Cyclodextrin-Based Optosensor for the Determination of Warfarin in Waters

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A flow-through optosensor for warfarin is described. The sensor is developed in conjunction with flow analysis systems and uses a commercial bound β-cyclodextrin material as the sensing phase. A strong fluorescence signal was observed as a result of the formation of an inclusion complex between warfarin and β-cyclodextrin. The analytical performance characteristics of the proposed sensor for analysis of low levels of warfarin were as follows: the detection limits for continuous and flow injection analysis systems were 2 and 19 ppb, respectively; the observed relative standard deviations at 0.5 ppm warfarin level were less than 2.3%. A study of the interference of other naphthalenic toxic substances was carried out. The continuous flow method was satisfactorily applied to the determination of the rodenticide in natural waters.

Keywords: *Optosensor; flow injection analysis (FIA); cyclodextrin; Warfarin*

INTRODUCTION

Pesticide contamination of soil, food, and waters has become a serious problem nowadays. Warfarin [3-(3-oxo-1-phenylbutyl)-4-hydroxycoumarin)] has found extensive use as rodenticide to held control rat population. It causes hypoprothrombinemia and vascular injury resulting in haemorrhage as the last cause of death (Proctor, 1988). Owing to their extensive use as anticoagulant in cardiovascular therapy several procedures have been reported for its determination, both in pharmaceutical and biological samples, including HPLC (Montgomery et al., 1996; Andersen et al., 1993), capillary electrophoresis (Yang and Hage, 1994; Gareil et al., 1993), spectrophotometric (Sastry et al., 1991), fluorimetric (Panadero et al., 1993), and room-temperature phosphorimetry techniques (Yang Su et al., 1984; Vanelly and Shulman, 1984; García Sánchez and Cruces Blanco, 1989). Humans impose warfarin on the environment through agricultural activities and only a few reports exist on its determination in environmental samples (Tang and Rowell, 1998; Márquez et al., 1990; Vichez Quero et al., 1996). The widespread use of warfarin has led to the need of developing new analytical methods for its determination in natural waters, where it could be accumulated due to agricultural runoff.

Cyclodextrins (CDs) are macrocyclic glucose oligomers consisting of six, seven, or eight $\alpha(1\rightarrow4)$-linked D-glucopyranose units (α-, β-, or γ- cyclodextrin, respectively). The oligosaccharide ring forms a torus, whose openings are of different size: the primary hydroxyl groups on C-6 of the glucose residue lie on the narrow side, while the wider opening contains the secondary hydroxyl groups on C-2 and C-3. The outer surface of the torus has a hydrophilic character; conversely, the inner cavity is hydrophobic.

Much of the interest in cyclodextrins hinges on their capacity to bind a variety of guest molecules inside the apolar cavity. A commonly accepted model for complex formation suggests that the complex forms when a suitable hydrophobic molecule displaces water from the cavity. Excellent reviews have appeared in the literature (Armstrong and DeMond, 1984; Szejtli and Osa, 1996) which address both the processes driving the inclusion complex formation with cyclodextrins and their many analytical applications. Recently, CDs have been used in environmental applications to improve the remediation of contaminated soil and aqueous systems (Fenyvesi et al., 1996; Murai et al., 1998; Wang et al., 1998).

Many studies have concentrated on unmodified CDs, specially β-CD, for reasons of cost and also because its cavity has a suitable size to include a variety of aromatic and aliphatic substances. Surprisingly, very few studies on molecular recognition with analytical purposes of pesticides have been attempted with CDs (Li and Purdy, 1992; Pospísil and Colombini, 1993; Pospísil et al., 1994, 1998; Szejtli, 1998).

Immobilized CDs have been synthesized to develop electrochemical and optical sensing devices, showing significant results for organic and biological molecules such as pyrene (Alarie and Vo-Dinh, 1991), cholic acids (He et al., 1997), and amino acids (Zhilong and Zhujun, 1996 a,b).

Following this line, the present study reports a flow through fluorimetric sensing approach (optosensor) based on the use of immobilized β-CD as the recognition phase for the sensitive determination of warfarin. The proposed method compares favorably with other luminescent methods in terms of sensitivity (Su et al., 1984; Márquez et al., 1990), making this sensing approach well suited for the direct measurement of warfarin in surface waters accumulated due to agricultural runoff.

EXPERIMENTAL PROCEDURES

Chemicals and Solutions. Cyclobond is the tradename used to describe a series of chemically bonded cyclodextrins to a high purity spherical silica gel. The Cyclobond I (β-cyclodextrin bonded), 40 μm, was obtained from Astec (Ad-

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10.1021/jf981330u CCC: $18.00
Published on Web 09/23/1999

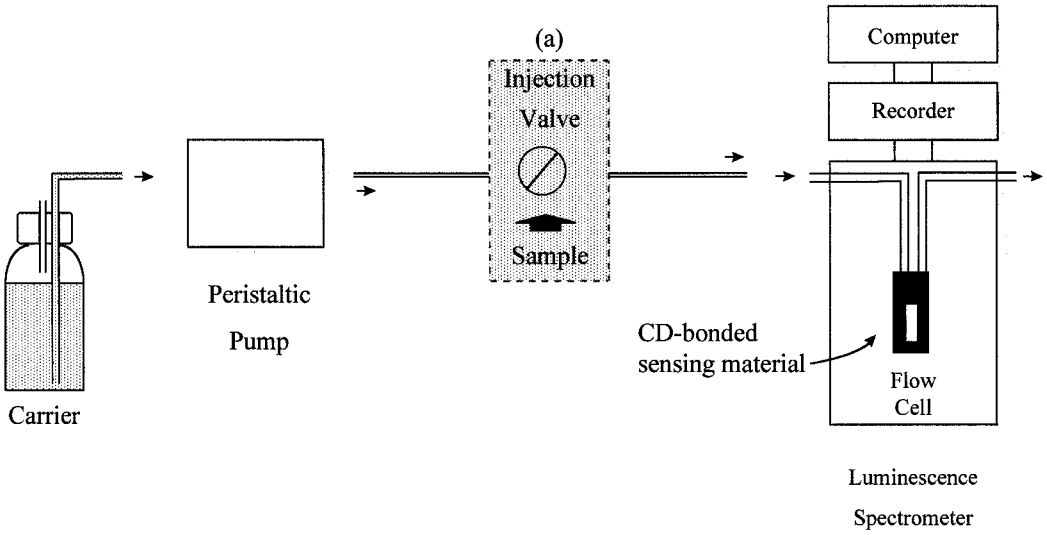

Figure 1. Schematic diagram of the continuous flow system. (a) Injection valve for FIA. Carrier: 0.02 M acetate buffer (pH 5.5 ± 0.2) 10% v/v methanol.

vanced Separation Technologies Inc.). Warfarin (WAR), 1-naphthylacetic acid (NAA), 1-naphthol (α) (NL), and β-cyclodextrin hydrate were purchased from Aldrich, and sodium acetate trihydrate was from Merck. Methanol and acetonitrile were obtained from Romil (Super Purity grade). All other reagents were super purity grade and were used without further purification unless stated otherwise.

All aqueous solutions were prepared using water obtained from a Milli-Q system (Millipore, Bedford, MA). WAR, NL, and NAA stock solutions (1×10^{-4} M) were prepared in methanol. When not in use, these solutions were stored at 4 °C. The standard solutions always were prepared using carrier buffer after an aliquot of stock methanolic solution was evaporated to dryness using an argon stream.

Instrumentation. Fluorescence emission measurements were carried out with a Perkin-Elmer LS 50B luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK), which employs a Xenon-pulsed (10 μs half-width, 50 Hz) excitation source. Instrumental parameters and processing data are controlled by the Fluorescence Data Manager software. The maximum excitation and emission wavelengths were set at 320 and 398 nm, respectively. Excitation and emission bandwidths were set both at 2.5 nm. A conventional Hellma flow-cell (model 176,52) of 25 μL was used. pH measurements were made with a Crison MicropH 2002 pHmeter (Crison, Barcelona, Spain). All experiments were carried out at 20 ± 2 °C.

Flow Manifold and General Procedure. The general set up for the continuous system is shown in Figure 1. For FIA a rotary valve was inserted in the system (Figure 1a). A four-channel Gilson Minipuls-3 peristaltic pump was used to generate the flow stream. A type 50 PTFE four-way rotary valve (Omnifit, Cambridge, UK), with a loop of 500 μL, was employed for sample introduction. PTFE tubing (0.8 mm i.d.) and fittings were used for connecting the flow-through cell, the rotary valve, and the carrier solution reservoirs.

The Cyclobond I was loaded with the aid of a syringe, as described elsewhere (Pereiro García et al., 1991). At the bottom of the flow-cell, a small piece of nylon net was placed to prevent particle displacement by the carrier while the other end of the cell was kept free. The packed flow-cell was connected to the flow system for 10 min allowing for the particles to settle. To secure that all of the packed solid material was in the light path, the Cyclobond I level was maintained up to the top part of the cell window. If not stated otherwise, the carrier buffer consisted of 0.02 M acetate buffer (pH 5.5 ± 0.2), 10% v/v methanol. The standard solutions and injected samples always were diluted with carrier buffer.

RESULTS AND DISCUSSION

Samples. Water samples were collected just below the surface at lakes (Enol and Ercina), rivers (Sella and Covadonga), and a spring (Pajares), all allocated in the province of Asturias (Spain). Samples were stored without any preservative, for as short time as possible, at 4 °C. Within 10 h and before measurement by the continuous flow optosensing approach, samples were filtered through 0.2 μm injection filters. It must be emphasized that water samples were taken in locations far from farming areas where warfarin is allegedly used.

Spectral Characteristics. In an aqueous continuous flow mode, the fluorescence spectrum of warfarin at pH 5.5 was characterized by an excitation band at 306 nm and a weak fluorescence maximum at 384 nm. The addition of soluble β-CD to the carrier solution produced no shifts in the spectral maximum but an enhancement in the fluorescence intensity of about 35%. These results pointed to the formation of inclusion complexes between warfarin and β-CD in solution.

Upon interaction of warfarin with the immobilized β-CD (Cyclobond phase), the fluorescence spectra of warfarin was red shifted. The excitation maximum was at 320 nm while the emission occurred at 398 nm. Also, an important increase in fluorescence intensity was observed which could be adscribed to two main factors (Ramamurthy and Eaton, 1988; Zhu et al., 1996; Szejtli, 1998). First, a local concentration of warfarin may take place in the Cyclobond phase (a preconcentration step). On the other hand, upon insertion of warfarin into the apolar CD cavity nonradiative pathways were minimized. The CDs offer a protective microenvironment and enhance the luminescence of the guest molecule by shielding the excited species from quenching and nonradiative decay processes that usually occur in the bulk aqueous solution.

Chemical Requirements and Experimental Conditions. The β-CD host is not ionizable over the pH 2−12; however, warfarin contains ionizable groups. Thus, to determine the working pH of the sensing approach a series of carrier solutions of different pH were prepared in the range of 3.5−7.5. It was found an optimum pH range between 4.5 and 6.5 for high signal/background ratio when an 8.7×10^{-7} M warfarin

Table 1. Analytical Characteristics of the Flow-Through Sensing Systems

	continuous sensing	FIA approach
DL	7.4×10^{-9} M (2 ppb)	6.3×10^{-8} M (19 ppb)
RSD	±1.0%	±2.3%
linear dynamic range	2.4×10^{-8} to 2.0×10^{-6} M	2.0×10^{-7} to 2.0×10^{-6} M
response time (min)	3	1
equation of the regression line	$I_F = 32.90 + 3.42 \times 10^8$ [WAR]	$I_F = 4.41 + 2.51 \times 10^8$ [WAR]
	$r = 0.998$	$r = 0.999$

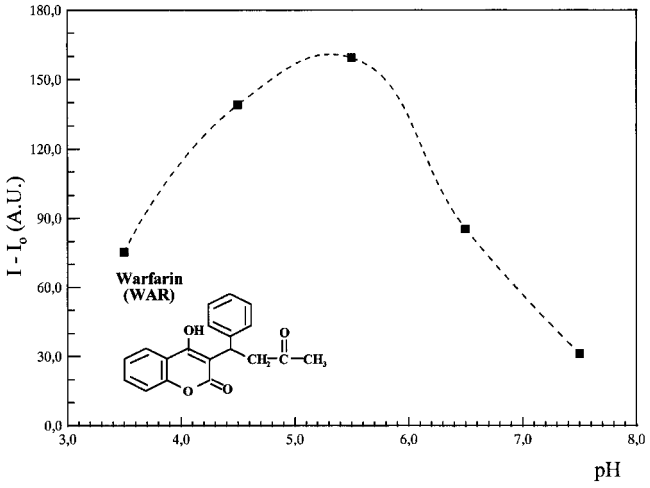

Figure 2. pH dependence of the fluorescence signal in a FIA approach (WAR, 8.7 10^{-7} M). Warfarin structure (inset).

solution was injected (Figure 2). Therefore, 0.01 M acetate buffer solution pH 5.5 was thoroughly used in this study.

Elution of warfarin from the Cyclobond I support was studied by using methanol, acetonitrile, and DMSO. Also, mixtures of these solvents with the carrier buffer were used in these experiments. Results demonstrated that as the organic solvent content in the carrier increased warfarin was slowly washed out of the Cyclobond I but a concomitant decrease in fluorescence intensity was observed. The time to recover the baseline was shorter when using methanol as carrier modifier. Therefore, a 10% v/v methanol mixture was selected as a compromise between sensitivity and time analysis. To minimize the regeneration time of the Cyclobond I sensing material, a cleaning solution consisting of an acetonitrile:carrier mixture (1:1) was injected to completely displace warfarin from the active surface. Typical response time for 90% of the final value fluorescence ranged from 3 min in a continuous flow mode (without using methanol in the carrier) to 1 min in the flow injection mode. Figure 3 shows the response profiles for warfarin using the flow through sensing approach in different experimental conditions.

The influence of the carrier flow rate was also studied over the range of 0.7−1.5 mL min^{-1}. As expected, as the carrier flow rate increased the analytical signal decreased slightly. Thus, a flow rate of 1.3 mL min^{-1} was finally selected as a compromise between sample throughput and sensitivity.

Interference Studies. 1-Naphthylacetic acid and 1-naphthol (α) have been selected as model compounds to study their influence on warfarin recognition. These two solutes have been chosen for their structural and chemical similarities. Both compounds contain naphthalene as their mainframe structure and thus exhibit similar luminescence behavior (Wu and Hurtubise, 1993; Delapeña et al., 1993). Similar positional substitution on the naphthyl moiety of these probes allows

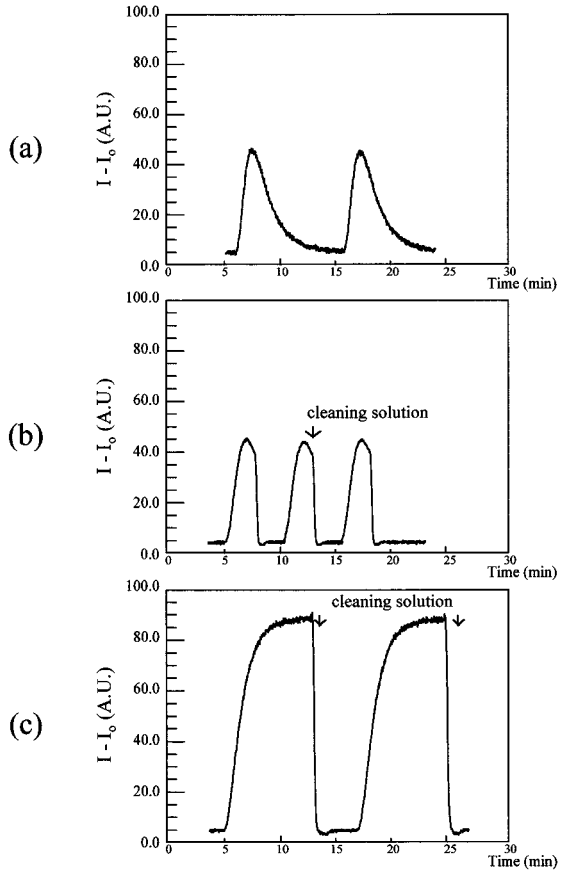

Figure 3. Response profiles for (a) FIA approach using a carrier buffer:methanol 10% v/v mixture, (b) as a after injecting a buffer:acetonitrile 1:1 cleaning solution, and (c) continuous flow mode using the aqueous carrier buffer (WAR, 1.6 10^{-6} M).

both molecules to fit similarly inside the β-CD cavity. Solutions of the compounds (1.6×10^{-6}M) were injected individually, and the signal obtained was compared with that for the same concentration of warfarin. In the optimal conditions for warfarin recognition, only 1-naphthol gave a signal 12% that for warfarin.

To check the effect of the concomitant species on the signal for warfarin, different molar concentration ratios of warfarin (1.6×10^{-6} M) to 1-naphthol and to naphthylacetic acid (1:0, 1:0.5, 1:1, 1:2) were studied. The results of analysis are shown in Table 2. Warfarin can be measured by the proposed system in the presence of 2-fold molar ratio interferents, as they are unable to expel the complexed warfarin from the β-CD cavity.

Analytical Performance. Analytical performance characteristics of the flow-through sensor operating in continuous or in FIA were evaluated under selected optimum experimental conditions. Results have been summarized in Table 1. The detection limits (DL) were defined as three times the standard deviation of the blank signal and the relative standard deviation (RSD) was evaluated for five determinations at 1×10^{-6} M

Table 2. Influence of NL and NAA on Warfarin Fluorescence Signal

naphthalene derivatives	molar ratio[a]	recovery (%)[b]
naphthol	1:0	100 ± 2
	1:0.5	107 ± 5
	1:1	108 ± 4
	1:2	110 ± 2
naphthol naphthylacetic acid	1:0	100 ± 2
	1:0.5	108 ± 3
	1:1	104 ± 3
	1:2	104 ± 4

[a] Warfarin, naphthalene derivatives. [b] Each value correspond to the mean of five determinations.

Table 3. Results Obtained in the Analysis of Spiked Surface Waters

sample		recovery (%)[a]
rivers	Sella	97 ± 2
	Covadonga	93 ± 4
lakes	Enol	101 ± 4
	Ercina	92 ± 1
spring	Pajares	101 ± 2

[a] Each value correspond to the mean of five determinations. In all cases the fortification level was set at 0.34 μg mL^{-1}.

warfarin level. The response was linear from the quantitation limit (defined as 3.3 times the detection limit) up to 2×10^{-6} M with a correlation coefficient of 0.998 for both systems. Calibration graphs were prepared from the results of triplicate responses of standard solutions.

After used, the sensing phase was cleaned flowing through it 10 mL of acetonitrile:carrier mixture (1:1) and 15 mL of water. It was found to be very stable for up 3 months (the maximum time tested), this being assessed by measuring a solution of warfarin of a given concentration once a week. No significative change in the intensity of fluorescence signal was found.

Applications. To assess the validity of the proposed optosensor, the continuous flow-through system was applied to the determination of warfarin in natural waters. The analysis by the proposed method did not reveal the presence of warfarin and the results were according to the water sampled locations. The spiked rodenticide (0.34 μg mL^{-1}) was determined in real matrixes without any previous treatment and recovery fluctuated between 92 and 101% in all cases, as shown in Table 3. This indicated that there were not other species in the water samples (organic matter) which could interfere warfarin recognition.

CONCLUSIONS

The continuous flow through sensor described in this paper proved to be an efficient tool for the fast, reliable, simple, and direct analytical environmental control of warfarin in natural water samples accumulated due to agricultural runoff. The integration of the sensing phase in a continuous flow approach reduce sampling preparation time as the complete extraction or the analyte preconcentration were not required as in traditional methods. On the other hand, the detection limit for warfarin using an flow injection analysis (FIA) system resulted to be not sensitive enough to directly measure warfarin in waters far away to farming areas. In the latter case, an extraction or a preconcentration step combined with the FIA system should be necessary. Further research is required to establish the ability of this CD sensing phase to complex other commonly used pesticides.

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We gratefully acknowledge financial support from CICYT (Proj. SAF96-1484). Received for review December 4, 1998. Revised manuscript received July 9, 1999. Accepted July 14, 1999.

JF981330U