http://informahealthcare.com/xen
ISSN: 0049-8254 (print), 1366-5928 (electronic)

Xenobiotica

Xenobiotica, 2014; 44(6): 498–510
 DOI: 10.3109/00498254.2013.866298

informa
healthcare

RESEARCH ARTICLE

High-resolution mass spectrometric investigation of the phase I and II metabolites of finasteride in pig plasma, urine and bile

Anna Lundahl[1]*, Annica Tevell Åberg[2,3], Ulf Bondesson[2,3], Hans Lennernäs[1], and Mikael Hedeland[2,3]

[1]Department of Pharmacy, Uppsala University, Uppsala, Sweden, [2]Department of Chemistry, Environment, and Feed Hygiene, National Veterinary Institute (SVA), Uppsala, Sweden, and [3]Division of Analytical Pharmaceutical Chemistry, Uppsala University, Sweden

Abstract

1. The metabolite profile of the 5α-reductase type II inhibitor finasteride has been studied in pig plasma, urine and bile using high-resolution mass spectrometry. The porcine biotransformation products were compared to those formed by human liver microsomes and to literature data of recently identified human *in vivo* metabolites. The objective of this study was to gain further evidence for the validity of using pigs for advanced, invasive drug–drug interaction studies that are not possible to perform in humans.
2. The use of high-resolution mass spectrometry with accurate mass measurements enabled identification of the metabolites by calculation of their elemental compositions as well as their fragmentation patterns.
3. There was an excellent match between the porcine and human metabolic profiles, corroborating the pig as a model of human drug metabolism. The glucuronides of the two recently described human hydroxylated metabolites MX and MY and the carboxylated metabolite M3 were identified as the major biotransformation products of finasteride in pig urine and bile.
4. Furthermore, the CYP enzymes involved in the formation of the hydroxylated metabolites were characterized. Human recombinant CYP3A4 could produce the two major hydroxylated metabolites MX and MY, whereas human recombinant CYP2D6 formed MY only.

Keywords

Bile, CYP3A4, finasteride, metabolism, pig

History

Received 13 September 2013
Revised 11 November 2013
Accepted 12 November 2013
Published online 9 December 2013

Introduction

Finasteride, [N-(2-methyl-2-propyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide] (Figure 1), is a synthetic 4-azasteroid and an inhibitor of 5α-reductase type II (Faller et al., 1993). It is used in the oral treatment of benign prostatic hyperplasia and androgenetic alopecia (male pattern hair loss; Drake et al., 1999; McConnell et al., 1992).

The metabolism of finasteride has been studied in humans, rats and dogs (Carlin et al., 1987, 1992, 1997; Ishii et al., 1992, 1994a,b). After oral administration of [^{14}C]-finasteride to humans, a majority of the dose was metabolized, and 39 and 57% of the dose was identified as metabolites in urine and feces, respectively. These human data indicated that biliary excretion was the major route of elimination for the finasteride metabolites and this was supported by rat data (Ishii et al., 1992). In humans, the main route of metabolism was cytochrome P450 (CYP) 3A4 mediated sequential transformation from ω-OH finasteride (M1, Figure 1) via finasteride ω-al (M2) to finasteride-ω-oic-acid (M3; Huskey et al., 1995). The 6α-OH finasteride (M4) was identified as a minor metabolite in humans (Carlin et al., 1988, 1992; Huskey et al., 1995). M1 and M3 were the major metabolites detected in plasma and urine, respectively, while the metabolites in feces were not structurally determined (Carlin et al., 1992).

In a human clinical drug–drug interaction study with finasteride, bile was collected with a single-pass perfusion technique (Bergman et al., 2006; Lundahl et al., 2009a,b). M3 was found to be the major metabolite detected in urine and low amounts of unchanged finasteride was excreted into bile and urine (<1%; Lundahl et al., 2009a). Unexpectedly, M1 was not present in quantifiable concentrations in that study. Instead, two other OH metabolites – denoted MX and MY – were identified in bile and urine (Lundahl et al., 2009b). Intact MY glucuronide was found in human bile and urine and we had indirect evidence (β-glucuronidase hydrolysis) to support that MX was also glucuronidated (for structures of finasteride and the metabolites; Figure 1). The conclusions from the previous study were that MX probably was a ring-closed lactol form of M1 and it could not be excluded that MY was identical to M4.

Recently we presented a drug–drug interaction study in an advanced pig model (Bergman et al., 2009;

*Present address: R&D Innovative Medicines, AstraZeneca R&D Mölndal, Mölndal, Sweden.

Address for correspondence: Mikael Hedeland, Department of Chemistry, Environment, and Feed Hygiene, National Veterinary Institute (SVA), 75189 Uppsala, Sweden. Tel: +46 18 674000. E-mail: mikael.hedeland@sva.se

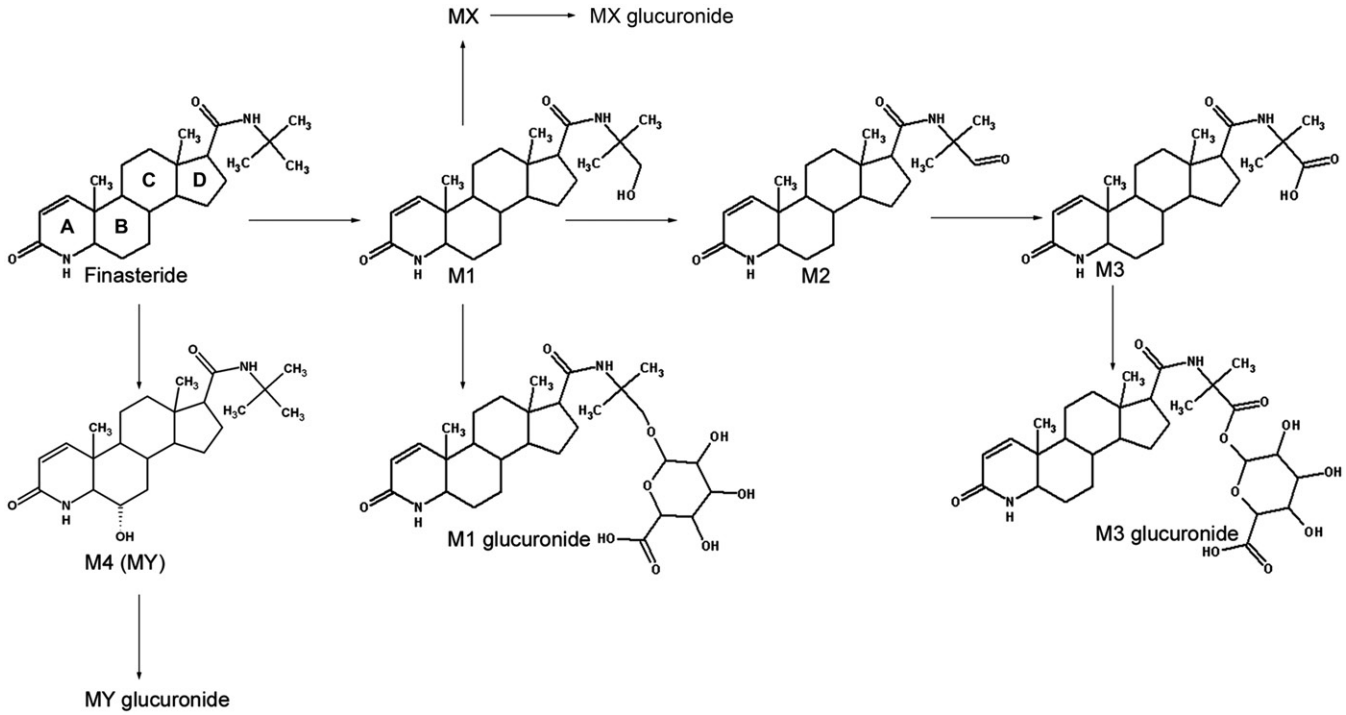


Figure 1. The chemical structures of finasteride and its metabolites.

Petri et al., 2006; Sjögren et al., 2012; Thörn et al., 2012) that enabled sampling directly from the biliary duct in which we followed the concentrations of finasteride, M1 and M3 over time (Lundahl et al., 2011). The pharmacokinetic profiles of these compounds were similar to those previously described in humans. However, a full comparison between the metabolite profiles in pigs and humans was not performed.

In this study, we present for the first time the metabolite profile of finasteride in porcine bile, urine and plasma samples collected in an advanced pig *in vivo* model. The metabolites found were compared to those previously described in humans in order to demonstrate the validity of using the pig model in drug metabolism studies (Lundahl et al., 2011). High-resolution mass spectrometry (Q-TOF) was used for structural elucidation of the phase I metabolites and their glucuronides. The human recombinant CYP enzymes that were involved in the formation of the hydroxylated metabolites were also investigated.

Materials and methods

Materials

Pooled HLM and human recombinant CYP Supersomes were purchased from BD Biosciences (Franklin Lakes, NJ). The metabolites, M1 and M3, were purchased from Toronto Research Chemicals (North York, Canada). NADPH (reduced tetrasodium salt) and finasteride were purchased from Sigma-Aldrich (St. Louis, MO). β-Glucuronidase (from *Escherichia coli* K12) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Acetonitrile, formic acid and methanol were purchased from Merck (Darmstadt, Germany). The water was purified using a MilliQ water purification system (Millipore, Bedford, MA) and all other chemicals used were of analytical grade or better and used without further purification.

In vivo samples

In vivo samples from pigs were selected from a previously reported pig study (Lundahl et al., 2011). The dose of finasteride was 0.8 mg/kg (directly to jejunum) and 0.2 mg/kg (iv) and samples were collected as described in Lundahl et al. (2011). The study was approved by the local ethics committee for animal research, Uppsala, Sweden (registration number C257/6).

Microsomal, HLM and recombinant CYP enzymes incubations

Finasteride (200 μM) was incubated with HLM (1 mg/mL) and NADPH (1 mM) in potassium phosphate buffer (0.1 M, pH 7.4). The samples were kept at 37 °C, in a shaking water bath, during the incubations. Additional aliquots of microsomes (1 mg) and NADPH (2 μmol) were added continuously, every hour or every second hour, to the incubation mixture (total volume 2–3 ml) for the first 8 h of the experiment. The reactions with HLM were carried out for 8 or 24 h. The turnover of finasteride was low in the HLM incubations and high finasteride concentrations and the long incubation times were needed to ensure production of relevant amounts of metabolites. Finasteride (100 μM) was also incubated with 50 pmol of each of seven different types of recombinant CYP enzymes; CYP3A4, CYP3A5, CYP2C8, CYP2C9*1 (Arg_{144}), CYP2C18, CYP2C19, CYP2D6*1, in the presence of NADPH (1 mM) in potassium phosphate buffer (0.1 M, pH 7.4) for 1 h at 37 °C. The shorter incubation times and lower finasteride concentrations were found to be enough for the qualitative assessment of the enzymes involved.

The total volume of the reaction mixture was 1 ml. The metabolic reactions were terminated by the addition of methanol (1:1 v/v) and the samples were put on ice for 10-15 min and then centrifuged (11 500 *g*, 10 min). The supernatants from the human recombinant CYP enzymes incubations were transferred to novel tubes and the solvent was evaporated under a stream of nitrogen while heated to 50 °C in order to concentrate the analytes. The concentrated samples were diluted with 100–150 µL of 50% methanol before LC-MS analysis. The supernatants from the HLM incubations were analyzed directly.

Sample treatment before analysis with UPLC-Q-TOF MS

The *in vitro* samples were transferred to vials and analyzed directly without any pre-treatment. The pig plasma samples (100 µL) were vortexed with acetonitrile (100 µL) and centrifuged (10 min at 12 000 *g*) to precipitate the plasma proteins. The supernatants were transferred to vials and analyzed. The pig urine and bile samples were analyzed both with and without hydrolysis with β-glucuronidase. Without hydrolysis: samples were diluted with MilliQ water (1:1, v:v), vortexed, and centrifuged (10 min at 12 000 *g*). The supernatants were transferred to vials and analyzed. With hydrolysis: the samples (100 µL) were mixed with 100 µL of potassium phosphate buffer (0.1 M, pH 6.05) and 5 µL β-glucuronidase and incubated in 60 °C for 2 h. The samples were centrifuged (10 min at 12 000 *g*) and the supernatants were transferred to vials and analyzed.

UPLC-Q-TOF MS

The chromatographic separation and accurate mass detection of finasteride and its metabolites were achieved on an Acquity UPLC system connected by an electrospray ion source to a Synapt G2 Q-TOF mass spectrometer from Waters Corporation (Milford, MA). The analytical column was an Acquity UPLC BEH C_{18} 1.7 µm (2.1 × 50 mm) and the mobile phase was delivered at 500 µL/min as a 10 min gradient with 0.1% formic acid in MilliQ water (A) or acetonitrile (B), respectively (5–95% B from 0 to 7 min; hold at 95% B for 0.5 min, back to 5% B in 0.5 min; then equilibration). The Synapt G2 was operated in the resolution mode with positive electrospray ionization. The capillary voltage was set to 0.75 kV in positive or 2.5 kV in negative ionization mode. The voltages of the sample and extraction cones were set to 40 and 4.0 V, respectively, in both positive and negative polarity. The source and desolvation temperatures were 120 and 450 °C, respectively, and the desolvation gas flow was set to 1000 L/h. The collision gas was argon, delivered at a flow rate of 2 L/h. Data was collected in the *m/z* 50–1200 scan range and the scan time was 0.3 s for MS/MS and 0.15 s for MS^E. The collision energy for MS/MS was ramped from 10 to 45 V or 25 to 45 V. In MS^E, the collision energy in the low energy trace was set to 6 V and for the high energy trace it was ramped from 15 to 35 V. The system was mass calibrated using sodium formate (0.5 mM) in 2-propanol:water (90:10; v/v)). Automatic lock-mass correction was applied using a solution of leucine–enkephalin (2 ng/µL) in acetonitrile: 0.1% formic acid in water (50:50; v/v). The instrument was controlled with the MassLynx software (version 4.1 SCN 781) from Waters Corporation (Milford, MA).

HPLC-ion trap MS

The HPLC autoinjector and binary pump [Agilent Technologies, Waldbronn, Germany (G1312A/1100)] was coupled to a Finnigan LCQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The mobile phase consisted of 0.1% formic acid in MilliQ water (A) and acetonitrile (B), and was delivered at a flow rate of 200 µL/min as a 22 min gradient (from 30 to 70% B from 0 to 12 min; then back to 30% B in 0.5 min; then equilibration at 30% B). The column used for the chromatographic separation was a Luna 5 µ C_{18}, 100 Å (50 × 2 mm) coupled to a C_{18} guard column (length 4 mm, i.d. 2 mm), both from Phenomenex® (Torrance, CA). The mass spectrometer was equipped with an electrospray ion source operated in the positive ion mode. The spray voltage was set to 3 kV, the capillary temperature 160 °C, the capillary voltage 33 V, and the sheath and auxiliary nitrogen gas flows were 80 and 1.5 arbitrary units, respectively. Instrument control, data acquisition and data processing were performed using the Xcalibur software version 1.3 (Thermo Fisher Scientific, Waltham, MA).

Results

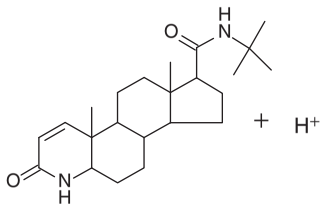
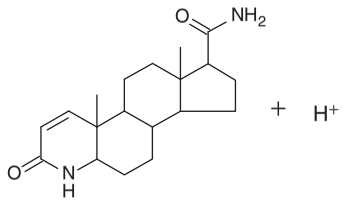
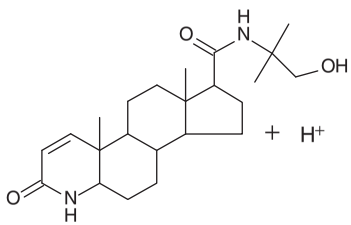
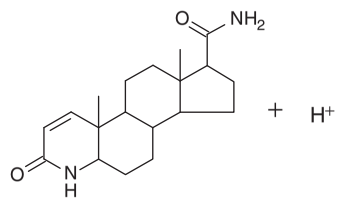
Structural elucidation of reference standards of finasteride, M1 and M3 by high-resolution mass spectrometry

Accurate mass measurements of finasteride and its metabolites were carried out with a Q-TOF mass spectrometer in order to obtain refined structural information as reference data for comparison with results from the human and porcine material (Table 1A–C). The accurate mass of the finasteride product at *m/z* 317 was in good agreement with a suggested loss of tertiary butene (–C_4H_8; Table 1A). However, the product at *m/z* 305 did not match the radical structure previously published by Constanzer et al. (1994). Our results rather imply a neutral loss of C_4H_4O, suggesting an even-electron product ion. In the H/D exchange experiment described in Lundahl et al. (2009b), it was demonstrated that the fragment corresponding to *m/z* 305 contained two deuterium atoms whereas the precursor contained four. Thus, it is likely that the loss of C_4H_4O occurred in the vicinity of a nitrogen, probably in the A ring.

For the reference standard of the hydroxylated metabolite M1, the loss of 17 Da yielding a product at *m/z* 372 was demonstrated to be formed by a loss of ammonia rather than by the previously suggested OH radical (c.f. Lundahl et al., 2009b; see Table 1(B) for suggested structures). The difference between the theoretical and experimental accurate masses for a loss of an OH radical would be −64.1 ppm as compared to −0.185 ppm for a loss of ammonia. The determined accurate masses of the other product ions closely matched the ones for the previously published formulae. The largest error (−13.5 ppm) was found for *m/z* 300; however this fragment had a very low intensity.

The fragments of the deprotonated carboxylated metabolite M3 were consistent with an initial decarboxylation yielding *m/z*

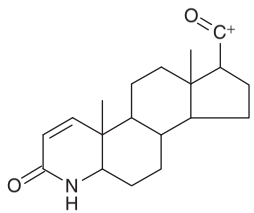
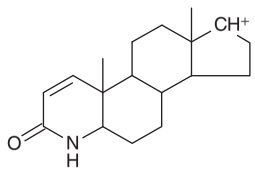
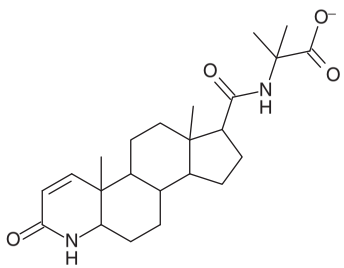
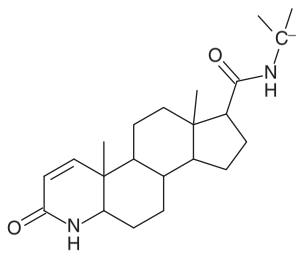
Table 1. Theoretical and experimental accurate masses of the protonated/deprotonated molecules and product ions of finasteride (A) obtained from reference standard, M1 (B) and M3 (C) obtained from reference standard and pig urine and bile samples.

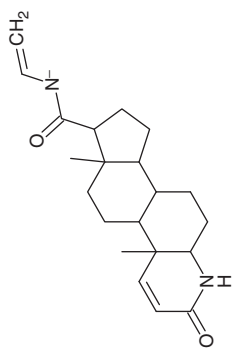
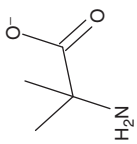
Precursor	Suggested formula of fragment	Suggested neutral loss	Theoretical *m/z*	Experimental *m/z*	Difference (ppm)	Reference standard		Pig urine sample		Pig bile sample	
						Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)
A. Protonated finasteride											
 $C_{23}H_{37}N_2O_2$			373.2855	373.2857	0.527						
	 $C_{19}H_{29}N_2O_2$	$-C_4H_8$	317.2229	317.2227	−0.641						
	$C_{19}H_{33}N_2O$	$-C_4H_4O$	305.2593	305.2586	−2.26						
B. Protonated M1											
 $C_{23}H_{37}N_2O_3$			389.2804	389.2803	−0.303	389.2806	0.467	389.2801	−0.817		
	$C_{23}H_{34}NO_3$	$-NH_3$	372.2539	372.2538	−0.185	372.2524	−3.95	372.2538	−0.185		
	$C_{19}H_{28}NO_3$	$-C_4H_6$	318.2069	318.2076	2.14	318.2061	−2.57	318.2116	14.7		
	 $C_{19}H_{29}N_2O_2$	$-C_4H_8O$	317.2229	317.2245	5.03	317.2243	4.40	317.2230	0.306		

(*continued*)

DOI: 10.3109/00498254.2013.866298

Table 1. Continued

			Reference standard		Pig urine sample		Pig bile sample	
			Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)
 $C_{19}H_{26}NO_2$	$-C_4H_{11}NO$	300.1964	300.1923	−13.5	300.1934	−9.84	300.1924	−13.2
 $C_{18}H_{26}NO$	$-C_5H_{11}NO_2$	272.2014	272.2010	−1.61	272.2016	0.591	272.2019	1.69
C. Deprotonated M3 (carboxy finasteride)								
 $C_{23}H_{33}N_2O_4$		401.2440	401.2433	−1.75	401.2408	−7.98	401.2444	0.997
 $C_{22}H_{33}N_2O_2$	$-CO_2$	357.2542	357.2548	1.68	357.2542	0.00	357.2539	−0.84

		Reference standard		Pig urine sample		Pig bile sample	
		Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)
	–$C_2H_4O_2$	341.2217	−3.52	341.2238	2.64	341.2233	1.17
$C_{21}H_{29}N_2O_2$							
	–$C_{19}H_{25}NO_2$	102.0554	−0.980	102.0554	−0.980	102.0557	−0.96
$C_4H_8NO_2$							

357 followed by a further net loss of methane to *m/z* 341 (Table 1C). The fragment at *m/z* 102 matched an amino acid product of the carboxylated tertiary butyl side chain.

High-resolution mass spectrometry for structural elucidation of phase I metabolites of finasteride formed by human liver microsomes

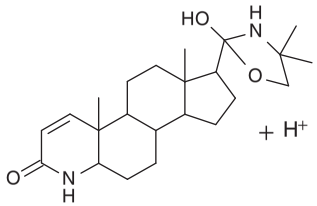
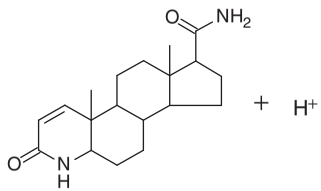
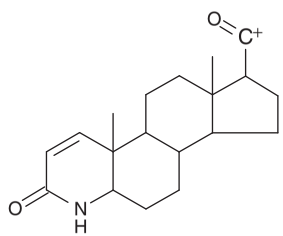
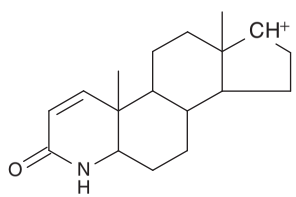
The results of the accurate mass measurements of the metabolites denoted MX and MY formed by human liver microsomes are shown in Table 2(A and B). For MX, the accurate mass of the $[M + H]^+$ at *m/z* 389 was only −1.07 ppm from the theoretical m/z of protonated OH finasteride. The product ion at *m/z* 372 was demonstrated to be formed by a loss of ammonia for this compound in the same way as for M1. The ion corresponding to *m/z* 305 for finasteride was represented by *m/z* 321 (loss of C_4H_4O, 305 + 16) for MX. The accurate mass of *m/z* 317 for MX indicated a loss of C_4H_8O which yielded the same fragment as the loss of the tertiary butyl group from protonated finasteride and the loss of tertiary butenol (C_4H_8O) from protonated M1. The product ions at *m/z* 300 and 272 both had accurate masses corroborating the previously suggested structures (c.f. Lundahl et al., 2009b, Table 2A). The formation of the fragments *m/z* 317, 300 and 272 from MX and the confirmation of their elemental composition by high-resolution MS/MS strongly suggested that MX has an unchanged steroid skeleton and that the oxidation must have occurred on the tertiary butyl side chain. Furthermore, the previous H/D exchange experiment (Lundahl et al., 2009b) precluded the possibility of an *N*-hydroxylation. The facts that all the methyl groups of the tertiary butyl are equivalent and that MX was obviously not identical to M1 strengthen the suggestion that MX is a ring-closed lactol form of M1.

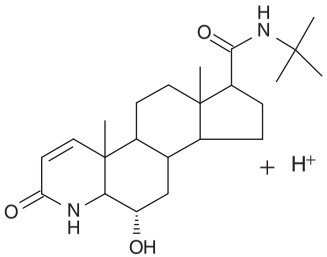
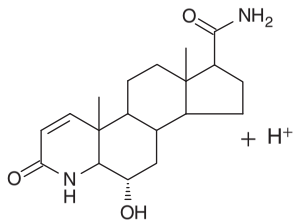
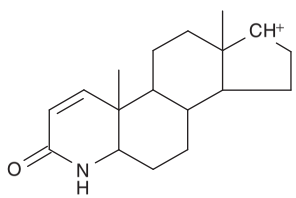
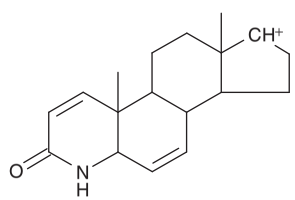
The determination of the accurate masses of the protonated molecule of MY yielded a value that was only 0.467 ppm from the theoretical one (Table 2B). The previously suggested structure of *m/z* 333, i.e. the hydroxylated correspondent of *m/z* 317 (317 + 16) formed by the loss of the tertiary butyl as C_4H_8 was strengthened as its accurate mass closely matched the expected value (difference 0.247 ppm). This fragment was unique to MY and its presence strongly suggested hydroxylation on the steroid skeleton. Furthermore, the results also corroborate the published structural proposals for *m/z* 315, 272 and 270. For *m/z* 298 and 280 that were not discussed in the previous paper, the accurate masses demonstrated loss of ammonia from *m/z* 315 and loss of water from *m/z* 298, respectively. The occurrence of the specific product ions *m/z* 333, 315, 298 and 270 and the supporting elemental compositions indicated that the hydroxylation of MY has taken place on the steroid skeleton.

Structural elucidation of pig *in vivo* finasteride metabolites with high resolution mass spectrometry

Plasma, bile and urine samples from pigs were analyzed for the presence of phase I and II metabolites of finasteride with and without β-glucuronidase treatment. In pig plasma, only traces of MX and M3 were observed (results not shown). After β-glucuronidase hydrolysis of urine and bile, three peaks with *m/z* 389 occurred in the chromatogram. A minor peak at *m/z* 389 with a retention time and product ion spectrum

Table 2. Theoretical and experimental accurate masses of the protonated molecules and product ions of the metabolites MX (A) and MY (B) obtained from human liver microsomes and pig urine and bile.

Precursor	Suggested formula of fragment	Suggested neutral loss	Theoretical m/z	Human liver microsomes		Pig urine sample		Pig bile sample	
				Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)
A. Protonated MX									
			389.2804	389.2800	−1.07	389.2807	0.724	389.2801	−0.817
$C_{23}H_{37}N_2O_3$									
	$C_{23}H_{34}NO_3$	$-NH_3$	372.2539	372.2546	1.96	372.2534	−1.26	372.2555	4.38
	$C_{23}H_{35}N_2O_2$	$-H_2O$	371.2699	371.2664	−9.30	371.2713	3.90	371.2703	1.20
	$C_{19}H_{33}N_2O_2$	$-C_4H_4O$	321.2542	321.2545	0.924	321.2539	−0.944	321.2539	−0.944
		$-C_4H_8O$	317.2229	317.2228	−0.325	317.2226	−0.955	317.2234	1.57
$C_{19}H_{29}N_2O_2$									
		$-C_4H_{11}NO$	300.1964	300.1969	1.82	300.1989	8.48	300.1961	−0.846
$C_{19}H_{26}NO_2$									
		$-C_5H_{11}NO_2$	272.2014	272.2011	−1.25	272.2014	−0.143	272.2014	−0.143
$C_{18}H_{26}NO$									

Precursor	Suggested formula of fragment	Suggested neutral loss	Theoretical m/z	Human liver microsomes		Pig urine sample		Pig bile sample	
				Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)	Experimental *m/z*	Difference (ppm)
B. Protonated MY									
			389.2804	389.2806	0.467	389.2797	−1.85	389.2798	−1.59
$C_{23}H_{37}N_2O_3$	$C_{23}H_{35}N_2O_2$	$-H_2O$	371.2699	371.2696	−0.682	371.2695	−0.952	371.2695	−0.952
		$-C_4H_8$	333.2178	333.2179	0.247	333.2171	−2.15	333.2177	−0.354
	$C_{19}H_{29}N_2O_3$								
	$C_{19}H_{27}N_2O_2$	$-H_2O$	315.2073	315.2082	3.00	315.2037	−11.3	315.2081	2.69
	$C_{19}H_{24}NO_2$	$-NH_3$	298.1807	298.1803	−1.36	298.1782	−8.40	298.1804	−1.02
	$C_{19}H_{22}NO$	$-H_2O$	280.1701	280.1707	2.142	280.1709	2.86	280.1703	0.714
		$-C_5H_{11}NO_2$	272.2014	272.2012	−0.878	272.2004	−3.82	272.2019	1.69
	$C_{18}H_{26}NO$								
		$-C_5H_{11}NO$	270.1858	270.1854	−1.48	270.1844	−5.18	270.1848	−3.70
	$C_{18}H_{24}NO$								

corresponding to that of the M1 reference standard was observed in both materials (Table 1B). However, the major two peaks at *m/z* 389 matched the MX and MY isomers described in human liver microsomes in terms of retention times and accurate mass spectra (Table 2A and B). The carboxylated metabolite M3 was identified in both pig urine and bile based on retention time and the accurate masses of its deprotonated molecule and three of its product ions (*m/z* 357, 341 and 102; Table 2C).

In the unhydrolyzed samples, two intact glucuronides, MX glucuronide and MY glucuronide, were identified in both bile and urine from pigs (Figure 2A–F and Table 3A and B). The initial formation of *m/z* 389 represented the characteristic loss of monodehydrogenated glucuronic acid (−176 Da) and the further products of *m/z* 389 were in agreement with those of MX and MY, respectively, *m/z* 321 being selective for MX and *m/z* 333 for MY (c.f. Table 2A and B). The mass accuracies were generally good, but for a few product ions with a very low ion count the mass error exceeded 5 ppm.

CYP enzymes involved in the formation of the hydroxylated metabolites MX and MY

A qualitative approach was applied to achieve more knowledge about the CYP enzymes involved in the formation of MX and MY. Finasteride incubations with human recombinant enzymes CYP3A4, CYP2D6, CYP3A5, CYP2C8, CYP2C9, CYP2C18 and CYP2C19 were performed and the samples were analyzed. The CYP3A4 enzymes resulted in the production of MX and MY (Figure 3A–C), while incubations with human recombinant CYP2D6 enzymes resulted only in the production of a metabolite with similar retention time and product ion spectrum as MY (Figure 3D, E). None of the other enzymes tested produced hydroxylated finasteride metabolites in detectable concentrations.

Discussion

Two metabolites of finasteride, M1 (ω-OH) and M3 (ω-COOH), were in an early study described as the major human plasma and urine biotransformation products (Carlin et al., 1992). However, in a later study by our group, contradictory results were found as M1 was not present in quantifiable concentrations in any of the examined human plasma, urine or bile samples whereas M3 was confirmed to be the major metabolite (Lundahl et al., 2009a,b). Instead of M1, two other hydroxylated metabolites, denoted MX and MY and the MY glucuronide were identified in human bile and urine (Lundahl et al., 2009b). Traces of M3 glucuronide could also be identified in human bile. Recently, we performed a drug–drug interaction investigation in pigs utilizing an advanced pig model that enabled bile sampling directly from the biliary duct (Lundahl et al., 2011). In that study, we aimed to follow the finasteride, M1, and M3 concentrations over time. However, the previously described main human metabolite M1 was not present in quantifiable concentrations in pig bile, plasma or urine which was in agreement with the latter human study (Lundahl et al., 2009a).

Previous knowledge from pigs suggests that the metabolism in this species has similarities to that of humans. In a recent report, the most abundant CYP subfamilies in pigs were found to be CYP2A, 2D, 2C and 3A (Achour et al., 2011). Enterocytes, hepatocytes, liver and intestinal microsomes from pigs have been used to study drug metabolism and similar activity and a corresponding rate of metabolism have been observed for typical CYP3A substrates such as testosterone and tacrolimus (Bader et al., 2000; Olsen et al., 1997; Skaanild & Friis, 1997; Thörn et al., 2011). Differences in metabolism between humans and pigs have also been observed for a few compounds, e.g. diclofenac and dextromethorphan (Thörn et al., 2011). Furthermore, *in vivo* pig experiments clearly demonstrated that raloxifene was metabolized to a high extent by intestinal glucuronidation in pigs and the metabolite formed was the same as that formed in humans (Thörn et al., 2012).

In the study presented in this article, we qualitatively identified phase I and phase II metabolites of finasteride in pig plasma, urine, and bile and compared the results with those obtained from an *in vitro* experiment using human liver microsomes. An important aim of this study was to examine the validity of the pig model in terms of the metabolite profile of finasteride as well as to confirm or reject previously published structural data based on results from low resolution MS. This is, to the best of the authors' knowledge, the first study on the metabolism of finasteride in pigs. We used state-of-the-art high resolution mass spectrometry [UPLC-Q-TOF-MS(/MS)] to obtain refined information on the structures of the metabolites found.

In our study from 2009, the structures of MX and MY were elucidated with a combination of the low resolution techniques tandem quadrupole MS/MS and ion trap MS^n. From the MS analyses, it was suggested that MX was a ring-closed lactol form of M1 and that MY could be identical to the previously described 6α-OH finasteride, M4 (Lundahl et al., 2009b). In this study, we had the possibility to use a more advanced Q-TOF mass spectrometer giving accurate mass data which can be used for calculation of elemental compositions of the intact compounds and their fragments. Interestingly, the metabolite profile of the pigs were in good agreement with that of the latest *in vivo* human study (Lundahl et al., 2009b) as well as with that of human liver microsomes. MX, MY and their intact glucuronides as well as M3 were identified in pig urine and bile (Figure 2 and Tables 1B, 2A and B, 3A and B). In this study, traces of M1 were found in the pig urine and bile samples. However, this apparent discrepancy with the previous human study was probably owing to the fact that a more sensitive mass spectrometer was used in this study. It might not have been possible to separate MX from M1 with the techniques used in the earlier studies and therefore M1 was described as the major metabolite in plasma (Carlin et al., 1992). The later quantitative investigations using more selective LC-MS/MS techniques were only directed towards finasteride, M1 and M3 (Lundahl et al., 2009a, 2011). A determination of the concentrations of MX and MY were not possible at the time as no reference standards of these compounds were available.

The high-resolution data obtained in this study corroborated most of the structural suggestions of the fragments that were previously obtained by our group with low resolution MS (Lundahl et al., 2009b). One important exception, though,

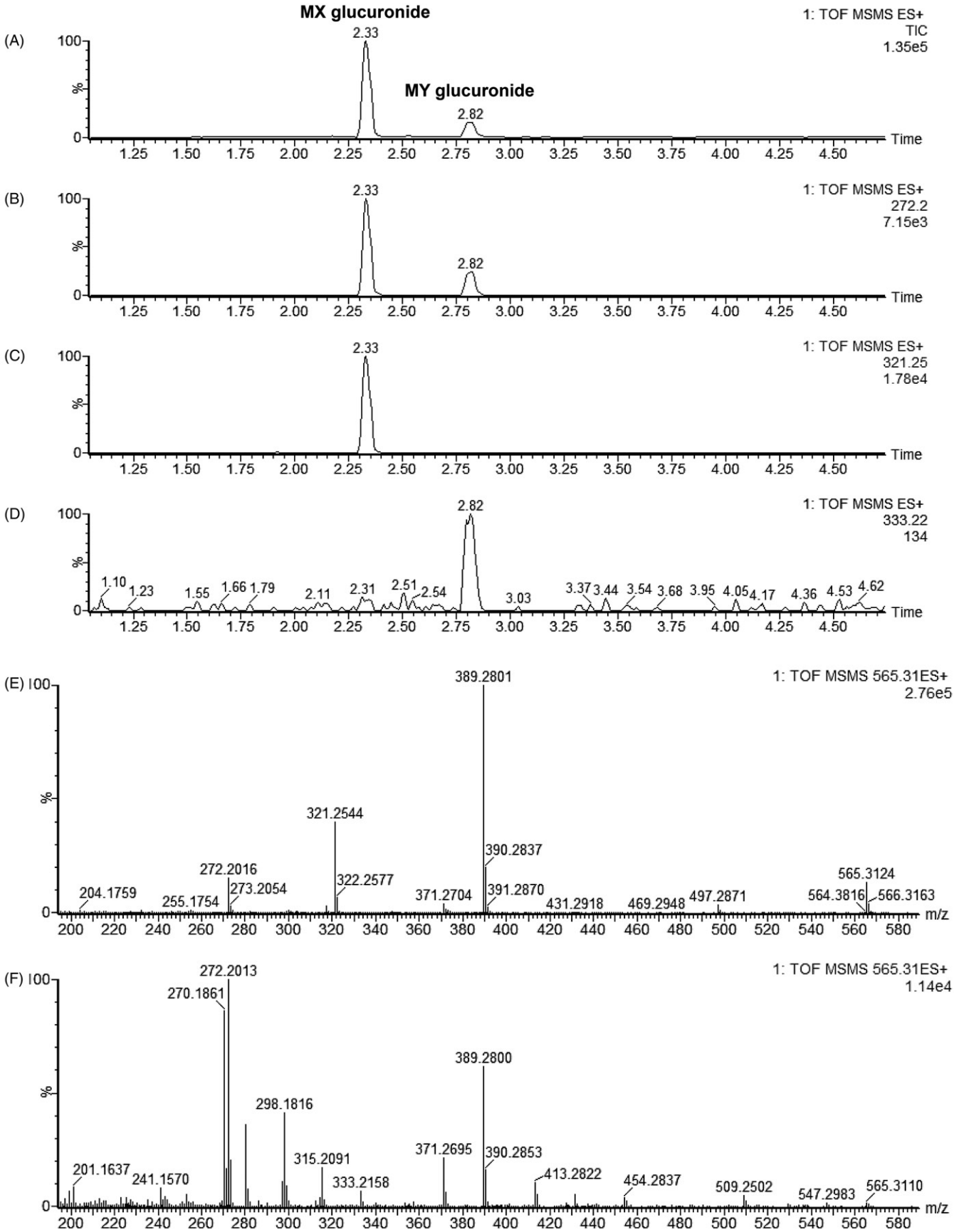
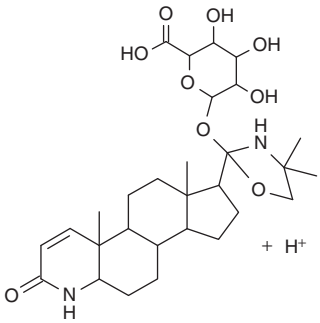
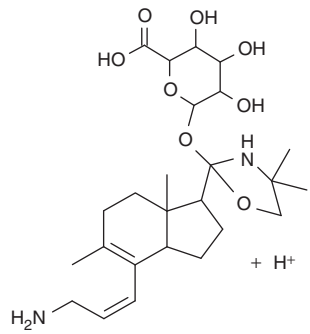
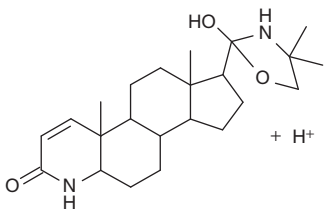
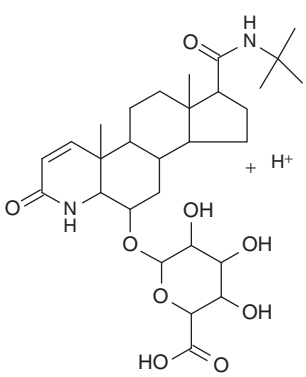
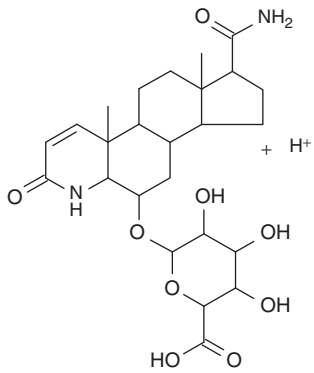
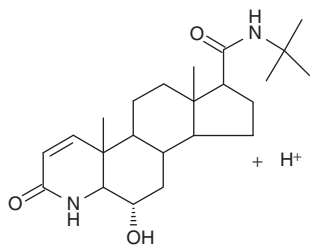
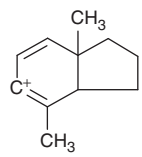


Figure 2. Chromatograms and spectra from an unhydrolyzed pig bile sample collected 120 min after finasteride dosage. MS/MS of *m/z* 565 with collision energy ramp from 35 to 75 eV, total ion chromatogram (A); extracted ions chromatograms for *m/z* 272.2 (B); *m/z* 321.3 (C); and *m/z* 333.2 (D); spectra from the peaks at 2.33 min corresponding to MX glucuronide (E); and 2.82 min corresponding to MY glucuronide (F).

Table 3. Theoretical and experimental accurate masses of the protonated molecules and product ions of the metabolites MX glucuronide (A) and MY glucuronide (B) obtained from pig urine and bile.

Precursor	Suggested formula of fragment	Suggested neutral loss	Theoretical *m/z*	Pig urine sample		Pig bile sample	
				Experimental *m/z*	Difference (ppm)	Experimental m/z	Difference (ppm)
A. Protonated MX glucuronide							
			565.3130	565.3107	−4.07	565.3124	−1.06
$C_{29}H_{45}N_2O_9$							
		–C_4H_4O	497.2868	497.2836	−6.41	497.2871	0.633
	$C_{25}H_{41}N_2O_8$						
		–$C_6H_8O_6$	389.2804	389.2781	−5.91	389.2801	−0.817
	$C_{23}H_{37}N_2O_3$						
	$C_{23}H_{35}N_2O_2$	–H_2O	371.2699	371.2609	−24.2	371.2704	1.47
	$C_{19}H_{33}N_2O_2$	–C_4H_4O	321.2542	321.2512	−9.34	321.2544	0.612
	$C_{19}H_{29}N_2O_2$[a]	–C_4H_8O	317.2229	317.2220	−2.84	317.2242	4.09
	$C_{19}H_{26}NO_2$[a]	–$C_4H_{11}NO$	300.1964	300.1967	0.999	300.1983	6.48
	$C_{18}H_{26}NO$[a]	–$C_5H_{11}NO_2$	272.2014	272.2006	−2.94	272.2016	0.591
B. Protonated MY glucuronide							
			565.3130	565.3152	3.89	565.3110	−3.54
$C_{29}H_{45}N_2O_9$							

(*continued*)

Precursor	Suggested formula of fragment	Suggested neutral loss	Theoretical *m/z*	Pig urine sample Experimental *m/z*	Pig urine sample Difference (ppm)	Pig bile sample Experimental m/z	Pig bile sample Difference (ppm)
	 $C_{25}H_{37}N_2O_9$	$-C_4H_8$	509.2494	509.2475	−3.73	509.2502	1.57
	 $C_{23}H_{37}N_2O_3$	$-C_6H_8O_6$	389.2804	389.2813	2.27	389.2800	−1.07
	$C_{23}H_{35}N_2O_2$	$-H_2O$	371.2699	371.2699	0.126	371.2695	−0.952
	$C_{19}H_{29}N_2O_3$[b]	$-C_4H_8$	333.2178	333.2173	−1.55	333.2158	−6.06
	$C_{19}H_{27}N_2O_2$	$-H_2O$	315.2073	315.2079	2.05	315.2091	5.86
	$C_{19}H_{24}NO_2$	$-NH_3$	298.1807	298.1806	−0.349	298.1816	3.01
	$C_{19}H_{22}NO$	$-H_2O$	280.1701	280.1713	4.28	280.1701	0.00
	$C_{18}H_{26}NO$[b]	$-C_5H_{11}NO_2$	272.2014	272.2014	−0.143	272.2013	−0.511
	$C_{18}H_{24}NO$[b]	$-C_5H_{11}NO$	270.1858	270.1859	0.370	270.1861	1.11
	 $C_{11}H_{15}$	$-C_7H_9NO$	147.1174	147.1172	−1.359	147.1173	−0.68

[a]For suggested structure of fragment, see Table 2A.
[b]For suggested structure of fragment, see Table 2B

was the product ion of protonated finasteride at *m/z* 305 for which the previously published structure (Constanzer et al., 1994) was demonstrated to be incorrect.

Furthermore, this study gave further insight into the CYP enzymes that are involved in the formation of MX and MY. Human recombinant CYP3A4 could produce both these metabolites while human recombinant CYP2D6 exclusively produced a metabolite with similar product ion spectrum as MY. Interestingly, CYP2D6 has not been previously mentioned to play a role in the metabolism of finasteride (Huskey et al., 1995). Previously, we found that human recombinant UGT1A4 and UGT1A3 were the major UGTs that were involved in the formation of M1 and M3 glucuronides, respectively (Lundahl et al., 2009b).

In summary, the pig formed the phase I metabolites MX and MY and their glucuronides in agreement with the previous human study. To the best of the authors' knowledge, this is the first study of the metabolite profile of finasteride in pigs and, interestingly, the qualitative match between these two species was excellent, demonstrating the validity of the pig as an *in vivo* model for human drug studies.

Conclusions

The metabolite profile of finasteride in pigs was for the first time examined in this study. The metabolites were in good agreement with those of humans and this demonstrates the value of performing advanced metabolism investigations in pigs. It is worth to highlight that the metabolites that were

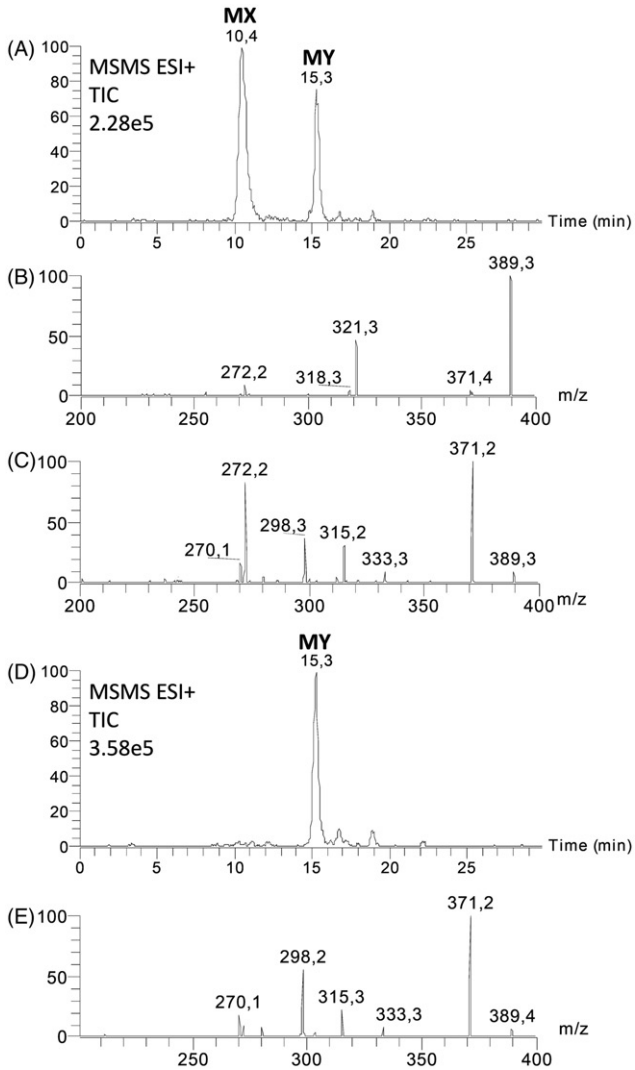


Figure 3. Chromatogram (A) and spectra of MX (B, peak at 10.4 min) and MY (C, peak at 15.3 min) produced by human recombinant CYP3A4; chromatogram (D) and spectrum (E, peak at 15.3 min) of the metabolite produced by human recombinant CYP2D6.

identified previously in human bile also could be identified in pig bile. The use of high resolution mass spectrometry enabled refined structural information of the OH metabolites (MX and MY) and their glucuronide conjugates. Furthermore, it was qualitatively identified that human recombinant CYP3A4 could produce MX and MY.

Declaration of interest

The authors report no declarations of interest.

References

Achour B, Barber J, Rostami-Hodjegan A. (2011). Cytochrome P450 pig liver pie: determination of individual cytochrome P450 isoform contents in microsomes from two pig livers using liquid chromatography in conjunction with mass spectrometry. Drug Metab Dispos 39: 2130–4.

Bader A, Hansen T, Kirchner G, et al. (2000). Primary porcine enterocyte and hepatocyte cultures to study drug oxidation reactions. Br J Pharmacol 129:331–42.

Bergman E, Forsell P, Tevell A, et al. (2006). Biliary secretion of rosuvastatin and bile acids in humans during the absorption phase. Eur J Pharm Sci 29:205–14.

Bergman E, Lundahl A, Fridblom P, et al. (2009). Enterohepatic disposition of rosuvastatin in pigs and the impact of concomitant dosing with cyclosporine and gemfibrozil. Drug Metab Dispos 37: 2349–58.

Carlin JR, Christofalo B, Arison C, et al. (1987). Metabolism and disposition of MK-906 in laboratory animals and man (abstract). Pharmacologist 29:149.

Carlin JR, Christofalo P, Arison BH, et al. (1997). Disposition and metabolism of finasteride in dogs. Drug Metab Dispos 25:100–9.

Carlin JR, Christofalo P, Vandenheuvel WJ. (1988). High-performance liquid chromatographic determination of N-(2-methyl-2-propyl)-3-oxo-4-aza-5 alpha-androst-1-ene-17 beta-carboxamide a 4-azasteroid in human plasma from a phase I study. J Chromatogr 427:79–91.

Carlin JR, Hoglund P, Eriksson LO, et al. (1992). Disposition and pharmacokinetics of [^{14}C]finasteride after oral administration in humans. Drug Metab Dispos 20:148–55.

Constanzer ML, Chavez CM, Matuszewski BK. (1994). Picogram determination of finasteride in human plasma and semen by high-performance liquid chromatography with atmospheric-pressure chemical-ionization tandem mass spectrometry. J Chromatogr B Biomed Appl 658:281–7.

Drake L, Hordinsky M, Fiedler V, et al. (1999). The effects of finasteride on scalp skin and serum androgen levels in men with androgenetic alopecia. J Am Acad Dermatol 41:550–4.

Faller B, Farley D, Nick H. (1993). Finasteride: a slow-binding 5 alpha-reductase inhibitor. Biochemistry 32:5705–10.

Huskey SW, Dean DC, Miller RR, et al. (1995). Identification of human cytochrome P450 isozymes responsible for the in vitro oxidative metabolism of finasteride. Drug Metab Dispos 23:1126–35.

Ishii Y, Mukoyama H, Hata S. (1994a). Metabolism of finasteride in rat hepatic microsomes: age and sex differences and effects of P450 inducers. Xenobiotica 24:863–72.

Ishii Y, Mukoyama H, Ohtawa M. (1994b). In vitro biotransformation of finasteride in rat hepatic microsomes, isolation and characterization of metabolites. Drug Metab Dispos 22:79–84.

Ishii Y, Mukoyama H, Ishii M, et al. (1992). Sex difference in the metabolism of finasteride in the rats (abstract). ISSX Proceed 2:109.

Lundahl A, Hedeland M, Bondesson U, et al. (2009a). The effect of St. John's wort on the pharmacokinetics metabolism and biliary excretion of finasteride and its metabolites in healthy men. Eur J Pharm Sci 36: 433–43.

Lundahl A, Hedeland M, Bondesson U, Lennernäs H. (2011). In vivo investigation in pigs of intestinal absorption hepatobilary disposition and metabolism of the 5-alpha reductase inhibitor finasteride and the effects of coadministered ketoconazole. Drug Metab Dispos 39: 847–57.

Lundahl A, Lennernäs H, Knutson L, et al. (2009b). Identification of finasteride metabolites in human bile and urine by high-performance liquid chromatography/tandem mass spectrometry. Drug Metab Dispos 37:2008–17.

McConnell JD, Wilson JD, George FW, et al. (1992). Finasteride an inhibitor of 5 alpha-reductase suppresses prostatic dihydrotestosterone in men with benign prostatic hyperplasia. J Clin Endocrinol Metab 74: 505–8.

Olsen AK, Hansen KT, Friis C. (1997). Pig hepatocytes as an in vitro model to study the regulation of human CYP3A4: prediction of drug-drug interactions with 17 alpha-ethynylestradiol. Chem Biol Interact 107:93–108.

Petri N, Bergman E, Forsell P, et al. (2006). First-pass effects of verapamil on the intestinal absorption and liver disposition of fexofenadine in the porcine model. Drug Metab Dispos 34: 1182–9.

Skaanild M, Friis C. (1997). Characterization of the P450 system in Gottingen minipigs. Pharmacol Toxicol 80:28–33.

Sjögren E, Bredberg U, Lennernäs H. (2012). The pharmacokinetics and hepatic disposition of repaglinide in pigs: mechanistic modeling of metabolism and transport. Mol Pharm 9:823–41.

Thörn HA, Lundahl A, Schrickx JA, et al. (2011). Drug metabolism of CYP3A4 CYP2C9 and CYP2D6 substrates in pigs and humans. Eur J Pharm Sci 43:89–98.

Thörn HA, Yasin M, Dickinson PA, Lennernäs H. (2012). Extensive intestinal glucuronidation of raloxifene in vivo in pigs and impact for oral drug delivery. Xenobiotica 42:917–28.