Identification of 7-Hydroxyfluphenazine as Major Metabolite of Fluphenazine-^{14}C in the Dog

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Abstract □ The major metabolite of fluphenazine-^{14}C {4-[1-^{14}C-3-[2-(trifluoromethyl)phenothiazin-10-yl]propyl]-1-piperazineethanol dihydrochloride} in dogs and rhesus monkeys is 7-hydroxyfluphenazine. This metabolite was isolated from the feces of dogs by a combination of solvent extraction, column chromatography, and preparative TLC. Mass spectral data and 220-MHz. NMR spectroscopy indicated that the metabolite was hydroxylated on either the C-7 or C-8 position of the phenothiazine ring system. Definitive identification of the metabolite as 7-hydroxyfluphenazine was provided by a comparison of the spectral and chromatographic properties of the metabolite with authentic samples of 7- and 8-hydroxyfluphenazine.

Keyphrases □ Fluphenazine-^{14}C metabolite—identification □ 7-Hydroxyfluphenazine—fluphenazine metabolite □ Fecal excretion—7-hydroxyfluphenazine □ Chromatography, column, thin-layer—isolation □ Mass spectroscopy—identification □ NMR spectroscopy—identification

A companion publication (1) reported the metabolic disposition of fluphenazine-^{14}C in the dog and rhesus monkey. That study pointed out that a major metabolite, designated "Metabolite C," was excreted in the feces of both species but was present in up to 73% of the dose in the feces of dogs. In addition, Metabolite C was present as the glucuronide conjugate in the bile of dogs but was found unconjugated in the feces.

This paper describes the isolation of Metabolite C from the feces of dogs and its unequivocal identification, with the aid of 220-MHz. NMR spectroscopy, as 7-hydroxyfluphenazine.

MATERIALS AND METHODS

Isolation of Metabolite C from Dog Feces—Dogs 143 and 144 (see *Reference 1*, Table II) were given 10 mg./kg. of fluphenazine-^{14}C orally. An average of 72% of the dose was excreted in the feces on the 4th day after dosing. These fecal samples were homogenized in 2–3 volumes of methanol; the homogenates were pooled and centrifuged, and the supernatant fluid (212 ml.) was recovered and saved. The remaining fecal residue was extracted with 212 ml. of chloroform–methanol (1:1) by shaking for 30 min. followed by centrifugation. The supernatant fluid was recovered and saved. The remaining residue was extracted a second time with 212 ml. of chloroform–methanol (1:1). The extracts were combined and evaporated at 30–40° under reduced pressure to about 100 ml. This procedure extracted 94% of the radioactivity present in the pooled fecal homogenate.

The concentrated extract was acidified to a pH of 1–2 with 6 *N* HCl. Enough alumina, grade 1 (Woelm), which had been previously washed with chloroform–methanol (5:1), was added to the concentrated extract to form a thick paste, which was dried overnight *in vacuo* at 37°. The dried sample was placed on top of a column of dry alumina, grade III (Woelm), 10 cm. high by 5 cm. wide, prepared, and eluted with chloroform–methanol (5:1) according to the method of Loev and Snader (2). Six fractions of 50 ml. each were collected. Fractions 2–4 were evaporated to about 8 ml., spotted on activated silica gel PF chromatograms (Brinkmann), 1 mm. thick, and developed in chloroform–100% ethanol–ammonia (80:10:1). The silica gel containing a UV-positive zone, migrating with an R_f of 0.25, was removed from the plate, packed into a small column, and eluted with chloroform–methanol (5:1). The eluate from the column was pooled and evaporated to about 3 ml.

To remove a number of fluorescent impurities, the sample was spotted on preparative thin-layer chromatograms coated with silica gel PF, 1 mm. thick, and developed in benzene–ammonia–dioxane (10:10:80). The silica gel containing a UV-positive zone, moving with an R_f of 0.4, was removed, packed into a short column, and eluted with chloroform–methanol (5:1). The concentrated eluate contained about 18 mg. of final product as the free base. This isolated metabolite, designated Metabolite C, was chromatographed in chloroform–100% ethanol–ammonia (80:10:1) and had a radiochemical purity of 93%. The presence of a radioactive component, representing 6% of the radioactivity applied to the plate, was also observed.

To determine whether the procedure described for the isolation of Metabolite C from the feces resulted in any degradation of the compound, authentic fluphenazine-^{14}C dihydrochloride or 7-hydroxyfluphenazine dihydrochloride was added to samples of fecal homogenates, obtained from dogs before dosing. The samples were extracted and then chromatographed, first on the alumina column and, subsequently, on the two silica gel chromatograms as described previously. This procedure for fluphenazine-^{14}C yielded a single peak, representing 98.5% of the radioactivity applied on the second silica gel plate and corresponded to the R_f value of authentic fluphenazine. Similarly, for 7-hydroxyfluphenazine, only a single UV-positive spot was found that corresponded to the R_f value of authentic 7-hydroxyfluphenazine in the two solvent systems used to develop the silica gel-coated plates. These results indicate that neither fluphenazine-^{14}C nor 7-hydroxyfluphenazine was degraded by the procedure used to isolate Metabolite C.

Identification of Metabolite C—NMR spectra were obtained on either a Varian Associates model A60 or HR220 spectrometer. Mass spectra were obtained on an Associated Electrical Industries

Table I—High-Resolution Mass Spectral Data for Metabolite C and Fluphenazine

Metabolite C		Fluphenazine	
Observed Mass	Formula[a]	Observed Mass	Formula[b]
453.1663	$C_{22}H_{26}N_3O_2SF_3$	437.1753	$C_{22}H_{26}N_3OSF_3$
435.1570	$C_{22}H_{24}N_3OSF_3$	419.1637	$C_{22}H_{24}N_3SF_3$
422.1492	$C_{21}H_{23}N_3OSF_3$	406.1550	$C_{21}H_{23}N_3SF_3$
409.1424	$C_{20}H_{22}N_3OSF_3$	393.1481	$C_{20}H_{22}N_3SF_3$
366.1025	$C_{18}H_{17}N_2OSF_3$	350.1053	$C_{18}H_{17}N_2SF_3$
352.0842	$C_{17}H_{15}N_2OSF_3$	336.0909	$C_{17}H_{15}N_2SF_3$
323.0587	$C_{16}H_{12}NOSF_3$	307.0632	$C_{16}H_{12}NSF_3$
322.0512	$C_{16}H_{11}NOSF_3$	306.0562	$C_{16}H_{11}NSF_3$
309.0443	$C_{15}H_{10}NOSF_3$	293.0486	$C_{15}H_{10}NSF_3$
296.0359	$C_{14}H_9NOSF_3$	280.0407	$C_{14}H_9NSF_3$
283.0268	$C_{13}H_8NOSF_3$	267.0332	$C_{13}H_8NSF_3$
282.0208	$C_{13}H_7NOSF_3$	266.0256	$C_{13}H_7NSF_3$
264.0640	$C_{14}H_9NOF_3$	248.0686	$C_{14}H_9NF_3$
171.1492	$C_9H_{19}N_2O$	171.1497	$C_9H_{19}N_2O$
157.1332	$C_8H_{17}N_2O$	157.1335	$C_8H_{17}N_2O$
143.1179	$C_7H_{15}N_2O$	143.1184	$C_7H_{15}N_2O$
125.1075	$C_7H_{13}N_2$	125.1070	$C_7H_{13}N_2$
113.1077	$C_6H_{13}N_2$	113.1084	$C_6H_{13}N_2$
100.0762	$C_5H_{10}NO$	100.0760	$C_5H_{10}NO$
98.0960	$C_6H_{12}N$	98.0958	$C_6H_{12}N$
98.0844	$C_5H_{10}N_2$	98.0843	$C_5H_{10}N_2$
84[c]	$C_5H_{10}N$	84.0810	$C_5H_{10}N$
70[c]	C_4H_8N	70.0653	C_4H_8N

[a] Calculated masses agreed within 2.3 millimass units of found masses, except for the M^+, which deviated by 3.6 millimass units. [b] Calculated masses agreed within 1.6 millimass units of found values. [c] Exact mass not measured.

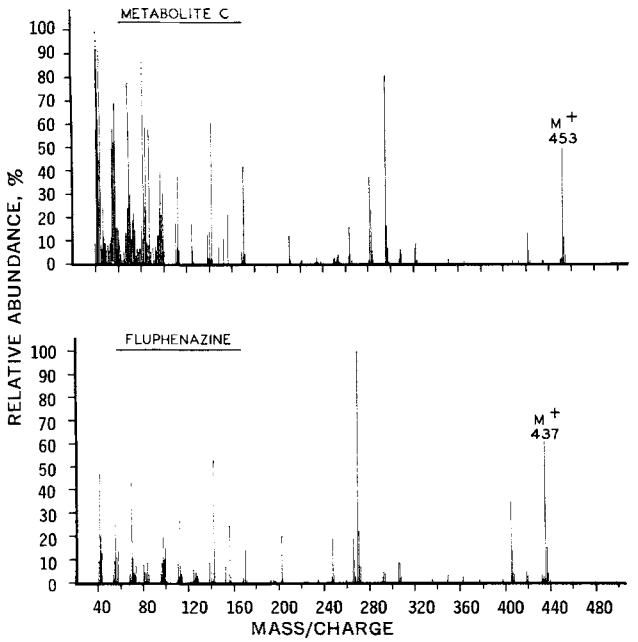

Figure 1—*Mass spectra of Metabolite C and fluphenazine.*

model MS902 spectrometer. Elemental compositions for selected mass spectral fragments were obtained either by peak-matching or by computer analysis of data recorded from the MS902 spectrometer.

RESULTS

The low-resolution mass spectra for Metabolite C and authentic fluphenazine dihydrochloride are shown in Fig. 1. The molecular ion for fluphenazine is found at *m/e* 437, which agrees with the calculated molecular weight for the free base. The molecular ion for Metabolite C is found at *m/e* 453, 16 a.m.u. higher than that of the parent molecule. In addition, prominent ions in Metabolite C are shifted 16 a.m.u. higher than the corresponding fragments observed for fluphenazine. A more detailed analysis for these fragments is provided by the data shown in Table I, which were obtained under conditions of high resolution.

By the reasoning of the mass spectrometric shift technique introduced by Biemann (3), a series of fragments occurs which is derived either from the side chain or from the tricyclic ring system. Those fragment ions arising exclusively from the alicyclic portion of the molecule, ions having *m/e* up to 171 a.m.u., were identical in both Metabolite C and fluphenazine. The empirical formulas obtained from the observed masses for Metabolite C and fluphenazine confirm that the additional 16 a.m.u. represents the introduction of an oxygen atom into the parent molecule. A series of fragments is observed having lower masses than the parent ions, which, for Metabolite C, contain an additional oxygen which is not present for the fragment ions of fluphenazine. Since the additional oxygen is not present on the piperazine-containing side chain, it must be located on the tricyclic ring system. The mass spectrum obtained with an authentic sample of fluphenazine sulfoxide indicated that Metabolite C was not the sulfoxide derivative.

Duffield *et al.* (4) reported that mass spectrometry is able to distinguish between oxygenation at C-3 *versus* C-6, C-7, and C-8 in chlorpromazine and 2-chlorophenothiazine. These authors also pointed out that the introduction of a side chain like dimethylaminopropyl to the phenothiazine ring system dramatically alters the mass spectral fragmentation process when compared with those compounds not having a side chain. In this study, the authors were unable to reason in an analogous manner from the fragmentation pattern reported for the oxygenated isomers of chlorpromazine (4), since fluphenazine contains a piperazine-containing side chain which results in a fragmentation pattern different from that observed when the phenothiazine ring system contains a dimethylaminopropyl side chain (5). Since they did not have a derivative of fluphenazine oxygenated at the C-3 position as a reference compound, they were not able to take an approach similar to that described for other substituted phenothiazines (4). Thus, localization of the additional oxygen atom of Metabolite C to either of the aromatic rings of the phenothiazine ring system was not possible on the basis of mass spectral data.

NMR spectroscopy was employed to obtain additional information regarding the location of the additional oxygen atom in Metabolite C. Examination of the 60-MHz. spectra for authentic fluphenazine base (not shown) and Metabolite C (Fig. 2) indicated that differences in the spectra had occurred only in the region of the aromatic proton resonances. This portion of the spectrum for Metabolite C contained six protons, whereas that for authentic fluphenazine contained seven protons, thus establishing Metabolite C as a phenolic compound. Since the three protons that resonate between 2.8 and 3.0τ correspond to the C-1, C-3, and C-4 protons of fluphenazine, it was possible to say that the hydroxyl group was not substituted on the ring bearing the trifluoromethyl group.

Craig and his coworkers (6, 7) reported that increased resolution was obtained for hydroxyl derivatives of chlorpromazine at 220 MHz., thus permitting positive structural assignments of these phenothiazine metabolites. The 220-MHz. spectrum for Metabolite C is shown in Fig. 3. The three protons resonating between 2.7 and 2.9τ were assigned to the ring containing the trifluoromethyl group, because the C-1 proton is coupled only to a *meta*-proton, while the C-4 proton is coupled only to an *ortho*-proton; the C-3 proton is coupled to both the C-1 and C-4 protons.

The remaining peaks in the spectrum, occurring from 3.1 to 3.4τ, are present as two sets of doublets and a quartet. The assignment of these three resonances is based on the fact that the low-field doublet at 3.13τ is coupled only to the intermediate-field quartet at 3.35τ which, in turn, is coupled to the *meta*-proton resonating at 3.40τ. The magnitude of the coupling constants between *ortho*-hydrogens is 7–9.2 Hz.; between *meta*-hydrogens, it is 1.1–3.1 Hz.; and between *para*-hydrogens, it is 0.0–0.7 Hz. (8). The coupling constants between the aromatic protons of Metabolite C, shown in Fig. 3, fall in the range given for *meta*- and *ortho*-coupled protons. These relationships among the protons only permit substitution of the hydroxyl group at C-7 or C-8.

To establish the structure of Metabolite C unequivocally, the IR and NMR spectra of Metabolite C were compared with those of authentic 7- and 8-hydroxyfluphenazine. Examination of the NMR spectra indicated that the aromatic proton resonances for 7- and 8-hydroxyfluphenazine were distinctly different at 60 MHz., whereas the spectra for 7-hydroxyfluphenazine and Metabolite C were superimposable at both 60 *and* 220 MHz. The IR spectra of 7-hydroxyfluphenazine and Metabolite C were also identical in all major features, except that more of the fine structure was exhibited by the authentic compound than by the isolated metabolite, a finding attributed to the greater purity and the crystalline properties of the former compound.

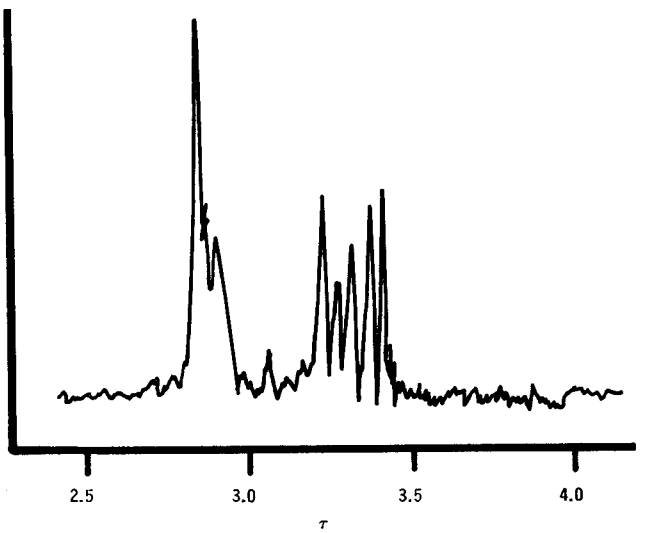

Figure 2—*NMR spectrum (60 MHz.) for the aromatic proton resonances of Metabolite C. The sample was dissolved in perdeuteriomethanol, with tetramethylsilane as a reference.*

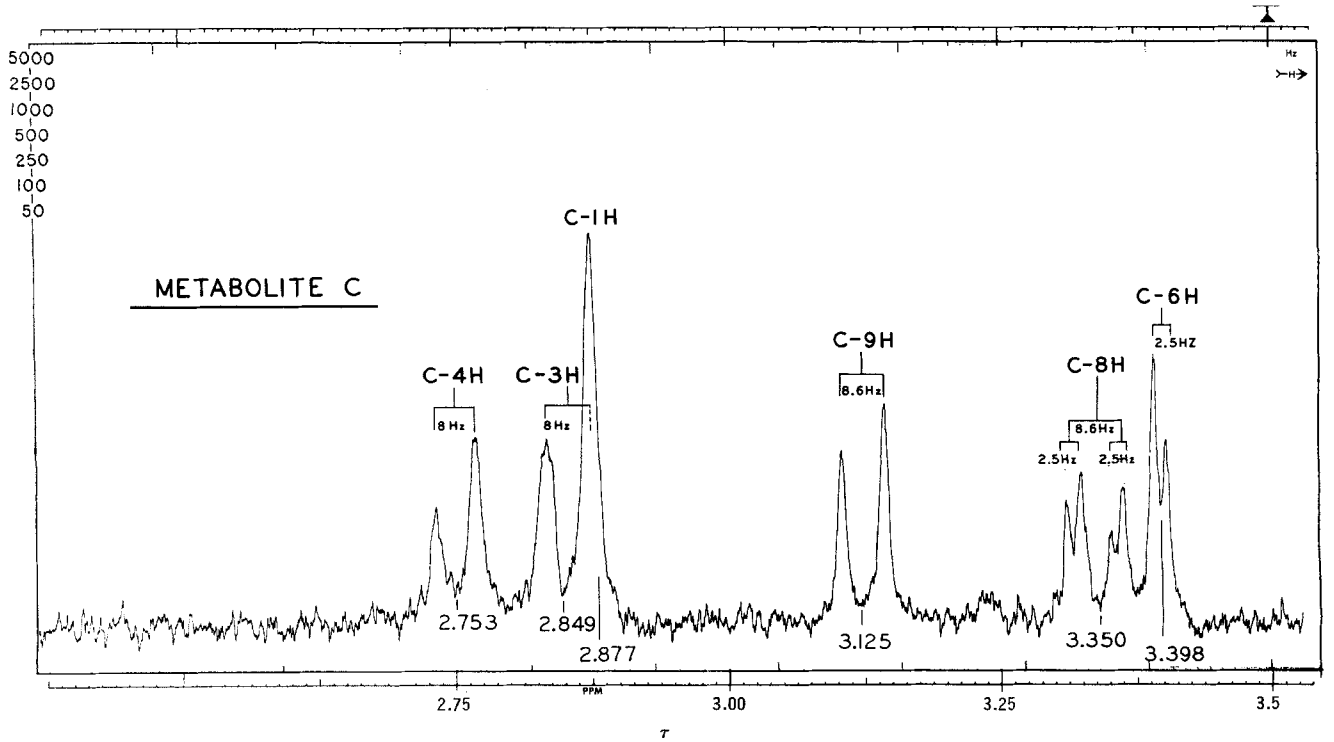

Figure 3—*NMR spectrum (220 MHz.) for the aromatic proton resonances of Metabolite C. The sample was dissolved in perdeuteriomethanol, with tetramethylsilane as a reference. The proton assignments are discussed in the text.*

TLC also confirmed the identity of Metabolite C as 7-hydroxyfluphenazine, since these compounds had identical R_f values in Solvent Systems 3, 4, and 6 (*Reference 1*, Table I). Both compounds exhibited the same reddish-brown color when the plates were allowed to stand in the laboratory for several hours. On the other hand, 8-hydroxyfluphenazine had a consistently lower R_f value than did Metabolite C in Solvent Systems 4 and 6 and, on standing, exhibited a yellow color.

DISCUSSION

The biotransformation of fluphenazine appears to resemble that previously reported for chlorpromazine (9–11). Both compounds are converted to the sulfoxide, a urinary metabolite, and to hydroxylated derivatives. In chlorpromazine, hydroxylation occurs in the dog and man primarily at C-7 and, to a lesser extent, at C-3. In the present study with fluphenazine (1), not all urinary and fecal metabolites were identified; however, if hydroxylation at C-3 does occur in the dog or rhesus monkey, it is a minor pathway. The metabolism of chlorpromazine and other substituted phenothiazines has also been studied in liver preparations *in vitro*. Under these conditions, hydroxylation of chlorpromazine at C-7 also occurred more readily than at C-3 in rat and human microsomal enzyme systems (12). Similarly, hydroxylation of various phenothiazine derivatives, including fluphenazine, with widely differing ring and side-chain substituents, occurred in rat liver preparations (13). Since fluphenazine contains a trifluoromethyl group on the phenothiazine ring system, instead of a chlorine atom as in chlorpromazine, it appears that this difference does not markedly influence the occurrence of hydroxylation at C-7. The same statement, by analogy, also applies to the lack of any marked influence of the nature of the side chain on ring hydroxylation in chlorpromazine and fluphenazine.

REFERENCES

(1) J. Dreyfuss, J. J. Ross, Jr., and E. C. Schreiber, *J. Pharm. Sci.*, **60,** 821(1971).

(2) B. Loev and K. M. Snader, *Chem. Ind.*, **1965,** 15.

(3) K. Biemann, "Mass Spectrometry—Organic Chemical Applications," McGraw-Hill, New York, N. Y., 1962.

(4) A. M. Duffield, J. C. Craig, and L. R. Kray, *Tetrahedron*, **24,** 4267(1968).

(5) A. I. Cohen, unpublished data.

(6) J. C. Craig and L. R. Kray, *Agressologie*, **IX,** 55(1968).

(7) J. C. Craig, D. E. Green, and S. K. Roy, *J. Med. Chem.*, **8,** 392(1965).

(8) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," vol. 2, Pergamon Press, London, England, 1966, p. 770.

(9) H. S. Posner, R. Culpan, and J. Levine, *J. Pharmacol. Exp. Ther.*, **141,** 377(1963).

(10) V. Fishman and H. Goldenberg, *Proc. Soc. Exp. Biol. Med.*, **112,** 501(1963).

(11) V. Fishman and H. Goldenberg, *J. Pharmacol. Exp. Ther.*, **150,** 122(1965).

(12) P. F. Coccia and W. W. Westerfeld, *ibid.*, **157,** 446(1967).

(13) A. E. Robinson, *J. Pharm. Pharmacol.*, **18,** 19(1966).

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