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Amperometric biosensor based on monoamine oxidase (MAO) immobilized in sol–gel film for benzydamine determination in pharmaceuticals

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Abstract

The development and application of a flow-through amperometric biosensor for benzydamine determination in anti-inflammatory drugs is described. The biosensor was obtained by physical entrapment of monoamine oxidase in a sol–gel film applied on platinum or carbon paste conducting support. The sol–gel membranes were prepared using an optimum concentration of 3-aminopropyltriethoxy silane, 2-(3,4-epoxycyclohexyl)ethyl-trimethoxy silane, double distilled water saturated with polyethylene glycol 6000 and HCl. The developed biosensors were incorporated in a single channel flow injection system to enable the determination of benzydamine in the concentration range of 0.05–2.5 mmol l^{-1} (with platinum based electrode) or within 0.1–2.5 mmol l^{-1} (carbon paste based electrode). The operational stability of the bioanalytical system developed was about 3 months permitting approximately 4700 substrate measurements. The flow injection system developed enables a sampling rate of 20–25 samples h^{-1} and relative S.D. of results less than 4%. The analytical usefulness of the proposed procedure was evaluated through analysis of commercial pharmaceutical products containing benzydamine, available on the Portuguese market. The results obtained did not differ significantly from the values resulting from analysis of the same products by the method described in the BP Pharmacopoeia.
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Keywords: Biosensor; FIA-amperometry; Monoamine oxidase (MAO); Benzydamine; Pharmaceutical formulations

1. Introduction

Benzydamine (1-benzyl-3-(3-dimethylamino-propoxy)-1H-indazole hydrochloride) is a tertiary amine (Fig. 1) with widespread therapeutic application due to its analgesic, anti-inflammatory and

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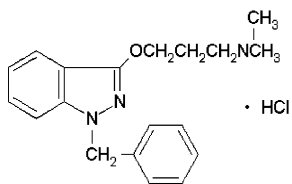


Fig. 1. Chemical structure of benzydamine.

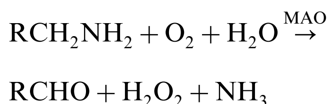
anti-pyretic activity, used either systemically or topically [1].

Several analytical methods for the assay of benzydamine have been proposed although suffering from different drawbacks when, routine non-expensive monitoring is intended. The non-aqueous titration method [2,3] is a tedious and time-consuming procedure, suffering from many organic impurities interference. Ultraviolet spectroscopy sometimes resorting to a previous solid phase extraction [4,5] has a narrow analytical response range and also lacks selectivity. Fourier-transform near-infrared spectroscopy [6] requires special and expensive instruments. High performance liquid chromatography, with peroxyoxalate chemiluminescence based detection [7] or spectrophotometric detection [8–10] proved to be sensitive and less susceptible to interference, although a sample clean-up procedure, including extraction and purification, is required prior to sample injection. The benzydamine potentiometric method [11,12] has the advantage that the equipment as well as the reagents required are simpler and less expensive. However, a major drawback is that the sample matrix affects the electrode response slope.

The development of amperometric biosensors constitutes a recent field of research and application in pharmaceutical control due to the possibility of simplified and highly specific analytical control procedures [13,14]. In this context, the immobilization of enzymes at the surface of electrodes with amperometric transduction is highlighted by the electrochemical generation of polymers [15–24] and sol–gel technology [25–30]. Sol–gel derived biocomposites provide a series of significant advantages relative to the former immobilization schemes once they can be produced using a wide range of compounds to entrap a large number of different biomolecules. Such materials also tend to preserve enzymatic activity, both initially and during long-term storage, and in some cases can lead to an improvement of stability for labile biomolecules [31].

Monoamine oxidase (MAO) has been scarcely used in analytical determinations. It was used in the estimation of fish quality by immobilization on to silanized electrodes by cross-linking with glutaraldehyde [32] and for determination of anti-depressant drugs, immobilized by electropolymerization of pyrrole [24].

In this work the immobilization of the enzyme MAO in a sol–gel film, cast on a platinum and/or carbon paste support, was carried out. The developed amperometric sol–gel MAO sensor was incorporated into a flow injection analysis system. The reaction is as follows



$$RCH_2NH_2 + O_2 + H_2O \overset{MAO}{\rightarrow}$$

$$RCHO + H_2O_2 + NH_3$$

Hydrogen peroxide was monitored electrochemically at +0.7 V in platinum and +0.8 V in carbon paste vs. Ag/AgCl electrode, respectively. Finally, the applicability of flow procedure was carried out in the determination of benzydamine in pharmaceutical formulations.

2. Experimental

2.1. Reagents and solutions

For the preparation of all solutions, reagents of p.a. quality or similar as well as deionised water (Millipore Milli-Q system) were used.

The MAO bovine plasma enzyme (I.U.B.1.4.3.6) was supplied by Worthington Biochemical Corporation (specific activity 22.5 UI mg^{-1}), while 3-aminopropyltriethoxy silane (3-APTES), 2-(3,4-epoxycyclohexyl)ethyl-trimethoxy silane (EETMOS) and polyethylene glycol 6000 (PEG 6000) were purchased from Fluka. The Sigma Chemical Co supplied benzydamine and also benzylamine (substract used in the evaluation of enzymatic activity).

The buffer solution used in the evaluation of the biosensor consisted of 0.1 mol l^{-1} $H_2PO_4^-$/ HPO_4^{2-} (pH 7.4). The same solution was also used as carrier in the FIA system developed.

The primary amino solution (benzylamine) used in the characterization of the biosensor was prepared by rigorous measurement of a certain volume of the corresponding liquid and its subsequent dissolution in phosphate buffer of pH 7.4. Stock solutions of 0.100 mol l^{-1} in benzydamine were prepared by careful weighing of the analyte and by subsequent dissolution in the same buffer solution. For the reference procedure, 0.025% (w/v) standard solutions were prepared by careful weighing of benzydamine and dissolution with ethanol (96% v/v).

2.2. Sample preparation

For the reference procedure [33] the samples of benzydamine cream were prepared by weighing an amount of product corresponding to 25 mg of benzydamine hydrochloride and dissolution with 50 ml of ethanol (96% v/v). After complete dissolution, the sample was placed in an ice-bath until a white precipitate was formed. It was then allowed to warm up to 20 °C, diluted to 100.0 ml with ethanol and then filtered. A 10.0 ml volume of the filtrate was diluted to 100.0 ml with ethanol and the absorbance measured at 308 nm.

The powder samples were prepared by removal of the contents of five sachets. About 9 g of the powder was transferred to a 100.0 ml volumetric flask, dissolved in 75 ml of ethanol (96% v/v), placed in an ultrasonic bath for 5 min, before being made up to a volume of 100.0 ml and filtered. A volume of 0.5 ml of the filtrate was then diluted to 100.0 ml with ethanol (96% v/v) and the absorbance measured at 308 nm.

The samples of mouth wash solution were prepared by dilution of 0.25 ml of the product with 100.0 ml of ethanol (96% v/v), followed by filtration before the absorbance was read at the same wavelength.

The tablet samples were prepared by powdering the content of five tablets in a mortar. About 1.5 g of the powder was transferred to a 100.0 ml volumetric flask, dissolved in 75 ml of ethanol (96%), placed in an ultrasonic bath for 5 min before the volume was made up to 100.0 ml. This was then filtered and centrifuged and the supernatant absorption measured at 308 nm.

The contents of 5 flasks of made-up gynaecological solution were mixed and 2.5 ml of this mixture was diluted to 100.0 ml with ethanol.

All samples analysed by FIA were prepared following the procedure described for the reference method without the need to proceed to centrifugation (in the case of tablets) and were dissolved in phosphate buffer (pH 7.4), so that, its concentration was comprised in the linear zone of the biosensor response.

2.2.1. Equipment

All electrochemical measurements were carried out using a potentiostat (PSTAT10 Echochemie/Autolab) controlled by GPES3 v.3.2 software. For general batch enzymatic studies, a 663 VA Metrohm Stand was used, consisting of a working platinum disk or carbon paste electrode (3-mm diameter), a reference Ag/AgCl, KCl (sat.) and an auxiliary electrode. For FIA experiments a flow cell (wall-jet 656 VA Metrohm Stand electrochemical detector) was used, consisting of a working platinum disk or carbon paste electrode (3-mm diameter), a reference electrode Ag/AgCl, KCl (sat.) and an auxiliary electrode.

The FI-system developed used a peristaltic pump (Gilson Minipuls 3) as propulsion system and an injection valve (Rheodyne 5020) for the intercalation of standard and sample solutions. The connections of all components of the FIA system were done with PTFE tubes (0.8-mm i.d.). A PTFE reactor with length of 40 cm was placed between the injection valve and the detector unit.

Sample analysis according to the method recommended by BP [33] was conducted using a Hitachi U-2000 spectrophotometer.

For solution pH measurements, a Metrohm ES20 potentiometer was used with a combined glass electrode of the same brand.

2.2.2. Construction of the biosensor

As support for the sol–gel membrane a platinum disk (from jewellery) or carbon paste disk was used. The carbon paste was prepared using a

mixture (0.2:0.4 w/w) of graphite (50 μ granulometry, Merck) and a non-conductive epoxy resin (Araldite M and HR hardener (Ciba-Geigy) [34], with a resistance $< 300\ \Omega$. In both cases the electrode body for biosensor construction was made from Teflon. A platinum disk or a carbon paste disk placed on a threaded aluminium rod was fitted in a hollow Teflon cylinder (5-mm diameter; 50-mm length) with its top exposed to the solution (Fig. 2). Three different diameters for the support materials (1, 2 and 3 mm) were studied. Before use, the platinum or carbon paste supports were manually polished with diamond spray Kemet® (3 and 1 μm), with polishing paper (both Kemet®, PSU8 type) and washed with deionised water.

The sol–gel membranes were prepared using an optimum concentration of 3-aminopropyltriethoxy silane (70 μl), 2-(3,4-epoxycyclohexyl)ethyl-trimethoxy silane (20 μl), double distilled water saturated with polyethylene glycol (PEG) 6000 (1400 μl) and HCl (7 μl), according to previous work [30]. A volume corresponding to 15 μl of the homogenized solution was cast on the platinum base or carbon paste support of the electrode body and about 1 h later 7 μl of MAO solution (0.5 mg ml^{-1}) was added to the layer of the sol–gel glass. The period before addition of the enzyme is necessary for adequate occurrence of polymerisation and condensation reactions which otherwise would be impaired by the addition of the naturally less acidic enzyme solution. Moreover, the addition of MAO over the ormosil guarantees saving in the amount of enzyme added to develop a biosensor unit. The electrodes were tested after a 24-h drying period at room temperature. When not in use, the electrodes were washed with 0.1 mol l^{-1} phosphate buffer (pH 7.4) and stored in the same buffer at 4 °C.

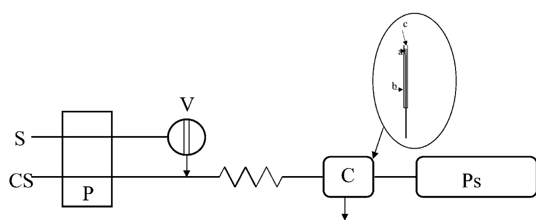


Fig. 2. FIA system and schematic diagram of the working electrode. Legend: platinum or carbon paste disk (a) placed on a threaded aluminium rod fitted in Teflon cylinder (5 mm diameter; 50 mm length) (b) with sol–gel layer (c). FIA system: S—sample; CS—carrier solution; P—peristaltic pump; V—injection valve; Ps—potentiostat; C—wall-jet cell.

2.3. Reference method

The reference method used for the determination of benzydamine in all formulations was performed by UV spectrophotometric determination at 308 nm.

British Pharmacopoeia [33] only describes benzydamine determination in cream, mouthwash and oromucosal spray formulations. As in Portugal there are other pharmaceutical forms, for which no reference method is described, it was decided to adopt the same spectrophotometric procedure as reference.

3. Results and discussion

A previous study was performed in order to evaluate the influence of the two types of membrane supports on the electrochemical response of the biosensors. Thus, the two types of electrodes with different surface areas corresponding to 1, 2 and 3 mm^2 with or without an inert sol–gel membrane were evaluated by cyclic voltammetry using a 10 mM potassium ferrocyanide solution prepared in potassium nitrate with 1 M ion strength. The voltammograms obtained with a scanning rate of 5 mV s^{-1} revealed peak diffusion currents proportional to surface area and 100 times greater for platinum support relatively to carbon paste support. In both cases the effect of the presence of sol–gel film was minimum considering the 3 mm^2 units. Hence it was decided to select the 3 mm^2 surface area electrodes for biosensor development, with sol–gel layer thickness of about 0.25 mm. The sol–gel film preparation was based on the use of 3-APTES and EETMOS as starting monomers, in a relative proportion previously optimised in order to provide adequate hydrophobic characteristics for enzyme entrapment [30]. The quantity of water present in the starting sol dispersion affects the monomer hydrolysis and condensation rates lead-

ing to the formation of gels with different pore sizes, affecting the efficacy of enzyme immobilization in sol–gel matrix [25,27]. Hence the influence of the amount of water added was studied in the interval from 500 to 1400 µl (R ratio (H_2O:Si) from 700 to 2000). The membrane formulation, which enabled the quickest and highest analytical response resulted of dropping on the electrode support an amount of 15 µl of a mixture made up of 3-aminopropyltriethoxy silane (70 µl), 2-(3,4-epoxycyclohexyl)ethyl-trimethoxy silane (20 µl), double distilled water saturated with polyethylene glycol (700 µl) and HCl (7 µl). After 1 h, a volume of 7 µl of MAO suspension (corresponding to 17.5 µIU) was added in order to enable enzyme physical entrapment under mildest chemical conditions.

The biosensors constructed were examined for their performance in absence and presence of benzylamine to investigate the nature of the amperometric response of MAO biosensors. These results are shown in Fig. 3a and b. There is an increase in the anodic current corresponding to the electrochemical oxidation of hydrogen peroxide.

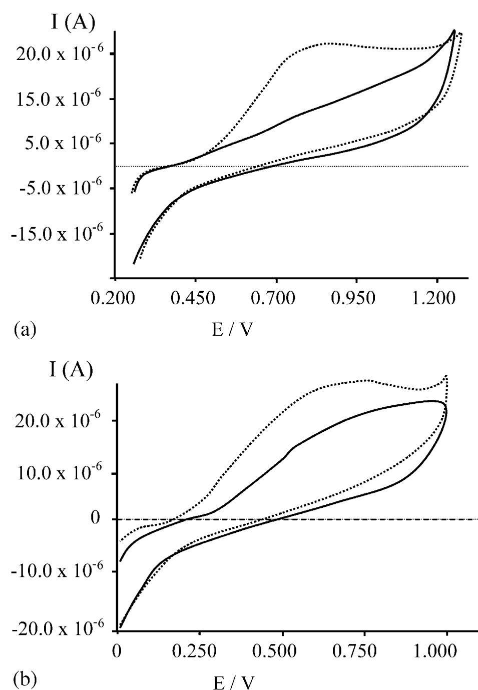


Fig. 3. Cyclic voltammograms of (a) carbon paste and (b) platinum based biosensors in absence (—) and presence (····) of benzylamine in phosphate buffer pH 7.4.

Cyclic voltammograms obtained after biosensors immersion in benzydamine solutions revealed displacement in the oxidation potential of benzydamine from +0.8 (carbon paste) to +0.7 V for platinum support based biosensors. The chemical conditions, which lead to maximum enzymatic activity, were settled by successive calibrations using standard solutions containing benzydamine up to 5 mM. In this way for both type of electrodes two different buffer solutions were studied, namely phosphate (0.1 mol l^{-1} phosphate buffer, pH 7.4) and HEPES (0.1 M *N*-[2-hydroxyethiyl] piperazine-*N′*-[2-ethanesulfonic acid] pH 7.4), with no difference having been verified in the cyclic curves obtained. Therefore it was decided to use phosphate buffer in subsequent studies. The amperometric response was evaluated and both electrodes responded rapidly (~ 10 s) but the carbon paste electrode presented a greater end current (200 µA) than that of the platinum (17.5 µA). The different end currents are probably due to different final sol–gel areas, because platinum is a smooth surface but graphite is neither smooth nor homogeneous, as a consequence graphite electrodes have a bigger biosensor surface thus leading to higher analytical signals, proportional to diffusion.

3.1. *Optimisation of the FIA system*

A single channel FIA manifold for benzydamine determination with amperometric detection incorporating the MAO biosensors developed was established (Fig. 2). The sample (200 µl) was inserted in a phosphate carrier solution (0.1 mol l^{-1} phosphate buffer, pH 7.4) and the amperometric current was monitored electrochemically using a three-electrode cell.

The influence of the different chemical and hydrodynamic parameters in enabling the measurements to be carried out within a wide concentration range, with good sensitivity and with a high sampling rate, were evaluated using the univariate method. For this purpose the height of analytical signal corresponding to 0.5 mM solution of benzydamine intercalated in the FIA system was measured.

The influence of the injected sample volume was assessed for values ranging from 75 to 500 μl. As expected, an increase in analytical signal intensity with an increase in injection volume was observed. With volumes greater than 200 μl, the variation in current intensity was of minor significance, but there was an increase in the width of the peak, which give rise to a fall-off in sampling rate. Therefore an injection volume of 200 μl was selected since it provided a good sensitivity as well as reproducibility and avoided the necessity of large sample consumption. The effect of flow rate was also evaluated. Low flow rates gave rise to a large interaction between the substrate and enzyme, favoring the bio-catalytic reaction and consequently leading to a greater amperometric response. For this reason, this parameter was studied in the 0.20–1.5 ml min^{-1} interval. No significant analytical signal variation was observed in the interval studied, although values greater than 0.83 ml min^{-1} yielded less reproducible analytical signals. Therefore, a flow rate of 0.83 ml min^{-1} was adopted, as a compromise between the sensitivity and the sampling rate obtained (20–25 samples h^{-1}).

The other factors that influenced the enzymatic activity such as pH of the buffer solution, temperature and ionic strength were fixed according to data obtained in cyclic voltammetric studies. The optimum pH was shown to be 7.4 and maximum sensitivity was observed when carrier solution temperature was settled at 37 °C.

Ionic strength influence on the analytical signal was evaluated as a function of the phosphate carrier solution concentration, in the 0.0025–0.01 mol l^{-1} range (Fig. 4). The best response was obtained with a 0.005 M phosphate buffer solution.

The dependence of the substrate concentration on the biosensor studied, in the range of 0.01–40 mM benzylamine showed a typical Michaelis–Menten constant behaviour. The response increased up to a concentration value of 20 mM and thereafter remained practically constant. The Michaelis–Menten constant value calculated was 5×10^{-3} M, which is lower than 0.75×10^{-3} obtained for the free enzyme in solution tested under static conditions [35] and lower than the previous obtained by enzyme electro immobilization into a polypyrrole film [24]. When considering benzydamine as analyte, linear responses were obtained within the concentration ranges of 0.05–2.5 mM, using platinum and 0.1–2.5 mM using carbon paste electrode, respectively, (Figs. 5 and 6). The higher detection limit obtained with carbon paste electrodes is a consequence of the higher end current observed for this type of biosensor.

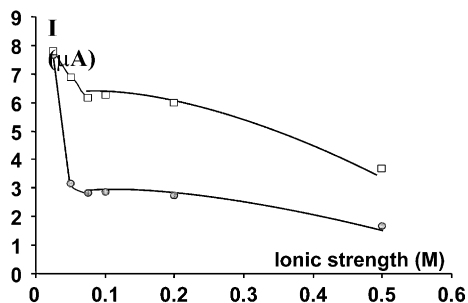


Fig. 4. Ionic strength effect platinum electrodes (●) and carbon paste electrodes (■).

Experiments were conducted to evaluate the biosensor stability over time. The analytical signal intensities obtained from the injection of a 1 mM standard benzydamine solution were verified during a period of 90 days. A fall-off in analytical signal to 85% of the initial value was shown after 30 days of use (720 substrate determinations), and 55% at the end of 3 months (4725 substrate determinations) for both types of biosensors constructed. When compared to the previous work [24] the number of analytical measurements increased more than 100 times stressing the robustness of sol–gel immobilization. The biosensor in which the membrane was deposited in carbon paste was unattached at the end of this period

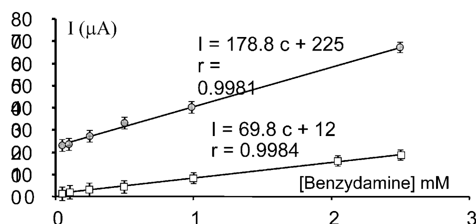


Fig. 5. Calibration curve for benzydamine (■) carbon paste electrodes; (◆) platinum electrodes.

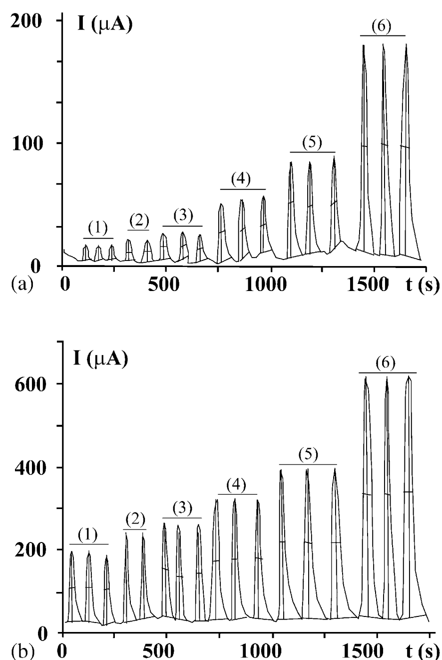


Fig. 6. (a) Platinum and (b) carbon paste based biosensors. (a) (1) 0.05 mM; (2) 0.1 mM; (3) 0.2 mM; (4) 0.5 mM; (5) 1 mM; (6) 2.5 mM benzydamine standard solutions. (b) (1) 0.05 mM; (2) 0.1 mM; (3) 0.25 mM; (4) 0.5 mM; (5) 1 mM; (6) 2.5 mM benzydamine standard solutions.

while the platinum based biosensors still worked. The biosensors constructed allowed more than 4700 substrate determinations to be carried out having been easily recycled at the end of their useful life.

3.2. Interferences study

The interference on the analytical signal of components commonly used as excipients such as manitol, lactose and glucose was also studied. These were evaluated by intercalation of solutions in the FIA system, with concentrations close to those normally present in pharmaceutical products, considering pure solutions of interfering specie and solutions of interfering specie prepared in 1 mM benzydamine. No significant variation in the analytical signal was observed except for NaCl, which showed interference for values higher than 0.5 M, giving rise to a decrease in response of 75%, probably due to ionic strength variations.

3.3. Determination of benzydamine in pharmaceutical formulations

The present system was used for benzydamine determination in eight samples. Table 1 shows the analytical data related to the analysis of eight formulations commercially available in Portugal. Accuracy of the results given by the FIA method (CF) was assessed by comparison with the results provided by the reference method (CR). A linear relationship was obtained and is expressed as follows: $C_B = 0.4745 + 0.9976\ C_R$ mol L^{-1}($R =$ 0.9997) for platinum based biosensors and $C_B = 1.5141 + 0.981\ C_R$ ($R = 0.9994$) for graphite based biosensors.

Table 1
Results obtained from the analytical determination of benzydamine in pharmaceutical products

Pharmaceutical formulation	Reference procedure	FIA procedure	
		Platinum	Carbon paste
Tantum gel[a]	31.31 ± 0.4	31.15 ± 1.3	30.93 ± 0.7
Rosalgin (washing liquid)[b]	114.37 ± 0.9	115.27 ± 1.9	115.47 ± 1.0
Tantum verde (Culotório)[b]	108.91 ± 2.6	109.51 ± 1.3	110.91 ± 2.3
Flugoral (Culotório)[b]	109.62 ± 3.6	107.91 ± 2.7	109.96 ± 2.6
Rosalgin (sachet)[a]	54.50 ± 3.3	52.52 ± 3.3	51.50 ± 3.3
Tantum rosa (sachet)[a]	54.43 ± 0.4	54.50 ± 3.3	52.45 ± 2.3
Tantum verde (tablets)	3.50 ± 0.7	3.50 ± 1.3	3.25 ± 0.8
Flogoral (tablets)[a]	3.15 ± 0.04	3.25 ± 0.08	3.47 ± 0.3

[a] mg g^{-1}.
[b] mg ml^{-1}.

A Student two-tail paired t-test was also applied yielding a value of 0.1353, which is less than the theoretical value of 1.624 for a confidence interval of 95%. It may thus be concluded that there are no significant differences between the proposed method and the reference procedure.

The within-run precision of FIA methodology was assessed by calculating the relative S.D.s after ten successive injections of a sample of 1.45 mM concentration. The values obtained showed good precision with a relative standard error less than 3.8% for platinum and 2.2% for carbon paste electrodes.

4. Conclusions

Sol–gel technology is both simple and inexpensive. Carbon paste based electrodes apart from being economic, allowed the attainment of MAO biosensors with greater analytical sensitivity than that obtained with platinum as the support matrix. This immobilization technique is of low-cost creating the possibility of employing it to other enzyme systems for electrochemical sensor development. The system developed here allows benzydamine determination over a wide concentration range, with an enzyme consumption less than 35 μg per biosensor, permitting up to 4700 determinations and with a useful life of approximately 90 days.

The proposed FIA system involves both a low reagent and sample consumption as well as a good sampling rate (20–25 samples h^{-1}).

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