

### Presence of an acid metabolite of amitriptyline in rabbit urine

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THE IDENTIFICATION and quantitative evaluation of hydroxylated and demethylated basic metabolites of amitriptyline in rabbit, have been previously reported by us.<sup>1-3</sup> This paper describes further investigations on amitriptyline metabolism which have led us to discover a carboxylic metabolite formed through the oxidative deamination of the drug.

#### EXPERIMENTAL

##### (1) Investigation of carboxylic metabolites

(a) *Urine*. The urines of six rabbits orally pretreated with 60 mg/kg of amitriptyline were pooled, every 8 hr over a 96-hr period and preserved by freezing. Then they were reduced to 150 ml by evaporation *in vacuo* at 38°, adjusted to pH 9 with conc. NaOH and centrifuged at 3500 g for half an hour. The precipitate was discarded because it did not show the presence of metabolites.

Subsequently the urines, adjusted to pH 1 with conc. HCl, were extracted with CCl<sub>4</sub> twice for 15 min: the combined CCl<sub>4</sub> layers, after concentration *in vacuo*, were washed a few times with 37% HCl to remove the interfering colouring material and then twice extracted with 1 N NaHCO<sub>3</sub>.

The alkaline aqueous layers, combined and acidified to pH 1 by addition of conc. HCl, were shaken with a (1.5:1) CCl<sub>4</sub>-cyclohexane mixture. The organic extract was evaporated to dryness and the residue, dissolved in ether, was analyzed by means of thin-layer chromatography<sup>1</sup> (TLC) on silica Gel G, in the solvent system: CHCl<sub>3</sub>, isopropyl alcohol, 0.1 N NaOH, NH<sub>4</sub>OH (68:30:1, 9:0:1) Running-time: 120 min.

(b) *Organs*. Liver and brain of three rabbits, orally treated with 15 mg/kg of amitriptyline, were taken 2 hr after drug administration, and homogenized in 0.01 N HCl. The material, adjusted to pH 1, was extracted with CCl<sub>4</sub> and the organic layer, evaporated *in vacuo* to 250 ml, was shaken twice with 1 N NaHCO<sub>3</sub>.

The aqueous phase was freed from lipids by several petroleum ether washings. After acidification to pH 1, it was re-extracted with a (1.5:1) CCl<sub>4</sub>-cyclohexane mixture.

The organic extract was evaporated and the residue, dissolved in ether, was used for chromatographic analysis with the following solvent system: benzene-ethyl alcohol (90:10). Running-time, 90 min.

The chromatographic detection in u.v. light (254 mμ) showed only one spot in urine as well as in organ extracts.

The same qualitative and quantitative chromatographic results, were obtained when urine and organ samples were submitted to enzymatic and acid hydrolysis.<sup>2</sup>

##### (2) Identification of the spot

The following proofs were employed for the spot identification.

(a) *Infrared absorption spectrum*. The spot detected after chromatographic separation of the urine extract was eluted in R.S. methyl alcohol and again submitted to chromatography in the same solvent system.

The infrared absorption spectrum on the eluate of the so purified spot was similar to that of amitriptyline except for an additional peak at 5.9 μ which indicated the presence of a carbonyl group.

(b) *Colorimetric tests*. (i) 2,4-dinitrophenylhydrazine for aldehydes and ketones:<sup>4</sup> negative.

(ii) Bromocresol green and silver nitrate-pyrogallol for acids:<sup>5, 6</sup> positive.

(iii) Alcohols, glycols and phenols, reagents:<sup>7-9</sup> negative.

(iv) Dragendorff's reagent:<sup>10</sup> negative.

These findings provide evidence that:

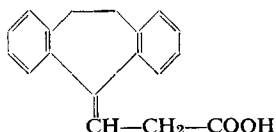
(i) The amino side-chain group of amitriptyline was not present.

(ii) The carbonyl band present in the i.r. spectrum was identifiable as a carboxyl group.

(iii) There were no hydroxyl groups in 10-11 position of ethylene bridge of amitriptyline, nor in the aromatic rings.

They suggest that the amino side-chain group of amitriptyline has been replaced by a carboxyl one, and that the oxidative rupture of the ethylene bridge with the formation of two carbonyl groups, as proposed by Hucker,<sup>11</sup> has not taken place.

The following structure therefore could be conceivably ascribed to the new identified metabolite.



### (3) Investigation on phenolic metabolites

This further investigation was performed to examine if amitriptyline metabolites with hydroxyl groups in the aromatic rings were present in rabbit urine.

A urine sample, prepared as described above, was subjected to enzymatic hydrolysis,<sup>2</sup> then adjusted to pH 5 and extracted with a (1:1) ether-chloroform mixture.

The organic phase was twice shaken with 0.1 N NaOH, and the resultant aqueous layer, acidified to pH 5, was extracted with chloroform.

The chloroform extract, concentrated to 2 ml, was freed from the coloured pigments which interfered with its thin-layer chromatographic analysis, by passage through an alumina column (type neutral 1 × 20 cm). We used 30 ml samples and the eluting solvents were: chloroform, ether, ethyl acetate, acetone, methyl alcohol and acetic acid, water and acetic acid.

Examination of these fractions by thin-layer chromatography in chloroform, isopropyl alcohol, 0.1 N NaOH, NH<sub>4</sub>OH (68:30:1.9:0.1) did not reveal the presence of phenolic metabolites.

The same results were obtained with the organs studied.

### (4) Quantitative evaluation of the carboxylic metabolite

The quantitative evaluation of the acid metabolite was performed according to the following procedure: known amounts of urine and organs extracts were subjected to chromatographic separation and the resulting spots were eluted three times from the silica gel with 5 ml of a (1:1) chloroform-methyl alcohol mixture.

The combined eluates, dried *in vacuo* and taken up in 3 ml of chloroform, were assayed by the H<sub>2</sub>SO<sub>4</sub> method of Beckett *et al.*<sup>12</sup>

The calibration curve was drawn employing, as standard, the 5-carboxy-methylidenedibenzo(a,d)-1,4)cycloheptadiene synthesized according to Winthrop *et al.*<sup>13</sup>

Increasing aliquots of the standard dissolved in 3 ml of chloroform were added to the same volume of 96% H<sub>2</sub>SO<sub>4</sub>. Each sample was shaken for 3 min, centrifuged, and absorption at 445 mμ of the correspondent aqueous phase was measured against a blank prepared in the same conditions.

The recoveries obtained after addition of known quantities of the standard to urine and organ samples, were about 90 and 85 per cent respectively.

The metabolite levels in urine and organs are shown in Table 1:

TABLE 1. METABOLITE LEVELS IN URINE AND ORGANS OF RABBITS

|                     | Urine          | Liver     | Brain |
|---------------------|----------------|-----------|-------|
|                     |                | (μg/10 g) |       |
| Amount recovered    | 12.7 μg/100 ml | traces    | —     |
| Per cent recoveries | 90%            | 85%       | 85%   |

The small amount of metabolites present in the urine provides evidence that, under normal conditions, the oxidative deamination of amitriptyline in rabbits is a minor metabolic pathway for the degradation of the drug.

However, preliminary experiments have shown that the urinary excretion of the acidic metabolite is about three times higher in rabbits treated with nor-amitriptyline than in amitriptyline-treated animals.

These results are consistent with the hypothesis that the oxidative deamination of amitryptiline might occur only after demethylation of the drug.

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#### REFERENCES

1. R. MAFFEI FACINO and G. L. CORONA, *Il Farmaco, Ed. Prat.* **23**, 366 (1968).
2. G. L. CORONA, R. MAFFEI FACINO, *Biochem. Pharmac.* **17**, 2045 (1968).
3. R. MAFFEI FACINO, G. L. CORONA, *J. Pharm. Sci.* **58**, 764 (1969).
4. E. STAHL, *Thin-Layer Chromatography* (Ed. E. STAHL), n. 52, p. 490. Springer Verlag, Berlin (1965).
5. E. STAHL, *Thin-Layer Chromatography* (Ed. E. STAHL), n. 22, p. 487. Springer Verlag, Berlin (1965).
6. E. STAHL, *Thin-Layer Chromatography* (Ed. E. STAHL), n. 141, p. 500. Springer Verlag, Berlin (1965).
7. T. KARIYONE and Y. HASHIMOTO, *Nature, Lond.* **168**, 511 (1951).
8. E. STAHL, *Thin-Layer Chromatography* (Ed. E. STAHL), n. 133, p. 500. Springer Verlag, Berlin (1965).
9. E. STAHL, *Thin-Layer Chromatography* (Ed. E. STAHL), n. 61, p. 491. Springer Verlag, Berlin (1965).
10. E. STAHL, *Thin-Layer Chromatography* (Ed. E. STAHL), n. 60, p. 491. Springer Verlag, Berlin (1965).
11. H. B. HUCKER, *Pharmacologist* **4**, 171 (1962).
12. A. H. BECKETT, M. A. BEAVEN and A. E. ROBINSON, *Biochem. Pharmac.* **12**, 782 (1963).
13. S. O. WINTHROP and M. A. DAVIS, *J. Am. Chem. Soc.* **80**, 4331 (1958).

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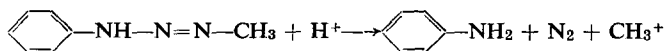
Biochemical Pharmacology, Vol. 19, pp. 1505-1508. Pergamon Press, 1970. Printed in Great Britain

#### Mechanism of carcinogenesis with 1-aryl-3,3-dialkyltriazenes—II *In vitro*-alkylation of guanosine, RNA and DNA with aryl-monoalkyltriazenes to form 7-alkylguanine

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1-ARYL-3-MONOALKYLTRIAZENES act as typical alkylating agents towards guanosine, RNA and DNA. Reaction *in vitro* resulted in the formation of 7-alkylguanine.

In part I of this series<sup>1</sup> a mechanism has been proposed for the carcinogenic action<sup>2</sup> of 1-aryl-3,3-dialkyltriazenes. Compounds of this type are *N*-dealkylated by microsomal fraction of the liver to form the corresponding aldehyde and 1-aryl-3-monoalkyltriazenes as proximate carcinogens. Aryl-monoalkyltriazenes are alkylating agents<sup>3-5</sup> (Equation 1):



We therefore proposed the alkylation of biopolymers as the first step in carcinogenesis by aryl-di-alkyltriazenes.<sup>1</sup> To verify this hypothesis, the reaction *in vitro* of phenylmonomethyl- and -monoethyltriene with guanosine, RNA and DNA has been investigated.