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New approach for the potentiometric-enzymatic assay of reversible-competitive enzyme inhibitors. Application to acetylcholinesterase inhibitor galantamine and its determination in pharmaceuticals and human urine

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ABSTRACT

A new kinetic-potentiometric method for the characterization and analytical determination of competitive reversible enzyme inhibitors was developed. The method is based on a mathematical approach, assuming that the reaction proceeds at the steady state, which permits calculation of a tentative substrate concentration to be used to determine low inhibitor concentrations and to obtain the value of inhibition constant corresponding to the inhibitor. The mathematical approach predicts a linear relationship between the inverse of the relative inhibition and the inverse of the inhibitor concentration. The method developed is applied to the acetylcholinesterase inhibitor galantamine, using an acetylcholine-selective electrode. A linear relationship for galantamine concentration from 2×10^{-8} to 1×10^{-6} M and a limit of detection of 5.4×10^{-9} M was found. A value for K_I^{Gal} of $2.0\times10^{-7}\pm0.1\times10^{-7}$ M was obtained. The effect of several other drugs and of the main galantamine metabolite excreted in urine was studied. The method was satisfactorily applied to the determination of galantamine in pharmaceuticals and human urine.

1. Introduction

The enzyme acetylcholinesterase (AChE) is considered to be very important since it terminates the transmission of impulses in cholinergic synapses through the rapid hydrolysis of acetylcholine (ACh), a neurotransmitter related with attention, learning, memory, consciousness, sleep and the control of voluntary movements [1]. AChE is the target of many neurotoxins and drugs that specifically bind to its active site [2]. Certain drugs, such as the alkaloid galantamine, produce reversible inhibition, interfering in substrate hydrolysis by transiently interacting with the enzyme, without forming covalent bonds [3]. This controlled inhibition has interesting therapeutic uses, including the treatment of Alzheimer's disease [4].

Other AChE inihibitors, such as organophosphate and carbamate compounds, are commonly used as pesticides [5]. Since new enzyme inhibitors obtained from natural plants and destined for the treatment of human diseases need to be evaluated [6–8], the availability of new tools that are simple, inexpensive and effective for the characterization of enzyme inhibitors is of great interest. Over recent decades, several kinetic analyses of different AChE inhibitors have been reported [9–12].

The indirect determination of drugs and pesticides by means of electrochemical enzymatic assays using AChE is recognized as a complementary screening technique to traditional spectrophotometric-kinetic and chromatographic methods and, in some cases, as an alternative to them if the sensitivity reached by the sensor is considered adequate [5]. The reason for this is that using a sensor is generally cheaper and more user-friendly than other methods, with the added advantage of possible miniaturization.

To our knowledge, only one ion-selective electrode (ISE) has been described for the indirect determination of AChE inhibitors, more specifically for the determination of an organophosphate pesticide, by monitoring the ACh concentration during its inhibited enzymatic catalyzed hydrolysis [13]. The ISE used by these authors consisted of a primitive liquid membrane electrode, which was applied for organophosphate pesticide analysis. Nowadays, this type of electrode has been totally replaced by plasticized polymeric membrane electrodes because of the many benefits they provide, including their mechanical stability, durability and simplicity. In this respect, we have recently published an article on a novel ACh-selective electrode suitable for use in the determination of ACh, as well as for enzymatic assays and characterization [14].

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The main goal of the present work was to develop a potentiometric method for the determination of the alkaloid galantamine based on its inhibition effect on the enzyme-catalyzed hydrolysis of ACh, by monitoring its concentration with the ACh-selective electrode recently described by our research group. The work has a wide scope since the ISE can be used not only for the determination of galantamine in pharmaceuticals and human urine, but also to characterize the type of inhibition and the corresponding inhibition constant for the inhibitor. For this purpose, a new type of calibration graph, from which the inhibition constant can be calculated, is presented, and a mathematical approach is developed on the assumption that the reaction proceeds at the steady state, to calculate a tentative substrate concentration to be used for determining the inhibitor. These two procedures can be used for any reversible competitive inhibition involving a given substrate. In view of the fact that many compounds exert this type of inhibition, the strategy here developed could be further applied in pharmacological, toxicological and environmental fields.

2. Materials and methods

2.1. Reagents, solutions and apparatus

All chemicals were of analytical reagent grade and Milli-Q water was used throughout.

Polyvinyl chloride (PVC) of high molecular weight, 2-nitrophenyl octyl ether (NPOE), potassium tetrakis [3,5-bis-(trifluoromethyl) phenyl]borate (KTPB), acetylcholine chloride and tetrahydrofuran (THF) were purchased from Fluka (Munich, Germany).

Acetylcholinesterase (type VI-S) from *Electrophorus electricus* (electric eel, EC 3.1.1.7, 288 U/mg solid) and galantamine hydrobromide from *Lycoris sp.* were obtained from Sigma-Aldrich (Munich, Germany).

The pharmaceuticals, Reminyl® 4 mg/ml oral solution and Reminyl® 8 mg extended-release capsules (Janssej-Cilag S.A., Madrid, Spain), were purchased from a local pharmacy.

Phosphate buffers of different pH values between 5.8 and 8.9 were prepared by accurately mixing 50 ml of 0.01 M KH_2PO_4 and appropriate volumes of 0.01 M NaOH and diluting to 100 ml with water.

A 0.1 M ACh stock solution was prepared by dissolving acetylcholine chloride in water. Working ACh solutions were prepared by diluting this with water. All these solutions were stored in a refrigerator at 4 °C.

An AChE stock solution was prepared by dissolving 0.46 mg of AChE in 2.5 ml of phosphate buffer and diluting with water to 5.0 ml in a calibrated flask, resulting in a solution of 25.8 U ml^{-1}. This solution was stored in a refrigerator at 4 °C.

A galantamine stock solution was prepared by dissolving galantamine hydrobromide in water. Working galantamine solutions were prepared by diluting this solution in water. All these solutions were stored in a refrigerator at 4 °C.

Potentiometric measurements were recorded using a home-made high-impedance data acquisition 16-channel box connected to a personal computer by USB (Universal Serial Bus). A Fluka (Munich, Germany) electrode body ISE and an Orion Ag/AgCl double-junction reference electrode (Orion 90-02) containing a 10^{-4} M KCl solution in the outer compartment, were used.

All potentiometric measurements were carried out with continuous magnetic stirring (400 rpm) in a thermostated vessel in a temperature range from 22 to 43 °C.

2.2. Membrane and electrode preparation

The membrane was prepared by dissolving 100 mg of PVC, 200 mg of the plasticizer NPOE and 1.5 mg of the ionic additive KTPB in 3 ml of THF. This solution was poured into a Fluka glass ring (inner diameter 28 mm, height 30 mm) on a Fluka glass plate, and allowed to settle overnight until all the THF had evaporated, to obtain a thin plastic membrane. A 6-mm-diameter piece was cut out with a punch and incorporated into a Fluka electrode body ISE containing 10^{-4} M KCl as internal filling solution. The electrode was conditioned in water until it reached a constant potential and, when not in use, the electrode was kept immersed in water.

2.3. Calibration of the ACh-selective electrode

The calibration graph of the ACh-selective electrode toward ACh was made by pipetting consecutive small volumes of the corresponding ACh working solution into 50.0 ml of the pH buffer to cover the concentration interval from 10^{-7} to 10^{-3} M for ACh. The steady-state potentials reached for each ACh concentration were then plotted *versus* the corresponding concentration logarithmic values. The data within the linear portion of the ACh calibrations (10^{-6} to 10^{-3} M) were fitted to $E=E^0+S\log C_{ACh}$, where E^0 and S are the standard potential and the slope of the electrode, respectively.

2.4. Kinetic monitoring of galantamine-inhibited acetylcholine hydrolysis

A volume of 50.0 ml of the corresponding pH phosphate buffer solution was transferred into the thermostated vessel. The ACh-selective electrode and the reference electrode were then immersed and potentiometric data acquisition was started. Once the potential stabilized, appropriate volumes of ACh and galantamine working solutions were injected. When the potential stabilized again, an appropriate volume of AChE stock solution was injected and the potential kinetic curve was left to develop. The uninhibited hydrolysis was monitored in the same way without adding galantamine.

The resulting curves were transformed into ACh-concentration kinetic curves using a calibration graph for ACh made for the corresponding pH phosphate buffer solution following the calibration procedure described above. The initial slopes, equivalent to the initial hydrolysis rates, were calculated from these kinetic curves.

2.5. Determination of galantamine in pharmaceuticals

The content of galantamine in Reminyl® oral solution was determined from a 1000-fold dilution in water and recording in triplicate the kinetic curve provided by 550 μl of this solution in 50.0 ml of pH 7.5 buffer solution in the presence of 4.7×10^{-5} M ACh and 0.3 U ml^{-1} AChE, following the procedure described above.

The amount of galantamine in Reminyl® extended-release capsules was determined by analyzing three capsules separately. The content of each capsule was introduced into a beaker with about 25 ml of water, which was placed in an ultrasonic bath for 3 h, ensuring that the bath temperature did not exceed 35 °C. In this way, the coating of the gelatine beads came off slowly, allowing the galantamine and other soluble excipients to be dissolved in water. The suspension obtained was filtered under vacuum with a 0.2 μm porosity filter and the filtrate was diluted to 100.0 ml with 7.5 pH buffer solution in a calibrated flask. Finally, the kinetic curve using 25.0 μl of this final solution in the presence of 4.7×10^{-5} M ACh and 0.3 U ml^{-1} AChE was recorded following the procedure described above.

2.6. Determination of galantamine in human urine

Two spiked urine samples were prepared by adding 18.0 and 27.0 μl of 1.0×10^{-3} M galantamine working solution to 25.0 ml of urine from two healthy volunteers. Then, 20.0 ml of these spiked urines were diluted to a final volume of 200.0 ml with 7.5 pH

buffer solution. Three aliquots of 50.0 ml of these solutions were analyzed by recording the corresponding kinetic curves in the presence of 4.7×10^{-5} M ACh and 0.3 U ml^{-1} AChE following the procedure described above.

3. Results and discussion

3.1. Potentiometric characterization of galantamine as AChE inhibitor from Michaelis–Menten and Dixon plots

In this paper, we used an ACh-ISE previously reported by our research group [14] for the potentiometric characterization and determination of the AChE inhibitor galantamine, which was selected as a model inhibitor.

The effect of galantamine on the kinetics of the AChE-catalyzed hydrolysis of ACh was studied by measuring the initial rates of the hydrolysis in different conditions, as described in Section 2. In a previous paper we showed the experimental kinetic curves for *E versus t* and the corresponding [ACh] *versus t* for the AChE-catalyzed hydrolysis of ACh together with the calculation of the initial rate [14].

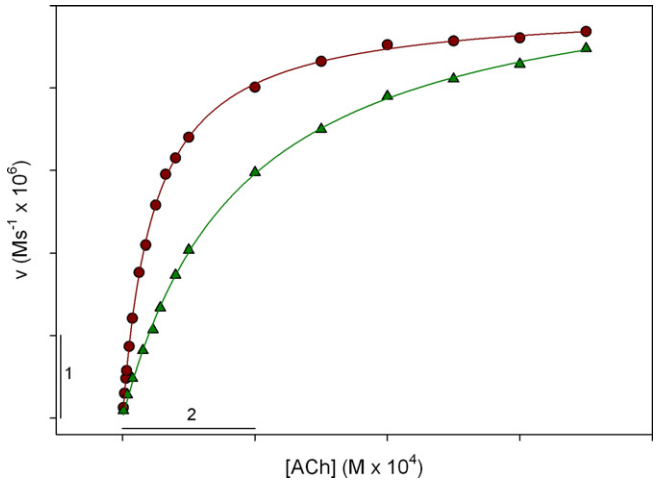

Fig. 1. Plot of the initial reaction rates obtained at several ACh initial concentrations in the absence (●) and in the presence (▲) of 2×10^{-8} M galantamine, fitted to the Michaelis–Menten equation. (The experiments were carried out at a pH of 7.5, 28 °C of temperature and at a 0.3 U ml^{-1} initial AChE concentration).

The influence of the ACh concentration on the initial hydrolysis rate was studied at pH 7.5 and 28 °C with an AChE concentration of 0.3 U mL^{-1} in the absence and in presence of a galantamine concentration of 6×10^{-7} M. The results obtained are shown in Fig. 1, where both data sets were fitted to the corresponding Michaelis–Menten equations for the uninhibited and the inhibited hydrolysis, Eqs. (A1) and (A2), respectively [15].

The value of K_m^{app} obtained for the inhibited hydrolysis, $17.9 \times 10^{-5} \pm 0.5 \times 10^{-5}$ M, was higher than the K_m respective value, $4.7 \times 10^{-5} \pm 0.1 \times 10^{-5}$ M, obtained for the uninhibited reaction, while the respective values obtained for v_{max} ($5.6 \times 10^{-6} \pm 0.5 \times 10^{-6}$ and $5.1 \times 10^{-6} \pm 0.5 \times 10^{-6}$ M s^{-1}) were similar. These results indicate a competitive inhibition by galantamine [9]. As can be seen in Fig. 1, the maximum difference between the initial reaction rates was found at an ACh concentration close to K_m.

In order to obtain the inhibition constant of the process, K_I^{Gal}, a series of kinetic experiments was carried out, in which the AChE concentration was kept constant at 0.3 U ml^{-1} and the galantamine concentration was varied between 2×10^{-8} and 6×10^{-7} M at ACh concentrations ranging from 6×10^{-5} to 2×10^{-4} M. The plots of the inverse of the initial rate *versus* galantamine concentration at the different ACh concentrations assayed (Dixon plot) are shown in Fig. 2a. As can be seen, the interception point of the straight lines is not located on the abscissa axis, which corroborates a competitive inhibition. The Dixon slopes obtained were plotted *versus* the inverse of ACh concentration (Fig. 2b). A straight line, (*Dixon slope* (M^{-1} s) $= -1.60 \times 10^{10}$ (M^{-1} s) $+ 3.74 \times 10^{7}$ (s) (1/[ACh](M))) with a good fitting ($r^2 = 0.9977$), was obtained and, K_I^{Gal} was calculated from the corresponding slope 3.74×10^7 s, using Eq. (1). A value of $2.2 \times 10^{-7} \pm 0.2 \times 10^{-7}$ M was obtained for K_I^{Gal}.

$$\frac{K_m}{(v_{max} K_I^{Gal})} = 3.74 \times 10^7 \quad (1)$$

3.2. Potentiometric assay of galantamine as AChE inhibitor using a new mathematical approach

Following, a new analytical method for the determination of galantamine is presented. This method provides in addition a

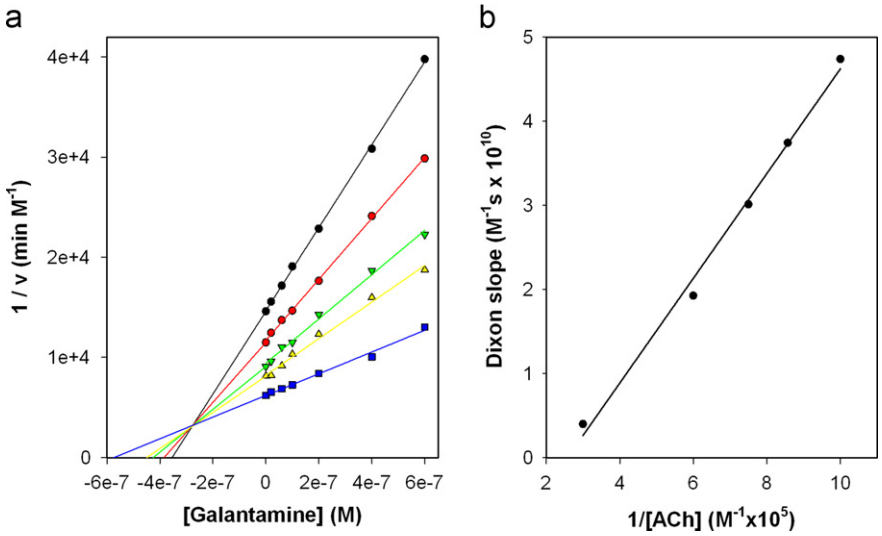

Fig. 2. (a) Dixon plots at different ACh concentrations: 6×10^{-5} (black, ●), 7×10^{-5} (red, ◦), 8×10^{-5} (green, ▾), 1×10^{-4} (yellow, ▲) and 2×10^{-4} M (blue, ▪). (b) Plot of the Dixon slopes *versus* the inverse of ACh concentration. (Experiments were carried out at pH 7.5, 28 °C of temperature and 0.3 U ml^{-1} AChE concentration). (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

straightforward way of obtaining its inhibition constant. This approach can be extended to other competitive inhibitors.

The approach used relies on Eq. (A6), deduced from common equations of the theory of competitive inhibition. Eq. (A6) establishes a linear relationship between the inverse of relative inhibition *Inh* and the inverse of the inhibitor concentration.

3.2.1. Influence of temperature and pH on the AChE inhibition

When working with enzymes, the pH and temperature are important factors that must be optimized. The effect of pH and temperature on the inhibition of AChE-catalyzed hydrolysis of ACh caused by galantamine was studied in a pH range of 5.8–8.9 and from 22 to 43 °C (Fig. 3). As can be seen, temperature had no appreciable influence on *Inh* values, while in the pH study, the highest values were obtained with pH values between 7.5 and 8.1. A pH value of 7.5 and temperature of 28 °C were therefore selected for further experiments since the highest *Inh* value was obtained under these conditions.

3.2.2. Influence of substrate and enzyme concentration on the determination of galantamine

As mathematically demonstrated in Apendix A, the substrate concentration at which the difference between the initial rates of the uninhibited and inhibited reactions is maximum for a low inhibitor concentration, corresponds to a value equal to K_m. Since the determination of low inhibitor concentration is one of the main aims of any analytical method, the K_m value can be used as tentative substrate concentration. In the case of galantamine, the difference between the experimental curves obtained at different [ACh] for the uninhibited and inhibited reactions (Fig. 1) was obtained. The curve obtained is shown in Fig. 4. Interestingly, 99% of the maximum difference between both rates is obtained at a value of ACh concentration equal to K_m (4.7×10^{-5} M) confirming the validity of the tentative concentration proposed.

As regards optimization of the enzyme concentration, [AChE], taking into account Eqs. (A1)–(A3), where the concentration of enzyme is included in v_{max}, it can be seen that an increase in the concentration of enzyme increases both the initial rates of the uninhibited and inhibited reactions and the difference between them. So, *a priori*, it is interesting to work at high enzyme concentrations, provided that the steady-state of the hydrolysis reaction is attained. On the other hand, a high enzyme concentration implies a high economic cost. From the analytical point of view, the best criterion for optimizing sensitivity is to evaluate the limits of detection (LOD) and quantification (LOQ) which represent the concentration of analyte corresponding to 3 and 10 times the standard deviation (SD) of the blank signal, respectively. In the case on inhibited reactions, LOD and LOQ can be obtained as the inhibitor concentration corresponding to a v_i equal to v_0−3SD and v_0−10SD, respectively. In the case of galantamine, experiments were carried out to determine LOD and LOQ using different AChE concentrations at pH=7.5 and 28 °C with an ACh concentration of 4.7×10^{-5} M. The values found, together with the corresponding initial rates and the inhibition percentage (*Inhx*100) values for each AChE concentration, are shown in Table 1. As can be seen, the best LOD and LOQ were obtained for an [AChE] of 0.3 U ml^{-1}, so this concentration was selected as optimum. Under this condition, a galantamine concentration as low as 1.8×10^{-8} M (6 ppb) could be quantified.

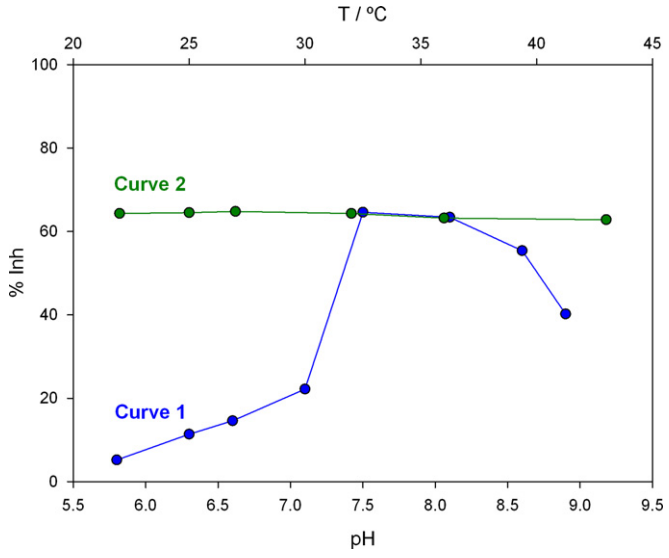

Fig. 3. Curve 1: Influence of pH on the inhibition percentage at 28 °C, 4×10^{-5} M ACh concentration and 0.3 U ml^{-1} AChE concentration. Curve 2: Influence of temperature on the inhibition percentage at pH of 7.5, 4×10^{-5} M ACh concentration and 0.3 U ml^{-1} AChE concentration.

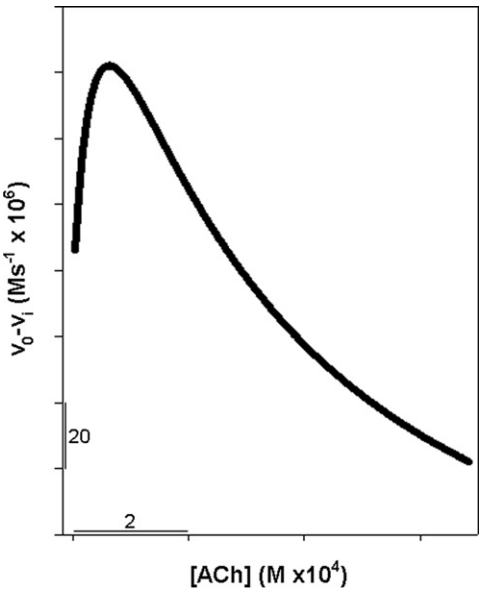

Fig. 4. Plot of the difference between fitted curves 1 and 2 of Fig. 1.

Table 1
Effect of the AChE concentration on the inhibition percentage, the initial rate of the inhibited reaction and on the limits of detection and quantification of galantamine[b].

[AChE] (U ml^{-1})	*Inh* × 100	$v_0 \times 10^6$ (Ms^{-1})[a]	LOD (M)	LOQ (M)
0.40	66.3	3.85±0.04	1.7×10^{-8}	5.5×10^{-8}
0.30	65.9	2.80±0.01	5.4×10^{-9}	1.8×10^{-8}
0.17	54.6	1.58±0.02	1.5×10^{-8}	4.9×10^{-8}
0.067	34.4	0.62±0.05	1.4×10^{-7}	4.7×10^{-7}
0.017	20.4	0.16±0.03	5.7×10^{-7}	1.9×10^{-6}

[a] Mean±SD (n=3).
[b] pH=7.5, T=28 °C and [ACh]=4.7×10^{-5} M.

Taking all these factors into account, the final conditions for the analytical method were: pH=7.5, 28 °C, [ACh]=4.7×10^{-5} M and [AChE]=0.3 U ml^{-1}.

3.2.3. Analytical performances of the method

When working with a substrate concentration equal to K_m, Eq. (A6) is simplified to Eq. (2).

$$\frac{1}{Inh} = 1 + 2\frac{K_I}{[I]} \quad (2)$$

According to this equation, by plotting (1/*Inh*) *vesus* (1/[*I*]), a straight line must be obtained, which can be used as the calibration graph for the determination of the competitive inhibitor and for the calculation of K_I^{Gal} from the experimental slope.

Fig. 5 shows the plot of (1/*Inh*) *versus* (1/[*Gal*]). As predicted by the theoretical equations, a linear relationship was obtained with a *y*-intercept very close to 1.

The reproducibility of the method was evaluated by making calibration graphs in triplicate with the galantamine concentration ranging from 2×10^{-8} to 1×10^{-6} M, on the same day and on nine different days during 1 month. The calibration parameters are shown in Table 2. Even after 1 month, the calibration parameters presented coefficients of variation of less than 3%.

The K_I^{Gal} value, calculated from the experimental slope, is also shown in Table 2. When the mean value obtained for K_I on different days, $2.0\times10^{-7}\pm0.1\times10^{-7}$, was statistically compared with the value obtained for K_I^{Gal} using Dixon plot, no significant difference was found at a 95% confidence level, confirming the accuracy of the method for calculating K_I. In addition, the new method is much faster.

3.3. Determination of galantamine in pharmaceuticals and human urine

The developed analytical method was applied to the determination of galantamine in pharmaceutical preparations and spiked human urine samples.

Two different pharmaceutical preparations containing galantamine were analyzed following the procedure described in Section 2 and the results obtained are summarized in Table 3. The galantamine content was found to be very similar to that labeled for the corresponding pharmaceutical. When a *t*-test was applied at a 95% confidence level, no significant differences were found between the values obtained and those labeled by the manufacturers. These good results point to the lack of interference on the part of the other compounds in the pharmaceuticals, at least at the corresponding concentrations they are present.

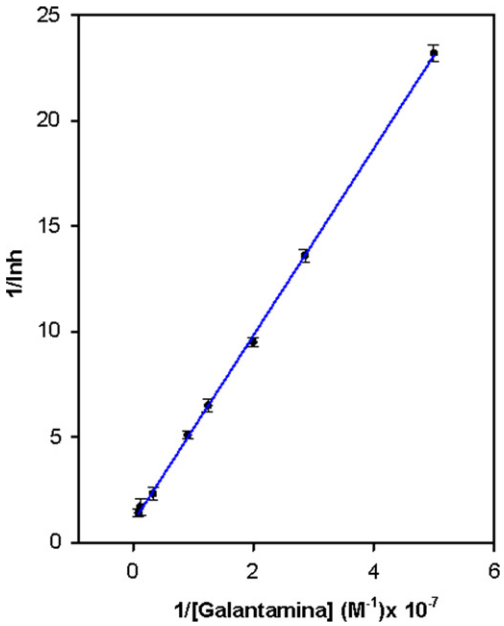

Fig. 5. Calibration graph obtained for galantamine ($n=3$).

Table 2
Calibration parameters obtained in repeatability and reproducibility studies.

	r^2	Intercept (M^{-1} s)	K_i^{Gal} ($M\times10^7$)
In the same day[a]	0.999±0.002	0.991±0.008	2.2±0.1
During 1 month[b]	0.997±0.006	0.958±0.025	2.0±0.1

[a] Mean±SD ($n=3$).
[b] Mean±SD ($n=9$).

Table 3
Determination of galantamine in pharmaceuticals and human urine.

Sample	Labeled	Present method[a]
Reminyl solution	4 mg/ml	4.06±0.04
Reminyl capsules	8 mg/capsule	7.90±0.18
Urine sample	Added (μg/l)	Found[a]
1	398	397±14
2	258	260±6

[a] Mean±SD ($n=3$).

For the determination of galantamine in spiked human urine, two different samples were prepared from the collected urine corresponding to 0–24 (2 l) and 0–72 h (6 l) of healthy volunteers, adding amounts of galantamine corresponding to the administration of 4 mg of galantamine and taking into account that, during the first 24 h about 20% of ingested galantamine is excreted and, during the first 72 h about 39%. Each sample was analyzed in triplicate following the procedure described in Section 2 and the results obtained are summarized in Table 3. In the analysis of both urines, recoveries of 99–101% were found.

The analysis of galantamine in real urine samples may be affected by the presence in the fluid of certain compounds which also act as inhibitors of AChE. This could be the case of some galantamine metabolites and some drugs administered to the patient. With regard to the first, galantamine is metabolized (about 75%) to pharmacologically inactive or less-active metabolites [16]. N-oxide galantamine, a phase I metabolite, is the main metabolite excreted in human urine, in whose formation cytochrome P450 3A4 is involved [17]. In the urine of 0–72 h the N-oxide galantamine content is about 17% (w/w) of the ingested galantamine [18]. Taking into account that the metabolite inhibition constant, $K_I^{N-oxide}$, is 1.1×10^{-6} M (value calculated as it is shown in Appendix B) and that the rates of the reactions inhibited by galantamine and by both galantamine and the metabolite are given by Eqs. (3) and (4), respectively, the hydrolysis rate in the presence of both would be reduced by about 3.3% compared with the rate for galantamine alone. This would produce a positive error of about 7% in the galantamine determined.

$$v_i=\frac{v_{max}[S]_0}{K_m(1+([Gal]/K_I^{Gal}))+[S]_0} \tag{3}$$

$$v_i=\frac{v_{max}[S]_0}{K_m(1+([Gal]/K_I^{Gal})+([N-oxide]/K_I^{N-oxide}))+[S]_0} \tag{4}$$

Besides galantamine, other pharmaceuticals may be administered and these too may act as AChE inhibitors. For this reason, quinidine (antiarrhythmic), paracetamol (analgesic), aspirin and ibuprofen (analgesic and antiinflammatory) and omeprazole (stomach protector) were studied as possible interferents. Their influence on the determination of galantamine was evaluated by kinetic experiments using galantamine and the foreign drug concentrations typically excreted in urine at 0–24 h [19]. The results obtained are shown in Table 4. It was considered that the tested drug produced a significant change in the hydrolysis rate when this change was more than 3 times the standard deviation of the hydrolysis rate obtained for galantamine alone. Quinidine,

Table 4
Effect of other drugs on the galantamine determination.

Drug	Molar ratio (drug/galantamine)	Error (%) in the galantamine determination
Quinidine	80	NI
Acetaminophen	180	NI
Ibuprofen	340	NI
Acetylsalicylic acid	2400	51
	240	21
	56	NI
Omeprazole	140	46
	14	14
	4	NI

NI=No interference.

acetaminophen and ibuprofen produced no error while acetylsalicylic acid and omeprazol gave positive errors in the determination of galantamine. For these two drugs, lower molar ratios (drug/galantamine) were tested and it was found that a ratio of 56 and 14, respectively, gave no error.

3.4. Comparison of the proposed method with those reported for the determination of galantamine

A survey of the literature concerning galantamine determination revealed that the most commonly used techniques are liquid and gas chromatography [20–29]. These methods require long analysis time and sometimes involve complicated methodologies, while the lowest detection limits reached was in ppb, the same level reached in the present paper. Some of these chromatographic methods took advantage of the fact that galantamine is a fluorescent compound [27]. Recently, the direct fluorimetric determination of galantamine following a method to enhance its fluorescence signal has been published, also reaching detection limits of ppb [30], but involving sophisticated second-order multivariate calibration.

The literature also mentions a spectrophotometric procedure [31] and several methods based on capillary electrophoresis [32–34]. Some of these procedures reached limits of detection of some tens of ppbs.

Two methods for the determination of galantamine based on amperometric immobilized-AChE biosensors are reported in the literature [35,36]. Du et al. applied the biosensor to the calculation of the K_m^{app} associated with the inhibition process caused by galantamine, while Stoytcheva et al. commented in a review on the use of an immobilized-AChE biosensor for the determination of galantamine based on the inhibition of the AChE hydrolysis. Although they reported a good limit of detection, the method was not applied to the determination of galantamine in real samples.

The potentiometric methodology here presented provides a simple, rapid and inexpensive way to determine galantamine at a ppb level, which is sufficient for its analysis in pharmaceuticals and urine. The method could also be applied to other reversible-competitive AChE inhibitors.

4. Conclusions

An ACh-selective electrode combined with a new mathematical approach, assuming that the reaction proceeds at the steady state, provides a straightforward method for the enzymatic characterization of reversible-competitive AChE inhibitors and for their analytical determination. The method has proven to be useful for galantamine and for its analytical determination in pharmaceuticals and human urine. The present work represents an important contribution to the field of enzymatic assays and drug analysis. Future perspectives include the extension of the presented methodology to other AChE-inhibitors and to inhibitors of other enzymes.

Acknowledgments

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Appendix A

Theoretical background

For an enzymatic hydrolysis involving a single substrate and single reaction product, the initial reaction rates of the uninhibited and inhibited reaction for a reversible, competitive inhibitor, v_0 and v_i, are given by Eqs. (A1) and (A2) [9].

$$v_0 = \frac{v_{max}[S]_0}{K_m + [S]_0} \tag{A1}$$

$$v_i = \frac{v_{max}[S]_0}{K_m^{app} + [S]_0} \tag{A2}$$

where v_0 and v_i are the initial rates of the catalyzed ACh hydrolysis in the absence and in the presence of the inhibitor, $[S]_0$ is the initial concentration of substrate, v_{max} is the maximum rate, and K_m and K_m^{app} are the corresponding Michaelis constants for the uninhibited and inhibited reactions, with $K_m^{app} = K_m(1+([I]_0/K_I))$.

An equation that linearly relates the inverse of the inhibition with the inverse of the inhibitor concentration can be obtained from the difference between the Eqs. (A1) and (A2). This difference $v_0 - v_i$ is given by Eq. (A3).

$$v_0 - v_i = v_{max}[S]_0 \left[\frac{K_m^{app} - K_m}{(K_m + [S]_0)(K_m^{app} + [S]_0)}\right] \tag{A3}$$

Dividing Eq. (A1) by Eq. (A3) and operating gives Eq. (A4):

$$\frac{v_0 - v_i}{v_0} = 1 + \left(1 + \frac{[S]_0}{K_m}\right)\frac{K_I}{[I]} \tag{A4}$$

By defining the relative inhibition (*Inh*) produced by an inhibitor, with *Inh* ranging from 0 for a situation of no inhibition to 1 for total inhibition (as Eq. (A5) indicates) an equation that relates the inverse of the inhibition produced with the inverse of the inhibitor concentration is obtained (Eq. (A6))

$$Inh = \left(\frac{v_0 - v_i}{v_0}\right) \tag{A5}$$

$$\frac{1}{Inh} = 1 + \left(1 + \frac{[S]_0}{K_m}\right)\frac{K_I}{[I]} \tag{A6}$$

From this equation, using a fixed $[S]_0$, knowing the value for K_m and accepting that the reaction proceeds in the steady state (considering the pure competitive character of the inhibition; working at $[S]_0 \gg [Enzyme]_0$, without causing inhibition due to excess substrate, and $[I]_0 \gg [Enzyme]_0$, neglecting the inhibitor bonded in form of an enzyme-inhibitor complex), a straight line can be obtained measuring $(1/Inh)$ at different inhibitor concentrations. Furthermore, the value of K_I can be calculated from the slope.

Calculation of a tentative substrate concentration

Deriving Eq. (A5) with respect to $[S]_0$ and equating to zero, the following equation can be reached:

$$K_m K_m^{app} - [S]_0^2 = 0 \tag{A7}$$

By substituting $K_m^{app} = K_m(1+([I]_0/K_I))$ and clearing, Eq. (A8) is obtained:

$$[S]_0 = K_m\sqrt{1+\frac{[I]_0}{K_I}} \tag{A8}$$

This equation permits us to calculate the substrate concentration at which the difference between the initial rates of the uninhibited and inhibited reactions for a given inhibitor concentration is maximum.

Since $\lim_{[I]\to 0}[S]_0 = K_m$, K_m is the value of the substrate concentration at which the inhibition corresponding to a very low inhibitor concentration is maximum.

Appendix B

The relationship between the concentration of a competitive inhibitor that produces a 50% of the inhibition of an enzyme, $[I]_{EC50}$, at a given initial concentration of substrate, $[S]_0$, and its corresponding inhibition constant, K_i, is given by Eq. (B1).

$$[I]_{EC50} = \left(1+\frac{[S]_0}{K_m}\right)K_I \tag{B1}$$

When another competitive inhibitor, I', with an inhibition constant, K_I', is studied at the same $[S]_0$, Eq. (B2) is obtained according to Eq. (B1).

$$[I']_{EC50} = \left(1+\frac{[S]_0}{K_m}\right)K_I' \tag{B2}$$

Dividing B1 by B2 gives Eq. (B3).

$$\frac{[I]_{EC50}}{[I']_{EC50}} = \frac{K_I}{K_I'} \tag{B3}$$

The values of $[Galantamine]_{EC50}$ and $[N\text{–}oxidegalantamine]_{EC50}$, referring to the enzyme AChE, have been reported in the literature [37]. Therefore, taking into account the value of K_I^{Gal} obtained in the present work and using Eq. (B3), the value of $K_I^{N-oxide}$ can be determined.

Taking into account that $[Galantamine]_{EC50} = 5.16\times10^{-6}$ M, $[N\text{–}oxidegalantamine]_{EC50} = 2.62\times10^{-5}$ M and $K_I^{Gal} = 2.2\times10^{-7}$ M, a value of $K_I^{N-oxide} = 1.1\times10^{-6}$ M is obtained.

References

[1] D. De Bundet, S. Sarre, A. Van Eeckhant, I. Smolders, Y. Mochotte, Sensors 8 (2008) 5171–5185.
[2] E. Milkani, C.R. Lambert, W.G. McGimpsey, Anal. Biochem. 408 (2011) 212–219.
[3] S. Darvesh, K.V. Darvesh, R.S. McDonald, D. Mataija, R. Walsh, S. Mothana, O. Lockridge, E. Martin, J. Med. Chem. 51 (2008) 4200–4212.
[4] B.M. Mcgkeenon, K.B. Dynan, A.P. Passmore, J. Clin. Pharmacol. 48 (1999) 471–480.
[5] J.S. Van Dyk, B. Pletschke, Chemosphere 82 (2011) 291–307.
[6] P.J. Houghton, Y. Ren, M.J. Howes, Nat. Prod. Rep. 23 (2006) 191–199.
[7] P. Anand, B. Singh, N. Singh, Bioorg. Med. Chem. 20 (2012) 1175–1180.
[8] P.K. Mukherjee, V. Kumar, M. Mal, P.J. Houghton, Phytomedicine 14 (2007) 289–300.
[9] G.G. Guibault, Enzymatic Methods of Analysis, 1st ed., Pergamon Press, Oxford, 1970.
[10] A.G. Hadd, S.C. Jacobson, J.M. Ramsey, Anal. Chem. 71 (1999) 5206–5212.
[11] A.G. Marangoni, Enzyme Kinetics. A Modern Approach. Wiley-Interscience, New Jersey, 2003.
[12] C.S. Pundir, N. Chauhan, Anal. Biochem. 429 (2012) 19–31.
[13] G. Baum, F.B. Ward, Anal. Chem. 43 (1971) 947–948.
[14] M. Cuartero, J.A. Ortuño, M.S. García, F. García-Cánovas, Anal. Biochem. 421 (2012) 208–212.
[15] L. Michaelis, M. Menten, Biochem. Z. 49 (1913) 333–369.
[16] R. Bachus, U. Bickel, T. Thomsen, I. Roots, H. Kewitz, Pharmacogenetics 9 (1999) 661–668.
[17] M.R. Farlow, Clin. Pharmacokinet. 42 (2003) 1383–1392.
[18] G.S.J. Mannens, C.A.W. Snem, J. Hendrickx, T. Verhaeghe, L. Le Jeune, W. Bode, L. Van Beijsterveldt, K. Lavrijsen, J. Leempoels, N. Van Osselaer, A. Van Peer, W. Meuldermans, Drug Metab. Dispos. 30 (2002) 553–563.
[19] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop, Clarkes's Isolation and Identification of Drugs, 2nd ed., The Pharmaceutical Press, London, 1986.
[20] K. Reddersen, T. Heberer, J. Sep. Sci. 26 (2003) 1443–1450.
[21] M. Gros, M. Petrovic, D. Barceló, Anal. Bioanal. Chem. 386 (2006) 941–952.
[22] S.D. Richardson, Anal. Chem. 81 (2009) 4645–4677.
[23] H.A. Claessens, M. Van Thiel, P. Westra, A.M. Soeterboek, J. Chromatogr. 275 (1983) 345–353.
[24] J. Tencheva, I. Yamboliev, Z. Zhivkova, J. Chromatogr. 421 (1987) 396–400.
[25] T. Verhaeghe, L. Diels, R. de Vries, M. De Meulder, J. de Jong, J. Chromatogr. 789B (2003) 337–346.
[26] N.R. Mustafa, I.K. Rhee, R. Verpoorte, J. Liq. Chromatogr. Relat. Technol. 26 (2003) 3217–3233.
[27] J. Maláková, M. Nobilis, Z. Svoboda, M. Lísa, M. Holčapek, J. Květina, J. Klimes, V. Palička, J. Chromatogr. B 853 (2007) 265–274.
[28] R.V.S. Nirogi, K.N. Vishwottam, M. Koteshwara, M. Santosh, J. Chromatogr. Sci. 45 (2007) 97–103.
[29] V. Ravinder, S. Ashok, A.V.S.S. Prasad, G. Balaswamy, Y.R. Kumar, B.V. Bhaskar, Chromatographia 67 (2008) 331–334.
[30] M.J. Culzoni, R.Q. Aucelio, G.M. Escandar, Talanta 82 (2010) 325–332.
[31] T. Ghous, A. Townshend, Anal. Chim. Acta 372 (1998) 379–386.
[32] R. Gotti, J. Fiori, M. Bartolini, V. Cavrini, J. Pharm. Biomed. Anal. 42 (2006) 17–24.
[33] A. Rizzi, R. Schuh, A. Brückner, B. Cvitkovich, L. Kremser, U. Jordis, J. Fröhlich, B. Küenburg, L. Czollner, J. Chromatogr. B 730 (1999) 167–175.
[34] L. Pokorna, A. Revilla, J. Havel, J. Patocka, Electrophoresis 20 (1999) 1993–1997.
[35] D. Du, S. Chen, J. Cai, D. Song, J. Electroanal. Chem. 611 (2007) 60–66.
[36] M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, M. Ovalle, Portugalie Electrochim. Acta 27 (2009) 353–362.
[37] R. Reyes-Chilpa, S. Berkov, S. Hernández-Ortega, C.K. Jankowski, S. Arsenau, I. Clotet-Codina, J.A. Esté, C. Codina, F. Viladomat, J. Bastida, Molecules 16 (2011) 9520–9533.