PERMANENT–ORIENTED ANTIBODY IMMOBILIZATION FOR DIGOXIN DETERMINATION WITH A FLOW–THROUGH FLUOROIMMUNOSENSOR

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ABSTRACT

Digoxin is a very important compound in clinical chemistry and is indicated in the treatment of congestive heart failure and artery disease.

The measurement of serum digoxin concentration is necessary owing to the narrow therapeutic range of this drug. Further, even with similar dosage regimens, the biological response of patients often results in very different concentrations of digoxin in serum. Concentrations of greater than 2.6 mmol/L are generally interpreted as toxic in adult patients.

Most methods for digoxin determination are based on gas chromatography or radiochemical and enzymatic immunoassay techniques. However, some of these methods are tedious and difficult to automate. Nowadays, they are being replaced by more practical immunoassay techniques, involving, for example, fluorescent immunosensors that allow rapid, automated and selective digoxin determinations.

This paper reports a new flow–through fluoroimmunosensor for digoxin determination, the function of which is based on antibodies immobilized on an inmunoreactor of controlled pore glass (CPG).

The immunosensor has a detection limit of 1.20 μg/L and provides high reproducibility (RSD = 4.5% for a concentration of 0.0025 mg/L, and RSD = 6.7% for 0.01 mg/L). The optimum working concentration range was found to be 1.2×10^{-3} - 4.0×10^{-2} mg/L. The lifetime of the immunosensor was about 50 immunoassays, if stored unused its

lifetime can be extended to three months. A sample speed of about 10–12 samples per hour can be attained. Possible interference from substances with structures similar to digoxin (morphine, heroine, tebaine, codeine, pentazocine and narcotine) was investigated. No cross-reactivity was seen at the highest digoxin:interferent ratio studied (1:100). The proposed fluoroimmunosensor was successfully used to determine digoxin concentrations in human serum samples.

Keywords: flow-through fluoroimmunosensor; digoxin; permanent immobilization; serum samples

1. INTRODUCTION

Digoxin (3- [o-2,6, dideoxy-β-D-ribo-hexapyranosil-(1→4) -0-2,6-dideoxy-β-D-ribo-hexapyranosil-(1→4)-0-2,6-dideoxy-β-D-ribo-hexapyranosil)oxy]-12,14-dihidroxy (3β,2β,12β)-card-20(22) is a cardiac or digitalic glycoside with specific effects on the myocardium. This drug, extracted from the leaves of *Digitalis lanatus*, is used in the treatment of congestive heart failure to increase circulation. It is also used in patients with atrial fibrillation and flutter to slow the ventricular rate. The measurement of serum digoxin concentration is necessary owing to the narrow therapeutic range of the drug; there is a thin line between therapeutic and toxic levels (0.05 – 0.2 μg/L) (1).

Many techniques have been used to determine blood concentrations of digoxin. These include gas chromatography (2), radioimmunoassays (3), enzyme immunoassay techniques (4,5), fluorescence polarization immunoassay (6), and electrochemical methods (7). Some are these methods are relatively tedious, often requiring sample pre-treatment, phase separation or even special technology to increase their sensitivity and selectivity (3).

Different and very sensitive radioimmunoassays exist for the measurement of digoxin in serum (8). However, radioisotopes are now being replaced by immunological labels, such as fluorescent antibodies, which allow simple, rapid techniques that can be automated.

When applied to immunoassay, flow injection (FI) offers some advantages, such as precise control of reaction times, the re-use of supports and reagents, and improved precision. Sensitivity is similar to that obtained with batch methods (9).

This paper presents the development and characterization of a flow-through fluoroimmunosensor for digoxin. This sensor uses an anti-digoxin polyclonal antibody immobilized on CPG in a direct competitive assay. Sensor optimization and analytical aspects are addressed. Finally, the optimized sensor was used to determine the digoxin concentrations of human serum samples.

2. EXPERIMENTAL

2.1. Apparatus and materials

- Perkin Elmer LS5 spectrofluorimeter controlled by an ATT computer.
- 18 μL Hellma flow cell (optical path 1 cm).
- Flow injection system consisting of a Gilson Minipulse 2 peristaltic pump, Omnifit injection valve (six ways) and PTFE tubes (0.5 mm id).
- E 516 Titriskop pH meter.

2.2. Chemicals and biochemicals

- Controlled pore glass (CPG) (Bio-Processing, Consett, Co. Durhan, UK), 460Å particle size 37-74 μm.
- Digoxin sheep polyclonal antibody (3.6 g/L) and digoxin labeled with fluorescent isothiocyanate (FITC) (15 μ mol/L) (Helena Bioscience, Sunderland, UK).
- Digoxin (95%) (Aldrich, Madrid, Spain).
- Phosphate buffer solution (PBS) pH=7.0 was prepared by adding 8 g NaCl, 0.2g KH_2PO_4, 0.2 g KCl, 2.9 g NaH_2PO_4, and 1 g $MgCl_2$ (Merck, Darmstadt, Germany) to 1L of deionized water.
- 0.5 M citric acid solution in 0.5 M NaCl pH=3.0 (Merck, Darmstadt, Germany).
- 3-aminopropyl triethoxylane (Merck, Shuchardt, Germany).
- Dialysis membrane Sigma D-9277 was used to separate the excess periodate.
- Deionized water from a Nanopure System (Barnstead, UK).

2.3. Procedures

2.3.1. Antibody immobilization by cross-linking to CPG

CPG was first alkylamined with 3-aminopropyl trietoxylane according to the León-Gonzalez and Townsend procedure (10) and the periodate method of Wilson and Nakane (11). 500 μg of oxidized antibody was allowed to react with 1.0 g of alkylamined CPG. The product was incubated overnight at room temperature. The immobilization yield was determined by measuring the fluorescence emission of the antibody solution at 340 nm (λ_{ex}= 298 nm) before and after coupling.

2.3.2. System design

A diagram of the flow-through immunosensor manifold is shown in Fig.1. The device consists of a peristaltic pump connected to a six-way valve with a 30μL injection coil. The immunoreactor is a flow cell whose optical path is filled with CPG-antidigoxin placed in the spectrofluorimeter for "in situ" fluorescence detection. A frit was placed in the optical path of the flow cell to prevent the carrier sweeping the CPG-antibody away.

2.3.3. Sample preparation

Serum samples from digoxin-treated patients were supplied by the Puerta de Hierro Hospital, Madrid, diluted (1:1) with PBS and analyzed using the proposed assay protocol.

2.3.4. Assay protocol

The proposed method is based on the principle of a heterogeneous competitive fluorescence immunoassay, where the antibody is covalently bound to the CPG. 30μl of a solution containing 0.2μmol L^{-1} labeled antigen (digoxin-FITC) and unlabeled antigen (digoxin) are injected into the carrier solution at a 0.2 mL min^{-1} flow rate (10 mM PBS, pH=7.2). Both antigens compete for the active sites of the antibodies, and antigen-antibody complexes are formed.

The excess antigen (labeled and unlabeled) was removed from the immunoreactor by the carrier solution. The fluorescence signal generated by the labeled antigen-antibody complex formation was measured "in situ" in the immunoreactor at λ_{em}=517nm and λ_{ex}= 496nm). Finally, a citric acid solution (0.5M, pH=3.0) was pumped into the flow cell for

immunoreactor regeneration. A competitive calibration curve was obtained by increasing the digoxin concentration to 0.05 mg L^{-1}.

3. RESULTS AND DISCUSSION

A suitable immunosensor should be able to perform analyses with high sensitivity, accuracy, speed, without interference, at low cost, and be of low maintenance. The choice of optimum assay conditions should take into account all these considerations. In many cases, a compromise must be adopted between assay sensitivity, speed and reusability (12).

3.1. Immunosensor optimization and characterization

Cross-linking efficiency was evaluated by analyzing the amount of unbound antibody present in all fractions collected during the immobilization procedure by spectrofluorimetry at 340nm (λ_{ex}= 280nm). In all cases, cross-linking efficiency was greater than 70% (see Table 1 for a summary of cross-linking efficiency).

The capacity of the immobilized antibodies to bind hapten depends of the amount of active sites available, that is, on good steric accessibility to active binding sites. This was expressed as the maximum amount of hapten bound to antibody immobilized on 1 g of CPG. For this purpose, 30μL of digoxin-FITC at different concentrations were injected and the fluorescence intensity of each monitored at 517nm (λ_{ex}= 496nm).

A systematic study was made of the parameters affecting the antibody-hapten reaction on the CPG. The effect of binding solution flow rate (0.1- 0.4ml min^{-1}) on the analytical signal was also studied. 0.2 mL min^{-1} was chosen as the optimum carrier flow rate. The influence of pH on the binding and regeneration solutions was investigated by varying the pH of both solutions within the ranges 6.8 to 8.5 and 2.0 to 3.5 respectively. Optimum results were obtained for pH=7.2 and pH=3.0.

Also to be optimized was the amount of antibody present in the reactor. This depends on the amount of CPG, on the antibody density described above, and the amount of antigen tracer used in each assay. Both parameters must be minimal if good assay sensitivity is to be attained, but they should also be large enough to procedure acceptable signals. Different competitive calibrations were tested using antigen tracer within the range 0.2 to 0.05 μmol L^{-1} (for results see Fig.2). The best compromise was found at 0.2 μmol L^{-1}

concentration of digoxin-tracer, the same value required to obtain an acceptable analytical signal.

Table 2. summarizes the optimum conditions for the immunosensor.

3.2. Analytical performance

Competitive calibration curves were produced under optimum conditions using standards at concentrations ranging from 0 to 0.05 mg L^{-1}. The normalized signals were plotted as (B/Bo) vs. digoxin concentration, where B is the speak of the fluorescent complex at different standard concentrations of digoxin and Bo is the blank sample. The experimental points were fitted to a quadratic polynomial equation.

The reproducibility of the method was tested by measuring two digoxin standard concentrations 6 times for several days. The relative standard deviation (RSD) of normalized signals for 2.5 μg L^{-1} and 10 μg L^{-1} digoxin standards were 4.5% and 6.7% respectively. Reproducibility between calibrates was determined on two different days. The graphs for these calibrates are almost identical (Fig. 2). Statistical comparison showed no significant differences at the 95% confidence level.

The detection limit for this assay, expressed as the least detectable dose (LDD), was determined as the concentration which provided twice the standard deviation from the mean blank signal measurement. The LDD under optimum conditions was 1.20μg/L digoxin. The assay's dynamic range (DR), defined as the analyte concentrations that inhibited the maximum signal by 20% and 80%, was between 1.20×10^{-3} -0.04mg/L.

For interference studies, competition curves were produced with digoxin-related (similar structures) compounds, and their corresponding I_{50} values determined. Cross-reactivity was then calculated as the ratio I_{50} digoxin/I_{50} related compound (both I_{50}s expressed in μg L^{-1}). The compounds studied were narcotine, heroine, tebaine, morphine, codeine and pentazocine. Fig. 3 shows calibration curves for each compound at concentrations 100 times higher than the analyte. In all cases, the cross-reactivity was less than 1%, which implies there is virtually no combination with the antibody over the range studied. No interference in the assay is therefore expected for this particular antigen.

The reusability of the immunosensor prepared on a solid support is a major problem in sensor development. Under optimum conditions, immunosurface activity remained

constant for 50 assays. When not in use, the immunosensor was kept at 4ºC in PBS, and was operative for at least 2 months. The total time required for each immunoassay was 300s.

3.3. Serum samples analysis

The proposed immunosensor was used to determine digoxin concentrations in serum samples from four patients. No sample treatment was necessary. The results, as well as those obtained by the radiochemical method used at the patients' hospital, are shown in Table 3. No significant differences were seen between the values obtained by these two methods (95% CI).

4. CONCLUSIONS

This paper proposes a fast and reliable flow-through fluoroimmnunosensor whose function is based on a heterogeneous competitive assay, to determine digoxin in serum samples. The proposed sensor is thought to be highly selective; cross-reactivity of compounds with structures similar to digoxin was negligible. The described method has advantages over other existing chromatographic techniques and allows sensitive and rapid sample assessment of digoxin without sample pre-treatment. Compared to other immunoassays for determining digoxin, it provides the benefits of being more rapid and requiring minimal sample handling.

The developed immunosensor was successfully used to determine digoxin in human serum samples.

Acknowledgements

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TABLES

TABLE 1: Efficiency of antibody loading on the solid support.

Immunosensor	μg Ac added/g CPG	μg Ac loaded/g CPG	% Efficiency
1	180	128	71.1
2	360	292	81.1
3	900	794	88.2
4	1800	1607	89.3
5	3600	3101	86.1

TABLE 2: Summary of optimum conditions for the digoxin fluoroimmunosensor

Parameter	Optimum value
Excitation wavelength (Ag*)	496 nm
Emission wavelength (Ag*)	517 nm
Excitation and emission slits	0.5, 2.5 nm respectively
Binding solution	10mmol PBS in 0.1 M NaCl
Binding solution pH	7.2
Regeneration solution	0.5M citric acid in 0.5 M NaCl
Regeneration solution pH	3.0
Binding flow rate	0.2 mL min^{-1}
Antibody concentration	50 μL (3.6g L^{-1})
Labeled antigen concentration	0.2 μmol L^{-1}
Fluorescence measurement time	300 s
Cross-linking efficiency	96-99 %

TABLE 3: Results of human serum analysis.

Sample	Fluoroimmnunosensor (X±SD) (mg L^{-1})	Reference method (X) (mg L^{-1})
1	$(4.05\pm0.10)\times10^{-3}$	4.00×10^{-3}
2	$(2.34\pm0.27)\times10^{-3}$	2.47×10^{-3}
3	$(1.43\pm0.51)\times10^{-3}$	1.70×10^{-3}
4	$(4.43\pm0.09)\times10^{-3}$	4.40×10^{-3}

FIGURE LEGENDS

Fig. 1: Flow-injection system.

Fig. 2: Calibration curves using different concentrations of antigen tracer.

Fig. 3: Calibration curves for the digoxin immunosensor performed on two different days.

Fig. 4: Cross-reactivity of digoxin with potential interferent substances.

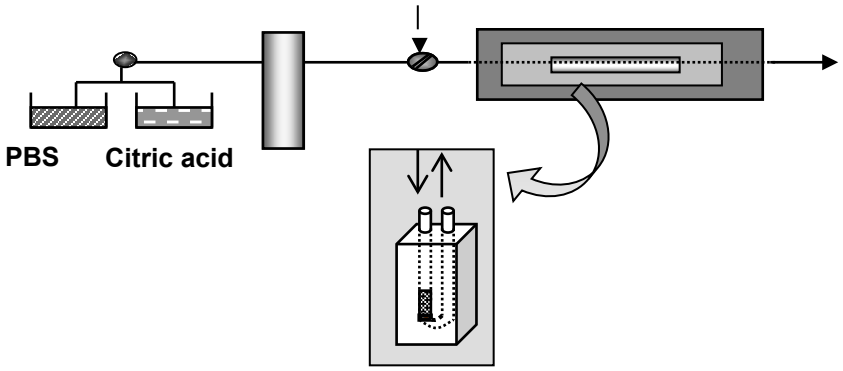

Figure 1

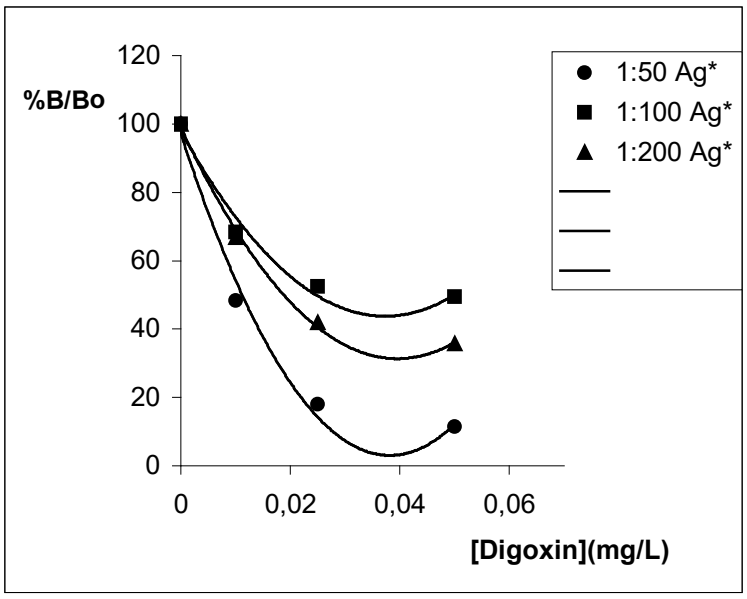
%B/Bo
120
100
80
60
40
20
0
0
0,02
0,04
0,06
[Digoxin](mg/L)
1:50 Ag*
1:100 Ag*
1:200 Ag*

Figure 2

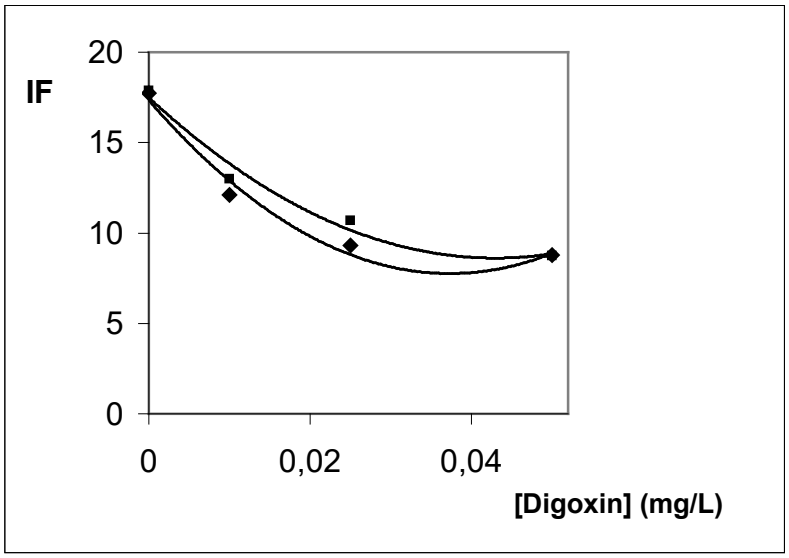

Figure 3

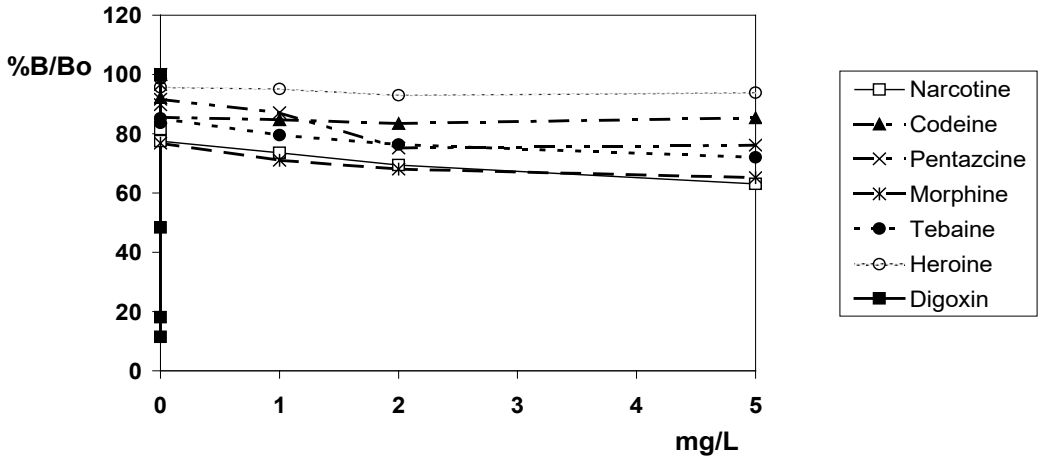
%B/Bo
120
100
80
60
40
20
0
0
1
2
3
4
5
mg/L
Narcotine
Codeine
Pentazcine
Morphine
Tebaine
Heroine
Digoxin

Figure 4