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Biosynthesis of 6β-hydroxymethyltestosterone using bovine hepatocyte cultures

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Abstract

Usually performed to investigate biotransformations of xenobiotics, in vitro liver models could become useful tools for the synthesis of not commercially available compounds. In this study, bovine hepatocyte cultures were used to biosynthesise, on the laboratory scale, one major metabolite of methyltestosterone: 6β-hydroxymethyltestosterone. After incubation of bovine hepatocytes with methyltestosterone for 24 h, culture medium was removed and stored at −20°C until analysis. The sample was extracted and purified on a reversed-phase HPLC system. The metabolite of interest was then analysed in LC-MS and GC-MS for structural identification. The purity and the isomery of the 6 and 17 positions were confirmed by NMR analyses. This first success in producing purified 6β-hydroxymethyltestosterone from bovine hepatocyte cultures allowed us to consider that in vitro liver models could be reliable tools for standard biosynthesis. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Biosynthesis; 6β-Hydroxymethyltestosterone; Bovine hepatocyte cultures

1. Introduction

Within the context of growth promoter control in animal production (European Union Directive 96/23), the knowledge of anabolic steroid metabolic pathways is essential. Indeed, the identification of metabolites can contribute to a more reliable control of the forbidden steroids. For that reason, several in vitro systems have been developed such as microsomal preparations or hepatocyte cultures for metabolism studies of growth promoting agents in food-producing animals [1–3]. Despite its prohibition, methyltestosterone (17α-methyl-17β-hydroxyandrost-4-en-3-one) (Fig. 1) is one of the anabolic steroids illegally used in cattle fattening. Compared to its natural homologue testosterone, the alkylation at the 17 position prevents the rapid oral inactivation and reduces androgenic side effects. Until now, limited information was available on methyltestosterone in vitro. In 1950, Levedahl and Samuels [4] compared the metabolism of methyltestosterone and testosterone in liver tissue of several species and evidenced the differences in the metabolism of the two androgens. In 1995, Welder et al. [5] demonstrated the toxic effect of some androgenic alkylsteroids included methyltestosterone in primary rat hepatic cell cultures under certain doses. However, no information refers to methyltestosterone biotransformations on bovine hepatocyte cultures. The metabolism of methyltestosterone has been widely studied in vivo in several species. It has been investigated by Rongone and Segaloff [6] and later by Quincey and Gray [7] in human who suffered from cancer. Epimerisation of this synthetic androgen was further determined in man by Schänzer et al. [8]. Templeton et al. [9] investigated the metabolic transformations of methyltestosterone in rabbit and they brought to the fore several metabolites in urine which were reduced and hydroxylated ones. As methyltestosterone is known to stimulate growth and produce sex reversal in some fishes species, metabolism studies have been undertaken on trout [10]. Furthermore, Schoene et al. [11] and Stanley et al. [12] investi-

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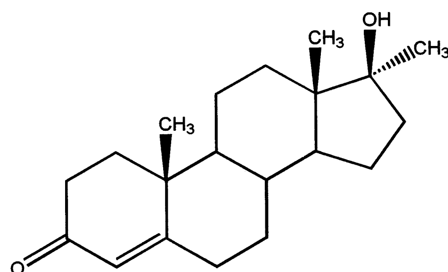

Fig. 1. Structure of 17α-methyltestosterone.

gated the metabolism of methyltestosterone in urine because of the widespread use of 17α-alkylsteroids in horse racing. Performing biotransformation studies implicates the identification of the metabolites evidenced. In any case, the identification of the metabolites requires corresponding standards for structural confirmation. Concerning methyltestosterone, only few potential metabolites are available as reference compounds on the market and chemical synthesis is often necessary. Within this context, the evidence of 6β-hydroxymethyltestosterone in several species, after methyltestosterone administration, led some authors to synthesise this compound [13,14].

The aim of this work was to show the feasibility of an in vitro model to biosynthesise and to produce, on the laboratory scale, one metabolite of methyltestosterone which was not commercially available: 6β-hydroxymethyltestosterone. To achieve this goal, the in vitro biotransformation was carried out using bovine hepatocyte cultures. The metabolite was purified on HPLC and then analysed by mass spectrometry and nuclear magnetic resonance.

2. Materials and methods

2.1. Apparatus

2.1.1. Gas chromatography-mass spectrometry

The GC-MS system (Hewlett-Packard, Palo Alto, CA, USA) consisted of a model 5890 gas chromatograph and a model 5971 mass spectrometer. The colufmn used was an OV-1 (Interchrom, Montluçon, France) (30 m × 0.25 mm id, film thickness 0.25 μm). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. A volume of 2 μl was injected in the splitless mode (1 min delay). The injector was maintained at 250°C. The GC conditions were as follows: 120°C (held for 2 min) increased at 15°C min^{-1} to 250°C (held for 0 min) and then 5°C min^{-1} to 300°C (held for 5 min). The transfer line temperature was maintained at 280°C. The electron beam energy was set at 70 eV in the electron ionisation (EI) mode.

2.1.2. Liquid chromatography-mass spectrometry

The analyser was a triple quadrupole LC Quattro (Micromass, Manchester, UK) equipped with a 'Z geometry' source design. A positive electrospray ionisation (ESI +) was performed. The sample was introduced by infusion using a capillary tube with a flow-rate of 10 μl min^{-1}. The applied parameters were 120°C for the source, 300°C for the desolvatation temperature, 32 V for the cone potential, 4.1 kV for the potential of capillary, 96 l h^{-1} for the nebulizer nitrogen flow-rate and 636 l h^{-1} for the desolvatation gas flow-rate. For MS^2 analysis, collision energy was set at 25 V and the argon gas pressure at 1.2×10^{-3} mbar. For experiments, sample was injected in ultrapure water/acetonitrile (56:44, v/v) containing 1% formic acid.

2.1.3. High performance liquid chromatography

The HPLC set-up used was a HP 1100 serie binary pump (HP, Waldbronn, Germany) with a HP 1100 serie autoinjector. The column was a 3 μm Spherisorb® S3 ODS1 (15 cm × 2.1 mm id) (Waters, Milford, MA, USA) with a 3.5 μm Spherisorb® S3 ODS1 guard column (10 × 2.1 mm). A HP serie 1050 UV detector operated at 254 nm was used to control roughly the HPLC separation. The flow-rate was 200 μl min^{-1}. The mobile phase was composed of acetonitrile (A) and ultrapure water (B) (pH adjusted at 4). Gradient elution was applied starting at 35% A increasing linearly to 44% A over 10 min, then an isocratic portion over 5 min and a linear decrease to the initial conditions over 5 min.

2.1.4. Nuclear magnetic resonance

The equipment used for the experiments consisted of a AMX 400 and a DRX 500 spectrometers (Brucker, Karlsrühe, Germany). Reference standards were dissolved in deuterochloroform. 6α-hydroxytestosterone was dissolved in acetone-d_6 for solubility.

2.2. Reagents and reference compounds

Most of the reagents and solvents used were of analytical grade quality and provided by Merck (Darmstadt, Germany) and by Solvants Documentation Synthèses (Peypin, France) except acetonitrile which was of HPLC grade quality (SDS). The solid-phase extraction C18 columns (2 g of phase) were purchased from Varian (Harbor City, CA, USA). The reference steroids methyltestosterone, testosterone, 6β-hydroxytestosterone were obtained from Sigma (Saint Quentin Fallavier, France) and 6α-hydroxytestosterone from Steraloids (Wilton, NH, USA). [^{3}H]-methyltestosterone was supplied by the Laboratory of Hormonology (Marloie, Belgium). The derivatisation reagents *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and trimethyliodosilane (TMIS) were provided by Fluka

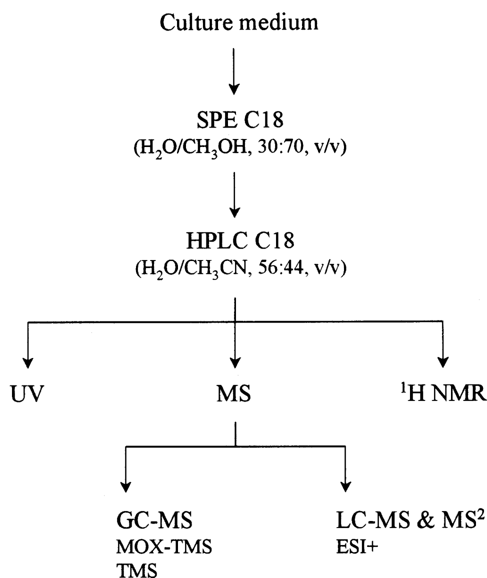

Fig. 2. Flow chart of metabolite preparation and identification.

(Buchs, Switzerland) and methoxylamine (MOX) by Pierce (Rockford, IL, USA).

2.3. Method

Calf hepatocytes were isolated and cultured as described previously [3,15]. Monolayer cultures were incubated with 100 μM methyltestosterone for 24 h. Medium was collected and stored at −20°C after addition of 1 mM ascorbic acid. For extraction, sample was applied to a previously conditioned C18 column (Fig. 2). Elution was performed using a water/methanol gradient from 90:10 (v/v) to 10:90 (v/v). Fractions of 5 ml were collected. The metabolite of interest was eluted with 5 ml of ultrapure water/methanol 30:70 (v/v). The fraction was reduced under a nitrogen flow and the remaining aqueous phase was extracted using 3 ml ethyl acetate. After evaporation of the organic phase, the residue was dissolved in the mobile phase water/acetonitrile and 50 μl were injected into the HPLC system. Fraction of interest was analysed in LC-MS and in GC-MS after evaporation and derivatisation. MOX-TMS derivatisation was performed by heating 60 min at 60°C with 25 μl of MOX-pyridine (2:98, w/v) and after evaporation of the reagent 60 min at 80°C with 25 μl of MSTFA-TMIS (1000:2, v/v). TMS derivatisation was realised by heating 15 min at 60°C in 25 μl of MSTFA. For NMR experiments, extraction was repeated several times and the HPLC purified extracts were combined. The fractions were reduced and extracted using diethylether. After evaporation, the residue was dissolved in $CDCl_3$ for analysis.

3. Results and discussion

Using bovine hepatocyte cultures for methyltestosterone metabolism study (still in progress), the aim of this work was to focus on a metabolite which can be produced as a reference compound for the laboratory needs. First, the use of a tritiated parent compound for investigating in vitro biotransformations allowed us to target the metabolite to be purified. One of the main metabolites was thus chosen. Then, non radioactive methyltestosterone was used and incubated on a larger scale in order to produce the metabolite in a sufficient amount. In this perspective, two experiments were performed using two liver pieces from two different male calves which enable to finally collect 500 ml of the culture medium containing the metabolites. The solvent gradient used for SPE contributed to eliminate a large part of interactions in the medium and to elute the metabolite of interest in the fraction water/methanol 30:70. Besides, SPE allowed the application of a large volume of medium per experiment which provides a significant time-saving for production. A reversed phase HPLC was then realised in order to purify the metabolite. Using the solvent gradient previously mentioned, the metabolite had a retention time of 10.5 min. Moreover, emission of a signal for the metabolite at 254 nm implied the presence of a chromophore on the molecule which probably corresponds to an enone group. The HPLC fraction corresponding to the metabolite elution was then analysed in mass spectrometry in order to ascertain the purity of the compound and to elucidate its molecular structure. The first step in LC-MS was the measurement in full scan mode. Fig. 3a shows the ESI mass spectrum of the metabolite which generated an abundant $[M+H]^+$ ion at $m/z = 319$. This pseudo-molecular ion was in accordance with the hypothesis of an hydroxylated metabolite of methyltestosterone. Cluster ions were also present at $m/z = 341$, 360 and 382 which correspond respectively to $[M+Na]^+$, $[M+CH_3CN]^+$ and $[M+CH_3CN+Na]^+$ ions. The CID ESI mass spectrum of the metabolite showed significant ions at $m/z = 301$ and 283, which indicated two sequential losses of water (Fig. 3b). We could therefore suppose that two hydroxy groups were present on the metabolite structure. Moreover, in reference to Williams et al. [16], the absence of the two ions at $m/z = 97$ and 109 (daughter ions of methyltestosterone) in the mass spectrum led us to eliminate some positions concerning the fixation of the second hydroxy. The structural elucidation was further studied by GC-MS. Acquisition was realised in the scan mode (100–650 uma) after EI ionisation. MOX-TMS derivatisation of the isolated metabolite gave a $M^{+\bullet}$ at $m/z = 491$ (Fig. 4). This molecular ion indicated the introduction of a nitrogen atom in the molecule by reaction with ketonic function leading to oxime formation. Moreover, this

m/z value was compatible with the presence of two trimethylsilylated hydroxy groups. In the mass spectrum, the base peak occurring at $m/z = 143$ was characteristic of the D ring scission with charge localisation on C-17 moiety. This significant fragment which was also present in the methyltestosterone spectrum excluded hydroxylation on 15 or 16 position. TMS derivatisation was also used for providing further information on the metabolite structure. The mass spectrum obtained revealed a specific loss of 56 ($m/z = 334$) (Fig. 5). Besides, the spectrum of a commercially available standard of 11α-hydroxymethyltestosterone did not show any loss of 56. We could therefore conclude that the metabolite was not hydroxylated in the 11 position. Comparing with 6α- and 6β-hydroxytestosterone, the spectrum showed the loss of the same fragment as that observed for the metabolite which could correspond to a fragmentation of the A ring. This would also imply that any hydroxylation occurs on the A ring. Combining the results of the two derivatisations, we suggested that the purified metabolite was hydroxylated in the six position. However, further analyses by NMR spectroscopy were essential to give additional information about the exact structure of the metabolite and confirm the purity. Indeed, GC-MS alone was not sufficient to make a clear distinction between the possible epimeric forms of the metabolite. Before analysis of the metabolite by NMR, related molecules were previously studied on a 400 MHz spectrometer in order to facilitate the spectral analysis of the compound. Providing methyltestosterone, 6α- and 6β-hydroxytestosterone analyses in ^{1}H and ^{13}C NMR and referring to data found in the literature, table of chemical shifts were established for these standards. In parallel, NMR identification of the metabolite implied a sufficient quantity of the product for analysis. Production was realised by using the method described, i.e. SPE and HPLC. 400 ml of medium were extracted and purified which corresponds to an important number of injections due to the lack of preparative HPLC system. After combining all the fractions which contain the purified metabolite, the sample was evaporated and extracted by diethylether for residual water and silice elimination. The extract was then evaporated and dissolved in $CDCl_3$ for 500 MHz NMR analysis. The quantity of the isolated metabolite was estimated at 500 μg. Considering the methyltestosterone introduced in the medium (about 12 mg in 400 ml of medium), this parent molecule was transformed for about 10% by the hepatocytes during the incubation period to give 6β-hydroxymethyltestosterone. By taking into account the metabolite as the starting material in the medium, the yield of the extraction/purification procedure was about 40%. The final quantity obtained was not far from the detection limit of ^{13}C NMR instrument used. However, only the ^{1}H NMR spectrum was recorded to demonstrate the purity of the product and to confirm its structure. The ^{1}H

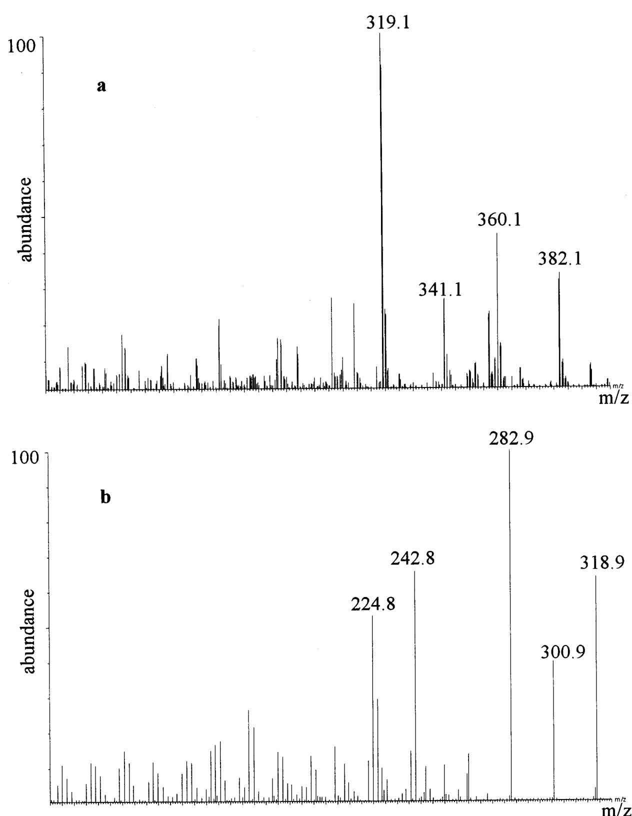

Fig. 3. ESI + mass spectrum of the metabolite in full scan acquisition (a) and MS^2 (b).

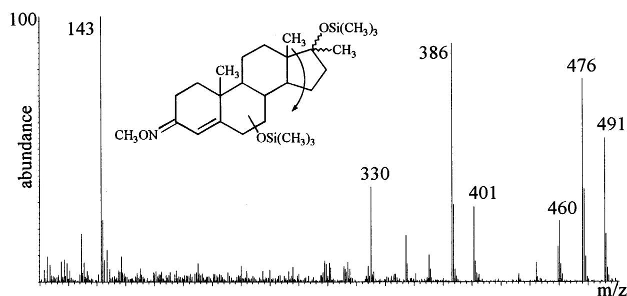

Fig. 4. Mass spectrum of the MOX-TMS derivative of the metabolite (EI mode, scan acquisition).

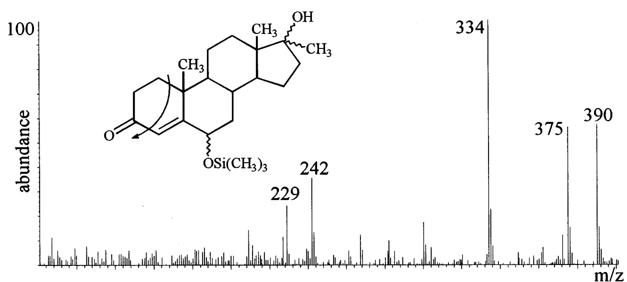

Fig. 5. Mass spectrum of the TMS derivative of the metabolite (EI mode, scan acquisition).

Table 1
^{1}H NMR chemical shifts (ppm) for standards (400 MHz) and metabolite (500 MHz)

Proton	6α-hydroxytestosterone C_3D_6O	6β-hydoxytestosterone $CDCl_3$	Methyltestosterone $CDCl_3$	Metabolite $CDCl_3$
H-4	6.11	5.82	5.73	5.80
H-6α	–	4.36	2.32	4.35
H-6β	4.30	–	2.40	–
CH_3-18	0.80	0.76	0.89	0.90

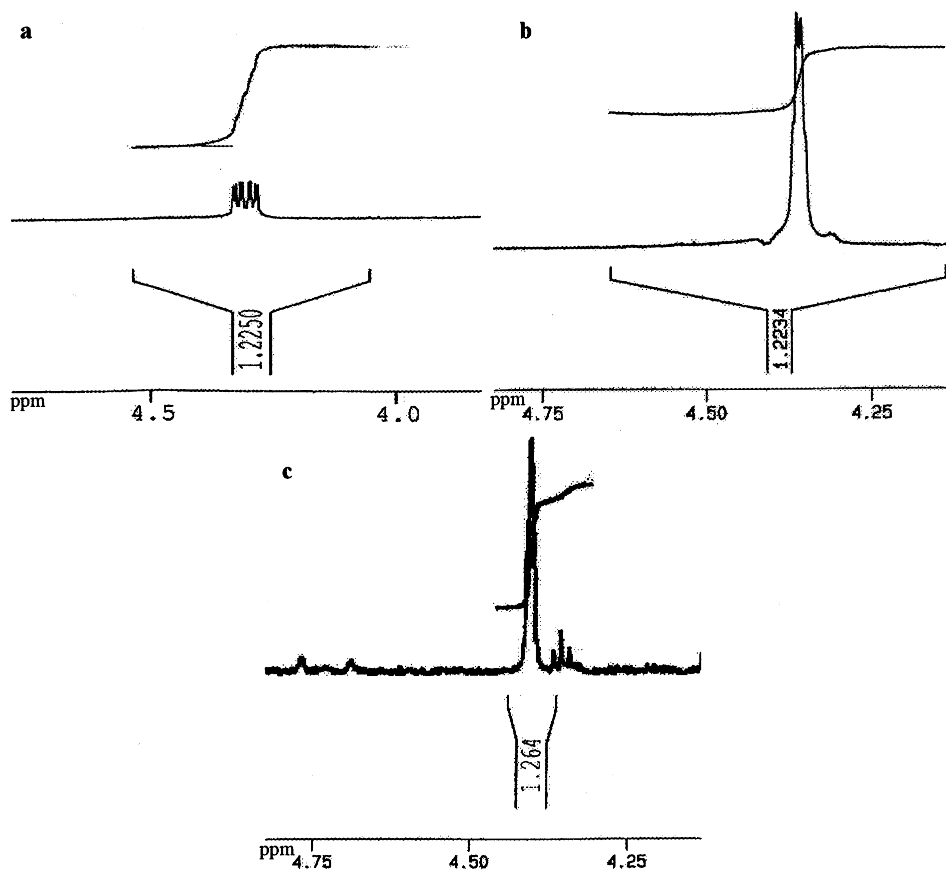

Fig. 6. ^{1}H NMR C-6 protons compared groups of 6α-hydroxytestosterone (a), 6β-hydroxytestosterone (b) and metabolite (c).

chemical shift values given in Table 1 confirmed the hydroxylation in the six position. Indeed, compared to methyltestosterone, the C-6 proton moves 2 ppm downfield when hydroxylation occurred in the six position. The H-4 signal is slightly shifted downfield by the 6β-hydroxy group. In contrast, the 6α-hydroxy group shifted the C-4 proton strongly downfield. Moreover, the ^{1}H NMR chemical shift of C-18 protons of the metabolite implied the 17α-position for the methyl as described in the literature [8,17]. The comparison of the group which corresponds to the C-6 proton shielding between 6α-, 6β-hydroxytestosterone and the metabolite allowed us to conclude on the 6β isomery for the compound (Fig. 6). The metabolite produced was finally assigned to be 6β-hydroxymethyltestosterone.

Through this study, we have demonstrated that it was possible to perform the biosynthesis of a steroid by using bovine hepatocyte cultures. HPLC purification of the metabolite was an essential stage for its further identification in mass spectrometry. The combination of separation (GC, LC) and ionisation (EI, ESI) techniques allowed us to characterise the hydroxylated metabolite. Purity and finale structure of the compound were then confirmed by NMR analysis. We have thereby shown the usefulness of an in vitro model to produce 'biostandard'. Considered as a first approach, this work could become a starting point for the production of other standards such as conjugated ones. This could be realised by checking the non toxicity of the parent molecule and its possible transformation by the hepatocytes.

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