyrosine were used as internal standards. This method was used to determine the plasma levels of deuterated and non-deuterated phenylalanine and tyrosine, after oral administration of [2′,3′,4′,5′,6′-$^2H_5$] L-phenylalanine to a healthy person.

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INTRODUCTION

A number of methods [1—12] have been reported for the determination of the compounds related to catecholamines and indoleamines in plasma, urine, cerebrospinal fluid, etc., since the proposal of the catecholamine hypothesis of affective disorders — investigations that have implicated catecholamines as aetiological factors. However, attempts to investigate the relationship between the levels of such compounds and the conditions of the disorders have not yielded uniform results.

Recently, many investigators have shown that gas chromatography—mass spectrometry (GC—MS), using a stable isotope compound as a tracer, is a powerful tool for the detailed investigation of metabolism in vivo. In a previous paper [13], we reported the GC—MS method for metabolic studies of phenylalanine and tyrosine using ring-deuterated phenylalanine. We also reported that a remarkable decrease in the turnover rate of tyrosine was found in a group of depressive patients.

During the course of further studies, it became necessary to develop a more sensitive method to determine deuterated and non-deuterated phenylalanine and tyrosine in plasma. This paper deals with the development of a highly sensitive method for the determination of such compounds using combined capillary GC and negative ion chemical ionization mass spectrometry (NICI-MS).

MATERIALS AND METHODS

Apparatus

A Finnigan 4000 gas chromatograph—mass spectrometer equipped with a pulsed positive ion negative ion chemical ionization accessory (Finnigan, CA, U.S.A.) was used in this work. Sample injections were performed with a solvent-cut injector (Gaskuro Kogyo, Tokyo, Japan). GC separation was carried out with an OV-101 coated fused-silica capillary column (25 m × 0.25 mm I.D.). Methane was used as the GC carrier gas and the chemical ionization reagent gas. The capillary column head pressure was held at 0.6 kg/cm^2, which gave a 1 ml/min flow-rate for methane. The ion-source pressure, the ion-source temperature, the electron energy and the emission current were maintained at 0.15 Torr, 250°C, 90 eV and 300 μA, respectively.

Reagents

[2′,3′,4′,5′,6′-$^2H_5$] L-Phenylalanine (Phe-d_5) was synthesized according to the method reported by Matthews et al. [14] with a slight modification. [2′,3′,5′,6′-$^2H_4$] L-Tyrosine was prepared according to the method reported by Tokuhisa et al. [15]. [2,2′,3,3,3′,4′,5′,6′-$^2H_8$] L-Phenylalanine (Phe-d_8) and [2,2′,3,3,3′,5′,6′-$^2H_7$] L-tyrosine (Tyr-d_7) were purchased from Merck Frosst Canada Inc. The percentage of deuterated species in the Phe-d_5 preparation was calculated from the relative intensities of isotopic peaks corresponding to $(M - PFB)^-$ measured for pentafluorobenzyl—trifluoroacetyl (PFB—TFA) derivatives of Phe-d_0 and Phe-d_5, assuming that the relative peak intensities of the isotopic ions $(M - PFB)^-$, $(M + 1 - PFB)^-$, $(M + 2 - PFB)^-$ and $(M + 3 -$

$PFB)^-$ of each species are analogous. Cation-exchange resin AG 50W-X2 (200-400 mesh) was obtained from Bio-Rad and used after treating with a 10-fold volume of 2 *M* hydrochloric acid at 80°C for 3 h and washing with redistilled water. The other reagents and solvents used in this work were reagent grade.

Administration of Phe-d_5 and analytical procedure

Phe-d_5 (5 mg/kg) was administered orally at 11.00 a.m. to a healthy person, and heparinized blood samples were obtained from the fore-arm vein at certain intervals. The blood samples were centrifuged at 10 000 *g* for 15 min in a refrigerated centrifuge. The plasma obtained was stored at −20°C until analysed.

Aliquots of 20 μl of plasma sample were deproteinized with 500 μl of 80% aqueous ethanol containing 200 ng each of Phe-d_8 and Tyr-d_7, and centrifuged. The supernatant was evaporated to dryness on a rotary evaporator and the residue was redissolved with 1 ml of 25 m*M* pyridine—formate buffer (pH 2.5). The solution was applied to a 150 mm × 5 mm I.D. glass column containing 0.3 ml of AG 50W-X2 (H^+ form). After the column had been drained, it was washed with 5 ml of 25 m*M* pyridine—formate buffer (pH 3.0). The amino acids were then eluted from the column with 6 ml of 25 m*M* pyridine—formate buffer (pH 4.5). An aliquot of the eluate was evaporated to dryness and the residue was redissolved with 200 μl of 80% ethanol. The solution was transferred into a siliconized glass ampoule for the derivatization and the solvent was evaporated in a nitrogen stream. A 50-μl aliquot of a mixture of trifluoroacetic anhydride (TFAA) and acetonitrile (1:1) was added to the residue. After 5 min of reaction at room temperature, the excess TFAA and acetonitrile were evaporated in a nitrogen stream. The residue was mixed with 50 μl of 2.5% pentafluorobenzylbromide (PFB-Br) in acetone for 5 min at room temperature in the presence of 4—5 mg of a mixture of sodium sulphate and sodium carbonate (1:1). The reaction mixture was diluted with 0.5 ml of *n*-hexane and the supernatant was transferred to another siliconized ampoule. After evaporation of the solvent and the excess reagent, the ampoule was placed for 30 min at room temperature in a vacuum sample-drying apparatus to remove the excess reagent as completely as possible. Then, 50 μl of the mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (4:1) were added to the residue. After 5 min of reaction at room temperature, the excess reagent and solvent were removed in a nitrogen stream. The residue was redissolved with 50 μl of ethyl acetate. A 1-μl aliquot of the resulting solution was injected into the gas chromatograph—mass spectrometer.

RESULTS AND DISCUSSION

The methane NICI mass spectrum of the PFB—TFA derivative of phenylalanine is shown in Fig. 1. As shown in a previous paper [16], this derivative gave good ionization efficiencies in the methane NICI mode and only 20 fg of the derivative were needed to generate a selected ion current profile for the $(M - PFB)^-$ ion, which had a signal-to-noise ratio of better than 3:1. This derivative also showed good properties in GC separation. The PFB—TFA derivative was used in the following work.

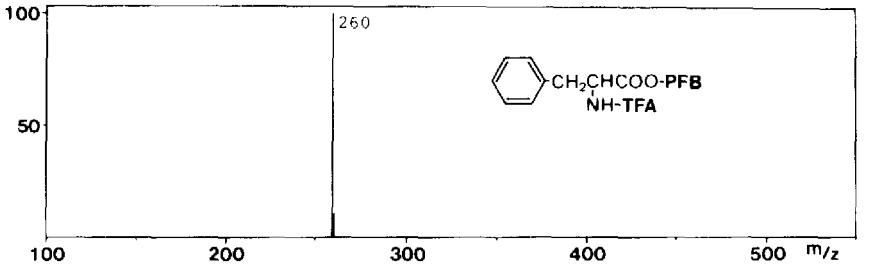

Fig. 1. Methane NICI mass spectrum of the PFB—TFA derivative of phenylalanine.

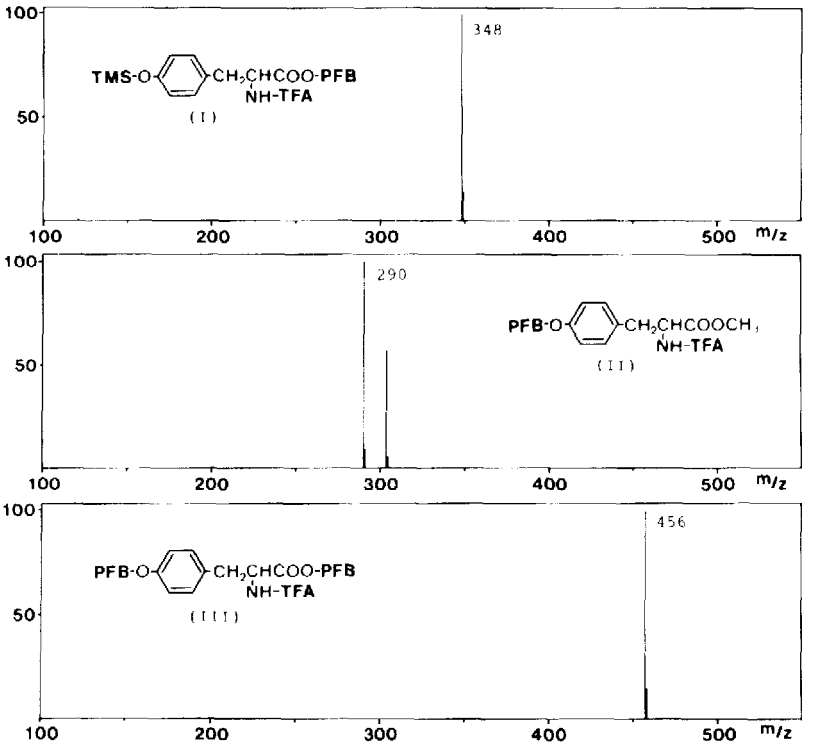

Fig. 2. Methane NICI mass spectra of PFB—TFA—TMS, PFB—TFA—methyl and PFB—TFA derivatives of tyrosine.

In the case of tyrosine, the derivatives shown in Fig. 2 were investigated. All of the derivatives showed good ionization efficiencies in the methane NICI mode compared with those in the methane positive ion chemical ionization (PICI) mode. However, the volatility of derivative III was relatively low and the sensitivity was not so good, probably due to a low yield in the derivatization. Although derivative II showed similar volatility and sensitivity to that of derivative I, derivative I was chosen for this work because it was possible to derivatize phenylalanine and tyrosine in the same sample to the PFB—TFA derivative and derivative I, respectively. The derivatization of phenylalanine and tyrosine was carried out according to the procedure described in the experimental section. Measurements of relative peak height at *m/z* 268 to that

at m/z 260 for phenylalanine after repeated derivatization for a sample containing certain amounts of Phe-d_0 and Phe-d_8 showed good reproducibility (S.D. = 0.98, n = 8). And the value did not change even if each reaction time was changed to about three times longer. Similar results were also obtained for a sample containing Tyr-d_0 and Tyr-d_7. These data suggest that no detectable ^2H—H exchange reactions occur in the derivatization reactions, not only for Phe-d_8 and Tyr-d_7 but also for Phe-d_5 and Tyr-d_4. These derivatives were stable for at least 2 days.

As described in the procedure, a cation-exchange column (AG 50W-X2) was used for the prepurification of phenylalanine and tyrosine. Pyridine—formate buffers were used in this step, because the buffer reagents could easily be evaporated. No detectable ^2H—H exchange reaction was observed in the prepurification.

In metabolic studies, the compounds used must suffer no loss of the labels. The deuterated phenylalanine, Phe-d_5, has been employed as a tracer to investigate the in vivo metabolism of phenylalanine and tyrosine by some investigators [17—19]. However, these reports contain no description relating to the ^2H—H exchange. An investigation of the ^2H—H exchange of Phe-d_5 and Tyr-d_4 in 0.01 *M* hydrochloric acid, 0.01 *M* sodium hydroxide and 0.01 *M* phosphate buffer (pH 7.0) was carried out to check the loss or scrambling of the labels under in vivo conditions. The peak-height ratios of Phe-d_5 and Tyr-d_4 (ring labelled) to their non-deuterated counterparts for the sample containing deuterated and non-deuterated amino acids, which was incubated at 37°C in these media, was in accord with that for the non-incubated sample. These data suggest that Phe-d_5 is useful for the investigation of the metabolic differences between patients and normal subjects.

Calibration curves for the determination of Phe-d_0, Phe-d_5 or Tyr-d_0 were prepared by plotting the corresponding peak-height ratio to that of internal standard (Phe-d_8 or Tyr-d_7) after correction for background due to the isotopic peak on the y-axis, and the amount (nmol) of each amino acid on the x-axis. The curves were linear with zero abscissa and ordinate intercept in a range from at least 1 pmol to 5 nmol of each amino acid. The curve for Tyr-d_4 was prepared in the following manner. When the calibration curve for Tyr-d_0 is expressed as $y = ax$, that for Tyr-d_4 is expressed as $y = (K_4/K_0)ax$, where K_4 or K_0 is the ratio of the ion abundance at m/z 352 or m/z 348, respectively, to total abundance of the isotopic ions corresponding to $(M - PFB)^-$ for the derivative of Tyr-d_4 or Tyr-d_0 sample. K_4 was calculated from the relative isotopic peak intensities for Tyr-d_0 and the percentage of deuterated species of the Phe-d_5 sample (d_0: 0.661%, d_1: 0.008%, d_2: 0.028%, d_3: 0.387%, d_4: 7.754%, d_5: 90.41%, d_6: 0.740, d_7: 0.014%), assuming that the isotopic peak-height ratios corresponding to $(M - PFB)^-$ of each species are analogous, deuterium was introduced equally to all positions of the benzene ring for Phe-d_5, and each species was equally metabolized to tyrosine in vivo. For the Phe-d_5 used in this work, K_4/K_0 was 0.920.

Fig. 3 represents the time course of plasma levels of Phe-d_0, Phe-d_5, Tyr-d_0 and Tyr-d_4 after the oral administration of Phe-d_5 (5 mg/kg), and Fig. 4 shows the mass fragmentograms obtained from the plasma sample 30 min after the administration. The plasma level of Phe-d_5 elevated rapidly and reached a

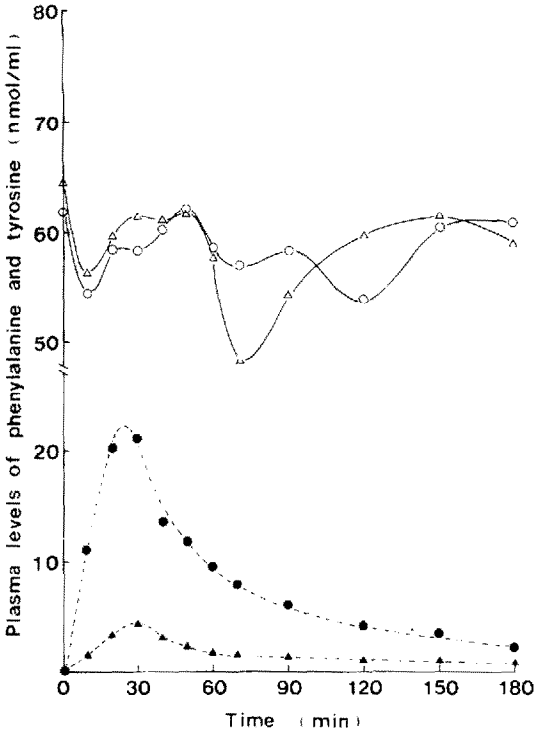

Fig. 3. Time course of plasma levels of Phe-d_0, Phe-d_5, Tyr-d_0 and Tyr-d_4 after oral administration of Phe-d_5 (5 mg/kg) to a normal adult. (○—○) = Phe-d_0; (●- - -●) = Phe-d_5; (△—△) = Tyr-d_0; (▲- - -▲) = Tyr-d_4.

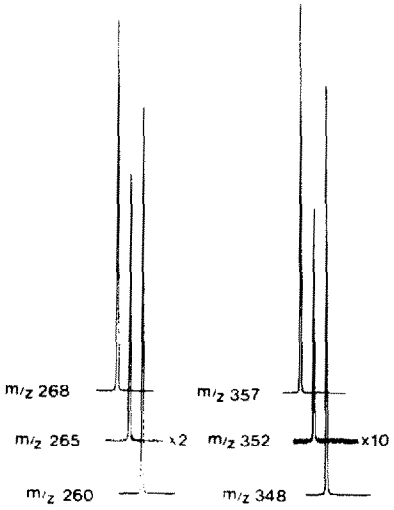

Fig. 4. Mass fragmentograms obtained from the plasma sample 30 min after the administration of Phe-d_5 (5 mg/kg) to a normal adult.

maximum about 25 min after administration. It then gradually decreased. The maximum Tyr-d_4 level was observed at about 30 min after administration. Compared with daily changes in these levels, the changes caused by the

administration of Phe-d_5 (5 mg/kg) may not be particularly large. Furthermore, this method seems to make it possible to carry out an investigation of the in vivo metabolism of Phe and Tyr under conditions closer to the physiological ones, i.e. using smaller amounts of Phe-d_5. Although similar methods have been reported by some investigators, they used large amounts of Phe-d_5 as a tracer, probably due to the low sensitivity of their methods. The administration of large amounts of tracer compound may not permit the metabolic investigation under physiological conditions, and is also expensive.

In tracer experiments using a deuterated compound as a tracer, biological isotope effects are often observed. This study did not fully investigate whether or not Phe-d_5 is affected in this way. However, Phe-d_5 may be usable for investigating relative metabolic differences between controls and patients.

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