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Biotransformation of linogliride, a hypoglycemic agent in laboratory animals and humans

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Abstract

Following oral administration of linogliride, a hypoglycemic agent, to rat (50 mg kg^{-1}), dog (30 mg kg^{-1}), and man (100 mg per subject), plasma, urine, and fecal extract sample pools were obtained. Nine metabolites plus unchanged linogliride were isolated and identified. The number of metabolites identified were: rat (5), dog (9), and man (1). In each species, more than 78% of the administered dose was recovered in the urine pools. Identified metabolites were estimated to account for >82% of the total amounts of drug-related sample in urine pools and >50% in plasma and fecal extract pools.

Formation of linogliride metabolites in the three species can be described by four proposed pathways: pyrrolidine hydroxylation, aromatic hydroxylation, morpholine hydroxylation, and imino-bond cleavage. Comparison of the proposed metabolic pathways among species reveals a similarity between rat and dog. In these two species, pyrrolidine hydroxylation was quantitatively the most important pathway, with 5-hydroxylinogliride and dominant hypoglycemic active metabolite in all sample pools. Further oxidation of 5-hydroxylinogliride resulted in the formation of five minor metabolites. The other three pathways appeared to be quantitatively unimportant.

Metabolism of linogliride in man occurred to a very limited extent. More than 90% of the total linogliride-related material in plasma was the unchanged drug. Greater than 76% of the administered dose was excreted unchanged in the urine. Only 5-hydroxylinogliride was identified in minor amounts in human samples.

Keywords: Biotransformation; Dog; Human; LC; Linogliride; Metabolite identification; MS; NMR; Rat

1. Introduction

Linogliride is a new oral hypoglycemic agent in type II diabetes, which differs both structurally and mechanistically from the biguanides and sulfonylureas [1-14]. The drug has been shown to be orally effective in fasted mice, rats, dogs, monkeys, and humans [2-14]. Linogliride stimulated insulin secretion in vivo and enhanced glucose-stimulated insulin secretion from perfused rat pancreas and isolated islets, but was inactive in pancreatectomized dogs [7]. Preliminary results from our laboratories on the disposition, pharmacokinetic, and metabolism of linogliride in Wistar rats, beagle dogs, and humans have been reported [15-19]. A very active hypoglycemic metabolite, 5-hydroxylinogliride, has been synthesized and patented [20,21]. This paper describes the isolation and identification of linogliride and nine metabolites

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in plasma, urine, and feces from rat, dog, and human. In addition, the metabolites in each sample pool were quantified and pathways by which linogliride was metabolized were proposed. These metabolic pathways were compared to establish their relative quantitative importance.

2. Materials and methods

2.1. Chemicals and materials

Linogliride - ^{14}C - fumarate - N - (1 - methyl - 2 - pyrrolidinylidene) - N′ - phenyl - 4 - morpholinecarbox-^{14}C-imidamide fumarate was synthesized at The R.W. Johnson Pharmaceutical Research Institute (Spring House, PA) with radiochemical purity >99% (by TLC and LC). Appropriate dilutions of the labeled material were made with unlabeled drug to obtain the desired specific activity for the various studies. The drug was used at a specific activity of 9.37 μCi mg^{-1} for the rat and 9.23 μCi mg^{-1} for the dog. Unlabeled linogliride fumarate was used in human studies.

Linogliride fumarate and reference compounds for metabolites Ia, Ib, Id, and II were synthesized at The R.W. Johnson Pharmaceutical research Institute [1,2,7,8,20]. The structures for these compounds are presented in Fig. 1.

Diazald, used to generate diazomethane, was purchased from Aldrich (Milwaukee, WI). Glusulase was obtained from Endo Laboratories (Wilmington, DE). Biofluor was purchased from New England Nuclear (Boston, MA). LC grade solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ), and glass distilled solvents were purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Other reagent grade chemicals were obtained from commercial sources and were used without further purification.

2.2. Sample source and preparation

Sample pools were obtained from ADME studies in eight rats (4M, 4F) and eight dogs (4M, 4F) [14–17], and from a metabolic study in 12 humans [18,19]. Plasma, urine and fecal samples were collected in polypropylene tubes or bottles and stored at −20 °C. Four working sample pools (plasma, untreated urine, Glusulase®-treated urine, and fecal extracts (except human)) were prepared from each study. The dose, sampling interval, and percentage of administered dose in each sample pool are shown in Table 1.

The Glusulase®-treated urine aliquots were adjusted to pH 5.1 with glacial acetic acid and buffered with 1 M sodium acetate solution (pH 5.1). Glusulase® (1% by volume) was added to this mixture and allowed to react overnight at 37 °C. The fecal extract pool for each species was prepared by combining equal percentages of the methanolic fecal extracts from each animal for the sampling interval reported in Table 1.

2.3. Analytical equipment

Total radioactivity in each sample before and after purification was determined by liquid scintillation counting of 0.1–0.5 ml aliquots of each sample in 10 ml Biofluor. The samples were counted in a Tracor Analytic 81 liquid scintillation counter (Tracor Instruments, Lebanon, NJ). Counting efficiency was determine to be >92% by the use of an external standard.

The thin-layer radiochromatographic analyses of the samples were conducted using a chloroform–acetone–ammonium hydroxide solvent system (20:30:1, v/v/v). The TLC plates were analyzed using a BID System 100 radiochromatogram Imaging System (Bioscan, Inc., Washington, DC).

A CRT-based gradient liquid chromatograph (model 344) equipped with variable wavelength UV detector (Model 165, Beckman Instrument, Columbia, MD) was used for all LC analyses. The LC guard and analytical column (13 cm × 4.6 mm i.d. packed with 10 μm LiChorsorb Rp-2 particles) used for LC analyses were purchased from Brownlee Laboratories, Inc. (Santa Clara, CA).

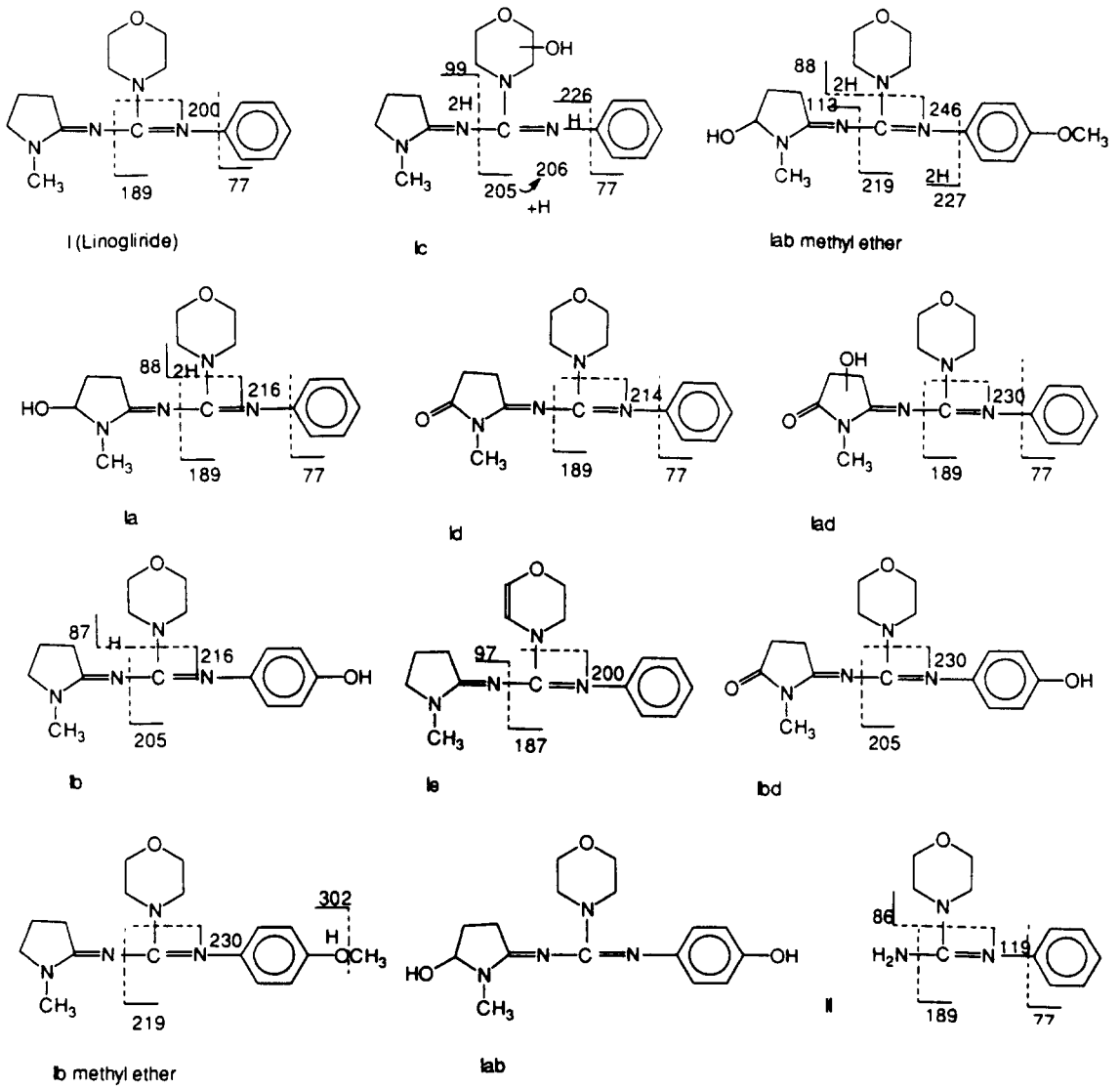
EI-MS and CI-MS were performed using a VG 7035 mass spectrometer (VG Micromass, Manchester, UK) and a Finnigan Model 3300 quadrupole mass spectrometer (Finnigan, Sunnyvale, CA). Methane was used as the reagent gas for the CI-MS. Samples were deposited on the Vg desorption CI probe tip (Ir–Pt alloy; pyrolyzed before use) before analysis.

All NMR spectra were determined using a Brucker Fourier tansform spectrometer (Model WM 360, Bruker Instruments, Inc., Manning

Park, Billerica, MA). The samples were dissolved in $CDCl_3$ and TMS was generally used as an interval standard. Chemcial shifts were reported in ppm (δ) downfield from TMS (0 ppm). The IR spectra were recorded using a Nicolet FT-IR model 60SX.

2.4. Metabolite isolation and identification

Prior to TLC and LC analysis, all samples were purified using the procedures shown in Table 1. Each LC analysis was performed at a wavelength of 254 nm. A gradient elution method was used for each 100 µl sample injected. Solvent A was water containing 0.005 M ammonium acetate with acetic acid at pH 4.5, while Solvent B was methanol containing the same percentage of buffers. The solvent flow rate was maintained at 1 ml min^{-1}. The gradient program was performed from 0% B to 100% B in 15 min, and maintained at 100% B for 1 min. All ^{14}C tracings were obtained using an on-line radioactive flow detector (Flo-one, Model HS). Table 2 contains the LC R_t values observed for linogliride and its metabolites. Each metabolite collected from the LC was reinjected into the LC and monitored (at 254 nm) for purity. Those requiring no further purification were evaporated to dryness to yield residues which were lyophilized to remove the ammonium acetate buffer. A representative LC biotransformation profile of dog urine is presented in Fig. 2. The dried residues were analyzed by IR, ^1H NMR, and MS. Fractions



(a)

Fig. 1(a).

Metabolite/derivative	CI [a]
I (linogliride)	($MC_2H_5^+$,6.8), 287(MH^+,100), 200(47.7), 189(43.2), 77(3.4)
Ia	331($MC_2H_5^+$,4.5), 303(MH^+,31.8), 285($MH^+ - H_2O$,100), 216(11.4), 189(11.4), 88(18.2)
Ib	303(MH^+,25), 302(100), 285($MH^+ - H_2O$,18.8), 216(18.8), 206(12.5)
Ib methyl ether	345($MC_2H_5^+$,8.2), 317(MH^+,100), 302(23.5), 230(49), 219(46.9)
Ic	303(MH^+,14.1), 285($MH^+ - H_2O$,19.7), 231(7), 206(100), 205(38), 99(91.5)
Id	329($MC_2H_5^+$,21.4), 301(MH^+,100), 214(35.7), 189(64.3)
Ie	313($MC_2H_5^+$,6.8), 285(90.9), 200(100), 187(22.7), 97(4.5)
Iab methyl ether	361($MC_2H_5^+$,1.9), 333(MH^+,25), 315($MH^+ - H_2O$,45), 246(10), 219(26.3), 113(26.3), 88(100), 86(43.8)
Iad	345($MC_2H_5^+$,6), 317(MH^+,100), 285(51.8), 230(39.8), 189(72.3), 139(14.5), 77(8.4)
Ibd	345($MC_2H_5^+$,7), 317(MH^+,15.7), 316(62.8), 230(27.9), 205(100), 113(39.5)
II	234($MC_2H_5^+$,9.3), 206(MH^+,100), 189(20.5), 175(6.8), 120(4.5), 88(52.3)
	EI [b]
I (linogliride)	288(M^+,59.1), 255(4.5), 200(90.9), 189(4.5), 124(34.1), 109(100), 77(43.2)
Ia	302($M^{\cdot +}$,34.1), 294($M^{\cdot +} - H_2O$,100), 216(31.8), 189(65.9), 140(11.4), 125(40.9), 77(52.3)
Ib	302($M^{\cdot +}$,45.2), 284(13.7), 271(4.1), 216(32.9), 205(19.2), 87(100)
Ic	302($M^{\cdot +}$,15.7), 284($M^{\cdot +} - H_2O$,20.5), 226(3.6), 205(61.4), 77(100)
Id	300($M^{\cdot +}$,96.4), 269(10.7), 214(100), 189(10.7), 138(25), 123(60.7), 77(60.7)
Ie	284($M^{\cdot +}$,8), 200(100), 97(9.1)
Iab methyl ether	332($M^{\cdot +}$,78.6), 314($M^{\cdot +} - H_2O$,71.4), 246(50), 227(100), 219(50), 86(78.6)
Iad	316($M^{\cdot +}$,86.9), 285(17.9), 230(59.9), 189(38.1), 139(90.5), 77(100)
II	205($M^{\cdot +}$,90.7), 174(18.6), 160(16.3), 148(30.2), 119(83.7), 77(100)

[a] Adduct M^+ and prominent fragments m/z (%RI).
[b] $M^{\cdot +}$ and prominent fragments m/z (%RI).
(b)

Fig. 1. (a) Structures and (b) summary of MS data for linogliride and its metabolties.

containing two or more unresolved components were further purified using the LC or TLC method. The radioactive bands from each TLC plate were individually removed by zone scraping and extracted with a mixture of ethyl acetate and methanol (80:20, v/v). The isolated material was then analyzed by LC, IR, NMR, and MS.

Table 1
Sources of pooled samples and purification procedures used for LC and TLC biotransformation profiles and metabolite isolation of linogliride in three species (% a = % of administered dose represented by sample. % b = % of sample recovered after purification)

	Source (Oral dose)	Plasma (0–24 h)			Urine [d] (0–48 h)			Fecal extract (0–48 h)		
		% a	Purif. proc.	% b [a]	% a	Purif. proc.	% b [a]	% a	Purif. proc.	% b
Rat	50 mg kg^{-1}	– [b]	A	81.4%	81.4%	A	97.9%	17.9%	C	50%
Dog	30 mg kg^{-1}	– [b]	A	100%	78.0%	A	86.2%	12.7%	C	47.5%
Man	100 mg per subject	– [b]	B	>90% [c]	>80% [e]	B	>90% [c]	– [f]	– [f]	– [f]

Purification procedures: A = lyophilized sample, extracted with methanol; B = extracted with ethyl acetate; C = evaporated sample to dryness, extracted the residue with acetic acid, washed with ether, basified with NH_4OH and extracted with ethyl acetate.
[a] The radioactive profiles were comparable before and after purification procedures; therefore, the purified sample is representative to the original sample.
[b] % a cannot be calculated; plasma pools were composed of equal aliquots from each sample collected during the sampling interval.
[c] Values were estimated by comparison of chromatograms (UV trace) before and after purification.
[d] Data for untreated and Glusulase-treated urine pools were identical.
[e] Values were estimated by summation of all metabolite peaks (including linogliride) in the chromatogram (UV trace).
[f] Fecal samples were not collected.

Table 2
TLC R_f values and LC R_t values of linogliride and linogliride metabolites

Metabolite	TLC R_f value	LC R_t interval (min)	Chemical abstract name
I	0.65–0.70	12.2–12.9	*N*-(1-Methyl-2-pyrrolidinylidene)-*N'*-phenyl-4-morpholinecarboximidamide
Ia	0.42–0.46	10.1–10.8	*N*-(5-Hydroxy-1-methyl-2-pyrrolidinylidene)-*N'*-phenyl-4-morpholinecarboximidamide
Ib	0.11–0.15	10.2–10.8	*N'*-(4-Hydroxyphenyl)-*N*-(1-methyl-2-pyrrolidinylidene)-4-morpholinecarboximidamide
Ic	0.17–0.22	9.5–10.5	2- or 3-hydroxy-*N*-(1-methyl-2-pyrrolidinylidene)-*N'*-phenyl-4-morpholinecarboximidamide
Id	0.85–0.90	15.5–16.0	*N*-(1-Methyl-5-oxo-2-pyrrolidinylidene)-*N'*-phenyl-4 morpholinecarboximidamide
Ie	0.76–0.81	14.3–14.6	3,4-Dihydro-*N*-(1-methyl-2-pyrrolidinylidene)-*N'*-phenyl-2*H*-1,4-oxazine-4-carboximidamide
Iab	0.00–0.05	8.0–8.8	*N*-(5-Hydroxy-1-methyl-2-pyrrolidinylidene)-*N'*-(4-hydroxyphenyl)-4-morpholinecarboximidamide
Iad	0.55–0.60		*N*-(3- or 4-Hydroxy-1-methyl-5-oxo-2-pyrrolidinylidene)-*N'*-phenyl-4-morpholinecarboximidamide
Ibd	0.30–0.35	–	*N'*-(4-Hydroxyphenyl)-*N*-(1-methyl-5-oxo-2-pyrrolidinylidene) -4-morpholinecarboximidamide
II	0.50–0.55	7.6–8.6	*N*-Phenyl-4-morpholinecarboximidamide

All metabolites were characterized by desorption CI-MS (methane as a reagent gas) and EI-MS. Mass spectra were compared with those of authentic samples, if available, for further structural confirmation. ^{1}H NMR and IR spectra were obtained for some of the metabolites along with those of authentic samples. Overall, each metabolite was identified using a combination of three or more characteristics obtained from TLC, LC, IR, NMR, and CI/EI-MS analyses. Some of the metabolites were derivatized using diazomethane and identified as the corresponding methyl ethers.

3. Results

Liquid chromatographic and TLC traces showing the biotransformation profile were obtained for a total of eight pooled samples from three species. The sources, sampling intervals, and percentages of administered dose represented by each sample are summarized in Table 1. The purification procedures utilized for each sample are also presented in Table 1.

Linogliride and nine metabolites were isolated, quantified, and identified. The structures of linogliride, its metabolites, and their MS data are shown in Fig. 1. The structures of linogliride metabolites have been divided into two types—linogliride analogs (I) and depyrrolidinated linogliride (II). The assignments a, b, and c for the Type I metabolites designate hydroxylated products at three different rings of the linogliride molecule, while "d" designates an "oxo" product and "e" an "olefinic" product. LC retention times and TLC R_f values for the metabolites are presented in Table 2.

The percentage of sample or dose represented by each metabolite, and the percentage of significant drug-related material (>5% sample or dose) identified in each sample pool, are summarized in Table 3. Metabolites accounting for <5% of sample are represented by single plus symbols. Identified metabolites and linogliride represent greater than 72% of the drug-related material in plasma and urine samples. However, about 40–50% of the total radioactivity in the fecal extract pools, representing 5–10% of the dose, was not identified.

Each metabolite was analyzed by CI–MS and/or EI-MS. The molecular ion, and the prominent fragments observed for each of the metabolites and their derivatives, are presented in Fig. 1. The ^{1}H NMR data observed for some of the metabolites are summarized in Table 4.

Unchanged linogliride (I) was isolated in all sample pools using LC. The identity of linogliride was confirmed by TLC, LC (Table 2), NMR (Table 4), and MS (Fig. 1) by comparison with an authentic sample [1]. The

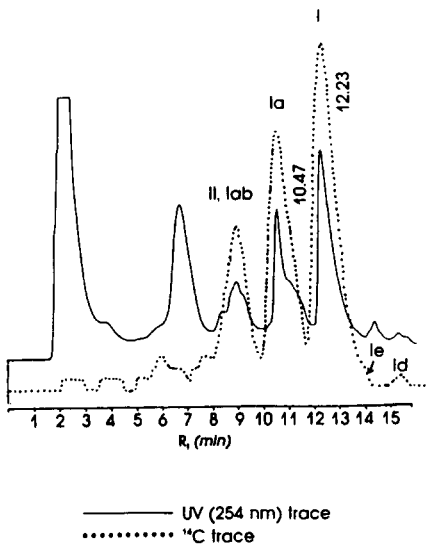


Fig. 2. LC profile of linogliride metabolites in dog urine.

CI-MS and EI-MS of the isolated material gave an intense protonated molecular ion and an apparent molecular ion at m/z 287 (100%) and m/z 286, respectively (Fig. 1). Several prominent ions at m/z 200, 189, 124, and 109 (CN-pyrrolidine ion) in both CI and EI are the characteristic fragments of linogliride. Linogliride was present in significant quantities in the plasma (>53%), urine (>33%), and fecal samples (>5%) of all three species (Table 3).

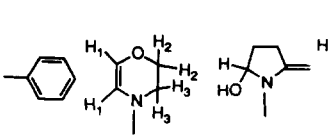
Metabolite Ia, a very active hypoglycemic metabolite, which showed equal potency as linogliride was isolated by LC and identified by IR, ^{1}H NMR, MS, and synthesis [20,21]. The IR spectrum of the isolated material showed a hydroxyl absorption band at 3300 cm^{-1}. Mass spectrometric analyses of Ia revealed an intense protonated molecular ion at m/z 303 and a molecular ion at m/z 302 in CI-MS and EI-MS, respectively (Fig. 1 and 3). The molecular ion is 16 u higher than linogliride, indicating the addition of an oxygen as a hydroxyl group, as the base peaks observed at m/z 285 ($MH^+ - H_2O$) and 284 ($M^{\cdot+} - H_2O$) for the CI-MS and EI-MS, respectively, were associated with the dehydration of an alcohol. Ions at m/z 189, 216, and 125 in both CI-MS and EI-MS indicated that the hydroxyl group was located on the pyrrolidine ring. The ^{1}H NMR data (Table 4) clearly attributed the hydroxyl group at the 5-position of the pyrrolidine ring to the presence of 5-methine proton at δ4.90 and the disappearance of a triplet at δ3.15 to two methylene protons present in the linogliride. Metabolite Ia was found as a major metabolite in plasma (>19%) and urine (>18%) samples from the rat and dog, and as a minor metabolite in plasma and urine samples from man and fecal samples from rat and dog (Table 3). Ia is present in a ring-closed form (carbinolamine) as evidenced by the ^{1}H NMR data (Table 4). This metabolite was identified as synthetic 5-hydroxylinogliride on the basis of their identical TLC, LC, NMR, and MS data [20,21].

Table 3
Amount of total drug-related material represented by linogliride and its metabolites in each sample

Sample species	Plasma$^{0-48\,h}$ (% sample)			Urine$^{0-48\,h}$ (% dose)			Feces$^{0-48\,h}$ (% dose)	
	Rat	Dog	Man [a]	Rat	Dog	Man [a]	Rat	Dog
I	63.5	53.6	>90.0	40.0	33.0	>76.0	+	5.4
Ia	25.1	19.2	+	26.1	18.8	+	8.8	+
Ib				+	+		+	+
Ic					+			
Id	+			+	+		+ [b]	+ [b]
Ie	+ [b]				+		+ [b]	+ [b]
Iab					+			
Iad					+			
Ibd								+
II	+ [b]	+ [b]			+		+	+ [b]
% Total drug-related material identified in each sample	>90	>73	>90 [a]	>82	>85	>97 [a]	>60	>50

+: amount present is less than 5% dose for urine and feces; % sample for plasma pools.
[a] Drug was not radiolabeled in the human study. Values were estimated by LC/UV.
[b] Metabolite identity confirmed by TLC and LC, but no spectroscopic data were generated.

Table 4
NMR spectral data for linogliride and its metabolites

Compound	Chemical shifts (δ)								
									
I (linogliride)	6.75-7.15(m)			3.47(t)	3.55(t)	3.17(t)	2.82(S)	2.15(t)	1.65(q)
Ia	6.78-7.15(t)		4.89(s)	3.70(t)	3.48(t)		2.82(S)	2.15(t)	1.95(q)
Id	6.80-7.15(m)			3.75(t)	3.48(t)		2.97(S)	2.38(S)	2.38(S)
Ie	6.95-7.20(m)	6.05(S),$2H_1$ 4.15(t),$2H_2$ 4.05(t),$2H_3$				3.30(t)	2.98(S)	2.35(t)	1.75(q)
II	6.90-7.10(m)			3.65(t)	3.32(t)				

Metabolite Ib was identified by LC, TLC, and MS anaylses in comparison with a synthetic sample. The CI and EI mass spectra of the isolated metabolite from dog urine showed an adduct ion at m/z 303 and a molecular ion at m/z 302, respectively (Fig. 1). Two significant fragment ions at m/z 216 (CI and EI) and 205 (EI) indicated the presence of a hydroxyl group at the phenyl ring. The isolated product was derivatized using diazomethane. The CI-MS of the resulting methyl ether product (Fig. 1) gave a base peak of m/z 317 for a protonated molecular ion and two prominent ions at m/z 230 and 219. These MS data clearly indicated the methoxy group on the aromatic ring. The NMR data of the methyl ether product revealed a well-defined AA′ BB′ quartet for four aromatic protons of the 4-methoxyphenyl ring. Metabolite Ib was present in minor amounts only in urine and fecal samples from both rat and dog (Table 3).

Metabolite Ic was isolated using LC and identified by MS. The mass spectra of Ic are shown in Fig. 1. CI-MS and EI-MS gave a protonated molecular ion and a molecular ion at m/z 303 and m/z 302, respectively. The prominent fragment ions at m/z ($MH^+ - H_2O$, CI), 284 ($M^{\cdot +} - H_2O$, EI), 226 (EI), 206 (CI), 205 (CI and EI), and 77 are consistent with the proposed structure, indicating a hydroxyl group attached to the morpholine ring of the molecule due to the fragment ions of m/z 205 and 77. The exact position of the hydroxyl group at the morpholine ring has not been confirmed. Metabolite Ic was found only in dog urine in minor quantities (Table 3).

Metabolite Id was isolated by LC and TLC and identified by IR, NMR, and MS. The EI-MS of Id exhibited an intense molecular ion at m/z 300. The MW of Id was further confirmed by CI-MS by the presence of a protonated ion at m/z 301 for MH^+. The MS data indicated that the isolated metabolite contained an oxo group (Fig. 1). Two key fragment ions at m/z 214 and 189 (CI and EI), along with two pyrrolidine ions at m/z 138 and 123 (EI) suggested an oxo group that was attached to the pyrrolidine ring. The IR spectrum also indicated an intense carbonyl absorption at 1656 cm^{-1}. The ^1H NMR spectrum for Id (Table 4) showed the disappearance of two methylene protons (at C_5 of pyrrolidine) at δ3.15 and two pairs of methylene protons (at C_4 and C_3 of pyrrolidine) at δ1.65 and δ2.15, respectively, present in the linogliride spectrum (Table 4). The latter four protons collapsed to give a singlet at σ2.40 (Table 4). The overall IR, MS, and NMR data indicated the structure for metabolite Id to be 5-oxo-linogliride, which was finally confirmed by synthesis [20,21]. Id was found in small quantities in the plasma samples from rat, and urine and fecal samples

from rat and dog (Table 3), which is a similar to the findings for bepridil [22,23].

Metabolite Ie was identified using IR, NMR, and MS. The IR spectrum of the isolated material indicated a carbon–carbon double bond absorption at 1623 cm^{-1}. The CI and EI mass spectra contained two diagnostic ions at m/z 200 (base peak in CI and EI) and 187 (CI), an intense protonated molecular ion (CI) at m/z 285 and a weak molecular ion (EI) and m/z 284 (Figs. 1 and 4). These MS data were used to assign a double bond in the morpholine ring. The ^1H NMR data (Table 4) clearly defined two olefinic protons of a morpholine ring at δ6.10. Metabolite Ie was observed in small quantities in rat plasma, dog urine, and rat and dog feces, and could be an artifact of metabolite Ic (Table 3).

Metabolite Iab was isolated in trace amounts as a methyl ether from dog urine by LC and TLC after derivatization using diazomethane. The structure of Iab was tentatively proposed on the basis of the CI-MS and EI-MS data (Fig. 1). A weak molecular ion (EI) was observed in m/z 332 and an apparent MH^+ ion (CI) at m/z 333. Intense dehydrated ions at m/z 315 (CI) and 314 (EI), and several important ions at m/z 246 (CI and EI), 227 (EI), 219 (CI and EI), and 113 (CI), clearly indicated two oxygenated (phenolic and alcoholic (at pyrrolidine)) sites of the metabolite. The proposed positions of the hydroxyl groups were based on the substitution positions of metabolites Ia and Ib.

The structure of metabolite Iad was tentatively proposed based on CI- and EI-MS data (Fig. 1). An intense molecular ion adduct at m/z 317 (100%, CI) and an apparent molecular ion at m/z 316 (EI) indicated the addition of an oxo and an alcoholic group to the linogliride molecule. The prominent ions at m/z 230, 189, and 139 (CN-OH-oxo-pyrrolidine ion) (CI and EI) led to assigned of both oxo and alcoholic groups to the pyrrolidine ring. The proposed position for the oxo group was based on the substitution position of metabolite Id. The exact position of the hydroxyl group is still unconfirmed. Iad was found to be a minor metabolite (< 5%) in dog urine (Table 3).

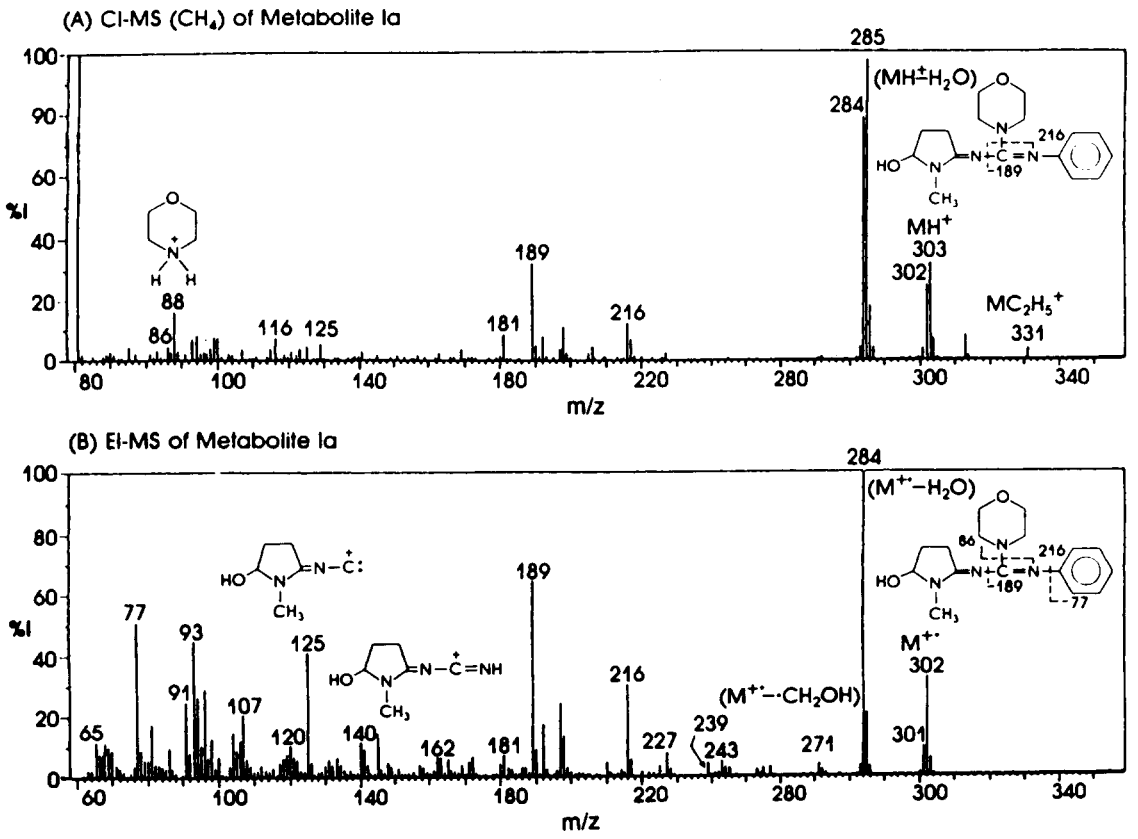


Fig. 3. CI-MS (methane) and EI-MS of metabolite Ia isolated from dog urine.

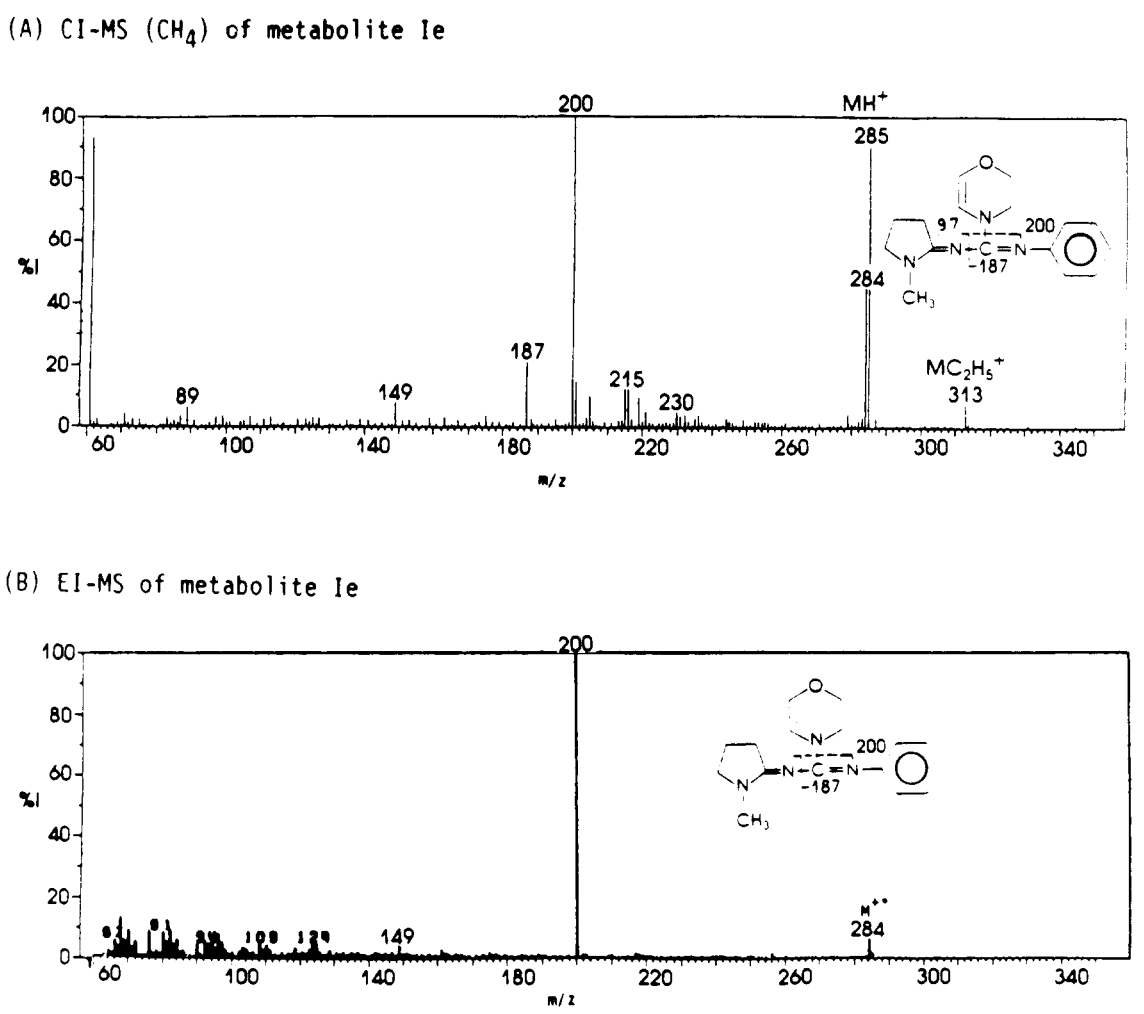


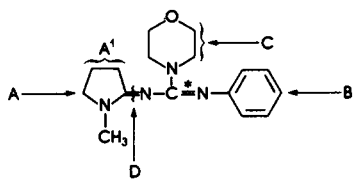
Fig. 4. CI-MS (methane) and EI-MS of metabolite Ie isolated from dog urine.

Metabolite Ibd was isolated in small amounts by LC and TLC from dog feces (Table 3). The CI-MS of the isolated substance displayed a protonated molecular ion at m/z 317 (Fig. 1). The prominent ions at m/z 230, 205, and 113 (H_2N-oxo-pyrrolidine ion) were consistent with the proposed structure. The proposed oxygenated positions of Ibd are based on the substitutional positions of Ib and Id.

Metabolite II was identified by comparing TLC, LC, NMR, and MS (CI and EI) data for the isolated material with those obtained for an authentic sample (C.R. Rasmussen, unpublished data). CI- and EI-MS provided an intense adduct molecular ion at m/z 206 (100% CI) and an apparent molecular ion at m/z 205 (EI). Several prominent ions were observed at m/z 189 (CI), 120 (CI, EI), 119 (EI), 88 (CI), and 77 (EI) (Fig. 1). The ^1H NMR data revealed two clearly-defined triplets at δ3.34 and δ3.70 attributed to eight morpholine protons, and a multiplet between δ6.90 and δ7.32 attributed to five aromatic protons (Table 4). Metabolite II was found in small quantities in rat and dog plasma, and feces and dog urine (Table 3). It could be an artifact from linogliride and/or metabolites Ia, Id and Iad.

4. Discussion

A total of nine linogliride metabolites and unchanged linogliride was identified in the three species studied. Formation of these metabolities in the three species can be described by four proposed pathways: hydroxylations at each of the three rings (pyrrolidine, phenyl, and morpholine) of the linogliride molecule and imino-bond cleavage (Fig. 5).



SITES OF REACTION	METABOLIC REACTIONS
A, A'	Pyrrolidine oxidation
B	Aromatic hydroxylation
C	Morpholine hydroxylation/dehydration
D	Imino-bond cleavage

* Site of ^{14}C label

Fig. 5. Sites of oxidation and corresponding metabolic reactions involving linogliride.

The Type I metabolites (linogliride series, Fig. 1) are the oxidation products at the three rings: A (pyrrolidine), B (phenyl), and/or C (morpholine). Further oxidation at ring A produced three "oxo" metabolites. The Type II metabolite is formed as a result of imino-bond cleavage via addition of water. Proposed metabolic pathways for linogliride for each species are shown in Fig. 6. Pyrrolidine hydroxylation at the 5-position of pyrrolidine was quantitatively the most important pathway [22,23], forming a hypoglycemic active metabolite, 5-hydroxyliongliride [20,21]. Biotransformation pathways for individual species are discussed as follows:

In the rat (Figs. 5 and 6; Table 3), pyrrolidine hydroxylation appears to be the only major pathway, resulting in the formation of significant amounts of Ia in plasma, urine, and feces. Further oxidation of Ia to form Id, and oxidation of I to form Ib and Ie are minor pathways [22,23]. The imino-bond cleavage via addition of water to form II is not important in the rat.

In the dog (Figs. 5 and 6; Table 3), similar to the rat, hydroxylation at the pyrrolidine ring to form 5-hydroxylinogliride (Ia) appears to be quantitatively the most important step. The subsequent steps for the formation of Id, Iab, Ibd, and Iad via further oxidation of Ia are less important. Again, aromatic hydroxylation and oxidation/dehydration at the morpholine ring seem to be minor pathways. The imino-bond cleavage to form a depyrrolidinated product (II) is not important in the dog.

In man (Figs. 5 and 6; Table 3), the major compound found in plasma and urine is unchanged linogliride. More than 76% of the administered dose is excreted unchanged in the

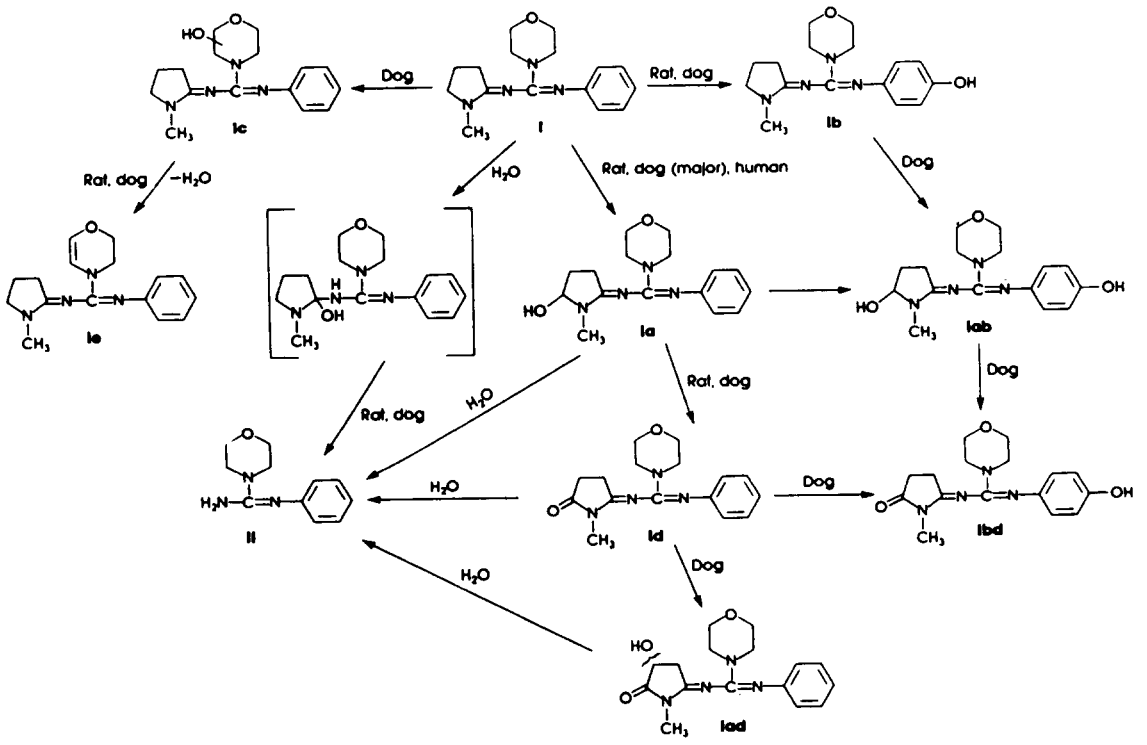


Fig. 6. Proposed metabolic pathways for linogliride in rat, dog and man.

urine [13,18,19]. Pyrrolidine hydroxylation is the only pathway observed in man, but the resulting metabolite (Ia) represented trace amounts of the sample in both plasma and urine.

Glusulase® hydrolysis of plasma, urine and fecal samples from all species did not reveal any conjugates, and therefore linogliride and its metabolities are believed to be present in unconjugated forms.

In conclusion, the rat has similar metabolic pathways to the dog; however, man is different from the other two species in that the extent of metabolism of linogliride in man is very limited.

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