pound was obtained with the molecular ion at *m/e* 363, corresponding to the *n*-butylboronate of the diol (Fig. 4). Furthermore, the TLC, GLC, and mass spectral properties of the diol were compared to those of a synthetic sample of the 10,11-dihydrodiol of protriptyline[7].

The second metabolite (third GLC peak) presented a spot at $R_f$ 0.08 and was identified as the 10-hydroxy (III) metabolite previously detected (6).

The mass spectrum (Fig. 2) showed a molecular ion at *m/e* 279 and a very intense peak at *m/e* 207, suggesting the introduction of an oxygen atom into the tricyclic part of the molecule during the biotransformation. In this case, however, the GLC analysis, after methylation of the substance with trimethylanilinium hydroxide, revealed a shift of the GLC peak (Fig. 1C). The mass spectrum showed a corresponding shift of the molecular ion of 14 amu (methylation), proving the presence of a hydroxylated metabolite.

The last metabolite, the 10,11-dihydrodiol, previously identified by Sisenwine *et al.* (6), had an $R_f$ 0.28 value and was the fourth GLC peak. The mass spectrum showed the molecular ion at *m/e* 297. The most important ions were at *m/e* 280 (−17 amu from the peak at *m/e* 297) and 179, arising from the ion at *m/e* 207 by loss of ethylene (the presence of a metastable ion confirmed this transition).

The study of fragmentation (Scheme III), the reaction of derivatization with *n*-butylboronic acid, as described here, and the comparison with the synthetic product established the structure of this metabolite.

A possible, but not proven, metabolic pathway of protriptyline is reported in Scheme IV. The drug may be transformed into the 10-hydroxy or 10,11-epoxide metabolite, which is metabolized to the 10,11-dihydrodiol. Both the mono- and dihydroxylated metabolites are also present in rat urines as glucuronides.

These findings are in agreement with the generally accepted hypothesis that epoxides represent the intermediates of aromatic and nonaromatic diols (7). In this investigation, the epoxide formed from protriptyline appears to be a stable metabolite, which could be isolated from and identified in urine.

[7] Donated by Italseber, Milan, Italy.

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# Identification of 10,11-Epoxide and Other Cyclobenzaprine Metabolites Isolated from Rat Urine


**G. BELVEDERE, C. PANTAROTTO, V. ROVEI, and A. FRIGERIO [x]**



**Abstract** □ Cyclobenzaprine (40 mg/kg ip) was administered to rats, and six urinary metabolites of this drug were identified. They were the 10,11-epoxide, the *N*-oxide, the desmethyl derivative, the hydroxylated and desmethylhydroxylated compounds, and the *N*-oxide hydroxylated at the 10- or 11-position. Mass spectrometric analysis confirmed their structures.



**Keyphrases** □ Cyclobenzaprine metabolites—six isolated from rat urine, TLC, GLC, and mass spectral identification □ Metabolites, cyclobenzaprine—six isolated from rat urine, TLC, GLC, and mass spectral identification □ Sedatives—cyclobenzaprine, six metabolites isolated from rat urine, TLC, GLC, and mass spectral identification


Previous studies in this laboratory established that tricyclic compounds such as carbamazepine (1), cyproheptadine (2), and protriptyline (3) can be transformed into stable epoxides in the 10,11-position and isolated from human and rat urine. This investigation was concerned with another compound with antidepressant activity and possessing a tricyclic structure, cyclobenzaprine (*N,N*-dimethyl-5*H*-dibenzo[*a,d*]cycloheptene-$\Delta^{5,\gamma}$-propylamine) (I) (4).

Six metabolites were identified by using TLC, GLC, and mass spectrometric techniques. They were the 10,11-epoxide (V), the *N*-oxide (II), the desmethyl derivative (III), the hydroxylated (VI) and desmethylhydroxylated (VII) compounds, and the *N*-oxide hydroxylated (IV) at the 10- or 11-position.

## EXPERIMENTAL

**Biological Samples**—Two male Sprague–Dawley rats, 200 g, were injected intraperitoneally with 40 mg/kg of cyclobenzaprine hydrochloride[1] dissolved in 0.9% NaCl. Urine was collected at various times over 48 hr following drug administration and frozen until analysis.

The drug, added to untreated rat urine and analyzed after 48 hr, did not show formation of any degradation products.

[1] Donated by Merck Sharp and Dohme, Rahway, N.J.

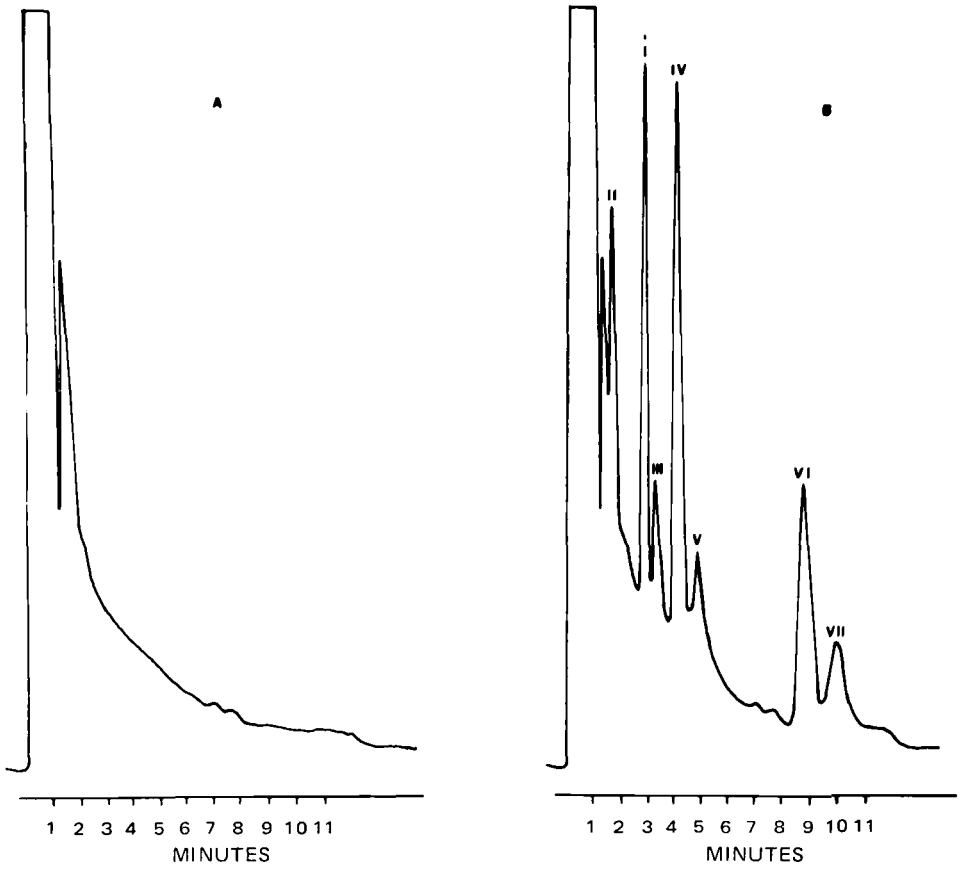



**Figure 1**—*Gas-liquid chromatograms of: A, urine blank; and B, urine of rats treated with I, 40 mg/kg ip.*

**Table I—$R_f$ Values of Cyclobenzaprine (I) and Its Metabolites on Thin-Layer Chromatograms**[a]

| Compound | $R_f$ in Solvent System[b] | |
|---|---|---|
| | A | B |
| I | 0.72 | 0.50 |
| Metabolite II | 0.16 | 0.00 |
| Metabolite III | 0.51 | 0.44 |
| Metabolite IV | 0.06 | 0.00 |
| Metabolite V | 0.68 | 0.40 |
| Metabolite VI | 0.46 | 0.35 |
| Metabolite VII | 0.19 | 0.23 |

[a] Silica gel F-254 (0.25 mm). [b] A = cyclohexane–2-propanol–methanol–diethylamine (60:25:10:5 v/v), and B = cyclohexane–diethylamine–toluene–methanol (75:10:10:5 v/v).

**Treatment of Biological Samples**—Urine samples (10–20 ml) were adjusted to pH 9 with 1 *N* NaOH and extracted with ethyl acetate (20–40 ml). The organic phase was concentrated to dryness in a water bath at 65° under a gentle stream of nitrogen. This material was then used for TLC, GLC, and mass spectral analyses.

**Incubation with β-Glucuronidase**—Incubation of urine samples was carried out after exhaustive extraction of the drug and the free metabolites with ethyl acetate. To 5 ml of urine adjusted at pH 4.5 with 1 *M* acetate buffer (5 ml) was added 0.5 ml of β-glucuronidase[2], and the samples were incubated at 37° for 12 hr. The incubate was extracted with ethyl acetate (5 ml), dried, and analyzed by the described techniques.

**General Procedures**—Thin-layer chromatograms were prepared on 5 × 20-cm glass plates precoated with silica gel F-254[3] and developed in the solvent systems shown in Table I. Cyclobenzaprine and the possible metabolites were visualized under UV light at 254 and 361 nm.

GLC determinations were performed on a gas chromatograph[4] equipped with a flame-ionization detector. The chromatographic column consisted of glass tubing, 2 m long and 4 mm i.d., packed with 100–120-mesh Gas Chrom Q and coated with 3% OV-17[5]. The operating conditions were: injection port temperature, 290°; oven temperature, 270°; nitrogen (carrier gas) flow rate, 50 ml/min; hydrogen flow rate, 20 ml/min; and air flow rate, 300 ml/min.

A GLC–mass spectrometer–computer system[6] was used at the following conditions: energy of the ionization beam, 70 ev; ion source temperature, 100°; and ionization current, 200 μamp. Sample introduction was carried out by a direct inlet system at a probe temperature of 120° or by a GLC procedure. This GLC procedure utilized a glass column, 2 m long and 4 mm i.d., packed with 3% OV-17 on 100–120-mesh Gas Chrom Q at the following conditions: injector temperature, 290°; oven temperature, 270°; and helium (carrier gas) flow rate, 30 ml/min.

**Synthesis of 10,11-Epoxide (V)**—Cyclobenzaprine hydrochloride (20 mmoles) was dissolved in water–methanol (1:1) (100 ml), and a mixture of 2 ml of acetonitrile and 5 ml of a 30% solution of hydrogen peroxide was added at various intervals. The pH was kept constant at 9–10 with 1 *N* NaOH until oxygen evolved.

After 48 hr at room temperature, the reaction mixture was extracted twice with an equal volume of methylene chloride, dried over sodium sulfate, and evaporated. To reduce the *N*-oxide obtained from V, a water solution of 1 *N* $Na_2S_2O_4 \cdot 7H_2O$ was added and the reaction was followed with the gas chromatograph.

The solution was then extracted twice with 15 ml of methylene chloride, and the organic layer was washed with 15 ml of 5% $NH_4Cl$. The organic phase was fractionated on a silica gel column, 60 × 2 cm, eluted with cyclohexane–chloroform–diethylamine (85:10:5 v/v). The fractions collected (2 ml) were examined by GLC, and those containing the epoxide were pooled and evaporated.

The epoxide yield, crystallized from methylene chloride, was 60%,

[2] Boehringer.
[3] Merck.
[4] Carlo Erba Fractovap G1.
[5] Applied Science Laboratories.
[6] Finnigan quadrupole model 3100 and computer model 6000.

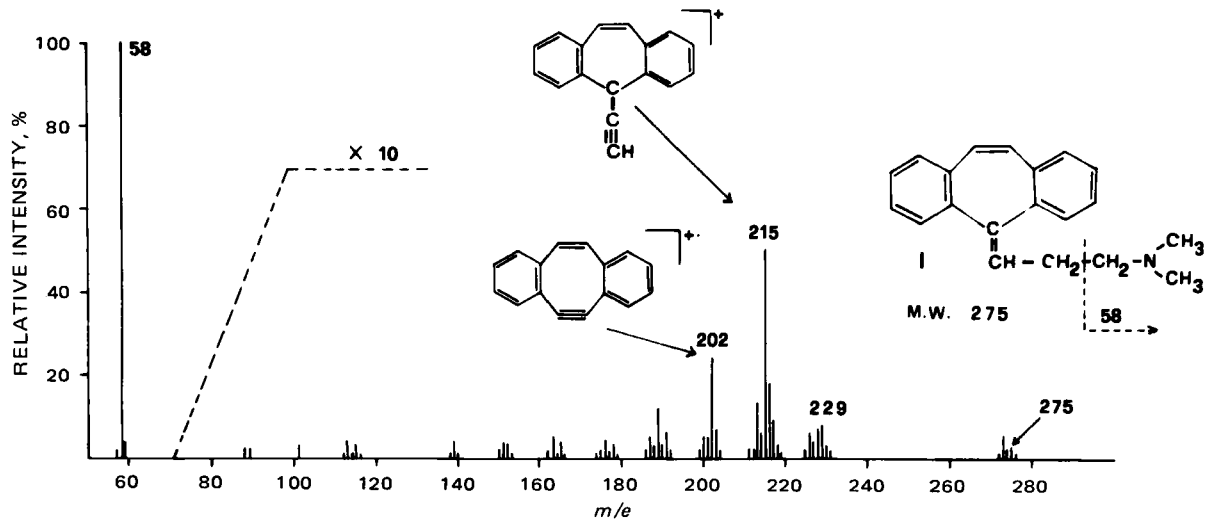



**Figure 2**—*Mass spectrum of authentic I. A similar spectrum was obtained by introducing the corresponding eluted TLC spots by the direct inlet system or GLC procedure.*

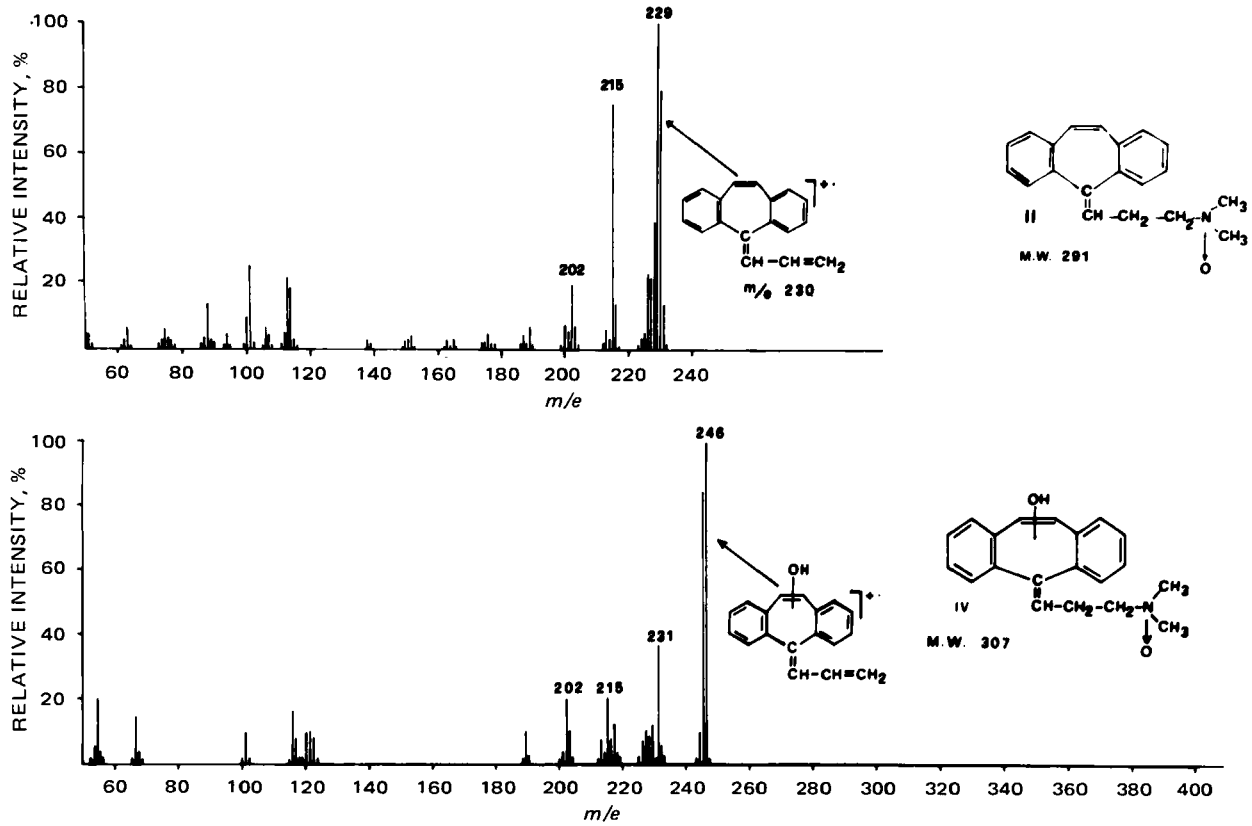



**Figure 3**—*Mass spectra of II and IV obtained by introducing the eluted TLC spots by the direct inlet system or GLC procedure.*

mp 97–100°. The compound showed $R_f$ values at 0.72 and 0.50 $R_f$ in Solvent Systems A and B (Table I), respectively; NMR[7] ($CDCl_3$): $\delta$ 4.2 (s, 2H, HCO epoxide), 2.8 [s, $N(CH_3)_2$], and 7.1–7.4 (m, 8H, aromatic proton).

## RESULTS

The ethyl acetate extract of urine obtained from rats treated with I (40 mg/kg ip) was concentrated to dryness, redissolved in 100 $\mu$l of methanol, spotted on a TLC plate, and developed in Solvent Systems A and B. In contrast to the extract obtained from urine of untreated animals, seven spots were obtained; their $R_f$ values are reported in Table I. Two metabolites (VI and VII) were also present in extracts obtained after $\beta$-glucuronidase treatment of the urine.

The extract of treated urine was also injected into the gas chromatograph, giving rise to seven peaks not present in the blank (Fig. 1).

The second GLC peak showed a retention time corresponding to that of authentic I. The $R_f$ values of 0.72 and 0.50 (Table I) in Solvent Systems A and B, respectively, were identical to those of the starting drug. Furthermore, the mass spectrum (Fig. 2) of the compound was indistinguishable from the one obtained with authentic I.

**Identification of Metabolites II and IV**—The mass spectrum of the methanolic eluate from the spot at $R_f$ 0.16 (Solvent System A) and the GLC–mass spectrum of the first peak exhibited a molecular ion at *m/e* 230 (Fig. 3). The structure elucidated by the mass spectrum

[7] Varian A60.

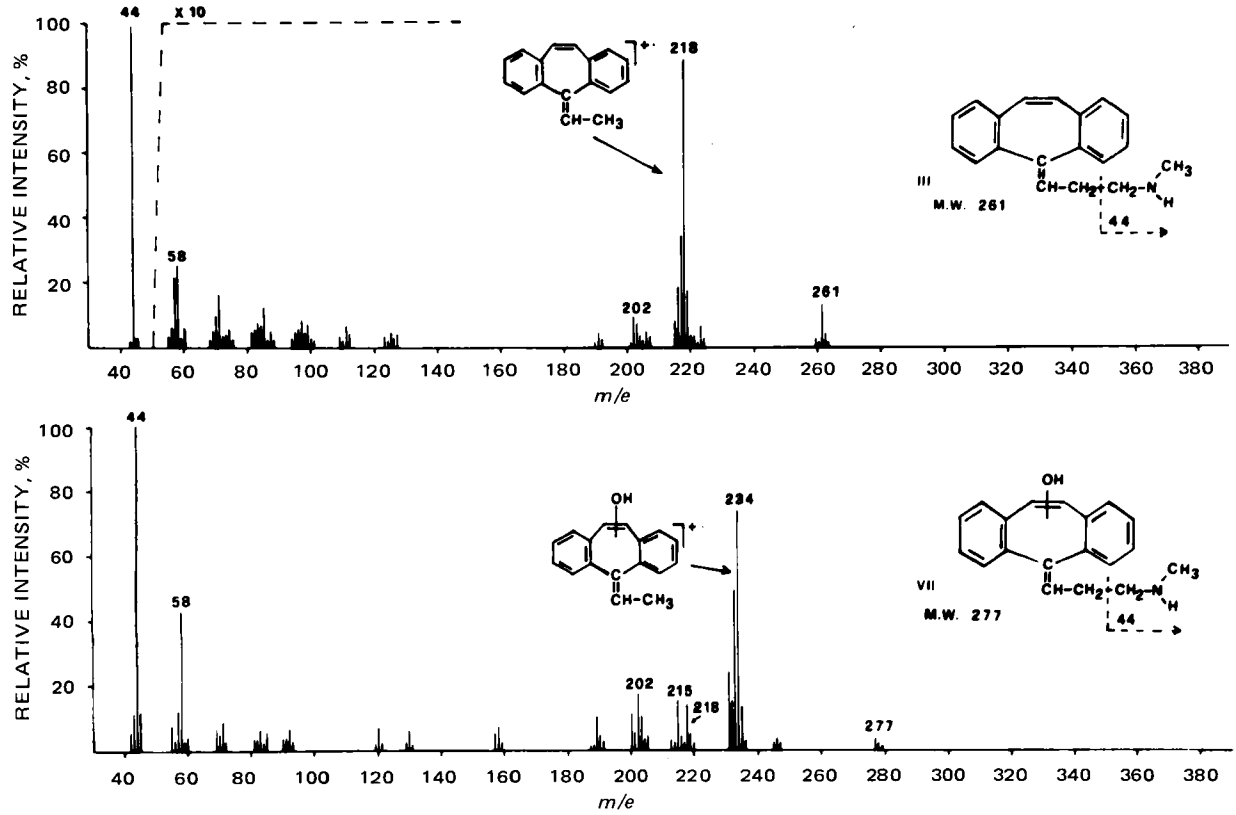



**Figure 4**—*Mass spectra of III and VII obtained by introducing the eluted TLC spots by the direct inlet system or GLC procedure.*

was the hydrocarbon VIII (Scheme I), which resulted from the Cope reaction of the *N*-oxide II in the injection port during GLC analysis (5). Therefore, the original identity of the TLC spot at $R_f$ 0.16 in Solvent System A was II.

The fourth GLC peak, obtained from the injection of the eluate of the spot at $R_f$ 0.06 (Solvent System A), gave rise to the mass spectrum in Fig. 3 when analyzed by GLC–mass spectrometry.

The higher polarity of this compound, demonstrated by a lower $R_f$

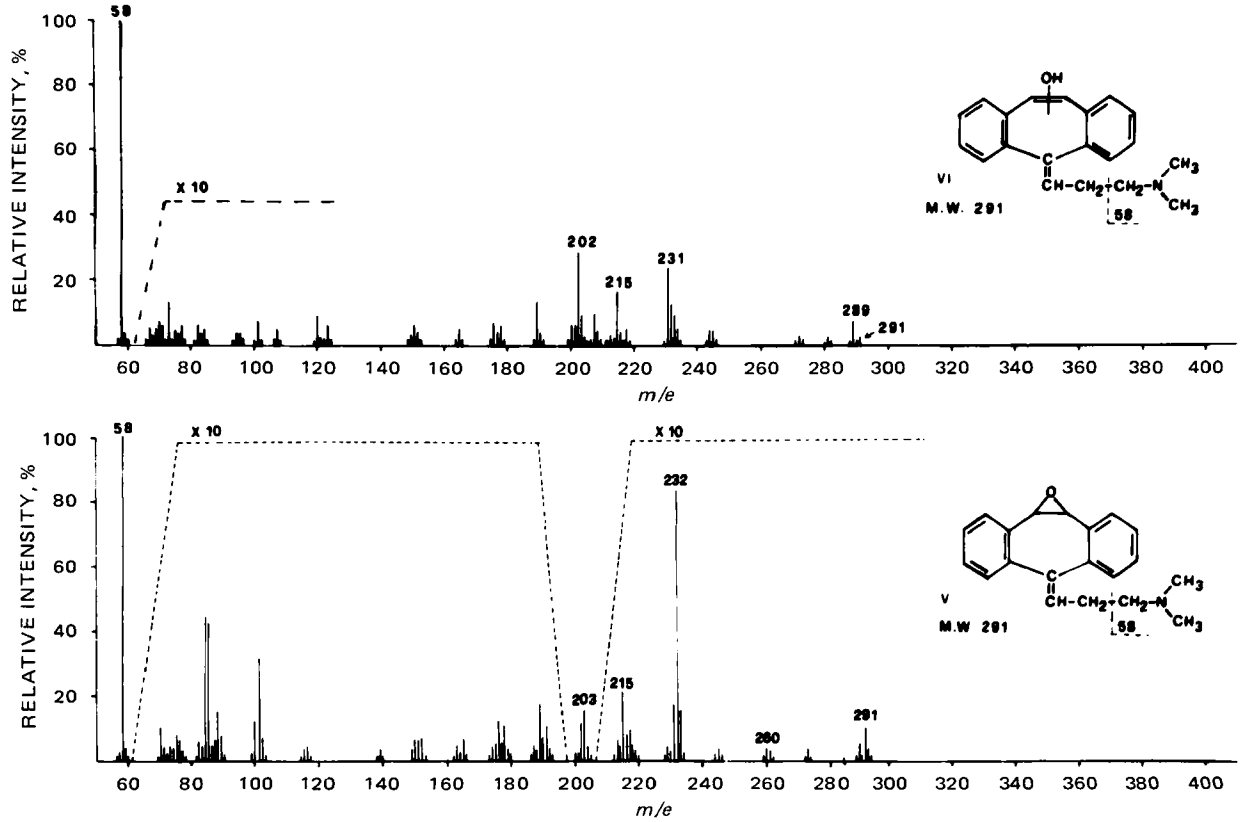
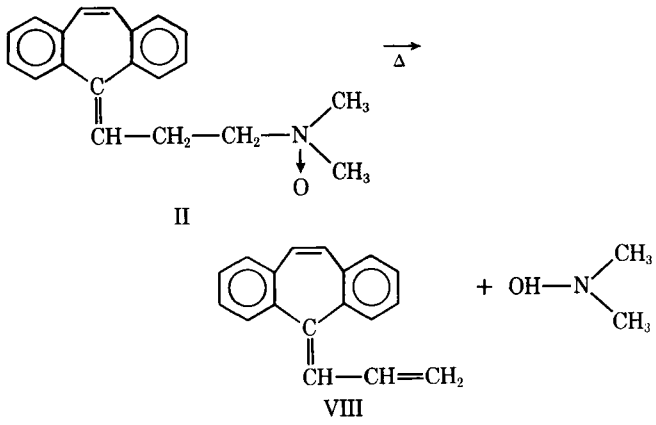



**Figure 5**—*Mass spectra of VI and the synthesized V obtained by introducing the eluted TLC spots by the direct inlet system or GLC procedure.*



II

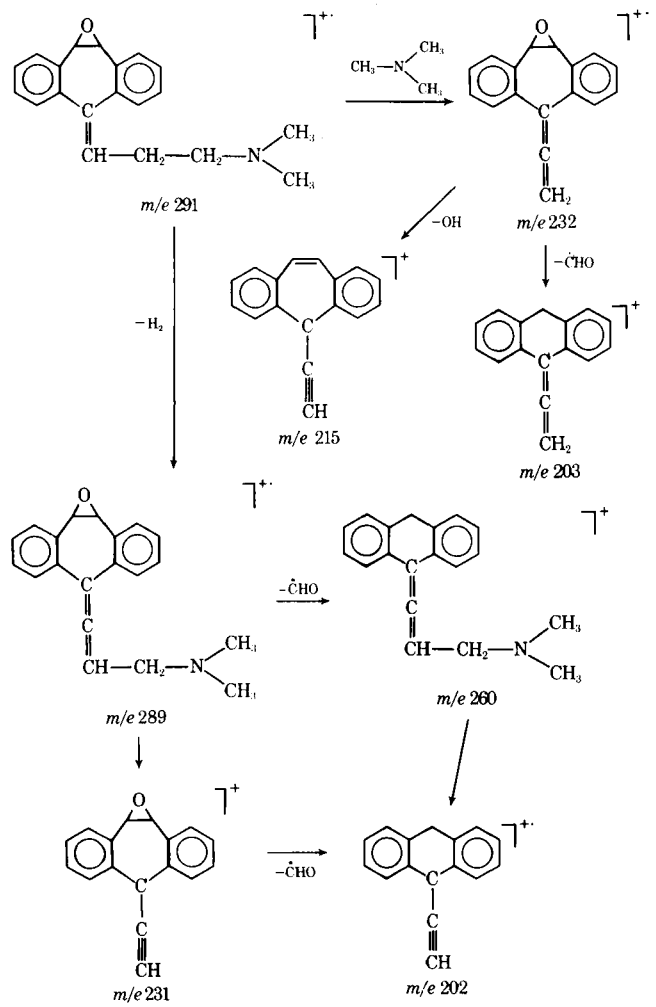
VIII

*Scheme I—Cope thermal degradation of cyclobenzaprine* N-*oxide*

value than that of the *N*-oxide and by a molecular ion at *m/e* 246 (16 amu more than the *N*-oxide), was consistent with the introduction of an oxygen atom into the 10- or 11-position of the *N*-oxide molecule, giving rise to IV. The presence of the hydroxyl group also was confirmed by on-column methylation with trimethylanilinium hydroxide.

This procedure gave rise to a GLC peak that, after mass spectral analysis, showed a mass spectrum in which the molecular ion was shifted to 14 amu, confirming the introduction of a methyl group.

As in the case of the *N*-oxide, the hydroxyl compound underwent the Cope reaction with the thermal loss of dimethylhydroxylamine.



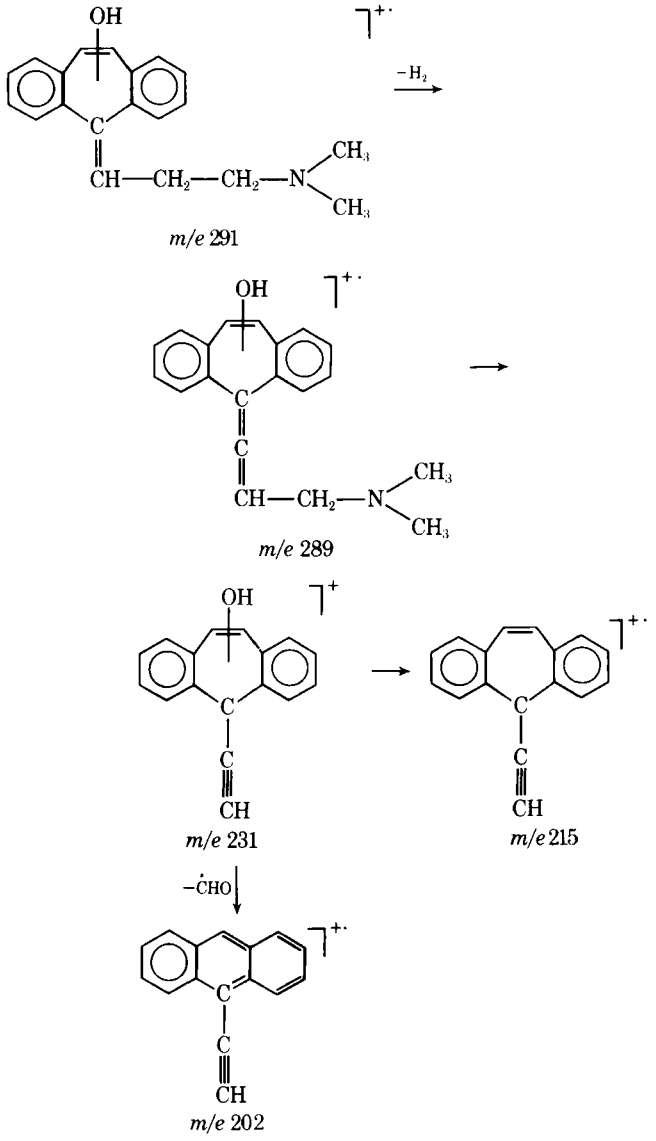
*Scheme II—Suggested fragmentation pathway of the 10,11-epoxide of cyclobenzaprine. (No exact mass measurements were included in the parent ions or fragments, and only a few metastable ions were available to support the fragmentation scheme.)*

**Identification of Metabolites III and VII**—The third GLC peak corresponded to a substance with $R_f$ values of 0.51 and 0.44 in Solvent Systems A and B, respectively. The mass spectrum obtained by GLC or the direct inlet system showed the molecular ion at *m/e* 261 (Fig. 4), suggesting the loss of a methyl group in respect to I. The fragmentation pathway, showing the base peak at *m/e* 44 and a radical ion at *m/e* 218 (Fig. 4), confirmed a structure corresponding to the desmethylcyclobenzaprine.

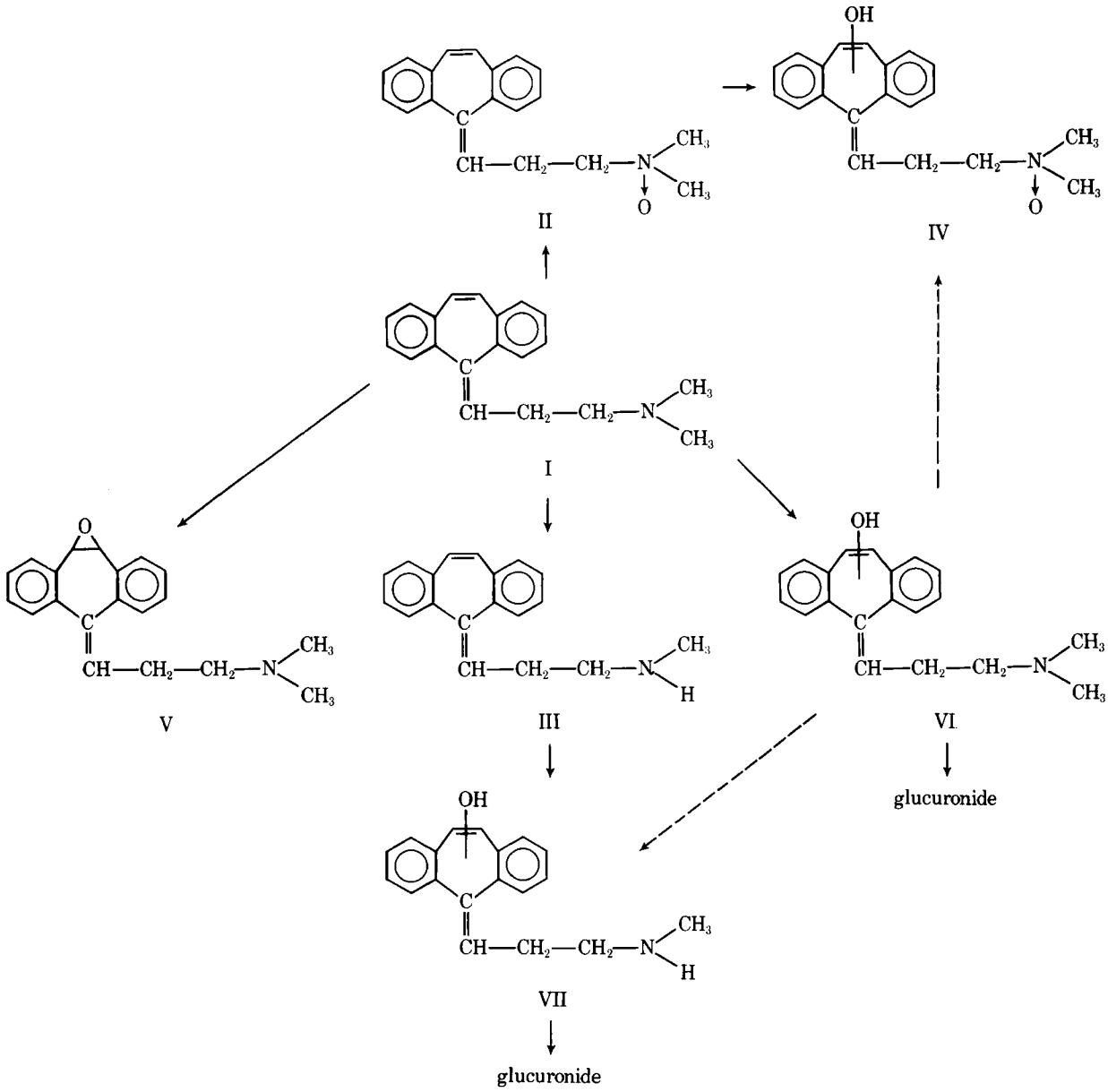
The hydroxydesmethyl derivative was identified as the seventh GLC peak and corresponded to the TLC spots at $R_f$ 0.19 and 0.23 in Solvent Systems A and B, respectively. The mass spectrum (Fig. 4) showed a molecular ion at *m/e* 277 due to the introduction of an oxygen atom into the 10- or 11-position of the drug molecule. After on-column treatment with trimethylanilinium hydroxide of the eluate of the TLC spots, a mass spectrum of the GLC peak was obtained with the molecular ion at *m/e* 305, confirming the structure of the metabolite. This compound also was present after incubation of the urine with β-glucuronidase.

**Identification of Metabolites V and VI**—The mass spectrum (Fig. 5) of the fifth GLC peak corresponded to a compound with the molecular ion at *m/e* 291 and $R_f$ values of 0.68 and 0.41 in Solvent Systems A and B, respectively.

The hypothesis of the presence of an epoxide derivative of I was



*Scheme III—Suggested fragmentation pathway of hydroxycyclobenzaprine. (No exact mass measurements were included in the parent ions or fragments, and only a few metastable ions were available to support the fragmentation scheme.)*



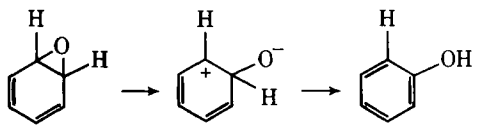

*Scheme IV—Metabolic pathway of cyclobenzaprine* in vivo.

supported by the fact that the molecular ion corresponded to the introduction of an oxygen atom into the drug molecule and by the failure in the methylation of the compound with trimethylanilinium hydroxide or diazomethane.

Moreover, the mass spectral, GLC, and TLC behavior of this metabolite was identical to that of a sample of the synthesized 10,11-epoxide of cyclobenzaprine. The fragmentation pathway of Metabolite V is reported in Scheme II.

The corresponding hydroxyl derivative of I gave rise to the sixth peak in the gas chromatogram. Its mass spectrum (Fig. 5) showed a molecular weight, 291, identical to that of the epoxide. However, there were different features in the fragmentation pathway (Scheme III) and, in addition, this compound reacted with trimethylanilinium hydroxide and formed a methylated product.

The hydroxycyclobenzaprine also was found in the $\beta$-glucuronidase-treated urine sample extracted with ethyl acetate.



*Scheme V*

## DISCUSSION

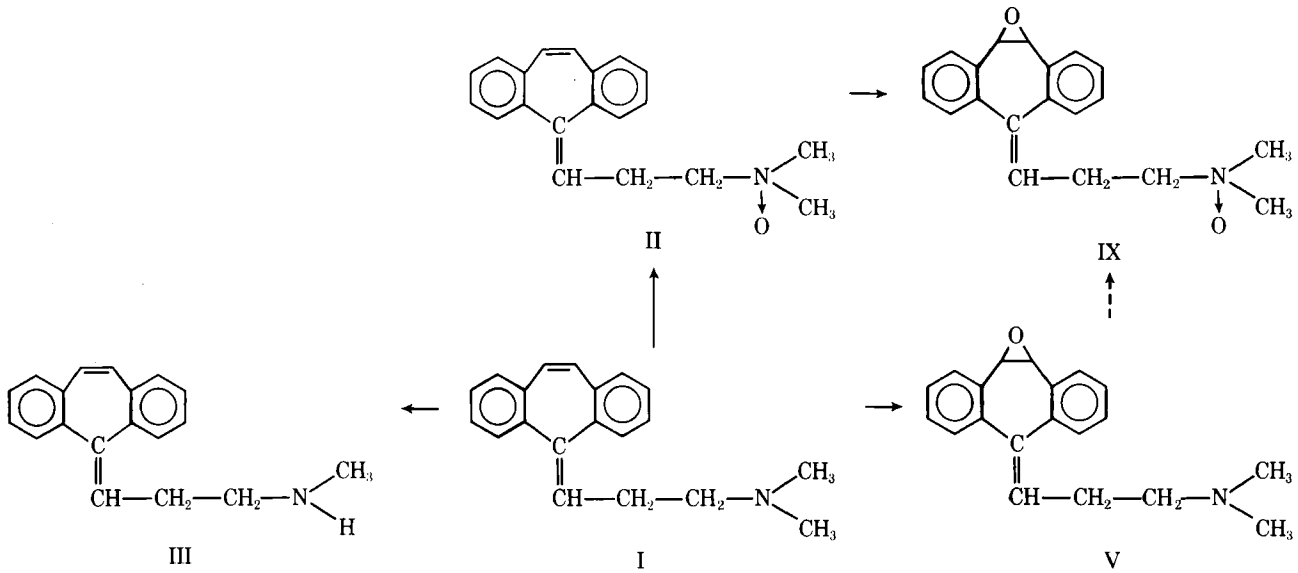
Scheme IV shows the metabolic pathway of cyclobenzaprine. The metabolic transformation of the drug probably occurs by oxidation of the nitrogen on the side chain to give II, by oxidation of the 10,11 double bond with formation of V and VI, and with loss of a methyl group giving rise to III. Metabolites II and III undergo a secondary transformation with oxygenation of the ethylenic double bond. Metabolites VI and VII also are present as glucuronide conjugates.

The loss of ĊHO to give the ions at *m/e* 260, 203, and 202 (Scheme II) seems to exclude the presence of the epoxide at the double bond of the aliphatic chain. Moreover, the TLC, GLC, and mass spectral properties of the metabolite are identical to those of the synthetic product which was unequivocally proven by NMR to be the cyclobenzaprine 10,11-epoxide.

The position of the hydroxyl group on the 10- or 11-carbon atom was assigned to Compounds IV, VI, and VII on the basis of methylation studies and mass spectral analysis. These compounds reacted immediately (5 min) with diazomethane, while prolonged (2 hr) reaction times were necessary for aromatic hydroxyl groups (6).

Moreover, the fragmentation pathway of V and VI (Schemes II and III) shows that both compounds give rise to the same radical ion at *m/e* 202 by loss of HĊO and contraction of the seven-membered ring.

Some reports (7, 8) have stated that the formation of monohydroxyl

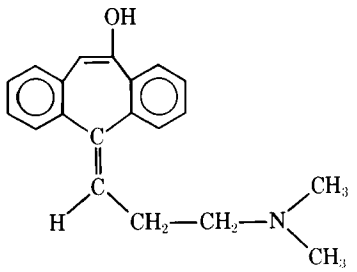


*Scheme VI—Metabolic pathway of cyclobenzaprine incubated with microsomes.*

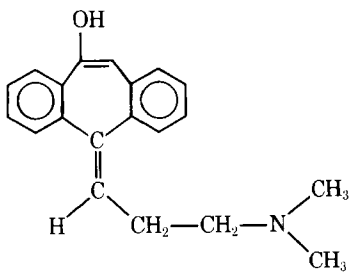
derivatives, when an epoxide was present in the metabolic pathway, occurred by a nonenzymatic rearrangement of the epoxide (Scheme V). In our case, however, it was established that the formation of the hydroxylated metabolites was not due to a chemical modification of the epoxide molecule. In fact, after incubating the synthetic epoxide with control rat urine for 48 hr at 37°, no degradation of the compound was observed with TLC, GLC, and mass spectral techniques.

An unresolved problem at the present time is the positioning of the hydroxyl group at the 10- or 11-carbon of the ethylenic bridge.

The chemical evidence of an isomerism of the hydroxyl group, in respect to the double bond in the side chain, is evident because two different structures, IX and X, can be drawn. Moreover, the thin-layer chromatogram has a spot that, when eluted with methanol, shows the identical GLC retention time and mass spectrum of Metabolite VI but a different $R_f$ value (0.27, Solvent System B).



IX



X

It can be argued from these data that the 10- and 11-positions are not the same from the chemical point of view but that the enzymatic introduction of oxygen is possible in both positions.

This investigation adds a new example of the formation of an epoxide when there is a double bond (10,11-position) of a tricyclic structure. This epoxide is obviously not highly reactive, since it can be isolated from urine and from liver microsomal enzymes (the metabolic pathway *in vitro* is reported in Scheme VI) (9). Studies in progress also indicate that this epoxide has pharmacological activity.

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