Metabolism of anabolic steroids by recombinant human cytochrome P450 enzymes

Gas chromatographic–mass spectrometric determination of metabolites

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Abstract

Metabolism of steroid hormones with anabolic properties was studied in vitro using human recombinant CYP3A4, CYP2C9 and 2B6 enzymes. The enzyme formats used for CYP3A4 and CYP2C9 were insect cell microsomes expressing human CYP enzymes and purified recombinant human CYP enzymes in a reconstituted system. CYP3A4 enzyme formats incubated with anabolic steroids, testosterone, 17α-methyltestosterone, metandienone, boldenone and 4-chloro-1,2-dehydro-17α-methyltestosterone, produced 6β-hydroxyl metabolites identified as trimethylsilyl (TMS)-ethers by a gas chromatography–mass spectrometry (GC–MS) method. When the same formats of CYP2C9 were incubated with the anabolic steroids, no 6β-hydroxyl metabolites were formed. Human lymphoblast cell microsomes expressing human CYP2B6 incubated with the steroids investigated produced traces of 6β-hydroxyl metabolites with testosterone and 17α-methyltestosterone only. We suggest that the electronic effects of the 3-keto-4-ene structural moiety contribute to the selectivity within the active site of CYP3A4 enzyme resulting in selective 6β-hydroxylation.

Keywords: Anabolic steroids; Cytochrome P450; Enzymes

1. Introduction

The cytochrome P450 enzymes (CYP enzymes) are involved in oxidative, peroxidative and reductive metabolic reactions of drugs, environmental chemicals and endogenous compounds. More than 500 CYP enzymes classified in a CYP superfamily of enzymes were identified, characterized and classified into 74 families, 14 of which are present in humans containing 35 CYP enzymes. A number of members of the CYP superfamily have been successfully expressed in bacterial, yeast, insect and mammalian cells providing an unlimited source of CYP enzymes for studying their structure, function and catalytic activity [1]. Of the identified human CYP enzymes, CYP1A, CYP2B6, CYP2C and CYP3A have been reported to catalyze hydroxylation of both endogenous and exogenous steroid hormones at different positions [2–5]. Of the enzymes present in human liver, the CYP3A and CYP2C subfamilies are most
abundantly expressed accounting for approximately 28% and 18% of the total microsomal cytochrome P450, respectively. Both CYP3A4 and CYP2C9 enzymes are the most abundant enzymes of their subfamilies and are of a considerable interest due to their involvement in metabolism of a relatively large number of drugs including anabolic steroids. Although present at the low level in human liver (about 0.2%), CYP2B6 enzyme was used in the present study as it was reported to catalyze hydroxylation of testosterone in the 16α- and 16β-positions [1,6].

Among the biotransformation reactions of endogenous and exogenous steroids studied, 6β-hydroxylation of testosterone by CYP3A4 has been the most extensively investigated. 6β-Hydroxylation is the major pathway of testosterone oxidation by human liver and has been frequently used as a marker reaction for studying the metabolic activity of CYP3A4 enzyme both in vivo and in vitro. However, this reaction is catalyzed also by CYP2C9 [1,6]. Because of a considerable interest in the metabolism of steroid hormones, we performed a systematic study on hydroxylation of 3-keto-4-ene anabolic steroids in vitro using recombinant human CYP enzymes.

In vitro metabolism studies using human liver microsomes and different formats of recombinant human cytochrome P450 enzymes are now widely used for drug metabolism research. For instance, microsomes prepared from human lymphoblastoid, yeast and insect cells infected with baculovirus systems, and reconstitution systems containing purified P450 enzymes are used. These model systems have been used to rationally predict metabolic reactions in vivo and/or to develop new analytical methods [1,7].

In the present study we investigated in vitro metabolism of the 3-keto-4-ene anabolic steroids, testosterone, 17α-methyltestosterone, metandienone, 4-chloro-1,2-dehydro-17α-methyltestosterone and boldenone with two formats of human recombinant CYP3A4 and 2C9 enzymes, and with one format of human CYP2B6. The formats of the recombinant CYP3A4 and CYP2C9 enzymes that were used are insect cell microsomes and reconstituted systems containing purified recombinant human enzymes expressed by Escherichia coli. For CYP2B6 human lymphoblast cell microsomes expressing the human enzyme were used. The structures of the metabolites were determined as trimethylsilyl derivatives by gas chromatography–mass spectrometry (GC–MS).

2. Experimental

2.1. Chemicals

Testosterone, 17α-methyltestosterone, metandienone, boldenone and NADPH were purchased from Sigma (Deisenhofen, Germany), and 4-chloro-1,2-dehydro-17α-methyltestosterone was a gift from Jenafarm (Jena, Germany). 6β-Hydroxyl metabolites of testosterone, 17α-methyltestosterone, metandienone, 4-chloro-1,2-dehydro-17α-methyltestosterone [8] and N'-methyl-N'-trimethylsilyl trifluoroacetamide (MSTFA) were synthesized in our laboratory according to the method described previously [9]. All other reagents and solvents were of analytical grade and water was glass distilled twice before use.

2.2. Formats of human recombinant CYP enzymes

Insect cell microsomes (Baculosomes) containing cDNA-expressed human CYP3A4 or CYP2C9, purified recombinant E. coli expressed (Reco system) human CYP 3A4 or CYP2C9 in a reconstituted system and control microsomes Baculosomes containing microsomes from insect cells that were infected with wild-type baculovirus, were a gift from PanVera (Madison, WI, USA). CYP2B6 available as human lymphoblast cell microsomes expressing human CYP2B6 was purchased from Gentest (Woburn, MA, USA). The enzyme preparations were received frozen under dry ice and were stored at −80°C until usage.

2.3. Procedure for metabolism studies

Enzymatic reactions were carried out in glass tubes in a total volume of 500 μl at pH 7.4. The buffer used was either sodium phosphate buffer, pH 7.4 (Baculosomes and human lymphoblast microsomes) or the Buffer Mix supplied with the purified recombinant system (Reco system). Stock solutions
of the anabolic steroids and of the authentic 6β-hydroxyl metabolites were prepared in methanol at concentration of 1 mg/ml and were stored at −20°C. Aliquots (50 μl) of the compounds investigated were placed in glass tubes for incubation (final concentration 200 μM). After solvent evaporation under vacuum, 50 μl of 1 M phosphate buffer, pH 7.4 and 10 μl of microsomes (7.7 mg protein/ml) were added to the glass tubes. The mixture was diluted with 434 μl distilled water and pre-incubated for 3 min at 37°C. The reaction was started by adding 6 μl of a 100 mM solution of NADPH. After an incubation period of 30 min at 37°C, addition of 100 μl acetonitrile stopped the reaction.

Incubation using the purified recombinant enzyme system (Reco system) was performed as described for Baculosomes with following modifications: the reaction mixture contained 100 μl of the recombinant enzyme system (Reco system), 100 μl of the Buffer Mix supplied with the system and 294 μl of distilled water. The reaction was started by addition of 6 μl of the 100 mM solution of NADPH.

In the control experiments performed as described for Baculosomes, the steroids investigated were incubated without the addition of NADPH to the incubation mixture, with addition of the buffer instead of the enzyme preparations and with the control microsomes. In addition, 5 μg of each steroid and the synthesized reference 6β-hydroxyl compound was derivatized as described in Section 2.4. The derivatized compounds were analyzed by the GC–MS in parallel with the samples for metabolism studies.

2.4. Sample extraction and derivatization

The steroids and the metabolites were extracted with 5 ml tert.-butylmethyl ether (distilled over calcium hydride) in the presence of 50 mg of a solid buffer sodium bicarbonate–potassium carbonate (2:1, w/w) mixture. After centrifugation at 3000 g, the ether layer was transferred into glass tubes and evaporated to dryness under vacuum.

To obtain TMS-ethers for GC–MS analysis, dry residue was derivatized with 60 μl of MSTFA–imidazole (100:2, v/w) and heated for 15 min at 60°C.

2.5. Gas chromatography–mass spectrometry

The electron impact (EI) mass spectra were registered with a GC–mass selective detection (MSD) system Hewlett-Packard (GC 5890/MS 5970), with the electron energy set to 70 eV. The assay for the steroids and the metabolites was carried out using mass chromatography (MC). The analyses were performed using a Hewlett-Packard, HP-Ultra-1 fused-silica capillary column (cross-linked methyl silicone, OV-1, 17 m×0.20 mm diameter, film thickness 0.11 μm). The carrier gas was helium (1 ml/min, split 10:1), and the temperature program was as follows: initial temperature 180°C for 0 min, program rate 10°C/min to 270°C, 40°C/min to final temperature 320°C, and at 320°C for 1 min. The injector temperature was set to 300°C.

3. Results and discussion

3.1. GC–MS detection and identification of metabolites

To identify the metabolites, the trimethylsilylated extracts of the incubation mixtures were analyzed by GC–MS. The recorded GC–MS full scan mode spectra were analyzed by extraction of the molecular ions, (M+ -15) ions and the expected major ions at m/z 129, 143, 209, 243, 281 and 315 of proposed metabolites. Fig. 1 shows the GC–MS ion chromatograms for detection of the metabolites with extracted m/z 448 for testosterone (Fig. 1A), m/z 462 for methyltestosterone (Fig. 1B), m/z 281 for metandienone (Fig. 1C), m/z 315 for 4-chloro-1,2-dehydro-17α-methyltestosterone (Fig. 1D) and m/z 446 for boldenone (Fig. 1E) metabolite. Fig. 2A shows a typical total ion current chromatogram of the trimethylsilylated extract of the incubation mixture with insect cell microsomes containing c-DNA expressed human CYP3A4 (Baculosomes) without addition of the steroids, thus indicating the level of the biological background present. Fig. 2B and C show the GC–MS ion chromatograms with extracted ions at m/z 448 and m/z 281 in the experiments using control microsomes incubated with testosterone and metandienone, respectively. These ion chromatograms clearly show that the peaks corre-
Fig. 1. GC–MS ion chromatograms of the bis-TMS derivatives of the metabolites detected after incubation of testosterone, ion extracted at $m/z$ 448 (A), methyltestosterone, ion extracted at $m/z$ 462 (B), metandienone, ion extracted at $m/z$ 281 (C), 4-chloro-1,2-dihydro-17α-methyltestosterone, ion extracted at $m/z$ 315 (D), and boldenone, ion extracted at $m/z$ 446 (E).

Responding to the metabolites identified in Fig. 1A and C were not either produced by the control microsomes or present as impurities. Similar results were obtained when other steroids were incubated with either the control microsomes or the enzyme preparations containing the expressed human enzymes. In
addition, Figs. 1C and 2C show the presence of the relatively high ion signal from the biological background when the ions at m/z 281 were extracted for identification of the metandienone metabolite.

EI mass spectra of the metabolites are presented in Fig. 3A–E. 6β-Hydroxy parent compounds (Fig. 4) were identified as the major metabolites identified when testosterone, 17α-methyltestosterone, metandienone, 4-chloro-1,2-dehydro-17α-methyltestosterone and boldenone were incubated with both insect microsomes expressing human CYP3A4 (Baculosomes) and purified reconstituted system containing the E. coli expressed human CYP3A4 (Reco system). To confirm the structure of the identified metabolites, the retention times and the fragmentation patterns of the identified metabolites were compared with those of the synthesized reference 6β-hydroxyl derivatives. This allowed us to confirm 6β-hydroxylation of testosterone (Fig. 3A), 17α-methyltestosterone (Fig. 3B), metandienone (Fig. 3C) and 4-chloro-1,2-dehydro-17α-methyltestosterone (Fig. 3D). Fragmentation of the low concentration metandienone metabolite (Fig. 3C) is characterized by the low intensity ions at m/z values above 300 (ions at m/z 460 and m/z 445 corresponding to M⁺ and M⁺-15, respectively). In addition, intense ions at m/z 143 (typical for D-ring fragmentation), and m/z 209 and 281 (sequential loss of two –OTMS groups from the molecular ion) are present. The mass spectrum of the synthetic reference 6β-hydroxy-4-chloro-1,2-dihydro-
Fig. 2. Total ion chromatograms of the trimethylsilylated extract of the incubation mixture with insect cell microsomes containing c-DNA expressed human CYP3A4 (Baculosomes) without addition of the steroids (A), and GC–MS ion chromatograms of the incubation mixtures after incubation of testosterone, ion extracted at \( m/z \) 448 (B) and metandienone, ion extracted at \( m/z \) 281 (C) with control microsomes.
Fig. 3. EI mass spectra of bis-TMS derivatives of 6β-hydroxytestosterone (A), 6β-hydroxymethyltestosterone (B), 6β-hydroxymetandienone (C), 6β-hydroxy-4-chloro-1,2-dihydro-17α-methyltestosterone (D), proposed 6β-hydroxyboldenone (E), synthesized reference 6β-hydroxymetandienone (F) and of the parent compound boldenone (G). The metabolites were identified after incubation of the parent compounds with insect cell microsomes containing c-DNA expressed human CYP3A4 (Baculosomes) or with reconstituted system containing purified recombinant human enzyme expressed by E. coli (Reco system).
17α-methyltestosterone. In the mass spectrum of the latter metabolite present are the fragments at m/z 243 and 315 (Figs. 3D and 4), which are the 4-chloro fragments structurally corresponding to the ions at m/z 209 and 281 of 6β-hydroxyl-metandienone (Figs. 3C and 4). As the authentic 6β-hydroxylated boldenone was not available, the structure of the identified metabolite is proposed based on the following considerations. The molecular ion m/z 446 in the spectrum of the identified metabolite (Fig. 3E) differs by 88 mass units from that in the spectrum of the parent compound boldenone (m/z at 358, Fig. 3G). This difference suggests the presence of an additional –OTMS group in the structure of the metabolite (Fig. 4). The mass spectrum of the boldenone metabolite is characterized by the ions at m/z 431 (indicating loss of a methyl group from the molecular ion) and the ions at m/z 356 and m/z 266, which could be assigned to the sequential loss of two –OTMS groups from the molecular ion. The relatively abundant ion at m/z 209 can be explained by an A/B ring fragmentation (Figs. 3E and 4). This fragmentation pattern corresponds well with that of the structurally similar 6β-hydroxyl metandienone (Fig. 3F). As expected, the difference in fragmentation of these two metabolites exists in the fragments formed by fragmentation of the ring D (Fig. 4). These consideration, together
Fig. 4. Structures of bis-TMS derivatives of 6β-hydroxy metabolites formed by incubation of 3-keto-4-ene anabolic steroids with recombinant human CYP3A4 enzyme, and of mono-TMS derivative of the parent compound boldenone.

with the results obtained with other 3-keto-4-ene steroids (Fig. 3A–D), allowed us to propose that also the metabolite identified in the incubation mixture containing boldenone and both formats of CYP3A4, is 6β-hydroxy boldenone as shown in Fig. 4. No metabolites could be detected in the extracts.
from the incubation mixtures containing the anabolic steroids investigated and the expressed CYP2C9 enzyme. The results thus obtained correspond to those obtained after analysis of the extracts from the experiments in which the steroids were incubated with control microsomes.

Lymphoblast cell microsomes expressing CYP2B6 enzyme produced traces of 6β-hydroxyl metabolites when incubated with testosterone or 17α-methyltestosterone only.

3.2. 6β-Hydroxylation of anabolic steroids

6β-Hydroxylation was reported as the major in vivo metabolic pathway in the metabolism of the anabolic steroids 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone and metandienone, whereas for testosterone, 17α-methyltestosterone, and boldenone, in vivo 6β-hydroxylation was negligible [8]. Both in vivo and vitro hydroxylation at 6β-position catalyzed by CYP3A4 enzyme was reported for testosterone [3,4,10,11], progesterone [4,5], cortisol [12] and androstenedione [13], as well as for the synthetic compounds, methandrostenolone (metandienone) [14], budesonide [15] and dexamethasone [16]. In the latter study, dexamethasone showed formation of 6α-hydroxylated isomer, in addition to the 6β-isomer, by catalytic activity of CYP3A4 enzyme. The ratio of 6β- to 6α-hydroxylated isomer produced was 3:1, showing also in this case predominant formation of 6β-hydroxyl metabolite.

Our study, performed by using two formats of human recombinant CYP3A4 enzyme (i.e., insect cell microsomes expressing human CYP enzymes and purified recombinant human CYP enzymes in a reconstituted system), confirmed the selectivity for 6β-hydroxylation of the anabolic steroids studied. As all steroids that are preferentially hydroxylated in 6β-position possess 3-keto-4-ene structure (so called Δ⁴-steroids) (Fig. 4), we suggest that the electronic effects of the 3-keto-4-ene structural moiety contribute to the selectivity of these substrates for the enzymatic 6β-hydroxylation. Cytochrome P450-catalyzed hydroxylation of hydrocarbons, presented schematically in Fig. 5A–C, is considered to occur by a mechanism involving hydrogen atom abstraction from the substrate (Fig. 5A) followed by rapid

![Fig. 5. Schematic presentation of the proposed mechanism for 6β-hydroxylation of the 3-keto-4-ene steroids.](image-url)
transfer of the iron-bound hydroxyl radical to the intermediate substrate radical (Fig. 5B). The proposed iron-oxo species (Fe^{IV}=O in Fig. 5A) represent one of possible active oxygen species that could be invoked during formation of hydroxylated metabolite in the cytochrome P450 catalytic cycle. This so-called oxygen rebound mechanism, proposed for hydroxylation of cytochrome P450 substrates, is consistent with experimental results obtained when investigating selectivity of hydroxylation of deuterated cyclohexene [17,18]. In the case of 3-keto-4-ene steroids, the selectivity for 6β-hydroxylation might be exerted through stabilization of the proposed substrate-radical intermediate formed, as well as by a lower stability of the C–H bond in the 6β-position. Based on these considerations, we propose the scheme presented as Fig. 5 as a favored path for the hydroxylation of the 3-keto-4-ene steroids in 6β-position. This suggestion is supported by the previously published results [8] showing that trimethylsilyl 3,5-dienol ethers of the steroids investigated in the present study, dissolved in ethanol, are easily auto-oxidized by direct sunlight preferentially to 6β-hydroxy derivatives.

To further clarify the role of the structural characteristics in metabolism of steroid hormones by CYP enzymes, we performed studies on the in vitro metabolism of 4-androstene-3,17-dione, androst-4-ene-3α and androst-4-ene-3β,17β-diols, androst-5-ene-3β,17β-diol, as well as of 5β- and 5α-androstane-3α,17β-diols, 5α-androstane-3α and 5α-androstane-3β,17β-diols and 5α-dihydrotestosterone (DHT).

In conclusion, we suggest that electronic effects at the 3-keto-4-ene structural moiety contribute to the selectivity for 6β-hydroxylation of endogenous and synthetic steroids as catalyzed by human CYP enzymes.

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