HUMAN LIVER S9 FRACTIONS: METABOLISM OF DEHYDROEPIANDROSTERONE, EPIANDROSTERONE, AND RELATED 7-HYDROXYLATED DERIVATIVES

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ABSTRACT:

Dehydroepiandrosterone (DHEA) and 3β-hydroxy-5α-androstan-17-one (epiandrosterone, EpiA) are both precursors for 7α- and 7β-hydroxylated metabolites in the human brain. These 7-hydroxylated derivatives were shown to exert anti-glucocorticoid and neuroprotective effects. When these steroids are administered per os to humans, the first organ encountered is the liver, where extensive metabolism takes place. The objective of this work was to assess the cofactor dependence and metabolism of DHEA, EpiA, and their metabolites in S9 fractions of human liver, using radiolabeled steroid substrate for quantification and gas chromatography-mass spectrometry for identification. The best transformation yields were obtained with NADPH and were larger in female than in male. Results showed that both DHEA and EpiA mainly transformed into their 17β-hydroxylated derivatives, 7- or 16α-hydroxylated metabolites under NAD(P)H conditions, and 5α-androstan-3,17-dione for EpiA under NAD(P)⁺ conditions. In turn, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA were partly transformed into each other via a 7-oxo-DHEA intermediate and were reduced into the 17β-hydroxy derivative, respectively. The same type of transformations occurred for 7α-hydroxy-EpiA and 7β-hydroxy-EpiA, except that no 7-oxo-EpiA intermediate was obtained. These findings determine the presence of enzymes responsible for the 7α- and 16α-hydroxylation in the human liver, the 11β-hydroxysteroid dehydrogenase type 1 responsible for the oxidoreduction of the 7-hydroxylated substrates, and the 17β-hydroxysteroid dehydrogenase responsible for the reduction of 17-oxo-steroids into 17β-hydroxysteroids.

Dehydroepiandrosterone (DHEA) has been termed “neurosteroid” after evidence was found for its production in the brain of gonadectomized and adrenalectomized rats (Corpechot et al., 1981), and this neurosteroid was also found in the human brain (Brown et al., 2000b; Lanthier and Patwardhan, 1986; Weill-Engerer et al., 2002). It is doubtful whether DHEA is important for neuroprotection because larger amounts exist in the cerebrospinal fluid of patients with neurodegenerative diseases (Brown et al., 2000a; Kim et al., 2003) and because of the absence of DHEA-mediated protective effects obtained in vitro (Pringle et al., 2003). This raised the question of DHEA metabolism and the putative neuroprotective effects of the metabolites produced. Previous research on the liver and brain showed that DHEA 7α-hydroxylation takes place in both organs (Doostzadeh and Morfin, 1996; Doostzadeh et al., 1997, 1998; Rose et al., 1997). All studies provided evidence for a P450-catalyzed process. The screening of hippocampal transcripts in rats, mice, and humans led to the identification of a new cytochrome P450 (termed CYP7B1) and to its cDNA production (Stapleton et al., 1995; Rose et al., 1997; Schwarz et al., 1997; Wu et al., 1999; Morfin and Starka, 2001; Trincal et al., 2002). The presence of CYP7B1 in the liver was demonstrated (Schwarz et al., 1998; Wu et al., 1999) and found to be responsible for the 7α-hydroxylation of most 3β-hydroxysteroids, including oxysterols, pregnenolone, DHEA, 5α-androstan-3β,17β-diol, 5α-androstan-3β,17β-diol, estradiol (Rose et al., 1997), 3β-hydroxy-5α-pregnan-20-one (Strömstedt et al., 1993), and epiandrosterone (EpiA) (Lafaye et al., 1999; Kim et al., 2004b).

Investigations of DHEA metabolism in mouse brain tissue provided evidence for the NADPH-dependent formation of almost equal quantities of both 7α-hydroxylated and 7β-hydroxylated transformation products (Doostzadeh et al., 1997; Doostzadeh and Morfin, 1997). No P450 was found to be responsible for 7β-hydroxy-DHEA production. Investigations with knockout mice for CYP7B1 showed that neither 7α-hydroxy-DHEA nor 7β-hydroxy-DHEA was produced (Lathe, 2002), which implied that the CYP7B1 was a requisite for their production. The question arising on the process responsible for 7β-hydroxy-DHEA has been tentatively addressed recently with data showing that in liver microsomes, the 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-HSD3 and unknown enzymes may be involved in an oxidoreduction process transforming 7α-hydroxy-DHEA into 7-oxo-DHEA and 7β-hydroxy-DHEA (Robinson et al., 2003; Kim et al., 2004a).

Neuroprotection exerted by 7α-hydroxy-DHEA was first suggested after work with rat hippocampal cell cultures and comparison with aromatization products (Jellinck et al., 2001). More recently, a test for this hypothesis was developed on a model of hypoxia-induced neu-
rodegeneration in organotypic rat hippocampal slice cultures (Pringle et al., 1997) and on an in vivo model of cerebral ischemia obtained in rats by four-vessel occlusion, which tested several 7-hydroxylated steroids (Pringle et al., 2003). It was then clearly shown that DHEA, EpiA, 7-keto-EpiA, 5α-androstan-3β,7β,17β-triol, and estradiol had no neuroprotective effects, whereas 7α-hydroxy-DHEA, 7α-hydroxy-EpiA, and 7β-hydroxy-EpiA prevented neuronal damage at 24 h posthypoxia (Pringle et al., 2003). The best results obtained in vivo were with 7β-hydroxy-EpiA (0.03 mg/kg injected in the femoral vein), which suggested that native 7β-hydroxysteroids may exert a neuroprotective function (Pringle et al., 2003; Dudas et al., 2004).

Possible pharmacological use of any of the neurosteroids described (i.e., DHEA, 7α-hydroxy-DHEA, 7β-hydroxy-DHEA, EpiA, 7α-hydroxy-EpiA, 7β-hydroxy-EpiA) implies that metabolism in the liver will determine drug transformation and subsequent availability of products to the brain. Our goals were to study the yields of these transformations in human liver S9 fractions and to identify the metabolites produced. To achieve this, we produced the labeled steroid substrates, incubated them under various conditions with human liver S9 preparations and then identified the metabolites by gas chromatography/mass spectrometry.

Materials and Methods

Steroids and Reagents. [4-14C]DHEA (55.5 mCi/mmol) and [4-14C]5α-dihydrotestosterone (53.6 mCi/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Paris, France). [4-14C]7α-hydroxy-DHEA, [4-14C]EpiA, [4-14C]7β-hydroxy-DHEA, [4-14C]7α-hydroxy-EpiA, and [4-14C]7β-hydroxy-EpiA were produced as previously described (Chalbot et al., 2002). DHEA, EpiA, 5α-androstan-3β,7β,17β-diol, 5α-androstan-3β,7β,17β-diol, 5α-androstan-3α,17β-diol, 17β-hydroxy-5α-androstane-3-one, testosterone, 4-androstene-3,17-dione, 5α-androstan-3,17-dione, 17β-estradiol, estrone, NADH, NAD+, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and buffer salts were obtained from Sigma-Aldrich (L’Isle d’Abeau Chesnes, France). The steroid references 7β-hydroxy-EpiA, 7α-hydroxy-EpiA, 6α-hydroxy-EpiA, and 7,8-oxo-EpiA were produced first from EpiA after incubation with the Rhizopus nigricans strain and purification according to Chambers et al. (1973). A custom chemical synthesis by Roosvin S.A. (Paris, France) provided milligram quantities of chemically pure 7α-hydroxy-DHEA, 7β-hydroxy-DHEA, 7α-hydroxy-EpiA, and 7β-hydroxy-EpiA. 5α-androstan-3β,7α,17β-triol and 5α-androstan-3β,7β,17β-triol were obtained after a KBH4 reduction of their respective 17-oxo precursors, 7α-hydroxy-DHEA, 16α-hydroxy-DHEA, and 3β,16α-dihydroxy-5α-androstane-17-one were obtained from Steraloids (Newport, RI). Both 5α-androstan-3β,7α,17β-triol and 5α-androstan-3β,7β,17β-triol were obtained after a KBH4 reduction of 7-oxo-DHEA. 3β,17β-Dihydroxy-5α-androstane-7-one and 7,8-oxo-EpiA were gifts from Dr. E. R. H. Jones (Oxford University, Oxford, UK). All solvents, Merck, Darmstadt, Germany) were of reagent grade.

Human Liver S9 Fractions. Both male and female human liver S9 pools transported in liquid nitrogen were from In Vitro Technologies Inc. (Baltimore, MD) and kept at −80°C before use. The female S9 pool derived from 10 donors with a 22 mg/ml protein content and a catalytic 7-ethoxycoumarin O-deethylase activity of 95 pmol/mg protein/min. The male S9 pool derived from 15 donors with a 21 mg/ml protein content and with a catalytic 7-ethoxycoumarin O-deethylase activity of 59 pmol/mg protein/min. The male S9 pool derived from 10-ml borosilicate glass tubes prior to the addition of the thawed human liver S9 fraction (0.75 mg of proteins) and either a 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, followed by a 0.25-mL solution in a buffer of the following: 1 mg of NADH, 1 mg of NADP+, 1 mg of NAD+, or a 0.65 mM concentration of the NADPH-regenerating system in 2% NaHCO3 containing NADP+, 7.09 mM glucose 6-phosphate, and 1.5 U/ml glucose-6-phosphate dehydrogenase. Identical conditions were used for control incubations with either 10-min boiled or unsupplemented S9 fractions. The tubes were left open during the course of incubation with shaking at 37°C for 20 min. Incubations were stopped after the addition of 0.5 ml of aceton followed by 4 ml of ethyl acetate. The extraction process continued with 4 ml of ethyl acetate and was repeated three times. Counting of the aqueous phase and dpm computation were carried out with an Amersham Biosciences AB (Uppsala, Sweden) 1209 rack beta liquid scintillation counter fitted with external standard equipment and automatic background subtraction (PerkinElmer, France).

Steroid Metabolite Separation and Quantification. Ethyl acetate extracts recovered from incubations with 4-14C-labeled steroids were analyzed by thin-layer chromatography (TLC) on ready to use silica-60-coated glass plates (Merck) developed once either in ethyl acetate or in benzene/ethanol (9:1 v/v). In these systems, the metabolites of each steroid substrate tested were clearly separated, except for the 7α-hydroxy-EpiA/7β-hydroxy-EpiA mixture. Radio-steroids were located on the plates by autoradiography, recovered by scraping with a razor blade, and eluted from the gel with methanol/ethyl acetate (1:9 v/v). Autoradiography of the thin-layer chromatograms with BioMax Light X-ray film (Eastman Kodak, Rochester, NY) was performed to locate the radio-steroids on TLC silica gel plates. Quantitative scanning of the plates was carried out with use of a Berthold automatic TLC-linear analyzer (PerkinElmer, France). High performance liquid chromatography (HPLC) was used in response to the poor TLC separation of the 7α-hydroxy-EpiA/7β-hydroxy-EpiA mixture. The Shimadzu LC-6A HPLC apparatus was fitted with a 100-μl injection loop and a 26-cm C18-coated silica (5-μm) column (Ultra- base; Société Française Chromato Colonne) from Interchim (Montluçon, France) that was maintained at 30°C. Elution was carried out at 0.7 ml·min−1 with methanol/water (6:4 v/v) and collection of 0.25-mL portions split for GC/MS identification and for quantification by counting.

Steroid Metabolite Identification (GC/MS Analysis). Rf values after TLC and retention times after HPLC were compared with those of authentic standards, and each major steroid metabolite was identified by GC/MS analysis with an Agilent Technologies (Massy, France) system including a 6890N network GC apparatus coupled with a 5973 network mass selective detector. Trimethylsilyl (TMS) ether derivatives of authentic steroid references and unidentified steroid metabolites were prepared just before analysis by reacting the dried steroids in 30 μl of pyridine with 20 μl of bis-silyl-trifluoroacetic acid containing 1% trimethylchlorosilane (Pierce Chemical, Rockford, IL) at 60°C for 60 min. TMS derivatives were dried and taken up in toluene for injection on an HP-5MS fused silica capillary column (30 m, 0.25-mm i.d.) coated at 5% with a phenylmethylsiloxane phase (0.25-μm thickness). The oven temperature was programmed from 80°C for 1 min and then with a temperature increment of 30°C/min, up to 240°C. After 5 min at 240°C, the temperature was increased again at 30°C/min up to 300°C and then maintained up to the end of the run. The column was directly coupled to the quadrupole mass spectrometer ionization chamber, where the source was set at 230°C and the energy of bombarding electrons was at 70 eV. The mass abundance was determined two times per second by scanning from m/z 50 to m/z 800. The identity of each of the major steroid metabolites produced by the human liver S9 fractions was confirmed by comparison of their retention time and mass spectra with those of the 25 authentic reference compounds. The assessment used the match-up function on the built-in library in the MSD ChemStation software D.01.00 (Agilent Technologies).

Results

Preliminary experiments were carried out with [4-14C]DHEA or [4-14C]EpiA and 0.75 mg of protein from human liver S9 fractions with use of either a 10 mM Tris-HCl buffer (pH 7.4) or a 66.7 mM phosphate buffer (pH 7.4), both containing 1 mM EDTA, and in the presence of the NADPH-regenerating system, or NADH, NAD+, and NADP+. The best yields were obtained with the phosphate buffer that was then selected for all other incubations. In these conditions, the control incubations carried out with 10-min boiled S9 fractions or in the absence of cofactors yielded no transformation products for any of the steroid substrates used.
DHEA Metabolism. After GC/MS comparison, the metabolites produced were identified with 7α-hydroxy-DHEA, 7β-hydroxy-DHEA, 7-oxo-DHEA, 16α-hydroxy-DHEA, and 5-androstene-3β,17β-diol authentic steroids (Table 1). One minor metabolite was not separated from 7-oxo-DHEA after TLC; however, after GC under a TMS derivative form of the mixture, it could not be formally identified. Fragments in its mass spectrum were those of a monohydroxylated DHEA derivative, thus leading to its qualification as n-hydroxy-DHEA (Table 1). The liver S9 fractions from both male and female donors transformed DHEA mainly under reductive cofactor supplementation. The largest yields in metabolism concerned the cofactor required. Both oxidized cofactors gave poor yields in the liver S9 fractions from both male and female donors transformed EpiA mainly under reductive conditions. The production of 5-androstene-3β,7α,17β-triol required the action of a 17β-hydroxysteroid oxidoreductase enzyme. This enzyme was almost as active with NADP(H) as with NAD(H), which indicates either that the 7α-hydroxy-DHEA substrate could be reduced by both cofactors or that two isomers of the same enzyme were present with near equivalent activities.

7β-Hydroxy-DHEA Metabolism. Only two metabolites were produced by female liver S9 fractions under NADPH supplementation, and these were identified with 7-oxo-DHEA and 5-androstene-3β,7β,17β-triol authentic steroids (Table 1). No trace of 7β-hydroxy-DHEA was detected, thus implying that the 7-oxo-DHEA produced could not be reduced back into the 7β-hydroxy-DHEA. The production of 5-androstene-3β,7α,17β-triol appeared to be a NADPH-driven oxidoreductase reaction. As for 7α-hydroxy-DHEA, the production of 5-androstene-3β,7β,17β-triol required the action of a 17β-hydroxysteroid oxidoreductase that was active under NADPH conditions (Table 2).

EpiA Metabolism. After a GC/MS comparison, the metabolites produced were identified with 7β-hydroxy-EpiA, 16α-hydroxy-EpiA, 5α-androstene-3β,17β-diol, and 5α-androstene-3,17-dione authentic steroids (Table 3). One minor metabolite was not formally identified because of the lack of appropriate reference and it was not investigated further (Table 3). The liver S9 fractions from both male and female donors transformed EpiA mainly under reductive conditions. The largest yields in metabolism were obtained after NADPH supplementation, except for 5α-androstene-3β,17β-diol and 5α-androstene-3,17-dione, where NADH and NADPH were the required cofactors, respectively (Table 4). No trace of 7α-hydroxy-EpiA was found.
obtained, in contrast to low but significant amounts of 7β-hydroxy-EpiA. In the female, both 16α-hydroxy-EpiA and 5α-androstane-3β,17β-diol were the major metabolites produced under NADPH and NADH supplementation, respectively. The same cofactor dependence occurred under oxidative conditions resulting in a larger production of 5α-androstane-3,17-dione in the female than in the male. This suggested that a 3β-hydroxysteroid oxidoreductase was present in larger quantities in the female liver as opposed to the male. The female liver S9 fractions were used for the studies of 7α- and 7β-hydroxy-EpiA metabolism because of the equivalent EpiA metabolic patterns in both sexes and the larger yields obtained in females.

### 7α-Hydroxy-EpiA Metabolism
Neither TLC nor HPLC permitted the separation of the two metabolites produced after incubation with female liver S9 fractions (Table 4). After a TMS derivative formation, the two metabolites were separated by GC and identified with 7β-hydroxy-EpiA and 5α-androstane-3β,7α,17β-triol authentic

### TABLE 2
Percentage transformation yields of 20 μM DHEA, 7α-hydroxy-DHEA, and 7β-hydroxy-DHEA by human liver S9 fractions in the presence of reduced or oxidized cofactors

All incubations were carried out with 0.75 mg of protein in 0.67 M phosphate buffer containing 1 mM EDTA at 37°C for 20 min. Only human female liver S9 fractions were used for 7α- and 7β-hydroxy-DHEA incubations. Data are the means ± S.D. obtained from triplicate experiments.

<table>
<thead>
<tr>
<th>DHEA Metabolites</th>
<th>Sex</th>
<th>NADPH</th>
<th>NADH</th>
<th>NADP+</th>
<th>NADP+ő</th>
<th>NAD+ő</th>
</tr>
</thead>
<tbody>
<tr>
<td>7α-Hydroxy-DHEA</td>
<td>Female</td>
<td>1.33 ± 0.18</td>
<td>0.79 ± 0.36</td>
<td>0.18 ± 0.02</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>1.18 ± 0.18</td>
<td>0.45 ± 0.07</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>7β-Hydroxy-DHEA</td>
<td>Female</td>
<td>3.32 ± 1.17</td>
<td>1.42 ± 0.18</td>
<td>0.37 ± 0.02</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.96 ± 0.18</td>
<td>0.46 ± 0.17</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>7α-hydroxy-DHEA + 7-oxo DHEA</td>
<td>Female</td>
<td>4.15 ± 1.05</td>
<td>1.70 ± 0.64</td>
<td>0.60 ± 0.06</td>
<td>0.74 ± 0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>1.09 ± 0.09</td>
<td>0.60 ± 0.13</td>
<td>0.39 ± 0.07</td>
<td>1.11 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>16α-Hydroxy-DHEA</td>
<td>Female</td>
<td>6.01 ± 2.03</td>
<td>2.14 ± 0.76</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.85 ± 0.14</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
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<tr>
<td>5-Androstene-3β,17β-diol</td>
<td>Female</td>
<td>1.77 ± 0.77</td>
<td>8.57 ± 2.51</td>
<td>0.72 ± 0.12</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2.43 ± 0.55</td>
<td>10.77 ± 0.58</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>7α-hydroxy-DHEA metabolites</td>
<td>Female</td>
<td>6.13 ± 0.06</td>
<td>5.08 ± 0.02</td>
<td>2.10 ± 0.00</td>
<td>1.53 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.13 ± 0.39</td>
<td>0.39 ± 0.13</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>7β-Hydroxy-DHEA</td>
<td>Female</td>
<td>6.09 ± 0.51</td>
<td>1.32 ± 0.15</td>
<td>2.46 ± 0.10</td>
<td>1.70 ± 0.12</td>
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<tr>
<td></td>
<td>Male</td>
<td>0.34 ± 0.14</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
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<tr>
<td>7-oxo-DHEA</td>
<td>Female</td>
<td>3.31 ± 0.33</td>
<td>3.20 ± 0.30</td>
<td>8.79 ± 0.16</td>
<td>4.50 ± 0.34</td>
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</tr>
<tr>
<td></td>
<td>Male</td>
<td>2.43 ± 0.55</td>
<td>10.77 ± 0.58</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

N.D., not detected.

a After use of the NADPH-regenerating system.

b 1 mM.

### TABLE 3
Identification of the steroid metabolites produced after incubation of 20 μM EpiA, 7α-hydroxy-EpiA, and 7β-hydroxy-EpiA with human liver S9 fractions

<table>
<thead>
<tr>
<th>Steroids</th>
<th>TLCc Rf</th>
<th>TLCb Rf</th>
<th>HPLC Rf</th>
<th>GCc Rf</th>
<th>Characteristic Mass Fragments of TMS Derivatives</th>
<th>Quality of Match</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>m/z</td>
<td></td>
<td></td>
<td>M⁺  M⁺-15  M⁺-73  M⁺-90  Major Fragments°F</td>
<td></td>
</tr>
</tbody>
</table>

References

EpiA 0.69 0.43 12.70 362 347 N.D. 272 347
6α-Hydroxy-EpiA 0.13 0.23 15.09 450 435 N.D. 360 450, 271, 73
7α-Hydroxy-EpiA 0.25 0.24 11.42 13.75 450 435 N.D. 360 450, 360, 270, 75, 73
7β-Hydroxy-EpiA 0.22 0.23 8.70 15.49 450 435 N.D. 360 450, 75, 73
16α-Hydroxy-EpiA 0.58 0.34 15.61 450 435 N.D. 360 216, 117, 106, 75
5α-Androstan-3α,17β-diol 0.67 0.37 11.92 436 421 N.D. 346 256, 214, 215, 129, 75, 73
5α-Androstan-3β,17β-diol 0.63 0.36 13.46 436 421 N.D. 346 421, 241, 129, 75, 73
5α-Androstan-3α,7α,17β-triol 0.22 0.19 8.80 13.61 524 509 N.D. 343 395, 73
5α-Androstan-3β,7α,17β-triol 0.25 0.19 6.65 15.80 524 509 N.D. 343 384, 393, 73
7-oxo-EpiA 0.44 0.34 15.52 376 361 N.D. 286 376, 329
5α-Androstan-3,17-dione 0.82 0.74 12.46 288 273 215 198 288
5α-Dihydrotestosterone 0.75 0.46 13.10 362 347 N.D. 272 347, 272, 257, 129, 73

EpiA Metabolites

7α-Hydroxy-EpiA Metabolites

EpiA 0.22 15.59 450 435 N.D. 360 435, 243, 73 99
Unknown 0.29 N.D. N.D. N.D. N.D. N.D. N.D. no match
16α-Hydroxy-EpiA 0.33 15.88 450 435 N.D. 360 216, 117 99
5α-Androstan-3β,17β-diol 0.36 13.66 436 421 N.D. 346 241, 129, 75, 73 99
5α-Androstan-3α,17β-dione 0.69 12.66 288 273 215 198 368, 244, 217 99
7α-Hydroxy-EpiA Metabolites

5α-Androstan-3α,7α,17β-triol 0.24 8.70 13.68 524 509 N.D. 343 395, 73 98
5α-Androstan-3β,7α,17β-triol 0.24 8.70 15.51 450 435 N.D. 360 450, 75, 73 99
7β-Hydroxy-EpiA Metabolites

5α-Androstan-3α,7β,17β-triol 0.23 6.65 15.78 524 509 N.D. 343 395, 73, 75, 73 98
7α-Hydroxy-EpiA 0.23 11.47 13.71 450 435 N.D. 360 450, 360, 270, 75, 73 98

Rt, retention time; N.D., not detected.

a Developed in ethyl acetate.
b Developed in benzene/ethanol (9:1 v/v).
c Developed in methanol/H₂O (6:4 v/v) at 30°C.
d Retention times of TMS derivatives.

EpiA, EpiA metabolites; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GC, gas chromatography; min, minutes; m/z, mass to charge ratio; N.D., not detected.
steroids (Table 3). The 7β-hydroxy-EpiA/5α-androstane-3β,7α,17β-triol ratio obtained after GC/MS separation indicated that 7β-hydroxy-EpiA only was produced in the presence of NADP⁺, whereas increasing amounts of 5α-androstane-3β,7α,17β-triol appeared under NADPH, NAD⁺, and NADH conditions. No trace of 7-oxo-EpiA was detected; therefore, the putative oxidation of 7α-hydroxy-EpiA into 7β-hydroxy-EpiA could only be suspected. Reductive conditions were necessary for a significant production of 5α-androstane-3β,7α,17β-triol by the two NADPH-dependent and NADH-dependent 17β-hydroxysteroid oxidoreductases.

**7β-Hydroxy-EpiA Metabolism.** Two metabolites were obtained after incubation with female liver S9 fractions and were identified with 7α-hydroxy-EpiA and 5α-androstane-3β,7α,17β-triol authentic steroids by GC/MS as shown in Table 3. Significant and major yields of 7α-hydroxy-EpiA were obtained under reductive and oxidative conditions, which led us to suspect an oxidoreductive mechanism (Table 4). The highest amounts of 5α-androstane-3β,7α,17β-triol were produced under NADH supplementation, thus inferring the presence of a NADH-dependent 17β-hydroxysteroid oxidoreductase.

**Discussion**

We used human liver S9 fractions that contained both hepatic microsomes and cytosol with active steroid-metabolizing enzymes. The age of the donors was not provided. Compared with microsomes and cytosol, S9 fractions offer more complete although lower representation of the steroid metabolism, since they contain both phase I and phase II activity (Brandon et al., 2003). Research into DHEA metabolism helped to link our data with those of others and consequently validate our approach for the studies of EpiA and 7-hydroxysteroid metabolism. The supplementation of S9 fractions with cofactors was necessary (Brandon et al., 2003) and allowed the sorting out of enzymes through their reductive or oxidative activities and dependence upon NAD(H) or NADP(H). The use of a NADPH-regenerating system helped with the hydroxylation process carried out by the microsomal cytochromes P450, which are well known in the liver. Thus, the 7α-hydroxylation of DHEA was already shown in human liver (Wu et al., 1999; Fitzpatrick et al., 2001; Miller et al., 2004) and described as resulting from catalysis by CYP3A4 (Fitzpatrick et al., 2001; Miller et al., 2004) and CYP7B1 (Wu et al., 1999). Since CYP7B1 carries out the 7α-hydroxylation on most of the 3β-hydroxysteroid substrates (Rose et al., 1997), it was surprising to find no trace of 7α-hydroxy-EpiA in EpiA digests. We have reported that other human tissue preparations 7α-hydroxylated EpiA (Lafaye et al., 1999) and found recently that the human CYP7B1 expressed in yeast microsomes carried out the 7α-hydroxylation of EpiA with a KM higher than that of DHEA (Kim et al., 2004b). The fact that 7β-hydroxy-EpiA was produced instead of 7α-hydroxy-EpiA suggests that one liver P450 carried out the 7β-hydroxylation of EpiA. At present, there is no documentation to support a P450-mediated 7β-hydroxylation of EpiA. However, the 7β-hydroxylation of DHEA by CYP3A4 is documented (Stevens et al., 2003; Miller et al., 2004), and this P450 may also carry out the 7β-hydroxylation of EpiA in the liver. We also observed a 7α/7β interconversion. This was reported as resulting from an oxidoreduction process catalyzed by 11β-hydroxysteroid dehydrogenase isofoms using both 7α-hydroxy-DHEA and 7β-hydroxy-DHEA via the 7-oxo-DHEA (Robinzon et al., 2003). Our data confirm such a paradigm, with DHEA yielding at least equivalent levels of 7α- and 7β-hydroxy-DHEA produced, together with large amounts of 7-oxo-DHEA. In turn, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA were both precursors for 7-oxo-DHEA. Concerning 7α-hydroxy-EpiA and 7β-hydroxy-EpiA, their interconversion was obtained without evidence for 7-oxo-EpiA production. It is then possible that either the 11β-hydroxysteroid dehydrogenase-catalyzed oxidoreduction process reported for 7-hydroxy-DHEA (Robinson et al., 2003) may extend to the 7-hydroxylated EpiA substrates, or that an unknown epimerase is involved in the interconversion process.

Our findings of significant amounts of 16α-hydroxy-DHEA and 16α-hydroxy-EpiA in DHEA and EpiA digests imply the presence of CYP3A4 and CYP3A5 in liver S9 fractions. These two P450s were described as responsible for DHEA 16α-hydroxylation (Niwa et al., 1998; Miller et al., 2004) rather than CYP3A7, which is found in human endometrium, placenta, and fetal liver (Miller et al., 2004). Even though the adult human liver carries out the 16α-hydroxylation of DHEA (Miller et al., 2004), no direct evidence is available for the 16α-hydroxylation of EpiA. We found a significantly larger production of 16α-hydroxy-DHEA and 16α-hydroxy-EpiA in females than...
in males, thus implying sex differences in the P450s responsible. Provided that EpiA 16α-hydroxylation is carried out by CYP3A4, this finding is in accordance with the higher levels of CYP3A4 found in the human female liver (Wolbold et al., 2003). In contrast, no unknown metabolite that could be 16α-hydroxylated was found in digests of 7α- and 7β-hydroxy-DHEA or 7α- and 7β-hydroxy-EpiA, thus inferring that the 7-hydroxylated derivatives of DHEA and EpiA were not substrates for the 16α-hydroxylase.

The human liver contains several isoforms of the 17β-hydroxysteroid oxidoreductase that differ in their dependence for the NAD(H) or NADP(H) cofactors (Blomquist, 1995). In this study, all of the substrates tested were 17-oxo-steroids and readily transformed into their 17β-hydroxy derivatives under the reductive conditions using either NADPH or NADH. This is in contrast with the results obtained with liver microsomes (Fitzpatrick et al., 2001; Miller et al., 2004), where no 17β-reduction of DHEA was obtained. Since the liver S9 fractions contain both microsomes and cytosol, we may suspect the 17β-reducing enzyme to be cytosolic. Thus, male and female livers contained both a NADP(H)-dependent and a NAD(H)-dependent 17β-hydroxysteroid oxidoreductase almost equivalently active on the DHEA substrate. In contrast, EpiA metabolism showed none of the NADP(H)-dependent 17β-hydroxysteroid oxidoreductase activity in the female, but a NAD(H)-dependent 17β-hydroxysteroid oxidoreductase activity was significantly larger in the female than in the male liver. Whether these differences result from different affinities of the steroid substrates for the 17β-hydroxysteroid oxidoreductases or from different 17β-hydroxysteroid oxidoreductase isoforms cannot be stated from the present results.

We found that EpiA was oxidized at the 3β-position. This is in contrast to DHEA, which was unchanged, in agreement with reports using human liver microsomes (Fitzpatrick et al., 2001; Miller et al., 2004). DHEA oxidation into 4-androstene-3,17-dione implies a two-step mechanism catalyzed by the NAD-dependent 3β-hydroxysteroid dehydrogenase/isomerase that is commonly found in steroidogenic tissues rather than in the liver. In contrast, the presence in human liver homogenates of a microsomal 3β-hydroxysteroid oxidoreductase has been reported (Pirog and Collins, 1999).

The present data obtained with the 7-hydroxylated EpiA and 7-hy-
droxylated DHEA metabolism in liver are of importance when the neuroprotective action of these steroids is considered. The putative use of 7β-hydroxy-DHEA as a neuroprotective agent is presently sought, and it is of importance to know whether, once administered per os, the liver enzymes will dramatically decrease the blood-borne quantities brought up to the brain. Our data show that more than 90% of the 7β-hydroxy-DHEA incubated are left intact, and that both the 5α-androstan-3β,7β,17β-triol and the 7α-hydroxy-DHEA produced may be transformed back into 7β-hydroxy-DHEA. The same findings apply to 7β-hydroxy-DHEA. One shortcoming of our present data concerns the putative action of phase II enzymes that would produce water-soluble conjugated derivatives. Recovery yields after ethyl acetate extraction of the digests were between 96 and 99%, and the possible steroid metabolites in the water phase were not examined. Yet, the formation of conjugates requires the addition of specific exogenous cofactors (Brandon et al., 2003), and this was not done in the present study. Nevertheless, the present findings are indicative of the metabolites produced in both sexes, even if transposable to neither whole homogenates nor an in vivo situation.

Our findings about the metabolism of DHEA, EpiA, and 7-hydroxylated derivatives by human liver S9 fractions are summarized in Fig. 1. The main originalities in these findings are that the 7-hydroxylated derivatives of DHEA and EpiA are not substrates for cytochrome P450-hydroxylating enzymes in human liver, and that both 7α- and 7β-epimers are converted into each other. The putative involvement of the microsomal 11β-hydroxysteroid dehydrogenases through an oxidoreductive process involving the production of a 7-oxo intermediate is appealing and justifies further investigations with use of the purified human enzymes.

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