be increased and buffered as desired without any significant change in the volume of the medium. The method may serve as a highly automated alternative to the rotating-bottle method described in the official compendia. It offers the following advantages:

1. The operation of the system is very simple, and the system permits variation of conditions as required by the assay method.

2. While the system is basically designed to test the pH dependence of the dissolution rate of solid dosage forms, it may be easily adapted for determining the influence of other factors. Its flexibility may be particularly useful if *in vitro* simulation of *in vivo* bioavailability data is desired.

REFERENCES

(1) "The National Formulary," 14th ed., Mack Publishing Co., Easton, Pa., 1975, pp. 985, 986.

(2) G. Dotevall, *Acta Med. Scand.*, **170**, 59(1951).

(3) P. L. Altman, in "Blood and Other Body Fluids," D. S. Dittmer, Ed., Biological Handbooks, Federation of American Societies for Experimental Biology, Washington, D.C., 1961, pp. 416, 417.

(4) L. C. Schroeter and J. G. Wagner, *J. Pharm. Sci.*, **51**, 957(1962).

(5) F. Sjuib, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **61**, 1381(1972).

(6) C. Cakiryildiz, P. J. Mehta, W. Rahmen, and D. Schoenleber, *ibid.*, **64**, 1692(1975).

(7) F. W. Goodhart, R. H. McCoy, and F. C. Ninger, *ibid.*, **62**, 304(1973).

(8) *Ibid.*, **63**, 1748(1974).

(9) H. Noyes and W. Whitney, *Z. Phys. Chem.*, **23**, 689(1897).

(10) J. G. Wagner, "Biopharmaceutics and Relevant Pharmacokinetics," 1st ed., Drug Intelligence Publications, Hamilton, Ill., 1971, pp. 115–120.

(11) J. E. Tingstad and S. Riegelman, *J. Pharm. Sci.*, **59**, 692(1970).

(12) R. E. Shepherd, J. C. Brice, and L. H. Luzzi, *ibid.*, **61**, 1152(1972).

(13) J. Cobby, M. Mayersohn, and G. C. Walker, *ibid.*, **63**, 725(1974).

(14) *Ibid.*, **63**, 732(1974).

(15) H. G. Kwee and G. A. Ulex, *Pharm. Ind.*, **36**, 576(1974).

(16) A. A. Ismail, M. W. Goudah, and M. M. Motawi, *ibid.*, **36**, 735(1974).

(17) J. B. Johnson, P. G. Kennedy, and S. H. Rubin, *J. Pharm. Sci.*, **63**, 1931(1974).

(18) J. B. Schwartz, A. P. Simonelli, and W. I. Higuchi, *ibid.*, **57**, 274(1968).

(19) *Ibid.*, **57**, 278(1968).

(20) F. Sjuib, A. P. Simonelli, and W. I. Higuchi, *J. Pharm. Sci.*, **61**, 1374(1972).

(21) M. Doubrow and M. Friedman, *ibid.*, **64**, 76(1975).

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Isolation of 10,11-Epoxide of Protriptyline in Rat Urine after Protriptyline Administration

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

Abstract □ The 10,11-epoxide, 10-hydroxy, and 10,11-dihydrodiol metabolites of protriptyline were identified in rat urine collected after the administration of 40 mg/kg ip of protriptyline. Mass spectrometric characterization confirmed the structure of these metabolites.

Keyphrases □ Protriptyline—three metabolites isolated from rat urine, characterized by TLC, GLC, and GLC–mass spectrometry □ Metabolites—protriptyline, isolated from rat urine, characterized by TLC, GLC, and GLC–mass spectrometry □ Antidepressant agents—protriptyline, three metabolites isolated from rat urine, characterized by TLC, GLC, and GLC–mass spectrometry

Epoxides are intermediates in the biological conversion of the aromatic ring system into the corresponding dihydrodiols (1). However, the instability of these metabolites has made their isolation and direct identification in biological fluids difficult.

The isolation from rat urine of the 10,11-epoxide of carbamazepine (2) after administration of carbamazepine, a drug with tricyclic structure, prompted a systematic investigation on the metabolism of other drugs with the same tricyclic structure. During this study, 10,11-epoxides and *N*-desmethyl metabolites of cyproheptadine (3) and cyclobenzaprine (4) were identified.

This report summarizes findings on the metabolism of protriptyline (*N*-methyl-5*H*-dibenzo[*a*,*d*]cycloheptene-5-propylamine) (I), a drug possessing antidepressant activity (5). The 10,11-epoxide, 10,11-dihydrodiol, and 10-hydroxy metabolites of protriptyline were isolated and characterized by means of TLC, GLC, and GLC–mass spectrometry in rat urine after protriptyline administration.

EXPERIMENTAL

Biological Samples—Two male Sprague–Dawley rats, 200 g, were injected with 40 mg/kg ip of protriptyline hydrochloride[1] dissolved in saline. Following drug administration, urine samples were collected at various times over 48 hr and kept frozen until analyses were performed.

After addition of drug to untreated rat urine and incubation at 37° for 48 hr, no degradation of the product occurred.

Treatment of Biological Samples—Urine samples (20 ml)

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

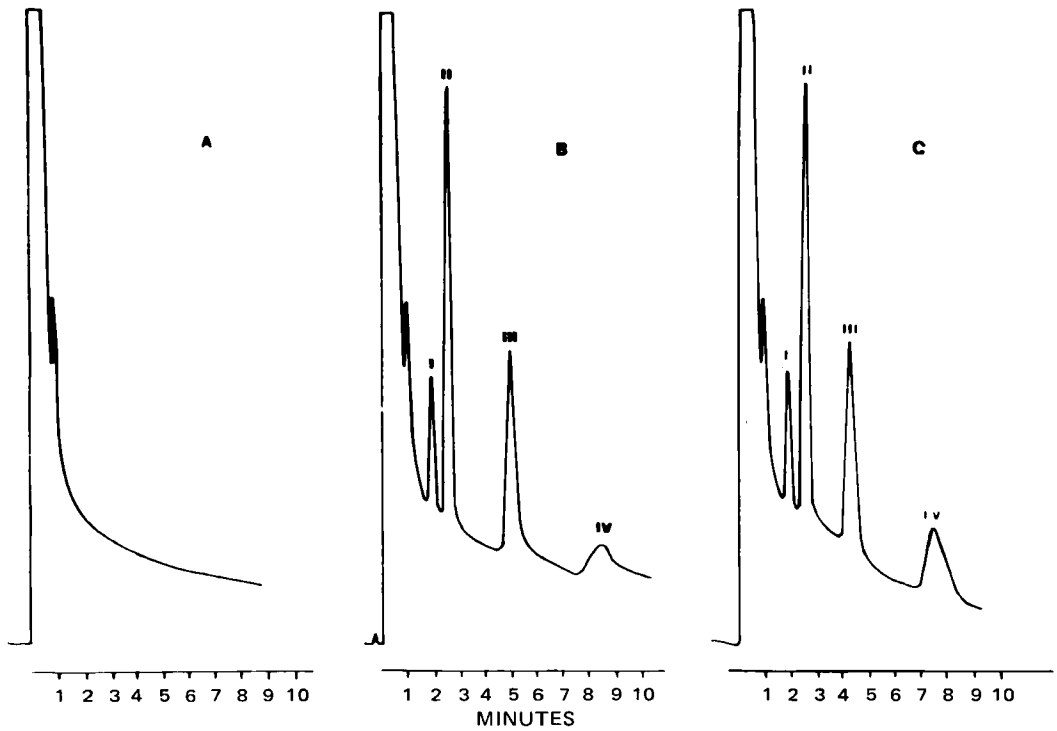


Figure 1—*Gas–liquid chromatograms. Key: A, urine blank; B, urine of rats treated with protriptyline (I), 40 mg/kg ip; and C, urine of rats treated with protriptyline after flash methylation with trimethylanilinium hydroxide.*

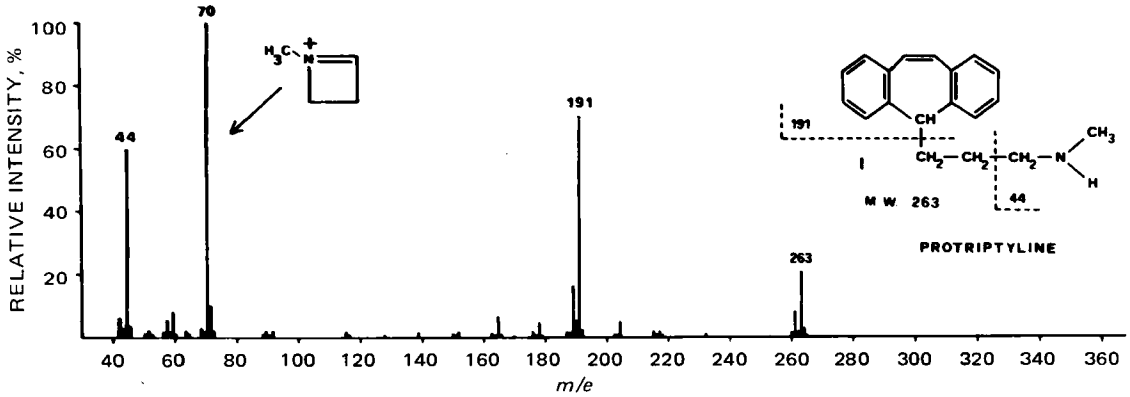


Figure 2—*Mass spectrum of authentic protriptyline (I). A similar mass spectrum was obtained by introducing the corresponding eluted TLC spot by a direct inlet system or GLC procedure.*

were adjusted to pH 9 with 1 *N* NaOH and extracted twice with ethyl acetate (20 ml).

The organic phase was concentrated to dryness under reduced pressure. After the addition of 0.2 ml of methanol, the material obtained was used for TLC, GLC, and mass spectral analysis.

Incubation with β-Glucuronidase—After extraction of free metabolites, the urine samples were incubated with β-glucuronidase[2]. Then 0.5 ml of the enzyme was added to 10 ml of urine, adjusted at pH 4.5 with acetate buffer (1 *M*), and the samples were incubated at 37° for 12 hr. The incubate was extracted twice with ethyl acetate (10 ml), evaporated to dryness, 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 at room temperature with *n*-hexane–diethylamine (50:50 v/v). Protriptyline and the possible metabolites were visualized with UV light at 254 and 361 nm (Table I).

GLC analyses 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, 300°; oven temperature, 290°; nitrogen (carrier gas) flow rate, 30 ml/min; hydrogen flow rate, 20 ml/min; and air flow rate, 300 ml/min.

Table I—R_f Values of Protriptyline and Its Metabolites on Thin-Layer Chromatogram[a]

Compound	R_f in Solvent System[b]
Protriptyline (I)	0.47
Metabolite II	0.36
Metabolite III	0.08
Metabolite IV	0.28

[a] Silica gel F-254 (0.25 mm). [b] *n*-Hexane–diethylamine (50:50 v/v).

[2] Boehringer.
[3] Merck.
[4] Carlo Erba Fractovap Gl.
[5] Applied Science Laboratories.

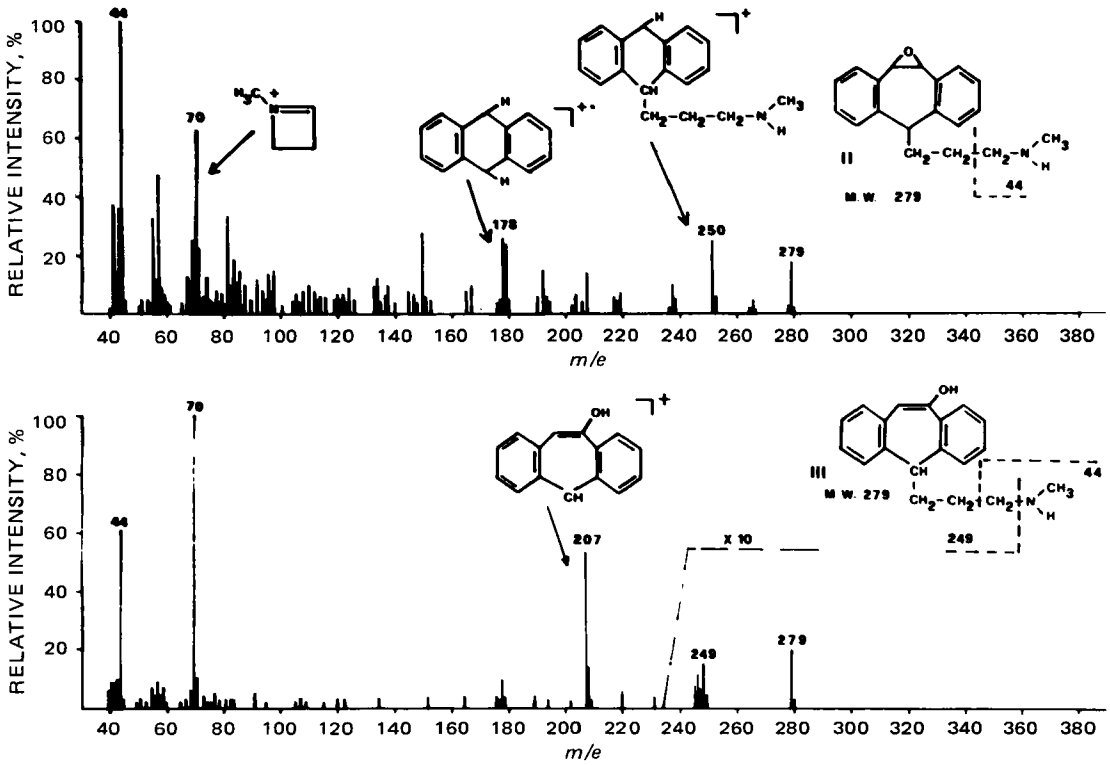
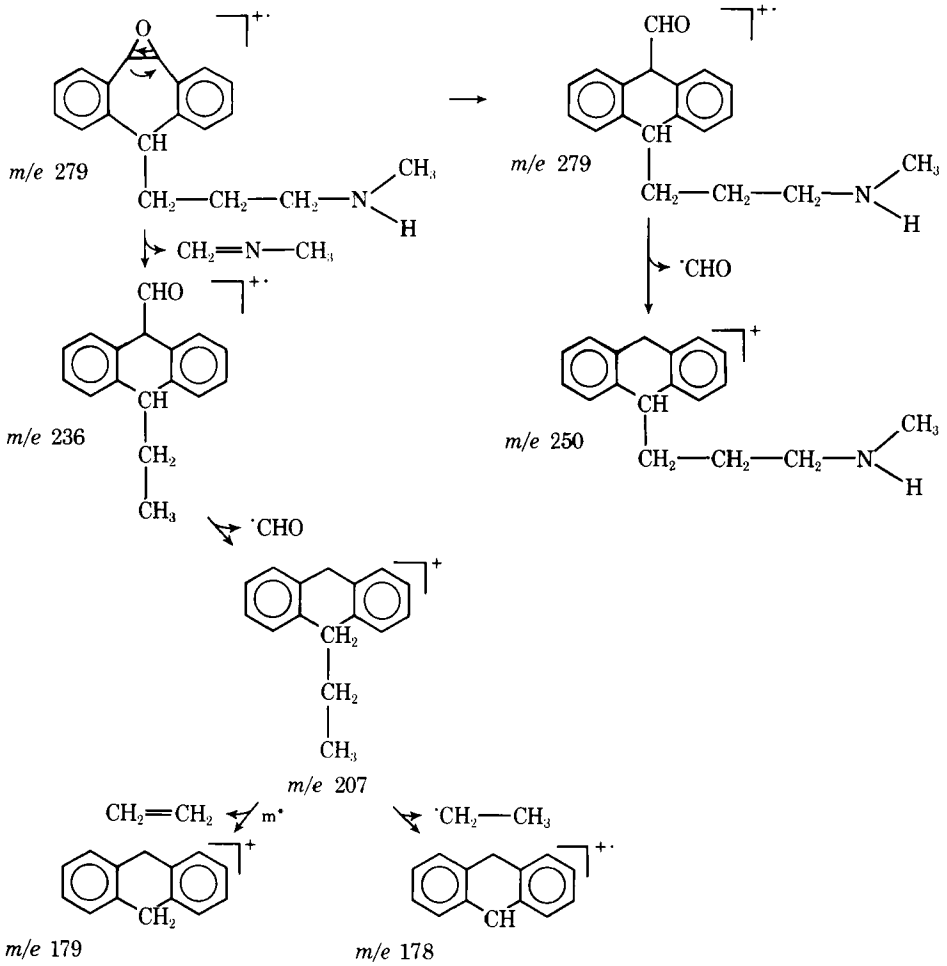


Figure 3—*Mass spectra of II (top panel) and III (bottom panel) obtained by introducing the corresponding eluted TLC spots by a direct inlet system or GLC procedure.*



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

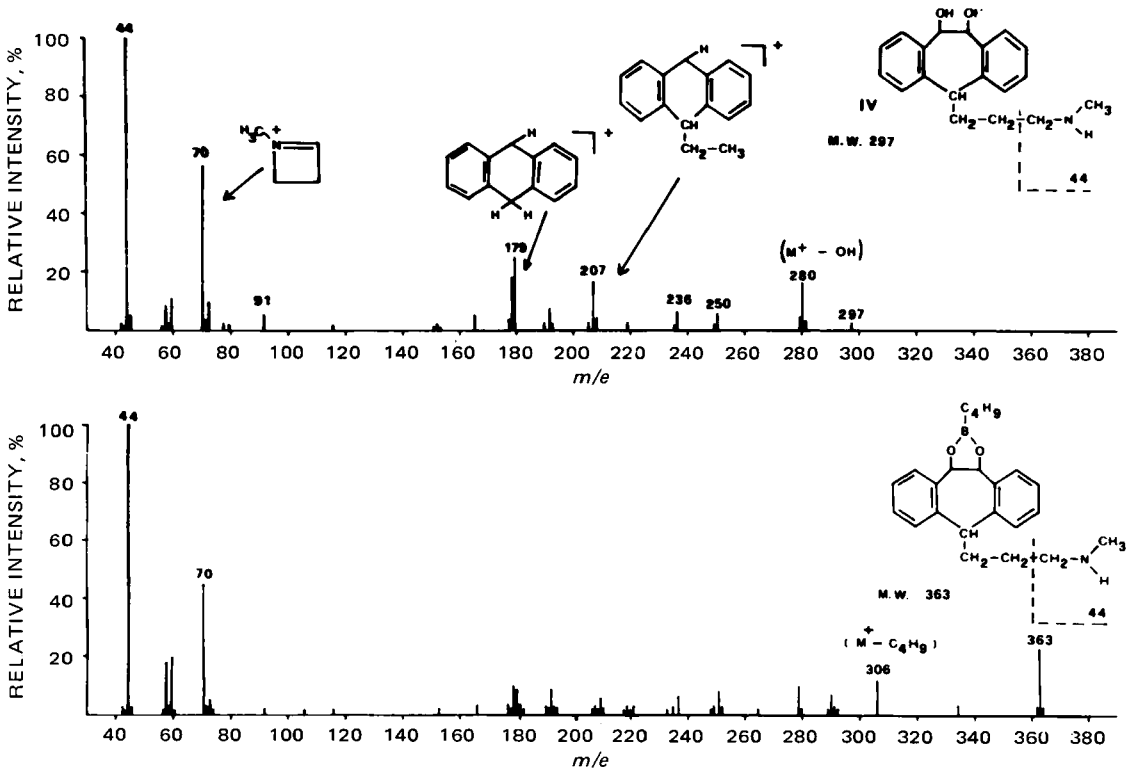


Figure 4—*Mass spectra of IV obtained by introducing the corresponding eluted TLC spot by a direct inlet system or GLC procedure (top panel) and after on-column reaction with* n*-butylboronic acid (bottom panel).*

A gas chromatograph–mass spectrometer[6] was used at the following conditions: energy of ionization beam, 70 ev; ion source temperature, 290°; and trap current, 60 μamp. Sample introduction was carried out by a direct inlet system at a probe temperature of 150° or by a GLC procedure utilizing a glass column (2 m long and 4 mm i.d.) packed with 3% OV-17 on 100–120-mesh Gas Chrom Q. The following conditions were used: injector temperature, 300°; oven temperature, 290°; and helium (carrier gas) flow rate, 30 ml/min.

RESULTS AND DISCUSSION

The ethyl acetate extract, obtained from 20 ml of rat urine, was concentrated to dryness, redissolved in 200 μl of methanol, spotted on a TLC plate, and developed with the described solvent system. The urine extracts of rats treated with the drug contained four spots, not present in the urine of control rats, with the R_f reported in Table I.

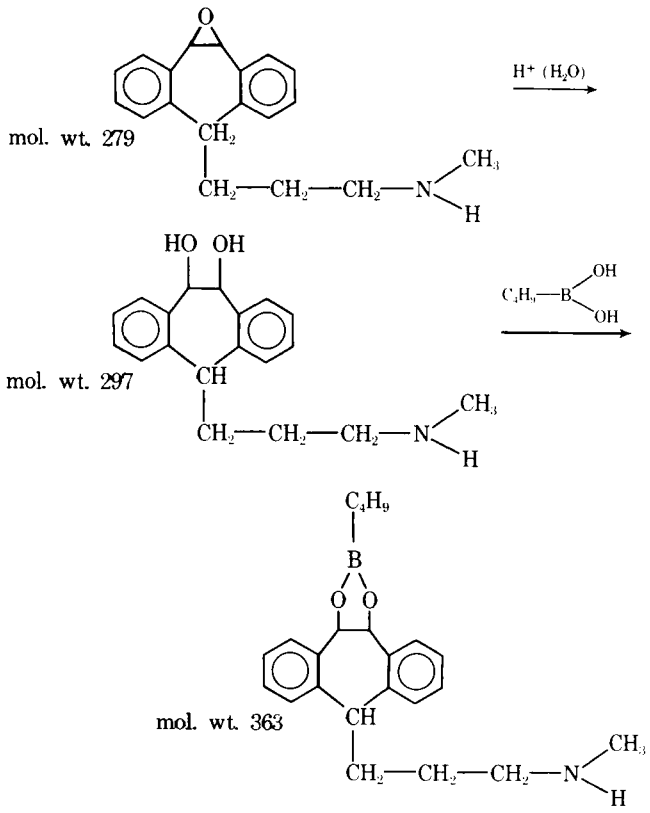
TLC of the extracts of the urine after incubation with β-glucuronidase showed two spots, with R_f 0.08 and 0.28, not present in the control urine extracts. The GLC analysis showed four peaks in the treated urine sample (Fig. 1).

Identification of the unchanged I (spot with R_f 0.47 and first GLC peak) was carried out by comparison with the mass spectrum of the standard drug (Fig. 2). The molecular ion is at *m/e* 263, and the peak at *m/e* 191 is characteristic of the tricyclic structure. The peak at *m/e* 44 and 70 (base peak) were due to the aliphatic chain cleavage.

The second GLC peak, corresponding to the spot with R_f 0.36, was identified as the 10,11-epoxide (II) of protriptyline (Fig. 3). The mass spectrum presented a molecular ion at *m/e* 279, which suggested the introduction of an oxygen atom into the drug molecule. The failure of this substance to react with trimethylanilinium hydroxide and the loss of 29 amu (ĊHO) from the molecular ion (Scheme I) to give an intense peak at *m/e* 250 suggested the presence of an epoxidic structure.

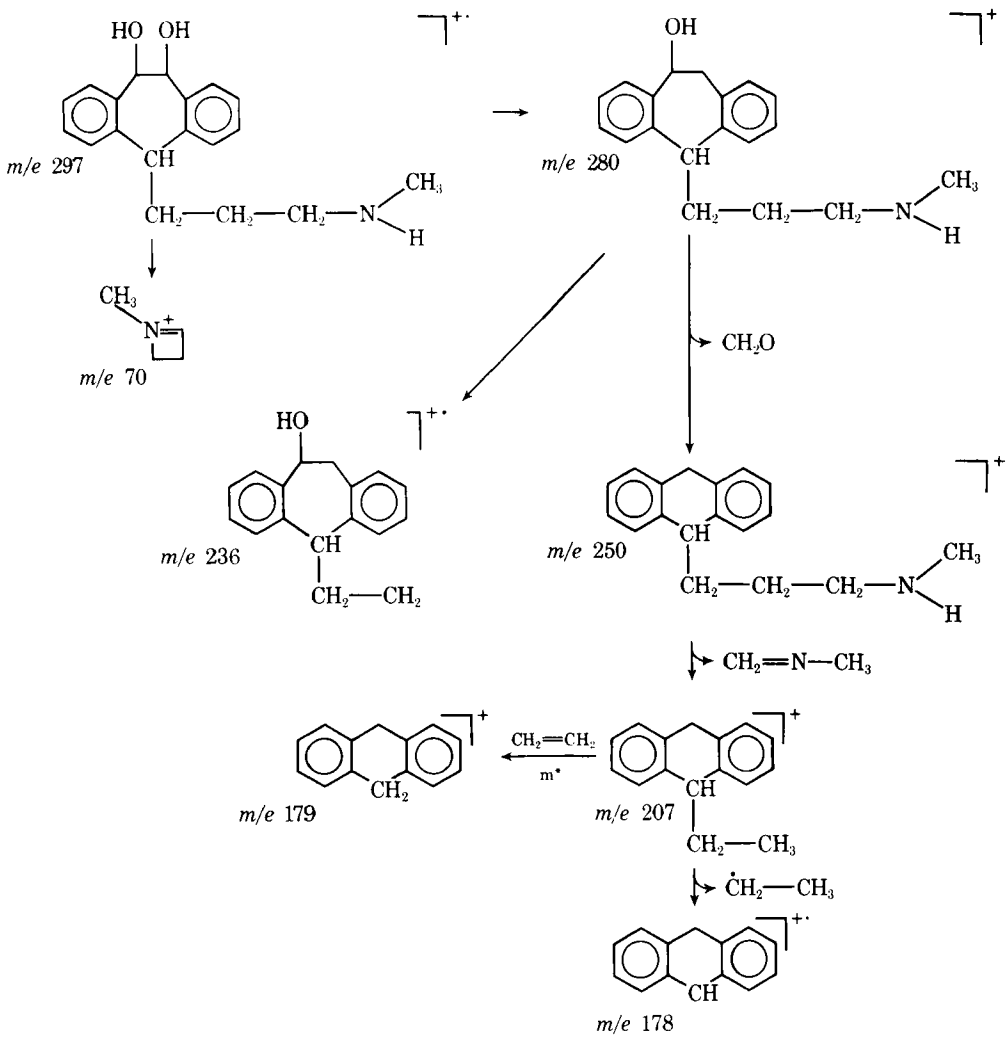
After isolation of II on TLC and treatment with 1 *N* HCl, it was possible to obtain a product with R_f 0.28. The spot was eluted with methanol. The material, analyzed by direct introduction into the ion source of the mass spectrometer, showed a spectrum corresponding to the 10,11-dihydrodiol (IV) of protriptyline (Fig. 4).

This spot was also analyzed by GLC–mass spectrometry after on-column reaction with *n*-butylboronic acid (Scheme II). A com-

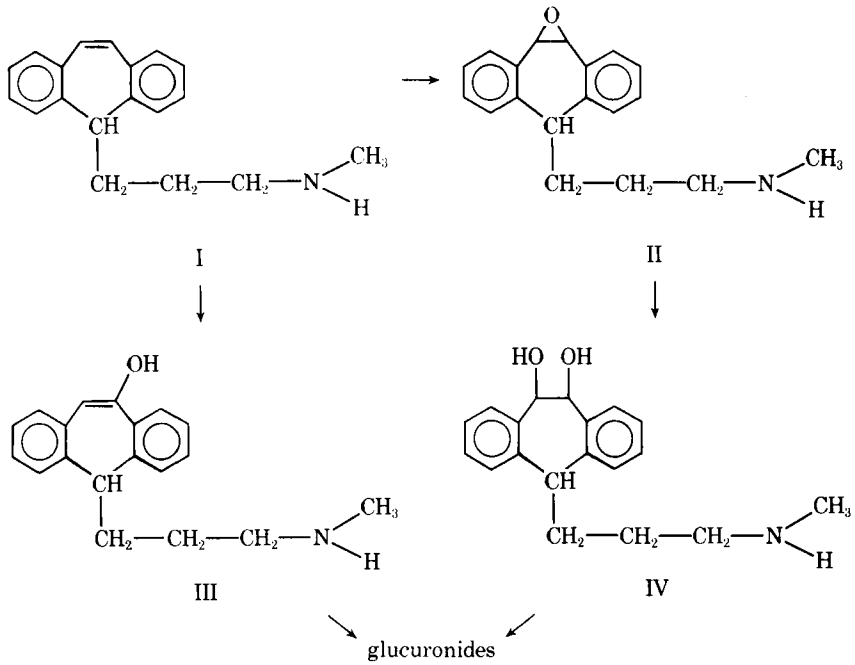


Scheme II—Reaction of the 10,11-dihydrodiol metabolite (obtained from the 10,11-epoxide of protriptyline) with n*-butylboronic acid.*

[6] LKB model 9000.



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



Scheme IV—Metabolic pathway of protriptyline (I).

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.

REFERENCES

(1) E. Boyland, *Biochem. Soc. Symp.*, **5**, 40(1950).

(2) A. Frigerio, R. Fanelli, P. Biandrate, G. Passerini, P. L. Morselli, and S. Garattini, *J. Pharm. Sci.*, **61**, 1144(1972).

(3) A. Frigerio, N. Sossi, G. Belvedere, C. Pantarotto, and S. Garattini, *ibid.*, **63**, 1536(1974).

(4) G. Belvedere, C. Pantarotto, V. Rovei, and A. Frigerio, *ibid.*, **65**, 815(1976).

(5) A. J. Ayd, Jr., *Lippincott's Med. Sci.*, **1968**, 54.

(6) S. F. Sisenwine, C. O. Tio, S. R. Shrader, and H. W. Ruelius, *J. Pharmacol. Exp. Ther.*, **175**, 51(1970).

(7) M. G. Horning, D. J. Harvey, E. C. Horning, and R. M. Hill, in "Proceedings of the International Symposium on Gas Chromatography–Mass Spectrometry," A. Frigerio, Ed., Isle of Elba, Italy, 1972, p. 403.

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

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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.