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Enzyme Immunoassays with an Electrochemical Detection Method Using Alkaline Phosphatase and a Perfluorosulfonated Ionomer-Modified Electrode. Application to Phenytoin Assays

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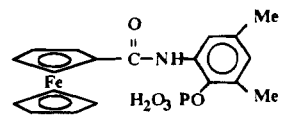
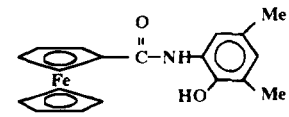
Enzyme immunoassays were performed with a Nafion-modified glassy carbon electrode as sensor, alkaline phosphatase as the enzyme label, and 6-(*N*-ferrocenoylamino)-2,4-dimethylphenyl phosphate (S^-) as the enzyme substrate. The three-step procedure required successive pH changes and was set up as a homogeneous or heterogeneous competitive immunoassay; phenytoin was selected as a model antigen. In step I, the competitive immunoreaction was performed in physiological neutral medium. In step II, the enzyme generation of 6-(*N*-ferrocenoylamino)-2,4-dimethylphenol (P) took place after addition of S^- in alkaline buffered solution (pH 10.2) containing a Mg^{2+} salt. In step III, the accumulation of P into the modified electrode proceeded by applying a potential of 0.6 V vs Ag/AgCl for 5 min in neutral medium (pH 7.5) and was followed by a square wave voltammetric scan. Substrate S^- was dianionic and therefore was repelled from the anionic Nafion film, whereas P was entrapped within the Nafion film as a ferrocenium salt, and so the sensitive electrochemical detection of P was possible (detection limit, 10^{-8} mol·L^{-1}). Both homogeneous and heterogeneous immunoassay techniques (methods A and B, respectively) were applied for the determination of phenytoin in clinical seric samples. Phenytoin at therapeutic concentrations could be sensitively determined in clinical samples with method B, but not so with method A, which can be envisioned only for semiquantitative assays (positive/negative tests).

Immunoassay, which combines the specificity of an antigen–antibody reaction with the sensitivity of an indicator system, is a very powerful technique for the determination of clinically important compounds in a variety of biological matrices. When a competitive immunoassay is carried out, a fixed quantity of labeled antigen (Ag*) is in competition with the unlabeled antigen (Ag) for a limited quantity of antibody binding sites (Ab). The determination of the amount of either the unreacted labeled antigen (Ag*) or the complex antibody/labeled antigen (Ab/Ag*) then allows the measurement of the unknown initial quantity of antigen. Enzymes[1] are the most widely used labels, because a single enzyme label can transform catalytically many molecules of substrate into product (10^1–10^4 molecules/min). This amplification effect provides the basis of very sensitive assays, and alkaline phosphatase (AP) has been successfully utilized due to its high activity, low cost, and nonspecificity. This enzyme is able to hydrolyze orthophosphoric monoesters into alcohols, and its activity is determined by measuring the amount of alcohol generated. Immunoassays with electrochemical detection carried out with AP as a label have been studied by several groups.[2–10] In these assays, the AP-labeled reactant transforms a phosphate ester substrate into an electroactive product, such as phenol[4,5] or 4-aminophenol,[6–10] which is detected at bare electrode. In most of the cases,[5–9] a flow injection system or HPLC is used to improve the sensitivity. However, these procedures are relatively sophisticated and therefore expensive. In order to lower the detection limit in a simpler manner, we have proposed very recently a new sensitive indirect electrochemical detection of AP on a Nafion-coated glassy carbon electrode using square wave voltammetry and the substrate/product (S^-/P) couple.[11]



S^-



P

The polyanionic perfluorosulfonated Nafion polymer is known for its ability to exchange selectively and accumulate cationic or procationic species[12–16] and concomitantly expel anionic compounds. Therefore, we have taken advantage of its permselectivity properties[12,16–18] and demonstrated that it is possible to accumulate

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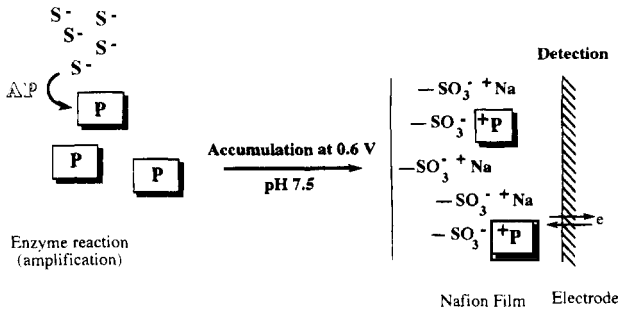

Figure 1. Schematic representation of the AP assay using a Nafion film.

the product P of the enzyme reaction in an hydrophobic cationic form (P^+), whereas the initial substrate cannot penetrate into the film due to its hydrophilic anionic form (S^-) (Figure 1). Both of the molecules S^- and P possess a ferrocenyl procationic group, which is reversibly oxidized into cationic ferrocenium near 0.4 V vs saturated calomel electrode. When a potential positive of 0.4 V (i.e., 0.6 V) is applied to the Nafion electrode, the neutral product P preconcentrates in the film in its oxidized ferrocenium form (P^+). Subsequent negative scan of the potential produces a reduction peak current with an amplified amplitude compared with a bare electrode, making it possible to detect low concentrations of the enzyme product and therefore trace amounts of AP (detection limit of 0.02 units·L^{-1}, equivalent to 2.8×10^{-14} mol·L^{-1} AP).[11] The accumulation and electrochemical detection of P must be performed at neutral pH for the following reasons. Substrate S^- possess one negative charge ($ROPO_3H^-$) in the pH range 2–6 and two negative charges ($ROPO_3^{2-}$) at pH > 6 in view of the two pK_a values of phosphate esters, i.e., about 2 and 6,[19] and so the repulsion effect of Nafion is reinforced in neutral or alkaline media. However, the pK_a value of the phenolic compound P is about 9, and so it is neutral at pH < 9. Hence, a pH range exists (about 6–9) in which the dianionic substrate ($ROPO_3^{2-}$) is repelled from the electrode and the neutral product (ROH) can penetrate into the film before being oxidized to P^+.

The objective of the present research is based on our previous investigation with the S^-/P couple[11] and is to demonstrate the feasibility of a simple electrochemical detection at Nafion film electrode sensor for competitive enzyme heterogeneous and homogeneous immunoassays. The results are reported here for a model antigen, phenytoin (5,5-diphenylhydantoin, DPH), and alkaline phosphatase as the enzyme label. This hapten is used in the treatment of epilepsy and various cardiac dysrhythmias and has a narrow therapeutic range (20–80 μmol·L^{-1}). Its concentration in serum is currently determined in laboratories by chromatography.[20] There are also available enzyme immunoassays for phenytoin that use spectrophotometric[21] and fluorometric[20] determinations or electrochemical detection involving biosensor[22] or unmodified electrode.[23] Eggers et al.[23] determined NADH at therapeutic levels by HPLC with electrochemical detection, using DPH labeled with enzyme glucose 6 phosphate dehydrogenase and NAD^+ as substrate. In their process, antibody and proteins were first removed to prevent adsorption and consequent fouling phenomena at the electrodes. A competitive homogeneous immunoassay technique using a Nafion film and cobaltocenium labeled phenytoin was previously reported by our group.[24] In this technique, measurements could be carried out without pretreatment of seric samples, because Nafion acts as a good antifouling barrier. However, the technique is limited to small antigens (haptens), because their labeled derivatives have to penetrate into the Nafion film through its narrow channels. Conversely, the techniques described below, which combine the advantages of Nafion electrode and the enzyme amplification, can replace the classical detection methods in enzyme immunoassays. Therefore, it can be envisaged for numerous kind of antigens detected by enzyme assays.

EXPERIMENTAL SECTION

Materials and Reagents. A 5 wt % Nafion solution (EW 1100) and phenytoin were purchased from Aldrich. Maxisorp Nunc tubes were obtained from Polylabo. The synthesis of compounds S^- and P was previously published.[11] Phenytoin–alkaline phosphatase complex (DPH-AP) was provided by Interchim (ref FIT 80-IP30). A few experiments were also carried out with free alkaline phosphatase (AP) from *Escherichia coli* provided by Sigma (ref P4252). Rabbit antiphenytoin serum (Ab) and rabbit normal serum (RNS) were obtained as previously described[25] and stored at −20 °C. To prepare the buffered aqueous solutions, deionized and doubly distilled water was used; tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma, Tween 20 from Fluka, and the other constituents from Prolabo.

Buffer Compositions. Aqueous NaCl solutions (5×10^{-3} mol·L^{-1}) and several buffers were used, i.e., $NaHCO_3$ (CB), phosphate (PB), Tris (TB), and Tris containing different amounts of $MgSO_4 \cdot 7H_2O$ (TB′ and TB″). A solution of HCl (4 mol·L^{-1}) was used to lower the pH value just before accumulation and electrochemical detection of P, since the enzyme reaction occurs in alkaline medium, whereas the detection at the Nafion electrode of product P must be carried out at pH 7.5.[11]

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Table 1. Experimental Conditions of Homogeneous and Heterogeneous Assays

step	homogeneous assay (method A)	heterogeneous assay (method B)
prep	no preparative step	(i) immobilization of 2 μL of antiserum 198 μL of PB on Maxisorp Nunc tubes at 4 °C for 15 h or more (ii) just before use, rinsing with PB containing 0.1% Tween 20
step I immunoreaction (neutral pH)	(i) mixing of 1 μL of serum containing DPH at unknown concentration 3 μL of antiserum 2 μL of RNS 234 μL of 5×10^{-3} mol.L^{-1} NaCl 10 μL of 10 000 units·L^{-1} DPH-AP in TB (ii) incubation at 37 °C for 30 min	(i) filling the tube with 1 μL of serum containing DPH at unknown concentration 189 μL of PB 10 μL of 5000 units·L^{-1} DPH–AP in TB (ii) incubation at 37 °C for 60 min
rinse	no rinsing step	rinsing with 2×200 μL of PB containing 0.1% Tween 20 2×200 μL of TB
step II enzymatic reaction (alkaline pH)	(i) addition of 10 μL of 10^{-3} mol·L^{-1} ethanolic solution of S^- 240 μL of TB′ (ii) incubation at 37 °C for 15 min	(i) filling the tube with 10 μL of 10^{-3} mol·L^{-1} ethanolic solution of S^- 190 μL of TB″ (ii) incubation at 37 °C for 15 min
step III detection of P (neutral pH)	(i) addition of 500 μL of CB 7 μL of 4 mol·L^{-1} HCl (ii) rotation of the electrode at 0.6 V for 5 min (600 rpm) (iii) SWV from 0.6 to 0 V vs Ag/AgCl and measurement of i_p	(i) mixing of the content of the tube Nunc (200 μL) 100 μL of 5×10^{-3} mol·L^{-1} NaCl 300 μL of CB 6 μL of 4 mol·L^{-1} HCl (ii) rotation of the electrode at 0.6 V for 5 min (600 rpm) (iii) SWV from 0.6 to 0 V vs Ag/AgCl and measurement of i_p

CB (pH 9.6): $NaHCO_3$ (45.5 mmol·L^{-1}), NaOH (9.1 mmol·L^{-1}), and NaCl (50 mmol·L^{-1}).

PB (pH 7.4): NaH_2PO_4 (8.7 mmol·L^{-1}), Na_2HPO_4 (30.4 mmol·L^{-1}), and NaCl (50 mmol·L^{-1}).

TB (pH 10.2): Tris (0.1 mol·L^{-1}) and NaCl (5 mmol·L^{-1}).

TB′ (pH 10.2): Tris (0.1 mol·L^{-1}), NaCl (5 mmol·L^{-1}), and $MgSO_4 \cdot 7H_2O$ (20 mmol·L^{-1}).

TB″ (pH 10.2): Tris (0.1 mol·L^{-1}), NaCl (5 mmol·L^{-1}), and $MgSO_4 \cdot 7H_2O$ (10 mmol·L^{-1}).

Apparatus and Electrodes. An EG&G PAR 273 potentiostat interfaced to an IBM XT 286 computer system with PAR M270 software was used for square wave voltammetry (SWV). The selected parameters for SWV are given in ref 14. Electrochemical experiments were carried out at 25 °C in a one-compartment glass cell that had a working volume of 1 mL.

Glassy carbon rods (3 mm diameter) were supplied by Carbone Lorraine. The Nafion film-coated GC electrode surface was prepared as already described.[14] In addition to the Nafion-coated electrode mounted on a Tacussel rotating-disk electrode, a platinum wire counter electrode and a Ag/AgCl (0.05 mol·L^{-1} NaCl) reference electrode were introduced into the cell. The final volume of the assay was 1 (homogeneous assays) or 0.6 mL (heterogeneous assays).

For each measurement, a Nafion rod electrode was pressure fitted into a narrow cylindrical hole of a Teflon tube in such a way that only the modified surface was exposed to the solution. This system was adapted for use as a rotating-disk electrode. For the accumulation procedure, the rotating-disk-modified electrode was exposed to the solution and immediately rotated at 600 rpm for 5 min. During this exposure time, the cationic exchange occurred with an applied potential of 0.6 V. Afterward, a rapid cathodic potential scan was applied at the stationary Nafion electrode, and the resulting cathodic peak current obtained by SWV was taken as the analytical response. A statistical analysis showed that the relative standard deviations between each modified electrode were on the order of 5–10%.[14]

Comparative Enzyme Activity of DPH-AP and AP. The enzyme reaction proceeded for 15 min at 37 °C in a mixture containing 10 μL of a TB solution of DPH-AP (5000 units·L^{-1}) or AP, 10 μL of an ethanolic solution of S^- (10^{-3} mol·L^{-1}), and 480 μL of TB″, and then 500 μL of CB and 7 μL of HCl (4 mol·L^{-1}) were added before electrochemical detection at pH 7.5.

Study of the Influence of the Serum Matrix. DPH-AP at different concentrations (400, 200, and 40 units·L^{-1}) in TB was maintained during 1 h at 37 °C in the presence of different volumes (x μL, $x = 0-10$) of RNS and ($240 - x$) μL of NaCl solution. After this period, 250 μL of TB′ containing S^- (4×10^{-5} mol·L^{-1}) was added, and the enzyme reaction proceeded for 15 min at 37 °C. Carbonate buffer (500 μL) and HCl solution (7 μL) were finally added before detection of P as previously described. In a second experiment, 500 μL of a 1/1 mixture of NaCl/TB′ containing 10^{-5} or 5×10^{-7} mol·L^{-1} P in the presence or in the absence of 6 μL of RNS was maintained for 15 min at 37 °C. CB (500 μL) and HCl solution (7 μL) were then added, and the accumulation proceeded.

Homogeneous Inhibition of DPH-AP by Antibody. To 224 μL of NaCl solution were added successively 20 μL of 5000 units·L^{-1} DPH-AP in TB, y μL ($y = 0-6$) of phenytoin antiserum, and ($6 - y$) μL of RNS. After thorough mixing, the solution was incubated for 1 h at 37 °C, and the subsequent steps were conducted as detailed in Table 1 (steps II and III of method A).

Antibody Immobilization and Coating Concentration. Immobilization of antibody on the walls of reagent tubes was carried out by contacting antiserum diluted in phosphate buffer within maxisorp Nunc tubes at 4 °C for 15 h or more, according to the manufacturing procedure. Series of five tubes were incubated, four containing dilute antiserum (dilutions 1/10, 1/100, 1/1000, and 1/10 000) and one pure PB. The volume of the solutions was 200 μL. Just before the immunoassay procedure, the supernatant was pipetted, and the tubes were carefully rinsed with PB containing 0.1% Tween 20 (rinsing solutions were maintained for 10 min in the tube). The tubes were then filled with DPH-AP

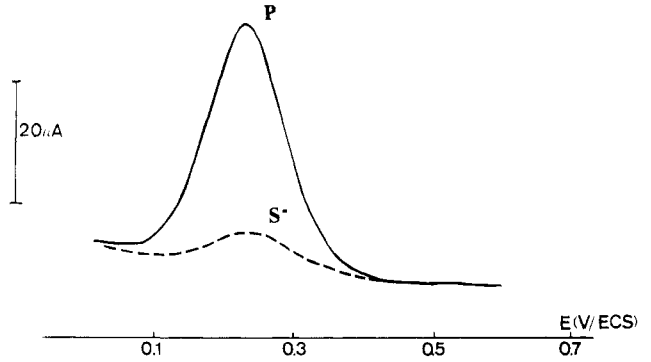

Figure 2. SWV curves on a Nafion electrode in PB for S^- (2×10^{-5} mol·L^{-1}, curve S$^-$) and P (10^{-6} mol·L^{-5}, curve P). Accumulation time, 5 min at 0.6 V, under 600 rpm.

($10\ \mu$L of a 5000 units·L^{-1} Tris solution) and phosphate buffer ($190\ \mu$L) and the solutions were incubated for 1 h at 37 °C. After this period, the tubes were rinsed with $2 \times 200\ \mu$L of PB containing 0.1% Tween 20 and $2 \times 200\ \mu$L of TB. The enzyme reaction (stepII) and the electrochemical detection of P (step III) were carried out as described in Table 1 (method B).

Homogeneous and Heterogeneous Immunoassay Procedures. These procedures are detailed in Table 1. The standard calibration curves were obtained in the presence of $1\ \mu$L of ethanolic DPH solution of various standard concentrations (C_{st}) instead of $1\ \mu$L of human seric samples containing DPH. Moreover, in the case of the homogenous assay, the standard calibration curve was performed with $3\ \mu$L of RNS instead of $2\ \mu$L, in order to have the same total serum content (RNS + antiserum) as for the assay of clinical seric samples.

RESULTS AND DISCUSSION

Nafion Film Selectivity. The specificity of the technique is based on the Nafion film selectivity and therefore on the preferential preconcentration of P (or P^+) compared with S^-. This is demonstrated in Figure 2, which gives the curves obtained by SWV at Nafion-modified electrodes immersed for 5 min in a S^- (2×10^{-5} mol·L^{-1}) or P (10^{-5} mol·L^{-1}) phosphate buffer solution (PB). A potential of 0.6 V was applied during the exposure period. In this neutral medium (pH 7.5), the phosphate ester S^- is dianionic, whereas the phenol derivative P is neutral. Both of the molecules possess a reversible redox system (ferrocene/ferrocenium) at ca. 0.35 V, and their electrochemical behavior is described in ref 11. Whereas at a naked electrode it is impossible to clearly differenciate S^- and P, at the Nafion electrode the peak amplitude (i_p) for S^- ($i_p = 5\ \mu$A) is definitively lower than that for P ($i_p = 40\ \mu$A). For P in the lowest concentration range, the increase of i_p with the concentration C is linear ($i_p = \varrho C$), as shown on the calibration curve presented in Figure 3. There is no further i_p increase above 10^{-6} mol·L^{-1}, suggesting the saturation of the available sulfonated sites of the Nafion by the ferrocenium moieties during the accumulation step. For S^- and P at low concentrations, the ratios $\varrho = i_p/C$ are calculated to be 272 and 0.25 A·L·mol^{-1}, respectively.[11] Hence, the corresponding selectivity factor $\gamma = \varrho(P)/\varrho(S^-)$ is 1100 in PB, which quantifies the excellent permselectivity of Nafion with respect to the S^-/P couple. The shape of the calibration curve and the γ values depend to some extent on the buffer composition[11] and the ethanol content, which corresponds to 1% for the calibration curve of Figure 3.

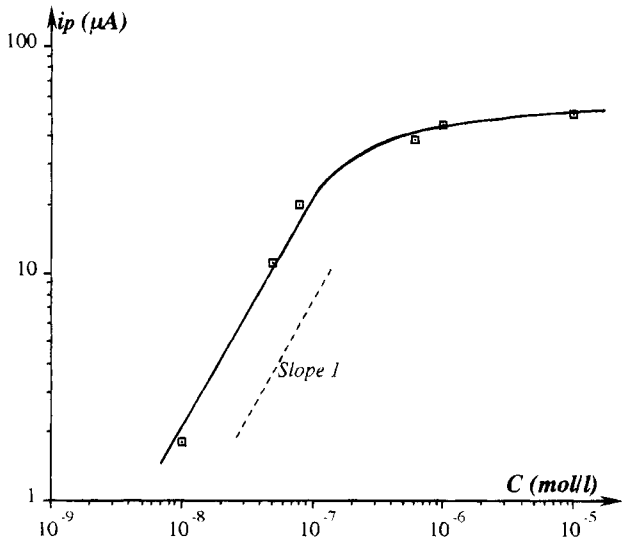

Figure 3. Variations of the SWV peak current intensity (i_p) with the concentration C of P in PB. Accumulation time, 5 min at 0.6 V, under 600 rpm.

Comparative Catalytic Activity of DPH-AP and AP. The calibration curve obtained for AP at the Nafion electrode indicates that it can be detected at very low levels (2.8×10^{-14} mol·L^{-1}).[11] It is well known that chemical linking of enzymes to other molecules can involve amino acid residues that are important in the enzyme activity or can cause steric hindrance to the active site, thus reducing their catalytic activities. So, it is necessary to compare the catalytic activities of alkaline phosphatase-labeled phenytoin (DPH-AP) and free AP with respect to substrate S^-. Their calibration curves under the same experimental conditions are shown in Figure 4. When the concentration of DPH-AP (curve A) or AP (curve B) was increased, the peak intensity i_p increased because the quantity of S^- which was hydrolyzed into P increased. Moreover, the similar increase of i_p with DPH-AP or AP concentrations shows that the enzyme activity of alkaline phosphatase conjugated to phenytoin is not affected significantly compared with AP.

Millimolar concentrations of substrate were commonly used in the previous AP assays at bare electrodes[2-10] to maintain zero-order enzyme kinetics and therefore high sensitivity. Such substrate concentrations lead to an appreciable anodic blank signal at the Nafion-coated electrode, which interferes to some extent with the product detection.[11] Therefore, several lower substrate concentrations were tested, and it was concluded that 10^{-5} mol·L^{-1} S^- is a good compromise between enzyme kinetics and blank signal.

The i_p value observed in Figure 4 in the absence of DPH-AP or AP ($i_p(0) = 2\ \mu$A, blank signal) is related to the non-negligible presence of S^- in the film. This value is lower compared with the blank signal obtained in Figure 2 ($5\ \mu$A) by accumulation in pure PB, because the detection of S^- depends on the buffer composition, as previously mentioned.[11] Furthermore, the $i_p(0)$ value remains constant whatever the incubation period (15 min–6 h), which shows that the spontaneous hydrolysis of S^- into P is not noticeable compared with the residual blank signal.

A few more experiments concerning the enzyme activity of DPH-AP were performed in a 1/1 mixture of NaCl solution and TB′ instead of pure TB″, followed as previously by addition of

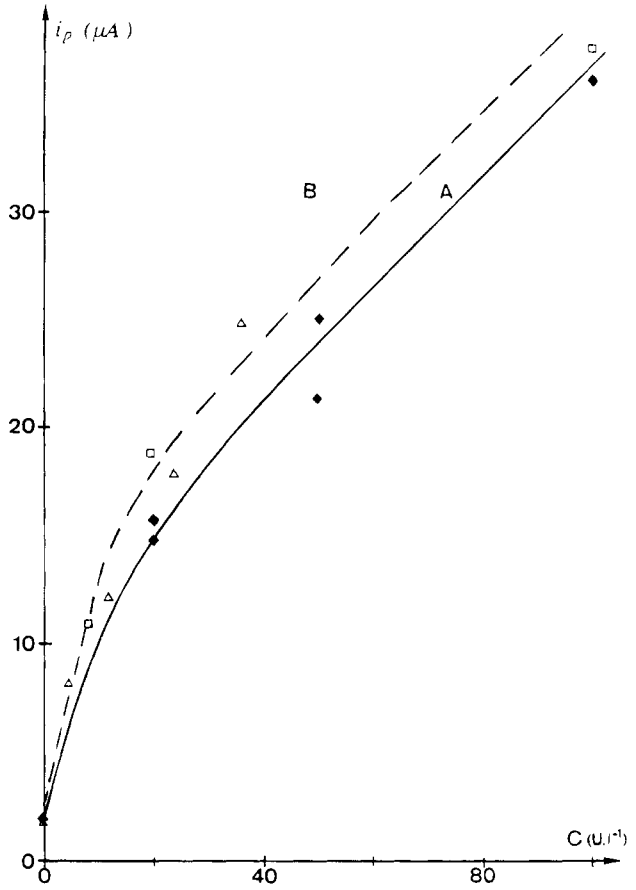

Figure 4. Variations of the SWV peak current intensity (i_p) of P generated enzymatically from S^- (2×10^{-5} mol·L^{-1}) and DPH-AP (curve A) or AP (curve B) at different concentrations (C) in 500 μL of TB″. Incubation was followed by addition of 500 μL CB and 7 μL HCl, and then 5 min accumulation at 0.6 V. The open squares were obtained when incubation of S^- and DPH-AP occurred in a mixture of 250 μL of NaCl (5×10^{-3} mol·L^{-1}) and 250 μL of TB′.

CB and HCl. The corresponding results (Figure 4, □) show no significant difference compared with the preceding ones.

Immunoassay Procedures. The general assay protocols are outlined in Figures 5 and 6 for the homogeneous and heterogeneous competitive immunoassays, respectively, and the experimental procedures are described in Table 1.

Considering first the homogeneous assay (method A), a fixed amount of DPH-AP (Ag-E) and a limited quantity of antibody (Ab) are incubated with a sample containing an unknown concentration of unlabeled DPH (Ag). When the immunoreaction takes place (step I), DPH competes with DPH-AP for the limited antibody binding sites. Insofar as the enzyme activity is inhibited in the complex Ab/Ag-E, the amount of P generated in step II is reduced. This inhibiting effect can be reversed by increasing the amount of unlabeled DPH (analyte) that competes for the available antibody binding sites. Thus the quantity of product generated in step II is increased with the concentration of analyte in solution. The immunoreaction takes place in physiological neutral medium (pH 7.5) to be efficient, and the enzyme reaction is performed, in the case of AP, in an alkaline buffered solution (pH 10.2) containing a Mg^{2+} salt[2-4,8,10] in addition to the substrate. The pH must be adjusted to 7.5 in step III before the modified electrode is immersed into the mixture, in order to be in the best Nafion film selectivity conditions.[11] Finally, the preconcentration of the product (P^+) occurs when an anodic potential is applied to generate the entrapped ferrocenium salt, and the resulting SWV peak current is related to the drug concentration in the sample. Such an assay can be successfully achieved as long as several conditions are fulfilled. It is necessary to verify that the catalytic hydrolysis of S^- into P by the complex Ab/DPH-AP is substantially inhibited. This is often the case for small antigens (haptens) conjugated to enzyme labels[1] such as glucose 6-phosphate dehydrogenase or malate dehydrogenase, but, to the best of our knowledge, it has never been described with alkaline phosphatase. Moreover, it is known that human seric samples contain some free AP,[26] and so conditions have to be selected in order that its influence is negligible compared with DPH-AP in step II.

In the heterogeneous assay (method B), the same successive pH changes are involved. In step I (pH 7.5), DPH (Ag) and DPH-AP (Ag-E) compete for the solid-phase antibody (-Ab) coated on the walls of the reagent tubes. Unreacted phenytoin (Ag) and labeled phenytoin (Ag-E) are then rinsed out. In step II (pH 10.2), the complex -Ab/Ag-E deposited on the tube walls transforms the substrate S^- into P, which is detected at the Nafion-modified electrode (pH 7.5) in step III, as in procedure A. The peak current now decreases when the amount of phenytoin present in the sample is increased, contrary to the homogeneous method. An advantage of the heterogeneous assay is the removal of potentially interfering components of the sample matrix prior to the determination of the enzyme-generated product.

A series of preliminary experiments were carried out concerning any of steps I–III before experimental conditions were defined as suitable for the enzyme immunoassays. Both of the competitive immunoassay methods required the evaluation of the antibody concentration and the DPH-AP dilution. A few more specific experiments were performed for the homogeneous assay, in order to study the potential interferences of the serum matrix and to quantify the antibody inhibition effect on DPH-AP activity. The competitive assay proceeded near the equilibrium (30–60 min) for each immunoassay reaction, and the enzyme reaction period was fixed to 15 min, which was a relatively good compromise between analysis time and sensitivity.

Influence of Serum Matrix on the Accumulation and Detection of P and on DPH-AP Activity. A clinical seric sample of antigen and a rabbit serum sample containing antibodies (antiserum) are introduced directly in the first step of the homogeneous immunoassay (Figure 5), without preliminary treatment. So, it is necessary to study the influence of serum matrix on the enzyme reaction (step II) and on the accumulation process (step III). This study was carried out with RNS.

Different concentrations of DPH-AP with increasing amounts of RNS were incubated for 1 h at 37 °C to evaluate the overall influence of RNS on steps II and III of the homogeneous assay. The enzyme reaction immediately followed under the conditions depicted in Table 1 (steps II and III). The variations of i_p with increasing amount of RNS are presented in Figure 7 for different concentrations of DPH-AP. The presence of RNS leads to a significant and continuous decrease of i_p, which suggests several possibilities. The presence of serum would decrease the enzyme activity of DPH-AP and/or would interfere with the accumulation of the generated product. Compound P was introduced directly in a mixture of NaCl solution and TB′ with 1.2% RNS and was maintained for 15 min at 37 °C to evaluate the influence of RNS

(26) Bretaudiere, J. P.; Spillman, T. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; VCH: Weinheim, 1986; Vol. IV, pp 75–92.

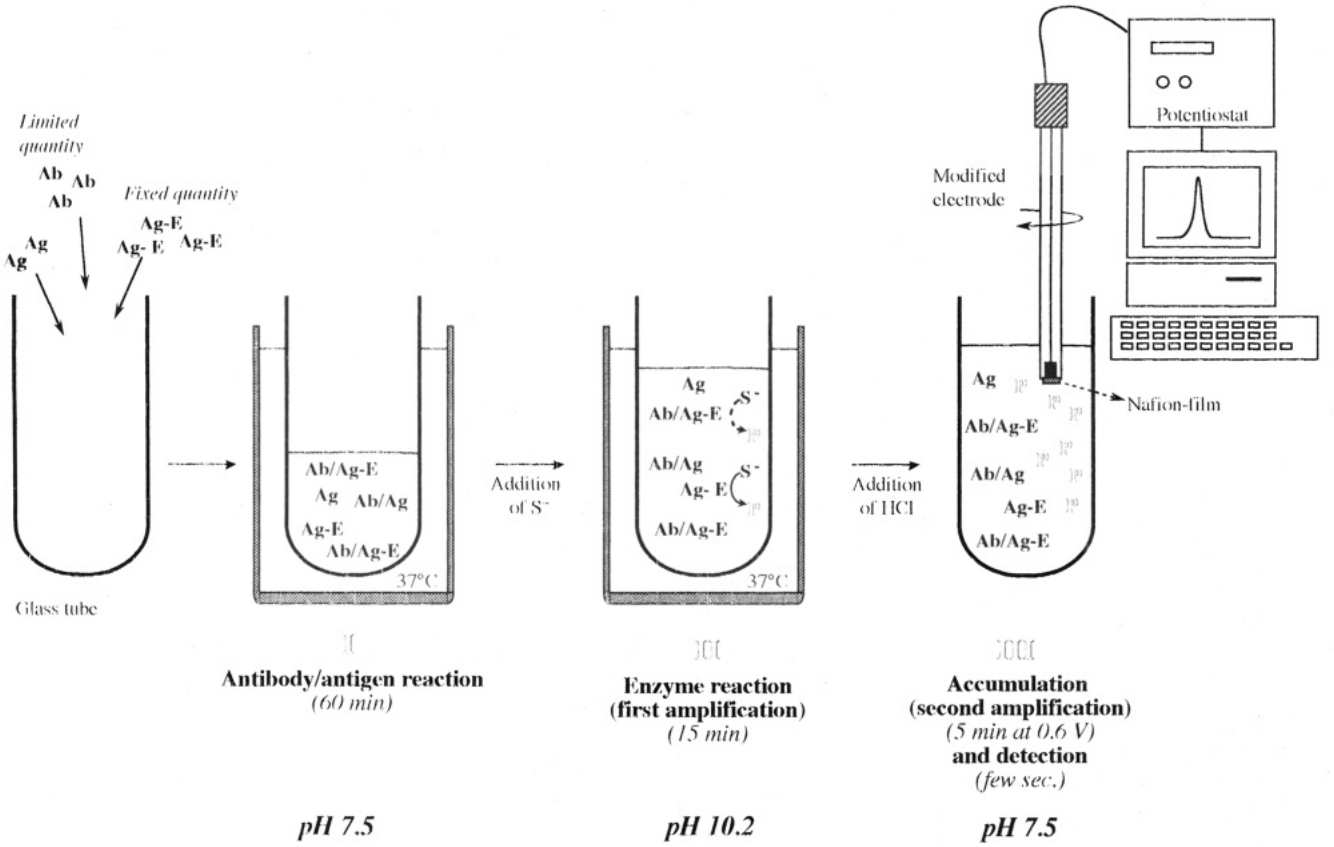

Figure 5. Schematic representation of homogeneous competitive enzyme immunoassay (method A) using a Nafion electrode. Antibody is represented by Ab. The antigen model phenytoin corresponds to Ag. The enzyme label E symbolizes AP.

on the accumulation process. The presence of RNS led to a slight decrease of i_p, about 10%, when the concentration of P was 10^{-5} mol·L^{-1}, but this decrease was reinforced at lower concentrations, reaching 80% for a concentration of 5×10^{-7} mol·L^{-1}. Such a phenomenon has been previously observed by our group for numerous ferrocene-labeled drugs,[27] and it would arise from hydrophobic interactions between the serum proteins and the hydrophobic ferrocene-labeled drugs, the diffusion of which is prevented through the narrow channels of the Nafion film. Indeed, it is well known that serum proteins can be associated with hydrophobic antigens by nonspecific interactions.[28]

These results indicate the limited sensitivity and poor precision of the method, since disparities in the contents of patient sera are expected. The experimental conditions corresponding to point a of curve A of Figure 7 were selected for the further studies, i.e., the immunoreaction took place in the presence of DPH-AP at a concentration 400 units·L^{-1} and a total serum content (RNS + antiserum) of 1.2%. The i_p value is still high enough under such conditions to allow further experiments with phenytoin antiserum, which will lead to an additional decrease of current.

Inhibition Study of DPH-AP by Antiphenytoin Serum. We verified that the enzyme activity of AP in Ab/DPH-AP was substantially reduced compared with DPH-AP, as it is required in method A. A fixed amount of DPH-AP (400 units·L^{-1}) was incubated with increasing volumes of antiphenytoin serum (y μL), while the total volume of serum (RNS + antiserum) remained constant (1.2%). The variations of i_p with y are presented in Figure 8. The peak current i_p decreases when the antiserum volume is increased, which confirms that the ability of DPH-AP to hydrolyze S^- into P decreases when it is bound to antibody. The presence of the latter would prevent the access of the substrate to the enzyme active site.[1] Conditions corresponding to point A of Figure 8 were further selected to attempt the competitive homogeneous immunoassay. The chosen volume of antiserum corresponded to a large inhibition. A 30 min incubation step was selected in the following studies, since it was observed that the i_p value corresponding to A was not significantly modified when the immunoreaction was either 30 or 60 min.

Competitive Homogeneous Immunoassay. The standard calibration curve of Figure 9 was obtained under the conditions indicated in Table 1 and in the Experimental Section. The examined concentration range corresponds to the therapeutic range (20−80 μmol·L^{-1}). The higher the standard concentration C_{st}, the higher the amount of DPH-AP unbound to AP, and the higher the amount of P generated during the enzyme step. Hence, the calibration curve of Figure 9 is typical of a competitive homogeneous immunoassay. However, the sensitivity of the method is limited to the i_p values within the range 5−9 μA, i.e., the C_{st} range 35−55 μmol·L^{-1}.

From the standard calibration curve of Figure 9, DPH concentration of human seric samples can be directly determine insofar as 1 μL of clinical seric sample is introduced in step I. However, this procedure is valid only for samples containing 35−

(27) Degrand, C.; Limoges, B.; Blankespoor, R. L.; Brossier, P. French Patent 9207089, 1992.

(28) Tillement, J. P.; Horrin, G.; Zini, R.; Urien, S.; Albengres, E.; Barré, J.; Sébille, B. In *Pharmacologie chimique—Base de la Thérapeutique 1*; Giroud, J. P., Ed.; Expension Scientifique Française: Paris, 1988; pp 1−24.

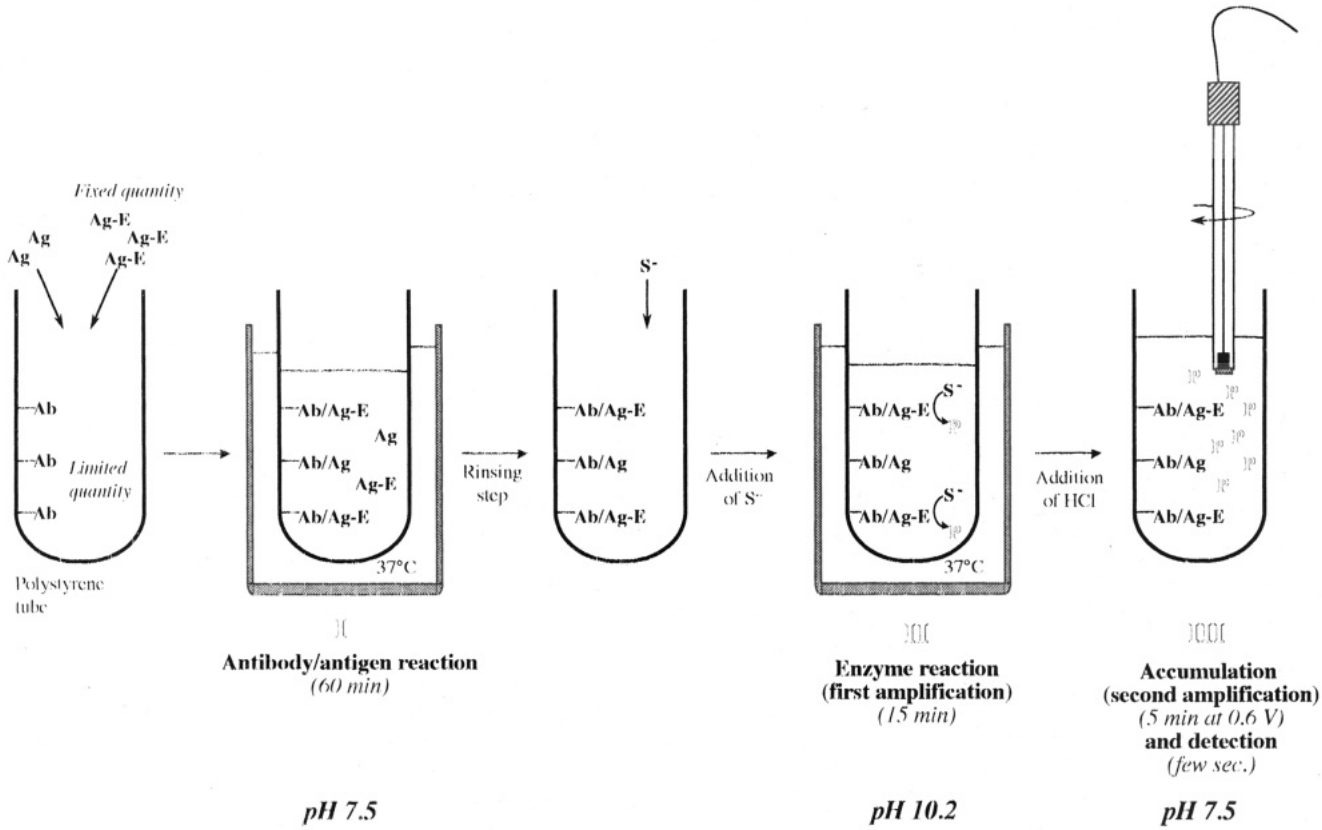

Figure 6. Schematic representation of heterogeneous competitive enzyme immunoassay (method B) using a Nafion electrode. Antibody is represented by Ab. The antigen model phenytoin corresponds to Ag. The enzyme label E symbolizes AP.

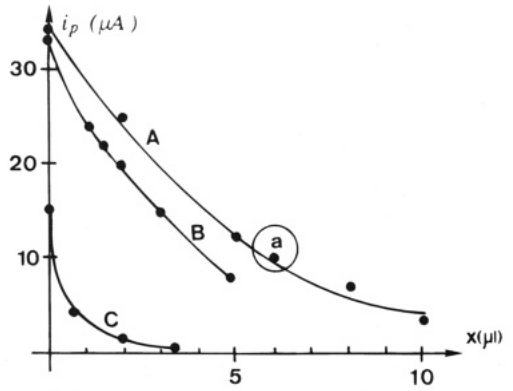

Figure 7. Influence of the RNS content (x) on the SWV peak current intensity (i_p). DPH-AP at concentrations of 400 (A), 200 (B), and 40 units·L^{-1} (C) was maintained for 30 min at 37 °C with x μL of RNS and (240 − x) μL of NaCl solution. Next, 250 μL of TB′ containing S^- (4×10^{-5} mol·L^{-1}) was added, and the enzyme reaction took place. The experimental conditions of the further steps corresponded to those described in Table 1.

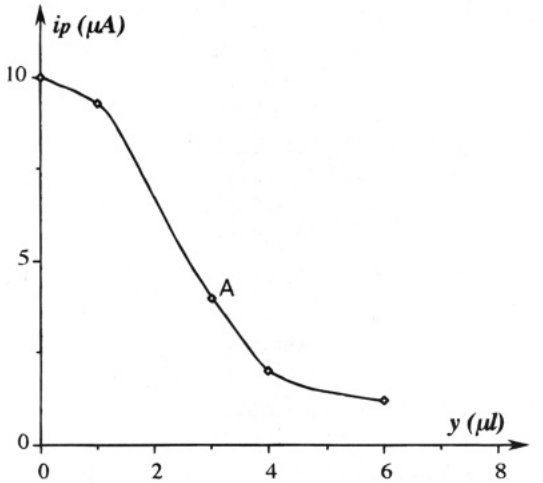

Figure 8. Influence of the added antiphenytoin serum volume (y μL) on the SWV peak current intensity (i_p). DPH-AP (400 units·L^{-1}) was maintained for 30 min at 37 °C with (6 − y) μL of RNS, y μL of Ab, and 234 μL of NaCl. The experimental conditions of the further steps corresponded to those described in Table 1.

55 μmol·L^{-1} of DPH, since the sensitivity domain of the calibration curve is very narrow and does not cover the whole therapeutic range. We have used this calibration curve to determine the phenytoin concentration of a clinical sample. Three experiments were necessary to successfully carry out this assay. The first two experiments, with 1 and 2 μL of a human seric sample, led to i_p values which were respectively below (4 μA) and above (10 μA) the narrow sensitivity range. In the third experiment, with 1.5 μL, the suitable i_p value (6 μA) allowed the determination of C (27 μmol·L^{-1}), which is close to the value determined for this sample in a hospital by HPLC (32 μmol·L^{-1}) and in our laboratory by a nonenzyme homogeneous electrochemical immunoassay (31 μmol·L^{-1}).[29]

The presence of AP in the seric sample can introduce an endogenous error, as was mentioned above. Although the AP concentration of human serum can reach 300 units·L^{-1},[26] dilution of the sample by 250 leads to a maximum endogenous AP concentration of 1.2 units·L^{-1}, which is far below the 400 units·L^{-1}

(29) Le Gal La Salle, A.; Anizon, J. Y.; Limoges, B.; Degrand, C.; Blankespoor, R. P. 44th Meeting of the International Society of Electrochemistry, Berlin, 1993; Comm. IV.2.1

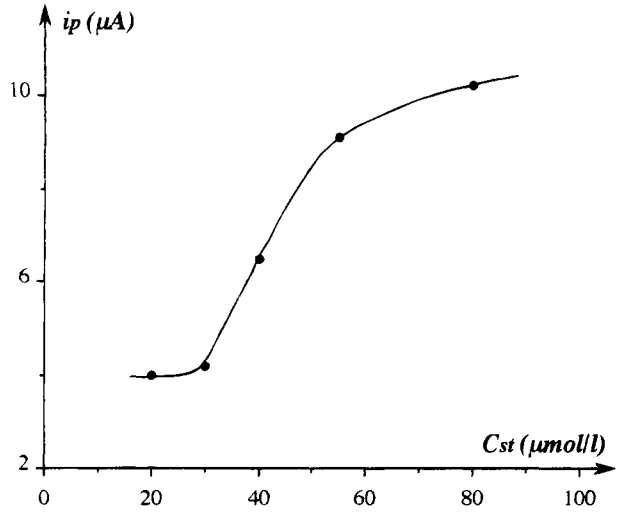

Figure 9. Standard calibration curve of the homogeneous immunoassay for phenytoin. For details, see Table 1. Phenytoin concentration refers to that in standard solution or clinical seric sample before dilution in the assay procedure.

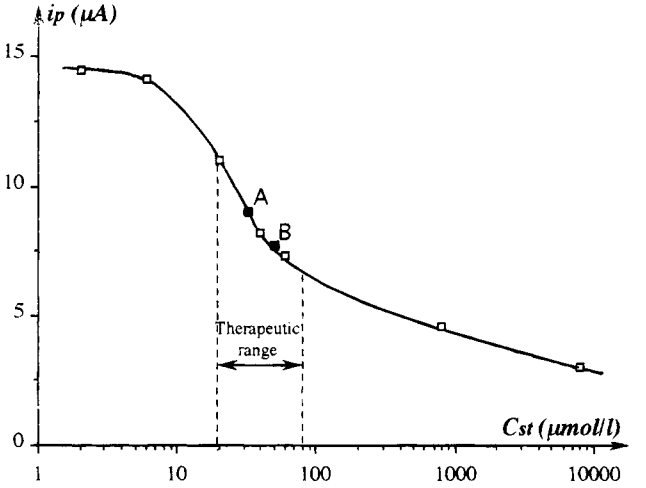

Figure 10. Standard calibration curve of the heterogeneous immunoassay for phenytoin. For details, see Table 1. Phenytoin concentration refers to that in standard solution or clinical seric sample before dilution in the assay procedure.

Table 2. Effect of Antibody Coating Dilution on Peak Current i_p[a]

entry	Ab dilution factor	i_p (μA)
1	pure PB	1.2
2	1/10 000	0.8
3	1/1000	3.0
4	1/100	13.0
5	1/10	13.0

[a] Assay performed as described in Table 1, but with no DPH in step I.

concentration of DPH-AP initially introduced. It can be concluded that the enzyme side activity of free AP is negligible, since it was shown previously that the enzyme activities of AP and DPH-AP are similar.

Optimization of the Antibody Coating Concentration for the Heterogeneous Immunoassay. It was necessary to define the limited antibody coating concentration on the tube walls prior to the competitive heterogeneous immunoassay. Hence, polystyrene tubes were coated with phenytoin antibody with several dilute antisera (dilutions 1/10, 1/100, 1/1000 and 1/10 000), and incubated with 250 units·L^{-1} DPH-AP. The resulting i_p values are given in Table 2. When no antibody was introduced (entry 1), no bound DPH-AP was present during the incubation step with S^-, and so the residual signal of low amplitude was due to the weak penetration of S^- into the Nafion film. When the Ab dilution factor was increased (entries 2–5), the amount of immobilized Ab, and therefore of Ab/DPH-AP, increased, which confirms that DPH-AP was still active when bound to Ab. Moreover, i_p reached a limit when the dilution was 1/100, which suggests that the maximum Ab adsorption capacity of the tubes was attained and each immobilized Ab units was bound to DPH-AP for this value. An antiserum dilution of 1/100 and a concentration of 250 units·L^{-1} DPH-AP were adopted for the subsequent studies to ensure that a limited quantity of antibody was present during the competitive immunoassay.

Competitive Heterogeneous Immunoassay. The DPH standard curve of Figure 10 was obtained according to the three-step procedure described in Table 1, except that in the first step, 1 μL of an ethanolic standard solution of DPH was used instead of 1 μL of human serum containing DPH. The intensity i_p decreases when the standard concentration C_{st} is increased, since DPH competes with DPH-AP for the available immobilized Ab sites. The most sensitive part of the calibration curve lies within the C_{st} range 10–100 μmol·L^{-1}, and so this curve is sensitive in the therapeutic domain and can be used directly to determine the concentration of phenytoin in 1 μL of clinical sample. To validate the method, experiments were performed with two clinical seric samples A and B, by introducing 1 μL of seric sample in the immunoreaction step. The two i_p values measured at the end of the assays are reported in Figure 10 (□). The corresponding C_{st} values are 32 (A) and 50 μmol·L^{-1} (B), which are in excellent agreement with the values previously determined by HPLC in a hospital (32 and 52 μmol·L^{-1}). It is worth noting that, in each assay, only 1 μL of seric sample was involved, and thus phenytoin amounts as low as 8 (A) and 12 ng (B) were accurately determined. Moreover, samples A and B contained eight and three more drugs, respectively, including phenobarbital in the same concentration range (47 μmol·L^{-1}) for sample A. Considering the structural similarity of phenobarbital and phenytoin, it is important to emphasize the phenytoin antiserum selectivity, which enhances the specificity of the method.

CONCLUSION

We have shown that the combination of a Nafion electrode, AP label, and S^-/P couple can find applications in heterogeneous enzyme immunoassay, and we have validated the concept for phenytoin as a model antigen. The technique is based on the Nafion selectivity, and so it is possible to discriminate the electrochemical detection of S^- and P, although they are oxidized at the same potentials. Hence, this concept can be extended to other enzymes and/or electroactive substrate/product couples, insofar as the electrochemical sensor is selective. Since the Nafion amplification in AP assay[11] provides a sensitivity comparable to those obtained with more sophisticated systems, such as electrochemical flow injection or electrochemical HPLC,[5–9] the simpler detection system developed here is expected to have similar performances. However, we have not yet optimized the enzyme

immunoassay parameters such as sensitivity and reproducibility. It is necessary to investigate in a further study the errors related to each step of the protocol assays summarized in Table 1, i.e., antibody coating, dilution, and time reproducibility. The competitive homogeneous immunoassay of phenytoin with AP label can be also performed, although the electrochemical signal is greatly influenced by the serum matrix. Moreover, the range of the standard calibration curve is narrow, and so the homogeneous method can only be envisioned for semiquantitative assays (positive/negative tests).

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