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Biosensor for the characterisation of hMAO B inhibitors and the quantification of selegiline

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ABSTRACT

A novel human monoamine oxidase B (hMAO B) based biosensor for inhibitory measurements was developed. It allows both the characterisation of the type of enzyme inhibition and the sensitive and simple determination of inhibitors like selegiline hydrochloride. The sensor consists of a screen printed carbon working electrode modified with 20% manganese dioxide (MnO_2) and the enzyme hMAO B, which was immobilised on the electrode via a dialysis membrane (regenerated cellulose, molecular weight cut-off 14000). Inhibition of hMAO B is evaluated by adding different concentrations of the inhibitor selegiline hydrochloride to the enzyme and applying a defined amount of the hMAO B substrate phenylethylamine (PEA). The enzymatically formed H_2O_2 is amperometrically detected at 0.4 V vs. Ag/AgCl in a flow injection analysis (FIA) system. With 100 µM PEA the sensor showed a linear correlation between peak height and inhibitor concentration in a range of 0.51–3.25 µg/mL selegiline hydrochloride. LOD and LOQ were determined to be 0.15 and 0.51 µg/mL, respectively. The sensor showed a repeatability of 3.7% and an intermediate precision of 8.1%. The inhibition-based biosensor was successfully employed to quantify selegiline hydrochloride in pharmaceutical samples. Kinetic studies via Lineweaver-Burk plot and enzyme quantity vs. current plot revealed that the inhibition is irreversible.

1. Introduction

In humans, monoamine oxidases (MAOs) occur in two isoforms, type A (hMAO A) and B (hMAO B). They are responsible for the oxidative deamination of biogenic and dietary amines [1]. While showing partially overlapping tissue distribution, the two isoforms differ in their substrate specificity [2]. hMAO A reacts specifically with serotonin and norepinephrine. The biogenic monoamines phenylethylamine (PEA) and benzylamine are degraded by hMAO B. Dopamine, tyramine and tryptamine are deaminated by both isoforms equally. Both enzymes react with their respective amines in the presence of oxygen and water, producing the corresponding aldehyde, ammonia and hydrogen peroxide [3]. In humans hMAO A and B are involved in various (patho)physiological processes. Therefore, the inhibition of MAOs is used for the treatment of monoamine related diseases such as Parkinson's disease (PD), Alzheimer's disease and depression. Inhibitors can be categorised according to their isoform selectivity, also including isoform non-selective substances, as well as their type of inhibiting the enzyme including reversible and irreversible inhibitors [4]. While inhibitors of hMAO A such as moclobemide are used as antidepressants, hMAO B selective inhibitors are applied for the treatment of PD with selegiline (deprenyl) being one of the most common substances [5]. In connection to neurodegenerative diseases especially hMAO B is part of ongoing investigations. Beside PD it was also shown that hMAO B inhibitors improve memory formation in Alzheimer's disease patients. For this reason there have been increased efforts to design and discover drugs inhibiting hMAO B for the treatment of PD, Alzheimer's disease and other disorders of memory and cognition [6]. In investigating inhibitors of hMAO B it is important to figure out the type of inhibition. Compared to reversible inhibitors irreversible substances show high efficiency to target disruption, an increased duration of action and less sensitivity toward pharmacokinetic parameters. Reversible inhibitors however show the advantage of saver profiles [7].

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In this connection inhibition-based biosensors can be novel interesting analytical tools as they can allow both, the possibility of investigating the type of inhibition and of determining the inhibitor concentration [8]. While the number of publications dealing with inhibition-based biosensors has been increasing during the last years [9], most of the reported sensors use acetylcholinesterase [10–13] or butyrylcholinesterase [14,15] for the determination of pesticides including paraoxon, malathion, monocrotophos and ethion. Furthermore, there are publications showing the quantification of active agents via inhibition of acetylcholinesterase including the drugs codeine [16], donepezil [17], galantamine and neostigmine [18]. Publications dealing with inhibition-based MAO biosensors are rare. Determinations of naturally occurring reversible MAO inhibitors [19] and of antidepressants [20] by applying a combination of MAO A and B as well as solely MAO A as bioreceptors were described. To the best of our knowledge the current sensor is the first one using well characterised human hMAO B expressed in *Pichia pastoris* for the determination of an irreversible inhibitor (selegiline hydrochloride) in pharmaceuticals. Existing methods for the determination of selegiline include chemiluminescence [21], gas chromatography with mass spectrometry (GC/MS) [22], high performance liquid chromatography with mass spectrometry (HPLC/MS) [23], spectrophotometry [24] and potentiometry [25]. While most of the described procedures show good sensitivity, they also often need high cost instrumentation and require complex and time-consuming measurements. Biosensors however share the advantages of being accurate, rapid and economical besides showing good sensitivity and are highly suitable for the quantification of target analytes in complex matrices [26]. Therefore, a simple and cost-effective sensor for the determination of selegiline is a useful approach with the additional benefit of being able to characterise parts of its pharmacological properties in humans in terms of the type of enzyme inhibition. For these reasons the aim of this work was the development of an inhibition-based MAO B sensor as novel analytical tool which allows both the characterisation of the kinetic behaviour and the quantification of inhibitors (selegiline) in real samples with the same biosensor design.

2. Materials and methods

2.1. Reagents

The enzyme solution contained hMAO B from *Pichia pastoris* in 50% glycerol (specific activity 0.54 U/mL) and was fermented and purified as described by Newton-Vinson et al. [27]. The purification was performed with slight modifications concerning the ion exchange column for the final step, where a High Q XK16-20 strong ion-exchange column was used. The dialysis membrane for immobilising the enzyme solution (regenerated cellulose, molecular weight cut-off 14000) was obtained from Spectrum Laboratories. Phenylethylamine hydrochloride, manganese dioxide (MnO_2, 99.99%), selegiline hydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Triton X-100 reduced were obtained from Sigma Aldrich. Na_2HPO_4 and KH_2PO_4 were purchased from Merck. Further chemicals included carbon ink (Electrodag 421 SS) from Acheson, silver conductive paint from Ferro as well as H_2O_2 (3% m/m), glycerol and “Selegiline Hexal 5 mg” tablets which were purchased from a local pharmacy. Pre-etched ceramic substrate alumina plates from Coors Ceramics were used to produce the working electrodes. For all solutions Milli Q high purity deionised water was used.

2.2. Apparatus

Flow injection analysis was performed using a Merck/Hitachi L-6200 A pump, a Perkin Elmer injection valve with a 20 µL sample loop, and a Metrohm Autolab PGSTAT128N electrochemical detector with Nova 1.8 software. The applied three electrode system consisted of a BASi MF-1092 cross-flow cell with integrated stainless steel auxiliary electrode, a Ag/AgCl 3 M KCl reference electrode, and a screen printed carbon working electrode. A cell gasket (thickness 125 µm) was used between working electrode and flow cell. For homogenisation of the carbon ink-mediator mixture an ELMA Transsonic 660/H ultrasonic bath was used. Enzyme and inhibitor solutions were mixed using an IKA MS 1 mixer. Centrifugation of the tablet solutions was performed by using a Labofuge 400 from Heraeus Instruments.

For the photometric measurements a Shimadzu UV-1800 UV/VIS spectrophotometer was used.

2.3. Production of the screen printed working electrodes by serigraphy

To print the electrodes, the alumina substrates were fixed together with a self-made template. For the production of 30 working electrodes 2 g of the printing ink mixture were prepared. For this purpose carbon ink was mixed with the respective amounts of MnO_2 to reach a mediator percentage between 5% and 35%. The respective mixtures were thoroughly homogenised in a beaker for 10 min and then put into the ultrasonic bath for 30 min. Following this, the respective mixture was applied on one side of the template and spread over to the other side. After removing the template the electrodes were dried for 24 h at room temperature. On one side of each electrode a small amount of silver conductive paint was applied to enable the connection to the electrochemical detector.

2.4. Investigation of time-dependency of selegiline hydrochloride

The time dependent behaviour of selegiline hydrochloride on hMAO B was investigated by photometric enzyme activity measurements as described in [27,28]. Briefly, a photometric activity assay measuring the production of benzaldehyde out of benzylamine was performed. The photometric measurements were carried out at 250 nm over a period of 300 s each and the respective activity was calculated according to the following formula: act [U/mL] = ΔA per min/ε*f (ε = 12.8 M^{-1} cm^{-1}, f = 200). For all measurements activity assay buffer (50 mM HEPES, 0.5% Triton X-100 reduced, adjusted to pH 7.5) and benzylamine solution (1%, v/v) were used. For the blank 970 µL activity assay buffer and 30 µL benzylamine solution were mixed. For the samples 960 µL activity assay buffer, 30 µL benzylamine solution and 10 µL of a mixture of hMAO B and inhibitor (1.0 µg/mL selegiline hydrochloride solution mixed with hMAO B 1:1 (v/v)) were used. For the determination of uninhibited hMAO B, 5 µL of the enzyme were mixed with 5 µL activity assay buffer. Measurements were performed immediately after mixing of enzyme and inhibitor solution and subsequently every 10 min (n = 2).

2.5. Preparation of inhibition-based biosensors

To prepare the biosensors, the enzyme solution was mixed on a vortex mixer (1000 U/min, 30 s) with the same amount (v/v) of buffer (Sørensen phosphate buffer, 33 mM, pH 7.5, produced by mixing aqueous solutions of Na_2HPO_4 and KH_2PO_4) or the respective inhibitor solution (0.50, 1.00, 3.25, 5.50, and 7.75 µg/mL selegiline hydrochloride). Hence selegiline hydrochloride, the actual analyte, already gets in contact with the enzyme while the respective electrode is prepared. 3.0 µL of the resulting homogenous solution were applied on one side of the electrode surface. The enzyme containing solution was spread over the rest of the electrode by covering it with a fitting slip of the dialysis membrane, which was stored in the buffer solution for at least 15 min before use. Following this immobilisation step the electrode was built into the flow injection analysis system (Fig. 1). After an overall incubation time of 30 min electrochemical measurements were carried out. This procedure was applied to all analyses.

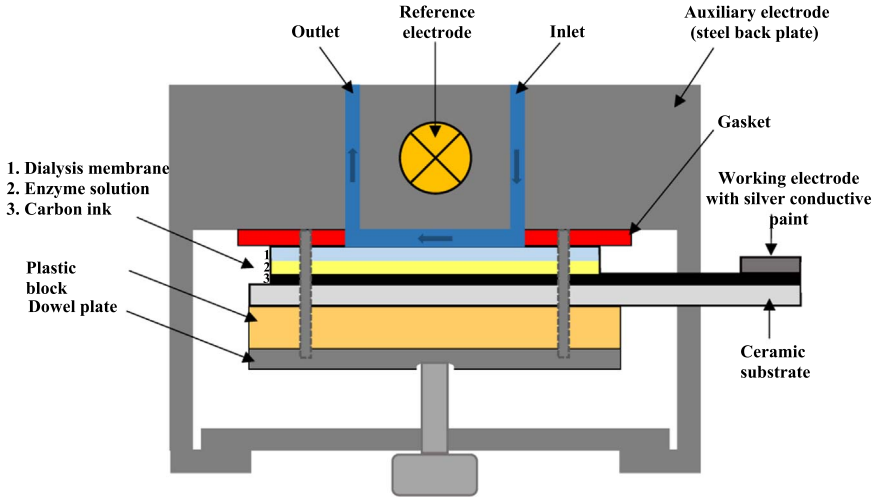

Fig. 1. Scheme of the three-electrode system (top view).

2.6. Electrochemical measurements

Sørensen phosphate buffer (33 mM, pH 7.5) was used for preparing solutions and for the storage of the dialysis membrane. It was also used as a carrier in FIA experiments. For the latter a flow rate of 0.5 mL/min and a fixed operating potential of + 400 mV was used. After achieving a stable baseline an activation procedure was performed by injecting hydrogen peroxide solution (30 µg/mL) for five times. This was performed once when a new electrode was used for the first time.

2.7. Inhibitor characterisation

Electrochemical inhibitor measurements were performed by using the above described MAO biosensors (0.50–7.75 µg/mL selegiline hydrochloride) and injecting 20 µL of different PEA concentrations to the FIA system. After finishing the measurement series (2.5–3000 µM PEA), the dialysis membrane was removed from the biosensor and a washing step of the screen printed working electrode by using ethanol and high purity deionised water was carried out. The cleaned working electrode was then reused for preparing further inhibition-based biosensors following the instructions given in 2.5. Additional measurements were done using varying amounts of enzyme solution (0.5, 1.0 and 1.5 µL; protein concentration 1.25 mg/mL) without inhibitor and the same amounts mixed with 1.0 µL of selegiline hydrochloride with a concentration of 0.5 µg/mL.

2.8. Samples

For each determination 2 tablets were pestled, dissolved in 50 mL buffer and stirred with a magnetic stirrer for 15 min. The solution was centrifuged at 3500 rcf for 15 min. The clear solution was diluted with buffer in order to get a calculated concentration of 0.5 µg/mL selegiline hydrochloride. This solution was used for preparing the biosensor as described above. For flow injection analysis a fixed PEA concentration of 100 µM was injected (n = 3). Standard addition method was carried out by spiking the tablet solution with 0, 0.69, 1.38, 2.06 and 2.75 µg/mL selegiline hydrochloride. The resulting solutions were used to prepare the appropriate biosensors.

3. Results and discussion

3.1. Functional principle

The principle of the developed biosensor is based on the formation of the product hydrogen peroxide, due to the reaction of hMAO B with its specific substrate PEA. The addition of inhibitor results in a reduced enzyme activity which leads to a decreased production of hydrogen peroxide. The latter one is then electrochemically detected at a MnO_2 modified screen printed working electrode at a fixed potential of + 400 mV (Fig. 2). With this type of sensor design it is possible to quantify hMAO B inhibitors as well as to characterise the mode of inhibition.

3.2. Optimisation of the transducer and FIA

As can be seen in Fig. 2, the products of the oxidative deamination catalysed by hMAO B are H_2O_2, ammonia and the respective aldehyde. In order to investigate a possible oxidation of enzymatically produced aldehydes and the necessity of the mediator for the detection of PEA, modified (20% MnO_2) and unmodified (absence of MnO_2) biosensors were tested by applying two common potentials (+ 400 and + 600 mV) (Fig. 3). The given results clearly show, that no oxidation of enzymatically produced products takes place at unmodified electrodes. As previously shown MnO_2 catalyses the oxidation of H_2O_2 [28]. If there is a contribution also of the aldehyde cannot be clearly answered.

To investigate the ideal concentration of the mediator MnO_2 for the screen printed working electrodes, carbon ink was mixed with different amounts of the mediator (5–35%, m/m). For each mediator percentage three biosensors were produced by immobilising the enzyme solution via a dialysis membrane onto the appropriate electrodes. Two different concentrations of the substrate (80 and 150 µM PEA) were applied in this optimisation step. These concentrations were chosen due to the

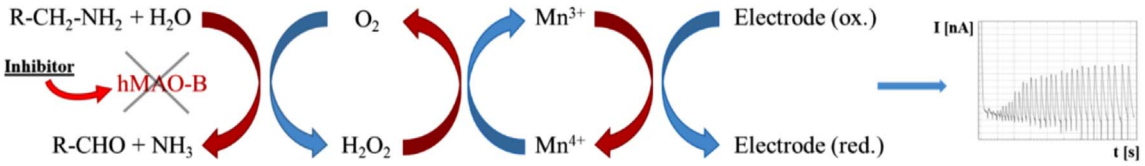

Fig. 2. Principle of the inhibition-based MAO sensor.

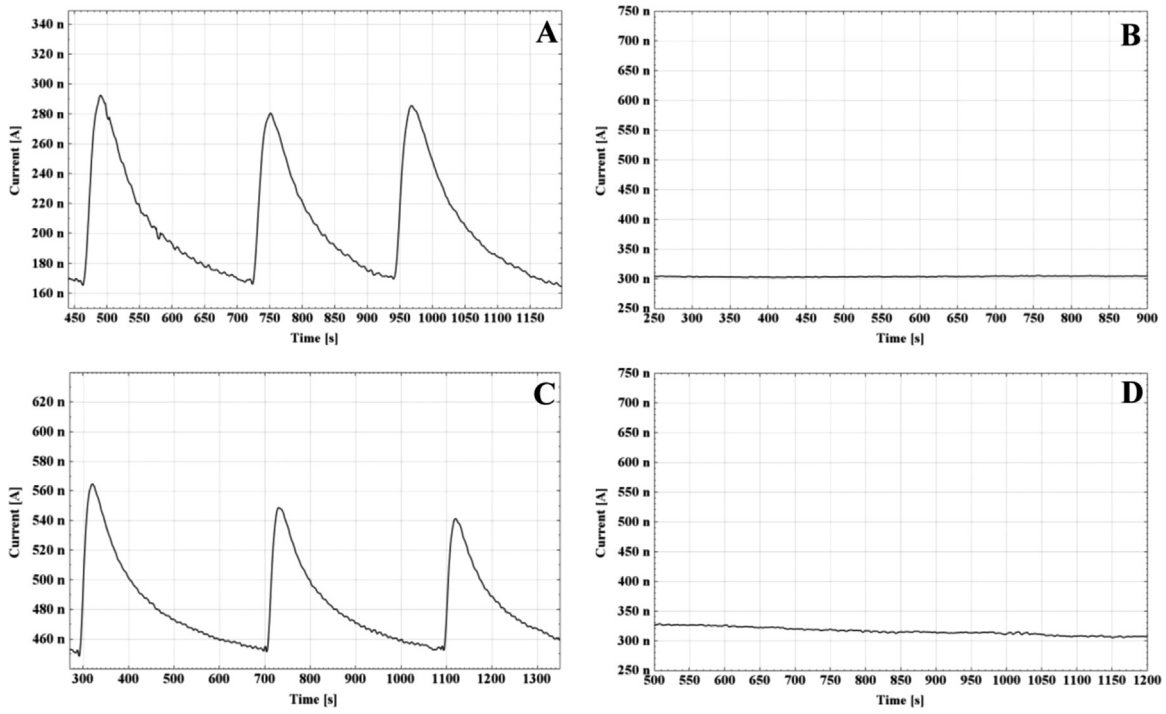

Fig. 3. Amperograms of 500 µM PEA (n = 3) measured with the biosensor in the presence (A, C) and absence (B, D) of MnO_2 as a mediator at + 400 mV (A, B) and + 600 mV (C, D).

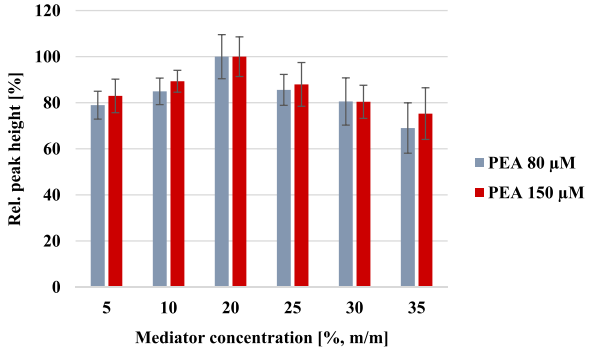

Fig. 4. Measurement of 80 µM and 150 µM PEA solutions at MAO electrodes with different mediator (MnO_2) concentrations. The mean peak height achieved with 20% MnO_2 electrodes is assumed as 100% (n = 3)."

fact that the selegiline hydrochloride quantification measurements were carried out within this concentration range. The results of these investigations are summarised in Fig. 4. As can be seen there, for both PEA concentrations the highest signals were achieved by electrodes with a mediator concentration (MnO_2) of 20%. The lowest response however was found with 35% modifier. Concerning precision, it can be assumed that apart from 30% and 35% MnO_2 modified electrodes with slightly higher standard deviations (S_{rel} 10%), all other modified electrodes showed similar standard deviations (S_{rel} 4–8%). Due to this behaviour the 20% MnO_2 modified SPE was selected as transducer system for the inhibition-based biosensor design. Immobilisation was achieved with a dialysis membrane according to our previous investigations. FIA was carried out using Sørensen phosphate buffer pH 7.5 in agreement with the pH optimum of the enzyme [29] and by applying previously optimised conditions including a flow rate of 0.5 mL/min and a potential of + 400 mV [28].

3.3. Investigation of inhibitor characteristics

For characterising inhibitors and designing the electrochemical sensor the effect of time on enzyme inhibition was evaluated. Former publications already reported on time-dependent inhibition of hMAO A [30] and hMAO B [6]. For the latter an incubation time of 30 min was fixed for their experiments. In order to verify this incubation time with the applied hMAO B and the inhibitor selegiline hydrochloride a photometric activity assay was performed by using benzylamine as substrate. These measurements showed, as expected, a time-dependent inhibition behaviour of selegiline hydrochloride. While about 75% of the final rate of inhibition were already achieved in the first measurement immediately after mixing, a constant value was reached after 30 min, which is in agreement with the references given above. Consequently, all experiments were preceded by an incubation period of 30 min.

Inhibition studies were performed electrochemically with inhibited hMAO B (0.50–7.75 µg/mL selegiline hydrochloride) sensors by using different PEA concentrations (2.5–3000 µM). The results of these measurements are shown in Fig. 5. As expected increasing inhibitor concentrations lead to lower signals. Furthermore, a steady rise of the peak height was observed within PEA concentrations < 500 µM for all curves, whereas the lower the inhibitor concentration the higher the increase was. However, with PEA concentrations ≥ 1000 µM the peak height remained constant.

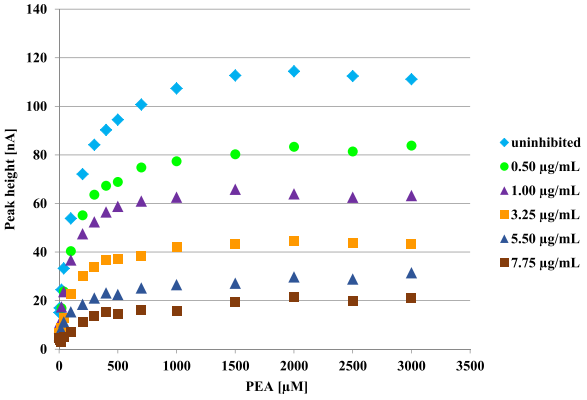

Fig. 5. PEA (2.5–3000 µM) measured at sensors inhibited with different concentrations of selegiline hydrochloride (n = 2).

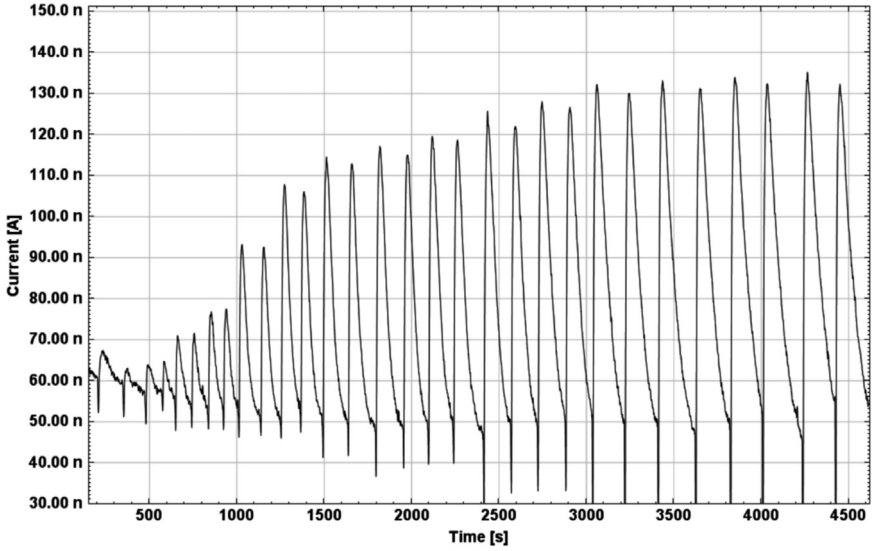

Fig. 6. Typical amperogram of different PEA concentrations (2.5, 5, 20, 40, 100, 200, 300, 400, 500, 700, 1000, 1500, 2000, 2500, 3000 µM) each measured twice at an electrode prepared with an inhibitor concentration of 0.50 µg/mL.

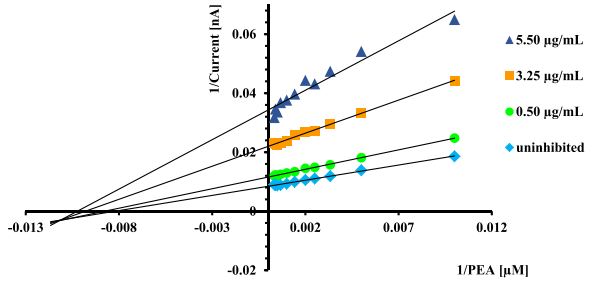

Fig. 7. Lineweaver-Burk plot of measurements of PEA (100, 200, 300, 400, 500, 700, 1000, 1500, 2000, 2500, 3000 µM) performed at uninhibited and inhibited (selegiline hydrochloride concentration 0.50, 3.25 and 5.50 µg/mL) electrodes (n = 2).

A typical amperogram of various PEA concentrations measured at an inhibition-based biosensor with an inhibitor concentration of 0.50 µg/mL is shown in Fig. 6.

For the representation of the results by Lineweaver-Burk the inverse current intensity is plotted versus the inverse substrate concentration. In the resulting depiction the intercept of the straight lines with the ordinate corresponds to the reaction velocity whereas Michaelis constant (K_M) can be read at the respective intercept with the x-axis. In Fig. 7 the plot obtained by measurements using an uninhibited and inhibited electrodes (selected selegiline hydrochloride concentration of 0.50, 3.25 and 5.50 µg/mL) is given. It shows that the maximum velocity (in this case current intensity) is decreased when a partly inhibited enzyme is used, whereas K_M remains unchanged. In the given case the lines intersect below the x-axis. This implies, that the inhibitor binds with different affinity to the free enzyme and the enzyme-substrate complex [31,32]. Under the given conditions a mean K_M was calculated to be about 100 µM PEA.

Due to the fact that non-competitive (reversible) and irreversible inhibitors show the same type of Lineweaver-Burk plot (decreased maximum velocity, unchanged K_M), further experiments were necessary to distinguish between these two types of inhibitors. These experiments were performed as described by Segel [33]. By plotting the total amount of enzyme vs. the maximum current, it is possible to differentiate between the two types of inhibitors. Compared to the control curve, a non-competitive inhibitor leads to a curve with a smaller slope and passing through the origin. The curve of an irreversible inhibitor however, has the same slope as the control curve and intersects the x-axis at a position equivalent to the irreversibly inhibited amount of hMAO B. These investigations were carried out by preparing electrodes with a fixed volume of enzyme containing solutions (3.0 µL), whereby the composition itself was varied. For the tests without inhibition different quantities of hMAO B solution (0.5–1.5 µL) were mixed with the respective amounts of Sørensen phosphate buffer. For the tests with inhibition a constant amount of selegiline hydrochloride solution (1.0 µL inhibitor solution; concentration of 0.5 µg/mL) was added to the proper hMAO B solutions (0.5–1.5 µL) and also mixed with buffer. The resulting solutions were applied to prepare electrodes and the respective maximum current was measured using a 1 mM PEA solution (n = 3). The results of these measurements are shown in Fig. 8.

The slopes of the two obtained curves (regression equation without inhibition I[nA] = 28.5 V[µL], R = 0.999 and with inhibition I[nA] = 29.1 V[µL] − 7.9, R = 0.999) are practically identical, hence these characteristics indicate clearly an irreversible inhibition behaviour. Consequently, these experiments show that the developed sensor is a valuable approach for investigating the kinetic behaviour of MAO B inhibitors.

3.4. Validation of the biosensor

Linear correlation between inhibitor concentration and measured signal (100 µM PEA) was found between 0.51 and 3.25 µg/mL selegiline hydrochloride with a regression equation of I[nA] = −11.9c[µg/mL] + 55.6 and a correlation coefficient of R = 0.997 (n = 3). Repeatability was measured by using 100 µM PEA (n = 6) with a

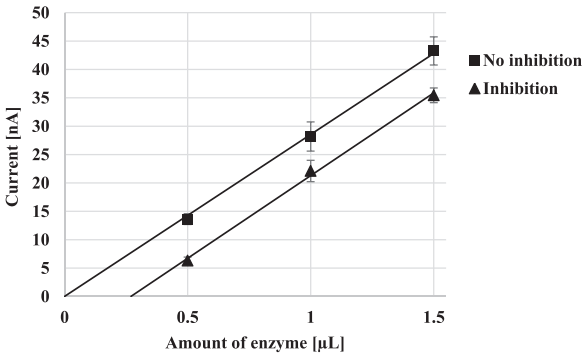

Fig. 8. Application of different amounts of enzyme solution (0.5, 1.0 and 1.5 µL) without inhibitor (upper straight line) and with inhibitor (1.0 µL, 0.5 µg/mL, lower straight line). Measurement of 1.0 mM PEA (n = 3; error bars represent the standard deviation).

Table 1
Summary of the validation parameters.

Parameter	Result
Linear range	0.51 – 3.25 µg/mL
Regression equation	I[nA] = −11.9c[µg/mL] + 55.6
R	0.997
LOD	0.15 µg/mL
LOQ	0.51 µg/mL
Repeatability	3.70%
Intermediate precision	8.12%

biosensor inhibited with 1.87 µg/mL selegiline hydrochloride (medium concentration range). Intermediate precision was investigated by preparing 3 different electrodes using the same inhibitor (1.87 µg/mL) and PEA (100 µM) concentrations. Repeatability and intermediate precision were determined to be about 4% and 8%, respectively. LOD was defined as three times the standard deviation of the blank. For this type of sensor, the 100 µM PEA solution measured at an uninhibited electrode was considered as the blank. With this method LOD was calculated to be 0.15 µg/mL selegiline hydrochloride. LOQ was calculated from 10 times the SD, giving a value of 0.51 µg/mL. A summary of the validation parameters is given in Table 1.

3.5. Application to samples

The validated sensor was applied to the quantification of selegiline hydrochloride in a pharmaceutical product ("Selegiline 5 mg" tablets). To exclude any possible matrix effects, the standard addition method was applied, which resulted in the following regression equation: I[nA] = −12.7c[µg/mL] + 49.7 (R = 0.999). When comparing the slopes of the linearity and the standard addition regression equation a difference of about 6% is given, which suggests that the matrix has negligible influence on the determination of the inhibitor (Fig. 9). However, standard addition was used to determine the recovery rate of the method, which resulted in a mean recovery rate of 105.2% (S_{rel} = 6.2%), which is shown in Table 2.

In total 3 determinations of selegiline hydrochloride in tablets were performed. As the application of standard addition method is not mandatory for the presented analysis, the quantification was carried out by using the calibration curve method and by considering the recovery rate. The determination of selegiline hydrochloride in tablets gave a mean value of 5.04 mg (S_{rel} = 5.6%) corresponding to 100.8% of the declared concentration (5 mg tablets).

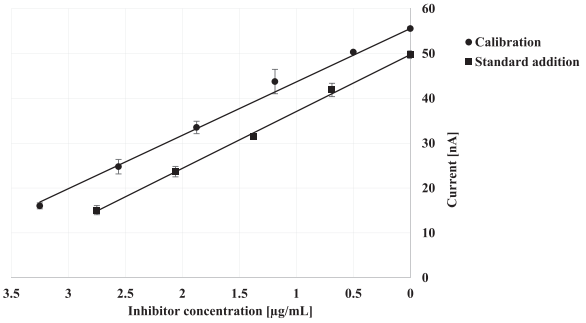

Fig. 9. Calibration curve and standard addition curve (error bars represent the standard deviation).

Table 2
Recovery rate of four different PEA concentrations (n = 3) and results.

Sample	Spiked PEA concentration [µg/mL]	Recovered PEA concentration [µg/mL]	Recovery rate [%]
1	0.69	0.66	96.42
2	1.38	1.54	112.06
3	2.06	2.19	106.40
4	2.75	2.91	105.95
		Mean recovery rate	**105.21**

4. Conclusion

The biosensor presented in this work is based on the inhibition of hMAO B. Besides allowing the sensitive and easy quantification of the hMAO B selective inhibitor selegiline hydrochloride it was also applied to figure out the type of enzyme inhibition by applying standard Lineweaver-Burk enzyme kinetic studies. To distinguish between non-competitive and irreversible inhibition enzyme quantity vs. current plots were performed. The sensor uses a simple and time-saving immobilisation procedure for the biocomponent hMAO B via dialysis membrane. The analytical parameters of the optimised sensor were determined and show both a good repeatability and intermediate precision. The developed sensor was successfully applied for the characterisation of the type of enzyme inhibition, proving an irreversible inhibition of the enzyme by selegiline hydrochloride. Furthermore, the applicability of the sensor in real samples was proven. Therefore, compared to existing well-approved methods the biosensor is characterised by both, the unique benefit of determining enzyme-inhibitor interactions and inhibitor quantification. It allows simple and time-saving electrode preparation and enables rapid and cost-effective measurements with a potential application for further hMAO B inhibitors.

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