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# Metabolic fate of the hypoglycemic agent pirogliride in laboratory animals and humans

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**Abstract**

Metabolism of the hypoglycemic agent, pirogliride, was investigated in the rat, dog, monkey and human. Unchanged pirogliride plus six metabolites were isolated and identified using solvent extraction, HPLC and CI and EI–MS from urine and fecal samples. Pirogliride was metabolized in man to a small extent by oxidation of the 4-position of the phenyl ring. The monkey metabolized pirogliride mainly by oxidation of the pyrrolidine rings, while oxidation of the phenyl ring was the minor pathway. In contrast to the monkey, the rat metabolized pirogliride primarily by oxidation of the phenyl ring. The dog showed a balance of oxidation between the phenyl and pyrrolidine rings. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Pirogliride; Rat; Dog; Monkey; Human; Urine; Feces; Drug metabolism; HPLC; MS

## 1. Introduction

Pirogliride, *N*-(1-methyl-2-pyrrolidinylidene)-*N′*-phenyl-1-pyrrolidine-carboximidamide (Fig. 1), tartrate is a new compound possessing oral hypoglycemic activity in mouse [1,2], rat [1–6,9], dog [1–3], monkey [1,2], and man [1,7–9]. It differs both structurally and mechanistically from the biguanides and sulfonylureas [2,3,5]. Preliminary metabolism and disposition reports in rat, dog, monkey, and man have indicated good absorption and rapid excretion of the compound [10–13]. The objectives of this study were to profile and identify the pirogliride metabolites and to understand the metabolic fate of pirogliride in these species.

## 2. Materials and methods

### 2.1. Chemical

$^{14}$C-Pirogliride was synthesized with the $^{14}$C-label at the position shown in Fig. 1. The specific activity of 3.1 μCi mg$^{-1}$ was used in animal studies; 0.314 μCi mg$^{-1}$ in human studies. The chemical and radiochemical purity of each lot was greater than 97% (TLC HPLC). Pirogliride and 4-hydroxyphenyl pirogliride (metabolite IV) (Fig. 1) were used as TLC, HPLC, and MS reference standard. Glusulase®, a mixture of arylsulfatase and β-glucuronidase (1:4 v/v) from *Helix pomatia*, was purchased from Endo. β-glucuronidase, type II, from *E. Coli* and sulfatase, type III, a mixture of phenol sulfatase and arylsulfatase from


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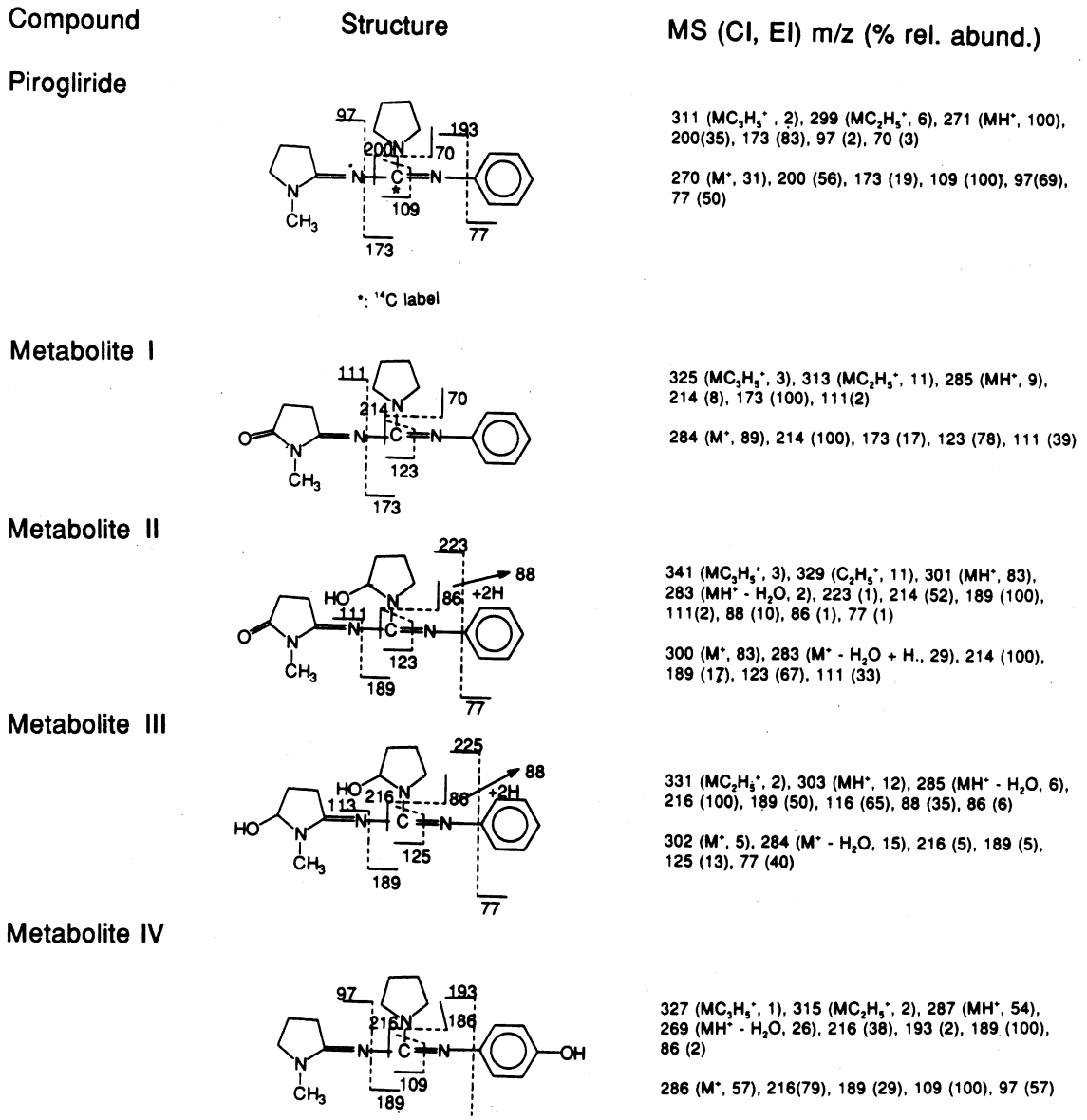


Fig. 1. Structures and mass spectral data for pirogliride, its metabolites and the dervatives.

limpets were obtained from the Sigma (St. Louis, MO). Diazald® was obtained from the Aldrich (Milwaukee, WI). Biofluor™ (New England Nuclear, Boston, MA) was used as a scintillation solution. PIC-B7 was purchased from Waters (Milford, MA). Glass distilled solvents were obtained from Burdick and Jackson (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 absorption and excretion studies in rats (3 M, 3 F, 100 mg $kg^{-1}$ dose), dogs (3 M, 3 F, 40 mg $kg^{-1}$ dose)

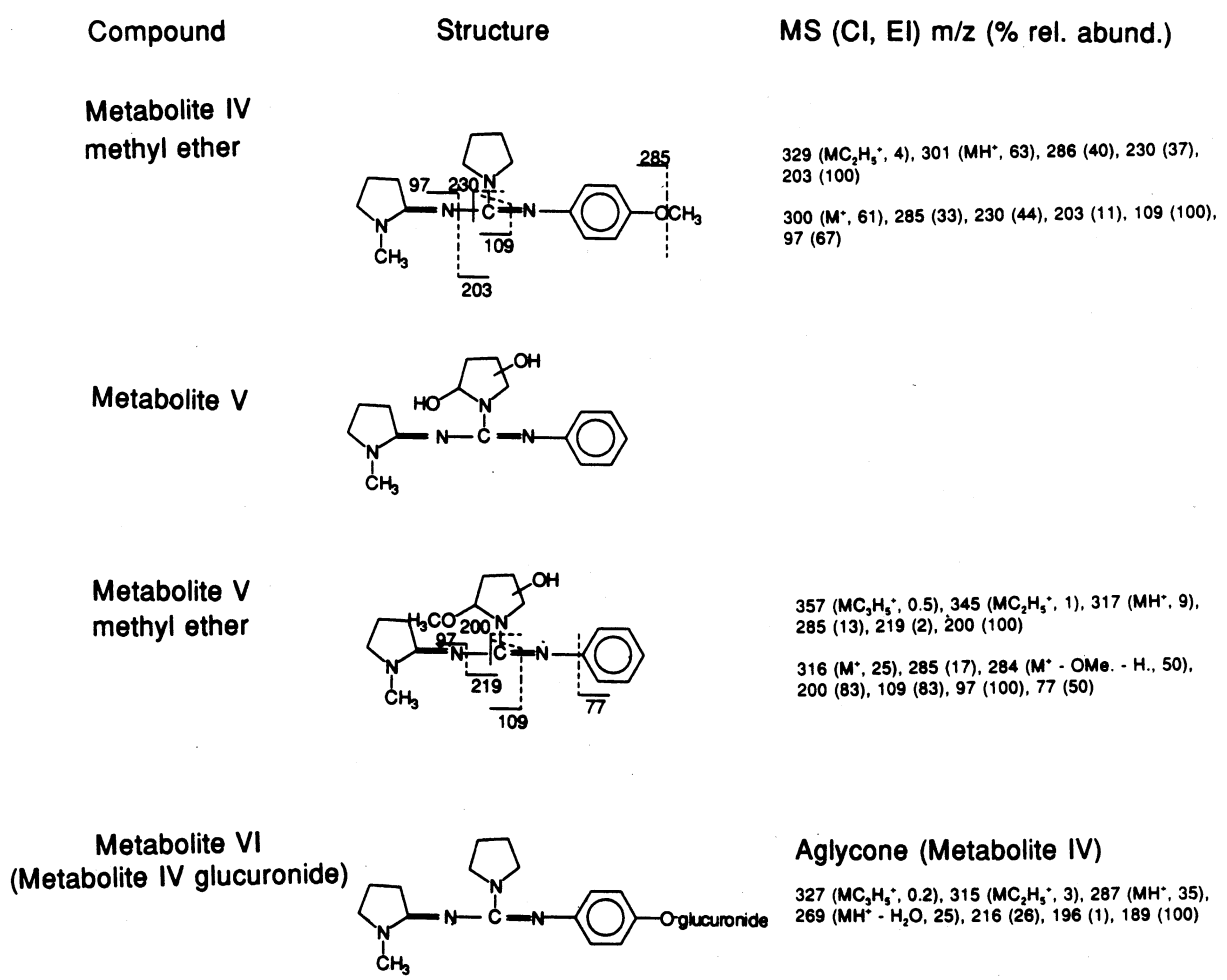


Fig. 1. (*Continued*)

and monkeys (3 M, 3F, 50 mg $kg^{-1}$ dose) [10]; and from a metabolic study in humans (8 M, 200 mg $subject^{-1}$) following single oral doses of $^{14}C$-pirogliride [10]. Urine (all species) and fecal extract (0–24 h, rat only) pools were prepared from each species for metabolite profiling, isolation and identification. Urine aliquots (50 ml) were adjusted to pH 5.1 with glacial acetic acid and buffered with 1 m sodium acetate solution (pH 5.1, 10% by volume). Glusulase (1% by volume) was added to this mixture and allowed to hydrolyze for 4 h at 37°C. The fecal extract (50 ml) was evaporated to dryness, reconstituted with methanol and the precipitate was removed by filtration. Recoveries of extractions from fecal samples were more than 90% of the sample.

### 2.3. Analytical equipment

Total radioactivity in each sample before and after purification was determined by liquid scintillation counting of 0.1 ml aliquots of each sample in 10 ml Biofluor. The samples were counted in a Searle Analytic 81 liquid scintillation counter. Counting efficiency was determined to be $>92\%$ by the use of an external standard. The thin-layer radiochromatographic analysis of the samples were conducted using ethanol/cyclohexane/diethyl

ether/ammonium hydroxide (55/15/25/5, by volume) as the solvent system. The silica gel plates were analyzed using a Berthold Radioscanner (LB 2760). HPLC system used was an Altex Scientific Model 100 Solvent Metering System equipped with a 254 nm detector (Altex Model 153). A 1 ml sample loop injection valve (Rheodyne) and a Tracor 980 A solvent programmer. HPLC column used was 30 cm $\times$ 4 mm i.d. $\mu$-Bondapak C18 packed column (Waters, Milford, MA). Mobile phases used were system A [acetonitrile–water (75/25, v/v)] and system B [acetonitrile–water (50/50, v/v)] with each liter of water containing 5 ml of PIC-B7 Reagent at 2 ml $min^{-1}$ flow rate. EI–MS analyses were conducted using an Hitachi Perkin Elmer mass spectrometer (Model RMU-6) by direct inlet at 70 eV. CI–MS analyses were performed by direct inlet with methane as the reagent gas using a Finnigan Model 9500–3300–6100 GC/MS/DS mass spectrometer system. The mass spectrometer was operated at an electron energy of 100 eV, source temperature 100°C and a source pressure of 1 torr for methane.

### 2.4. Metabolite isolation and identification

Urine samples (3 ml) from each species studied were mixed with 3 ml of methanol and centrifuged. Aliquots (50 μl) of the supernatant (extraction efficiency: $>80\%$) from each urine sample were injected onto the HPLC column. Each metabolite collected from the HPLC was reinjected into the HPLC and monitored at 254 nm for purity. Those requiring no further purification were evaporated to remove acetonitrile, basified with ammonium hydroxide, and extracted with ethyl acetate (6 ml). Recoveries of extractions were more than 80% of the sample. The ethyl acetate extract residues were analyzed by CI–MS and EI–MS. The Glusulase-hydrolyzed urine and fecal extract samples were also separated as above. All metabolites were characterized and identified by desorption CI (methane as a reagent gas) and EI mass spectrometry. Mass spectra were compared with those of synthetic samples, if available, for further confirmation. Some of the metabolites were derivatized as methyl ethers using ethereal diazomethane [14].

## 3. Results and discussion

HPLC and TLC biotransformation profiles were obtained for the urine pool (0–24 h) from each species and for the fecal extract (0–24 h) from the rat. HPLC $R_t$ times and TLC $R_f$ values for unchanged pirogliride and its metabolites are presented in Table 1. Representative HPLC profiles for the rat urine and fecal extract and human urine are presented in Fig. 2. HPLC and TLC profiles were comparable between untreated and solvent extracted samples. Pirogliride and six metabolites were isolated and identified. The structures of pirogliride, its metabolites, and their MS data are shown in Fig. 1.

Unchanged pirogliride was isolated and identified as one of the major components in urine (all species) and rat fecal extract ($>30\%$ of the sample). The CI-mass spectrum of this product exhibited an intense protonated molecular ion at $m/z$ 271 with two adduct ions at $m/z$ 299 ($MC_2H_5^+$) and $m/z$ 311 ($MC_3H_5^+$), consistent with the formula $C_{16}H_{22}N_4$ (Fig. 1). The two prominent fragment ions at $m/z$ 200 and 173 from CI–MS are also observed with authentic pirogliride (Fig. 1). The EI–mass spectrum confirmed the findings of the CI–MS analysis (Fig. 1). Further confirma-

Table 1
HPLC $R_t$ and TLC $R_t$ values of pirogliride and its metabolites

| Compound | HPLC $R_t$ (min) | TLC $R_t$ |
|---|---|---|
| Pirogliride | 5.8 | 0.38–0.43 |
| Metabolite I | 3.0 | 0.87–0.92 |
| Metabolite II | 3.0 | 0.71–0.78 |
| Metabolite III | 2.9 | 0.11–0.16 |
| Metabolite IV | 4.0 | 0.06–0.11 |
| Metabolite IV conjugate (metabolite VI) | 1.5 | 0.01–0.05 |
| Metabolite IV methyl derivative | — | 0.14–0.19 |
| Metabolite V | 3.9 | 0.05–0.10 |
| Metabolite V methyl derivative | — | 0.45–0.50 |

HPLC mobile phase: acetonitrile–water (75:25 v/v) (system A) each liter of water contained 5 ml of PIC-B7 reagent.
HPLC column: $\mu$-Bondapak® $C_{18}$ packed column (Waters).
TLC solvent system: ethanol/cyclohexane/ether/ammonium hydroxide (55/15/25/5 by volume).
TLC plate: silica gel GF (5 $\times$ 20 cm, 250 m).

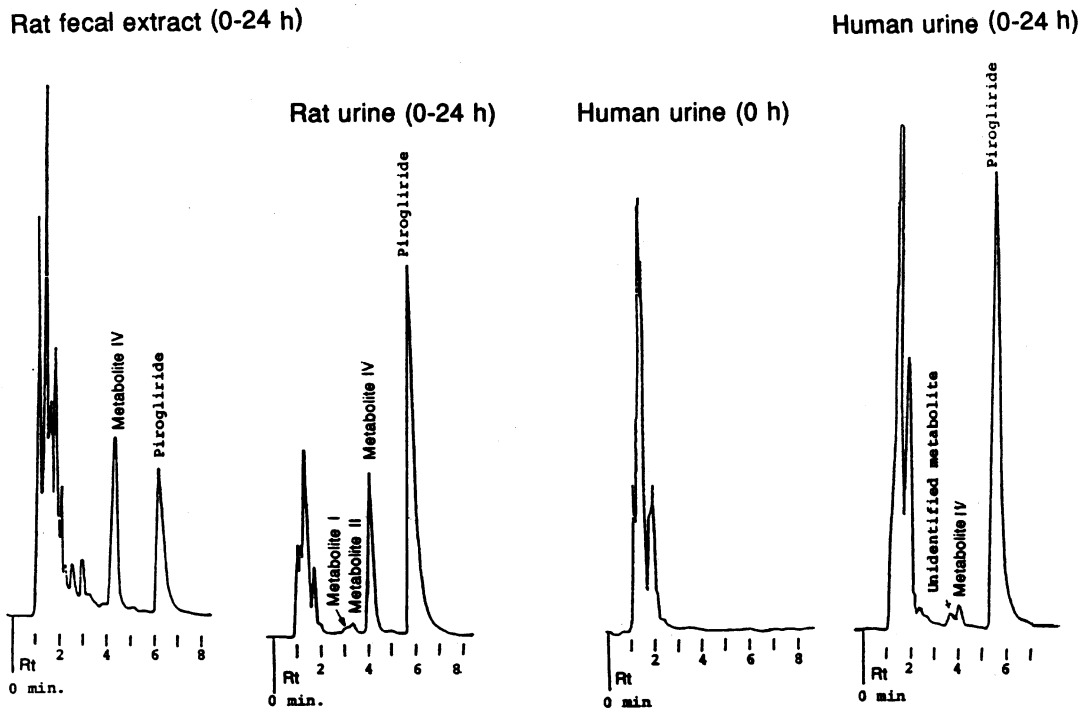


Fig. 2. HPLC metabolic profiles for the rat and human samples.

tion was obtained by direct TLC and HPLC comparison of the urinary product to authentic pirogliride.

Metabolite I, *N*-(1-methyl-5-oxo-2-pyrrolidinylidene)-*N'*-phenyl-1-pyrrolidinecarboximidamide, was isolated as a major product from monkey (22% of the sample) and dog urine (10% of the sample). The CI mass spectrum (Fig. 1) showed an intense protonated molecular ion at *m/z* 285 with two adduct ions at *m/z* 313 ($MC_2H_5^+$) and 325 ($MC_3H_5^+$) corresponding to the formula of $C_{16}H_{20}N_4O$, 14 amu higher than pirogliride. The two intense and significant fragment ions at *m/z* 214 and 173, when compared with the fragment ions at *m/z* 200 and 173 from authentic pirogliride suggested the structure of metabolite I tentatively assigned (Fig. 1). The EI–MS (Fig. 1) also revealed a molecular ion at *m/z* 284 and the two intense ions at *m/z* 214 and 173.

Metabolite II, 2-Hydroxy-*N*-(1-methyl-5-oxo-2-pyrrolidinylidene)-*N'*-phenyl-1-pyrrolidinecarboximidamide, was isolated as a major urinary metabolite in both monkey (12% of the sample) and dog (8% of the sample). The compound gave a negative phenol test [15]. The structure of the metabolite was tentatively proposed by means of CI and EI–MS. The EI mass spectrum contained a molecular ion at *m/z* 300 and the CI mass spectrum showed an intense protonated molecular ion at *m/z* 301 together with two adduct ions at *m/z* 329 ($MC_2H_5^+$), and 341 ($MC_3H_5^+$). These data are consistent with the formula $C_{16}H_{20}N_4O_2$, 30 amu higher than pirogliride and 16 amu higher than metabolite I (Fig. 1). The two intense and significant fragment ions at *m/z* 214 and 189 present in both CI and EI–MS supported the structure proposed for the metabolite, in which a ketone is located on the *N*-methyl-pyrrolidine ring and an alcohol is located on the other pyrrolidine ring (Fig. 1). An alternative structure for metabolite II was carboxy-pirogliride ($M_W$ 300) which was formed via further oxidation of 2-hydroxy-

pyrrolidinyl ring of pirogliride. However, it was eliminated, since the MS fragmentation patterns did not support the structure of carboxy-pirogliride. Metabolites I and II were detected by HPLC as trace amounts in rat urine (Fig. 2).

Metabolite III, 2-Hydroxy-*N*-(5-hydroxy-1-methyl-2-pyrrolidinylidene)-*N'*-phenyl-1-pyrrolidinecarboximidamide, was present as a major metabolite in both monkey (24% of the sample) and dog (12% of the sample) urine. The compound gave a negative phenol test. The EI mass spectrum gave a molecular ion at $m/z$ 302 and the CI mass spectrum revealed an intense protonated molecular ion at $m/z$ 303 with an adduct ion at $m/z$ 331 ($MC_2H_5^+$) corresponding to a formula $C_{16}H_{22}N_4O_2$, 32 amu higher than pirogliride and 2 amu higher than metabolite II (Fig. 1). A rather intense ion appearing at $m/z$ 285 ($MH^+ - H_2O$), was strongly indicative of a hydroxy group on the molecule (Fig. 1). The two ions at $m/z$ 216 and 189 indicate that the molecule was a diol (Fig. 1), oxidation having occurred on both pyrrolidine rings.

Metabolite IV, *N'*-(4-hydroxyphenyl)-*N*-(1-methyl-2-pyrrolidinylidene)-1-pyrrolidinecarboximidamide was identified by TLC, HPLC, and MS as a major metabolite in the rat urine (52% of the sample) and fecal extract (30% of the sample) and as a minor product in man ($< 5\%$ of the sample), monkey ($< 5\%$ of the sample) and dog ($< 5\%$ of the sample) (Table 2). A conjugate of metabolite IV (metabolite VI) (12% of the sample) constituted one of the major urinary products in the dog. It appeared as a polar metabolite on TLC. Glusulase® hydrolysis of the polar conjugate led to the isolation of a less polar aglycone. The isolated aglycone and metabolite IV showed a molecular ion at $m/z$ 286 in EI–MS and a rather intense protonated molecular ion with two adduct ions at $m/z$ 287 ($MH^+$), 315 ($MC_2H_5^+$) and 327 ($MC_3H_5^+$) in CI–MS, consistent with the formula of $C_{16}H_{22}N_4O$, 16 amu higher than pirogliride (Fig. 1). The intense fragment ions at $m/z$ 216, 193 and 189 present in both CI and EI–MS were strongly suggestive of a phenolic group (Fig. 1). A positive phenolic test for the hydrolyzed product supported the structure. Final confirmation was made by direct comparison of TLC mobility and both CI and EI–MS from the hydrolyzed product to an authentic sample (Table 1 and Fig. 1). Individual treatment of the aglycone and authentic sample with ethereal diazomethane solution yielded methylated products, which gave identical CI and EI–MS. The methyl derivatives showed apparent molecular ions at $m/z$ 300 in EI–MS and an intense protonated molecular ion with an adduct ion at $m/z$ 301 ($MH^+$) and 329 ($MC_2H_5^+$) in CI–MS (Fig. 1). These findings indicate the formation of methyl ether as proposed in Fig. 1. The two important fragment ions at $m/z$ 230 and 203 appeared in both CI and EI–MS and were consistent with the structure of the product assigned (Fig. 1). A very minor amount of 4-hydroxyphenylpirogliride conjugate was detected in monkey urine by direct comparison of HPLC profiles, before and after Glusulase® hydrolysis of urine. No significant changes in urinary HPLC profile were observed in humans after Glusulase® hydrolysis.

Metabolite V, 2,3 (or 5)-dihydroxy-*N*-(1-methyl-2-pyrrolidinylidene)-*N'*-phenyl-1-pyrrolidinecarboximidamide, was present as a major urinary metabolite in the dog (18% of the sample) and was isolated as a methyl derivative after diazomethane treatment. The CI–MS revealed an intense protonated molecular ion with two adduct ions at $m/z$ 317 ($MH^+$), 345 ($MC_2H_5^+$), and 357 ($MC_3H_5^+$) (Fig. 1). These data are in agreement with the formula $C_{17}H_{24}N_4O_2$. The important fragment ions present in CI–MS were consistent with the structure tentatively assigned (Fig. 1). The EI–MS also showed a molecular ion at $m/z$ 316 and a series of important ions similar to those present in the CI–MS (Fig. 1).

Table 2
Percent of sample for pirogliride and its metabolites in urines

| Compound | Rat | Dog | Monkey | Human |
|---|---|---|---|---|
| Pirogliride | 60 | 30 | 28 | 90 |
| Metabolite I | 2 | 10 | 22 | — |
| Metabolite II | 3 | 8 | 12 | — |
| Metabolite III | — | 12 | 24 | — |
| Metabolite IV | 30 | 5 | 5 | 4 |
| Metabolite V | — | 18 | — | — |
| Metabolite VI | — | 12 | — | — |
| Total % of sample identified | 95 | 94 | 91 | 94 |

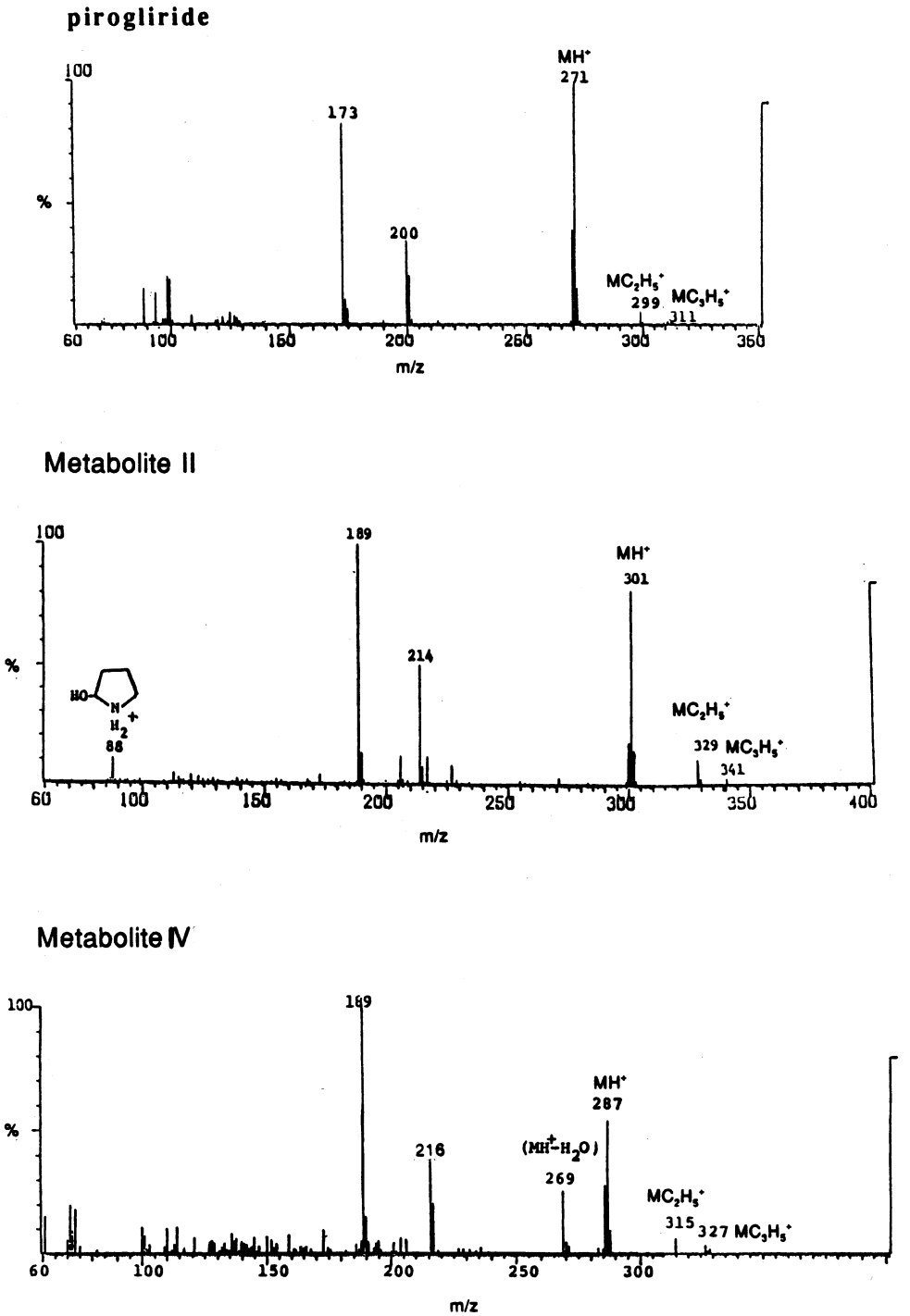


Fig. 3. Representative CI-mass spectra for pirogliride and metabolites II and IV.

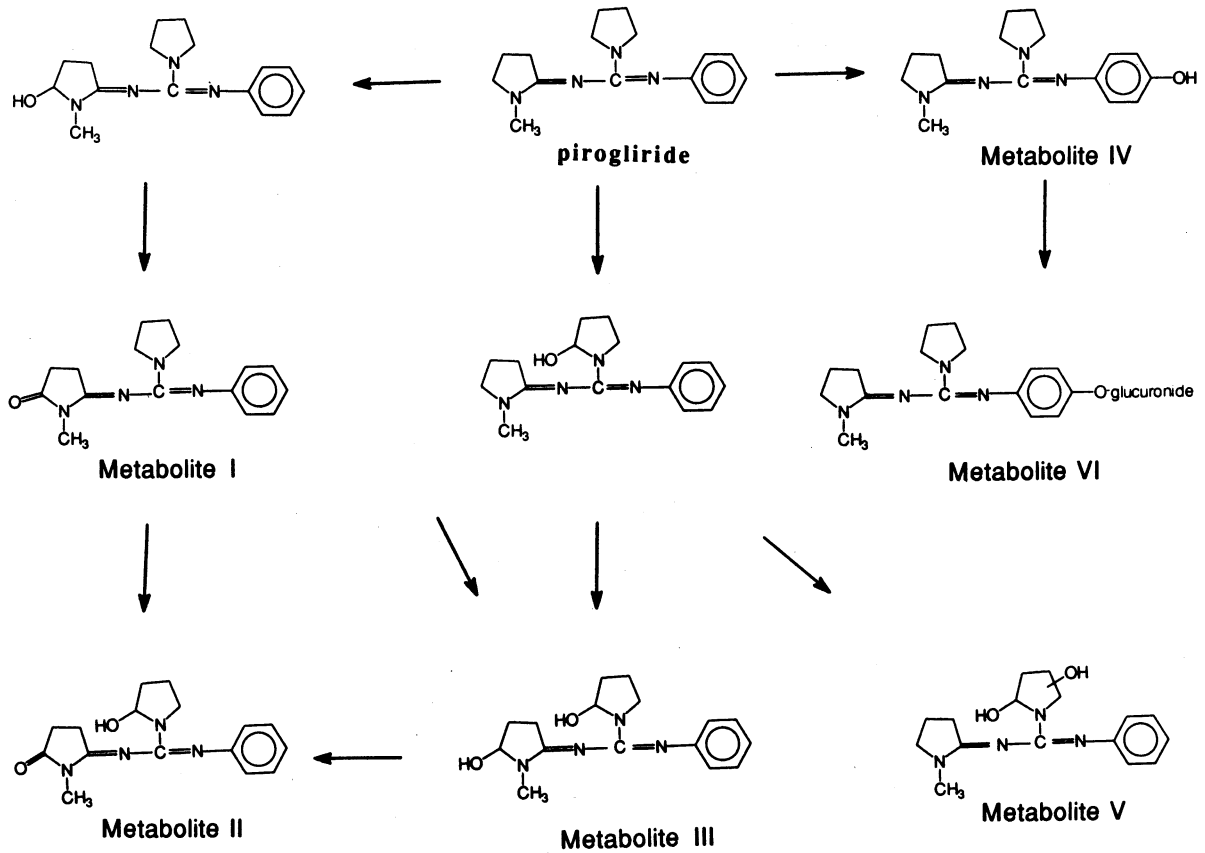


Fig. 4. Proposed metabolic pathways for pirogliride in the rat, dog, monkey and human.

Representative CI-mass spectra for pirogliride and two metabolites (II and IV) are presented in Fig. 3.

A total of six metabolic products of pirogliride tartrate, metabolites I, II, III and IV, a conjugate of metabolite VI and metabolite V were isolated and characterized from healthy human subjects, monkeys, dogs, and rats. The proposed biotransformation pathways for pirogliride in these species are shown in Fig. 4.

## 4. Conclusion

In the rhesus monkey and the dog, one of the initial metabolic pathways would appear to be the formation of a monoalcohol probably at the position adjacent to the nitrogen on either of the two pyrrolidine rings; however, these metabolites were not found in this investigation. Further oxidation of the proposed monoalcohol compounds forms either a monoketone derivative, i.e. metabolite I, or oxidative attack at the other pyrrolidine ring or at the same ring forms diol derivatives, i.e. metabolite III, or V, respectively. All of these compounds have been isolated and characterized as metabolic products. Further oxidation of metabolite I at the second pyrrolidine ring or oxidation of metabolite III forms metabolite II as a final product.

A second pathway is the oxidation of the aromatic ring to form 4-hydroxyphenylpirogliride (metabolite IV) a minor product in monkey and man, but a major product (metabolite VI) in rat and dog. This metabolite (IV) was excreted either free (rat) or conjugated (dog and monkey).

Unchanged pirogliride was observed in all species. Pirogliride is not extensively metabolized in humans which is quite different from the dog, rat, and monkey.

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