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A SENSITIVE METHOD FOR DETERMINATION OF 5-S-CYSTEINYLDOPA

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Abstract. A sensitive and highly specific method is described for the fluorimetric determination of 5-S-cysteinyldopa. The method is suitable for determination of 5-S-cysteinyldopa in pigment-forming tissues and can also be used for determination of the urinary excretion of this catechol in patients with melanoma.

Cysteinyldopa is considered to be an intermediary substance in the formation of the pigment of red hair (5). With the use of primarily fluorimetric methods for the detection of 5-S-cysteinyldopa, substantial amounts of this catechol were recently discovered in a melanoma metastasis in a red-haired man (3). 5-S-cysteinyldopa has since been demonstrated in several other melanomas in Caucasians and in three Negroes (8). These findings raised the question whether the presence of 5-S-cysteinyldopa is related to the malignant growth or to the pigment genetics of the patient with melanoma. Better knowledge of the formation and occurrence of this catechol derivative in various melanomas and in normal pigment-forming tissues is desirable.

The fluorimetric method hitherto used for determining cysteinyldopa is not extremely sensitive and other catechol thioethers may interfere with the determination of 5-S-cysteinyldopa (7). For our further work we needed a more sensitive and specific method. The previously described oxidation procedure used to detect 5-S-cysteinyldopa gives a peak not only at 485 nm, but also a shoulder at a shorter wave-length (7).

We decided to try to develop oxidation conditions leading to the formation of one fluorophore only, the one producing the peak at 485 nm or the other producing the shoulder at the shorter wavelength.

MATERIAL AND METHODS

5-S-cysteinyldopa was a gift from Professor G. Prota (Department of Organic Chemistry, University of Naples, Italy).

Glutathione-dopa, cysteinyl-dopyl-glycyl-glycine, glutathione-dopyl-glycyl-glycine and cysteinyl-dopamine were synthesized enzymatically (7). The polyphenoloxidase (EC 1.10.3.1.) used was obtained from Sigma Chem. Corp. (activity 3 690 U/mg solid).

Glutathione-dopamine was synthesiezed by incubation of the following mixture for 4 hours at room temperature: 2 mg tyramine (Fluka AG) and 3.1 mg glutathione in 2.3 ml of a 0.1 M phosphate buffer, pH 6.5, containing 0.3 mg of polyphenoloxidase. The incubation was stopped by addition of 0.23 ml of 4 N perchloric acid, and the volume was then made up to 20 ml with 0.4 N perchloric acid. The purest commercially available chemicals were used.

Adsorption of catechols onto Al_2O_3 and elution were performed as described previously (1, 6). Unless otherwise stated, all eluates were adjusted to pH 4 with 0.5 N K_2CO_3 before oxidation. Fluorimetry was performed as described previously (7).

OXIDATION PROCEDURES AND RESULTS

Oxidation of 5-S-cysteinyldopa with $K_3Fe(CN)_6$ at pH 6.5, as described by Bertler, Carlsson & Rosengren (2) for determination of noradrenaline and adrenaline, resulted in the formation of a fluorescent compound with maximum excitation at 365 nm and maximum emission at 485 nm. This fluorescence was the same as that of dopa oxidized in the same way, but the intensity of the fluorophore of cysteinyldopa was only 4% of that of dopa fluorophore.

Oxidation of cysteinyldopa giving a fluorophore with pure emission at 485 nm seemed to be a fairly insensitive, non-specific method.

We therefore changed the oxidation conditions

Table I. *Schedule for determination of 5-S-cysteinyldopa*

Volumes in ml	Standard	Sample	Tissue blank	Reagent blank
Buffer, 0.2 M acetate buffer, pH 4.0	0.20	0.20	0.20	0.20
Eluate	—	0.20	0.20	—
Cysteinyldopa 0.5 μg/ml	0.20	—	—	—
H_2O	0.80	0.80	1.05	1.00
I. Iodine solution. 0.02 N[a]	0.05	0.05	—	0.05
II. 5 N NaOH added 2 min after I	0.20	0.20	0.20	0.20
III. 10 N acetic acid added 2 min after II	0.50	0.50	0.50	0.50
0.2 M sodium sulfite	0.20	0.20	—	0.20

[a] Dissolve 0.254 g iodine and 5 g KJ in 100 ml H_2O.

in order to obtain the fluorophore which we had found to produce a shoulder slightly above 400 nm when cysteinyldopa was oxidized according to Anton & Sayre (1). The procedure shown in Table I was arrived at after numerous modifications of the oxidation conditions. The fluorophore obtained had a maximum excitation at 325 nm and maximum emission at 405 nm (Fig. 1).

The fluorescence intensity was studied systematically for variation with the following parameters of the method: pH at oxidation, duration of oxidation, amount of alkali and duration of treatment with alkali after oxidation, amount of iodine for oxidation, amount of acid added to interrupt treatment with alkali. The procedure found to give the optimum fluorescence is that given in Table I. The smallest amount of 5-S-cysteinyldopa in a sample that can be determined with the method is 0.025 μg.

The fluorescence was proportional to the concentration of 5-S-cysteinyldopa up to 5 μg per sample.

The fluorescence did not decrease within 1 hour, which was the longest time studied.

Table II shows that the method can distinguish 5-S-cysteinyldopa from other catechols, indoles and analogues of these compounds. Thus the method is of high specificity.

Table III shows that the method also detects the cysteine thioether of an *N*-terminal dopa peptide, but no other catechol thioethers.

The recovery of the method was studied by addition of 5 μg authentic 5-S-cysteinyldopa to extracts of 2–4 g of whole white mice, except skeleton. The recovery in 9 experiments was $56 \pm 3.5\,\%$.

The corresponding figure in six experiments, where 0.5 μg was added to similar extracts, was $55 \pm 2.1\,\%$.

The fluorescence obtained after oxidation of extracts from total body of white mice minus skeleton did not differ from that of tissue blanks.

Table II. *Relative fluorescence of some catechol- and indole-derivatives*

Cysteinyldopa	100.0
Dopamine	0.0
Dopa	0.0
Noradrenaline	0.0
Normetanephrine	0.0
Adrenaline	0.0
α-methyldopa	0.0
3-methoxydopa	0.0
4-methoxydopa	0.0
3-methoxydopamine	0.0
DOPAC	0.0
HVA	0.0
Vanillin	0.0
Serotonine	0.0
Trytophan	4[a]
Tryptamine	13[a]

[a] Fluorescence maxima 320/370; the compound is not adsorbed onto aluminium oxide, for which reason it does not interfere with the determination of cysteinyldopa.

DISCUSSION

Cysteinyldopa can be histochemically detected by the Falck-Hillarp formaldehyde method for demonstrating catechols (3). The compound can also be determined quantitatively by a previously described oxidation method (6, 7). The methods hitherto available have made it possible to demonstrate cysteinyldopa in certain melanomas, which

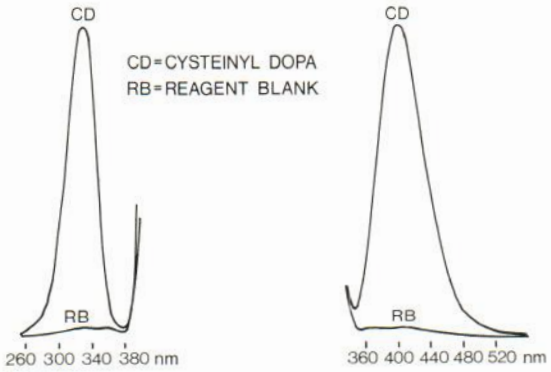

Fig. 1. Excitation and emission spectra for the fluorophore of 5-S-cysteinyldopa.

may contain considerable amounts of this catechol.

The method described here is 30 times more sensitive than the oxidation method previously used and is also more specific. The fluorescence of tissue blanks is weak. The new method can facilitate investigation of the role played by cysteine in normal pigmentation. It permits identification of cysteinyldopa in melanomas even when present in only small amounts (8). A classification of melanomas, based on their catechol content, whill thus become possible. The method is also suitable for detection of cysteinyldopa in the urine (8). In preliminary experiments large amounts of this catechol derivative have been found in urine from patients with melanoma (4).

Table III. *Relative fluorescence of fluorophores of some catechol-thioethers*

The fluorescence of cysteinyldopa = 100. The amounts of the catechol thioethers were calculated on the assumption that they had the same molar absorbance at 292 nm as cysteinyldopa

Compound	Relative fluorescence	Excitation and emission maxima (nm)
Cysteinyldopa	100	325/405
Glutathionedopa	0.0	
Cysteinyl dopyl-glycyl-glycine	94	325/405
Glutathione dopyl-glycyl-glycine	0.2	335/405 (slope at 370)
Cysteinyl-dopamine	0.1	355/440
Glutathione-dopamine	0.2	355/440

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