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5-S-Cysteinyldopac in Human Urine*

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5-S-Cysteinyldopac, a compound hitherto demonstrated only in brain tissue, has been isolated and quantified in urine. The urines from 12 individuals were found to contain 20 ± 9.1 μg 5-S-cysteinyldopac/24 hours. Incubation of 5-S-cysteinyldopamine with MAO-containing tissue did not give any formation of 5-S-cysteinyldopac, indicating that this compound is formed by nucleophilic addition of cysteine directly to dopac. The findings give further evidence for a small but significant non-specific oxidation of endogenous catechol derivatives in vivo, a fact to be considered when using 5-S-cysteinyldopa as a measure of pigment metabolism. ***Key-words: Dopac; Dopamine; Autoxidation; 5-S-Cysteinyldopa; Melanin.***

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Cysteinyldopas are important metabolites of the melanocyte but there is also evidence that these compounds can be formed outside the melanin forming cells. All cysteinyldopas are produced by a nucleophilic addition of cysteine to dopaquinone; in the melanocyte formed by the enzyme tyrosinase but in other cells by other oxidative systems.

This oxidation can be brought about by free radicals (1, 2). It is not known to what extent such reactions occur in living tissue, but it seems quite possible that the pseudotyrosinase activity of metalloproteins may cause oxidation of catechols also in vivo (3). The quinones possibly formed should be highly reactive and damaging to vital macromolecules in the cell. Quinones however react rapidly by addition of glutathione or cysteine and these compounds therefore act as protective agents. Findings of cysteinyladducts in ganglion stellatum and in brain (4, 5, 6) are indicative of oxidation of catechol derivatives in nervous tissue.

The mechanism of oxidation of catechol derivatives and addition of thiols to the resulting quinones has been most extensively studied in the case of dopa (7, 8). The occurrence of cysteinyladducts of catecholic compounds in brain of several species (1, 2) and the finding of cysteinyldopamine in the urine of some melanoma patients and in some control urines (9), show that the quinone scavenging by thiols is an important metabolic pathway for metabolism of other catecholic substances than dopa.

3, 4-Dihydroxyphenylacetic acid (dopac) is a catechol derivative formed by the oxidative deamination of dopamine, a reaction catalysed by monoamineoxidase (MAO). Dopac originates exclusively in the nervous system, in dopaminergic neurons but also in noradrenergic neurons. Most of the dopac formed is 3-O-methylated by the action of catechol-O-methyltransferase (COMT) to homovanillic acid (HVA), the major metabolite of dopamine (10).

Dopac has until recently been considered to be a metabolite without pharmacological effects, but in vitro studies have demonstrated cytotoxic effects on melanoma cells (11, 12). These effects could be due to formation of quinones. Reaction of the quinone of dopac with cysteine, leading to cysteinyldopac, would be a marker of such quinone formation.

We present here the finding of 5-S-cysteinyldopac in urine from healthy individuals indicating the oxidation of dopac in the body. In addition we present evidence that 5-S-cysteinyldopac is formed directly by the addition of cysteine or glutathione to dopacquinone, and not from 5-S-cysteinyldopamine through the action of MAO.

MATERIAL AND METHODS

5-S-Cysteinyldopac and 5-S-cysteinyldopamine were prepared as earlier described (4).

* These results have previously been presented as a poster at the Second meeting of the European Society for Pigment Cell Research at Uppsala June 18–21, 1989.

Estimation of 5-S-cysteinyldopac

Twenty-four-hour urine specimens were collected in plastic bottles containing 1 g sodium metabisulphite and 50 ml 100% acetic acid, from 8 women and 4 men, 26 to 44 years of age, all healthy and free from medication. The study was performed during the months of January and February and recent sun exposure had not occurred. To a 20 ml aliquot of the urine 5 μg 5-S-D-cysteinyl-L-dopa was added as an internal standard (13). Proteins were precipitated by adding 1 ml 4 M perchloric acid and the sample was centrifuged for 10 min at 15,000 rpm. After centrifugation, 200 mg alumina, 200 mg EDTA and 10 mg sodium metabisulphite were added to the supernatant. The pH of the mixture was adjusted to 8.6 with sodium hydroxide. The samples were shaken for 10 minutes, centrifugated at 4,000 rpm for 5 min, washed three times with water, and the catechol derivatives were then eluted with 1 ml of a solution containing 0.125 M citric acid and 0.25 M boric acid. The eluate was put onto a column (4.5×0.5 cm) containing Dowex 50W-X4 in H^+-form. Before utilization the resin was washed in a solution containing 0.05% EDTA and 0.5% sodium metabisulphite in 0.1 M sodium phosphate buffer, pH 7.5, in order to remove impurities such as iron and manganese ions which otherwise would readily oxidize the catechol derivatives put on the column. After application of the sample the column was washed with 5 ml water and 4 ml 0.1 M sodium citrate buffer, pH 4, and elution performed with a buffer containing 0.2 M citric acid and 0.4 M disodium hydrogen phosphate, pH 4.0. One ml fractions were collected and monitored by HPLC. Fractions 3–5 contained all the cysteinyldopas and cysteinyldopacs present in the effluent.

HPLC

HPLC analysis was performed as previously described (4), using a 15 cm×4.6 mm id Supelcosil LC-18 column (Supelco, Belafonte, Pa USA) with particle size 3 μ. The mobile phase contained 3 g H_3PO_4 and 6 g methane sulphonic acid per litre of water. The pH was adjusted to 1.75 with sodium hydroxide. Flow rate 1.5 ml/min. Runs were made with several systems, with varying pH values and with varying amounts of methanol.

Recovery determination

A solution of 5-S-D-cysteinyl-L-dopa and 5-S-cysteinyldopac in 0.4 M percloric acid was processed as above on alumina, EDTA and metabisulphite and after elution put on the Dowex column. The eluates from this column were analysed on HPLC.

Isolation of dopac was carried out using the first steps in the method for preparation of cysteinyldopac and cysteinyldopa. Alfa-methyldopamine was chosen as internal standard. Dopac was measured from the alumina eluates by HPLC with electrochemical detection; column: Supelcosil LC 18, 15 cm×4.6 mm id, particle size 3 μ, mobile phase: 30 mg sodium octane sulphonate, 1.7 g H_3PO_4 in 1 l water. The pH was adjusted to 2.25 with sodium hydroxide.

Determination of the identity of urinary cysteinyldopac by Liquid Chromatography-Mass Spectrometry

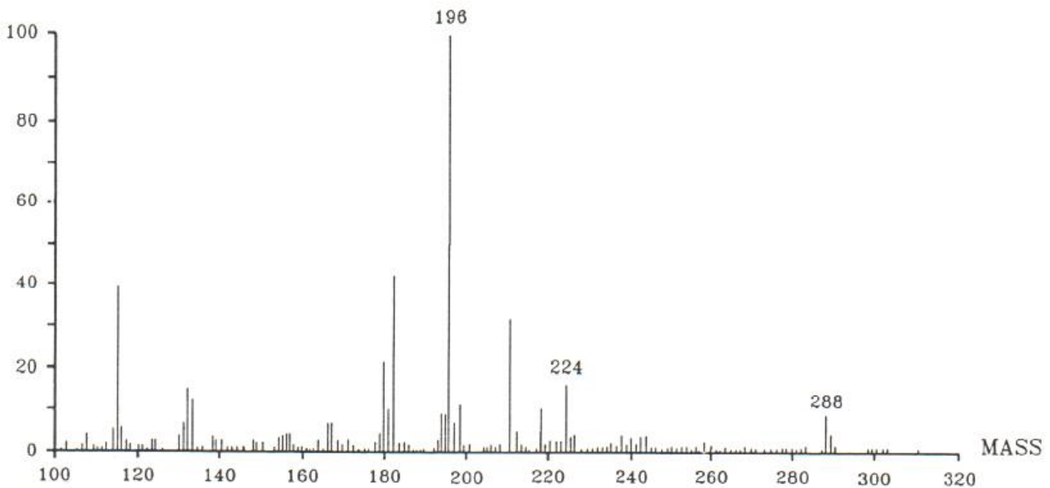
Sample preparation. The sample preparation described could be used succesfully in the purification of urine before analysing cysteinyldopac with HPLC and electrochemical detection. The eluates obtained contained, however, impurities which prevented analysis by liquid chromatography-mass spectrometry and a further clean up was needed. Optimal conditions for such a preparation were as follows; Cysteinyldopac was extracted from 200 ml urine in 20 ml aliquots, isolated on alumina. The combined eluates were purified by ion exchange chromatography on four Dowex columns as described. To the pooled eluates containing cysteinyldopac, sodium hydroxide was added and the pH adjusted to 6.0. The sample was then applied to a phenylboronate column (3×1 cm), equilibrated with 0.1 M sodium citrate buffer, pH 6.0. After washing with water elution was performed with 0.1 M trifluor-acetic acid, and fractions of 1 ml were collected. Fractions 7–11 contained cysteinyldopac. These fractions were combined and 0.1 ml portions were injected repeatedly onto a Supelcosil LC 18 column with particle size 3 μ. Mobile phase: 0.1% trifluor acetic acid, 17% methanol in water. Detection UV at 292 nm. The eluates containing cysteinyldopac were collected and the combined eluates evaporated in a rotary evaporator until almost dry. After dilution with mobile phase, the sample was put onto another HPLC Supelcosil LC 18 column mounted in the LC-MS- apparatus. Mobile phase; 0.1 M HAc, 5% methanol, pH was adjusted to 5.5 with ammonium solution.

Mass Spectrometry. The effluent from the HPLC column was introduced into a VG Trio 3 quadropole mass spectrometer via the standard LC-MS thermosprayplasmaspray probe. In the thermospray studies, post-column addition of ammonium acetate buffer, (0.3 ml/min) was employed to give a final buffer concentration of approximately 0.1 M. The vaporizer capillary had a temperature of 210°C and the plasma spray discharge current was 210 μA. A standard VG 11/253 data system configuration was employed to acquire mass spectral data.

MAO incubation. Fourteen g fresh rat liver was homogenised with a glass-glass homogeniser in 35 ml 0.1 M phosphate buffer pH 7.0. The homogenate was centrifugated at 17,000 rpm for 10 min. The pellet was suspended in 35 ml of the same buffer and 0.5 ml was then incubated with 0.5 ml of a 0.01 mM cysteinyldopamine solution under oxygen bubbling, 37°C. The possible appearance of 5-S-cysteinyldopac was followed with HPLC as above. 0.5 ml of the same homogenate was then incubated with 0.4 ml buffer and 100 μg dopamine under oxygen bubbling, 37°C. The appearance of dopac was followed on HPLC.

RESULTS AND DISCUSSION

On the chromatograms from HPLC analysis of the purified samples, peaks appeared with retention times identical to that of authentic 5-S-cysteinyldo-



pac. The fraction from HPLC supposed to contain 5-S-cysteinyldopac was tested in several HPLC systems and found to contain a compound that behaved as authentic 5-S-cysteinyldopac in all systems. The eluates from HPLC containing the relevant peak were analysed by means of LC-MS. The mass spectrum obtained from 4 μg of authentic 5-S-cysteinyldopac is shown in Fig. 1. The $M + H^+$-ion (288) is small. Two major fragments are shown: m/z 224 and m/z 196, consistent with loss of a molecule H_2O and the carboxyl group, and with a further loss of CO respectively. The small amount of 5-S-cysteinyldopac recovered from HPLC-separation of urine was analyzed with the mass spectrometer arranged for selected ion monitoring (SIM) for these fragments. The HPLC retention time and the fragmentation pattern were identical with those of autenthic 5-S-cysteinyldopac.

The results of the determination of dopac, 5-S-cysteinyldopa and 5-S-cysteinyldopac are given in Table I. The mean value of 5-S-CD is higher than those earlier reported by us. This is due to the extremely high value of one individual, a value found to have been constantly high for several years.

Recovery of 5-S-cysteinyldopac was 120 ± 1.8% of that of 5-S-D-cysteinyl-L-dopa after a mixture of equal amounts of these two compounds had been absorbed onto alumina, eluted and passed onto a Dowex column and then analysed on HPLC.

Incubation of 5-S-cysteinyldopamine with homogenised rat liver did not lead to any formation of 5-S-cysteinyldopac. When incubating the same homogenate with dopamine, dopac was formed.

The results show the presence in human urine of 5-S-cysteinyldopac a substance so far only detected in brain postmortally. The identity of the compound is established by identical retention times and coeluation from HPLC in several different systems with a synthetically prepared standard, and by identical mass-fragmentograms from a biological sample and the standard.

5-S-cysteinyldopa was first detected in malignant melanoma tissue and the urinary excretion of 5-S-cysteinyldopa has been extensively used as a marker of metastases of malignant melanoma (14). Normal pigment-producing melanocytes also form cysteinyldopa. Several recent reports indicate, however, an extramelanosomal origin for some excreted 5-S-cysteinyldopa. Thus the occurrence of 5-S-cysteinyldopa in tissues not containing melanocytes (15) and in the urine of albinos (16–19) indicates a certain non-melanocytic oxidation of dopa.

During the univalent reduction of oxygen in the body, hydrogen peroxide is formed as are several species of reduced oxygen molecules. The cell utilises different enzyme systems: dismutases, catalase, glutathione peroxidase to protect itself against these toxic substances (20).

Nucleophilic addition of glutathione to quinones

Table I. *Urinary excretion of dopac, 5-S-cysteinyldopa and 5-S-cysteinyldopac. All values in μg compound excreted per 24 hour. n = 12. Mean ± SD.*

Dopac	870±380
5-S-cysteinyldopa	205±154
5-S-cysteinyldopac	20± 9.1

may also be an important scavenging system for catecholic compounds oxidized outside the melanocyte (21). Basal levels of 5-S-cysteinyldopa excretion do not correlate with constitutionally determined pigmentation (22), and the rise of urinary 5-S-cysteinyldopa in human urine after exposure to UV-irradiation is not correlated with the induced pigmentation or melanocyte proliferation (23). Increased cysteinyldopa excretion after UV-irradiation can thus be due to damage to melanocytes and/or other cells in epidermis or dermis.

The present study further illustrates the importance of thiols as protective agents against reactive quinones. The finding of cysteinyldopac in urine establishes the presence of a new metabolic pathway for dopac, and thus for dopamine, and demonstrates the in vivo formation of a cysteinyl adduct hitherto only found postmortally. The lack of cysteinyldopac formation when incubating cysteinyldopamine with MAO-containing tissue, demonstrates that cysteinyldopac is formed in vivo from thiol addition to the quinone formed by oxidation of dopac.

The quantity of dopac metabolised via thiol addition to the quinone is small compared to that metabolised to HVA. However, the occurrence of cysteinyldopac offers evidence of a potentially deleterious oxidative reaction in cells of the nervous system and the protection by the scavenging action of thiols.

Excretion of conjugated cysteinyldopac may be larger than that of the free compound, thus it is possible that the quantity of free cysteinyldopac demonstrated by us only represents a minor fraction of oxidized dopac.

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