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THE EFFECT OF FLUORINE SUBSTITUTION ON THE HEPATOTOXICITY AND METABOLISM OF PARACETAMOL IN THE MOUSE

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Abstract—The widely used analgesic paracetamol (P) produces fulminant hepatocellular necrosis in humans when taken in overdose. The toxicity is mediated by drug oxidation and depletion of hepatic glutathione. We have, therefore, explored the effects of fluorine substitution on the hepatotoxicity of P in female CD1 mice. 3-Fluoro-4-hydroxyacetanilide (1FPO), 3,5-difluoro-4-hydroxyacetanilide (2FPO), 2,6-difluoro-4-hydroxyacetanilide (2FPN) and 2,3,5,6-tetrafluoro-4-hydroxyacetanilide (4FP) were synthesized, characterized and investigated for their potential to cause hepatotoxicity in the mouse. Introduction of fluorine into P increases the oxidation potential of the drug. The oxidation potentials of paracetamol and its fluorinated analogues were measured by cyclic voltametry and found to increase in the order P < 1FPO < 2FPO < 2FPN < 4FP.

Serum transaminase (ALT) and hepatic glutathione were measured 24 and 6 hr, respectively, after administration of a single dose (2.65 mmol/kg) of each compound to female CD1 mice. There was significant elevation of ALT in mice given P, 1FPO and 2FPO, but not in those which received either 2FPN or 4FP. Hepatic glutathione was reduced significantly by administration of P and IFP, but not after administration of 2FPO, 2FPN or 4FP. Accordingly, glucuronide and sulphate conjugates, but not thioether metabolites, were detected in urine after administration of ^{14}C-labelled 2FPO, 2FPN and 4FP. These data indicate that introduction of fluorine into the 2 and 6 positions increases the oxidation potential of paracetamol which in turn reduces the propensity of the molecule to undergo oxidative bioactivation, and thereby reduces the *in vivo* toxicity of the molecule.

Although adverse drug reactions to paracetamol (P¶) are rare, overdose of the drug can cause severe centrilobular necrosis in humans which can often prove fatal [1, 2]. P-induced hepatotoxicity can be reproduced in experimental animals, mice and hamsters being especially sensitive to the hepatotoxic effects of the drug [3]. The hepatotoxicity is thought to be caused by an electrophilic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), generated within the endoplasmic reticulum of hepatocytes by the cytochrome P450 enzymes, CYP1A and CYP2E [4–6]. However, the metabolite NAPQI has not been isolated from biological systems because of its chemical instability [7]. NAPQI reacts with glutathione to form a 3-glutathionyl conjugate, and with thiol groups of proteins to give paracetamol protein conjugates [8]. The precise mechanisms involved in hepatic necrosis have not been established, but it is thought that the quinoneimine initiates the events leading to cell death by oxidation of, and/or covalent binding to, essential cellular thiols e.g. the Ca^{2+}-ATPases which are involved in maintaining the calcium gradient across the cell membrane [9, 10].

After administration of a therapeutic dose of P the major pathways of metabolism are glucuronidation and sulphation [11]. Oxidation is a minor pathway of metabolism and any NAPQI formed is normally inactivated by conjugation with hepatic glutathione. However, after an overdose the sulphation pathway becomes saturated, hepatic glutathione is depleted and there is an accumulation of the toxic quinoneimine within the hepatocyte [12]. Thus, increasing the dose of the drug leads to an unfavourable change in the balance between intoxication and detoxication pathways of drug metabolism.

In order to explore further the relationship between the metabolism and the toxicity of P, we have studied a series of fluorinated analogues (Fig. 1). The introduction of fluorine into a molecule can have pronounced effects on the biological activity and the physio-chemical properties of aromatic compounds. These changes are a consequence of the enhanced stability of the carbon–fluorine bond (compared with the carbon–hydrogen bond),

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¶ Abbreviations: P, paracetamol (4-hydroxyacetanilide); NAPQI, *N*-acetyl-*p*-benzoquinoneimine; 1FPO, 3-fluoroparacetamol (3-fluoro-4-hydroxyacetanilide); 1FPN, 2-fluoroparacetamol (2-fluoro-4-hydroxyacetanilide); 2FPO, 3,5-difluoroparacetamol (3,5-difluoro-4-hydroxyacetanilide); 2FPN, 2,6-difluoroparacetamol (2,6-difluoro-4-hydroxyacetanilide); 4FP, tetrafluoroparacetamol (2,3,5,6-tetrafluoro-4-hydroxyacetanilide); 3FP, trifluoroparacetamol (*N*-trifluoroacetyl-4-hydroxyaniline); GSH, reduced glutathione; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); ALT, alanine aminotransferase; MOPS, 3-[*N*-morpholino]propanesulphonic acid; PEG 200, Polyethylene glycol 200.

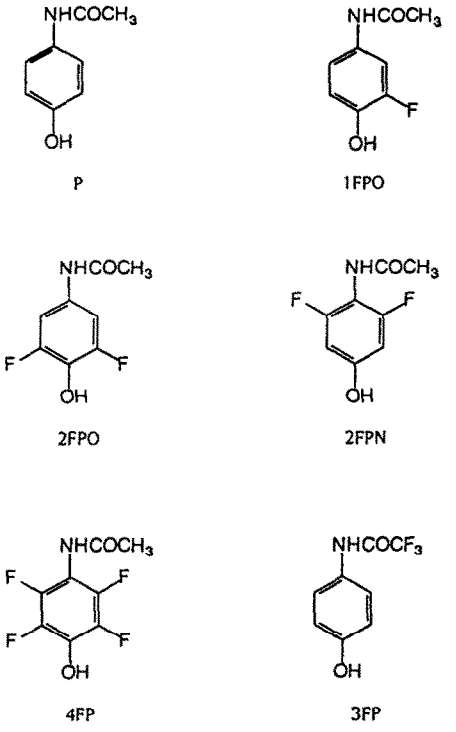

Fig. 1. Chemical structures of P, 1FPO, 2FPO, 2FPN, 4FP and 3FP.

increased lipid solubility, increased hydrogen bonding potential and alterations in the pK_a of phenolic groups [13]. In addition, we have found that fluorine-substitution alters the oxidation potential of P [14]. It was therefore of interest to determine whether fluorine-substitution might reduce the oxidative bioactivation of paracetamol *in vivo* and thereby reduce hepatotoxicity.

MATERIALS AND METHODS

Materials

[^{14}C]Acetic anhydride and NCS tissue solubilizer were obtained from Amersham International (Amersham, U.K.). CD1 mice were obtained from Bantin and Kingman (Hull, U.K.). β-Glucuronidase (Glucurase), aryl sulphatase (Type VIII), β-glucuronidase (Type H_2), D-saccharic-acid-1,4-lactone, ring labelled [^{14}C]P, EDTA, 3-[*N*-morpholino]propanesulphonic acid (MOPS) and the alanine aminotransferase Kit (Kit No. 505) were obtained from the Sigma Chemical Co. (Poole, U.K.). Preparative TLC plates, polyethylene glycol 200 (PEG 200), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and hydrogen peroxide (30% w/v) were obtained from BDH Chemicals (Poole, U.K.). Type GS filters (0.22 micron) and type HV filters (0.45 micron) were obtained from Millipore (Watford, U.K.). HPLC columns were obtained from Millipore or HPLC Technology (Macclesfield, U.K.). P metabolites (P glucuronide, sulphate, cysteine and mercapturate) were gifts from Dr G. Hawksworth, University of Aberdeen. 1FP (fluoroparacetamol), 2FPO (3,5-difluoro-4-hydroxyacetanilide), 2FPN (2,6-difluoro-4-hydroxyacetanilide), and 4FP (2,3,5,6-tetrafluoro-4-hydroxyacetanilide) were synthesized as described previously [14].

Synthesis of radiolabelled P analogues

[^{14}C]*Acetyl-3,5-difluoro-4-methoxyacetanilide.* [^{14}C]Acetyl-3,5-difluoro-4-methoxyacetanilide was prepared from 3,5-difluoro-4-methoxyaniline (0.15 g, 0.94 mmol) in dry chloroform (2 mL) and [^{14}C]acetic anhydride [500 μCi, 29.3 mCi/mmol (9.8 μL, 20% w/w in toluene) diluted with unlabelled acetic anhydride (1.15 mmol)]. The crude product was washed with light petroleum (b.p., 40–60°), dried under vacuum and used without further purification.

[^{14}C]*Acetyl-2FPO.* [^{14}C]Acetyl-3,5-difluoro-4-methoxyacetanilide (0.18 g, 0.9 mmol) in dry dichloromethane (1.5 mL) was demethylated using boron tribromide [14]. The crude product was purified by flash column chromatography on silica using ethyl–acetate (9:1) as eluent, to give [^{14}C]-acetyl-2FPO as off-white crystals, 0.14 g (84%); *m/z* 187 (M^+, 30%), 145 (100%), 97 (20%). The radiochemical purity was assessed by HPLC using (a) a Spherisorb 5 micron ODS column eluted with water–acetonitrile (19:1), retention time 29 min; 99.2%, and (b) a μBondapak 10 micron ODS column eluted with a linear gradient of methanol (5–40% over 20 min) in 0.05 *M* sodium acetate buffer (pH 3.6), retention time 16 min; >99.5%. The specific activity of the product was 0.75 μCi/mg (radiochemical yield 21%).

[^{14}C]*Acetyl-2,6-difluoro-4-methoxyacetanilide.* [^{14}C]Acetyl-2,6-difluoro-4-methoxyacetanilide was prepared from 2,6-difluoro-4-methoxyaniline (0.15 g, 0.94 mmol) in dry chloroform (2 mL) and [^{14}C]acetic anhydride [500 μCi, 10 mCi/mmol] diluted with unlabelled acetic anhydride (1.1 mmol). The crude product was washed with light petroleum (b.p., 40–60°), dried under vacuum and used without further purification.

[^{14}C]*Acetyl, 2FPN* The demethylation of [^{14}C]-acetyl-2,6-difluoro-4-methoxyaccranilide (0.185 g, 9.92 mmol) in dry dichloromethane (1.5 mL) was carried out using boron tribromide [14]. The crude product was purified by flash column chromatography on silica using chloroform–ethanol (11.5:1) as eluent. The fractions were monitored radiometrically. [^{14}C]Acetyl-2FPN was obtained as off-white crystals, 0.138 g, 80%; *m/z* 187 (M^+, 7%), 145 (100%), 43 (22%). The radiochemical purity was assessed by HPLC using a Spherisorb 5 micron ODS column eluted with (a) water–acetonitrile (24:1), retention time 15 min 20 sec; >99.5%, and (b) a linear gradient of methanol (5–40% over 20 min) in 0.05 M sodium acetate buffer (pH 4.4), retention time 11 min 40 sec; >99.5%. The specific activity was 1.39 μCi/mg (radiochemical yield, 38%).

[^{14}C]*Acetyl-2,3,5,6-tetrafluoro-4-methoxyacetanilide.* [^{14}C]Acetyl-2,3,5,6-tetrafluoro-4-methoxyacetanilide was prepared from 2,3,5,6-tetrafluoro-4-methoxyaniline (0.1 g, 0.52 mmol), and [^{14}C]acetic anhydride [250 μCi, 24.8 mCi/mmol (5.72 μL,

20% w/w in toluene) diluted with unlabelled acetic anhydride (0.75 mmol)] in toluene (1.5 mL). The crude off-white solid was washed with light petroleum (b.p., 40–60°), dried under vacuum and used without further purification.

[^{14}C]*Acetyl-4FP*. The demethylation of [^{14}C]acetyl-2,3,5,6-tetrafluoro-4-methoxyacetanilide (0.11 g, 0.46 mmol) in dry dichloromethane (1 mL) was carried out using boron tribromide [14]. The crude product was purified by preparative TLC using ethyl acetate–hexane (11:9) as eluent to give [^{14}C]acetyl-4FP 0.083 g, 81%; *m/z* 223 (M^+, 4%), 181 (100%), 133 (12%). The radiochemical purity was assessed by TLC, ethyl acetate–hexane (11:9); >99.5%, and by HPLC, using a Spherisorb 10 ODS column eluted with a non-linear gradient of methanol (5–50%) in 0.05 M sodium acetate buffer (pH 3.6) retention time 14 min; >99.5%.

Biological methods

General procedure for toxicological experiments. All mice were starved overnight prior to the experiments. The drugs were administered intraperitoneally (i.p.), between 10 and 12 a.m. Groups of 10 mice (CD1, female, 20–30 g body weight) received various doses of P and its fluorinated analogues. The drugs were dissolved in 1:1 PEG 200–saline (8 mL/kg) so that each mouse received a 0.15–0.25 mL final volume. Control mice received 1:1 PEG 200–saline only (0.15 mL). After dosing, the mice were allowed free access to food and water. Any changes in behaviour were noted and deaths recorded. Surviving mice were killed humanely after 6 or 24 hr. Blood was collected and serum prepared for measurement of serum alanine aminotransferase (ALT). Livers were removed and either frozen immediately in liquid nitrogen for the measurement of GSH levels, or stored in buffered formalin for histological examination.

The measurement of serum ALT. Serum ALT was determined 24 hr after administration of the drug or vehicle. Serum was stored at 4°, and determination of ALT was made within 2 days of collection, using a Sigma diagnostic kit. The method is based on that of Reitman and Frankel [15].

The determination of hepatic GSH. GSH was determined 6 hr after administration of the drug or vehicle. The excised livers were frozen immediately in liquid nitrogen and stored at −80° until assayed for glutathione content. The method is based on the procedure described by Akerboom and Sies [16] using the colour reagent DTNB [17].

Frozen liver (0.5–1 g) was homogenized in ice cold 1 M perchloric acid/0.02 M EDTA (5 mL). The homogenates were centrifuged (1000 *g*, 15 min, 4°) and an aliquot (1 mL) of the supernatant neutralized with 2 M potassium hydroxide/0.3 M MOPS (1.2 mL). The pH of each solution was then checked and if necessary adjusted to pH 6.8–7 by adding more potassium hydroxide/MOPS. The neutralized mixture was then centrifuged (1000 *g*, 2 min, 4°) and an aliquot (100 μL) of supernatant added to 0.1 M potassium phosphate buffer (pH 7), 1 mM EDTA (1.86 mL), prior to the addition of DTNB (60 μg) in 0.5% (w/v) sodium bicarbonate (40 μL). The absorbance was measured at 412 nm and the glutathione concentration determined from a standard curve for GSH (0–5 mM).

Liver histology. Livers were removed immediately post mortem and fixed whole in 10% buffered formalin. A thin block of tissue was trimmed from the edge of the largest liver lobe for further fixation. Sections (6 micron thick) were prepared from paraffin-embedded blocks of liver and examined by light microscopy [18]. The severity of liver injury was scored according to the extent of parenchymal cell injury:

0 = no histological abnormality;
1 = focal parenchymal cell necrosis;
2 = coalescing parenchymal cell necrosis in the centrilobular area (Zone 3);
3 = extensive parenchymal cell necrosis involving the majority of an affected lobule.

Investigation of drug metabolism in vivo

Groups of four mice were administered [^{14}C]P or the fluorinated analogues (3)–(5) in (1:1) PEG 200–saline (i.p. 8 mL/kg). The compounds were administered at 2.65 mmol/kg (5–10 μCi/mouse). Each animal was placed in an individual glass metabolism cage. Urine was collected (at room temperature) at 6 and 24 hr after dosing. After each collection the cage was washed thoroughly with distilled water, the washings and the urine were mixed and stored at −70° until required. After 24 hr the animals were killed and livers removed and analysed for radioactive content.

The estimation of radioactivity in urine and liver. After each collection, three weighed aliquots of urine (100 μL) were taken and scintillation fluid (4 mL) added. The radioactive content of the samples was determined by liquid scintillation spectrometry.

The estimation of radioactivity in the liver. In order to solubilize the tissue completely, a suspension of weighed tissue (50–100 mg), water (0.25 mL) and tissue solubilizer (1 mL) was left at 50° overnight. Upon cooling, hydrogen peroxide (30% w/v, 0.3 mL) was added and the solution was maintained at 50° for a further 30 min. Scintillation fluid (20 mL) was then added to the cooled samples and the radioactive content was determined by liquid scintillation spectrometry.

The separation and characterization of urinary metabolites. In order to characterize the urinary metabolites of P, and the fluorinated analogues, samples of urine were hydrolysed in an equal volume of sodium acetate buffer (0.2 M, pH 5) containing β-glucuronidase (5000 U/mL) or Type H_2 β-glucuronidase (5000 U/mL, from *Helix pomatia*, also containing aryl-sulphatase activity). Hydrolysis with aryl-sulphatase (Type VIII, 100 U/mL) was carried out in the presence of D-saccharic-acid-1,4-lactone (20 mM in sodium acetate buffer, 0.2 M, pH 5) to inhibit the β-glucuronidase present in the sulphatase preparation. Control hydrolysis of urine samples was carried out in an equal volume of buffer only. The incubations were carried out overnight (18 hr) at 37°. Excess protein was removed from the enzyme mixture by the addition of methanol and centrifugation.

Urinary metabolites of ring-labelled [^{14}C]P were separated by reverse phase HPLC and quantified by

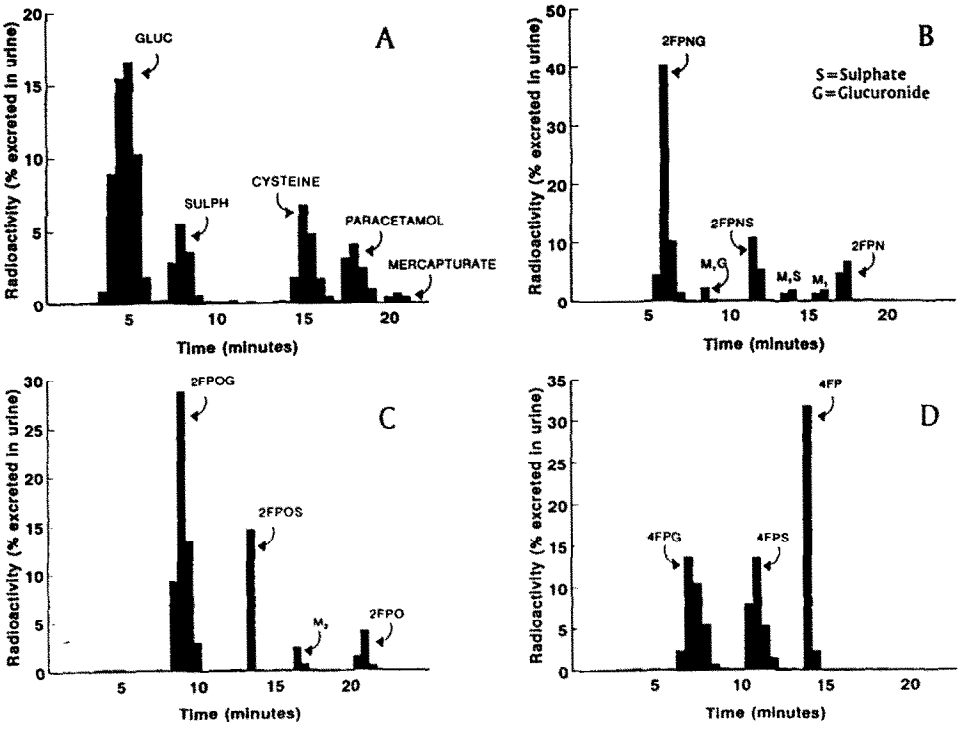

Fig. 2. Radiometric chromatograms of 24-hr urine samples obtained from mice after administration of ^{14}C-labelled compounds. (A) P, (B) 2FPN, (C) 2FPO, and (D) 4FP.

liquid scintillation spectrometry. The identity of the metabolites was confirmed by co-chromatography with authentic standards and by enzyme hydrolysis. Filtered, diluted urine samples were injected directly onto a Spherisorb 5 micron ODS column. Mobile phase (8% methanol in 0.05 M sodium acetate, pH 4.4) was delivered at a rate of 1 mL/min. The following retention times were noted for P and metabolites: P glucuronide, 4.8 min; P sulphate, 8.1 min; P cysteine, 14.8 min; P 17.7 min; and P mercapturate, 20.2 min.

Urinary metabolites of [^{14}C]acetyl-2FPO were separated by reverse phase HPLC and quantified by liquid scintillation counting. Filtered, diluted urine samples were injected directly onto a Spherisorb 5 micron ODS column. The column was eluted with a linear gradient of methanol (5–40% over 20 min) in sodium acetate buffer (0.05 M, pH 4.4) at a flow rate of 1 mL/min. Parent compound was extracted from urine with ethyl acetate and further purified by the above HPLC method. Buffer salts were removed by HPLC using a mobile phase consisting of water, adjusted to pH 4 with acetic acid, prior to the identification of parent compound by mass spectrometry. The conjugates were characterized by enzyme hydrolysis of the individual isolated conjugate followed by HPLC analysis and mass spectrometry of the aglycone.

Urinary metabolites of [^{14}C]acetyl 2FPN were separated, quantified and characterized as described for 2FPO, except that an Ultratech 5 micron ODS column was used. The column was eluted with a gradient of methanol (1–40%) in sodium acetate buffer (0.05 M, pH 4.4).

Urinary metabolites of [^{14}C]acetyl-4FP were separated, quantitated and characterized as described for 2FPO except that a Spherisorb 10 micron ODS column was used. The column was eluted with a gradient of methanol (5–50%) in sodium acetate buffer (0.05 M, pH 3.6). The isolated sulphate conjugate was purified by elution from a C18 Sep-pak cartridge using methanol, prior to identification by mass spectrometry.

Table 1. The comparative toxicity of P and some fluorinated analogues: doses which cause 50% mortality*

	mg/kg	mmol/kg
P	510	3.38
1FP	670	3.96
2FPO	750	4.01
2FPN	>1100	>5.88
4FP	720	3.23
3FP	290	1.41

* Deaths were recorded 24 hr after administration (N = 10–30).

RESULTS

The effect of fluorine substitution on the gross toxicity of P

P and the fluorinated analogues 1FP, 2FPO and

Table 2. Serum transaminase (ALT) activity 24 hr after i.p. administration of P and the fluorinated analogues 1FP, 2FPO, 2FPN and 4FP to mice*

Compound	Dose (mmol/kg)	ALT activity (SF units/mL)
Control	—	35 ± 13‖
P	1.33	57 ± 34
	2.65	3077 ± 1522§
	3.64	5153 ± 1543§
1FP	1.33	38 ± 20
	2.65	2567 ± 1495§
2FPO	1.33	30 ± 9
	2.65	648 ± 390‡‖
2FPN	1.33	29 ± 5
	2.65	30 ± 6‖
	3.64	39 ± 7‖
	4.14	43 ± 12‖
	4.96	46 ± 10‖
	5.96	52 ± 22†‖
4FP	1.33	35 ± 12
	2.65	41 ± 6‖
	3.15	45 ± 15‖
	3.64	55 ± 23†‖

* Results are expressed as mean ± SD (N = 20).
† $P < 0.05$, ‡$P < 0.01$, §$P < 0.001$ vs control, ‖$P < 0.001$ vs paracetamol (2.65 mmol/kg).
Analysed by Mann–Whitney U Rank Sum Test.

Table 3. Hepatic glutathione (GSH) 6 hr after i.p. administration of P and the fluorinated analogues, 1FP, 2FPO, 2FPN and 4FP to mice*

Compound	Dose (mmol/kg)	GSH (μg/g liver)
Control	—	1806 ± 190§
P	2.65	967 ± 340‡
	2.65	
1FP		1127 ± 592†
2FPO	2.65	1960 ± 438§
	3.64	933 ± 595†
2FPN	2.65	2072 ± 380§
	3.64	2010 ± 440§
	4.96	1887 ± 211§
4FP	2.65	1588 ± 328§

* Results are expressed as mean ± SD (N = 10–18).
† $P < 0.01$, ‡$P < 0.001$ vs control, §$P < 0.001$ vs paracetamol (2.65 mmol/kg).
Analysed by Mann–Whitney U Rank Sum Test.

4FP caused 50% mortality at comparable doses (Table 1). 2FPN did not cause 50% mortality at any of the doses tested.

The effect of fluorine substitution on the hepatotoxicity of P

At a dose of 1.33 mmol/kg, P and the fluorinated analogues (IFPO, 2FPO, 2FPN, 4FP) did not raise serum ALT significantly above those of vehicle treated control animals, indicating that this dose is non-hepatotoxic for all the compounds investigated. However, on doubling the dose (2.65 mmol/kg) changes in ALT, indicative of hepatic damage, were seen for some compounds (Table 2). The rank order of hepatotoxicity based on ALT and hepatic GSH levels (Tables 2 and 3) was P > 1FP > 2FPO > 4FP > 2FPN. A summary of the occurrence and severity of hepatic damage in mice receiving P and the fluorinated analogues as assessed by histological examination, is given in Table 4. P caused severe parenchymal cell necrosis in zone 3 in seven out of 10 animals. The rank order of hepatotoxicity according to ALT and hepatic GSH levels is supported by the histological results. Up to 4.14 mmol/kg 2FPN did not raise ALT levels above those of control animals. At 4.96 and 5.96 mmol/kg 2FPN significantly ($P < 0.05$) raised ALT levels, possibly indicating some enzyme leakage although no hepatic damage was observed on histological examination after a dose of 4.96 mmol/kg (Table 4). However, at all doses tested 2FPN was significantly ($P < 0.001$) less hepatotoxic than P. A similar trend was observed for 4FP. At 3.64 mmol/kg 4FP significantly ($P < 0.05$) raised ALT levels above control animals but at all the doses tested 4FP was significantly ($P < 0.001$) less hepatotoxic than P.

The effect of fluorine substitution on the depletion of hepatic glutathione by P

P and 1FP significantly ($P < 0.001$) depleted hepatic GSH levels after a dose of 2.65 mmol/kg (Table 3). Hepatic GSH levels were also significantly ($P < 0.001$) depleted in 2FPO treated animals but a higher dose (3.64 mmol/kg) was required. Hepatic GSH was not significantly depleted by either 2FPN or 4FP at the doses investigated.

The effect of fluorine substitution on the disposition of P

After administration of P, 2FPO, 2FPN and 4FP at a single dose (2.65 mmol/kg) more than 85% of the administered radioactivity was recovered in the urine in 24 hr. The residual radioactivity in the liver 24 hr after dosing was significantly greater ($P < 0.01$) in mice treated with radiolabelled P compared to 2FPO, 2FPN and 4FP (Table 5).

After administration of [^{14}C]P (2.65 mmol/kg) 97% of the radioactivity excreted in the urine (24 hr) was accounted for by five metabolites (Table 6 and Figs 2 and 3). HPLC analysis of β-glucuronidase/aryl-sulphatase hydrolysed urine samples revealed that 67% of the administered dose was excreted as free drug plus glucuronide and sulphate conjugates. The remainder of the administered dose (19%) was excreted as thioether conjugates (cysteine and mercapturate) reflecting reactive metabolite formation.

After administration of [^{14}C]2FPO, [^{14}C]2FPN and [^{14}C]4FP (2.65 mmol/kg) greater than 90% of the radioactivity excreted in urine (24 hr) was accounted for by three components in each case (Table 6 and Fig. 2). Selective enzyme hydrolysis of isolated peaks revealed that these components were, in each case, the parent compound together with a sulphate and glucuronide conjugate (Table 6 and Figs 2 and 3). In the case of 2FPN an unidentified minor metabolite (9%), M1, was excreted as free compound and as a glucuronide and a sulphate indicating that it is a

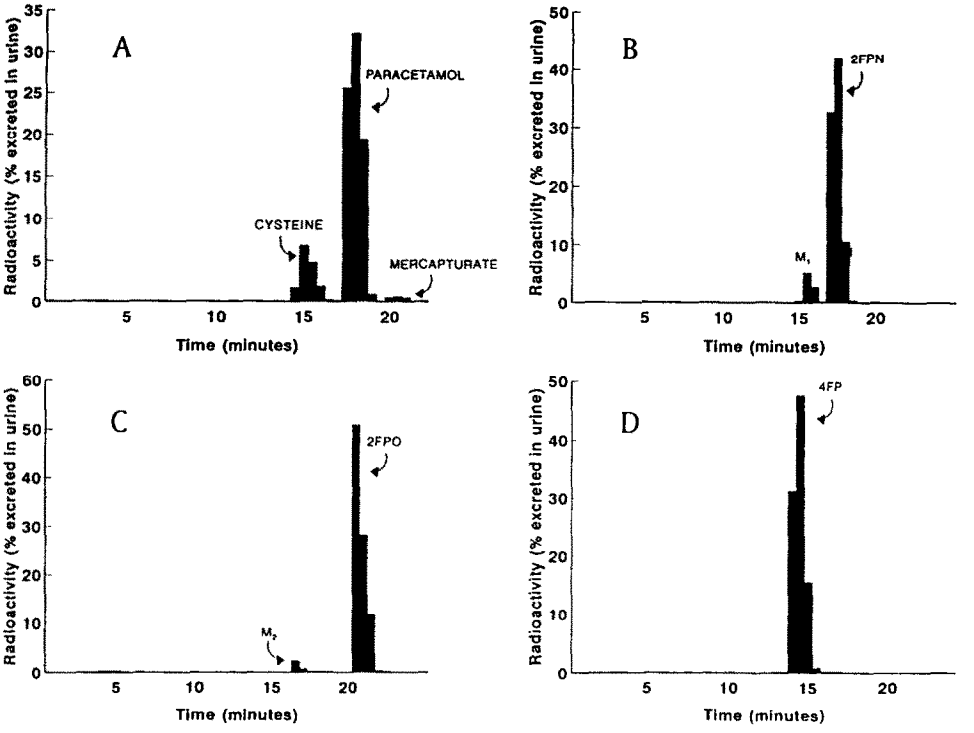

Fig. 3. Radiometric chromatographs of hydrolysed (β-glucuronidase/aryl-sulphatase) 24-hr urine samples obtained from mice after administration of ^{14}C-labelled compounds. (A) P, (B) 2FPN, (C) 2FPO and (D) 4FP.

Table 4. A summary of the occurrence and severity of liver injury in mice receiving P and some fluorinated analogues

Compound	Dose (mmol/kg)	Number	Severity score* 0	1	2	3
Control	—	10	9	1	0	0
P	2.65	10	2	1	4	3
1FP	2.65	9	6	0	3	0
2FO	2.65	10	6	4	0	0
2FPN	2.65	10	10	0	0	0
2FPN	4.96	9	9	0	0	0
4FP	2.65	10	7	2	1	0

* Severity scores: 0 = no histological abnormality; 1 = focal parenchymal cell necrosis; 2 = coalescing parenchymal cell necrosis in Zone 3; 3 = extensive parenchymal cell necrosis involving majority of individual lobes.

Table 5. Residual radioactivity in the liver 24 hr after mice had been treated with 2.65 mmol/kg [^{14}C]P, 2FPO, 2FPN and 4FP

Compound	Residual radioactivity*
P	0.41 ± 0.072
2FPO	0.28 ± 0.0208†
2FPN	0.16 ± 0.0096†
4FP	0.23 ± 0.022†

* Data are expressed as percentage of dose and represent the mean (N = 4) ± SD.

† $P < 0.01$ vs paracetamol (one-way ANOVA, differences between treatment groups were tested by Scheffe's method).

hydroxylated metabolite of 2FPN. For 2FPO a minor (*ca.* 2% of the dose), unidentified metabolite (M2) was excreted in urine (Figs 2 and 3). In contrast to the thioether conjugates of P, which could not be extracted from urine at neutral pH, the unknown metabolite could be extracted from urine at neutral pH with ethyl acetate indicating that it was not a thioether conjugate.

The metabolism of 3FP was complex and will not be reported in detail here. Mass spectrometry and HPLC indicated that P sulphate and P glucuronide were major metabolites indicating that detrifluoroacetylation to 4-aminophenol followed by acetylation had occurred.

Effect of fluorine substitution on the physico-chemical properties of P

The chemical synthesis and the determination of the oxidation potentials, pK_as and log K_os for P, 1FP, 2FPO, 2FPN and 4FP have been reported elsewhere [14] and are summarized in Table 7. A correlation matrix for the various physico-chemical

Table 6. The metabolism of P and some fluorinated analogues (2.65 mmol/kg) in the mouse*

Compound	P	2FPO	2FPN	4FP
Unchanged drug	10.1 ± 0.6	6.2 ± 1.3	9.4 ± 2.3	44.1 ± 9.8
Glucuronide	49.1 ± 0.9	54.4 ± 2.3	58.2 ± 1.2	23.7 ± 7.1
Sulphate	16.9 ± 0.9	29.0 ± 3.2	16.2 ± 1.2	27.4 ± 3.8
Cysteine	19.0 ± 1.2	—	—	—
Mercapturate	1.9 ± 0.3	—	—	—

Results are expressed as percentage of the dose excreted in urine (mean ± SD, N = 4).

* ^{14}C-Labelled compounds were administered i.p. and urine collected for 24 hr. Metabolites were separated and identified as described in the Materials and Methods.

Table 7. Some physico-chemical properties of P and some fluorinated analogues

Compound	pK_a	Oxidation* potential (V)	Log K_o (pH 3)	Log K_o (pH 7.4)	UV Shift (nm)†
P	9.53	1.14	0.65	0.65	15
1FP	8.1	1.25	0.85	0.76	13
2FPO	7	1.33	1.05	0.7	9
2FPN	9.05	1.52	0.67	0.65	−3
4FP	4.75	1.74	0.79‡	−0.03	−8
3FP	9.14	1.37	1.00	1.00	23

* VS Saturated Calomel electrode.

† The shift (nm) between the primary UV absorbance band of the amides and their corresponding amines. The shift (nm, bathochromic or hypsochromic) represents a measure of the degree of conjugation of the acetyl group. A negative value indicates loss of conjugation.

‡ Measured at pH 2.5.

Table 8. The correlation between mean ALT levels (2.65 mmol/kg) and various physico-chemical parameters for P and some fluorinated analogues

	pK_a	Log K_o (pH 3)	Log K_o (pH 7.4)	Oxidation potential (V)	Degree of acetyl-amide conjugation (UV shift, nm)
r	0.56	−0.201	0.52	−0.87	0.88
P	0.32	0.75	0.38	0.056	0.048

Results were analysed by linear regression. Compounds, P, 1FP, 2FPO, 2FPN and 4FP, were included in the correlation.

measurements and mean ALT levels is given in Table 8.

DISCUSSION

The pathological and biochemical changes associated with P-induced liver damage include dramatic increases in the activities of serum enzymes alanine aminotransferase and aspartate aminotransferase, decreased hepatic glutathione concentrations and extensive marked necrosis around centrilobular veins with coalescence of necrotic areas, as indicated by liver histology [19].

Female mice were used in this study because it has been reported that they are more susceptible than male mice to the hepatotoxic effects of P [20]. Administration of a single dose of 2.65 mmol/kg of P produced significant hepatotoxicity at 24 hr in the mouse, as measured by a significant increase in ALT accompanied by marked changes in liver histology. The effects on ALT and the histological changes observed in this study were the same as those reported in previous studies of P hepatotoxicity [21–23]. There was also a significant 2-fold decrease in hepatic glutathione, measured 6 hr after drug administration. Using this single dose (2.65 mmol/

kg) it was possible to make a quantitative assessment of the comparative hepatotoxicities of the novel fluorinated P derivatives, as this dose was hepatotoxic (for some compounds) but non-lethal.

From Tables 2, 3 and 4 it can be seen that introduction of the fluorine into the aromatic nucleus significantly reduced the hepatotoxicity of P. The extent of reduction in toxicity was dependent upon both the extent and position of fluorine substitution. Introduction of a single fluorine atom had little effect on the hepatotoxicity of P. The gross toxicity for 1FPO was similar to that of P, and the changes in ALT and hepatic glutathione produced by the two compounds were also similar. We have also found that 2-fluoro-4-hydroxyacetanilide (1FPN) has similar effects on serum transaminase (unpublished data). The histological appearances of the livers from 1FPO group were more variable than those in the P group (Table 4) but where liver injury was observed, it was found to be similar in nature for the two compounds.

Introduction of two fluorine atoms into P, into either the 3,5-positions (2FPO) or the 2,6-positions (2FPN) significantly reduced the elevation of serum ALT seen with P itself. Substitution ortho to the amide group was considerably more effective than substitution ortho to the hydroxyl group, indeed, doses as high as 5.96 mmol/kg of 2FPN did not raise ALT activity above control and no histological changes in liver structure were observed at 2.65 or 4.96 mmol/kg (Table 4). The gross toxicity for 2FPN was much less than that of P (Table 1). At 2.65 mmol/kg neither 2FPO nor 2FPN significantly depleted hepatic glutathione levels at 6 hr despite the fact that the former produced a significant increase in serum ALT levels compared with control animals at 24 hr. However, a more thorough investigation of the time-course of GSH depletion is required to determine whether the dissociation between GSH depletion and enzyme leakage is real or apparent. Importantly, it was found that 2FPO did deplete hepatic GSH after a dose of 3.64 mmol/kg, whereas 2FPN did not, even after a dose of 4.96 mmol/kg.

Complete fluorine substitution on the aromatic ring (4FP) also significantly reduced the hepatotoxicity produced by P as illustrated by normal ALT and hepatic glutathione after administration of 2.65 mg/kg. However, a remarkable histological feature of this group of livers was the presence of hepatic capsular fibrinous exudate suggesting an inflammatory response to a tissue irritant. This may be due to the highly acidic nature of the compound, the pK_a of the phenolic group was 4.75 which is similar to the pK_a of acetic acid. The gross toxicity of 4FP was greater than that of either of the two difluorinated analogues.

Investigation of the hepatotoxicity of the trifluoroacetyl analogue of P (3FP) *in vivo* was thwarted by the low LD_{50} which was a consequence of CNS toxicity.

Hepatotoxicity observed after administration of large doses of P is thought to be caused by metabolic oxidation to form NAPQI [24]. The results reported here support this concept in as much as fluorine substitution increases the oxidation potential of P and thus decreased the hepatotoxic reaction. There is a good correlation between mean ALT levels after 2.65 mol/kg and the oxidation potential of the compounds measured by cyclic voltametry ($r = -0.87$, $P = 0.056$) given the small number of compounds investigated (Table 8). The studies of hepatic glutathione depletion add further support to the hypothesis; since GSH depletion mirrored the ability of the compounds to cause elevations of serum ALT.

Thus it appears that introduction of fluorine into P, and into the 2- and 6-positions in particular, reduces hepatotoxicity by blocking the *in vivo* bioactivation of P by the cytochrome P450 enzymes. We therefore investigated the metabolism of P and three of the fluorinated analogues, *in vivo* in the mouse at 2.65 mmol/kg. P, 2FPO, 2FPN and 4FP are excreted rapidly (0–24 hr) and over 85% of the administered radioactivity is recovered in the urine. P underwent extensive glucuronidation, sulphation and also oxidation, as evidenced by the excretion of the thioether metabolites, the cysteine and mercapturate conjugates. In contrast neither the two difluoro analogues, nor the tetrafluoro analogue formed thioether conjugates *in vivo*. 4FP underwent extensive renal clearance, which can be accounted for by the increased pK_a of the phenolic group. The major metabolites formed from 2FPO and 2FPN were the corresponding glucuronide and sulphate conjugates. There was no evidence for thioether conjugates, although both compounds did appear to undergo limited phase I metabolism, presumably to catechol or resorcinol metabolites.

Thus it can be seen that introduction of fluorine into the aromatic nucleus of P can block the *in vivo* oxidation, and therefore the hepatotoxicity of paracetamol, provided that the oxidation potential of the derivative is sufficiently greater than that of P. One final question to be asked is the reason for the greater effect of fluorine substitution, ortho to the amide group (2,6-positions) compared with substitution ortho to the hydroxyl group (3,5-positions), especially as 3,5 substitution blocks the positions through which glutathione (and proteins) normally conjugate to P. It can be seen (Table 7) that introduction of fluorine increases the oxidation potential of P, because of the electron-withdrawing effect of the element. However, the fluorines in the 2 and 6 positions have a second effect. An electronic interaction with the acetyl carbonyl group causes the acetyl group to twist out of conjugation with the aromatic nucleus, thus further disfavouring quinoneimine formation. Thus, there is a significant correlation between the mean ALT levels at 2.65 mol/kg and the degree of conjugation of the acetyl group ($r = 0.88$, $P = 0.048$). Similar arguments may be advanced to explain the reduced hepatotoxicity of 2,6-dimethyl-P despite the contrasting inductive effects of the fluorine and methyl groups [25–27].

The rank order of toxicity found *in vivo* correlates with that found *in vitro* using an established model system, i.e. isolated hepatocytes from 3-methylcholanthrene induced rats [28]. This suggests that the differences in hepatotoxicity observed *in vivo* are not simply caused by alterations in pharmacokinetics due to the introduction of fluorine.

In contrast to the effect of fluorine-substitution in the aromatic ring, introduction of fluorine into the amide group of P produces a highly lipophilic compound (3FP) and leads to a shift in toxicity from the liver to the CNS and to markedly different routes of metabolism, involving extensive de-trifluoroacetylation.

In conclusion, these studies on the effect of modification of P toxicity by fluorine-substitution have provided further insight into the role of metabolic activation in drug induced hepatotoxicity, and have illustrated how chemical modification can influence the critical balance between drug activation and drug detoxication, for drugs with the potential to form toxic quinone metabolites *in vivo*.

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