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PYRIDOGLUTETHIMIDE [3-ETHYL-3-(4-PYRIDYL)-PIPERIDINE-2,6-DIONE], AN ANALOGUE OF AMINOGLUTETHIMIDE

METABOLISM AND PHARMACOKINETICS

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Abstract—Pyridoglutethimide [3-ethyl-3-(4-pyridyl)piperidine-2,6-dione] has been developed as an analogue of aminoglutethimide [3-(4-aminophenyl)-3-ethyl-piperidine-2,6-dione] possessing specific aromatase activity with potency comparable to aminoglutethimide. This study investigates the pharmacokinetics of pyridoglutethimide in the rat and the rabbit: the plasma half-life is 6 hr in the rat and 16.4 hr in the rabbit. The sole metabolite found in urine (rat) and plasma (rat and rabbit) is pyridoglutethimide *N*-oxide.

Pyridoglutethimide [3-ethyl-3-(4-pyridyl)piperidine-2,6-dione] (PyG) [1] is an analogue of aminoglutethimide [3-(4-aminophenyl)-3-ethyl-piperidine-2,6-dione] (AG), a drug currently in use for the treatment of hormone-dependent metastatic breast carcinoma [2, 3]. It is thought to inhibit tumor growth by blocking oestrogen synthesis, specifically the conversion of androstene-3,17-dione and testosterone into oestrone and oestradiol mediated by the enzyme complex aromatase [4]. However, AG also inhibits desmolase, the enzyme complex responsible for the conversion of cholesterol into pregnenolone [5], thus depleting corticosteroid production. Hence hydrocortisone must usually be given as replacement therapy [6]. PyG is a strong inhibitor of the aromatase enzyme (K_i 1.1 μM) [1] but is slightly less active than AG (K_i 0.6 μM). The apparent K_i for AG against desmolase has been reported as 14 μM [7], whereas PyG does not inhibit desmolase at the highest concentration tested (50 μg/ml) [1]. Therefore, unlike AG, PyG is a selective inhibitor of aromatase.

Major side effects observed with AG in the clinic reflect its CNS toxicity (drowsiness, ataxia). In standard tests in mice PyG produces little or no CNS side effects (sedation, ataxia, anticonvulsant activity) at 300 mg/kg, whereas AG produces pronounced CNS effects at 100–200 mg/kg [8]. Therefore, assuming that therapeutic doses in humans will be similar for AG and PyG, PyG should produce fewer CNS-related side effects.

Metabolism plays a major role in the inactivation of AG [7, 9] and a component of this metabolism is drug-induced [10]. We now report on the metabolism of PyG in the rat and in a human volunteer together with pharmacokinetic studies in the rat and rabbit, with a view to assessing the potential of PyG as a more effective compound than AG for the treatment of hormone-dependent breast carcinoma.

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MATERIALS AND METHODS

Animals. Female Wistar rats (150–200 g) obtained from Olac., Oxon., U.K. were used throughout the animal studies. The dose of PyG used for metabolism studies was 0.165 mmole/kg (s/c, in suspension) whereas that used for pharmacokinetic studies was 0.110 mmole/kg (orally in DMSO + corn oil, 1:5, 0.3 ml; i/v in DMSO 0.1 ml). Female New Zealand white rabbits (Olac., Oxon.) 3–3.5 kg were administered PyG, 0.110 mmole/kg as the HCl salt in water for injection (1.0 ml) i/v into the ear vein.

Human. One adult male volunteer took 50 mg PyG orally, in aqueous solution (in the absence of further toxicology studies a more extended study at higher doses was deemed inadvisable).

Chromatography. Reversed phase thin-layer chromatography (RPTLC) was carried out using 20 × 20 cm Whatman $KC_{18}F$ glass plates with acetonitrile—5% aqueous sodium chloride (3:7, v/v) as the developing solvent (Solvent I). Normal phase TLC employed 5 × 20 cm (0.25 mm) silica gel 60 F-254 (Merck) plates with chloroform–methanol (9:1, v/v) as the solvent (Solvent II). U.v.-absorbing components were detected using a Hanovia Chromatolite at 254 nm.

High pressure liquid chromatography (HPLC) was carried out using a Waters Model ALC/GPC 204 liquid chromatograph equipped with a Model 6000A solvent delivery system, a U6K injector, a Model 440 dual channel absorbance detector at 254 and 280 nm for metabolism studies and 254 and 229 nm for pharmacokinetic studies. Analyses were carried out using a Trivector Trilab Model III computing integrator.

Mass spectrometry. Samples were eluted from developed plates using methanol and concentrated onto the direct insertion probe. Mass spectra were determined in the electron impact (EI) mode (ionizing voltage 70 eV, trap current 100 μA) at an ion-

source temperature of 160° using a VG 7070H spectrometer and 2235 data system.

In vivo *metabolism studies*. The rat was kept in a standard metabolism cage and allowed free access to food and water. Urine was collected at 24 hr intervals (pre- and post-treatment) for rat and human and stored at −20° prior to extraction. The extracted urinary metabolites of PyG and PyG itself, from rat and human were examined by TLC and quantified by HPLC.

Extraction for TLC examination. Aliquots of pre- and post-treatment urines from rat (5 ml), or human (50 ml) were acidified with an equal volume of M HCl and extracted with 2 volumes of dichloromethane (acid extract). The aqueous residue was saturated with Na_2HPO_4 and re-extracted with an equal volume of dichloromethane (basic extract). The organic extracts were then dried over anhydrous Na_2SO_4, concentrated on a rotary film evaporator and the residue dissolved in dichloromethane (200 μl). Aliquots (50 μl) of each extract were subjected to RPTLC using Solvent I as the eluant, and synthetic PyG and PyG *N*-oxide (pyridoglutethimide *N*-oxide) [1] as standards. U.v.-absorbing components in the urine extracts with R_f values corresponding to the standard materials (R_fs: PyG—0.35, PyG *N*-oxide—0.66) were removed and eluted with methanol. The eluate was divided in two, half to be subjected to mass spectrometry and half for TLC on silica gel (Solvent II) alongside synthetic standards. U.v.-absorbing bands of interest were again removed, eluted and subjected to mass spectrometry.

Quantification of pyridoglutethimide. HPLC was used throughout for quantification.

Urine extraction. Aliquots of urine from the rat (2 ml), and human (5 ml) were saturated with Na_2HPO_4 and extracted with an equal volume of dichloromethane. The organic phase was dried over anhydrous Na_2SO_4 and concentrated on a rotary film evaporator.

Rat urine samples. The residues were dissolved in acetonitrile–H_2O (30:70, v/v, 200 μl) and aliquots (4 μl, in duplicate) were subjected to HPLC (μBondapak C_{18}, 30 cm × 3.9 mm i.d., acetonitrile–H_2O, 30:70, 1.5 ml/min). Internal standard (AG, a = 100 μg/ml, b = 124 μg/ml) was added to post-treatment urine prior to extraction. Control (pre-treatment) urine (2 ml) containing PyG, PyG *N*-oxide and AG (200 μg of each in ethanolic solution) was extracted and the HPLC profile compared with those of extracts of control urine to which no compounds had been added and with the post-treatment urine extracts.

Human urine samples. Residues were dissolved in acetonitrile–0.01 M $(NH_4)_2HPO_4$ (20:80 v/v, 200 μl) and the samples filtered through a 0.45 μm membrane prior to HPLC (μBondapak C_{18}, 30 cm × 3.9 mm i.d., acetonitrile–0.01 M $(NH_4)_2HPO_4$, 20:80, 1.5 ml/min) of aliquots (15 μl, duplicate). Internal standard (AG, 5.0 μg/ml) was added to post-treatment urine prior to extraction. Standard solutions of control urine (5 ml) containing (i) AG (5.05 μg/ml) and (ii) PyG (2.02 μg/ml), PyG *N*-oxide (5.13 μg/ml) and AG (5.05 μg/ml) were extracted and HPLC profiles compared with that of an extract of control urine.

Pharmacokinetic studies. Calibration curves were constructed for PyG and PyG *N*-oxide using standard solutions (0.01 mg/ml, 0.1 mg/ml, 1.0 mg/ml). Aliquots (4 μl, in triplicate) were subjected to HPLC (NovaPak C_{18} 15 cm × 3.9 mm i.d., acetonitrile–0.01 M phosphate buffer, pH 7.0, 13:87 v/v, 1.5 ml/min) and the area under the curve (AUC) for each component plotted against concentration.

Recoveries were determined using pre-treatment plasma (1 ml) to which PyG and PyG *N*-oxide (5.0 μg of each) were added.

Plasma extraction. Total blood (5–6 ml) was collected from rats at intervals after dosing [5 min (i/v), 15, 30, 60 min, 2, 4, 6 hr (i/v and p.o.)] by cardiac puncture, centrifuged and the plasma stored at −20° prior to analysis. Rabbits were bled via the ear vein (opposite ear to that used for injection), 3 ml blood samples being collected at each of the

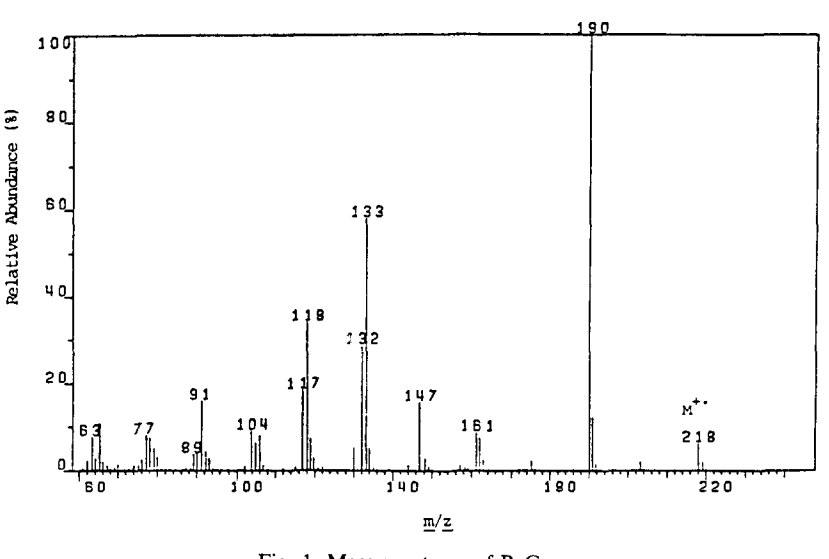

Fig. 1. Mass spectrum of PyG.

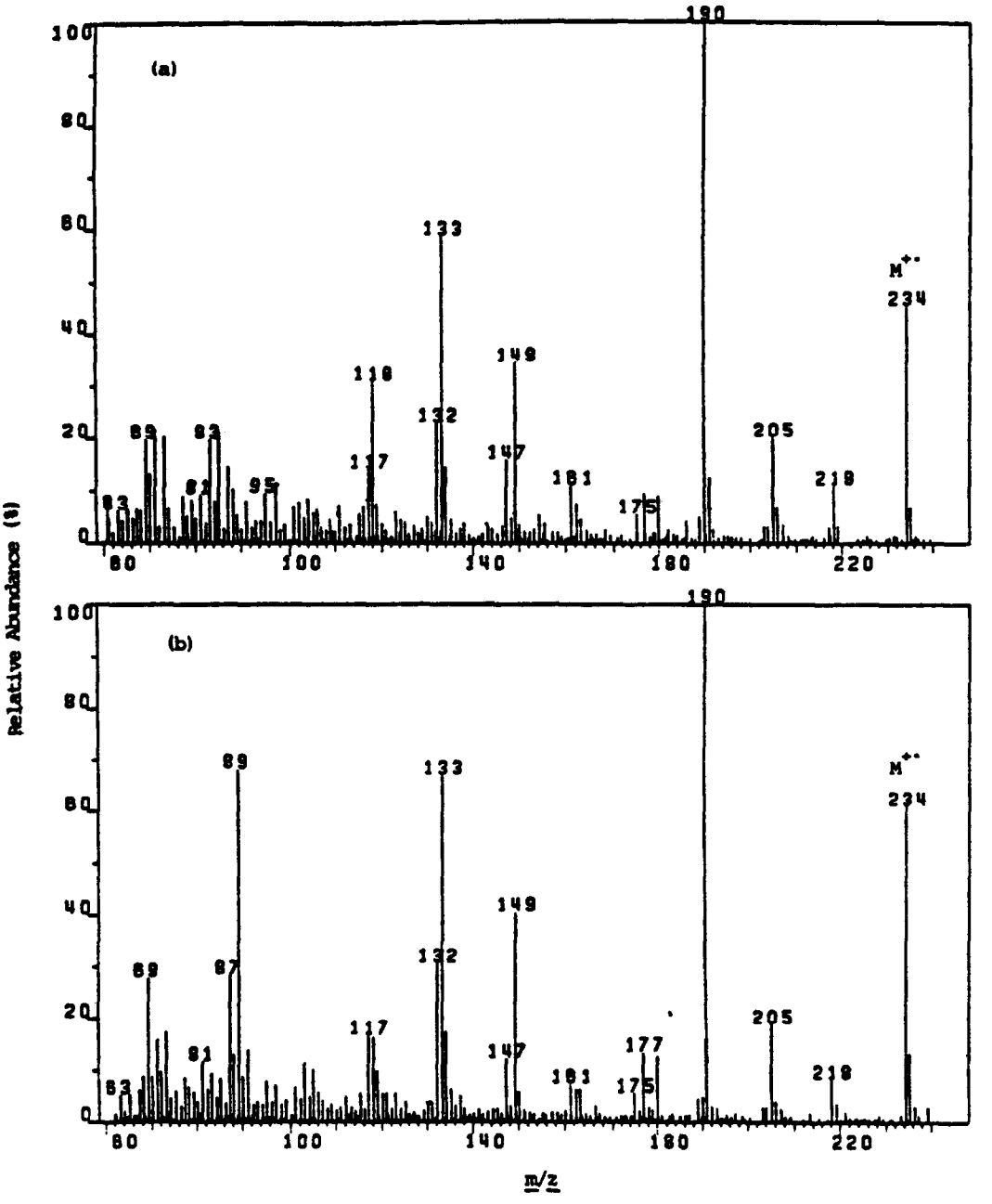

Fig. 2. Mass spectra of PyG *N*-oxide: (a) synthetic compound; (b) isolated from the urine of a rat treated with PyG.

above-timed intervals. Aliquots of plasma (1 ml rat; 0.5 ml plasma plus 0.5 ml water, rabbit) were extracted with ethyl acetate (3 × 7 ml), in duplicate, the combined organic phase concentrated on the rotary film evaporator and the residue dissolved in acetonitrile–H_2O (50:50 v/v, 200 μl). Aliquots (4 μl, in triplicate) were subjected to HPLC and the plasma concentration of PyG and PyG *N*-oxide determined from AUC after correction for recovery.

RESULTS

The metabolic profiles of PyG in 24 hr post-treatment urine from rat and human were initially compared by RPTLC. The acid extracts of urine from both species showed no drug-derived components when compared with PyG and PyG *N*-oxide synthetic standards. In the basic extracts of rat and human urine, drug-derived components corresponded to PyG (R_f 0.35) and PyG *N*-oxide (R_f 0.66) on RPTLC (Solvent I). These structures were confirmed by mass spectrometry. Following RPTLC the component with an R_f value corresponding to PyG *N*-oxide appeared to contain more than one component by mass spectrometry. Re-chromatography on silica gel (Solvent II) separated the components which were shown by mass spectrometry to be PyG *N*-oxide and a normal urinary component.

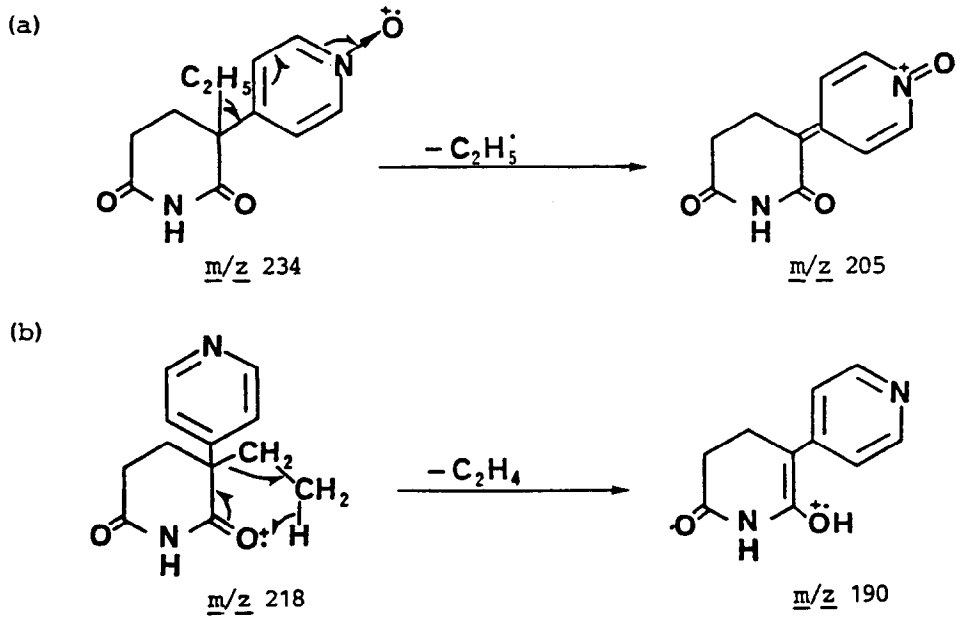

Fig. 3. Proposed mechanisms for (a) the loss of the ethyl radical from the molecular ion of PyG *N*-oxide and (b) the loss of ethylene from the molecular ion of PyG under electron impact.

Mass spectra

Figure 1 shows the mass spectrum of PyG and Fig. 2 those of synthetic PyG *N*-oxide and the metabolite isolated from rat urine. The *N*-oxide gives an abundant molecular ion at m/z 234 but readily loses oxygen to give the molecular ion of PyG at m/z 218, the fragmentation of which dominates the spectrum. The peak at m/z 89 in the mass spectrum of the metabolite is attributable to a urinary component; it is present in extracts of urine from rats which have not received PyG. A noteworthy difference between PyG and PyG *N*-oxide is the loss of an ethyl radical from the molecular ion of PyG *N*-oxide to give the ion m/z 205 whereas that of PyG itself loses ethylene to give m/z 190. This difference in the initial fragmentation of the molecular ion has been noted before for other 3-aryl-3-ethylglutarimides [10] and has been rationalised in terms of the influence of aryl ring substituents on this fragmentation. Here, it can be ascribed to the electron-accepting properties of the N—O bond in the *N*-oxide (Fig. 3a) but, in the absence of this structural feature in PyG, preference for an alternative fragmentation pathway (Fig. 3b) is observed.

Quantification of urinary PyG *and* PyG *N-oxide*

Extracts of 24 hr post-treatment urine from rat and human were quantified by HPLC using AG as the internal standard. The presence of PyG and PyG *N*-oxide in these extracts was confirmed by a comparison of retention time (R_T) with those of synthetic standards. By comparing pre- and post-treatment urine extracts, HPLC systems were developed in which PyG and PyG *N*-oxide were separated from normal urinary components, (rat: PyG = 260 sec, PyG *N*-oxide = 132 sec, AG = 325 sec; human: PyG = 445 sec, PyG *N*-oxide = 175 sec, AG = 650 sec).

The percentages of the administered dose extracted as PyG and PyG *N*-oxide from 24 hr post-treatment urine (rat and human) are given in Table 1.

Table 1. HPLC quantification of pyridoglutethimide (PyG) and pyridoglutethimide *N*-oxide (PyG *N*-oxide) in rat and human 24 hr urine

	PyG		PyG *N*-oxide	
	μg/ml	% dose in 24 hr urine	μg/ml	% dose in 24 hr urine
Rat 0.165 mM/kg	75.3 (72.7–78.3)	14.5%	48.6 (47.1–50.3)	8.7%
Human 50 mg dose	1.4 (1.2–1.5)	4.9%	9.4 (9.3–9.6)	31.7%

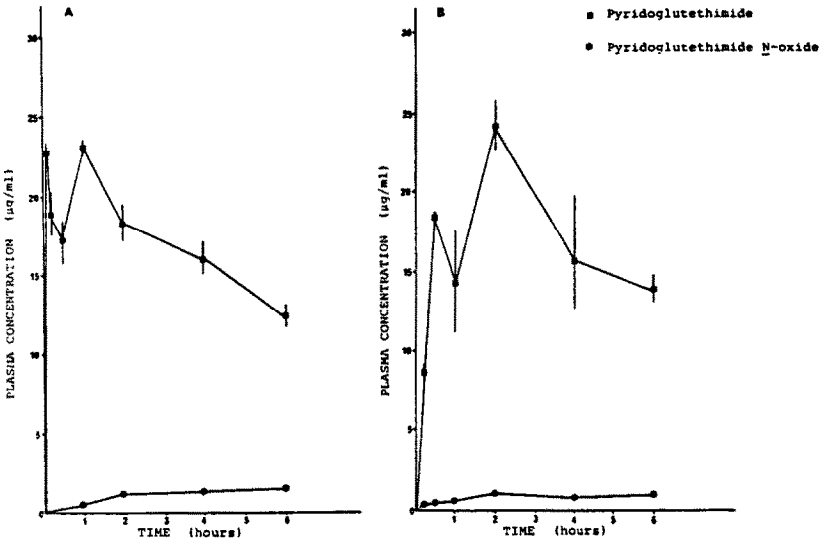

Fig. 4. Plasma concentration–time profile in rats of pyridoglutethimide (0.110 mmole/kg) and its *N*-oxide metabolite after (A) i/v and (B) oral administration.

Pharmacokinetic studies

The calibration curves constructed for PyG and PyG *N*-oxide showed the relationship between concentration and AUC to be linear over the concentration range under consideration. The limit of detection at 254 nm was 10 ng per injection for PyG and 3–5 ng for PyG *N*-oxide. Recoveries from plasma were repeatable and for rat were 88% for PyG and 63% for PyG *N*-oxide and for rabbit were 85% for PyG and 35% for PyG *N*-oxide. The retention times of PyG *N*-oxide and PyG in the HPLC system were 85 sec and 335 sec respectively. Comparison with control plasma extracts confirmed there to be no interference from plasma components.

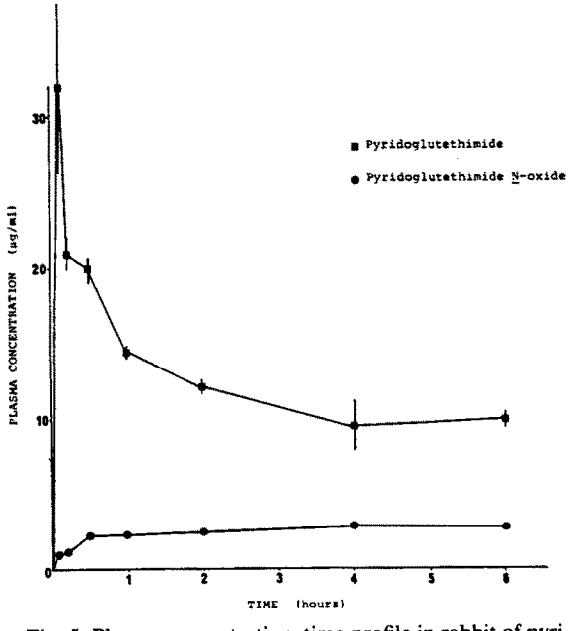

Fig. 5. Plasma concentration–time profile in rabbit of pyridoglutethimide (0.110 mmole/kg) and its *N*-oxide metabolite after i/v administration.

Plasma levels of PyG and PyG *N*-oxide in the rat after i/v or p.o. administration of PyG are shown in Figs 4a and 4b respectively. PyG reached a maximum plasma concentration of 23.2 µg/ml (i/v) and 24.0 µg/ml (p.o.) 1–2 hr after administration. Plasma levels were well maintained with PyG having a calculated half-life (i/v) of 6 hr. As previously found for the urine extracts, the only plasma metabolite of PyG detected was PyG *N*-oxide. This reached maximum plasma concentrations of 1.0–1.5 µg/ml over the 6 hr test period. Similarly, after i/v administration to the rabbit (Fig. 5) the maximum plasma concentration attained for PyG was 32 µg/ml at 5 min and the calculated plasma half life was 16.4 hr. The *N*-oxide was the only plasma metabolite detected, reaching a concentration of 2.5–3.0 µg/ml at 4–6 hr.

DISCUSSION

Pyridoglutethimide (PyG) is a selective inhibitor of the aromatase enzyme complex [11] and lacks, experimentally, the side effects associated with aminoglutethimide (AG) [8]. In this study we have investigated the metabolism of PyG *in vivo*. The urinary metabolism profile was determined for PyG in rat and human and a preliminary study has been made of its pharmacokinetics in the rat and rabbit. The sole metabolite detected in extracts from rat and human urine and rat and rabbit plasma was the *N*-oxide (PyG *N*-oxide). This compound has been synthesised and shown to be inactive against aromatase [1]. Although the metabolism of PyG, inasmuch as the *N*-oxide was the only detectable metabolite in urine, appears qualitatively identical for both species, quantitative differences are seen. From the HPLC results obtained for the urine extracts (Table 1) a greater proportion of the dose of PyG in the human was excreted as the *N*-oxide over a 24 hr period, compared with the rat. This could be a reflection of interspecies variation in enzyme(s) responsible for *N*-oxidation. However, as the human was

exposed to a much lower dose of PyG per kg body weight, more complete metabolism of the substrate might be expected.

Determination of the concentration of PyG in rat plasma at known intervals after administration showed there to be a higher proportion of PyG in plasma relative to PyG *N*-oxide. Maximum plasma levels for PyG were 23.2–24.0 μg/ml 1–2 hr after dosing, and the half-life, 6 hr (i/v). PyG *N*-oxide reached plasma levels of 1.0–1.5 μg/ml but this is probably unrepresentative of total metabolic turnover due to more rapid urinary excretion of this polar product. After i/v administration of PyG to rabbit, similar results were obtained, with slightly more *N*-oxide (2.5–3.0 μg/ml) being detected and a plasma half life for PyG of 16.4 hr. The observed rebound rise in plasma levels of PyG in the rat (i/v and p.o.) may be due to enterohepatic cycling of the compound; faecal metabolites of PyG have not yet been investigated. A comparison of alternative routes of administration of PyG to rats in these pharmacokinetic studies revealed the bioavailability of the compound to be excellent with rapid absorption from the gastro-intestinal tract and no detectable first pass effect.

It appears that PyG does undergo metabolism (inactivation) *in vivo,* but to a lesser extent than observed with AG. This, together with the relatively high plasma levels attained, long half-life and lower CNS toxicity indicate that PyG may be a useful improvement over AG clinically. To date we have investigated only those urinary metabolites which are readily extractable with organic solvents. As the results of this study indicate, this accounts for less than 100% of the substrate over a 24 hr period. The metabolic formation of more soluble conjugates cannot be discounted and these may be excreted in urine and faeces. A further search for more polar metabolites is in progress in our laboratory.

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