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ZETIDOLINE METABOLISM BY RAT LIVER MICROSOMES

FORMATION OF METABOLITES WITH POTENTIAL NEUROLEPTIC ACTIVITY

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Abstract—1-(3′-Chlorophenyl)-3-[2-(3,3-dimethyl-1-azetidinyl)ethyl] imidazolidin-2-one, zetidoline, a new neuroleptic agent, when incubated with rat liver microsomes was rapidly metabolized to six free (mets B, D, I, L, M and N) and two conjugated metabolites (mets E and F). Sites of the metabolic attack (oxidation) were primarily the aromatic moiety, then the imidazolidinone and the azetidine rings.

The metabolites were purified and structures assigned by means of EI-MS, ^{1}H-NMR and chemical synthesis (mets B, D, L and M).

The main metabolites, zetidoline, some chemical analogues and a few known dopamine antagonists were tested as *in vitro* inhibitors of ^{3}H-zetidoline and ^{3}H-spiperone binding to rat striatal membranes, and as *in vivo* inducers of prolactin release in female rats (inhibition of the estrus cycle). Two zetidoline metabolites, namely 4′-hydroxy zetidoline (met. B) and 5-hydroxy zetidoline (met. L), were found to have both *in vitro* and *in vivo* activities comparable to those of the parent drug.

Identification of these active hydroxylated metabolites appears important both in the search of new leads of neuroleptics and for designing pro-drugs derivatives with improved pharmacokinetic profiles.

Zetidoline,§ 1-(3-chlorophenyl)-3-[2-(3,3-dimethyl-1-azetidinyl)ethyl]imidazolidin-2-one hydrochloride, is a new selective dopamine antagonist [1] which has been shown active in animal models [2–4] and in clinical trials [6–9]. As compared to other neuroleptics zetidoline proved to be more potent than chlorpromazine, less than haloperidol, but it has reduced unwanted effects. Interestingly, evidence has been shown that zetidoline binds a population of dopaminergic receptors partly different from that labelled by ^{3}H-spiperone and its receptor binding distribution in cerebral areas significatively differs from that of butyrophenones [10]. Very likely, zetidoline and sulpiride share instead a common population of binding sites [10].

Studies of the pharmacokinetics and metabolism of zetidoline have been carried out in the rat and the dog [11, 12], and in man [13]. In these species, the compound is rapidly absorbed and is extensively metabolized in a first-pass, followed by an equally rapid elimination of metabolites via the kidneys. The main phase I reactions are two successive hydroxylations of the aromatic ring of zetidoline at carbons 4 and 6, followed by a chemical rearrangement and/or by conjugation with glucuronic acid (rat, dog) or sulphate (man).

Three free metabolites, 4-hydroxy zetidoline (met. B), 1-(3-chlorophenyl)-3-[2(3,3-dimethyl-1-azetidinyl)2-oxoethyl]imidazolidin-2-one (met. C) and 1-[2(3,3-dimethyl-1-azetidinyl)ethyl]imidazolidin-2-one (met. D), plus 4 conjugated metabolites, the 4-β-D-glucuronide or sulphate ester of met. B (mets. F and G) and the 6-hydroxy-4-β-D-glucuronide or sulphate ester of met. B (mets. E and H), were isolated and purified [12, 13]. Among them only the free metabolites were tested in classic animal models predictive for a potential neuroleptic activity in man, and met. B was proved as active as zetidoline [14]. This overall evidence suggests that zetidoline may also act as a pro-drug for the active met. B. With the aim of assessing whether other metabolic pathways may lead to pharmacologically active derivatives, in the present study further zetidoline metabolites were produced *in vitro* by rat liver microsomes. Following purification, identification and chemical synthesis, their anti-dopaminergic (i.e. potential neuroleptic activity) activity was determined *in vitro* by their ability to displace ^{3}H-zetidoline and ^{3}H-spiperone specific binding to rat striatal membranes and *in vivo* by their ability to stimulate prolactin release from the pituitary.

Few other synthetic zetidoline analogues were also comparatively assayed in these tests to provide additional information on the structure–activity relationships of this new class of neuroleptic agents.

MATERIALS AND METHODS

Chemicals. [2-^{14}C]zetidoline, specific activity 1.22 mCi/mmole, with a radiochemical purity exceeding 99%, as detected after TLC, autoradio-

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§ International nonproprietary name (WHO), also referred to elsewhere as DL 308-IT

graphy and liquid scintillation counting, was synthesized in our Laboratories as previously described [11]. [4,5-^{3}H]zetidoline, specific activity 27.5 Ci/mmole and radiochemical purity of 95.5–99%, was synthesized at New England Nuclear Dr, F.R.G., by catalytic reduction of 4,5-dehydro imidazolin-2-one derivative (L15643) with tritium gas in the presence of 5% rhodium on carbon catalyst.

[Benzene ring-^{3}H]spiperone, specific activity 24.5 Ci/mmole and radiochemical purity exceeding 98% was purchased from New England Nuclear.

1 - (3 - Chloro - 4 - methoxyphenyl) - 3 - [2 - (3,3 - dimethyl - 1 - azetidinyl)ethyl] - imidazolidin - 2 - one (L14619), 1-(3-chloro-6-methoxyphenyl)-3-[2-(3,3-dimethyl - 1 - azetidinyl)ethyl]imidazolidin - 2 - one (L16270), 1-(3-chlorophenyl)-3-[2-(3,3-dimethyl-1-azetidinyl)ethyl]-4,5-dehydro-imidazolidin-2-one-(L15643) and 1-(3-chlorophenyl)-3-[2-(3,3-di-t-butyl-1 - azetidinyl)ethyl]imidazolidin - 2 - one (L9310) besides (±)sulpiride from Gruppo Lepetit (Milan, Italy), chloropromazine from Farmitalia (Milan, Italy), haloperidol and spiperone from Janssen Pharmaceutica (Beerse, Belgium) were used as reference compounds.

Synthesis of metabolites B and D. 1-(3-Chloro-4-hydroxyphenyl) - 3 - [2 - (3,3 - dimethyl - 1 - azetidinyl)ethyl] imidazolidin-2-one (met. B) and 1-[2-(3,3-dimethyl-1-azetidinyl)ethyl] imidazolidin-2-one (met. D) were prepared in our laboratories as already reported [12].

Synthesis of metabolite L. 1-(2-Aminoethyl)-3,3-dimethyl-azetidine and 2-bromo-1,1-diethoxyethane were refluxed in 95% ethanol in the presence of $NaHCO_3$ for 20 hr. The reaction mixture was filtered, the ethanol solution was evaporated under moderate vacuum and the oil residue was purified by fractional distillation at 90–110° and 1 mm Hg. The resulting 3,3-dimethyl-1-[2-(2,2-diethoxyethyl)amino] ethyl azetidine in diethyl ether was added to a solution of 3-chlorophenyl isocyanate in diethyl ether. The reaction mixture was refluxed for 3 hr, then the solvent was evaporated under vacuum. The crude *N*-(3 - chlorophenyl)-*N'*-(2,2-diethoxyethyl)-N'-(3',3'-dimethyl-1-azetidinyl) ethyl urea was dissolved in an excess of 10% HCl, the aqueous solution was washed with diethyl ether, made alkaline with conc. NH_4OH and then extracted with diethyl ether. 1-(3-Chlorophenyl)-5-hydroxy-3[2-(3,3-dimethyl-1-azetidinyl) ethyl] imidazolidin-2-one (met. L) was crystallized from the slightly alkaline diethyl ether solution (Fig. 1); m.p. 189–190°, elemental analysis for $C_{16}H_{22}ClN_3O_2$.

Synthesis of metabolite M. 1-(1'-Chloroethyl)-3,3-dimethyl azetidine added to 1-(3-chloro-6-methoxyphenyl) imidazolidin-2-one in the presence of NaH in dimethylformamide yields 1-(3-chloro-6-methoxyphenyl)-3[2-(3,3-dimethyl-1-azetidinyl)ethyl] imidazolidin-2-one [1]. This compound was added quickly to a suspension of sodium ethanethiolate and NaH in dimethylformamide.

The reaction mixture was then heated at 100° for 4 hr, cooled, concentrated under vacuum, poured into an excess of 20% HCl and filtered. The resulting acidic solution was washed with diethyl ether, made alkaline with conc. NH_4OH and finally extracted with diethyl ether. Evaporation of the organic layer gave, after crystallization of the residue from ethyl acetate, met. M. 1-(3-Chloro-6-methoxyphenyl)-3-[2-(3,3-dimethyl-1-azetidiny)ethyl] imidazolidin-2-one. HCl (Fig. 1); m.p. 176°, elemental analysis for $C_{17}H_{25}Cl_2N_3O_2$.

In vitro metabolism. Male Sprague–Dawley rats weighing 150–175 g at arrival were purchased from Charles River Italy (Calco, Italy). Before treatment the animals were acclimatizated for one week in a temperature (20 ± 2°) and humidity (50–60%) controlled room with a 12 hr dark-light cycle.

After overnight fasting they were killed by decapitation and the livers placed in ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 25 mM KCl. The tissue was weighed, minced and homogenized with 4 vol./g of the same medium in a glass Potter-Elveyhjem tissue grinder with a 4000 rpm motor-driven Teflon pestle. The homogenate was then fractionated by centrifugation: 3000 *g* for 10 min, 20,000 *g* for 20 min and 105,000 *g* for 60 min. The final pellet was decanted, resuspended in the original volume of 50 mM Tris–HCl buffer, pH 7.4, and used as the microsomal fraction.

Microsomal protein was determined by the Bradford method [15], using a commercial dye reagent (Bio-Rad Labs, Missisanga, Canada) and bovine serum albumin as standard.

Incubation was carried out at 37° in an air atmosphere in a Dubnoff shaking incubator, at 120 swings/min, for 5, 15, 30 or 60 min, in 5 ml of solution containing 3 mg/ml of microsomal protein, 5 mM $MgCl_2$, 0.8 mM $NADP^+$ (Sigma Chem. Co. St. Louis, MO), 8 mM glucose-6-phosphate, 3.5 U/ml of glucose-6-phosphate dehydrogenase (Boehringer Mannheim, F.R.G.) and 10^{-3} M [2-^{14}C]zetidoline, added last in 50 µl of buffer. Control flasks were also prepared with heat killed enzyme systems. After incubation the reactions were stopped by the addition of 1 volume of boiling water.

For preparative purposes only, 300 mg of [2-^{14}C]-zetidoline were incubated for 2 hr in a microsomal preparation (800 ml) from the livers of 24 rats.

Assay and purification of the metabolites. After the incubation, aliquots (1 ml) of the microsomal preparations taken at different time intervals were lyophilized, extracted three times with CH_3OH and the extracts submitted to TLC on silica gel F_{254} plates, 0.25 mm thick (Merck, Darmstadt, F.R.G), solvent system $CHCl_3$:CH_3OH:NH_4OH (80:20:1, v/v).

TLC-autoradiographs were obtained by exposing the plates to Kodirex-ray-films (Kodak Ltd, London), and for the assay of zetidoline and its metabolites, individual zones of radioactivity detected by autoradiography were scraped off the plates and measured by liquid scintillation counting in a dioxane M19 cocktail (Packard Inst., Downers Grove, IL) The recovery figures for [2-^{14}C]zetidoline added (10^{-3} and 10^{-4} M) to heat-killed microsomal samples at the two concentrations tested were 88 ± 2% and 86 ± 3%.

For preparative scale production and isolation of the metabolites, the lyophilized microsomal preparation was extracted 3 times with 500 ml of methanol, radioactivity recovery of 96%. The organic extract was concentrated under vacuum, adsorbed on 10 g of silica gel 60 (230 mesh) and chro-

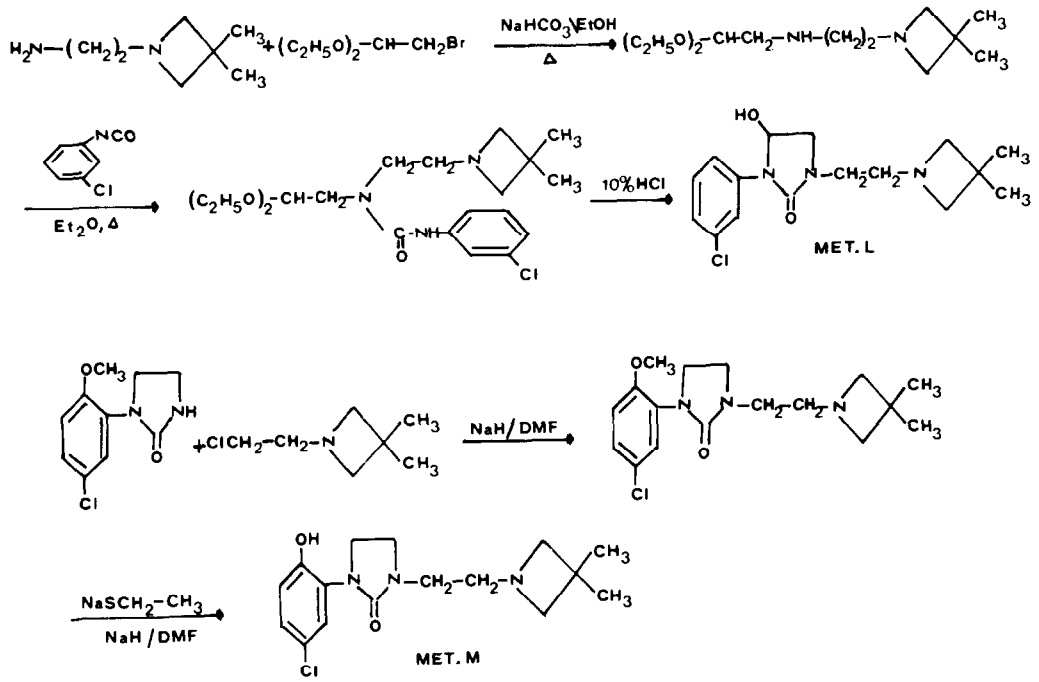


Fig. 1. Synthetic routes to metabolites L and M. For details see the experimental section.

matographed on a silica gel 60 column (3.5 × 70 cm), eluted first with 250 ml of $CHCl_3$ then with increasing concentrations of CH_3OH in $CHCl_3$ and finally with $CHCl_3$:CH_3OH:NH_4OH, 80:20:2 (Fig. 2). Eight radioactive peaks were collected (fractions I–VIII) and individually submitted to TLC, solvent systems: 1st run $CHCl_3$:CH_3OH, 8:2; 2nd run $CHCl_3$:CH_3OH:NH_4OH, 80:20:1. TLC autoradiographs showed nine main products (zetidoline and metabolites B, D, E, F, I, L, M, and N), which were scraped off the plates and re-chromatographed separately on silica gel 60 columns (1 × 15 cm), with one-step elution with mixtures of $CHCl_3$:CH_3OH or $CHCl_3$:CH_3OH:NH_4OH selected according to the chromatographic profile shown in Fig. 2.

In vitro *binding studies*. *In vitro* binding conditions

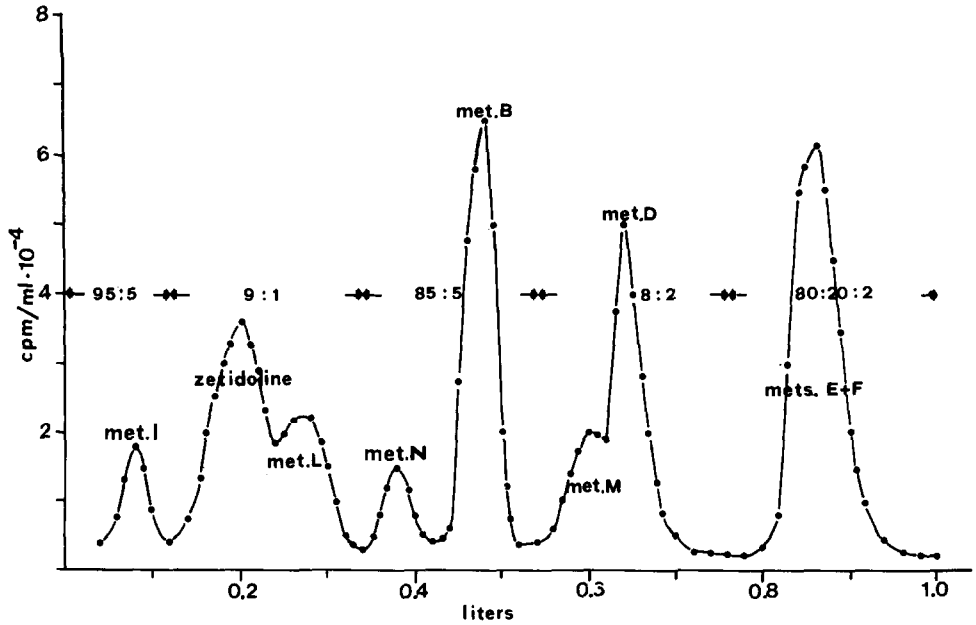


Fig. 2. Radioactivity tracing (cpm 10^{-4} ml^{-1}) of the microsomal extract chromatographed on a silica gel 60 column (3.5 × 70 cm). Stepwise elution with $CHCl_3$,$CHCl_3$:CH_3OH 95:5, 9:1, 85:5, 8:2 and $CHCl_3$:CH_3OH:NH_4OH, 80/20/2. Arrows indicate the change of the elution mixture.

for both ^{3}H-zetidoline [3, 4, 10] and ^{3}H-spiperone [16] have already been published. Briefly, fresh or frozen rat striata were sonicated in 100 vol. (v/w) of cold 50 mM Tris–HCl buffer, pH 7.4, with a Polytron PT10 microhomogenizer (20 sec, setting 6) and centrifuged twice at 50,000 *g* for 10 min at 2°. The intermediate pellet was sonicated in the same volume of cold buffer. The buffer for the sonication of the final pellet and for the incubation of the cerebral membranes was that commonly used for the *in vitro* binding of ^{3}H-neuroleptics [16, 17]: 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% ascorbic acid, pH 7.4 at 25° for ^{3}H-spiperone and pH 7.8 at 25° for ^{3}H-zetidoline.

^{3}H-Spiperone (0.2 nM) was incubated with striatal membranes (0.15–0.25 mg/ml) at 37° for 15 min and ^{3}H-zetidoline (0.8 nM) in melting ice for 45 min. Incubation was stopped by rapid *in vacuo* filtration of the entire contents of each test-tube through Whatman GF/B filters, using a multiple semi-automatic apparatus (Skatron Flow Labs, Norway) [18]. After washing for 10 sec with the same ice-cold incubation buffer the total radioactivity bound to the filtered tissue was measured in 10 ml of Biofluor (New England Nuclear). Total protein concentration was determined by the method of Bradford [15] using globulin proteins as standard.

For both ligands the specific binding was that displaced by 3 μM (+) butaclamol and was 85% of total for ^{3}H-zetidoline and 95% for ^{3}H-spiperone. IC_{50} values (concentrations of the drug causing a 50% inhibition of the specific binding) were assessed from 6–9 concentrations run in triplicate. The % inhibition points corresponding to the different drug concentrations were submitted to a non-linear fitting analysis (model logistic equation) on a Hewlett Packard HP85 minicomputer, according to the "Recept Program" described by Benfenati and Guardabasso [19].

In order to clarify the nature of the inhibition of ^{3}H-spiperone binding by zetidoline and its derivatives, saturation studies of ^{3}H-spiperone binding were carried out in the presence of zetidoline, haloperidol, chlorpromazine or (±) sulpiride as reference compounds and the data were evaluated by Scatchard analysis [20]. To label preferentially dopaminergic binding sites [10] a concentration range between 0.01 and 0.4 nM of ^{3}H-spiperone was chosen: eight different concentrations of ^{3}H-spiperone were incubated in triplicate with or without the listed drugs added, dissolved in the buffer, at their IC_{50} concentrations (non-specific binding was that displaced by 3 μM (+) butaclamol). The Student *t*-test was used to evaluate the significance of the differences in intercepts of the resulting regression curves.

Effects on prolactin release. Adult female (220–250 g) Sprague–Dawley rats from Charles River, Italy, were housed in an air conditioned room with a 14 hr light : 10 hr darkness photoperiod. They had free access to standard diet and tap water. The effect on prolactin release was biologically assayed by the method of Galliani [21]. The animals, whose daily vaginal smears indicated two or more consecutive 4-day estrous cycles, were given the test compounds or vehicle i.p., dissolved or suspended in 0.5% methocel (2 ml/kg). Administration was done during the diestrus-1 phase of the cycle at 5 p.m., when the corpora lutea are maximally sensitive to the luteotrophic effect of prolactin [21]. Vaginal smears were taken and evaluated daily until normal estrous cycles were resumed. Post-treatment cycles lasting more than 4 days were considered to have been interrupted. The dose required to interrupt the estrous cycle in 50% of the animals (ED_{50}) was determined by linear regression analysis of data obtained from groups of 6–20 animals at 3–5 doses.

Instrumentation. Radioactivity was measured with an Intertechnique model SL 4000 spectrometer. The electron impact mass spectra (EI-MS) were obtained with an Hitachi RMU-6L instrument, at 70 eV, with direct inlet system, at 120–150°, ion source temperature 200°. Proton nuclear magnetic resonance spectra (^{1}H-NMR) were recorded at 270 MHz, in dimethylsulphoxide-d_6 (DMSO-d_6) with tetramethylsilane (TMS) as internal reference, on a Brucker W-270 FT cryospectrometer equipped with 36 K BNC-12 computer and disk unit.

A Beckman DB-GT spectrophotometer, an LKB mod 7000 A fraction collector and an Edwards PTB III lyophilizer were used as ancillary equipment.

RESULTS

Elucidation of structure

Metabolites B, D, E, F, I, L, M and N, isolated and purified as described in the experimental section, were submitted to physicochemical analyses to determine their structures.

Metabolites B, D, E and F were already known and their identities were confirmed by comparing their EI-MS and ^{1}H-NMR spectra with those of the metabolites isolated from rat and dog urine [10]. Met. B is 4-hydroxy-zetidoline, met. D is de-arylated zetidoline, met. E is the 4-0-glucuronide of 6-hydroxy zetidoline, and met. F is the 4-0-glucuronide of zetidoline (see Fig. 3). The mass spectrum for met. L (Table 1) shows the molecular ion at m/z 323/325, i.e. 16 mass units more than that of zetidoline, indicating the presence of one additional oxygen, which is not located on the azetidine moiety, as suggested by the presence of the fragment at m/z 98 as in zetidoline. The losses of OH and H_2O (ions at m/z 306/308 and 305/307), which are usually not observed in chlorinated phenol spectra (see met. B ref. 10), suggest the presence of a hydroxyl group on the imidazolidinone ring. The ^{1}H-NMR spectra of met. L (Table 2) show that the pattern of the aromatic protons is the same as that of zetidoline, while the presence of a mobile proton at δ 6.65 and the downfield shift of H-5 indicates the hydroxylation of the C-5 of the imidazolidinone ring. Position 5 is preferred to position 4 because the shift of H-4 has not changed and a slight downfield shift of the aromatic hydrogens was observed.

The mass spectrum of met. M (Table 1) shows the molecular ion at m/z 323/325 and its pattern of fragmentation is similar to that of met. B, indicating the presence of an hydroxyl group on the phenyl moiety. The ^{1}H-NMR spectrum (Table 2) also indicates the introduction of a hydroxyl group into the

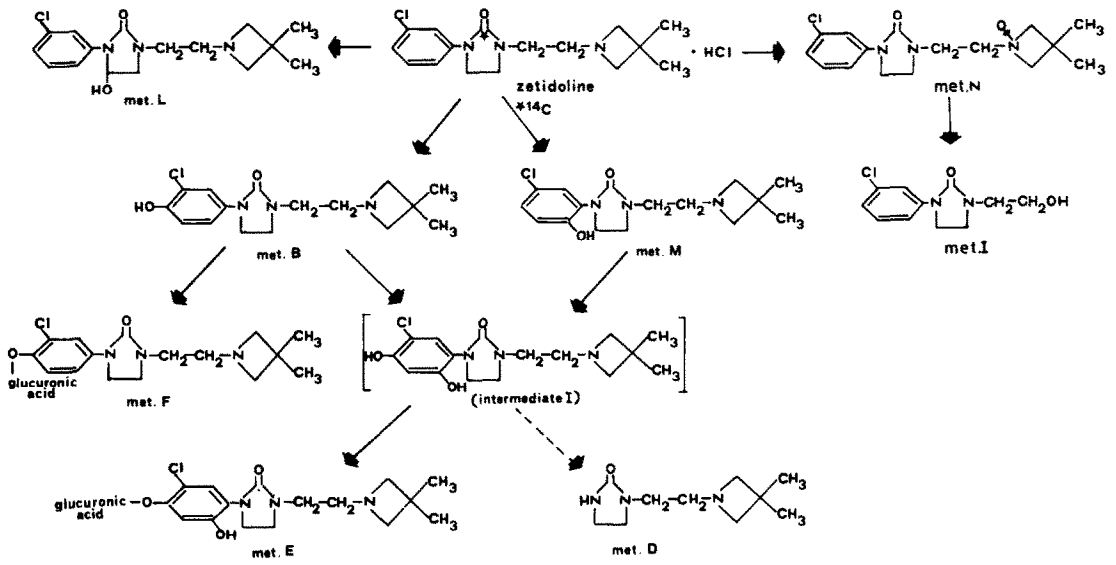


Fig. 3. Proposed metabolic pathways for zetidoline in rat liver microsomes. Dashed arrow indicates a possible chemical transformation.

aromatic ring, but the pattern is different from that of met. B; the assignment of the OH at C-6 is based on the aromatic shifts obtained by additivity rules. The spectral data for both met. L and M are the same as those recorded for the two authentic compounds obtained by chemical synthesis.

Because of the small amounts and their lower degrees of purity, metabolites I and N could be analysed only by EI-MS (Table 1). The structure of met. I as proposed in Fig. 3 is suggested by the following evidence: (i) it has a molecular ion at m/z of 240/242 containing a chlorine atom and its elementary composition, calculated on the basis of the isotopic contribution, is $C_{11}H_{13}ClN_2O_2$; (ii) the fragmentation pattern clearly shows the absence of the azetidine moiety (lack of the ion at m/z 98, $C_6H_{12}N|^+$); (iii) the base peak at m/z 209/211 (metastable at m/z 182) and the peak at m/z 196 indicate losses of the $^{\cdot}CH_2OH$ radical and C_2H_4O radicals; (iv) the peak at m/z 111/113 ($C_6H_4Cl|^+$) confirms the presence of the unchanged aromatic moiety.

Met. N has a molecular ion at m/z 323/325 and contains a chlorine atom. Although it has the same elementary composition as metabolites B, M and L, its fragmentation pattern is markedly different. In fact, the base peak at m/z 114 suggests the introduction of an additional oxygen atom on the azetidine moiety, an hypothesis which is reinforced by the presence of the ion at m/z 209/211 corresponding to $C_{10}H_{10}ClN_2O|^+$. Three key-fragments at m/z 196, 127 and 111 further support the structure proposed in Fig. 3: the first and the second, corresponding to

Table 1. Selected mass spectral data (electron impact, 70 eV) for zetidoline and Metabolites I, L, M and N: ions m/z (relative intensity), and attribution

Zetidoline	Attribution	Metabolite I	Metabolite L	Metabolite M	Metabolite N
307 (3)	$M^{+\cdot}$	240 (25)	323 (1.5)	323 (9.5)	323 (1.5)
251 (1.5)	$[M-C_4H_8]^{+\cdot}$			267 (0.9)	
	$[M-^{\cdot}C_5H_{10}N]^+$			239 (1.3)	
	$[M-^{\cdot}C_5H_{10}NO]^+$				223 (4)
	$[M-H_2O-C_5H_9N]^+$		222 (6.7)		
209 (9)	$[M-^{\cdot}C_6H_{12}N]^+$			225 (4.6)	
	$[M-^{\cdot}CH_2OH]^+$	209 (100)			
	$[M-^{\cdot}C_6H_{12}NO]^+$				209
	$[M-C_2H_4O]^{+\cdot}$	196 (6)			
196 (4)	$[M-C_7H_{13}N]^{+\cdot}$				
	$[M-C_7H_{13}NO]^{+\cdot}$			196 (2.5)	196 (2)
	$[M-H_2O-C_7H_{13}N]^{+\cdot}$		194 (5.8)		
	$[C_6H_4Cl]^+$	111/113 (7)			
	$[C_7H_{13}NO]^{+\cdot}$				127 (7)
111 (16)	$[C_7H_{13}N]^{+\cdot}$		111 (60)	111 (8)	111 (10)
	$[C_6H_{12}NO]^+$				114 (100)
98 (100)	$[C_6H_{12}N]^+$		98 (100)	98 (100)	98 (8)

Table 2. ^{1}H-NMR data for zetidoline (base) and its Metabolites L and M at 270 MHz in DMSO-d_6 (chemical shifts δ, ppm; coupling constants J,Hz)

Compound	$2CH_3$	H-4	H-5	H-6	H-7	H-8,9	H-2'	H-4'	H-5'	H-6'	OH
Zetidoline (base)	1.15 s	3.52 t,J = 7	3.80 t,J = 7	3.14 t,J = 6	2.52 t,J = 6	2.88 s	7.80 dd J = 1.5,2	7.04 ddd J = 2,2,8	7.35 dd J = 8,8	7.43 ddd J = 1.5,2,8	—
Met. L	1.20	3.30 and 3.73 J gem = −11	5.63 J = 6	3.30	2.75	3.30	7.88	7.10	7.40	7.55	6.65
Met. M	1.20	3.52	3.78	3.20	2.75	3.36	7.25 (7.40)*	7.13 (7.01)*	6.91 (6.81)*	—	N.D.

* Calculated values for OH at C-6'.
n.d. = not detected.

$C_9H_9ClN_2O|^{+\cdot}$ and $C_7H_{13}NO|^{+\cdot}$, represent the two complementary fragments 3-chlorophenyl-imidazolidin-2-one and 3-dimethyl, ethylene azetidine N-oxide, while the ion at m/z 111 seems to have been generated from the ion 127 by loss of oxygen [22].

In vitro *metabolism of zetidoline*

When incubated with rat liver microsomes, zetidoline underwent rapid biotransformation to several products. Six of these metabolites B, D, E + F, L and M, were quantified after TLC separation (Table 3): conjugated metabolites E and F and the free metabolite B were the major ones while the minor metabolites D, L and M were produced in almost equivalent amounts. About 20% of the total ^{14}C was accounted for as unidentified products plus metabolites I and N. The time courses of both the disappearance of zetidoline and the formation of the metabolites proceeded linearly up to the 30th minute of incubation. In parallel experiments, heat-killed microsomes did not metabolize the drug.

In vitro *binding studies*

Both ^{3}H-zetidoline and ^{3}H-spiperone are known to label at least two populations of binding sites with decreasing affinity [10]. Reported K'_D and K''_D values, calculated by Scatchard analysis, were of 0.11 and 2.49 nM for zetidoline and of 0.06 and 0.74 nM for spiperone. Two series of compounds were evaluated as inhibitors of ^{3}H-zetidoline and ^{3}H-spiperone binding at concentrations (0.8 and 0.2 nM respectively) corresponding to one-fifth of those saturating both binding sites and surely all the dopaminergic ones [10]. The first series included a few known antagonists at cerebral dopamine receptors while the second series included some chemical analogues along with zetidoline and its metabolites (Table 4).

Metabolites I and N were not synthesized for testing their potential dopaminolytic activity, the first because thought inactive, the second because of methodological difficulties.

For both ^{3}H-ligands the rank order for the inhibition of the specific binding for the reference drugs was: spiperone > haloperidol > chlorpromazine > (±) sulpiride.

Zetidoline and related compounds were more active in displacing ^{3}H-zetidoline than ^{3}H-spiperone. The main discrepancy in the IC_{50} rank order within this group was represented by L9310, which was shown to be the most potent inhibitor of ^{3}H-spiperone binding (35.9 nM) whereas against the ^{3}H-zetidoline binding it was weaker (8.51 nM) than zetidoline (2.56 nM), met. B (3.61 nM) and met. L (4.86 nM).

Among the different metabolites tested, metabolites B and L had IC_{50} values comparable to those of the parent molecule, met. M showed a low affinity for both [^{3}H]zetidoline and [^{3}H]spiperone receptor sites and metabolites C and D were completely ineffective in displacing the two tritiated ligands. Methylation of the m-hydroxy group of met. M (L16270) and unsaturation of the imidazolidinone ring of zetidoline (L15643) led to almost inactive compounds, whereas the affinity of the *p*-methoxy derivative (L14619) was about one-fifth (^{3}H-zetidoline) and one-tenth (^{3}H-spiperone) that of met. B.

Table 3. Zetidoline disappearance and metabolite formation by rat liver microsomes. Zetidoline (10^{-3} M) was incubated at 37° in 5 ml solution containing 3 mg/ml of microsomal protein, 5 mM $MgCl_2$, 0.8 mM $NADP^+$, 8 mM G1-6P and 3.5 U/ml of G1-6P-DH.

	n moles/mg proteins ± S.E.			
	Incubation time (min)			
Compound	5	15	30	60
Zetidoline	*261 ± 11	217 ± 10	133 ± 8	75 ± 8
Met. B	17 ± 1	30 ± 2	57 ± 5	73 ± 6
Met. D	5 ± 0.7	10 ± 1	17 ± 1.2	23 ± 2.1
Met. L	6 ± 0.8	10 ± 0.9	13 ± 1.0	18 ± 1.5
Met. M	6 ± 1.2	9 ± 0.7	13 ± 1.1	16 ± 1.2
Mets E + F	27 ± 2	40 ± 3	80 ± 6	113 ± 10
Others	5 ± 0.6	12 ± 0.9	16 ± 1.4	20 ± 1.8

* Each value is the mean ± standard error for triplicate experiments.

Zetidoline, met. B, L9310 and reference compounds incubated at their IC_{50} concentrations displaced ^{3}H-spiperone competitively since only the affinity of the complex ^{3}H-spiperone-striatal dopamine receptor was significantly lower ($P < 0.01$) in their presence, while the maximum number of binding sites for ^{3}H-spiperone (B_{max}) was not affected by any of them (Table 5).

Effect on prolactin release

Zetidoline, its metabolites and analogues, with haloperidol, chloropromazine and (±)sulpiride as reference compounds, were evaluated *in vivo* for their prolactin releasing activity in female rats. As shown in Table 4, zetidoline (ED_{50} = 2.3 μmoles/kg) was about one-sixteenth as potent as (±)sulpiride and one-eighth as potent as haloperidol, but five times more effective than chloropromazine. Met. B and L9310 had the same activity (ED_{50} = 2.8 and 2.5 μmoles/kg) as the parent drug, met. L and L14619 were a half to one-third as effective and the other compounds had negligible activity (met. M) or none.

DISCUSSION

The results of this *in vitro* metabolic study are fully consistent with those obtained *in vivo* in the rat [12], as they demonstrate that zetidoline is metabolically oxidized mainly on the aromatic moiety of the molecule. The first phase-I reactions on the phenyl ring occur at either carbon 4 (met. B) or 6 (met. M). The dihydroxylated derivative [intermediate I] has never been isolated or detected, probably because it is highly unstable. The hypothesis is that [intermediate I] undergoes chemical degradation to met. D unless stabilized by conjugation with glucuronic acid (met. E).

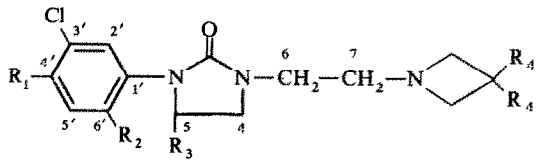
As already seen *in vivo*, phase II reactions account completely for the glucuronidation (metabolites E and F) while sulfoconjugation, which is the major detoxication pathway in man [13], does not seem to occur *in vitro*.

A novel minor route of biotransformation involves the imidazolidinone ring, which is hydroxylated at C-5 to form met. L. Another site for metabolic attack might also be the nitrogen atom of the azetidine ring, which is oxidized to give rise first to met. N then, after hydrolysis, to met. I.

The *in vitro* study of displacement of ^{3}H-zetidoline and ^{3}H-spiperone from their striatal binding sites showed that hydroxylation of either the phenyl (carbons 4 and 6) or imidazolidinone (carbon 5) rings of zetidoline leads to derivatives that still fully (metabolites B and L) or partly (met. M) retain the binding properties of the parent compound. Oxidation of the aliphatic chain at C-7, like the loss of the aromatic moiety, completely abolished the binding affinity (metabolites C and D). Methylation of the m-hydroxyl group of metabolite M and 4,5 unsaturation of the imidazolidinone ring led to compounds (L16270 and L15643) much less effective in displacing both tritiated ligands, while the methylated met. B (L14619), although only one-fifth to one-tenth as potent as the parent molecule still displayed appreciable binding capacity. When the two alkyl chains of the azetidine ring are lengthened, the resulting analogue, L9310, displayed less affinity than zetidoline for the receptors labelled by ^{3}H-zetidoline whereas, it was one of the most effective compounds in displacing ^{3}H-spiperone. The pharmacological profile of L9310 in the rat has already been shown to differ considerably from that of zetidoline, as it was much less effective in the normal conditioned response test and showed poor inhibitory activity on the anticipatory response [1].

The results obtained *in vivo* for zetidoline and its analogues are fully consistent with the *in vitro* results, the only minor exception being L14619 (methylated met. B), which in spite of its reduced ability to displace *in vitro* both ^{3}H-zetidoline and ^{3}H-spiperone, was only one third as effective as zetidoline in stimulating the prolactin release. This finding can be explained, however, by the occurrence of a metabolic first-pass, involving the demethylation of the *p*-methoxy group, allowing a fraction of the

Table 4. *In vitro* inhibition (IC_{50}) of specific ^{3}H-zetidoline and ^{3}H-spiperone binding to rat striatal membranes and prolactin releasing effect (ED_{50}) for reference neuroleptics, zetidoline and its metabolites



Compound	R_1	R_2	R_3	R_4	IC_{50} (nM) on ^{3}H-zetidoline*	IC_{50} (nM) on ^{3}H-spiperone†	ED_{50} i.p. (μ moles/kg)
Zetidoline	H	H	H	CH_3	2.56 ± 0.20 (1)‡	48.3 ± 2.5 (2)	2.3
Met. B	OH	H	H	CH_3	3.61 ± 0.22 (2)	69.5 ± 2.2 (3)	2.8
Met. C§	H	H	H	CH_3	~ 36,000 (9)	~ 36,000 (10)	> 30¶
Met. D	—	—	H	CH_3	> 36,000 (10)	~ 30,000 (9)	> 500¶
Met. L	H	H	OH	CH_3	4.86 ± 0.62 (3)	91.0 ± 3.4 (4)	5.6
Met. M	H	OH	H	CH_3	80.8 ± 3.9 (7)	1,300 ± 90 (6)	> 15¶
L9310	H	H	H	nC_4H_9	8.51 ± 0.44 (4)	35.9 ± 1.5 (1)	2.5
L14619	OCH_3	H	H	CH_3	15.9 ± 1.3 (5)	700 ± 25 (5)	7.4
L16270	H	OCH_3	H	CH_3	8,946 ± 899 (8)	~ 26,000 (8)	14.8
L15643	H	H	Δ4,5‖	CH_3	75.9 ± 5.1 (6)	5,100 ± 355 (7)	n.t
Spiperone					0.18 ± 0.01	0.29 ± 0.03	n.t
Haloperidol					2.03 ± 0.05	3.32 ± 0.20	0.27
Chlorpromazine					4.21 ± 0.40	11.8 ± 0.6	14.1
(±)sulpiride					5.08 ± 0.35	98.8 ± 4.8	0.15

* At 0° per 45 min.
† At 37° per 15 min.
‡ Rank order
§ C-7 is C = 0.
‖ Double bond between carbons C-4 and C-5
¶ Ineffective at the dose shown

Table 5. Scatchard analysis of ^{3}H-spiperone binding to rat striatal membranes in the presence of reference compounds, zetidoline, metabolite B and L9310, at their IC_{50} concentrations

Drug	Concentration (nM)	B_{max} (fmoles specifically bound/mg protein)	K'_a (nM^{-1})	$K'_d \pm$ S.E. (nM)	Regression coefficent
Control	—	0.494 ± 0.015	10.0	0.100 ± 0.010	0.990
Haloperidol	3.3	0.500 ± 0.020	6.25*	0.160 ± 0.015*	0.980
Chlorpromazine	11.8	0.486 ± 0.015	7.14*	0.140 ± 0.010*	0.990
Zetidoline	48.3	0.480 ± 0.010	5.88*	0.170 ± 0.017*	0.985
Met. B	69.5	0.522 ± 0.030	5.48*	0.182 ± 0.020*	0.986
L9310	35.9	0.494 ± 0.019	6.07*	0.160 ± 0.020*	0.995

* $P < 0.01$ vs control (Student t-test).

dose to be bioavailable as met. B. Neither the *in vitro* nor the *in vivo* data here reported provide direct evidence that zetidoline metabolites reach the striatal dopamine receptors after crossing the blood–brain barrier. It is a fact, however, that met. B was found active *in vivo* in classic pharmacological tests [14] and that met. L retains intermediate physicochemical properties between those of zetidoline and met. B. This would suggest, in conclusion, that also met. L displays anti dopaminergic activity.

The previous hypothesis that in animals or in man [12, 13] zetidoline might act as a pro-drug for met. B, has been disproved by the present evidence, since both zetidoline and met. B are highly effective *in vitro*. Pro-drugs could be instead designed for both metabolites B and L, that besides retaining a high dopaminolytic activity, bear hydroxyl groups that can be used for a number of chemical linkages (i.e. esther bonds with long chain fatty acids) readly hydrolized by serum enzymes.

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