SYNTHESIS OF DEUTERIUM LABELLED EUMELANIN (PRECURSOR) METABOLITES

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SUMMARY

Starting from 2,5,6-trideutero-3,4-dihydroxyphenylethylamine, a mixture of 4,7-dideutero-5,6-dihydroxyindole, 4,7-dideutero-5-hydroxy--6-trideuteromethoxyindole and 4,7-dideutero-6-hydroxy-5-trideuteromethoxyindole was prepared. A similar procedure for the preparation of 4,7--dideutero-5,6-dihydroxyindolyl-2-carboxylic, 4,7-dideutero-5-hydroxy-6--trideuteromethoxyindolyl-2-carboxylic and 4,7-dideutero-6-hydroxy-5--trideuteromethoxyindolyl-2-carboxylic acids from methyl ester of 2,5,6-trideutero-L-3,4-dihydroxyphenylalanine is described. The procedures are designed for the production of deuterium labelled internal standards for mass fragmentographic analysis of eumelanin (precursor) metabolites in urine. The methods can also be used for the synthesis of non-deuterated analogs.

Key Words: Deuterium, indoles, melanin precursors, melanoma markers.

INTRODUCTION

Recently, we have reported the gas chromatographic - mass spectrometric identification of five indolic compounds - 5,6-dihydroxyindole (5,6DHI), 5-hydroxy-6-methoxyindole (5H6MI), 6-hydroxy-5-methoxyindole (6H5MI), 5-hydroxy-6-methoxyindolyl-2-carboxylic and 6-hydroxy-5-methoxyindolyl-2-carboxylic acids (5H6MI2C and 6H5MI2C) - extracted from the urine of patients with malignant melanoma (1). Since 1967 two of them, 5H6MI2C and 6H5MI2C, have been known to be present in melanotic urine (2).

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For the simultaneous quantitative measurements of these eumelanin related compounds, a mass fragmentographic method has been developed using structurally related compounds as internal standards (unpublished results). However, in the most favourable case the mass fragmentographic assays require the use of suitable isotopically labelled internal standards enabling us to perform isotope - dilution analyses.

In this paper we describe the preparation of deuterium labelled 5,6DHI, 5H6MI, 6H5MI from ring-deuterated 3,4-dihydroxyphenylethylamine (dopamine - DA) and deuterium labelled 5,6-dihydroxyindolyl-2-carboxylic (5,6DHI2C) as well as isomeric 5H6MI2C and 6H5MI2C from ring-deuterated L-3,4-dihydroxyphenylalanine (DOPA). The method can be also used for the preparation of the naturally occuring non-deuterated compounds, for which no simple synthesis has yet been described.

MATERIAL AND METHODS

DOPA and DA were purchased from Sigma Chemical Co.; N-methyl-d_3-nitroso-p-toluensulphonamide (diazomethane-d_2 precursor) was from Merck, Sharp & Dohme. Ethyl acetate (gas chromatographic-spectroscopic quality) was from Baker Chemicals (Deventer, The Netherlands). Pentafluoropropionic anhydride (PFPA) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were from Pierce Chemical Company. All other chemicals and solvents were purchased from Merck.

Gas chromatography (GC) was performed on a Packard - Becker Model 417 gas chromatograph equipped with a 25 m x 0.26 mm (I.D.) fused silica column coated with CP-Sil-5 (0.14 μm film thickness) from Chrompack (Middelburg, The Netherlands). Gas flow was 0.9 ml He x min^{-1}, split ratio 1:30, flame ionisation detector temperature 260°C, injector temperature 240°C. Oven temperature was programmed from 120°C to 220°C at 5°C x min^{-1}.

Gas chromatography - mass spectrometry (GC-MS) was performed using a Varian Model 3700 gas chromatograph coupled to a Varian MAT 44S mass spectrometer with a SS 200 data system. The gas chromatograph contained a 15 m x 0.25 mm (I.D.) glass capillary column coated with SE-54 from Franzen Analysen-Technik, Bremen, F.R.G. Injector temperature was 240°C, interface temperature 250°C and source temperature 250°C. Helium flow-rate was 1 ml x min^{-1}, ionisation energy 70 eV. The temperature program was from 120 - 230°C at 10°C x min^{-1}.

Derivatization of indolic acids was carried out with 150 µl of a 1:2 (v/v) mixture of HFIP and PFPA. Indoles containing no carboxy groups were treated with 100 µl of PFPA. After heating at 60°C for 10 min, the solvents were evaporated under nitrogen at 40°C and the residues dissolved in 100 µl of freshly prepared ethyl acetate with 5% of PFPA.

During the synthetic procedures, each step was checked by GC and GC-MS analysis.

EXPERIMENTAL

1. Synthesis of deuterium labelled 5,6DHI, 5H6MI and 6H5MI

a/ Preparation of 2,5,6-trideuterodopamine (DA-d_3)

The hydrogen-deuterium exchange reaction of DA was performed under similar conditions as have been described for other catecholamines (3). 25 mg of dopamine hydrochloride were dissolved in 4 ml of 1.37 mol x l^{-1} DCl in D_2O and the solution heated in a sealed tube in a heating block at 80°C for 20 hours. The solvent was evaporated in vacuo, the residue redissolved in 4 ml of DCl/D_2O solution (see above) and heated another 20 hours under the same conditions. The efficiency of deuterium incorporation was established by GC-MS.

b/ Synthesis of 4,7-dideutero-5,6-dihydroxyindole (5,6DHI-d_2) from DA-d_3

The solution of DA-d_3 was evaporated in vacuo and the residue dissolved in 4 ml of D_2O. 1.5 ml of 0.30 mol x l^{-1} potassium ferricyanide in 0.50 mol x l^{-1} sodium bicarbonate was added. The solution immediately turned red. The content of the tube was briefly mixed and 2.0 ml of a 0.45 mol x l^{-1} zinc acetate solution were added. The suspension was than extracted with 4 x 10 ml of ethyl acetate. Pooled ethyl acetate extracts were dried over anhydrous sodium sulphate.

c/ Synthesis of 4,7-dideutero-5-hydroxy-6-trideuteromethoxyindole (5H6MI-d_5) and 4,7-dideutero-6-hydroxy-5-trideuteromethoxyindole (6H5MI-d_5)

The ethyl acetate solution of 5,6DHI-d_2 was bubbled with diazomethane-d_2 for several minutes, the tube was tightly closed and stored at room temperature in the dark. The process of methylation was frequently checked by GC analysis. After 18 hours the ethyl acetate was evaporated and the residue dissolved in distilled methanol. The solution was kept under nitrogen at -20°C until use.

2. Synthesis of deuterium labelled 5,6DHI2C, 5H6MI2C and 6H5MI2C

a/ Preparation of 2,5,6-trideuterodopa (DOPA-d_3)

Deuterium labelled DOPA was prepared via the similar procedure as described for DA-d_3. 70 mg of DOPA was dissolved in 4 ml of 2.74 mol x l^{-1} DCl in D_2O and the solution was heated at 80°C for 20 hours. After evaporation the same procedure was repeated.

b/ Preparation of methyl ester of DOPA-d_3

The solution of deuterated DOPA was dried in vacuo, and the residue dissolved in 2 ml of ca 0.1 mol x l^{-1} DCl in CH_3OD (prepared by mixing

of CH_3OD with acetylchloride). The solution was allowed to stay at room temperature for 4 hours.

c/ Preparation of methyl ester of 4,7-dideutero-5,6-dihydroxyindolyl-2--carboxylic acid (5,6DHI2C)

After the evaporation in vacuo, the methyl ester of DOPA-d_3 was dissolved in 8 ml of D_2O. To that solution a 4.5. ml of potassium ferricyanide (0.30 mol x l^{-1}) in 0.50 mol x l^{-1} sodium bicarbonate were added. The red methyl ester of dopachrome was immediately formed. After a short mixing, 5 ml of a 0.45 mol x l^{-1} zinc acetate solution was added and formed suspension was extracted into 4 x 25 ml of ethyl acetate. Pooled ethyl acetate extracts were dried with anhydrous Na_2SO_4 and an aliquot of about 0.5 ml was used for GC-MS analysis.

d/ Trideuteromethylation of 5,6DHI2C-Me-d_2

Pooled ethyl acetate extracts were saturated with diazomethane-d_2 for several minutes. The solution was stored at room temperature in the dark. The process of methylation was frequently checked by GC. After 24 hours, ethyl acetate was evaporated.

e/ Hydrolysis of methyl esters of 4,7-dideutero-5,6-dihydroxyindolyl-2--carboxylic (5,6DHI2C-d_2), 4,7-dideutero-5-hydroxy-6-trideuteromethoxyindolyl-2-carboxylic (5H6MI2C-d_5) and 4,7-dideutero-6-hydroxy-5--trideuteromethoxyindolyl-2-carboxylic (6H5MI2C-d_5) acids.

The residue after the evaporation was dissolved in 2 ml of 2.5 mol x l^{-1} sodium hydroxide which contained 1 mmol of sodium meta-bisulfite. The solution was kept under the nitrogen in the shaking waterbath at 37°C. After two hours the solution was acidified by several drops of 37% DCl in D_2O to pH 1 and free acids were extracted with 3 x 10 ml of ethyl acetate. The ethyl acetate extracts were kept under nitrogen at -20°C until use.

RESULTS AND DISCUSSION

1. Synthesis of deuterium labelled 5,6DHI, 5H6MI and 6H5MI.

As has been described in the experimental part, each reaction step was checked by GC or GC-MS. The mass spectra of pentafluoropropionyl (PFP) derivatives of DA-d_0 together with its deuterated analog are shown in Fig. 1A. The differences in m/e of characteristic fragments of DA-d_3 $(PFP)_3$ showed that by analogy to the other catecholamines three hydrogen atoms were exchanged per molecule. To minimize possible reexchange, the next steps were performed in deuterated solvents.

Several methods for the synthesis of indolic compounds by cyclization of o-dihydroxyphenyl derivatives (e.g. DOPA, DA, epinephrine, norepinephrine) have been described (1,4-7). Our synthesis was based on the reccomendation of Harley-Mason and Bu'Lock (8), to use potassium ferricyanide and zinc acetate for the oxidative-reductive reaction and rearrangement of indole molecule. The formation of the pyrrole ring, however, results in the loss of one deuterium atom, leading to 5,6DHI-d_2 (Fig. 1B). To increase the content of deuterium in the isomeric hydroxymethoxyindoles, diazomethane-d_2 has been used for methylation. The analysis of their mass spectra showed that the majority of methoxy groups contained three deuterium atoms (Fig. 1C). This fact can be explained by the presence of exchangeable deuterium in the solvent.

With respect to the yield of the synthesis, the oxidation-reduction rearrangement of dopamine to 5,6DHI seems to be the most critical step. The ratio of synthetized isomeric hydroxymethoxyindoles gradually increased during the methylation. The average yield of 5,6DHI-d_2, 5H6MI-d_5 and 6H5MI-d_5 was 2 - 10 %.

2. Synthesis of deuterium labelled 5,6DHI2C, 5H6MI2C and 6H5MI2C.

The synthesis of the deuterium labelled indolic acids was started with deuterated DOPA prepared by exchange reaction in DCl/D_2O solution.

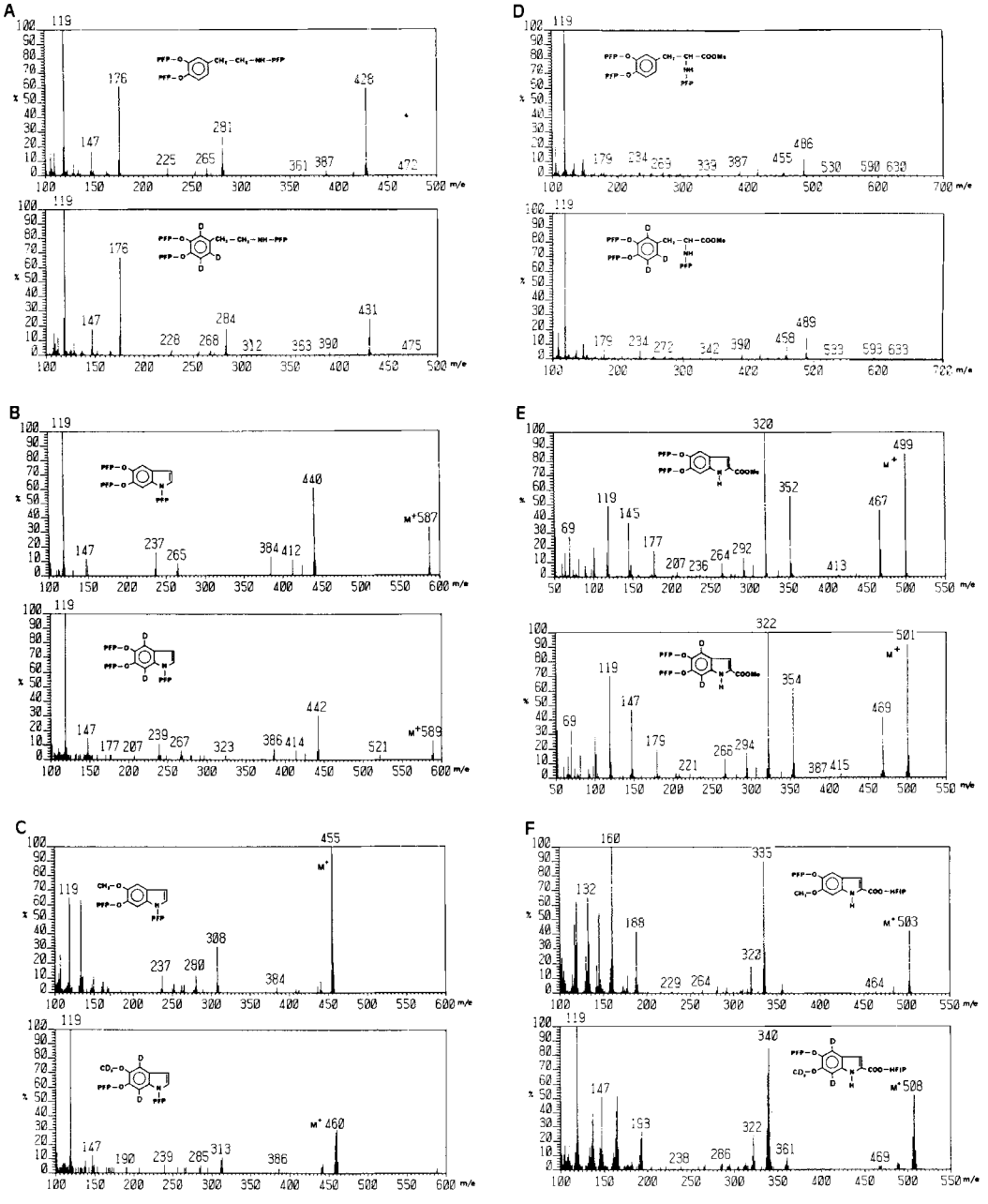

Fig. 1. Mass spectra of PFP (PFP-HFIP) derivatives of deuterated and non-deuterated DA (A), 5,6DHI (B), 5H6MI (C), DOPA-Me (D), 5,6DHI2C-Me (E), and 5H6MI2C (F).

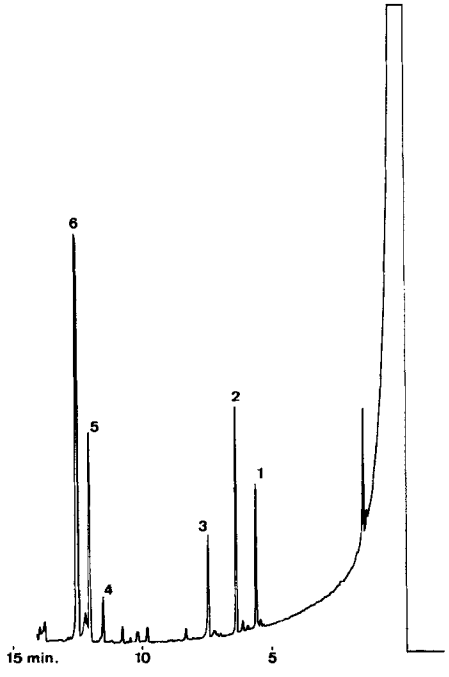

Fig. 2. Gas chromatogram of PFP (PFP-HFIP) derivatives of a mixture of the synthetized indolic compounds. 1 - 5,6DHI-d_2, 2 - 5H6MI-d_5, 3 - 6H5MI-d_5, 4 - 5,6DHI2C-d_2, 5 - 6H5MI2C-d_5, 6 - 5H6MI2C-d_5.

As in the case of DA, three deuterium atoms were exchanged per molecule. In Fig. 1D a mass spectrum of methyl ester of DOPA-d_3 is shown together with a mass spectrum of non-deuterated analog.

Experience has shown that during the cyclisation reaction of DOPA under different conditions, a variable fraction of the DOPA was decarboxylated. This process was found to be difficult to control. To avoid this side-reaction, the methyl ester of deuterated DOPA was prepared. In addition, the formed 5,6DHI2C-Me-d_2 (Fig. 1E) was easily extractable with ethyl acetate, in which the methylation could take place.

The hydrolysis of methyl esters of deuterated indolic acids was performed under the antioxidative protection of sodium meta-bisulfite. Two hours

of alkaline hydrolysis were shown to be sufficient as checked by gas chromatography. The yield of indolyl-2-carboxylic acids was in the range of 10 - 30 %. In the Fig. 1F, the mass spectra of deuterated 5H6MI2C together with non-deuterated analog are shown.

Gas chromatography analysis during the methylation procedures provided evidence that methylation preferentially took place at 6-O-position (Fig.2). This fact is in accordance with the observation of Axelrod and Lerner (7), who described the predominance of 6-O-methylation of 5,6DHI and 5,6DHI2C during the enzymatic methylation using catechol-O-methyl transferase. The quantitative estimation of indolic compounds in melanotic urine confirmed the preference for 6-O-methylation in vivo (9,10).

The presented method of preparing isotopically labeled indolic compounds is useful for the small scale preparation of eumelanin (precursor) metabolites, because it avoids complicated synthetic procedures. The method is simple and results in relatively high yields. Although the indolic compounds were not purified further, no interference was observed in the course of mass fragmentographic analysis of urines, in which they were used as internal standards.

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