



Where does hydrolysis of nandrolone decanoate occur in the human body after release from an oil depot?



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ABSTRACT

Long-term therapy of nandrolone (N) is recommended to increase mineral density and muscle strength. Using a parenteral sustained release drug formulation with nandrolone decanoate (ND), therapeutic N levels can be achieved and maintained. Until now, it is unknown if hydrolysis of ND into N occurs in tissue at the injection site or after systemic absorption. Therefore, hydrolysis studies were conducted to investigate the location and rate of ND hydrolysis after its release from the oil depot.

ND hydrolysis was studied in porcine tissues, to mimic the human muscular and subcutaneous tissues. Additionally, the ND hydrolysis was studied in human whole blood, plasma and serum at a concentration range of 23.3–233.3 μ M.

ND hydrolysis only occurred in human whole blood. The hydrolysis did not start immediately, but after a lag time. The mean lag time for all studied concentrations was 34.9 ± 2.5 min. Because of a slow penetration into tissue, hydrolysis of ND is found to be very low in surrounding tissue. Therefore the local generation of the active compound is clinically irrelevant.

It is argued that after injection of the oil depot, ND molecules will be transported via the lymphatic system towards lymph nodes. From here, it will enter the central circulation and within half an hour it will hydrolyse to the active N compound.

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1. Introduction

Androgens can be used to increase bone mineral density and muscle strength (Crawford et al., 2003; Erdtsieck et al., 1994; Frisoli et al., 2005; Notelovitz, 2002). For this purpose, long-term therapy of nandrolone is recommended. Therapeutic nandrolone levels in blood can be maintained using a parenteral sustained release drug formulation (Bagchus et al., 2005; Kalicharan et al., 2016c; Minto et al., 1997; Wijnand et al., 1985). An example of such a parenteral drug formulation is an oil depot. In general, slow release from oil depots is a result of the high partition coefficient of lipophilic compounds; The release rate decreases when the compound is more lipophilic. Increased lipophilicity can be accomplished

through esterification with a fatty acid. For nandrolone, the decanoate has been selected as the appropriate moiety. In contrast to nandrolone (the active parent compound), nandrolone decanoate (ND) is an inactive prodrug. Oil depots with ND have been applied in several clinical studies, in which they were administered by intramuscular (i.m.) (Bagchus et al., 2005; Minto et al., 1997; Wijnand et al., 1985) or subcutaneous (s.c.) routes (Kalicharan et al., 2016c).

Although pharmacokinetic profiles of nandrolone depots have been published, the fundamental mechanisms of drug release and absorption into the central circulation have hardly been studied. In theory, ND is released from the oil depot into the interstitial (tissue) fluid. The rate at which this occurs is largely determined by the compound concentration in the oil formulation and its partition coefficient. Subsequently, ND is hydrolysed into nandrolone. Until now, it is generally assumed that ND is hydrolysed in serum and not in the tissue (fluid) at the site of injection (Wijnand et al., 1985).

Recently, we have demonstrated that there exists a delay (lag time) in the appearance of nandrolone in the central circulation

Abbreviations: BOH, benzyl alcohol; HB, human whole blood; HP, human plasma; HS, human serum; N, nandrolone; ND, nandrolone decanoate; NBCS, new born calf serum.

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(Kalicharan et al., 2016c). Since nandrolone has a $\log P$ of 3.0 (“ChemSpider,” 2015) and therefore will be absorbed relatively rapidly, this observed lag time indicates that immediate hydrolyses of ND does not occur. The lag time can be affected by several factors, such as diffusion in tissue fluid, cell membrane adsorption and cell absorption (Kalicharan et al., 2016c). On the one hand, at the site of injection, interstitial fluid transport is slower than blood flow. On the other hand, this transport is faster than diffusion. Another factor, cell membrane adsorption is relevant, because lipophilic prodrugs have high affinity with lipophilic cell structures such as cell membranes and membrane proteins. Due to the adherence to the cell membrane, cell absorption seems a logical consequence. Once absorbed, the lipophilic prodrug can be hydrolysed by esterases (if present) localized in cytosol and microsomes (Jewell et al., 2007; Li et al., 2005; Prusakiewicz et al., 2006). After hydrolysis, efflux of the active parent compound out of the cell must occur in order to reach the central circulation. All these factors may contribute to a prolonged residence time of the lipophilic prodrug in tissues and fluids around the injection site.

Hydrolysis can occur via chemical processes or by carboxylesterases (Imai and Ohura, 2010; Jewell et al., 2007; Prusakiewicz et al., 2006). These enzymes hydrolyse a different ester prodrug, haloperidol decanoate (Nambu et al., 1987; Oh-E et al., 1987). Because the ester bond in this prodrug is similar to the ester bond in ND, it is likely that ND hydrolysis also occurs by carboxylesterases. To our knowledge, this has however never been published yet and will be studied in this paper.

Interestingly, carboxylesterases are inhibited by benzil (Hatfield and Potter, 2011). This compound shows great similarity on molecular structure with a commonly added oil depot additive: benzyl alcohol (BOH). Although BOH is processed in a significant quantity in oil depots of 1–10% (m/v) (Bagchus et al., 2005; Kalicharan et al., 2016c; Minto et al., 1997; Van Weringh et al., 1994; Wijnand et al., 1985), any inhibitory effect of BOH on carboxylesterases is yet unknown but can be clinically relevant if it inhibits carboxylesterases.

The time period between ND release from the oil depot and metabolism in the liver may account for the complete lag time, but it is also well possible that hydrolysis occurs earlier in the absorption phase. Until now, this has never been unambiguously demonstrated.

The aim of this research was to determine whether ND hydrolysis occurs at the injection site after its release from an oil depot. This paper covers the ND hydrolysis in human blood, plasma, and serum. Hydrolysis in interstitial fluid was also indirectly studied, as interstitial fluid originates from blood plasma (Charman and Stella, 1992; Wiig et al., 2012). Furthermore, the rate of ND hydrolysis is studied in muscle and subcutaneous tissue from pigs to mimic the injection site in respectively human muscle and subcutaneous tissue. Usability of carboxylesterases from porcine liver was evaluated in an experimental setup using new born calf serum (NBCS). This was also used to check whether carboxylesterases from porcine could induce ND hydrolysis in human intravascular fluids, as a positive control in the experimental setup. The evaluation took into account the effects of BOH on carboxylesterases.

2. Method

2.1. Chemicals and reagents

Nandrolone decanoate (ND) (Ph. Eur. quality) was purchased from MSD (Oss, the Netherlands). Nandrolone (N) (analytical standard), spironolactone (analytical standard), benzyl alcohol (BOH) (analytical standard), zinc sulphate (analytical standard), RIPA-buffer and carboxylesterases from porcine liver (lyophilized

powder, ≥ 15 units/mg solid) were purchased from Sigma Aldrich, USA. HPLC-grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) was manufactured under current Good Manufacturing Practice conditions in the hospital pharmacy at the University Medical Center Utrecht, the Netherlands.

2.2. Biological materials

New born calf serum (NBCS) was purchased from Life Technologies (Carlsbad, CA).

Human blood (HB) was obtained by drawing blood into a BD Vacutainer[®] with anticoagulant (18.0 mg K₂EDTA). Human plasma (HP) was obtained by centrifuging HB for 10 min at 4000 rpm. Human serum (HS) was obtained by drawing blood into a BD Vacutainer[®] (REF 367896) with clot activator (micronized silica particles).

Muscle (gluteus maximus) and subcutaneous tissues were obtained from porcine (hind left leg) at the Central Laboratory Animal research facility (Utrecht University, the Netherlands). Before euthanizing with pentobarbital (barbiturate), esketamine (anaesthetic) and midazolam (benzodiazepine) were administered to the animal. Directly after termination, the tissues were cryopreserved with liquid nitrogen until usage.

2.3. HPLC system and conditions

The reversed phase-HPLC system was from Agilent Technologies 1100 series with a UV–vis detector (G1314A VWD). A Phenomenex guard column (C18, 4 × 2 mm ID, 5.0 μ m particle size) was used to filter out contaminants from each injection. Compound separation was carried out on a LiChrospher 100 RP-C18 column (125 × 4 mm ID, 5.0 μ m particle size). The column temperature was kept at 30 °C during analysis. The autosampler kept the samples at 4 °C. A volume of 15 μ L for samples originating from serum/plasma or 30 μ L for samples originating from blood were injected per run of 10 min. The flow rate was set at 1.0 mL/min. The eluted peaks were subsequently detected at a wavelength of 240 nm. The mobile phase was set as a gradient with a mixture of methanol absolute/distilled water, as listed in Table 1. Software used for equipment control and data acquisition was Chromeleon, version 7.1.3.2425 from ThermoFisher Scientific (Waltham, MA).

2.4. Standards

A calibration curve of the mixed stock solution was made of 2, 25, 100, 250, 500 and 1000 μ g/mL for both ND and N in ethanol absolute. Internal standard (IS) spironolactone was prepared as a 25 μ g/mL acetonitril solution. A stock concentration of 300 units/mL carboxylesterases (originating from porcine liver) was prepared in 10 mM borate buffer (pH 6.0) conform protocol Sigma

Table 1

Schematic representation of the used gradient. The gradient allowed elution within 10 min of the three substances: spironolactone, nandrolone and nandrolone decanoate.

Time (minutes)	Distilled water (%)	Methanol (%)
0.00	34.0	66.0
3.30	34.0	66.0
3.40	2.0	98.0
7.00	2.0	98.0
7.10	34.0	66.0
10.00	34.0	66.0

Aldrich. A freeze and thaw experiment of the carboxylesterases was evaluated on enzyme activity conform protocol Sigma Aldrich. Freeze-thaw cycles from -70°C to ambient temperature in 10 mM borate buffer were performed 4 times, whereby samples were stored 6 h at ambient temperature.

2.5. Incubation studies

2.5.1. New born calf serum

Ten microliters of ND solution was added to 490 μL NBCS. To each studied NBCS sample, 100 μL 10 mM borate buffer was added. The borate buffer was replaced with 100 μL carboxylesterase solution for the positive control samples. Final ND concentrations were 212.3, 396.6, 977.6 and 1957.5 μM .

2.5.2. Human intravascular fluids

Five microliters of ND solution was added to 245 μL human serum, plasma or blood. To the studied samples, 50 μL 10 mM borate buffer was added and to the positive control samples 50 μL carboxylesterase solution was added. Final ND concentrations were 23.3, 58.3, 116.7 and 233.3 μM .

Inhibition studies with BOH were performed by replacing a volume fraction of NBCS or human intravascular fluids by a percentage (v/v) of BOH.

2.5.3. Porcine tissues

Whole muscle (690 mg) and subcutaneous (590 mg) tissues were incubated in 50 mL tubes (polypropylene, sterile and with screw cap, Greiner bio one, Germany). Each tissue was washed with approximately 5 mL PBS. Subsequently, to lose potential lysate of leaking cells, the solution was gently centrifuged at 300g for 5 min, and the supernatant was poured away. This wash procedure was repeated three times in total. Hereafter, 4917 μL PBS and 83 μL ND solution were sequentially added. Final ND concentration was 700.1 μM . To positive control samples, 50 μL PBS was replaced with 50 μL carboxylesterase solution.

All solutions were incubated at 37°C . Incubation period for NBCS was 90 min. For human intravascular fluids samples and porcine fluids, the incubation period was 300 min. All experiments were conducted in triplicate.

2.6. Sample preparation

2.6.1. New born calf serum

One hundred microliters of incubation fluid in NBCS was mixed with 200 μL IS in 1.5 mL mixing tubes (Eppendorf, Hamburg, Germany). Hereafter, the tubes were vortexed for 10 s to precipitate serum proteins. Subsequently, samples were centrifuged for 5 min at 14,000 rpm. Supernatant was analysed using HPLC.

2.6.2. Human intravascular fluids

Fifty microliters of incubation fluid in human serum/plasma was mixed with 100 μL IS in 1.5 mL mixing tubes (Eppendorf, Hamburg, Germany). Hereafter, the tubes were vortexed for 10 s to precipitate serum proteins. For human blood samples, 50 μL blood was sequentially mixed with 50 μL zinc sulphate and 200 μL IS. Human blood samples were vortexed for 40 s. Subsequently, samples were processed as described above.

2.6.3. Porcine tissues

Fifty microliters of incubation fluid was mixed with 50 μL Radio-Immuno precipitation Assay (RIPA)-buffer and 100 μL IS in 1.5 mL mixing tubes. The mixtures were vortexed for 40 s. Subsequently, samples were processed as described above.

2.7. Data analysis

All data will be depicted as mean \pm SD. Michaelis-Menten parameters and plots were obtained using a non-linear fit. To analyse the inhibition studies with BOH to ND hydrolysis in NBCS, a one-way ANOVA test was conducted to compare the control sample (0.0% BOH) to the samples with 0.5, 1.0 and 2.0% BOH. For the inhibition study with BOH to ND hydrolysis in human blood, an unpaired *t*-test was conducted to compare the control sample (0.0% BOH) to the sample with 1.0% BOH.

In both statistical studies, a *P*-value less than 0.05 was considered as significantly different to the control samples. All data and statistical analysis were performed in GraphPad Prism version 6.02.

Lag time was calculated by extrapolating the trend line of the last 3 sample points to the x-axis. The lag time was defined as this intercept.

3. Results

3.1. Hydrolysis of nandrolone decanoate in newborn calf serum

To study whether ND is hydrolysed by carboxylesterases, the rate of hydrolysis from ND into N was determined without and in the presence of porcine carboxylesterases (50 units/mL) in NBCS. A range of 212.3–1957.5 μM ND was chosen because of the good detection of ND and N, not for any clinical relevance.

Hydrolysis to N was only observed in the presence of carboxylesterases. A typical hydrolysis profile is given in Fig. 1. Michaelis-Menten kinetics was observed with a maximum reaction rate of 7.2 $\mu\text{M}/\text{min}$ and a Michaelis constant of 483.1 μM (Fig. 2). No hydrolysis appeared in samples without carboxylesterases.

3.2. Hydrolysis in human intravascular fluids

Incubation studies in human intravascular fluids were carried out according to the same method as was used for the hydrolysis assay of ND in NBCS. The rate of hydrolysis of ND was examined over a period of 5 h in human blood (HB), human plasma (HP) and human serum (HS). To these fluids, an amount of ND in a range between 23.3–233.3 μM was added.

In Fig. 3 is shown the hydrolysis of ND to N at four different concentrations in HB. During 5 h of incubation, ND partially hydrolysed to N in HB. The hydrolysis of ND did not start immediately, in contrast to the positive control samples. Only a very low amount of N was observed after 30 min of incubation time. This lag time lasted 34.9 ± 2.5 min ($n=12$) in all studied

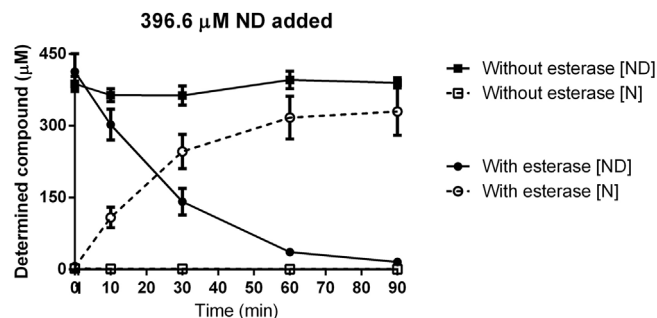


Fig. 1. Hydrolysis of 396.6 μM ND in new born calf serum ($n=3$). Hydrolysed ND samples (■) without carboxylesterases resulted in N (□), whereas hydrolysed ND samples (●) with added carboxylesterases from porcine liver resulted in N (○). Incubation studies were conducted at 37°C for 90 min. Results expressed as mean \pm SD, unless SD is smaller than the symbol.

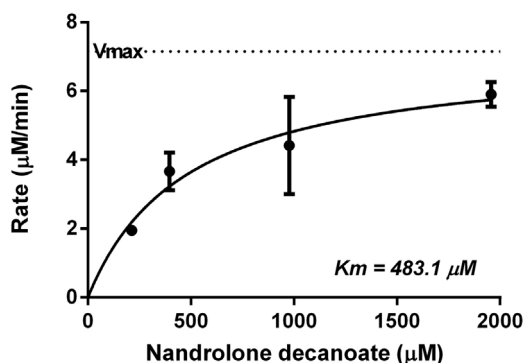


Fig. 2. Typical Michaelis-Menten plot of ND kinetics catalysed by carboxylesterases from porcine liver ($n=3$). Results expressed as mean \pm SD, unless SD is smaller than the symbol.

concentrations. There was no relationship between lag time and ND concentration. After one hour of incubation, a significant amount of N (approximately 2.3%) was measured. During the first 5 h, approximately 30% of ND was converted to N in HB, regardless of the added ND concentration. In contrast to the results obtained with carboxylesterases in NBCS where Michaelis Menten kinetics were observed (Fig. 2), a linear correlation between ND concentration and hydrolysis rate was obtained (Fig. 4).

No hydrolysis of ND to N occurred in HP or in HS. Carboxylesterases from porcine liver (50 units/mL) were added

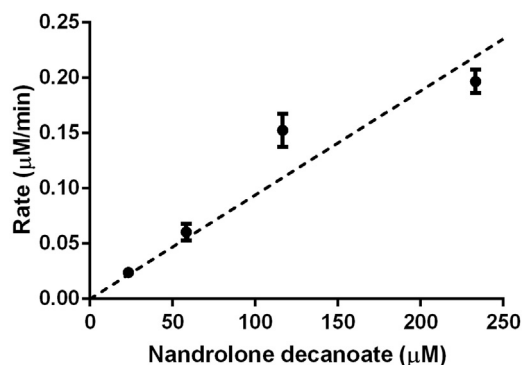


Fig. 4. Hydrolysis rate plot of ND kinetics in human blood ($n=3$). Results expressed as mean \pm SD, unless SD is smaller than the symbol. Dotted line represents the linear regression line (forced through 0,0) with equation: $Y = 0.0009414 * X - 0.0$.

to the lowest (23.3 μ M) and highest (233.3 μ M) ND sample as a positive control to check whether hydrolysis occurred in specific medium (positive controls, see Fig. 3A and D). In all HP and HS positive controls, ND was also converted to N (data not shown).

3.3. Hydrolysis in porcine muscle and subcutaneous tissue

To study hydrolysis of ND in muscle and subcutaneous tissue whole porcine tissues were incubated to mimic human tissues. These incubation studies were carried out according to the same

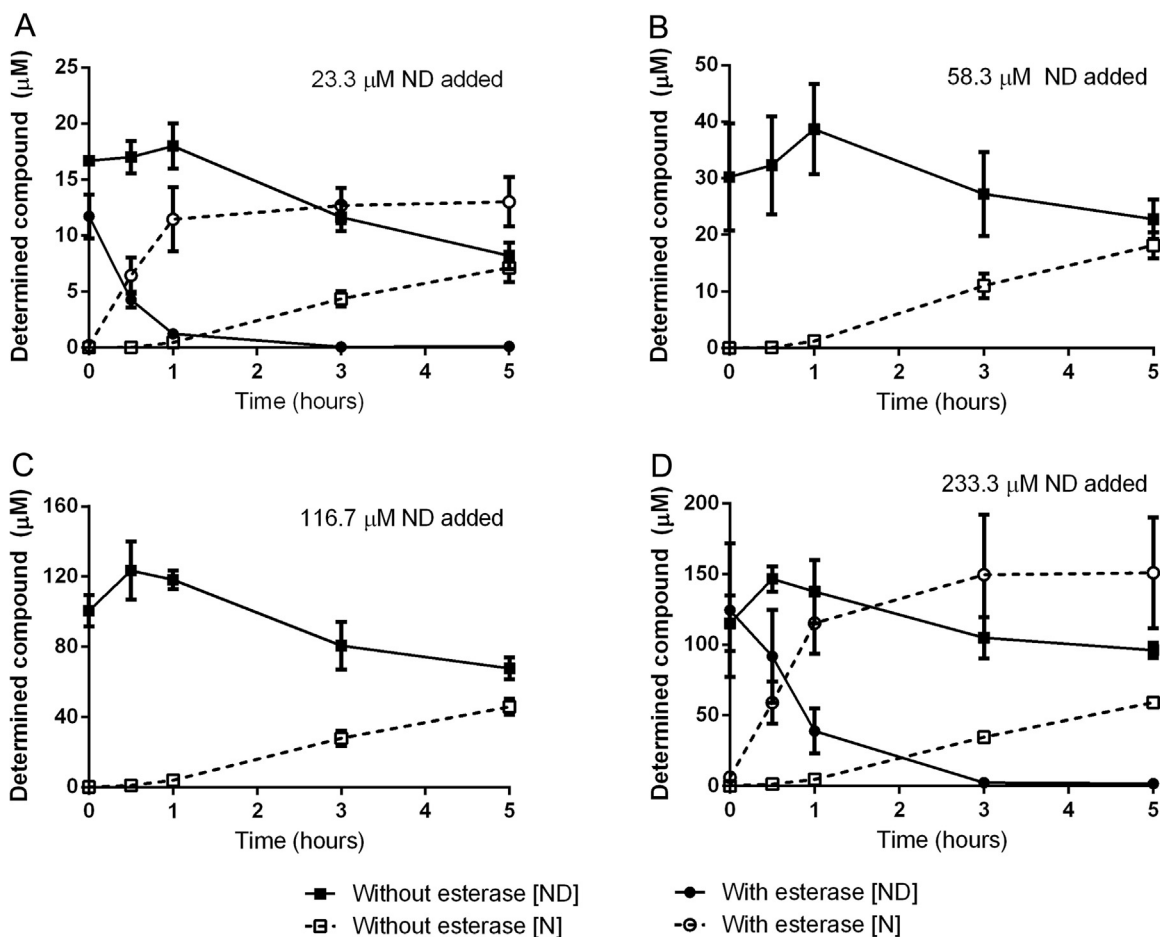


Fig. 3. Hydrolysis of ND in human whole blood for four different concentrations ($n=3$). Hydrolysed ND samples (■) without carboxylesterases resulted in N (□), whereas hydrolysed ND samples (●) with added carboxylesterases from porcine liver resulted in N (○). Incubation studies were conducted at 37 °C for 5 h. Results expressed as mean \pm SD, unless SD is smaller than the symbol.

method that was used for the hydrolysis assay of ND in NBCS and human intravascular fluids. The realistic situation was to expose the tissues to an oil depot with ND, but preliminary experiments showed that there was no ND released from an oil depot in aqueous media (data not shown). Therefore, ND solution (ethanol absolute) was added to the experimental setup, to determine any hydrolysis activity in these tissues. Concentrations ND below $700.1 \mu\text{M}$ were not hydrolysed after 5 h of incubation.

Fig. 5 shows the percentage recovery of N in the muscle and subcutaneous tissues. In this figure, N recovery in HB is added to compare the relevance of ND hydrolysis in these tissues. During 5 h of incubation, nandrolone was just above the detection limit in the incubation tissue media: Less than 1% N was recovered during incubating ND with porcine muscle tissues. No N was obtained in ND incubated porcine subcutaneous tissues.

Carboxylesterases from porcine liver (50 units/mL) were added in all media as a positive control to check whether ND hydrolysis occurred in the specific medium. In all media, the positive controls yielded full N within 5 h incubation. A control freeze-thaw experiment with carboxylesterases from porcine liver did not affect the hydrolysis activity to obtain N from ND (data not shown).

3.4. Inhibitory effect of BOH on hydrolysis

To study the inhibitory effect of BOH on ND hydrolysis, NBCS containing ND was incubated with carboxylesterases. Upon addition of relatively high amounts of BOH to NBCS solutions, precipitation was visually noticeable. Full precipitation, presumable of protein aggregates, was seen upon addition of 10.0% BOH. Therefore, it was decided not to proceed with 10.0% BOH in the inhibition study. Precipitation was not visible after adding 2% BOH or less.

The inhibitory effect of 0.5, 1.0 and 2.0% (v/v) BOH on the ND hydrolysis activity of porcine liver carboxylesterases (50 units/mL) in NBCS is given in Fig. 6. Complete inhibition of ND hydrolysis was examined with the addition of 2.0% BOH during 90 min of incubation ($P=0.011$) (Fig. 6). One percent of BOH resulted in a significant inhibition of ND hydrolysis ($P=0.035$) whereas the addition of 0.5% BOH exhibited only a slight effect on ND hydrolysis. In the 1.0% BOH control sample without added carboxylesterases, no ND hydrolysis was observed (negative control). Additional control experiments (Fig. 1), demonstrated that in NBCS in the presence of carboxylesterase ND is hydrolysed

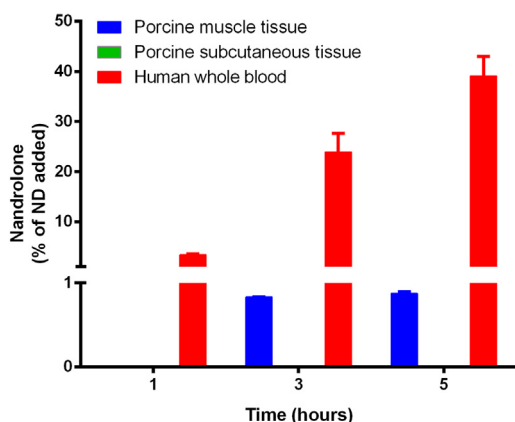


Fig. 5. Bars represent the percentage recovery of N after 1, 3 and 5 h of incubation (37°C) corrected for the amount ND added. Red bars show the recovery of N obtained from human whole blood (originated from Fig. 3C.), whereas blue and green bars represent respectively porcine muscle and subcutaneous tissues. Results expressed as mean \pm SD, unless SD is smaller than the symbol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

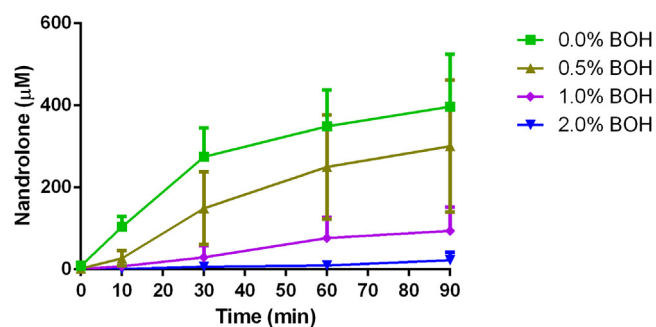


Fig. 6. Inhibitory effect of BOH on $977.6 \mu\text{M}$ ND hydrolysis in new born calf serum ($n=3$). The ND hydrolysis is inhibited by a raising BOH concentration. Results expressed as mean \pm SD, unless SD is smaller than the symbol.

to N. Moreover, no spontaneous ND hydrolysis was observed in NBCS (Fig. 1). Therefore, this medium was suitable to study the inhibitory effect of BOH on carboxylesterase with respect to the conversion of ND to N.

Similar to the BOH inhibition study in NBCS, 1.0% BOH significantly inhibited the conversion to N in HB ($P<0.01$) (Fig. 7). Without BOH, the obtained concentration of N was $45.8 \pm 2.6 \mu\text{M}$ ($n=3$), whereas the N concentration with 1.0% BOH was $5.7 \pm 1.5 \mu\text{M}$ ($n=3$). Both conditions contained the initial ND concentration of $117 \mu\text{M}$. No precipitation was noticed.

4. Discussion

This research aimed to study whether locally injected ND as an oil depot is hydrolysed at the site of injection. Drug absorption from a locally injected oil depot into the central circulation exhibits a delayed absorption pattern, which is defined as *lag time* (Kalicharan et al., 2016c). About 8 h after a s.c. ND oil depot injection, N is observed in the central circulation (Kalicharan et al., 2016c). Remarkably, this lag time is much shorter when the oil depot is injected in the vastus lateralis muscle (2.7 h), about the same when injected in the gluteal muscle (11–12 h) and much longer when injected in the deltoid muscle (26.4 h) (Kalicharan et al., 2016c). This phenomenon of various lag times will be discussed in another article. This lag time is most likely caused by the postponed ND hydrolysis which would mean that hydrolysis does not take place in the tissue around the site of injection, but rather occurs in the central circulation or in the liver. This research elucidates whether the hydrolysis of N occurs at the injection site, or in the central circulation.

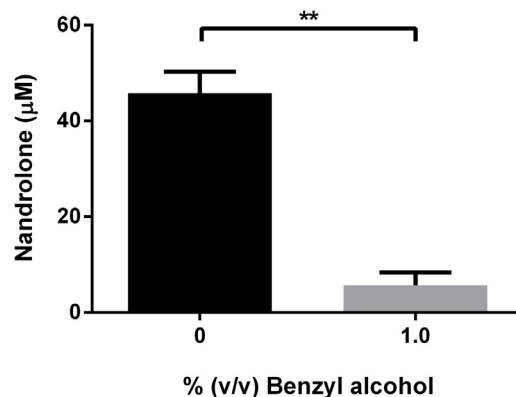


Fig. 7. 1.0 % Benzyl alcohol resulted in a significant inhibition of $116.7 \mu\text{M}$ ND hydrolysis in human blood after 5 h of incubation ($n=3$). **, $P<0.01$. Results expressed as mean \pm SD.

In literature, a half-life time hydrolysis of 4 min for $2.5 \times 10^{-3} \mu\text{mol/mL}$ ($1 \mu\text{g/mL}$) nandrolone phenyl propionate in rat plasma has been reported (14). Based on these data, the duration time to hydrolyse $2.3 \times 10^{-5} \mu\text{mol/mL}$ ($=0.01 \mu\text{g/mL}$) ND in HS was proposed by Wijnand et al. (Wijnand et al., 1985) to be below one hour. However, as reported by Li et al. and Rudakova et al. (Li et al., 2005; Rudakova et al., 2011), no carboxylesterases are present in HP. This implies that no enzymatic hydrolysis of ND to N can occur in HP and HS, which is confirmed in this current study: In human serum and plasma, no hydrolysis of ND occurred during 5 h of incubation. Also, no chemical hydrolysis of ND occurred in these media. Moreover, this entails that ND hydrolysis does not occur in interstitial fluid since interstitial fluid originates from blood plasma. It contains water and molecules less than approximately 40 kD molecular weight (Charman and Stella, 1992; Wiig et al., 2012). This means that even when carboxylesterases would be present in human blood plasma, they would not appear in the interstitial fluid as the molecular weight of human carboxylesterases is approximately 60 kD (Imai and Ohura, 2010).

This paper demonstrates that ND hydrolysis does occur in human blood. Apparently human blood contains carboxylesterases. The appearance of N was observed after an average lag time of 34.9 ± 2.5 min. Noticeable was the absence of Michaelis-Menten behaviour. Instead of nonlinear enzyme kinetics, a linear rate of hydrolysis was seen (Fig. 4). These observations suggest that the carboxylesterases, involved in ND hydrolysis, must be present in the blood cells since the distinction between whole blood and plasma is the presence of cells. Hydrolysis can occur either on the blood cell membrane, or occurs intracellularly. Because a lag time of N appearance was noticed, carboxylesterase activity on the cell membrane is apparently not very pronounced. Otherwise, the N recovery should be seen instantaneously. It can therefore be concluded that hydrolysis occurs after membrane diffusion and, subsequently intracellularly in erythrocytes cytosol (Quon and Stampfli, 1985) and probably in leukocytes. This is in line with literature, where it is reported that carboxylesterases are present in the endoplasmic reticulum and cytosol in human cells.

In porcine muscle and subcutaneous tissues, little hydrolysis of ND occurred in porcine muscle tissue during 5 h of incubation (Fig. 5). The tissues were chosen to be originated from a pig instead of other species (such as rats) to prevent false-positive results. Similar to human tissues, interstitial fluid between porcine tissue cells does not contain carboxylesterases (Li et al., 2005). Because the penetration depth of lipophilic molecules into tissue is very low (Lerner et al., 2006), it is relevant to study the carboxylesterase activity in cells that are in the direct proximity of the oil depot. The results with tissue conclude that hydrolysis in porcine muscle and subcutaneous cells occurs slowly, indicating that only little ND has reached an enzyme. In line with hydrolysis in HB, this also suggests that in vivo hydrolysis only occurs intracellularly, because immediate (within 30 min) N appearance was absent.

Before intracellular hydrolysis can occur, ND must diffuse through the cell membrane. As can be seen in Fig. 4, the enzymatic conversion is proportional to the concentration of the substrate.

This suggests that there must be another process that plays a role. This process is the diffusion of ND through the cell membrane which is the rate-limiting step. Otherwise, Michaelis-Menten kinetics would have been observed and a lag time absent. As ND is more lipophilic ($\log P = 8.1$ ("ChemSpider," 2015)) than N ($\log P = 3.0$ ("ChemSpider," 2015)), we assume that the limiting step in mass transfer is the cell influx of ND rather than the cell efflux of N. Therefore, the N efflux will be neglected in the following estimation. With the value of lag time being roughly 2100 s and the literature value of the erythrocyte membrane thickness (h) ($7 \times 10^{-7} \text{ cm}$ (Changjun Liu et al., 2003)), the diffusion coefficient (D) of ND through this membrane can be estimated to be $3.9 \times 10^{-17} \text{ cm}^2/\text{s}$ according to the following equation:

$$\text{Lagtime} = \frac{h^2}{6D}$$

This calculated value of the diffusion coefficient is much lower than other steroid diffusion coefficients. For example, the D of testosterone in percutaneous absorption has been reported to be $1.95 \times 10^{-11} \text{ cm}^2/\text{s}$ (Scheuplein et al., 1969). Calculated lag time for this testosterone would be $4.2 \times 10^{-3} \text{ s}$ (h was kept constant), which gives a negligible corrected (for the N efflux) diffusion coefficient for ND in current study (D remains $3.9 \times 10^{-17} \text{ cm}^2/\text{s}$). There is a significant difference between the values of ND and testosterone and this may be a source of some discussion. However, it is at least a reflection of the large difference that exists between a prodrug having a $\log P = 8$ and a parent drug having a $\log P = 3$. The conclusion of this observation is that ND hardly does permeate through tissue. This indicates that ND must be absorbed into the central circulation via other routes, as direct absorption is excluded due to the high partition coefficient. It is assumed that ND subsequently adheres to small proteins ($<40 \text{ kD}$) and migrates into lymph vessels to be absorbed into the central circulation.

Recently, it was shown that the in situ surface of a 0.5 mL injected oil depot is 755.4 mm^2 (Kalicharan et al., 2016a). As can be seen in Table 2, the amount of cells per 1000 mm^2 tissue is around 10^5 – 10^8 cells, which is negligible with the amount of cells in blood (10^{15} cells/mL). Another advantage of blood, is that it is continuously refreshed (sink conditions).

Benzyl alcohol (BOH) is commonly used in oil depots at a concentration of 1–10% (m/v) (Bagchus et al., 2005; Kalicharan et al., 2016c; Minto et al., 1997; Van Weringh et al., 1994; Wijnand et al., 1985). It is used as viscosity reducer, local anaesthetic and as co-solvent in oil depots (Rowe et al., 2009). Recently, BOH was reported to have a very different absorption profile than N (Kalicharan et al., 2016c). In contrast to N, BOH was detected in the bloodstream within minutes after injection. Furthermore, whereas N was measured for 5 weeks after injection, BOH was cleared from the central circulation within 36 h (Kalicharan et al., 2016b).

The BOH molecular structure shows great similarity to the one of benzil (Table 3), which is an inhibitor of carboxylesterases (Hatfield and Potter, 2011). The inhibition is due to steric interaction of the benzene ring of benzil with the pocket of human carboxylesterase 1 (hCE1) (Harada et al., 2009). This was

Table 2

Estimation of amount of cells in studied media nandrolone decanoate may encounter during absorption into the central circulation.

Type	Amount of cells	Amount of cells available for ND at tissue surface ^c
Subcutaneous cells ^a	$2.6 \times 10^3 \text{ cells/mg tissue}$	$1.4 \times 10^2 \text{ cells/mm}^2$
Muscle cells ^b	$9.3 \times 10^4 \text{ cells/mg tissue}$	$3.2 \times 10^5 \text{ cells/mm}^2$
Erythrocytes	4.2 – $5.5 \times 10^{15} \text{ cells/mL blood}$	–



^a The volume of human adipose cells is $660 \times 10^{-6} \text{ mm}^3$ (26). The 590 mg porcine subcutaneous tissue had a volume of approximately 1 cm^3 ($=1000 \text{ mm}^3$).

^b No dimensions of human muscle cells are published. The following data is used: rat myocytes have a volume of $15.6 \times 10^{-6} \text{ mm}^3$ (27). The 690 mg porcine muscle tissue had a volume of approximately 1 cm^3 ($=1000 \text{ mm}^3$).

^c the diameter of human subcutaneous cells and muscle cells are respectively 94 and $2 \mu\text{m}$ (28,29).

Table 3

Summary of benzyl alcohol and benzil and their molecular properties.

Component name	Molecular formula	Molecular structure	Molecular weight (Da)	Log P
Benzyl alcohol	C ₇ H ₈ O		108.1	1.03
Benzil	C ₁₄ H ₁₀ O ₂		210.2	3.38

Source: [http://www.chemspider.com/\(9\)](http://www.chemspider.com/(9)), visited at July 15, 2016.

the reason to study the influence of BOH on ND hydrolysis. As observed, the addition of 1.0% BOH significantly inhibits the ND hydrolysis in human blood.

Although not investigated, the effect of BOH on the carboxylesterases in porcine muscle and subcutaneous tissues can be predicted. This can be estimated by calculating the amount of BOH locally using the *log P* of BOH. The *log P* of BOH is 1.03 ("ChemSpider," 2015) which indicates a distribution in the water phase (*interstitial fluid*) of approximately 10% of the total amount injected BOH. The processed amount BOH in oil depots is 10% (m/v), which would result in a concentration less than 1% in the water phase. In the current study, 1.0% BOH significantly inhibited carboxylesterases in whole blood. Therefore, it is possible that this inhibition will also occurs in the proximity of the oil depot. This is only temporary however, since nearly all BOH in the oil depot is released within 36 h. Indicating a large amount of BOH that enter the interstitial fluid and diffuses through the tissues near the injection site, and, consequently, it will temporary inhibit the carboxylesterases in these tissues.

This study reveals that ND is stable in NBCS during incubation for 90 min at 37 °C (Fig. 1). This implies the absence of chemical and enzymatic degradation of ND in NBCS (Fig. 1). It also has been shown that carboxylesterases from porcine liver are active in tissues from other species than pigs since porcine carboxylesterase also induced hydrolysis in new born calf serum (NBCS) and in human blood. This experimental setup was therefore suitable for use with human intravascular fluids.

5. Conclusion: contribution of nandrolone decanoate hydrolysis to nandrolone absorption

After injection of the oil depot, the released ND molecules appear in the interstitial tissue. Due to the high partition coefficient (*log P*=8.1 ("ChemSpider," 2015)) of ND, it is likely that ND adheres to small proteins (< 40 kD) in the interstitial fluid. As argued above, the prodrug is neither hydrolysed in the interstitial fluid nor is able to diffuse in a significant way into the tissue. The interstitial fluid is drained via the lymph vessels, and therefore ND absorption into the central circulation via the lymphatic system is in fact the only likely route. Therefore, it can be reasoned that ND is hydrolysed when entering the central circulation. Alternatively, it is possible that the cellular components in the lymphatic system take care of (a part of) the hydrolysis.

Once entered in the central compartment, it will take about half an hour before ND hydrolysis will occur. All these phenomena, transport through the lymph and diffusion through cell membranes, contribute to the lag time observed after the injection of an oil depot.

Although this article focusses on the location and rate of ND hydrolysis, the outcome is also applicable for other esterified prodrugs processed in oil depots and i.m. or s.c. injected, such as haloperidol decanoate (Van Weringh et al., 1994), fluphenazine decanoate (Soni et al., 1988) or testosterone undecanoate (Morgentaler et al., 2008).

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Conflicts of interest

The authors declare that there is no conflict of interest.

Author contributions

R.K, C.O, H.V wrote the manuscript; R.K, M.B, C.O and H.V designed the study; R.K and M.B analysed the data.

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