J. steroid Biochem. Vol. 22, No. 5, pp. 623–629, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

PHARMACOKINETICS OF 19-NORTESTOSTERONE ESTERS IN NORMAL MEN

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(*Received* 6 *September* 1984)

Summary—A reliable method for the isolation of 19-nortestosterone (NT), testosterone (T) and dihydrotestosterone (DHT) by high-performance liquid chromatography (HPLC) and quantitation of the individual steroids by radioimmunoassays is described. The method was used to measure serum concentrations of NT, T and DHT in a pharmacokinetic study and in a clinical trial for male fertility control. Following intramuscular injection of either 50 mg 19-nortestosterone-3-(*p*-hexoxyphenyl)-propionate (NP) or 50 mg 19-nortestosterone-decanoate (ND) serum NT increased rapidly to maximal concentrations of 4.6 ± 3.2 and 2.0 ± 1.3 nmol/l ($\pm$SD), respectively, in the 6 volunteers. The half-life time was 8 days for ND and 21 days for NP. Based on these findings a clinical trial with NP was performed. NP was given to 5 healthy men in doses of 100 mg/week for the first 3 weeks followed by 200 mg/week for 10 further weeks. Serum NT levels increased gradually and maximal concentrations were reached in the 13th treatment week (20.2 ± 3.4 nmol/l). Measurable amounts of NT were detectable for 19 weeks after the last injection. The study shows that NT accumulates under this treatment regime and wider spacing of the injection intervals may be possible in future trials.

INTRODUCTION

The anabolic steroid 19-nortestosterone (NT) has the potential to serve as a means for male fertility control since it suppresses spermatogenesis, replaces testosterone action and has not shown serious toxic side-effects in over 20 years of clinical use [1]. The design of appropriate clinical trials requires the measurement of NT in serum. Radioimmunoassays [2–6] and a chemiluminescent assay [7] as well as methods based on high-performance liquid chromatography [8] and gas chromatography–mass spectrometry [9] have been developed to detect the illegal use of NT in athletes, racehorses and cattle. HPLC methods have been evaluated for separation of adrenal steroids in serum [10] and urine [11, 12] using gradient systems, as well as for androgens in serum [13, 14]. We have developed a method for the separation of NT, T and DHT by HPLC which allows the radioimmunological quantitation of the 3 steroids separately, using an isocratic system. The method was used to measure serum steroid concentrations of the three androgens in a pharmacokinetic study with a single injection of two NT-esters and in a clinical trial for male fertility control with repeated high-dose injections. While the clinical results of the latter study have been published elsewhere [1], the serum NT levels are presented here.

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EXPERIMENTAL

Cross-over study

Six healthy men (26–30 years old) participated in the pharmacokinetic study. They gave written informed consent to participate in the study after its aims and risks had been explained in detail. The study was approved by the Ethics Committee of the University of Münster. The study was designed as an open cross-over trial. Two commercially available NT-esters were used: 19-nortestosterone-3-(*p*-hexoxyphenyl)-propionate (NP) (Pharma Leo, Düsseldorf, F.R.G.) and 19-nortestosterone-17-decanoate (ND) (Organon GmbH, Oberschleißheim, F.R.G.). Each preparation contained 50 mg of the ester in 1 ml oil. The amount of NT in the preparation was 27.1 mg for ND. Three of the six subjects chosen at random first received 50 mg ND in a single i.m. injection. Blood samples were taken 7 days before the study and immediately prior to the injection. Further blood samples were obtained 2, 4, 8 and 24 h after the injection and then on day 2, 3, 4, 6, 8, 10, 15, 20, 25 and 30. Forty-four days after the ND-injection 50 mg NP were injected intramuscularly. Blood samples were taken at the same intervals as before. The other 3 volunteers received NP first followed by ND and blood samples were taken as in the other volunteers.

Clinical trial

Five healthy men (21–25 years old) participated in the clinical trial. They received 100 mg NP per week

i.m. for the first 3 weeks and then 200 mg per week for 10 further weeks. Blood samples were obtained at weekly intervals during treatment and in week 1, 4, 8, 16, 18, 20 and 24 after treatment. For further details of the study see Schürmeyer *et al.*[1]. In the serum samples obtained from this study NT and T (but not DHT) were measured.

Solvents and reagents

The organic solvents diethylether (Hoechst, Frankfurt, F.R.G.) and methanol (Merck, Darmstadt, F.R.G.) were of analytical grade. *n*-Hexane and 2-propanol were of HPLC grade (LiChrosolv, Merck) and were degassed with helium before use. The assay buffer used was normal saline, containing 1 mg bovine immunoglobulin (Behringwerke AG, Marburg, F.R.G.), The charcoal solution used in the radioimmunoassays was 500 mg charcoal (Merck) in 100 ml dextran T 70 solution.

Non-radioactive steroids testosterone (17β-hydroxy-4-androsten-3-one, T) and dihydrotestosterone (17β-hydroxy-5-androstan-3-one, DHT) were purchased from Merck, 19-nortestosterone (17β-hydroxy-4-estren-3-one, NT) from Leo AB (Helsingborg, Sweden).

Standard solutions were prepared in methanol and stored at −20°C (stock solution 1 mg/ml). Working standards were prepared in assay buffer, starting at 1000 pg/tube in RIA standard curves.

[1,2-^{3}H]Dihydrotestosterone (sp. act. = 9.25 MBq) and [1,2-^{3}H]testosterone (sp. act. = 9.25 MBq) were purchased from New England Nuclear Corp. (Dreieich, F.R.G.). [1,2-^{3}H]19-nortestosterone (sp. act. = 33,03 MBq) was a generous gift from Organon (Oss, Netherlands). Labelled testosterone and dihydrotestosterone were purified by thin-layer chromatography, 19-nortestosterone by HPLC using a 125 mm Diol column (Bischof, Berlin, Germany), according to the method and system described below.

HPLC equipment

The HPLC equipment was purchased from Waters Associates (Königstein, F.R.G.), using a gradient system (M 27), a data module (M 730), 2 pumps (M 6000 and M 45), an automatic sample processor (Wisp 710-B), a fixed u.v. detector (M 441) and a variable u.v. detector (Pye-Unicam PU 4020). All operations could be time-programmed, including the collection of the effluent by a fraction collector (Ultrorack 2070, LKB, Turku, Finland).

The packing material used for the column was 5 μm Diol (Bischof), column dia 250 × 4.6 mm (i.d.), precolumn 18 × 4.6 mm (i.d.), 10 μm material.

Chromatography

Tracer amounts of labelled steroids (2000 cpm) each were added to 0.5 or 1 ml serum samples and after 30 min steroids were extracted in 5 ml diethylether. After evaporation the extracts were redissolved in 220 μl *n*-hexane and transferred into HPLC sample mini-vials from which 200 μl were injected. The following HPLC conditions were used: flow rate 1.4 ml/min, column pressure 620 psi, isocratic separation (pump A) of the 3 steroids with *n*-hexane–2-propanol (96:4; v/v), elution of the more polar serum compounds with pump B (*n*-hexane–2-propanol (75:25, v/v). Running time was 45 min. Retention time of the steroids was determined in a run with standards (Fig. 1) and this profile was checked routinely every week.

Radioimmunoassays

After chromatography the effluent steroid fractions from the serum samples were collected automatically using the pre-determined retention times. The radioimmunoassays were then performed with the evaporated fractions. The T and DHT radioimmunoassays were carried out as previously described [15, 16].

The NT fraction was redissolved in 1500 μl assay buffer. Duplicates of 500 μl were used in the assay, while 300 μl were taken for recovery measurement. The antiserum for the NT radioimmunoassay was raised in rabbits using 19-nortestosterone-3-(*O*-carboxymethyl)-oxime coupled to bovine serum albumin as antigen. The final dilution of the antibody was 1:50,000; the total assay volume was 1000 μl. Dextran coated charcoal was used to separate bound and free steroids. Calculation of radioimmunoassay data was performed by a logit-log transformation computer program. Data from the clinical trials were statistically analyzed using the Student's *t*-test.

In the cross-over study the elimination constant of NT (k) was determined by linear regression analysis between 6 and 30 days (in one subject k was determined between 10 and 30 days). In the clinical study k was determined by linear regression analysis starting at day 7 after the last injection. The area under the serum concentration-time curve, $AUC_{0\ldots}$, was calculated for each subject using the trapezoidal rule with the residual area estimated as the area under the regression line after the last observed serum concentration. Calculation of relative bioavailability of NT were based on $AUC_{0\rightarrow x}$-values of NT and the doses of NP and ND.

RESULTS

HPLC

The method described here provides a good separation of the steroids. The u.v. chromatogram (Fig. 1) shows the separation of NT, T, DHT and the 2 NT esters, ND and NP. Baseline separation was achieved even between T and NT.

The fraction between the steroids was collected and measured for radioactivity (NT and T). For the radioimmunoassays of NT and T only runs were accepted where no radioactivity was found between the two fractions.

After separation the more polar serum components were eluted from the column with a gradient system (*n*-hexane–2-propanol, 75:25; v/v). Total run-time

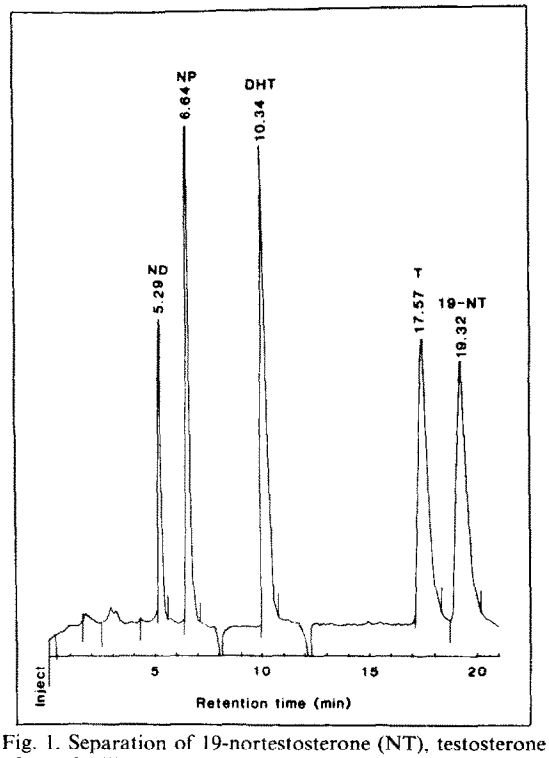

Fig. 1. Separation of 19-nortestosterone (NT), testosterone (T) and dihydrotestosterone (DHT) from 19-nortestosterone-decanoate (ND) and 19-nortestosterone-propionate (NP) by HPLC using a DIOL 5 μm column, solvent system hexane–2-propanol, 96:4 (v/v), flow 1.4 ml/min, pressure 620 psi, u.v. detection at 254 and 206 nm (DHT).

was 45 min. The mean retention times and the overall recovery after extraction and chromatography are summarized in Table 1. The contamination of the column between two different samples as measured by radioactivity in the individual fraction was less than 0.5%.

Reliability of the radioimmunoassays

Sensitivity. The sensitivity of the individual steroid standard curves was evaluated using the 2 SD range of B_0 value (Table 2). The theoretical sensitivity of the assays (column 4) was calculated using the mean percent recovery and the sensitivity of the standard curve.

Accuracy. The accuracy of the assay was determined by adding known amounts of the steroids to charcoal-stripped "steroid free" serum. Linear regression analysis of the measured steroid concentrations is summarized in Table 3.

Precision. The intra- and interassay coefficient of variation, measured in replicate determinations from a male control serum which was substituted by two different concentrations of NT (NT I and II) is shown in Table 4.

Specificity. The specificity of the method is determined by the efficiency of the steroid separation (see above) and the cross-reactivity of the antiserum. The NT antiserum showed a 8% cross-reactivity with T and 14% with DHT, as determined according to Abraham[17].

Table 1. Recoveries of [^{3}H]steroids (2000 cpm each) after HPLC, retention time and range of individual steroids (Values were evaluated from 20 runs)

Steroid	Percentage [^{3}H]steroid recovery after HPLC (mean ± SD)	Retention time min (mean ± SD)	Range min
DHT	54.4 ± 4.4	10.68 ± 0.19	10.2–11.0
T	61.2 ± 3.3	17.59 ± 0.29	17.3–18.1
NT	63.4 ± 4.2	19.20 ± 0.44	18.7–19.9

Table 2. Standard curve parameters and theoretical sensitivity of the steroid radioimmunoassays (Values were evaluated from 12 assays)

Steroid	Sensitivity of standard curve fmol (mean ± SD)	50% intercept fmol (mean ± SD)	Blank of buffer nmol/l (mean ± SD)	Sensitivity of the assay nmol/l
NT	15.4 ± 2.8	576 ± 91	0.05 ± 0.09	0.09
T	40.1 ± 6.2	649 ± 125	0.17 ± 0.13	0.62
DHT	15.2 ± 2.4	266 ± 40	0.04 ± 0.04	0.15

Table 3

DHT		T		NT	
Added nmol/l	Found (mean ± SD)	Added nmol/l	Found (mean ± SD)	Added nmol/l	Found (mean ± SD)
0.09	0.09 ± 0.04	1.73	1.86 ± 0.07	0.91	0.72 ± 0.09
0.17	0.14 ± 0.02	3.47	3.36 ± 0.34	0.82	1.44 ± 0.09
0.34	0.35 ± 0.04	6.94	6.14 ± 0.05	3.64	3.10 ± 0.22
0.69	0.67 ± 0.06	17.35	16.72 ± 0.60	7.29	7.16 ± 0.19
1.72	1.71 ± 0.06	34.70	33.34 ± 2.39	18.23	17.62 ± 0.55
3.44	3.05 ± 0.10			36.45	34.72 ± 0.52
$y = 0.89\ x - 0.03$		$y = 0.94\ x + 0.58$		$y = 0.96\ x + 0.04$	
$r = 0.977$		$r = 0.989$		$r = 0.995$	

Accuracy of the three steroid assays after HPLC ($y = a_o + bx$, where y represents nmol/l of steroid measured and x nmol/l steroid added), r = coefficient of correlation. Values were measured in triplicate.

Table 4. Steroid concentration in two standard control sera

Steroid	Concentration nmol/l (mean ± SD)	Intra-assay variation CV (%)	Inter-assay variation CV (%)
NT I	2.37 ± 0.15	6.3	13.3
NT II	8.96 ± 0.64	7.7	11.0
T	11.9 ± 1.79	7.2	9.1
DHT	0.84 ± 0.14	9.8	11.4

Precision was evaluated from 15 assays.

Pharmacokinetic study

Serum NT concentrations rose rapidly following i.m. injection of 50 mg NP or ND in the 6 subjects studied, reaching maximal serum levels (± SD) of 4.6 ± 3.2 nmol/l (2.8–9.7) (ND) and 2.0 ± 1.3 nmol/l (1.3–4.5) (NP). Individual data show a high variability when maximal concentrations were reached (Table 5). Thereafter the decrease of serum NT was approximately linear in all subjects. In five out of six men, the elimination of NT was slower after administration of NP than ND. The mean half-life (±SD) of NP was 21 ± 12 days (4–34 days) and 8 ± 5 days (5–17 days) for ND. No significant differences in the calculated NT half-life between subjects receiving NP injections first or second was found. The serum concentrations of NT, T and DHT are shown in Figs 2 and 3. After administration of NP and ND they always showed mirror image serum concentrations. However, the effect on T and DHT was more pronounced in the group treated with NP, but only T serum concentrations in the NP group were suppressed to a statistically significant degree ($P < 0.05$) compared to pre-treatment values on days 1, 6 and 10. The same trend was found in the group treated with ND but no statistical significance was calculated.

When serum concentrations of NT were below 1 nmol/l in both treatment groups (days 10–15 for ND and days 15–20 for NP), suppressed T concentration rose significantly ($P < 0.02$) to pre-treatment values. Thus the NT threshold serum concentration for significant rise of the T levels is 1 nmol/l in both groups.

The AUC (Table 5) represents the bioavailability of the two injected esters. There is no difference for the AUC values between the two preparations. The bioavailability of NT was on average 1.2 times higher following NP than after ND administration. The difference was, however, not statistically significant.

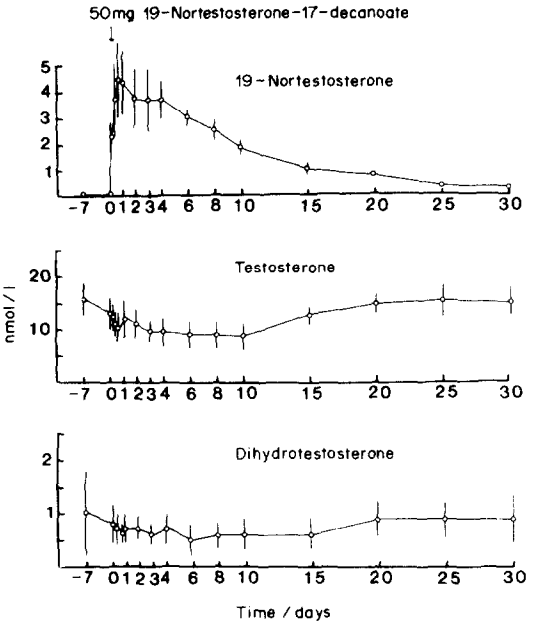

Fig. 2. Serum concentration (nmol/l) of NT, T, and DHT in the randomized pharmacokinetic study after i.m. administration of 50 mg 19-nortestosterone-decanoate (mean ± SEM). NT serum concentrations are significantly ($P < 0.01$) different from pre-treatment values up to day 20 and ($P < 0.05$) on day 25.

Clinical trial

In all 5 subjects NT serum levels rose steadily reaching peak levels in the 13th week of treatment (Fig. 4). This is in agreement with the long half-life time determined in the cross-over study. The concentration then declined gradually and remained significantly different from basal values through the 19th week post-treatment. A significant increase ($P < 0.01$) of suppressed T levels was found in the 24th week and NT levels at that time were 1 nmol/l. The half-life for NP after discontinuation of NP treatment was calculated from the serum disappearance rate and was even longer, 38 days as on average. NT serum concentrations are significantly different ($P < 0.01$) from pre-treatment values for 18 weeks after the last injection. At the 20th post-treatment week NT levels (±SD) were 0.82 ± 0.41 and a subsequent rise of suppressed serum T levels was observed.

Table 5. Pharmacokinetic parameters of NT (mean ± SD, range) after a single i.m. administration of 50 mg 19-nortestosterone-decanoate (ND) or 50 mg 19-nortestosterone-3-*p*-(hexoxyphenyl)-propionate (NP)

Ester	NP	ND
C_{max} (nmol/l)	2.0 ± 1.3 (1.3–4.5)	4.6 ± 3.2 (2.8–9.7)
T_{max} (hours)	8	24
$T_{1/2}$ (days)	21 ± 12 (4–35)	8 ± 5 (5–17)
$AUC_{0-\infty}$ (nmol/l + days)	54 ± 29 (24–107)	53 ± 13 (37–75)

The elimination half-life ($T_{1/2}$), and area under the serum concentration time curve ($AUC_{0-\infty}$) were calculated as described in the Experimental section. T_{max} indicates the time until peak concentrations are reached and C_{max} gives the maximal serum concentrations.

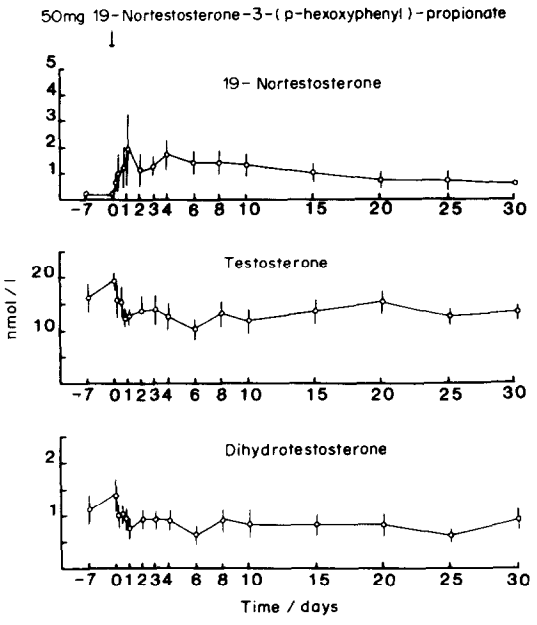

Fig. 3. Serum concentration (nmol/l) of NT, T, and DHT in the randomized pharmacokinetic study after i.m. administration of 50 mg 19-nortestosterone-3-*p*-(hexoxyphenyl)-propionate (mean ± SEM). NT serum concentrations are significantly ($P < 0.01$) different from pre-treatment values over the whole period; T concentrations are significantly ($P < 0.05$) suppressed on day 1, 6 and 10.

DISCUSSION

Full assessment of the clinical effectiveness of NT esters require pharmacokinetic studies based on appropriate analytical methods. The HPLC method described in this paper provides complete separation of DHT, T and NT from the NT esters. The chromatographic purification step prior to radioimmunoassay is necessary despite relatively specific antisera. HPLC has been shown to be as efficient in the separation of steroids as gas chromatography

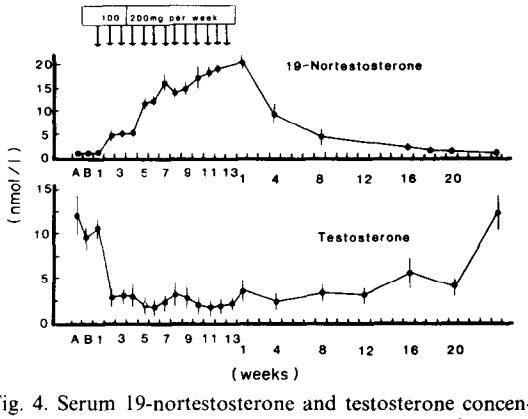

Fig. 4. Serum 19-nortestosterone and testosterone concentration (nmol/l) after weekly i.m. administration of 100 mg 19-nortestosterone-3-*p*-(hexoxyphenyl)-propionate in the first 3 weeks and further 200 mg/week for the remaining 10 weeks (mean ± SEM). Significant differences ($P < 0.01$) from control values are found for 18 weeks after the last injection.

[18, 19], but has the advantage of not requiring prior derivatisation. The combination HPLC/radioimmunoassay provides the same sensitivity as mass spectroscopy methods [20]. However, the available HPLC detector systems with sensitivities of about 5 ng do not allow direct quantitation of serum steroids in physiological concentrations [21] since only samples containing 5 ng (about 14 nmol/l NT/T) or more could be reliably distinguished from the background.

For separation of the compounds we used an isocratic system and not a gradient system as preferred by other authors for separation of adrenal or gonadal steroids [10, 13]. Baseline separation of T and NT with high reproducibility of retention times such as provided by the isocratic system is important since the antisera showed some cross-reactivity. The column is then purified of polar substances by 2-propanol. The relatively low recoveries after HPLC are caused by procedural losses at the injection system and by cutting narrow fractions in order to avoid any overlapping of fractions.

The accuracy of the method is satisfactory. Inter- and intra-assay variation are in the range of steroid assays without chromatography, although the technique includes more steps than simple radioimmunoassays, which could be an additional source of systematic errors.

The injection of either NP or ND led to a rapid increase of NT in serum. The absorption of ND was faster than of NP and maximal serum concentrations were achieved within 8 h, while this occurred after 24 h with NP. The peak values of NT were, however, higher after administration of ND, whereas the elimination of NT was slower when NP was given. The data show that NT is released from both esters at a relatively constant rate over a 4 day (ND) and 10 day (NP) period. The decrease in serum NT begins thereafter, but circulating levels of NT are detectable for 25 days (ND) and more than 30 days (NP). Extension of the half-lives to infinite times led to calculation of AUC, which represents the bioavailability of the esters. The areas were nearly identical, but NP application led to prolonged bioavailability of NT. The part of NT in NP is 18% lower than in ND, thus the availability of NT is not only prolonged but slightly higher.

Pharmacokinetic studies with different norethisterone esters [22, 23] have shown that physicochemical properties of the substances is the factor determining the absorption rate, rather than the release rates after cleavage of the ester. Structure–activity relationships indicate that NP would be the formulation with more biological activity [24]. This was demonstrated by suppression of testosterone levels for only 10 days, indicating that NP has a longer duration and enhanced intensity [25]. ND and NP application resulted in decreased serum T concentrations, however, this decrease was significant only after NP injection.

The reason for the longer half-life of NT after NP administration is not known, but it may be speculated that higher lipophility of NP in comparison with ND may result in a slower release from the intramuscular depot. Furthermore, higher protein binding of NP or a lower affinity of this ester for the hydrolytic enzymes may lead to lower susceptibility to enzymatic hydrolysis and thereby higher stability of NP in the body.

However, irrespective of the underlying mechanism it seems that the terminal half-life of NT is longer after administration of NP than ND. This has to be taken into account when dosage regimens are designed. If similar doses of ND and NP are given with similar intervals, larger variations of NT concentrations in the body could be expected after ND administration.

The 19-NT dosage administered in the clinical trial for male fertility control was higher than in the pharmacokinetic study to achieve suppression of gonadotrophins.

The NT serum concentration accumulated during the 10 weeks of multiple dosing of NP. This is in good agreement with the long half-life, since the rate of accumulation is dependent on the half-life of a compound. When linear kinetics are followed 94% of the plateau is reached within 4 half-lives. The calculated half life of NP is 21 days, and 4 half-life times (84 days) led to saturation of serum levels (91 days).

Serum levels were significantly different from pretreatment up to 18 weeks after the last injection. This rather long presence in circulation is not only of interest for the clinician, but also for athletes using NT and for doping control agencies.

In the volunteers participating in the pharmacokinetic study basal serum NT concentrations were below the detection limit of the assay. In the clinical trial, however, three of the five volunteers had low, yet detectable serum NT concentrations before treatment. Although NT has been reported to occur under physiological conditions in horses [27], this has not been demonstrated in humans. Since all volunteers were active in strenuous athletics it may be possible that they had residual NT deposits from any previous injections. Considering the long half-life of the esters this may be a more likely explanation than considering its presence in these volunteers as a physiological phenomenon.

Acknowledgements—The authors are indebted to Dr M.S. Moss, Racecourse Security Services Limited Laboratories, England, for kindly donating antiserum against 19-nortestosterone-3(*O*-carboxymethyl)-oxime coupled to bovine serum albumin, and Dr Anneke Bosch, Organon, Oss, Netherlands for the NT tracer. The technical assistance of Ms A. Guderjahn and secretarial help of Ms I. Oberschachtsiek and Ms A. Jung-Dieckheuer is gratefully acknowledged.

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