Identification of Finasteride Metabolites in Human Bile and Urine by High Performance Liquid Chromatography/Tandem Mass Spectrometry

Anna Lundahl
Hans Lennernäs
Lars Knutson
Ulf Bondesson
Mikael Hedeland

Department of Pharmacy, Uppsala University, Sweden (A.L., H.L.); Department of Surgical Sciences, Uppsala University Hospital, Sweden (L.K.); Department of Chemistry, National Veterinary Institute (SVA), Uppsala, Sweden and Department of Medicinal Chemistry, Analytical Pharmaceutical Chemistry, Uppsala University, Sweden (U.B., M.H.)
Running title: Identification of Finasteride Metabolites in Human Bile and Urine

Corresponding author

Mikael Hedeland, National Veterinary Institute (SVA), Department of Chemistry

SE-751 89 Uppsala, Sweden, Phone numbers: 00 46 18 674 209 or 0046 70 657 16 63,
Fax number: 0046 18 674 099, E-mail address: Mikael.Hedeland@sva.se

Number of text pages: 36

Number of tables: 1

Number of figures: 6

Number of references: 37 (max 40)

Number of words in the abstract: 249 (max 250)

Number of words in the introduction: 741 (max 750)

Number of words in the discussion: 1471 (max 1500)

Abbreviations

Area under plasma concentration-time curve (AUC), benign prostatic hyperplasia (BPH), collision-induced dissociation (CID), dihydrotestosterone (DHT), electrospray ionization (ESI), European Medicines Agency (EMEA), U.S. Food and Drug Administration (FDA), hydrogen/deuterium (H/D), high performance liquid chromatography (HPLC), liquid chromatography quadropole (LCQ), ω-hydroxy finasteride (M1), finasteride ω-al (M2), finasteride-ω-oic acid (M3), 6α-hydroxy finasteride (M4), mass spectrometry (MS), adenosine 3’-phosphate 5’-phosphosulphate (PAPS), Prostate Cancer Prevention Trial (PCPT), selected reaction monitoring (SRM), triple-stage quadropole (TSQ), UDP-glucuronic acid (UDPGA), UDP-glucuronosyltransferases (UGTs)
Abstract

The objective of this study was to further investigate the metabolism of the 5α-reductase inhibitor, finasteride, and to identify previously unknown phase I and phase II metabolites \textit{in vitro} and \textit{in vivo}, in human bile and urine. Healthy volunteers were administered 5 mg finasteride, directly to the intestine, and bile and urine were collected for 3 and 24 hours, respectively. A single-pass perfusion catheter, Loc-I-Gut, was used for drug administration and bile collection from the proximal jejunum, distal to papilla of Vater. Incubations with human liver microsomes/S9 fractions and different co-factors were performed with finasteride and the previously known metabolites, ω-hydroxy finasteride (M1) and finasteride-ω-oic acid (M3). Liquid chromatography coupled to triple quadrupole mass spectrometry with positive/negative electrospray ionization (LC-ESI-MS/MS) and ion trap with MS\textsuperscript{n} measurements were used for structural investigations and identification of metabolites. Two hydroxy-metabolites of finasteride, other than M1, and one intact hydroxy-finasteride glucuronide were identified \textit{in vitro} and in bile and urine. The glucuronide and at least one of the hydroxy-metabolites were previously unidentified. M1 and M3 were glucuronidated \textit{in vitro} by specific human UDP-glucuronosyltransferases, UGT1A4 and UGT1A3, respectively. M1 glucuronide was not identified \textit{in vivo} and M3 glucuronide, an acyl-glucuronide, was present in low amounts in bile from a few individuals. In conclusion, previously undescribed metabolites were identified, \textit{in vitro} and in human urine and bile. Bile collection using the Loc-I-Gut technique followed by sensitive LC-MS/MS analysis led to the discovery of novel, both phase I and phase II, finasteride metabolites in human bile.
Introduction

Finasteride, [N-(2-methyl-2-propyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide], is a synthetic 4-azasteroid, used in the oral treatment of benign prostatic hyperplasia (BPH) and androgenetic alopecia (male pattern hair loss). By inhibition of the 5α-reductase type II enzyme that converts testosterone to dihydrotestosterone (DHT), the intraprostatic DHT levels are reduced, leading to a decreased prostate volume and relief of BPH symptoms (McConnell et al., 1992, Gormley et al., 1992, Rittmaster et al., 1989, Drake et al., 1999). In addition, in one extensive study, the Prostate Cancer Prevention Trial (PCPT), finasteride treatment was proven to prevent or delay the development of prostate cancer, but the patients who developed cancer had a higher risk to develop a more aggressive form (Thompson et al., 2003). The use of finasteride for chemoprevention has been discussed and evaluated since the PCPT study was presented. Recently, the American Society of Clinical Oncology and the American Urological Association jointly came up with recommendations for the use of 5α-reductase inhibitors for prostate cancer prevention (Kramer et al., 2009). Based on this, it is likely that clinicians will prescribe finasteride more frequently to a wider population.

The expected increased prescription of this drug has made it important to more fully characterize its metabolism. Finasteride is a lipophilic drug (log K_{ow}=3.2) that is well absorbed and completely, but slowly metabolized (Loftsson and Hreinsdottir, 2006, Steiner, 1996, Sudduth and Koronkowski, 1993). The phase I metabolism of finasteride has previously been investigated but only partly characterized. In hepatic liver microsomes from male rats (phenobarbital treated for induced metabolism), four metabolites were formed and identified (Ishii et al., 1994). The metabolites found were
two alcohols (M1 and 6α-hydroxy finasteride (M4)), an aldehyde (finasteride ω-al, (M2))
and a carboxylic acid (finasteride-ω-oic-acid, (M3)) (Fig. 1). Cytochrome P450 3A4 was
later identified as the major enzyme involved in the sequential biotransformation of
finasteride to M1, M2 and M3 (Huskey et al., 1995). In dogs, the oral bioavailability of
finasteride was high (92%) and at least six metabolites (M1, M3, M4 and three
dihydroxy-metabolites) were found in plasma, urine and feces. Finasteride and M1 were
the major components in dog plasma (Carlin et al., 1997). When healthy human
volunteers were orally administered [14C]-finasteride, 39% of the dose was recovered in
urine and 57% in feces (7 days sampling). A very low proportion (<1%) of the finasteride
dose was excreted unchanged. M1 was the major identified metabolite in plasma (~12%
of total radioactivity) and M3 was the major metabolite in urine (18%) (Carlin et al.,
1992). The metabolites excreted in feces were not structurally described, indicating the
possible presence of unidentified metabolites in human bile.

With the unique possibility of accessing human bile via a single-pass perfusion
technique (Loc-I-Gut) (Bergman et al., 2006, Persson et al., 2006), a clinical herb-drug
interaction study was performed with administration of finasteride before and after St.
John’s wort treatment. Pharmacokinetic data based on quantification of finasteride, M1
and M3 in bile, urine and plasma by liquid chromatography coupled to a mass
spectrometer (LC-MS/MS) were reported in a previous publication (Lundahl et al., 2009).
As reported, finasteride was not excreted unchanged to urine and extremely low amounts
of parent drug were identified in bile (~0.01%). This supported the theory that finasteride
was eliminated primarily by metabolism. In total, approximately 22% of the given dose
was recovered in the study, the majority of which was M3 excreted to urine 0-24 hours
after parent drug administration. Surprisingly, and in contrast to data published by Carlin et al., 1992, M1 was not present in quantifiable concentrations in any of the examined body fluids (Lundahl et al., 2009). If it is assumed that at least 70% of the given finasteride dose was absorbed into the enterocytes and 22% of the dose was quantified after 24 hours sampling of urine and 3 hours sampling of bile, it follows that almost 50% of the dose remains to be identified. The St. John’s wort treatment significantly reduced the area under plasma concentration-time curve (AUC) for finasteride, but the AUC for M3 remained unchanged. This indicated that both the formation and elimination of M3 were induced by the St John’s wort treatment or the presence of other inducible parallel metabolic pathways.

The major objective of this study was to further investigate the metabolism of finasteride and to identify previously unknown phase I and phase II metabolites excreted to human bile and urine. The unique possibility of sampling using Loc-I-Gut enabled us to directly search for metabolites in human bile.
Methods

Chemicals and Enzyme Preparations. Acetic acid-OD (99 atom% D), alamethicin (from *Trichoderma viride*), β-glucuronidase (Type H1 from *Helix pomatia*), β-NADPH (reduced tetrasodium salt), D-saccharolactone, deuterium oxide (99.9 atom% D), finasteride, magnesium chloride, potassium phosphate, UDP-glucuronic acid (UDPGA) (trisodium salt), adenosine 3’-phosphate 5’-phosphosulphate (PAPS) and GSH were all purchased from Sigma-Aldrich. The two phase I metabolites, M1 and M3, were purchased from Toronto Research Chemicals (North York, ON, Canada). Finasteride tablets (Proscar®), 5 mg, were produced by Merck Sharp & Dohme and purchased via a Swedish pharmacy. Human liver microsomes, male pool (n=10) were purchased from BD Biosciences (Woburn, MA, USA). BD Gentest supersomes, human UDP-glucuronosyltransferases (UGT) 1A1, 1A3, 1A4, 1A7, 1A6, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Solvents and other reagents i.e., acetonitrile, formic acid and methanol were of analytical reagent grade and purchased from Merck (Darmstadt, Germany).

Microsomal Incubations with NADPH. Finasteride (final concentration 100 µM) was incubated with human liver microsomes (2.5 mg/ml) in a medium containing potassium phosphate buffer (0.1 M, pH 7.4) and NADPH (1 mM) at 37°C. The methanol content in the incubation mixture did not exceed 0.5%. Before starting the reaction, 100 µL (0 hour sample) of the mixture was withdrawn and transferred to a vial containing methanol (300 µL). After starting the reaction with NADPH, aliquots (250 µL) were withdrawn after 1, 2 and 4 hours or 8, 12 and 24 hours and the enzymatic reaction was terminated with
methanol (750 µL). Each sample was centrifuged (10,000 g, 10 minutes) and the supernatant was separated and frozen at -20°C pending analysis.

Microsomal Incubations with UDPGA. M1 and M3 (final concentrations 100 µM) were incubated with human liver microsomes and UDPGA for 24 hours. The incubations were carried out according to the method described by Fisher et al., 2000. First, the microsomes (2 mg) were activated with alamethicin (25 µg) in potassium phosphate buffer (0.1 M, pH 7.1). After 15 minutes on ice, the substrate was added together with saccharolactone (5 mM, final concentration) and magnesium chloride (1 mM, final concentration) and pre-incubated for 6 minutes at 37°C. To start the reaction, UDPGA (5 mM, final concentration) was added and the final volume of the reaction mixture was 200 µL. MilliQ-water was added to the 0 hour-samples instead of UDPGA. The methanol content in the incubation mixture did not exceed 0.5%. The incubation was carried out at 37°C and the reaction was stopped with ice cold acetonitrile (150 µL). After 30 minutes on ice, the samples were centrifuged (10,000 g, 10 minutes) and the supernatant separated and frozen at -20°C pending analysis. In addition, finasteride (final concentration 200 µM) was incubated with human liver microsomes (8 mg/ml) according to the same protocol with two co-factors, NADPH (1 mM) and UDPGA (5 mM).

Incubations with UGT Supersomes. M1 and M3 were incubated with all 12 commercially available UGT supersomes. The incubations were carried out identically for all the different supersomes (37°C for 24 hours). Each incubation mixture, total volume 200 µL, contained the substance (200 µM), supersomes (1 mg/ml), potassium
phosphate buffer (0.1 M, pH 7.4), magnesium chloride (5 mM) and UDPGA (5mM). Water was added instead of UDPGA for the 0 hour sample. The methanol content in the incubation mixture did not exceed 1%. The reaction was stopped by adding 150 µL ice cold acetonitrile to the sample. After 30 minutes on ice the samples were centrifuged (10,000 g, 10 minutes) and the supernatant separated and frozen at -20°C pending analysis.

**Incubations with PAPS and GSH.** For sulphate conjugation, incubations with 100 µM of M1, M3 and finasteride in human liver S9 fractions (2.5 mg/ml) in the presence of PAPS (4 mM) were performed according to the method described by van der Woude et al., 2004. For glutathione conjugation of the metabolites or possible reactive intermediates, incubations with 100 µM of M1, M3 and finasteride with human liver microsomes (2 mg/ml) in the presence of GSH (5 mM) and NADPH (1 mM) were performed according to the method described by Kang et al., 2007. A 0.1 M potassium phosphate buffer (pH 7.4) was used for both the GSH and PAPS incubations. The enzymatic reactions were terminated with acetonitrile (1:1) followed by centrifugation (10,000 g, 10 minutes) and separation of the supernatant, which was then frozen at -20°C pending analysis.

**Subjects and Study Design.** Details and results of the investigation of the clinical pharmacokinetics of finasteride and the interaction of St. John’s wort with finasteride are reported elsewhere (Lundahl et al., 2009). After providing written, informed consent, fifteen healthy male volunteers were enrolled in the study (mean age 28 years (range 20-
43 years), mean weight 78 kg (range 65-95 kg) weight and mean body mass index 24 kg/m² (range 21-27 kg/m²). A clinical examination prior to the study showed that all participants had normal clinical and laboratory values. Twelve of the 15 enrolled subjects completed the study. All subjects were required to abstain from consuming grapefruit and its juices during the study. Nicotine, alcohol and caffeine use were prohibited at least 24 hours prior to and during the study days. With the exception of the study drugs, no other medication was allowed during the study.

The clinical study was performed at the Clinical Research Department, University Hospital, Uppsala, Sweden in accordance with the Declaration of Helsinki and was consistent with European Medicines Agency (EMEA) guidelines for good clinical practice (CPMP/ICH/135/95). The study protocol was approved by the regional ethics committee at Uppsala University and by the Swedish Medical Products Agency, Uppsala, Sweden.

**Drug Administration and Bile Sampling.** Finasteride (5 mg) was administered directly to the proximal jejunum as a suspension via the intestinal perfusion tube, Loc-I-Gut (Bergman et al., 2006, Knutson et al., 1989, Lennernas et al., 1992). On arrival at the clinic in the morning after an overnight fast of 10-14 hours, the Loc-I-Gut tube was introduced orally and positioned in the distal duodenum/proximal jejunum below the papilla of Vater. The position of the tube was verified by fluoroscopy. The tube comprised two inflatable balloons and in this study the proximal balloon was inflated with 20-25 ml of air. Finasteride was administered into the jejunum together with the non-absorbable marker, [¹⁴C]-polyethylene glycol 4000, distally to the inflated balloon.
and bile was quantitatively collected proximal to the balloon. A vacuum pump was connected to the tube to facilitate the bile collection. Bile was collected in 20 minutes intervals for a total of 3 hours after finasteride administration. Blank bile samples were collected before finasteride was administered. The samples were kept on ice during collection and then weighed, fractionated and frozen at -20 °C pending analysis. In this study, bile from 7 individuals was analyzed for the presence of phase I and phase II metabolites.

Urine Sampling. Urine was quantitatively collected into BD Vacutainer® Collection Containers, 3 L, at intervals of 0-3, 3-6, 6-9, 9-12 and 12-24 hours after finasteride administration. One blank sample was collected before finasteride was administered. Each sample was weighed, fractionated and frozen at -20 °C pending analysis. In this study, urine from 6 individuals was analyzed for the presence of phase I and phase II metabolites.

Sample Preparation and Hydrolysis. For hydrolysis of urine or bile samples, 100 Sigma units of β-glucuronidase in 100 µL acetate buffer (0.5 M, pH 4.9) were added to 100 µL of urine/bile and the samples were placed in a 37°C water bath for 1, 2 or 24 hours. The reaction was stopped with 100 µL of acetonitrile and a centrifugation (12,000 g, 6 minutes) was carried out prior to analysis. The supernatant was used for the LC-MS/MS analysis. The hydrolysis was performed according to the method described by Shibasaki et al., 2001. Non-hydrolysed urine samples were directly analysed without any sample preparation. Bile was diluted (1:2) with MilliQ water and centrifuged (12,000 g, 6
minutes) before analysis. Following centrifugation and removal of proteins, the in vitro samples were pure enough to be directly injected.

**Liquid Chromatography-Triple Quadrupole Mass Spectrometry (TSQ).** A volume of 10 µl of the sample was injected by an HTC PAL auto sampler (CTC Analytics AG, Zwingen, Switzerland). The mobile phase was delivered by a Surveyor MS pump (Thermo Electron Corp., San Jose, CA, USA) at a flow rate of 0.2 ml/min. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). For hydrogen/deuterium exchange the mobile phase A consisted of 0.1% acetic acid-OD in deuterium oxide. The gradient program was: 0 to 2 minutes 5% of B, 2 to 8 minutes 5 to 90% of B, 8 to 15 minutes 90% of B, 15 to 15.1 minutes 90 to 95% of B and 15.1 to 17 minutes 95 to 5% of B. For the LC, a Phenomenex Luna C18 chromatographic column (length 50 mm, I.D. 2.00 mm, particle size 5 µm) coupled to an ODS-octadecyl guard column C18 (length 4 mm, I.D. 2.00 mm) were used. Both columns were supplied by Scandinavian GeneTech, Västra Frölunda, Sweden.

The MS detection was carried out using a Finnigan TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Electron Corp.) with positive or negative ESI. When running MS/MS, the collision cell was filled with argon (1.5 mTorr). To study the presence of phase I metabolites and glucuronides, different scan methods were applied. In positive ESI, Q1 was set to transmit the precursor ions [M+H]⁺ m/z 389 for M1 and its isomers, m/z 387 for M2, m/z 373 for finasteride and m/z 405 for dihydroxy-finasteride. The collision energies used were 20-40 V and the Q3 scan range was from m/z 50 to about 10 m/z units higher than that of the respective precursor ion. M3 was
detected using negative ESI with Q1 set for m/z 401 and a collision energy of 35 V and a Q3 scan range of m/z 50-500.

In the search for M1 glucuronide, selected reaction monitoring (SRM) with three parallel transitions was used (positive ESI): m/z 565 to m/z 230, 270 and 372. In the search for M3 glucuronide, SRM was used (negative ESI) with the parallel transitions m/z 577 to m/z 175, 193 and 401. Full product ion scans for hydroxy-finasteride glucuronides were performed (positive ESI) with the precursor ion m/z 565, a collision energy of 35 V, and a Q3 scan range of m/z 50-600. Other conjugates were sought by neutral loss scans of 129 and 307 u for glutathione and of 80 u for sulphate. The scan time was 1 second in all the analyses above, and the peak widths for Q1 and Q3 were set to 0.7 full width of half maximum.

The MS system was tuned for sensitivity by constant infusions of solutions of finasteride, M1 and M3 connected to the mobile phase flow by a T coupling. For analysis of finasteride, M2, dihydroxy-finasteride and M1, its isomers and their glucuronides, a spray voltage of 5 kV, a sheath and auxiliary pressure of 30 and 5 mTorr and a capillary temperature of 281°C were used. For analysis of M3 and its glucuronide, a spray voltage of -3.5 kV, a sheath and auxiliary pressure of 30 and 10 mTorr and a capillary temperature of 264°C were used. To avoid adduct formation with formic acid in the mobile phase, a skimmer voltage (-8 V) was applied. The search for the metabolites was performed manually by interpretation of mass spectra and chromatograms and by comparing spectra from standard solutions, in vitro and in vivo samples. Instrument control, data acquisition and data processing were performed using Xcalibur software, version 2.0 (Thermo Electron Corp.)
Ion Trap Mass Spectrometry, LCQ. MS experiments with finasteride standard solution, M1 standard solution and MX and MY produced in vitro were performed with a Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corp., San José, CA, USA). The standard solutions were directly infused into the MS, by a T coupling, or first separated on a LC column. All the in vitro samples were separated with LC prior to MS detection. For the LC, a Phenomenex Luna C18 chromatographic column (length 50 mm, I.D. 2.00 mm, particle size 5 µm) coupled to an ODS-octadecyl guard column C18 (length 4 mm, I.D. 2.00 mm) were used. Both columns were supplied by Scandinavian GeneTech, Västra Frölunda, Sweden. Twenty µl of sample was injected by an Agilent Autosampler (G1312A/1100) and the mobile phase was delivered by a binary Agilent pump (Agilent (G1313A/1100), Technologies, Waldbronn, Germany) at a flow rate of 0.3 ml/min. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The gradient program was: 0 to 8.0 minutes 30 to 65% of B, 8.0 to 8.1 minutes 65 to 70% of B and 8.1 to 13.0 minutes 70 to 30% of B. For hydrogen/deuterium exchange, the mobile phase A consisted of 0.1% acetic acid-OD in deuterium oxide (D2O). To generate m/z spectra, different scan methods and collision energies were applied. The isolation width was 2 m/z or less, the collision energy ranged from 25 to 48%, and the activation time was 30 msec.

The MS system was tuned for sensitivity by constant infusion of M1 solution (10 µg/ml) connected to the mobile phase flow by a T coupling. A spray voltage of 3 kV, a sheath gas flow rate of 80, a capillary voltage of 33 V and a capillary temperature of
160°C were used. Instrument control, data acquisition and data processing were performed using Xcalibur software version 1.3 (Thermo Finnigan).

To concentrate MX and MY, samples from the *in vitro* incubations were pooled together and dried at 65°C under nitrogen. Prior to analysis, the samples were diluted in 75%/25% water/acetonitrile and centrifuged for 10 minutes at 2000 g.
Results

Mass Spectral Properties of Finasteride, M1 and M3. MS/MS analysis of standard solutions of finasteride, M1 and M3 were carried out in order to obtain reference spectra for comparison with those obtained from the metabolites found in the *in vitro* and *in vivo* samples. There were no differences between the fragmentation patterns from the triple quadrupole and the ion trap instruments. Full product ion scan of finasteride standard with the precursor ion \(m/z\) 373 corresponding to \([M+H]^+\), resulted in two main product ions at \(m/z\) 317 and 305, see Table 1 for proposed structures and Fig. 2a for a spectrum. Hydrogen/deuterium (H/D) exchange using deuterium oxide (D₂O) in the mobile phase, was used to elucidate the number of exchangeable protons of the studied ions, in order to facilitate structural determination. Finasteride standard gave product ions at \(m/z\) 320 (317+3) and 307 (305+2) upon H/D exchange, indicating that \(m/z\) 317, as shown in Table 1, contained three labile protons, i.e. the two bound to the two nitrogens and the one carrying the charge. The shift for \(m/z\) 305 was also in accordance with the suggested structure, as it was protonated and contained only one nitrogen.

Full product ion scan of M1 standard with the precursor ion \(m/z\) 389 corresponding to \([M+H]^+\), resulted in the main fragments at \(m/z\) 372, 318/317, 300, 272 and 72, see proposed structures in Table 1 and a spectrum in Fig. 2b. The ion at \(m/z\) 372 (-17 u from 389) could be formed by the loss of an OH radical or NH₃. The option that this fragment was formed by the loss of ammonia from the A ring was considered impossible, as MS³ experiments with \(m/z\) 389 → 372 → scan gave the main fragments \(m/z\) 300 and 272 (results not shown) c.f. the suggested structures in Table 1. A loss of NH₃ from the C-17 amide would imply a rearrangement, attaching the tertiary butanol...
side-chain to the structure to form m/z 372. M/z 318 was further studied with MS$^3$ m/z 389 → 318 → scan. The spectrum was unrelated to the ones obtained from m/z 389 and m/z 372, suggesting that m/z 318 was formed by a completely different mechanism compared to the other main fragments. H/D exchange of the M1 standard resulted, as expected, in the precursor ion at m/z 393 (389+4), which indicated the presence of four exchangeable protons including the one carrying the charge. The first major products were a double peak at m/z 374/373 (372+2/1), corresponding to losses of 19 and 20 u, indicating a mixture of mechanisms. A loss of an OD radical would give -18 u. If the formation of the neutrals 19 and 20 u represent ammonia in the forms of NHD$_2$ and ND$_3$, it was considered highly unlikely that the loss would emanate from the A ring, based on the previously mentioned MS$^3$ experiments (m/z 389 → 372 → scan). The other major products of m/z 393, were at m/z 321 (318+3), 301(300+1), and 273(272+1). For the first one, the presence of two nitrogens and a proton carrying the charge was indicated and for the two latter ones, a shift of only one was observed as they were carbocations (no protonation) and only contained one nitrogen each. The H/D exchange experiments thus helped to strengthen the structural suggestions of the fragments and were thereby useful in the structural elucidation of the metabolites, as presented below. MS$^4$ experiments with 389 → 372→272→scan for M1 gave information on the fragmentation of the ion at m/z 272 and were used for comparison with the other hydroxylated metabolites.

Full product ion scan of M3 standard with the precursor ion m/z 401 corresponding to [M-H]$^-$, resulted in the main fragments m/z 357, 341, 314, 256 and 102, see Table 1 for proposed structures of the fragments and Fig. 2c for product ion spectrum.
Negative ESI gave the highest sensitivity for M3, probably owing to its carboxylic acid function.

**Mass Spectral Properties of Phase I Metabolites Produced In Vitro.** When finasteride was incubated with human liver microsomes and NADPH, five suspected phase I metabolites were detected of which three were hydroxylated forms with the same nominal \( m/z \) as M1. The hydroxylated metabolites that were not identical to M1 are referred to as MX and MY below. Interestingly, only a low signal in the mass chromatogram at the retention time of M1 was observed. M3 with \( m/z \) 401 (negative ESI) and M2 with \( m/z \) 387 (positive ESI) were both identified as metabolites of finasteride (results not shown).

MX and MY eluted after 7.4 and 7.7 minutes, respectively, compared with M1 standard that eluted after 6.2 minutes with the same method. See Fig. 3a for chromatograms and spectra of MX and MY. Full product ion scan of MX with the precursor ion \( m/z \) 389 corresponding to \([M+H]^+\), resulted in the main fragments at \( m/z \) 372, 321, 317, 300 and 272. It is notable that this compound also lost 17 u, corresponding to an OH radical, rather than water. However, it was concluded from the difference in retention time and product ion spectra that MX was structurally different from M1. The product ion spectrum was similar to the one of M1, but with one main difference; the fragment at \( m/z \) 321 was only found as a product ion for MX. This fragment did most probably correspond to the finasteride fragment at \( m/z \) 305 (loss of A ring) plus 16 for oxygen. MS³ analyses of MX (\( m/z \) 389 -> 321 -> scan) and finasteride (\( m/z \) 373 -> 305 -> scan) gave further evidence for these theories as the spectra contained
the same ions at the low mass end, see Fig. 4a and 4b. This means that the hydroxyl group of MX was not situated on the A ring. H/D exchange experiments resulted in a precursor ion at \(m/z\) 393 (389+4) for MX, which was identical to the shift of M1 and demonstrated that the hydroxylation had taken place on a carbon rather than on a heteroatom. The main resulting product ions of MX when using D\(_2\)O in the mobile phase were, \(m/z\) 325 (321+4), \(m/z\) 301(300+1) and 273(272+1). The first one probably formed by exchanging the hydrogens on the remaining two heteroatoms (oxygen and nitrogen), one carrying the charge and one formed by transfer of a deuterium to the B ring upon cleavage of the A ring. The latter two product ions were probably identical to the corresponding ions formed from M1 (c.f. Table 1). Furthermore, it was concluded from MS\(^3\) measurements that \(m/z\) 272 was the same fragment for both M1 and MX (results not shown). In summary, the mass spectral data mentioned above indicated that MX was hydroxylated on a carbon, and not on the steroid skeleton. However, all the methyl groups on the tertiary butyl group are identical, which means that no side chain hydroxylation different from that of M1 could be formed. Another plausible step might be that MX is a ring-closed form of M1, where the hydroxyl group has attacked the amide carbon, forming a five-membered ring lactol-type structure.

Full product ion scan of MY with the precursor ion \(m/z\) 389 corresponding to [M+H]\(^+\), resulted in the main fragments at \(m/z\) 371, 333, 315, 298, 272 and 270. Thus, MY was cleaved differently compared to M1 and MX. The product ion at \(m/z\) 371, corresponded to a loss of 18 u (H\(_2\)O) in contrast to the losses of 17 u for MX and M1. The formation of \(m/z\) 333 (\(m/z\) 317 of finasteride +16) was probably a result of the loss of the tertiary butyl group and suggested that the OH group was located on the steroid
skeleton and not on the tertiary butyl group, as for M1. The fragment \( m/z \) 315 was probably formed by a loss of water (-18 u) from \( m/z \) 333, the fragment \( m/z \) 298 was suggested to be formed by a loss ammonia (-17 u) from \( m/z \) 315. Furthermore, MY gave both \( m/z \) 272 and 270. \( M/z \) 272 must have had a structure different from the fragment of M1 with the same nominal mass as MS3 measurements of MY with 389→272→scan generated a slightly different fragmentation pattern compared to \( m/z \) 272 formed by M1. The ion at \( m/z \) 270 was suggested to be the same as \( m/z \) 272 from M1 and MX with an extra unsaturation formed by a loss of water from the steroid skeleton (\( m/z \) 272 of M1 –2 u). H/D exchange experiments gave further evidence for the above suggested structures.

MY gave the precursor ion at \( m/z \) 393(389+4), which was a shift identical to those for M1 and MY, which was in line with the suggestion that the hydroxylation had taken place on a carbon on this metabolite as well. The main resulting product ions after H/D exchange for MY were; \( m/z \) 373 (371+2), indicating the loss of D₂O (-20) from \( m/z \) 393, \( m/z \) 337 (333+4) indicating the presence of four exchangeable protons (on three heteroatoms and one protonation) and \( m/z \) 317 (315+2), corresponding to the loss of D₂O from \( m/z \) 337.

As the mass spectral data suggest that MY is hydroxylated on the steroid skeleton, it is likely that this metabolite is identical to the previously described M4. However, no reference compound was available for complete confirmation.

**Phase I Metabolites Detected In Vivo in Human Bile and Urine.** M3 has previously been quantified in plasma, bile and urine (Lundahl et al., 2009). M1, which has been described as a main metabolite in humans (Carlin et al., 1992), was not detected in any of the body fluids in this or the previous study and was not identified after \( \beta \)-glucuronidase
hydrolysis. Instead, two other compounds with \( m/z \) corresponding to hydroxy-finasteride were identified. Their retention times (7.5 and 7.7 minutes) and CID mass spectra matched those of MX and MY from the in vitro experiments (Fig. 3). The intensities of the peaks increased after \( \beta \)-glucuronidase hydrolysis, suggesting that these compounds were mainly excreted as glucuronic acid conjugates to both bile and urine. Identification of an intact conjugate will be presented below.

In addition, bile and urine samples were analyzed for the presence of the aldehyde, M2, and dihydroxy-metabolites. There was no clear evidence for the presence of either of those forms, neither with nor without hydrolysis.

**Phase II Metabolites Produced In Vitro.** The results from this study show that glucuronidation was the most important conjugation pathway for the finasteride phase I metabolites. One glucuronide with the precursor ion \( m/z \) 565 (389 +176 u) was produced when finasteride was incubated with NADPH and UDPGA for 24 hours in alamethicin-treated human liver microsomes (Fig. 5a). After full product ion scan of \( m/z \) 565 (CID, 35V) the glucuronide (7.5 minutes peak) gave the fragments \( m/z \) 389, 371, 333, 315, 298, 272 and 270. The spectrum corresponded to that of MY and it was concluded that the identified conjugate was MY glucuronide.

M1 and M3 were both glucuronidated after incubation of alamethicin-treated human liver microsomes in the presence of UDPGA. Two typical chromatographic patterns for M1 glucuronide (Fig. 6a) and M3 glucuronide (Fig. 6b) were found, using SRM with the transitions for protonated/deprotonated intact glucuronides and the three major product ions of each metabolite (see Materials and Methods for more details). To
determine the most important human UGT form for glucuronidation of M1 and M3, these metabolites were incubated with twelve different UGT supersomes. By SRM measurements and comparison of intensities of peaks it was obvious that UGT1A4 and UGT1A3 were the main enzymes responsible for the production of M1 and M3 glucuronide, respectively.

There was no clear evidence for sulphate conjugation after incubation of M1, M3 or finasteride with PAPS and S9 fractions of human liver. In addition, there was no clear evidence for glutathione conjugation when M1, M3 or finasteride were incubated with NADPH and GSH in human liver microsomes.

**Intact Glucuronides Identified In Vivo in Human Bile and Urine.** SRM was used to search for intact M1 and M3 glucuronides in bile and urine samples. The M1 glucuronide was not found and there were only traces of M3 glucuronide in bile from two of four examined individuals. Another glucuronide, with the precursor ion \(m/z\) 565 (positive ESI), and that eluted after 7.5 minutes was detected in both bile and urine (Fig. 5b and 5c). The spectrum of the peak included fragments corresponding to those of MY glucuronide, produced in vitro, with product ions of \(m/z\) 389, 371, 333, 315, 298, 272 and 270. The MY glucuronide was present in all examined bile and urine samples. The other peaks in the chromatograms (Fig. 5) did not contain any fragments matching those of the finasteride metabolites.
Discussion

In this study, novel metabolites of finasteride were identified, \textit{in vivo}, in human bile and urine. Two hydroxy-metabolites, MX and MY, were identified. MX is described for the first time in this paper and it is possible that it is a ring-closed form of M1. It was considered likely that MY was identical to the previously found M4. Furthermore, MY glucuronide, was identified in this study. It was the first time that intact glucuronides of finasteride were identified. MY glucuronide was the major glucuronide in human bile and urine. M1 was glucuronidated \textit{in vitro} but the glucuronide was not identified in the \textit{in vivo} samples. M3 was also glucuronidated \textit{in vitro} and trace amounts of the glucuronide was identified in human bile.

Previously, the phase I metabolism of finasteride has been investigated and four major metabolites identified, M1-M4 (Fig.1), of which two are hydroxy-metabolites (M1 and M4) (Ishii et al., 1994, Huskey et al., 1995). Another hydroxy-metabolite (11-α-hydroxy finasteride) was produced \textit{in vitro}, after incubation with green algae (\textit{Selenastrum capricornutum}), but was not identified in humans (Venkataramani et al., 1994). M4 was described as a minor metabolite in human plasma (Carlin et al., 1987). M1 was previously identified as a major metabolite in human and dog plasma and the fraction of formed M1 was lower in humans than in dogs. The M1 concentrations were determined by high performance liquid chromatography with ultraviolet detection (HPLC-UV) after oral administration of high doses of finasteride (10 or 80 mg/kg to dogs and 38 mg to humans) (Carlin et al., 1992, Carlin et al., 1997). For comparison, in this study, the healthy volunteers were administered 5 mg of finasteride which is the therapeutic dose for BPH treatment. The results obtained in this investigation, in
combination with those described in the previous publication (Lundahl et al., 2009) suggest that M1 is not a principal metabolite in humans. It was not detected when a more sensitive and selective analytical method was applied (LC-MS/MS) and when the therapeutic dose of finasteride was administered. The identification of MX and MY has broadened the knowledge of the metabolism of finasteride in male subjects. However, the finding of MX and MY indicates that there are at least one other parallel pathway of metabolism competing with the formation of M3 (Fig. 1).

In this study, both MX and MY were identified in human bile and this is the first time that metabolite identification for finasteride has been performed directly in human bile. Metabolite identification in rat bile has been performed and preliminary data have been reported (Ishii et al., 1992). In male rats, biliary excretion of metabolites accounted for approximately 64% of the oral finasteride dose. In humans, almost 60% of a radioactive dose was recovered in feces as metabolites and this indicated that biliary excretion was probably the major route of elimination for these metabolites (Carlin et al., 1992). However, the metabolites found in feces were not identified. Radioactively labeled drug detected in feces has been traditionally used to estimate the amount of drug excreted via bile in humans (Ghibellini et al., 2006a). This mass balance approach, however, does not distinguish between amount of unabsorbed parent drug and amount unchanged drug excreted in bile. The approach is further limited by additional factors such as: i) biliary clearance is impossible to estimate with feces sampling, ii) feces collection results in a difficult matrix for metabolite identification and iii) unstable metabolites, for example glucuronides that are being hydrolyzed by enzymes along the intestine, can be difficult to quantify properly. By collecting bile directly from the small intestine (proximal jejunum)
it is possible to estimate biliary clearance (Bergman et al., 2006, Ghibellini et al., 2006a) and directly search for metabolites, including intact glucuronides in a relatively simple matrix. This can provide a more accurate prediction of the exposure of the drug and its metabolites to the liver, gall bladder and biliary duct. One previous example of metabolite identification directly in bile is the identification of a new glucuronide of piperacillin after sampling from healthy volunteers via an oroenteric tube (Ghibellini et al., 2006b). Another example of a more advanced and complicated method for bile collection and metabolite identification is the application of a nasobiliary drainage tube to cholangiocarcinoma patients (Teichert et al., 2009). However, this advanced method can not be applied to healthy volunteers.

In this study, the sampling of bile from healthy volunteers was performed with an intestinal single-pass perfusion technique, Loc-I-Gut, which has been used for many years by this research team with the main purpose to study drug permeability, absorption and dissolution (Lennernäs et al., 1992, Knutson et al., 1989, Bonlokke et al., 1999). More recently, the method has been utilized to investigate the biliary excretion of drugs and metabolites after collection of the biliary fluid secreted to the upper part of the jejunum (Bergman et al., 2006, Persson et al., 2006). Bile was collected for 3 hours after finasteride administration and M1, M3 and finasteride were quantified with LC-MS/MS and reported in a pharmacokinetic herb-drug interaction study (Lundahl et al., 2009). As reported, M1 was not detected in bile. Finasteride and M3 were present in bile but the amount found represented only a low fraction of the dose (~0.01% and ~0.7%, respectively). It is likely that the detected fraction of M3 excreted to bile would have been higher if it had been possible to collect bile for a longer period of time. However,
we believed it was likely that other metabolites could be detected in the bile and this material was thus used for metabolite identification. The results presented here illustrate how the Loc-I-Gut technique, in combination with sensitive LC-MS/MS analyses has the potential to contribute to safety assessments of new chemical entities by generating valuable knowledge of metabolites excreted to bile.

The objective of this study was not only to search for phase I metabolites but also to identify phase II metabolites excreted to bile and/or urine. Phase II metabolism for finasteride has been briefly reported. In dog, there was indirect evidence (after hydrolysis) for glucuronide formation and in rat, one glucuronide was found in bile (Carlin et al., 1997, Ishii et al., 1992). In this study, MY glucuronide, was discovered in both bile and urine from all investigated subjects. Identification of this glucuronide in bile indicates that MY can probably undergo enterohepatic circulation, after hydrolysis and intestinal re-absorption, leading to possible increased hepatobiliary exposure and an expected long terminal plasma half-life. Today, there is no information on the safety aspects of this finding. Finasteride has a terminal half-life of ~5 hours in humans but its pharmacodynamic effect lasts up to 72 hours (Ohtawa et al., 1991). Contribution of an active metabolite with a long half-life could explain this observation. However, an alternative explanation may be that finasteride binds irreversibly to 5α-reductase and that the biosynthesis of new enzyme takes up to 72 hours (Tian, 1996, Faller et al., 1993). M1 and M3 were both glucuronidated in vitro in the present study by human liver microsomes and supersomes. UGT1A4 and UGT1A3 were the most active enzymes in the glucuronidation of M1 and M3, respectively. However, M1 glucuronide was not identified in vivo and M3 glucuronide was present at very low amounts in bile of only a
few individuals. M3 glucuronide is an acyl-glucuronide, an electrophilic conjugate that can cause toxicity by binding to proteins or DNA (Benet et al., 1993, Shipkova et al., 2003). The identification of M3 glucuronide may be an important finding and should thus be further investigated.

Finasteride has been on the market for almost two decades and is considered a safe drug in long-treatment effect studies (Lowe et al., 2003, Vaughan et al., 2002). However, few published investigations examine the pharmacokinetics and metabolism of the drug. Recently, a clinical practice guideline was published with recommendations on the use of 5α-reductase inhibition in prostate cancer chemoprevention (Kramer et al., 2009). If finasteride will come to be used for chemoprevention in healthy men and prescribed to a wider population, we believe that it is important to have a broader understanding of the metabolism of this drug. With this study new information about the metabolism of finasteride has been given and novel metabolites have been identified in human bile and urine. Further investigation is needed to understand the importance of this finding both from a safety and efficacy perspective.

In summary, M3, two hydroxy-metabolites, MX and MY, and one intact glucuronide, MY glucuronide, were identified in both human bile and urine. For the first time, intact glucuronides of finasteride were identified. MY glucuronide was the main glucuronide, found both in bile and urine and low amounts of M3 glucuronide, an acyl-glucuronide, were identified in human bile. To our knowledge, this is the first time that human bile has been used for the identification of previously unknown metabolites of finasteride.
References


Shibasaki H, Tanabe C, Furuta T, Kasuya Y (2001) Hydrolysis of conjugated steroids by the combined use of beta-glucuronidase preparations from helix pomatia and...


Legends for figures

FIG. 1: Chemical structure of finasteride and the metabolites discussed in this paper. M1-M4 have previously been described (Huskey et al., 1995).

FIG. 2: Full scan product ion spectra after collision induced dissociation (CID) of synthetic standards for A, finasteride (100 µg/ml), m/z 373 positive ion mode (CID 25 V), B, M1 (99 µg/ml), m/z 389 positive ion mode (CID 25 V), and C, M3 (103 µg/ml), m/z 401 negative ion mode (CID 35 V). Measurements were performed at the triple quadrupole mass spectrometer.

FIG. 3: Extracted ion chromatograms and spectra after full product ion scan of the precursor m/z 389 (CID 25V). Two hydroxylated metabolites (MX) (the first peak) and (MY) (the second peak) A, formed in vitro after 1 hour incubation of finasteride (100 µM) with human liver microsomes and NADPH and B, identified in urine, here from one representative sample 3 hours after finasteride administration. The urine sample was treated with β-glucuronidase for 2 hours prior to analysis. Measurements were performed at the triple quadrupole mass spectrometer. The arrow at 6.2 minutes indicates the retention time of M1.

FIG. 4: A, MS³ (373 (40 V)→305 (35 V)→scan (m/z 80-400) of finasteride standard solution, B, MS³ (389 (35 V)→321 (30 V)→scan (m/z 85-400) of MX produced in vitro and C, MS³ (389 (35 V)→333 (40 V)→scan (m/z 90-400) of MY produced in vitro. Measurements were performed at the LCQ ion trap instrument.
FIG. 5: Extracted ion chromatograms of \textit{m/z} 389 and spectra after full product ion scan of the precursor ion \textit{m/z} 565 (CID 35 V) corresponding to MY glucuronide identified A, \textit{in vitro} B, in urine and C, in bile. Measurements were performed at the triple quadrupole mass spectrometer.

FIG. 6: Ion chromatograms after full scan for the precursor ion in combination with single reaction monitoring (SRM) for specific fragments of A, M1 glucuronide (precursor ion \textit{m/z} 565, positive ion mode) and B, M3glucuronide (precursor ion \textit{m/z} 577, negative ion mode). Both glucuronides were formed \textit{in vitro} after M1/M3 (100 µM) incubation with alamethicin activated human liver microsomes and UDPGA for 24 h. Measurements were performed at the triple quadrupole mass spectrometer.
Table 1 Proposed structures for the most abundant fragments from MS/MS analysis of finasteride (precursor ion \( m/z \) 373, positive scan), M1 (precursor ion \( m/z \) 389, positive scan) and M3 (precursor ion \( m/z \) 401, negative scan).

<table>
<thead>
<tr>
<th>Finasteride</th>
<th>M1</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>( m/z(+D)^b )</td>
<td>Proposed fragments</td>
<td>( m/z(+D)^b )</td>
</tr>
<tr>
<td>373 (+3)</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>389 (+4)</td>
</tr>
<tr>
<td>317(^a) (+3)</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>372 (+2/1)</td>
</tr>
<tr>
<td>305(^a) (+2)</td>
<td><img src="image7.png" alt="Structure" /></td>
<td>317 (+3)</td>
</tr>
<tr>
<td>300 (+1)</td>
<td><img src="image10.png" alt="Structure" /></td>
<td>314</td>
</tr>
<tr>
<td>272 (+1)</td>
<td><img src="image12.png" alt="Structure" /></td>
<td>256</td>
</tr>
<tr>
<td>72</td>
<td><img src="image14.png" alt="Structure" /></td>
<td>102</td>
</tr>
</tbody>
</table>

\(^a\)Structures previously suggested by Constanzer et al., 1994  \(^b\)Increase in \( m/z \) after hydrogen/deuterium exchange
Figure 1

Finasteride → M1 → M2 → M3

MX

M4 (MY) → M1 glucuronide → MY glucuronide

M3 glucuronide
Figure 6

(A) RT: 5.0 - 9.0

Relative Abundance

5.9 6.7 7.2 7.8 8.6

6.5

NL: 5.73E3

m/z = 229.50-230.50

RT: 6.5 - 7.6

Relative Abundance

6.6 6.9

7.2

7.1

NL: 4.54E4

m/z = 174.50-175.50

(B) RT: 6.5 - 7.6

Relative Abundance

6.6 6.9 7.2 7.2

7.1

NL: 2.74E5

m/z = 192.50-193.50

RT: 7.1

Relative Abundance

6.8 7.0

7.3 7.5

NL: 4.63E5

m/z = 400.50-401.50

Time (min)

5 6 7 8 9

6.2 6.3 6.7 7.9 7.8

Time (min)

6.6 7.0

7.2 7.4