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SHORT REPORTS

Diastereomers of 5-S-Cysteinyldopa

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Abstract. Diastereomers of 5-S-cysteinyldopa formed from D-dopa and L-cysteine or from L-dopa and D-cysteine can be separated from 5-S-cysteinyldopa formed from L-dopa and L-cysteine by liquid chromatography. The diastereomers have a great potential as internal standards in the analysis of 5-S-cysteinyldopa. They can be used as reference substances in the differentiation of stereospecific enzymatic oxidation of dopa from non-specific oxidation.

Cysteinyldopas are amino acids produced by nucleophilic addition of cysteine to dopaquinone (4). Cysteine may add at all positions in the aromatic ring, but addition in the 5-position is predominant (2, 4). Cysteinyldopas have been intensely studied in the past decade and have been defined as intermediates in melanin synthesis and as major excretion products of the melanocytes (2, 4, 5).

The naturally occurring cysteinyldopas are formed from L-dopa and L-cysteine. For investigative studies on cysteinyldopas we have found it of value to use diastereomers formed from L-dopa and D-cysteine or from D-dopa and L-cysteine. The preparation, identification and separation of these compounds will be described.

MATERIAL AND METHODS

Three diastereomers of 5-S-cysteinyldopa were prepared, 5-S-L-cysteinyl-L-dopa, 5-S-L-cysteinyl-D-dopa and 5-S-D-cysteinyl-L-dopa. The method for preparation of cysteinyldopas has been described previously (1), but some modifications have been made and therefore the procedure is given below.

Preparation of 5-S-D-cysteinyl-L-dopa

0.24 g D-Cysteine (Sigma) and 0.2 g L-dopa (Merck, Darmstadt) were dissolved in 100 ml 0.5 M Sörensen's phosphate buffer, pH 6.5. 75 mg Mushroom tyrosinase (2230 U/mg, Sigma) was added and the solution was allowed to stand at room temperature while being gently stirred. The progress of cysteinyldopa formation was recorded by measurement of the UV absorption of aliquots taken from the incubation mixture at 30-min intervals. During the incubation the absorption peak of dopa at 280 nm gradually decreased and absorption peaks at 292 nm and 255 nm related to cysteinyldopas increased. Experience had shown that the yield of cysteinyldopas was satisfactory when the ratio of absorbance at 292 nm and 255 nm was 0.7 in the samples taken from the reaction mixture. When this ratio was attained after about 3 hours the reaction was stopped by adding 4 M perchloric acid until the pH of the solution was 1.0. The solution was chilled in an ice bath. The precipitates of tyorsinase and $KClO_4$ were removed by centrifugation. The supernatant was put on a Dowex 50 W-X4 column (200×18 mm) in H^+-form. The column was washed with 200 ml M HCl and elution performed with 2 M HCl.

The effluent was collected in 25 ml fractions and UV absorption spectra were measured. Fractions 20–30 showed absorption peaks which indicated the presence of 5-S-cysteinyldopa. These fractions were pooled and evaporated to dryness under reduced pressure. The violet-blue powder obtained was dissolved in the mobile phase described below (1 mg/ml) and further purified on a semipreparative liquid chromatography column (μBondapak G8, Waters Ass.) equipped with a 0.6 ml loop. Mobile phase: MilliQ purified water containing 6 g methane sulphonic acid (Fluka AG) and 3 g H_3PO_4 per litre; pH 1.75. Flow rate 2 ml/min. The separation was monitored with a Varian Vari-Chrom UV detector working at 290 nm.

A major peak which appeared 11 min after injection was collected and found to have UV spectral data similar to 5-S-L-cysteinyl-L-dopa. New injections were performed until all of the original solution was used up and the material of the peak appearing after 11 min was collected. The combined fractions were passed through a Dowex 50 W-X4 column (50×5.4 mm) in H^+-form. After careful washing with water, elution was performed with M HCl in 5-ml portions. Fractions 3–9 contained a substance with spectral data similar to 5-S-L-cysteinyl-L-dopa. They were pooled and evaporated to dryness under reduced pressure. The colourless residue had a weight of 137.5 mg.

Identification

Elemental analysis showed the molecular formula H_{16}, C_{12}, N_2, O_6, S, 2HCl (found: H 4.9, C 34.5, N 6.5, S 7.5 and Cl 16.4% calculated H 5.2, C 33.9, N 6.6, S 7.5 and Cl 16.7%).

Strong absorption peaks at 292 nm (ε=2 800) and 255 nm (ε=3 450) were observed. The spectral data of the substance were close to those of 5-S-L-cysteinyl-L-dopa reported by Ito and Prota (3).

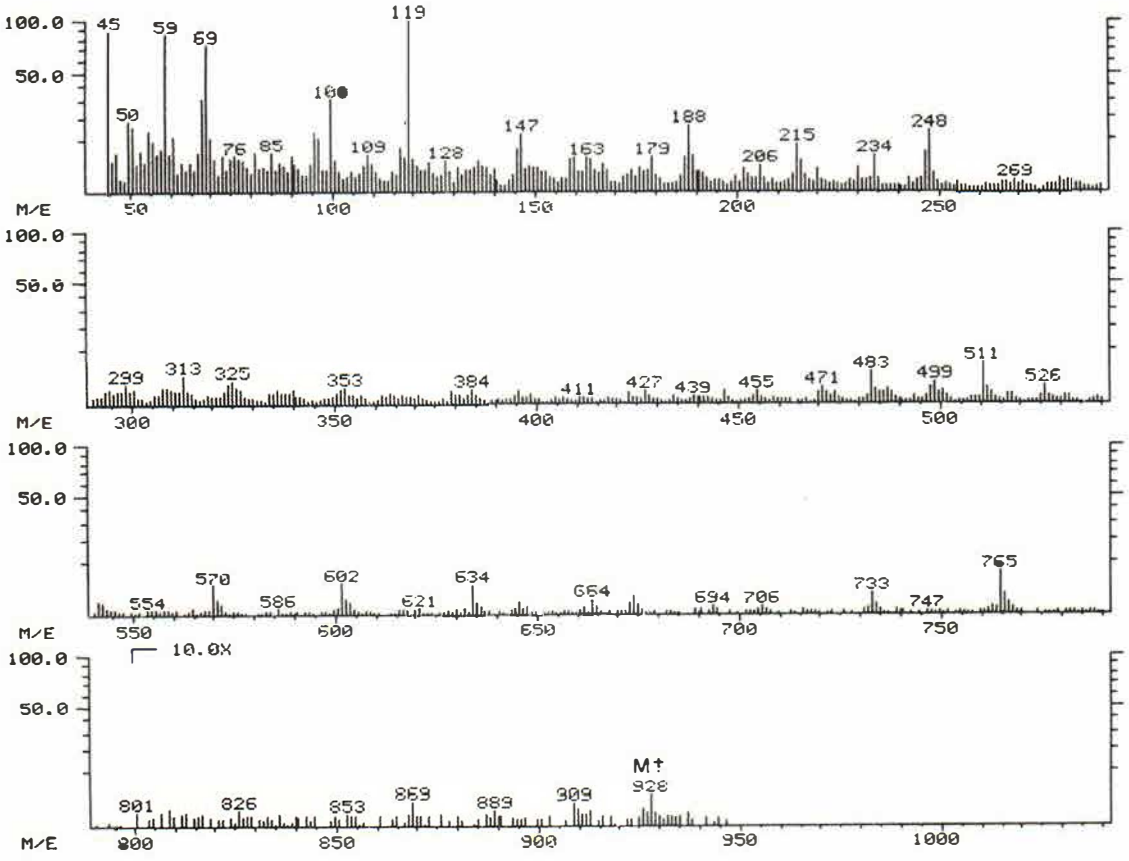

Fig. 1. Mass spectrogram of the PFPA-methyl derivative of 5-S-D-cysteinyl-L-dopa.

NMR spectra were recorded on a Jeol MA100, with 1 M DCl in D_2O as solvents, and TMS capillary as standard.

5-S-D-cysteinyl-L-dopa: 7.49 (1 H, d, aromatic), 7.39 (1 H, d, aromatic), 4.91–4.68 (2 H, m, -CH), 4.03–3.56 (4 H, m, $-CH_2$), J aromatic = 2.1 Hz.

5-S-L-cysteinyl-L-dopa: 7.50 (1 H, d, aromatic), 7.38 (1 H, d, aromatic), 4.95–4.70 (2 H, m, -CH), 4.05–3.60 (4 H, M, $-CH_2$), J aromatic = 2.1 Hz.

For *gas chromatography–mass spectrometry* earlier described fractions were evaporated. 2 mg of the powder was dissolved in 25 ml 0.1 M HCl and an aliquot corresponding to 10 μg was evaporated to dryness and the residue was dissolved in 150 μl methanol-HCl prepared by passing dry HCl gas into methanol to a concentration of 3 N HCl. The sample was then heated for 15 min at 100°C in a sealed tube. Next, the methanol–HCl was evaporated using a stream of dry nitrogen. 30 μl of pentafluorpropionic anhydride (PFPA) was then added, and the tube was heated at 60°C for 30 min in the tapered reaction vial. The PFPA was evaporated with dry nitrogen, and the residue was dissolved in 30 μl 1% PFPA in ethyl acetate.

Mass spectrum of the derivative of 5-S-D-cysteinyl-L-dopa showed the same fragmentation as previously reported for 5-S-L-cysteinyl-L-dopa with molecular ion at 928 (Fig. 1).

Liquid chromatographic (HPLC) separation of diastereomers of 5-S-cysteinyldopa

A Varian model 5000 (Varian, Palo Alto, Calif., USA) solvent delivery system was used.

Samples were injected with a valve injector Rheodyne model 7120 (Rheodyne, Berkeley, Calif., USA) equipped with a 100-μl loop. A model LC-10 amperometric detector (Bioanalytical Systems Inc., West Lafayette, Ind., USA) was used for detection. The detector potential was +0.75 V vs. the Ag/AgCl (3 M NaCl) reference electrode.

The working electrode was prepared from carbon paste (CPO) material. Columns were packed in 250×4.6 mm stainless steel tubes with a chemically bonded 5 μm C_{18} material (Nucleosil C_{18}, Machery, Nagel and Co., Düren, GFR). The mobile phase contained 6.0 g methane sulphonic acid and 3.0 g orthophosphoric acid per litre of MilliQ purified water. pH was adjusted to 3.00. The separation of 5-S-L-cysteinyl-L-dopa from 5-S-D-cysteinyl-L-dopa is shown in Fig. 2. 5-S-L-cysteinyl-D-dopa was not separable from 5-S-D-cysteinyl-L-dopa.

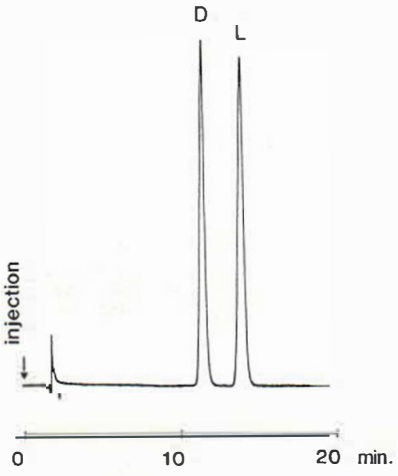

Fig. 2. Separation of 5-S-L-cysteinyl-L-dopa from 5-S-D-cysteinly-L-dopa. Mobile phase: Water, 3.0 orthophosphoric- and 6.0 g methanesulphonic acid per litre. pH 3.00. Column: Nucleosil C_{18} (5 μm), 250×46 mm. Flow rate: 1.5 ml/min. Peak identity: D = 5-S-D-cysteinyl-L-dopa, L = 5-S-L-cysteinyl-L-dopa.

DISCUSSION

The finding of easily separable diastereomers of 5-S-cysteinyldopa may be of great practical value in the study of cysteinyldopas and related substances.

Analysis of catecholic amino acids of importance in pigment metabolism, such as 5-S-cysteinyldopa, meets with considerable difficulties because such compounds are easily oxidized. Falsely low serum values of 5-S-cysteinyl-dopa may be obtained because of oxidation at sample collection, during sample handling, storage, or quantitation. Falsely high blood levels of cysteinyldopa may be seen in patients on dopa medication due to artificial oxidation of a minute portion of the large quantities of dopa present in the serum. To avoid errors of the type mentioned, different reducing agents may be added to the blood samples. If the reductant added is similar to the substance to be analysed the added reducing agent may also serve as an internal standard in the analytical procedure. Now that it is possible to analyse diastereomers of 5-S-cysteinyldopas separately, an ideal internal standard has been found.

Quantitation of the cysteine addition products of D- and L-dopa will be especially helpful in the investigation of tyrosinase activity in Man, since tyrosinase shows great stereospecificity in higher animals.

The diastereomers formed from D-cysteine may be used in the investigations of cysteine metabolism, e.g. for quantitation of L-cysteine levels in body fluids and tissues.

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Scanning Electron Microscopic Visualization of Rolled Hair (Poils en Spirale)

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Abstract. A patient suffering from recalcitrant pemphigoid developed rolled hairs after prolonged oral corticosteroid