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Assessment of the biotransformation of the cardiotonic agent piroximone by high-performance liquid chromatography and gas chromatography–mass spectrometry

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ABSTRACT

^{14}C-labelled piroximone was administered to rats at a dose of 10 mg/kg body weight. Of the total radioactivity administered, 74.9 ± 7.9% ($n=4$) and 87.8 ± 1.7 ($n=3$) were recovered in the 8-h urine collection after oral and intravenous administration, respectively. Two major metabolites, M_1 and M_2, were detected in methanol extracts and accounted for 7.1 ± 1.2% ($n=4$) (M_1) and 4.3 ± 0.4% ($n=4$) (M_2) in response to oral administration and 5.7 ± 0.8% ($n=3$) (M_1) and 6.7 ± 2.0% ($n=3$) (M_2) in response to intravenous administration. In addition, three minor metabolites were detected; M_3 and M_4 in the 8-h urine collection and M_5 in the 12-h urine collection. Separation of piroximone and metabolites was achieved by high-performance liquid chromatography on a C_{18} column by gradient elution with 0.05 *M* ammonium acetate (pH 7) using 0–60% methanol over 20 min at a flow-rate of 1 ml/min, followed by isocratic elution with 60% methanol for 10 min. M_1 and M_2 were isolated by fraction collection following the addition of 1 m*M* tetrabutylammonium acetate in the mobile phase. Between each injection a column re-equilibration time of 45 min was necessary to achieve optimum collection of M_1 and M_2 fractions. Gas chromatography–mass spectrometry of M_1 provided evidence for a molecular structure consistent with isonicotinic acid methyl ester. Corroborative evidence for this identification was obtained by comparison with a synthetic standard. Isonicotinic acid is assumed to be the actual metabolite while esterification with methanol had occurred as a result of the work-up procedure. *In vitro* studies carried out with rat liver microsomes resulted in a mean total metabolite formation rate of 94.3 pmol/mg microsomal protein/min.

INTRODUCTION

Piroximone [4-ethyl-1,3-dihydro-5(4-pyridinylcarbonyl)-2*H*-imidazol-2-one; MDL 19205] (Fig. 1), is a cardiotonic agent with positive inotropic properties [1–3]. It is a specific inhibitor of a high-affinity cAMP phosphodiesterase type III [4] and is being developed for the treatment of congestive heart failure [5]. The pharmacokinetics of piroximone in healthy volunteers and patients with congestive heart failure have been reported previously [6]; however, its metabolism and the unequivocal identification of metabolites remained to be evaluated.

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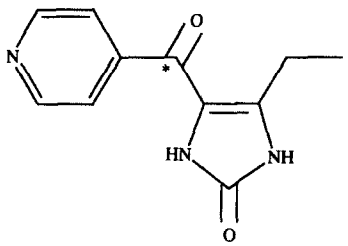

* 14C

Fig. 1. Structure of piroximone and ^{14}C-label position.

The present report presents results on the separation and tentative identification of *in vivo* and *in vitro* biotransformation products of piroximone.

EXPERIMENTAL

Reagents and chemicals

Unlabelled piroximone was provided as ampoules containing 1.5 mg/ml in physiological saline (total volume 20 ml) and was packed by the Marion Merrell Dow Research Institute (Winnersh, UK). ^{14}C-labelled piroximone with a specific activity of 17.4 Ci/mol was synthesized by Dr. Eugene R. Wagner (Marion Merrell Dow Research Institute, Indianapolis, IN, USA). The position of the radiolabelled carbon atom is shown in Fig. 1. Hydroxypiroximone (MDL 20770) was obtained from Marion Merrell Dow Research Institute (Cincinnati, OH, USA), isonicotinic acid and ammonium acetate were purchased from Sigma (St. Louis, MO, USA), methanol (spectroscopic grade) from Merck (Darmstadt, Germany) and tetrabutylammonium acetate from Aldrich (Milwaukee, WI, USA).

In vivo metabolic study

Adult male Sprague–Dawley rats with an average body weight of 200 g were obtained from Charles River (Cléon, France). They were fasted and acclimatized to metabolic cages 16 h before dosing and subsequent collection of urine and faeces.

Each rat received a single dose of 10 mg/kg body weight of piroximone (*i.e.*, 46 μmol/kg) containing 100 μCi of ^{14}C-labelled piroximone in a 0.9% NaCl isotonic saline solution. The dose solution was administered alternatively by gastric intubation or intravenously into a tail vein. The volume infused did not exceed 1.3 ml. Food and water were given *ad libitum*. Urine and faeces were collected over 48 h at time intervals of 0–8, 8–12, 12–24 and 24–48 h. All samples were stored at −20°C prior to analysis.

In vitro metabolic study

Induction in vivo of drug metabolizing enzymes. Adult female Sprague–Dawley rats (n = 3) with an average body weight of 200 g were treated at 10 a.m. with a single intraperitoneal injection of 600 μmol/kg body weight of Aroclor 1254 dissolved in corn oil and were killed 5 days later. Aroclor 1254 is a mixture of polychlorinated biphenyls (PCBs). The latter induce distinctively isoforms of cytochrome P-450 dependent monooxygenases, as detailed previously [7].

Preparation of hepatic microsomes. Control and treated rats were killed and the livers were weighed and washed free of superficial blood, cooled to 4°C and used within 1 h. All of the operations described below were performed at 4°C.

The liver was finely chopped with scissors and homogenized for 2 min in an MSE homogeniser with 0.15 *M* KCl (3 ml/g wet weight of liver). The homogenate was then centrifuged at 11 000 g_{av} for 30 min using an 8 × 50 or an 8 × 14 ml titanium angle-head rotor in an MSE 3 Prepspin 50 or a Sorvall OTD 50B ultracentrifuge. The supernatant was centrifuged at 104 000 g_{av} for 60 min and the microsomal pellet resuspended in 0.15 *M* KCl using a Potter–Elvejhem glass–Teflon homogenizer. The microsomal suspension was then washed by recentrifugation at 104 000 g_{av} for a further 30–60 min and the pellet was resuspended in 0.25 *M* sucrose–20 m*M* Tris buffer–5 m*M* EDTA (pH 7.4) at a final concentration of *ca.* 20 mg protein/ml [9].

In vitro metabolic assay. Hepatic microsomes that were isolated from control and treated rats were incubated with 0.55 μmol of piroximone containing 2.2 μCi of ^{14}C-labelled piroximone by adjusting the microsomal protein concentration to *ca.* 10 mg (equivalent to 0.5 g of liver) per flask. The incubations were carried out in a shaking water-bath at 37°C for 20 or 40 min with a buffer system containing 1.8 mmol of Tris buffer (pH 7.4), 0.6 mmol of $MgCl_2$, 2.2 mmol of nicotinamide and 0.2 ml of an NADPH-generating system consisting of 21.2 μmol of glucose-6-phosphate, 4.6 μmol of $NADP^+$ and 1.6 I.U. of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49). The enzymatic reactions were stop-

ped by protein precipitation with 40% trichloroacetic acid (Merck) [8].

Sample treatment

Radioactivity was measured by liquid scintillation using an LS 5000 CE counter (Beckman, San Ramon, CA, USA) and Aquasol-2 (Dupont de Nemours, France). Urine samples and liver supernatant samples were added to the scintillator directly. Radioactivity in faecal samples was measured after treatment with Lumasolve (Lumac, Landgraaf, Netherlands) according to the manufacturer's recommendations.

Piroximone and metabolites were extracted from urine samples with the addition of methanol, followed by centrifugation at 10 500 *g* for about 10 min (Zentrifuge 3200, Eppendorf, Hamburg, Germany); the supernatant contained >99% of radioactivity. A similar recovery was obtained with other biological samples.

High-performance liquid chromatography (HPLC)

The chromatograph consisted of a WISP Model 710 B automatic sample injector (Waters, Milford, MA USA), two pumps (Waters Model 6000), a gradient programmer (Waters Model 660), a Waters Model 450 variable-wavelength UV detector and a Flo-one HP-HS radioactivity detector (Radiomatic, Tampa, FL, USA). Quickszint Flow 302 (Zinsser Analytic, Maidenhead, UK) was used as the scintillator at a flow-rate of 4 ml/min using a liquid cell with a volume of 1.6 ml. Samples were separated on an IP-Ultrasphere C_{18} (5-μm particles size) column (25 cm × 4.6 mm I.D.) (Beckman, San Ramon, CA, USA) that was connected with a Waters μBondapack C_{18} guard column.

UV detection of piroximone and metabolites was carried out at 230 mm [9]. The separation of piroximone and metabolites was achieved using a combination of gradient and isocratic elution as follows: the two major metabolites (M_1 and M_2) were eluted by concave gradient elution (curve type 9) using 0.05 *M* ammonioum acetate (pH 7) and 0–60% methanol over a period of 20 min at a flow-rate of 1 ml/min followed by an isocratic elution of the two minor metabolites (M_3 and M_4) and of piroximone with 60% methanol for a period of 10 min.

The HPLC conditions were optimized by an addition of 1 m*M* tetrabutylammonium acetate (TBA) to the mobile phase to increase the retention times of M_1 and M_2. They were isolated automatically with a FOXY fraction collector (Roucaire, Lincoln, NE, USA). TBA was removed from the fractions by cation-exchange chromatography using a Dowex 50W-X8 resin (Bio-Rad Labs, Richmond, CA, USA). The resin was conditioned prior to use as described previously [10]. A 0.5-ml volume of resin suspended in water was used to elute M_1 and M_2 using 4.5 and 5.5 ml of 0.2 *M* NaCl, respectively. The purified M_1 and M_2 fractions were lyophilized and concentrated in methanol. Sodium chloride was removed by sedimentation at 10 500 *g* for 10 min.

Gas chromatography–mass spectrometry (GC–MS)

Mass spectrometric analysis was carried out with QMD 1000 GC–MS system (Erba Sciences, Massy, France). The chromatographic column was a 5% phenylmethylsilicone fused-silica capillary column (12.5 m × 0.32 mm I.D.) with a film thickness of 0.52 μm (Hewlett Packard, les Ulis, France).

The column temperature was kept at 70°C for 1 min and then programmed to 280°C at 35°C/min. The pressure of the injector head was set at 70 kPa. Injection was carried out in the splitless mode and the injector port was kept at 250°C. The interface of chromatograph and mass spectrometer was maintained at 250°C and the ion source temperature was set at 180°C.

Mass spectra were obtained in the positive-ion chemical ionization (PICI) mode with an electron energy of 70 eV and a filament current of 300 μA. Ammonia was used as the reagent gas. The instrument was scanned over the mass range 90–600 a.m.u.

RESULTS AND DISCUSSION

Of the total radioactivity administered, within 48 h after dosing, 82.4 ± 8.3% (n = 4) and 92.2 ± 2.5 (n = 3) were recovered in urine samples in response to oral and intravenous treatment, respectively. Approximately 2% of the total radioactivity was recovered in faeces. These results indicate the importance of renal excretion as compared with biliary excretion into the gut and subsequent elimination by faeces.

Of the total radioactivity administered orally,

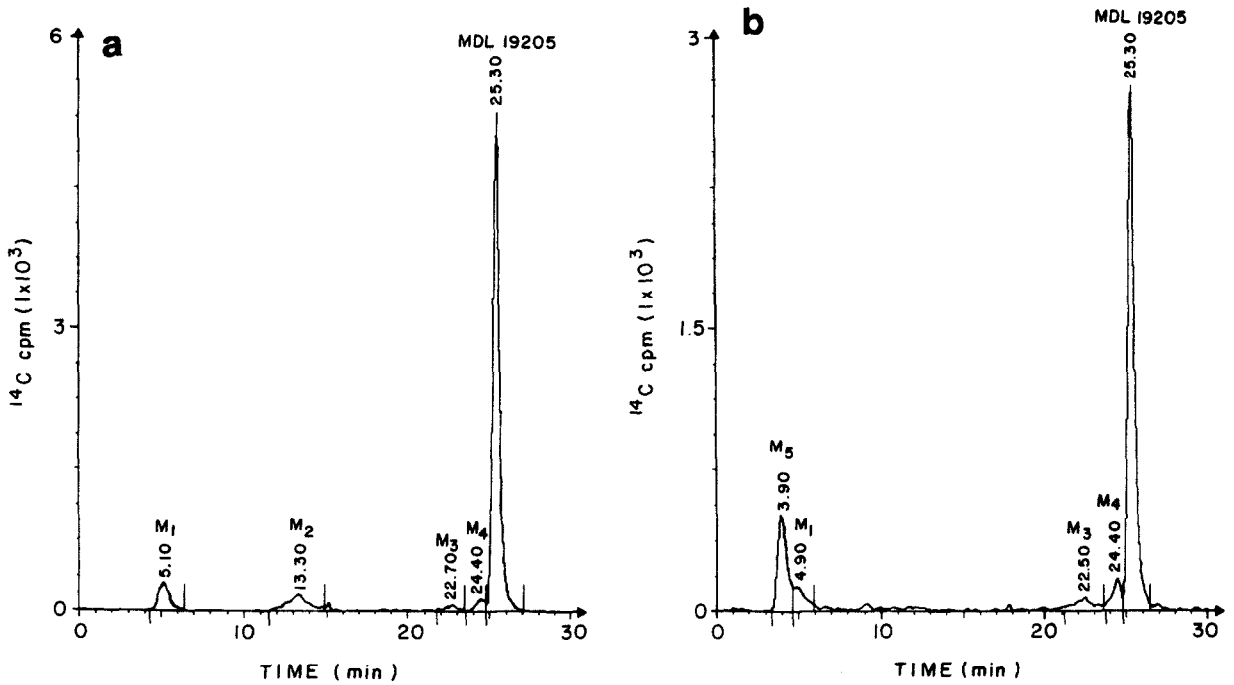

Fig. 2. HPLC of ^{14}C-labelled piroximone and metabolites in a urinary extract: (a) 8 h post-treatment; (b) 12 h post-treatment.

74.9 ± 7.9% (n = 4) was recovered in the first 8 h of urine collection, which corresponds to a urinary excretion rate of 34.5 ± 3.6 μmol/kg body weight per 8 h. Two major (M_1 and M_2) and two minor (M_3 and M_4) metabolites were detected (Fig. 2a). M_1 and M_2 accounted for 7.1 ± 1.2% (n = 4) and 4.3 ± 0.4% (n = 4) (Fig. 3), thus implying urinary excretion rates of 3.3 ± 0.6 and 2.0 ± 0.2 μmol/kg body weight per 8 h for M_1 and M_2, respectively.

In contrast, of the total radioactivity administered intravenously, 87.8 ± 1.7% (n = 3) was recovered in the first 8 h of urine collection. This corre-

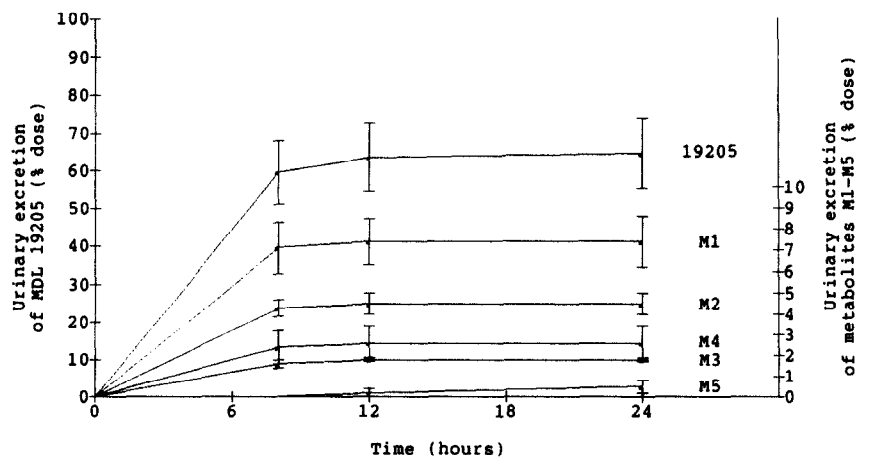

Fig. 3. Cumulative urinary excretion of MDL 19205 and metabolites after oral administration of ^{14}C-labelled piroximone.

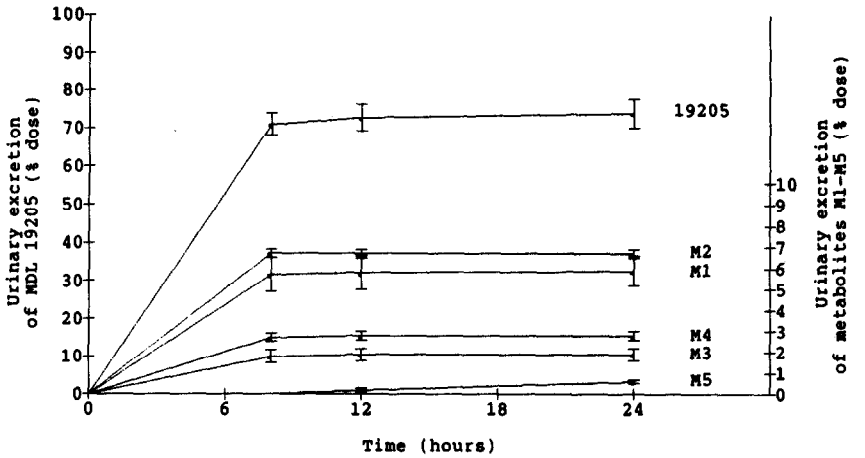

Fig. 4. Cumulative urinary excretion of MDL 19205 and metabolites after intravenous administration of ^{14}C-labelled piroximone.

sponds to a urinary excretion rate of 40.4 ± 0.8 μmol/kg body weight per 8 h. Two major (M_1 and M_2) and two minor (M_3 and M_4) metabolites were detected (Fig. 2a). M_1 and M_2 accounted for 5.7 ± 0.8% (n = 3) and 6.7 ± 2.0% (n = 3) (Fig. 4), thus implying urinary excretion rates of 2.6 ± 0.4 and 3.1 ± 0.9 μmol/kg body weight per 8 h for M_1 and M_2, respectively.

The urine analysis shows that piroximone and its metabolites were essentially excreted within the first 8 h following treatment. The ratio of metabolites M_1 and M_2 was 1.7 for oral administration and 0.9 for intravenous administration. This suggests increased formation of M_2 following intravenous administration. The reasons for these differences due to alternate administration routes are unknown. A minor metabolite (M_5) was detected in the 12-h urine collection (Fig. 2b). The cumulative urinary excretion of piroximone and its metabolites after oral administration is shown in Fig. 3 and after intravenous administration in Fig. 4.

When TBA was added to the mobile phase the retention times of M_1 and M_2 increased. TBA is a lipophilic positive counter ion which increases the retention time of the negative ions on a reversed-phase column [11,12]. With 1 mM TBA there is a relationship between the re-equilibration time of the column and the retention times of M_1 and M_2. It was found that a column re-equilibration time of 45 min between sample injections resulted in optimum purity of the M_1 and M_2 fractions for subsequent mass spectrometric analysis.

CI-MS of M_1 yielded a molecular ion at m/z 138, corresponding to the MH^+ of isonicotinic acid methyl ester (Fig. 5a and b). In addition, the observed GC retention time of M_1 and of a standard sample of isonicotinic acid methylester were identical (Fig. 6a and b), suggesting that the *in vivo* biotransformation of piroximone led to the formation

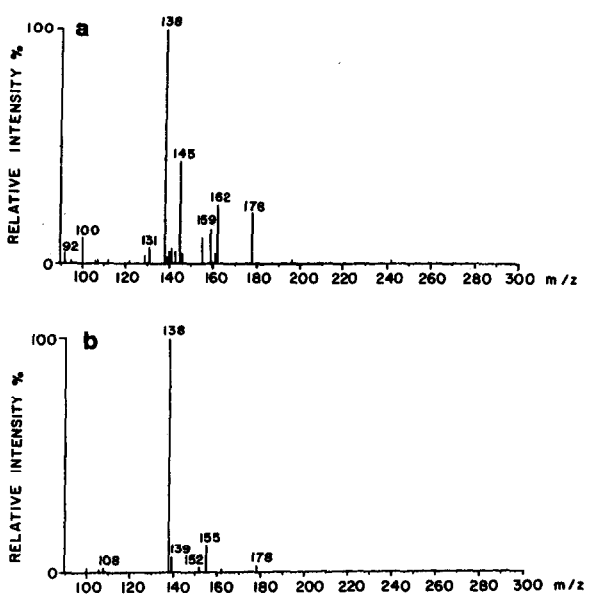

Fig. 5. PICI mass spectra of (a) M_1 and (b) isonicotinic acid methyl ester.

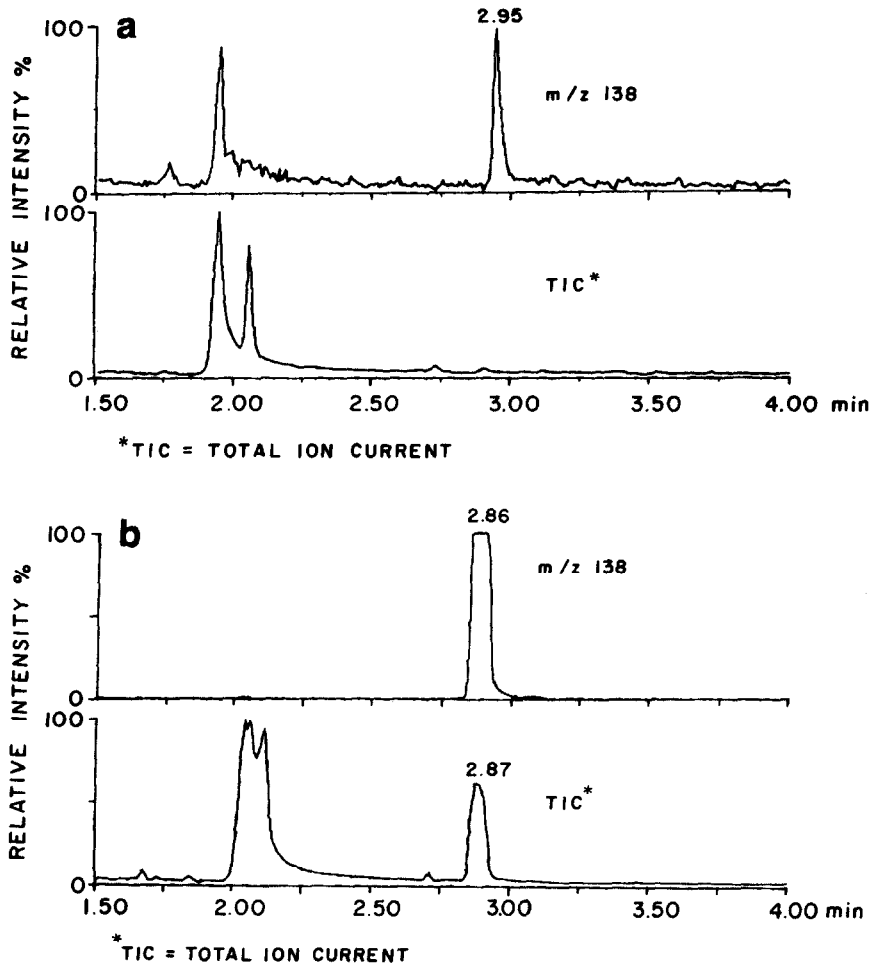

Fig. 6. Ion chromatograms (PICI) of (a) M_1 and (b) isonicotinic acid methyl ester.

of isonicotinic acid. Moreover, isonicotinic acid is the only degradation product of piroximone under basic conditions [0.1 *M* NaOH (pH 13) at 60°C] and was found to be the major degradation product in 1% hydrogen peroxide solution [10]. Isonicotinic acid methyl ester could, however, be the result of non-enzymatic methylation, probably owing to the work-up procedure with methanol.

TABLE I

METABOLISM WITH RAT LIVER MICROSOMES

	Microsomal protein (mg/g liver)[a]	Metabolite (pmol/ml microsomal protein/min)[a]	
		M_1	M_4
Control	22.9 ± 1.8	72.6 ± 13.7	21.7 ± 5.0
PCB	23.1 ± 1.1	74.4 ± 13.4	14.9 ± 1.1

[a] Mean ± S.D. (n = 4).

In vitro metabolic studies of piroximone with rat hepatic microsomes resulted in a total metabolite formation of 94.3 pmol/mg microsomal protein/min (Table I). This represents an approximate mean rate of metabolism of 13.7 μmol in the liver per 8 h, which compares favourably with the above-calculated urinary excretion rate. It is noteworthy that only M_1 and M_4 could be detected in *in vitro* incubations using hepatic microsomal membranes. Induction of hepatic cytochrome P-450-dependent

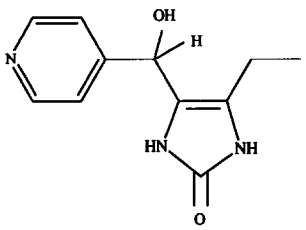

Fig. 7. Structure of hydroxypiroximone.

monooxygenases by treatment with Aroclor 1254 [9] did not increase the rate of M_1 and M_4 formation (89.3 pmol/mg microsomal protein/min) (Table I), and consequently induced isoforms of hepatic cytochrome P-450-dependent monooxygenases by PCB are not involved in the formation of M_1 and M_4.

CONCLUSION

Piroximone is metabolized *in vivo* and *in vitro*. The *in vivo* biotransformation of piroximone resulted in the formation of two major metabolites (M_1 and M_2) representing approximately 15% of the administered dose. M_1 was identified as isonicotinic acid.

Preliminary studies in dogs (data not shown) provided evidence for a large interspecies variation in the metabolism of piroximone. Unlike rats, M_4 was found to be the major metabolite in dogs. This metabolite co-chromatograms (UV detection) with the synthetic standard hydroxypiroximone (Fig. 7), thus providing evidence for interspecies variation in the metabolism of piroximone.

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