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IN VIVO METABOLISM OF NORETHISTERONE-3-OXIME IN RABBITS

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SUMMARY

[4-^{14}C]-Norethisterone oxime was synthesized and separated into the *syn-* and *anti*-isomers. The purified isomers and the racemic mixture were administered intraperitoneally to rabbits. About 50% of the dose was recovered in urine and about 10% in the faeces. Excretion was rapid, most of the urinary radioactivity being recovered within 24 h of injection. More than half of the urinary radioactivity was extractable after enzymic (β-glucuronidase) hydrolysis. The major compound detected in urinary extracts was unchanged norethisterone oxime. The *syn*-isomer appeared to be converted *in vivo* into the *anti*-isomer. Plasma levels of radioactivity declined rapidly from a value of about 3.5% of the dose per 100 ml at 2 h after injection to 0.5% of the dose per 100 ml at 24 h.

INTRODUCTION

Norethisterone and derivatives such as the acetate and oenanthate, produced by esterification of the 17β-hydroxyl group in the D ring, are widely used as steroidal contraceptives either alone or in conjunction with a synthetic oestrogen. However, little attention has been given to derivatives of norethisterone involving the 4-en-3-one group in ring A. Shroff *et al.*[1] showed that whereas norethisterone-17-acetate-3-oxime had a similar activity to that of norethisterone in the Clauberg test in the rabbit, it had a much greater antifertility activity in rats. In view of our interest at that time in the relative rates of metabolism of derivatives of norethisterone, the metabolism of norethisterone-3-oxime was investigated in rabbits *in vivo*.

MATERIALS AND METHODS

Preparation of [4-^{14}C]-*norethisterone-3-oxime* (*NET-OX*). Norethisterone (NET, 29.8 mg), [4-^{14}C]-NET (5 μCi, S.A. 3.8 μCi/mg) and hydroxylamine hydrochloride (7.3 mg) were dissolved in 0.25 ml anhydrous redistilled pyridine and warmed on a steam bath for 2.5 min. 1 NHCl was added to the mixture placed in an ice-bath, and the precipitate obtained was dissolved in sodium carbonate, then acidified with 0.1 NHCl and extracted with redistilled ether. The NET-OX obtained was crystallised from methanol. The product was a mixture of approximately equal amounts of the *syn-* and *anti*-isomers. Some of this preparation was used in the investigations. The remainder was separated into the two isomers by thin-layer chromatography on 250 μ thick silica gel plates (Merck, Darmstadt, Germany). The eluted isomers were crystallised from methanol. The assignment of configuration to the isomers was based on the finding [2] that the anti-oxime is less polar than the syn-oxime (R_F *anti*-isomer 0.51; *syn*-isomer 0.3 using the solvent system toluene–acetone (4:1, v/v) and on nuclear magnetic resonance spectra. The oxime OH-proton in the anti-form would be relatively deshielded by the double bond and would resonate at a lower field (10.32) than the *syn*-isomer (10.11). Conversely the vinyl proton of the *anti*-isomer would be relatively shielded by the OH-proton and would resonate at a higher field (5.78) than the *syn*-isomer (6.39). The purity of all three preparations was established by elementary analysis, U.V. spectroscopy (NET, λ_{max} 258 mμ; NET-OX racemate, λ_{max} 264 m) and infrared spectroscopy (KBR disc, NET 1650 cm^{-1}, NET-OX 1630 cm^{-1}). In addition treatment of the prepared [4-^{14}C]-NET-OX with 1 NHCl for 30 min at 37°C gave a compound similar in chromatographic properties to NET. All preparations of [4-^{14}C]-NET-OX used in the investigations were at least 98% pure. The specific activity of all preparations was 0.15 μCi/mg.

Administration of steroids and sample collection. Female New Zealand white rabbits (body weight 2.5–4.0 Kg) received a single dose of 1.5 μCi [4-^{14}C]-NET-OX or the separate isomers dissolved in 0.1 ml ethanol and 0.9 ml 0.9% saline by intraperitoneal injection. The animals were kept in metabolic cages with free access to food and water. Urine and faeces were collected daily over a period of five days. Blood samples (10–12 ml) were collected into heparinised containers from two rabbits 2, 4, 7 and 24 h after receiving either *anti-* or *syn*-NET-OX. Plasma was obtained by centrifugation.

Estimation of radioactivity. Total radioactivity in urine and plasma was estimated as described previously [3] except that Triton-X100 based scintillant was used. Samples of urine (1 ml) and plasma (0.5 ml) were counted directly in the scintillator. Radioactivity in faeces was extracted as described previously [4]. The residue was partitioned between hexane:70% aqueous methanol. The aqueous methanol phase was evaporated to a small volume, diluted with water and extracted with chloroform. Radioactivity was assayed in a Beckman Model No.1550 liquid scintillation spectrophotometer.

Processing of urine. Samples of each urine collection were extracted with 2 vol. $CHCl_3$. The $CHCl_3$ phase was washed once with 0.1 vol. water, dried with anhydrous sodium sulphate and evaporated to dryness to yield an extract containing freely extractable steroids. The aqueous phase remaining after the removal of the non-conjugated radioactivity was processed [5] on Amberlite XAD-2. The methanolic eluates obtained were evaporated to dryness under reduced pressure, the residues dissolved in water and the pH adjusted to 4.7 [3]. 5 M Acetate buffer pH 4.7 (1 ml) and 10,000 units/ml of a β-glucuronidase preparation were added. The β-glucuronidase preparation from limpets [6] had an activity of 2×10^6 units/g. After incubation at 37°C for 48 h, the urine was extracted with chloroform and treated as described for the freely extractable fraction.

For solvolysis the aqueous phase remaining after enzymic hydrolysis was treated either as described by Edwards *et al.*[7] or with pyridinium sulphate [8]. In each case, the organic extract was evaporated, the residue dissolved in 5 ml dioxane and kept at 38°C for 24 h. Chloroform (20 ml) was added to the solvolysis mixture. The chloroform phase was washed with water (2 ml), 2 N NaOH (5 ml) and water (5 ml) and evaporated to dryness.

Extraction of plasma radioactivity. Plasma (2 ml) was extracted with 10 ml chloroform [3]. The non-conjugated radioactivity (chloroform phase) and conjugated radioactivity (aqueous phase) were estimated.

Chromatography of urine and faecal extracts. Metabolite patterns in urine and faecal extracts were obtained by thin layer chromatography using the solvent system cyclohexane–ethyl acetate (1:1, v/v) or by paper chromatography using the formamide: $CHCl_3$ system[9]. Radioautographs of the plates were prepared on Kodirex-Autoprocess X-ray film [10]. Thin layer and paper chromatograms were scanned for radioactivity using a Packard Model No. 7200 radiochromatogram scanner. The relative proportions of the metabolites were calculated by triangulation and the relevant areas were eluted in ethanol.

Gas chromatography of NET-OX. The trimethyl silyl ether of NET-OX was formed according to Chambaz and Horning[11]. A Pye 104 gas chromatograph was used with flame ionisation detection. The 150 × 0.3 cm glass column contained 3% SE-30 on Gas-chrom Q (80–1000 mesh). The oven temperature was 230°C and the nitrogen flow rate was 50 ml/min.

RESULTS

Excretion of radioactivity in urine and faeces

After intraperitoneal administration of [4-^{14}C]-NET-OX about 50% of the dose was recovered in urine over a 5 day period and 80% of this radioactivity was recovered within the first 24 h after administration (Table 1). There was no difference in the rate of excretion of radioactivity after administration of the *syn*- and *anti*-isomers or of the mixture. Although there was no difference between the mixture and the *syn*-isomer in the amount of urinary radioactivity in a freely-extractable and in a conjugated form (Table 2) after administration of the *anti*-isomer more of the metabolites were freely extractable and extractable after solvolysis with a consequent decrease in the amount extracted after enzymic hydrolysis. For all three preparations of NET-OX the major part of the excreted radioactivity was recovered after enzymic hydrolysis suggesting that the metabolites were excreted as glucuronides and the proportion so excreted tended to increase with time after administration.

Only small amounts of the dose were excreted in the faeces, the mean values (± S.D.) for the total recovery of radioactivity in the faeces for the three forms of norethisterone oxime were: mixture 7.9 ± 0.7%, *anti*-isomer 7.5 ± 2.1%, *syn*-isomer 13.2 ± 1.2%. The mean value for the *syn*-isomer was significantly higher ($P < 0.05$) than for the mixture and the anti-isomer. Most (63–81%) of the faecal radioactivity was excreted within 24 h of administration, predominantly in a non-conjugated form.

Table 1. Excretion of radioactivity in urine after intraperitoneal injection of [4-^{14}C] norethisterone oxime

Day after administration	NET-OX administered		
	Mixture	*anti*-isomer	*syn*-isomer
1	44.2 ± 3.5	43.4 ± 2.5	40.6 ± 2.8
2	6.3 ± 1.5	6.5 ± 1.2	6.8 ± 3.4
3	2.3 ± 2.6	3.0 ± 1.4	2.8 ± 0.5
4	2.3 ± 3.4	1.6 ± 0.3	2.8 ± 1.5
5	1.1 ± 1.5	1.6 ± 1.2	2.7 ± 3.5
Total	56.3 ± 7.7	56.2 ± 1.9	55.7 ± 6.2

Value are the mean ± S.D. for three rabbits, expressed as % of dose.

Table 2. Extraction of radioactivity from pooled urine (days 1 and 2) from rabbits receiving [4-^{14}C] norethisterone oxime

Hydrolysis procedure	NET-OX administered		
	Mixture	*anti*-isomer	*syn*-isomer
None	3.4 ± 1.8	13.4 ± 3.4	6.2 ± 2.2
Enzymic	65.1 ± 13.0	49.5 ± 6.8	73.4 ± 5.1
Solvolysis	20.1 ± 3.6	29.5 ± 1.6	16.9 ± 1.4

Values are the mean% ± S.D. of total urinary radioactivity for 3 rabbits in each group. As indicated in the text after administration of the *syn*-isomer, the *anti*-isomer is found in urine.

Table 3. Radioactivity in rabbit plasma after administration of *anti*- and *syn*-[4-^{14}C]-norethisterone oxime

	Isomer administered					
	anti-isomer			*syn*-isomer		
Time after administration (h)	% dose per 100 ml	% total radioactivity free	conjugated	% dose per 100 ml	% total radioactivity free	conjugated
2	3.2	65.6	34.3	3.8	52.5	47.3
4	2.1	57.1	42.8	2.4	58.3	41.6
7	1.0	50.0	45.0	1.6	75.0	25.0
24	0.5	40.0	60.0	0.8	62.5	37.6

Values are means for dupicate determinations from single samples.

Plasma levels of radioactivity

Plasma levels of radioactivity measured 2, 4, 7 and 24 h after administration of the *syn*-or *anti*-isomers to rabbits are shown in Table 3. There was no significant difference between the values for the two isomers at any time studied. The amount of radioactivity present in plasma decreased rapidly with time; the half-life from 2 to 7 h being approximately 3¼ h for both isomers and that from 7 to 24 h being about 17 h. However, these values may be misleading since, as shown in Table 3, the amounts of radioactivity present in freely extractable and conjugated forms differ between the two isomers. Whereas for the *anti*-isomer the amount freely extractable decreased with time after administration with a concommitant increase in the conjugated form, for the *syn*-isomer there was no such relationship. The amount present in the freely extractable form tended to increase slightly with time after administration and that present in a conjugated form decreased slightly. For the freely extractable radioactivity in plasma after administration of the *anti*-isomer the half-life was 2½ h from 2 to 7 h and 12½ h from 7 to 24 h.

Examination of urinary metabolites

The nature of the metabolites present in the freely extractable, enzyme hydrolysed and solvolysable fractions of urine from animals receiving the three different preparations of norethisterone oxime were determined by thin-layer and paper chromatography. For the freely extractable fraction the pattern was similar for the mixture, *anti*- and *syn*-isomers. On paper chromatography two main areas of radioactivity were seen. The major area, representing 81.5 ± 3.0% of detectable radioactivity for the mixture, 79.3 ± 4.8% for the *anti*-isomer and 81.5 ± 1.9% for the *syn*-isomer, had a chromatographic mobility similar to norethisterone oxime. The major area also had a similar retention time to norethisterone oxime on gas–liquid chromatography. On hydrolysis with 1 N HCL at 37°C it gave rise to a product which on both thin-layer and gas–liquid chromatography behaved like norethisterone. The second area of radioactivity did not move from the origin and represented 18.4 ± 1.5% of detectable radioactivity for the mixture, 20.5 ± 5.7% for the *anti*-isomer and 12.2 ± 3.6% for the *syn*-isomer.

The pattern on paper chromatography of metabolites present in the enzyme hydrolysed fraction of urine was also similar for the three forms of norethisterone oxime. One area, which accounted for 36.9 ± 8.5% of this fraction for the mixture, 54 ± 1.9% of the *anti*-isomer and 57.4 ± 6.9% for the *syn*-isomer, had a chromatographic mobility similar to norethisterone oxime. Two other more polar areas of radioactivity were present. The major area of radioactivity was eluted and subjected to thin-layer chromatography to separate the two isomers of norethisterone oxime. Irrespective of which isomer had been administered to the rabbit, the *anti*-form was the only one detected on the thin-layer chromatograms. The absence of the *syn*-isomer was also confirmed by gas–liquid chromatography. Thus the *syn* portion in the mixture and the pure *syn*-isomer were converted *in vivo* into the *anti*-form.

In the solvolysable fraction, three areas of radioactivity were present. One corresponded to norethisterone oxime and after administration of the mixture accounted for 38.2% of the sample. Of the two other areas one was less polar than norethisterone oxime and accounted for 29.3% and the second was more polar than the oxime and accounted for 34.8%.

Paper chromatographic examination of an extract of faeces from rabbits receiving norethisterone oxime showed that the major area of radioactivity, accounting for 65.6% of the sample, corresponded in chromatographic mobility to norethisterone oxime. Two other more polar metabolites were also present.

DISCUSSION

The finding that norethisterone-17-acetate-3-oxime was a more potent antifertility agent in the rat than norethisterone acetate [1] itself was confirmed by later studies [12] indicating that addition of the methoxime groups to norethisterone increased the antifertility potency of this steroid in the female rat about 10-fold. Clinical studies suggested that norethisterone acetate oxime was well tolerated in humans [13]. At the time the present investigation was started there were no reports regarding the metabolism *in vivo* of the steroid oximes.

After administration of norethisterone oxime the amount of the dose found in urine, slightly more than 50%, agrees with the values obtained in the rabbit using other related 19-norsteroids. Thus Arai *et al.*[14] recovered 31–62% of norethynodrel, Kamyab *et al.*[3] recovered about 50% of norethisterone and norgestrel and Yamamoto[15] recovered about 35% of lynestrenol. All of the progestogens appeared to be rapidly excreted, the major proportion of the dose being excreted on day 1. The amounts of the oxime found in the enzyme hydrolysed fraction (49–73%) are higher than those found with other gestagens (21% for norgestral, 31% for norethisterone, 37% for norethynodrel and 43% for lynestrenol). However the higher recovery of metabolites in the enzyme hydrolysed fraction may be due to the different methodology used in this investigation compared with previous ones. The purification of the conjugated fraction of urine by ion-exchange chromatography is known to remove a number of inhibitors of the enzyme β-glucuronidase [16]. As with the other gestagens the faecal route of excretion of metabolites was relatively unimportant.

The levels of total radioactivity in plasma during the 24 h after administration of norethisterone oxime were similar to those observed with norethisterone itself. However there were differences between the two isomers of norethisterone oxime. Whereas the amount of *anti*-isomer present in the freely extractable state decreased with time after administration and that present in the conjugated form increased, there was no such clear relationship with the *syn*-isomer. The rate of decrease of freely extractable radioactivity in plasma after administration of the *syn*-isomer differed markedly from the *anti*-isomer. Whereas for the latter it was possible to calculate the half-life, no such calculation was possible from the available data for the *syn*-isomer. One possible explanation could be that metabolism of the *syn*-isomer was slow and that conversion to the *anti*-isomer was necessary before it was metabolised. Although the *anti*-isomer without any of the *syn*-isomer was present in urine after administration of the *syn*-oxime showing that complete conversion had occurred, the nature of the isomer present in blood after administration of the *syn*-oxime was not determined. The relative biological activities of the two isomers appears not to have been determined.

The resistance of norethisterone oxime to metabolism and even to hydrolysis was shown by the predominance of the unchanged administered oxime in both urine and faeces. No radioactivity was detected in the norethisterone area of chromatograms. Although a small amount was detected in the area corresponding to tetrahydronorethisterone, this area was not positively identified. Steroid oximes are readily hydrolysed under acidic conditions [2, 17] even at 37°C and hydrolysis may therefore occur readily in the stomach after oral administration. In the present investigations the oximes were administered intraperitoneally. In a recent study in Rhesus monkeys [18] a large proportion of norgestrel-17-acetate-3-oxime administered intragastrically was converted to noregestrel.

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REFERENCES

1. Shroff A. P., Harper C. H., Allen G. O. and Blye R. P.: Synthesis and antifertility activity of some oximinoandrostens. *J. med. Chem.* **16** (1973) 113–115.
2. Huettemann R. E. and Shroff A. P.: Hydrolysis of steroid oximes: mechanism and products. *J. Pharm. Sci.* **63** (1974) 74–77.
3. Kamyab S., Littleton P. and Fotherby K.: Metabolism and tissue distribution of norethisterone and norgestrel in rabbits. *J. Endocr.* **39** (1967) 423–435.
4. Reed M. J., Fotherby K. and Steele S. J.: Metabolism of ethynyloestradiol in man. *J. Endocr.* **55** (1972) 351–361.
5. Bradlow L. H.: Extraction of steroid conjugates with a neutral resin. *Steroids* **11** (1968) 265–272.
6. Fotherby K. and Love D. N.: A modified method for the estimation of pregnanetriol in urine. *J. Endocr.* **20** (1960) 157–162.
7. Edwards R. W. H., Kellie A. E. and Wade A. P.: The extraction and oxidation of urinary steroid conjugates. *Mem. Soc. Endocrinol.* No. **2** (1953) 53–69.
8. McKenna J. and Norymberski J. K.: The extraction and measurement of urinary 17-oxosteroid hydrogen sulphates. *Biochem. J.* **76** (1960) 60p.
9. Zaffaroni A.: Micromethods for the analysis of adrenocortical steroids. *Recent Prog. Horm. Res.* **8**, (1953) 51–86.
10. Berg A. and Gustafsson J-Å.: Regulation of hydroxylation of 5α-androstane-3α,17β-diol in liver microsomes from male and female rats. *J. biol. Chem.* **248** (1973) 6559–6567.
11. Chambaz E. M. and Horning E. C.: Conversion of steroids to trimethylsilyl derivatives for gas phase analytical studies. *Analyt. Biochem.* **30** (1969) 7–12.
12. Kärkkäinen J., Ohisalo J. J. and Luukkainen T.: Steroid methoxime antifertility activity of NET-3-methoxime in the rat. *Contraception* **12** (1975) 505–509.
13. McQuarrie H. G., Harris J. W., Pasquale S. and Santella P. J.: *OBGYN Digest* **21** (1972) 1.
14. Arai K., Golab T., Layne D. S. and Pincus G.: Metabolic fate of orally administered [H^3]-norethynodrel in rabbits. *Endocrinology* **71** (1962) 639–648.
15. Yamamoto H.: Metabolism of 17α-ethynyl-estrenol. *Folia Endocr. Jap.* **44** 1968) 1309–1319.
16. Abul-Fadl M. A. M.: Inhibition and activation of β-glucuronidase in urine. *Biochem. J.* **65** (1957) 16p–17p.
17. Buhler D. R., Schroeter L. C. and Wechter W. J.: The modification of 11β-hydroxy-11α-methyl-5β-pregnane-2,30-dione dioxime under acidic conditions. *J. med. Chem.* **8** (1965) 215–219.
18. Sisenwine S. F., Liu A. L., Kimmel H. B. and Ruelius H. W.: The conversion of D-norgestrel-3-oxime-17-acetate to D-norgestrel. *Contraception* **15** (1977) 25–37.