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1. Title page

UGT2B7 and UGT2B17 display converse specificity in testosterone and epitestosterone glucuronidation, whereas UGT2A1 conjugates both androgens similarly

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2. Running title page

a. Running title

Human UGTs and stereoselectivity in androgens glucuronidation

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Number of words in the Introduction: 745

Number of words in the Discussion: 1109

d. Abbreviations

GC/C/IRMS, gas chromatography/combustion/isotope ratio mass spectrometry; T/E, urinary ratio of testosterone to epitestosterone; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-α-D-glucuronic acid;

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Abstract

Testosterone and epitestosterone are endogenous steroids that differ in the configuration of the hydroxyl-bearing carbon at the C-17. Testosterone is the predominant male sex hormone while the role of epitestosterone is largely unclear. In humans, both androgens are mainly excreted as glucuronide conjugates and the urinary ratio of testosterone to epitestosterone (T/E), used to expose illicit testosterone abuse by male athletes, indicates the relative concentrations of the respective glucuronides. Some male athletes have T/E above 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 positives" in doping tests was low epitestosterone glucuronide concentration, not 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 similar affinity as testosterone. Epitestosterone glucuronidation is mainly catalyzed by UGT2B7 and the K_m of this reaction is significantly lower than the K_m of UGT2B17 for testosterone. While UGT2B7 and UGT2B17 exhibited high, although converse, stereoselectivity in testosterone and epitestosterone glucuronidation, UGT2A1, an extrahepatic enzyme that is mainly expressed 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.

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(Introduction)

Testosterone (4-androsten-17β-ol-3-one) is an endogenous sexual hormone that is primarily secreted 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 mainly present in the urine as the respective glucuronide conjugates, but sulfate conjugates and even the unconjugated form of these steroids were found (Dehennin, 1994; Borts and Bowers, 2000; Jakobsson Schulze et al., 2008a).

The interest in testosterone glucuronidation is often linked to studies on anabolic steroids abuse by male athletes (Dehennin 1994; Jakobsson Schulze et al., 2008a and 2008b). The urinary testosterone to epitestosterone concentration ratio, the T/E, serves as a marker for possible testosterone abuse among male athletes (Donike et al., 1982; World Anti-Doping Agency 2004). Presently, a T/E above 4.0 is considered suspicious and requires further examination according to the World Anti-Doping Agency Guideline for elevated T/E (2006). The T/E value, the analytical response ratio of testosterone and epitestosterone in urine samples following treatment with β-glucuronidase, is practically equivalent to the glucuronide ratio of the two steroids. Therefore, identifying the individual 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.

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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 UDP-glucuronosyltransferases (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, that are divided into 3 subfamilies UGT1A, UGT2A and UGT2B (Mackenzie et al., 2005). These proteins are found is 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 understanding of the interactions of individual UGTs with different compounds, including the regio- and stereoselectivity of these enzymes.

Testosterone and epitestosterone are diastereomers 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 Bichlmaier and co-workers on the interactions of selected UGTs with different chiral compounds led to the development of high-affinity and high specificity inhibitors for UGT2B7 (Bichlmaier et al., 2007) 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 (Itäaho 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).

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In agreement with a major role for UGT2B17 in testosterone glucuronidation, it was found that people lacking UGT2B17 due to a common genetic deletion, excrete significantly less testosterone glucuronide (Jakobsson et al., 2006). 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 (Green and Tephly, 1996; Jedlitschky et al., 1999; Turgeon et al., 2001; Kuuranne et al., 2003; Bowalgaha et al., 2007), but no systematic analysis of all the human UGTs for their ability to glucuronidate testosterone has been reported.

While 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 activity 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, deepen our understanding of the interaction of human UGTs with different steroids.

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Materials and Methods

Materials

Methyltestosterone and testosterone glucuronide were from Steraloids Inc. (Newport, RI, USA) and epitestosterone glucuronide sodium salt was from National Analytical Reference Laboratory (NARL, Pymble, AU). Deuterated d_3-testosterone and d_3-epitestosterone were from National Measurement Institute (NMI, Pymble, Australia). Testosterone (4-androsten-17β-ol-3-one), epitestosterone (4-androsten-17α-ol-3-one), uridine-5-diphospho glucuronic acid trisodium salt (UDPGA), D-saccharic acid-1,4-lactone, ammonium iodide and dithioerythritol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Magnesium chloride hexahydrate, disodium hydrogenphosphate, potassium dihydrogenphosphate, potassium carbonate, 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 Haën (Seelze, Germany). Trimethylchorosilane and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were from Fluka (Sigma-Aldrich, Steinheim, Germany). β-glucuronidase from *E. coli* K12 was from Boehringer-Mannheim (Mannheim, Germany). All the solvents were HPLC grade, and the eluents were filtered through a 0.22 µm filter for UPLC analysis.

Recombinant UGTs

Recombinant human UGTs of subfamilies UGT1A and UGT2B 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

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of their cloning will be published elsewhere (Sneitz, Court, Zhang, Ding and Finel, manuscript in preparation). The recombinant UGTs carried a short C-terminal fusion peptide ending with 6 His residues, a His-tag. The latter provides the possibility to compare the expression level of each UGT in the insect cells microsomal preparations and, despite large differences between them in this respect, to evaluate the relative activity of the different enzymes, the so-called normalized activity. Protein concentrations were determined by the BCA method (Pierce Biotechnology Inc., Rockford, IL, USA). 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™ Gentest, BD Biosciences, MA, USA) 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 to the other UGTs in this study.

Analytical methods

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

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Analytical methods calibration and validation

Stock solutions were prepared in 70% (v/v) acetonitrile (epitestosterone 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 0.25-100 µM. The analytical method was validated with respect to specificity, accuracy, precision, and limit of quantification in line with previous recommendation (Shah et al., 2000). The limits of detection and quantification were determined at signal to 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 Woolf, 2007).

Incubation conditions for UGTs screening

The assays to identify the human UGT isoforms that are active in testosterone and epitestosterone glucuronidation 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 $MgCl_2$. 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 DMSO solution so that the final DMSO concentration was 5% in all assays. It may be noted here that since this was a screening experiment, the conditions were not optimized for each UGT. Control incubations were carried out in the presence of the highest aglycone substrate concentration and in the absence of UDPGA, as well as in the in the absence of aglycone substrate, but in the presence of UDPGA. The reactions were started

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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 16000 *g*, and aliquots of the supernatants were used for the UPLC analyses. The reactions were carried out in triplicate, except the negative controls that were single samples.

The glucuronidation rate was expressed as the amount of glucuronides formed (pmol) per protein amount (mg) 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 the expression level of UGT2B7 as 1.0.

Kinetic analyses

Kinetic assays of testosterone and epitestosterone glucuronidation by UGT2B7 and UGT2B17, respectively, and both substrates by UGT2A1, were carried out. The reaction conditions were similar to those in the screening assays, except that protein concentrations and incubation times were selected to ensure that the product formation was within the linear range with respect to both these parameters. The substrate concentration range in the kinetic assays was from 2.5 to 200 μM. The kinetic constants K_m and V_{max} were obtained by fitting kinetic models to experimental data using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA). The Michaelis-Menten and Hill equations were

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tested, and the best model was chosen on the grounds of r^2 values, parameter standard error estimates, and 95% confidence intervals.

The inhibitory effects of epitestosterone on testosterone glucuronidation by UGT2B17 and testosterone on epitestosterone 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:

$$Y = V_{\max} * X / (K_m(1 + [I]/K_i) + X)$$

where X is the substrate concentration in μM, Y is the measured activity in pmol/mg protein/min, V_{max} is maximum velocity in pmol/mg protein/min, K_m is the Michaelis-Menten constant, I is the inhibitor concentration in μM, and K_i is the inhibition constant. The data fit to the competitive inhibition model was verified by Eadie-Hofstee plot.

Urinary steroid profiles

Urine samples were selected based on their T/E and subjected to steroid profile analysis. The samples were anonymous and obtained from routine doping control samples with a written consent allowing the use for research purposes. To achieve deeper insight into testosterone and epitestosterone conjugation in male athletes, we have selected samples for 3 groups, each with a different level of T/E. The as follow:

1. "low", individuals with T/E lower than 0.2 (n=20)
2. "normal", individuals with T/E in the range of 0.8 - 1.2 (n=20)
3. "high", individuals with T/E above 4.0 (n=14)

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It should be highlighted here that samples with T/E above 4.0, (group "high") were only taken from individuals for whom there was no indication of drug abuse, as determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analysis.

The samples were analyzed for testosterone and epitestosterone glucuronides, as well as the sulfate conjugates of the two steroids. Sample preparation for the glucuronides 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_3-testosterone and d_3-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 β-glucuronidase solution, corresponding to 7 international units of β-glucuronidase activity, and incubating the samples at 50°C for 60 min. After adding 0.5 ml of aqueous potassium carbonate:potassium bicarbonate solution (1:1, 20%, v/w) and 2 g of sodium sulfate, the hydrolysate was vortex-mixed with 5 ml of diethyl ether for 30 s. Following 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, USA), which was pre-conditioned with methanol and water. The column was first rinsed with 5 ml of water and 2.5 ml of *n*-heptane and then

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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 adding 3 ml of aqueous mixture of 2 M NaOH and 4 M NaCl, the hydrolysate 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 MSTFA/ammonium iodide/dithioerythritol (1000:2:4, v/w/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 Inc, Palo Alto, USA) and an Agilent 5973N mass selective detector (Agilent Technologies Inc, Palo Alto, USA). Steroids were separated on an Agilent HP-1 fused silica capillary column (16 m, 0.2 mm i.d., film thickness 0.11µm). Injection of 2 µl was done in split mode (1:15) at 280°C. 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. MS was operated in electron ionization mode (70 eV) using selected ion monitoring (SIM). For each analyte one specific ion was measured with the dwell time of 10 ms.

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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 whom declared as negative for testosterone abuse in routine analysis. In order to get deeper insight into the results, the urine samples were divided into 3 groups, based on the screened T/E, according to the following criteria: “low” (T/E < 0.2), “normal” (T/E = 0.8 - 1.2), and “high” (T/E > 4.0). The results (Fig. 2) reveal, as expected, that samples in the “low” group contain very low concentration of testosterone glucuronide, potentially due to inactive UGT2B17 (Jakobsson Schulze et al., 2008a) or to limitations in the transport of testosterone glucuronide.

Another noteworthy finding came from the “high” group, namely urinary samples that had T/E values above 4.0 and, therefore, subjected to the laborious and time-consuming, GC/C/IRMS analysis, but found not to have abused exogenous testosterone. The levels of testosterone glucuronide in these samples of group “high” was practically the same as in group “normal”, but their epitestosterone glucuronide level was lower. Hence, the high T/E in the samples of group “high” was due to very low concentration of epitestosterone glucuronide, not exceptionally high level of testosterone glucuronide (Fig. 2).

Most of the urinary testosterone was present as glucuronide, not sulfate conjugates (Fig. 2, groups “normal” and “high”). 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

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these experiments, we have anticipated that athletes with low concentration of urinary testosterone glucuronide will have higher concentration of testosterone sulfate. Intriguingly, the results proved us wrong and the sharp decrease in the concentration of testosterone glucuronide (group "low") was not accompanied by an increase in the concentration of testosterone sulfate (Fig. 2).

We have embarked on identifying the enzymes that are active in testosterone and epitestosterone glucuronidation in order to gain 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. Following incubations, glucuronides were detected using the UPLC system (ultra performance liquid chromatography) 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 μM and 0.25 μM, respectively (S/N >3 and 10, n=5). The accuracy and precision of the method were acceptable with respect to the guidelines for bio-analytical method validation (Shah et al. 2000). The between assays coefficient of variation for testosterone glucuronide and epitestosterone glucuronide were less than 11%.

Several human UGTs exhibited detectable glucuronidation activity towards 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

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diastereomers and highly similar in their physicochemical properties, they are largely glucuronidated by two different enzymes, 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 since this enzyme is mainly expressed 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 the commercially available UGT2B15 (see also Itäaho et al., 2008). Therefore, we have tested here both our preparation (the expression level of which can be compared with that of the other recombinant UGTs) and UGT2B15 from a commercial source. The results show that (also) the commercial 2B15 is much less active in testosterone glucuronidation than UGT2B17 (Fig. 3), a finding that is in good agreement with results from another laboratory (Bowalgaha et al., 2007).

In the case of epitestosterone, we have found that in addition to the high glucuronidation rate of UGT2B7 toward this steroid, there are few other enzymes that could catalyze such a conjugation reaction. The epitestosterone glucuronidation activity of UGT1A4 and UGT2B4 was detectable, but very low (Fig. 4). Higher activity rate was exhibited by UGT2A2, whereas the normalized epitestosterone glucuronidation activity of UGT2A1 was particularly

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high (Fig. 4). Hence, UGT2A1 is perhaps the only human enzyme that can glucuronidate both testosterone and epitestosterone at considerable rates.

It is worth noting here that UGT2B17, the most active enzyme in testosterone glucuronidation, revealed no epitestosterone glucuronidation activity (Fig. 4). To find out whether or not UGT2B17 binds epitestosterone, we have turned to kinetic analyses. The testosterone glucuronidation activity of UGT2B17 followed Michaelis-Menten kinetics (Fig 5, Table 2). The presence of increasing concentrations of epitestosterone in the testosterone glucuronidation assay lead to increased K_m values, but not V_{max} values, indicating that epitestosterone is a competitive inhibitor of UGT2B17 catalyzed testosterone glucuronidation. The competitive inhibition model was further verified by fitting the data to Eadie-Hofstee plots (Fig. 5, inset). Moreover, the derived K_i value for the competitive inhibition by epitestosterone, 10.6 ± 0.9 μM, is nearly identical to the K_m value of UGT2B17 for testosterone (in the absence of inhibitor), 10.0 ± 0.8 μM (Table 2). 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 ± 0.2 μM, 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 due to the high affinity of UGT2B7 for epitestosterone and the lower quantitation limit of the analytical

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method, obtaining reliable rate values in the presence of less that 2.5 µM 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 is comparable, if not identical (Fig. 7, Table 2). The glucuronidation reactions of UGT2A1 followed Michaelis-Menten kinetics and revealed higher substrate K_m in this enzyme for both steroids, in comparison to the respective values in UGT2B7 and UGT2B17 (Table 2). Hence, the observed lack of stereoselectivity in testosterone and epitestosterone glucuronidation by UGT2A1 appears to be linked to lower substrate affinity than of the stereoselective enzymes UGT2B7 and UGT2B17.

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Discussion

We have analyzed the testosterone and epitestosterone conjugates in the urine of male athletes that did not abuse anabolic steroids. Among them, the main reason for T/E values above the accepted limit of 4 was 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 WADA Technical document (2004). The athletes in the "low" group, T/E < 0.2, had very low level 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, since 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 in order 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 steroids specificity of the human UGTs. Screening all 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). Interestingly, UGT2A1 exhibited substantial activity towards both steroids, something no other human UGT is capable of (Fig. 7, Tables 1 and 2). While the latter results may not be highly

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relevant for doping control since UGT2A1 is mainly expressed in the nasal epithelium (Jedlitschky et al., 1999), they nevertheless extend our knowledge on the complex substrate specificity of the UGTs and provide new tools to study their aglycone binding site.

The current findings concerning the testosterone glucuronidation activity of the human UGTs explain the observations on the effect of UGT2B17 deletion on the T/E in males (Jakobsson et al., 2006). Our results demonstrate the central role of UGT2B17 in testosterone glucuronidation in humans and, therefore, the large decrease in urinary testosterone glucuronide concentration in individuals who are homozygous carriers of the UGT2B17 genetic deletion (Jakobsson et al., 2006). On the other hand, the detected low level of testosterone glucuronidation activity by the liver UGTs 1A3, 1A4, 1A9, 2B7 and 2B15 (and perhaps also the extrahepatic UGTs 1A8, 1A10 and 2A1) (Fig. 3), can account for the low, but measurable, concentrations of urinary testosterone glucuronide even in individuals that lack active UGT2B17 (Jakobsson et al., 2006).

From the UGTs substrate specificity point of view, the results of this work are in line with the importance of substrates stereochemistry for many glucuronidation reactions (Bichlmaier et al., 2006a and 2006b; Itäaho et al., 2008). The chemical structure (Fig. 1) and physicochemical properties of these two steroids are very similar and, therefore, it was challenging to find out whether or not the strict specificity of UGT2B17 for testosterone glucuronidation is due to exclusion of epitestosterone from its substrate binding site. The latter possibility was examined using kinetic assays and the results clearly showed that epitestosterone is a competitive inhibitor of testosterone glucuronidation by UGT2B17 (Fig.

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4). Moreover, the inhibition constant (K_i) for 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 is not accessible to conjugation, probably due to its spatial positioning with respect to the co-substrate UDPGA.

UGT2B7 exhibited low testosterone glucuronidation activity alongside high rate of epitestosterone glucuronidation. Hence, while the (converse) substrate specificity of UGT2B7 with respect to these steroids is not as strict as in UGT2B17, it is clearly high. We have carried out 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 (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 carried out a study on the stereoselectivity of the human UGTs in β-estradiol and epiestradiol glucuronidation, two steroids that have the same D-ring stereochemistry as testosterone and epitestosterone, respectively (Itäaho et al., 2008). One of the observations in that study was the exceptionally high affinity of UGT2B7 for epiestradiol, but not for β-estradiol. It is also noteworthy that UGT2B17 was found to be strictly specific for β-estradiol, whereas UGT2B7 exhibited a clear preference, but not absolute specificity, for the glucuronidation of the 17-α-OH in epiestradiol (Itäaho et al., 2008). The results of the present study on testosterone and epitestosterone glucuronidation, in combination with the

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recent findings from the estradiols glucuronidation study, suggest that the stereoselectivity of UGT2B7, but not UGT2B17, stems from large difference in the enzyme affinity to testosterone and epitestosterone due to the configuration of the carbon 17 bound to the nucleophilic hydroxyl group of the substrate. An alpha configuration for this group, as in epitestosterone and epiestradiol, significantly increases the affinity of UGT2B7 for the compound. Future studies with other steroids and related compounds will show if this is indeed a general rule for the substrate specificity of UGT2B7, and how atoms or functional groups at other stereocenters in related steroids affect the substrate specificity of this and other UGTs.

In summary, analyses of testosterone and epitestosterone conjugates in urine samples from athletes revealed that low concentrations of epitestosterone glucuronide often lead to higher than threshold T/E. We have also observed an apparent link between very low testosterone glucuronide and very low testosterone sulfate concentrations in the same urine samples, a finding that is not easily explained merely by the genetic deletion of UGT2B17. A screen of all the human UGTs of subfamilies 1A, 2A and 2B revealed that UGT2B17 is by far the most important enzyme in testosterone glucuronidation whereas UGT2B7 is the main contributor to epitestosterone glucuronidation in humans. Both UGT2B17 and UGT2B7 exhibited high stereoselectivity for one over the other of these two androgens. The reasons governing that stereoselectivity, however, differ between these two homologous UGTs. UGT2A1, in contrast to either UGT2B7 or UGT2B17, glucuronidates both testosterone and epitestosterone similarly, an interesting observation from the point of view of structure-function relationships in the UGTs, even if due to its restricted expression among the human

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tissues, UGT2A1 may not play a major role in determining the urinary concentration ratio of testosterone and epitestosterone.

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Footnote

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Figure legends

Figure 1. Chemical structure of testosterone and epitestosterone.

Figure 2. Relations between testosterone and epitestosterone glucuronides and sulfate conjugates in the urine. The urinary testosterone and epitestosterone conjugates profile of the 55 male athletes that were selected for this study. All these anonymous samples tested negative for testosterone abuse. The samples were divided into three groups, based on their concentration ratio of testosterone glucuronide to epitestosterone glucuronide (panel A). In samples of group "Low" that glucuronides concentration ratio was lower than 0.2, in group "Normal" it was 0.8-1.2, and in group "High" it was above 4.0. The concentrations of the different metabolites were corrected with respect to the specific gravity of the sample. For further details and study protocol see Materials and Methods.

Figure 3. Testosterone glucuronidation by recombinant human UGTs. A. The measured glucuronidation rates (pmol/mg protein/min) without correction for expression levels. B. Normalized activity, namely glucuronidation rates were corrected according to the relative expression level of each UGT. The expression level of UGT2B7 was the reference value. *2B15c is from BD Biosciences (UGT2B15 Supersomes^{TM}). This enzyme is not included in panel B since its expression level could not be compared with the other UGTs. See Materials and Methods for further details.

Figure 4. Epitestosterone glucuronidation by recombinant human UGTs. See legend to Fig. 3 for more details.

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Figure 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 3 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 ± SD. For further details, see Materials and Methods.

Figure 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 ± SD.

Figure 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 15 min. The points represent an average of triplicate samples ± SD.

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Table 1. Testosterone and epitestosterone glucuronidation activity screen of all the recombinant human UGTs of subfamilies 1A, 2A and 2B. The values are average of three replicates ± SD. Rates were not corrected for expression level. ND, no detectable activity.

UGT	Glucuronide formation, pmol/mg 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*	63 ± 2.9	n.d.
2B17	**508.9 ± 19.6**	n.d.
2B28	n.d.	n.d.

*2B15c is from BD Biosciences (UGT2B15 Supersomes™)

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Table 2. Kinetic parameters (± S.E.) for testosterone and epitestosterone glucuronidation by recombinant human UGTs 2A1, 2B7 and 2B17.

	Testosterone		Epitestosterone	
	V_{max} *(pmol/mg/min)*	K_m *(μM)*	V_{max} *(pmol/mg/min)*	K_m *(μM)*
UGT2A1	427 ± 9.5	38.7 ± 2.7	271 ± 6.7	11.6 ± 1.2
UGT2B7			337 ± 4.8	1.7 ± 0.2
UGT2B17	1002 ± 16.7	10.0 ± 0.8		

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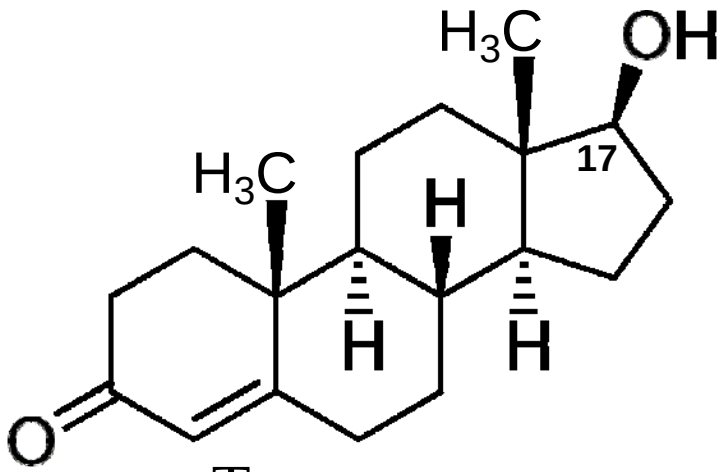
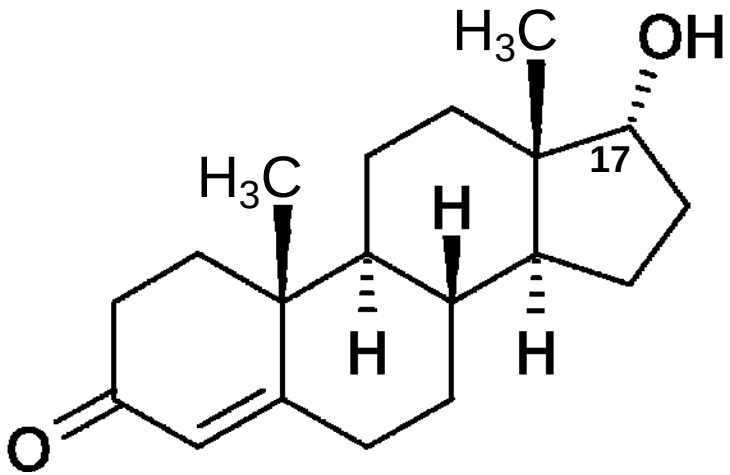


Testosterone



Epitestosterone

Fig. 1

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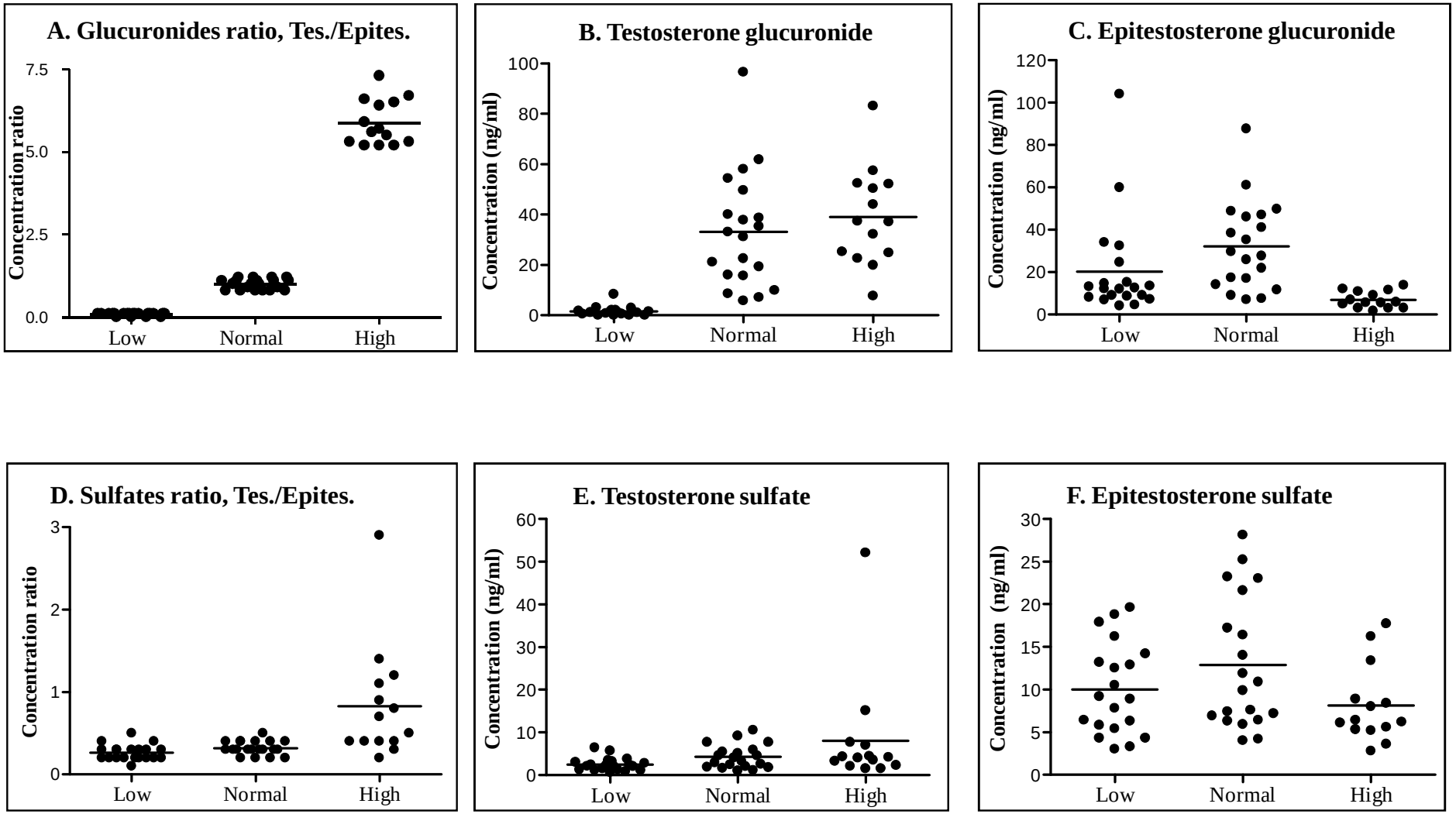

Fig 2

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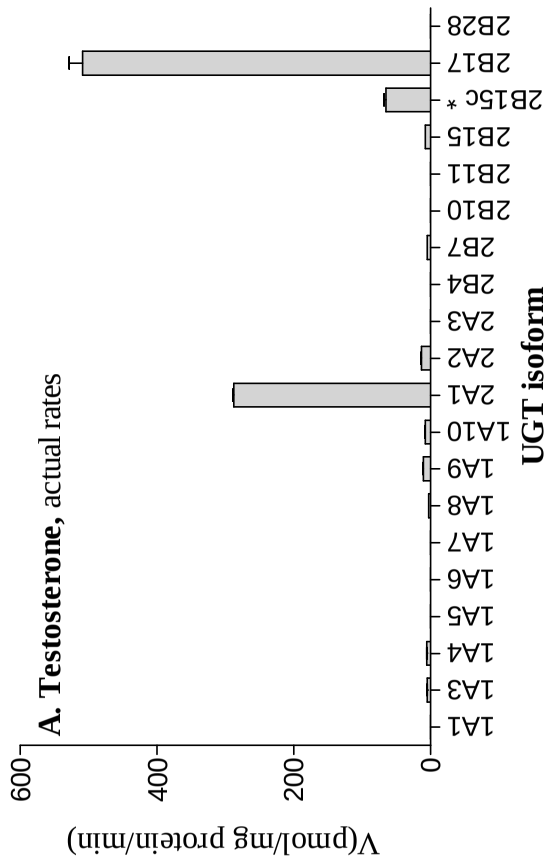

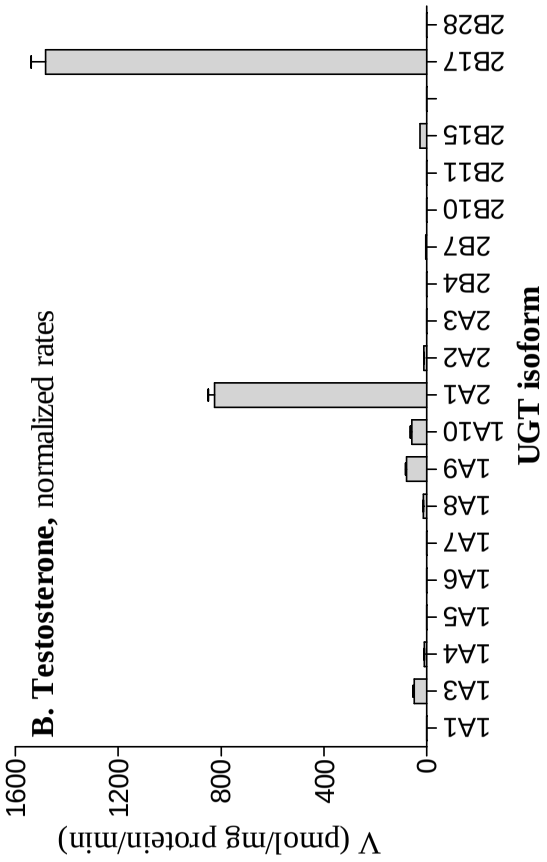

Fig. 3

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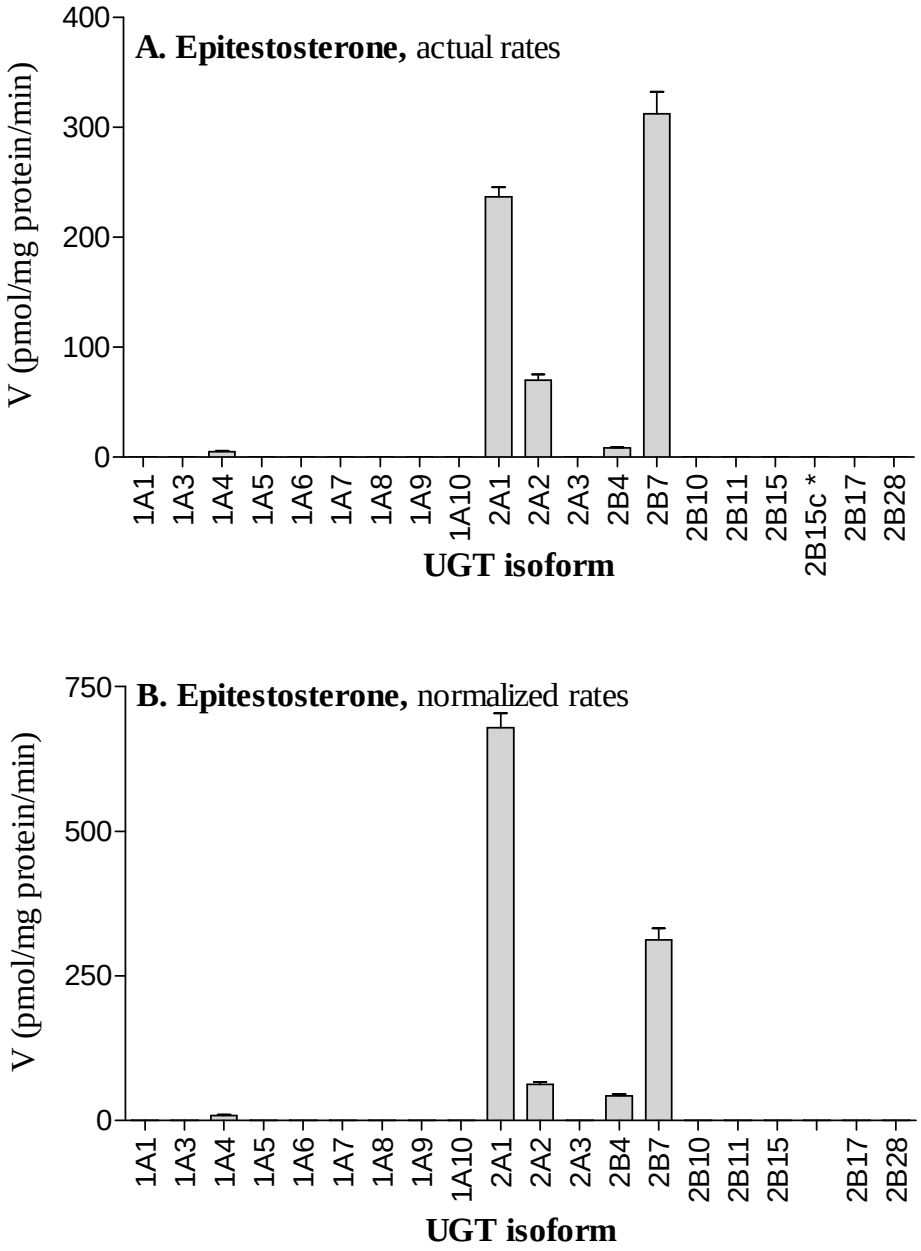

Fig. 4

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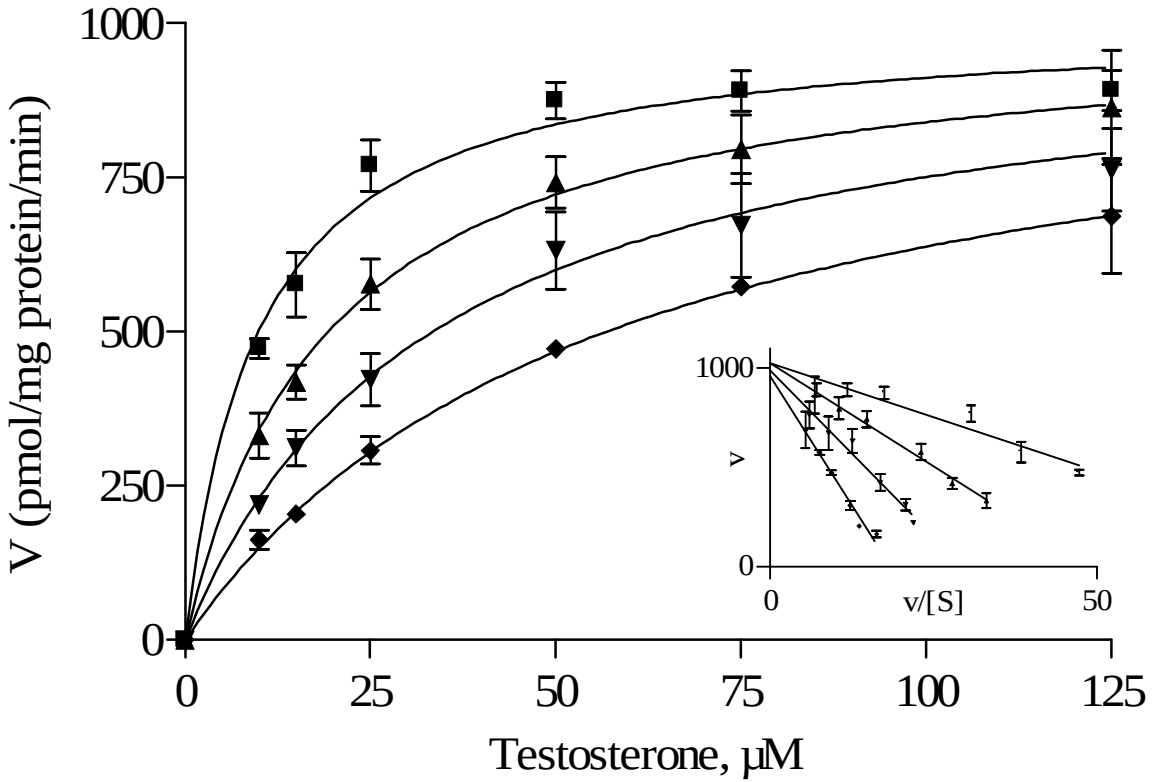

Fig. 5

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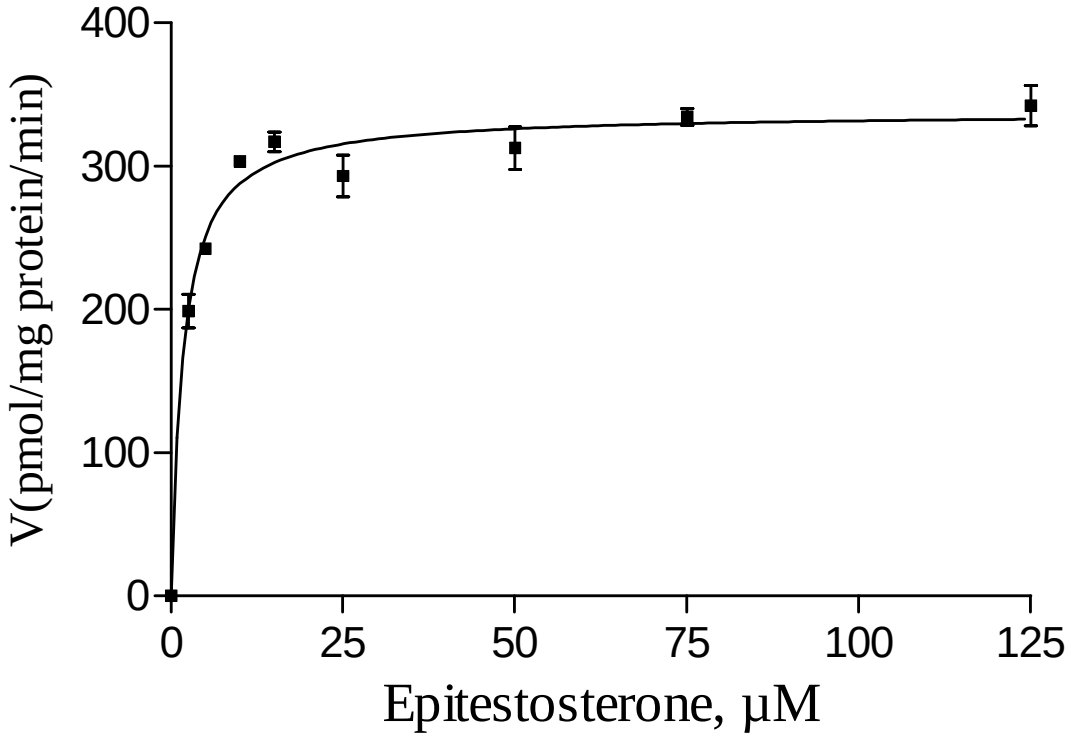

Fig. 6

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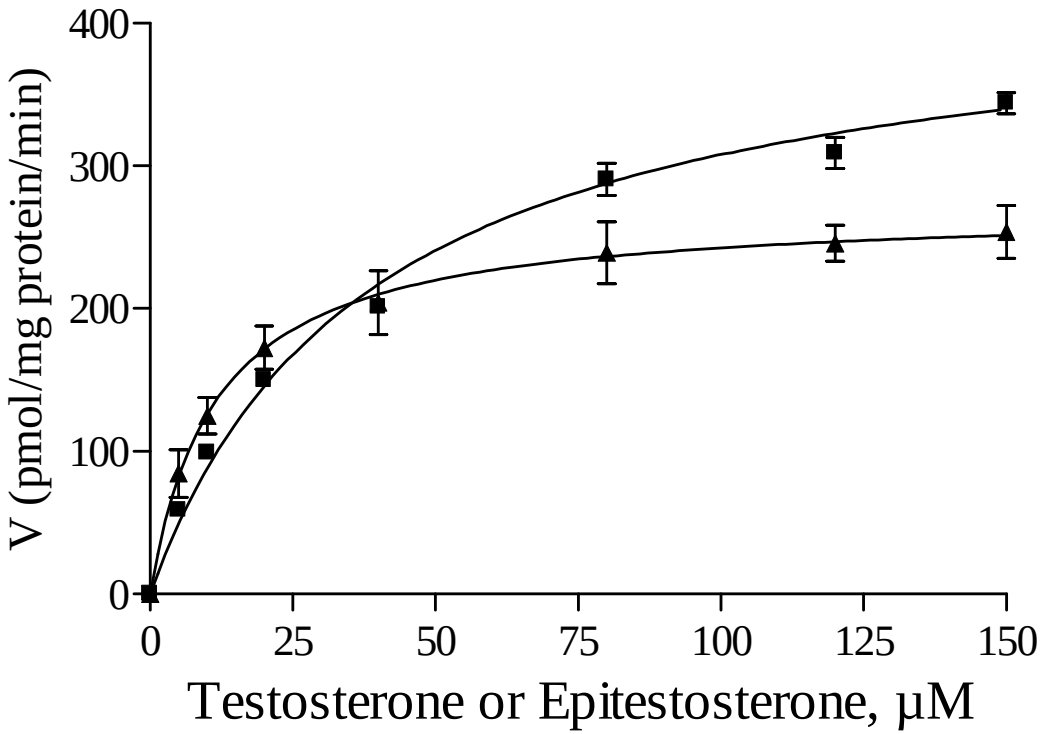

Fig. 7

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