ACTA CHEMICA SCANDINAVICA 19 (1965) 1854–1858

Oxidative Conversion of Homovanillic Acid to a Fluorescent Compound

HANS CORRODI and BENGT WERDINIUS

Research Laboratories, AB Hässle, Göteborg and Department of Pharmacology, University of Göteborg, Göteborg, Sweden

When homovanillic acid is oxidized with potassium ferricyanide in alkaline medium, a strong blue fluorescence develops. This is the basis of an already described fluorometric method for the determination of homovanillic acid, applicable to biological material. The method has now been studied in closer detail. The fluorescent oxidation product has been isolated, and its chemical structure established as 2,2′-dihydroxy-3,3′-dimethoxy-biphenyl-5,5′-diacetic acid.

In the living organism, 3-methoxy-4-hydroxyphenylacetic acid or homovanillic acid (HVA) is an important metabolite to the catecholamine dopamine (3,4-dihydroxyphenylethylamine), the immediate precursor of noradrenaline and adrenaline.

Several methods have been employed for the separation and determination of HVA in biological material, *e.g.* paper chromatography,[1–5] thin layer chromatography on silica gel [6] or polyamide,[7] high voltage electrophoresis,[8,9] gas-liquid partition chromatography,[10,11] demethylation to 3,4-dihydroxyphenylacetic acid and subsequent colorimetric analysis.[12] All the methods mentioned, with the possible exception of thin layer chromatography, are either complicated and time-consuming, semiquantitative, or not sensitive or specific enough to permit analysis of the minute amounts of HVA found, *e.g.* in the brain, the sensitivity being generally limited by the colorimetric procedure employed.

Fluorometric methods are, in general, more sensitive than colorimetric methods. Recently and independently, two quantitative methods for HVA determination in biological tissue appeared, founded on the fluorescence exhibited after oxidation with ferric chloride in acid medium and subsequent alkalinization,[13] or with potassium ferricyanide in alkaline medium.[14] In the present paper the latter method has been studied in closer detail. The fluorescent compound has been isolated, and its chemical structure established.

Table 1. Schedule for the oxidation of HVA. RB = Reagent Blank; TB = Tissue Blank; S− = Sample; S+ = Sample with HVA added to the homogenate for control of the recovery through the entire procedure; IS− and IS+ = S− and S+, respectively, with added HVA for control of the oxidation procedure.

	Standard								
	2 μg	1 μg	RB	S−	IS−	TB	S+	IS+	TB
H_2O ml	—	0.1	0.2	0.2	—	0.2	0.2	—	0.2
Tris buffer 0.05 M, pH 7.5	1.0	1.0	1.0	—	—	—	—	—	—
L-Cysteine 0.1 %	—	—	—	—	—	0.2	—	—	0.2
$K_3Fe(CN)_6$ 0.01 %	—	—	—	—	—	0.2	—	—	0.2
HVA [a]	0.2	0.1	—	—	0.2	—	—	0.2	—
Tris buffer sample	—	—	—	1.0	1.0	1.0	1.0	1.0	1.0
NH_3 5 N	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$K_3Fe(CN)_6$ 0.01 %	0.2	0.2	0.2	0.2	0.2	—	0.2	0.2	—
4 minutes									
L-Cysteine 0.1 %	0.2	0.2	0.2	0.2	0.2	—	0.2	0.2	—

[a] Containing 10 μg of HVA per ml.

Methods

The technique has been described earlier in detail [14] and will be outlined here only briefly.

After tissue homogenization and protein precipitation (perchloric or metaphosphoric acid) the centrifuged and filtered extract is purified from lipids and other interfering material by means of shaking with chloroform or heptane at neutral pH. After reacidification to pH 1−2 and saturation with sodium chloride, the acid products, among them HVA, are transferred to an ether phase which is further purified by deepfreezing. The acid products are reextracted into a small volume of "tris" buffer (trishydroxymethylaminomethane 0.05 M, pH 8.5); about 70 % of the original HVA is recovered in the aqueous buffer phase.

The tris buffer is then treated with potassium ferricyanide to oxidize the HVA, and the oxidation is interrupted by cysteine addition after 4 min. A schedule for the procedure is given in Table 1. The fluorescence obtained is read in an Aminco-Bowman spectrophotofluorometer, with the activation and fluorescence wavelengths set at 315 and 425 mμ, respectively (uncorrected instrumental values). The fluorescence is linear in the range 0−10 μg HVA, and is stable for several hours.

Discussion of method

Activation and fluorescence spectra (uncorrected instrumental recordings) of oxidized HVA are given in Fig. 1 (curve A), together with spectra of the isolated fluorophor in a complete reagent blank (ammonia + potassium ferricyanide + cysteine) as well as in ammonia alone (curves B and C, respectively). It is seen that the presence of the reagent blank does not affect the fluorescence intensity at all at the activating wavelength maximum employed (315 mμ), whereas the smaller activation maximum at about 260 mμ disappears due to absorption by the reagent blank.

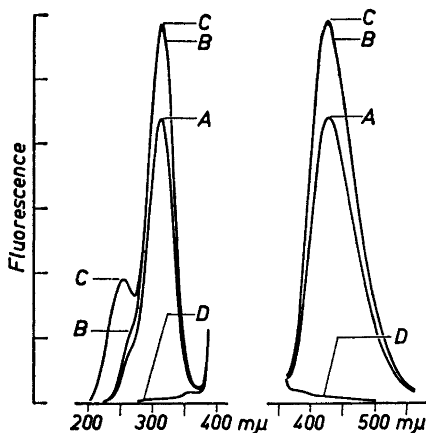


Fig. 1. Activation and fluorescence spectra of the fluorophor, recorded with an Aminco-Bowman spectrophotofluorometer and a Moseley model 135 C X—Y recorder. Left: activation spectrum, with the fluorescence wavelength set at 425 mμ. Right: fluorescence spectrum, with the activation wavelength set at 315 mμ. Fluorescence is given in arbitrary units. A: 2 μg HVA oxidized. B: 2 μg isolated fluorophor in a complete reagent blank (ammonia + potassium ferricyanide + cysteine). C: 2 μg isolated fluorophor in ammonia of the same strength as in B. D: Reagent blank.

The pH of the solution to be oxidized was preferably kept at 11—12. If the ammonia was replaced by sodium hydroxide, the fluorescence was both weaker and less stable to UV irradiation. Acidification quite abolished the fluorescence, but realkalinization completely restored it again.

Of different oxidants tested, potassium ferricyanide turned out to be the simplest to use, since its reduced equivalent, potassium ferrocyanide, did not disturb the reading of the fluorescence. If ferric chloride is employed,[13] it has to be removed by precipitation with sodium hydroxide and centrifugation, before reading can be performed. The resulting fluorescence is reported to be rather sensitive and unstable to the activating UV-light, which might partly be due to the high pH.

As reducing agent, cysteine was preferred to the commonly used ascorbic acid, since the latter absorbed the activating light to a substantial degree. In the preparative synthesis of the fluorophor, on the other hand (see below), ascorbic acid could advantageously be used.

The ionic strength in the sample to be analyzed must be kept low. A weak tris buffer was finally shown to be a suitable medium, preferable to *e.g.* phosphate buffer. Even small amounts of different anions, *e.g.* chloride ions, diminish the oxidation yield considerably.

The specificity of the oxidation seems to be quite good. A fluorescence of the same nature is exhibited by the dopamine metabolite 3-methoxytyramine. With the previous extraction procedure employed, however, amines are not extracted. Some α-alkylated HVA-derivatives, *e.g.* α-propyl-HVA, and their corresponding amides also give fluorescence.[15] Serious interference is caused by salicylic acid, which gives a blue fluorescence in alkaline medium with maximal activating and fluorescent wavelengths (295 and 410 mμ, respectively) that only insignificantly deviate from those of oxidized HVA.

With some additional purification steps, the method can be applied for HVA assay in urine.[16]

Isolation and identification of the fluorescent substance

In order to evaluate the chemical nature of the fluorescent substance, some preliminary paper chromatographic experiments were performed. A standard, oxidized after the usual method but omitting the tris buffer, was acidified with hydrochloric acid, saturated with sodium chloride and extracted with ether. The ether phase was freed from water by deep freezing, evaporated to dryness and dissolved in ethanol. Subsequent paper chromatography of

the extract (solvent butanol:pyridine:water 14:4:5, 15 h), revealed two spots, one of them consisting of unchanged HVA (R_F 0.60, grey-blue colour with diazotized *p*-nitroaniline), the other located much nearer the starting point (R_F = 0.33, negative to diazo reagents, but visible under UV lamp after exposition to ammonia vapors as a blue fluorescent spot). If salt saturation was omitted, the extraction of the fluorescent substance into the ether phase was much poorer, whereas HVA was nearly as well extracted. The fluorophor thus seemed to be much more hydrophilic than HVA. Further it was probably acidic in nature as attempts to extract it at alkaline pH gave no results.

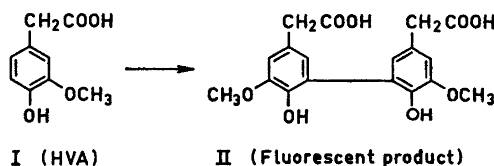
The oxidation procedure was then performed on a large scale in order to obtain large amounts of the fluorescent substance for structural determinations.

EXPERIMENTAL

3.0 g of HVA were dissolved in 30 ml of water and 5 ml of concentrated ammonia. A solution of 6.0 g of potassium ferricyanide was added in small portions. After 5 min 1.0 g of ascorbic acid was added, the solution acidified with hydrochloric acid and extracted with ethyl acetate. The extract was dried over sodium sulfate and evaporated to dryness. The residue was recrystallized from ethanol-ethylacetate. Yield 1.8 g. M.p. 230°C.

STRUCTURE OF THE FLUORESCENT PRODUCT

The pure recrystallized product showed microanalytical data consistent with $C_{18}H_{18}O_8$, suggesting a dimerization product of HVA by loss of two hydrogen atoms. (Found: C 59.38; H 5.14; O 35.01; mol. wt. 349. Calculated for $C_{18}H_{18}O_8$: C 59.66; H 5.01; O 35.33; mol. wt. 362).



I (HVA) II (Fluorescent product)

The mass spectrum, recorded with an Atlas model CH4 mass spectrometer, showed fragments of molecular weight 318 and 274 (decarboxylation products). The UV-spectrum showed maxima at 287 mμ (ε = 5200) in ethanol, at 230 mμ (ε = 32 700) and 306 mμ (ε = 7000) in 1 N NaOH.

The nuclear magnetic resonance (NMR) spectrum of the sodium salt in D_2O, recorded on a Varian A60 instrument, shows a typical fourline AB pattern for the aromatic protons centered at 2.12 ppm downfield from HOD, with J_{AB} = 2 cps. This is characteristic for an aromatic *meta* coupling [17] and is thus a rather strong indication that the dimerization has occurred at the position *ortho* to the phenolic hydroxyl group in HVA. The methoxyl and methylene protons appear at 0.82 and 1.24 ppm, respectively, upfield from the HOD peak. The NMR spectrum is fully consistent with the proposed biphenyl structure (II).

Acknowledgements. The authors would like to thank Dr. R. Carter, Dept. of Organic Chemistry, University of Göteborg, for recording and interpreting the NMR-spectrum and for checking the English.

REFERENCES

1. Armstrong, M. D., Shaw, K. N. F. and Wall, P. E. *J. Biol. Chem.* **218** (1956) 293.
2. Shaw, K. N. F., McMillan, A. and Armstrong, M. D. *J. Biol. Chem.* **226** (1957) 155.
3. Tompsett, S. L. *J. Pharm. Pharmacol.* **13** (1961) 747.
4. Duchon, J. and Gregora, V. *Clin. Chim. Acta* **7** (1962) 443.
5. Bernheimer, H. *Nature* **204** (1964) 587.
6. Sankoff, I. and Sourkes, T. L. *Can. J. Biochem. Physiol.* **41** (1963) 1381.
7. Segura-Cardona, R. and Soehring, K. *Med. Exptl.* **10** (1964) 251.
8. v. Studnitz, W. *Scand. J. Clin. Lab. Invest.* **12** (1960), *Suppl.* **48.**
9. v. Studnitz, W. *Klin. Wochschr.* **40** (1962) 163.
10. Williams, C. M. and Sweeley, C. C. *J. Clin. Endocrinol. Metab.* **21** (1961) 1500.
11. Sweeley, C. C. and Williams, C. M. *Anal. Biochem.* **2** (1961) 83.
12. Ruthven, C. R. J. and Sandler, M. *Biochem. J.* **83** (1962) 30P.
13. Sharman, D. F. *Brit. J. Pharmacol.* **20** (1963) 204.
14. Andén, N.-E., Roos, B. E. and Werdinius, B. *Life Sci.* **2** (1963) 448.
15. Corrodi, H. and Werdinius, B. *In preparation.*
16. Werdinius, B. *In preparation.*
17. Pople, J. A., Schneider, W. C. and Bernstein, H. J. *High Resolution Nuclear Magnetic Resonance*, McGraw-Hill, New York 1959, p. 193.

Received April 15, 1965.