UDP-Glucuronosyltransferases (UGTs) 2B7 and UGT2B17 Display Converse Specificity in Testosterone and Epitestosterone Glucuronidation, whereas UGT2A1 Conjugates Both Androgens Similarly

Taina Sten, Ingo Bichlmaier, Tiia Kuuranne, Antti Leinonen, Jari Yli-Kauhaluoma, and Moshe Finel

Centre for Drug Research (T.S., M.F.) and Division of Pharmaceutical Chemistry (T.S., I.B., J.Y.-K.), Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; and Doping Control Laboratory, United Laboratories Ltd., Helsinki, Finland (T.K., A.L.)

ABSTRACT:
Testosterone and epitestosterone are endogenous steroids that differ in the configuration of the hydroxyl-bearing carbon at C-17. Testosterone is the predominant male sex hormone, whereas the role of epitestosterone is largely unclear. In humans, both androgens are excreted mainly as glucuronide conjugates and the urinary ratio of testosterone to epitestosterone (T/E), used to expose illicit testosterone abuse, indicates the relative concentrations of the respective glucuronides. Some male athletes have T/E values greater than the accepted threshold value (4.0), even without testosterone abuse. We have analyzed athletes’ urine samples and found that the main reason for such false-positive results in doping tests was a low epitestosterone glucuronide concentration not at a high level of testosterone glucuronide. Sulfate conjugates of both testosterone and epitestosterone were also detected in the different urine samples. Glucuronidation assays with the 19 human UDP-glucuronosyltransferases (UGTs) of subfamilies UGT1A, UGT2A, and UGT2B revealed that UGT2B17 is the most active enzyme in testosterone glucuronidation. UGT2B17 does not glucuronidate epitestosterone, but inhibition studies revealed that it binds epitestosterone with affinity similar to that of testosterone. Epitestosterone glucuronidation is catalyzed mainly by UGT2B7, and the $K_m$ of this reaction is significantly lower than the $K_m$ of UGT2B17 for testosterone. Although UGT2B7 and UGT2B17 exhibited high, although converse, stereoselectivity in testosterone and epitestosterone glucuronidation, UGT2A1, an extrahepatic enzyme that is expressed mainly in the nasal epithelium, catalyzed the glucuronidation of both steroids at considerable rates and similar kinetics. The results shed new light on the substrate specificity and stereoselectivity of human UGTs.

Testosterone (4-androsten-17β-ol-3-one) is an endogenous sexual hormone that is secreted primarily in the testes of males but also in the adrenal gland, and, therefore, it is also found in females. Testosterone is used for androgen-replacing therapy, and it has been abused in sports (Beg et al., 2008; Sjöqvist et al., 2008). Epitestosterone (4-androsten-17α-ol-3-one) is the 17α-epimer of testosterone (Fig. 1), and much less is known about its biological role, as well as biosynthesis and elimination (Starka, 2003). Testosterone and epitestosterone are present mainly in the urine as the respective glucuronides, but sulfate conjugates and even the unconjugated form of these steroids were found (Dehennin, 1994; Borts and Bowers, 2000; Schulze et al., 2008a). The interest in testosterone glucuronidation is often linked to studies on anabolic steroid abuse by male athletes (Dehennin 1994; Schulze et al., 2008a,b). The urinary testosterone to epitestosterone concentration ratio, T/E, serves as a marker for possible testosterone abuse among male athletes (Donike et al., 1983; World Anti-Doping Agency, WADA Technical Document—TD2004EAAS, 2004, http://www.wada-ama.org/rtecontent/document/end_stereoids_aug_04.pdf). Presently, a T/E >4.0 is considered suspicious and requires further examination according to the World Anti-Doping Agency Guidelines for elevated T/E (2006, http://www.wada-ama.org/rtecontent/document/GuidelineReportingManagementElevatedTERatios.pdf). The T/E value, the analytical response ratio of testosterone and epitestosterone in urine samples after treatment with β-glucuronidase, is practically equivalent to the glucuronide ratio of the two steroids. Therefore, identification of the individual UDP-glucuronosyltransferases (UGTs) that catalyze the glucuronidation of testosterone and epitestosterone is required for better interpretation of the T/E and the factors that affect its value, such as genetic polymorphism.

Glucuronidation is a phase II metabolic reaction, in which the glucuronic acid moiety of UDP-α-d-glucuronic acid (UDPGA) is conjugated to various aglycones, thereby increasing aqueous solubility and stimulating their excretion into urine or bile. Glucuronidation is catalyzed by the UGTs, a family of membrane-bound enzymes of
the endoplasmic reticulum (King et al., 2000; Tukey and Strassburg, 2000). The human genome encodes some 19 UGT isoforms, which are divided into three subfamilies (UGT1A, UGT2A, and UGT2B) (Mackenzie et al., 2005). These proteins are found in several different tissues, and many UGTs, but not all, are highly expressed in the liver. The substrate specificity of the different UGTs is very complex. One of the major goals in current glucuronidation research is to understand the interactions of individual UGTs with different compounds, including the regio- and stereoselectivity of these enzymes.

Testosterone and epitestosterone are diasteromers that differ only in the configuration of the carbon 17, the one bound to the hydroxyl group that undergoes conjugation during the enzyme-catalyzed glucuronidation reaction. A series of studies by Bichlmair et al. (2007) on the interactions of selected UGTs with different chiral compounds led to the development of high-affinity and high-specificity inhibitors for UGT2B7 and set us on the route to the present work. In addition, we have recently shown that the configuration of C17 in another important steroid, estradiol, has a major effect on its glucuronidation by different UGTs (Iitahiro et al., 2008).

In a previous study of four UGTs of subfamily 2B, namely 2B4, 2B7, 2B15, and 2B17, only the latter enzyme exhibited high testosterone glucuronidation activity (Turgeon et al., 2001). In agreement with a major role for UGT2B17 in testosterone glucuronidation, it was found that people lacking UGT2B17 owing to a common genetic deletion excrete significantly less testosterone glucuronide (Jakobsson et al., 2002). Nevertheless, even the deletion carriers had detectable testosterone glucuronides in their urine, indicating that other UGTs are also capable of testosterone glucuronidation. In addition to UGT2B17, few other UGTs were shown to catalyze testosterone glucuronidation in their urine, indicating that other UGTs are also capable of testosterone glucuronidation. In addition to UGT2B17, few other UGTs were shown to catalyze testosterone glucuronidation (Green and Tephly, 1996; Jedlicky et al., 1999; Turgeon et al., 2001; Kuuranne et al., 2003; Bovill and Bowlin, 2007), but no systematic analysis of all the human UGTs for their ability to glucuronidate testosterone has been reported.

Although the urinary concentration of epitestosterone glucuronide is an integral part of the T/E, the glucuronidation of epitestosterone by recombinant UGTs has been reported only rarely, implicating UGT1A4 and UGT2B7 in this activity (Green and Tephly, 1996; Coffman et al., 1998). In the present study we have examined the testosterone and epitestosterone glucuronidation activities of the 19 human UGTs, revealing the central roles of UGT2B17 and UGT2B7, respectively, in these activities. UGT2A1, on the other hand, may not play a major role in determining the T/E, but it exhibited interesting activity that, together with the converse stereoselectivity of UGT2B7 and UGT2B17, deepens our understanding of the interaction of human UGTs with different steroids.

Materials and Methods

Materials. Methyltestosterone and testosterone glucuronide were obtained from Steraloids (Newport, RI), and epitestosterone glucuronide sodium salt was from the National Analytical Reference Laboratory (Pymble, Australia). Deuterated δ7-testosterone and δ5-epitestosterone were from the National Measurement Institute (Pymble, Australia). Testosterone (4-androsten-17β-ol-3-one), epitestosterone (4-androsten-17α-ol-3-one), uridine-5′-diphosphoglucuronic acid trisodium salt, D-saccharic acid-1,4-lactone, ammonium iodide, and dithioerythritol were purchased from Sigma-Aldrich (St. Louis, MO). Magnesium chloride hexahydrate, disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium bicarbonate, sodium sulfate, diethyl ether, n-heptane, perchloric acid, sodium acetate, sodium hydroxide, and sodium chloride were from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and formic acid, analytical grade, were from Riedel-de Haen (Seelze, Germany). Trimethylchlorosilane and N-methyl-N-trimethylsilyl trifluoroacetamide were from Fluka (Sigma-Aldrich, Steinheim, Germany). β-Glucuronidase from Escherichia coli K12 was from Roche Diagnostics (Mannheim, Germany). All of the solvents were high-performance liquid chromatography grade, and the eluents were filtered through a 0.22-μm filter for UPLC analysis.

Recombinant UGTs. Recombinant human UGTs of subfamilies UGT1A1 and UGT2B7 were expressed in baculovirus-infected insect cells as described previously (Kurkela et al., 2007, and references therein). The human UGTs of subfamily 2A were expressed in the same system, but the details of their cloning will be published elsewhere (N. Sneitz, M. H. Court, X. Ding, and M. Finel, manuscript in preparation). The recombinant UGTs carried a short C-terminal fusion peptide ending with six His residues, a His-tag. The latter provides the possibility of comparing the expression level of each UGT in the insect cell microsomal preparations and, despite large differences between them in this respect, of evaluating the relative activity of the different enzymes, the so-called normalized activity. Protein concentrations were determined by the BCA method (Pierce Biotechnology, Rockford, IL). The relative expression level of each recombinant UGT was determined by dot-blot analyses using tetra-His antibodies (Qiagen, Hilden, Germany), as detailed elsewhere (Kurkela et al., 2007). Commercial UGT2B15 (BD Supersomes; BD Gentest, Woburn, MA) was also used for the activity screens. The latter enzyme, like our recombinant UGTs, is expressed in baculovirus-infected insect cells, but it does not carry a His-tag. Hence, its expression level could not be compared with that of the other UGTs in this study.

Analytical Methods. A Waters Acquity UPLC system (Waters, Milford, MA) with Empower 2 software was used for the analysis. Solvents were 0.1% HCOOH (aqueous) (A) and 0.1% HCOOH in acetonitrile (B) with gradient elution of 0 to 3 min B (10–55%), 3 to 3.5 min B (55–90%), and 3.5 to 3.6 min B (90–100%), followed by an equilibrium time of 2.4 min. The flow rate was 0.2 ml/min, and column temperature was 50°C. An Acquity BEH Shield RP18 column (100 × 0.1 mm i.d. with 1.7-μm particle size; Waters) was used for the separation, combined with UV detection at 246 nm. The injection volume was 3 μl.

Analytical Method Calibration and Validation. Stock solutions were prepared in 70% (v/v) acetonitrile (epitesterone glucuronide, 1.0 mM) or methanol (testosterone glucuronide, 2.6 mM) and stored at −20°C. Standard calibration working solutions of each analyte were prepared separately in acetonitrile. The samples for the calibration curves were prepared as duplicates by evaporating the solvent of the standard solution and reconstituting with blank incubation matrix to obtain glucuronide concentrations within the range of 0.25 to 100 μM. The analytical method was validated with respect to specificity, accuracy, precision, and limit of quantification in line with the previous recommendation (Shah et al., 2000). The limits of detection and quantification were determined at signal/noise ratios of 3 and 10, respectively. In the case of analytes that were to be frozen before analysis, analyte stability during freeze-thaw cycles was assessed as recommended by Nowatzke and Wood (2007).

Incubation Conditions for UGT Screening. The assays to identify the human UGT isoforms that are active in testosterone and epitestosterone glu-
curonidation were performed in the presence of 5 mM UDPGA, 5 mM D-saccharic acid-1,4-lactone, 50 mM Na-K-phosphate buffer (pH 7.4), and 5 mM MgCl₂. The protein concentration was between 0.2 and 1.25 mg/ml in a total reaction volume of 50 μl. The aglycone was added as a DMSO solution so that the final DMSO concentration was 5% in all assays. It may be noted here that because this was a screening experiment, the conditions were not optimized for each UGT. Control incubations were performed in the presence of the highest aglycone substrate concentration and in the absence of UDPGA, as well as in the absence of aglycone substrate but in the presence of UDPGA. The reactions were started by the addition of UDPGA, and the samples were incubated at 37°C in a shaking incubator for 120 min. The reactions were terminated by the addition of ice-cold 4 M perchloric acid, 10% of the reaction volume, and transfer to ice. The mixtures were then centrifuged for 10 min at 16,000 g, and aliquots of the supernatants were used for the UPLC analyses. The reactions were carried out in triplicate, except for the negative controls that were single samples. The glucuronidation rate was expressed as the amount of glucuronides formed (picomoles) per protein amount (milligrams) and the reaction time in minutes. The results were also “normalized,” or corrected, with respect to the relative expression level of the UGT in each sample. The normalization was done by dividing the measured glucuronidation rate of a given recombinant UGT by its relative expression level (Kurkela et al., 2007). In this study we have used 1.0 as the expression level of UGT2B7.

Kinetic Analyses. Kinetic assays of testosterone and epitestosterone glucuronidation by UGT2B7 and UGT2B17, respectively, and both substrates by UGT2A1, were performed. The reaction conditions were similar to those in the coruronidation by UGT2B7 and UGT2B17, respectively, and both substrates by UGT2A1, and obtained from routine doping control samples with written consent allowing the use for research purposes. To achieve deeper insight into testosterone and obtained from routine doping control samples with written consent allowing the use for research purposes. To achieve deeper insight into testosterone

Kinetic Analyses. Kinetic assays of testosterone and epitestosterone glucuronidation by UGT2B7 and of testosterone on epitestosterone glucuronidation by UGT2B7 were studied using incubation conditions as described above. The substrate concentration ranged from 10 to 125 μM, and the “inhibitory” diastereomer concentration was either 10, 25, or 50 μM. The raw data points were fitted into the competitive inhibition model equation

\[ F = V_{\text{max}} \cdot X/(K_m(1 + [I]/K_I) + X) \]

where \( X \) is the substrate concentration (micromolar), \( F \) is the measured activity in picomoles per milligram of protein per minute, \( V_{\text{max}} \) is maximum velocity in picomoles per milligram of protein per minute, \( K_m \) is the Michaelis-Menten constant, \( I \) is the inhibitor concentration (micromolar), and \( K_I \) is the inhibition constant. The data fit to the competitive inhibition model was verified by an Eadie-Hofstee plot.

Urinary Steroid Profiles. Urine samples were selected on the basis of their T/E and subjected to steroid profile analysis. The samples were anonymous and obtained from routine doping control samples with written consent allowing the use for research purposes. To achieve deeper insight into testosterone and epitestosterone conjugation in male athletes, we selected samples for three groups, each with a different level of T/E: 1) “low,” individuals with T/E <0.2 (n = 20); 2) “normal,” individuals with T/E in the range of 0.8 to 1.2 (n = 20); and 3) “high,” individuals with T/E >4.0 (n = 14). It should be highlighted here that samples with T/E >4.0 (high group) were only taken from individuals for whom there was no indication of drug abuse, as determined by gas chromatography/combustion/isotope ratio mass spectrometry analysis. The samples were analyzed for testosterone and epitestosterone glucuronides and for the sulfate conjugates of the two steroids. Sample preparation for glucuronide determination was based on the procedure of Geyer et al. (1994). A 2-ml aliquot of urine was spiked with 20 μl of internal standard (solution containing methyltestosterone, \( d_4 \)-testosterone, and \( d_4 \)-epitestosterone at concentration levels of 50, 3, and 3 μg/ml, respectively) and buffered with 0.75 ml of phosphate buffer (2 M, pH 7.0). Enzymatic hydrolysis was performed by adding 50 μl of β-glucuronidase solution, corresponding to 7 IU of β-glucuronidase activity and incubating the samples at 50°C for 60 min. After addition of 0.5 ml of aqueous potassium carbonate-potassium bicarbonate solution (1:1, 20%, v/v) and 2 g of sodium sulfate, the hydrolysat was vortex-mixed with 5 ml of diethyl ether for 30 s. After centrifugation, the organic phase was separated and evaporated to dryness.

Sample preparation for the determination of sulfate conjugates was based on a published procedure (von Kuk and Schänzer, 2004). A 2-ml aliquot of urine was treated as described above. After separation of the organic phase, 1 ml of sodium acetate buffer (1 M, pH 4.9) was added to the aqueous phase. The mixture was then applied to a C18 solid-phase extraction cartridge (SEP-PAK; Waters), which was preconditioned with methanol and water. The column was first rinsed with 5 ml of water and 2.5 ml of n-heptane and then eluted with 3 ml of methanol. The eluent was evaporated to dryness and then dissolved in 0.5 ml of anhydrous hydrogen chloride in methanol (1 M). Hydrolysis of the sulfate conjugates was performed at 60°C for 15 min. After addition of 3 ml of aqueous mixture of 2 M NaOH and 4 M NaCl, the hydrolysat was vortex-mixed with 5 ml of diethyl ether for 30 s, and after subsequent centrifugation the organic phase was separated and evaporated to dryness. Isolated steroid fractions were derivatized with 50 μl of N-methyl-N-trimethylsilyl trifluoroaceticamid-ammonium iodide-dithioerythritol (1000:2:4, v/v/w) at 60°C for 15 min.

Gas Chromatography/Mass Spectrometry. Gas chromatography/mass spectrometry was performed on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) and an Agilent 5973N mass selective detector (Agilent Technologies). Steroids were separated on an Agilent HP-1 fused silica capillary column (16 m, 0.2 mm i.d., film thickness 0.11 μm). Injection of 2 μl was done in split mode (1:15) at 280°C. The carrier gas was helium (0.5 ml/min, constant flow mode). The oven was first ramped from 120 to 230°C at 3°C/min and then up to 310°C at 30°C/min and held at the final temperature for 3.5 min. The mass spectrometer was operated in electron ionization mode (70 eV) using selected ion monitoring. For each analyte one specific ion was measured with the dwell time of 10 ms.

Results

We have examined the absolute and relative concentrations of urinary testosterone and epitestosterone glucuronides and sulfate conjugates from selected doping control samples, all of which were declared as negative for testosterone abuse in routine analysis. To get deeper insight into the results, the urine samples were divided into three groups, based on the screened T/E, according to the criteria as described under Urinary Steroid Profiles. The results (Fig. 2) revealed, as expected, that samples in the low group contained very low concentration of testosterone glucuronide, potentially due to inactive UGT2B17 (Schulze et al., 2008a) or to limitations in the transport of testosterone glucuronide.

Another noteworthy finding came from the high group, namely athletes having urine samples with T/E >4.0. These samples had, therefore, been subjected to laborious and time-consuming gas chromatography/combustion/isotope ratio mass spectrometry analysis, but the athletes were found not to have abused exogenous testosterone. The levels of testosterone glucuronide in these samples from the high group were practically the same as those from the normal group, but their epitestosterone glucuronide level was lower. Hence, the high T/E in the samples from the high group was due to a very low concentration of epitestosterone glucuronide not an exceptionally high level of testosterone glucuronide (Fig. 2).

Most of the urinary testosterone was present as glucuronide not as sulfate conjugates (Fig. 2, normal and high groups). On the other hand, a significant portion of the epitestosterone in the urine, some 30 to 50%, was present as epitestosterone sulfate (Fig. 2). At the outset of these experiments, we anticipated that athletes with a low concentration of urinary testosterone glucuronide will have a higher concentration of testosterone sulfate. Intriguingly, the results proved us wrong, and the sharp decrease in the concentration of testosterone sulfate...
glucuronide (low group) was not accompanied by an increase in the concentration of testosterone sulfate (Fig. 2).

We embarked on identifying the enzymes that are active in testosterone and epitestosterone glucuronidation to gain a better understanding of the substrate specificity of the human UGTs as well as to characterize factors that affect the T/E and detection of anabolic steroid abuse. After incubations, glucuronides were detected using a UPLC system, and the retention times of testosterone glucuronide and epitestosterone glucuronide were 2.4 and 2.6 min, respectively. The retention times of the unconjugated testosterone and epitestosterone were 3.1 and 3.3 min, respectively. The limits of detection and quantitation for both testosterone and epitestosterone glucuronides were 0.1 and 0.25 μM, respectively (signal/noise ratio > 3 and 10, n = 5). The accuracy and precision of the method were acceptable with respect to the guidelines for bioanalytical method validation (Shah et al., 2000). The between-assay coefficients of variation for testosterone glucuronide and epitestosterone glucuronide were less than 11%.

Several human UGTs exhibited detectable glucuronidation activity toward either testosterone or epitestosterone. Nevertheless, each of these two androgens was primarily glucuronidated by a single enzyme: testosterone by UGT2B17 and epitestosterone by UGT2B7 (Table 1; Figs. 3 and 4). Hence, although testosterone and epitestosterone are diastereomers and highly similar in their physicochemical properties, they are largely glucuronidated by two different enzymes, the UGTs of subfamily 2B that are 76% identical to each other in their primary protein structure. The other human UGTs that displayed detectable testosterone glucuronidation activity were 1A3, 1A4, 1A8, 1A9, 1A10, 2A1, 2A2, 2B7, and 2B15 (Fig. 3). The activity of most of them, with the exception of UGT2A1, was very low (see more about UGT2B15 below). As for UGT2A1, it should be noted here that because this enzyme is expressed mainly in the nasal epithelium, it is unlikely to contribute significantly to the urinary concentration of testosterone and epitestosterone.

In the case of UGT2B15 we have noticed that the specific activity of our recombinant enzyme is significantly lower than that of commercially available UGT2B15 (Itäahio et al., 2008). Therefore, here we tested both our preparation (the expression level of which can be compared with that of the other recombinant UGTs) and UGT2B15 from a commercial source. Our results also show that the commercial

| Table 1 |
|------------------|------------------|
| **Testosterone and epitestosterone glucuronidation activity screen of all the recombinant human UGTs of subfamilies 1A, 2A, and 2B** |
| Values are averages of three replicates ± S.D. Rates were not corrected for expression level. |
| UGT | Glucuronide formation pmol/mg of protein/min |
| Testosterone | Epitestosterone |
| 1A1 | n.d. | n.d. |
| 1A3 | 4.6 ± 0.3 | n.d. |
| 1A4 | 5.5 ± 0.3 | 4.9 ± 0.8 |
| 1A5 | n.d. | n.d. |
| 1A6 | n.d. | n.d. |
| 1A7 | n.d. | n.d. |
| 1A8 | 2.7 ± 0.1 | n.d. |
| 1A9 | 10.4 ± 0.5 | n.d. |
| 1A10 | 7.9 ± 0.8 | n.d. |
| 2A1 | 253 ± 5.4 | 172 ± 0.9 |
| 2A2 | 13.5 ± 0.5 | 69.7 ± 5.4 |
| 2A3 | n.d. | n.d. |
| 2B4 | n.d. | 8.1 ± 0.6 |
| 2B7 | 4.6 ± 0.1 | 312.5 ± 20.0 |
| 2B10 | n.d. | n.d. |
| 2B11 | n.d. | n.d. |
| 2B15 | 7.9 ± 0.1 | n.d. |
| 2B15c | 65 ± 2.9 | n.d. |
| 2B17 | 508.9 ± 19.6 | n.d. |
| 2B28 | n.d. | n.d. |

n.d., no detectable activity.

*UGT2B15 Supersomes.*
increased epitestosterone in the testosterone glucuronidation assay led to increased glucuronidation activity of UGT2B17 followed Michaelis-Menten kinetics (Fig. 5; Table 2). The presence of increasing concentrations of epitestosterone, we turned to kinetic analyses. The testosterone glucuronidation activity of UGT2B17 was particularly high (Fig. 4). To find out whether or not UGT2B17 binds testosterone glucuronidate both testosterone and epitestosterone at considerable rates. Hence, UGT2A1 is perhaps the only human enzyme that can glucuronidate both testosterone and epitestosterone at considerable and similar rates (Figs. 3 and 4). The kinetics of testosterone and epitestosterone glucuronidation by UGT2A1 are comparable, if not identical (Fig. 7; Table 2). The glucuronidation reactions of UGT2A1 followed Michaelis-Menten kinetics (Fig. 6). Interestingly, the $K_m$ value in this case (1.7 $\pm$ 0.2 $\mu$M) is much lower than the respective value for testosterone glucuronidation by UGT2B17 (Table 2). In addition and in contrast to our expectation, the epitestosterone glucuronidation activity of UGT2B7 was not sensitive to inhibition by testosterone (results not shown). It may be added here that because of the high affinity of UGT2B7 for epitestosterone and the lower quantitation limit of the analytical method, obtaining reliable rate values in the presence of less than a 2.5 $\mu$M concentration of substrate was not feasible, and, hence, the $K_m$ value in this case was derived by extrapolation.

UGT2A1 is the only human UGT that catalyzes the glucuronidation of both testosterone and epitestosterone at considerable and similar rates (Figs. 3 and 4). The kinetics of testosterone and epitestosterone glucuronidation by UGT2A1 are comparable, if not identical (Fig. 7; Table 2). The glucuronidation reactions of UGT2A1 followed Michaelis-Menten kinetics and revealed higher substrate affinities, even though it was not sensitive to inhibition by testosterone (results not shown). It may be added here that because of the high affinity of UGT2B7 for epitestosterone and the lower quantitation limit of the analytical method, obtaining reliable rate values in the presence of less than a 2.5 $\mu$M concentration of substrate was not feasible, and, hence, the $K_m$ value in this case was derived by extrapolation.

UGT2A1 is the only human UGT that catalyzes the glucuronidation of both testosterone and epitestosterone at considerable and similar rates. These results suggest that UGT2B17 binds testosterone and epitestosterone at the same site and with similar affinities, even though it can only catalyze the glucuronidation of one of them (testosterone).

Epitestosterone glucuronidation by UGT2B7 also followed Michaelis-Menten kinetics (Fig. 6). Interestingly, the $K_m$ value in this case (1.7 $\pm$ 0.2 $\mu$M) is much lower than the respective value for testosterone glucuronidation by UGT2B17 (Table 2). In addition and in contrast to our expectation, the epitestosterone glucuronidation activity of UGT2B7 was not sensitive to inhibition by testosterone (results not shown). It may be added here that because of the high affinity of UGT2B7 for epitestosterone and the lower quantitation limit of the analytical method, obtaining reliable rate values in the presence of less than a 2.5 $\mu$M concentration of substrate was not feasible, and, hence, the $K_m$ value in this case was derived by extrapolation.

UGT2A1 is the only human UGT that catalyzes the glucuronidation of both testosterone and epitestosterone at considerable and similar rates (Figs. 3 and 4). The kinetics of testosterone and epitestosterone glucuronidation by UGT2A1 are comparable, if not identical (Fig. 7; Table 2). The glucuronidation reactions of UGT2A1 followed Michaelis-Menten kinetics and revealed higher substrate affinities, even though it was not sensitive to inhibition by testosterone (results not shown). It may be added here that because of the high affinity of UGT2B7 for epitestosterone and the lower quantitation limit of the analytical method, obtaining reliable rate values in the presence of less than a 2.5 $\mu$M concentration of substrate was not feasible, and, hence, the $K_m$ value in this case was derived by extrapolation.

UGT2A1 is the only human UGT that catalyzes the glucuronidation of both testosterone and epitestosterone at considerable and similar rates. These results suggest that UGT2B17 binds testosterone and epitestosterone at the same site and with similar affinities, even though it can only catalyze the glucuronidation of one of them (testosterone).

Epitestosterone glucuronidation by UGT2B7 also followed Michaelis-Menten kinetics (Fig. 6). Interestingly, the $K_m$ value in this case (1.7 $\pm$ 0.2 $\mu$M) is much lower than the respective value for testosterone glucuronidation by UGT2B17 (Table 2). In addition and in contrast to our expectation, the epitestosterone glucuronidation activity of UGT2B7 was not sensitive to inhibition by testosterone (results not shown). It may be added here that because of the high affinity of UGT2B7 for epitestosterone and the lower quantitation limit of the analytical method, obtaining reliable rate values in the presence of less than a 2.5 $\mu$M concentration of substrate was not feasible, and, hence, the $K_m$ value in this case was derived by extrapolation.

UGT2A1 is the only human UGT that catalyzes the glucuronidation of both testosterone and epitestosterone at considerable and similar rates (Figs. 3 and 4). The kinetics of testosterone and epitestosterone glucuronidation by UGT2A1 are comparable, if not identical (Fig. 7; Table 2). The glucuronidation reactions of UGT2A1 followed Michaelis-Menten kinetics and revealed higher substrate affinities, even though it was not sensitive to inhibition by testosterone (results not shown). It may be added here that because of the high affinity of UGT2B7 for epitestosterone and the lower quantitation limit of the analytical method, obtaining reliable rate values in the presence of less than a 2.5 $\mu$M concentration of substrate was not feasible, and, hence, the $K_m$ value in this case was derived by extrapolation.
We analyzed the testosterone and epitestosterone conjugates in the urine of male athletes who did not abuse anabolic steroids. Among them, the main reason for T/E values greater than the accepted limit of 4 was a very low concentration of urinary epitestosterone glucuronide not high levels of testosterone glucuronide (Fig. 2). This observation is in line with a previous report (Dehennin, 1994) and suggestions in the World Anti-Doping Agency (WADA Technical Document TD2004EAAS, 2004, http://www.wada-ama.org/rtecontent/document/end_steroids_aug_04.pdf). The athletes in the low group (T/E <0.2) had very low levels of urinary testosterone glucuronide, suggesting that these individuals may carry the genetic deletion in UGT2B17 that was previously found to dramatically reduce the T/E (Jakobsson et al., 2006). The situation may be more complex, however, because the samples in this group also contained very low levels of testosterone sulfate. The apparent linkage between low testosterone glucuronide and low testosterone sulfate in the urine of male athletes who did not abuse anabolic steroids. Among them, the main reason for T/E values greater than the accepted limit of 4 was a very low concentration of urinary epitestosterone glucuronide not high levels of testosterone glucuronide (Fig. 2). This observation is in line with a previous report (Dehennin, 1994) and suggestions in the World Anti-Doping Agency (WADA Technical Document TD2004EAAS, 2004, http://www.wada-ama.org/rtecontent/document/end_steroids_aug_04.pdf). The athletes in the low group (T/E <0.2) had very low levels of urinary testosterone glucuronide, suggesting that these individuals may carry the genetic deletion in UGT2B17 that was previously found to dramatically reduce the T/E (Jakobsson et al., 2006). The situation may be more complex, however, because the samples in this group also contained very low levels of testosterone sulfate. The apparent linkage between low testosterone glucuronide and low testosterone sulfate in the urine requires further research to validate it statistically and then to trace its origin.

Studying testosterone and epitestosterone glucuronidation was expected to yield new insight into how stereochemistry affects the steroid specificity of the human UGTs. Screening all of the human UGTs of subfamilies 1A, 2A, and 2B for testosterone and epitestosterone glucuronidation activity revealed that several enzymes could catalyze these reactions at detectable rates. Nevertheless, the results strongly suggest that UGT2B7 is the main contributor to epitestosterone glucuronidation in humans, whereas UGT2B17 plays the corresponding role in testosterone glucuronidation (Figs. 5 and 6; Table 1). It is interesting to note that UGT2A1 exhibited substantial activity toward both steroids, something no other human UGT is capable of.

### Discussion

The kinetics of testosterone glucuronidation by UGT2B17 in the absence or the presence of three epitestosterone concentrations in the incubation mixture, either 10 µM (▲), 25 µM (■), or 50 µM (●) was examined. The data are presented as both Michaelis-Menten and Eadie-Hofstee plots. The protein concentration in the assays was 0.2 mg/ml, and the incubation time was 15 min. The points represent an average of triplicate samples ± S.D. For further details, see Materials and Methods.

**FIG. 5.** Kinetics of testosterone glucuronidation by UGT2B17 and its inhibition by epitestosterone. The kinetics of testosterone glucuronidation by UGT2B17 in the absence (●) or the presence of three epitestosterone concentrations in the incubation mixture, either 10 µM (▲), 25 µM (■), or 50 µM (●) was examined. The data are presented as both Michaelis-Menten and Eadie-Hofstee plots. The protein concentration in the assays was 0.2 mg/ml, and the incubation time was 20 min. The points represent an average of triplicate samples ± S.D.

**FIG. 6.** Epitestosterone (▲) glucuronidation kinetics by UGT2B7. The data were fitted to the Michaelis-Menten equation (see Table 2 for kinetic parameters). The protein concentration in the assays was 0.2 mg/ml, and the incubation time was 15 min. The points represent an average of triplicate samples ± S.D.

**FIG. 7.** Testosterone (■) and epitestosterone (▲) glucuronidation kinetics by UGT2A1. The data were fitted to the Michaelis-Menten equation (see Table 2 for kinetic parameters). The protein concentration in the assays was 0.2 mg/ml, and the incubation time was 15 min. The points represent an average of triplicate samples ± S.D.

### Table 2

<table>
<thead>
<tr>
<th>Testosterone</th>
<th>Epitestosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (pmol/mg/min)</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>UGT2A1</td>
<td>427 ± 9.5</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>1002 ± 16.7</td>
</tr>
</tbody>
</table>

Values are means ± S.E.
epitestosterone was practically the same as the $K_m$ of the enzyme for testosterone, indicating that the affinity of UGT2B17 for both steroids is very similar. It may thus be concluded that although epitestosterone can bind to the active site of UGT2B17, its 17α-OH group is not accessible to conjugation, probably because of its spatial positioning with respect to the cosubstrate UDPGA.

UGT2B7 exhibited low testosterone glucuronidation activity alongside the high rate of epitestosterone glucuronidation. Hence, although the (converse) substrate specificity of UGT2B7 with respect to these steroids is not as strict as in UGT2B17, it is clearly high. We have performed a corresponding inhibition study in UGT2B7 and found that, contrary to the situation in UGT2B17, the epitestosterone glucuronidation activity of this enzyme is not sensitive to inhibition by testosterone (data not shown).

An important finding with respect to the stereoselectivity of UGT2B7 was its very high affinity for epitestosterone, much higher than the affinity of UGT2B17 for testosterone (Table 2).

We have recently performed a study on the stereoselectivity of the human UGTs in glucuronidation of $\beta$-estradiol and epistrediol, two steroids that have the same D-ring stereochemistry as testosterone and epitestosterone. For skillful technical assistance and Waters Finland for providing us with the UPLC equipment.

We thank Johanna Mosorin and Sanna Sisonen for skilful technical assistance and Waters Finland for providing us with the UPLC equipment. We thank Johanna Mosorin and Sanna Sisonen for skilful technical assistance and Waters Finland for providing us with the UPLC equipment.

**References**


Schuls J, Landmark J, Garle M, and Rane A (2008a) Doping tests result in genotype of UGT2B7, the major enzyme for testosterone glucuronidation. *J Clin Endocrinol Metab* 93:2500–2506.


