Biochemical Pharmacology, Vol. 18, pp. 1235–1238. Pergamon Press. 1969. Printed in Great Britain

SHORT COMMUNICATIONS

The action of catechol-*O*-methyltransferase on 7,8-dihydroxychlorpromazine—Formation of 7-hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine

(*Received* 19 *August* 1968; *accepted* 4 *October* 1968)

DURING the course of investigations on possible hydroxylated metabolites of chlorpromazine,[1–4] the action of microsomal enzymes on various monohydroxylated chlorpromazines was studied.[5] *N*-demethylation was the major metabolism, but *ortho*-dihydroxychlorpromazines were also formed and were detected through the use of catechol-*O*-methyltransferase and S-adenosylmethionine-methyl-^{14}C.[6] 7-Hydroxychlorpromazine, a major hydroxylated metabolite of chlorpromazine in humans,[7–9] on incubation with rabbit liver microsomes formed trace amounts of an *O*-methylated metabolite. On the basis of chromatographic evidence, this metabolite was proposed to be a 7,8-hydroxymethoxychlorpromazine.[5] In this paper we wish to present evidence that this metabolite *in vitro* of 7-hydroxychlorpromazine is a mixture of 7-hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine.

The chlorpromazine derivatives used in this study were synthesized for the Psychopharmacology Research Branch, National Institute of Mental Health, by the Research Institute of Temple University (under Contract No. SA-43-ph-3748) and by the Regis Chemical Co. (under Contract No. SA-43-ph-3021). 7,8-Dimethylmethylenedioxychlorpromazine was converted to 7,8-dihydroxychlorpromazine with 2 N HCl for 1 hr at 70° in a sealed tube under nitrogen.[5] The mixture was then lyophilized and the product dissolved in ethanol. 7,8-Dibenzyloxychlorpromazine could also be converted to 7,8-dihydroxychlorpromazine by reduction with hydrogen at 45 psi in ethanol solution with 10% palladium on charcoal catalyst. Reduction was approximately 80 per cent complete in 20 hr. The solution was then filtered and concentrated to dryness *in vacuo* and the product redissolved in ethanol. The 7,8-dihydroxychlorpromazine obtained by either of these alternate methods gave the same results on enzymatic methylation but some loss of chlorine during hydrogenolysis is possible.

Rabbit liver microsomes were prepared as previously described.[5] Microsomal preparations corresponding to 1 g liver were incubated at 37° for 15 min in a mixture containing the following components (micromoles): substrate, 2·0; phosphate buffer, pH 7·8, 1000; magnesium chloride, 10; NADP, 3·0; glucose 6-phosphate, 15; S-adenosylmethionine-methyl-^{14}C (180,000 cpm), 5; and glucose 6-phosphate dehydrogenase (5 units) in a final volume of 6 ml. The incubations with 7-hydroxychlorpromazine, 8-hydroxychlorpromazine or 7,8-dihydroxychlorpromazine contained 7-hydroxy-8-methoxychlorpromazine, 0·1 and 8-hydroxy-7-methoxychlorpromazine, 0·1.

Rat liver supernatant fraction (100,000 *g*, 1 hr), 5 ml corresponding to 1·5 g liver, was incubated for 30 min at 37° in a mixture with the following components (micromoles): 7,8-dihydroxychlorpromazine, 5·0; phosphate buffer, pH 7·8, 1000; magnesium chloride, 10; and S-adenosylmethionine-methyl-^{14}C (180,000 cpm), 10, in a final volume of 10 ml.

Incubations were terminated with 5 ml of 0·5 M borate buffer, pH 10·0, and extracted with 2 vol. of ethyl acetate. The extract was dried over sodium sulfate, concentrated *in vacuo* and aliquots were chromatographed in three thin-layer chromatographic systems with appropriate reference compounds. The thin-layer chromatoplates (silica gel-GF, Analtech, Inc.) were scanned for radioactivity in a Vanguard thin-layer chromatoplate scanner. Color development of phenothiazines was with 50% sulfuric acid–ethanol (1:1).

In Table 1 are given R_f values for reference compounds and the major radioactive peaks obtained when 7-hydroxychlorpromazine, 8-hydroxychlorpromazine or 7,8-dihydroxychlorpromazine was incubated with liver microsomes under the conditions described. It was found necessary to add carrier 7-hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine in order to prevent oxidation of the trace amounts of *O*-methylated metabolites during purification and

TABLE 1. R_f VALUES AND COLOR REACTIONS OF HYDROXYCHLORPROMAZINES

Compound	Color with 50% H_2SO_4–ethanol (1:1)	R_f value*		
		Solvent 1	Solvent 2	Solvent 3
7-Hydroxychlorpromazine	purple	0·73	0·80	0·35
8-Hydroxychlorpromazine	blue–green	0·69	0·68	0·45
7,8-Dihydroxychlorpromazine	red–purple	decomp.	decomp.	decomp.
7-Hydroxy-8-methoxychlorpromazine	blue–purple	0·51	0·70	0·25
7-Hydroxy-8-methoxynorchlorpromazine	blue–purple	0·35	0·41	0·25
8-Hydroxy-7-methoxychlorpromazine	blue–purple	0·46	0·59	0·28
(^{14}C)-methoxyhydroxychlorpromazines				
From 7,8-dihydroxychlorpromazine with microsomes		0·48†	0·70, 0·57‡	0·26†
From 7-hydroxychlorpromazine with microsomes		0·47†	0·70, 0·59‡	0·27†
From 8-hydroxychlorpromazine with microsomes		0·48†	0·70, 0·57‡	0·27†

* Thin-layer chromatography (SiO_2). Solvent 1: acetone–isopropanol–1% ammonium hydroxide (9:7:4); solvent 2: ethyl acetate–acetone–methanol–diethylamine (68:2:20:15); solvent 3: benzene–ethanol–methanol–triethanolamine (70:20:10:0:25). R_f values previously reported[5] for (^{14}C) methoxychlorpromazines in solvents 2 and 3 represent oxidation products.

† Broad peak indistinguishable from a mixture of 7-hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine.

‡ Two peaks corresponding to 7-hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine.

chromatography in solvents 2 and 3. When the carrier was not added, oxidation to compounds exhibiting low R_f values occurred, especially with solvents 2 and 3.

With 7-hydroxychlorpromazine and 8-hydroxychlorpromazine, *N*-demethylation was a major metabolic pathway, as reported previously.[5] 7-Hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine on incubation with liver microsomes formed metabolites which were judged to be the corresponding desmethyl derivatives based on color reactions and R_f values. In addition, radioactive metabolites were formed from both 7-hydroxychlorpromazine and 8-hydroxychlorpromazine on incubation with liver microsomes and S-adenosylmethionine-methyl-^{14}C (Table 1 and Fig. 1). The same major products were obtained when 7,8-dihydroxychlorpromazine was incubated with S-adenosylmethionine-methyl-^{14}C and the catechol-*O*-methyl transferase contained in rabbit liver microsomes (Table 1) or the soluble catechol-*O*-methyltransferase from rat liver (Fig. 1). Incubation of the 7,8-dihydroxychlorpromazine with the soluble catechol-*O*-methyltransferase from rat liver and S-adenosylmethionine allowed positive identification (R_f values and color reactions) of the products as 7-hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine. These *O*-methylated compounds formed in a ratio of approximately 7:3, as judged from visual estimation and confirmed by integration of the area of the respective radioactive peaks. No carrier methoxyhydroxychlorpromazines were added in this case.

The metabolism of chlorpromazine *in vitro* with liver microsomes has been shown to be predominately by *N*-demethylation,[5,10–12] while *N*-oxidation, sulfoxidation and ring hydroxylation in positions 3 and 7 represent minor pathways.[12] Ring hydroxylated phenothiazines such as 7-hydroxychlorpromazine undergo further hydroxylation to *ortho*-dihydroxyphenothiazines,[5] which have been demonstrated by using an assay based on the selective methylation of such compounds with S-adenosylmethionine-methyl-^{14}C and catechol-*O*-methyltransferase.[6] Since 7-hydroxylation is a significant pathway for chlorpromazine metabolism in man[7–9] and 8-hydroxychlorpromazine has been suggested as a metabolite of chlorpromazine,[13] the tentative identification of a 7,8-hydroxymethoxychlorpromazine as a metabolite *in vitro* of 7-hydroxymethoxychlorpromazine and 8-hydroxychlorpromazine[5] was of some interest. These preliminary findings have now been confirmed. It has been shown by comparison with authentic compounds that 7,8-dihydroxychlorpromazine forms mainly 7-hydroxy-8-methoxychlorpromazine and lesser amounts of 8-hydroxy-7-methoxychlorpromazine on incubation with catechol-*O*-methyltransferase and S-adenosylmethionine.

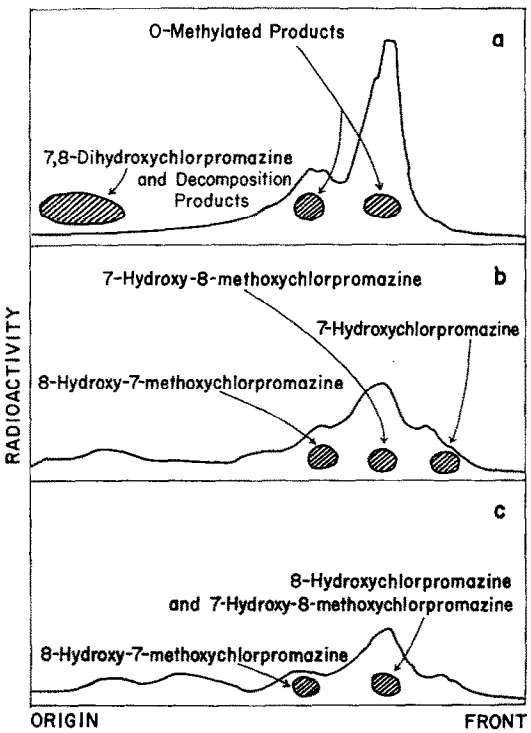

FIG. 1. Thin-layer chromatoplate (solvent system 2, see Table 1) of [^{14}C]-methoxyhydroxychlorpromazines. (a) 7,8-Dihydroxychlorpromazine incubated with soluble catechol-*O*-methyltransferase from rat liver. No carrier methoxyhydroxychlorpromazines were added. (b) 7-Hydroxychlorpromazine incubated with rabbit liver microsomes. 7-Hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine were added as reference compounds. (c) 8-Hydroxychlorpromazine incubated with rabbit liver microsomes. 7-Hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine were added as reference compounds. The thin-layer chromatoplates were scanned for radioactivity in a Vanguard thin-layer chromatoplate scanner. Scaler settings were full scale 10,000 for (a) and 1000 for (b) and (c). Similar chromatograms were obtained in two other incubations. No radioactive peaks were detected in (b) and (c) when cofactors necessary for oxidation were omitted The methylated products from 7,8-dihydroxychlorpromazine (a) showed the same color reactions with sulfuric acid (blue turning slowly purple) and the same R_f values as 7-hydroxy-8-methoxychlorpromazine and 8-hydroxy-7-methoxychlorpromazine.

Other *ortho*-dihydroxy compounds have been reported to give mixtures of *O*-methylated isomers on incubation with catechol-*O*-methyltransferase.[14–19] By using the method of Kuehl *et al.*,[19] it has been found that catechol-*O*-methyltransferase from liver microsomes or soluble supernatant of rabbit, mouse, guinea pig or rat gives approximately the same ratio of isomers (7 parts of 4-hydroxy-3-methoxyphenethylamine and 1 part of 3-hydroxy-4-methoxyphenethylamine) on methylation of dopamine.* Therefore, the finding that the soluble rat liver catechol-*O*-methyltransferase and the microsomal rabbit liver catechol-*O*-methyltransferase both give the same mixture of *O*-methylated products from 7,8-dihydroxychlorpromazine is not surprising.

7-Hydroxychlorpromazine or 8-hydroxychlorpromazine gave at least two radioactive products when incubated with liver microsomes, cofactors and S-adenosylmethionine-methyl-^{14}C. No radio-

* J. DALY, unpublished results.

active products were detected when the cofactors for microsomal oxidation were omitted.[5] The principal product cochromatographed with 7-hydroxy-8-methoxychlorpromazine. The lesser product appeared to be 8-hydroxy-7-methoxychlorpromazine. These (^{14}C)-methoxyhydroxychlorpromazines were found to undergo oxidation in solvents 2 or 3 (Table 1) unless carrier amounts of standard compounds were added. The results previously reported[5] for (^{14}C)-methoxyhydroxychlorpromazines for solvents 2 and 3 are, therefore, incorrect and represent R_f values of decomposition products. In the case of 7-hydroxychlorpromazine and 8-hydroxychlorpromazine, the minor peaks mentioned in the previous publication[5] do in fact correspond rather well with 7-hydroxy-8-methoxychlorpromazine and its 8-hydroxy-7-methoxy-isomer.

In conclusion, a new but perhaps minor pathway of chlorpromazine metabolism has been confirmed. This pathway leads from either 7-hydroxy-chlorpromazine or 8-hydroxychlorpromazine via the extremely labile 7,8-dihydroxychlorpromazine to a mixture of *O*-methylated compounds consisting of 7-hydroxy-8-methoxychlorpromazine and lesser amounts of 8-hydroxy-7-methoxychlorpromazine. Further investigations will be needed to determine whether this represents a significant pathway *in vivo* in humans and whether such a pathway might be involved in abnormal 7-hydroxychlorpromazine metabolism and hyperpigmentation that is occasionally observed with patients on long-term chlorpromazine therapy.[20]

National Institute of Arthritis and Metabolic Diseases,
National Institutes of Health,
Bethesda, Md. 20014, *U.S.A.*

JOHN W. DALY

and

National Institute of Mental Health,
Health Sciences and Mental Health Administration,
Chevy Chase, Md. 20203, *U.S.A.*

ALBERT A. MANIAN

REFERENCES

1. A. A. MANIAN, D. H. EFRON and M. E. GOLDBERG, *Life Sci.* **4**, 2425 (1965)
2. E. A. NODIFF, S. INA, N. ODA, T. HAYAZAKI, S. NISHIBE, T. KOHNO, M. HAUSMAN and A. A. MANIAN, *J. heterocyclic Chem.* **4**, 239 (1967).
3. H. M. GROTTA, T. F. PAGE, JR., C. J. RIGGLE and A. A. MANIAN, *J. heterocyclic Chem.* **4**, 611 (1967).
4. E. A. NODIFF, N. ODA, T. HAYAZAKI, S. INA, T. ITO, S. NISHIBE, T. UEDA, K. SUZUKI, M. HAUSMAN and A. A. MANIAN, *J. heterocyclic Chem.* **5**, 165 (1968).
5. J. W. DALY and A. A. MANIAN, *Biochem. Pharmac.* **16**, 2131 (1967).
6. J. AXELROD, J. K. INSCOE and J. DALY, *J. Pharmac. exp. Ther.* **149**, 16 (1965).
7. V. FISHMAN and H. GOLDENBERG, *Proc. Soc. exp. Biol. Med.* **112**, 501 (1963).
8. H. GOLDENBERG and V. FISHMAN, *Biochem. Biophys. Res. Commun.* **14**, 404 (1965).
9. V. FISHMAN and H. GOLDENBERG, *J. Pharmac. exp. Ther.* **150**, 122 (1965).
10. A. E. ROBINSON and V. H. BEAVEN, *J. Pharm. Pharmac.* **16**, 342 (1964).
11. A. E. ROBINSON, *J. Pharm. Pharmac.* **18**, 19 (1966).
12. P. F. COCCIA and W. W. WESTERFELD, *J. Pharmac. exp. Ther.* **157**, 446 (1966).
13. P. TURANO, C. CANTON, W. J. TURNER and S. MERLIS, *Agressologie* **9**, series 2, 193 (1968).
14. S. SENOH, J. DALY, J. AXELROD and B. WITKOP, *J. Am. chem. Soc.* **81**, 6240 (1959).
15. J. W. DALY, J. AXELROD and B. WITKOP, *J. biol. Chem.* **235**, 1155 (1960).
16. R. KNUPPEN and H. BREUER, *Hoppe-Seyler's Z. physiol. Chem.* **346**, 114 (1966).
17. J. FISHMAN, M. MIYAZAKI and I. YOSHIZAWA, *J. Am. chem. Soc.* **89**, 7147 (1967).
18. J. AXELROD and A. B. LERNER, *Biochim. biophys. Acta* **71**, 650 (1963).
19. F. A. KUEHL, JR., M. HICHENS, R. E. ORMOND, M. A. P. MEISINGER, P. H. GALE, V. J. CIRILLO and N. G. BRINK, *Nature, Lond.* **203**, 154 (1964).
20. A. C. GREINER and K. BERRY, *Can. med. Ass. J.* **90**, 663 (1964).