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Mass Spectrometric Identification of an *N*-Oxide formed by Incubation of *N,N*-Dimethyl-*5H*-dibenzo[*a,d*]cycloheptene-$\Delta^{5,\gamma}$-propylamine with Rat Liver Microsomes

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Abstract—On incubation with rat liver microsomes, *N,N*-dimethyl-5*H*-dibenzo[*a,d*]cycloheptene-$\Delta^{5,\gamma}$-propylamine is converted to the *N*-oxide. The identification of this metabolite has been achieved by the use of gas chromatography, mass spectrometry and thin-layer chromatography.

Introduction

THE FORMATION of tertiary amine *N*-oxides is one of the most common pathways for drug *N*-oxidation.

The significance of *N*-oxide formation was underestimated until recently, but since 1960 there has been increasing interest in this field,[1] because several *N*-oxides have been shown to be carcinogens[2–4] or to exhibit pronounced toxicological action such as methemoglobin formation.[5]

N,N-dimethyl-5*H*-dibenzo[*a,d*]cycloheptene-$\Delta^{5,\gamma}$-propylamine (**1**) is an antidepressive drug[6] structurally related to imipramine; the present '*in vitro*' study shows that the formation of the *N*-oxide is an enzymatic process not due to the occurrence of chemical artifacts.[7,8]

Experimental

Materials

The drug was kindly donated by Merck Sharp and Dohme, Pavia, Italy. The following reagents were used: glucose 6-phosphate, glucose-6-phosphate dehydrogenase and NADP (Boehringer); nicotinamide (British Drug Houses); $MgCl_2$ (Merck). The solvents used were of analytical grade.

Incubation system

Microsomes were obtained from the liver of male Sprague Dawley rats (120 to 140 g), with a procedure involving centrifugation (17,000 g) of the liver homogenate at low speed in a sucrose-EDTA mixture.[9] Final concentration of constituents of the mixture, consisting of cofactors and microsomes, in 0.2 mol dm^{-3} phosphate buffer pH 7.4 were: **1** added as an aqueous solution of its chlorhydrate (0.1 μmol dm^{-3}), NADP (0.3 mmol dm^{-3}), glucose 6-phosphate (10 mmol dm^{-3}), glucose-6-phosphate dehydrogenase (0.3 U/ml), $MgCl_2$ (5 mmol dm^{-3}), nicotinamide (10 mmol dm^{-3}). The mixture was heated on a water bath with gentle shaking for 2 h at 37 °C.

Extraction procedure

The incubation mixture (3 ml) was adjusted to pH 9 with 1 N NaOH and extracted twice with 5 ml of methylene chloride. The organic phase was evaporated to dryness under reduced pressure and the material obtained redissolved in methanol, then subjected to g.c.m.s.

Thin-layer chromatography

This was carried out on glass plates coated with silica gel G (Merck) 0.25 mm thick. Chromatograms were developed at room temperature in an isopropyl alcohol + diethylamine (95:5) solvent system.

Gas chromatography

The gas chromatographic analysis was performed on a Carlo Erba Fractovap G 1 gas chromatograph equipped with a flame ionization detector. The gas chromatographic column was glass tubing (1 m long, 4 mm i.d.) packed with 100 to 120 mesh Chromosorb Q coated with 3% OV 17. The operating conditions were: injection port temperature 285 °C; oven temperature 250 °C; nitrogen (carrier gas) flow rate 50 ml/min; hydrogen flow rate 20 ml/min and air flow rate 300 ml/min.

Gas chromatography-mass spectrometry

A gas chromatograph-mass spectrometer (LKB 9000) was used at the following conditions: energy of the ionization beam 70 eV; ion source temperature 250 °C; trap current 60 μA. Sample introduction was carried out either by a direct inlet system at a probe temperature ranging from 20 to 120 °C or by a g.c. procedure with the helium (carrier gas) flow rate 30 ml/min and other conditions as described above.

Synthesis of *N,N*-dimethyl-5*H*-dibenzo[*a,d*]cycloheptene-$\Delta^{5,\gamma}$-propylamine *N*-oxide

The drug free base (20 mmol) was dissolved in methanol (50 ml) and a 30% solution of hydrogen peroxide (10 ml) was added dropwise to the stirred solution. After 2 h hydrogen peroxide (5 ml) were added

and 2 h later the aqueous mixture was extracted twice with methylene chloride + ethyl acetate 4:1 solution (20 ml). The yield of *N*-oxide, crystallized from methylene chloride, was 90% (melting point 103 to 105 °C) and the compound showed an R_f of 0.1 in the above-mentioned t.l.c. system.

In the n.m.r. spectrum $(CDCl_3)$,$N(CH_3)_2$ appear as a singlet at 2.6 δ in the drug and as two singlets at 2.9 δ and 3.0 δ in the *N*-oxide; a narrow singlet at 6.8 δ is due to 2H in positions 10, 11 and at 7.2 to 7.4 δ there is a multiplet due to eight aromatic hydrogens.

Results and discussion

The gas chromatograms obtained from the methylene chloride extracts of microsomes incubated with **1**, and the control sample prepared by the same procedure but incubated without the drug, are shown in Fig. 1. The methanolic solution of the incubation mixture extracts was also spotted on a t.l.c. plate and three spots, not present in the blank and with an R_f different from that of the starting drug (R_f 0.51), were obtained after development in the above described solvent system.

The spot at R_f 0.1 eluted with methanol and injected into the gas chromatograph, showed a peak at a retention time corresponding to the first one reported in Fig. 1. This peak, when analysed by g.c.m.s., showed the molecular ion at *m/e* 230, while the second corresponded to the intact drug (Fig. 2). The similarity of its mass spectrum to that of **1** was consistent with the hypothesis that the first peak could be a metabolic degradation product of the drug. The absence of the peak at *m/e* 58 and the even molecular weight of the possible metabolite were in agreement with a modification of the sidechain containing the nitrogen atom. However, the loss of 45 a.m.u. from the drug could not be related to a metabolic process and so the possibility of a thermal degradation of the metabolite during the g.c. analysis was taken into consideration. To confirm that this type of degradation occurred in our case, we investigated the effect of the flash heater temperature on the thermal degradation of the metabolite. Lowering the flash heater and column temperature, to 250 and 230 °C, respectively, we obtained a peak with the same retention time and with a mass spectrum identical to that obtained at higher temperature. Furthermore, in order to eliminate any interference due to the g.c. process, the material isolated from the spot at R_f 0.1 was also introduced directly into the ion source of the mass spectrometer. Mass spectra were obtained at the lowest possible temperature and when the probe was unheated spectra were scanned immediately after probe insertion. The mass spectrum obtained with this technique was identical with that obtained after g.c. analysis.

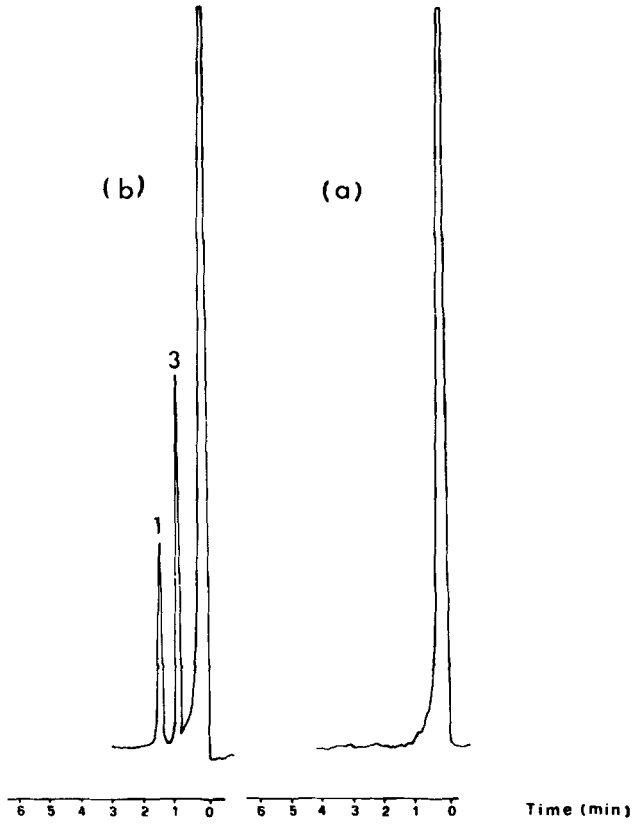
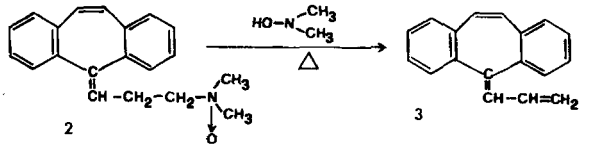


FIG. 1. Gas chromatograms of the methylene chloride extract of microsomes: (a) control sample; (b) microsomes incubated with the drug. The first peak corresponds to the thermal degradation product (3) of the drug *N*-oxide, while the second peak corresponds to the added drug (1).

All this evidence is in agreement with the hypothesis of an *N*-oxide of the drug as the metabolite. In fact the most reasonable decomposition process which might be in agreement with the molecular weight of the compound obtained, and which occurred at a rather low temperature,[10] was found to be the Cope rearrangement. This process occurs by thermal loss of dialkylhydroxylamine from tertiary amine *N*-oxides (Scheme 1).[11,12] Moreover a sample of **1** *N*-oxide was synthetized and subject to t.l.c., g.c., g.c.m.s. and m.s. analysis using the direct inlet system; in all cases its behaviour was similar to that of the metabolite. In order to ascertain that the formation of this *N*-oxide was not due to a chemical reaction occurring within the incubation mixture, but to an enzymatic process, the drug was incubated under various experimental conditions as described in Table 1. As shown, with inactivated microsomes, after boiling, or in the absence of NADP, in the incubation mixture, *N*-oxide formation was not observed, proving that the reaction occurs only in the presence of the complete microsomial system.



SCHEME 1. The Cope thermal degradation of the drug *N*-oxide (2).

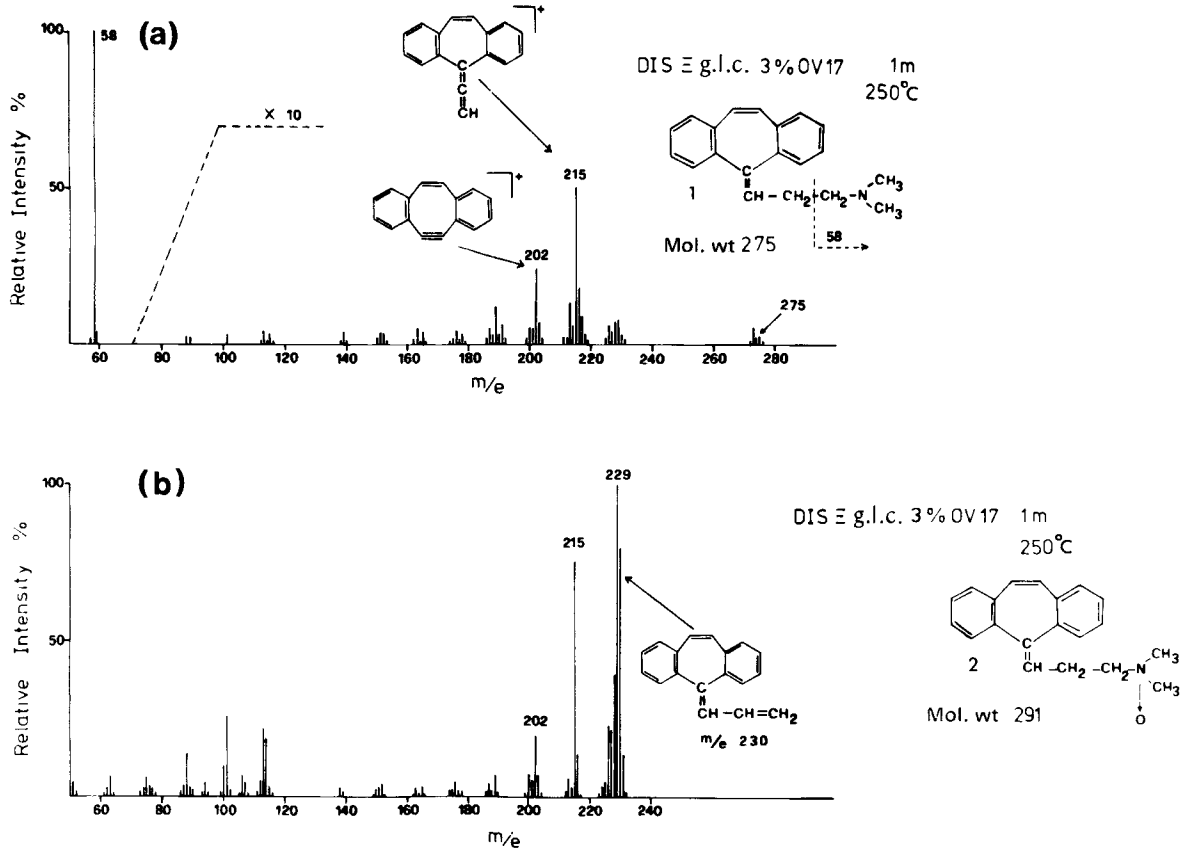


FIG. 2. Mass spectrum of (a) the drug (**1**) and (b) the drug *N*-oxide (**2**).

TABLE 1. Experimental conditions for *N*-oxide formation

Experimental conditions	*N*-oxide formation
Microsomes + cofactors	+
Boiled microsomes[a] + cofactors	−
Microsomes + cofactors − NADP	−
Cofactors only	−

[a] Boiled microsomes were prepared heating microsomes, resuspended in phosphate buffer 0.2 mol dm^{-3}, at 100 °C for 10 min.

The drug was also incubated with cofactors alone and the formation of the *N*-oxide was not observed, proving that this was not due to the occurrence of chemical artifacts. Preliminary quantitative data, obtained by comparing the area of the metabolite peak to the area of the peak formed by injecting a known amount of synthetic *N*-oxide, showed that the *N*-oxide recovered from the incubation mixture (recovery 85 ± 3%) accounts for about 15% of the drug added to microsomes.

The g.c. behaviour and the identification of other metabolites, whose presence is suggested by the observation of the t.l.c. spots and the ratio of the *N*-oxide peak to that of **1**, is still under investigation.

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