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RADIOIMMUNOASSAY OF A NEW PROGESTAGEN, ORG 2969, AND ITS METABOLITE

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SUMMARY

A method for the determination of a new potent progestagen, Org 2969 (13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-17-ol) and its metabolite, 3-keto-Org 2969 (13-ethyl-17-hydroxy-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3-one) by radioimmunoassay is described. The unconjugated steroids were extracted from 1–2 ml serum samples with 2 × 4 ml diethyl ether–ethyl acetate (1:1, v/v), and the metabolites fractionated on 0.5 gr Lipidex-5000™ columns. The saturation analysis was performed using 3-keto-Org 2969-3-(O-carboxymethyl)-oxime [^{125}I]-histamine as tracer and an antibody of negligible cross reactivity with endogenous steroids. The sensitivity of the assay was 160 pg/ml for Org 2969 and 15 pg/ml for 3-keto-Org 2969. The coefficients for inter/intraassay variation were 19.9%/15.7% and 10.4%/6.8% for Org 2969 and 3-keto-Org 2969, respectively at 1 ng/ml concentration level. A serial analyses of serum concentrations of Org 2969 and 3-keto-Org 2969 in samples obtained from a volunteer who had ingested 2.5 mg of Org 2969 gave the maximum concentration of 12.7 ng/ml for 3-keto-Org 2969 and 0.7 ng/ml for Org 2969 1.5 h after ingestion, which is interpreted to support the hypothesis that a biotransformation of Org 2969 to 3-keto-Org 2969 occurs *in vivo*.

INTRODUCTION

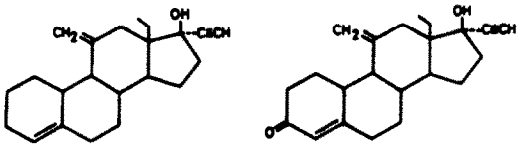
Org 2969 (13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-17-ol) is a new synthetic progestagen synthesized by Organon Int., B.V., Oss, The Netherlands. It has proved to be a very potent progestational agent in animal experiments [1], and inhibits ovulation in most volunteers at a 0.015 mg daily dose [2].

Although the biological activity of Org 2969 is very high, its affinity to the myometrial progesterone receptor is comparably low, only 16% and 18% of that of progesterone in human and rabbit, respectively. The introduction of a 3-keto group to the molecule increases the affinity to 150% (human) and 280% (rabbit) [3]. This, and what is known about the metabolism of another 3-deoxyprogestagen, lynestrenol [4, 5] have led to the hypothesis that the biological activity of Org 2969 might be mediated by biotransformation to its 3-keto derivative, (13-ethyl-17-hydroxy-11-methylene-18, 19-dinor-17α-pregn-4-en-20-yn-3-one). A similar mechanism of bioactivation has also been suggested for lynestrenol [6].

The aim of the present investigation was to develop a radioimmunoassay for the determination of both the parent compound, Org 2969, and the presumed biologically active metabolite, 3-keto-Org 2969 with a view to be used in the evaluation of the pharmacokinetics of Org 2969.

EXPERIMENTAL

Solvents and reagents. Diethyl ether (analytical grade; Merck AG, Darmstadt, Federal Republic of Germany) was twice distilled. Chloroform (Merck) was distilled, 1% ethanol was added and stored in a dark bottle. Petroleum ether (BDH Ltd., Poole, England), was distilled and the fraction boiling at 64–69° was used. Histamine HCl (Merck), tri-*n*-butylamine (BDH), isobutylchloroformate (Sigma Chemical Co., St. Louis, MO) were used as supplied. The bovine serum albumin, BSA (fraction V. Koch-Light Laboratories Ltd., Colnbrooks, Bucks, U.K.) was treated with charcoal to remove free fatty acids



Org 2969 3-keto-Org 2969

Fig. 1. The structure of Org 2969 and 3-keto-Org 2969.

The following codes and trivial names were used: Org 2969 = 13-ethyl-11-methylene-18.19-dinor-17α-pregn-4-en-20-yn-17-ol: 3-Keto-Org 2969 = 13-ethyl-17-hydroxy-11-methylene-18.19-dinor-17α-pregn-4-en-20-yn-3-one. 3β-Hydroxy-Org 2969 = 13-ethyl-11-methylene-18.19-dinor-17α-pregn-4-en-20-yne-3β.17-diol. 3-Keto-5αH-dihydro-Org 2969 = 13-ethyl-11-methylene-18.19-dinor-5α,17α-pregn-20-yn-3-one. 3β-Hydroxy-5αH-dihydro-Org 2969 = 13-ethyl-11-methylene-18.19-dinor-5α,17α-pregn-20-yne-3β,17-diol. Lynestrenol = 19-nor-17α-pregn-4-en-20-yn-17-ol: 11-Deoxycortisol = 17α,21-dihydroxypregn-4-ene-3.20-dione. 5α-Dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one. 17-Hydroxyprogesterone = 17α-hydroxy-pregn-4-ene-3,20-dione. Pregnenolone = 3β-hydroxypregn-5-en-20-one.

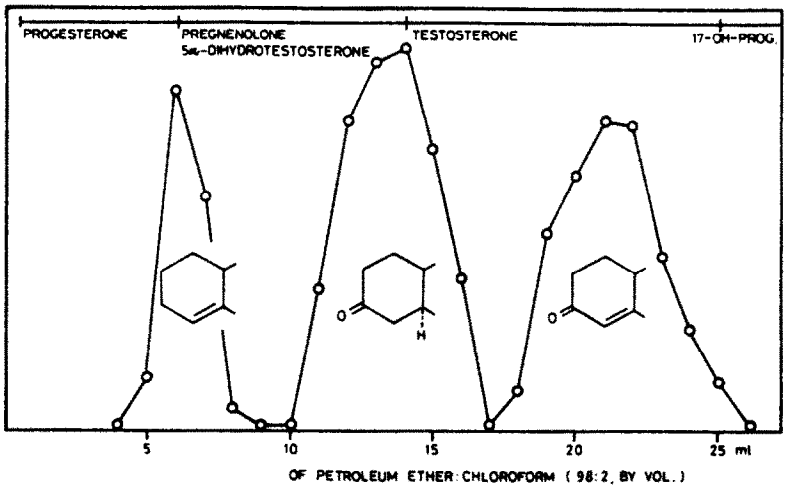


Fig. 2. The elution pattern of Org 2969, 3-keto-5αH-dihydro-Org 2969, 3-keto-Org 2969 and some endogenous steroids in Lipidex 5000™-chromatography used in the assay of Org 2969 and 3-keto-Org 2969. The hydroxylated metabolites of Org 2969 are eluted considerably later.

according to the method of Chen[7]. Lipidex-5000™ (hydroxyalkoxypropyl Sephadex) and Instagel® were purchased from Packard-Becker, B.V., Groningen, The Netherlands. Na^{125} I was purchased from the Radiochemical Center, Amersham, U.K.

Steroids and steroid derivatives. Org 2969, 3-keto-Org 2969-3-(O-carboxymethyl)-oxime, 3-keto-5αH-dihydro-Org 2969, 3β-hydroxy-5αH-dihydro-Org 2969, 3β-hydroxy-Org 2969 as well as [16-^{3}H]-Org 2969, S.A. 11.3 Ci/mmol, were donated by Organon Int., B.V. Other nonlabeled steroids were obtained from Ika-pharm, Ramat-Gan, Israel, or Steraloids Inc., Wilton, NH 03086, U.S.A.

Assay buffer. Fifteen percent of ethanol was added into phosphate buffered saline (PBS, 8) to avoid the strong adsorption of Org 2969 on the surface of glassware. This amount of ethanol disturbed the binding reaction to some extent, but was essential for the recovery of Org 2969.

Antisera. 3-keto-Org 2969-3-(O-carboxymethyl)-oxime was coupled to BSA by the mixed anhydride reaction[9]. The steroid: BSA-ratio obtained was 27 as measured by U.V.-absorption at 240 nm. The immunization was performed in rabbits by the method of Vaitukaitis *et al.*[10]. The working dilution of 1:30,000 was obtained within four months.

Material for analysis. Blood samples were drawn from one female volunteer, who ingested 2.5 mg of Org 2969 as a single dose. The samples were collected in glass tubes, allowed to clot, and then centrifuged. The serum was stored at −20°C until analyzed.

Extraction. About 5000 d.p.m. of [^{3}H]-Org 2969 was added to 2 ml serum samples and after 30 min equilibration at room temperature, the samples were extracted twice with ethyl acetate; diethyl ether (1:1, v/v.). The combined organic phases were evaporated to dryness under nitrogen.

Lipidex-5000™ chromatography. The features of the chromatography setup have been described in detail earlier [11]. The dry residue after evaporation was dissolved in three 0.2 ml portions of petroleum ether–chloroform (98:2, v/v) and applied onto 0.5 g Lipidex-5000™ columns (320 × 2.6 mm) packed in one ml pipettes. The elution pattern of Org 2969, two of its possible metabolites and the endogenous steroids eluting in the same area, are presented in Fig. 2. The fraction collected for Org 2969 analysis was eluted between 4 and 9 ml and for 3-keto-Org 2969 analysis between 17 and 27 ml. The fractions were then evaporated under a stream of nitrogen, and dissolved in 1 ml of ethanol.

Saturation analysis. For the saturation analysis, aliquots from the ethanol fraction were evaporated to dryness and 0.1 ml of diluted antibody was added to the dry residues. After 15 min preincubation at room temperature, about 15,000 c.p.m. of iodinated 3-keto-Org 2969-3-(O-carboxymethyl)-oxime-histamine, prepared according to Nars and Hunter[12] was added, and the reaction mixture was incubated for 2 h at room temperature. The bound and unbound ligands were then separated by a dextran coated charcoal suspension [8] and the bound fraction was counted with an LKB-Wallac 8000 Gamma Sample Counter (Wallac, SF-90520 Turku 52, Finland). Fifty percent of the Org 2969 fraction was used to count the recovery of the trititiated tracer with LKB-Wallac Model 81000 Counter in 10 ml of Instagel®, in order to check the recovery of the procedure. The actual concentrations were calculated on the basis of standards analyzed in parallel with the unknowns, by a Nova 840 computer belonging to a System Olli 4000 hospital data processing system using logit plot linearized standard curves [13].

RESULTS AND DISCUSSION

The specificity of the antibody is presented in Table 1. As can be seen, the cross reactivity of endogenous

Table 1. The specificity of the antibody used for the radioimmunoassay of Org 2969 and 3-keto-Org 2969

Ligand	Relative affinity
Org 2969	14
3-Keto-Org 2969	100
3β-Hydroxy-Org 2969	89
3-Keto-5αH-dihydro-Org 2969	46
3β-Hydroxy-5αH-dihydro-Org 2969	46
Pregnenolone	<0.006
5α-Dihydrotestosterone	<0.003
Testosterone	<0.003
Progesterone	<0.003
17α-Hydroxyprogesterone	<0.003
11-Deoxycortisol	<0.003
Cortisol	<0.003
Estradiol	<0.003

Table 2. The characteristics of the radioimmunoassay of Org 2969 and 3-keto-Org 2969

(%)	Recovery of added steroid	*Recovery of added ^{3}H-tracer	Rec. of cold steroid after correction of the losses	Blank (mean ± S.D.) (pg/ml)	Sensitivity (pg/ml)	Intra-assay variation (%)	Inter-assay variation (%)
ORG 2969	77.9%	85.6%	91.0%	72 ± 45	160	15.7%	19.9%
(n)	(10)	(10)	(10)	(9)		(10)	(15)
3-keto-Org 2969	97.6%	—	—	5.6 ± 4.9	15	6.8%	10.4%
(n)	(10)			(7)		(10)	(16)

* After extraction and chromatography (S.D. ± 4.4%). n = number of analysis.

steroids is negligible, and since the complete separation of the cross reacting metabolites of Org 2969 is accomplished by the Lipidex-chromatography, they do not influence the results. Subsequently the radioimmunoassay of both Org 2969 and 3-keto-Org 2969 can be considered very specific. The equilibrium association constant of the antiserum used was $0.7 \times 10^9\ M^{-1}$ for Org 2969 and $3.4 \times 10^9\ M^{-1}$ for 3-keto-Org 2969 calculated from Scatchard-type plot [14]. The difference in affinities suggests the importance of the 3-keto-function in the binding reaction.

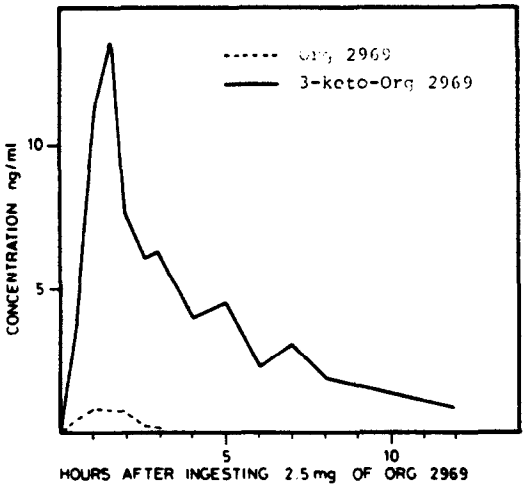


Fig. 3. The concentrations of Org 2969 and 3-keto-Org 2969 in a volunteer, who had ingested 2.5 mg of Org 2969 as a single dose.

The characteristics of the radioimmunoassay of Org 2969 and 3-keto-Org 2969 are presented in Table 2. From the data presented, it is evident that the correction of the Org 2969 values with the aid of an internal standard improves the recovery of the assay considerably, whereas in the case of 3-keto-Org 2969, no correction is needed. The greater assay variations and blank problems as well as the lower sensitivity of the Org 2969 assay can possibly be explained by the lower affinity of the antibody to Org 2969 and by the high adsorption of Org 2969 to glass surfaces. Fifteen percent of ethanol was added into the buffer to avoid this adsorption. This ethanol concentration was found to be the best possible compromise between attempt to keep Org 2969 in solution and the loss of binding ability of the antiserum.

The results of analysis of Org 2969 and 3-keto-Org 2969 in samples taken from a volunteer who had ingested orally 2.5 mg of Org 2969 as a single dose, are presented in Fig. 3. The results, the mathematical analysis of which will be published later, support the hypothesis that Org 2969 is converted rapidly to 3-keto-Org 2969 because the concentrations of Org 2969 are much lower than the 3-keto-Org 2969 con-

centration in all samples analyzed and the highest 3-keto-Org 2969 concentration is measured as soon as 1.5 h after the dose. Before further conclusions about the pharmacokinetics of Org 2969 can be drawn, more data, also with lower doses, must be collected. Because the method described in this paper allows the determination of the serum concentration of Org 2969 after a 0.5 mg oral dose and the concentration of 3-keto-Org 2969 after a 0.005 mg oral dose of Org 2969, good possibilities for such investigations exist.

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