The Effect of Fluorine Substitution on the Physicochemical Properties and the Analgesic Activity of Paracetamol

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Abstract—The physicochemical properties and analgesic action of six fluorinated analogues of 4-hydroxyacetanilide (paracetamol) have been investigated. Fluorine substitution adjacent to the hydroxyl group increased lipophilicity and oxidation potential whilst substitution adjacent to the amide had little effect on lipophilicity but led to a greater increase in oxidation potential. Lack of coplanarity and conjugation of the amide group and aromatic ring was also apparent with the analogues that had fluorine in the 2 and 6 positions. Introduction of fluorine into the amide group of paracetamol increased the lipophilicity 4-fold and also increased the oxidation potential of paracetamol. ED50 values for analgesic activity in the phenylquinone-induced abdominal constriction test on male Swiss White mice showed that ring substitution by fluorine reduced activity, especially at the 2,6-positions. Introduction of fluorine into the amide group enhanced activity significantly. Correlation of the analgesic activity with the physicochemical properties indicated that conjugation (and planarity) of the amide group with the aromatic ring is essential for activity and that ease of oxidation may also be an important factor.

Paracetamol (1) (4-hydroxyacetanilide) is a widely used antipyretic analgesic. It is most effective with low grade pain and has little effect on visceral pain or pain due to wounding. Unlike aspirin, paracetamol has no anti-inflammatory action. The mechanism of paracetamol-induced analgesia has not yet been fully elucidated. It appears to act by blocking prostaglandin biosynthesis by inhibition of the enzyme cyclo-oxygenase which catalyses a bis-dioxygenation of arachidonic acid to form the short-lived endoperoxide prostaglandin G_2 (PGG$_2$). Over-production of certain prostaglandins is implicated in a wide range of disorders including pain, fever, and inflammation (Ferreira 1972). Paracetamol is a weak inhibitor of cyclo-oxygenase from peripheral tissues whilst it is more effective against cyclo-oxygenase derived from the central nervous system (CNS). This finding may explain the differential ability of paracetamol to produce analgesia and antipyresis without a concomitant anti-inflammatory effect. Aspirin, a stronger inhibitor of cyclo-oxygenase, inhibits prostaglandin generation in peripheral and CNS tissues to the same extent (Flower & Vane 1972; Piletta et al 1991). Evidence suggests that the catalysis of prostaglandin biosynthesis by cyclo-oxygenase involves a peroxide-initiated free-radical mechanism (Hemler & Lands 1980; Lands 1981, 1985). The chain reaction is initiated by the interaction of peroxides and the haem prosthetic group on the enzyme, and is then propagated by subsequent radical rearrangement reactions.

Inhibition of cyclo-oxygenase by paracetamol is thought to involve antioxidant or free radical-trapping properties. Antioxidants inhibit prostaglandin biosynthesis by removing the intermediate radicals essential to the cyclo-oxygenase mechanism. When peroxide concentrations are high, parace-

tamol is found to be a weak inhibitor of cyclo-oxygenase, however, when the peroxide concentrations are reduced to those found intracellularly in-vivo, the potency of paracetamol against cyclo-oxygenase is increased (Lands 1985). The oxidation potential of paracetamol is also relevant to the toxicity of paracetamol, since an oxidative metabolite, N-acetyl-p-benzoquinoneimine, is responsible for the hepatotoxicity induced by paracetamol overdose (Vermeulen et al 1992).

To investigate further the pharmacology of paracetamol, compounds 2–7 (Fig. 1) were synthesized in order to determine the effect of fluorine substitution, in the aromatic ring and in the acyl group of paracetamol, on the analgesic (antinociceptive) potency and to relate any observed changes in potency to the physicochemical properties of the compounds. Fluorine has a similar size to hydrogen, but can markedly alter the oxidation potential of a molecule because of its strong electron withdrawing properties (Hewitt & Silvester 1988).

Materials and Methods

Male Swiss White mice, 25–36 g, were obtained from Charles River, Margate, Kent, UK. 2-Phenyl-1,4-benzoquinone and polyethylene glycol 200 were obtained from Sigma, Poole, Dorset, UK. Pentafluoronitrobenzene, 1,3,5-trifluorobenzene, 3-fluoro-4-methoxyaniline and 2,6-difluorophenol were obtained from Aldrich Chemical Company, Gillingham, Dorset, UK.

Chemistry
Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Melting points were determined on a Köfler hot stage apparatus and are uncorrected. Proton NMR spectra were recorded using Jeol (60 and 270 MHz), Perkin Elmer R34 (220 MHz) and Bruker (200, 250 and 400 MHz) instruments

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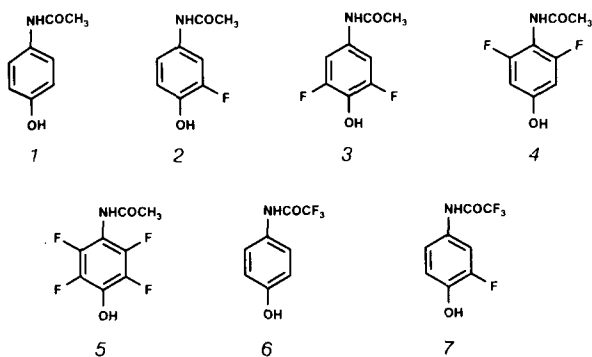
Fig. 1. Paracetamol (1) and fluorinated analogues.

1·15 g (88%); mp, 76–77°C (Wall et al (1963), 75–76·5°C).

2,3,5,6-Tetrafluoro-4-methoxyacetanilide (10). Acetylation of the fluorinated methoxyanilines was carried out by refluxing with acetic anhydride or trifluoroacetic anhydride (1·5–2 molar equivalents) in a dry solvent (toluene, chloroform, dichloromethane) under nitrogen. Acetic anhydride (1·57 g, 15·38 mmol) was added to a stirred solution of 2,3,5,6-tetrafluoro-4-methoxyaniline (1·5 g, 7·69 mmol) in dry toluene (50 mL) and the solution was then heated under reflux under nitrogen for 2·5 h. The toluene was evaporated under reduced pressure and the residue was washed three times with light petroleum (bp, 40–60°C), and then recrystallized from water to give 2,3,5,6-tetrafluoro-4-methoxyacetanilide, 1·7 g (93%); mp, 119°C (Burdon et al (1965), 116–117°C).

with $(CH_3)_4$ Si as internal standard. Fluorine NMR spectra were recorded using Jeol (56·4 MHz) and Bruker (235·3 and 376·4 MHz) instruments and unless otherwise stated CF_3CO_2H was used as an external standard. Mass spectra were recorded on a VG 7070E mass spectrometer and ultraviolet spectra were recorded on a Hewlett Packard 8452A spectrometer. Cyclic voltammograms were recorded using a TR70/3A potentiostat with a type RB2 waveform generator, a Chessell BD91 x-y recorder and a Keithly 168 autoranging DMM voltmeter.

TLC was performed using Merck Kieselgel 60 F_{254} precoated silica plates and column chromatography using Merck 9385 silica gel. HPLC was carried out using an LKB 2150 HPLC pump and LKB 2152 pump controller. In all cases the flow rate was 1 mL min⁻¹ and the eluent was monitored at 254 nm using a Gilson 3B detector. Elemental analysis (C, H, N) was carried out by the University of Liverpool Micro-Analysis Laboratory. Where analyses are indicated only by the symbols of the elements, analytical results for the elements are within 0·4% of the theoretical values.

2,3,5,6-Tetrafluoro-4-methoxynitrobenzene (8). 2,3,5,6-Tetrafluoro-4-methoxynitrobenzene was prepared from pentafluoronitrobenzene by displacement of fluorine with sodium methoxide, according to the method of Allen et al (1965).

2,3,5,6-Tetrafluoro-4-methoxyaniline (9). Reduction of the fluorinated nitromethoxybenzenes was carried out using ammonium formate and 10% palladium charcoal, by the general method of Ram & Ehrenkaufer (1988). Ammonium formate (1·0 g, 33·5 mmol) was added to a stirred suspension of 2,3,5,6-tetrafluoro-4-methoxynitrobenzene (1·5 g, 6·7 mmol) and 10% palladium-charcoal (0·2–0·3 g) in methanol at room temperature (21°C). The resulting mixture (slightly exothermic and effervescent) was stirred at room temperature and the reduction followed by TLC (usually complete in 30–40 min). The catalyst was removed by filtration through a Celite pad and the filtrate was evaporated under reduced pressure. The residue was triturated with water and the product extracted into ether and dried (MgSO₄). Evaporation of the ether gave the crude product which was purified by sublimation (≈ 80°C, 2 mmHg) to give colourless needles,

2,3,5,6-Tetrafluoro-4-hydroxyacetanilide (5). Demethylation of the fluorinated aryl methylethers was carried out using boron tribromide, according to the general method of McOmie et al (1968). Boron tribromide solution (1 M in dichloromethane, 16·8 mL, 16·8 mmol) was added to a stirred solution of 2,3,5,6-tetrafluoro-4-methoxyacetanilide (2 g, 8·4 mmol) in dry dichloromethane (50 mL) under argon. The resulting solution was stirred at room temperature overnight. Water (75 mL) was added to hydrolyse the excess of boron tribromide and boron complexes and the solution was stirred at room temperature for a further 30 min. The layers were separated and the aqueous layer extracted with ether. The combined organic layers were dried (MgSO₄) and evaporated. The crude product was purified by flash column chromatography using ethyl acetate-light petroleum (2:1) as eluent, to give 5 as off-white crystals, 1·68 g (89%); mp, 182·5–184°C; IR 3240 [υ(NH)], 3220–2640 [broad, υ(OH)], 1650 [υ(C=O)] cm⁻¹; ¹H NMR (DMSO-d₆, 270 MHz); δ 11·62 (1H, br. exch. OH), 9·83 (1H, sharp exch. NH) 2·18 (3H, s, -COCH₃); ¹⁹F NMR (D₂O, 376·4 MHz, external reference CFCl₃); δ -91 (2F, d, J_{F-F} = 16 Hz, Ar-F), −105·13 (2F, d, J_{F-F} = 16 Hz, Ar-F); mass spectrum m/z 223 (M^+, 4%). Anal. ($C_8H_5F_4NO_2$) C,H,N.

2,6-Difluoromethoxybenzene (11). Methylation of 2,6-difluorophenol was carried out using diazomethane. Diazald (N-methyl-N-nitrosotoluene-p-sulphonamide, 17 g) was dissolved in dry ether (230 mL) and cooled in ice. To this, a solution of potassium hydroxide in 96% ethanol (3·2 g, in 80 mL) was added dropwise. After 5 min the ethereal diazomethane solution was distilled from a water bath into a solution of 2,6-difluorophenol (6 g, 46 mmol) in dry ether (30 mL), cooled in ice. The resulting yellow solution was left to stand overnight. The excess of diazomethane was destroyed by the addition of glacial acetic acid and the ether was evaporated to give 2,6-difluoromethoxybenzene, 6·3 g (95%). The product was purified by distillation, bp, 50°C ≈ 25 mmHg (Niemann et al (1941), 62°C 40 mmHg).

3,5-Difluoro-4-methoxynitrobenzene (12). Nitration of 2,6-difluoromethoxybenzene was carried out using the method of Niemann et al (1941) to give the nitro compound, mp, 37·5–39°C (Niemann et al (1941), 37–38°C).

3,5-Difluoro-4-methoxyaniline (13). Reduction of 3,5-difluoro-4-methoxynitrobenzene (3 g, 15·9 mmol) was carried out as described for 9. The crude product was purified by sublimation ($\simeq 75°C$, 1 mmHg) to give 3,5-difluoro-4-methoxyaniline as colourless needles, 2·3 g (91%); mp, 77·5–78°C (Niemann et al (1941), 78·5–79°C).

3,5-Difluoro-4-methoxyacetanilide (14). 3,5-Difluoro-4-methoxyaniline (2·5 g, 15·7 mmol) was acetylated as described for 10 by heating under reflux in dry toluene (50 mL) for 1 h. The product was purified by sublimation ($\simeq 100°C$, 1 mmHg) to afford colourless crystals, 2·9 g (91%); mp, 107·5–109°C; IR 3280 [v(NH)], 1670 [v(C=O)] cm^{-1}; ¹H NMR (CDCl₃, 250 MHz); δ 7·49 (1H, br. exch. -NH), 7·15 (2H, d, $J_{H-F} = 9·8$ Hz, Ar-H), 4·05 (3H, s, -OCH₃), 2·17 (3H, s, -COCH₃); ¹⁹F NMR (CDCl₃, 235·3 MHz); δ -50·04 (d, $J_{H-F} = 9·8$ Hz); mass spectrum m/z 201 (M^+, 34%). Anal. (C₉H₉F₂NO₂) C,H,N.

3,5-Difluoro-4-hydroxyacetanilide (3). The demethylation of 3,5-difluoro-4-methoxyacetanilide (3 g, 14·9 mmol) was carried out as described for 5 except that the product was extracted from the aqueous layer with ethyl acetate. The crude product was purified by flash column chromatography using ethyl acetate-ethanol (9:1) as eluent, and then by distillation ($\simeq 240°C$, 1 mmHg), 2·28 g (82%) and was obtained as colourless crystals; mp, 192–194°C; IR 3360 [v(NH)], 3205–2400 broad, v(OH)], 1675 [v(C=O)]; 1625 cm^{-1}; ¹H NMR (DMSO-d₆, 250 MHz); δ 10·02 (1H, s, exch.), 9·81 (1H, br.exch.), 7·28 (2H, d, $J_{H-F} = 9·85$ Hz, Ar-H), 2·03 (3H, s, -COCH₃); ¹⁹F NMR (DMSO-d₆, 235·3 MHz); δ -53·6 (d, $J_{H-F} = 9·85$ Hz, Ar-F); mass spectrum m/z 187 (M^+, 30%). Anal. (C₈H₉F₂NO₂) C,H,N.

2,4,6-Trifluoronitrobenzene (15). 1,3,5-Trifluorobenzene was stirred at 0°C, and a mixture of fuming nitric acid (3·1 g, 2·05 mL, 49 mmol) and concentrated sulphuric acid (2·6 g, 1·41 mL, 27 mmol) was added cautiously, and the solution left stirring at room temperature overnight. Water (50 mL) was added and the crude product extracted with ether and purified by distillation (bp, 172°C) to give a pale yellow liquid, 5·76 g (86%).

The reaction of sodium methoxide with 2,4,6-trifluoronitrobenzene. A solution of sodium (0·76 g, 33 mmol) in dry methanol (34 mL) was added to a stirred solution of 2,4,6-trifluoronitrobenzene (5·6 g, 32 mmol) in dry methanol (75 mL), over 2 h. The solution was then left stirring for a further 2 h. The methanol was removed under reduced pressure and water (300 mL) was added to the residue. The resulting solution was then extracted with ether, the combined ether layers dried (MgSO₄), and the ether evaporated to give a yellow residue. GC analysis of the residue revealed two peaks in the ratio 2·2:1. GC/MS indicated the two peaks were isomers, M^+ 189 (i.e. replacement of one fluorine by methoxide in each case). Fluorine NMR analysis of the mixture revealed that the major isomer was the one where a fluorine *ortho* to the nitro group had been replaced (i.e. 2,4-difluoro-6-methoxynitrobenzene (16)), whilst the minor isomer was the one where *para* fluorine had been replaced (i.e. 2,6-difluoro-4-methoxynitrobenzene (17)). The two isomers could not be separated by TLC or by recrystalliza-

tion but were assigned on the basis of the demethylation described below.

The reaction of the difluoromethoxynitrobenzenes with boron tribromide. Boron tribromide (1 M in dichloromethane, 48 mL, 48 mmol) was added to a solution of the mixed isomers (6 g, 31·7 mmol), in dry dichloromethane (150 mL). The resulting solution (deep red) was stirred at room temperature overnight. Work-up (as described for 5) gave a brown crystalline residue. GC analysis of the residue revealed two peaks in the ratio 2·2:1. GC/MS gave M^+ 175 for the major peak (6·5 min, methoxy group cleaved) and M^+ 189 for the minor peak (10·5 min, methoxy group intact). The residue was taken up into ether (200 mL) and was washed with 2 M sodium hydroxide (4 × 50 mL) and water (3 × 50 mL). The organic solution was then dried (MgSO₄) and the ether evaporated to afford a yellow-brown residue. This was crystallized from light petroleum to give 2,6-difluoro-4-methoxynitrobenzene (17) (one peak on GC) as pale yellow cubes, 1·4 g; mp, 23–24°C; IR 1630, 1595, 1530 cm^{-1}; ¹H NMR (CDCl₃, 220 MHz); δ 6·57 (2H, d, $J_{H-F} = 10$ Hz, Ar-H), 3·9 (3H, s, -OCH₃); ¹⁹F NMR (CDCl₃, 56·4 MHz); δ -37·94 (d, $J_{H-F} = 10$ Hz, Ar-F); mass spectrum m/z 189 (M^+, 87%). Anal. (C₇H₅F₂NO₃) C,H,N.

The combined sodium hydroxide and water washings were neutralized with 6 M hydrochloric acid and extracted with ether. The ether was dried (MgSO₄) and evaporated to leave a yellow residue which was purified by steam distillation to give 3,5-difluoro-6-nitrophenol (18) as bright yellow needles, 3·1 g; mp, 40–42°C; IR 3240–2550 [broad, v(OH)], 1630, 1555, 1365 cm^{-1}; ¹H NMR (CDCl₃, 220 MHz); δ 10·83 (1H, br. exch., -OH), 6·67 (1H, dd, $J_{H-F} = 9·9$ and $J_{H-H} = 2·7$ Hz, Ar-H), 6·55 (1H, dt, $J_{H-F} = 9·9$ Hz and $J_{H-H} = 2·7$ Hz, Ar-H); ¹⁹F NMR (CDCl₃, 56·4 MHz); δ -16·56 (1F, m, Ar-F), -31·78 (1F, t, J_{H-F}, $J_{F-F} = 9·95$ Hz, Ar-F); mass spectrum m/z 175 (M^+, 100%). Anal. (C₆H₃F₂NO₃) C,H,N.

2,6-Difluoro-4-methoxyaniline (19). Sodium borohydride (1.2 g, 31·8 mmol) was added over a period of 10 min to a stirred suspension of 2,6-difluoro-4-methoxynitrobenzene (2 g, 10·6 mmol) and 10% palladium-charcoal (0·3 g) in water-ethanol (60 mL, 1:1). The suspension was stirred at room temperature and the reduction monitored by TLC. The catalyst was removed by filtration through a Celite pad. The filtrate was acidified with 6 M hydrochloric acid to destroy any residual borohydride, neutralized with 2 M sodium hydroxide, and then extracted with ether. The combined ethereal extracts were dried (MgSO₄) and evaporated to afford an off-white solid which was purified by sublimation ($\simeq 30°C$, 1 mmHg) to give colourless crystals, 1·52 g (90%); mp, 32–33°C; IR 3410 [v(NH)], 3330 [v(NH)], 1605 cm^{-1}; ¹H NMR (CDCl₃, 200 MHz); δ 6·46 (2H, d, $J_{H-F} = 9·95$ Hz, Ar-H), 3·74 (3H, s, -OCH₃), 3·4 (2H, br.exch. -NH₂); ¹⁹F NMR (CDCl₃, 235·3 MHz); δ -52·96 (d, $J_{H-F} = 9·95$ Hz, Ar-F); mass spectrum m/z 159 (M^+, 26%). Anal. (C₇H₇F₂NO) C,H,N.

2,6-Difluoro-4-methoxyacetanilide (20). 2,6-Difluoro-4-methoxyaniline (1·5 g, 9·4 mmol) was acetylated as described for *10*, by heating under reflux in dry chloroform (30 mL) for 30 min. The chloroform was removed under reduced pressure to leave a light pink solid which was purified by

sublimation ($\simeq$150°C, 1 mmHg) to give colourless crystals, 1·8 g (95%); mp, 145–146°C; IR 3260 [v(NH)], 1670 [v(C=O)], 1640, 1540 cm^{-1}; ^{1}H NMR (CDCl$_3$, 250 MHz); δ 6·83 (1H, br.exch. -NH), 6·51 (2H, d, J_{H-F}=9·1 Hz, Ar-H), 3·77 (3H, s, -OCH$_3$), 2·18 (3H, s, -COCH$_3$); ^{19}F NMR (CDCl$_3$, 235·3 MHz); δ -39·58 (d, J_{H-F}=9·1 Hz); mass spectrum m/z 201 (M^+, 14%). Anal. (C$_9$H$_9$F$_2$NO$_2$) C,H,N.

2,6-Difluoro-4-hydroxyacetanilide (4). Demethylation of 2,6-difluoro-4-methoxyacetanilide (2·5 g, 12·4 mmol) was carried out as described for 5, except that the solution was left stirring at room temperature for 60 h, and during the work-up the product was extracted from the aqueous layer with ethyl acetate. The residue was purified by flash column chromatography using ethyl acetate-ethanol (9:1) as eluent to give off-white crystals, 2·1 g (91%); mp, 156–157°C; IR 3240 [v(NH)], 3110–2540 [broad, v(OH)], 1625 [v(C=O)], 1610, 1550 cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 250 MHz); δ 10·33 (1H, exch.), 9·31 (1H, s, exch.), 6·52 (2H, d, J_{H-F}=9·5 Hz, Ar-H), 2·03 (3H, s, -COCH$_3$); ^{19}F NMR (DMSO-d$_6$, 235·3 MHz); δ -40·15 (d, J_{H-F}=9·5 Hz, Ar-F); mass spectrum m/z 187 (M^+, 8%). Anal. (C$_8$H$_7$F$_2$NO$_2$) C,H,N.

N-*Trifluoroacetyl-3-fluoro-4-methoxyaniline* (22). Acetylation of 3-fluoro-4-methoxyaniline (2·5 g, 17·7 mmol) was carried out using trifluoroacetic anhydride (7·43 g, 35·4 mmol) in dry dichloromethane by heating under reflux for 45 min. After evaporation of the solvent the powdery solid was sublimed ($\simeq$ 100°C, 1 mmHg) to give colourless needles, 3·94 g (94%); mp, 108·5–109°C; IR 3290 [v(NH)], 1705 [v(C=O)], 1600, 1550 cm^{-1}; ^{1}H NMR (CDCl$_3$, 250 MHz); δ 8·13 (1H, br. exch., -NH), 7·46 (1H, dd, J_{H-F}= 12 Hz and J_{H-H}=2·4 Hz, Ar-H), 7·24 (1H, dd, J_{H-H}=8·9 and 2·4 Hz, Ar-H), 6·96 (1H, t, J_{H-H} and J_{H-F}=8·9 Hz, Ar-H) and 3·9 (3H, s, -OCH$_3$); ^{19}F NMR (CDCl$_3$, 235·3 MHz); δ 1·93 (s, -COCF$_3$) and -54·55 (dd, J_{H-F}= 12 and 8·9 Hz, Ar-F); mass spectrum m/z 237 (M^+, 100%). Anal. (C$_9$H$_7$F$_4$NO$_2$) C,H,N.

N-*Trifluoroacetyl-3-fluoro-4-hydroxyaniline* (7). The demethylation of N-trifluoroacetyl-3-fluoro-4-methoxyaniline was carried out as described for 5. The crude product was purified by flash column chromatography using ethyl acetate-ethanol (9:1) as eluent, and then by recrystallization (twice from water) to give colourless needles, 1·86 g (79%); mp, 150–151°C; IR 3360–2672 [broad, v(OH)], 3283 [v(NH)], 1707[v(C=O)], 1556cm^{-1}; ^{1}H NMR (d$_6$-DMSO, 270 MHz); δ 11·25 (1H, br. exch.), 10·12 (1H, br. exch.), 7·6 (1H, dd, J_{H-F} = 11·8 Hz and J_{H-H}=2·2 Hz, Ar-H), 7·38 (1H, dd, J_{H-H}=8·9 and 2·2 Hz, Ar-H) and 7·06 (1H, t, J_{H-H} and J_{H-F}=8·9 Hz Ar-H); ^{19}F NMR (376·4 MHz, external reference CFCl$_3$); δ -75·6 (s, -COCF$_3$) and -118·7 (dd, J_{H-F}=11·8 and 8·9 Hz); mass spectrum m/z 223 (M^+, 60%). Anal. (C$_8$H$_5$F$_4$NO$_2$) C,H,N.

3-Fluoro-4-methoxyacetanilide (21). 3-Fluoro-4-methoxyaniline (3 g, 21·3 mmol) was acetylated as described for *10* by refluxing in dry toluene (90 mL) for 45 min. The toluene was removed under reduced pressure and the residue purified by flash column chromatography using ethyl acetate-light petroleum (1:3) as eluent, mp, 112–113°C (Elderfield et al (1946), 112–112·5°C).

3-Fluoro-4-hydroxyacetanilide (2). The demethylation of 3-fluoro-4-methoxyacetanilide (3·25 g, 17·8 mmol) was carried out as described for 5 except that the product was extracted from the aqueous layer with ethyl acetate. The product was purified by flash column chromatography using acetone-ethanol (9:1) as eluent to give off-white crystals, 2·5 g (83%); mp, 190–191°C; IR 3316 [v(NH)], 3108–2591 [broad, v(OH)], 1641 [v(C=O)], 1519 cm^{-1}; ^{1}H NMR (d$_6$-DMSO, 270 MHz); δ 9·95 (1H, sharp exch.), 9·63 (1H, br. exch.), 7·6 (1H, dd, J_{H-F}= 12·6 Hz and J_{H-H}=2·2 Hz, Ar-H), 7·14 (1H, dd, J_{H-H}=8·9 and 2·1 Hz, Ar-H), 6·94 (1H, t, J_{H-H} and J_{H-F}=8·9 Hz, Ar-H) and 2·08 (3H, s, -COCH$_3$); ^{19}F NMR (D$_2$O), 376·4 MHz, external reference CFCl$_3$); δ -118·1 (dd, J_{H-F}=12·9 and 8·8 Hz, Ar-F); mass spectrum m/z 169 (M^+, 33%). Anal. (C$_8$H$_8$FNO$_2$) C,H,N.

N-*Trifluoroacetyl-4-hydroxyaniline* (6). *p*-Aminophenol (2 g, 18·3 mmol) was acetylated in a similar fashion to that described for *10* by heating with trifluoroacetic anhydride (3·9 mL, 27·6 mmol) in dry dichloromethane (60 mL) under reflux for 30 min. The residue was recrystallized from water to give colourless needles, 3·5 g (93%); mp, 173–174°C (Porter & Gompf (1962), 173·5–174°C).

Measurement of the conjugation of the acetyl/amide group

The degree of conjugation of the acetyl-amide group was determined by UV spectroscopy. The shift (nm, bathochromic or hyposochromic) between the UV absorption band of the amine and that of the amide for each compound (e.g. *p*-aminophenol vs paracetamol) represents a measure of the conjugation of the acetyl/amide group for that compound.

Determination of phenolic ionization constants

The pK$_a$ of paracetamol and its fluorinated analogues was determined by potentiometric titration against a standard solution of carbonate-free potassium hydroxide.

Measurement of log K$_o$

The lipophilicity of paracetamol and the fluorinated analogues was evaluated by determining the log K$_o$ values by reversed phase HPLC using a 25 cm C$_{18}$ 5μ Spherisorb ODS column according to the method of McCall (1975).

The capacity ratio K of a compound can be related to its retention according to equation 1.

$$K = \frac{t_R - t_o}{t_R} \tag{1}$$

where t_R = the retention time of the compound in question and t_o = the retention time of an unretained compound.

The retention time of each compound was measured (at least in duplicate) at four different fractions of methanol in buffer. The fractions of methanol used were between 5 and 35% (v/v). The determination of t_o was made using methanol as the unretained compound.

For each compound the capacity ratio (K) was calculated at each fraction of methanol used. The log K$_o$ (i.e. log K at 0% methanol) was obtained from interpolation of the straight line obtained from plotting log K against the fraction of methanol used.

Log K$_o$ values were determined at pH 3 (pH 2·5 for 5) using ammonium dihydrogen orthophosphate buffer

(0·43 M) and pH 7·4 using potassium dihydrogen orthophosphate buffer (0·5 M).

Cyclic voltammetry

The oxidation potentials of paracetamol and its fluorinated analogues were measured by cyclic voltammetry. The electrochemical cell had the standard three-electrode configuration with a platinum working electrode, a saturated calomel reference electrode and a platinum foil counter electrode. Each voltammogram was generated using a freshly prepared electrode surface.

Analytical grade acetonitrile was distilled from calcium hydride and then passed through a column of active neutral alumina to remove any protic impurities.

Tetrabutylammonium hexafluorophosphate (supporting electrolyte) was dried under vacuum at 100°C for 24 h before use. A solution of the supporting electrolyte (0·1 M) in acetonitrile was prepared and degassed with argon for 15 min. This solution was then stored under argon. The concentrations of the samples to be analysed were 1–5 mM. The oxidation potentials were determined from the first oxidation wave recorded during cyclic voltammetric scanning. The scan rate was 400 mV s^{-1}.

The number of electrons involved in the oxidation of paracetamol was determined by comparison with 1,4-dimethoxybenzene, a compound known to undergo reversible one-electron oxidation. The number of electrons involved in the oxidation of the fluorinated analogues 2–7 was determined by comparison with paracetamol.

2-Phenyl-1,4-benzoquinone-induced abdominal constriction test

The method is based on that described by Siegmund et al (1957), modified by Milne & Twomey (1980).

Male Charles River mice (Swiss-strain), 25–36 g, were allowed free access to food and water, and were randomized into groups of ten. Test compounds were dissolved in 50% PEG 200-water, and were administered by the subcutaneous route in a final volume of 10 mL kg^{-1}. Control animals received 10 mL kg^{-1} of vehicle alone. Following a pretreatment period of 20 min, mice were injected intraperitoneally with 2-phenyl-1,4-benzoquinone, 2 mg kg^{-1} at 37°C in a final volume of 10 mL kg^{-1}. The mice were then placed, in groups of three, in a compartmented Perspex box maintained at room temperature and were observed for 8 min. During this period the number of abdominal constriction responses per animal were recorded where constriction consists of an

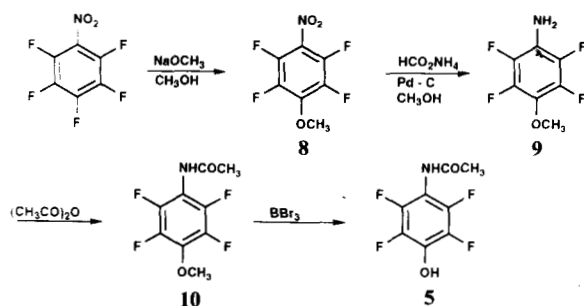intermittent contraction of the abdomen associated with hind leg extension.

The degree of inhibition (antinociceptive protection) afforded by the test compound was determined from the mean number of constriction responses observed in the treated group (T) expressed as a percentage of the mean
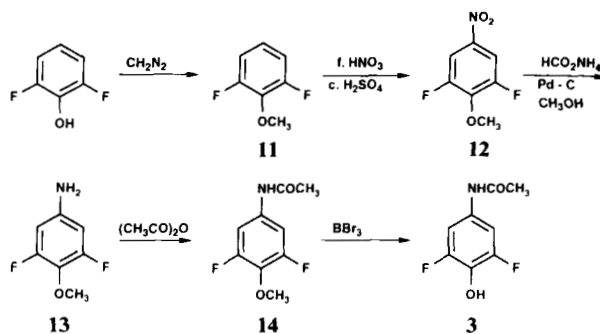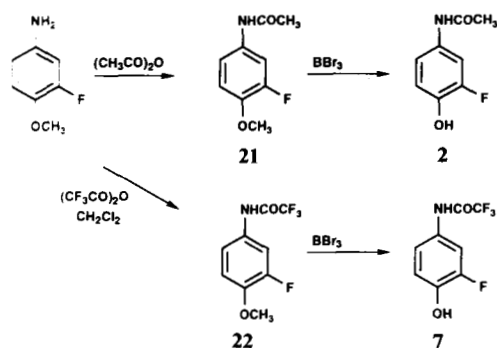

SCHEME 2
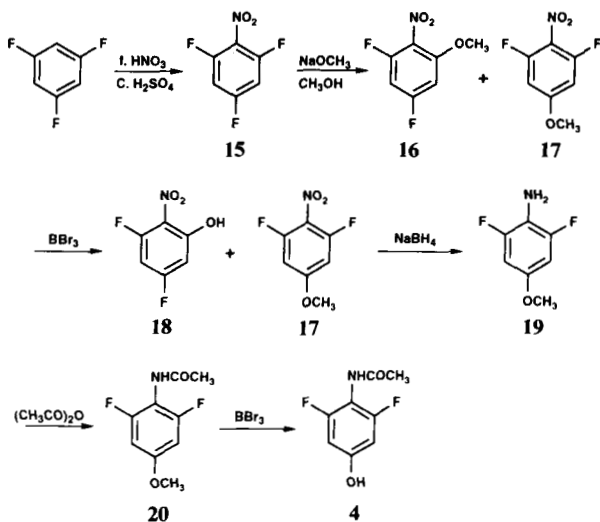
SCHEME 3

SCHEME 1

SCHEME 4

number of constriction responses in the control group (C) according to equation 2.

$$\% \text{ Inhibition} = [1 - (T/C)] \times 100 \qquad (2)$$

The percentage quantal inhibition was determined as the number of mice failing to show any responses to 2-phenyl-1,4-benzoquinone expressed as a percentage of the total number of mice in the group (eqn 3).

$$\% \text{ Quantal protection} =$$

$$\frac{\text{Number of mice failing to constrict}}{\text{Total number of mice per group}} \times 100 \qquad (3)$$

Results

The synthesis of compounds 2–5 and 7 was carried out using the commercially available fluorinated precursors pentafluoronitrobenzene, 2,6-difluorophenol, 1,3,5-trifluorobenzene and 3-fluoro-4-methoxyaniline. The synthetic routes used are illustrated in Schemes 1–4. Compound 6 was synthesized in one step from p-aminophenol using trifluoroacetic anhydride to acetylate the amino-group.

The primary UV absorption band of the amides 1, 2, 3, 6, 7 shows a bathochromic shift relative to the corresponding amines indicating that the acetyl/amide group was conjugated. The primary UV absorption band represents the $\pi-\pi^*$ transition. For amides 4 and 5 there was a marked hypsochromic shift relative to the corresponding amines indicating the loss of conjugation of the acetyl/amide group.

All the fluorinated analogues studied were more acidic, i.e. had lower pK_a values than paracetamol itself (pK_a 9·53). Fluorine substitution on the aromatic ring adjacent to the hydroxyl group had the greatest effect (analogues 2, 3, 5 and 7 (Table 1)). 2,3,5,6-Tetrafluoro-4-hydroxyacetanilide, 5, was the most acidic analogue with a pK_a value of 4·75, making it as strong an acid as acetic acid (pK_a 4·4). Indeed, 5 liberates carbon dioxide from a bicarbonate solution.

The intrinsic lipophilicity (measured in the absence of ionization) of paracetamol 1 and the fluorinated analogues 2–7 is illustrated by the log K_o values at pH 3 in Table 1. 3-Fluoro and 3,5-difluorosubstitution (i.e. adjacent to the hydroxyl group (2 and 3)) increased the lipophilicity of paracetamol and the effect was additive. However, 2,6-difluorosubstitution (i.e. adjacent to the amide group (4)) had no effect on the lipophilicity of paracetamol, whilst 2,3,5,6-tetrafluoro-substitution (5) increased lipophilicity a

little, although the increase was less than that due to monofluorosubstitution adjacent to the hydroxyl group. The most lipophilic compound, 7, which contains the trifluoromethyl group, was four times more lipophilic than paracetamol.

The effective lipophilicity (taking into account ionization) of paracetamol and the fluorinated analogues at pH 7·4 is also illustrated by the log K_o values in Table 1. At pH 7·4 there is no effect on the lipophilicity of paracetamol 1 and 6 and little effect on the lipophilicity of 4 and 7. The greatest effect is observed with 5 which has a log K_o value of -0.03 at pH 7·4 and is thus seven times less lipophilic at pH 7·4 than at pH 2·5 and five times less lipophilic than paracetamol at pH 7·4.

Paracetamol (1) and the fluorinated analogues, 2–7, undergo a two electron oxidation in one wave. The number of electrons involved in the oxidation of paracetamol and the fluorinated analogues was determined by comparison with 1,4-dimethoxybenzene, a reference compound known to undergo one electron oxidation. In all cases the electrochemical behaviour was irreversible, i.e. no reduction wave was seen. All of the fluorinated analogues were harder to oxidize, i.e. had higher peak potentials than paracetamol itself (Table 1). In particular, fluorine substitution adjacent to the amide nitrogen (compounds 4, 5, 6 and 7) had the greatest effect. Compound 5 had the highest oxidation potential (1·74 V).

Using the abdominal constriction test the antinociceptive activity of a compound is determined by the degree of inhibition of abdominal constriction produced by an intraperitoneal injection of a chemical irritant, 2-phenyl-1,4-benzoquinone. Paracetamol was positive in this test and comparable results (mean ED50 values) were obtained on three separate occasions (2·29, 2·28 and 2·08 mmol kg^{-1}). A compound is said to provide quantal protection when the animals show no response to the chemical irritant. Up to 800 mg kg^{-1} (5·3 mmol kg^{-1}) paracetamol failed to completely protect any of the mice from the noxious effects of the chemical stimulus (Table 2).

All of the fluorinated derivatives tested had antinociceptive (analgesic) activity except for tetrafluoroparacetamol (5) which showed only slight but statistically significant activity at both 800 and 1000 mg kg^{-1} (3·6 and 4·5 mmol kg^{-1}), and 2,6-difluoroparacetamol (4) which was devoid of activity up to 800 mg kg^{-1} (4·3 mmol kg^{-1}) (Table 2). The rank order of potency based on the ED50 value is $7 > 6 > 1 > 2 > 3 > 4 \cong 5$.

Clearly from a potency standpoint the most interesting compound is analogue 7 which is six times more active than paracetamol (1) in this test. In addition, 7 completely protected 2/5 animals from the noxious effects of the chemical stimulus at 200 mg kg^{-1} (0·9 mmol kg^{-1}) and thus caused 40% quantal protection.

Attempts were made to correlate the antinociceptive activity expressed as ED50 (mmol kg^{-1}) and the physicochemical parameters log K_o (at pH 3 and pH 7·4), oxidation potential, and the degree of conjugation of the acetyl/amide group. The data were examined by linear regression analysis and statistical significance was established at a level of $P < 0.05$. Compounds 1, 2, 3, 6 and 7 were included in the analysis (Table 3). No significant correlation was found between the ED50 and the lipophilicity parameter log K_o at

Table 1. Some physicochemical properties of paracetamol (1) and the fluorinated analogues (2–7).

Compound	pK_a	Oxidation potential[a] (V)	Log K_o[b] at pH 3	Log K_o[b] at pH 7·4
1	9·53	1·14	0·65	0·65
2	8·1	1·24	0·85	0·76
3	7	1·33	1·05	0·7
4	9·05	1·52	0·67	0·64
5	4·75	1·74	0·79*	-0.03
6	9·14	1·37	1·00	1·00
7	8·4	1·47	1·25	1·21

[a] vs saturated calomel electrode. [b] The log K_o measured at pH 3 is related to the partition coefficient, log P, whilst that measured at pH 7·4 is related to the distribution coefficient, log D. * Measured at pH 2·5.

Table 2. The antinociceptive activity of some fluorinated analogues of paracetamol in the mouse.

Compound	Dose (mg kg⁻¹)	Inhibition[a] (%)	ED50 (mg kg⁻¹)	ED50 (mmol kg⁻¹)	Quantal protection[b] (%)
Paracetamol (1)	100	21·2	313·7	2·08	0
1	200	35·4***	(192·5–511·2)		0
	400	43·4***			0
	800	87·6***			0
2	200	25·7*	427	2·53	0
	400	52·2***	(202·4–901)		0
	800	66·4***			20
3	200	26·5*	781·7	4·18	0
	400	31·9**	(273·9–2231)		0
	800	53·1***			0
4	200	7·8	>800	>4·28	0
	400	10·4			0
	800	12·2			
5	600	7	>1000	>4·48	0
	800	27*			0
	1000	33*			
6	50	19·7	98·2	0·48	0
	100	53·6***	(66·8–144·4)		0
	200	79***			10
7	25	71.0	85·6	0·38	0
	50	32·7**	(57·2–128·1)		0
	100	42·5**			0
	200	89·4***			40

* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$ vs control, Student's t-test ($n = 5$).

[a] Inhibition = $[1 - (T/C)] \times 100$, where T = mean number of contraction responses in treated animals and C = mean number of responses in control animals.

[b] Quantal protection = $\left(\dfrac{\text{n of mice failing to contract}}{\text{total n of mice per group}} \right) \times 100$.

Table 3. The correlation between analgesic activity (ED50, mmol kg⁻¹) and various physicochemical parameters, for paracetamol and some fluorinated analogues.

	log Kₒ pH 3	log Kₒ pH 7·4	pKₐ	Oxidation potential (V)	Degree of amide conjugation
r	−0·316	−0·82	−0·663	−0·47	−0·97
P	0·61	0·089	0·223	0·424	0·0065

Data examined by linear regression analysis. Compounds included in the analysis were 1, 2, 3, 6 and 7, n = 5. r = Pearson's correlation coefficient.

Table 4. UV absorption data for paracetamol (1), the fluorinated analogues (2–7) and their corresponding amines[a].

Compound	Primary UV absorption band for amine (nm)	Primary UV absorption band for amide (nm)	Shift (nm)
1	235	250	15
2	236	249	13
3	239	248	9
4	231	228	−3
5	229	221	−8
6	235	258	23
7	236	256	20

[a] The shift (nm, bathochromic or hypsochromic) between the UV absorption band of the amine and that of the corresponding amide represents a measure of the degree of conjugation of the acetyl/amide group. A negative shift indicates loss of conjugation.

pH 3, the pKₐ, or the oxidation potential. However, a significant ($P = 0·0065$, r = −0·97) correlation was found between the ED50 and the degree of conjugation of the acetyl/amide group of paracetamol and its fluorinated analogues.

Discussion

It can be seen from Table 1 that fluorine-substitution has profound effects on the physicochemical properties of paracetamol. The electron-withdrawing effect of fluorine is reflected in the increased acidity of the phenolic group in compounds 2–7 compared with paracetamol itself. However, the effect was greater than expected for 5 where the pKₐ was five units lower than that of paracetamol. This may possibly be explained by a greater solvation of the ionized molecule. The ability of fluorine to increase the acidity of neighbouring

hydroxyl groups has also been demonstrated by other workers (Kirk 1976; Kirk et al 1979). For example, 5-hydroxytryptamine has a pKₐ of 10·73 whilst 6-fluoro- and 4,6-difluoro- analogues have pKₐ values of 9·07 and 7·97, respectively. The biological significance of lowering of pKₐ by fluorine substitution is an increase in the amount of phenolate anion present at physiological pH.

Fluorine substitution may also influence the conjugational interaction between the amide group and the benzene ring (Table 4). The importance of charge-separated canonical forms (Fig. 2) in non-hindered acetanilides accounts for the long wave-lengths of their primary aromatic π–π^* absorption bands compared with the corresponding amines (Dear-

den & O'Hara 1978). For example, the absorption band of paracetamol in ethanol (250 nm) is at a longer wavelength than that of p-aminophenol (235 nm).

This shift of absorption maximum to longer wavelength of the amides relative to the amines should not be observed when conjugation between the amide group and aromatic ring is lost. Indeed, it has been shown that for 2,6-dimethyl-4-hydroxyacetanilide the absorption maximum is at 220 nm, similar to that of 3,5-xylenol, indicating that the conjugative interaction between the amide and benzene ring is absent (Dearden & O'Hara 1978). In this case, the steric effect of the ortho methyl groups twists the adjacent amide out of the plane of the benzene ring so reducing the conjugation. A similar effect is observed with compounds 4 and 5. The absorption maximum for 4 in ethanol is at 228 nm compared with 231 nm for 2,6-difluoro-4-methoxyaniline, a hypsochromic shift of 3 nm, whereas the absorption maximum of 5 in ethanol is at 221 nm compared with 229 nm for 2,3,5,6-tetrafluoro-4-methoxyaniline, a hypsochromic shift of 8 nm (Table 4). Thus ortho fluorine substituents also appear to reduce conjugation between the amide group and the aromatic ring. Simple steric effects seem unlikely to be responsible for a lack of planarity in this case in view of the small size of fluorine, although in a solvent such as ethanol (or water in-vivo) H-bonding interactions with the solvent may increase its effective size. Other factors which may play a part are electrostatic interactions and unfavourable dipole alignments.

Fluorine substitution also has an effect on the lipophilicity of the paracetamol derivatives (Table 1). At pH 7·4 the effective lipophilicities are similar to the intrinsic lipophilicities except for compound 5, which is fully ionized at pH 7·4. The measurement for 5 at pH 2·5 should reflect its true intrinsic lipophilicity since ionization of this abnormally acidic phenol should be suppressed under these conditions.

Fluorine adjacent to the amide group has little effect on the lipophilicity (Table 1, compounds 4 and 5). Since fluorine would normally be expected to increase lipophilicity this lack of effect may be attributable to a compensating reduction in lipophilicity due to loss of conjugation of the amide (increased conjugation tends to be associated with increased lipophilicity). Fluorine adjacent to the hydroxyl group causes a marked increase in lipophilicity (Table 1, compounds 2 and 3) although the intrinsic lipophilicity of 5 is lower than that of 3, presumably because of loss of conjugation of the acetamido group.

The trifluoromethyl group is lipophilic (Welch 1987), and this is illustrated by the fact that 6 and 7, which both contain the trifluoromethyl group, are the most lipophilic of the fluorinated analogues investigated.

The number of electrons involved in the oxidation of paracetamol was determined by comparison with a reference compound known to undergo one-electron oxidation. The oxidation occurred in one wave, was irreversible and

Fig. 2. Canonical forms of acetanilide.

involved two electrons. Although the electrochemical oxidation of aromatic amides has received little attention, irreversible one-wave oxidations involving two electrons have been reported (Ohmori et al 1981) including that of paracetamol (Miner et al 1981). The two electrons are probably removed sequentially. The first electron is removed (from oxygen or nitrogen) to give a cation radical and then the second electron removed so rapidly that the oxidation appears as a single two-electron transfer, the driving force being the formation of an electrochemically neutral compound after the loss of two protons. The electrochemical oxidation of paracetamol is irreversible because the electrochemical reactions are almost certainly followed by a chemical reaction. N-Acetyl-p-benzoquinoneimine (NAPQI) has been shown to react with paracetamol in an initial comproportionation reaction to give N-acetyl-p-benzosemiquinoneimine (NAPSQI) which then reacts to form dimers and polymers. NAPQI may also be hydrolysed to benzoquinone by any residual water (Potter & Hinson 1986).

The electrochemical behaviour of the fluorinated analogues, 2-7, was similar to that of paracetamol. All the compounds underwent irreversible two-electron oxidation in one wave. However, the introduction of fluorine leads to derivatives with decreased ease of oxidation. This is expected because of the electron withdrawal by the fluorine atoms and is consistent with the observed increase in phenol acidities. Similar decreases in the ease of electrochemical oxidation have been observed with the 4- and 7-fluoro-dihydroxytryptamines compared with dihydroxytryptamine (Kawase et al 1990). The fluorines adjacent to the amide group (compounds 4 and 5) have the greatest effect on the oxidation potential of paracetamol as would be expected if oxidation essentially involves initial removal of an electron from nitrogen. The biological implications of the introduction of fluorine in terms of oxidation are that the fluorinated analogues are less likely to be oxidized to quinoneimine metabolites by either cyclo-oxygenase or cytochrome P450.

The antinociceptive activity of paracetamol and the fluorinated analogues was determined using an abdominal constriction test in the mouse. The method is based upon antagonism by analgesics of the typical syndrome produced by intraperitoneal injection of a chemical irritant (e.g. formic acid, acetic acid, bradykinin). The irritant used in the present study was 2-phenyl-1,4-benzoquinone. The test is simple and quantitative in that an ED50 is determined. The constriction test is an in-vivo model for analgesic action and therefore the ED50 obtained is a function of both the pharmacokinetic and the pharmacodynamic properties of the compound. The ED50 obtained for paracetamol was 2·1 mmol kg^{-1} and is in good agreement with the ED50 obtained by Starmer et al (1971) with the abdominal constriction test using formic acid as the irritant.

The effect of fluorine substitution on the analgesic potency (ED50) did not exhibit a simple pattern. Both the nature of the group and its position appeared to influence analgesic potency. For example, analogues 6 and 7 which contain the trifluoroacetyl group, were five to six times more active than paracetamol in this test and this may be related to their increased lipophilicity compared with paracetamol; the most potent compound (7) is also the most lipophilic both at pH 3
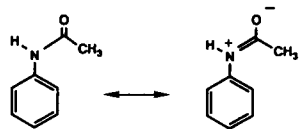
and pH 7·4. Dearden & O'Hara (1976) have found a good parabolic relationship between analgesic activity and log P with some alkyl derivatives of paracetamol. However, we have found that, at least for compound 6, the increased lipophilicity does not lead to a general increase in brain levels of the compound compared with paracetamol (Barnard 1991). Therefore the increased analgesic potency of 6 is most likely to be due to a more effective combination of the drug at the site of action.

For the ring-fluorinated analogues, substitution at the 2- and 6-positions (compounds 4 and 5) completely removes activity whereas substitution at the 3- and 5-positions has less effect but also reduces activity. Fluorine substitution at the 2- and 6-positions also has a greater effect on the oxidation potential than at the 3- and 5-positions. Thus, those derivatives which are less easily oxidized show less analgesic activity. This adds some support to the suggestion by Hemler & Lands (1980) that the antioxidant properties of paraceta- mol are involved in preventing the peroxide-initiated free-radical mechanism of prostaglandin synthesis. However, the enhanced activity of the trifluoroacetyl derivatives 6 and 7, which are also more difficult to oxidize than paracetamol, indicates that the ease of oxidation is not the only factor. There is an excellent correlation between analgesic potency and the degree of conjugation of the amide group (Table 3). All the active compounds show evidence of conjugation and hence planarity of the amide group and aromatic ring as indicated by bathochromic shifts of the UV absorption bands of the amides relative to the amines (Table 4), whereas lack of activity is associated with nonplanarity in compounds 4 and 5. It has also been reported that alkyl substituents at the 2- and 6-positions lead to reduced activity and again this has been rationalized by steric effects causing lack of planarity (Dearden & O'Hara 1976, 1978). Thus it appears that both steric compatibility with the active site and ease of oxidation may be involved in analgesic activity.

Acknowledgements

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