TESTICULAR STEROID SULFATASE
Substrate Specificity and Inhibition

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ABSTRACT

Kinetic studies of the cleavage of dehydroepian-
drosterone-sulfate (l) and androstenediol-3-sulfate by
a particulate enzyme preparation from a rat testicular
microsomal fraction gave $K_m$ values of $2.04 \times 10^{-6} \text{M}$ for
DHA-S and $0.935 \times 10^{-6} \text{M}$ for androstenediol-3-sulfate
with identical $V_{\text{max}}$ values. Inhibition studies with
equimolar concentrations of substrate and inhibitor
demonstrated that $\Delta_4\Delta_5$-androstane-3,17-diol was the
most potent inhibitor among fifteen C-19 and C-18
unconjugated steroids investigated. Substitution of:
1) a $\Delta^4$ or $\Delta^5$ bond or phenolic A ring for a saturated A
ring, 2) $17\alpha$-hydroxyl group for a $17\beta$-hydroxyl group, or
3) a $3\alpha$-hydroxyl group for a $3\beta$-hydroxyl group, markedly
decreased the inhibitory effect of the steroid. $K_i$
values of $1.7 \times 10^{-6} \text{M}$, $3.3 \times 10^{-6} \text{M}$ and $11.8 \times 10^{-6} \text{M}$ were
found with $\Delta_4\Delta_5$-androstane-3$\beta$,17$\beta$-diol, $5\alpha$-androstane-3$\alpha$,
17$\beta$-diol and testosterone, respectively. The kinetic
data related to inhibition are consistent with partial
competitive inhibition.

In previous studies of the comparative gonadal
metabolism of dehydroepiandrosterone (DHA) and
dehydroepiandrosterone-sulfate (DHA-S) it was found
that a soluble extract of rat testes converted DHA-S to
androstenediol-3-sulfate ($\Delta^5$-diol-3-S) four times as
fast as DHA to androstenediol (2). In similar
experiments (3) in which $^3$H-DHA-S and $^{14}$C-DHA were
incubated with a microsomal preparation of rabbit ovaries, DHA-S was converted to \(\Delta^5\)-dion-3-S and the following free steroids: testosterone, androstenedione, 5\(\alpha\)-androstane-3\(\beta\),17\(\beta\)-dion (3\(\beta\)-A-dion), dihydrotestosterone and epiandrosterone. To elucidate further the role of \(\Delta^5\)-dion-3-S in the biosynthesis of gonadal androgens, the cleavage of DHA-S and \(\Delta^5\)-dion-3-S by a particulate fraction from rat testes was compared.

The recent report by Notation and Ungar (4) that DHA-S cleavage occurring in a rat testicular homogenate was inhibited by free DHA and testosterone led us to examine the degree and type of inhibition of these and other free steroids, especially the 5\(\alpha\)-reduced C-19 steroids which have been demonstrated to be products of the DHA-S and DHA metabolic pathway (3,5).

**Experimental**

**Materials:** The ammonium salt of DHA-7\(\alpha\)-\(\text{H}\)-S (10.4 c/mmole) was purchased from New England Nuclear Corporation. It was purified by column partition chromatography on Celite in system AIT-1 as described by Calvin et al. (6). \(\Delta^5\)-dion-3\(\text{H}\)-3-S was prepared from DHA-7\(\alpha\)-\(\text{H}\)-S by reduction with potassium borohydride in 95% methanol, followed by purification on ITLC as described previously (7). Radiochemical purity of both compounds was established by recrystallization of aliquots to constant specific activity (SA).

DHA-S and \(\Delta^5\)-dion-3-S were prepared as the potassium salts as described previously (2). DHA, estrone, 17\(\beta\)-estradiol and estriol were purchased from Sigma Chemical Co., epitestosterone from Steraloids, Inc., 11\(\beta\)-hydroxyestradiol (U-6814) was a gift from
Dr. John Babcock, Upjohn Co. All other steroids were obtained from Mann Research Laboratories, Inc.

**Tissue Preparations:** Testes were obtained from mature Holtzman rats. The testes were decapsulated and homogenized in 4 volumes of 0.25 M sucrose in a teflon-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 15,000 g for 20 minutes. The supernatant fluid was centrifuged at 105,000 g for 1 hr. The resulting supernate was discarded and the pellets were combined in ice-cold water, homogenized briefly and lyophilized. The lyophilized preparation was homogenized again (unless otherwise indicated) in 0.25 M sucrose and centrifuged for 90 min. at 105,000 g. The pellets were combined in ice-cold water, homogenized and stored in 1-ml aliquots at -20°C. The protein concentration was approximately 5 mg/ml. This solution was diluted with water prior to incubation. No loss of sulfatase activity was observed over 2.5 months.

**Enzyme Assay:** The substrate was dissolved in 1 ml buffer solution and warmed to 37°C, the incubation temperature. For inhibition studies the substrate and free steroid were dissolved in 0.05 ml absolute methanol prior to the addition of 0.95 ml buffer solution. One ml of diluted enzyme preparation (0.43 - 0.46 mg/ml) was added and the mixtures were incubated for a period of 20 minutes unless otherwise indicated. A 0.5 ml aliquot was removed at the appropriate time, and delivered into a culture tube and sulfatase activity immediately assessed as described by Burstein and Dorfman (8). Enzyme activity (v) is expressed as μmoles of free steroid released per minute per mg protein. Controls lacking enzyme were processed simultaneously with the experimental samples. The minute amount of radioactivity found in the toluene extract from these controls was subtracted from that of the experimental samples. Protein was determined by the method of Lowry et al (9).

**Results**

**pH Optimum:** To determine optimal conditions for carrying out the kinetic studies, pH optima were determined for rat testis sulfatase activity using both DHA-S and Δ⁵-diol-3-S as substrates. As seen
in Fig. 1, the optimum with $\Delta^5$-dol-3-S is between pH 6.4 and 6.5, while the optimum with DHA-S is at pH 6.2. Tris-HCl buffer (0.05M) was used for pH levels between 7.0 and 8.0 and imidazole -HCl buffer (0.05M) between 5.8 and 7.0. Tris-HCl buffer was used at some pH levels below 7.0 and no difference in enzyme activity was observed between the two buffers at the same pH.

![Fig. 1. Effect of pH on the rate of DHA-S (△) and $\Delta^5$-dol-3-S (●) cleavage. Each reaction mixture contained 1.68 mg lyophilized microsomal protein and 4.8 x 10^{-6}M substrate. The buffers were 0.05M Imidazole-HCl at pH values up to 6.8 and 0.05M Tris-HCl from 7.0 to 7.9. $v = \mu$mole/min/mg protein.](image)

The pH optimum observed in the present study was considerably lower than previously reported by Burstein and Dorfman (8). It seemed possible that lyophilization had solubilized the enzyme and that this accounted for the observed difference. Thirty-five mg of lyophilized microsomal fraction was homogenized in 6.5 ml of 0.25 M sucrose and centrifuged for 90 min. at 105,000 g. Over
90 per cent of the enzyme activity was recovered in the pellet. Although this treatment did not yield a solubilized enzyme, it did increase the specific activity of the particulate preparation by approximately two-fold. Therefore, all subsequent kinetic studies were carried out using the recentrifuged lyophilized enzyme preparation.

**Enzyme Activity:** Fig. 2 shows the variation in enzyme activity during a 60 minute incubation. Linearity was observed up to 20 min. after which there was some deviation from linearity. The concentration of substrate (3.62 x 10^-7 M) was the minimum concentration used in any of the experiments reported in this study. At higher substrate concentrations linearity was observed up to 60 min. It should be noted that the line passes through the origin and therefore incubation periods up to 20 min. reflect initial velocity. The relationship between amount of enzyme and reaction velocity is illustrated in Fig. 3. A linear relationship between velocity and mg protein was observed between 0.24 and 2.6 mg. All subsequent assays were carried out with approximately 0.45 mg of protein.

**Substrate Specificity and Inhibition:** Enzyme activity was measured at various substrate concentrations utilizing DHA-S and Δ⁵-diol-3-S in 0.1M imidazole-HCl
buffer at pH 6.5. As illustrated in Fig. 4, both substrates appeared to be cleaved by the same enzyme since they yielded an identical $V_{\text{max}}$. However, the apparent $K_m$ of $0.935 \times 10^{-6} \text{M}$ calculated for $\Delta^5$-diol-3-S is considerably lower than the $K_m$ of $2.04 \times 10^{-6} \text{M}$ for DHA-S. Substrate inhibition at high substrate concentration ($9 \times 10^{-5} \text{M}$) was observed with both substrates. When the same experiment was carried out in 0.1M Tris-HCl buffer at pH 7.4 the apparent $K_m$ values for
Fig. 4. Relation of enzyme activity to concentration of substrate at pH 6.5. Lineweaver-Burk plots with DHA-S as substrate, and with Δ⁵-diol-3-S as substrate.

Δ⁵-diol-3-S and DHA-S were 2.63 x 10⁻⁶M and 6.67 x 10⁻⁶M respectively. Again the V_max was the same for both substrates, but the substrate inhibition was not observed.

Various C-19 and C-18 free steroids were tested for their effect on sulfatase activity. The particular steroids were chosen either because they are immediate or distant products of metabolism of the substrate, or because their substitution and configuration at C-17 allowed some evaluation of specificity. Table I presents the percent inhibition observed when DHA-S or Δ⁵-diol-3-S was incubated in the presence of equimolar concentration of each steroid. Inhibition was determined at pH 7.4 and at pH 6.5.
The greatest inhibition by all steroids studied was observed at pH 7.4 with DHA-S serving as the substrate. At both pH levels and with both substrates, the degree of inhibition depended on the group at C-3 and C-17 and the saturation of ring A. In all cases, the "diols" inhibited the sulfatase activity to the greatest degree. Of the "diols", 3β-A-diol was the most potent inhibitor. Substituting a 3α-hydroxyl for a 3β-hydroxyl decreased the percent inhibition (i.e. compare 2 with 3 and 9 with 10, Table I). Also, substituting a Δ⁵, Δ⁴ or a phenolic ring A for a saturated ring A decreased the percent inhibition (i.e. 4 vs 5 with DHA-S as substrate and 1 or 12 vs 3, Table I). The same pattern was observed with substitution of a 17α-hydroxyl for a 17β-hydroxyl as seen by comparing the inhibition caused by epitestosterone vs testosterone (4 vs 6, Table I) and 17α-estradiol vs 17β-estradiol (12 vs 13, Table I). Addition of a 16α-hydroxyl, i.e. estriol, or an 11β-hydroxyl group to 17β-estradiol obviates the inhibition demonstrated with 17β-estradiol (14 and 15 vs 12, Table I). Δ⁴-androstenedione which has neither a 3 nor a 17-hydroxyl group caused essentially no inhibition (7, Table I).

To determine the type of inhibition effected by the free steroids, varying concentrations of DHA-S were
Table I. Inhibition of rat testicular steroid sulfatase by free steroids*

<table>
<thead>
<tr>
<th>Steroid</th>
<th>pH 7.4</th>
<th>DHA-S</th>
<th>pH 6.5</th>
<th>DHA-S</th>
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<tr>
<td></td>
<td>$\Delta^5$-diol-3-S</td>
<td>DHA-S</td>
<td>$\Delta^5$-diol-3-S</td>
<td>DHA-S</td>
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<tr>
<td>1. Androstenediol</td>
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<td>47</td>
<td>14</td>
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<tr>
<td>2. 5α-androstane-3α,17β-diol</td>
<td>31</td>
<td>50</td>
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<tr>
<td>3. 5α-androstane-3β,17β-diol</td>
<td>42</td>
<td>71</td>
<td>31</td>
<td>53</td>
</tr>
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<td>4. Testosterone</td>
<td>10</td>
<td>28</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>5. Dihydrotestosterone</td>
<td>--</td>
<td>42</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>6. Epitestosterone</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7. Androstenedione</td>
<td>None</td>
<td>10</td>
<td>None</td>
<td>7</td>
</tr>
<tr>
<td>8. Dehydroepiandrosterone</td>
<td>10</td>
<td>19</td>
<td>5</td>
<td>11</td>
</tr>
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<td>9. Epiandrosterone</td>
<td>15</td>
<td>30</td>
<td>8</td>
<td>16</td>
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<tr>
<td>10. Androsterone</td>
<td>7</td>
<td>--</td>
<td>None</td>
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<td>22</td>
<td>7</td>
<td>11</td>
</tr>
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<td>14. Estriol</td>
<td>3</td>
<td>7</td>
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<td>3</td>
</tr>
<tr>
<td>15. 11β-hydroxyestradiol</td>
<td>--</td>
<td>5</td>
<td>4</td>
<td>3</td>
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</tbody>
</table>

* Two or more incubations were carried out in 0.1M Tris-HCl buffer, pH 7.4, or in 0.1M Imidazole-HCl buffer, pH 6.5 with the appropriate substrate at 4.52 x $10^{-6}M$ and the appropriate free steroid at 4.34 x $10^{-6}M$. The values presented are averages.

incubated in the presence of several levels of 5α-androstene-3α,17β-diol (3α-A-diol). Plots of 1/v against 1/S are shown in Fig. 5. The lines intersect the ordinate at the same point ($V_{max}$) indicating competitive inhibition. Similar results were obtained when varying concentrations of $\Delta^5$-diol-3-S were incubated with several levels of testosterone and when varying concentrations
Fig. 5. (Left) Inhibition of DHA-S cleavage by 5α-androstane-3α,17β-diol. Incubation carried out for 15 min. at pH 6.5. Inhibitor concentration: none, •; 6.94 x 10^{-6} M, □; 13.9 x 10^{-6} M, △; 27.8 x 10^{-6} M, ○; 41.7 x 10^{-6} M △△.

Fig. 6. (Right) Replot of data from Fig. 5. Substrate concentration: 3.65 x 10^{-7} M, •; 5.91 x 10^{-7} M, □; 10.4 x 10^{-7} M, △△; 23.1 x 10^{-7} M, △.

of DHA-S were incubated with several levels of 3β-A-diol. This indicates that these two steroids also act as competitive inhibitors. Fig. 6 (1/v vs inhibitor concentration at various substrate concentrations) is a replot of the data presented in Fig. 5. The resulting hyperbolic curves are consistent with partial competitive inhibition (10). The data in Fig. 7 and 8 are plotted in the same manner as in Fig. 6. As can be noted, testosterone concentration plotted against 1/v at a given concentration of DHA-S (3.62 x 10^{-7} M also yields a
hyperbolic curve (Fig. 7). When 3β-A-diol is used as the inhibitor, there appear to be two distinct slopes instead of a hyperbolic curve (Fig. 8). A very abrupt change in slope occurs at inhibitor concentrations between 5.21 $\times$ 10^{-6}M and 6.95 $\times$ 10^{-6}M. With inhibitor concentrations of 6.95 $\times$ 10^{-6}M to 2.78 $\times$ 10^{-5}M there is a second much more gradual slope. The enzyme activity in the presence of 6.95 $\times$ 10^{-6}M of 3β-A-diol is greater than at the lower inhibition concentration of 5.21 $\times$ 10^{-6}M. The experiment was repeated 5 times with similar results.

Determination of $K_i$ values (10) from the data presented in Fig. 5 - 8 were: 3β-A-diol, 1.7 $\times$ 10^{-6}M; 3α-A-diol, 3.3 $\times$ 10^{-6}M; and testosterone, 11.8 $\times$ 10^{-6}M.
Discussion

The cleavage of DHA-S by a microsomal extract or homogenates of rat testis has been reported by Burstein and Dorfman (8) and Notation and Ungar (4,11). The present study was carried out with a particulate preparation obtained by recentrifuging a lyophilized microsomal fraction. This procedure doubled the specific enzyme activity of the lyophilized preparation. The kinetic studies indicate $K_m$ values for DHA-S cleavage that are considerably lower than those previously reported (8, 11). They also show $\Delta^5$-dil-
3-S is a better substrate than DHA-S and therefore may be an important intermediate in the formation of free steroids from DHA-S. In an earlier study (2) a soluble extract of rat testis was observed to convert DHA-S to $\Delta^5$-dol-3-S four times as fast as free DHA was converted to androstenediol under identical incubation conditions. The microsomal fraction from rat testis, in the presence of appropriate cofactors, converted DHA-S to $\Delta^5$-dol-3-S, testosterone, and other free metabolites (12). Similar results have been obtained when incubating DHA and DHA-S simultaneously with either a soluble or microsomal fraction obtained from rabbit ovaries (3). Pérez et al (13) when studying the synthesis of testosterone from DHA and DHA-S in homogenates of testis obtained from a patient with the testicular feminization syndrome, isolated both androstenediol and $\Delta^5$-dol-3-S as intermediates. Slaunwhite and Burgett (14) have reported the rapid conversion of androstenediol to testosterone in rat testicular homogenates. The conversion of DHA-S to testosterone has been reported by Aakvaak et al (15) to occur in perfused canine testis and ovaries. These investigators were unable to detect any free DHA in the spermatic vein after infusion of DHA-S into the spermatic artery, suggesting that DHA-S may have been reduced to $\Delta^5$-dol-
3-S prior to the cleavage of the sulfate ester. The finding in the present study that the $K_m$ for $\Delta^5$-diol-3-S is less than half the $K_m$ for DHA-S provides support for the view that $\Delta^5$-diol-3-S may be an important intermediate in the conversion of DHA-S to testosterone in testis and possibly in other tissues.

The apparent $K_m$ of $2.04 \times 10^{-6}$M observed in this study for DHA-S cleavage is considerably lower than the $K_m$ for DHA-S cleavage by rat testicular microsomal fractions or homogenates reported by Burstein and Dorfman (8) and Notation and Ungar (11). The former authors studied DHA-S cleavage in 0.1M Tris-acetate buffer, pH 7.2, and reported apparent $K_m$ values ranging from 0.85 to $2.5 \times 10^{-5}$M. In the studies of Notation and Ungar, DHA-S cleavage was carried out in 0.1M bicarbonate buffer, pH 7.4, in the presence of ATP, $\beta$-DPN and $K_2SO_4$. They reported a $K_m$ value for DHA-S cleavage of $1 \times 10^{-5}$M. As shown in the present study, the pH optimum for DHA-S cleavage with a lyophilized rat testicular microsomal fraction was approximately 6.2 and that for $\Delta^5$-diol-3-S was approximately 6.5. At pH 7.2 or 7.4 the activity of the enzyme changes sharply with variation in pH (Fig. 1) and this variation may complicate the measurement of $K_m$ values. In the present study $K_m$ values measured in 0.1M Tris-HCl
buffer at pH 7.4 were $6.7 \times 10^{-6}$ M for DHA-S and $2.6 \times 10^{-6}$ M for $\Delta^5$-dion-3-S. The value for DHA-S cleavage at pH 7.4 is closer to, but still lower than, previously published values. This variation could be due to the somewhat purer enzyme preparation used in this study. Some variation with different enzyme preparations was observed similar to that reported by Burstein and Dorfman (8). Preliminary studies suggest that there may be a relationship between $K_m$ and the age of the rats, the higher values being observed in testes obtained from older rats. However, further studies need to be done to establish this relationship. In all instances the relative difference for DHA-S and $\Delta^5$-dion-3-S cleavage was observed.

Of the various free steroids tested as inhibitor of the cleavage of DHA-S or $\Delta^5$-dion-3-S, 3β-A-diol was the most potent when added at levels equimolar to that of the substrate (Table 1). Three other steroids exhibited a lesser but significant capacity to inhibit the reaction. These were 3α-A-diol, androstenediol and 17β-estradiol. DHA and testosterone were much less effective as inhibitors while $\Delta^4$-androstenedione was practically without effect.

Notation and Ungar (11) recently reported inhibition of DHA-S cleavage by several of the free
steroids that were examined in the present study. When a ratio of inhibitor to substrate concentration of 13 to 1 was employed, the magnitude of inhibition was the same or less than that presently reported for equimolar concentrations of inhibitor and substrate. Cofactors used in the incubation medium by Notation and Ungar may have affected the degree of inhibition observed. They noted that DHA in the presence of DPN was rapidly metabolized; it is likely that androstenediol, estradiol and testosterone were also metabolized in the presence of this cofactor. Furthermore, ATP, K_2SO_4 and Mg^{++} in their incubation medium could have resulted in sulfurylation of 3β-hydroxysteroids as they reported previously for DHA (4).

The demonstration in this study that very low concentrations of the 5α-A-diols are needed for inhibition of the sulfatase activity, as reflected in the low K_i values, suggest that either of these compounds might control androgen synthesis from DHA-S in testis. Gower (5) reported the isolation of 3α-A-diol from rat testis incubated with DHA. Over a four hour incubation period there was a rapid increase in formation of this compound. This "diol" and the 3β-A-diol were also identified as products when a rat testis homogenate was incubated with testosterone (16).
The identification of the 3β-A-diol as a product of DHA-S and of Δ5-diol-3-S metabolism in testes is presently being investigated. This compound has been identified as a product of DHA-S and DHA metabolism by rabbit ovary microsomal fraction (3).

The kinetic data observed for the inhibition of DHA-S cleavage by 3α-A-diol suggest that this inhibitor acts competitively but at a site distinct from the catalytic site. If it was acting competitively at the catalytic site one would expect to obtain a linear relationship when plotting the reciprocal of the velocity against concentration of inhibitor. None of the three inhibitors that were more thoroughly investigated gave such a linear relationship. The hyperbolic curves that result when plotting 1/v against concentration of 3α-A-diol or testosterone are indicative of allosteric inhibition. The same type of plot for 3β-A-diol as the inhibitor did not yield either a continuous straight line or a hyperbolic curve. It seems unlikely that the kinetics observed with these free steroids are a reflection of a decrease in solubility at higher inhibitor concentrations since deviation from linearity in the 1/v vs inhibitor concentration plots occurs at markedly different concentrations of the three inhibitory steroids investigated.
At present there is no ready explanation for the kinetics observed with the 3β-A-diol as the inhibitor. It was recrystallized and checked for purity in three separate chromatography systems and yielded a single compound in each. It had a melting point of 165° which agrees well with published melting point (17). A possible explanation for the unusual kinetics is that a conformational change of the enzyme occurs at a critical concentration of the inhibitor. Enzymes exhibiting allosteric effects are usually oligomers or polymers involving several interacting subunits. A molecular weight of $>600,000$ has been reported by Burstein (18) for a rat liver microsomal steroid sulfatase that cleaves DHA-S. This high molecular weight is consistent with the possibility of this enzyme existing as a polymer and therefore exhibiting the tendency for conformational flexibility. Solubilization of the testicular enzyme will be required preliminary to its rigorous characterization and further studies of the mode of inhibition.

The kinetic data, presented in this report, illustrating inhibition by a distant product (i.e. a 5α-A-diol) of the DHA-S androgenic pathway and inhibition by high concentrations of substrate are consistent with the possibility that this testicular steroid sulfatase may
act as a "control" enzyme (11). DHA-S which is present in relatively high concentration in plasma (19) and can be synthesized within the testis (13, 20) could serve as the inactive precursor of active hormones.

Acknowledgments

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References

1. The following trivial names and abbreviations have been used:

Dehydroepiandrosterone (DHA), 3β-hydroxy-5-androsten-17-one; Dehydroepiandrosterone sulfate (DHA-S), 17-oxo-5-androsten-3β-yl sulfate; Androstenediol, 5-androstene-3β,17β-diol; Androstendiol-3-sulfate (Δ5-diol-3-S), 17β-hydroxy-5-androstene-3β-yl sulfate; Androstenedione, 4-Androstene-3,17-dione; Testosterone, 17β-hydroxy-4-androsten-3-one; Dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; Epitestosterone, 17α-hydroxy-4-androsten-3-one; Epitestosterone, 3α-hydroxy-5α-androstan-17-one; Androsterone, 3α-hydroxy-5α-androstan-17-one; 3β-A-diol, 5α-androstane-3β,17β-diol; 3α-A-diol, 5α-androstane-3α,17β-diol; Estrone, 3-hydroxy-1,3,5 (10)-Estratriene-17-one; 17β-Estradiol, 1,3,5 (10)-Estratriene-3-17β-diol; 17α-Estradiol, 1,3,5 (10)-Estratriene-3-17α-diol; Estriol, 1,3,5 (10)-Estratriene-3,16α,17β-triol; 11β-hydroxyestradiol, 1,3,5 (10)-Estratriene-3,11β,17β-triol.


