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Screen-printed enzymatic biosensor modified with carbon nanotube for the methimazole determination in pharmaceuticals formulations

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ABSTRACT

This paper describes the development of a screen-printed sensor, modified with carbon nanotubes for the rapid and sensitive quantification of methimazole (MT) in pharmaceuticals formulations. Tyrosinase [EC 1.14.18.1], immobilized on a rotating disk, catalyzed the oxidation of catechols to *o*-benzoquinone, whose back electrochemical reduction was detected on graphite screen-printed electrodes modified with carbon nanotubes at −150 mV. Thus, when MT was added to the solution, this thiol-containing compound participate in Michael type addition reactions with *o*-benzoquinone to form the corresponding thioquinone derivatives, decreasing the reduction current obtained proportionally to the increase of its concentration. This method could be used to determine MT concentration in the range of 0.074–63.5 μM ($r = 0.998$). The determination of MT concentration was possible with a detection limit of 0.056 μM in the processing of as many as 25 samples per hour. The biosensor has a reasonable reproducibility (R.S.D. < 3.50%) and a very stable amperometric response toward this compound (more than 1 month). The application of this analysis to different pharmaceutical samples containing MT supports the utility this biosensor.

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1. Introduction

Methimazole (MT, 1-methyl-2-mercaptoimidazole, tapazole) is an orally active drugs used in the therapy of hyperthyroidism. MT is absorbed by the gastrointestinal tract and concentrates in the thyroid gland [1]. MT is widely used in medicine for treatment of hyperthyroidism and even as model substance for endocrine disruption in physiological and genomic studies. Its action is to slow iodide integration into tyrosine and thus inhibits the production of thyroid hormones.

Methimazole is used as a drug to manage hyperthyroidism associated with Grave's disease, but it has side effects as possible decrease of white blood cells in the blood [2]. MT has also been employed to promote growth in animals for human consumption.

In human body, methimazole is metabolized to *N*-methylimidazole and sulfite via sulfenic and sulfinic acid intermediates that are associated with the cytotoxic effects [3]. Substantial portion of orally taken drug is excreted with urine [4]. It has been reported that methimazole may also cause side effects, such as nephritis, liver cirrhosis, irritation of the skin, allergies and pharyngitis with fever [5].

Several analytical procedures have been described for the determination methimazole in different samples. Techniques used were gas chromatography–mass spectrometry (GC–MS) [6-8], high-performance liquid chromatography–mass spectrometry (HPLC–MS) [9,10], HPLC with ultraviolet detection [11,12], potentiometric [13], titrimetric [14] and flow-injection with ultraviolet detection [15].

Screen-printing technique seems to be one of the most promising approaches allowing simple, rapid and inexpensive biosensors production [16]. The biosensors based on screen-printed electrodes have been extensively used for detections of biomolecules, pesticides, antigens and anions [17]. Electrochemical biosensors based on screen-printed electrodes are in tune with the requirements of in situ screening devices, since all the equipment needed for the electrochemical analysis is portable. They have all the major performance characteristics of biosensors, among them the minimum sample preparation, the simplicity of the apparatus, the obtaining of fast results, moreover they are cost effective, small and becoming miniaturized with new technologies [18].

Carbon nanotubes (CNTs) are a novel type of carbon material and can be considered as the result of folding graphite layers into carbon cylinders. There are two groups of carbon nanotubes, multi-walled carbon nanotubes (MWCNT) and single-walled carbon nanotubes (SWCNT) [19]. The CNTs have generated great interest in future applications based on their field emission and electronic transport properties [20], their high mechanical strength and their chemical properties [21].

The research has been focused on their electrocatalytic behaviours toward the oxidation of biomolecules and their perfor-

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mance has been found to be much superior to those of other carbon electrodes in terms of reaction rate, reversibility and detection limit [22]. The uses of CNTs for preparation of biosensors based on CNT-modified screen-printed electrodes have been reported previously [23–27].

Tyrosinase a two copper-containing enzyme, catalyzes the *o*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols (Q) to *o*-quinones (P) (diphenolase activity) [28–30]. Over the past decades, several reports on the tyrosinase action mechanism have been published [31–34]. This enzyme has been used extensively in the development of biosensors for the detection of phenolic compounds [35–37]. To the best of our knowledge, no study involving an enzymatic biosensor behaviour for MT has been reported. Thus, in this paper, we present and discuss for the first time the electrochemical and enzymatic reaction for MT determination, resulting in a single, fast and inexpensive analytical method as well as very sensitive devise based on tyrosinase rotating biosensor systems.

In this paper, we performed a screen-printed enzymatic sensor modified with MWCNT for rapid and sensitive quantification of MT in pharmaceutical preparations. Tyrosinase immobilized on a rotating disk, catalyzed the oxidation of catechol (Q) to *o*-benzoquinone (P), whose back electrochemical reduction was detected on graphite screen-printed electrodes (GSPE) at −150 mV versus Ag/AgCl/NaCl 3 M. Thus, when MT was added to the solution, this thiol-containing compound participate in Michael type addition reactions with P to form the corresponding thioquinone derivatives, decreasing the reduction current obtained proportionally to the increase of its concentration. A large number of samples can be processed by means of the proposed method, which shows adequate sensitivity, low cost, versatility, simplicity and effectiveness. Our aim was to develop a new method able to analyze pharmaceuticals formulations, avoiding or minimizing the number of steps needed to assess the concentration of the MT.

2. Materials and methods

2.1. Reagents and solutions

All reagents used were of analytical reagent grade. The enzyme tyrosinase (from mushroom, EC 1.14.18.1, 2000 U mg^{-1}) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The enzyme concentration was determined taking the value of M_r as 120,000. Glutaraldehyde (25% aqueous solution) was purchased from Merck, Darmstadt. 3-Aminopropyl-modified controlled-pore glass, 1400 Å mean pore diameter and 24 m^2 mg^{-1} surface area, was from Electro-Nucleonics (Fairfield, NJ) and contained 48.2 mol g^{-1} of amino groups. GSPE was purchased from Eco-BioServices&Researches S.r.l. (Fienze, Italy). Catechol and MT were purchased from Sigma Chemical Co., St. Louis, and all other reagents employed were of analytical grade and used without further purifications. Aqueous solutions were prepared using purified water from a Milli-Q-system.

2.2. Flow-through reactor/detector unit

The main body of the cell was made of Plexiglas. Fig. 1 illustrates the design of the flow-through chamber containing the rotating disk and the detector system. The GSPE is on the top of the rotating reactor. The rotating reactor is a disk of Plexiglas into which a miniature magnetic stirring bar has been embedded. Rotation of the lower reactor was effected with a laboratory magnetic stirrer with control of temperature (Metrohm AG, Herisau, Switzerland) and controlled with a variable transformer with an output between 0

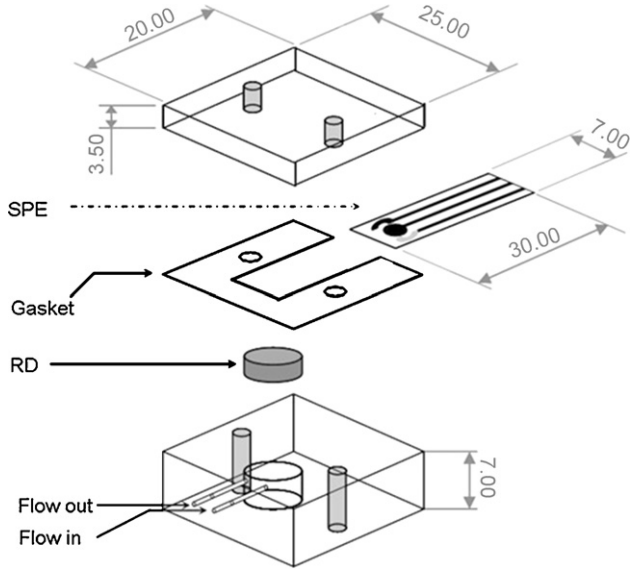

Fig. 1. Schematic representation of components in the bioreactor flow cell. SPE: Screen-printed electrode, RD: rotating disk. All measurements are given in millimetres. Gasket: Teflon, thickness: 0.3 mm.

and 250 V and maximum amperage of 7.5 A (Waritrans, Argentina). All solutions and reagents were conditioned to 37 °C before the experiment, using a laboratory water bath Vicking Mason Ii (Vicking SRL, Argentina).

Amperometric detection was performed using a BAS LC-4C potentiostat and BAS 100 B/W (electrochemical analyzer Bioanalytical System, West Lafayette, IN) was used to voltammetric determinations.

A pump (Wilson Minipuls 3 peristaltic pump, Gilson Electronics, Middleton, WI, USA) was used for pumping, introducing the sample, and stopping the flow. Fig. 2 illustrates schematically the components of the single-line continuous-flow setup. The pump tubing was Tygon (Fisher Accu Rated, 1.0 mm i.d., Fisher Scientific, Pittsburgh, PA, USA), and the remaining tubing used was Teflon (1.0 mm i.d. from Cole-Parmer, Chicago, IL, USA).

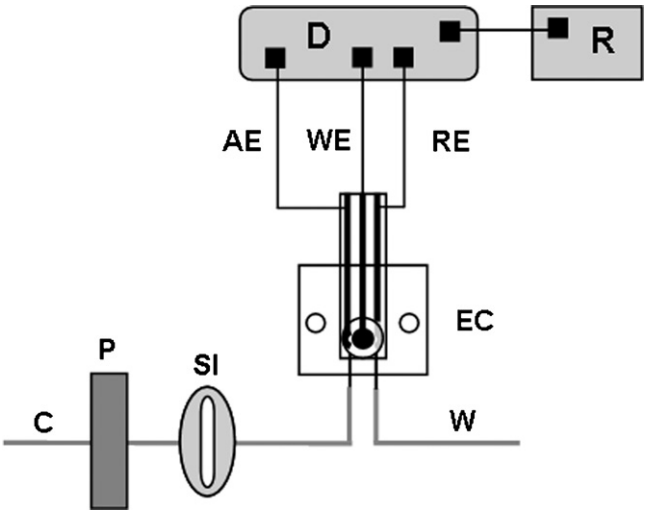

Fig. 2. Block diagram of the continuous-flow system and detection arrangement. P: Pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics, Inc. Middleton, WI); C: carrier buffer line; SI: sample injection; W: waste line; EC: cell containing the rotating disk and GSPE; WE: GSPE; RE: pseudo-reference electrode; AE: auxiliary electrode; D: BAS LC-4C potentiostat (Bioanalytical Systems, West Lafayette, IN, USA); R: recorder.

All pH measurements were made with an Orion Expandable Ion Analyzer (model EA 940, Orion Research, Cambridge, MA, USA) equipped with a glass combination electrode (Orion Research).

2.3. Preparation of the CNTs-modified GSPE

An electrode pretreatment was carried out before each voltammetric experiment in order to oxidize the graphite impurities and to obtain a more hydrophilic surface [38], with the aim of improving the sensitivity and reproducibility of the results. The graphite electrode surface is pretreated applying a potential +1.6 V (versus Ag-SPE) for 120 s and +1.8 V (versus Ag-SPE) for 60 s in 5 ml of 0.25 M acetate buffer, containing 10 mM KCl (pH 4.75), under stirred conditions. Then, the electrodes were washed using 0.01 M phosphate buffered saline (PBS), pH 7.2 and stored in the same buffer at 4 °C.

One milligram of MWCNT was dispersed with the aid of ultrasonic stirring for 45 min in methanol/water (50:50, v/v) in an aqueous 0.1% Nafion solution. A 5 μl aliquot of this dispersion was dropped on the screen-printed graphite working electrode surface and then the solvent was evaporated under an infrared heat lamp [39].

2.4. Tyrosinase immobilization

The rotating disk reactor (bottom part) was prepared by immobilizing tyrosinase on 3-aminopropyl-modified controlled-pore glass (APCPG). The APCPG, smoothly spread on one side of a double-coated tape affixed to the disk surface, and was allowed to react with an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 M carbonate) for 2 h at room temperature. After washing with purified water and 0.10 M phosphate buffer of pH 7.00, the enzyme (5.0 mg of enzyme preparation in 0.25 ml of 0.10 M phosphate buffer, pH 7.00) was coupled to the residual aldehyde groups in phosphate buffer (0.10 M, pH 7.00) overnight at 5 °C. The immobilized enzyme preparation was finally washed with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C between uses. The immobilized tyrosinase preparations were perfectly stable for at least 1 month of daily use.

2.5. Analysis of pharmaceutical samples

Ten tablets or the contents of 10 tablets were weighed form each dosage forms and powdered. Equivalent amount to one tablet was weighed and transferred to a 100 ml volumetric flask. The flask was sonicated for 10 min and filled with 0.05 M phosphate buffer, pH 7.0. Appropriated solutions were prepared by taking suitable aliquots of the clear supernatant and diluting them with 0.05 M phosphate buffer, pH 7.0; and injected in to sample loop by means of a peristaltic pump. Aliquots of pharmaceutical samples were added into a 15 ml thermostated glass cell, homogenized with the aid of a magnetic stirrer, degassed with nitrogen for 1 min and the amperometric measurements were performed at −0.15 V and the resulting cathodic current was displayed on the *x–y* recorder.

2.6. Preparation of synthetic tablet samples

Synthetic tablet samples were prepared into a 100 ml calibrated flasks by spiking a placebo (mixture of tablet excipients, cellactose, sodium croscaramelose, magnesium estearate) with accurately amount of MT at a concentration similar to formulation concentration (5.0–20 mg.). Then, the procedure described above was followed.

2.7. Dosage forms of MT

(1) Danantizol 5 mg tablets (Gador) and (2) danantizol 20 mg tablets (Gador).

3. Results and discussions

3.1. Study of the enzymatic process

Reactions catalyzed by enzymes have long been used for analytical purposes in the determination of different analytes, such as substrates, inhibitors and also the enzymes. In this paper, we apply a tyrosinase biosensor for a highly sensitive determination of MT in pharmaceutical formulations. The measuring principle of this biosensor for the determination of MT is shown in Fig. 3. First, the tyrosinase immobilized on a rotating disk catalyzes the oxidation of Q to P [40,41], whose electrochemical reduction back to Q was obtained at peak potential of −150 mV. Second, the detection of the MT is accomplished by suppressing the substrate recycling process between tyrosinase and the electrode (denoted by the dotted arrow), decreasing the peak current obtained proportionally to the increase of MT concentration. Therefore, the detection principle is similar to biosensors based on substrate competition [42–44].

Nevertheless, when thiol-containing compound is added to the solution, readily undergo reaction with quinone derivative P, through the Michael type addition, decreasing the peak current obtained proportionally to the increase of thiol-containing compound concentration.

The initial reaction in the sequence (Q = P) is well established [45–47] with NMR, pulse radiolysis and a number of electrochemical techniques used to probe the mechanism. The potential analytical utility offered by the second step (1) as a method of detecting MT is explored in this paper.

3.2. Cyclic voltammetry of Q and MT on GCE

Cyclic voltammetry (first cycle) of 1 mM of Q in aqueous solution containing 0.05 M phosphate buffer pH 7.0, shows one anodic (A_1)

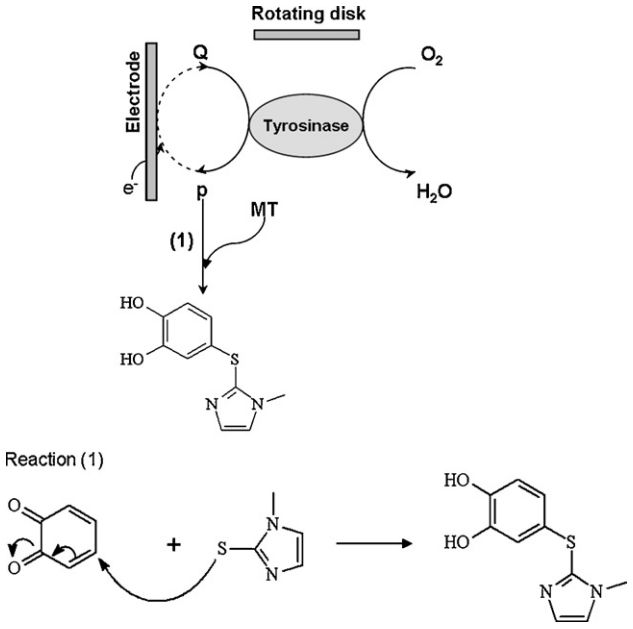

Fig. 3. Schematic representations of the reduction wave of the enzymatic process between catechol (Q), benzoquinone (P), methimazole (MT) and tyrosinase.

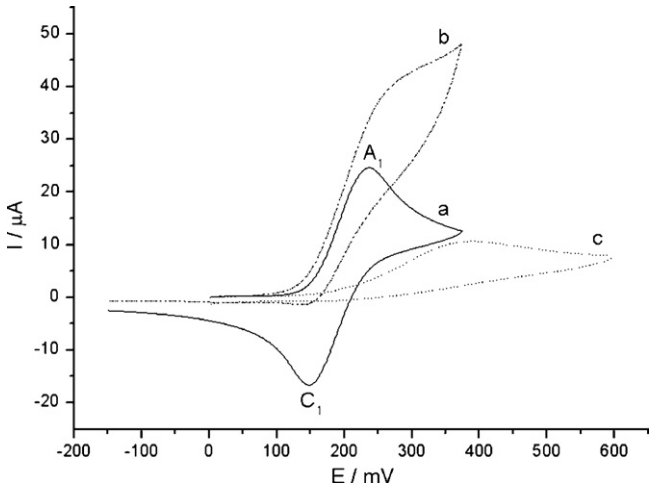

Fig. 4. Cyclic voltammograms of 1 mM Q (a) in the absence; (b) in the presence of 1 mM MT; (c) 1 mM MT in the absence of Q, at glassy carbon electrode (3 mm diameter) in aqueous solution containing 0.05 M phosphate buffer (pH 7.00). Scan rate: 25 mV s^{-1}; T: 25 ± 1 °C.

and corresponding cathodic peak (C_1), which corresponds to the transformation of Q to P and vice versa within a quasi-reversible two-electron process (Fig. 4, curve a). A peak current ratio (I_{C_1}/I_{A_1}) of nearly unity, particularly during the recycling of potential, can be considered as criteria for the stability of Q produced at the surface of electrode under the experimental conditions. In other word, any hydroxylation [48–51] or dimerization [52,53] reactions are too slow to be observed in the time scale of cyclic voltammetry.

The oxidation of Q in the presence of MT as sample in aqueous solution containing 0.05 M phosphate buffer pH 7.0 was studied in some details. The height of the oxidation peak was found to increase with increasing additions of MT with the loss of the corresponding reduction peak consistent with the ECE type mechanism proposed in Fig. 3 (Fig. 5 curves a–d). Hence, the increase in the oxidation peak height is attributed to the oxidation of Q-MT adducts that arises through the electrochemically initiated reaction (Fig. 3). In fact, once P is formed, could react with a variety of nucleophilic reagents, as those possessing sulfhydryl (SH) groups [54].

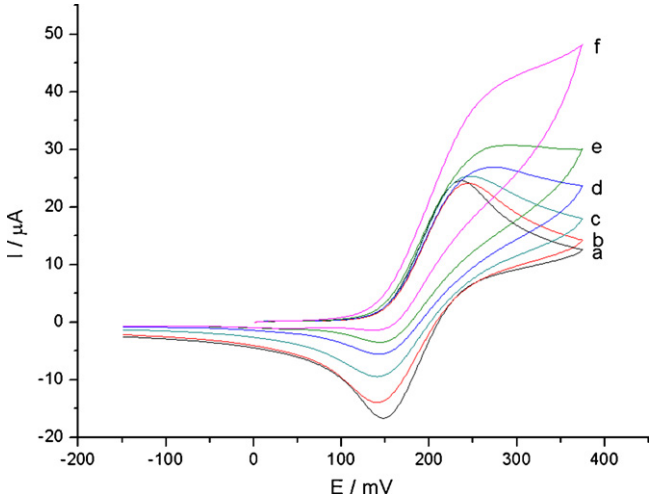

Fig. 5. Typical voltammograms of 1 mM of Q at a glassy carbon electrode (3 mm diameter) in aqueous solution containing 0.05 M phosphate buffer (pH 7.00) at various MT concentrations, C_{MT}: (a) 0.0, (b) 0.05, (c) 0.3, (d) 0.5, (e) 0.75 and (f) 1.0 mM; scan rate: 25 mV s^{-1}, T: 25 ± 1 °C.

Given that the direct oxidation of this thiol at the electrode does not occur within the potential window studied (Fig. 4, curve c), the increase in the magnitude of the Q oxidation peak can be attributed solely to the re-oxidation of the Q-MT adduct. Furthermore, the consequent decrease on the height of the P reduction peak can be ascribed to the fact that increasing concentration of MT scavenges the oxidized form of Q leaving little available for electro-reduction. The peak current ratio (I_{C_1}/I_{A_1}) versus concentration for a mixture of Q and MT confirms the reactivity between both, appearing as a decrease in the height of the cathodic peak C_1 at higher concentration of MT (Fig. 5).

3.3. Effect of reactor rotation and continuous-flow/stopped-flow operation

To optimise the proposed method is necessary to have an understanding of the effect of the parameters governing the system. The implementation of continuous-flow/stopped-flow programming and the location of two facing independent reactors (Fig. 1) permits: (a) utilization of relatively low enzyme loading conditions, (b) instantaneous operation under high initial rate conditions, (c) easy detection of accumulated products and (d) reduction of apparent Michaelis-Menten constant, K'_M. A more complete reagent homogenization is achieved, because the cell works as a mixing chamber by facilitating the arrival of substrate at the active sites and the release of products from the same sites. The net result is high values of current. The main advantages of this system are its simplicity, and the ease with which it can be applied to the determination of MT at low levels.

The effect of the rotation rate was evaluated in a range of 100–900 rpm. A significant increase of electric signal was observed in overall the range of rotation rate but insignificant differences were obtained for greater rotation velocities, in fact the current is constant, and chemical kinetics controls the overall process. Therefore, a rotation velocity of 900 rpm was used.

If the rotating disk in the cell is devoid of rotation, the response is lower because diffusional reactions are too slow to be observed in the time scale of electrochemical analysis. If a rotation of 900 rpm is imposed on the rotating disc at the bottom of the cell, the signal is dramatically enlarged.

As noted, rotation is expected to decrease the values of the apparent Michaelis-Menten constant, K'_M, since the catalytic efficiency is increased. K'_M which differ substantially from that measured in homogeneous solution, is not an intrinsic property of the enzyme, but of the system. This constant characterizes the reactor, not the enzyme itself. It is a measure of the substrate concentration range over which the reactor response is linear [55–57].

3.4. Effect of cell volume and sample size

Depending on the volume of the cell the overall process becomes controlled by diffusion (large volumes) or by the chemical kinetics of the enzyme-catalyzed reactions (small volumes). The cell volume was changed from 20 to 100 μl. The rate of response, as expected, decreased linearly with an increase in cell volume, due to the dilution effect favoured by rotation, and the fact that the measured current is directly proportional to bulk concentration. The smallest cell volume of 20 μl was adopted for further studies.

The sample size was studied in the range 5–50 μl. Sensitivity is almost tripled in the range between 5 and 20 μl (Fig. 6). Insignificant differences were obtained for greater sample size. A sample size of 20 μl was used to evaluate other parameters.

The rate of enzymatic response under flow conditions was studied in the pH range 4–8 and show a maximum value of activity at pH 7.0. The influence of pH on peak potential (E_p) of the reaction

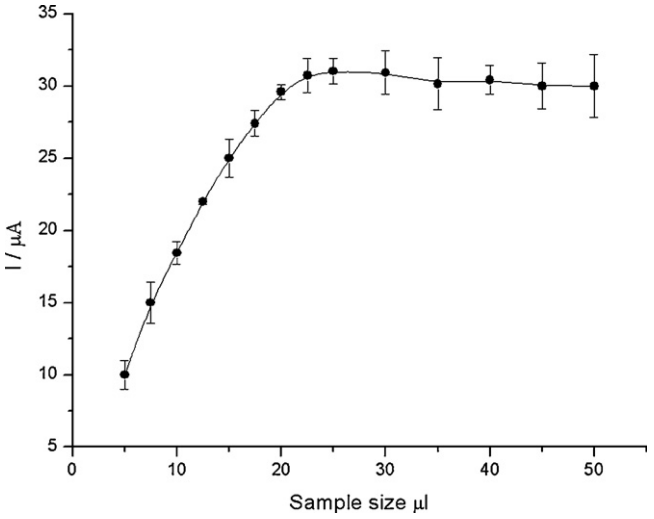

Fig. 6. Effect of sample size. Solution containing 1 mM of Q in 0.05 M phosphate buffer pH 7.00. Flow rate 100 μl min^{-1}. Each value of initial rate based on triplicate of six determinations.

was assessed through examining the electrode response to Q-MT obtained in solutions buffered between pH 4 and 8. Therefore, the pH value used was 7.00 in 0.05 M phosphate buffer in concordance with the steadier pH of the enzyme.

3.5. MT measurement with tyrosinase biosensor

The performance of the biosensor for the measurement of MT concentrations was characterized. For MT measurement a solution containing 1 mM Q was injected into the screen-printed biosensor and the flow was detained, thus, a large reduction current was observed due to the quinone derivative and after 1 min the flow was started again; after that a solution containing 1 mM Q, and several MT concentrations were injected into the rotating biosensor; and the reduction current was measured. The addition of MT resulted in a current decrease.

A linear relation (Eq. (1)) was observed between the rate of response and the MT concentration in the range of 0.074–63.5 μM.

$$\text{rate of response (mA/min)} = 29.52 - 269.33[C_{MT}] \quad (1)$$

The correlation coefficient for this type of plot was typically 0.998. Detection limit (DL) is the minimal difference of concentration that can be distinguished from the signal of the pure Q solution. The DL was calculated as the amount of MT required to yield a net peak that was equal to three times the S.D. of the pure Q signal, the DLs for amperometric detection 0.056 μM. Reproducibility assays were made using repetitive standards ($n = 5$) of 30 μM; the relative standard deviation was less than 3.72%.

The long-term stability of the enzymatic system to pharmaceutical formulations was study. In this experiment, after every five samples, a standard of 30 μM MT is injected to test the electrode response. In the FIA system using an enzymatic reactor, there is practically no decay in the catalytic current after six samples.

Table 1
Specificity results of the proposed method[a]

Sample no.	Pure sample 30 (μM)	Synthetic tablet sample ($n = 5$) X (μM)
1	30.02	30.26
2	29.97	30.18
3	30.07	29.92
4	29.98	29.90
5	29.94	30.13
$X \pm$ S.D.	29.96 ± 0.35	30.07 ± 0.47

[a] X (μM), mean ± S.D., standard deviation.

Table 3
Within-assay precision (five measurements in the same run for each control sample) and between-assay precision (five measurements for each control sample, repeated for three consecutive days)

Added[a] (mg L^{-1})	Within-assay		Between-assay	
	Mean	CV%	Mean	CV%
5	4.96	3.42	5.19	4.76
10	10.09	2.18	10.36	3.21
20	20.20	1.38	19.84	3.54

[a] mg L^{-1} MT.

3.6. Determination of MT in pharmaceuticals formulations

Specificity is the ability of the method to measure the analyte response in the presence of all the potential interference. For the specificity test, FIA of standard solution of tablet excipients were recorder at selected conditions. The response of the analyte with excipients, were compared with the response of pure MT. It was found that assay results were not changed. Therefore, the excipients did not interfere with the quantization of MT as such in synthetic as commercial tablet samples. In Table 1, the results are shown.

Recovery studies were performed by adding a synthetic mixture prepared according to the manufacturer's batch formula to known amount of MT. The recovery was 101.13%. The results are shown in Table 2.

The precision for MT was <3.5% within the range 5.00–20.0 mg (Table 2). Precision studies were performed by adding a synthetic mixture prepared according to the manufacturer's batch formula to known amount of MT. The accuracy for MT was <1.2% (Table 2).

The precision of the electrochemical assay was checked with control samples 5.0, 10.0 and 20.0 mg L^{-1} MT concentrations. The within-assay precision was tested with five measurements in the same run for each sample. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The results obtained are presented in Table 3. The MT assay showed good precision; the CV within-assay values were below 3.5% and the between-assay values were below 5%. There are no significant differences in the results, indicating that the analysis of MT tablets by the proposed method is reproducible.

Table 2
Accuracy and precision dates for MT obtained by amperometric measure

Added (mg)	Found (mg)	Recovery (%)	Precision (mg)	Accuracy[b] (% relative error)
5.00	4.96	99.20	X[a] = 4.96 ± 0.17, VC = 3.42%	−0.8
10.0	10.09	100.9	X = 10.09 ± 0.22, VC = 2.18%	0.9
15.0	15.17	101.13	X = 15.17 ± 0.45, VC = 2.96%	1.13
20.0	20.20	101.0	X = 20.20 ± 0.28, VC = 1.38%	1

[a] X = mean ± standard deviation.
[b] Accuracy = [(found − added)/added] × 100.

Table 4
Determination by the developed method of amount of MT contained in commercial, based on triplicate of six determinations

Sample no.	Danantizol 5 mg tablets (mg/capsule)	Danantizol 20 mg tablets (mg/capsule)
1	5.08	20.26
2	4.98	19.84
3	4.82	20.18
4	5.11	19.89
5	4.88	20.04
6	5.14	19.92
Average	5.01	20.02
S.D.	0.13	0.17

The developed FIA-biosensor method for the MT determination was applied to two commercial preparations (Table 4). There is no need for any extraction procedure before FIA analysis. No change of the peak height in the presence of the excipients was observed.

4. Conclusions

The usefulness of CNT-modified screen-printed electrodes enzymatic sensor as a determiner of very low concentrations of MT was demonstrated. The biosensor developed in this work is the first one developed for MT determination. This type of detection (addition reaction on cosubstrates) shows promise with regards to biological and pharmacological sensing.

In practice, the biosensor developed in this work is able to operate as a fast, selective and sensitive detection unit when is incorporated into a FIA system, also minimizes the wastage of expensive reagents, shows physical and chemical stability, low background current, wide working potential range, accuracy and does not require highly skilled technicians or expensive and dedicated equipment. This method is very simple and straightforward, it is the good applicability in pharmaceutical industry as a routine method.

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