Pergamon

0960-0760(94)E0021-C

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 2/3, pp. 227–231, 1994
Elsevier Science Ltd. Printed in Great Britain

Aromatization and Hydrolysis of Norethisterone-3-oxime in Rabbit

Qi-gui Li

Department of Pharmacology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100, U.S.A.

Two pharmacologically active metabolites, norethisterone (NET) and ethinylestradiol (EE_2), were detected by HPLC and HPLC-RIA methods in rabbit plasma following single i.v. and i.g. administration at a dose of 1 mg/20 μCi/kg of [^{3}H]norethisterone-3-oxime (NETO). Approximately 48% (i.v. injection) and 91% (i.g. administration) of the NETO dose were hydrolyzed to NET. Although only 0.35% of the NETO dose was aromatized to EE_2, due to its high estrogenic potency, EE_2 might contribute to the overall pharmacological pattern of NETO in the rabbit.

J. Steroid Biochem. Molec. Biol., Vol. 49, No. 2/3, pp. 227–231, 1994

INTRODUCTION

Norethisterone-3-oxime (NETO), a new derivative of norethisterone (NET), was synthesized and shown to be very effective in preventing implantation of ova in rats and rabbits [1–3]. McQuarrie *et al.* [4] confirmed that NETO is metabolized to NET *in vivo*. Thus NETO appears to be a prodrug of NET. Hüttemann and Shroff [5] and Mitra *et al.* [6] showed that the hydrolysis of the steroid oxime including NETO and NETO acetate occurred in acidic solution *in vitro*. Khan and Fotherby [7] demonstrated that the major compound in urine extracts was unchanged NETO, when NETO was administered to rabbits. These results seem to indicate that the hydrolysis of NETO proceeds slowly *in vivo*. In addition, Li and Gao [8, 9] found that like NET, NETO possessed a high estrogenic efficacy in rats and rabbits. One explanation for the estrogenic effects of NET would mean that NET itself can bind to the estrogen receptor and thereby acts as an estrogen. However, binding of NET to the estrogen receptor is rather low [10, 11] and cannot explain the pharmacological effects. These effects can be characterized by a high estrogenic efficacy in the rat and a progestogenic efficacy in the rabbit, which is different from progesterone [12, 13]. In man there is some clinical evidence for an estrogenic activity of NET [14, 15]. In patients deficient of androgen and progesterone receptors NET was able to lower LH levels, which is interpreted as interfering with the estrogen receptor (positive feedback mechanism) mediated by the putative metabolite ethinylestradiol (EE_2) [16, 17].

Whether EE_2 can arise from NET in man has been controversial for about 30 years now. Breuer *et al.* [18] reported EE_2 in human urine after administration of NET. It was estimated that about 3–10% of the NET dose could be converted into phenolic metabolites [19, 20]. Results were confirmed by others [21, 22]. In 1970, however, Breuer [23] acknowledged that his earlier results could be artificial as indicated by Townsley and Brodie [24, 25]. Strong alkaline or acidic treatment of urine containing 1-β-hydroxylated metabolites will aromatize the A-ring [26]. Avoiding extreme pH-conditions, low EE_2 levels were demonstrated in human urine [27]. Results from *in vitro* tests were controversial. No aromatization was found for NET with human placenta homogenate [28], but this was detected using placental microsomes [29–31]. Some studies in rat [32] and men [33] *in vivo* were in agreement with the hypothesis of EE_2 as an *in vivo* metabolite. Recently, EE_2 was detected in human hepatocytes and other human tissues after incubation with NET [34–36]. Our data also confirmed that NETO was biotransformated to EE_2 *in vivo* in monkeys [37].

The aim of the present study was to evaluate the pharmacokinetic parameters of NETO in New Zealand white rabbits and to specifically look at two steps in biotransformation, i.e. the conversion into NET and the generation of EE_2.

Received 21 Sept. 1993; accepted 31 Jan. 1994.

EXPERIMENTAL

Study design

Three 3 female New Zealand white rabbits of body wt 3.8–4.6 kg were used. The animals received an intravenous (i.v.) and intragastric (i.g.) treatment with 1 mg/20 μCi/kg of [^{3}H]NETO separated by a wash-out period of 3 weeks. For i.v. injection the drug was dissolved in 30% (v/v) propyleneglycol in water at a concentration of 1 mg/ml. For i.g. administration (Nasogastric tube, 12FR) a microcrystalline suspension in physiological saline at a concentration of 1 mg/ml was available. Prior to dosing and 10 (i.v.), 15 (i.g.), 20 (i.v.), 30, 60 min, 1.5, 3, 6, 8, 24 and 48 h thereafter 3–4 ml blood was drawn from ear veins and plasma prepared.

Assay of NETO and NET

Plasma sample (0.15–0.5 ml) was prepared by deproteinization and liquid–liquid extraction and subjected to HPLC assay [37]. The HPLC equipment consisted of two pumps and an automated gradient controller (Waters Assoc., U.S.A.). Injection was carried out by means of Rheodyne U6K (200 μl loop). The system was connected to a spectroflow 783 (Applied Biosystems, U.S.A.) which was set to a wavelength of 254 nm. A Spherisorb ODS-2, 5 μm particle size (250 × 4.6 mm) column was used. Elution was performed by a linear gradient from A to B within 30 min with A = 30% (v/v) methanol in 0.001 M phosphate saline buffer (PSB), pH 7, and B = 90% (v/v) methanol in PSB at a flow of 1.5 ml/min. Eluent was fractionated by means of an ISCO fraction collector (5 fractions/min). Alternatively NETO and NET were sampled according to their retention times. [^{3}H]NETO and [^{3}H]NET served as reference compounds for determination of NETO and NET. Radioactivity of eluted samples was counted off line without quench control and dpm transformation after addition of 4 ml of Atomlight per fraction.

HPLC-RIA assay of EE_2

Plasma sample (0.5 ml) was prepared by deproteinization and liquid–liquid extraction and subjected to HPLC-RIA assay [37]. The HPLC equipment of Knauer (Germany) consisted of two model FR-30 pumps, an automated gradient controller and a Rheodyne model U6K injector fitted with a 200 μl loop. The use of the UV detector was the same as the assay of NETO and NET. The column used was a Spherisorb ODS-2, 3 μm particle size (125 × 4.6 mm). Mobile phases for linear gradient elution from A to B within 30 min consisted of A = 45% (v/v) methanol–acetonitrile (9:1, v/v) in 0.001 M PSB, pH 7, and B = 65% (v/v) methanol–acetonitrile (9:1, v/v) in PSB. The flow rate was 1.5 ml/min. About 0.8–1 ml of eluent (starting at 18.8 min) was collected by the ISCO fraction collector (Fig. 1). At this retention time EE_2 was eluted. The eluent collected was evaporated to dryness under N_2 and the residue used for EE_2 radioimmunoassay [37].

RESULTS AND DISCUSSION

Pharmacokinetic data for unchanged NETO and the two metabolites (NET, EE_2) are given in Table 1. From plasma level determinations a mean clearance rate of 27.2 ml/min/kg was calculated for NETO which can be regarded as metabolic clearance because only trace amounts of unchanged NETO are excreted [38]. Normal plasma liver flow (PLF) in rabbit is about 26 ml/min/kg and therefore the metabolic clearance rate of 27.2 ml/min/kg is close (104%) to PLF. The comparison of total radiolabel plasma levels and NETO levels revealed that only about 3% of the total radiolabel can be assigned to NETO after i.v. and i.g. administration, suggesting that an intensive metabolism of NETO occurs in the rabbit.

NETO does not act as a progestin itself but is a prodrug for NET [37]. NET is a major metabolite of i.v. and i.g. administered NETO in the rabbit. A rough estimate of the NET dose coming from 1 mg NETO/kg in the rabbit can be made on the basis of published data which showed that an i.v. dose of 85 μg NET/kg generated an AUC of 40.6 ± 2.5 ng·h·ml^{-1} in rabbit [39]. This would be equivalent to a NET dose of 0.48 mg/kg for i.v. and 0.91 mg/kg for i.g. administration in the present study. Thus, approx. 48 and 91% of the i.v. and i.g. NETO dose were hydrolyzed to NET, respectively. In addition, the $t_{1/2}$ of elimination and mean residence time (MRT) were 2- or 3-times longer for NET than those for NETO (Table 1). Therefore, NETO seems to be a prodrug of NET in rabbits, at least after oral administration. On average NET AUC after i.g. treatment was 2-fold higher than the AUC after i.v. treatment (Fig. 2). It is suggested that gut wall metabolism and/or acid hydrolysis of NETO [5, 6] in the stomach might account for the differences observed.

In a previous study [30, 37] it was demonstrated that NET can be converted into EE_2 by A-ring aromatization. In the present study, EE_2 levels were also determined using the same HPLC-RIA method as described previously [37]. The sensitivity of radioimmunoassay was 15 pg/ml for EE_2 [40], and a high accuracy ($92 \pm 8\%$), precision ($5.6 \pm 3.8\%$) and low bias (-3.2 ± 1.1) were shown for this method. The cross-reactions of EE_2 with endogenous estrogens and other steroids were not present to any significant degree [40, 41]. Specifically measured EE_2 plasma levels were in the range of 80–1260 pg/ml (26 ± 9 pg/ml for blank value). EE_2 was detected after both routes of administration. EE_2 levels were much lower than those of NETO or NET and therefore EE_2 can be regarded as a minor metabolite. Interestingly, the total availability of EE_2 (measured as AUC_{0-6h}) was higher after i.v. as

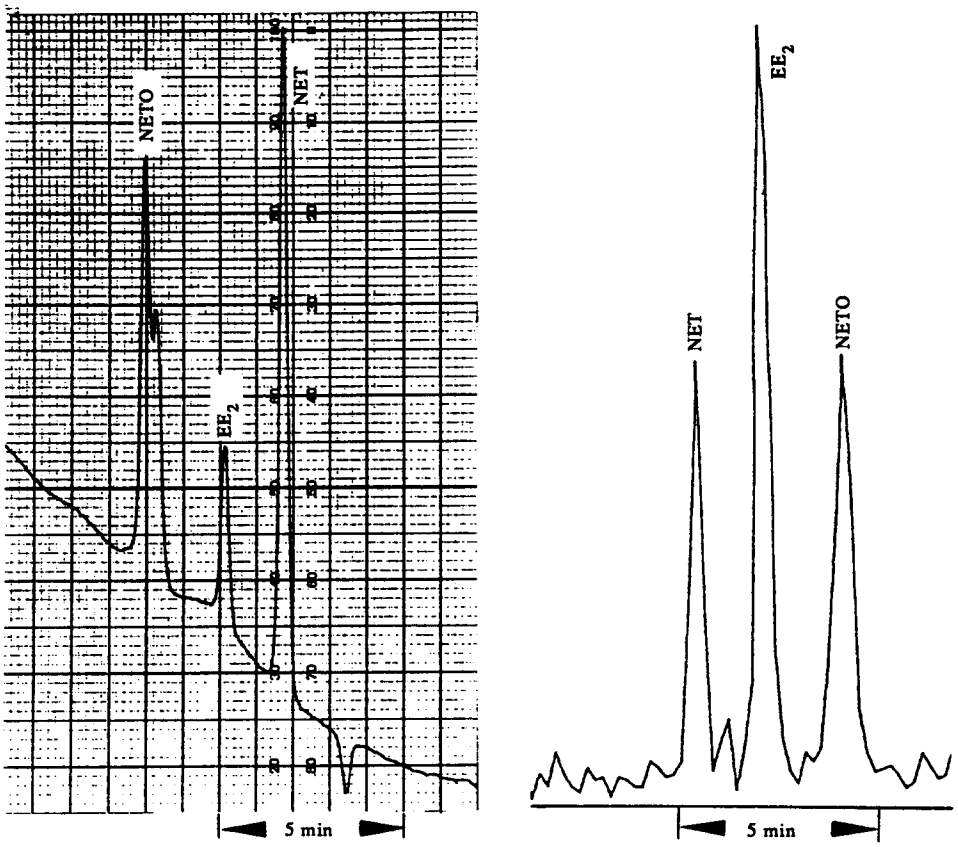

Fig. 1. Separation of EE_2 from NET and NETO by HPLC, UV-chromatogram of standard compounds (left) and radiochromatogram of standard tracers (right). For chromatographic detects see text.

compared to i.g. treatment (Table 1), which might be explained by the presystemic metabolic degradation of NETO following this route of administration.

Concerning the total EE_2 dose generated from 1 mg NETO/kg a similar estimate can be made for NET. Previously published data [42] showed that the i.v. injection of 0.1 mg EE_2/kg to New Zealand white rabbits produced an AUC of 46.3 $ng \cdot h \cdot ml^{-1}$. Thus 1 mg NETO/kg will develop an EE_2 dose of approx. 3.3 μg/kg after i.v. and 2.2 μg/kg after i.g. treatments, which is <0.35% of the NETO dose. The demonstration of EE_2 as a NETO metabolite could serve as an explanation for the estrogenic activity of NET and NETO in the rabbit [8, 9].

Table 1. Pharmacokinetic parameters of NETO, NET (HPLC determinations) and EE_2 (HPLC-RIA determination) in female rabbits after a 1.0 mg/kg dose of [^{3}H]NETO by i.v. and i.g. administration

	i.v. (mean ± SD)			i.g. (mean ± SD)		
Parameters	NETO	NET	EE_2	NETO	NET	EE_2
C_{max} (ng/ml)	333 ± 37	109 ± 10	1.05 ± 0.28	39.0 ± 25.5	44.0 ± 32.9	0.39 ± 0.23
T_{max} (min)	10	10	13.3 ± 5.8	78.0 ± 18.0	110 ± 62	130 ± 87
$AUC_{0-6\,h}$ (ng·h/ml)	—	—	1.52 ± 0.61	—	—	1.00 ± 87
$AUC_{0-8\,h}$ (ng·h/ml)	473 ± 102	188 ± 5	—	199 ± 208	186 ± 165	—
$AUC_{inf.}$ (ng·h/ml)	595 ± 116	386 ± 60	2.96 ± 1.62	452 ± 480	737 ± 586	3.11 ± 0.86
CL (ml/min/kg)	27.2 ± 5.0	52.5 ± 8.6	37.5 ± 9.9	31.9 ± 8.8	48.5 ± 4.2	36.4 ± 9.6
$t_{1/2}$ α-phase (h)	0.19 ± 0.14	0.50 ± 0.17	0.46 ± 0.05	1.16 ± 0.85	1.24 ± 0.82	0.76 ± 0.44
$t_{1/2}$ β-phase (h)	4.18 ± 1.31	13.3 ± 8.7	3.31 ± 0.5	12.2 ± 2.75	26.0 ± 6.32	3.75 ± 0.43
MAT (h)	—	—	—	0.69 ± 0.43	1.15 ± 0.53	5.41 ± 0.62
MRT (h)	4.6 ± 1.1	14.2 ± 9.3	3.58 ± 0.96	14.8 ± 3.0	34.4 ± 9.2	7.79 ± 2.21
Bioavailability (%)		—	—	77.9 ± 80.1	—	—
AUC_{NETO}/AUC_{NET} (0–8 h)	2.52 ± 0.58			0.98 ± 0.20		

—, No measurement or calculation, NETO = norethisterone-3-oxime, NET = norethisterone, EE_2 = ethinylestradiol, MAT = mean arrival time, MRT = mean residence time.

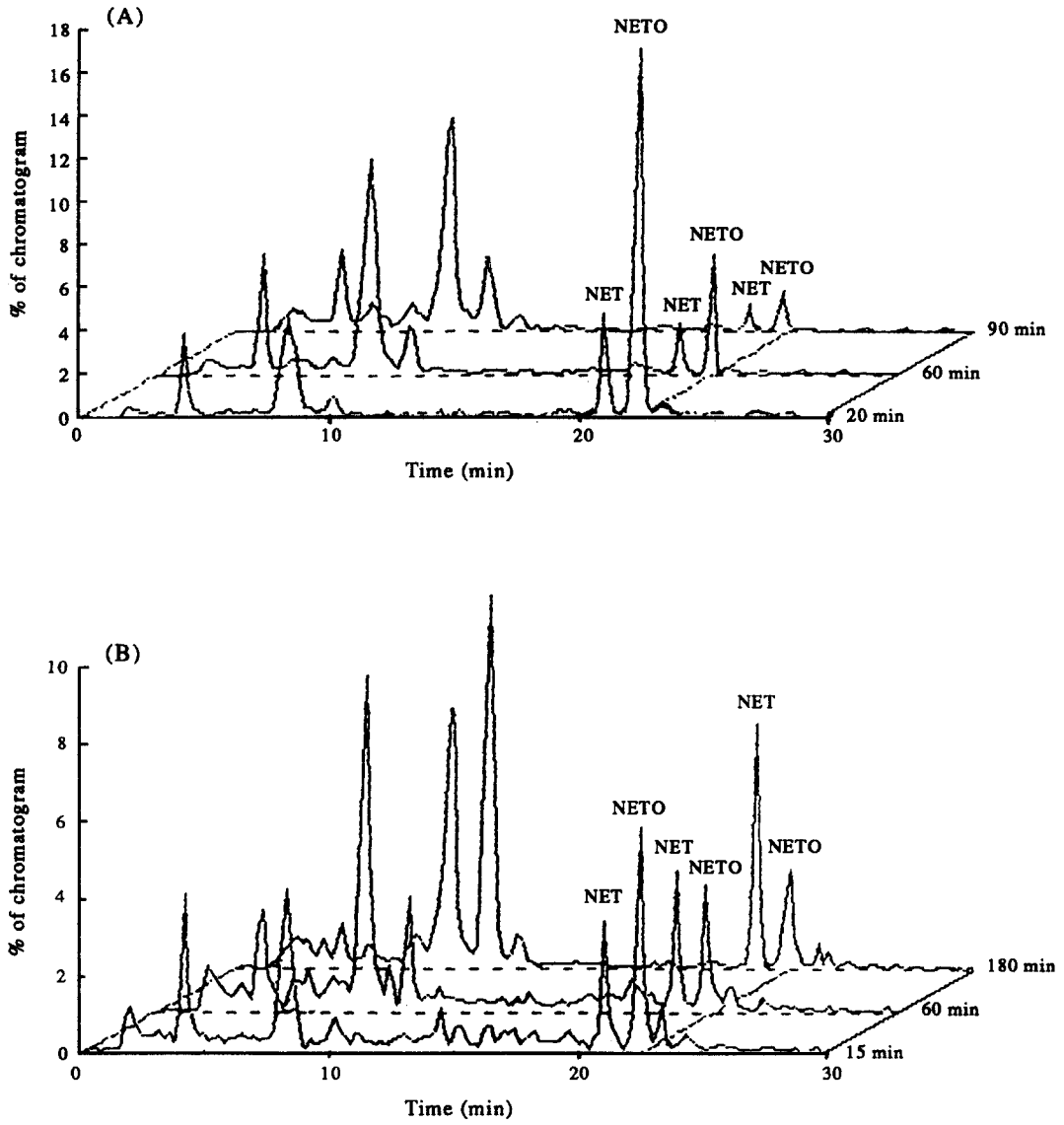

Fig. 2. Patterns of plasma metabolites after intravenous injection (A) and intragastric administration (B) of 1 mg NETO/kg in the female rabbit. Sampling times are given at the right and running time for HPLC was 30 min. NETO = norethisterone-3-oxime, NET = norethisterone.

Acknowledgement—This study was conducted while the author was in receipt of a WHO Research Training Grant at Schering AG.

REFERENCES

1. Fotherby K.: Pharmacokinetics of contraceptive steroids in humans. *Proc. 5th Int. Congr. Pharmac.* 1 (1973) 220–239.
2. Shroff A. P., Harper C. H., Allen G. O. and Blye R. P.: Synthesis and antifertility activity of some oximinoandrostenes. *J. Med. Chem.* 16 (1973) 113–115.
3. Li D.-Z. and Li Q.-G.: Study of the mechanism of antiimplantation action of norethisterone-3-oxime. *J. Tongji Uni.* 6 (1986) 76–80.
4. McQuarrie H. G., Harris J. W., Pasquale S. and Santella P. J.: Study of 17α-ethinyl-17β-acetoxy-19-norandrost-4-en-3-one oxime in women. *Obstet. Gynec. Digest.* 21 (1972) 1–5.
5. Hüttemann R. E. and Shroff A.: Hydrolysis of steroid oximes; Mechanism and products. *J. Pharmac. Sci.* 63 (1974) 74–77.
6. Mitra S., Van Dress M., Anderson J. M., Petersen R. V., Gregonis D. and Feijen J.: Pro-drug controlled release from polyglutamic acid. *Polym. Prepr. Am. Chem.* 20 (1979) 32–35.
7. Khan F. S. and Fotherby K.: *In vivo* metabolism of norethisterone-3-oxime in rabbits. *J. Steroid Biochem.* 9 (1978) 229–232.
8. Li Q.-G. and Li D.-Z.: Analysis of anti-implantational action of norethisterone-3-oxime. *Contraception* 36 (1987) 667–676.
9. Gao X.-L. and Li D.-Z.: Antiprogestational action of norethisterone-3-oxime in rabbits. *Zhonggou Yaoli Yuebao.* 7 (1986) 260–264.
10. Terenius L.: Affinities of progesterone and estrogen receptors in rabbit uterus for synthesis progestogens. *Steroids* 23 (1974) 909–919.
11. Edgren R. A.: Relative protencies of oral contraceptive. In *Controversies in Contraception* (Edited by K. S. Moghissi). Williams and Wilkins Co., Baltimore (1979) pp. 1–27.
12. Besch P. L., Watson D. I., Vorys N. and Barry R. D.: Aromatization *in vivo* of 3β, 17β-bisacetoxy-17α-ethinyl-19-norandrost-4-ens (SC-11800). *Biochim. Biophys. Acta* 69 (1963) 417–418.
13. Pausen C. A., Leach R. B., Lanman J. L., Goldston N., Maddock W. O. and Heller C. G.: Inherent estrogenicity of norethindrone and norethynodrel: comparison with other synthetic progestins and progesterone. *J. Clin. Endocr. Metab.* 22 (1962) 1033–1039.

14. Orme M. L'E., Back D. J. and Breckenridge A. M.: Clinical pharmacokinetics of contraceptive steroids. *Clin. Pharmac.* **8** (1983) 95–139.
15. Ranney R. E.: Comparative metabolism of 17α-ethinyl steroids used in oral contraceptives. *J. Toxic Environ. Hlth* **3** (1977) 139–166.
16. Jones R. C. and Edgren R. A.: The effects of various steroids on the vaginal histology in the rat. *Fert. Steril.* **24** (1973) 284–291.
17. Larrea F., Moctezuma O. and Pèrez-Palacios G.: Estrogen-like effects of norethisterone on the hypothalamic pituitary uint of ovariectomized rats. *J. Steroid Biochem.* **20** (1984) 841–847.
18. Breuer H., Dardenne U. and Nocke W.: Ausscheidung von 17-ketosteroiden, 17-ketogenen Steroiden und Östrogenen beim Menschen nach Gabe von 17α-ethinyl-19-nor-testosteron-estem. *Acta Endocr. (Kbh.)* **33** (1960) 10–26.
19. Fotherby K. and James F.: Metabolism of synthetic steroids. In *Advances in Steroid Biochemistry and Pharmacology* (Edited by M. H. Brigs and G. A. Christa). Academic Press, London, Vol. 3 (1972) p. 67.
20. Sisenwine S. F., Liu A. L., Kimmel H. B. and Ruelius H. W.: Phenolic metabolites of dl-norgestrel: a method for the removal of 1-hydroxylated metabolites, potential sources of phenolic artifacts. *Acta Endocr.* **76** (1974) 789–800.
21. Ryan K. J.: Biological; aromatization of steroids. *J. Biol. Chem.* **234** (1959) 268–272.
22. Mills T. M., Lin T. J., Hemandez-ayup S., Greenblatt R. B., Ellegood J. O. and Mahesh V. B.: The metabolic clearance rate andurinary excretion of oral contraceptive drugs. *Am. J. Obstet. Gynec.* **120** (1974) 764–772.
23. Breuer H.: Metabolism of progestagens. *Lancet* **2** (1970) 615–616.
24. Townsley J. D. and Brodie H. J.: Mechanism of estrogenbiosynthesis III. The stereochemistry of aromatization of C19 and C18 steroids. *Biochemistry* **7** (1968) 33–40.
25. Townsley J. D. and Brodie H. J.: Studies on the mechanism of estrogen biosynthesis. IV. Ovarian metabolism of estr-4-ene-3,17-dione. *Biochim. Biophys. Acta* **144** (1967) 440–445.
26. Fotherby K.: Metabolism of 19-norsteroids to oestrogenic steroids. *Excerpt Med. Int. Congr. Ser. [Int. Norgestrel Symp., Proc., 2nd]* **344** (1974) 30–34.
27. Townsley J. D. and Brodie H. J.: Low conversion of 19-nortestosterone to urinary oestrogens. *Lancet* **2** (1970) 1039.
28. Starka L., Breuer H. and Cedard L.: Biosynthesis of equilin and related ring B unsaturated oestrogens in perfused human placenta. *J. Endocr.* **34** (1966) 447–456.
29. Hobe G., Dänhardt S. and Busch W.: Umwandlung von 19-Norgestagenen in östrogene durch Mikroorganismen des Rinderpansens. *Archs Exp. Veterinaermed.* **33** (1979) 495–498.
30. Barbieri K. L., Petro Z., Canick J. A. and Ryan K. J.: Aromatization of norethisterone to ethinyl estradiol by human placental microsomes. *J. Clin. Endocr. Metab.* **57** (1983) 299–303.
31. Okada H., Honlo H., Ohono Y. and Yasuda J.: Receptors of synthetic steroids. *Int. Congr. Ser. Exerperta Med. (Horm. Recept. Dis.)* **603** (1983) 145–152.
32. McPherson J. C., Costoff A., Eldrigde J. C. and Mahesh V. B.: Effects of various progestational preparation on gonadotropin secretion in ovariectomized immature female rats. *Fert. Steril.* **25** (1974) 1063–1070.
33. Braselton W. E., Liu T. J., Mills T. M., Ellegood J. O. and Mahesh V. B.: Identification and measurement by gas chromatography-mass spectrometry of norethindrone and metabolites in human urine and blood. *J. Steroid Biochem.* **8** (1977) 9–18.
34. Yamamoto T., Yoshiji S., Yasuda J., Shiroshita K., Kitawaki J., Fujii M., Urabe M., Honjo H. and Okada H.: Rapid communication, aromatization of norethisterone to ethinylestradiol in human adult liver. *Endocr. Jap.* **33** (1986) 527–531.
35. Yoshiji S.: Aromatization of C-19 norsteroid in human placenta, liver, ovary and adipose tissues. *Kyoto-furitsu Ika Daigaku Zasshi* **96** (1987) 969–980.
36. Yamamoto T., Kitawaki J., Shiroshita K., Urabe M., Sasaki J., Fukuoka M., Naitoh K., Honjo H. and Okada H.: The confirmation of norethisterone aromatization in primary human hepatocytes by the vitafiber-II cell culture system. *Acta Obstet. Gynec. Jap.* **40** (1988) 87–89.
37. Li Q.-G. and Hümpel M.: Pharmacokinetics, hydrolysis and aromatisation of norethisterone-3-oxime in female cynomolgus monkey. *Eur. J. Drug Metab. Pharmacokinet.* **17** (1992) 281–291.
38. Li Q.-G.: Study on norethisterone-3-oxime. In *Investigations on the Metabolism of Five Progestogens of the 19-Nortestosterone Type in Various Laboratory Animal Species* (Edited by Q.-G. Li). Papyrus-Druck GmbH Berlin (1989) pp. 142–182.
39. Back D. J., Breckenridge A. M., Crawford F. E., Orme M. L'E. and Rowe P. H.: Phenobarbitone interaction with oral contraceptive steroids in the rabbit and rat. *Br. J. Pharmac.* **69** (1980) 441–452.
40. Li Q.-G. and Nieuwboer B.: Antiserum development and characteristics of levonorgestrel and ethinylestradiol in rabbits. *J. Tongji Med. Uni.* **9** (1989) 65–73.
41. Jung-Hoffmann C. and Kuhl H.: Interaction with the pharmacokinetics of ethinylestradiol and progestogens contained in oral contraceptives. *Contraception* **40** (1989) 299–312.
42. Düsterberg B., Kühne G. and Täuber U.: Half-lives in plasma and bioavailability of ethinylestradiol in laboratory animals. *Arzneim.-Forsch./Drug Res.* **36** (1986) 1187–1190.