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Characterisation of metabolites of 3-ethyl-3-(4-pyridyl)-piperidine-2,6-dione, a potential breast cancer drug

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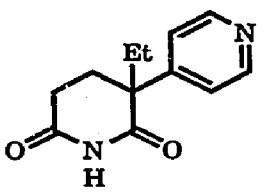
ABSTRACT

The identification of metabolites from the pyridylglutarimide 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione (PG, Rogletimide) was achieved using liquid chromatography–mass spectrometry with a thermospray interface (LC–TSP–MS). The urinary metabolites include PG N-oxide, the products of 4- and 5-hydroxylation in the piperidine residue (4- and 5-hydroxy-PG) and a γ-butyrolactone derived via terminal hydroxylation in the ethyl residue. In addition to the above metabolites, several products of glutarimide ring-opening could be detected in the plasma extracts after multiple-dose treatment. Thus LC–TSP–MS is potentially a simple and rapid technique in studies of drug metabolism for the important glutarimide class of drug.

INTRODUCTION

The pyridylglutarimide, 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione (PG, Rogletimide, **1**, Fig. 1) is a new analogue of aminoglutethimide, 3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione (AG, **2**, Fig. 1) [1]. AG is currently used to treat postmenopausal women suffering from estrogen-dependent breast carcinoma [2]. The drug inhibits the enzyme aromatase (estrogen synthetase) which is responsible for



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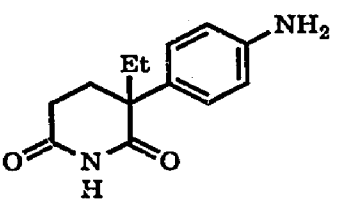

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Fig. 1. Structures of PG (**1**) and AG (**2**).

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the conversion of androgens into estrogens, thus depleting the estrogen level in the plasma and retarding tumour growth. However, AG also inhibits desmolase, the enzyme which blocks the cholesterol side-chain cleavage [3]. A consequence of unselective inhibition by AG is the need to replace glucocorticoid by administering hydrocortisone daily. Moreover, patients suffer adverse side-effects from AG, such as dizziness, lethargy and ataxia. In view of all these problems, more specific inhibitors of aromatase are currently being developed, and PG is one of those currently undergoing clinical trial. PG is a more selective inhibitor *in vitro* towards aromatase [1], has better retention in the plasma of the rat and rabbit [4], produces less central nervous system (CNS)-related toxic side-effects in mice [5] and it has retained a potency for aromatase inhibition comparable to that of AG [1].

Of the known metabolites of AG in the rat [6] and human [7,8], seven out of nine metabolites involved the amino group. In PG, the amino functional group is absent and therefore less metabolism would be envisaged. The metabolism of PG has only previously been studied in rats, a healthy male volunteer [4] and several patients [9]. Only one major metabolite, namely PG N-oxide (3), was identified, using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) followed by mass spectrometry (MS).

In recent years HPLC on line coupling to MS using a thermospray (TSP) interface has become an invaluable technique for the identification of drugs and their metabolites in biological fluid [10-12]. We utilized this technique, and for the first time have studied the metabolism of PG in postmenopausal patients suffering from breast carcinoma.

EXPERIMENTAL

Chemicals

Ethyl 4-pyridylacetate was purchased from Lancaster Synthesis (Morecambe, UK). Other chemicals were supplied by Aldrich (Poole, UK). Solvents were analytical or HPLC grade (BDH, Gillingham, UK).

Isolation of metabolites from urine samples

Patients' urine samples before and 24 h after PG treatment (250 mg intravenously) were collected and stored at −20°C until analysis. Each sample (5 ml) was acidified using 1 *M* HCl (500 μl) and extracted with ethyl acetate (3 × 10 ml). The organic phase was discarded and the aqueous phase was neutralised by slowly adding 1 *M* NaOH (500 μl) followed by ethyl acetate (3 × 10 ml) extraction. The organic phase was concentrated to dryness and then redissolved in methanol (100 μl). Aliquots of this sample (1 and 10 μl) were analyzed by HPLC and LC–MS, respectively.

Isolation of metabolites from plasma samples

Plasma samples were collected before a dose, 6 h after a single dose and 6 h after multiple doses of PG treatment (1 g/day orally for seven days). Each plasma sample (1 ml) was passed through a C_8 Bond Elut cartridge (1 ml capacity) and eluted with methanol (2 × 0.5 ml). The organic eluate was dried under a stream of nitrogen and redissolved in methanol (40 μl). Aliquots (2 and 10 μl) of each sample was analyzed by HPLC and LC–MS, respectively.

In order to obtain enough samples for nuclear magnetic resonance (NMR) or MS analysis, certain urine and plasma extracts were repeatedly subjected to HPLC, and the appropriate peaks were collected for analysis.

Mass spectrometry and NMR analysis

Direct insertion mass spectra were recorded on a VG 7070H mass spectrometer (Altrincham, UK), operating at 70 eV in the electron-impact (EI) mode, with an ion source temperature of 180°C and equipped with a VG 2235 data system. NMR spectra were obtained at 250 MHz using a Bruker AC-250 spectrometer (Switzerland Spectrospin, Faellander, Switzerland).

LC–MS analysis

The HPLC equipment was manufactured by Applied Chromatography Systems (HPLC Technology, Manchester, UK) and consisted of a gradient controller (Model 351/04) with a variable-wavelength UV detector (Model 702/12). The column used was a Spherisorb S3 ODS-2 (HiChrom, Reading, UK), 150 mm × 4.9 mm, particle size 3 μm. The mobile phases were methanol–0.1 *M* ammonium acetate (20:80, v/v) for the ethyl acetate urine samples (system 1) and (30:70 v/v) for the plasma sample (system 2), both at a flow-rate of 1 ml/min isocratic.

The mass spectrometer used was a Vestec Model 201 (Vestec, Houston, TX, USA) fitted with a Vestec TSP interface, operated in the positive ion mode. Full scan mass spectra were acquired in the range of *m/z* 180–400, at 1.5 s/scan. The vaporiser temperature was 280°C and the block temperature was at 300°C, and these were optimized daily with the mobile phase.

Synthesis of reference compounds and metabolites

PG *N*-oxide (**3**) was obtained as described previously [1].

Synthesis of 3-[2-(aminocarbonyl)ethyl]-3-(4-pyridyl)-5,6-dihydrofuran-2-(3H)-one (4). To a stirred solution of ethyl 4-pyridylacetate (1.69 g, 10.2 mmol) and 2-(tetrahydropyran-2-yloxy)-1-iodoethane (2.66 g, 10.4 mmol) in *tert.*-butanol (20 ml) at 25°C under nitrogen was added potassium *tert.*-butoxide (1.34 g, 11 mmol). After 10 min, acrylamide (1.09 g, 11 mmol) and further potassium *tert.*-butoxide (1.34 g, 11 mmol) were added [13]. After 30 min, the mixture was partitioned between dichloromethane (3 × 80 ml) and aqueous sodium hydrogen carbonate (1 *M*, 100 ml). The organic layers were dried (Na_2SO_4), concentrated, and the residue chromatographed on silica gel (Merck 15111). Elution with 1:10

triethylamine–diethyl ether gave 3-(4-pyridyl)-3-[2-(tetrahydropyran-2-yloxy)-ethyl]piperidine-2,6-dione as a viscous oil (1.73 g, 53%). δ_H ($CDCl_3$) *inter alia* 4.46 (1H, m, OC*H*O), 7.22 (2H, m, pyridyl), 8.20 (1 H, br, NH), 8.62 (2H, m, pyridyl), which was hydrolysed without further purification. A portion of this imide (342.7 mg) was dissolved in methanol (2 ml). The solution was stirred at room temperature and 50% concentrated HCl was added (0.5 ml). After 10 min, the mixture was partitioned between dichloromethane (5 × 15 ml) and aqueous potassium carbonate (2 *M*, 15 ml). The combined organic layers were concentrated and the residue was crystallised from 2:1 toluene–methanol to give 3-[2-(aminocarbonyl)ethyl]-3-(4-pyridyl)5,6-dihydrofuran-2-(3*H*)-one as crystals (93 mg, 37%), m.p. 133–135°C. Elemental composition found was: C, 61.4; H, 6.1; N, 12.0%. $C_{12}H_{14}N_2O_3$ requires: C, 61.5; H, 6.0; N, 12.0%. υ_{max} (neat film from tetrahydrofuran, THF) 1700 (amide C=O) and 1764 (lactone C=O) cm^{-1}. δ_H (Me_2SO-d_6) 1.83-2.28 (4H, m, $CH_2CH_2CONH_2$), 2.50 (1H, dt, J = 13.2, 7.7 Hz, CH_2CH_2O), 2.68 (1 H, ddd, J = 4.9, 7.2, 13.2 Hz, CH_2CH_2O), 4.14 (1H, dt, J = 9.0, 7.4 Hz, CH_2CH_2O), 4.38 (1 H, ddd, J = 4.9, 7.9, 9.0 Hz, CH_2CH_2O), 6.3–6.7 and 6.7–7.2 (v.br., $CONH_2$), 7.45 (1 H, m. pyridyl *meta* to N), 8.57 (1H, m, pyridyl *ortho* to N). Mass spectrum: *m/z* 234 (M^+, 50%), 190 ($[M-CONH_2]^+$, 95%), 162 ($[M-CH_2CH_2CONH_2]^+$, 100%), 134 (96%).

*Synthesis of 3-ethyl-3-(4-pyridyl)piperidin-5-ol-2,6-ones (**5** and **6**). A solution of lithium diisopropylamide was prepared by adding a solution of n*-butyllithium in hexanes (1.6 *M*, 2.31 ml, 3.7 mmol) to a stirred solution of diisopropylamine (373 mg, 3.7 mmol) in dry THF (4 ml) under argon at 0°C, and was added to a stirred solution of PG (366 mg, 1.677 mmol) in dry THF (4 ml) at 20°C under argon. The resulting yellow suspension was stirred for 15 min, then oxodiperoxymolybdenum(pyridine)hexamethyl-phosphoramide (MoOPH) (874 mg, 2.01 mmol) was added as the solid. After 20 h, the mixture was concentrated onto silica gel (Merck 15111; 2 g) and applied to a column of this silica (30 g). Elution with 1:20 methanol–dichloromethane gave 3-ethyl-3-(4-pyridyl)piperidin-5-ol-2,6-one as a mixture of stereoisomers (*ca.* 3.5:1) (240 mg, 61% yield).

Crystallisation from isopropanol gave the major isomer (**5**) [relative stereochemistry (3*R*,5*S*)], m.p. 194–197°C. Elemental composition found was: C, 61.9; H, 6.1; N, 11.7%. $C_{12}H_{14}N_2O_3$ requires: C, 61.5; H, 6.0; N, 12.0%. δ_H (Me_2SO-d_6 at 320 K) 0.79 (3H, t, J = 7.4 Hz, CH_3CH_2), 1.88 (2H, m, CH_3CH_2), 2.20 [1H, dd, J = 12.3, 13.6 Hz, diminished 2.9% upon pre-irradiation at δ 7.33, H-4 (axial)], 2.56 [1 H, dd, J = 5.0, 13.6 Hz, enhanced by 3.7% upon pre-irradiation at δ 7.33, H-4 (equatorial)], 3.78 (1H, m, becomes dd after D_2O exchange, J = 5.0, 12.3 Hz, enhanced 6% upon pre-irradiation at δ 5.5 and 2.4% upon pre-irradiation at δ 7.33, H-5), 5.54 (1 H, d, J = 5.0 Hz, disappears on D_2O exchange, O*H*), 7.33 (2H, m, pyridyl H *meta* to N), 8.57 (2H, m, pyridyl H *ortho* to N), 10.99 (1H, br, s, disappears on D_2O exchange, N*H*). Mass spectrum: *m/z* 234 (M^+, 58%), 206 ($[M-C_2H_4]^+$, 14%), 190 ($[M-CONH_2]^+$, 77%), 182 (85%), 134 (100%).

In order to isolate the minor isomer a separate experiment was carried out. This gave a lower yield of product (556 mg) 5-hydroxy-PG (5-OHPG) as a 3.5:1 mixture of stereoisomers from 1.87 g PG (28% yield). After crystallisation of the major isomer, the mother liquors were concentrated and subjected to HPLC (5 μm Apex II ODS 250 mm × 10 mm column, methanol–0.01 *M* ammonium acetate, 30:70, v/v, flow-rate 3.5 ml/min). The major isomer was eluted at 10.9 min and the minor isomer (**6**) was eluted at 7.4 min [relative stereochemistry (3*R*,5*R*)], δ_H ($CDCl_3$) 0.88 (3H, t, J = 7.4 Hz, CH_3CH_2), 2.22 (2H, m, CH_3CH_2), 2.35 [1H, t, J = 13 Hz, H-4 (axial)], 2.58 [1H, dd, J = 5.7, 13.5 Hz, H-4 (equatorial)], 4.58 (1 H, dd, J = 5.7, 12.1 Hz, H-5) 7.40 (br., pyridyl), 8.66 (v.br., pyridyl), δ_H (Me_2SO-d_6 at 320 K), 0.76 (3H, t, J = 7.4 Hz, CH_3CH_2), 2.10 (2H, q, J = 7.4 Hz, CH_3CH_2), 2.26 (2H, m, H-4), 4.38 (1 H, m, H-5), 5.5 (1H, d, J = 5 Hz, OH), 7.37 (2H, m, pyridyl), 8.46 (2 H, m, pyridyl), 8.58 (s, NH). Mass spectrum: *m/z* 234 (M^+, 52%), 206 ($[M-C_2H_4]^+$, 42%), 190 ($[M-CONH_2]^+$, 68%), 162 ($[M-CONH_2CO]^+$, 68%), 134 (100%).

*Synthesis of 2-ethyl-2-(4-pyridyl)-5-carboxypentanamide (**8**).* To a stirred solution of compound **1** (20 mg, 0.1 mmol) in methanol (10 ml) was added sodium methoxide (5 mg). The mixture was stirred at room temperature for 4 h. At the end of the reaction, the mixture was centrifuged. The supernatant was concentrated *in vacuo* and the crude mixture was purified by 20 cm × 20 cm silica gel 60 F_{254} TLC glass plates, chloroform–methanol (10:1, v/v) as the eluting solvent. Four products were obtained. The band with the lowest R_F value (0.1) was removed and eluted with methanol to give compound **8**. δ_H (CD_3OD) 0.88 (3 H, t, J = 7.4 Hz, CH_3CH_2), 2.22 (4H, m, 2 × CH_2) 2.4 (2H, t, J = 7.4 Hz, CH_2COOH), 7.5 (2H, d, J = 5.9 Hz, pyridine), 8.6 (2H, d, J = 5.9 Hz, pyridine). Mass spectrum: *m/z* 236 (M^+, 3%), 218 ($[M-H_2O]^+$, 4%), 190 (42%), 134 (100%).

*Synthesis of 2-ethyl-2-(4-pyridyl)-5-hydroxypentanamide (**9**) and 4-ethyl-4-(4-pyridyl)-5-hydroxypentanamide (**10**).* To a stirred solution of 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione (2.0 g, 9.2 mmol) in ethanol (20 ml) at room temperature was added sodium borohydride (378 mg, 10 mmol). The mixture became slightly warm. After 1 h, the mixture was concentrated and the residue chromatographed on a column of silica gel. Elution with 5:1 chloroform–methanol gave a 3:2 mixture of compound **9** [2-ethyl-2-(4-pyridyl)-5-hydroxypentanamide] and compound **10** [4-ethyl-4-(4-pyridyl)-5-hydroxypentanamide] as a viscous oil (1.60 g, 79%). The components gave, respectively, δ_H ($CDCl_3$) *inter alia* δ 3.64 (2H, t, J = 6.9 Hz, CH_2OH) and δ 3.76 (2H, ABq, J = 12 Hz, CH_2OH). Trituration under diethyl ether afforded a solid which was a 1:1 mixture of regioisomers. A small sample of the 2-ethyl-2-(4-pyridyl) isomer (**9**) was obtained following careful further chromatography and was a solid, m.p. 148–150°C. Elemental composition found was: C, 64.46; H, 8.15; N, 12.53%; $C_{12}H_{18}N_2O_2$ requires; C, 64.84; H, 8.16; N, 12.60%; δ_H (Me_2SO-d_6) 0.66 (3H, t, J = 7.2 Hz, CH_3CH_2), 1.0–1.16 (2H, m, CH_3CH_2), 1.8–2.0 (4H, m, 2 × CH_2), 3.35 (2H, q, J = 6 Hz; reduces to t,

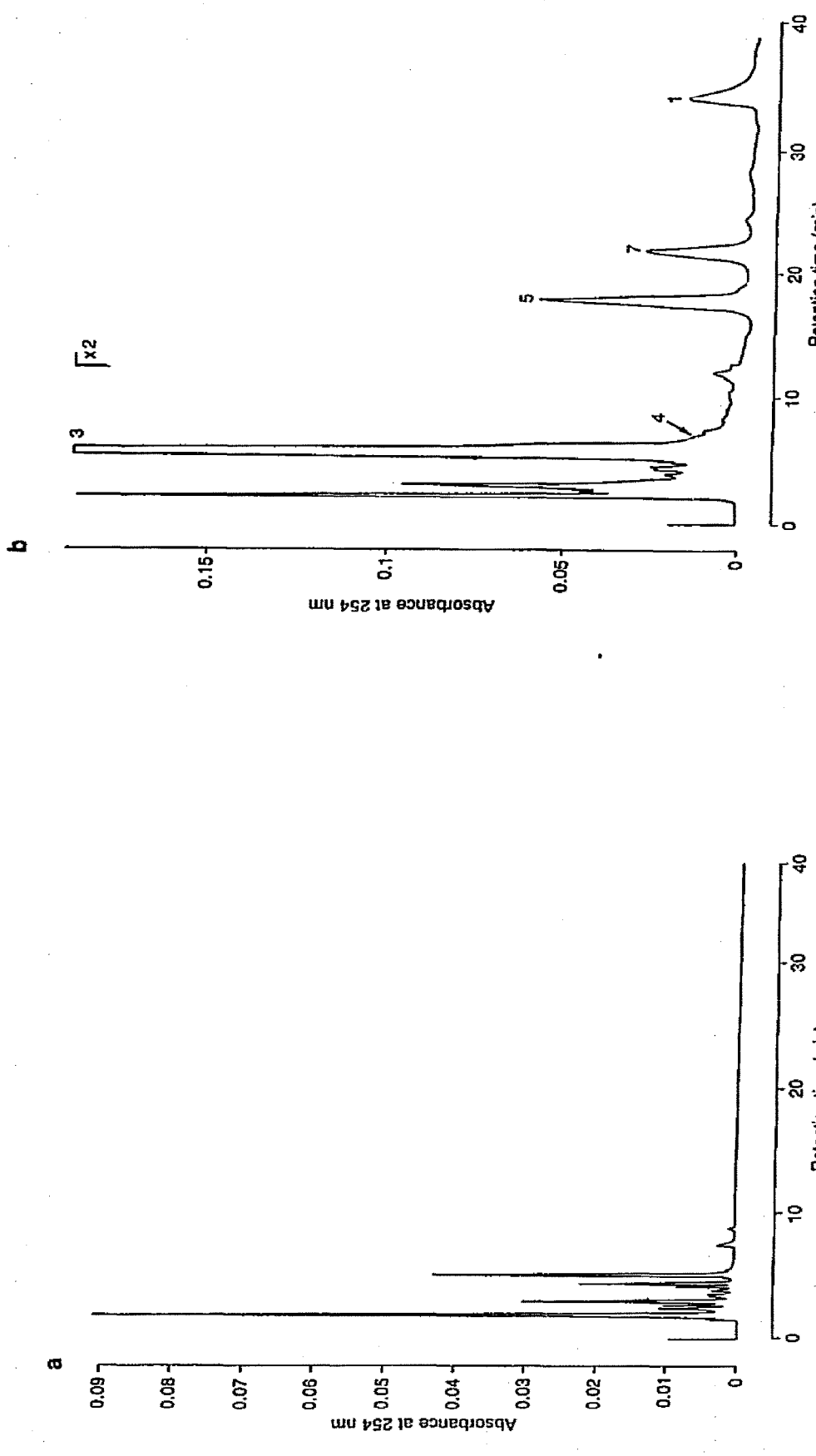

Fig. 2. HPLC profiles of (a) pre-treatment neutral urine extracts and (b) 24 h post-treatment (250 mg/m^2 PG intravenously) neutral urine extracts. Peak 1 = parent drug; peaks labelled 3, 4, 5 and 7 correspond to metabolites 3, 4, 5 and 7, respectively.

J = 6.3 Hz upon irradiation at δ 4.24, HOCH_2), 4.24 (1H, t, J = 5.2 Hz, OH), 6.82 (br.s., amide NH_2), 7.24 (2H, d, J = 5.9 Hz, pyridine), 8.49 (2H, d, J = 5.9 Hz, pyridine). Mass spectrum for **9**: m/z 222 (M^+, 6%), 194 ([$M-C_2H_4$]$^+$, 17%), 179 ([$M-CH_2CHOH$]$^+$, 57%), 178 ([$M-CONH_2$]$^+$, 53%), 160 (79%), 134 (100%).

These compounds were analysed by LC–MS using the appropriate chromatographic system.

RESULTS AND DISCUSSION

In order to evaluate the response of PG under LC–TSP–MS ionisation, PG was introduced by loop injection in the column bypass mode, using 0.1 *M* ammonium acetate and methanol (80:20, v/v) as the mobile phase. It showed a strong [M + H]$^+$ molecular ion and the minimum amount required to obtain a full scan spectrum was 800 pg.

The HPLC profiles of the ethyl acetate extracts from the urine samples before and after PG treatment are shown in Fig. 2a and b. Based on the retention times, compound **1** was the unchanged parent drug (M^+ = m/z 218) and compound **3** was the major metabolite PG *N*-oxide (M^+ = m/z 234). These were confirmed by

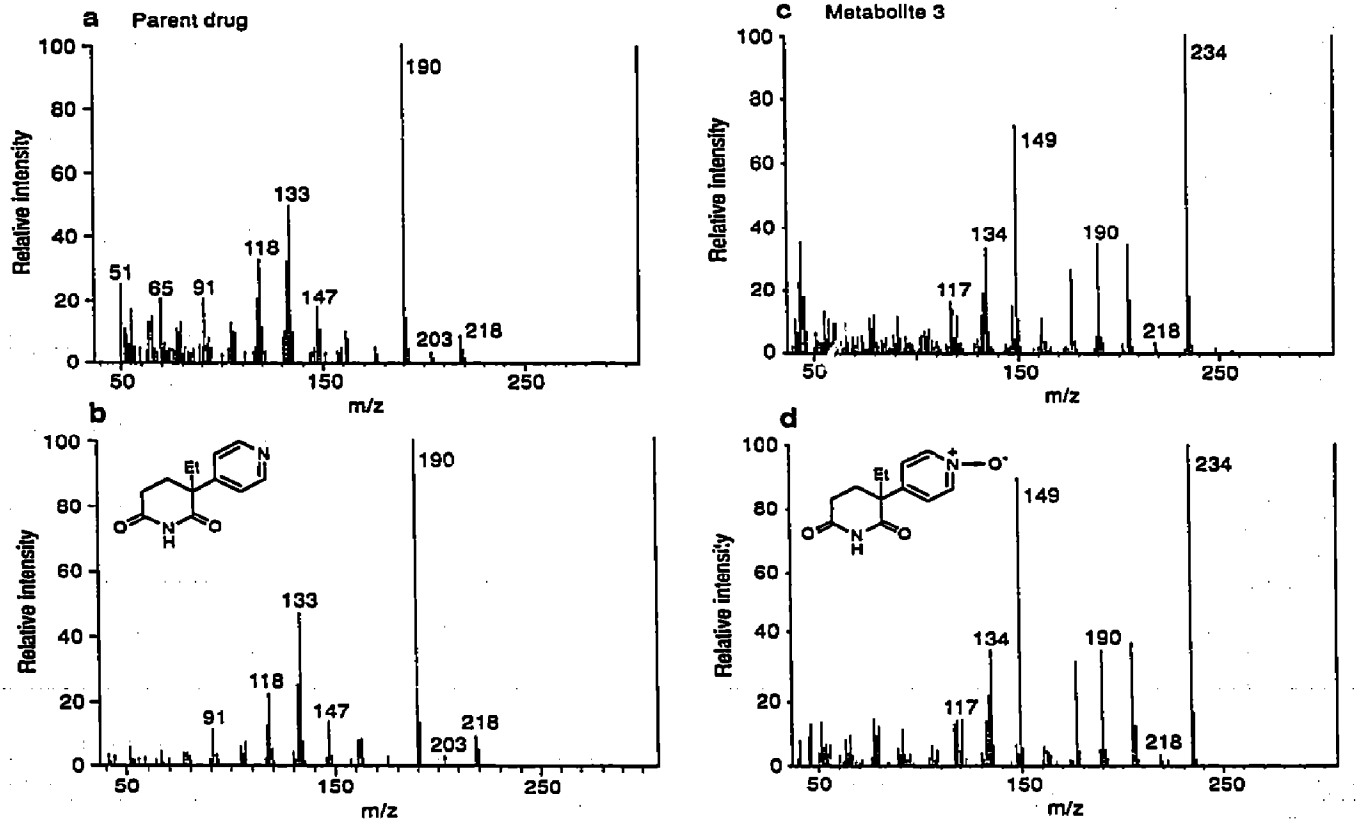

Fig. 3.

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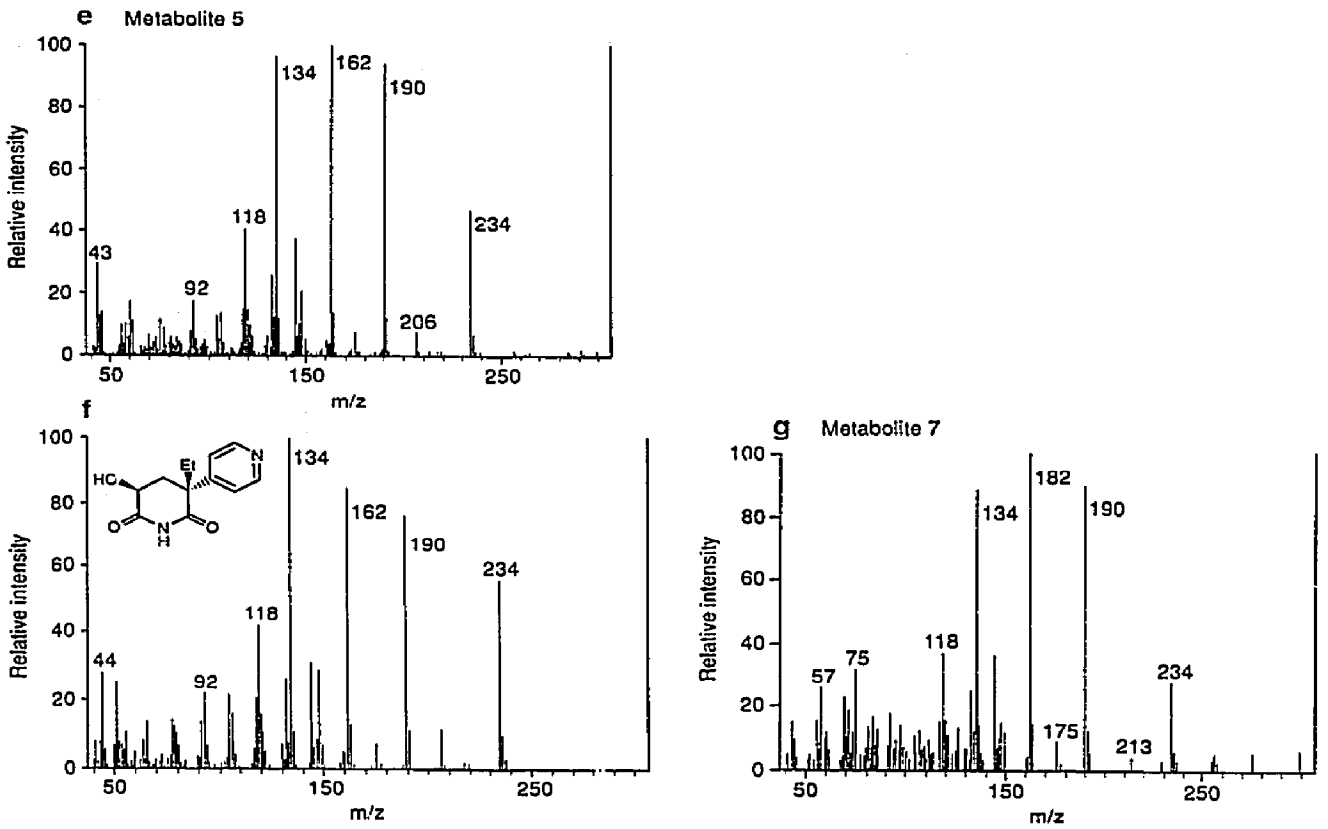

Fig. 3. (a) EI spectrum of compound 1 isolated from urine extracts; (b) EI spectrum of authentic parent drug PG; (c) EI spectrum of compound 3 isolated from urine extracts; (d) EI spectrum of authentic PG *N*-oxide; (e) EI spectrum of compound 5 isolated from urine extracts; (f) EI spectrum, of authentic 5-OHPG; (g) EI spectrum of compound 7 isolated from urine extracts.

their EI mass spectra (Fig. 3a–d). The fragments at *m/z* 190 represented a loss of 28 units from the parent compound. Accurate mass measurement, by peak matching at a resolution of 10 000 on the fragment ion at *m/z* 190, gave a value of 190.07134, indicated that it was the loss of C_2H_4 from the ethyl side-chain (theoretical value 190.074228). In LC–TSP–MS using selected-ion monitoring (SIM) on *m/z* 235 $([M + H + 16])^+$), *m/z* 219 $([M + H]^+)$ and *m/z* 191 $([M + H - 28]^+)$, it was possible to detect the parent drug PG (Fig. 4a, peak 1) and other PG metabolites (Fig. 4b, peaks labelled 4, 5 and 7). From the ion chromatogram (Fig. 4b), there was a component **4** eluted at a retention time (t_R) of 7.7 min, with an $[M + H]^+$ of *m/z* 235. This compound could be a hydroxylated PG metabolite. Since it was present in small quantity and eluted very closely to the PG *N*-oxide in LC–TSP–MS, no attempt was made to isolate this compound. However, it had an identical retention time with the authentic compound **4** namely 3-[2-(aminocarbonyl)ethyl]-3-(4-pyridyl)-5,6-dihydrofuran-2-(3H)-one which was formed by hydroxylation of the ethyl side-chain followed by rearrangement to give a lactone (**4**, Fig. 4c).

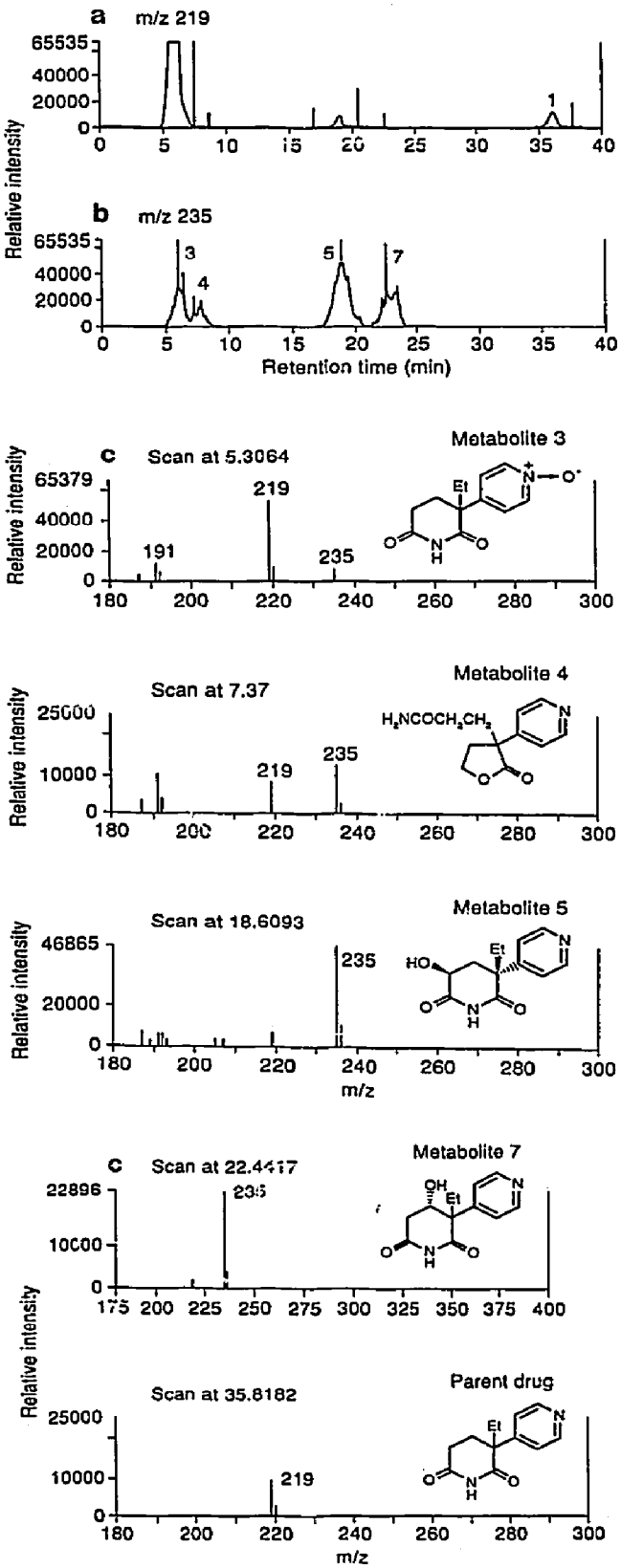

Fig. 4. Extracted ion chromatogram of (a) m/z 219 and (b) m/z 235 of the neutral urine extracts indicating compounds 3, 4, 5 and 7 have an $[M + H]^+$ of m/z 235; (c) LC–TSP–MS spectra of PG metabolites isolated from neutral urine extracts (HPLC system 1).

The ion chromatograms and EI mass spectra of compounds **5** and **7** were very similar except for a fragment at *m/z* 206 present in compound **5** [a loss of 28 mass units] (Fig. 3e–g). However, there were significant differences in their NMR spectra which could distinguish these two compounds and provided their structural information.

The ^{1}H NMR of compounds **5** and **7** ($CDCl_3$) showed, respectively, a double doublet at 4.08 ppm and a triplet at 4.9 ppm which were absent in the spectrum of PG. These downfield signals indicated the protons could be adjacent to a hydroxyl group. The aromatic protons at 8.05 ppm and 8.78 ppm retained the *para*-substitution pattern, indicating hydroxylations were not on the pyridine ring. They could be at the C-4 and C-5 positions of the glutarimide ring or on the ethyl side-chain. For the hydroxy group to be present at C-5, the proton at C-5 should be coupled to the axial proton at C-4 (2.20 ppm) and to the equatorial proton at C-4 (2.74 ppm). Indeed, the double doublet at 4.08 ppm (J = 5.0 and 12.5 Hz) observed for the metabolite is coupled to the axial proton at C-4 (2.20 ppm, dd, J = 5.5 and 13.1 Hz) and to the equatorial proton at C-4 (dd, J = 12.5 and 13.1 Hz). The data for the authentic compound **4** confirm this structure, not only by close resemblance between the respective NMR spectra, but also by MS and HPLC. The large coupling constant between the proton on C-5 and one of the adjacent protons shows that this C-5 proton is axial and the C-5 OH is equatorial. The isomer **6** (*rel*-3*R*,5*R*) with a retention time of 8 min (system 1) is absent in the urine sample. Relating the stereochemistry at C-5 with that at C-3 was achieved by a nuclear overhauser study on the synthetic sample of **5**. Pre-irradiation of the proton *meta* to nitrogen on the pyridine ring enhanced the equatorial proton at C-4 (3.7%) and that on C-5 (2.4%) showing that these protons are *cis* to the pyridyl ring and therefore that the stereochemistry at C-4 is *rel*-3*R*,5*S*[a].

The ^{1}H NMR spectrum of metabolite **7** showed a triplet at 4.9 ppm (J = 2.8 Hz) and a signal at 2.9 ppm (d, CH_2CHOH). This compound is tentatively identified as 4-OHPG (**7**). Since the authentic compound representing this metabolite is not available, structural determination has to rely on the NMR evidence. Assignment of its stereochemistry is based on the published data [5] that an equatorial proton at C-4 would give symmetrical couplings to the vicinal protons, whereas an axial proton usually shows divergent couplings and an upfield chemical shift. The relative stereochemistry of the pyridine ring and ethyl side-chain is not known.

The HPLC profiles of the plasma extracts before treatment and 6 h after a single dose of PG treatment (HPLC system 2) are shown in Fig. 5a and b. The

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[a] For **5** this places the pyridyl ring in an axial position. This compound corresponds to a metabolite reported for aminoglutethimide [δ_{II} (Me_2SO) 3.80 ppm [6], *cf.* **5** has δ_{II} 3.78 ppm, but **6** has δ_{II} 4.38 ppm for H-5], thus the reported aminoglutethimide metabolite was *rel*-3*R*,5*S* and not as previously reported [6,8] where an equatorial position of the pyridyl ring was assumed incorrectly.

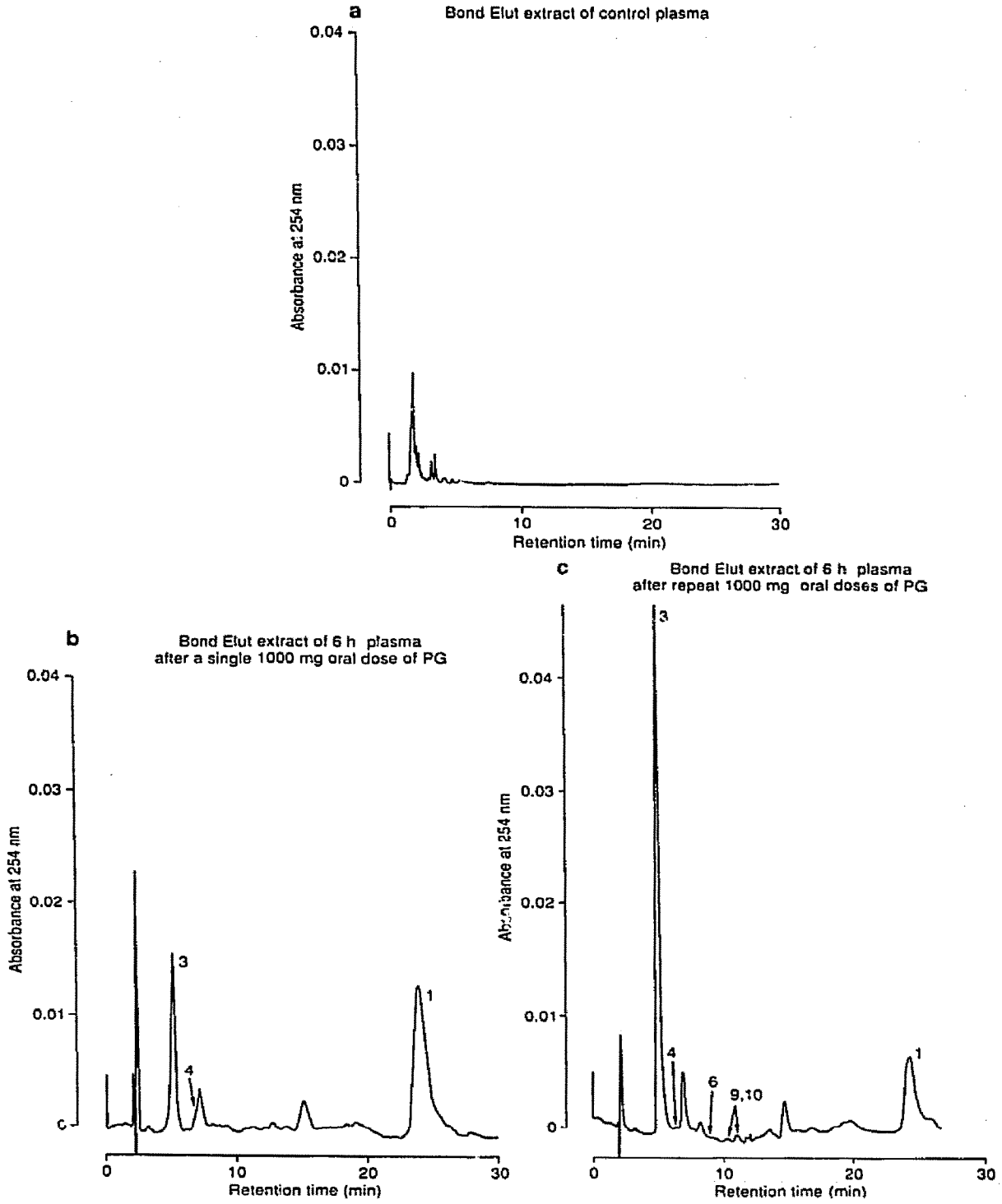

Fig. 5. HPLC profiles (system 2) of (a) pre-treatment plasma extracts and (b) 6 h single-dose post-treatment plasma extracts; peak 1 = parent drug; peaks labelled 3 and 4 correspond to the metabolites **3** and **4**, respectively; (c) 6 h multiple-dose post-treatment plasma extracts; peaks labelled 3, 4, 6, 9 and 10 correspond to the metabolites **3**, **4**, **6**, **9** and **10**, respectively.

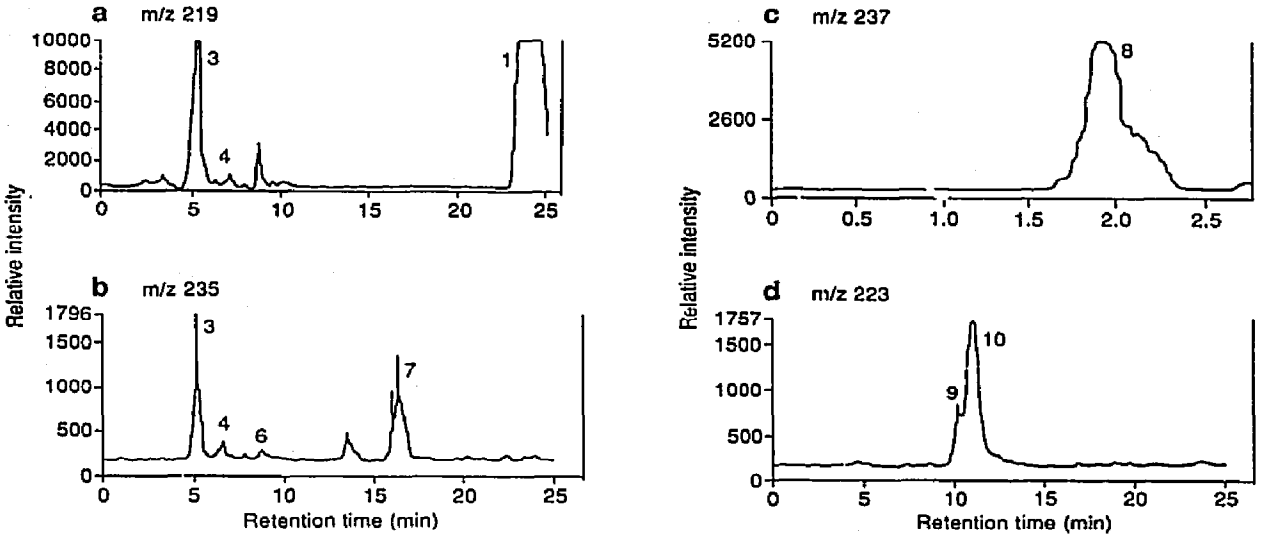

Fig. 6. Extracted ion chromatograms of (a) *m/z* 219, (b) *m/z* 235, (c) *m/z* 237 and (d) *m/z* 223 of plasma extracts after multiple-dose treament of PG (HPLC system 2).

parent drug was detected at 24 min. The metabolite PG *N*-oxide (3) gave an $[M + H]^+$ of *m/z* 235 at 5 min. At the same retention time, an endogenous material with *m/z* 181 was present in a substantial quantity. This endogenous material was also present in the pre-treatment plasma extract. The component which eluted at 14.4 min gave an $[M + H]^+$ of *m/z* 195 and could have been an endogenous material, since it was also present in the blank plasma.

The HPLC profile and the ion chromatogram (HPLC system 2) of the plasma extracts 6 h after multiple doses of PG administration were shown in Figs. 5c and 6, respectively. At an early scan, around 2.5 min, an LC–TSP–MS spectrum showed a component with *m/z* of 219 (Fig. 7a). Early elution indicated that this had been a polar conjugate, but decomposed under TSP conditions to give the PG moiety. Structural identification of this polar component as an intact molecule may be achieved by using fast atom bombardment MS [14]. The reconstructed ion chromatogram showed that there were four metabolites with protonated ions of *m/z* 235 (Fig. 6b). They were PG *N*-oxide (3) at 5 min, compound **4** at 6.5 min, the 5-OHPG (**6**, Fig. 7b) at 8.6 min and compound **7** tentatively at 16 min, respectively. No 5-OHPG (**5**) was observed. A metabolite with $[M + H]^+$ of *m/z* 237 was present at 2.2 min (Fig. 6c). It has an identical retention time to a sample of 2-ethyl-2-(4-pyridyl)-5-carboxypentanamide (**8**, Fig. 7c), and it was detected using a different LC–MS system[a]. To confirm that this compound was not formed

[a] The HPLC system used was a Waters Model 600 multisolvent delivery system, the column was a 5 μm ODS cartrige column (Alltech Assoc.), 15 cm × 4.6 mm I.D. Methanol–0.1 *M* ammonium acetate (20:80, v/v) was the eluting solvent at a flow-rate of 1.25 ml/min. The mass spectrometer used was a VG 30–250 quadrupole mass spectrometer (VG Mass Lab, Altrincham) with a VG TSP interface; the source and capillary temperature were 245 and 210°C, respectively.

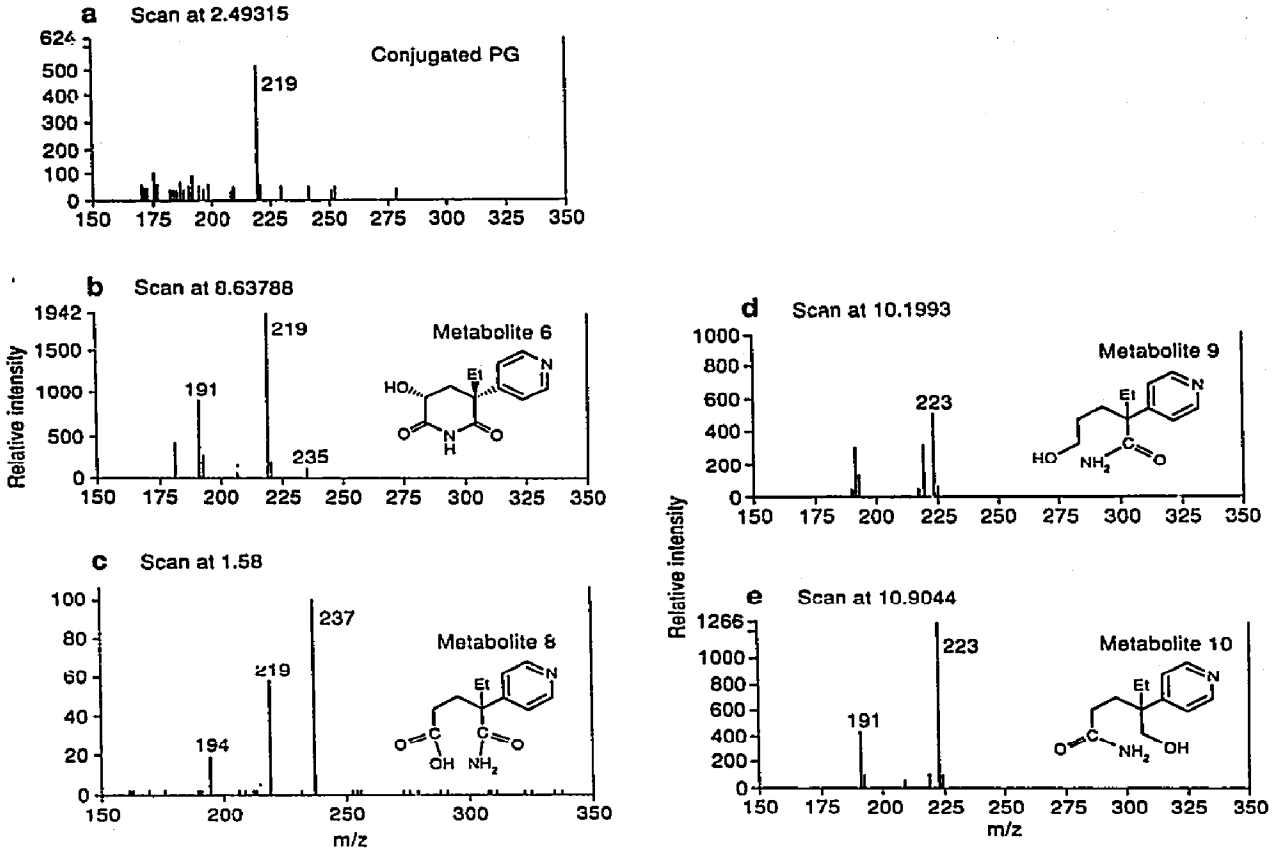

Fig. 7. LC–TSP–MS spectra of plasma extracts after multiple doses of PG (HPLC system 2).

from PG during the extraction procedures of the plasma, a blank plasma (1 ml) was incubated with PG at 37°C for 30 min, then followed by the same extraction procedure as the patients' plasma. No component of *m/z* 236 was present in this sample. Two peaks (**9** and **10**, Fig. 6d) eluted very closely together at 10.2 and 10.9 min both with an $[M + H]^+$ of *m/z* 223, 219 and 191 (Fig. 7d and e). These compounds had identical retention times to the two isomers formed from PG by reductive opening of the glutarimide ring (**9** and **10**).

CONCLUSION

PG was extensively biotransformed in the patients, and several metabolites were detected in the urine and plasma extracts (Fig. 8). PG *N*-oxide (**3**) was a major urinary metabolite and was also present in the plasma after a single dose of PG. Several hydroxylated PGs were present. Hydroxylation took place at the 4 and 5 positions of the glutarimide ring or at the ethyl side-chain. 5-OHPG (**5**) and 4-OHPG (**7**) were both present in the urine extracts in substantial quantity but **5** was not detected in the plasma. Only trace levels of 5-OHPG (**6**) and 4-OHPG (**7**) were present in the plasma after repeated doses of PG. This finding follows a

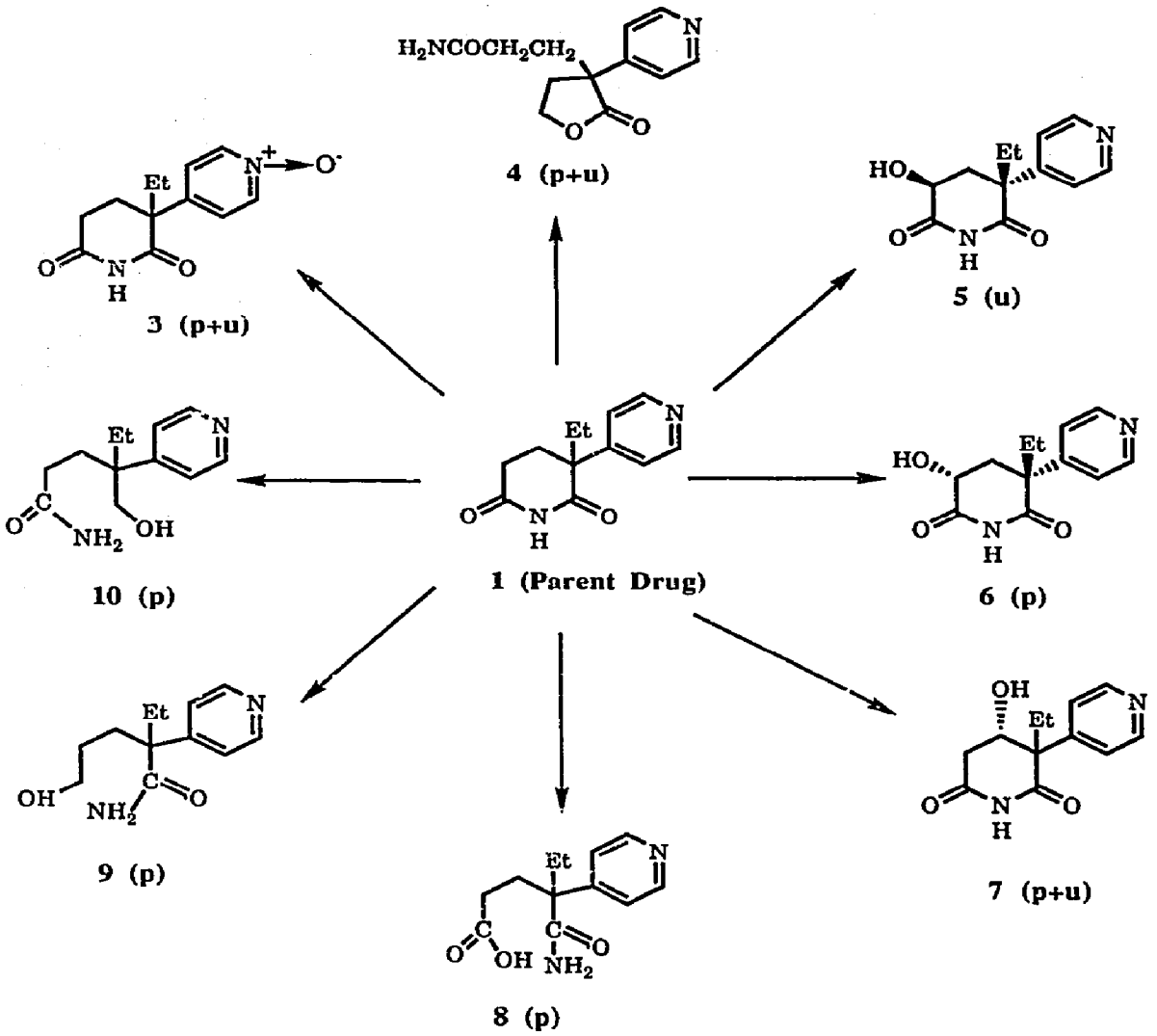

Fig. 8. Proposed scheme for metabolism of PG metabolites; p = detected in the plasma extracts; u = detected in the urine extracts.

similar pattern from previous reports of hydroxylated metabolites detected in the rat, dog and man after glutethimide ingestion [15,16].

The γ-butyrolactone metabolite (**4**) formed by the terminal hydroxylation of the ethyl side-chain, followed by intramolecular cyclization, was a feature in common with the metabolism of AG [6]. This metabolite could be detected consistently both in urine and plasma samples.

The two isomers of **9** and **10** were formed by hydrolytic cleavage of the glutarimide ring. LC–TSP–MS could easily separate and identify these two components, their structures being confirmed by comparison with the standard reference compounds.

Generally speaking, the metabolism of PG resembles the metabolism of AG

[4,6]. Replacement of the aniline ring with a pyridine ring resulted in *N*-oxidation of the pyridine moiety instead of acetylation [6] and N-hydroxylation [7] of the amino function. Other metabolites (**5–10**), which resembled the metabolites of AG, had not been detected previously [4,9].

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