Biochemical Pharmacology, Vol. 43, No. 5, pp. 985–994, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
Pergamon Press plc

THE METABOLISM OF 2- AND 4-FLUORO-17β-OESTRADIOL IN THE RAT AND ITS IMPLICATIONS FOR OESTROGEN CARCINOGENESIS

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(*Received* 5 *September* 1991; *accepted* 21 *November* 1991)

Abstract—2-Fluoro-[6,7-^{3}H]17β-oestradiol ([^{3}H]2-FE_2) and 4-fluoro-[6,7-^{3}H]17β-oestradiol([^{3}H]4-FE_2) were synthesized by the fluorination and reduction of [^{3}H]oestrone and purified by HPLC. [^{3}H]2-FE_2 and [^{3}H]4-FE_2 (72.5 μg/kg; 0.25 μmol/kg) were administered i.v. to anaesthetized female and male Wistar rats (N = 4) with biliary cannulae. Bile was collected for 6 hr. Female rats administered [^{3}H]2-FE_2 excreted 85% of the dose into bile over 6 hr whilst male rats excreted 77%. After the administration of [^{3}H]4-FE_2, female and male rats excreted 72 and 83% of dose into bile over 6 hr, respectively. The biliary metabolites were glucuronides in all cases. The principal metabolite of [^{3}H]2-FE_2 liberated from biliary conjugates by β-glucuronidase was 2-fluoroestrone in both female rats (64% of dose) and male rats (57%). No 2-hydroxylated, i.e. oxidatively defluorinated, metabolites were detected in either sex. In contrast, 2-hydroxylation of [^{3}H]4-FE_2 did occur, but only in female rats: 2-hydroxy-4-fluoro-oestrone (22%) and 2-methoxy-4-fluoroestrone (17%) were identified as biliary aglycones. However, the major metabolite was 4-fluoroestrone (4FE_1; 38%). In male rats, 4-FE_1 and 4-fluoro D-ring-oxygenated products were the principal biliary aglycones. The differences in metabolism between the two fluoro analogues and oestradiol are discussed with particular reference to the possible involvement of 2- and 4-hydroxy (catechol) oestrogens in oestrogen toxicity.

Synthetic and natural oestrogens have been associated in humans with a variety of vaginal, hepatic and cervical cancers [1–6]. In rats and mice, oestrogens induce mammary, pituitary, cervical and uterine tumours [7]. In Syrian hamsters, the synthetic oestrogen diethylstilbestrol and the natural oestrogen 17β-oestradiol (E_2‡) have equal ability to induce renal carcinoma [8]. Although the mechanism of carcinogenesis has not been elucidated, it has become clear that hormonal potency cannot be directly correlated with the carcinogenic activity of oestrogens [8–10]. It has been suggested that oxidative metabolism plays an important role in the carcinogenicity of steroid oestrogens [11, 12]. The natural oestrogens, E_2 and E_1, in common with a synthetic oestrogen, EE_2, are known to undergo extensive oxygenation to 2- and 4-hydroxylated (catechol) metabolites in man [13, 14] and experimental animals [15, 16]. Catechols can be oxidized to chemically reactive species (quinones and semiquinones) [17–20] which can react irreversibly with proteins and DNA [13, 21–23]. It has been proposed that reactive metabolites can initiate oestrogen carcinogenesis via such a mechanism [24, 25]. Thus, the preparation of steroid oestrogens functionalized at either C-2 or C-4 such that aromatic hydroxylation is blocked whilst the physicochemical and physiological properties of the molecule remain essentially unaltered is of particular interest.

In order to differentiate between the contributions of metabolic activation and hormonal activity towards carcinogenesis by oestrogens, Liehr and coworkers [26, 27] investigated the oestrogenic and carcinogenic activities of 2- and 4-FE_2 in the Syrian hamster. These compounds, by virtue of the strength of the C—F bond, were presumed to be unable to undergo 2- and 4-hydroxylation, respectively. 2-FE_2 was almost as oestrogenic as E_2 but noncarcinogenic and this was taken as further evidence for the involvement of catechol metabolites in oestrogen carcinogenesis. However, Li *et al.* [28] subsequently demonstrated that 2-FE_2 undergoes oxidative defluorination by hepatic microsomes from castrated male hamsters and hence questioned the usefulness of fluorinated oestrogens as metabolic probes of oestrogen carcinogenicity.

Nevertheless, since the conjugation reactions which limit the extent of oxidative metabolism of an oestrogen *in vivo* [29] would have been absent from this microsomal system, only studies using intact animals will enable a thorough assessment of the biotransformation of A-ring fluorinated derivatives.

The present study of the metabolic fates of [^{3}H]-2-FE_2 (2-fluoro-[6,7-^{3}H]17β-oestradiol) and [^{3}H]4-FE_2 (4-fluoro-[6,7-^{3}H]-17β-oestradiol) in female and male rats was undertaken to determine the influence

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‡ Abbreviations: E_1, oestrone; E_2, 17β-oestradiol; EE_2, 17α-ethynyloestradiol; FE_1, fluoro-E_1; FE_2, fluoro-E_2; FEE_2, fluoro-EE_2; OHE_1, hydroxy-E_1; OHE_2, hydroxy-E_2; $MeOE_1$, methoxy-E_1; $MeOEE_2$, methoxy-EE_2; FE_1Ac, FE_1, acetate; 2-OH-4-FE_1, 2-hydroxy-4-FE_1; 2-MeO-4-FE_1, 2-methoxy-4-FE_1; 2-F-15α-OHE_1, 2-fluoro-15α-OHE_1; 2F-16α-OHE_1, 2-fluoro-16α-OHE_1; MeO-15α-OHE_1, methoxy-15α-OHE_1; MeO-16α-OHE_1, methoxy-16α-OHE_1; 2-F-16ketoE$_2$, 2-fluoro-16ketoE$_2$; 4-F-15α-OHE_1, 4-fluoro-15α-OHE_1; 2-F-D-ring kerols, 2-fluoro-D-ring ketols; EI, electron impact.

of A-ring fluorination on the balance between phase I and phase II biotransformations of oestrogens. The bile-cannulated, anaesthetized Wistar rat was used in these studies for several reasons: the biliary metabolites of E_2 in the rat have been fully characterized [15] and are similar to those in man [14]; there is a high recovery of metabolites in bile using this method and thus sufficient mass of metabolites is readily obtained for characterization; the sole study in hamsters investigated only urinary metabolites of E_2 [16], whereas it is preferable to study biliary metabolites due to the complications of enterohepatic circulation and faecal biotransformations which may be encountered during the analysis of urinary metabolites.

MATERIALS AND METHODS

Chemicals and enzymes

[^{3}H]E_1 ([6,7-^{3}H]E_1; 54.6 Ci/mmol; radiochemical purity, 99%) was purchased from NEN Research Products (Dreieich, Germany). Unlabelled E_1, ethanol (99.7–100%, v/v) and oestrogen standards were obtained from the Sigma Chemical Co. (Poole, U.K.). *N*-Fluoropyridinium triflate was purchased from the Aldrich Chemical Co. (Gillingham, U.K.). HPLC-grade solvents were products of Fisons plc (Loughborough, U.K.). General laboratory reagents were from BDH Ltd (Poole, U.K.). β-Glucuronidase–arylsulphohydrolase (Type H-2, 100×10^3 U/mL β-glucuronidase and 4.2×10^3 U/mL of arylsulphohydrolase) and β-glucuronidase from beef liver (Glucurase, 5×10^3 U/mL) were from Sigma.

Synthesis of [^{3}H]2-FE_2 *and* [^{3}H]4-FE_2

Preparation of [^{3}H]2- *and* [^{3}H]4-*FE_1*. Radiolabelled 2- and 4-FE_1 were prepared by a variation of the method of Bulman-Page *et al.* [30, 31]. [^{3}H]E_1 (550 μCi), E_1 (3.30 mg, 12.2 μmol) and *N*-fluoropyridinium triflate (6.50 mg, 24 μmol) were dissolved in dry 1,1,2-trichloroethane (3 mL) and stirred at 100° for 24 hr. The solvent was evaporated under N_2 at 50° and organic material extracted with ethyl acetate (3×2 mL). Radiometric HPLC analysis of a methanol solution showed a 47% conversion to a mixture of [^{3}H]2- and [^{3}H]4-FE_1 (R_t 26 min; isomers irresolvable on C_{18} column). This mixture was isolated by eluting aliquots (100 μL) from a μBondapak C_{18} column (30×0.39 cm i.d., 10 μm; Waters Associates, Hertford, U.K.) with acetonitrile in $NH_4H_2PO_4$ (43 mM, pH 3.0): 20–75% at 1.5 mL/min over 40 min. The eluate was monitored at 280 nm with a Spectroflow 773 absorbance detector (Kratos Analytical Instruments, Manchester, U.K.). The [^{3}H]2-/4-FE_1 was extracted from the eluate with ethyl acetate. [^{3}H]2-/4-FE_1 (0.86 mg, 3 μmol; 137 μCi) was recovered in 24% yield from [^{3}H]E_1 and was 90% radiochemically pure. It yielded the correct EI spectrum: *m/z* 288 (M^+, 100%) and *m/z* 231 ([M-$CH_2CH_2C{=}OH$]$^+$, D-ring fragmentation, 36%).

Preparation of [^{3}H]2- *and* [^{3}H]FE_1*Ac*. The mixture of [^{3}H]2-FE_1 and [^{3}H]4-FE_1 was acetylated with acetic anhydride (2 mL) and pyridine (2 mL) by stirring at 110° for 3 hr. The product was extracted with ethyl acetate. Radiometric HPLC showed complete turnover to [^{3}H]2- and [^{3}H]4-FE_1Ac (R_t 34.5 min); the products co-chromatographed with authentic 2- and 4-FE Ac on a C_{18} column (R_t 34.5 min).

Separation of [^{3}H]2-FE_1*Ac and* [^{3}H]4-*FE_1Ac by normal-phase HPLC*. [^{3}H]2-FE_1Ac and [^{3}H]4-FE_1Ac were separated by injecting aliquots (25–35 μL) of an ethyl acetate–cyclohexane (50:50, v/v) solution (1 mg/mL, 860 μL) onto a silica column (25×0.46 cm i.d., 5 μm; HPLC Technology Ltd, Macclesfield, U.K.) and eluting with cyclohexane–ethyl acetate–acetic acid (100:2:1, by vol.) at 1.5 mL/min. The eluate was monitored at 280 nm. The isomers (1:2 ratio of [^{3}H]4-FE_1Ac and [^{3}H]2-FE_1Ac; R_t 32 and 39 min, respectively) co-chromatographed with authentic 4-FE_1Ac and 2-FE_1Ac, respectively. Radiometric HPLC showed [^{3}H]2- and [^{3}H]4-FE_1Ac to be 99 and 98% pure, respectively. [^{3}H]2-FE_1Ac (0.38 mg, 1.14 μmol; 52.2 μCi) and [^{3}H]4-FE_1Ac (0.18 mg, 0.56 μmol; 26 μCi) were recovered in 38 and 19% yield, respectively, from [^{3}H]2-/4-FE_1.

Preparation of [^{3}H]2-*FE_2 and* [^{3}H]4-*FE_2*. [^{3}H]2-FE_1Ac (0.38 mg, 1.14 μmol; 52.2 μCi) and [^{3}H]4-FE_1Ac (0.18 mg, 0.56 μmol; 26 μCi) were dissolved in methanol (3 mL) and excess K_2CO_3 (*ca.* 200 mg) added. The mixture was stirred at 60–70° for 3 hr. The products were extracted with ethyl acetate. HPLC showed that [^{3}H]2-FE_1 and [^{3}H]4-FE_1 were 94 and 93% radiochemically pure, respectively, and were recovered quantitatively from their acetates. They were dissolved in tetrahydrofuran–H_2O (2.5:0.5 mL), excess sodium borohydride (*ca.* 300 mg) was added, and the mixture stirred at 45–50° for 2.5 hr. The products were extracted with ethyl acetate. Radiometric HPLC showed quantitative conversion to [^{3}H]2-FE_2 and [^{3}H]4-FE_2 (R_t 23.6 min). These were purified by chromatographing aliquots (100–150 μL) of a methanol solution on a C_{18} column as detailed for [^{3}H]-2/4-FE_1 and recovered from the eluate as before. [^{3}H]2-FE_2 and [^{3}H]4-FE_2 were 98 and 97% radiochemically pure, respectively. Their EI spectra were identical to those of the authentic unlabelled isomers: *m/z* 290 (M^+, 100%), *m/z* 231 ([M-$CH_2CH_2CH_2OH$]$^+$, D-ring fragmentation, 80%).

The synthesis afforded [^{3}H]2-FE_2 (0.28 mg, 0.98 μmol, 47.3 μCi) and [^{3}H]4-FE_2 (0.12 mg, 0.42 μmol, 20.5 μCi) in 8.6 and 3.7% yield, respectively, from [^{3}H]E_1.

Animal experiments

Male [208 ± 22 g (mean ± SD) body wt; N = 4] and female (216 ± 9 body wt; N = 4) Wistar rats were anaesthetized with urethane (1.4 g/mL in isotonic saline; 0.2 mL/200 g given i.p.) and the jugular vein and common bile duct were cannulated. [^{3}H]2-FE_2 (81.1 ± 0.8 μg/kg, 0.25 μmol/kg; 2.44–3.04 μCi) and [^{3}H]4-FE_2 (82.8 ± 0.7 μg/kg, 0.25 μmol/kg; 2.03–3.08 μCi), dissolved in isotonic saline:ethanol (7:3), were injected into the jugular vein. Bile was collected in 1 hr fractions for 6 hr and sampled for determination of radioactivity; the remainder was stored at −30°. The rats were killed

by cervical dislocation and major organs were removed for determination of residual radioactivity.

Determination of radioactivity in bile and tissues

Aliquots of bile (5–25 μL) were mixed with 4 mL of Aqua Luma Plus scintillation fluid (Lumac bv, Landgraaf, The Netherlands) for scintillation counting. Samples of tissues (85–130 mg) were solubilized and assayed for radioactivity as described previously [29]. Quench correction was performed by external standardization.

Analysis of biliary metabolites by HPLC

Aliquots of bile (5–25 μL) and the deconjugated biliary metabolites of [^{3}H]2-FE_2 and [^{3}H]4-FE_2 (5–50-μL methanol solutions) were analysed on a Waters μBondapak C_{18} column linked to an LKB 2249-2152 pump-controller. Samples (10–100 × 10^3 dpm) were eluted with a gradient of methanol (20–60% over 40 min) in ammonium acetate (10 mM, pH 6.9) for bile and a gradient of acetonitrile (20–75% over 40 min) in $NH_4H_2PO_4$ (43 mM, pH 3.0) for deconjugated metabolites. The flow rate was 1.5 mL/min. Eluate was monitored at 280 nm. Radiolabelled metabolites were quantified either by collecting 0.5-min eluate fractions and dissolving them in scintillant (4 mL) for off-line counting or by on-line analysis with a Flo One Beta radiometric detector (Canberra Packard, Pangbourne, U.K.).

Deconjugated metabolites (50 × 10^3 dpm) were also analysed on a diol column (30 × 0.39 cm i.d., 10 μm; HPLC Technology) with propan-1-ol in hexane (5–30%, 30 min) at 1.5 mL/min. The eluate was monitored at 280 nm, collected in 0.5-min fractions, and dissolved in scintillant (4 mL) for measurement of radioactivity.

Recoveries of chromatographed radiolabelled materials were 80–100%.

Enzyme hydrolysis of the biliary conjugates of [^{3}H]-2-FE_2 *and* [^{3}H]4-FE_2 *in rats*

For metabolite analysis, bile (10–200 μL; 50–150 × 10^3 dpm, 50–125 ng equivalent 2- and 4-FE_2) was mixed with buffer (0.5–0.65 mL 0.1 M sodium acetate, pH 5.0) and incubated with either H-2 enzyme preparation (β-glucuronidase–arylsulphohydrolase, *ca.* 2 × 10^3 U and 100 U, respectively) or β-glucuronidase (5 × 10^3 U) at 37° for 16 hr. Incubations contained 10 mM ascorbic acid to prevent the degradation of catechols. Control incubations were performed in buffer alone. Incubations were extracted with diethyl ether (3 × 2 mL). The combined extracts were evaporated to dryness under nitrogen at 30°, and reconstituted in 100–200 μL of methanol for determination of radioactivity and HPLC analysis.

Isolation and identification of major metabolites of [^{3}H]2-FE_2 *and* [^{3}H]4-FE_2 *in rats*

Anaesthetized male and female rats (200–250 g) with biliary cannulae were given [^{3}H]2-FE_2 or [^{3}H]4-FE_2 (1.78 mg/kg, 1.25 μCi) i.v. via the jugular vein. Bile was collected for 3 hr. Aliquots (0.8–1.0 mL; 0.21 μmol equivalent) were adsorbed onto C_{18} Sep-pak cartridges (Water Associates, Milford, MA, U.S.A.), and eluted with methanol; the eluate was evaporated to dryness under nitrogen at 40°. Hydrolysis of the concentrated metabolites was carried out with β-glucuronidase–arylsulphohydrolase (6 × 10^3 and 300 U, respectively) in 0.1 M acetate buffer containing 10 mM ascorbic acid at 37° for 16 hr. After extraction with diethyl ether (3 × 2 mL) and evaporation of the solvent under nitrogen at 30°, the extract was reconstituted in methanol (200 μL) and analysed by radiometric HPLC on a μBondapak column to ensure the metabolic profile was unchanged from the low-dose study. UV peaks corresponding to the major deconjugated metabolites (R_t determined by radiometric analysis) were isolated on a μBondapak column using the acetonitrile-ammonium dihydrogen orthophosphate gradient. The metabolites were extracted with diethyl ether (3 × 2 mL), concentrated to dryness under N_2 and redissolved in methanol (3 mL) for determination of radiochromatographic purity.

The minor metabolite of [^{3}H]2-FE_2 in females was identified after elution from a diol column (R_t 23.3 min) with propan-1-ol in hexane (5–30%, 30 min; flow rate 1.5 mL/min). The solvent was evaporated and the material reconstituted in methanol.

Samples (*ca.* 5 μg) of the isolated metabolites were analysed by EI mass spectrometry.

Mass spectrometry

Authentic steroids and isolated metabolites were analysed by EI mass spectrometry as described previously [32].

RESULTS

Excretion and tissue residues of radiolabelled material

The radiolabelled metabolites of [^{3}H]2-FE_2 (72.5 μg/kg) were rapidly and extensively excreted in bile by both sexes: 57.6 ± 4.5 (mean ± SD, N = 4) and 52.3 ± 2.8% of the dose during the first hour by females and males, respectively, and 85.4 ± 2.3 and 76.7 ± 1.1% over 6 hr. After 6 hr, only residual quantities of radiolabelled material remained in the tissues: 0.9 ± 0.1% of the dose in the livers of male and female rats and <0.1% per organ(s) in the kidneys, heart, spleen, lungs and brain.

[^{3}H]4-FE_2 (72.5 μg/kg) was also rapidly and extensively excreted in bile: 50.0 ± 6.6 (mean ± SD, N = 4) and 58.3 ± 12.1% of the dose during the first hour by females and males, respectively, and 71.4 ± 10.0 and 82.6 ± 11.4% after 6 hr. In the liver of female and male rats, 1.0 ± 0.15 (mean ± SD, N = 4) and 0.9 ± 0.2% of the dose, respectively, remained and <0.1% of the dose per organ(s) was present in the kidneys, heart, spleen, lungs and brain.

Biliary metabolites

Female rats administered [^{3}H]2-FE_2 (72.5 μg/kg; 0.25 μmol/kg) excreted one major (R_t 19.5 min, 66% of dose) and two minor (R_t 11.5 min, 5%; R_t 22.5 min, 7%) conjugated metabolites, which underwent essentially complete hydrolysis by β-glucuronidase to yield two aglycones. It was found that the profiles from the 0–1 hr bile collections were

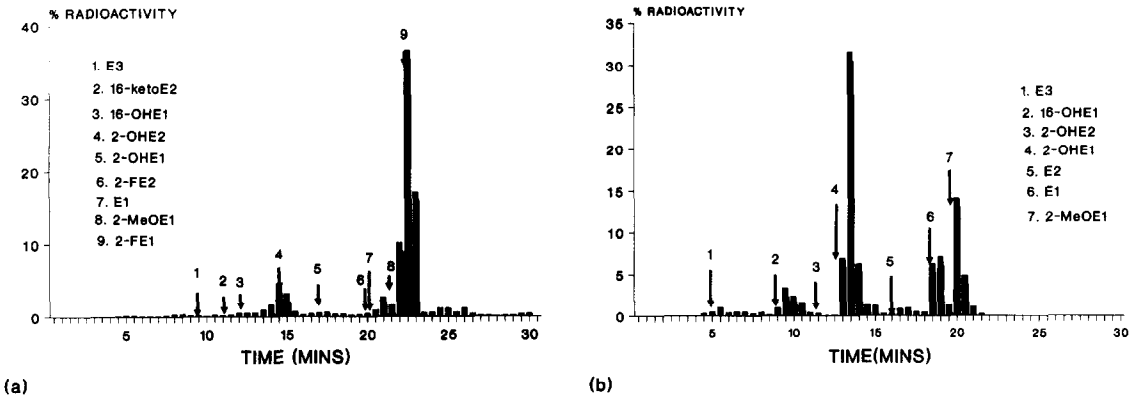

Fig. 1. High performance liquid radiochromatograms of the deconjugated metabolites of (a) [^{3}H]2-FE$_2$ and (b) [^{3}H]E$_2$ (results from Maggs *et al.*, *J Steroid Biochem Mol Biol*, in press [36]) in female rats at 0–1 hr. [^{3}H]2-FE$_2$ and [^{3}H]E$_2$ were administered i.v. to anaesthetized female rats. Hydrolysed bile was analysed on a reversed-phase column eluted with a gradient of acetonitrile in ammonium dihydrogen orthophosphate buffer.

Table 1. Comparison of the principal fragments of EI mass spectra of biliary aglycone metabolites of [^{3}H]2-FE$_2$ in the rat and authentic oestrogens

Biliary aglycone metabolites	Authentic oestrogens
[^{3}H]2-FE$_1$ (R_t 22.5 min, 64% of dose, Female): m/z 288 = M$^{+\cdot}$ (100%, Relative Intensity), 231 = M$^{+\cdot}$-57, [M—$CH_2CH_2C{=}OH]^{+\cdot}$, D-ring fragmentation, (35); 217(10); 190(25); 164(25).	2-FE$_1$: 288(M$^{+\cdot}$, 100); 231(34); 217(9) 190(28); 164(23).
[^{3}H]2-FE$_1$ (R_t 22.5 min, 57%, Male): 288(M$^{+\cdot}$,100) 231(36); 217(12); 190(22); 164(15).	E$_1$: 270(M$^{+\cdot}$,100); 213(17).
[^{3}H]2-F-16α-OHE$_1$ (R_t 15 min, 20%, Male): 304 (M$^{+\cdot}$,30) 232(M$^{+\cdot}$-72, [M-$CH_2CH(OH)C{=}O]^{+\cdot}$,40); 217(10) 190(23); 177(20); 164(15).	16α-OHE$_1$: 286(M$^{+\cdot}$,100); 214(90); 199(23) 172(50); 159(46); 146(26).
[^{3}H]2-F-D-ring ketols (R_t 13 min, 11%, Male): 304(M$^{+\cdot}$,100); 286(40); 232(25); 229(27); 204(28); 190(31); 177(53); 164(12).	16-keto: 286(M$^{+\cdot}$,25); 268(100) 211(29); 148(48).
[^{3}H]2-F-16α-OHE$_1$, (R_t 23.3 min, 9%, Female): 304(M$^{+\cdot}$,46); 232(60); 217(12); 190(28); 164(12).	15α-OHE$_1$: 286(M$^{+\cdot}$,100); 268(3); 214(20) 211(8); 172(17); 159(75); 146(46).

All metabolites isolated on a reversed-phase column eluted with a gradient of acetonitrile in ammonium dihydrogen orthophosphate buffer except for [^{3}H]2-F-16α-OHE$_1$ from female rats which was isolated on a diol column eluted with a gradient of propan-1-ol in hexane.

representative of those from pooled bile samples. This is to be expected considering that approximately 70% of the recovered radioactivity is excreted in the 0–1 hr collections. The major (R_t 22.5 min, 64% of dose) aglycone co-eluted with authentic 2-FE$_1$ (Fig. 1a). This identification was confirmed by the EI mass spectra (m/z 288 [M$^{+\cdot}$], 100%, and m/z 231 [M-$CH_2CH_2C{=}OH]^{+\cdot}$, D-ring fragmentation, 36%) of metabolite isolated from the bile of a female rat given a high dose of [^{3}H]2-FE$_2$ (1.7 mg/kg), which was identical to the mass spectrum of authentic 2-FE$_1$ (Table 1). The metabolite profile of [^{3}H]2-FE$_2$ in female rats can be compared to the corresponding profile of [^{3}H]E$_2$ (Fig. 1b). The other deconjugated metabolite in females (R_t 14.5 min, 9%) co-chromatographed with authentic 2-OHE$_2$ (Fig. 1a) on a C_{18} column. It was isolated on a diol column and gave an EI mass spectrum characteristic of a D-ring hydroxyfluoroestrone (m/z 304 [M]$^{+\cdot}$, 46% and m/z 232 [M-$CH_2CH(OH)C{=}O]^{+\cdot}$, D-ring fragmentation, 60%); cf. spectra of authentic 16α-OHE$_1$ and 2-FE$_1$ (Table 1). This confirmed that defluorination had not taken place and the metabolite was the product of D-ring biotransformation.

Male rats given [^{3}H]2-FE$_2$ excreted four (R_t 6 min, 4.5%; 10 min, 24%; 15.5 min, 6% and 18.5 min, 40%) glucuronide conjugates. Upon hydrolysis with β-glucuronidase these yielded three deconjugated metabolites. The major aglycone (R_t 22.5 min, 57%) co-chromatographed with authentic 2-FE$_1$ (Fig. 2a). Again this identification was confirmed by the EI mass spectrum of the isolated metabolite from the

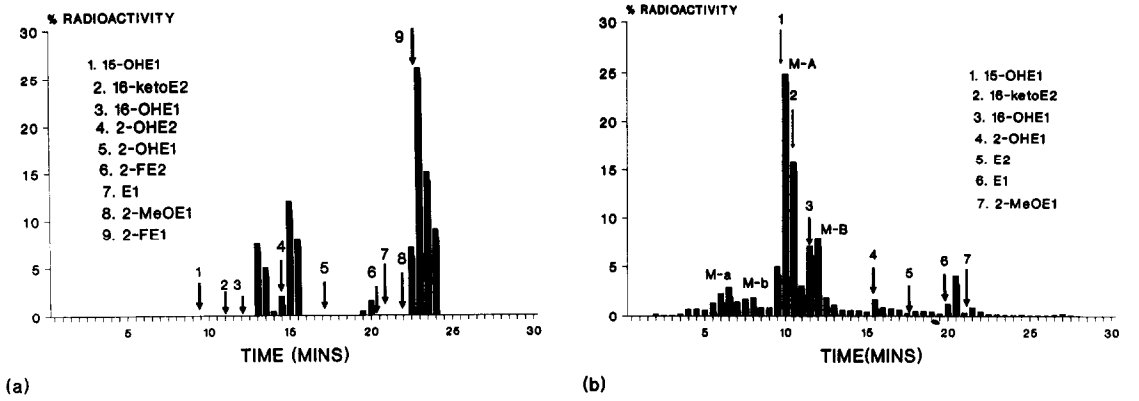

Fig. 2. High performance liquid radiochromatograms of the deconjugated metabolites of (a) [^{3}H]2-FE_2 and (b) [^{3}H]E_1 (results from Maggs *et al.*, *J Steroid Biochem Mol Biol*, in press [36]) in male rats at 0–1 hr. (M-a and M-b were identified as 2,15α-dihydroxyE_1 and 2,16α-dihydroxyE_1, respectively; M-A as a mixture of 15α-OHE_1 and 2-MeO-15α-OHE_1, and M-B as a mixture of 16α-OHE_1 and 2-MeO-16α-OHE_1). [^{3}H]2-FE_2 and [^{3}H]E_2 were administered i.v. to anaesthetized male rats. Hydrolysed bile was analysed on a reversed-phase column eluted with a gradient of acetonitrile in ammonium dihydrogen orthophosphate buffer.

high dose experiment (Table 1). The two minor metabolites (R_t 13 min, 11% and 15 min, 20%) eluted (Fig. 2a) between the D-ring oxygenated oestrogen standards 16α-OHE_1, 16-ketoE$_2$ and 15α-OHE_1 and the catechol oestrogen standards (2-OHE_2 and 2-OHE_1). EI mass spectral analysis of the isolated metabolites showed that the less polar of the two (R_t 14.5 min) gave a spectrum (m/z 304 [$M^+\cdot$], 30% and m/z 232 [M-$CH_2CH(OH)$-C=O]$^+\cdot$, D-ring fragmentation, 40%) analogous to that of 16α-OHE_1, i.e. its analogue peaks were 18 atomic mass units heavier (Table 1). The more polar of the two also gave the molecular ion of an hydroxyfluoroestrone (m/z 304) but its fragmentation pattern (m/z 286 [M-H_2O]$^+\cdot$, dehydration, 40%) was distinct from that of the isomeric metabolite. Although this metabolite remains to be fully characterized, several of its fragment ions are analogous to those of 15α-OHE_1 (Table 1); furthermore, 15α-OHE_1 elutes just before 16α-OHE_1 (Fig. 2b). However, the prominent dehydration peak at m/z 286 is inconsistent with the fragmentation of 15α-OHE_1 and might arise from co-eluting 2-F-16-ketoE$_2$; authentic 16-ketoE$_2$ undergoes substantial dehydration (Table 1) and elutes between the isomeric ketols (Fig. 2b).

In the case of [^{3}H]4-FE_2, females and males excreted four glucuronide metabolites. Their R_t were, for females, 21.5 (31%), 23 (13%), 24.5 (26%) and 29 min (11%); and for males, 7 (15%), 9 (41%), 16 (7%) and 21.5 min (22%). β-Glucuronidase liberated four deconjugated metabolites from female bile (Fig. 3a). The major metabolite in females (R_t 22.5 min, 38%) was identified as 4-FE_1 on the basis of co-chromatography with authentic standard and EI mass spectrometry (Table 2). The least abundant

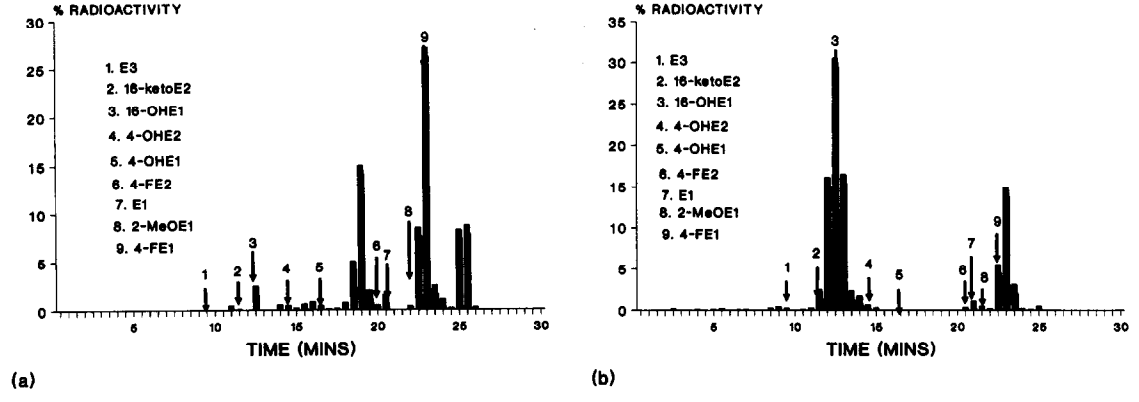

Fig. 3. High performance liquid radiochromatograms of the deconjugated metabolites of [^{3}H]4-FE_2 in (a) female rats at 0–1 hr and (b) male rats at 0–1 hr. [^{3}H]4-FE_2 was administered i.v. to anaesthetized rats. Hydrolysed bile was analysed on a reversed-phase column eluted with a gradient of acetonitrile in ammonium dihydrogen orthophosphate buffer.

Table 2. Comparison of the principal fragments of EI mass spectra of biliary aglycone metabolites of [^{3}H]4-FE$_2$ in the rat and authentic oestrogens

Biliary aglycone metabolites	Authentic oestrogens
[^{3}H]4-F-2-MeOE$_1$ (R_t 25 min, 17% of dose, Female): m/z 318 = $M^{+}\cdot$,100% (Relative Intensity); 261(15); 194(14); 155(21).	2-MeOE$_1$: 300($M^{+}\cdot$,100); 243(5); 176(18); 137(18).
[^{3}H]2-FE$_1$ (R 22.5 min, 38%, Female): 288($M^{+}\cdot$,100); 231 ($M^{+}\cdot$-57, [M—CH_2CH_2C=OH]$^{+}\cdot$, D-ring fragmentation, 35).	
[^{3}H]2-OH-4-FE$_1$ (R_t 18.5 min, 22%, Female): 304($M^{+}\cdot$,85); 247($M^{+}\cdot$-57, [M—CH_2CH_2C=OH]$^{+}\cdot$, 48).	2-OHE$_1$: 286($M^{+}\cdot$,100); 229($M^{+}\cdot$-57,[M—CH_2CH_2C=OH]$^{+}\cdot$, 16).
[^{3}H]4-F-x-OHE$_1$ (R_t 12.5 min, 2%, Female): 304($M^{+}\cdot$,11); 286($M^{+}\cdot$-18, [M—H_2O]$^{+}\cdot$, 48) 243(14); 229(22).	6β-OHE$_1$ (and 16-ketoE$_2$): 286($M^{+}\cdot$,25); 268($M^{+}\cdot$-18,[M—H_2O]$^{+}\cdot$,100); 225(17); 211(30).
[^{3}H]4-FE$_1$ (R 22.5 min, 23%, Male): 288($M^{+}\cdot$,100); 231(33).	
[^{3}H]4-F-x-OHE$_1$ (R 12.5 min, 69%, Male): 304($M^{+}\cdot$,100); 286($M^{+}\cdot$-18, [M—H_2O]$^{+}\cdot$, 22); 232($M^{+}\cdot$-72, [M—$CH_2CH(OH)C$=O]$^{+}\cdot$, 24).	

All metabolites isolated on a reversed-phase column eluted with a gradient of acetonitrile in ammonium dihydrogen orthophosphate buffer. x = unidentified position of OH group.

and most polar metabolite (R_t 12.5 min, 2%) had a R_t indicative of non-A-ring hydroxylation. Its mass spectrum (m/z 304 [$M^{+}\cdot$], 11% and m/z 286 [M-H_2O]$^{+}\cdot$, dehydration, 48%) confirmed that it was an hydroxyfluoroestrone and the fragmentation pattern was consistent with B-ring or D-ring oxygenation (Table 2). The other two metabolites (R_t 18.5 min, 22%; 25 min, 17%) were identified as 2-oxygenated-4-fluoro derivatives from their R_t and mass spectra. The more polar of the two (R_t 18.5 min), which eluted between 4-OHE$_1$ and 2-MeOE$_1$ standards, had a R_t indicative of A-ring hydroxylation. It gave a mass spectrum (m/z 304 [$M^{+}\cdot$], 85% and m/z 247 [M-CH_2CH_2C=OH]$^{+}\cdot$, D-ring fragmentation, 48%) which confirmed the presence of an hydroxyl group and a fluorine atom in the A-ring (Table 2). The least polar metabolite (R_t 25 min) eluted after 4-FE$_1$ and its mass spectrum (m/z 318 [$M^{+}\cdot$], 100%) was indicative of a methylated catechol metabolite (Table 2).

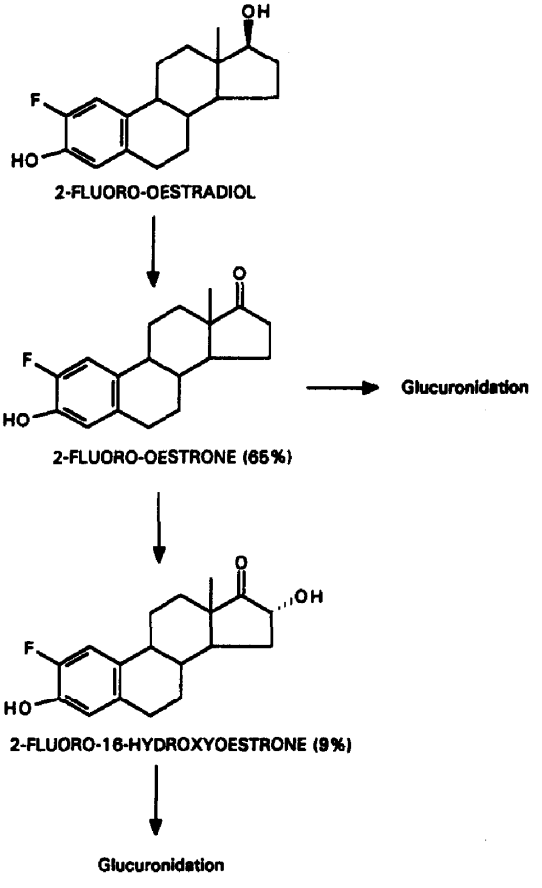

Fig. 4. Metabolism of [^{3}H]2-FE$_2$ in female rats.

In males administered [^{3}H]4-FE$_2$, β-glucuronidase liberated two aglycone metabolites (Fig. 3b). The major metabolite (R_t 12.5 min, 69%) gave a mass spectrum (m/z 304 [$M^{+}\cdot$], 100%, m/z 286 [M-H_2O]$^{+}\cdot$, dehydration, 22% and m/z 232 [M-$CH_2CH(OH)C$=O]$^{+}\cdot$, 24%) which suggested a mixture of D-ring oxygenated products including 4-F-15α-OHE$_1$ (Table 2). The other metabolite (R_t 22.5 min, 23%) co-chromatographed with 4-FE$_1$ standard and its EI mass spectrum confirmed this identification (Table 2).

DISCUSSION

The main findings of this study are that whilst C-2 fluorination completely blocks the 2-hydroxylation and partially restricts the D-ring hydroxylation (males only) of E$_2$ in male and female rats, it has no apparent effect upon oestrogen 3-O-glucuronylation.

The general characteristics of the elimination of 2-FE$_2$ and 4-FE$_2$ are similar to those of E$_2$ [15] and other natural and synthetic oestrogens [32–35]. Thus, there is rapid and extensive biliary excretion of conjugated metabolites: the elimination of administered radiolabel by female rats over 6 hr is

86% for 2-FE_2 and 72% for 4FE_2 compared with 76% for E_2 [36]. The essentially complete hydrolysis by β-glucuronidase of the biliary metabolites of both fluoroestrogens indicated that the major conjugates are glucuronides.

The metabolite profile of 2-FE_2 (Fig. 1a) in female rats is remarkably simple compared with that of E_2 [36] (Fig. 1b). The metabolism of E_2 is more complex because of extensive oxygenation and partial O-methylation at C-2 following dehydrogenation to E_1. Between 40 and 60% of E_2 undergoes 2-hydroxylation and less than 16% is excreted as conjugates of E_2 and E_1 [36]. In contrast, the major pathway for 2-FE_2, in both male and female rats, is dehydrogenation to 2-FE_1 which then undergoes glucuronylation. Only one other discernable metabolite was excreted by females given 2-FE_2: its mass spectrum indicated that it was 2-F-16α-OHE_1 (Table 1). A metabolic scheme for [^{3}H]2-FE_2 in female rats is shown in Fig. 4.

The metabolic profiles of 2-FE_2 (Fig. 2a) and E_2 (Fig. 2b) have more in common in male rats than in female rats. This reflects the low level of 2-OHE_1 excreted by males. The predominance of D-ring oxygenated metabolites in males [36] can be attributed to male-specific P450 with oestrogen 16α- and 15α-hydroxylase activity [37, 38]. 2-FE_2 and E_2 are oxidized to their oestrone derivatives, 2-FE_1 (57%) and E_1 (7%) [36], and then undergo D-ring oxygenation. The other metabolites of 2-FE_2 in male rats were 2-F-16α-OHE_1 (20%) and what appears to be a mixture of 2-F-D-ring ketols (11%): possibly F-15α-OHE_1 and 2-F-16-ketoE_2 (Table 1). 16α-Hydroxylation is a male-predominant pathway of E_2 metabolism in rats [37–40]. 15α-Hydroxyoestrogens are minor products of endogenous oestrogens [41] but major metabolites of 11β-MeO EE_2 in man [42]. In Wistar rats, 15α-hydroxylation is a male-specific biotransformation and 15α-OHE_1 the principal phase I metabolite [36].

With 4-FE_2, 2-hydroxylation does occur in female rats but to a lesser extent than with E_2. 4-FE_1 (38%)

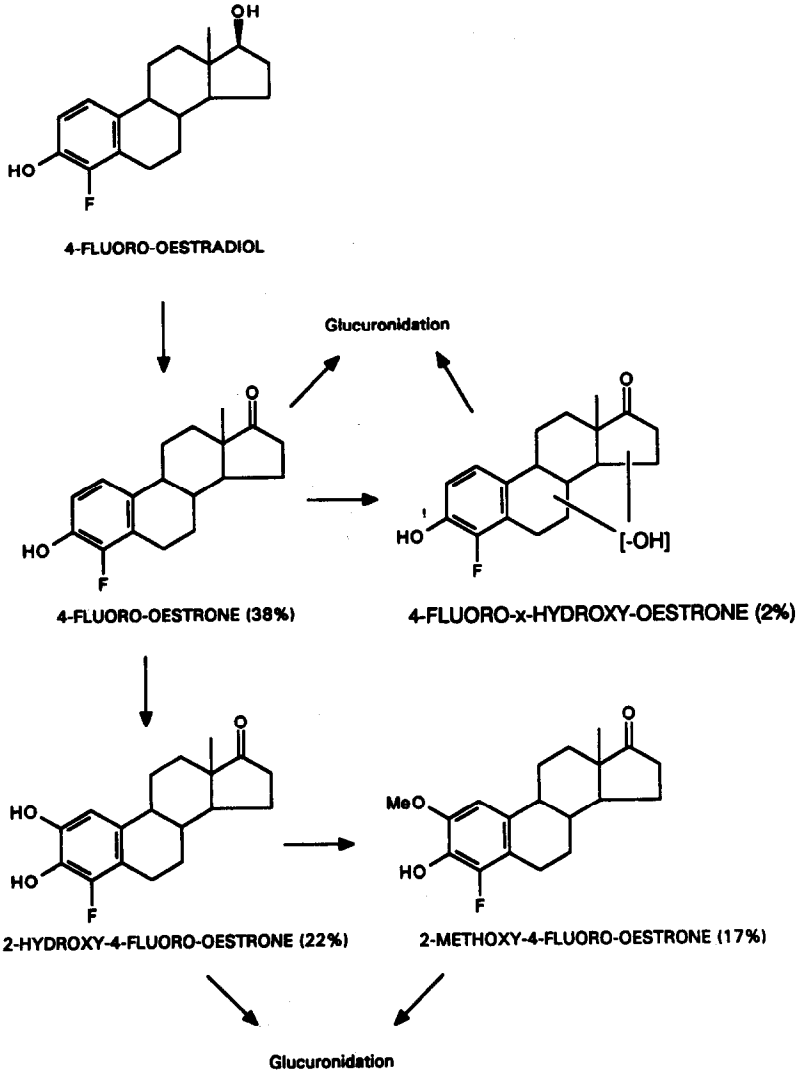

Fig. 5. Metabolism of [^{3}H]4-FE_2 in female rats.

is the major metabolite, with 2-OH-4-FE_1 and 2-MeO-4-FE_1 present at 22 and 17%, respectively (Fig. 3a). A schematic representation of [^{3}H]4-FE_2 metabolism in female rats is shown in Fig. 5. In males, again only dehydrogenation and D-ring oxygenation occur (Fig. 3b). The single polar fraction appears to be a mixture of D-ring ketols, (Table 2). This is similar to E_2 in terms of D-ring oxygenation and can again be attributed to male-specific P450 [37, 38].

Comparison of the metabolic profiles in male rats of E_2 (Fig. 2b), 2-FE_2 (Fig. 2a) and 4-FE_2 (Fig. 3b) suggests the fluorine substituents exert regioselective effects on D-ring oxygenation: C-2 fluorination selectively restricts 15α-hydroxylation whereas C-4 fluorination appears to abolish 16α-hydroxylation.

The blockade of A-ring hydroxylation effected by 2,4-dibromination of either EE_2 [32] or E_2 [43] can be rationalised on grounds of steric hindrance. Such arguments cannot explain the effects of fluorine substitution since fluorine is isosteric with hydrogen. However, the energy of the C—F bond is 116 kcal/mol compared to 99.5 kcal/mol for the C—H bond [44]. Thus the additional strength of the C—F bond could divert metabolism from oxidation at C-2 to glucuronylation at O-3. This would prevent A-ring oxidation and subsequent formation of the reactive intermediates (quinones and semiquinones) of which catechol oestrogens are the precursors [17–20, 45].

The metabolic fates of 2-FE_2 and 4-FE_2 provide a rationale for the observations of Liehr and co-workers [26, 27], namely that 2-FE_2 is non-carcinogenic but retains oestrogenicity. Fluorine at C-2, because of its size, would not be expected to impede receptor binding [46] but, because of its electronic properties, can block hydroxylation. Liehr [26] showed that 4-FE_2 was carcinogenic but required a longer induction period than E_2. Accordingly, 4-FE_2 undergoes 2-hydroxylation in female rats to a much lesser extent than E_2. Again this can be accounted for by the high electronegativity of fluorine reducing electrophilic attack at the A-ring. The C-4 fluorine could also interfere with catalytic interactions at the active site, either through deactivation of the aromatic ring or hydrogen bonding interactions, such that C-2 oxygenation and the consequent formation of reactive intermediates is retarded. This rationalization assumes that the effects of A-ring fluorine substitution on metabolism in rats are operative in hamsters. However, the metabolite profile of E_2 is similar in female rats [15] and Syrian hamsters [16].

The partial divertion by fluorine of phase I metabolism from the A-ring to the D-ring accords with the known effects of substitutions on the regioselectivity of oestrogen hydroxylation in rats. For example, 16α-hydroxylation is the only appreciable hydroxylative biotransformation of 2,4-dibromo-E_2 in male and female rats [43]. Although the C-2 and C-4 oxygenation of 2-FE_2 obtained with hamster hepatic microsomes [28, 47] does not occur in rats, the more extensive C-2 oxygenation of 4-FE_2 by hamster microsomes operates *in vivo*.

The present studies highlight the usefulness of fluorine substitution in diverting metabolism from activation to detoxification pathways; fluorination has a similar effect with a number of carcinogens [48, 49]. Nevertheless, as subsequently discovered with 2-F-EE_2 [50], appreciable oxidative dehalogenation of 2-fluoroestrogens can be engendered by certain substituents.

Acknowledgements—P.M. was supported by the Department of Education, Northern Ireland and F.H. by the North West Cancer Research Fund. B.K.P. is a Wellcome Principal Research Fellow. The TS-250 mass spectrometer in the Dept. of Pharmacology and Therapeutics was purchased by means of generous grants from the Wellcome Trust and the University of Liverpool. We thank Miss S. Newby for her technical assistance.

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