DISPOSITION AND METABOLISM OF A NOVEL PROSTANOID ANTIGLAUCOMA MEDICATION, TAFLUPROST, FOLLOWING OCULAR ADMINISTRATION TO RATS

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Running Title: ADME of Tafluprost in the Rat after Ocular Administration

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Number of text pages: 29

Number of tables: 10

Number of figures: 9

Number of references: 24

Total number of words in the Abstract sections: 247

Total number of words in the Introduction sections: 637

Total number of words in the Discussion sections: 1378

Non-standard abbreviations used: tafluprost, 1-Methylethyl \((5Z)-7-\{(1R,2R,3R,5S)-2-[(1E)-3,3-difluoro-4-phenoxy-1-butenyl]-3,5-dihydroxycyclopentyl\}-5-heptenoate; tafluprost acid, \((5Z)-7-\{(1R,2R,3R,5S)-2-[(1E)-3,3-difluoro-4-phenoxy-1-butenyl]-3,5-dihydroxycyclopentyl\}-5-heptenoic acid; IOP, intraocular pressure; 7-EC, 7-ethoxycoumarin; 7-HC, 7-hydroxycoumarin; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid; PGF\(_{2\alpha}\), prostaglandin F\(_{2\alpha}\); prostanoid FP-receptor, receptor whose internal ligand is PGF\(_{2\alpha}\)
ABSTRACT

The disposition and metabolism of tafluprost, an ester prodrug of the 15,15-difluoro-PGF$_{2\alpha}$ antiglaucoma agent, have been studied in rats after ocular administration. Radioactivity was absorbed very rapidly into the eye and systemic circulation after a single ocular dose of 0.005% $[^3]$Htafluprost ophthalmic solution, with maximum levels in plasma and most eye tissues occurring within 15 minutes. The absorption ratio of radioactivity was about 75%, suggesting the high availability of ocular administration of tafluprost. About 10% of the dose was present in cornea at this time, and radioactivity concentrations in this tissue exceeded those in aqueous humour and iris/ciliary body throughout the 24-hour study period. After repeated daily ocular doses, radioactivity levels remained greatest in cornea, followed by iris/ciliary body that replaced aqueous humour as the eye tissue containing the second highest radioactivity concentration. In female rats, radioactivity was excreted equally between urine and faeces after a single ocular dose, whereas in males more was excreted in faeces, reflecting the greater biliary excretion in males (50% dose) compared with females (33% dose). Tafluprost was extensively metabolised in the rat, such that intact prodrug was not detected in plasma, tissues or excreta by radio-HPLC. On the other hand, the active moiety, tafluprost acid, was the only noteworthy radioactive component in cornea, aqueous humour and iris/ciliary body for at least 8 h after the ocular dose, and was also a major plasma metabolite in early time points. The gender differences in conjugation reactions resulted in the differences in the excretion.
INTRODUCTION

Glaucoma is a group of degenerative eye diseases for which the major risk factor is excessive intraocular pressure (IOP) leading eventually to irreversible damage to the optic nerve. Reducing IOP is the only approach in the current therapy of the glaucoma (Ishida et al., 2006). Several synthetic PGF$_{2\alpha}$ analogues with potent and long-lasting IOP-lowering properties are widely used as first-line therapy of glaucoma or ocular hypertension. These agents include latanoprost (Stjernschantz et al., 1995), unoprostone isopropyl (Taniguchi et al., 1996), travoprost (Sharif et al., 1999) and bimatoprost (Woodward et al., 2001). It is believed that these prostanoid FP-receptor agonists reduce IOP by increasing the uveoscleral outflow of aqueous humour (Kaufman and Crawford, 1989; Nilsson et al., 1989). Although they have no general side effects they do not always produce satisfactory results and long-term use can result in unwanted local side effects (Scherer, 2002; Watson, 1998).

Recent studies with 15-monofluorinated- and 15,15-difluorinated prostanoids showed that replacement of the hydroxyl group on that position with the halogen atom(s) can increase the desired FP-receptor-related activities while decreasing the side effects (Nakajima et al., 2003). Tafluprost [1-methylethyl (5Z)-7-{(1R,2R,3R,5S)-2-[(1E)-3,3-difluoro-4-phenoxy-1-butenyl]-3,5-dihydroxycyclopentyl}-5-heptenoate; Fig. 1] is such a difluorinated prostanoid FP-receptor agonist (Matsumura et al., 2004) that has been developed as an ophthalmic drug for the treatment of glaucoma and ocular hypertension. The ester is a lipophilic prodrug of the carboxylic acid of tafluprost, which is the pharmacologically active form of the drug. Esterification of the carboxyl group on the $\alpha$-side chain of these prostaglandins greatly enhances their penetration into the cornea (Camber and Edman, 1987; Camber et al., 1986), and the presence of esterase activity in the cornea and sclera capable of hydrolysing these
derivatives to the corresponding acids for uptake during absorption into aqueous humour is also well-established (Redell et al., 1983).

Tafluprost decreases the IOP in vivo mainly by increasing the uveoscleral outflow by up to 65% (Takagi et al., 2004). Studies have shown that tafluprost is superior to latanoprost in several important pharmacological criteria (Nakajima et al., 2003; Takagi et al., 2004). Thus, in both ocular normotensive and laser-induced ocular hypertensive monkeys, a single instillation of 0.0025% tafluprost solution lowered IOP significantly more than 0.005% latanoprost. Once daily for five-day applications of 0.001%-0.005% tafluprost to the eyes of normotensive monkeys had the advantage of a reduced IOP at the trough time of 24 h after dosing, whereas 0.005% latanoprost did not. Tafluprost also has less stimulating effects on melanogenesis in vitro, while tafluprost acid possesses a greater affinity for the prostanoid FP-receptor than the acid form of latanoprost (Takagi et al., 2004). Recent results of a clinical study in healthy volunteers also have shown well-tolerability of 0.0025% and 0.005% tafluprost ophthalmic solutions and greater IOP reduction by 0.005% tafluprost than 0.005% latanoprost (Sutton et al., 2007).

The aims of this study were to investigate: (1) the absorption and distribution of tafluprost into ocular tissues and plasma after single and repeated topical ocular administrations of an ophthalmic solution of [3H]tafluprost in rats; (2) the nature of the resultant radiolabeled material in selected ocular and systemic tissues and excreta to confirm that the active drug tafluprost acid was released from its prodrug tafluprost after absorption; (3) the rates and routes of excretion of radioactivity in bile, urine and faeces after ocular doses of the tritium-labelled drug; and (4) the in vitro metabolism of [3H]tafluprost in rat, dog, cynomolgus monkey and human hepatocytes. To assist the metabolic investigations, an in vitro system was used to generate glucuronic acid conjugates of likely Phase I chain-shortened metabolites of tafluprost. Since there is obvious potential for an ocularly administered compound to be
absorbed into the systemic circulation via the conjunctiva or nasal mucosa, the systemic tissue
distribution, placental transfer and milk secretion of the drug were also investigated in rats,
the main species used in its toxicological evaluation.
MATERIALS AND METHODS

Chemicals

[3H]tafluprost (Fig. 1) was synthesised by Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan) and provided as a solution in ethanol which was stored at −70°C. Specific activity of [3H]tafluprost was 1.18-1.79 GBq/mg. Non-radiolabeled tafluprost and synthetic samples of the putative metabolites tafluprost acid (AFP-172), 1,2-dinor-AFP-172, 1,2,3,4-tetranor-AFP-172, and 1,2,3,4-tetranor-AFP-172 lactone were supplied by Asahi Glass Co. Ltd. (Tokyo, Japan). Reagents and solvents were obtained from Sigma-Aldrich Chemical Co. Ltd. (Poole, UK), Fisher Scientific UK Ltd. (Loughborough, UK) or VWR International (Lutterworth, UK), and were of analytical or HPLC grade.

Ophthalmic solutions of [3H]tafluprost for topical application to the eyes of rats were prepared by mixing appropriate volumes of the radiolabeled and non-radiolabeled compound solutions in ethanol, then removing the solvent under nitrogen and dissolving the residue in the tafluprost dose vehicle (pH 6.0) to total drug concentrations of 0.05%, 0.005%, or 0.0005% (w/v). Radiochemical purity of [3H]tafluprost in each dose formulation was 98% by HPLC.

In vivo Metabolism Studies in the Rat

All procedures involving animals were performed in compliance with the relevant guidelines issued by the UK Home Office, from whom Project and Personal Licences were obtained as specified by the Animals (Scientific Procedures) Act 1986 following the European Union Directive 86/609/EEC which requires ethics review of the Licences and their Amendments.
Such animal studies conducted at Huntingdon Life Sciences (HLS) are subject to review for approval by the HLS Ethical Review Process Committee.

Sprague-Dawley CD strain rats were obtained from Charles River UK Ltd (Margate, UK) and allowed free access to standard laboratory diet (VRF1C) and drinking water in a controlled and continuously monitored environment according to Standard Operating Procedures. After dosing, the animals were group-housed according to gender in either stainless-steel cages (plasma level/tissue distribution studies) or singly in glass metabolism cages (mass balance studies) or plastic Bollman-type cages (biliary excretion and enterohepatic circulation studies), while nursing rats were housed singly with their respective litters in plastic solid-floor cages (milk secretion study). For the biliary excretion study, each rat had a flexible plastic cannula surgically inserted into its common bile duct to collect the bile, and another inserted into its stomach under isoflurane/oxygen anaesthesia. Bile duct-cannulated rats had free access to water containing 4% glucose instead of water, and sodium taurocholate solution was infused into the stomach cannula to replace lost bile salts. In the enterohepatic circulation study, linked rats were used whereby the biliary cannula from one bile duct-cannulated rat was surgically implanted into the duodenum of a second cannulated rat of the same sex, such that the bile from the dosed (‘donor’) rat was continuously administered to the ‘recipient’ rat while the total bile from the latter was being collected. Bodyweights at the time of dosing/first dose were in the approximate ranges 170-340 g for non-pregnant rats, 260-390 g for pregnant females, and 280-380 g for nursing dams.

Ocular doses of \[^{3}H\]tafluprost ophthalmic solution were administered to rats as single or 7, 14 or 21 daily 5 µL instillations to the cornea of both eyes using a pre-calibrated electronic pipette. After completion of the experimental period, rats were killed by cervical dislocation under isoflurane/oxygen anaesthesia.


Study designs

Single-dose studies

For the preliminary dose-ranging study, terminal blood samples were collected from pairs of male rats at 1, 2, 4, 8, 12 and 24 h after single 5 µL ocular doses of 0.05%, 0.005% and 0.0005% [3H]tafluprost ophthalmic solutions to both eyes, and the separated plasma analysed for radioactivity concentrations. All the other single- and multiple-dose studies utilised 5 µL ocular doses of 0.005% (w/v) [3H]tafluprost ophthalmic solution only, i.e. a total dose to each rat of 0.5 µg tafluprost (ca 17 µCi; 630 kBq).

In the single-dose tissue distribution study, ocular doses of [3H]tafluprost were administered to male and female rats, and the animals were killed at 5, 15, and 30 min, 1, 2, 4, 6, 8, 12 and 24 h (n = 6 per sex per time point) when blood samples and ocular and systemic tissues/organs were collected (see below). The individual ocular tissues were combined from the eyes of 2 rats of the same sex at each sacrifice time, to provide 3 sets of each pooled tissue per sex per time point. Other rats (n = 20 per sex) were dosed similarly and the following samples collected for metabolite profiling at selected times post-dose: plasma, aqueous humour, cornea, iris/ciliary body, kidneys, liver and lungs.

To determine its excretion profile, rats (n = 4 per sex) received single ocular doses of [3H]tafluprost, and urine and faeces (the former into solid CO₂ cooled containers) were collected at suitable intervals for 7 days post-dose. Volatile radioactivity in the expired air was collected in traps of purified water. The animals were killed at 168 h and the carcasses retained for analysis with the excreta, expired air traps, and cage washes. In the biliary excretion study, single ocular doses of [3H]tafluprost were administered to bile duct-cannulated rats (n = 4 per sex) after recovery from surgery and anaesthesia, then bile, urine and faeces (the former into solid CO₂ cooled containers) were collected for 2 days. The rats were killed at 48 h for analysis of their excreta, blood, liver, gastrointestinal tract, remaining

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carcasses, and cage washes. To determine the extent of reabsorption of total tafluprost-derived material excreted in bile, single ocular doses of [3H]tafluprost were administered to bile duct-cannulated rats linked to other such rats via the biliary cannula (n = 4 linked pairs per sex) after recovery from surgery and anaesthesia. Bile was collected into solid CO2 cooled containers from each ‘recipient’ rat, and urine and faeces were collected from all the ‘donor’ and ‘recipient’ rats for 2 days. All rats were killed at 48 h after dosing for analysis of their excreta, blood, liver, gastrointestinal tract, remaining carcasses, and cage washes.

For the milk secretion study, single ocular doses of [3H]tafluprost were administered to lactating rats at 10-13 days after parturition, milk samples (ca 0.3-0.5 mL) were collected at each of the following times: 0.5, 1, 2, 4, 8, 24, 48 and 96 h (n = 4 per time point). The rats were then killed and blood samples obtained. Pups were removed from the dams at 3 h before the intended time of milk sampling and oxytocin (1 unit/kg; i.p.) was administered about 15 min before sampling to stimulate milk production.

To investigate its placental transfer, single ocular doses of [3H]tafluprost were administered to 20 pregnant rats on Day 12 of gestation and to another 20 on Day 18 of gestation. The rats were killed at 0.25, 1, 4, 12, and 24 h (n = 4 per time point), and foetuses and/or foetal tissues collected (as appropriate). Foetal tissues were pooled by litter.

To assist the identification of in vivo metabolites, single intravenous doses of [3H]tafluprost (100 µg/kg; ca 30 µCi (1100 kBq) per animal) were administered to 9 rats as a solution in 0.05% Tween 80 in isotonic saline. Rats were killed in groups of 3 at 20 min, 1 and 24 h post-dose, and urine, faeces and terminal blood samples collected as described above.

Repeat-dose studies
After repeated daily ocular doses of $[^{3}H]$tafluprost to rats, animals were killed ($n = 6$ per sex per time point) at 0.5, 1, 2, 8 and 24 hours (7 and 14 daily doses) or at 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 hours (21 daily doses). Blood samples and ocular and systemic tissues were collected (and pooled, where appropriate) as described previously. Other rats ($n = 4$ per sex) were administered 21 daily ocular doses of $[^{3}H]$tafluprost, and urine and faeces collected at intervals during the dosing period and for 7 days after its completion. The animals were killed at 168 h after the final dose and the carcasses retained for analysis with the excreta and cage washes.

**In vitro Synthesis of Glucuronic Acid Conjugates of Phase I Metabolites**

Chemically synthesised compounds of tafluprost Phase I metabolites, namely 1,2-dinor-AFP-172, 1,2,3,4-tetranor-AFP-172, and 1,2,3,4-tetranor-AFP-172 lactone, were incubated with pooled human ($n = 10$) liver microsomes for synthesis of the glucuronic acid conjugates of the Phase I metabolites. The human liver microsomes were purchased from Xenotech LLC (Kansas City, KS, USA). The reaction mixture for each compound comprised potassium phosphate buffer (100 µM, pH 7.4, containing 5 mM saccharic acid-1,4-lactone and 1 mM MgCl$_2$), microsomal protein [2 mg/mL, pre-treated with alamethicin (50 µg/mg protein) at 0°C for 15 min], NADPH (2 mM), and UDPGA (16 mM) in a total volume of 10 mL. After brief mixing and pre-incubation for 3 min at 37°C, the substrate (1 mg of each compound) was added, then incubation was continued for 18 h before being terminated by the addition of acetonitrile (10 mL). Control incubations performed in the absence of liver microsomes (total incubation volume = 0.5 mL) showed no deterioration of the substrate.

Terminated samples were centrifuged at 3000g to sediment the microsomal protein. The acetonitrile present in the supernatants was removed under a stream of gas. Each concentrated aqueous solution was then fractionated by solid-phase extraction using Strata-
X® cartridge (33 µm polymeric sorbent; Phenomenex Ltd., Macclesfield, UK), which was eluted with water followed by water containing an increasing proportion of acetonitrile (1, 5, 20 and 50%). The glucuronidated metabolites were isolated by repeated injection of the relevant fractions into the chromatograph using HPLC Conditions I (see below), and samples of each were isolated for structural characterisation by mass spectrometry.

In vitro Metabolism Studies with Isolated Hepatocytes

[3H]tafluprost (2.5, 25 or 100 µM) was incubated with cryopreserved hepatocytes (In Vitro Technologies Inc, Baltimore, MD, USA or Xenotech LLC) from male SD rat, beagle dog, cynomolgus monkey and human (n = 5) which had been previously treated with Williams’ Medium E. The incubations were performed in polypropylene tubes under an atmosphere of 95% O2/5% CO2 using an orbital shaking water bath set at 37°C. The incubation medium was Williams’ Medium E supplemented with HEPES (10 mM), foetal calf serum (10%, v/v), dexamethasone (1 µM) and SPITE (a concentrated liquid medium supplement solution containing selenium, pyruvate, insulin, transferrin and ethanolamine). Cell density determined by haemocytometer was about 1 × 10⁶ viable cells/mL. A trypan blue exclusion test was conducted on the hepatocytes to confirm the viability of each preparation prior to incubation. The incubations were terminated at suitable times by cooling in ice and homogenising with an ultrasonic probe, then stored at about −20°C until analysed. Aliquots of each sample were also subject to enzymatic deconjugation by incubation with β-glucuronidase/arylsulphatase (type H-1 from Helix pomatia) at pH 5.0 for at least 1 h at 37°C. Control incubations were performed in the absence of hepatocytes or [3H]tafluprost. All samples were then treated with a small volume of a solution of either tafluprost and tafluprost acid in ethanol or 7-EC and 7-HC in methanol (depending on sample type: see below) and centrifuged at 10000g for 15 minutes to remove particulate matter prior to metabolite profiling by radio-HPLC. Incubation
of the isolated hepatocytes with $[^{14}C]7$-EC (50 µM) as substrate was used as a positive control test. These samples were analysed using an HPLC method based on that described by Walsh et al. (1995) for liver slices.

**Measurement of radioactivity**

All biological samples were weighed prior to storage at about $-20^\circ$C, apart from whole-blood which was stored at about $+4^\circ$C.

In tissue distribution studies, the following procedures were used to remove the ocular tissues after sacrifice: whilst still in the orbit, each eye was rinsed well with saline and the excess removed with paper. The eyeball was removed from the carcass, washed with saline and blotted dry, then the total conjunctiva was separated from the enucleated eyeball and washed similarly. The aqueous humour was collected using a 25 µL glass microsyringe, and the total volume noted. The cornea, lens, and iris/ciliary body were separated from the sclera, then the vitreous humour and choroid/retina were removed. Systemic tissues and organs were obtained by conventional dissection techniques. Terminal blood samples were taken from the dorsal aorta into heparinised tubes and centrifuged with the minimum delay to separate the plasma.

Gastrointestinal tracts from rats used in tissue distribution studies were divided into the stomach, duodenum, ileum and large intestine, then the contents were removed from each section, the walls were washed with saline and the washings mixed with the corresponding contents. In the biliary excretion studies, the entire gastrointestinal tract was processed in the same manner. Livers, intact foetuses and foetal carcasses were homogenised using an Ultra-Turrax laboratory blender (Semat Technical (UK); St Albans, UK). Larger systemic and foetal organs were homogenised by scissor-mincing, while smaller organs were analysed in their entirety after subdivision into portions where necessary. Carcasses of rats used in
excretion studies were solubilised at 55°C for 24 or 48 hours in a mixture of Triton X–405, sodium hydroxide, methanol and distilled water. Faeces were homogenised with distilled water (1:1, w/v).

Serial samples of plasma, urine, bile, cage washings, contents of expired air traps, carcass digests, and HPLC fractions were mixed with Ultima Gold Scintillator (Canberra Packard Ltd., Pangbourne, UK) for liquid scintillation analysis. Weighed portions or entire samples (as appropriate) of ocular, systemic, and foetal tissues/homogenates, amniotic fluid, terminal plasma samples (untreated and lyophilised to determine total and non-volatile radioactivity respectively), whole-blood, milk, and faeces homogenates were burned in oxygen using a Packard 307 automatic sample oxidiser (Canberra Packard). The products of combustion were absorbed in Monophase S water absorber/scintillator (Canberra Packard) for measurement of radioactivity concentrations using a Wallac 1409 automatic liquid scintillation counter (Perkin Elmer Life & Analytical Sciences, Cambridge, UK). The combustion efficiency of the sample oxidiser determined by processing tritium-standards was generally greater than 95%.

Metabolite profiling

Profiles of radioactive components in the biological samples generated during these investigations were determined by HPLC with radioactivity detection. Chromatography was performed using a Waters Alliance 2690 Separations Module (Waters Ltd, Watford, UK) with built-in autosampler and membrane degassing system or a Thermoseparation products modular system. Radiometabolites were separated using a Hypersil ODS-5 (250 × 4.6 mm) column maintained at 50°C, and a 0.1% formic acid (A)/acetonitrile (B) mobile phase eluted at a flow rate of 1 mL/min using the following gradient (Conditions I):
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<th>Time (minutes)</th>
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Quantification of the separated radioactive components was performed using the online β-RAM radioactivity detector (LabLogic Systems Ltd, Sheffield, UK) with Monoflow 3 scintillant or by offline fraction collection/liquid scintillation counting, depending on the sample radioactivity concentration. Under these conditions, tafluprost and tafluprost acid eluted with retention times of approximately 37 and 30 minutes respectively.

Tissue samples were extracted with methanol (20 vols.), faeces homogenates with acetonitrile (20 vols.), and plasma [untreated and after incubation with β-glucuronidase/sulphatase (Sigma type H–1, from Helix pomatia) in pH 5 acetate buffer for 16 h at 37°C] with acetonitrile (4 vols.), then each was centrifuged and evaporated to low volume prior to mixing with mobile phase. Urine samples (untreated and after incubation with β-glucuronidase/sulphatase) were centrifuged to remove particulate matter and analysed directly.

For metabolite identification, urine from intravenously dosed rats was concentrated and fractionated by solid-phase extraction using Strata-X® cartridges (33 µm polymeric sorbent; 500 mg per 6 mL; Phenomenex Ltd., Macclesfield, UK). After conditioning with acetonitrile and water, cartridges were loaded and eluted successively with water, followed by water containing 1, 5, 10, 20 and 50% acetonitrile (10-20 mL). Aliquots of each eluate were analysed by HPLC then, after further concentration, by LC-MS where appropriate.

**Structural Characterisation of Metabolites**
The identities of tafluprost metabolites formed \textit{in vitro} and \textit{in vivo} were investigated by liquid chromatography-mass spectrometry (LC-MS) using a Finnigan TSQ 7000 spectrometer (Thermo-Finnigan, San Jose, CA, USA) with an electrospray ionisation source operating in the negative ion (ESI-) mode. Spectra were acquired with the spray voltage and capillary temperature set to +4.5 kV and 250°C respectively, while various scan ranges were used from \textit{m/z} 120 to 900 at a scan rate of 1 sec/scan. In the MS/MS product ion mode, argon was used as collision gas (collisional energy 25 eV) at a pressure of 2 mTorr. The LC conditions were those used for metabolite profiling but using the following gradient (Conditions II):

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<th>Time (minutes)</th>
<th>Solvent A (%)</th>
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<td>75</td>
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Under these conditions, tafluprost and tafluprost acid eluted at approximately 52 and 65 min respectively. Metabolites of tafluprost were identified from their mass spectra (Q1, product ion, selected reaction monitoring, or neutral loss monitoring) and, where appropriate, by comparison of their spectra and/or retention times with those of synthetic reference compounds (including the effect of enzyme treatment on glucuronide or sulphate conjugates).

**Pharmacokinetic Data Processing**

Maximum concentrations of total and non-volatile radioactivity in plasma and tissues (C\textsubscript{max}) and the times of their occurrence (T\textsubscript{max}) were determined by visual inspection of the time-concentration data. Areas under the time-plasma concentration curves up to 24 h post-dose were calculated using the linear trapezoidal rule. The highest dose of drug-related material
that a rat pup was likely to receive by ingestion of maternal milk was estimated from \[C_{\text{max}} \times (M/P) \times V_m\], where M/P is the milk : plasma AUC_{0-96} ratio, and \(V_m\) is the average daily milk intake of a pup (assumed to be 1 mL per day) (Wilson et al., 1980).

The extent of exchange of the \(^3\text{H}\) radiolabel was calculated as \((C_{24} \times V)/D\), where \(C_{24}\) is the mean concentration of volatile radioactivity (total radioactivity – non-volatile radioactivity; tritiated water) in plasma at 24 h, \(V\) is the volume of exchangeable bodywater (assumed to be 60\% of the mean rat bodyweight; Richmond et al., 1962), and \(D\) is the dose.
RESULTS

Preliminary Dose-Ranging Study

Following single 5 µL ocular doses of 0.05%, 0.005% or 0.0005% (w/v) \([^3]H\)tafluprost ophthalmic solution to both eyes of male albino rats (ie totals of 5, 0.5 and 0.05 µg tafluprost respectively), radioactivity was absorbed rapidly into the systemic circulation and penetrated all the major anterior and posterior eye tissues. After each dose, plasma and most ocular tissue radioactivity concentrations attained peak values at 1 h, ie the first sacrifice time in this study. Both the rate and extent of exposure of rats to drug-derived material – as characterised by the plasma \(C_{\text{max}}\) and \(AUC_{0-24}\) respectively – increased proportionately with increasing dosage over the nominal dose level range 0.025 to 2.5 µg tafluprost per eye (Table 1). Radioactive concentrations in the tissues also increased in an approximate dose-proportional manner (Table 2).

Based on associated pharmacological data, all other single- and multiple-dose studies described in this communication were performed in male and female albino rats using a 0.005% (w/v) \([^3]H\)tafluprost ophthalmic solution, with 5 µL (ie 0.25 µg tafluprost) applied to both eyes of each animal.

Plasma radioactivity kinetics

Radioactivity was rapidly absorbed after a single ocular instillation of \([^3]H\)tafluprost to rats, with maximum plasma levels occurring at 5 min post-dose (Table 3, Fig. 2). Concentrations appeared to decline biexponentially thereafter. There was little difference in \(C_{\text{max}}\) for male and female rats after a single ocular dose, but \(AUC_{0-24}\) for females was greater than that for the males. The corresponding curves for male and female rats after 21 daily ocular doses of
[\textsuperscript{3}H]tafluprost exhibited a secondary peak, in this case at 6 h for both sexes. \(C_{\text{max}}\) and \(AUC_{0-24}\) for total radioactivity and \(C_{\text{max}}\) for non-volatile radioactivity were all higher in females than in males, although \(AUC_{0-24}\) for non-volatile radioactivity was similar for each sex. The exposure to tafluprost-derived radioactivity was therefore greater in female rats than in males after single and repeat ocular dosing.

The \(AUC_{0-24}\) values for plasma total radioactivity after 7, 14 and 21 daily ocular doses of [\textsuperscript{3}H]tafluprost to rats were 2.7-, 3.3- and 3.5-fold greater respectively than after a single dose in males and 2.6-, 3.0- and 3.1-fold higher respectively in females (Table 3). Although this accumulation ratio appears to increase with the number of daily doses administered, the increase was only slight and may have been due to the smaller number of sampling times utilised after the 7 and 14 daily doses. Steady-state conditions had therefore apparently been attained by the 14-day dosing period.

The proportion of the drug-derived material in plasma present as volatile radioactivity (presumably tritiated water) increased with time, until at 24 h it accounted for approximately 90%. The calculated extent of exchange of the tritium radiolabel with bodywater ranged from 3.0 to 15.1% in both sexes (Table 3).

**Tissue Distribution of Radioactivity**

Ocular tissue radioactivity pharmacokinetic data following single ocular doses of [\textsuperscript{3}H]tafluprost to rats are given in Table 4. Radioactivity was present in all the tissues and fluids examined, but mean concentrations in most were maximal at 5 min, apart from those in the lens which were noticeably later (\(T_{\text{max}}\) 1 or 2 h). At 5 min, radioactivity concentrations were highest in the cornea (which contained about 10% of the total dose), moderate levels in the conjunctiva, iris/ciliary body, sclera and aqueous humour, and relatively low levels in the choroid/retina and vitreous humour; lowest levels of all at this time were present in the lens.
Radioactivity concentrations in the ocular tissues declined thereafter (Fig. 3), those in the cornea with a terminal half-life of 6.6 h, but all were still measurable at 24 h when highest values were recorded in the lens and cornea. Among the systemic tissues analysed after the single dose, highest concentrations of radioactivity were generally associated with the liver, kidney, gastrointestinal tract and urinary bladder (ie the main organs of drug absorption and excretion) and the Harderian gland (which is located immediately behind the eyeball – the site of administration) (Table 5).

Following the last of 7 or 14 daily ocular doses of [³H]tafluprost, mean total radioactivity levels in the ocular tissues/fluids were maximal at 30 min, apart from those in the lens which peaked at 1 or 2 h (Table 4). Highest levels were present in the cornea at 30 min, moderate levels in the iris/ciliary body and aqueous humour, and low levels in sclera, choroid/retina, conjunctiva and vitreous humour; lowest levels of all were in the lens. Concentrations declined subsequently, but all were still measurable at 24 h when values were greatest in the lens. At 30 min after the final dose to both of these groups of rats, total radioactivity levels in the systemic tissues were greatest in the liver, kidney, gastrointestinal tract, urinary bladder and Harderian gland; others were generally less than plasma levels. Concentrations declined thereafter, but all were still measurable at 24 h when those in the gastrointestinal tract were highest.

After 21 daily ocular doses of [³H]tafluprost to rats, concentrations of radioactivity in the ocular tissues/fluids were generally greatest at 15 min, apart from those in the lens which peaked at 2 h (Table 4). At the former time, highest levels were present in the cornea, moderate levels in the iris/ciliary body, aqueous humour and sclera, and low levels in the conjunctiva, vitreous humour and choroid/retina; lowest levels of all were found in the lens. Concentrations declined subsequently, but all were still measurable at 24 h when they were greatest in the lens. At 15 min after the 21st dose, total radioactivity levels in the systemic...
tissues and organs were greatest in the liver, kidney, gastrointestinal tract, urinary bladder and Harderian gland, while the rest were generally less than plasma levels (Table 5). Concentrations declined thereafter, but all were still measurable at 24 h when those in the gastrointestinal tract were highest.

Radioactivity concentrations in selected maternal tissues and foetuses of pregnant rats dosed ocularly with [\(^{3}\)H]tafluprost on Day 12 of gestation were greatest at 15 min (the first sacrifice time), apart from those in amniotic fluid and foetuses which peaked at 4 h (Table 6). During the 24-h study period, highest levels were recorded in maternal liver and kidney, while those in the foetuses and the other maternal organs analysed were generally less than plasma levels. The proportion of the radioactive dose located in all the foetuses was maximal at 24 h, although it only accounted for 0.013% of the dose.

In pregnant rats dosed on Day 18 of gestation, certain individual foetal organs were analysed in addition to intact foetuses and maternal tissues. Radioactivity was present in measurable concentrations in all organs and tissues for 24 h after dosing, the greatest being in maternal kidney and liver, while the rest were less than plasma levels (Table 6). Initially, radioactivity levels in the foetal liver were about twice those in the other foetal organs, but by 24 h levels in all the foetal tissues (including foetal blood) were similar to each other and to maternal blood. The proportion of the maternal dose located in all the foetuses was 0.20% at 15 min increasing to 0.54% at 24 hours, corresponding to about 0.02% and 0.05% dose per individual foetus respectively.

In both sets of pregnant rats, totals of about 23% and 3% dose were present in the liver and kidneys respectively at 15 min, declining to ca 0.3% and 0.04% at 24 h.

Radioactivity concentrations in the milk, plasma and whole-blood of nursing rats administered single ocular doses of [\(^{3}\)H]tafluprost are illustrated in Fig. 4. Radioactivity
levels in plasma and whole-blood peaked at 30 min, while concentrations in milk were maximal at 2 h when they were more than twice as high as plasma levels; concentrations in all three fluids were still measurable at 96 h post-dose. If the daily milk consumption of a neonate is assumed to be 1 mL, it may be calculated that a maximum of 0.1% of the maternal dose of tafluprost would be ingested by a suckling pup each day during Segment III reproductive toxicity studies.

**Excretion**

Radioactivity was excreted in equal proportions in urine and faeces by female rats after a single ocular dose of [³H]tafluprost by 168 h post-dose, whereas in males the ratio was about 4 : 6; the total overall recovery at 168 h was 98 ± 1% of the radioactive dose in both cases (Table 7A). Excretion in urine was rapid, as >90% of the total urinary radioactivity was recovered within 24 h. Approximately 2% of the dose remained in the animal carcasses at 168 h, indicating that excretion was almost complete by this time.

After single ocular doses of [³H]tafluprost to rats with cannulated bile ducts, biliary excretion of radioactivity was extensive, particularly in males where 50% dose was eliminated by this route, compared with 33% dose in females (Table 7B). Urinary excretion was also extensive and correspondingly greater in female rats, such that the combined biliary and urinary excretion of the radioactivity accounted for about 75% of the dose to both sexes. The extent of enterohepatic circulation of the ocular dose of [³H]tafluprost was investigated in linked pairs of bile duct-cannulated rats, when it was found that about 20% of the radioactivity dosed to a ‘donor’ rat was reabsorbed to be subsequently excreted in the bile and urine of the ‘recipient’ rat (data not shown).

During the dosing period and for 168 h after the last of 21 daily ocular doses of [³H]tafluprost to rats, 87% of the total radioactivity administered was excreted in urine and faeces, and less
than 1% remained in the carcasses (Table 7A). In male rats, radioactivity was excreted in urine and faeces in the ratio 3:7, while in females excretion was divided more evenly, as was the situation after a single dose. The daily rate of excretion of radioactivity in urine remained in the range 24.3-26.5% and 37.6-40.1% of the cumulative dose in males and females respectively (Fig. 5). The daily faecal excretion of radioactivity spanned a greater range during the dosing period, viz 42.3-57.4% and 31.5-43.3% of the cumulative dose in male and female rats respectively, but after Day 3 the ranges spanned less than 5%. These results therefore indicate that excretion of total drug-derived material in both urine and faeces after each daily ocular dose of [3H]tafluprost was largely complete by the time of administration of the next dose.

Metabolite Profiles

In vitro

[3H]tafluprost (2.5 and 25 μM) incubated with hepatocytes of rat, dog, monkey and human for 4 h was transformed up to 12 components. The predominant metabolite, which corresponded to the retention time of tafluprost acid on the radiochromatograms, was detected with 28%-45% (monkey), 38%-65% (rat), 50%-66% (human), and 96%-97% (dog) of injected radioactivity (Table 8). The next major peaks were observed in rat, monkey and human, whose retention times corresponded to 1,2,3,4-tetranor-AFP-172 and 1,2-dinor-AFP-172, however those were detected very little in dog.

In vivo

HPLC analysis of rat plasma, ocular and systemic tissues, and excreta showed that unchanged tafluprost was not present in any sample. Two major metabolite peaks were detected in plasma soon after dosing, namely M7 and M11 (Fig. 6), whose retention times corresponded
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...to 1,2,3,4-tetranor-AFP-172 and tafluprost acid respectively. The proportions of these two components declined with time until they were no longer detected at 24 and 8 h respectively, whereas the proportion of more-polar metabolites increased with time until they accounted for virtually the entire plasma radioactivity at 24 h. The proportion of M2 in plasma was higher in males than in females (Table 9), and declined with β-glucuronidase/sulphatase treatment.

In the ocular tissue extracts examined, M11 (tafluprost acid) was the only major radioactive metabolite in cornea and aqueous humour (Fig. 6) during 8 h post-dose. Although up to 12 minor metabolites were detected in these tissues, at no time did any one account for more than 5% of the sample radioactivity. M11 was also one of three major metabolites in the iris/ciliary body (Fig. 6). On the other hand, M11 was essentially absent from the liver and kidney, although it was present in lung tissue. As was the case with plasma, the abundance of the less-polar metabolites in the systemic tissues tended to decline gradually with time, while that of the polar metabolites increased. Neither tafluprost acid nor tafluprost was detected in the urine of the dosed rats, but the former was present in faeces, where it accounted for less than 1% of the dose. M1 was more abundant in bile, urine and faeces of males than in those of females (Table 9), and the portion of the M1 declined by treatment with β-glucuronidase/sulphatase.

**Mass Spectrometric Identification of Metabolites**

*In vitro*

Incubation of [³H]tafluprost at a concentration of 100 µM with human hepatocytes for 18 h resulted in the formation of about 10 metabolites (Fig. 7). LC-MS and MS/MS analysis showed by comparison of their mass spectra with those of synthetic reference compounds that these were tafluprost acid and two glucuronides thereof, 1,2-dinor-AFP-172 and two glucuronides thereof, and 1,2,3,4-tetranor-AFP-172 and one glucuronide thereof. (Table 10);
the location of the glucuronide moiety in each case could not be deduced from the available data. The two remaining metabolites appeared to be mono-hydroxylated derivatives of 1,2-dinor-AFP-172 and 1,2,3,4-tetranor-AFP-172, and the absence of the characteristic \( m/z \) 93 (phenoxy) ion and presence of ions at \( m/z \) 108 and 109 in both spectra indicated that the substituent was located in the phenyl ring.

*In vivo*

LC-MS/MS (ESI-) analysis of selected SPE fractions generated from a 0-6 h urine pool from male rats dosed intravenously with \([\text{\textsuperscript{3}}\text{H}]\text{tafluprost}\) using the technique of selected reaction monitoring (transition \( m/z \) 355 to \( m/z \) 179) confirmed the presence of 1,2,3,4-tetranor-AFP-172 as a minor metabolite. However, a [M-H]⁻ ion at \( m/z \) 371 and analysis of its product ion spectrum indicated that the most abundant metabolite corresponded to the same phenyl-ring hydroxylated derivative of 1,2,3,4-tetranor-AFP-172 observed in human hepatocytes *in vitro* (Fig. 8). A [M-H]⁻ ion at \( m/z \) 451 and neutral loss monitoring (80 amu; \( \text{SO}_3 \)) by LC-MS/MS demonstrated that a sulphate conjugate of the hydroxylated acid was also a major metabolite. Treatment of the rat urine sample with arylsulphatase (type V from *Patella vulgata*) confirmed the presence of a sulphate conjugate as a major metabolite in the rat. A more polar metabolite (Fig. 8) could not be identified.
DISCUSSION

The pharmacological efficacy of antiglaucoma agents applied topically to the eye is dependent on their ability to penetrate the cornea and access the anterior ocular tissues, particularly the iris/ciliary body, which is a presumed target of action. In the case of PGF$_{2\alpha}$ analogues, esterification of the prostaglandin $\alpha$-chain carboxyl group sufficiently increases the lipophilicity of the molecule for it to traverse the cornea and/or sclera, where it is enzymatically hydrolysed back to the active acid form for delivery to the ocular tissues and fluids (Madhu et al., 1998). In accord with such findings, the present studies have demonstrated that the novel 15,15-difluorinated PGF$_{2\alpha}$ ocular hypotensive agent tafluprost (Nakajima et al., 2003) acts as an efficient prodrug for tafluprost acid when applied topically to the eyes of albino rats, just as it does with related compounds such as latanoprost (Sjöquist et al., 1998). Hydrolysis of the ester to the pharmacologically active acid was both rapid and complete after ocular administration of tafluprost: The tritiated prodrug was not detected in eye tissues or plasma by radio-HPLC, whereas the acid form was effectively the only radioactive component in the cornea, aqueous humour and iris/ciliary body for at least 8 h post-dose.

At 5 minutes after a single ocular administration of $[^3]$Htafluprost to the rat, radioactivity concentrations in the ocular tissues followed the rank order: cornea>conjunctiva>iris/ciliary body>sclera~aqueous humour>choroid/retina~vitreous humour>>lens. Radioactivity levels in the cornea were highest of all studied ocular tissues during 12 h after administration, and were measurable for a further 12 h, suggesting that the cornea would act as a slow-release depot for the drug into the anterior tissues. A similar situation has been reported for a related prostanoid, latanoprost (Stjernschantz et al., 1995).
Absorption of the ocular dose of [3H]tafluprost into the systemic circulation was rapid and extensive in the rat. Maximum radioactivity levels in plasma and most eye tissues occurred within 15 min of administration, and the absorption ratio of radioactivity, which was calculated from bile and urine excretion ratios in bile duct-cannulated rats after the ocular dose (Table 7B), was about 75% in both sexes. Plasma radioactivity levels were considerably lower than ocular tissue levels, particularly those comprising the uveal tract, suggesting that a portion of the topically-applied drug penetrated into the posterior tissues by a local route (eg via the cornea and aqueous humour) rather than by a systemic one. The quantitative similarity in the radioactivity concentration-time curves of the iris/ciliary body and aqueous humour suggests rapid exchange of drug-derived material between the two tissues. Such exchange would assist in increasing uveoscleral outflow, which is considered to be the main mechanism for the ocular hypotensive effect achieved by tafluprost (Takagi et al., 2004), as it is for related PGs (Stjernschantz et al., 1995).

The systemic exposure of the treated rats to drug-derived material (as reflected in C_{max} and AUC_{0-24} values) tended to be greater in female rats than in males, although there was no evidence for gender-specific differences in ocular tissue radioactivity levels. The apparently greater exposure in females was reflected in the less extensive hepatobiliary route of excretion of radioactivity and correspondingly greater renal elimination exhibited by rats of this sex. Repeated ocular administration of [3H]tafluprost did not affect excretion profiles, and the data obtained indicated that by about Day 5 each day’s dose had been largely excreted before the next one was administered, ie accumulation of drug-related material during such a dosing regimen is unlikely to be extensive. AUC_{0-24} values for plasma radioactivity concentrations after 7, 14 and 21 daily ocular doses were 2-3-fold higher than after a single dose for both sexes, and so indicate that steady-state conditions had been attained before the end of the 21-day dosing regimen.
Whereas tafluprost-derived material persisted in the eye for at least 24 h after ocular administration, there was no accumulation in the systemic tissues and organs during the same period. At 24 h after the last of 21 daily doses, systemic tissue radioactivity levels were all about 4-5 times greater than after a single dose, the only notable exceptions being thyroid and fat which were up to 9-fold greater. Highest concentrations of radioactivity at early sacrifice times occurred in the Harderian gland (reflecting its proximity to the site of administration) and kidney, whereas at later times those in the liver and gastrointestinal tract predominated. Generally, therefore, tissue levels were only greater than plasma levels in the main organs of drug absorption and elimination. The tissue data also showed that tafluprost and/or its metabolites did not readily cross the blood/brain barrier or associate extensively with the erythrocytes. The presence of radioactivity in the reproductive organs of male and females rats shows that these organs would be exposed to the drug and/or its metabolites during reproductive toxicity studies, and radioactivity levels in bone marrow provides evidence of its exposure during a micronucleus test. The placental transfer of radioactivity after the ocular dose of [\(^{3}\)H]tafluprost was less than 0.1% dose per foetus, indicating that the placenta was an effective barrier in the uptake of the drug and/or its metabolites into the foetus. Radioactivity levels in milk tended to be greater than those in plasma, but AUC\(_{0-96}\) was similar for both fluids, indicating that tafluprost and/or its metabolites readily diffused into milk, which may be expected in view of the non-extensive binding of radioactivity to rat plasma proteins \textit{ex vivo} (<80%).

Incubation of [\(^{3}\)H]tafluprost with human hepatocytes indicated that the acid of tafluprost and its 1,2-dinor and 1,2,3,4-tetranor analogues were the main \textit{in vitro} biotransformation products of this fluoroprostanoid. Such \(\beta\)-oxidation degradations of the \(\alpha\)-chain are well-documented metabolic pathways for related prostaglandins such as 15-deoxy PGF\(_{2\alpha}\) (Higaki et al., 1995), latanoprost (Sjoquist et al., 1998) etc, as they are for long-chain fatty acids in general.
(Johnson et al., 1972). Two minor in vitro metabolites also identified by mass spectrometry were phenyl ring-hydroxylated derivatives of the above side-chain shortened acids. Tafluprost acid was also the primary in vivo systemic metabolite of tafluprost, being the main radiocomponent in plasma shortly after ocular administration, but this was rapidly superseded by a metabolite whose chromatographic mobility indicated it was the 1,2,3,4-tetranor acid. The latter compound was also the main in vivo metabolite in well-perfused tissues such as liver and lung. However, tafluprost acid was the only noteworthy metabolite in the anterior ocular tissues for at least 8 h post-administration. Presumably, some oxidation of the cyclopentyl carbon bearing the tritium radiolabel must also have occurred, as formation of tritiated water was quite extensive in vivo. No other metabolite on the ω-chain was detected in this study. Tafluprost is 15,15-difluorinated and has no hydroxyl group on the position. This chemical structure would prevent extensive transformations of the ω-chain by a prostaglandin dehydrogenase (15-PGDH), which are shown in many prostaglandins including latanoprost (Sjöquist et al., 1999), and therefore this might keep the active metabolite, tafluprost acid, remaining more and prolong the pharmacological efficacy in the eyes.

There were gender differences in the biotransformation of tafluprost in the rat after the ocular doses (Table 9). M1 in bile, urine, and faeces and M2 in plasma, which were higher in male than in female, appeared to contain conjugates from the incubation results with β-glucuronidase/sulphatase. Thus, the gender differences in exposure and excretion of radioactivity as mentioned previously could reflect the conjugation diversity. The urine metabolite profile of rat after intravenous administration consisted principally of the phenyl ring-hydroxylated tetranor acid and its sulphate conjugate, together with the tetranor acid itself. It has been suggested (without being demonstrated) that cytochrome P-450 catalysed aromatic hydroxylation of latanoprost might be a minor metabolic pathway in the rabbit cornea (Sjöquist et al., 1998).
In conclusion, [³H]tafluprost was very rapidly absorbed into the eye and systemic circulation after ocular administration to rats. Hydrolysis of tafluprost to the pharmacologically active acid metabolite, tafluprost acid, was fast and no parent tafluprost was detected in plasma, tissues or excreta. Proposed metabolic pathway of tafluprost in rat and other species is shown in Fig. 9. Following ocular dose of [³H]tafluprost, the excretion of radioactivity into the faeces was predominant in male rats as the biliary excretion rate was high, whereas the difference was not observed in females. This study has shown the entire picture of disposition and metabolism of tafluprost in a primary preclinical species after ocular administration.
ACKNOWLEDGEMENTS

We acknowledge the contribution of Mr. Brian John and Dr. Ian Midgley of Huntingdon Life Sciences Ltd. to this work. We also thank Dr. Padma Bezwada of Santen Inc. and Dr. Pertti Pellinen of Santen Oy for their kind assistance in the preparation of this manuscript.
REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1
Chemical structures of [3H]tafluprost and synthetic reference compounds.

Figure 2
Concentrations of total radioactivity in plasma after single or 21 daily ocular doses of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to rats; n = 6.

Figure 3
Concentrations of total radioactivity (± SD) in aqueous humour, choroid/retina, conjunctiva, cornea, iris/ciliary body and lens after single ocular doses of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to rats; n = 3 pairs of rats.

Figure 4
Concentrations of total radioactivity (± SD) in milk, plasma, and whole-blood after a single ocular dose of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to lactating maternal rats; n = 4.

Figure 5
Cumulative rates of excretion of radioactivity (± SD) in urine and faeces during 21 daily ocular doses of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to rats; n = 4.

Figure 6
HPLC radiohistograms (Conditions I) of extracts of plasma at 15 min, cornea at 2 h, aqueous humor at 2 h, and iris/ciliary body at 2 h after a single ocular administration of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to male rats.

Figure 7
HPLC radiochromatogram (Conditions II) of an incubation mixture of [3H]tafluprost (100 µM) in human hepatocytes after 18 hours. See Table 10 for peak assignment.
Figure 8
HPLC radiochromatograms (Conditions II) of 0-6 h male rat urine after single 100 µg/kg intravenous doses of [3H]tafluprost.

Figure 9
Proposed biotransformation pathways of tafluprost in vivo (rat) and in vitro (rat, dog, monkey, and human).
TABLE 1

Radioactivity pharmacokinetic parameters in plasma following a single ocular administration of \([ ^3 H ] \)tafluprost ophthalmic solution (0.05%, 0.005%, or 0.0005%, w/v; 5 µL to both eyes) to male rats

<table>
<thead>
<tr>
<th>Dose concentration</th>
<th>( C_{\text{max}} ) (ng eq./g)</th>
<th>( T_{\text{max}} ) (h)</th>
<th>( t_{1/2} ) (h)</th>
<th>( \text{AUC}_{0-24} ) (ng eq.h/g)</th>
<th>Tritium exchange (% dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05%</td>
<td>4.08</td>
<td>1</td>
<td>25(^a)</td>
<td>31.1</td>
<td>2.6</td>
</tr>
<tr>
<td>0.005%</td>
<td>0.355</td>
<td>1</td>
<td>57(^a)</td>
<td>3.29</td>
<td>2.9</td>
</tr>
<tr>
<td>0.0005%</td>
<td>0.043</td>
<td>1</td>
<td>24(^a)</td>
<td>0.346</td>
<td>2.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose concentration</th>
<th>Achieved dose level (µg tafluprost/eye)</th>
<th>Dose level Ratio(^*)</th>
<th>( C_{\text{max}} ) Ratio(^*)</th>
<th>( \text{AUC}_{0-24} ) Ratio(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05%</td>
<td>2.28</td>
<td>92</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td>0.005%</td>
<td>0.248</td>
<td>10</td>
<td>8.3</td>
<td>9.5</td>
</tr>
<tr>
<td>0.0005%</td>
<td>0.0247</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Estimated value (linear regression analysis of the terminal decline phase did not meet one or more of the acceptance criteria)

* Each value shows the ratio of the achieved dose level, \( C_{\text{max}} \), or \( \text{AUC} \) in 0.05% or 0.005% to that in dose of 0.0005%. 

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TABLE 2

Total radioactivity pharmacokinetic parameters in ocular tissues following a single ocular administration of [3H]tafluprost ophthalmic solution (0.05%, 0.005%, or 0.0005%, w/v; 5 µL to both eyes) to male rats

<table>
<thead>
<tr>
<th>Tissue/Fluid</th>
<th>Ophthalmic solution concentration</th>
<th>0.05%</th>
<th>0.005%</th>
<th>0.0005%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous humour</td>
<td></td>
<td>488 (1)</td>
<td>115 (1)</td>
<td>10.5 (1)</td>
</tr>
<tr>
<td>Choroid/retina</td>
<td></td>
<td>68.5 (1)</td>
<td>7.64 (1)</td>
<td>1.33 (1)</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td></td>
<td>99.7 (1)</td>
<td>24.6 (1)</td>
<td>3.10 (1)</td>
</tr>
<tr>
<td>Cornea</td>
<td></td>
<td>2130 (1)</td>
<td>572 (1)</td>
<td>64.0 (1)</td>
</tr>
<tr>
<td>Iris/ciliary body</td>
<td></td>
<td>971 (1)</td>
<td>173 (1)</td>
<td>33.1 (1)</td>
</tr>
<tr>
<td>Lens</td>
<td></td>
<td>36.0 (2)</td>
<td>8.29 (1)</td>
<td>0.965 (1)</td>
</tr>
<tr>
<td>Sclera</td>
<td></td>
<td>114 (1)</td>
<td>26.5 (1)</td>
<td>3.61 (1)</td>
</tr>
<tr>
<td>Vitreous humour</td>
<td></td>
<td>104 (1)</td>
<td>18.3 (1)</td>
<td>1.73 (1)</td>
</tr>
</tbody>
</table>

Results are presented as C<sub>max</sub> (ng eq./g) and T<sub>max</sub> (h) respectively, and represent the mean of 2 animals per time point. Sacrifice times were 1, 2, 4, 8, 12 and 24 hours.
TABLE 3

Radioactivity pharmacokinetic parameters in plasma following single, 7, 14 and 21 daily ocular doses of [³H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to male and female rats

<table>
<thead>
<tr>
<th>Total radioactivity</th>
<th>Number of daily doses</th>
<th>Cₘₐₓ (ng eq./g)</th>
<th>Tₘₐₓ (h)*</th>
<th>AUC₀₋₂₄ (ng eq.h/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>1</td>
<td>1.73 ± 0.27</td>
<td>1.91 ± 0.15</td>
<td>0.083</td>
<td>0.083</td>
</tr>
<tr>
<td>7</td>
<td>0.723 ± 0.080</td>
<td>0.969 ± 0.143</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>0.793 ± 0.039</td>
<td>0.992 ± 0.068</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>21</td>
<td>1.15 ± 0.19</td>
<td>1.67 ± 0.23</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-volatile radioactivity</th>
<th>Number of daily doses</th>
<th>Cₘₐₓ (ng eq./g)</th>
<th>Tₘₐₓ (h)*</th>
<th>AUC₀₋₂₄ (ng eq.h/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>1</td>
<td>1.80**</td>
<td>2.00**</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>7</td>
<td>0.397 ± 0.028</td>
<td>0.500 ± 0.133</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>0.380 ± 0.044</td>
<td>0.574 ± 0.046</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>21</td>
<td>0.684 ± 0.146</td>
<td>1.13 ± 0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Results represent the mean (± SD) of 6 animals per sex per time point.

It was not possible to calculate accurately the terminal rate constant (and therefore the derived parameters t½ or AUC), due mainly to the occurrence of a secondary peak and the relatively short measurement period relative to the estimated half-life.

* In each case, Tₘₐₓ was the first sampling/sacrifice time
** Samples were pooled from 6 animals per sex per time point

a Sacrifice times were 5, 15, and 30 minutes, 1, 2, 4, 6, 8, 12 and 24 hours
b Sacrifice times were 30 minutes, 1, 2, 8 and 24 hours
c Sacrifice times were 15 and 30 minutes, 1, 2, 4, 6, 8, 12 and 24 hours

<table>
<thead>
<tr>
<th>Tritium exchange</th>
<th>Number of daily doses</th>
<th>Tritium exchange (% dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>9.8</td>
<td>10.8</td>
</tr>
<tr>
<td>14</td>
<td>11.3</td>
<td>11.2</td>
</tr>
<tr>
<td>21</td>
<td>15.1</td>
<td>14.9</td>
</tr>
</tbody>
</table>
Accumulation ratio

<table>
<thead>
<tr>
<th>Number of daily doses</th>
<th>Accumulation ratio</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total radioactivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2.7</td>
<td>2.6</td>
<td>1.1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>3.3</td>
<td>3.0</td>
<td>1.7</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>3.5</td>
<td>3.1</td>
<td>1.4</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 4

Ocular tissue total radioactivity pharmacokinetic data following single and 7, 14 and 21 daily ocular doses of [3H]tafluprost ophthalmic solution (0.005\%, w/v; 5 µL to both eyes) to male and female rats

<table>
<thead>
<tr>
<th>Tissue/fluid</th>
<th>Single dose(^a)</th>
<th>7 Daily doses(^b)</th>
<th>14 Daily doses(^b)</th>
<th>21 Daily doses(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Aqueous humour</td>
<td>369 (0.5)</td>
<td>477 (0.25)</td>
<td>215 (0.5)</td>
<td>218 (0.5)</td>
</tr>
<tr>
<td>Choroid/retina</td>
<td>83.2 (0.083)</td>
<td>91.4 (0.083)</td>
<td>15.7 (0.5)</td>
<td>20.2 (0.5)</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>624 (0.083)</td>
<td>973 (0.083)</td>
<td>10.5 (0.5)</td>
<td>13.0 (0.5)</td>
</tr>
<tr>
<td>Cornea</td>
<td>3490 (0.083)</td>
<td>4210 (0.083)</td>
<td>1540 (0.5)</td>
<td>1490 (0.5)</td>
</tr>
<tr>
<td>Iris/ciliary body</td>
<td>445 (0.083)</td>
<td>573 (0.5)</td>
<td>305 (0.5)</td>
<td>273 (0.5)</td>
</tr>
<tr>
<td>Lens</td>
<td>8.35 (1)</td>
<td>8.06 (2)</td>
<td>7.86 (1)</td>
<td>7.47 (2)</td>
</tr>
<tr>
<td>Sclera</td>
<td>263 (0.083)</td>
<td>330 (0.083)</td>
<td>136 (0.5)</td>
<td>51.0 (0.5)</td>
</tr>
<tr>
<td>Vitreous humour</td>
<td>55.4 (0.25)</td>
<td>55.1 (0.083)</td>
<td>10.3 (0.5)</td>
<td>11.6 (0.5)</td>
</tr>
<tr>
<td>Plasma</td>
<td>1.73 (0.083)</td>
<td>1.91 (0.083)</td>
<td>0.723 (0.5)</td>
<td>0.969 (0.5)</td>
</tr>
</tbody>
</table>

Results are presented as C\(_{\text{max}}\) (ng eq./g) and T\(_{\text{max}}\) (h) respectively, and represent the mean of 6 animals per sex per time point.

\(^a\) Sacrifice times were 5, 15, and 30 minutes, 1, 2, 4, 6, 8, 12 and 24 hours
\(^b\) Sacrifice times were 30 minutes, 1, 2, 8 and 24 hours
\(^c\) Sacrifice times were 15 and 30 minutes, 1, 2, 4, 6, 8, 12 and 24 hours
TABLE 5

Concentrations of total radioactivity in systemic tissues at 30 minutes and 24 hours after single and 21 daily ocular doses of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to male and female rats

<table>
<thead>
<tr>
<th>Tissue/organ</th>
<th>Single dose</th>
<th></th>
<th>21 daily doses</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male 30 min</td>
<td>Male 24 h</td>
<td>Female 30 min</td>
<td>Female 24 h</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.488</td>
<td>0.101</td>
<td>0.550</td>
<td>0.118</td>
</tr>
<tr>
<td>Whole-blood</td>
<td>0.313</td>
<td>0.092</td>
<td>0.364</td>
<td>0.107</td>
</tr>
<tr>
<td>Aorta</td>
<td>0.254</td>
<td>0.070</td>
<td>0.343</td>
<td>0.134</td>
</tr>
<tr>
<td>Vena cava</td>
<td>0.308</td>
<td>0.055</td>
<td>0.408</td>
<td>0.120</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.088</td>
<td>0.086</td>
<td>0.119</td>
<td>0.095</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.081</td>
<td>0.085</td>
<td>0.128</td>
<td>0.103</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.113</td>
<td>0.073</td>
<td>0.174</td>
<td>0.090</td>
</tr>
<tr>
<td>Heart</td>
<td>0.238</td>
<td>0.076</td>
<td>0.612</td>
<td>0.094</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.06</td>
<td>0.094</td>
<td>5.33</td>
<td>0.113</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>2.01</td>
<td>0.080</td>
<td>1.19</td>
<td>0.085</td>
</tr>
<tr>
<td>Liver</td>
<td>13.4</td>
<td>0.141</td>
<td>10.4</td>
<td>0.124</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.337</td>
<td>0.085</td>
<td>0.329</td>
<td>0.094</td>
</tr>
<tr>
<td>Trachea</td>
<td>0.329</td>
<td>0.075</td>
<td>0.312</td>
<td>0.077</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.270</td>
<td>0.082</td>
<td>0.278</td>
<td>0.086</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.123</td>
<td>0.087</td>
<td>0.165</td>
<td>0.096</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>0.166</td>
<td>0.082</td>
<td>0.191</td>
<td>0.086</td>
</tr>
<tr>
<td>Harderian gland</td>
<td>4.74</td>
<td>0.102</td>
<td>4.87</td>
<td>0.136</td>
</tr>
<tr>
<td>Lacrimal gland</td>
<td>0.492</td>
<td>0.091</td>
<td>0.253</td>
<td>0.104</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0.151</td>
<td>0.084</td>
<td>0.161</td>
<td>0.101</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>0.198</td>
<td>ND</td>
<td>0.259</td>
<td>0.078</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>0.165</td>
<td>0.080</td>
<td>0.192</td>
<td>0.091</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.105</td>
<td>0.084</td>
<td>0.151</td>
<td>0.102</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.364</td>
<td>0.064</td>
<td>0.265</td>
<td>0.067</td>
</tr>
<tr>
<td>Prostate or Ovaries</td>
<td>0.636</td>
<td>0.085</td>
<td>0.441</td>
<td>0.088</td>
</tr>
<tr>
<td>Testes or Uterus</td>
<td>0.123</td>
<td>0.090</td>
<td>0.518</td>
<td>0.108</td>
</tr>
<tr>
<td>Bone</td>
<td>0.049</td>
<td>0.015</td>
<td>0.062</td>
<td>0.022</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0.096</td>
<td>0.062</td>
<td>0.150</td>
<td>0.106</td>
</tr>
<tr>
<td>Fat</td>
<td>0.312</td>
<td>0.026</td>
<td>0.096</td>
<td>0.017</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.114</td>
<td>0.086</td>
<td>0.143</td>
<td>0.099</td>
</tr>
<tr>
<td>Skin</td>
<td>0.161</td>
<td>0.087</td>
<td>0.198</td>
<td>0.098</td>
</tr>
<tr>
<td>Stomach wall</td>
<td>3.03</td>
<td>0.274</td>
<td>4.23</td>
<td>0.279</td>
</tr>
<tr>
<td>Duodenum wall</td>
<td>5.55</td>
<td>0.295</td>
<td>8.96</td>
<td>0.201</td>
</tr>
<tr>
<td>Ileum wall</td>
<td>5.68</td>
<td>0.335</td>
<td>5.13</td>
<td>0.239</td>
</tr>
<tr>
<td>Large intestine wall</td>
<td>1.92</td>
<td>0.670</td>
<td>1.95</td>
<td>0.694</td>
</tr>
</tbody>
</table>

Results are expressed as ng eq./g and represent the mean of six rats per time point.

ND No radioactivity detected in tissue for at least 50% of the animals in a group
### TABLE 6

Maternal and foetal tissue total radioactivity concentration data following single ocular doses of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to pregnant rats on Days 12 and 18 of gestation

<table>
<thead>
<tr>
<th>Tissue/organ</th>
<th>Day 12</th>
<th>Day 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.799 (0.25)</td>
<td>0.887 (0.25)</td>
</tr>
<tr>
<td>Whole-blood</td>
<td>0.524 (0.25)</td>
<td>0.588 (0.25)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.139 (0.25)</td>
<td>0.140 (0.25)</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.131 (0.25)</td>
<td>0.103 (0.25)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.750 (0.25)</td>
<td>0.743 (0.25)</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.46 (0.25)</td>
<td>9.10 (0.25)</td>
</tr>
<tr>
<td>Liver</td>
<td>8.66 (0.25)</td>
<td>7.56 (0.25)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.556 (0.25)</td>
<td>0.623 (0.25)</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>0.098 (4)</td>
<td>0.117 (4)</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>0.267 (0.25)</td>
<td>0.342 (0.25)</td>
</tr>
<tr>
<td>Ovary</td>
<td>0.656 (0.25)</td>
<td>1.40 (0.25)</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.233 (0.25)</td>
<td>0.168 (0.25)</td>
</tr>
<tr>
<td>Uterus</td>
<td>0.351 (0.25)</td>
<td>0.309 (0.25)</td>
</tr>
<tr>
<td>Foetus</td>
<td>0.089 (4)</td>
<td>–</td>
</tr>
<tr>
<td>Foetal blood</td>
<td>–</td>
<td>0.116 (1)</td>
</tr>
<tr>
<td>Foetal brain</td>
<td>–</td>
<td>0.113 (4)</td>
</tr>
<tr>
<td>Foetal kidney</td>
<td>–</td>
<td>0.114 (1)</td>
</tr>
<tr>
<td>Foetal liver</td>
<td>–</td>
<td>0.197 (1)</td>
</tr>
<tr>
<td>Foetal lung</td>
<td>–</td>
<td>0.117 (4)</td>
</tr>
<tr>
<td>Foetal carcassb</td>
<td>–</td>
<td>0.114 (4)</td>
</tr>
</tbody>
</table>

Results are presented as $C_{\text{max}}$ (ng eq./g) and $T_{\text{max}}$ (h) respectively, and represent the mean of 4 animals per sex per time point. Sacrifice times were 0.25, 1, 4, 12 and 24 hours.

* Intact foetuses (Day 12 rats only)
* Carcass remaining after removal of the specified foetal organs (Day 18 rats only)
### TABLE 7

(A) Excretion of radioactivity during 7 days after single and the last of 21 daily ocular doses of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to intact male rats

<table>
<thead>
<tr>
<th>Biological sample (Collection period)</th>
<th>Single dose</th>
<th>21 daily doses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>(0-24 h)</td>
<td>(0-168 h)</td>
</tr>
<tr>
<td>Urine</td>
<td>33.1</td>
<td>44.9</td>
</tr>
<tr>
<td>(0-168 h)</td>
<td>35.6</td>
<td>48.3</td>
</tr>
<tr>
<td>Faeces</td>
<td>48.6</td>
<td>32.7</td>
</tr>
<tr>
<td>(0-168 h)</td>
<td>58.7</td>
<td>46.4</td>
</tr>
<tr>
<td>Expired air</td>
<td>0.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Total excretion(^a) (0-168 h)</td>
<td>95.8</td>
<td>96.0</td>
</tr>
<tr>
<td>Carcass (168 h)</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Total recovery (168 h)</td>
<td>97.9</td>
<td>98.2</td>
</tr>
</tbody>
</table>

Results are expressed as % total radioactive dose and represent the mean of 4 animals per sex.

NM  Not measured

(B) Excretion of radioactivity during 48 hours after single ocular doses of [3H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to rats with cannulated bile ducts

<table>
<thead>
<tr>
<th>Biological sample (Collection period)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile (0-6 h)</td>
<td>42.4</td>
<td>26.1</td>
</tr>
<tr>
<td>(0-48 h)</td>
<td>50.2</td>
<td>32.8</td>
</tr>
<tr>
<td>Urine (0-6 h)</td>
<td>17.2</td>
<td>19.8</td>
</tr>
<tr>
<td>(0-48 h)</td>
<td>25.3</td>
<td>41.2</td>
</tr>
<tr>
<td>Faeces (0-48 h)</td>
<td>3.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Total excretion(^a) (0-48 h)</td>
<td>79.3</td>
<td>79.8</td>
</tr>
<tr>
<td>Liver (48 h)</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>GIT (48 h)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Carcass (48 h)</td>
<td>4.8</td>
<td>6.5</td>
</tr>
<tr>
<td>Total retention (48 h)</td>
<td>5.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Total recovery (0-48 h)</td>
<td>89.4</td>
<td>88.9</td>
</tr>
</tbody>
</table>

Results are expressed as % radioactive dose and represent the mean of 4 animals per sex.

GIT  Gastrointestinal tract

\(^a\) Total excretion results includes cage washings
TABLE 8

Proportions of major metabolites in rat, dog, monkey and human hepatocytes after 4 hour incubation of [3H]tafluprost (2.5 and 25 μM)

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Rat</th>
<th>Dog</th>
<th>Monkey</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 μM</td>
<td>25 μM</td>
<td>2.5 μM</td>
<td>25 μM</td>
</tr>
<tr>
<td>TetrnorAFP-172</td>
<td>0.3</td>
<td>0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hydroxylated 1,2-dinor-AFP-172</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1,2,3,4-tetrnor-AFP-172</td>
<td>35.8</td>
<td>15.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1,2-dinor-AFP-172</td>
<td>12.1</td>
<td>7.4</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>AFP-172 glucuronic acid</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tafluprost acid</td>
<td>37.5</td>
<td>65.1</td>
<td>96.3</td>
<td>97.5</td>
</tr>
<tr>
<td>Tafluprost</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Results are expressed as % injected radioactivity.

ND  Not detected as a radioactive component on radiochromatogram
# TABLE 9

Proportions of metabolites in pooled plasma, bile, urine and faeces after single ocular doses of $[^3]$H]tafluprost ophthalmic solution (0.005%, w/v; 5 µL to both eyes) to rats

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Plasma $^a$ (1 h post-dose)</th>
<th>Bile $^b$ (0-24 h)</th>
<th>Urine $^c$ (0-24 h)</th>
<th>Faeces $^c$ (0-48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>M1</td>
<td>9</td>
<td>12</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>M2</td>
<td>23</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>M3</td>
<td>1</td>
<td>ND</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>M4 (hydroxylated 1,2-dinor-AFP-172)</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>M5</td>
<td>1</td>
<td>ND</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>M6</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>M7 (1,2,3,4-tetranor-AFP-172)</td>
<td>44</td>
<td>52</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>M8</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>M9 (1,2-dinor-AFP-172)</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M10</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M11 (Tafluprost acid)</td>
<td>6</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M12 (Tafluprost)</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

ND  Not detected as a radioactive component on radiochromatogram

$^a$% of sample radioactivity; samples were obtained from intact rats

$^b$% of radioactivity dose; samples were obtained from bile duct-cannulated rats

$^c$% of radioactivity dose; samples were obtained from intact rats
### TABLE 10

**LC-MS/MS data (HPLC conditions II; negative ESI) for metabolites of [3H]tafluprost (100 µM) after 18 hours incubation in vitro with human hepatocytes (see Fig. 7).**

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Retention time (min)</th>
<th>[M-H]⁻ (m/z)</th>
<th>Key ions (m/z)</th>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.1</td>
<td>371</td>
<td>179, 159, 109, 108</td>
<td><img src="image1" alt="Structure" /> (Phenyl-ring) hydroxylated 1,2,3,4-tetranor-AFP-172</td>
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<tr>
<td>2</td>
<td>32.7</td>
<td>531</td>
<td>355, 317, 249, 193</td>
<td>Glucuronide of 1,2,3,4-tetranor-AFP-172</td>
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<tr>
<td>3</td>
<td>34.2</td>
<td>397</td>
<td>289, 269, 251, 109, 108</td>
<td><img src="image2" alt="Structure" /> (Phenyl-ring) hydroxylated 1,2-dinor-AFP-172</td>
</tr>
<tr>
<td>4</td>
<td>39.3</td>
<td>557</td>
<td>539, 193, 175</td>
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</tr>
<tr>
<td>5</td>
<td>39.4</td>
<td>355</td>
<td>179, 159, 93</td>
<td>1,2,3,4-tetranor-AFP-172</td>
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<tr>
<td>6</td>
<td>42.2</td>
<td>557</td>
<td>539, 193, 175</td>
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<tr>
<td>7</td>
<td>44.8</td>
<td>585</td>
<td>409, 193, 175, 113</td>
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<tr>
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<td>9</td>
<td>47.2</td>
<td>585</td>
<td>409, 175, 133, 113</td>
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<tr>
<td>10</td>
<td>51.8</td>
<td>409</td>
<td>295, 277, 257, 233, 213, 187, 93</td>
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<tr>
<td>11</td>
<td>63.7</td>
<td>451</td>
<td>NM</td>
<td>Tafluprost</td>
</tr>
</tbody>
</table>

NM Not measured (the ester tafluprost did not ionise under the LC-MS (ESI-) conditions used)
Figure 1

[3H]Tafluprost (3H indicates the position of the tritium radiolabel)

Tafluprost acid (AFP-172)

1,2-Dinor-AFP-172

1,2,3,4-Tetranor-AFP-172

1,2,3,4-Tetranor-AFP-172 lactone
Figure 6

[Graph showing radioactivity over time for different samples including plasma, cornea, aqueous humor, and iris/ciliary body.]
Figure 7

![Graph showing radioactivity over time](image-url)
Figure 8

[Graph showing radioactivity levels over time, labeled with peaks for Hydroxylated 1,2,3,4-tetranor-AFP-172 sulphate, Hydroxylated 1,2,3,4-tetranor-AFP-172, Unknown, and 1,2,3,4-tetranor-AFP-172.]