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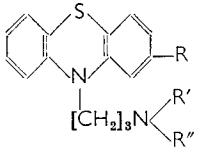
Hydroxylation *in vitro* of pharmacologically active phenothiazine derivatives

ANN E. ROBINSON AND VIDA H. BEAVEN

The hydroxylation of promazine, chlorpromazine, chlorproethazine, triflupromazine and promethazine by a rat liver preparation is described. It is concluded that promazine is converted to 3-hydroxypromazine by this system.

PREVIOUS studies have demonstrated that chlorpromazine (I, R = Cl, R′ = R″ = Me) and related phenothiazine derivatives are oxidised



(I)

to the sulphoxide and that the dialkylamino-group (if present) is dealkylated on incubation with certain liver homogenate systems (see reviews by Emmerson & Miya, 1963; Schenker & Herbst, 1963). The products resulting from the *in vivo* metabolism of these compounds indicate that hydroxylation of the phenothiazine moiety precedes the formation of a glucosiduronic acid derivative (Emmerson & Miya, 1963; Fishman & Goldenberg, 1963; Posner, Culpan & Levine, 1963; Schenker & Herbst, 1963; and references there cited). The present communication reports the *in vitro* hydroxylation of chlorpromazine and related compounds and demonstrates that promazine (I, R = H, R′ = R″ = Me) is probably hydroxylated in the 3-position.

Methods

The livers of adult male albino Wistar rats (200–300 g) were homogenised in the cold with two volumes of potassium chloride solution (1·15% w/v). The homogenate (2 ml) was incubated in a reaction mixture (total volume, 8 ml) which was modified from that described by Mueller & Miller (1953) by substituting glucose-6-phosphate for glucose-1,6-diphosphate and adding reduced nicotinamide adenine dinucleotide phosphate (100 μg) and the phenothiazine derivative (2·5 μmoles). The reaction vessels (50 ml conical flasks) were incubated at 37° with shaking for 1 hr. Trichloroacetic acid (to 5% w/v) was added to a volume of the incubation mixture, containing at least 5 μmoles of the phenothiazine derivative, and the precipitated protein was hydrolysed by heating with sodium hydroxide solution (30 ml of 3% w/v) on a boiling water-bath for 1 hr. The supernatant fluid (acid-soluble fraction) obtained after removal of the protein precipitate was made alkaline. This and the

From the School of Pharmacy, Chelsea College of Science and Technology, Manresa Road, London, S.W.3

hydrolysis mixture were separately extracted with washed diethyl ether, and the ether extracts were evaporated almost to dryness. The concentrated extracts were chromatographed on paper using the solvent system of Eisdorfer & Ellenbogen (1960) and on thin-layer plates prepared from Aluminium oxide G (Merck) using chloroform containing absolute ethanol (10%, by volume) as the solvent. The chromatograms were examined as described previously (Beckett, Beaven & Robinson, 1963). Paper chromatographic separation resolves mixtures of the phenothiazine compounds and their sulphoxide analogues, while the thin-layer method satisfactorily separates the parent compounds from their dealkylated analogues (see Table 1).

TABLE 1. RESULTS OF THIN-LAYER CHROMATOGRAPHY ON ALUMINA PLATES

Solvent: chloroform containing 10% v/v ethanol. The solvent moved 100 mm. All spots showed a blue fluorescence when irradiated with ultra-violet light

Compound	Distance from base line in mm*	50% v/v H_2SO_4	Reaction with $HCl/NaNO_2$
Chlorpromazine	87–92	pink	pink
Desmonomethylchlorpromazine	40–44	pink	pink
Desdimethylchlorpromazine	10–15	pink	pink
Promazine	90–95	orange-pink	pink
2-Hydroxypromazine	42–48	blue	pink
Extracts of chlorpromazine incubation mixture:			
acid-soluble fraction	85–90	pink	pink
	55–60	blue-purple	pink
	40–45	pink	pink
	7–10	pink	pink
Alkali treated fraction	85–90	pink	pink
	40–45	pink	pink

* The distances given are for the rear and advanced edges of the spots.

Spectroscopic measurements were made under optimum conditions using a Beckman DK2 recording spectrophotometer.

Results

CHLORPROMAZINE

Paper chromatography of the acid-soluble fraction showed the presence of desmonomethylchlorpromazine, (I, R = Cl, R′ = Me and R″ = H) and to a lesser extent desdimethylchlorpromazine (I, R = Cl and R′ = R″ = H) and unchanged chlorpromazine. Chlorpromazine sulphoxide was not detected. Thin-layer chromatograms confirmed the presence of desmonomethyl- and desdimethyl-chlorpromazine and chlorpromazine itself (Table 1). In addition, a fourth component which gave a purple colour with 50% v/v sulphuric acid was not present in sufficient quantity to permit a satisfactory test for the amine group. The ultra-violet absorption curves of the acid solutions of the extracts of the acid-soluble fraction showed peaks at 260, 290 and 340 mμ approximately; chlorpromazine sulphoxide (λ_{max} 237, 273, 298 and 340 mμ) was not present. Differential measurements using chlorpromazine solutions in the reference beam indicated the presence of oxidised and reduced nicotinamide adenine dinucleotide phosphate in the extracts. After treating the acid-soluble fraction extract with 50% v/v sulphuric acid,

a purple colour (λ_{max} 555–60 mμ) was obtained. In contrast, chlorpromazine treated similarly gives a red colour (λ_{max} 525–7 mμ). The alteration in the position of the absorption peak was considered to result from aromatic hydroxylation of the compound by the liver homogenate. Since this could not be confirmed because the appropriate reference compounds were not available, the *in vitro* hydroxylation of promazine was studied. The extracts of the alkaline hydrolysate gave two spots on thin-layer chromatograms which corresponded with chlorpromazine (which predominated) and desmonomethylchlorpromazine. The ultraviolet absorption curve in dilute acid solution and the visible absorption curve after sulphuric acid treatment were similar to those for chlorpromazine. This fraction was normally devoid of the hydroxylated component.

PROMAZINE (I, R = H, R′ = R″ = Me)

Thin-layer chromatograms of the extracts of the acid-soluble fraction from incubation mixtures containing promazine yielded at least two phenothiazine spots; of these, one corresponded with unchanged promazine and the other moved approximately the same distance as 2-hydroxypromazine and gave a purple spot with the sulphuric acid reagent (promazine gives a pink-orange colour). The demethylated analogues were not available, but, by analogy with chlorpromazine and by comparison with 2-hydroxypromazine, the hydroxylated compound probably retains the tertiary amine group. The ultra-violet absorption curves of the dilute acid solutions of the extracts again indicated the presence of cofactors in the acid-soluble fraction and the absence of sulphoxide in either fraction. After treatment of the acid-soluble fraction extract with sulphuric acid, the main absorption peak was at 555 mμ with minor peaks at 345 and 375 mμ whereas promazine gives a peak at 510 mμ under these conditions. In some instances, when the extract contained an appreciable proportion of "unchanged" promazine the visible absorption peaks were not well-defined, however, differential spectrophotometry, using sulphuric acid-treated promazine solutions in the reference cuvette, clearly confirmed the presence of the hydroxylated component. The latter possessed a main absorption peak at 555 mμ, minor peaks at 345 and 375 mμ and a shoulder at 450–470 mμ. This absorption spectrum is similar to those of analogous compounds in ethanolic sulphuric acid, viz 3-methoxypromazine (342, 372, 565 mμ), 3-hydroxyphenothiazine (342, 369 (443), 549 mμ) and differs from that of 2-hydroxypromazine (343, 558 mμ) by the presence of the additional peak at 375 mμ (Beckett & Curry, 1963). There was no similarity with the spectra of 1-hydroxypromazine or 4-hydroxypromazine. The presence of ethanol in the sulphuric acid does not alter the positions of the absorption peaks significantly.

OTHER COMPOUNDS

The relevant absorption data for chlorproethazine (I, R = Cl, R′ = R″ = Et) triflupromazine (I, R = CF_3, R′ = R″ = Me) and

promethazine (10-(2-dimethylamino-2-methyl ethyl) phenothiazine) and their metabolites are given in Table 2.

TABLE 2. VISIBLE ABSORPTION PEAKS OF PHENOTHIAZINE DERIVATIVES IN 50% v/v SULPHURIC ACID BEFORE INCUBATION AND AFTER RECOVERY FROM THE ACID-SOLUBLE FRACTION OF THE INCUBATION MIXTURE

Compound	λ_{max} in mμ	
	Before incubation	Recovered compound(s)
Promazine	510	345, 375, 555
Chlorpromazine	525–8	555–60
Chlorproethazine	525	565
Triflupromazine	498	533
Promethazine	514	564

Discussion

The lack of suitable analytical methods for the detection of hydroxyl groups attached to the phenothiazine ring sytem has made it difficult to determine the biological fate of these compounds in animals and in isolated tissue systems. However, the 30–40 mμ bathochromic shift in the absorption peak obtained on treatment with sulphuric acid affords a distinction between the parent compounds and their hydroxylated analogues (Beckett & Curry, 1963). Incubation of promazine, chlorpromazine, chlorproethazine, triflupromazine and promethazine in the liver homogenate system yielded metabolites which, in 50% v/v sulphuric acid, showed similar shifts in the locations of the absorption peaks when compared with those of the starting compounds. It is probable therefore, that all these drugs are hydroxylated *in vitro*.

In addition, experiments with thin-layer chromatography also indicate that promazine is hydroxylated, since one of the promazine metabolites moved approximately the same distance as 2-hydroxypromazine. We believe that promazine is hydroxylated in the 3-position because this metabolite yields an absorption peak at 375 mμ in 50% v/v sulphuric acid, which is not found with either 1-, 2- or 4-hydroxypromazine.

The hydroxylated chlorpromazine metabolite was detected on thin-layer chromatograms, but lack of reference compounds hindered adequate identification of the product. The most likely sites for biochemical hydroxylation appear to be the 3- and 7-positions.

The *in vitro* hydroxylation of chlorpromazine is in accord with the finding that these drugs are excreted in human urine as *O*-glucuronides.

A large proportion of the phenothiazine-positive material remains bound to the liver tissue after incubation, but could be recovered after alkaline hydrolysis. The hydroxylated products were found in the acid-soluble fraction (before hydrolysis) or in the supernatant solution after centrifuging the incubation mixture at 140,000 rcf for 30 min. If hydroxylation is effected by enzymes associated with liver microsomes, then release of the hydroxylated compound into the medium after the reaction is in accord with the more polar nature of the product.

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