Identification and Evaluation of Amitriptyline and Its Basic Metabolites by Thin-Layer Chromatography in Rabbit Organs

R. MAFFEI FACINO and G. L. CORONA

Abstract □ The conditions for the identification and for a quantitative evaluation, in rabbit organs, of amitriptyline and its basic metabolites (10-hydroxyamitriptyline, 10,11-hydroxyamitriptyline, noramitriptyline, 10-hydroxynoramitriptyline, and 10,11-hydroxynoramitriptyline) are reported. After a suitable purification of organ extracts a chromatographic separation of amitriptyline and its metabolites is achieved by using thin-layer chromatography. Quantitative colorimetric analyses of metabolites are run.

Keyphrases □ Amitriptyline, basic metabolites—analysis, rabbit organs □ TLC—separation □ UV light—chromatographic spot visualization □ Colorimetric analysis—spectrophotometer

In a previous publication the identification and evaluation of amitriptyline and its basic metabolites in rabbit urine was reported (1).

This study describes a practical method for the purification of the organ extracts which allows the identification and determination of amitriptyline and its basic metabolites.

MATERIALS AND METHODS

Rabbit organs, pretreated orally with amitriptyline (15 mg./kg. body wt.), were homogenized with *N* 100 HCl (5 ml. acid/g. of tissue). The homogenate was divided in two portions.

One portion, consisting of one-tenth of the volume, was adjusted to pH 9.5–10 with 1 *N* sodium hydroxide and extracted with *n*-heptane. Unchanged amitriptyline present in the extract was evaluated by means of the methyl orange and bromocresol methods (1). The other portion consisting of nine-tenths of the volume was adjusted to pH 4.9 with acetic acid and buffered with 0.1 *M* sodium acetate (1); then incubated for 16 hr. at 37° with arylsulfatase and β-glucuronidase (100 I.U./g. each). The hydrolyzed material was then adjusted to pH 9.5–10 with 1 *N* NaOH and extracted with *n*-heptane.

One-half of this extract was used for the evaluation of the total amount of amitriptyline and its basic metabolites by means of the bromocresol, methyl-orange, and $CuSO_4$–CS_2 methods (I); the recoveries from the tissues, performed using standards of amitriptyline and noramitriptyline, were about 95% (Table I).

The other half, for TLC, was reduced to 1-ml. volume *in vacuo* at 40°.

Since fatty material present in the concentrated extract interfered with the chromatographic separation of amitriptyline and its metabolites, the fatty material was removed by the following method.

The heptane extract was shaken in a separator with a third of its volume of *N* 10 HCl for 20 min. After centrifugation and paper filtration of the aqueous layer, the heptane layer together with the filter was again extracted with *N* 10 HCl. The aqueous layers were combined, adjusted to pH 10, and extracted with three volumes of benzene. The benzene extract was then evaporated *in vacuo* at 35°, and the residue taken five times with 5-ml. portions of methyl alcohol. The alcoholic solution was concentrated to 1-ml. volume and used for chromatographic analysis.

Thin-layer Chromatography—Glass plates (10 × 20 cm.) were coated with a 250-μ thick layer of Silica Gel G containing 2.5% of a fluorescent green indicator.[1] They were oven-dried at 110° for 30 min. and subsequently cooled in closed containers and stored in a dust free atmosphere. Before use each plate was oven-activated at 110° for 30 min.

The solvent mixture was benzene–dioxane–32% ammonium hydroxide–water (62.5 : 35 : 0.5 : 2) and the time of run was 90 min. (2).

Amitriptyline and metabolites were detected by means of UV light (254 mμ). Amitriptyline and noramitriptyline were identified by means of the standards, while the identification of the other metabolites was achieved using the methods reported in the authors' previous work (1).

Investigations performed prolonging the time of run to 120 min. in chromatograms of some freshly extracted organs, allowed the detection of another spot, which was identified as the isomer of 10-hydroxyamitriptyline (Fig. 1).

The identification was carried out as follows: (*a*) the spot reacted positively with the tests for 10-hydroxyamitriptyline (1); (*b*) the

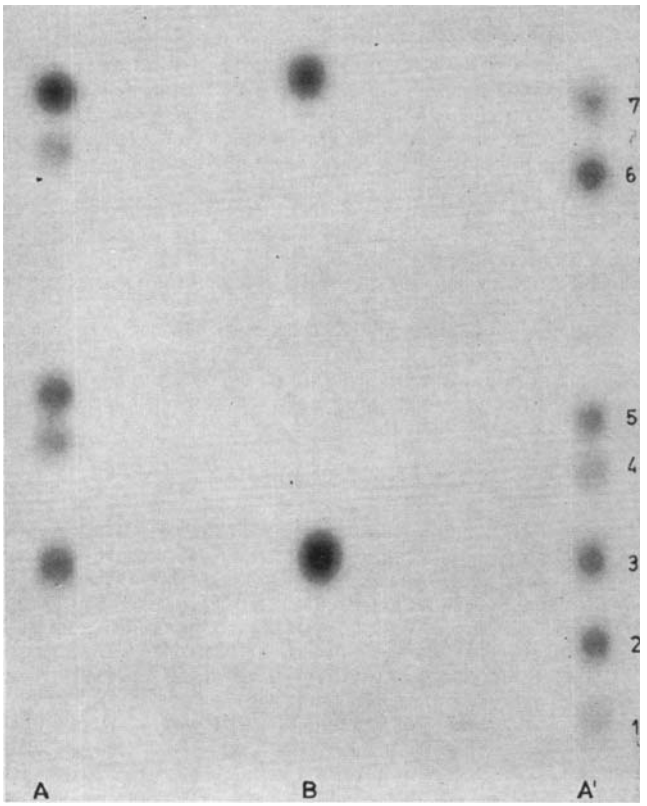

Figure 1—*Thin-layer chromatogram of brain samples on Silica Gel G. Solvent: benzene–dioxane–32% ammonium hydroxide–water (62.5 : 35 : 0, 5 : 2). Time of run: 120 min. Key: A, bulbus and pons; A', cerebellum; B, standards; 1, 10-hydroxynoramitriptyline; 2, 10,11-hydroxynoramitriptyline; 3, noramitriptyline; 4, 10-hydroxyamitriptyline; 5, 10-hydroxyamitriptyline isomer; 6, 10,11-hydroxyamitriptyline; 7, amitriptyline.*

[1] Wolme.

Table I—Levels of Amitriptyline and Metabolites in Brain and Liver of Rabbits

Compd., mcg/10 g.	Direct Evaluations of Heptane Extracts			Evaluations of Heptane Extracts after Chromatographic Separation		
	White Matter	Gray Matter	Liver	White Matter	Gray Matter	Liver
Amitriptyline	6.1	4.4	2.6	5.80	3.76	2.50
10,11-Hydroxyamitriptyline				—	—	0.45
10-Hydroxyamitriptyline isomer	3.3	3.5	3.6	0.58	0.45	1.35
10-Hydroxyamitriptyline				2.03	2.51	1.50
Noramitriptyline				2.91	2.68	1.08
10,11-Hydroxynoramitriptyline	3.6	2.5	1.2	0.48	0.26	0.07
10-Hydroxynoramitriptyline						
Total	13.0	10.4	7.4	11.80	9.66	6.95
Recoveries, %		95[a]		90.7[b]	95.7[b]	93.9[b]

[a] Percent of recovery of the drug directly added to the fresh tissue. [b] Percent of recovery calculated from the amount of amitriptyline and metabolites directly estimated on the heptane extracts.

substance present in the original extract disappeared progressively after some days; (*c*) the quantitative determination of both 10-hydroxyamitriptyline derivates showed a reciprocal transformation of one metabolite in the other.

These results demonstrate that the two metabolites must be isomers of each other and agree with the previous findings on 10-hydroxynoramitriptyline isomers of McMahon (3).

The chromatogram of two samples of rabbit's brain is shown in Fig. 1.

Quantitative Determination—The silica gel of the spots of amitriptyline and metabolites was scraped-off and extracted three times with 3 ml. of a mixture of *N* 10 HCl and methyl alcohol (1 : 2). Each extract was concentrated to 1 ml. *in vacuo*.

The quantitative determination of these eluates was carried out according to the methods previously described (4).

The recovery of amitriptyline and its metabolites was between 90 and 95% of the total amitriptyline and metabolites found in the heptane extracts before and after hydrolysis.

RESULTS AND DISCUSSION

The method described above shows that it was possible to evaluate by means of colorimetric methods the amount of unchanged amitriptyline present in the heptane extracts before hydrolysis, and after hydrolysis, together with amitriptyline, also the metabolites that were glucuron-conjugated (1).

It was also possible to perform, after a purification of organ extracts, an identification and a quantitative evaluation, by TLC, of amitriptyline and each of its metabolites. These possibilities are proved in Table I, in which examples of the quantitative evaluations of amitriptyline and metabolites in the brain and in the liver of rabbits, killed 4 hr. after the drug administration, are reported.

The good chromatographic separation shown in Fig. 1 and the almost quantitative recoveries obtained after elution and colorimetric evaluation of the spots, make this procedure very useful for the metabolism of amitriptyline in organs.

This method probably could be extended to the study of compounds whose chemical structure is strictly related to that of amitriptyline.

SUMMARY

A method for the identification and the quantitative evaluation of amitriptyline and its basic metabolites in rabbit organs is presented. By this method it is possible to perform a colorimetric evaluation of amitriptyline and of its basic metabolites directly in the organ extracts. After a suitable purification of these extracts, it is possible to achieve a chromatographic analysis for the identification and the quantitative evaluation of amitriptyline and each metabolite.

REFERENCES

(1) G. L. Corona and R. Maffei Facino, *Biochem. Pharmacol.*, **17**, 2045(1968).

(2) G. B. Cassano, S. E. Sjöstrand, and E. Hansson, *Psychopharmacologia*, **8**, 9(1965).

(3) R. E. McMahon, F. J. Marshall, H. W. Culp, and W. M. Miller, *Biochem. Pharmacol.*, **12**, 1207(1963).

(4) R. Maffei Facino and G. L. Corona, *Il Farmaco*, **23**, 366 (1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 4, 1968, from *Cattedia di Farmacologia & Farmacognosia, University of Pavia, Pavia, Italy.*

Accepted for publication December 2, 1968.