IN VITRO AND IN VIVO METABOLISM OF THE PROGESTAGEN ORG 30659 IN SEVERAL SPECIES


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ABSTRACT:

The metabolism of Org 30659 [(17α)-17-hydroxy-11-methylene-19-norpregna-4,15-dien-20-yn-3-one], a new potent progestagen currently under clinical development by NV Organon for use in oral contraceptive and hormone replacement therapy, was studied in vivo after oral administration to rats and monkeys and in vitro using rat, rabbit, monkey, and human hepatocytes. After oral administration of [7-3H]Org 30659 to rats and monkeys, Org 30659 was extensively metabolized in both species. Fecal excretion appeared to be the main route of elimination. In rats, opening of the A-ring, resulting in a 2-OH,4-carboxylic acid, 5α-H metabolite of Org 30659, was the major metabolic route in vivo. Other metabolic routes involved the introduction of an OH group at C15β, followed by a shift of the Δ15-double bond to a 16/17-double bond with subsequent removal of the OH group at C17 and reduction of the 3-keto,Δ4 moiety followed by sulfate conjugation of the 3-OH substituent. These metabolic routes observed in vivo were also major routes in incubations with rat hepatocytes. In rat liver microsomes, Org 30659 was metabolized by reduction of the 3-keto,Δ4 moiety. Rat hepatocyte incubations with Org 30659 were more representative of the in vivo metabolism of Org 30659, compared with rat microsomal incubations. Both in vitro and in vivo, the majority of the metabolites were 3α-OH,4,5α-dihydro derivatives. In monkeys, Org 30659 was mainly metabolized at the C3- and C17-positions in vivo. The 3-keto moiety was reduced to both 3β-OH and 3α-OH substituents. In addition to phase I metabolites, glucuronic acid conjugates were observed in vivo. In monkey liver microsomes, the 6β-OH metabolite of Org 30659 was the major metabolite present. Similar to the monkey liver microsomes, rabbit and human liver microsomes converted Org 30659 to the 6β-OH metabolite. This metabolite was also the major metabolite in incubations with human hepatocytes.

Org 30659 [(17α)-17-hydroxy-11-methylene-19-norpregna-4,15-dien-20-yn-3-one] (fig. 1) is a new potent progestagen currently under clinical development by NV Organon for use in oral contraceptive and hormone replacement therapy. In pharmacological studies in rats and rabbits, the progestational activity of Org 30659 (as determined in the ovulation inhibition test) was shown to be of the same order as the activity of etonogestrel and was much higher than the activities of norethisterone and levonorgestrel. In addition, a lack of androgenic activity (according to the Hershberger test) distinguishes Org 30659 from other progestagens. Except for some weak estrogenic activity, Org 30659 is devoid of other hormonal activities, such as glucocorticoid and antiguargocorticoid activity (Deckers et al., 1992).

The present investigation was performed to study the metabolism of Org 30659 in vivo and in vitro in several species. The metabolism of Org 30659 was studied in vivo in female rats and female cynomolgus monkeys, the species used in the preclinical safety studies. To study the in vivo metabolism of Org 30659, the rats were dosed with 10 mg/kg [3H]Org 30659 and the monkeys with 3 mg/kg [3H]Org 30659. In vitro studies were performed with rat, rabbit, monkey, and human liver microsomes and rat and human hepatocytes. Metabolites were isolated from microsomal incubations and urine and feces samples by HPLC. Identification of the metabolites was performed by NMR, MS, and IR spectroscopy.

In vitro metabolic routes observed in incubations with human liver microsomes and human hepatocytes were used to predict the in vivo metabolic routes of Org 30659 in humans. The in vitro-in vivo correlation obtained for rats and monkeys in this study was taken into account to strengthen these extrapolations.

Materials and Methods

Chemicals. [7-3H]Org 30569 (radiochemical purity, ≥99%; specific radioactivity, 888 GBq/mmol) was prepared by the Organic Synthesis Section of the Department of Process Chemistry of NV Organon (Oss, The Netherlands). Unlabeled Org 30659 was synthesized by the Department of Process Chemistry, NV Organon. All other chemicals were obtained from local commercial sources and were of analytical grade.

In Vivo Studies in Rats and Monkeys. Animals. Female Wistar rats (approximately 185 g, HSD/CPB:WU) were obtained from Harlan CPB (Zeist, The Netherlands). Each rat received standard pelleted food (diet RMH-B; Hope Farms BV, Woerden, The Netherlands) ad libitum.

Female cynomolgus monkeys (2–4 kg) were obtained from Inveresk Research (Tranent, Scotland). Each monkey was offered 0.2 kg/day of a complete dry diet of known formulation (SDS Mazuri Diet; Special Diet Services, Witham, Essex).

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Tap water was available ad libitum from drinking bottles and was refreshed each day. During sampling of urine and feces, the rats and monkeys were housed individually in stainless steel metabolism cages in a room under standard conditions (temperature range, 17–24°C; relative humidity range, 35–70%).

Animal Treatment and Sampling. Six female rats were treated with a single oral dose of 10 mg/kg (12.6 MBq/kg) [14C]Org 30659. Four female cynomolgus monkeys received unlabeled Org 30659 once daily for 14 days, at a dose level of 3 mg/kg. On day 15, [1H]Org 30659 (834 kBq/kg) was administered at the same dose level.

Urine and feces samples were collected in fractions up to 168 hr after dosing. Urine samples were collected in chilled containers. Rat blood samples were taken from the tail vein at 0.5, 1, 2, 4, 7, and 24 hr after administration of the radioactive dose. Cynomolgus monkey blood samples were taken from the femoral vein at 0 (before the dose), 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hr after radioactivity administration. All samples were stored at −20°C until required for analysis.

In Vitro Incubation Studies with Liver Microsomes and Hepatocytes. Liver Tissue. Liver tissue was obtained from female Wistar rats (HSD/Cpb: WU; Harlan CPB), female cynomolgus monkeys (Sanofi Recherche, Montpellier, France), and female New Zealand White rabbits (HSD/Cpb:NZW; Broekman Instituut, Someren, The Netherlands). Human liver microsomes and hepatocytes (female donors) were prepared from surgical waste liver tissue, after consent from legal authorities and patients had been obtained, in cooperation with the Groningen Human Liver Group, University of Groningen, The Netherlands. Human livers 1, 2, and 3 were used for the preparation of microsomes and human livers 4 and 5 for the isolation of hepatocytes.

Preparation of Liver Microsomes. Liver samples were homogenized using a Potter-Elvehjem homogenizer at 0°C in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. Microsomes were prepared by centrifugation (20,000 N/kg; supernatant, 2°C until required) and washed in 2 ml of potassium phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl₂, 20 mM glucose-6-phosphate, 1 mM NADP⁺, 1.5 units/ml glucose-6-phosphate dehydrogenase, and 10 μM (37 kBq/ml) [1H]Org 30659, at 37°C. After a preincubation period of 2 min at 37°C, incubations were started by the addition of the test compound. Incubations were carried out under a gentle stream of 95% O₂/5% CO₂. Incubations were stopped after 2 hr of incubation by freezing (solid CO₂/ethanol, approximately −80°C). All samples were stored at −20°C until required for analysis.

Hepatocyte Isolation. A female Wistar rat was anesthetized with Nembutal, an abdominal midline incision was made, and the bile duct, vena porta, and thoracic vena cava inferior were cannulated. Hepatocytes were further isolated by collagenase perfusion (Braakman et al., 1989). Human hepatocytes were isolated at the Department of Pharmacokinetics and Drug Delivery, Groningen Utrecht Institute for Drug Exploration, University of Groningen, by collagenase perfusion of a liver section with a single cut surface (Olinga et al., 1998b).

Hepatocyte Incubations. Hepatocytes were suspended in Krebs-Henseleit buffer, supplemented with bovine serum albumin (pH 7.4), at a cell density of 3–4 × 10⁷ cells/ml (total volume, 1 ml). After preincubation for 15 min at 37°C in a shaking water bath under a gentle stream of 95% O₂/5% CO₂, [1H]Org 30659 was added at a final concentration of 215–580 kBq/ml (equivalent to approximately 75–201 ng/ml). Hepatocytes were incubated with the test compound for a total time period of 3 hr at 37°C. The hepatocyte suspension was then centrifuged for 1 min in an Eppendorf centrifuge, and the pellet (cells) and supernatant (cell medium) were separated. Cell and cell medium samples were frozen at approximately −80°C until required for analysis.

Sample Analysis. Determination of Radioactivity Concentrations. The concentrations of radioactivity in plasma, urine, and incubation samples were determined by liquid scintillation counting (Tri-Carb 2500 TR/2; Canberra Packard, Groningen, The Netherlands). The concentrations of radioactivity in feces were determined by combustion in a Sample Oxidizer 306 or 387 (Canberra Packard), followed by liquid scintillation counting. Feces samples were homogenized with Milli-Q water before combustion.

Determination of Metabolite Profiles. Pooled plasma was analyzed by HPLC without pretreatment. Pooled rat urine was dried in a Speed Vac concentrator (Dunne, The Netherlands); the residue was dissolved in Milli-Q water and used for HPLC analysis.

Monkey pooled plasma and urine samples were applied to C₁₈ solid-phase extraction columns. Columns were washed twice with Milli-Q water and eluted with methanol. The methanol extracts were evaporated to dryness in a Speed Vac concentrator. Residues were dissolved in methanol or Milli-Q water/ methanol for HPLC analysis.

Pooled feces homogenates from rats and monkeys were extracted with acetonitrile. The extracts were concentrated in a Speed Vac concentrator, diluted with Milli-Q water, and subsequently applied to C₁₈ solid-phase extraction columns. The columns were washed with Milli-Q water and eluted with methanol. The methanol eluents were dried, subsequently dissolved in methanol, and used for HPLC analysis.

Microsomal incubation samples were used for HPLC analysis without pretreatment. The cell media of the rat and human hepatocyte incubations were passed through 0.45-μm filters before HPLC analysis of the radioactivity profiles.

HPLC Analysis of Metabolite Profiles. HPLC analysis of the plasma, urine, feces, and incubation samples was performed using a μ-Bondapak C₁₈ column (7.8 × 300 mm) and a gradient of ammonium acetate buffer (0.1 M, pH 4.2) (solvent A) and methanol (solvent B). Elution was performed with a linear gradient of 10−90% (v/v) solvent B in 35 min, at 50°C. The flow rate was 2.5 ml/min.

HPLC analysis was performed with an HP1090 liquid chromatograph equipped with an HP1040 diode-array detector (Hewlett Packard, Walbron, Germany) and a Flo-one [B]eta model A525 on-line radioactivity detector (Canberra Packard). Samples were spiked with unlabeled Org 30659 as a reference for retention time (UV signal at 254 nm).
Metabolite numbers were assigned on the basis of retention times. Metabolites from rat plasma, urine, and feces (R), monkey plasma, urine, and feces (C), microsomal incubations for all species (M), and rat and human hepatocyte incubations (H) were numbered independently.

Isolation of Metabolites from Urine, Feces, and In Vitro Incubation Samples. Urine and Feces. Rat urine was applied to C18 solid-phase extraction columns. Monkey urine was concentrated in a Speed Vac concentrator and then applied to C18 solid-phase extraction columns. The columns were washed with Milli-Q water and eluted with methanol. The methanol effluents were concentrated by vacuum centrifugation and subjected to HPLC.

Feces homogenates from rats and monkeys were extracted with acetonitrile. The extracts were concentrated in a Speed Vac concentrator, diluted with Milli-Q water, and subsequently applied to activated C18 solid-phase columns. The columns were washed with Milli-Q water and eluted with methanol. The methanol effluents were dried, dissolved in methanol, and subjected to HPLC analysis.

### Table 1

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Rats (N = 3)</th>
<th>Monkeys (N = 4)</th>
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<tr>
<td></td>
<td>Urine (%)</td>
<td>Feces (%)</td>
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<tr>
<td>0–24</td>
<td>30.3 ± 6.3</td>
<td>17.4 ± 5.9</td>
</tr>
<tr>
<td>24–48</td>
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<td>3.0 ± 3.2</td>
<td>9.4 ± 6.5</td>
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<tr>
<td>72–96</td>
<td>0.4 ± 0.2</td>
<td>2.9 ± 2.6</td>
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<tr>
<td>96–120</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.5</td>
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<tr>
<td>120–144</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>144–168</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>0–168</td>
<td>43.0 ± 2.3</td>
<td>50.8 ± 0.9</td>
</tr>
<tr>
<td>Total</td>
<td>93.8 ± 3.2</td>
<td>71.2 ± 14.2</td>
</tr>
</tbody>
</table>

Data are expressed as percentages of the administered dose (mean ± SD of individual data).

**Fig. 2A.** Metabolite profiles for Org 30659 in plasma, urine, and feces from female rats.
The chromatographic conditions for the urine and feces samples were as described in HPLC Analysis of Metabolite Profiles. The monkey feces samples were eluted with a linear gradient of 10–90% solvent B in 30 min, instead of 10–90% solvent B in 35 min. The effluent was collected in fractions. Fractions constituting a peak of radioactivity were pooled and dried in a Speed Vac concentrator or under a gentle stream of nitrogen. The residues isolated from monkey urine were desalted and further processed, as appropriate, for NMR and MS analysis.

Residues from rat urine and feces and monkey feces were dissolved in methanol and/or Milli-Q water and then subjected to a second HPLC analysis using a μ-Bondapak phenyl column (3.9 × 300 mm) and a gradient of ammonium acetate buffer (0.1 M, pH 4.2) (solvent A) and methanol (solvent B). Elution was performed with a linear gradient of 5–60% (v/v) solvent B in 30 min, at 50°C. The flow rate was 1.5 ml/min. Elution for the feces samples from monkeys was performed with 5–80% methanol in 35 min.

The HPLC effluent was collected in fractions at the approximate retention times.
times of the eluting peaks of radioactivity. Fractions constituting a peak of radioactivity were pooled and then dried. The residues were further processed, as appropriate, for NMR and MS analysis and, if necessary, for IR analysis.

Preparative Incubation Samples. The incubation mixture from the preparative microsomal incubation was applied to 6-ml Bakerbond SPE C18 solid-phase extraction columns. The columns were washed with Milli-Q water and eluted with methanol. The methanol effluents were concentrated by vacuum centrifugation, diluted with Milli-Q water, and again subjected to C18 solid-phase extraction. The methanol effluents were concentrated to an appropriate volume and subjected to HPLC. The chromatographic conditions used for HPLC analysis were as described in HPLC Analysis of Metabolite Profiles. The HPLC effluent was collected in fractions. Fractions constituting a peak of radioactivity were pooled and dried. Residues were further processed, as appropriate, for NMR and MS analysis and, if necessary, for IR analysis.

Identification of Metabolites. NMR Spectroscopy. The 1H spectra were recorded at 400 and 600 MHz with Bruker DRX-400 and DRX-600 instruments (Bruker Spectrospin AG, Fällanden, Switzerland), respectively, under standard conditions. The chemical shifts are given in parts per million. The samples were dissolved in deuteromethanol. The CH3OD was used as a reference and was set to 3.3 ppm.

MS. Electron ionization spectra were recorded at 70-eV electron energy with an HP-5989B mass spectrometer (Hewlett Packard, Palo Alto, CA), using an HP-59890B particle-beam interface. Sample introduction was performed by HPLC, using a Prodigy ODS column (5 µm, 2 x 150 mm) and a gradient of Milli-Q water (solvent A) and acetonitrile (solvent B). Isocratic elution was performed with 40% solvent B for 5 min, followed by a linear gradient of 40–100% (v/v) solvent B in 10 min. The flow rate was 0.4 ml/min. The ion source temperature was 250°C, and the desolvation chamber was maintained at 60°C. Particle-beam chemical ionization spectra were recorded using methane gas as the reagent gas, at 200-eV electron energy.

The metabolites were also analyzed by HPLC/ion-spray MS, using a LC-200 Quad pump (Perkin-Elmer Scieix) and a Perkin-Elmer ISS-200 autosampler. Ion-spray mass spectra were recorded in positive- and negative-ion modes, using the ion-spray interface of the PE-Sciex API-100 mass spectrometer (Perkin-Elmer). The PE-Sciex API-100 mass spectrometer was operated at positive and negative ion-spray voltages of approximately 5800 and –4500 V,
respectively, with a nebulizer Org gas flow of air at 10 bar (prepressure, approximately 4 bar) and a curtain gas flow of nitrogen at 8 bar (prepressure, approximately 3 bar). The orifice was held at 25 and −25 V, respectively, whereas the ring was held at 275 and −275 V, respectively. Mass spectra were recorded from approximately 200 to 1000 amu, with a step size of 0.1 amu and a dwell time of 0.35 msec.

Sample introduction was performed by HPLC, using a Prodigy ODS column (5 μm, 2 × 150 mm) and a gradient of 5 mM ammonium acetate solution (solvent A) and methanol (solvent B). Isocratic elution was performed with solvent B (10%) for 1 min, followed by a linear gradient of 10–90% (v/v) solvent B in 10 min. Isocratic elution was then performed with solvent B (90%) for 9 min. The flow rate was 0.4 ml/min.

IR Spectrometry. IR spectra were recorded using a Bio-Rad FTS-60 spectrometer, which was continuously purged with nitrogen. DRIFT spectra were obtained using the Barnes DRIFT accessory. The samples were dissolved in a small amount of methanol and were transferred, using a syringe, onto a DRIFT mirror. The spectra were recorded in Kubelka-Munk mode, with a resolution of 2 cm⁻¹ and 128 scans.

Results

Excretion of Radioactivity in Urine and Feces from Rats and Monkeys. Data on the excretion of radioactivity after an oral dose of [³H]Org 30659 to rats and monkeys are given in table 1. The total excretion of radioactivity in the 0–168-hr period after a single oral administration of 10 mg/kg [³H]Org 30659 to female Wistar rats was 93.8 ± 3.2% (mean ± SD of individual samples, N = 3). The 0–168-hr excretion in the urine was 43.0 ± 2.3% and that in the feces was 50.8 ± 0.9%.

The total excretion of radioactivity (0–168 hr) after 2 weeks of daily oral doses of 3 mg/kg Org 30659, followed by a single oral dose of 3 mg/kg [³H]Org 30659, to female cynomolgus monkeys was 71.2 ± 14.2%, of which 27.3 ± 4.7% was excreted in the urine and 43.9 ± 16.5% in the feces. The total recovery of radioactivity in urine and feces from the monkeys was not quantitative. During the study, the feces from the monkeys were of a liquid nature, resulting in high cage wash values (range, 2.4–29.4%) (results not shown). The liquid nature of the feces was presumably the result of the stress caused by the dosing and handling of the animals.

Metabolites of Org 30659 Present in Plasma, Urine, and Feces from Rats and Monkeys. Rat plasma metabolite profiles after a single oral dose of 10 mg/kg [³H]Org 30659 contained nine compounds, i.e., R1, R6, R8, R10, R13–R16, and R18. The metabolites R13 and R15, followed by R8, were the major metabolites present in the plasma samples at 0.5, 1, and 2 hr. On the basis of the retention times of the metabolites isolated from urine and feces, the putative structures of R13 and R14 were the A-ring-opened 2-OH,4-carboxylic acid,5α-H metabolite of Org 30659 and the A-ring-opened 2-OH,4-carboxylic acid,5α-H,15β-OH,Δ10γ,17-ethyl metabolite of Org 30659, respectively (see below). The putative structure of compound R15 was the 3α-OSE,5α-H metabolite of Org 30659, based on comparison with the retention time of a metabolite isolated from the liver perfusate after circulatory perfusion of Org 30659 in rat liver (results not shown). The structures of the other compounds present in plasma remained unidentified. The HPLC metabolite profile of rat plasma (at 0.5 hr, the time of the maximal plasma concentration) is given in fig. 2A.

Monkey plasma metabolite profiles after 2 weeks of daily oral dosing with unlabeled Org 30659, followed by a single oral dose of [³H]Org 30659, contained 14 compounds, i.e., C4, C9–C15, Org 30659, C16–C19, and C21. The metabolite profiles in plasma showed gradual changes with time. At 0.5 hr after dosing, the time point at which the peak mean concentration of Org 30659 plus metabolites was measured, metabolite C12 was the main compound observed. Metabolite C12 was a major compound present at 1 hr after dosing, it was not particularly prominent at 1.5 and 2 hr after dosing, and it was again a major compound at 3 hr after dosing. This finding suggested that metabolite C12 possibly represented two different compounds, one at the early time points and the other at the later time points.

C14 was a major metabolite at 2, 3, 4, 6, and 8 hr after dosing. C15 and C17 were major metabolites at 1.5 hr after dosing but not at any of the other time points studied. Metabolite C18 was a main metabolite at 1, 1.5, 2, 3, and 4 hr after dosing.

On the basis of comparisons of retention times with metabolites isolated from urine and feces, some of the compounds in plasma could be identified. The putative structures are given in table 2. HPLC metabolite profiles for monkey plasma at three different time points, i.e., 0.5, 2, and 4 hr, after dosing are given in fig. 2B.

Org 30659 was extensively metabolized after oral administration to rats and monkeys; no Org 30659 was present in urine and feces samples of either species. The compounds present in urine and feces from rats and monkeys are given in table 2. The metabolites present in urine and feces from rats were identified as R1–R20 and those from monkeys as C1–C22. The HPLC metabolite profiles for Org 30659 in pooled (0–96 hr) urine and feces samples from rats and monkeys are given in fig. 2.

Major metabolites identified in rat urine were R13 and R14. Compound R13 was also the major compound present in rat feces. Monkey urine samples showed C12 and C14 as the major compounds present, whereas C17 and C18 were the major compounds present in monkey feces. For the identification of the metabolites, see below.

Identification of the Metabolites Present in Urine and Feces from Rats and Monkeys. Analysis. The major compounds present in urine and feces samples from rats and monkeys were isolated and identified by NMR and MS analysis. In addition, IR analysis was performed for the identification of compound R13. Minor metabolites remained unidentified because the isolated amounts of metabolites were insufficient or the mixtures were too complex for NMR and MS analysis. The NMR and MS (and IR, if available) data for the
metabolites isolated from urine and feces from rats and monkeys are summarized in table 3.

A-Ring-Opened 2-OH,4-Carboxylic Acid,5α-H Metabolite of Org 30659 (R13). The presence of a C10-CH2-CH2OH moiety, the absence of the 3-keto,Δ4 moiety, and the chemical shifts of the 4-H (2H) protons were indications that the A ring was opened and a carboxylic acid group at C4 and an OH substituent at C2 were formed. In addition, the IR spectrum showed the presence of a carboxylic acid substituent.

A-Ring-Opened 2-OH,4-Carboxylic Acid,5α-H,15β-OH,Δ16,17-Ethinyl Metabolite of Org 30659 (R14). The shift of the H15 signal, the change in the multiplicity of the H16 signal, and the shifts of the 17-ethinyl-H (which is now probably under the moisture peak) and H18 signals were indications for 17-ethinyl-Δ16 and 15β-OH substituents. The presence of a C10-CH2-CH2OH moiety, the absence of the proton signals at C3, and the shifts of the H4 proton signals indicated that the A-ring was opened and a carboxylic acid group at C4 and a OH substituent at C2 were formed.

17β-O-Glucuronide of Org 30659 (C12A). Metabolite C12 consisted of two metabolites, C12A and C12B. The presence of the anomeric proton at 4.99 ppm together with the glucuronide signals at 3.28–3.62 ppm suggested a glucuronic acid conjugate. All of the signals, compared with Org 30659, were present, so the glucuronic acid is probably conjugated at the 17β-OH group.

O-Glucuronide of the 2α-OH,3α-OH,5α-H Metabolite of Org 30659 (O-Glucuronide at Either C2 or C3) (C12B). The presence of a broad multiplet at 3.85 ppm indicated a 2α-OH substituent. This signal was coupled with the narrow multiplet at 3.46 ppm. No signal was present at 5.87 ppm, which indicated the absence of the Δ4 moiety. Together with the glucuronide signals between 3.20 and 3.57 ppm and the anomeric proton at 4.37 ppm, the presence of a 3α-glucuronide was indicated. The position of the O-glucuronide could be at either C2

Fig. 3. Metabolite profiles after 30 min of incubation of Org 30659 with rat liver (A), rabbit liver (B), monkey liver (C), and human liver 2 (D) microsomes.
Metabolites isolated from microsomal incubations are summarized in Table 5. Minor metabolites remained unidentified because the isolated amounts of metabolites were insufficient or the mixtures were too complex for NMR and MS analysis.

**Identification of the Metabolites Present in Microsomal Incubations.** Analysis. The NMR, MS, and IR (only M7) data for the metabolites isolated from microsomal incubations are summarized in Table 5. Minor metabolites remained unidentified because the isolated amounts of metabolites were insufficient or the mixtures were too complex for NMR and MS analysis.

**β-OH Metabolite of Org 30659 (M4).** The 4-H multiplicity and the presence of 10-H at 3.07 ppm indicated a substituent at C6β. A triplet at 4.35 ppm pointed to the presence of a β-OH substituent. 15β,16β-Epoxide Metabolite of Org 30659 (M7). The signals for 15-H and 16-H were observed at 3.48 ppm, which indicated the presence of a 15,16-epoxy group. In the 3-H-NMR NOE difference spectrum, a NOE contact was found between 15-H and 14α-H. No NOE contact was observed between 18-methyl and 15-H and/or 16-H. This indicated the presence of a 15β,16β-epoxide moiety.

Metabolites M9 and M10 were identified based on comparisons with the retention times of metabolites isolated from the liver perfusate after circulatory perfusion of Org 30659 in rat liver (results not shown). The putative structure of M9 was the 3β-OH,5α-H metabolite of Org 30659 and/or the 5α-H metabolite of Org 30659, whereas the putative structure of metabolite M10 was the 3α-OH,5α-H metabolite of Org 30659.

**Metabolites Present in Hepatocyte Incubations.** The cell medium contained the major fraction (>82%) of radioactivity after 3 hr of incubation of [3H]Org 30659 with rat and human hepatocytes. At least 26 compounds (H1–H26), in addition to Org 30659, were observed in the HPLC radioactivity profiles.

Org 30659 was extensively metabolized by rat hepatocytes; no Org 30659 was present in the cell medium, and <2% of the integrated radioactivity in the cell medium eluted at the retention time of Org 30659. In human hepatocyte incubations, Org 30659 was reasonably well metabolized: approximately 37% (human liver 4) and 53% (human liver 5) was accounted for by Org 30659 in cell media from human hepatocyte incubations. H11, H15, and H17 were the major metabolites present in cell media from rat samples, and H13 and Org 30659 were the major compounds present in cell media from human liver 4 and human liver 5 samples. The metabolites present in incubation samples were identified by comparison with the retention times of metabolites isolated from urine and feces from rats, from the preparative incubation samples with rabbit liver microsomes, or from the liver perfusate after a circulatory perfusion of Org 30659 in rat liver (results not shown). The compounds present in HPLC metabolite profiles of cell media from incubations with rat and human hepatocytes and the putative structures are given in Table 6.

**Discussion**

**In Vivo Metabolism of Org 30659 in Rats and Monkeys.** Org 30659 was extensively metabolized after oral administration to rats and monkeys; no unchanged Org 30659 was observed in urine and feces samples from rats and monkeys. The feces appeared to be the main route of excretion.

In rats, opening of the A-ring was the major metabolic route for Org 30659. The A-ring-opened 2-OH,4-carboxylic acid,5α-H metabolite of Org 30659 was a major metabolite present in plasma (0.5 hr), urine, and feces. A possible mechanism for the opening of the A-ring is via Baeyer-Villiger oxidation. Baeyer-Villiger oxidation is an important reaction in organic synthesis for the oxidation of ketones to esters or lactones. In Baeyer-Villiger oxidation, a carbonyl compound

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<th>NMR (ppm)</th>
<th>Ref.</th>
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<td>2α-H/2β-H</td>
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*Ref.* Reference compound (Org 30659).

*MS (m/z)* Chemical shifts are given in ppm.

*EI [M+]* Electron ionization.
is oxidized by a per oxy compound in the presence of an acid catalyst, in a manner involving insertion of an oxygen atom into one of the carbon-carbon bonds at the carbonyl group (Carey and Sundberg, 1990). In the literature, Baeyer-Villiger oxidation reactions of several substrates, such as camphor and cyclohexanone, have been reported to be catalyzed by bacterial FMOs (Walsh and Chen, 1988; Fang et al., 1989; Hrycko et al., 1995; Ryerson et al., 1982; Alphand et al., 1990; Levitt et al., 1990). These enzymatic Baeyer-Villiger reactions require a bound flavin cofactor in the presence of NADPH and oxygen. The flavin 4α-hydroperoxide intermediate reacts as a nucleophile with the carbonyl group of the substrate (Walsh and Chen, 1988; Fang et al., 1995; Ryerson et al., 1982).

The cleavage of the side chain of progesterone to form androstenedione is also assumed to occur via a Baeyer-Villiger rearrangement. Cytochrome P450 17, a microsomal steroidogenic cytochrome P450 enzyme located in the adrenal cortex, ovary, and testis, is proposed to catalyze this side chain cleavage of progesterone (Swinnen and Mak, 1994).

Chen et al. (1995) purified FMO1 from pig liver microsomes. Pig liver FMO1 catalyzes the Baeyer-Villiger oxidation of salicylaldehyde to pyrocatechol. The mechanism of this C-oxidation of salicylaldehyde is probably the same as the mechanism of the Baeyer-Villiger oxidation catalyzed by bacterial enzymes. Demonstrating the involvement of pig liver FMO1 in the C-oxidation of salicylaldehyde, Chen et al. (1995) suggested that the oxidation of ketones or aldehydes by Baeyer-Villiger reactions catalyzed by FMO isoforms should be considered as a possibility. By analogy with the mechanism of Baeyer-Villiger oxidation reactions catalyzed by bacterial monooxygenases, a possible mechanism for the A-ring opening of Org 30659 is proposed in fig. 4.

In rats, Org 30659 was also metabolized at the C3-position. At the C3-position, 3α-OH was observed in combination with the reduction of the Δ4 double bond to 5α-H. Metabolites of etonogestrel (structurally related to Org 30659) were also mainly 3α-OH, 5α-H derivatives, so it seems that reduction of the 3-keto moiety to a 3α-OH group is preferred in rats. The 3α-OH, 5α-H metabolite was further metabolized to a 3α-OH, 5α-H metabolite of Org 30659. An additional major metabolite present in rat urine showed introduction of an OH group at C15 (15β-OH), a shift of the Δ15-double bond to a 16/17-double bond, and subsequent removal of the OH group at C17 (the Δ15-shifted metabolite of Org 30659).

In monkeys, Org 30659 was mainly metabolized at the C3- and the C17-positions. The 3-keto moiety was reduced to 3β-OH and 3α-OH groups. No specific preference for reduction of the 3-keto moiety to 3α-OH or 3β-OH was observed. The 3-OH metabolites were present in combination with a 5α-H group in most cases, but 5β-H derivatives were also observed.

In monkey urine, conjugates of 3-OH metabolites with glucuronic acid were present. The formation of a 2α-O-glucuronide derivative has not been proven but cannot be excluded as a possibility, because the position of the glucuronic acid in metabolite C12B could be at either C2 or C3. Conjugation with glucuronic acid at the 17-OH group was also observed, yielding a major metabolite excreted in the urine.

The majority of the metabolites isolated from monkey urine and
feces were glucuronides, so it seems that glucuronidation is the predominant form of steroid conjugation in cynomolgus monkeys. Guillemette et al. (1996) studied the levels of glucuronidated and sulfated steroids, e.g. androstane-3α,17β-diol glucuronide, in plasma of humans, cynomolgus monkeys, and rats. High levels of circulating glucuronidated steroids were found in plasma of cynomolgus monkeys, whereas no glucuronic acid conjugates could be detected in plasma of rats. The levels of glucuronidated steroids in human plasma samples were 10-fold lower than those in cynomolgus monkey samples. With respect to the sulfated steroids circulating in plasma, very low levels were detected in plasma of cynomolgus monkeys, compared with humans.

In Vitro Metabolism of Org 30659. In rat liver microsomes, Org 30659 was metabolized very rapidly by reduction of the 3-keto, Δ4 moiety to yield a 3α-OH,4,5α-dihydro derivative and a 3β-OH,4,5α-dihydro and/or 4,5α-dihydro derivative. The reduction of the 3-keto moiety was mainly to the 3α-OH group, as was observed in vivo. In rat hepatocyte incubations, these 3α-OH,5α-dihydro derivatives and the sulfate conjugates of these derivatives were minor metabolites.

In rats in vivo, the opening of the A-ring was the major metabolic routes for Org 30659. This A-ring opening reaction was not observed in the incubations of Org 30659 with rat liver microsomes. Assuming that FMO is involved in the opening of the A-ring of Org 30659, this latter finding is unexpected. Possible explanations include preferential reduction of the 3-keto, Δ4 moiety of Org 30659 by 5α-reductase and 3α-HSD, as a result of differences in the affinities of these enzymes (compared with FMO) for Org 30659 under the conditions used and/or differences in the levels of expression of these enzymes in female liver microsomes. Further study is clearly required to establish both the mechanism and the enzyme(s) involved in the formation of an A-ring-opened metabolite of Org 30659 in rats.

In contrast to the microsomal incubations, hepatocyte incubations showed the presence of the A-ring-opened metabolites of Org 30659. All of the metabolic routes for Org 30659 observed in vitro were also observed in vivo, with the exception of reduction of the 3-keto moiety to a 3β-OH group (yielding a minor metabolite), which was observed in vitro but not in vivo. However, the metabolism of Org 30659 in rat hepatocyte incubations was a better reflection of the in vivo metabolism of Org 30659 in rats, compared with the metabolism of Org 30659 in rat microsomal incubations. The metabolic routes of Org 30659 found in rats are given in fig. 5.

In monkey liver microsomes, the 6β-OH metabolite of Org 30659 was the major metabolite present, which was unexpected, because the 6β-OH metabolite of Org 30659 was not observed in vivo. As can be seen from the in vivo data, glucuronidation is a major metabolic route for Org 30659 in monkeys. Glucuronidated conjugates formed in the liver can be easily excreted in the bile or urine. Probably the glucuronidation of Org 30659 in monkeys is so fast that hydroxylation at C6 in vivo does not take place to a major extent. In general, the
metabolism of Org 30659 in monkey liver microsomes was not representative of the in vivo metabolism of Org 30659 in monkeys. The metabolic routes of Org 30659 observed in monkeys are shown in fig. 5.

Similar to the monkey liver microsomes, rabbit and human liver microsomes mainly converted Org 30659 to the 6β-OH metabolite of Org 30659. The 6β-OH metabolite of Org 30659 was also the major metabolite after incubations with human hepatocytes. The 6β-hydroxylation is a known metabolic route for steroids, e.g. testosterone, androstenedione, and progesterone, in human liver microsomes and is primarily catalyzed by cytochrome P450 3A4 (Waxman et al., 1988). In monkey and human hepatocytes, 6β-hydroxylation was also observed as a major metabolic route for testosterone (Olinga et al., 1998a; Kanter et al., 1998).

In addition to the 6β-OH metabolite of Org 30659, a 15β,16β-epoxy metabolite of Org 30659 (M7) was observed after incubation with rabbit liver microsomes. The metabolic routes of Org 30659 in rabbit liver microsomes are shown in fig. 5.

In human liver microsomes, 3-keto,Δ4-reduction of Org 30659 was not observed. Early studies investigating the 3α-HSD enzyme in human liver (Iyer et al., 1992) showed no activity with 3-ketosteroids containing a Δ4 double bond. However, the 3α-HSD enzyme reduced 5α-H and 5β-H dihydro derivatives at similar rates. The Δ4-reductase responsible for the reduction of the 4/5-double bond is predominantly found in the cytosolic fractions of human liver (Ward and Back, 1993). Hence, the 3-keto,Δ4-reduced metabolites of Org 30659 were not present in microsomes.

In human hepatocyte incubations, 3α-OH,5α,H derivatives and possibly 3β-OH,5α-H derivatives were present as minor metabolites. Other minor metabolites of Org 30659 in human hepatocyte incubations were glucurononic acid and sulfate conjugates of the 3α-OH,5α-H derivatives. Also, the A-ring-opened metabolite of Org 30659 (H15) and the 15β,16β-epoxide metabolite of Org 30659 (H18) were observed as minor metabolites in human hepatocyte incubations. The metabolism of Org 30659 in human liver microsomes and hepatocytes is shown in fig. 5.

Considering the in vitro-in vivo correlation of Org 30659 in rats, the rat hepatocyte incubations with Org 30659 were more representative of the in vivo metabolism of Org 30659 in rats than were rat microsomal incubations. In addition, the metabolism of Org 30659 in monkey liver microsomes was not representative of the in vivo metabolism of Org 30659 in monkeys. The major metabolic routes for Org 30659 in human microsomal incubations (6β-hydroxylation) and human hepatocyte incubations were similar. Hence, extrapolating the in vitro metabolic routes observed in incubations with human liver microsomes and human hepatocytes, it is likely that in vivo hydroxylation at the C6-position (6β-OH) would occur.

In addition, there is the possibility of finding D-homo metabolites, in which the five-membered D-ring is expanded to a six-membered D-ring, in humans; Abdel Aziz and Williams (1970) isolated a D-homo metabolite of ethinylestradiol from human urine after oral administration of ethinylestradiol to humans. Düsterberg et al. (1987) demonstrated the formation of a D-homo metabolite of gestodene (a steroid structurally related to Org 30659).

Also, 3-keto,Δ4-reduced metabolites can be expected to be formed in vivo, because the hepatocyte incubations showed the presence of 3α-OH,5α,H derivatives and possibly 3β-OH,5α-H derivatives. In addition, major urinary metabolites of gestodene (a steroid structurally related to Org 30659) in humans were the tetrahydro-reduced metabolites (Ward and Back, 1993). Phase II metabolism is also likely to occur in vivo, because sulfate and glucuronide conjugates of Org 30659 were observed in incubations with human hepatocytes.

Various phase I reactions, such as carbon hydroxylation, opening of the A-ring, and reduction of the 3-keto,Δ4 moiety, are observed in the metabolism of Org 30659 in several species. Cytochrome P450 is known to catalyze a large number of phase I reactions. 3-Keto,Δ4-reduction of Org 30659 may be catalyzed by 5α- and/or 5β-reductase and 3α- and/or 3β-HSDs. The identification of the enzymes responsible for the phase I metabolism of Org 30659 will be an interesting subject for further investigations.

References


