

## METABOLISM OF 5-S-CYSTEINYLDOPA BY O-METHYLATION

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**Abstract.** 5-S-cysteinyl-dopa is metabolized by O-methylation on incubation with a liver extract and S-adenosyl methionine. O-methylated 5-S-cysteinyl-dopa isolated from melanoma urines by ion exchange and paper chromatography was identified by gas chromatography-mass spectrometry and NMR.

**Key words:** 5-S-cysteinyl-dopa; Melanoma; O-Methylation; 3-Methoxytyrosine

Recent studies have demonstrated that the amino acid 5-S-cysteinyl-dopa, formed by the interaction of dopaquinone with cysteine or glutathione in the melanocytes, is an important metabolite of these cells (1, 4, 6, 7). 5-S-cysteinyl-dopa is excreted in the urine (5), and its renal plasma clearance is high (2).

The present investigation was performed in order to find metabolites of 5-S-cysteinyl-dopa. Dopa, another substance of central importance in the melanocytes, is metabolized in the human body to a large number of compounds (10). O-methylation of dopa, leading to formation of 3-methoxytyrosine, is one pathway for dopa metabolism. It was decided first to investigate the possibility of O-methylation of 5-S-cysteinyl-dopa. Dopa is to a large extent decarboxylated to form dopamine. The possible occurrence of decarboxylation of 5-S-cysteinyl-dopa was also studied.

### MATERIAL AND METHODS

5-S-cysteinyl-dopa was prepared as described earlier (11). To investigate the possible *O-methylation* of 5-S-cysteinyl-dopa, 11.9 g of liver tissue from a male rat was homogenized in 16 ml 1/15 M KCl and centrifuged for 1 hour at 100 000 g. To 5 ml of the supernatant, 5 ml 0.5 M potassium phosphate buffer (pH 8.4) containing  $10^{-4}$  M ascorbic acid and 20 mg of Mg Cl<sub>2</sub> was added. The mixture was exposed to N<sub>2</sub> for 10 minutes at 37°C. 400 µg of 5-S-cysteinyl-dopa and 7.4 mg S-adenosylmethionine dis-

solved in 0.6 ml H<sub>2</sub>O were then added, and the sample was incubated for 1 hour at 37°C. The incubation was stopped by the addition of 1 ml 4 N perchloric acid. The samples were centrifuged, and the clear supernatant was examined for O-methylated 5-S-cysteinyl-dopa. As control, 5 ml of the liver extract was boiled for 10 minutes and then incubated with 5-S-cysteinyl-dopa as above.

To obtain a preparation of *dopa decarboxylase* 5 g of guinea-pig kidney was homogenized in 10 ml 1/15 M phosphate buffer (pH 7.5), centrifuged at 100 000 g for 10 minutes, and filtered. 0.1 mg 5-S-cysteinyl-dopa was incubated in 2 ml of the kidney extract together with 0.1 mg pyridoxal-5-phosphate for 2 hours at 37°C. As control for decarboxylase activity, 0.1 mg dopa was also incubated with 2 ml extract and 0.1 mg pyridoxal-5-phosphate. Controls were obtained by addition of 15 ml 0.4 N perchloric acid before incubation. Catechols were adsorbed onto Al<sub>2</sub>O<sub>3</sub>. The possible formation of 5-S-cysteinyl-dopamine was examined by GC-MS using enzymatically synthesized samples as reference. 5-S-cysteinyl-dopamine was prepared from tyramine and cysteine by the method described for 5-S-cysteinyl-dopa (11). To confirm dopa decarboxylase activity in our system, incubation with dopa was performed and the formation of dopamine was checked with GC-MS authentic dopamine as reference.

Urine specimens from two men, aged 46 (HN) and 26 (HG), with widespread melanoma metastases were examined. 24-hour specimens were collected in plastic bottles containing 50 ml glacial acetic acid and 1 g sodium metabisulphite. Dopa+dopamine and 5-S-cysteinyl-dopa were determined by methods described previously (8, 11, 12).

For *purification of O-methyl-cysteinyl-dopa*, supernatant of the incubates or 20-ml specimens of urine were put on a column, 27×5 mm, containing Dowex 50 W X4, H<sup>+</sup> form. Elution was performed with 2 N HCl, and the effluent was collected in 5-ml fractions. The 5th fraction was further investigated by combined gas chromatography and mass spectrometry (GC-MS).

*Gas chromatography-mass spectrometry* was performed by a procedure described previously (3). The sample 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 minutes at 100°C in a sealed tube. Next the methanol-HCl was evaporated using a

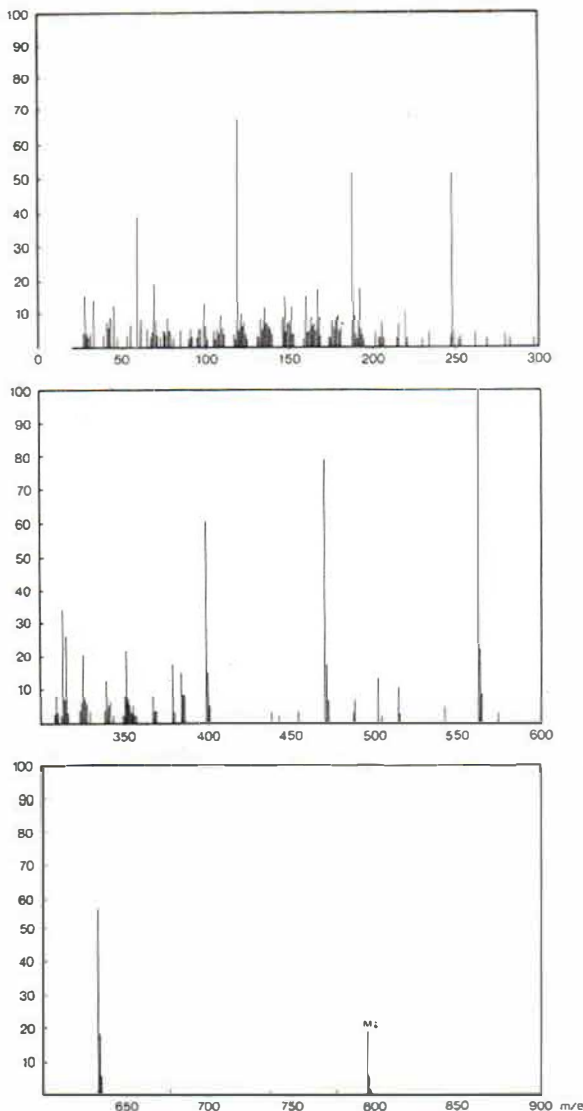


Fig. 1. Mass spectrum of material isolated from the urine of a melanoma patient.

stream of dry nitrogen. 30  $\mu$ l of pentafluoropropionic anhydride (PFPA) was then added, and the tube was heated at 60°C for 30 minutes in the tapered reaction vial. The PFPA was evaporated with dry nitrogen, and the residue was dissolved in 30  $\mu$ l 1% PFPA in ethyl acetate.

Nuclear magnetic resonance spectra were recorded on a Varian XL-100-15 NMR-spectrometer.

For isolation of *O*-methyl-cysteinyl-dopa 24-hour samples of urine from two patients with high urinary excretion of 5-S-cysteinyl-dopa (HN 124 and HG 76 mg/24 h) were treated as follows.

Acidification to pH 1 with 6 N HCl. Centrifugation at 17 000 g for 10 minutes. Adsorption on a 15 $\times$ 2 cm column of Dowex 50 W X4, H<sup>+</sup> form. 450 ml of N HCl was passed

through the column and the effluent discarded. Elution was performed with 2 N HCl; the peak of *O*-methyl-5-S-cysteinyl-dopa appeared after 310–330 ml of 2 N HCl.

*O*-methyl-5-S-cysteinyl-dopa was further purified by paper chromatography in *n*-propanol/N HCl (3:2). The R<sub>f</sub>-value was found to be 0.56. The compound was identified on a strip of the paper by spraying with diazotized *p*-nitroaniline (13) which gave a pinkish red spot, and *O*-methyl-5-S-cysteinyl-dopa was eluted from the paper with 0.1 N HCl. UV-spectrometry was performed in 0.1 N HCl. After drying, the isolated material was weighed.

## RESULTS

Incubation of 5-S-cysteinyl-dopa with the rat liver extract containing large amounts of catechol *O*-methylating enzyme and S-adenosyl methionine led to the formation of a methyl derivative of 5-S-cysteinyl-dopa, identified by GC-MS.

The methyl derivative formed was also detected in large amounts in the urine of two melanoma patients. The methyl derivative could be crystallized, and the amounts obtained from 24 h urine samples from the two melanoma patients (HN and HG) were 16 and 10 mg *O*-methyl-5-S-cysteinyl-dopa, respectively. The UV-spectrum of the isolated compound showed maxima at 255 nm and 290 nm.

Fig. 1 shows the mass spectrum of the *O*-methylated 5-S-cysteinyl-dopa isolated from melanoma urine. The mass spectrum was identical with that of the *O*-methylated product formed on incubation of 5-S-cysteinyl-dopa with liver extract. Fig. 2 shows the probable fragmentation pattern. NMR-spectra of the substance isolated from the urines with sodium 3-(trimethylsilyl)-1-propane-

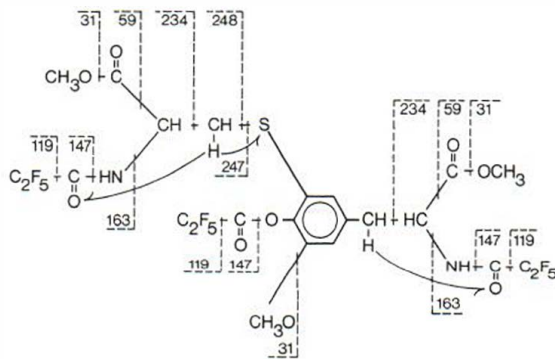


Fig. 2. Fragmentation of *O*-methyl-5-S-cysteinyl-dopa derivative. For clarity, only one of the isomers is presented.

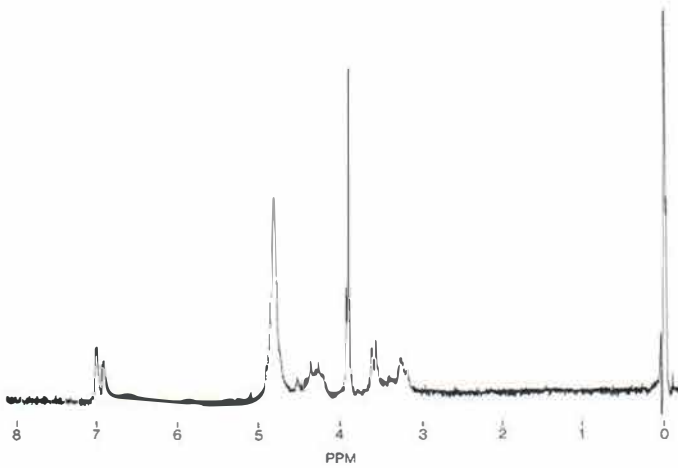


Fig. 3.  $^1\text{H}$  NMR spectrum of material isolated from the urine of a melanoma patient.

sulphonate as internal standard ( $\delta=0.00$  to TMS) were consistent with the structure of O-methyl-5-S-cysteinyldopa.

$^1\text{H}$  NMR (100 MHz,  $\text{D}_2\text{O}$ ): 3.20 (d, 2H,  $J$  6.9 Hz,  $\text{ArCH}_2$ ), 3.57 (d, 2H,  $J$  5.4 Hz,  $\text{ArSCH}_2$ ), 3.89 (s, 3H,  $\text{CH}_3\text{O}$ ), 4.09–4.47 (m, 2H,  $\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$ ), 6.88 and 6.97 (AB-system, 2H,  $J$  1.8 Hz,  $\text{ArH}$ ). See Fig. 3.

Incubation of 5-S-cysteinyldopa with the guinea-pig kidney extract containing dopa decarboxylase in large amounts did not result in formation of 5-S-cysteinyldopamine detectable by GC-MS. Nor could any 5-S-cysteinyldopamine be demonstrated by GC-MS of the urines of the two melanoma patients.

## DISCUSSION

Catechol-O-methylation is of great importance in the metabolism of dopa, and a high excretion of 3-methoxytyrosine has recently been demonstrated by mass fragmentography in patients with melanoma (3). A substance identical with that formed by incubation of 5-S-cysteinyldopa with rat liver extract was demonstrated in the urine of the two melanoma patients studied by GC-MS.

The proton resonance spectrum of the methylated cysteinyldopa shows an AB-quartet in the aromatic region. The doublet splittings of 1.8 Hz show clearly that the two ring-protons responsible for the signals are *meta* to one another. The product must therefore be a 1,3,4,5-tetra substituted benzene. The signals from the methoxyl protons show one strong, sharp singlet at  $\delta$  3.89 and a sec-

ond, weak, but sharp singlet 2.5 Hz down-field with an intensity ratio of 5.4:1. This indicates that the methylation has occurred to about 85% at one of the phenolic hydroxyls and to about 15% at the other. This is a clear difference from the isomer ratio for monomethylated dopa, and shows that the methylation occurs on the 5-S-cysteinyldopa.

The findings, which indicate O-methylation of 5-S-cysteinyldopa in the presence of catechol-O-methyl-transferase *in vitro*, suggest that 5-S-cysteinyldopa is also a substrate for this enzyme.

O-methylation of dopa in the body occurs mainly in the *meta* position (9). Insufficient material is yet available to establish the site of O-methylation of 5-S-cysteinyldopa. It must be borne in mind that the thioether bond may determine the site of methylation.

With the methods used, 5-S-cysteinyldopamine could not be detected in incubates after incubation of 5-S-cysteinyldopa with dopa decarboxylase or in melanoma urines. Of course, our findings do not preclude the possibility of some decarboxylation, but if it does occur it must be much less than the decarboxylation of dopa. Some decarboxylation of 5-S-cysteinyldopa may actually occur, since the mass spectrograms obtained from urines suggest the presence of an O-methylated and decarboxylated 5-S-cysteinyldopa metabolite. Work is in progress to isolate and define this substance.

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