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Excretion balance and metabolism of the progestagen Org 30659 in healthy postmenopausal women

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Abstract

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 contraception and hormone replacement therapy, was studied in vivo after oral administration to healthy postmenopausal women. After oral administration of [^{14}C]-Org 30659 to postmenopausal women, the compound was extensively metabolized. The dosed radioactivity was predominantly excreted via urine. Org 30659 was to a large extent metabolized at the C3- and the C17-positions. Phase II metabolism, and in particular conjugation with glucuronic acid at the 17β-hydroxy group, is the major metabolic route for Org 30659 in vivo. Not only phase II metabolism was observed for Org 30659 after oral administration to postmenopausal volunteers, but also metabolism in the A-ring occurred, especially reduction of the 3-keto-Δ^4 moiety to give 3α-hydroxy, 5α(β)-dihydro and 3β-hydroxy, 5α-dihydro derivatives. Oxidative metabolism (6β-hydroxylation) observed in human liver preparations in vitro, was not observed to a significant extent in vivo. So, in vitro human metabolism is different from the in vivo metabolism, indicating that the in vitro–in vivo extrapolation is far from straightforward, at least when only liver preparations are used. The proper choice of the in vitro system (e.g., microsomes, hepatocytes, slices or individually expressed enzymes) and the substrate concentration can be very important determinative factors for the predictability of the in vitro system for the in vivo situation. Species comparison of the metabolic routes of Org 30659 after oral administration indicated that the monkey seems to be a better representative species than the rat for the metabolism of Org 30659 in humans.

1. Introduction

Org 30659 ((17α)-17-hydroxy-11-methylene-19-norpregna-4,15-dien-20-yn-3-one) shown in Fig. 1 is a new potent progestagen currently under clinical development by NV Organon for use in contraception and hormone replacement therapy. Org 30659, a derivative of 19-nortestosterone, shows a very high progestagenic activity in pharmacological studies of rats and rabbits, compared to the known progestagens 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 in rats, Org 30659 is devoid of other hormonal activities, such as glucocorticoid and antiglucocorticoid activities [1].

Recently, the metabolism of Org 30659 was studied in vivo after oral administration to rats and monkeys, and in vitro using rat, rabbit, monkey and human liver microsomes and rat and human hepatocytes [2]. Rats and monkeys are generally used as the test species in preclinical studies of drugs, which affect human repro-

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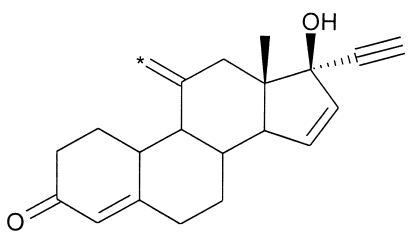
ductive processes. Rats are used because of the wealth of historical data that exists, whereas the monkey is used because of the belief that due to similar reproductive anatomy and physiology, the monkey would be the best representative of man [3]. After oral administration of [^{3}H]-Org 30659 to rats and monkeys, Org 30659 was extensively metabolized in both species [2]. Fecal excretion appeared to be the main route of elimination.

The major metabolic route for Org 30659 in vivo in rats was opening of the A-ring, resulting in a 2-hydroxy,4-carboxylic acid,5α-H metabolite of Org 30659. Other metabolic routes involved the introduction of a hydroxy group at C15β, followed by a shift of the Δ^{15} double bond to a 16/17 double bond and then removal of the hydroxy group at C17 and furthermore, the reduction of the 3-keto, Δ^4 moiety, followed by sulfate conjugation of the 3-hydroxy 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, Δ^4 moiety with the majority of the metabolites being 3α-hydroxy, 5α-H derivatives.

In monkeys, Org 30659 was mainly metabolized at the C3- and the C17-positions in vivo. The 3-keto moiety was reduced to both a 3β- and 3α-hydroxy substituent. In addition to phase I metabolites, glucuronic acid conjugates were observed in vivo, i.e., the 17β-*O*-glucuronide, 3α(β)-*O*-glucuronide, and 5α-H metabolites of Org 30659. In monkey liver microsomes, the 6β-hydroxy metabolite of Org 30659 was the major metabolite present. Similar to the monkey liver microsomes, rabbit and human liver microsomes converted Org 30659 into the 6β-hydroxy metabolite. This was also the major metabolite in incubations with human hepatocytes.

The present investigation was performed to study the excretion balance and metabolism of Org 30659 in healthy postmenopausal women as part of the clinical development of the compound. For this purpose, five healthy postmenopausal female volunteers received a single oral dose of [^{14}C]-Org 30659 after three days of daily oral treatment with unlabeled Org 30659. Based on the data obtained from this study, the predictive value of previous human in vitro studies [2] was evaluated. Moreover, the human biotransformation data were compared with the data obtained from in vivo metabolism studies in rats and monkeys.



* position of [^{14}C]-label

Fig. 1. Structure of Org 30659.

2. Material and methods

2.1. Chemicals and reference compounds

[^{14}C]-Org 30659 (label at C11 methylene, radiochemical purity ≥ 98%) was prepared by the Organic Isotope Group of the Department of Process Chemistry of NV Organon, Oss, The Netherlands. Unlabeled Org 30659 and the reference compounds Org 39412 ((5α,17α)-17-hydroxy-11-methylene-19-norpregna-15-en-20-yn-3-one), Org 39413 ((5α,17α)-2,17-dihydroxy-11-methylene-19-nor-2,3-secopregna-15-en-20-yn-3-oic acid), Org 39386 ((15β)-15-hydroxy-11-methylene-19-norpregna-4,16-dien-20-yn-3-one) and Org 38585 ((6β,17α)-6,17-dihydroxy-11-methylene-19-norpregna-4,15-dien-20-yn-3-one) were synthesized by the Department of Process Chemistry, NV Organon.

β-Glucuronidase/arylsulfatase obtained from *Helix Pomatia* and β-glucuronidase isolated from *Escherichia coli* K 12 were purchased from Boehringer Mannheim, Almere, The Netherlands. All other chemicals were obtained from commercial sources and were of analytical grade.

2.2. Subjects, dosing and sample collection

The clinical part of this study was performed in the clinical research centre of Pharma Bio-Research, Assen, The Netherlands. Five healthy postmenopausal female volunteers received an oral administration of one capsule of 240 μg non-radiolabeled Org 30659 (which is in the range of the expected therapeutic dose), daily for three consecutive days in order to attain steady state plasma concentrations. Based on kinetic data (results not shown), steady state for Org 30659 was observed after three days (plasma half-life ~12 h). On the fourth day, they were given orally, one capsule of 243 μg/555 kBq [^{14}C]-labeled Org 30659. Blood samples were collected pre-dose and at 0.25–0.5–1–1.5–2–3–4–6–8–24-h and every 24 h post radioactive dose until discharge from the clinic. Plasma was prepared by centrifugation. Urine samples were collected in 8-h portions during the first 24 h and in 24-h portions thereafter until discharge from the clinic. Feces samples were collected in separate portions every 24-h post radioactive dose until discharge from the clinic. In addition, pre-dose urine and feces samples

were collected. Based on data obtained with Org 30659 in animals, it was anticipated that the study would conclude on the morning of Day 11. From the ninth day onwards, excretion of radioactivity in urine and feces was examined on a daily basis in order to determine the elimination of radioactivity. If the level of radioactivity was below 50 dpm/ml in urine and 75 dpm/400 mg feces sample, the subjects were discharged from the clinic. Plasma, urine and feces samples were stored at −20°C until shipment. Samples were shipped in deep-frozen condition to NV Organon, Oss, The Netherlands for further analysis.

2.3. Determination of radioactivity in plasma, urine and feces

The amount of radioactivity in plasma and urine samples was determined by liquid scintillation counting (LSC) (Tri-Carb 2500 TR/2, Canberra Packard, Belgium). The amount of radioactivity in feces samples was determined by combustion in a Sample Oxidizer 387 (Canberra Packard) followed by LSC. Feces samples were homogenized with approximately two volumes of Milli-Q water before combustion.

2.4. Pharmacokinetics

All calculations were performed with WinNonlin Standard, version 1.5 (Scientific Consulting, Cary, NC, USA) and Microsoft EXCEL version 5.0, running under Windows NT version 4.0. A plasma concentration-time curve was constructed for each individual. The data were fitted with a noncompartmental method and various compartmental methods. The model which resulted in the best fit was chosen to calculate the AUC_{0-inf}, C_{max}, t_{max}, K01-HL (absorption half-life), K10-HL (elimination half-life) and the alpha and beta half-lifes associated with the first and second elimination phases of the plasma concentration–time curve.

2.5. Pooling of samples for metabolite profiling

For metabolite profiling, plasma samples containing sufficient radioactivity were pooled per sampling time point (0.5–1–1.5–2–3–4 h post dosing). The plasma samples of later time points did not contain enough radioactivity for metabolite profiling purposes. For urine and feces samples, pools were composed per subject, per sampling time interval (8- or 24-h fractions) for those time intervals representing an excretion of at least 5% of the administered dose.

2.6. Sample treatment for the analysis of metabolite profiles

Plasma and urine were applied to 6 ml pretreated Bakerbond SPE C_{18} solid-phase extraction columns. Columns were washed with ammonium acetate (0.1 M, pH 4.2) and eluted with methanol. The methanol effluents were concentrated by vacuum centrifugation and subjected to HPLC analysis. Feces samples were extracted with two volumes of acetonitrile. The extracts were concentrated in a Speed Vac concentrator. The samples were then centrifuged for 5 min at 4000 *g*. Supernatant was decanted and subjected to HPLC analysis.

2.7. HPLC analysis of metabolite profiles in plasma, urine and feces

HPLC analysis of the plasma, urine and feces samples was performed using a μ-Bondapak C_{18} column (internal diameter: 7.8 mm; internal length: 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 a HP1040 diode array detector (Hewlett Packard, Germany). Radioactivity in the HPLC effluent was determined by the collection of fractions followed by liquid scintillation counting. Samples were spiked with unlabeled Org 30659, Org 39386 and Org 38585 prior to HPLC analysis as references for retention time (UV signal at 254 nm).

Radioactive peaks in the HPLC metabolite profiles were characterized as a metabolite if the peak area was at least the average area of five times 1-min integrated background noise + 3∗SD (standard deviation), the peak height was at least three times the average peak height of five times 1-min integrated background noise and the peak duration was at least 0.5 min. Metabolite numbers were assigned on the basis of retention times. Metabolites from plasma (**P**), urine (**U**) and feces (**F**) were numbered independently.

2.8. Isolation of metabolites from urine

Urine of subject 5 (0–8 h) was taken for the isolation of metabolites from urine, because major metabolites assigned in urine samples of postmenopausal women were clearly present in this sample. Urine of subject 5 (0–8 h, 198 ml) was applied to pretreated C_{18} solid-phase extraction columns. The columns were washed with ammonium acetate (0.1 M, pH 4.2) and eluted with methanol. The methanol effluents were concentrated in a Speed Vac concentrator, diluted with Milli-Q water and then purified once more by solid-phase extraction as described above. The latter solid-phase extraction procedure was repeated twice. The methanol effluent obtained finally was concentrated by

vacuum centrifugation and subjected to HPLC analysis using the chromatographic conditions as described in Section 2.7. The effluent was collected in fractions. Fractions constituting a peak of radioactivity were pooled and dried in a Speed Vac concentrator. The residues were analyzed by LC-MS analysis.

2.9. Isolation of metabolites from feces

Feces homogenate of subject 1 (48–72 h) was taken for the isolation of metabolites from feces, because all metabolites assigned in feces samples of postmenopausal women were present in this sample. Feces homogenate of subject 1 (48–72 h, 235 g) was extracted with acetonitrile. The extract was concentrated and then applied to C_{18} solid-phase extraction columns. The columns were washed with ammonium acetate (0.1 M, pH 4.2) and eluted with methanol. The eluates were concentrated, diluted with Milli-Q water and were applied again to C_{18} solid-phase extraction columns as described above. The methanol effluent was concentrated, diluted with methanol and subjected to HPLC analysis using a μ-Bondapak C_{18} column (internal diameter: 3.9 mm; internal length: 150 mm) and a gradient of ammonium acetate (0.5 mM, pH 4.2) (solvent A) and methanol (solvent B). Elution was performed with a linear gradient of 10–80% solvent B in 30 min, further increasing to 90% solvent B in 1 min, followed by 90% solvent B for 3 min (isocratic) at 50°C. The flow rate was 1 ml/min. The effluent was collected in fractions. Fractions constituting a peak of radioactivity were pooled and concentrated in a Speed Vac concentrator. The residues were dissolved in methanol and then subjected to a second HPLC separation using a Symmetry C_{18} column (internal diameter: 3.9 mm: internal length: 150 mm) and a gradient of ammonium acetate buffer (0.1 mM, pH 4.2) (solvent A), acetonitrile (solvent B) and methanol (solvent C). Elution was performed (flow rate 1 ml/min) with 5% B and 15% C for 5 min (isocratic), followed by a linear gradient from 5 to 20 % B and 15 to 60% C in 25 min, further increasing to 70% C in 1 min (linear), followed by 20% B and 70% C for 3 min at 50°C. The effluent was collected in fractions. Fractions constituting a peak of radioactivity were pooled and dried in a Speed Vac concentrator. The residues were analyzed by LC-MS analysis.

2.10. Hydrolysis of the urinary metabolites

Metabolite U5, isolated from urine, was divided between two tubes. To each tube, 5 ml ammonium acetate buffer (0.1 M, pH 4.9) and either 20 μl of β-glucuronidase/arylsulfatase or 20 μl of buffer (control) were added. The tubes were capped and incubated in a shaking water bath at 37°C for 18 h. Urine of subject 5 (0–8 h) was taken for hydrolysis by β-glucuronidase. The urine sample (5 ml) was incubated in a shaking water bath for 5 h at 37°C in the presence or absence (control) of 100 μl of β-glucuronidase.

Incubation mixtures were applied to 6 ml solid-phase extraction columns. The columns were washed with 6 ml ammonium acetate (0.1 M, pH 4.2) and eluted with methanol (6 ml). The eluates were evaporated to dryness, dissolved in methanol, and subsequently subjected to HPLC analysis using the HPLC system as described in Section 2.7.

2.11. Identification of the metabolites

The metabolites isolated from urine, feces, and in preclinical studies and the reference compounds were subjected to LC-MS analysis. LC-MS analysis of the metabolites was performed using a μ-Bondapak C_{18} column (internal diameter: 3.9 mm; internal length: 150 mm) as described in Section 2.9 with the exception that HPLC analysis was performed at 40°C instead of 50°C. Atmospheric Pressure Chemical Ionization (APCI) spectra were recorded with a Perkin Elmer Sciex API 300 Mass Spectrometer. MS-spectra were recorded in the positive and/or the negative mode. The samples were introduced into the Mass Spectrometer with a HP 1100 liquid chromatograph (Hewlett Packard, Germany).

3. Results

3.1. Radioactivity in plasma

The concentration of Org 30659 plus metabolites, expressed in ng-equivalent/g plasma, was calculated from the concentration of total radioactivity in plasma samples and the specific activity of the radioactive dose formulation. Pharmacokinetic curve fitting of the data reflected a two-compartment model. Modeling of the parameters resulted in values of kinetic parameters given in Table 1.These data do not reflect the kinetics of Org 30659 only, but also the kinetics of a mixture of the parent compound and its metabolites. After administration of 243 μg of [^{14}C]-Org 30659 to female human volunteers, it was shown that the oral absorption of [^{14}C]-Org 30659 was fast (half-life of 12 ± 4 min). The mean C_{max} observed was 5.8 ng-equivalent/g plasma, which was reached at 0.7–1.2 h post radioactive dose. The mean total area of radioactivity under the curve (AUC_{0-inf}) was 102 ng-equivalent/h/g plasma. The elimination part of the plasma concentration–time curve (total radioactivity) showed two phases; a first elimination part, which is faster than the second one. The overall elimination half-life ranged from 7 to 12 h but the terminal phase of the plasma

Table 1
Values of kinetic parameters (±SD) after oral administration of [^{4}C]-Org 30659 to postmenopausal women

Subject	Parameter[a]						
	$AUC_{0\text{-inf}}$ (ngeq h/g)	C_{max} (ngeq/g)	t_{max} (h)	K01-HL (min)	K10-HL (h)	Alpha-HL (h)	Beta-HL (h)
S1	160 ± 43	6.2 ± 0.3	1.2 ± 0.2	16.2 ± 10.7	12.2 ± 5.2	1.0 ± 0.6	43 ± 17
S2	84 ± 20	5.5 ± 0.2	1.1 ± 0.1	14.0 ± 2.9	8.0 ± 2.1	1.6 ± 0.5	31 ± 14
S3	83 ± 15	6.1 ± 0.2	0.7 ± 0.1	8.8 ± 2.4	7.0 ± 1.7	0.8 ± 0.3	20 ± 5
S4	85 ± 27	5.1 ± 0.3	1.0 ± 0.3	_[b]	_[b]	_[b]	20 ± 10
S5	97 ± 17	6.0 ± 0.3	0.7 ± 0.1	7.2 ± 1.8	8.9 ± 1.9	1.0 ± 0.3	23 ± 6
Mean	102 ± 33	5.8 ± 0.5	0.9 ± 0.2	11.6 ± 4.2	9.0 ± 2.2	1.1 ± 0.3	28 ± 10

[a] $AUC_{0\text{-inf}}$, area under the plasma concentration–time curve (total radioactivity), calculated till infinity; C_{max}, the maximum concentration of Org 30659 plus metabolites after dosing; t_{max}, the time at which C_{max} was reached; K01-HL, absorption half-life; K10-HL, elimination half-life; Alpha- and beta-HL, Half-lifes associated with the first and second elimination phase of the plasma concentration–time curve, respectively.
[b] modeling failed: no values available.

concentration-time curve demonstrated a longer half-life of 20–40 h. This may be due to a slower elimination of the parent compound from a peripheral compartment or to the slower elimination of one or more of the metabolites. The mean residence time of radioactivity (calculated by means of a noncompartmental model) was 52 ± 11h.

3.2. Excretion of radioactivity in urine and feces

Data on the excretion of radioactivity in urine and feces (0–168 h) are given in Table 2. Urine was sampled up to 168- h or 192-h post radioactive dose. The 0–168 h recovery of radioactivity in urine (mean ± SD) was 73.1 ± 3.8%. Feces was sampled up to 144-216- or 240-h post radioactive dose. For the time interval of sampling of both urine and feces (0–168 h), the recovery of radioactivity in feces (mean ± SD) was 21.2 ± 5.1% of the dose. The total recovery of radioactivity within 168-h post radioactive dose averaged at 94.3%.

Table 2
Percentage of the dose excreted in urine and feces of postmenopausal women after oral administration of [^{14}C]-Org 30659

Time interval[a] (h)	Radioactivity in urine and feces (% of dose)[b]	
	Urine	Feces
0–8	29.8 ± 2.8	[c]
8–16	12.0 ± 1.2	[c]
16–24	7.1 ± 0.7	0.3 ± 0.5[c]
24–48	14.6 ± 2.5	9.4 ± 7.9
48–72	5.0 ± 1.7	4.9 ± 4.7
72–96	2.5 ± 1.1	3.5 ± 1.5
96–120	1.0 ± 0.5	1.4 ± 1.3
120–144	0.6 ± 0.2	0.8 ± 0.5
144–168	0.5 ± 0.3	0.8 ± 1.4
Total 0–168	73.1 ± 3.8	21.2 ± 5.1
Total 0–168 (U + F)		94.3 ± 5.8

[a] Time post radioactive dose.
[b] Data are expressed as mean ± SD (n = 5 subjects).
[c] Feces samples were collected in 0–24 h fractions.

3.3. HPLC metabolite profiles in plasma

Data on the metabolite profiles in pooled plasma samples obtained from postmenopausal female volunteers after oral administration of [^{14}C]-Org 30659 are given in Table 3. Org 30659 and its metabolites were quantified as a percentage of extracted radioactivity from the plasma pools. A representative HPLC metabolite profile in plasma (2 h) is shown in Fig. 2.

On the basis of the integration criteria, at least nine compounds (P1–P9) were observed in plasma. At 0.5 h post dosing, compound P8, followed by P9 were the main compounds observed. Compound P8, eluting at the retention time of Org 30659, was also a major compound present at the time points 1-, 1.5-, 2-, 3- and 4- h post dosing. The highest contribution of Org 30659 (41.1% of total radioactivity in plasma) was found 0.5 h after dosing, which decreased to 9.2% of total radioactivity 4 h after dosing. Besides P8, compounds P2, P4, P7 and P9 were major compounds present at 1-, 1.5-, 2-, 3- and 4- h post radioactive dose.

3.4. HPLC metabolite profiles in urine and feces

Data on the metabolite profiles of the urine and feces samples obtained from postmenopausal women after oral administration of [^{14}C]-Org 30659 are given in Tables 4 and 5, respectively. Org 30659 and its metabolites were quantified as a percentage of dosed radioactivity. Representative HPLC metabolite profiles are shown in Fig. 3 (urine, 0–8 h, subject 5) and Fig. 4 (feces, 48–72 h, subject 1).

On the basis of the integration criteria, at least five compounds each were assigned in urine (U1–U5) and feces (F1–F5). The main compound present in all the

Table 3
HPLC metabolite profiles of Org 30659 in plasma pools after oral administration of [^{14}C]-Org 30659 to postmenopausal women[a,d]

Time interval[b] (h)	Plasma concentration[c]	P1	P2	P3	P4	P5	P6	P7	P8	P9	R
0.5	4.1 ± 1.2	6.1	5.8	–	13.4	–	11.2	8.0	41.1	14.5	–[e]
1	5.8 ± 0.2	–[e]	11.6	9.3	11.7	7.9	9.2	17.4	17.5	15.4	–[e]
1.5	5.0 ± 0.7	5.4	12.2	4.0	18.5	6.6	8.4	15.3	19.6	10.2	–[e]
2	4.3 ± 0.6	7.0	8.4	10.4	18.3	5.9	7.1	17.3	17.1	8.5	–[e]
3	3.4 ± 0.3	4.8	16.2	17.7	15.1	6.8	–[e]	17.1	14.6	7.8	–[e]
4	2.9 ± 0.6	9.2	15.5	15.7	11.8	3.5	6.3	16.1	9.2	10.0	2.8

[a] Org 30659 and its metabolites were quantified as a percentage of extracted radioactivity from the plasma pools.
[b] Time post radioactive dose.
[c] ng-equivalent/g plasma (concentration of Org 30659 plus metabolites).
[d] R, remainder, a minor metabolite (unknown); P4, 17β-*O*-Gluc metabolite of Org 30659; P7, 3α-hydroxy, 5α(β)-H, 17β-*O*-Gluc metabolite of Org 30659; P8, Org 30659; P9, 3β-hydroxy, 5α-H and/or 3-keto, 5α-H metabolite of Org 30659. The structures of compounds P1–P3, P5 and P6 remained unidentified.
[e] Not present.

urinary fractions analyzed was U2, followed by U5. Org 30659 itself was not observed.

F3 and F4 were the major compounds present in the feces of all subjects. Compound F2 was also a major compound present in feces fractions of subjects 1, 2 and 5 and compound F1 in feces fractions of subject 1. Compound F5 was only present in metabolite profiles of subjects 1 and 4. No unchanged Org 30659 was observed.

3.5. Identification of the metabolites

Based on co-chromatographic analysis (using one or two different HPLC systems) with metabolites isolated and identified in preclinical studies [2] and authentic reference compounds, it is inferred that the 17β-*O*-glucuronide (*O*-Gluc) metabolite of Org 30659 (P4/U2), Org 30659 itself (P8), the 3β-hydroxy, 5α-H and/or 3-keto, 5α-H metabolite of Org 30659 (P9/F3) and the 3α-hydroxy, 5α-H and/or 3α-hydroxy, 5β-H metabolite of Org 30659 (F4) were present in plasma, urine and feces samples of postmenopausal women after oral administration of [^{14}C]-Org 30659.

In addition, the metabolites in urine and feces samples were isolated using one or two different HPLC separations for identification of the unknown metabolites by LC-MS analysis. However, the concentration of the metabolites isolated from urine and feces was very low and almost all isolated metabolites contained too many endogenous impurities for structure identification by LC-MS analysis. Only for compound U2 a reliable MS spectrum was obtained. In the LC chromatogram of compound U2, a peak with a retention time of 18.16 min was observed. The APCI spectrum in the positive mode showed an ion at m/z 485.4 $[M+1]^+$ and a fragment ion was observed at m/z 309.3 $[M+1-Gluc]^+$. Based on the M + 1 peak and the fragment ion, the proposed structure for this compound is the 17β-*O*-Gluc metabolite of Org 30659.

Hydrolysis of metabolite U5 with β-glucuronidase or β-glucuronidase/arylsulphatase demonstrated that this metabolite could be hydrolyzed by glucuronidase. The newly generated peak was identified on the basis of co-chromatography analysis with metabolites isolated and identified in preclinical studies as the 3α-hydroxy, 5α(β)-H metabolite of Org 30659. Furthermore, the retention time of the untreated metabolite U5 was compared with the retention time of the

Table 4
HPLC metabolite profiles of Org 30659 in urine pools after oral administration of [^{14}C]-Org 30659 to postmenopausal women (Data are given as a percentage of dosed radioactivity)[a]

Subject	Time interval (h)	% of the dose[b]	U1	U2	U3	U4	U5	R
1	0–72	72.2	5.7	21.3	3.2	4.7	19.2	18.4
2	0–48	65.4	6.0	24.2	3.6	4.5	10.1	17.3
3	0–48	62.1	6.0	17.9	3.2	2.1	12.6	20.3
4	0–48	62.5	5.3	16.1	3.3	3.3	11.6	23.1
5	0–72	68.9	6.0	21.4	2.6	3.3	17.1	18.5

[a] R, remainder, several minor metabolites (unknown); U2, 17β-O-Gluc metabolite of Org 30659; U5, 3α-hydroxy, 5α(β)-H, 17β-O-Gluc metabolite of Org 30659.
The structures of compounds U1, U3 and U4 remained unidentified.
[b] Percentage of the radioactive dose excreted in each fraction.

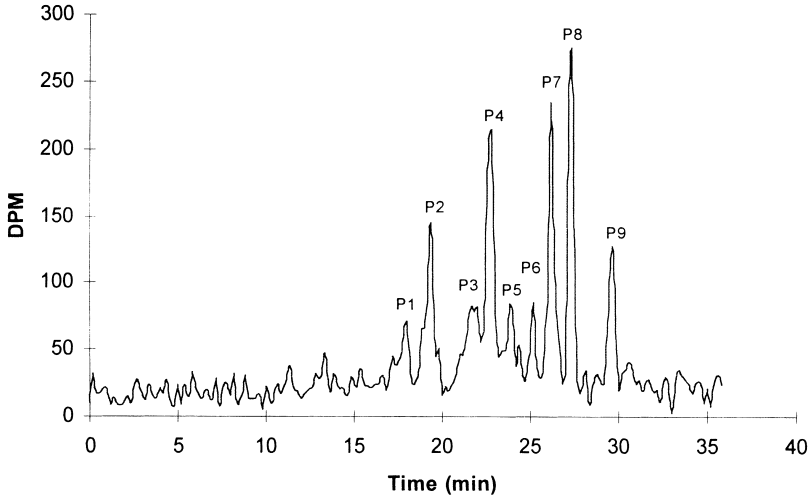


Fig. 2. HPLC metabolite profile of Org 30659 in plasma (2 h pool) after oral administration of [^{14}C]-Org 30659 to postmenopausal women.

3α(β)-*O*-Gluc, 5α-H metabolite of Org 30659. Based on these results, compound U5 was identified as the 3α-hydroxy, 5α(β)-H metabolite of Org 30659 conjugated with glucuronic acid at the 17-position. Compound P7 observed in plasma eluted at the same retention time as compound U5 in urine.

Based on the metabolites identified in the present study, the proposed biotransformation of Org 30659 in postmenopausal volunteers is given in Fig. 5.

4. Discussion

The results of the metabolism study with Org 30659 in postmenopausal women after oral administration of Org 30659 showed that Org 30659 is metabolized extensively. No unchanged Org 30659 was observed in urine and feces of the postmenopausal women. The dosed radioactivity was predominantly excreted via urine.

In postmenopausal women Org 30659 was to a large extent metabolized at the C3- and the C17-positions. Phase II metabolism, and in particular conjugation with glucuronic acid at the 17β-hydroxy group, is the major metabolic route for Org 30659 in vivo. The uridine glucuronosyl transferase (UGT) enzymes are a family of enzymes responsible for the conjugation with glucuronic acid to a large number of drugs. They are divided in two different families: the UGT1 family and the UGT2 family. Particularly the UGT2B subfamily seems to be active towards steroids and their metabolites [4]. Four human hepatic UGT enzymes of the UGT2B subfamily, e.g., UGT2B7, UGT2B11, UGT2B15 and UGT2B17, have been shown to have the capacity to glucuronidate C_{19} steroids specifically at the 3α-hydroxy and/or the 17β-hydroxy groups [5,6,7]. In contrast, 3β-hydroxy steroids are poor substrates for the UGT enzymes of the UGT2B subfamily and are generally conjugated with sulfate in vivo [5,7].

Sisenwine et al. [8] studied the urinary metabolites of levonorgestrel, a steroid structurally related to Org 30659. After oral administration of 1.5 mg [^{14}C]-levonorgestrel, most of the isolated metabolites were present as conjugates of which the major metabolites were glucuronide conjugates. Rittmaster et al. [9] have demonstrated that androstanediol (Adiol) incubated in human liver homogenate could either be glucuronidated at the 17β-hydroxy or at the 3α-hydroxy group. Glucuronidation at the C17- or the C3-position was apparently catalyzed by different enzymes. Approximately 90% of the glucuronidated Adiol, formed after incubation in human liver homogenate, was conjugated at the C17-position. The data of Rittmaster et

Table 5
HPLC metabolite profiles of Org 30659 in feces pools after oral administration of [^{14}C]-Org 30659 to postmenopausal women (Data are given as a percentage of dosed radioactivity)[a]

Subject	Time interval (h)	% of the dose[b]	F1	F2	F3	F4	F5	R
1	48–96	15.0	4.8	2.0	3.2	2.4	1.8	0.8
2	24–48	14.5	1.2	5.0	4.6	2.9	–[c]	0.8
3	24–48	20.7	3.1	1.9	8.1	7.6	–[c]	0.0
4	24–72	15.3	2.2	1.9	6.7	2.4	1.4	0.8
5	48–72	5.8	–[c]	2.6	1.6	1.3	–[c]	0.3

[a] R, remainder, several minor metabolites (unknown); F3, 3β-hydroxy, 5α-H and/or 3-keto, 5α-H metabolite of Org 30659; F4, 3α-hydroxy, 5α-H and/or 3α-hydroxy, 5β-H metabolite of Org 30659. The structures of compounds F1, F2 and F5 remained unidentified.
[b] Percentage of the radioactive dose excreted in each fraction.
[c] not detected.

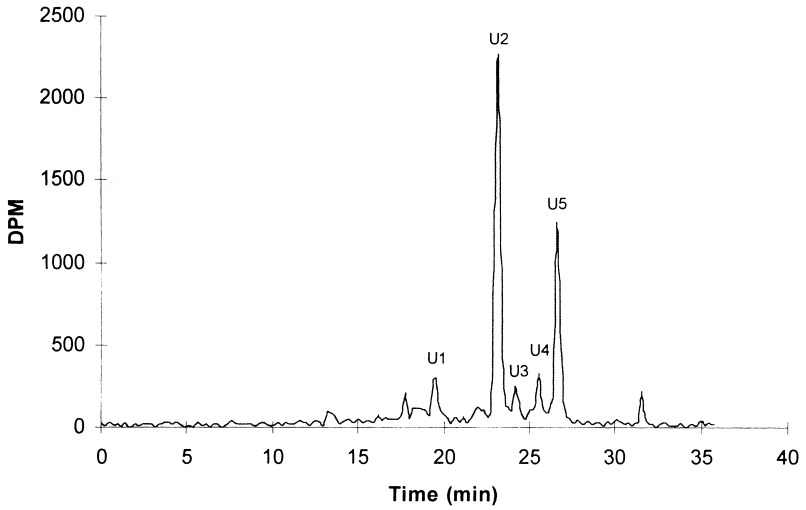


Fig. 3. HPLC metabolite profile of Org 30659 in urine after oral administration of [^{14}C]-Org 30659 to postmenopausal women.

al. support the findings in the present study in which conjugation with glucuronic acid was also predominantly observed at the C17-hydroxy group instead of conjugation at the C3-hydroxy group.

Not only phase II metabolism was observed for Org 30659 after oral administration to postmenopausal women, but also metabolism in the A-ring occurred by reduction of the 3-keto Δ^4 moiety to 3α-hydroxy, 5α (β)-dihydro and 3β-hydroxy, 5α-dihydro derivatives. No specific preference for reduction of the 3-keto moiety to the 3α-hydroxy or the 3β-hydroxy was observed. A reduction of the 3-keto Δ^4 moiety was also observed for steroids with a structure similar to Org 30659, gestodene and levonorgestrel. Major urinary metabolites of structurally related steroids, gestodene and levonorgestrel, in humans were the tetrahydro reduced metabolites [8,10,11]. Furthermore, gestodene was oxidized at the C1-,C6- and C11-positions,whereas levonorgestrel was hydroxylated at the C2- and C16-positions. For Org 30659, oxidative metabolism was not observed to a significant extent in vivo.

Abdel Aziz and Williams [12] isolated a D-homo metabolite of ethinylestradiol from human urine after oral administration of ethinylestradiol to humans. A D-homo metabolite is a metabolite in which the 5-membered D-ring is expanded to a 6-membered D-ring. In addition, Düsterberg et al. [11] have demonstrated the formation of a D-homo metabolite of gestodene. Since Org30659 possesses a 17α-ethinyl moiety like EE_2 and gestodene (a prerequisite for the D-homoannulation reaction [13]), it is noticeable that no D-homo metabolites were observed for Org 30659 after administration to postmenopausal women.

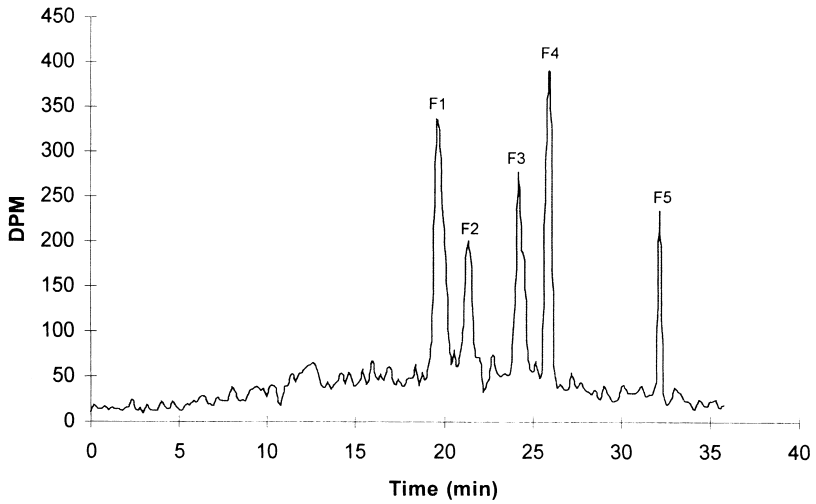


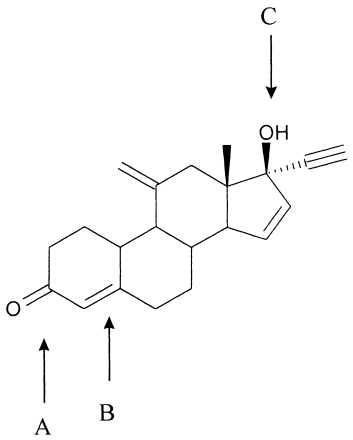
Fig. 4. HPLC metabolite profile of Org 30659 in feces after oral administration of [^{14}C]-Org 30659 to postmenopausal women.

4.1. Species differences

Metabolism of Org 30659 was found to differ between species [2]. Metabolic routes of Org 30659 in rats showed considerable differences compared to the metabolic routes of Org 30659 in humans. Opening of the A-ring resulting in a 2-hydroxy,4-carboxylic acid, 5α-H metabolite of Org 30659, was the major metabolic route for Org 30659 in vivo in rats, a metabolic route which was not observed in the present study in postmenopausal volunteers. In contrast, metabolic routes of Org 30659 in monkeys were very similar to that of Org 30659 observed after oral administration to female postmenopausal volunteers. In monkeys in vivo, Org 30659 was mainly metabolized at the C3- and the C17-positions, which were also important positions for metabolism of Org 30659 in humans. The 3-keto moiety was reduced to a 3β- and a 3α-hydroxy. The 3-hydroxy metabolites were present in combination with a 5α-H group in most cases, but 5β-H derivatives were also observed. Similar to human, the majority of the metabolites isolated from monkey urine were conjugated with glucuronic acid. However, in monkeys Org 30659 was glucuronidated at both the C3- and C17-hydroxy groups.

4.2. In vitro–in vivo comparison

Org 30659 was previously incubated with human liver microsomes and hepatocytes [2]. After in vitro incubations, the 6β-hydroxy metabolite of Org 30659 was the major metabolite present. In a previous study, it was determined that CYP3A is the major P450 family involved in the 6β-hydroxylation of Org 30659 [14]. In contrast to the in vitro results, glucuronidation was the major metabolic route for Org 30659 after oral administration of Org 30659 to postmenopausal volunteers. Furthermore, no oxidative metabolites were identified in this study.



A. Reduction of the 3-keto moiety to a 3α- or 3β-hydroxy

B. Reduction of the Δ^4-double bond to a 5α-H or 5β-H

C. Glucuronidation of the 17β-hydroxy group

Fig. 5. Metabolic routes for Org 30659 in postmenopausal women.

When using in vitro systems to study metabolism, the choice of the substrate concentration is very important. The major metabolic pathways may be shifted, depending on the drug concentration used as a result of differences in the relative affinities of the substrate (K_m) for the various enzymes. A possible explanation for in vitro–in vivo discrepancy includes a preferential glucuronidation of Org 30659 by the uridine diphosphate (UDP)–glucuronosyltransferases (UGTs) in vivo, at the concentration of Org 30659 in liver obtained after oral administration of Org 30659 to humans, as a result of differences in affinity of Org 30659 for these enzymes (as compared to CYP3A). After oral administration of 250 μg/subject the maximum plasma concentration of Org 30659 itself was approximately 8 nM (unpublished results), whereas human hepatocytes were incubated at final concentrations of 240–650 nM, approximately 30–80 times higher. In a previous study [14], for the 6β-hydroxylation of Org 30659 an apparent K_m of 41μM was observed in CYP3A4 supersomes, which is several orders of magnitude above the maximum human plasma concentration of Org 30659 (8 nM) after oral administration. In order to further support this explanation for the discrepancy, studies on the apparent V_{max} and K_m values for the 17β-*O*-glucuronidation of Org 30659 with the individual expressed UGT enzymes are required.

Another possible explanation for the observed discrepancy between the in vitro and in vivo metabolism of Org 30659 is,that Org 30659 is conjugated by the UGT enzymes in extra-hepatic tissues, i.e., gastrointestinal tract or kidney, instead of liver. The UGT enzymes are primarily found in mammalian liver, but expression of the UGTs is also observed in extra-hepatic tissues, including kidney, lung, gastrointestinal tract, breast, prostate, testis, spleen, skin, ovary and brain [7,15]. Further study with Org 30659 in human slices of extra-hepatic tissues/organs is required to establish the possible involvement of these tissues/organs in the glucuronidation of Org 30659 after oral administration.

In summary, Org 30659 was well absorbed and extensively metabolized. Org 30659 and its metabolites were predominantly excreted via the urine. Metabolism of Org 30659 was dominated by direct glucuronidation of the 17β-hydroxy group and reduction of the 3-keto, Δ^4 moiety. The oxidative metabolism observed in human liver preparations in vitro was not observed in

vivo, although it cannot be excluded that oxidative metabolism occurred to a minor extent, since several of the minor metabolites remained unidentified. Comparison of the metabolic routes of Org 30659 after oral administration in different species indicated that the metabolism of Org 30659 in rats differed from human metabolism, whereas the metabolism of Org 30659 in monkeys showed several similarities. Based on the available data, the monkey seems to be a better representative than the rat for the metabolism of this compound in humans. However, the data clearly demonstrate that extrapolation of metabolism of Org 30659 from animals to human is fairly difficult. For Org 30659, in vitro human liver metabolism is different from the in vivo metabolism, indicating that the in vitro–in vivo extrapolation for this steroid and possibly also for other synthetic steroidal hormones is far from straightforward, at least when only liver preparations are used.

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