# SYNTHESIS AND CHARACTERIZATION OF THE ENZYMATIC MARKER NANDROLONE-3-CARBOXYMETHYLOXIME-ALKALINE PHOSPHATASE TO BE USED IN ELISA TECHNIQUE FOR ASSAYS OF NANDROLONE FROM BIOLOGICAL SAMPLES

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*Abstract*. The procedure of obtaining nandrolone-3-CMO-phosphatase alkaline, the most important reagent to be used in ELISA technique for assay of nandrolone from biological sample, was described. This paper presents the synthesis of this enzymatic marker and enzymatic and immunological characteristics. There are also presented the effect of a substrate concentration on the enzyme kinetics, the time stability of the marker and also the positive reaction of the marker with antinandrolone antibodies.

*Key words*: nandrolone-3CMO-alkaline phosphatase, ELISA, $K_M$, antinandrolone antibody.

## INTRODUCTION

Nandrolone is an anabolic steroid that can be found in the human body in small quantities. The synthesis rate of endogenous nandrolone is regulated by some cellular receptors (sensitive to human chorionic gonadotropin [7]). A concentration of 0.01 − 0.02 μg/L of endogenous nandrolone derivatives in urine samples collected from healthy donors was found [7]. On the other hand, exogenous nandrolone is found to have beneficial effect in modulating the muscle mass growth [11]. As a consequence, nandrolone is illegally used by athletes and bodybuilders for improving of their performances in professional competition. The International Olympic Committee has set of 2 ng per ml of urine as the upper limit, beyond which an athlete is suspected of doping.

Nandrolone was found to influence many other metabolic processes. The rate of neural stem cell proliferation induced with epidermal growth factor in rat was decreased by the presence of nandrolone [2]. Two weeks of nandrolone decanoate administration caused a significant decrease of the basal level of dopamine in rat [1]. Also administration of the nandrolone decanoate to female rats causes

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alterations in the morphology of their uterus and a reduction in reproductive capacity [5]. This compound has positive effects including appetite stimulation and increased red blood cell production and bone density. Some of these effects have medical applications as the remodeling and improving the biomechanical properties of bio-artificial tissues [11]. Because nandrolone is not broken down into dihydratotestosterone (DHT), this steroid has effects such as gynaecomastia, erectile dysfunction and cardiovascular damages as well a decreasing of luteinizing hormone level by negative feedback [9].

Nandrolone use is indirectly detectable in urine by testing for the presence of 19-nortestosterone, a metabolism product of this molecule. Due to the extremely low concentration in biological sample, the most proper technique to measure nandrolone concentration is the gas chromatography with a mass spectrometer as detector [8]. This technique being expensive, cheaper and easy to use the methods based on ELISA (enzyme linked immunosorbent assay) were purposed [3, 6, 8, 13]. ELISA is used mainly in immunology to detect the presence of an antibody or an antigen in a sample. This technique uses specific reagents as antibodies and enzymatic markers that can be obtained by covalent linking of the antibody or antigen to an enzyme.

The paper presents a procedure for obtaining and characterization of the nandrolone-3-carboxymethyl oxime-alkaline phosphatase (Nand-3-CMO-PhA), the main reagent that will be used in an assay of nandrolone from biological samples.

## MATERIALS AND METHODS

The structure of nandrolone (17β-Hydroxyestra-4-en-3-one or $C_{18}H_{26}O_2$) is given in Figure 1:

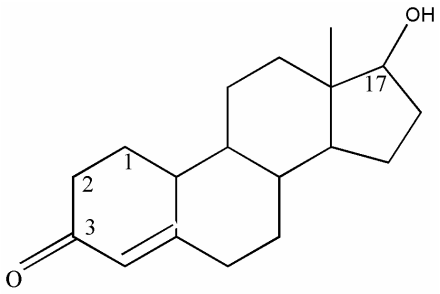


Fig. 1. Nandrolone molecular structure.

Alkaline phosphatase and nandrolone were achieved from Sigma-Aldrich CO, USA. Nandrolone-3-carboxymethyl oxime (Nand-3-CMO) was prepared by condensation of nandrolone with aminooxyacetic acid according to the general

procedure described by Erlanger *et al*. [4]. Nandrolone and oxyaminoacetic acid were refluxed in ethanol in the presence of NaOH. The formed product, Nandrolone-3-carboxymethyloxime, was purified by thin layer chromatography and used as derivative for coupling with alkaline phosphatase.

## COUPLING OF NANDROLONE-3-CMO TO ALKALINE PHOSPHATASE

The reaction between Nand-3-CMO and alkaline phosphatase (presented in Fig. 2) was produced using a protocol as follows: a mixture of 1 mg nandrolone-3-CMO in dioxane (90.5 ml) and 1 ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1 mg) in water (0.5 ml) was stirred for 30 minutes to activate the carboxy group of the nandrolone derivative. 50 µl of this activated mixture was added to a solution of alkaline phosphatase (1 mg) in water (1 ml). The resulted solution was then stirred for 20 hours and the product of interest, nandrolone-3-CMO-alkaline phosphatase, was purified by Sephadex G50 column (30 × 1 cm) having as eluent 0.05 M phosphate buffer pH 7.8. The collected fractions (2 ml) were measured from the point of view of the absorbance at 250 nm (the steroid has a specific peak at 250 nm). The enzymatic activity of fractions was also analyzed.

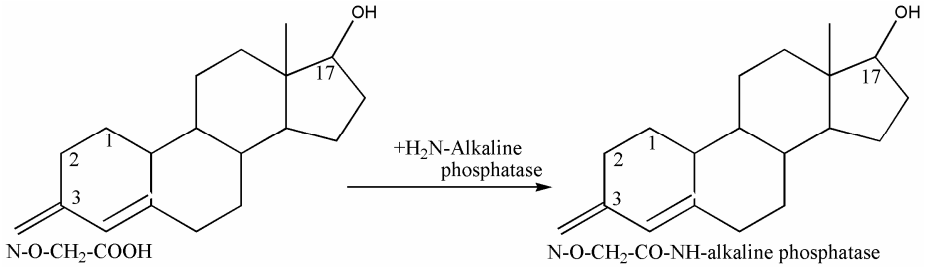


Fig. 2. Reaction of Nand-3-CMO with alkaline phosphatase.

## ENZYMATIC CHARACTERISTICS (OR PROPERTIES) OF NANDROLONE-3-CMO-ALKALINE PHOSPHATASE

Alkaline phosphatase catalyzes the cleavage of phosphate group from a variety of compounds, including the p-nitrophenylphosphate [9, 10]. The solution of this substrate is colorless, but in the presence of the enzyme one of the products of the enzymatic reaction, p-nitrophenol, is yellow in basic solutions as can be detected analyzing by spectrophotometer the optical absorption at $\lambda = 400$ nm. The reaction between enzyme and p-nitrophenyl phosphate is presented in Figure 3. The intensity of yellow color indicates the degree to which the substrate was transformed by the enzyme.

The reaction between Nand-3-CMO-PhA ($M_E$) and p-nitrophenylphosphate (S), according to the steady-state theory, is given by the relation:

$$M_E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} M_ES \xrightarrow{K_{+2}} M_E + \text{products}$$

the product of interest which can be measured being p-nitrophenol resulted from the enzymatic reaction by the action of the enzyme on the substrate. $M_ES$ is the enzyme-substrate complex and $K_{+1}$, $K_{-1}$, $K_{+2}$ are the rate constants.

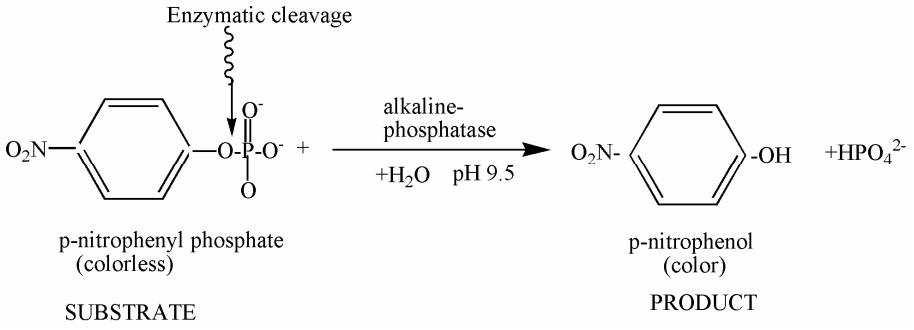


Fig. 3. Reaction of Nand-3-CMO-PhA with the substrate p-nitrophenyl phosphate resulting in the yellow colored product p-nitrophenol.

If $e$ is the concentration of $M_E$, $S$ is the concentration of substrate, $x$ is the concentration of $M_ES$ then the rate of formation of $M_ES$ will be $K_{+1}(e-x)S$ and the rate of breakdown into $M_E$ and $S$ and into $M_E$ and products, namely $K_{-1}x$ and $K_{+2}x$, respectively. Thus the rate of change of $x$ will be:

$$\frac{dx}{dt} = K_{+1}(e-x)S - (K_{-1} + K_{+1})x \tag{1}$$

For the steady-state, when $\frac{dx}{dt} = 0$, it results:

$$x = \frac{eS}{\frac{K_{-1} + K_{+2}}{K_{+1}} + S} \tag{2}$$

The rate of the enzyme reaction as measured by the formation of the product p-nitrophenol will be:

$$v = K_{+2}x = \frac{K_{+2}eS}{K_M + S} \tag{3}$$

where $K_M$ is the Michaelis constant:

$$K_M = \frac{K_{-1} + K_{+2}}{K_{+1}} \tag{4}$$

The maximum velocity is given by:

$$V = K_{+2}e \tag{5}$$

By replacing equation (5) into (3) it results:

$$v = \frac{VS}{S + K_M} \tag{6}$$

The relation (6) was used for the experimental determination of the enzymatic characteristics of the Nand-3-CMO-PhA.

## IMMUNOLOGICAL CHARACTERISTICS

The enzymatic marker Nand-3-CMO-PhA, due the presence of the steroid nandrolone linked on the surface of the protein alkaline phosphatase, is able to react with antinandrolone antibodies. In the ELISA technique the antibody antigen reaction is produced on solid surfaces (usually a plastic surface). For this reason one of the reagents of the immunological reaction has to be linked on that surface. In our case, when incubated with ELISA plastic plate (96 wells) antinandrolone antibodies will bind to the surface of the wells. The ELISA plates 12 × 8 were supplied by Nunc InterMed, Denmark, and antinandrolone antibodies from Abcam plc, UK. All other reagents were obtained commercially and were of reagent grade. Incubation of wells with antibodies diluted 1:100 with 0.05 M phosphate buffer pH 7.8 for 24 hours at 4 °C was followed by three rinses with 0.05 M phosphate buffer pH 7.8. A second incubation was done with nandrolone-3-CMO-alkaline phosphatase (200 µl) plus competing antigen standard nandrolone (range 0.1 ng to 10 ng/well) in 0.1% bovine serum albumin for 24 hours at room temperature. The wells were three times rinsed with distilled $H_2O$ and finally with 200 µl/well p-nitrophenylphosphate 500 µl/ml, substrate for alkaline phosphatase in 0.2 M $Na_2CO_3$ (pH 9.8). The reaction was stopped with 100 µl 1M NaOH after one hour and the absorbance read at 400 nm.

## RESULTS AND DISCUSSIONS

Procedure of obtainment of the enzymatic marker was realized by coupling of nandrolone-3-carboxymethyloxime activated with carbodiimide to phosphatase alkaline. Enzymatic marker nandrolone-3-carboxymethyloxime-phosphatase alkaline was then purified on Sephadex G50 column and the results of the measurements are shown in Figure 4. A specific peak for the enzymatic conjugate was obtained. Since the concentration of marker in the mixture is very low the absorption of marker (nandrolone-3-CMO-PhA) at 250 nm is unobservable in comparison with the absorption of the uncoupled nandrolone-3-CMO.

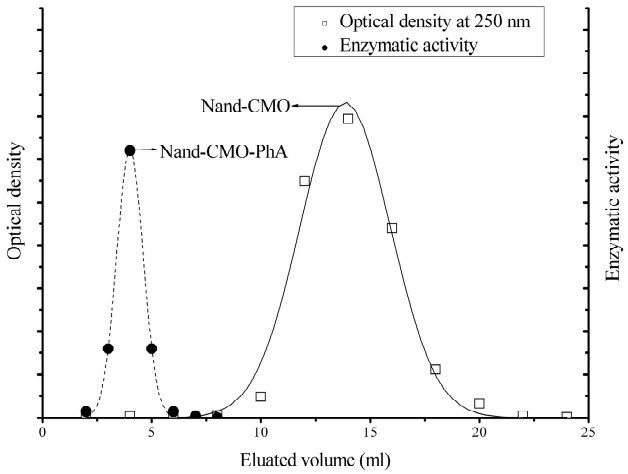


Fig. 4. Chromatography of the Nand-3-CMO-PhA on Sephadex G50.

The enzymatic activity of the fractions was measured with p-nitrophenyl phosphate used as substrate. The transformation of p-nitrophenyl phosphate into product p-nitrophenol by nandrolone-3-CMO-alkaline phosphatase is described by the absorption spectra in Figure 5. During the reaction the specific peak of the substrate ($\lambda$ = 310 nm) decrease and a new peak (specific to the product) occurs at $\lambda$ = 400 nm. Evaluating the transformed substrate mass by the enzymatic conjugate in comparison with the pure enzyme the specific enzymatic activity of the marker was measured to be ~ 530 u/mg.

The evolution of the enzymatic reaction at various substrate concentrations as well the effect of the substrate concentration on the velocity of reaction are presented in Figure 6. The velocity of the reaction was evaluated from the initial slope of the time optical density curves (Fig. 6). The velocity represented against the substrate concentration is represented in Figure 7. Michaelis constant $K_M$ = 9.51 mM and maximum enzymatic velocity $V_{max}$ = 0.184 mM/min for the experiment was estimated by fitting of the experimental data with analytical hyperbola described by Eq. (6).

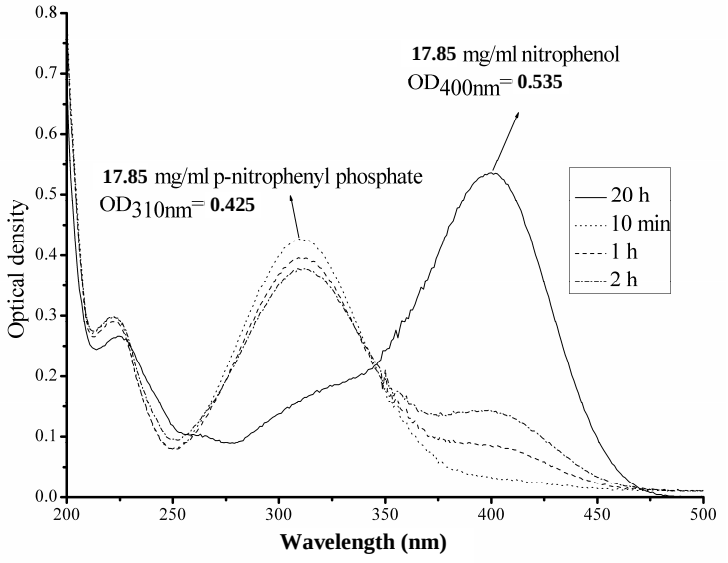


Fig. 5. Absorption spectra of p-nitrophenyl phosphate and the enzymatic product p-nitrophenol.

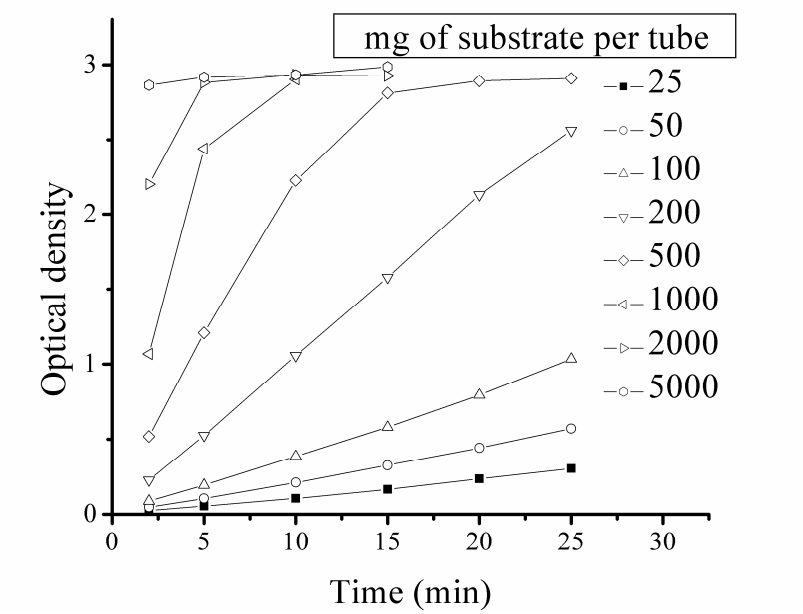


Fig. 6. Time evolution of enzymatic reaction at various amounts of substrate.

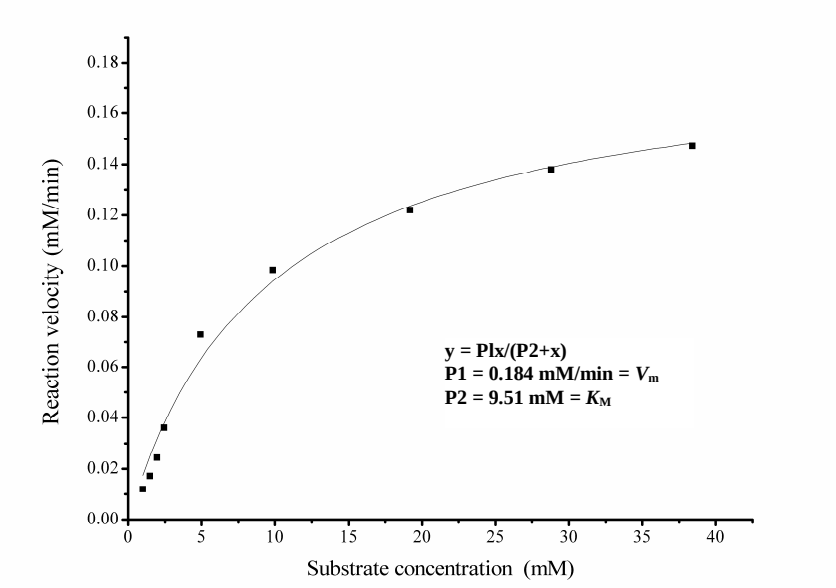


Fig. 7. The reaction velocity *versus* concentration of the substrate.

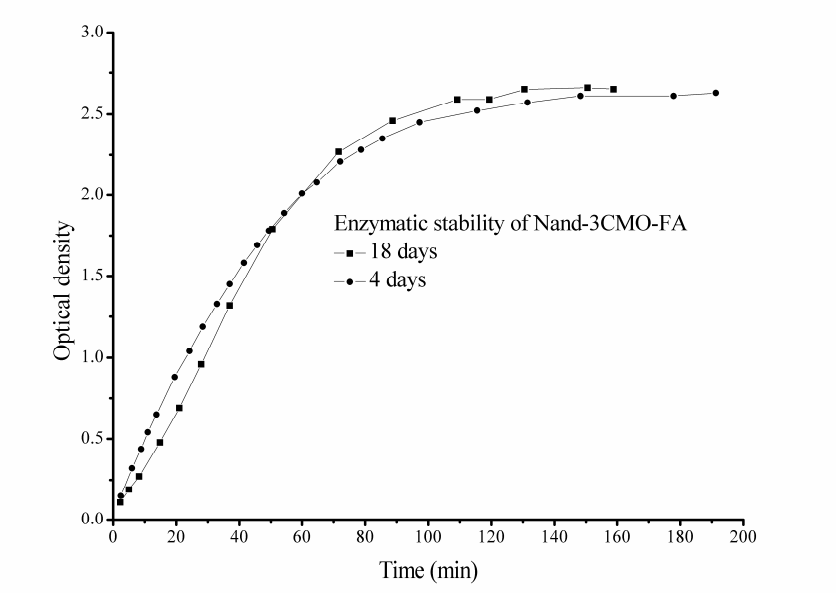


Fig. 8. Time stability of the enzymatic marker Nand-3-CMO-PhA.

Immunological properties of the marker were determined in a competitive reaction of the nandrolone-3-CMO-phosphatase alkaline and nandrolone with antinandrolone antibodies used as reagents in an ELISA system. The results of this experiment are given in Table 1.

*Table 1*

Optical absorbance at 400 nm for nandrolone-3-CMO-phosphatase alkaline-antinandrolone antibody system

| Sample | Nandrolone (ng/well) | Enzymatic markers (µl) | Absorbance at 400 nm (arbitrary units) |
|---|---|---|---|
| 1 | 0 | 100 | 2544 |
| 2 | 0.1 | 100 | 2576 |
| 3 | 0.5 | 100 | 1832 |
| 4 | 1.0 | 100 | 1104 |
| 5 | 5.0 | 100 | 763 |
| 6 | 10.0 | 100 | 756 |
| 7 * non-specific | – | 100 | 815 |

*Wells without antibodies

For a very low amount of nandrolone the antibodies linked on the well bottom react only with enzymatic markers and the concentration of the enzymatic product (evaluated here by the absorbance at 400 nm) is high. Increasing the nandrolone amount the competition with enzymatic conjugate occurs and a smaller number of enzymatic marker molecules are coupled to antibodies resulting in a lower level of absorbance.

## CONCLUSIONS

The enzymatic characteristics: maximum velocity of the enzyme reaction, $V_{max}$ = 0.184 mM/min, Michaelis constant, $K_M$ = 9.51 mM, the specific enzymatic activity and the time stability are defining the quality of the enzymatic marker which is going to be used in ELISA technique for assay of nandrolone from biological samples. Therefore, enzymatic marker nandrolone-3-CMO-phosphatase alkaline presents both properties: enzymatic, by reaction of marker with p-nitrophenylphosphate, substrate for alkaline phosphatase, and immunological by reaction with antinandrolone antibodies. Concluding the presented reagent can be used as enzymatic marker in ELISA technique.

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