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IDENTIFICATION AND EVALUATION OF AMITRYPTILINE AND ITS BASIC METABOLITES IN RABBIT'S URINE

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Abstract—The metabolism of amitryptiline has been studied in rabbits. 10-hydroxy amitryptiline, 10,11-hydroxy amitryptiline, nor-amitryptiline, 10-hydroxy nor-amitryptiline and 10,11-hydroxy nor-amitryptiline were detected in rabbit's urine and studied by means of TLC. In addition, a quantitative determination of amitryptiline and some of its metabolites is reported.

IN ORDER to study the transformation and distribution of amitryptiline[1, 5] and its metabolites in rabbit's urine, some practical methods for the identification of these compounds were established.

MATERIALS AND METHODS

Extraction

The investigation was carried out on rabbits orally-treated with 65 mg/Kg of amitryptiline. Urine samples of two animals were taken for 96 hr, centrifuged and preserved by freezing. Then were reduced to 250 ml by evaporation *in vacuo* at 38° and recentrifuged.

The evaporated samples were adjusted to pH 4·9 with glacial acetic acid and buffered with 25 ml of M/10 sodium acetate, and subjected to enzymatic hydrolysis for 24 hr by adding β-glucuronidase (60 I.U./ml) and arylsulfatase (80 I.U./ml) every 8 hr.[3]

Then the samples were adjusted to pH 9·5–10 with 1 N NaOH and extracted three times with 500 ml of *n*-heptane by shaking for 15 min. The heptane extracts were then concentrated *in vacuo* to 4 ml and analyzed by means of chemical and chromatographic methods.

Thin-layer chromatography (TLC)

Glass plates (10 cm × 20 cm) were coated with a layer of Silicagel G, 250 μ thick and dried at 110° for 30 min (before use every plate was oven-activated at 110° for 30 min). The chromatographs were performed in suitable glass tanks with the chromatoplates inclined at 45°

The solvent was a mixture of chloroform, isopropyl-alcohol and 5 per cent NH_4OH in the volume proportions of 74·4:25:0·6. The normal run was 60 min; however we increased it to 90 min in order to achieve better spot-resolution.[6]

After development the chromatographs were oven-dried at 110° for 2 min and sprayed with Dragendorff's reagent,[7] amino group detector.

Results similar to those obtained by using enzymes, although qualitative, were achieved by means of basic hydrolysis. This was carried out by adding 3 g of solid NaOH for each 100 ml of concentrated urine and heating for 1 hr on a steam bath. The samples were adjusted to pH 9·5–10 with concentrated HCl and extracted by means of the method described above.

Identification of the spots

The following procedures were used to identify the six spots observed on the chromatographs treated with Dragendorff's reagent.

(A) *Investigation of amino groups*

(1) *Test for primary amines.* All spots eluted from a chromatograph with N/10 HCl, were assayed with paradimethylaminobenzaldehyde.[8] The test for primary amines was negative.

(2) *Test for secondary amines.* Equal amounts of heptane extract were applied as two separate bands on a Silicagel G chromatoplate: after chromatographic run, one half of the plate was sprayed with Dragendorff's reagent and the other with sodium nitroprusside,[9] reagent for secondary amines. In this way we identified three secondary amines in the 1st, 2nd and 3rd spots from the starting line.

(3) *Test for tertiary amines.* In another chromatograph the bands corresponding to the 4th, 5th and 6th spots on the unsprayed portion were scraped off, eluted from the Silicagel with N/10 HCl and tested with methyl-orange.[10] All bands were positive for tertiary amines.

In addition by means of the standards it was shown that the 3rd and 6th spots corresponded respectively to nor-amitryptiline and amitryptiline.

(B) *Investigation of hydroxyl groups*

To achieve better identification of the 1st, 2nd, 4th and 5th spots whose aminic groups were determined, we performed various analyses to confirm the presence, as reported by some authors[1, 5] of alcohols or glycols in 10 and 11 positions of the ethylene bridge of amitryptiline.

(1) *Alcoholic groups.* (a) The four spots reacted positively with oxidizing reagents such as phosphomolybdic acid[11] and benzidine-sodium metaperiodate.[12] The reaction was not ascribed to phenolic groups because the paranitroaniline diazo[13] and ferric chloride[14] tests were negative for the four spots. Also the absorption maxima at 238 mμ presented by the 1st, 2nd, 4th and 5th bands, did not shift in alkali showing that the hydroxyl groups were not phenolic.[15] (b) The tests for alcohols of Karyone–Hashimoto[16] and Lederer–Summerfield[17] were positive for the four substances.

In addition the eluates of the four spots collected were rechromatographed on Silicagel G plates with two solvent mixtures generally used for the separation of alcoholic compounds: *n*-butanol-H_2O-acetic acid (4:5:1) and *n*-butanol-H_2O (9:1). In this way spot-resolution was greatly improved.

(2) *Glycol groups.* (a) The four spots on one chromatograph were sprayed with a mixture of concentrated HCl and ethyl alcohol[18] and heated in an oven at 90°. The 2nd and 5th spots reacted positively with a pink color. (b) In addition, the 2nd and 5th spots eluted from a chromatograph with N/10 HCl, reacted positively with

periodic acid,[19] test for vicinal glycols. An ultra-violet spectrum of each of the four substances was taken first in N/10 HCl and then in concentrated HCl, after incubation at 100° for 15 min in order to determine the position of the alcohol and glycol groups on the ethylene bridge of amitryptiline.

It was observed that every spot eluted with N/10 HCl (1st, 2nd, 4th and 5th) presented an absorption maximum at 238 mμ, either amitryptiline or nor-amitryptiline.

In concentrated HCl however, the absorption spectra of the 1st and 4th were different from those of the 2nd and 5th. The 1st and 4th showed respectively absorption maxima at 225 mμ and 290 mμ and a minimum at 265 mμ, typical of 10,11-dihydro nor-amitryptiline and 10,11-dihydro amitryptiline,[20] ascribed to a probable dehydration of a secondary alcohol.

The 2nd and 5th showed absorption maxima at 255 mμ, whose presence could be ascribed to a benzoil group. It is highly probable that the maxima of the 1st and 4th substances correspond to 10-hydroxy nor-amitryptiline and 10-hydroxy amitryptiline respectively, while the maxima of the 2nd and 5th to 10,11-hydroxy nor-amitryptiline and 10,11-hydroxy amitryptiline.[21]

Quantitative determination

The following methods were employed for the quantitative evaluation of amitryptiline and some of its metabolites.*

(1) Bromocresol-purple method for the determination of primary, secondary and tertiary amines.[22]

In order to evaluate the total amount of amitryptiline and some of its metabolites, two calibration curves were made because different ratios of secondary and tertiary amines were present in the urine extracts.

In effect since it was determined that the ratio of secondary to tertiary amines varied from (3:10) to (1:1) and that the difference in the slope of the calibration curves for this concentration interval was not considerable, one of the calibration curves was made with a 30 per cent and the other with a 50 per cent, content of secondary amines on the total.

Reagents

(a) A 0·1% aqueous solution of bromocresol-purple (Merck) (the ethanol solution reported in the original method was substituted by an aqueous solution). 100 mg of bromocresol-purple were dissolved in 100 ml of hot water: the solution was allowed to cool and then washed five times with 50 ml of redistilled chloroform until the optical density at 410 mμ of the washing liquid was about 0·01.

(b) Buffer solution of M/10 sodium citrate in N/10 HCl (pH 3·5).

(c) Redistilled chloroform.

Amitryptiline and nor-amitryptiline were employed for the calibration curves reported in Fig. 1(a).

(2) Methyl-orange method for the quantitative determination of tertiary amines.[10]

(3) Copper sulfate-carbon disulfide method for the evaluation of secondary amines.[23]

Secondary amines reacted in basic solution with $CuSO_4$ and CS_2 giving a yellow or dark-brown coloured salt: the coloration extracted with benzene was proportional, according to Beer's law, from 1 to 8 μg to the secondary amine content.

* The evaluation of amitryptiline and each metabolites after chromatographic separation is reported in ref. 6.

Reagents

(a) 5% aqueous solution of copper sulfate.

(b) N/10 HCl.

(c) 10% NH_4OH.

Procedure. The secondary amine was dissolved in 0·5 ml of N/10 HCl and placed in a test tube furnished with a glass ground stopper. 0·5 ml of copper sulfate was

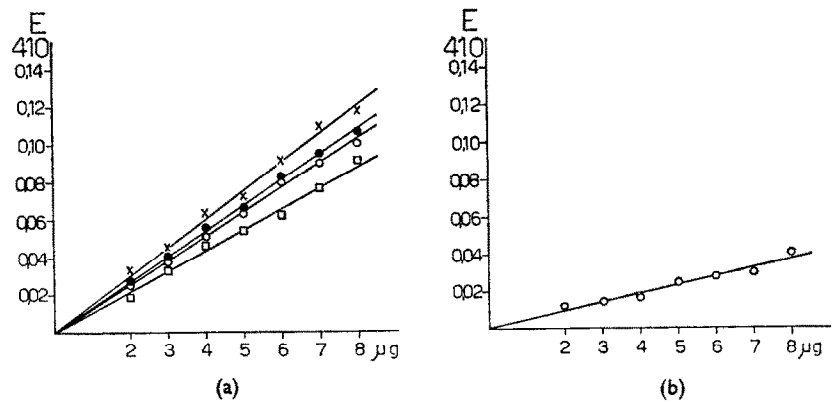

FIG. 1. Calibration curves. (a) Bromocresol-purple method: × tertiary amines; ● mixture (3:10) of secondary amines and tertiary amines; ○ mixture (1:1) of secondary amines and tertiary amines; □ secondary amines. (b) $CuSO_4$–CS_2 method: ○ secondary amines.

added to the solution and the mixture alkalinized with a few drops of 10% NH_4OH to the appearance of the color of copper-ammonium complex. Three ml of a benzene-carbon disulfide solution (3:1) were added, shaken for 3 min and centrifuged for 10 min. The organic extract was read at 410 mμ with a blank serving as control.

Nor-amitryptiline was employed for the calibration curve reported in Fig. 1(b).

RESULTS AND DISCUSSION

These methods were employed for the quantitative determination of amitryptiline and some of its metabolites in rabbit's urine before and after enzymatic hydrolysis.

Before hydrolysis. Three ml of urine were placed in a 50 ml glass-stoppered centrifuge tube, the pH adjusted to 9·5–10 with 1 N NaOH and extracted with 20 ml of *n*-heptane by shaking for 20 min.

Nine ml of the heptane extract was transferred to a vacuum flask containing 4 ml of buffer solution (pH 3·5) and evaporated *in vacuo* at 40°, the bromocresol-purple method was carried out on 2 ml of aqueous residue.

The other 9 ml was transferred to a glass-stoppered tube containing 6 ml of N/10 HCl for the methyl-organge method.

After hydrolysis. Three 30 ml samples of the total heptane extract were transferred to three vacuum flasks containing respectively 4 ml of buffer solution (pH 3·5), 6 ml of N/10 HCl and 1 ml of N/10 HCl.

The organic layers were evaporated and the aqueous residues tested with bromocresol, methyl-orange and $CuSO_4$-CS_2 method. The results are reported in Table 1.

We evaluated by native urines extraction the unchanged amitryptiline amount, while after hydrolysis together with unchanged amitryptiline, also the glucuron-conjugated metabolites.[24]

TABLE 1. VALUES OF AMITRYPTILINE AND SOME METABOLITES IN RABBIT'S URINE $\beta g/\omega l$

In the heptane extract	A			B		
	Methods			Methods		
	Bromo-cresol purple	$CuSO_4$-CS_2	Methyl-orange	Bromo-cresol purple	$CuSO_4$-CS_2	Methyl-orange
Before hydrolysis amitryptiline	9·90		9·33	21·66		21·56
After hydrolysis						
Secondary amines: nor-amitryptiline, 10-hydroxy nor-amitryptiline, 10,11-hydroxy nor-amitryptiline		6·93			46·13	
Tertiary amines: amitryptiline, 10-hydroxy amitryptiline, 10,11-hydroxy amitryptiline		22·93				46·66
Total amines	30·40	29·86		93·33	92·79	

We found remarkable differences in the amount of amitryptiline and metabolites excreted from several animals: by this reason the determinations of groups of metabolites together with amitryptiline, allowed us to give quantitative data about differences of amitryptiline metabolism.

In addition, preliminary investigations on urines of rats proved that amitryptiline in animals dosed i.m. with the same amount of drug, underwent, although qualitative, the same metabolic fate of rabbit. On the contrary, these results would be hardly checked by the evaluation in urine of only amitryptiline.

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