Article

Br. J. Anal. Chem., **2019**, *6* (23) pp 12-19
DOI: 10.30744/brjac.2179-3425.AR.137-2018

Development of Spectrophotometric Method for the Determination of 17α-Methyltestosterone

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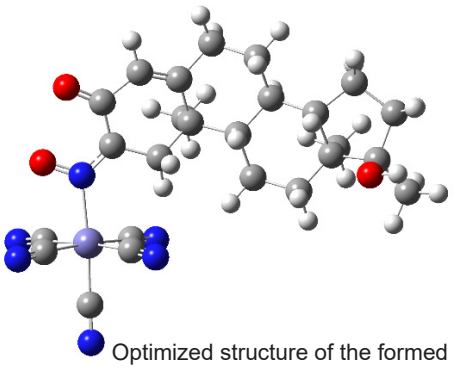
Graphical Abstract



Optimized structure of the formed compound between nitroprusside ion and 17α-methyltestosterone

Contamination of waters by 17α-methyltestosterone (MT) has increased in recent years due to the increase in the fish farms production. This work describes the determination of the MT hormone by a new spectrophotometric method in the visible region, after the reaction with potassium nitroprusside with the generation of a compound having maximum absorbance at 400 nm. To understand the process of the compound formation, geometry optimizations were performed at MPW1PW91/6-311+G (2d,2p) level of theory and the calculations showing that the NO group of nitroprusside bonds to the carbon with double bond adjacent to the carbonyl of 17α-methyltestosterone. In this method, MT was determined at the concentration between 8.0 x 10^{-8} and 2.7 x 10^{-7} mol L^{-1} with a coefficient of determination of 0.9946. The relative standard deviation for the repeatability in nine replicates was 2.37% for [MT] = 1.50 x 10^{-7} mol L^{-1}. Limit of detection (LOD) was calculated as 1.75 x 10^{-8} mol L^{-1} and the limit of quantification (LOQ) was established as the lowest value of the linear concentration range (8.0 x 10^{-8} mol L^{-1}). This spectrophotometric method was suitable according to the analysis of analytical figures of merit, it has low cost and can be applied to samples of fish farm waters with portable spectrophotometers for *in situ* measurements, increasing the environmental control with rapid analysis.

Keywords: hormone, fish farm, analytical method.

INTRODUCTION

The use of the synthetic hormone 17α-methyltestosterone (MT) (Figure 1) in fish farms to induce male sex cultures has been going on for a long time [1]. Male specimens are of greater economic interest since they have higher growth rate given that high-energy losses associated with female gonadal development and reproduction are avoided [2].

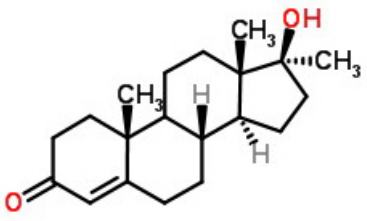


Figure 1. Molecular structure of 17α-methyltestosterone.
Source: ChemSpider (Copyright Royal Society of Chemistry, 2018)

However, the intensive use of MT added to feed (60 mg kg^{-1}) in fish farms, still poses concerns regarding the risks to consumers and environmental health [2]. The routes of contamination of water involve uneaten food and elimination of the non-metabolized hormone [3]. Steroid hormones such as MT are quickly absorbed into the sediments due to their hydrophobic nature and its elimination in water after its application is due to adsorption in sediment, biotransformation or photodegradation processes and are dependent on several factors such as pH and organic content of the medium, presence of Fe (III) and salinity [2-6].

The hormone 17α-methyltestosterone to induce several developmental and biochemical effects in zebrafish early life stages. The most important developmental effects included tail malformations, edemas, abnormal development of the head and hatching delay [7]. The sexual changes caused by MT in crocodiles in their habitat demonstrated the increase in the proportion of males in relation to the control with the introduction of this hormone coming fish farms [8,9], indicating the need for control and rapid determination of MT in environmental compartments.

Therefore, the need to determine the levels of MT in water and its implication in environmental, emphasizes the importance of appropriate analytical methods that can quantify MT with reliability, speed and low cost. The most widely used analytical methods for quantifying MT are based on chromatographic techniques with UV detection or coupled mass spectrometry [10-15] or chemiluminescence [16], as well as electrochemical methods in the determination of 17α-methyltestosterone [17].

These methods have versatility, sensitivity and efficiency, but it has as disadvantages the need for using potentially toxic solvents or reagents or harmful to the environment and instrumentation with high cost, often not portable, leading to the search for more environmentally friendly methods that can be readily accessible.

Spectrophotometry in the visible region is a technique that can be used directly in the productive sector of fish farming due to the lower cost, ease of operation and staff training besides the portability, allowing the control of MT levels directly in the property. There are few studies involving the determination of MT by spectrophotometry among which methods in the UV region at 241 nm [18,19], but several organic molecules present in the sample matrix can absorb in the ultraviolet region, with decreasing the resolution of the analytical method. Methods that use the visible region can have less interference and lower cost and are essential for application in several fields of the productive sector.

In this work, the development of a spectrophotometric method for the determination of MT was based on the reaction with the NO group of the potassium nitroprusside ($[Fe(CN)_5NO]^{2-}$) in alkaline medium to give the anion compound [20] and the formed compound absorbs in the visible region. To provide a better understanding to the experimental UV-VIS spectrum, a theoretical study was carried on MT-nitroprusside, since there is no mention of this compound in the literature.

MATERIALS AND METHODS

Chemical reagents were of highest purity (>99%). The water used for preparing stock solutions or dilutions was distilled and purified by reverse osmosis (ADAMO, water resistance: 10 MΩ cm^{-1} at 25.0 °C). The stock solution of MT was prepared from pharmaceutical standard (101.5 ± 2.0% purity) by diluting in ethanol (99%, Sigma-Aldrich).

Appropriate volumes of stock solution were transferred to volumetric flasks using micropipette (Labmate, ± 0.82%), adding then enough potassium nitroprusside solution (5% w/v) to 1:1 stoichiometry with the MT [20], and diluted with NaOH solution (30% w/v) with final pH of 13.0. Measures have been taken in spectrophotometer Shimadzu UV-1601 PC, double beam spectrophotometer (resolution of ± 1 nm).

The spectra of methyltestosterone and nitroprusside solutions were obtained before and after the

formation of the product at wavelength between 200 and 700 nm to characterize and evaluate each possible overlapping band. The stability of the nitroprusside solutions and the formed compound were evaluated by measuring the absorbance of the solutions over time.

Geometry optimizations were performed at MPW1PW91/6-311+G (2d,2p) level of theory. The stationary points on the potential energy surface were characterized by calculating the Hessian matrix and analyzing the vibrational normal modes. The excitation transitions of MT-nitroprusside compound in implicit water were calculated using time-dependent density functional theory (TD-DFT) at the MPW1PW91/6-311+G (2d,2p) level of theory [21]. This functional was used as it provides good estimates for the excited states of interest for this work [22]. To obtain the excitation spectra of the studied compound, six excited states were calculated [23].

The solvent (water) was simulated by means of the self-consistent reaction field (SCRF) method based on the polarizable continuum model (PCM) [24,25]. All calculations were performed with the Gaussian 09 software [26].

The selectivity of the method was evaluated from study of interfering of organic functional groups in reaction with the nitroprusside of the proposed method for 17α-methyltestosterone for the substances: acetic acid, acetone, methylethylketone and ethanol. For this, 1.0 mL of pure substance (>99.0% purity) was mixed with 1.0 mL of 5% (w/v) sodium nitroprusside and diluted in 30% (w/v) sodium hydroxide solution in volumetric flask and the absorbance this solution was read in spectrophotometer (between 200 and 700 nm). The 17α-methyltestosterone solution (4.0×10^{-6} mol L^{-1}) was used as a control and the mixture of the reagents was used as blank. The interference of iron, aluminum and copper ions was evaluated during the recovery study with a sample of fishpond water.

The linearity was assessed by means of standard 17α-methyltestosterone curves in water with the points of the curve measured in quintuplicate. To determine the limit of detection (LOD), it was used the equation of the calibration curve data of higher sensitivity, obtained from the standard deviation of the arithmetic mean of the absorbance of the blank and the slope of the calibration curve multiplied by 3.3. The limit of quantification (LOQ) was established as the lowest value of the linear concentration range.

The precision of the method was evaluated by estimating the repeatability in nine replicates at the concentration of 1.50×10^{-7} mol L^{-1} of 17α-methyltestosterone and for three levels of 17α-methyltestosterone concentration (1.2×10^{-7} mol L^{-1}, 2.0×10^{-7} mol L^{-1} and 2.7×10^{-7} mol L^{-1}).

Accuracy was assessed by methyltestosterone pattern recovery studies added in samples of fishpond water in which there was no use of the hormone. The samples were collected in fish farm and characterized by the content of total iron, total copper and total aluminum by X-Ray Fluorescence, S2 Picofox, with Mo K radiation, 50 kV voltage and current of 602 μA. These metals could interact with the nitroprusside and could interfere with the analytical signal [27-29]. The fishpond water conductivity was measured by a LUTRON CD-4303 conductivity meter (2% accuracy of the full scale), calibrated with standard KCl solution (± 0.5%); the pH was measured by a LABMETER PHS-3B pHmeter (resolution ± 0.01) and combined glass electrode, calibrated with buffer solutions pH 7.0 (± 0.05) and pH 4.0 (± 0.02); and the turbidity was measured using a Tecnopon TB1000 digital turbidimeter (resolution 0.8) calibrated with standards between 0.1 and 1,000 NTU.

The proposed method was compared with the method of determination of 17α-methyltestosterone by UV from the analytical curves obtained from solutions of the standard.

RESULTS AND DISCUSSION

Figure 2 shows the absorption spectrum of the reaction product between nitroprusside and MT in alkaline medium, in which the maximum absorption is observed at 400 nm.

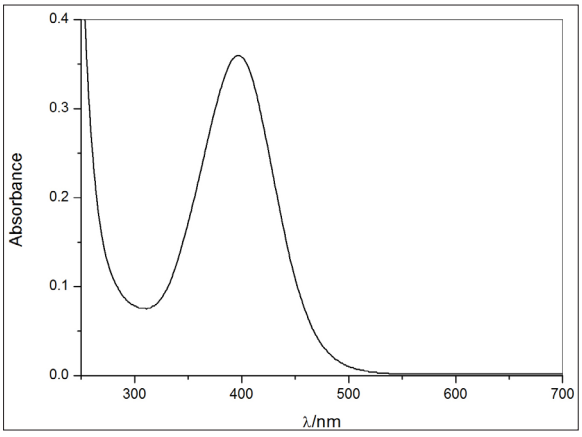


Figure 2. Visible spectrum of compound between nitroprusside solution (5% w/v) and MT (2.0 x 10^{-7} mol L^{-1}). Alkaline medium (pH = 13.0) and temperature of 25.0 °C.

Spectrophotometric measurements for potassium nitroprusside (5.0 x 10^{-5} mol L^{-1}) showed absorption at 271 nm concerning the nitro group and at 222 nm attributed to the strong absorption of the cyan group. The MT showed absorption at 241 nm and refers to enone, characteristic for the π-π* electron transition of unsaturated ketones, with ε = 17000 L mol^{-1} cm^{-1} [30]. These results demonstrate that pure MT solution and pure nitroprusside solution do not show absorption in the visible region and do not interfere with the measurements.

Stability tests indicated that the absorbance of the MT-nitroprusside compound has a decay constant of 0.0039 min^{-1} (Figure 3). The absorbance in 271 nm of the 5% (w/v) sodium nitroprusside solution drops to 3.65% day^{-1}, showing that the solution should be used on the same day of preparation.

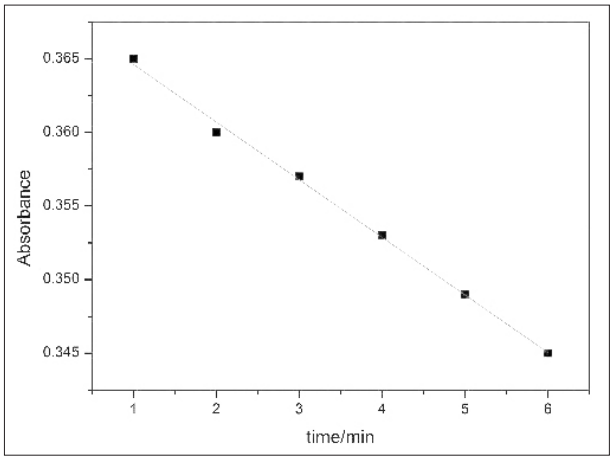


Figure 3. Decay of the MT-nitroprusside compound absorbance (2.04 x 10^{-7} mol L^{-1}) in alkaline medium (pH = 13.0) and 25.0 °C. R^2 = 0.9967.

In the alkaline medium, the nitroprusside ion is in equilibrium according to Equation 1 [31]. In the same way, the enolization of acetone group occurs (Equation 2) and the reaction between them generates the product $[Fe(CN)_5ON{=}CHCOCH_3]^{4-}$, which is also in agreement as proposed by Feigl [20].

$$[Fe(CN)_5NO]^{2-} + 2\ OH^- \leftrightarrows [Fe(CN)_5NO_2]^{4-} + H_2O \quad (1)$$

$$CH_3COCH_3 + OH^- \leftrightarrows CH_3COCH_2^- + H_2O \quad (2)$$

The proposal is that 17α-methyltestosterone follows the same compound formation mechanism with nitroprusside with the attack of the NO group on carbon with double bond adjacent to the carbonyl.

Figure 4 shows the optimized structure of the formed compound between nitroprusside ion and MT. To understand the absorption spectra observed, it was analyzed through theoretical calculations the most important electronic excitation in MT-nitroprusside compound, within the vertical approximation of Franck-Condon. Our calculations showing that the NO group of nitroprusside bonds to the carbon with double bond adjacent to the carbonyl of 17α-methyltestosterone.

Figure 5 illustrates the main molecular orbitals involved in the electronic transition studied. The HOMO (-1) orbital is mainly localized on the metallic cation, while the LUMO orbital is more delocalized in NO and C–C=O. The position of the orbitals in this compound may help to explain the electronic excitation. There is apparently a superposition of the orbitals HOMO (-1) and part of the LUMO located on NO fragment, which is expected to facilitate the excitation. In other words, the HOMO (-1) that has a large percentage of 3d(Fe) atomic orbital character overlaps with π*(NO). Similar vertical excitation has been detected both theoretically and experimentally for ground state of the nitroprusside compound [23,32].

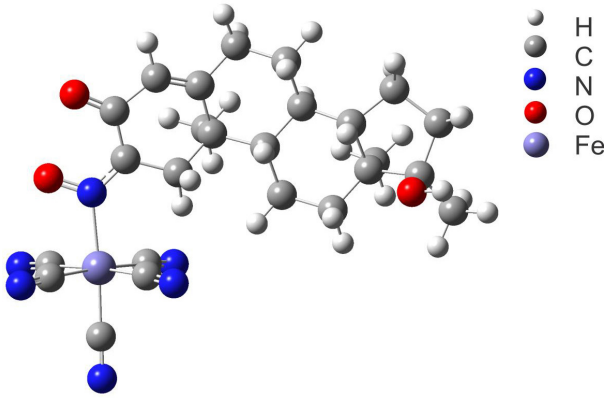


Figure 4. MT-nitroprusside compound in implicit water (● oxygen); (● nitrogen); (● iron); (● carbon)

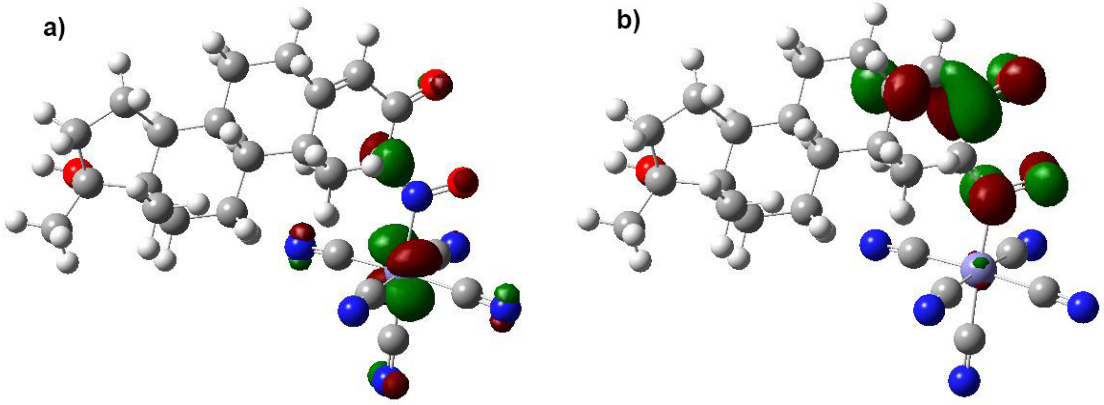


Figure 5. (a) ■ HOMO (-1) and (b) ■ LUMO orbitals of the MT-nitroprusside compound in implicit water (● oxygen); (● nitrogen); (● iron); (● carbon)

The interference of organic functional groups in the reaction with nitroprussiate showed the maximum absorbance for the ketone (λ_{max} = 403 nm), methylketone (λ_{max} = 396 nm) and alcohol groups (λ_{max} = 399 nm), but not of carboxylic groups (λ_{max} = 215 nm). This result points to the careful selection of substances

used in the extraction process of 17α-methyltestosterone in different matrices to avoid agents that may interfere with the analytical signal. However, considering possible 17α-methyltestosterone degradation processes in the environment, several studies in the literature indicate that there is no formation of ketones that could interfere with the analytical signal obtained by the proposed method [33-36].

Figure 6 shows the analytical curve obtained for different concentrations of MT, after the reaction with nitroprusside at λ= 400 nm.

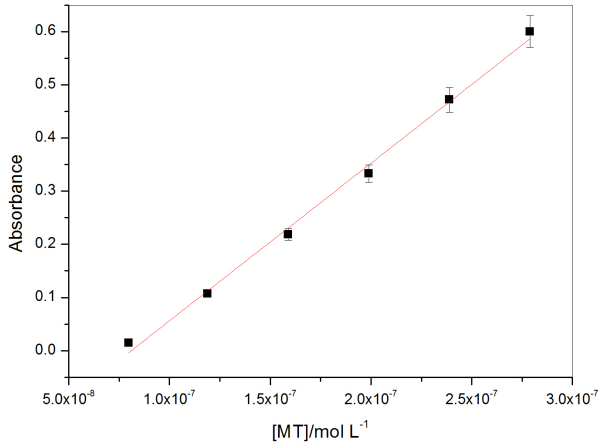


Figure 6. 17α-methyltestosterone analytical curve (quintuplicate). Temperature of 25.0 °C. Linear equation: y = -0.24 + 2.96325x10^6[MT]. Error bars of 5%.

The linear concentration range is between 8.0 x 10^{-8} and 2.7 x 10^{-7} mol L^{-1} (24.2 µg L^{-1} and 81.7 µg L^{-1}) with determination coefficient of 0.9946. This concentration range is adequate because the levels of methyltestosterone in the environment may be very low, between 5.1 x 10^{-8} and 3.3 x 10^{-7} mol L^{-1} [37].

The relative standard deviation for the repeatability in nine replicates (1.50 x 10^{-7} mol L^{-1}) was 2.37%. For three levels of concentration and in triplicate, tests of repeatability of MT showed that the values of relative standard deviation were 7.8% (1.2 x 10^{-7} mol L^{-1}), 1.9% (2.0 x 10^{-7} mol L^{-1}) and 1.5% (2.7 x 10^{-7} mol L^{-1}). Limit of detection (LOD) was calculated as 1.75 x 10^{-8} mol L^{-1} (5.29 µg L^{-1}). The limit of quantification (LOQ) was established as the lowest value of the linear concentration range (8.0 x 10^{-8} mol L^{-1}). Table I presents the comparison of LOD for MT in waters in some articles.

Table I. LOD values of MT in water reported in the literature

Method	LOD (µg L^{-1})	Reference
UV-HPLC	10.0	[5]
Luminescence	10.0	[16]
Hg electrode	3.07	[17]
UV-HPLC	3.60	[38]
visible	**5.29**	**This work**

The mean recovery was calculated as 101.5 ± 0.5% (triplicate) for the MT concentration of 2.4 x 10^{-7} mol L^{-1} added in surface water collected from fishpond (pH = 6.3, conductivity = 40.8 µS cm^{-1}, turbidity = 8.7 NTU and ionic strength of 0.01 mol L^{-1}) and indicated that the concentration of 1.46 x 10^{-3} mol L^{-1} of Fe^{3+}, 1.89 x 10^{-5} mol L^{-1} of Cu^{2+} and 4.64 x 10^{-3} mol L^{-1} of Al^{3+} found in this sample do not interfere with the measurements.

The results of the determination of 17α-methyltestosterone in water samples obtained by the proposed method were compared with a direct spectrophotometric method in the ultraviolet region and are presented in Figure 7. The results were evaluated by Student's t-test for differences in the confidence level of 95% and showed that there was no statistically significant difference between the results. The slope of the linear curve near 1 indicates a high correlation between the methods.

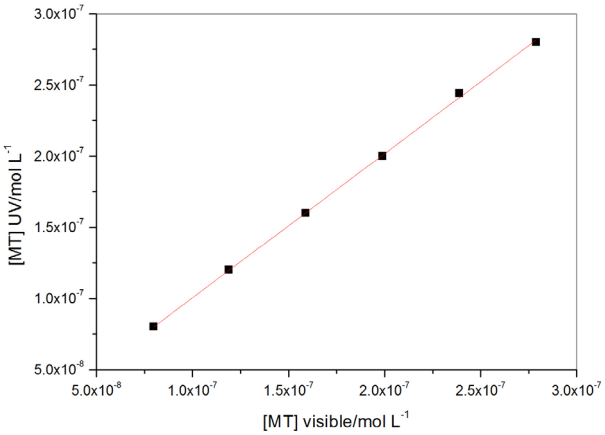


Figure 7. Comparison between the proposed method (visible) and the direct UV method. R^2 = 0.9998 with slope = 1.012.

CONCLUSION

The reaction between nitroprusside and 17α-methyltestosterone resulted in a yellow colored product that can be used *in situ* spectrophotometric determinations in the visible region and considered suitable according to the analytical figures of merit. Theoretical calculations reveal that the absorption spectrum observed for MT-nitroprusside compound is mainly due to 3d(Fe)→π*(NO) electronic transition. This method can be applied *in situ* for analyses of fish farms water samples, with portable and low cost spectrophotometers, which increases environmental control by rapid analysis.

Manuscript submitted: Dec. 12, 2018; revised manuscript submitted: March 13, 2019; manuscript accepted: April 8, 2019; published online: June 11, 2019.

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