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A novel conjugate as a major metabolite of bromperidol in man

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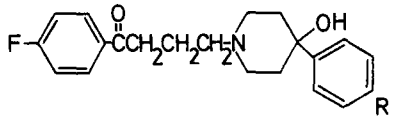
The major urine metabolites of the neuroleptic drug, bromperidol, after oral doses to rats and dogs are *p*-fluorophenylacetic acid and its glycine conjugate resulting from oxidative *N*-dealkylation. While the same metabolites were also detected in human urine, also present was a major unknown component representing 50% of the total urine metabolites, which apparently was not formed by rats and dogs to any extent. Mass spectroscopic investigations of the metabolite showed that it could be thermally degraded to bromperidol and contained a substituent attached to the tertiary hydroxyl group. The mass spectrum of the metabolite after trifluoroacetylation was consistent with an *O*-glucofuranosiduronolactone conjugate of bromperidol.

Bromperidol (I, R = Br) is one of a series of butyrophenone neuroleptic agents (Niemegeers & Janssen 1974; Parent & Fleischhauer 1978) of which haloperidol (I, R = Cl) is the best known. Metabolism of haloperidol in animals (Soudijn et al 1967) and in man (Forsman et al 1977) proceeds by *N*-dealkylation giving rise to *p*-fluorobenzoylpropionic acid which is further metabolized to *p*-fluorophenylacetic acid and its glycine conjugate. The rat and dog produce the same metabolites from bromperidol (Chasseaud 1978), and while human urine contains small amounts of such components, the major metabolite is different and this paper investigates its structure.

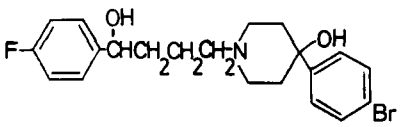


(I)

MATERIALS AND METHODS

Materials

Urine 0–6 h was obtained from humans after a 2 mg oral dose of [^{3}H]bromperidol (Chasseaud 1978) and after a 5 mg oral dose of non-radioactive bromperidol. Urine samples from patients receiving multiple oral doses of 60 mg bromperidol were supplied by Cilag Ltd, Schaffhausen, Switzerland, as were *p*-fluorophenylacetic acid, *p*-fluorophenylaceturic acid, *p*-fluorobenzoylpropionic acid, and bromperidol *N*-oxide. 1-*p*-Fluorophenyl-4-[4^1-(*p*-bromophenyl)-4^1-hydroxypiperidin-1-yl]-butan-1-ol (II), the reduced form of bromperidol, was a gift from Ortho Pharmaceutical Corporation, New Jersey, U.S.A.



(II)

Urine extraction

Urine (100 ml) was evaporated to dryness under reduced pressure at 37 °, the residue dissolved in methanol and insoluble salts removed by centrifugation. The methanol (containing 98% of the urinary radioactivity) was evaporated and the residue taken up in distilled water (10 ml). Filtered aliquots (1 ml) were subjected to h.p.l.c. and, for isolation of the major metabolite, aliquots (10 ml) were applied to an Amberlite XAD-2 column (20 × 2 cm), which was washed with distilled water (150 ml) and metabolites eluted with methanol (100 ml). After evaporation of the methanol, the residue in distilled water (5 ml) was acidified with HCl (1 M, 1 ml) and extracted with hexane–ether (1:4, 2 × 20 ml). The organic layers were discarded and the aqueous layer treated with NaOH (4 M, 0·5 ml) and extracted with hexane–ether (1:4, v/v, 20 ml). The organic layer was discarded and the aqueous layer was evaporated to dryness. The residue was dissolved in methanol and salts removed by centrifugation. The methanol was finally evaporated to dryness and the residue dissolved in distilled water (5 ml), filtered and aliquots (1 ml) taken for h.p.l.c.

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Hydrolysis experiments

For enzymic hydrolysis, urine (10 ml) and acetate buffer (0·2 M, pH 5) (10 ml), were treated with a β-glucuronidase/sulphatase preparation (10 000 units, Type H-1 from *Helix pomatia;* Sigma Chemical Co. Ltd, Dorset, U.K.) and incubated (37°, 24 h): this was continued (24 h) following addition of a further 10 000 units. Methanol extracts were then prepared for analysis by h.p.l.c. as above.

For chemical hydrolysis, samples (3 ml) of the desalted concentrated urine were mixed with either 1 M NaOH (1 ml) or 1 M H_2SO_4 (1 ml) and heated (100 °C, 1 h); the samples were then neutralized, evaporated to a small volume and analysed by h.p.l.c.

Measurement of radioactivity

Samples of urine, aqueous extracts or fractions from h.p.l.c. were diluted to 1 ml with water and mixed with a toluene:Triton X-100 based scintillator system (10 ml, Fisons Scientific Apparatus Ltd, Loughborough, U.K.). Radioactivity was measured using a Philips Liquid Scintillation Counter.

High-performance liquid chromatography (h.p.l.c.)

Reversed-phase h.p.l.c. was carried out using a Waters model ALC 202 chromatograph fitted with a stainless-steel column (30 cm × 0·4 cm i.d.) prepacked with μBondapak C_{18} (mean particle diameter 10 μm) (Waters Associates Ltd) and equipped with an ultraviolet absorption detector (254 nm) and an on-line radioactivity detector system which mixed scintillator with the column eluent. For isolation of the metabolite, the column eluent was collected automatically in fractions. Chromatograms were developed using a linear gradient of aqueous sodium acetate (0·5%, w/v, pH 3·6) containing 25% (v/v) methanol, increasing to 99% (v/v) methanol during 20 min, at a flow rate of 2 ml min^{-1}. For chromatograms generated by the radiochemical detector, an isocratic mobile phase of 40% methanol in aqueous 0·5% (w/v) sodium acetate (pH 3·5) (flow rate of 1 ml min^{-1}) was used.

For purification of the major metabolite, fractions from up to five h.p.l.c. separations, containing the metabolite in about 100 ml of urine, were combined and evaporated. The residue was dissolved in water (1 to 2 ml), the solution injected into the chromatograph and eluent fractions corresponding to the metabolite collected. Fractions containing the largest amounts of radioactivity were combined, evaporated to dryness and the residue dissolved in distilled water (1 ml). This solution was then extracted with freshly redistilled isobutanol (2 × 2 to 3 ml), the organic layers combined and evaporated to dryness in a silylated glass tube. The residue was dissolved in methanol (Distol grade, Fisons Scientific Apparatus Ltd) and an aliquot measured for radioactivity. This measurement showed that up to 90% of the radioactivity was extracted into isobutanol. The remaining sample was stored at −20 °C until taken for mass spectrometry.

Mass spectrometry (m.s.)

Mass spectra were obtained using a VG Micromass 16F mass spectrometer linked to a VG Display Digispec data system (VG Analytical Ltd, Altrincham, U.K.). Electron impact (EI) mass spectra were obtained at 70 eV (trap current 100 μA): for chemical ionization (CI) spectra, isobutane was used at 50 eV (emission current 200 μA). In each case, the source temperature was 200–220 °C with samples introduced by direct insertion probe, and spectra recorded while the sample temperature increased from 30 ° to about 250 °C.

Gas chromatography-mass spectrometry (g.c.-m.s.) used a 1·5 m × 2 mm i.d. glass column packed with 3% OV17 on Diatomite CQ (100/120 mesh), with helium as carrier gas (flow rate of 20 ml min^{-1}). The oven, separator and source temperatures were 290 °, 240 ° and 200 °C respectively. Mass spectra were acquired at 10 s (g.c.) or 20 s (probe) intervals and stored for subsequent examination.

High resolution m.s. was carried out at the Physicochemical Measurements Unit, Harwell, U.K., using a Kratos MS50 double-focusing mass spectrometer (Kratos Ltd, Manchester, U.K.). It was operated at a resolving power of 10 000 under EI conditions at 70 eV (trap current 500 μA) and a source temperature of 250 °C using the direct insertion probe for sample introduction.

Chemical derivatization procedures

Methylation was accomplished by bubbling diazomethane through a cold methanolic solution of the metabolite until a permanent yellow colour persisted.

Trimethylsilyl derivatives were prepared using *N,O*-bis-(trimethylsilyl)-acetamide in pyridine (40 °C; 30 min).

Perfluoroacyl derivatives were prepared using trifluoroacetic acid anhydride or pentafluoropropionic acid anhydride (40 °C; 30 min).

For deuteration, the metabolite was dissolved in deuteromethanol (1 ml) and deuterated water (0·5 ml) (20 °C; 1 h).

RESULTS AND DISCUSSION

The available reference compounds were all separated by h.p.l.c. Their retention times were: *p*-fluorophenylaceturic acid, 4·4 min; *p*-fluorophenylacetic acid, 7·5 min; *p*-fluorobenzoylpropionic acid, 8·5 min; 'reduced' bromperidol, 12·3 min; bromperidol, 13·6 min and bromperidol *N*-oxide, 15·2 min. A 0–6 h urine extract (13% dose) contained three major radioactive components. Two components, associated with 28% and 21% of the total radioactivity respectively, corresponded to *p*-fluorophenylaceturic acid and *p*-fluorophenylacetic acid. None of the components corresponded to bromperidol. The third component (40% radioactivity) had the same retention time as 'reduced' bromperidol, but was not this compound. The pattern of metabolites in 0–24 h urine (22% dose) was similar.

Only 36% and 1% of the sample radioactivity was extracted sequentially from urine with hexane–ether under acidic and alkaline conditions respectively. The unextracted portion consisted of *p*-fluorophenylaceturic acid and the unknown metabolite whereas 'reduced' bromperidol was readily extractable from aqueous solution at alkaline pH. After heating with dilute acid or alkali, the unknown metabolite was essentially unchanged although there was some formation of a component corresponding to bromperidol after acid hydrolysis. The use of stronger alkali or especially acid was inappropiate due to the instability of bromperidol itself to these conditions. After incubation with β-glucuronidase/sulphatase, the metabolite was unaffected.

The EI mass spectrum of the metabolite was similar to that of bromperidol (Fig. 1). One apparent difference was that the metabolite invariably gave a spectrum with two bromine doublets in the molecular ion region (*m/z* 421/423 and *m/z* 419/421). The relative intensities of these doublets (*m/z* 419/421 corresponding to bromperidol) varied according to the sample. The occurrence of the M + 2 peaks is believed to be due to the presence of sample impurities resulting in formation of a rearrangement product in the mass spectrometer. The CI spectra of the same samples of the metabolite only showed a pseudomolecular ion (M + 1) doublet at *m/z* 420/422 and the spectra were always similar to that of bromperidol.

The EI and CI mass spectra of the metabolite were different from those of 'reduced' bromperidol, although *m/z* 421/423 in the metabolite EI spectrum would correspond to the molecular ion of 'reduced' bromperidol. The mass spectrum of bromperidol contained two characteristic bromine-containing fragments at *m/z* 268/270 and *m/z* 281/283 formed by cleavage of the *p*-fluorobenzoylpropionyl side-chain.

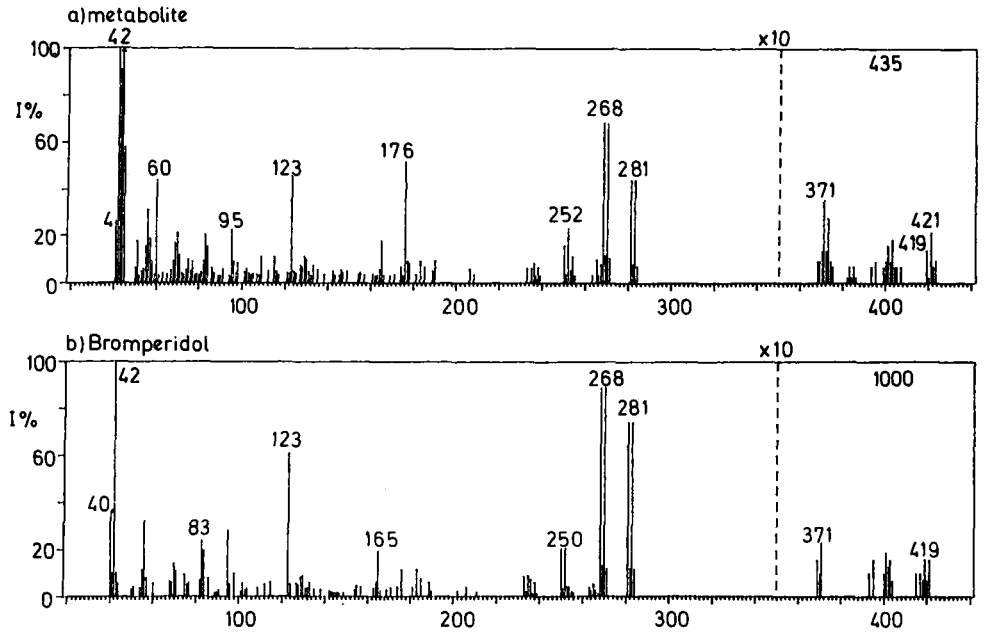
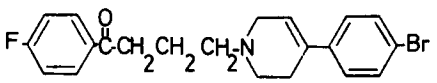


FIG. 1. Electron impact mass spectra of bromperidol and the major human urine metabolite. Samples were introduced by the direct insertion probe and spectra recorded at probe temperatures of 200 °C (metabolite) and 100 °C (bromperidol).

The latter fragment was produced by a McLafferty rearrangement involving the keto group and consequently was not present as an intense ion in the spectrum of 'reduced' bromperidol but it was an intense ion in the metabolite spectrum.

The sample temperature required to obtain the mass spectrum of the metabolite was considerably higher (200 °C) than that required for bromperidol (100 °C), thus indicating that the former was a derivative of the latter and decomposed at high temperature to bromperidol.

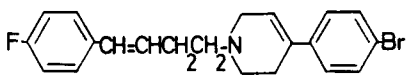
G.c.-m.s. of the metabolite and bromperidol resulted in a single peak with the same retention time and similar mass spectra, presumably because the metabolite decomposed during g.c. G.c.-m.s. of trimethylsilyl (TMS) derivatives also showed the presence of the same component for the metabolite and bromperidol, the mass spectrum of which was consistent with a bis-TMS derivative (M^+ 563, M^+-CH_3 548) at the tertiary hydroxyl group and presumably at an enolic form of the keto group. These results confirmed that the metabolite could be converted to bromperidol, both thermally and during derivatization, and suggested that it possessed a conjugate structure. Furthermore the mass spectrum of bromperidol, after treatment with $MeOD/D_2O$, indicated quantitative isotopic exchange of the



(III)

hydroxylic proton since bromine doublet fragment ions (*m/z* 269/271 and *m/z* 282/284) were each one mass unit higher than that in the spectrum of the untreated compound. However, this did not occur after similar treatment of the metabolite, suggesting that it contained the bromperidol structure with a substituent on the tertiary hydroxyl group.

The EI mass spectrum of bromperidol, after treatment with trifluoroacetic anhydride (TFA), showed a high mass doublet at *m/z* 401/403 (*m/z* 402/404 after CI) consistent with the molecular ion of the dehydration product (III) formed by loss of the tertiary hydroxyl (or a trifluoroacetyl group) in the piperidine ring. Similarly the EI spectrum of derivatized, 'reduced' bromperidol showed a high mass doublet at *m/z* 384/386 consistent with the M-H ions of the unsaturated compound (IV) which would result from

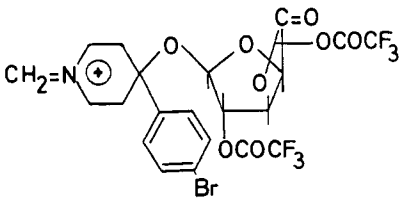


(IV)

the loss of two molecules of trifluoroacetic acid from the bis-TFA derivative.

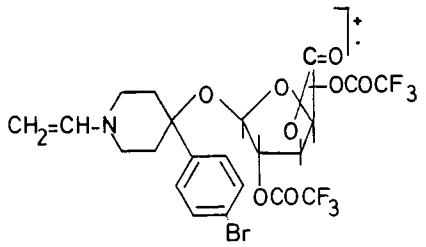
As stated above, two characteristic pairs of bromine doublets (*m/z* 268/270 and *m/z* 281/283) occur in the EI mass spectrum of bromperidol (and the metabolite) and corresponding doublets were observed in the spectra of the TFA derivative (*m/z* 618/620 and *m/z* 631/633) and in the pentafluoropropionyl (PFP) derivative (*m/z* 718/720 and *m/z* 731/733) of the metabolite. The mass difference in these two derivatives is 100 units, corresponding to two CF_2 groups, indicating that the metabolite forms a bis-perfluoroacyl derivative.

By analogy with the fragmentation pathway of bromperidol, the doublet ions *m/z* 618/620 and *m/z* 631/633 must differ by a CH group and must contain one bromine atom and two trifluoroacetyl groups. Accurate mass measurement (high resolution m.s.) gave values of 618·0208 and 631·0277 for the first ion of each doublet and the only sensible possibilities for the molecular formulae of these ions were $C_{22}H_{19}NO_8F_6{}^{79}Br$ and $C_{23}H_{20}NO_8F_6{}^{79}Br$ respectively. Within the constraints imposed by the other evidence, the only meaningful structures that can be assigned to these ions are those of the bis-TFA derivative of glucuronolactone attached via a glycosidic link to the tertiary hydroxyl group of the piperidine ring (V and VI). The formation of these two ions and the presence of the ions at *m/z* 123 and *m/z* 165 (*p*-fluorobenzoyl side-chain), indicated that the structure (VII) could be assigned to the derivatized metabolite. Support for this structure was obtained from the CI mass spectrum of the same derivative which showed a pseudomolecular ion doublet (M + 1) at *m/z* 770/772. In addition, the EI spectrum of the underivatized metabolite showed (Fig. 1) an intense fragment ion, *m/z* 176, which can be assigned to glucofuranosiduronolactone.

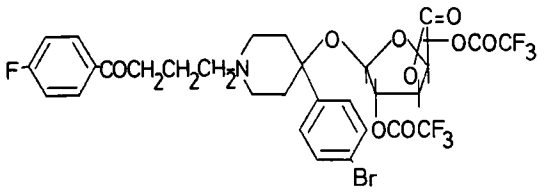


(V, m/z 618)

A glucopyranosiduronic acid conjugate is not able to lactonize due to the relative configurations of the C-3 hydroxyl and carboxylic acid groups. Although it is possible that the metabolite could be an *O*-glucofuranosiduronic acid and that lactone formation occurs during subsequent extraction, this is



(VI, m/z 631)



(VII)

unlikely. The 3,6-lactone can form when glucofuranosiduronic acids are heated at high temperature and more readily after esterification of the carboxylic acid group (Kato et al 1964a), but the bromperidol metabolite was isolated and characterized under mild conditions. The metabolite was identified after trifluoroacetylation, and this derivatization of the free hydroxyl group in a glucuronic acid conjugate would essentially block subsequent lactone formation. Furthermore the metabolite was resistant to hydrolysis by β-glucuronidase, whereas Kato et al (1964b) have shown that both β-D-glucofuranosiduronic acid and β-D-glucopyranosiduronic acid conjugates of 2-naphthol are hydrolysed by this enzyme. Since glucuronide conjugates are believed to exist in the β-D-pyranose form, it seems unlikely that a conjugate of bromperidol would be formed exclusively in the β-D-furanose form. It is only as the lactone that a glucuronic acid moiety is locked in the furanose form. We are not aware of any reports of glucofuranosiduronolactone conjugates of exogenous compounds, and although Hignite et al (1981) have shown that glucofurano- and glucopyranosiduronic acid ester conjugates of clofibrate are excreted by man, there was no evidence to suggest that the clofibrate furanose conjugate was converted to the lactone during isolation. Kuenzle (1970) has identified several disaccharide conjugates of bilirubin present in human bile, one of which was an acyl 4-*O*-β-D-glucofuranosyluronic acid acid/D-glucopyranoside. Unless glucuronide conjugates are identified by direct techniques such as mass spectrometry, it is unlikely that other procedures will distinguish between pyranose and furanose forms.

This human urine metabolite of bromperidol represents the first reported example of a glucuronolactone conjugate and it is surprising that others have not been detected, particularly after large doses of some compounds that are metabolized extensively by glucuronidation. However, more rigorous examination of unknown conjugates, often reported in the literature, could lead to other examples of this biotransformation pathway. It is interesting that this metabolite was not formed by rat and dog to any extent, but assessment of the species specificity of this pathway must await further examples. The formation of a polar bromperidol metabolite in human but not in dog plasma could be inferred from the results of studies using radioimmunoassay for the measurement of bromperidol (Van den Eeckhout et al 1980). For human samples, lower values were obtained when plasma extracts were assayed than when untreated plasma was assayed, whereas, for dog samples, values were similar for untreated plasma or plasma extracts. This result was also observed with the related compound, haloperidol. To account for cross-reactivity in this radioimmunoassay of human plasma, it is likely that the plasma metabolite is formed by a minor structural modification of bromperidol and it could correspond to the conjugate identified in this study. A similar metabolite of haloperidol would also be expected.

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