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A MIP-BASED FLOW TROUGH FLUOROIMMUNOSENSOR AS AN ALTERNATIVE TO IMMUNOSENSORS FOR THE DETERMINATION OF DIGOXIN IN SERUM SAMPLES

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Abstract This work reports a comparative study of two automated flow-through fluorosensors for the determination of digoxin in serum samples: an immunosensor with an anti-digoxin polyclonal antibody as the reactive phase permanently immobilized on CPG, and a sensor with a selective reaction system based on a methacrylic molecularly imprinted polymer (MIP) synthesized by bulk polymerisation. The variables affecting the sensitivity and dynamic range of the sensors (e.g., the carrier and elution solutions, flow-rates, pH and reagent concentrations) were optimised and the binding characteristics of their reactive phases compared in a competitive fluorescent assay. Digoxin was reproducibly determined by both sensors at the mg L^{-1} level (detection limit= 1.20×10^{-3} mg L^{-1} and RSD= 4-7% for the immunosensor; detection limit= 1.7×10^{-5} mg L^{-1} and RSD= 1-2% for the MIP sensor). No cross reactivity with digoxin-related compounds was seen for either sensor at a digoxin:interferent ratio of 1:100. The lifetime of the immunosensor was about 50 immunoassays; its shelf life, when unused, is about three months. The lifetime of the MIP sensor was over 18 months. Both sensors were used to determine the digoxin concentration of human serum samples with satisfactory results.

Keywords Digoxin · Permanent immobilization · Molecular imprinting · Flow-through fluorosensor · Serum analysis.

Introduction

Immunosensors represent an important development in the field of immunochemistry, and expectations regarding the clinical advantages they offer are high. Many biological materials, e.g., antibodies, proteins or nucleic acids, and even living biological systems, including cells, tissues or whole organisms, have been used as specific recognition elements in the development of sensors for use in medicine, environmental pollution monitoring, and for the control of food quality and safety [1]. However, such technology has important limitations related to the poor chemical and physical stability of the biocomponents used, shortages in the supply of these components, and the high cost many demand, particularly antibodies [2].

Synthetic molecular receptors with properties similar to biorecognition systems have attracted considerable attention. Molecular imprinting technology has been of particular interest since it provides a means of making recognition systems that do not require antibodies or any other biocomponent. Molecularly imprinted polymers (MIPs) are robust, relatively cheap, show good physical and chemical stability, and are more easily prepared than antibody-based sensors; certainly they require no sacrifice of animals [3]. Moreover they often function better in organic solvents -environments in which antibodies perform poorly- than under aqueous conditions. Initially, large molecules could not be imprinted [4, 5], but rapid advances have been made in terms of MIP formats; films, coatings and microbeads on which the binding sites are more readily accessible are now available. These formats also allow for an easier integration of MIPs into sensing devices.

A current criticism of MIPs is the large quantity of template molecules needed to prepare a useful amount of imprinted polymer, a potential problem if the molecule to be imprinted is expensive or difficult to obtain. However, this is compensated by the high stability of MIPs and the possibility of using them repeatedly over long periods of time. In addition, dramatic advances are being made in reducing the quantity of template molecules required [6, 7].

This paper reports a comparative study of two flow-through fluorosensors for the determination of digoxin: an immunosensor and a MIP-based sensor. The reactive phase of the immunosensor [8] involved the orientated, chemically permanent immobilization of anti-digoxin polyclonal antibodies on controlled-pore glass (CPG) (immobilization efficiency for optimum amount of immobilized antibody was always >88%), while the

recognition element of the MIP-based sensor [9] was a methacrylic polymer obtained by bulk polymerisation. Competitive assays of these systems were undertaken in which fluorescein isothiocyanate-labelled digoxin (FITC-digoxin) and unlabelled digoxin competed for the active recognition sites. The fluorescence signal measured was that generated by the labelled digoxin remaining attached to the immuno or MIP reaction phase. The physical and chemical variables affecting the performance of both sensors were optimised, and the binding and elution stages compared. The selectivity of the two fluorosensors with respect to a number of compounds with structures analogous to digoxin was compared, as was the sensitivity achieved in the determination of digoxin in human serum samples. The reproducibility of the results provided by these systems, their working ranges and detection limits are discussed. Taking into account the MIP-based sensor seems to perform much better than the immunosensor. The results obtained, show molecularly imprinted materials can be used as a proper alternative to immunosorbents.

Experimental

Instrumentation and materials

A Perkin-Elmer LS-5 spectrofluorometer controlled by an AAT computer was used to measure the fluorescence intensity of the reactive phase of each system. The immunoreactor consisted of a 100 µl flow cell with a 3 mm optical path (Hellma; Jamaica, NY, USA) filled with the CPG-antibody or digoxin MIP reactive phase. This was placed in a spectrofluorometer for *in situ* detection. The flow injection system involved a Gilson Minipulse-2 peristaltic pump and an Omnifit injection valve (six way). PTFE tubes (0.5 mm i.d) were employed to build the manifold. A Metrohm 654 pH meter was used to monitor the pH. Radiation for polymer synthesis was provided by an ultraviolet lamp (Vilber Lourmat CN-6T) at 365 nm. Template extraction was performed using a soxhlet extractor system with cellulose extraction thimbles. Imprinted and control polymers were ground in a glass mortar (Aldrich, Madrid, Spain) and then passed through CISA standard sieves (355-600 µm) (Afora, Madrid, Spain).

Chemical and biochemical reagents

Digoxin (95%), sodium azide and sodium tetrahydridoborate were purchased from Sigma-Aldrich (Madrid, Spain). Sheep anti-digoxin polyclonal antibodies (3.6 g L^{-1}) were purchased from Helena Bioscience (Sunderland, UK); these were immobilized on CPG (particle range 37-74 μm) (Bio-Processing, Consett, Co Durham, UK). FITC-digoxin (10 μmol L^{-1}) was supplied by MicroPharm (Newcastle Emlyn, Carmarthenshire, UK). Ethylene glycoldimethacrylate (EDMA), methacrylic acid (MAA), sodium dodecylsulphate (SDS) and 3-aminopropyl triethoxysilane were obtained from Merck (Darmstadt, Germany). Sodium docecylsulphate solutions at different concentrations (4, 6, 8, 10 mM) were prepared in 10 mL of Milli-Q water. 2-2´-*azobis*-isobutyronitrile (AIBN) was purchased from Fluka (Buchs, Switzerland). Phosphate buffer solution (PBS, pH between 6.8-8.5) was prepared in 1.0 L of Milli-Q water (Millipore Ibérica, Madrid, Spain) containing 0.1 mM NaCl, 1.4 mM KH_2PO_4, 2.7 mM KCl, 8 mM NaH_2PO_4 and 21.3 mM $MgCl_2$ (Merck, Darmstadt, Germany). Citric acid solutions (0.5 M) were prepared in 0.5 M NaCl made in 500 mL of Milli-Q water at pH between 2-3.5. Acetonitrile and methanol (HPLC grade) were provided by Scharlab (Barcelona, Spain). Deionised water (18.3 MΩ cm) produced by a Milli-Q water system was used for the preparation of all aqueous solutions.

Procedures

Anti-digoxin antibody immobilization by cross-linking to CPG

The antibody was oxidized according to the method of Wilson and Nakane [10], and the CPG alkylaminated with 3-aminopropyl triethoxysilane according to León-González and Townshend [11]. Anti-digoxin antibodies were subjected to site-directed immobilization by covalent attachment of the generated carbonyl groups to the amine groups produced on the surface of the alkylaminated CPG. CPG is a widely used support for enzyme and antibody immobilization since undesired interactions with other compounds do not occur. Different volumes of oxidized antibody (5, 10, 25, 50 and 100 μL) were then allowed to react with 0.1 g of the treated CPG for 24 h at room temperature in complete darkness. The immobilization yield was determined by measuring the fluorescence emission of the antibody solution at 340 nm (λ_{exc}= 298 nm)

before and after coupling. Studies showed the efficiency of the antibody immobilization to be greater than 70%; the results obtained for different batches were reproducible [8].

After immobilization, the product was incubated for 45 min at 4ºC with 4 mg mL^{-1} $NaBH_4$ and then stored in PBS (pH= 7.2) containing 0.02% NaN_3 as a preservative. Under these conditions the immunosensor was stable for at least three months without detectable loss of activity.

Synthesis of the digoxin MIP

The template molecule (digoxin, $2 \cdot 10^{-3}$ mmol), functional monomer (MAA, 2 mmol), crosslinker (EDGMA, 10 mmol) and initiator (AIBN, $6 \cdot 10^{-2}$ mmol) were placed in a 25 mL glass tubes and dissolved in 10 ml acetonitrile (ACN) as porogen. The mixture was then purged with nitrogen for 10 min before transferral of the tubes to the photochemical reactor, where they were kept under UV light at 365 nm for 24 h at 10ºC to allow polymerisation. The bulk polymer obtained was ground in a mechanical mortar and sieved into particles in the size range 355-600 μm. The template was extracted over 20 h using the soxhlet system and employing MeOH:ACN (50:50 v/v). Non-imprinted polymer (NIP), prepared by omitting the template molecule, was used as a control.

Flow injection system

The immunoreactor consisted of a flow cell, filled with either the CPG-anti-digoxin or digoxin MIP, placed in a spectrofluorometer to provide *in situ* fluorescence detection; the reaction and detection systems were therefore integrated. A frit was placed in the bottom of optical path of the flow cell to prevent the carrier sweeping away the reactive phases. Figure 1 shows a diagram of the flow-through immunosensor manifold.

Sample preparation

Serum samples containing digoxin were supplied for analysis by the Puerta de Hierro Hospital (Madrid, Spain). For analysis using the MIP sensor system, aliquots (650 μL) were mixed with 900 μL of ACN and centrifuged for 30 min at 3500 rpm to precipitation the serum proteins. Digoxin samples solutions were prepared by adding

170 µL of conjugated FITC-digoxin in ACN (dilution 1/50, 0.2 µmol L^{-1}) to 1700 µL of the correspondingly treated serum. For the immunosensor system, no prior treatment of serum samples was necessary; rather, they were simply diluted (1:1) with PBS (10 mM, pH= 7.2).

Competitive fluorescent assay for the determination of digoxin in human serum samples

Digoxin binding was measured using a fluorescent competitive assay reaction between FITC-digoxin and free digoxin. The proposed methods are based on the principle of competitive fluorescence assays where labelled (FITC-digoxin) and unlabeled (digoxin) antigens compete for the actives sites of the antibody (which is covalently bound to the CPG) and the antigen-antibody complexes are formed. In MIP sensor both antigens compete for the complementary cavities of the polymer.

For the immunosensor, tracer solution was prepared from commercial FITC-digoxin by diluting 1/50 in PBS (10 mM, pH= 7.2), and stock digoxin solution (1000 µL, 0.5 mg L^{-1}) was also prepared in PBS. To prepare digoxin solution for the MIP sensor, a mixture of Na_2CO_3 (0.1 M, pH= 8) and ACN (50:50, v/v) was used. To obtain the standard response curve of the reactive phases for digoxin, standard solutions of different digoxin concentrations were prepared up to $5 \cdot 10^{-2}$ mg L^{-1} for use with the immunosensor and from 0 to $4 \cdot 10^{-3}$ mg L^{-1} for use with the MIP sensor. For this, 100 µL of tracer solution (1/50, 0.2 µmol L^{-1}) and the corresponding aliquot of stock digoxin solution were added to each of the above, and the final volume made up to 1000 µL with PBS (pH= 7.2) or ACN as required. For the assay the corresponding solutions were injected into the carrier PBS (10 mM, pH= 7.2) for the immunosensor, and ACN (pH= 9) for the MIP sensor; 30 µl were injected for the immunosensor assay, and 150 µL for the MIP sensor assay. The fluorescence signals were measured in the immunoreactor at an excitation wavelength of 496 nm and emission wavelength of 517 nm in both cases. The sensors were then regenerated, with citric acid (0.5 M, pH= 3) for the immunosensor, and MeOH/ACN (90:10, v/v) for the MIP sensor.

Results and discussion

The experimental conditions for both sensors were optimised taking into account that any suitable sensor system should be highly sensitive and selective, have a short response time, and be free from interference. A systematic study was made of the variables (type of solvent, pH, and the flow rates of the binding and elution solutions) that affected the efficacy of the retention process and the amount of conjugated digoxin required. These assays were carried out by spectrofluorimetry at λ_{exc}= 496 nm and λ_{em}= 517 nm.

Initially, the efficiency of the immobilization processes were calculated by analyzing, by spectrofluorimetry at 340 nm (λ_{exc}= 298 nm), the amount of unbound antibody present in the wash fractions collected during the washing of 0.1 g of CPG after the immobilization procedure and this was greater than 70% in all cases.

For the immunosensor, digoxin tracer (FITC-digoxin) concentrations of 0.05, 0.10 and 0.20 μmol L^{-1} were tested for each immobilized antibody volumes of 5, 10, 25, 50 and 100 μL corresponding to amounts of antibody (18, 36, 90, 180 and 360 μg) on 0.1 g of CPG support in order to obtained a good assay sensitivity. For studies 30 μL of FITC-digoxin were injected and the fluorescence intensity monitored at the wavelengths mentioned. The optimum values for FITC-digoxin and antibody were 0.2 μmol L^{-1} and 50 μL, ~~respectively~~. The optimum volume of immobilized antibody (50 μL) corresponding to 1800 μg antibody added/ g CPG (A) and 1607 μg antibody loaded/ g CPG (B) being the efficiency of antibody loading on the solid support (B/A×100) of 89.3%.

A compromise between a suitable assay speed and the fluorescence signal was necessary in order to reduce the total assay time, thus the effect of the flow rate (0.1 to 0.4 mL min^{-1}) (Fig. 2) and the pH (6.8-8.5) (Fig.3) of the PBS binding solution on the analytical signal was studied. A flow rate of 0.20 ml min^{-1} and a pH of 7.2 were selected as optimum.

For the MIP sensor, the polymer composition (amounts of functional monomer MAA, crosslinker EDMA, nature solvents) and the conditions of polymerisation and extraction process were studied in order to obtain the optimum MIP for digoxin analyte [9]. 11.5 mg of imprinted polymer were packed in the flow-cell to carry out the assays. To obtain a good sensitivity, binding studies on molecular imprinted polymer for

different digoxin tracer concentrations (studied range $7.5 \cdot 10^{-3}$ to 0.3 μ mol L^{-1}) and carrier solutions (ACN, MeOH and aqueous phosphate buffer solution PBS 10 mM at pH 7.5) were carried out. For these studies the effect of the binding solution flow rate was studied over the range of 0.1 to 0.6 mL min^{-1}(Fig. 2). The optimum FITC-digoxin concentrate was 0.2 μmol L^{-1} and ACN was chosen as the best binding solution at 0.27 mL min^{-1} optimum flow rate. The effect of the pH binding solution ACN (from 3 to 9) was tested by varying of binding solution pH with citric acid, Na_2CO_3 or NaCl at different pH according to the pH value required. ACN at pH= 9.0 was selected as the best binding solution (Fig.3). In order to increase the intensity of fluorescence signal the influence of a tensactive substance, sodium dodecylsulphate salt (SDS), at different concentrations (between 4 and 10 mM) was also tested. The addition of this at a concentration (8 mM) close to the critical micellar concentration (cmc= 8.1 mM) increased the fluorescence signal considerably.

The regeneration solution must be able to break the bindings between analyte and antibody or MIP, but neither affecting the properties nor binding ability of reactive phases. Studies for optimization of parameters that affect the regeneration process (pH, washing time, compositions of solutions) were carried out. For immunosensor a citric acid solution was used as regeneration solution and the pH effect was studied by varying the pH within the range 2.0 to 3.5 and for washing times between 100-130 seconds. In case of MIP sensor the effect of nature and composition of elution solutions were tested: MeOH:ACN (70:30, 50:50, 30:70, 90:10, v/v), H_20, MeOH and MeOH:ACN:H_2O (80:10:10, v/v) and the washing time from 70 to 90 seconds were studied. For the immunosensor, the best regeneration solution was citric acid (0.5 M in NaCl 0.5 M) at pH 3 and during 120 s. Under these conditions no loss of immunosurface activity was seen for 50 assays (reusability of the immunosurface is one of the main problems encountered in immunosensor development). Its storage time (unused) was three months -although storage must be at 4ºC in PBS. For the MIP sensor, the optimum regeneration solution was a mixture of MeOH and ACN (90:10 v/v), this solution showed the greatest elution percentage (99%), and the optimum washing time was 80 s. This sensor showed great stability and could be used repeatedly for over a year. Further, no special storage conditions were required while not in use. Table 1 shows the optimum regeneration conditions for each sensor.

Sensitivity was estimated under optimum conditions using competitive calibration curves. These were constructed using digoxin standards over a concentration

range of 0 to 4×10^{-3} mg L^{-1} for the MIP sensor, and up to 0.05 mg L^{-1} for the immunosensor (Fig. 4). The normalized signals expressed as B/Bo (where B is the peak of the fluorescence complex at different standard concentrations of digoxin and Bo is the blank sample) were plotted against digoxin concentration. In immunoassay the limit of detection is the least detectable dose (LDD), commonly used to define sensitivity. LDD is measured by assaying of the zero standard (e.g., 5-10 replicates) and calculating the mean counts bound and standard deviation. The mean is used for the standard curve, and the response, (mean – 2 SD) read in mass or concentration from the standard curve is the LDD, that is the smallest dose that is not zero with 95% confidence [12]. In accordance with this, the detection limits were calculated using five replicates of zero digoxin standards and expressed as the least detectable dose of digoxin. The obtained values were 1.20×10^{-3} mg L^{-1} and 1.7×10^{-5} mg L^{-1} for the immunosensor and the MIP sensor respectively. For the immunosensor, the reproducibility shown for the 2.5×10^{-3} and 1×10^{-2} mg L^{-1} digoxin standards was RSD= 4.5 and 6.7% respectively (n= 6), while for the MIP sensor the reproducibility was RSD= 1.0 % and 1.8% for 1×10^{-3} 4×10^{-3} mg L^{-1} respectively (n= 6) (Table 2).

Specificity is the ability of an assay to produce a measurable response only for the analyte of interest. For the interference study, competitive curves were constructed for several substances with structures analogues to digoxin (narcotine, heroine, codeine, pentazocine, morphine and tebaine) (see Fig. 5). The cross reactivity or selective binding was tested. A displacement curve for cross-reacting/interfering substance is compared to the standard curve (only digoxin). The concentration at 50% displacement (standard) divided by the concentration of competitor (interfering) is the cross reactivity in concentration units.

$$\text{Cross reactivity at 50\% displacement} = \frac{\text{concentration of standard}}{\text{concentration of competidor}} \text{x100}$$

In this work, the interfering calibrations curves were carried out for concentrations of interfering 100 times higher than digoxin in standard calibration curve.With all potential interfering, and under the optimum conditions for both sensors, no cross reactivity at 50% were measurable, which means that no interference was detected (Fig. 6).

When using the MIP sensor, the analysis of the serum samples was performed in an organic medium; however, the addition of this medium, required to precipitate the serum proteins, is a simple process. For the immunosensor this step was not necessary; analysis was performed in an aqueous medium. Table 3 shows the results obtained with each fluorosensor. No significant differences were seen between the values obtained by the immunosensor and MIP-sensor with respect to the Hospital methods (95%).

Conclusions

Combining biosensor technology with molecular imprinting is an interesting alternative to the traditional method of immunosensor construction. This paper shows that the replacement of antibodies by MIPs as the selective molecular recognition system in fluorescent assays is feasible and offers several advantages. For example, the reactive phase produced is more stable can be used under harsher conditions (such as in organic environments). Further, the MIP sensor tested was more sensitive and provided more reproducible results than the immunosensor. Both proposed fluorosensors were, however, highly selective and their sensitivity was high enough to be used in the determination of serum digoxin with no complicated pre-treatment of the samples. In the case of the immunosensor, no sample treatment was required at all. Cross reactivity was negligible for both sensors. The highly automated system used allowed for rapid assays; the total assay time for both sensors was around 300-400 s.

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TABLES

Table 1 Summary of the optimum conditions for the tested sensors

Variable	Optimum values for the immunosensor	Optimum values for the MIP sensor
Excitation and emission slits	0.5 / 2.5	5 / 10
Binding solution	10 mM PBS in 0.1 M NaCl (pH= 7.2)	ACN/SDS (8 mM) (50:1) (pH= 9)
Regeneration solution	0.5 M citric acid in 0.5 M NaCl (pH= 3)	MeOH/ACN (90:10)
Flow rate	0.20 ml min^{-1}	0.27 mL min^{-1}
Concentration of the reactive phase	Antibody (50 µL; 3.6 g L^{-1})	MIP (~11.5 mg)
FITC-digoxin	0.20 µmol L^{-1}	0.20 µmol L^{-1}
Assay time	300s	400 s

Table 2 Analytic characteristics of the developed digoxin fluorosensors

	Immunosensor	MIP sensor
Optimum working concentration range	1.2×10^{-3} - 4×10^{-2} mg L^{-1} digoxin	1.7×10^{-5} - 4×10^{-3} mg L^{-1} digoxin
Detection limit	1.2×10^{-3} mg L^{-1}	1.7×10^{-5} mg L^{-1}
RSD	4–7% (standards, n= 6)	1–2% (standards, n= 6)
Calibration curve	%B/Bo= 6×10^{4} C^2 – 5×10^{3} C + 97 (n= 4, r= 0.994)	%B/Bo= 9×10^{6} C^2 – 5×10^{4} C + 96 (n= 5, r= 0.990)
Assay frequency	10-12 assays/hour	7-8 assays/hour
Lifetime	50 assays (shelf life 3 months)	approximately 18 months

Table 3 Results of human serum analysis performed with the studied sensors (n= 6)

Sample	Immunosensor (mg L^{-1})	Radiochemical reference method (mg L^{-1})
1	$(4.1 \pm 0.1) \times 10^{-3}$	$(4.0 \pm 0.1) \times 10^{-3}$
2	$(2.3 \pm 0.3) \times 10^{-3}$	$(2.5 \pm 0.2) \times 10^{-3}$
3	$(1.4 \pm 0.5) \times 10^{-3}$	$(1.7 \pm 0.3) \times 10^{-3}$
4	$(4.4 \pm 0.1) \times 10^{-3}$	$(4.4 \pm 0.1) \times 10^{-3}$
	MIP sensor (mg L^{-1})	Reference method (Microparticle Immunoenzyme Assay) (mg L^{-1})
5	$(7.6 \pm 0.8) \times 10^{-4}$	$(8.0 \pm 0.3) \times 10^{-4}$
6	$(2.5 \pm 0.2) \times 10^{-4}$	$(3.0 \pm 0.2) \times 10^{-4}$
7	$(21.0 \pm 2.1) \times 10^{-4}$	$(24.2 \pm 1.3) \times 10^{-4}$
8	$(9.1 \pm 0.6) \times 10^{-4}$	$(9.0 \pm 0.3) \times 10^{-4}$
9	$(15.2 \pm 0.9) \times 10^{-4}$	$(19.0 \pm 2.1) \times 10^{-4}$
10	$(8.4 \pm 0.7) \times 10^{-4}$	$(8.0 \pm 0.4) \times 10^{-4}$

FIGURE LEGENDS

Fig. 1 Flow injection sensor

Fig. 2 Variation of the fluorescence signal with different binding solution flow rates for two sensors studied using as carrier solutions PBS (10 mM, pH= 7.2) and ACN (pH= 9) for immunosensor an MIP sensor, respectively. FITC-digoxin concentration= 0.2 μmol L^{-1}.

Fig. 3 Variation of the fluorescence signal with pH binding solutions (PBS and ACN) at 0.20 and 0.27 mL min^{-1} flow rates for immunosensor and MIP sensor respectively. FITC-digoxin concentration= 0.2 μmol L^{-1}.

Fig. 4 Calibration curves for both sensors under optimum conditions. %B/Bo refers to the fluorescence intensity of digoxin, where B is the peak of the fluorescence complex at different standard concentrations of digoxin and Bo is the blank sample

Fig. 5 Chemical structures of interfering compounds and digoxin.

Fig. 6 Cross reactivity at 50% for each interfering substance using 100 times higher concentration of interfering than digoxin, under optimum conditions. (a) Immunosensor. (b) MIP sensor.

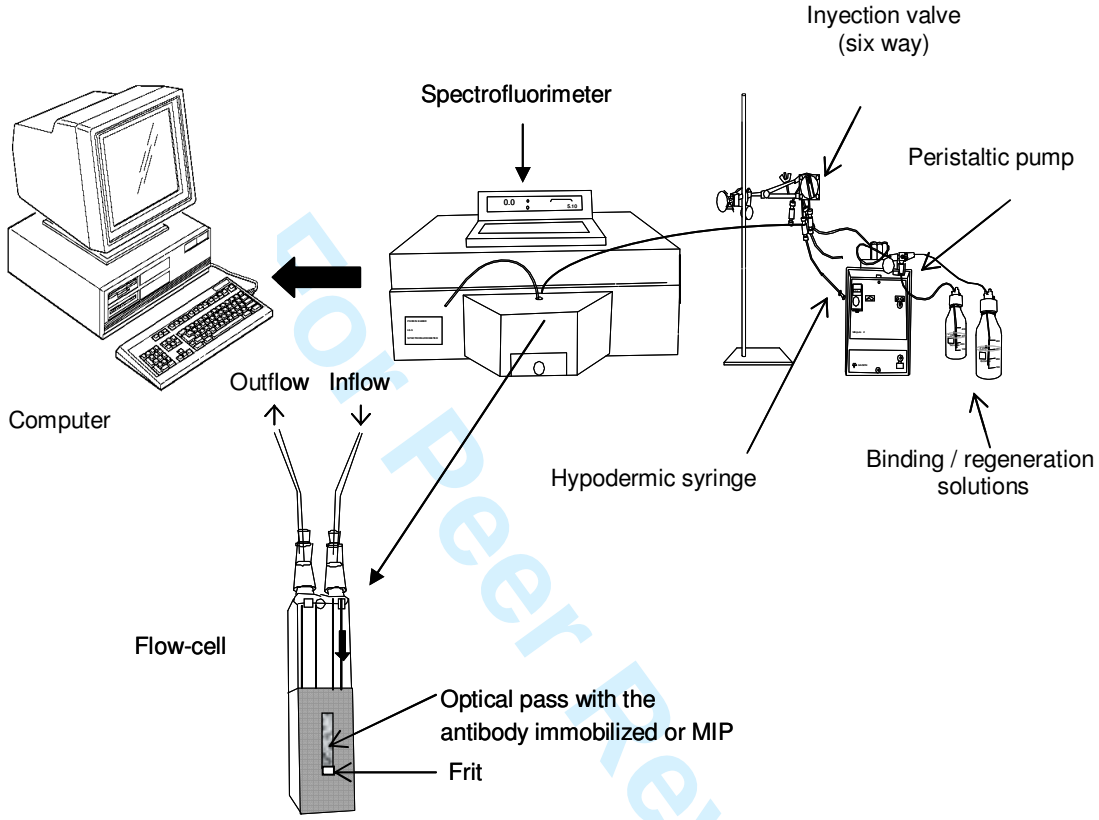


Fig. 1

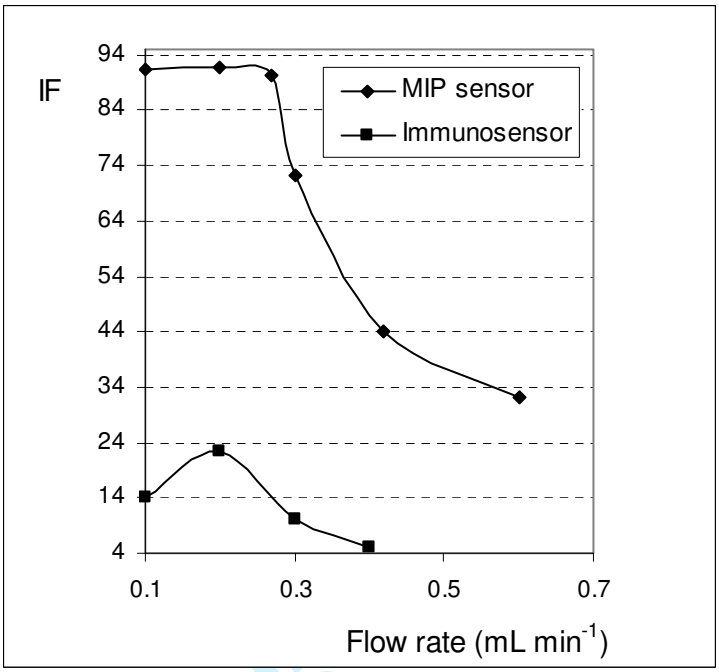


Fig. 2

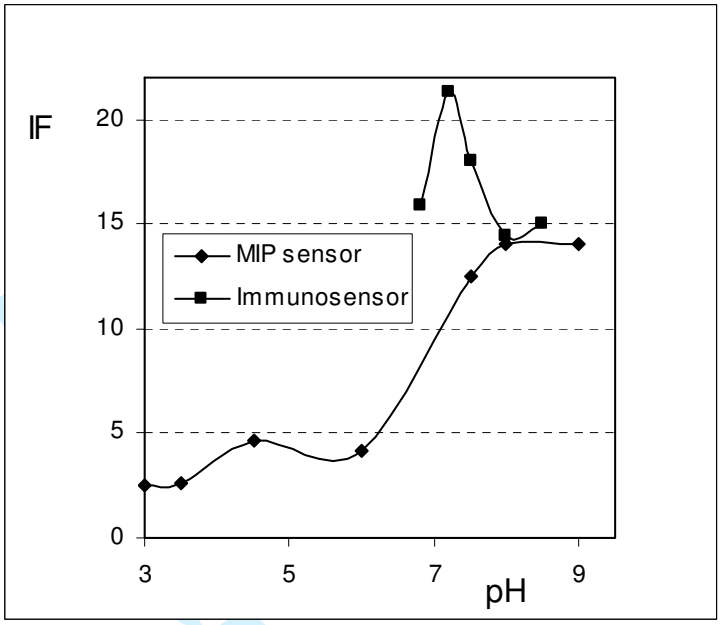


Fig. 3

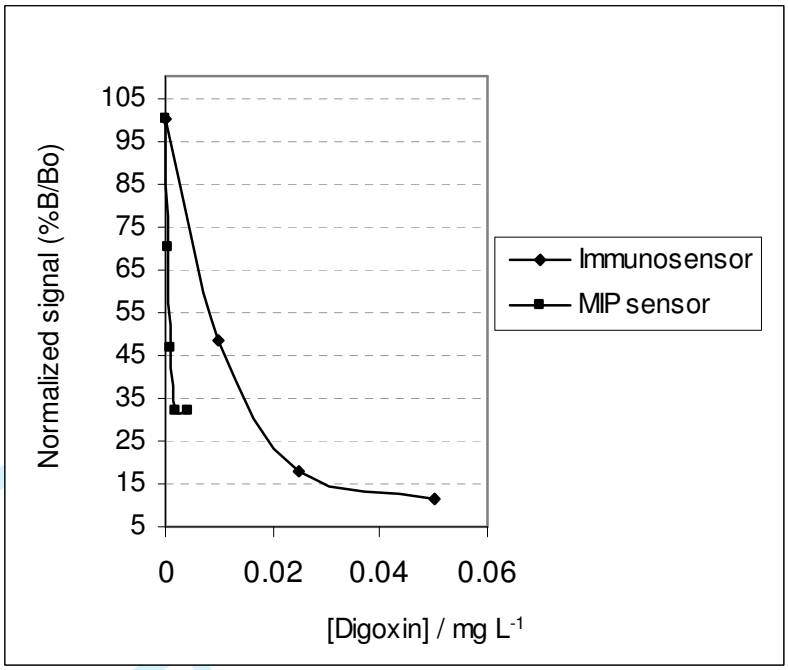


Fig. 4

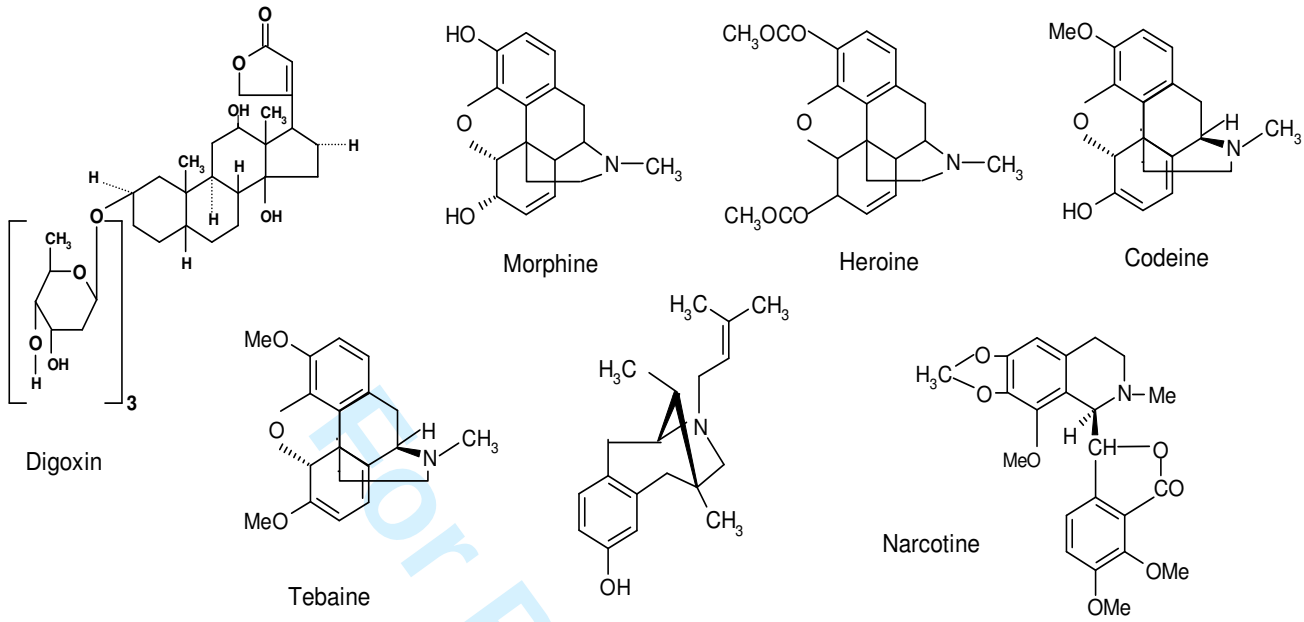
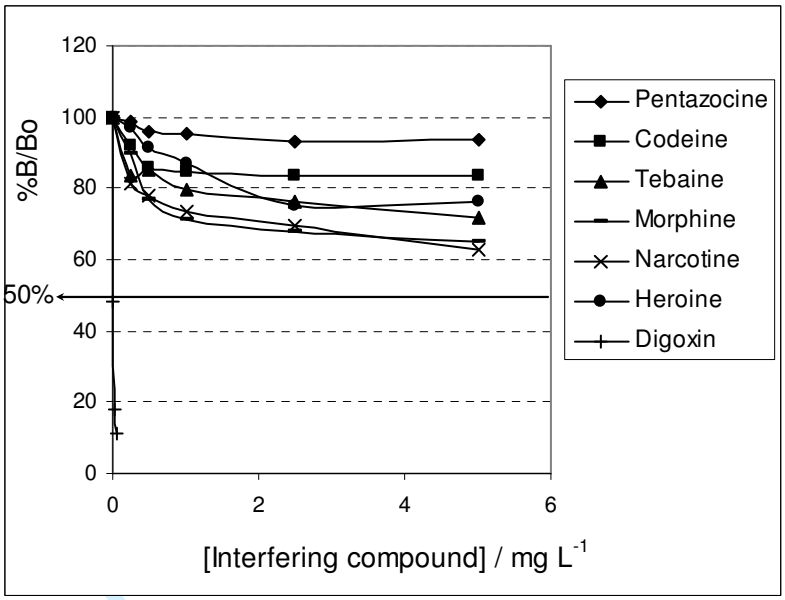
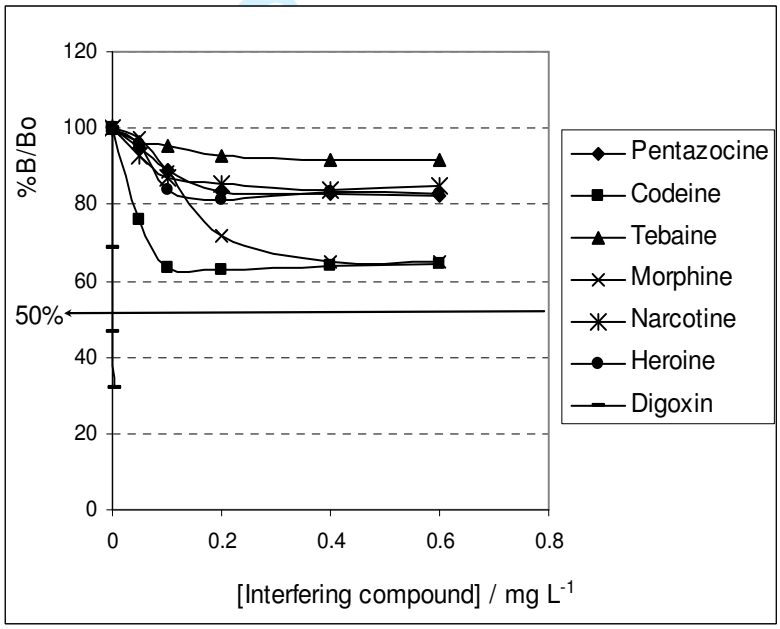


Fig. 5



(a) Immunosensor



(b) MIP sensor

Fig. 6

Table 1 Summary of the optimum conditions for the tested sensors

Variable	Optimum values for the immunosensor	Optimum values for the MIP sensor
Excitation and emission slits	0.5 / 2.5	5 / 10
Binding solution	10 mM PBS in 0.1 M NaCl (pH= 7.2)	ACN/SDS (8 mM) (50:1) (pH= 9.0)
Regeneration solution	0.5 M citric acid in 0.5 M NaCl (pH= 3.0)	MeOH/ACN (90:10)
Flow rate	0.20 ml min^{-1}	0.27 mL min^{-1}
Concentration of the reactive phase	Antibody (50 µL; 3.6 g L^{-1})	MIP (~11.5 mg)
FITC-digoxin	0.20 µmol L^{-1}	0.20 µmol L^{-1}
Assay time	300s	400 s

Table 2 Analytic characteristics of the developed digoxin fluorosensors

	Immunosensor	MIP sensor
Optimum working concentration range	1.2×10^{-3} - 4×10^{-2} mg L^{-1} digoxin	1.7×10^{-5} - 4×10^{-3} mg L^{-1} digoxin
Detection limit	1.2×10^{-3} mg L^{-1}	1.7×10^{-5} mg L^{-1}
RSD	4–7% (standards, n= 6)	1–2% (standards, n= 6)
Calibration curve	%B/Bo= 6×10^{4} C^2 – 5×10^{3} C + 97 (n= 4, r= 0.994)	%B/Bo= 9×10^{6} C^2 – 5×10^{4} C + 96 (n= 5, r= 0.990)
Assay frequency	10-12 assays/hour	7-8 assays/hour
Lifetime	50 assays (shelf life 3 months)	approximately 18 months

Table 3 Results of human serum analysis performed with the studied sensors (n= 6)

Sample	Immunosensor (mg L^{-1})	Radiochemical reference method (mg L^{-1})
1	$(4.1 \pm 0.1) \times 10^{-3}$	$(4.0 \pm 0.1) \times 10^{-3}$
2	$(2.3 \pm 0.3) \times 10^{-3}$	$(2.5 \pm 0.2) \times 10^{-3}$
3	$(1.4 \pm 0.5) \times 10^{-3}$	$(1.7 \pm 0.3) \times 10^{-3}$
4	$(4.4 \pm 0.1) \times 10^{-3}$	$(4.4 \pm 0.1) \times 10^{-3}$
	MIP sensor (mg L^{-1})	Reference method (Microparticle Immunoenzyme Assay) (mg L^{-1})
5	$(7.6 \pm 0.8) \times 10^{-4}$	$(8.0 \pm 0.3) \times 10^{-4}$
6	$(2.5 \pm 0.2) \times 10^{-4}$	$(3.0 \pm 0.2) \times 10^{-4}$
7	$(21.0 \pm 2.1) \times 10^{-4}$	$(24.2 \pm 1.3) \times 10^{-4}$
8	$(9.1 \pm 0.6) \times 10^{-4}$	$(9.0 \pm 0.3) \times 10^{-4}$
9	$(15.2 \pm 0.9) \times 10^{-4}$	$(19.0 \pm 2.1) \times 10^{-4}$
10	$(8.4 \pm 0.7) \times 10^{-4}$	$(8.0 \pm 0.4) \times 10^{-4}$

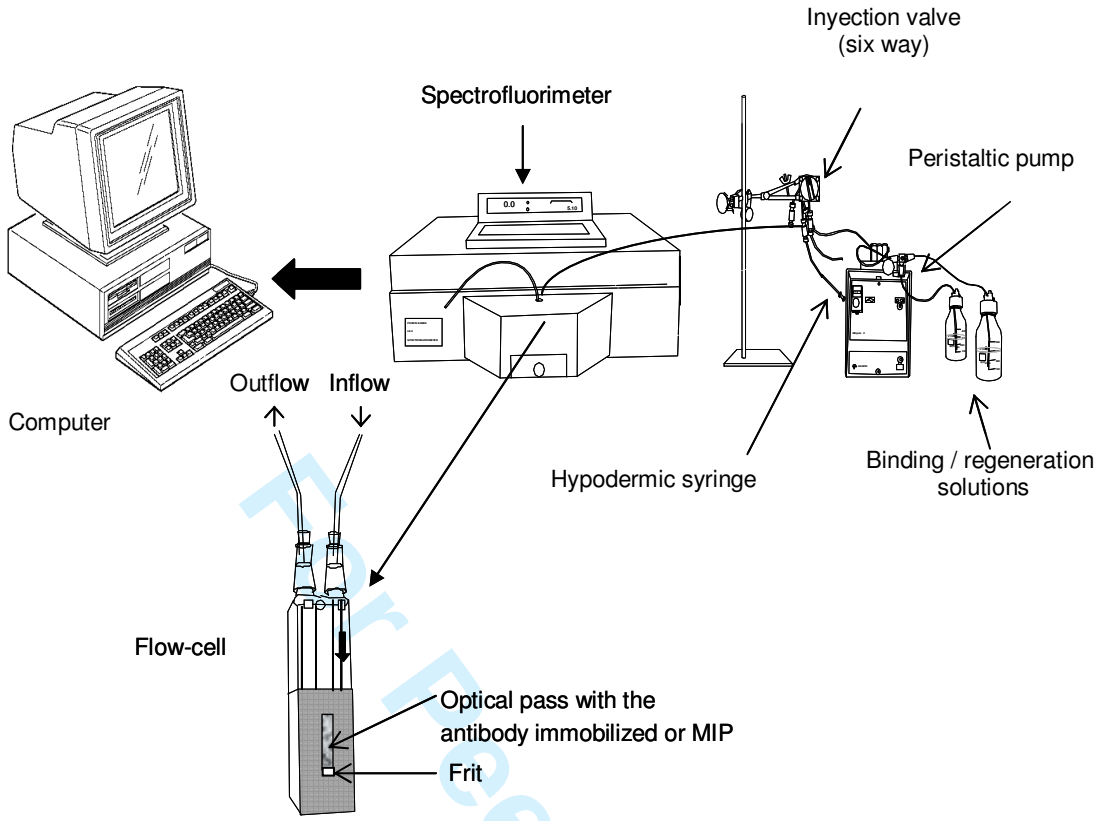
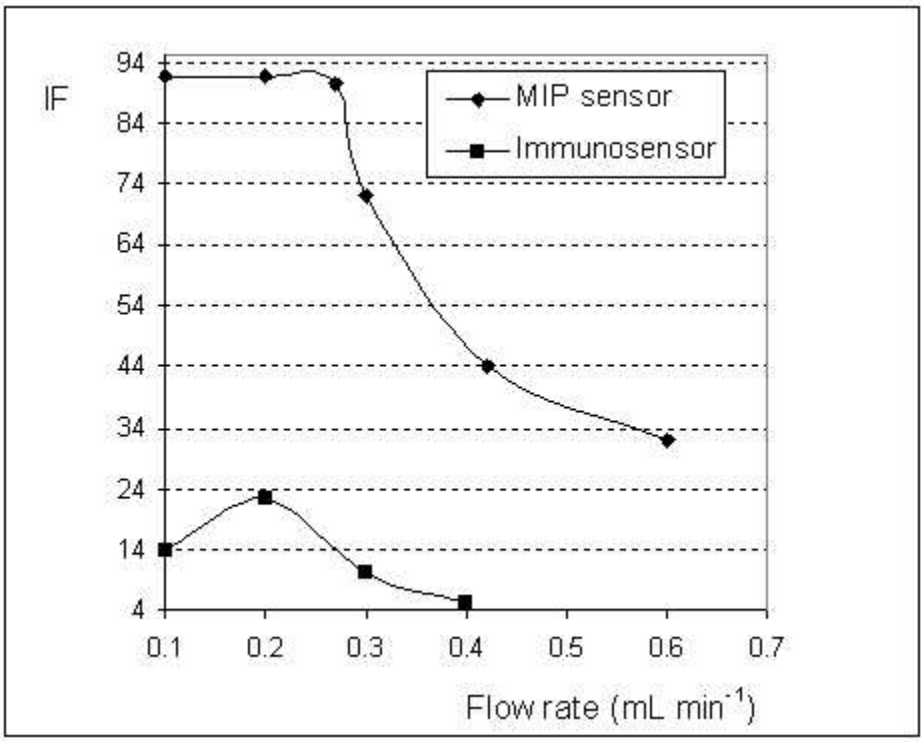


Fig.1



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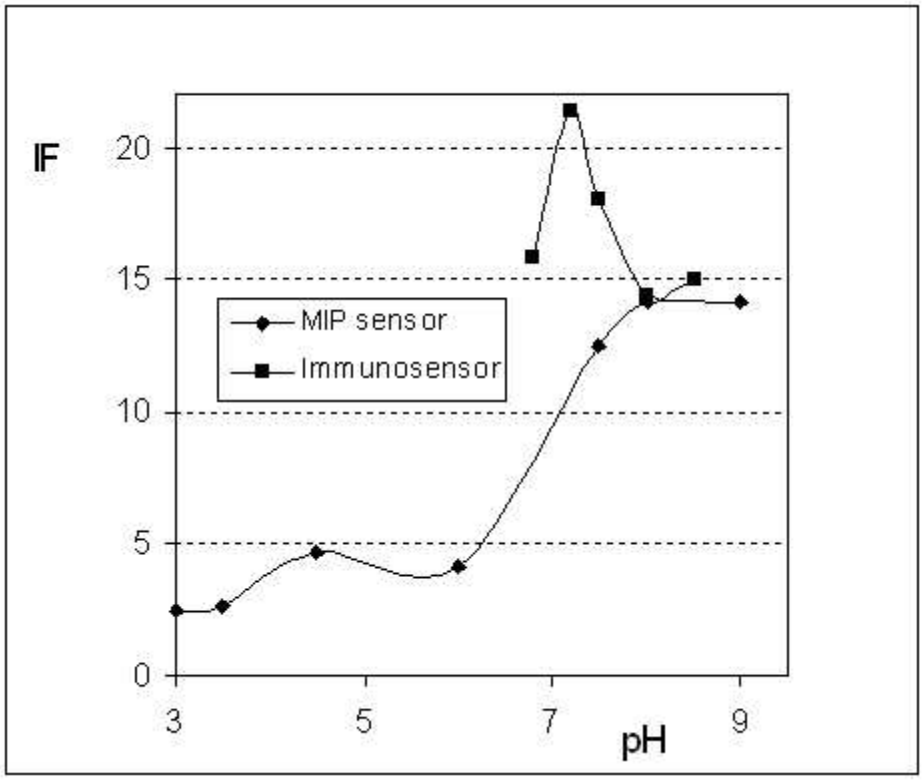

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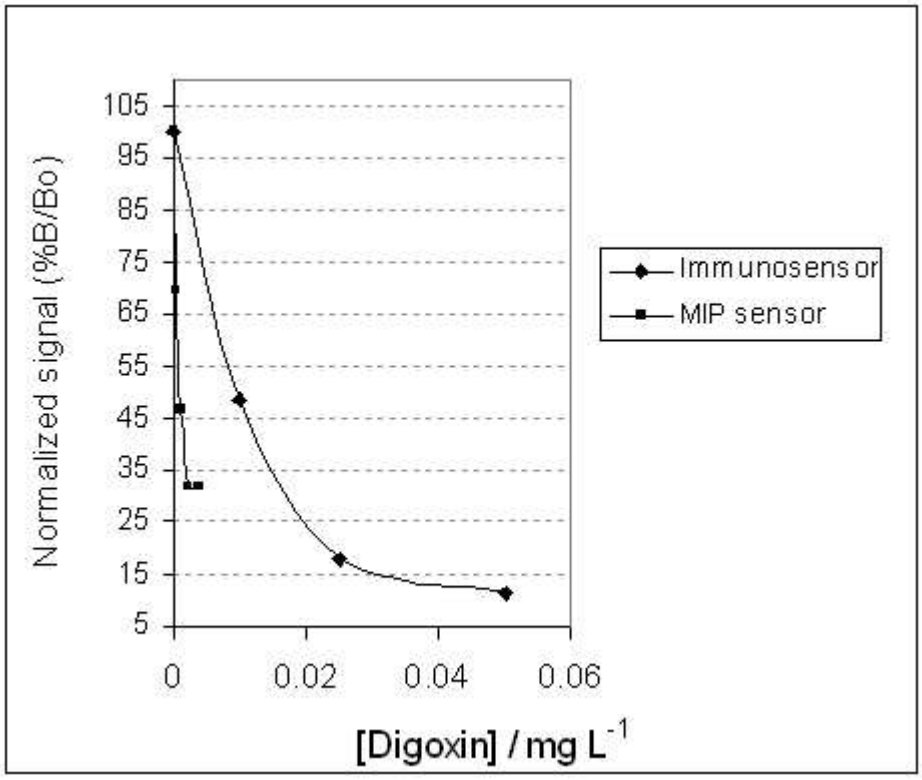

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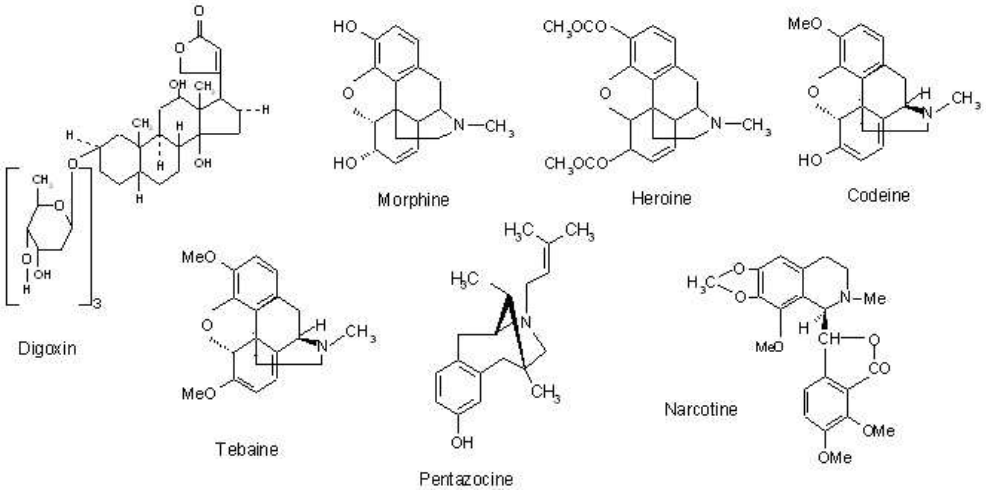

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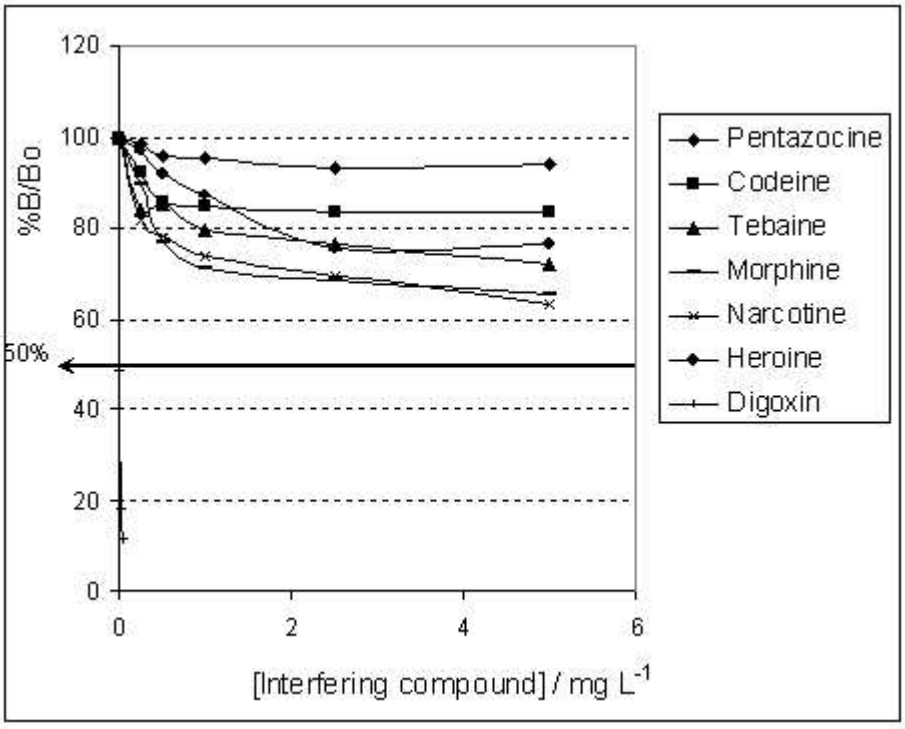

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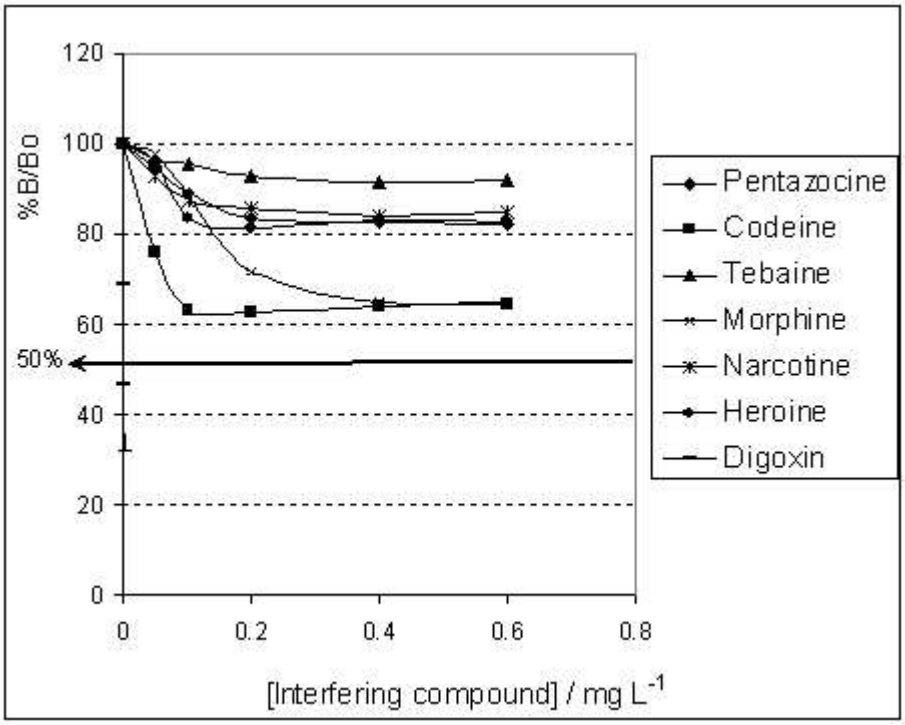

161x129mm (72 x 72 DPI)

FIGURE LEGENDS

Fig. 1 Flow injection sensor

Fig. 2 Variation of the fluorescence signal with different binding solution flow rates for two sensors studied using as carrier solutions PBS (10 mM, pH= 7.2) and ACN (pH= 9) for immunosensor an MIP sensor, respectively. FITC-digoxin concentration= 0.2 μmol L^{-1}.

Fig. 3 Variation of the fluorescence signal with pH binding solutions (PBS and ACN) at 0.20 and 0.27 mL min^{-1} flow rates for immunosensor and MIP sensor respectively. FITC-digoxin concentration= 0.2 μmol L^{-1}.

Fig. 4 Calibration curves for both sensors under optimum conditions. %B/Bo refers to the fluorescence intensity of digoxin, where B is the peak of the fluorescence complex at different standard concentrations of digoxin and Bo is the blank sample

Fig. 5 Chemical structures of interfering compounds and digoxin.

Fig. 6 Cross reactivity at 50% for each interfering substance using 100 times higher concentration of interfering than digoxin, under optimum conditions. (a) Immunosensor. (b) MIP sensor.