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Homogeneous Electrochemical Immunoassay Using a Perfluorosulfonated Ionomer-Modified Electrode as Detector for a Cationic-Labeled Hapten

Benoit Limoges and Chantal Degrand*

Laboratoire d'Electrochimie Organique, Thermodynamique et Electrochimie en Solution, URA 434, Université Blaise Pascal de Clermont-Ferrand, 24 Avenue des Landais, 63177 Aubière, France

Pierre Brossier

Faculté de Pharmacie, Unité d'Immunoanalyse, Université de Bourgogne, 7 Boulevard Jeanne d'Arc, 21033 Dijon, France

Ronald L. Blankespoor

Department of Chemistry, Calvin College, 3201 Burton SE, Grand Rapids, Michigan 49546

A Nafion film-modified electrode has been used as an electroanalytical sensor to detect a drug containing a cationic redox label by square wave voltammetry. A tracer, composed of the model drug amphetamine covalently attached to a cobaltocenium redox label, was synthesized and used in a competitive immunoassay technique. In this assay, only the positively charged tracer not bound to the antibody preconcentrated in the anionic film. Consequently, it was not necessary to separate free from antibody-bound labeled amphetamine, thereby allowing the assay to be carried out in homogeneous media at the electrode surface. Using an optimized procedure to prepare reproducibly the Nafion-modified electrode a detection limit in the nanomolar concentration range was obtained for the cationic-labeled drug with a working range linear between 2.5 nM and 1.0 μM. This procedure combines for the first time a Nafion film electrode with a homogeneous competitive immunoassay of a redox-labeled drug. The advantages and limitations of this electrochemical technique are discussed.

INTRODUCTION

Due to its specificity and high sensitivity, immunoassay is often the method of choice for trace analyses of clinically important compounds contained in complex biological matrices[1] and of substances found in the environment.[2] Radioisotopic labels have played an important role in the development of immunoassay in the last three decades. However, the disadvantages associated with the use of radioactive materials have led to the implementation of a wide range of new labels, first by enzymatic methods and then by fluorescent,[3,4] chemiluminescent,[5,6] and IR-active[7,8] labels or markers which are composed of or contain chelates,[9] isoluminol derivatives,[10] and organometallic compounds.[11,12] Although these labels certainly have their own specific advantages, they also have characteristics that limit their usefulness in assaying compounds from both practical and commercial viewpoints. There is a continuing effort, therefore, to develop new immunoanalytical procedures, particularly in the field of the organometallic tracers. Organometallic labels provide many analytical methods suitable for the detection and quantitative determination of the label by atomic absorption spectrometry,[13,14] infrared spectroscopy,[7,8] and electrochemical techniques.[15] By analogy to the radioimmunoassay, this type of procedure was named metalloimmunoassay by Cais.[13]

Immunoassay with electrochemical detection offers several advantages over the more widely used spectrophotometric techniques. It is relatively inexpensive, easy to operate, and can be used to make measurements in turbid media. A number of studies have utilized amperometric or potentiometric detection of electroactive species such as NADH,[16,17] phenol,[18] O_2,[19,20] H_2O_2,[21,22] and NH_3[23] generated catalytically by an enzyme label on an antigen. The high sensitivity of

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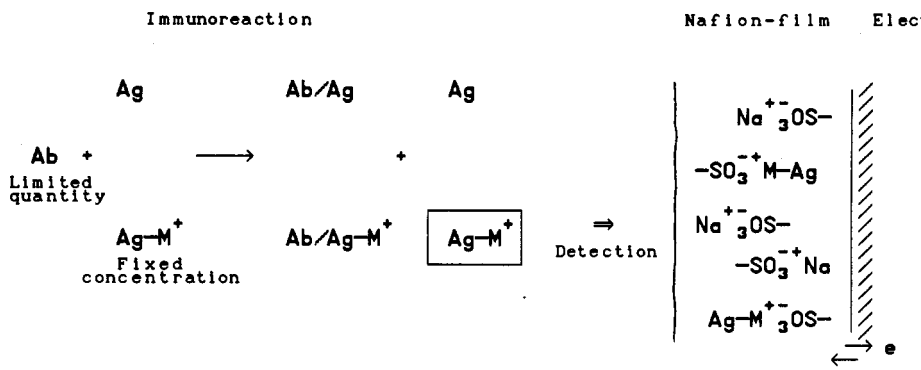

Figure 1. Schematic representation of the overall assay systems. (The principle is explained in the text.)

this method makes it competitive with that of radioimmunoassay.[18] Another application of electrochemistry is the detection of a redox group that is a label on an antigen.[24-28] Earlier studies in this field have shown that this method is not very sensitive because the amperometric detection of redox-labeled molecules is limited to the micromolar range.[24,25] However, more recent work using a bare electrode and coupling the redox reaction with an enzymatic amplification system[26,27] has shown that it is possible to improve the detection limit of a redox-labeled drug such as ferrocenyl lidocaine.[26] Use of a similar approach, but with an electrode modified with a redox polymer, resulted in an immunoassay that was more sensitive.[28] With this method the redox-labeled antigen not only displays immunoreactivity but also acts like a mediator between the enzyme and the redox polymer. The charge that accumulates during the catalytic reaction by the redox polymer is measured for a fixed time. Another original amplification of the electrochemical signal was demonstrated by employing an anion-exchange modified electrode with an anionic electroactive species encapsulated within a liposome label.[29,30] When the antibody binds the label antigen, each liposome can release about 10^4 anionic redox centers, which are then accumulated and detected by the sensor electrode.

The use of modified electrodes for trace analyses[31-39] and, more specifically, for the detection of electroactive drugs[37,38] is well documented, especially when the electrodes are coated with ion-exchange polymers.[38-43] One of them, Nafion (trademark of E. I. DuPont de Nemours and Co. Inc.)[38,39,43] a polyanionic perfluorosulfonate ionomer, not only has the ability to accumulate cations but also displays permselectivity. That is, large hydrophobic cations rather than small hydrophilic ones accumulate in the polymer via cationic exchange.[44] This preconcentration of hydrophobic cations such as $Ru(NH_3)_6^{3+}$ in a Nafion film-coated electrode can provide detection limits on the order of 10^{-9} M.[45] The permselectivity of Nafion can also result from the polyanionic nature of the film, which serves as an electrostatic barrier toward anions,[46] or from its microscopic structure consisting of narrow channels[47] which prevent the penetration of bulky molecules such as proteins.[39,48]

These remarkable properties of Nafion suggested to us a new immunoelectroanalytical technique, one that uses a Nafion film-coated electrode as a sensor to preconcentrate a cationic redox-labeled antigen (Ag-M^+) or hapten (the terms hapten and antigen will be used interchangeably in the remaining text) in the very simple format of a homogeneous competitive immunoassay. The technique is illustrated in Figure 1. When the immunological reaction takes place, a fixed quantity of labeled antigen (Ag–M^+) is in competition with the unlabeled antigen (Ag), for a limited quantity of antibody binding sites (Ab). In contrast to Ag and Ag-M^+ and because of its large size, the antibody-bound labeled antigen (Ab/Ag-M^+) is not expected to penetrate the Nafion film. Thus, when the concentration of Ag (i.e., the analyte) increases, the concentration of free Ag-M^+ available for anion exchange in the film increases, and as a consequence, more Ag-M^+ is incorporated into the film. The current corresponding to the oxidation or reduction of Ag-M^+ in the film then will depend upon the concentration of Ag in the bulk solution, and the actual concentration of Ag will be known from calibration curves.

In this paper we demonstrate that the above conditions can be fulfilled, and we develop a new immunoassay method that is both simple and sensitive. To evaluate this new immunoelectroanalytical technique, we have chosen amphetamine as model and we have carried out its quantitative determination in a buffered solution at physiological pH 7.4. Amphetamine is a powerful and widely abused sympathomimetic drug. Because this is an illicit substance, its detection

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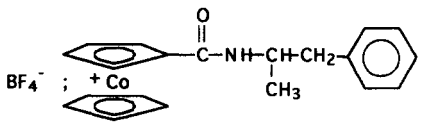
in urine must occasionally be carried out in a simple and rapid manner[36,49-51] (positive response for a sample concentration >2 μM).[50] Our choice of amphetamine as analyte was also based on the functionality present in this compound. As an amine it can be readily attached covalently to a label via an amide linkage. The cobaltocenium ion was chosen as the redox label because it is stable, cationic, and reversibly reduced (1e⁻) to cobaltocene at approximately −1.1 V (vs SCE) at pH 7.4.

First, we have prepared the tetrafluoroborate salt of the cobaltocenium-labeled amphetamine (1) and demonstrated that the Nafion-modified electrode is suitable for its quantitative measurement in both buffer and dilute serum. In the subsequent step, we have investigated the homogeneous immunoassay of amphetamine at physiological pH 7.4. In view of serum analysis, the influence of different parameters was studied, including the serum constituents, and amphetamine related and unrelated drugs.



1

EXPERIMENTAL SECTION

Materials and Reagents. A 5 wt % Nafion solution (EW 1100) and 4-acetamidophenol was purchased from Aldrich Chemical Co. *dl*-Amphetamine sulfate, *d*-methamphetamine hydrochloride, *dl*-norepinephrine, carbamazepine, and nortriptyline hydrochloride were supplied by Sigma Chemical Co., and *l*-(−)-epinephrine was from by Janssen Chemica. Glassy carbon (GC) rods (3-mm diameter) were obtained from Carbon Lorraine. Normal serum was taken from a rabbit. Phosphate-buffered saline (PBS) (8.7 mM NaH_2PO_4, 30.4 mM Na_2HPO_4, and 56 mM NaCl, pH 7.4) was used in all experiments. A stock solution of amphetamine sulfate was prepared with PBS and stored at 0–4 °C. All reagents were of analytical grade, and water was deionized and doubly distilled.

Synthesis of N-Succinylated Amphetamine. Amphetamine sulfate (1 g, 5.44 mmol) was transformed to free base amphetamine with 0.1 M NaOH and extracted with ethyl acetate. Then 285 mg (2.11 mmol) of amphetamine was reacted with succinic anhydride (219 mg, 2.19 mmol) in ethanol (25 mL) by the procedure of Hubbard et al.[52] The reaction yielded 379 mg (76.3%) of amphetamine succinate.

Preparation of Amphetamine Immunogen. A solution of amphetamine succinate (21 mg, 0.09 mmol) in a mixture of 3 mL of water and 1 mL of 0.2 M sodium acetate (pH 5.5) was prepared at room temperature. To this stirred solution was added 84 mg (0.44 mmol) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide followed by a solution of 142 mg (0.02 mmol) of bovine serum albumin (BSA as carrier) in 20 mL of water which was added in 1-mL portions. After the reaction mixture was stirred for 24 h at room temperature in the dark, it was dialyzed at 4 °C against distilled water for 2 days. The retenate was lyophilized to obtain the amphetamine immunogen (amphetamine-BSA). The number of amphetamine residues per mole of carrier was determined by the UV method.[53] This was found to be 22 mol of amphetamine/mol of bovine serum albumin.

Antiserum Production. Following the method recommanded by Landon and Moffat,[54] three "fauve de Bourgogne" rabbits were immunized with the amphetamine–BSA conjugate prepared above. Into each flank was administered an initial subcutaneous injection of 0.250 mg of the immunogen in 1 mL of H_2O/Freund's complete adjuvant (1:3) emulsion. The booster immunizations were given at intervals of 1 month with the same amounts of immunogen but Freund's complete adjuvant was replaced by Freund's incomplete adjuvant. Serum of each rabbit was obtained from the marginal ear vein at 12–15-day intervals after the third injection. The harvested serum obtained after centrifugation was frozen in small aliquots at −20 °C for future use. The specificity of the antisera used in the studies described below will be published separately.[55]

Preparation of (Amphetaminecarbonyl)cobaltocenium Tetrafluoroborate (1). The starting material, carboxycobaltocenium hexafluorophosphate (125 mg, 0.330 mmol), was prepared and converted in situ to the acid chloride using an excess of $SOCl_2$, according to the literature method.[56] After the bulk of excess $SOCl_2$ was removed by distillation, the remaining $SOCl_2$ was evaporated using a stream of N_2. To this crude (chlorocarbonyl)cobaltocenium hexafluorophosphate was added a solution of amphetamine (113 mg, 0.837 mmol) in 15 mL of dry THF (freshly distilled from CaH_2). After stirring overnight at room temperature, the solvent was removed under reduced pressure and the residue was dissolved in 20 mL of H_2O/acetone (9:1). To remove excess amphetamine and its salt, the aqueous solution was treated with 3 mL of 1.0 M NaOH and extracted with ether (2 × 20 mL). The aqueous layer was then neutralized with aqueous HCl and combined with a solution of $NaBF_4$ (1.8 g) in 5 mL of H_2O. The cobaltocenium tetrafluoroborate salt was extracted into CH_2Cl_2 (2 × 20 mL), and the CH_2Cl_2 was removed under reduced pressure giving a yellow solid. Chromatography of this solid on silica gel followed by elution with acetone/H_2O (4:1) gave a small amount of carboxycobaltocenium tetrafluoroborate. Elution with acetone/water/$NaBF_4$ (100 mL:100 mL:200 mg) gave a second yellow band which contained (amphetaminecarbonyl)cobaltocenium tetrafluoroborate. After the acetone in the mixture was removed under reduced pressure, $NaBF_4$ (1.0 g) was added to the remaining aqueous solution and the cobaltocenium salt was extracted using CH_2Cl_2 (2 × 15 mL). The CH_2Cl_2 extracts were combined, dried over $NaBF_4$, and evaporated to dryness in a rotary evaporator. Further drying (60 °C, 0.1 Torr, 30 min) gave a yellow solid which was recrystallized from 2-propanol to give 65 mg (45%) of the desired cobaltocenium: mp 140–141 °C; IR (Nujol) 3320, 3130, 1645 (s), 1570 (s), 1320 (w), 1240 (w), 1205 (w), 1145 (w), 1080 (s), 1055 (s), 1030 (s), 1025, 880 (w), 870 (w), 820 (w), 760 (w), 710 (w); ^1H NMR (300 MHz, acetone-d_6) δ 1.32 (d, 3 H), 2.88–3.03 (m, 2 H), 3.08 (d, 1 H), 4.41–4.54 (m, 1 H), 5.67 (s, 5 H), 5.95 (s, 1 H), 5.97 (s, 1 H), 6.23 (s, 1 H), 6.28 (s, 1 H), 7.17–7.42 (m, 5 H), ^{13}C NMR (300 MHz, acetone-d_6) δ 20.7, 42.5, 48.1, 84.3, 85.3, 87.0, 95.2, 127.1, 129.2, 130.1, 140.3, 161.3; MS (EI, 70 eV) *m/e* (relative intensity) 350 (100), 232 (57), 216 (12), 188 (100), 187 (29), 129 (21), 124 (19), 93 (12), 91 (26), 65 (18). Anal. Calcd for $C_{20}H_{21}BCoF_4NO$: C, 54.95; H, 4.84; B, 2.47; Co, 13.48; N, 3.20. Found: C, 54.92; H, 4.87; B, 2.28; Co, 13.02; N, 3.37.

Electrode Preparation. GC rods were sanded flat with 1200 grit silicon carbide paper and polished with 0.05-μm aqueous alumina suspension (ESCIL). Immediately after being polished, the electrodes were ultransonically cleaned in ethanol, rinsed with doubly distilled water, and dried at 100 °C in an oven.

The Nafion film-coated electrode surface (GC/Nafion) was prepared according to the following procedure. First, 0.4 mL of the Nafion solution (Aldrich) was combined with 19.28 mL of *N,N*-dimethylformamide (DMF) and 0.32 mL of aqueous 0.05 M LiOH to give the Li^+ salt of the Nafion. The Nafion coating was made by applying 5 μL of this diluted solution to the polished surface of the GC electrode and removing the bulk of the solvent at 140 °C for 5 min under an atmosphere saturated with DMF vapor. To assure complete removal of solvent, the electrode was placed in an oven for 10 min at 140 °C.

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For each measurement, a GC/Nafion rod 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. A film thickness of 0.4 μm was calculated by assuming a density of 1.58 g/cm^3.[57]

Apparatus. An EG&G PAR 273 potentiostat interfaced to a IBM XT 286 computer system with a PAR M270 software was used for square wave voltammetry (SWV). Electrochemical experiments were carried out at 22 °C in a one-compartment glass cell that had a working volume of 2 mL. In addition to the Nafion film-coated electrode mounted on a Tacussel rotating-disk electrode, a platinum wire counterelectrode and an Ag/AgCl (0.056 M NaCl) reference electrode were introduced in the cell.

Accumulation Procedure. The samples were prepared in vials before being transferred into the electrochemical cell. Then, the rotating-disk modified electrode was exposed to the solution and immediately rotated at 600 rpm for 5 min. During this exposure time, the ion exchange occurs under electrical open circuit. Afterward, a rapid scan of square wave potential was applied and the resulting peak current was taken as the analytical response.

Antibody Titration Curve Procedure. To 935 μL of buffer were added in order 5 μL of 1 (1.82×10^{-5} M in PBS), y μL (0–60) μL of amphetamine antiserum, and $(60 - y)$ μL of normal serum. After thorough mixing, the solution was incubated for 1 h at 37 °C. The solution was then assayed using the accumulation procedure.

Immunoassay Procedure. To $(955 - x)$ μL of buffer was added 5 μL of 1 (1.82×10^{-5} M in PBS) followed by x μL (0–100) μL of amphetamine sulfate (1.3×10^{-4} M in PBS) or N-succinylated amphetamine (2×10^{-4} M in PBS). After thorough mixing, 40 μL of amphetamine antiserum was added and the contents were incubated at 37 °C for 1 h. For the reference tube, x was equivalent to zero and the antiserum was replaced by normal serum. Thereafter, the accumulation procedure was followed.

RESULTS AND DISCUSSION

Square Wave Voltammetry. SWV is not only one of the most sensitive electroanalytical techniques that is employed to detect reversible redox systems, but it also has the important advantage of being rapid.[58] When this technique is utilized with an ion-exchange modified electrode, it seems appropriate to refer to this method of analysis as square wave ion-exchange voltammetry (SWIEV), based upon the literature usage of square wave anodic stripping voltammetry (SWASV) defined by Osteryoung and co-workers[59] and ion-exchange voltammetry (IEV) defined by Whiteley and Martin.[45] In SWV the peak current depends on various parameters including the potential step increment (δE), square wave amplitude (E_{SW}), and frequency (f). Taking into account the theoretical prediction for a reversible one-electron transfer,[58] an E_{SW} value of 50 mV was chosen in order to have the best ratio of peak current to peak width. Sensitivity also increases with frequency, and a good current response was found for f = 100 Hz, and δE = 2 mV.

Nafion-Coated Electrodes. Figure 2 shows the square wave voltammograms obtained at both bare and Nafion-coated electrodes immersed in a solution containing 0.91 μM 1. Under the same experimental conditions, the peak current (i_p) is greatly enhanced at the modified electrode (about 40 times). However, the modified electrode cannot be reused for further experiments since the reduced form of 1, which is neutral, remains in the Nafion film and cannot be easily expelled. It was necessary, therefore, to prepare a series of modified electrodes and demonstrate that they give reproducible results when used in assaying for 1 under identical conditions. Four series of 10 Nafion-coated GC electrodes were prepared, processed at 140 °C, and used to assay a solution of 0.91 μM 1. Statistical analysis showed that the relative standard deviations for intra- and interassay precision were on the order of 5 and 10%, respectively. Thus, the process at 140 °C not only increases the Nafion film stability, as previously noted,[60] but also gives reliable data, even at high rotation speeds. Interestingly, the sensitivity was 2 times greater for Nafion films processed at the higher temperature than those processed at room temperature.

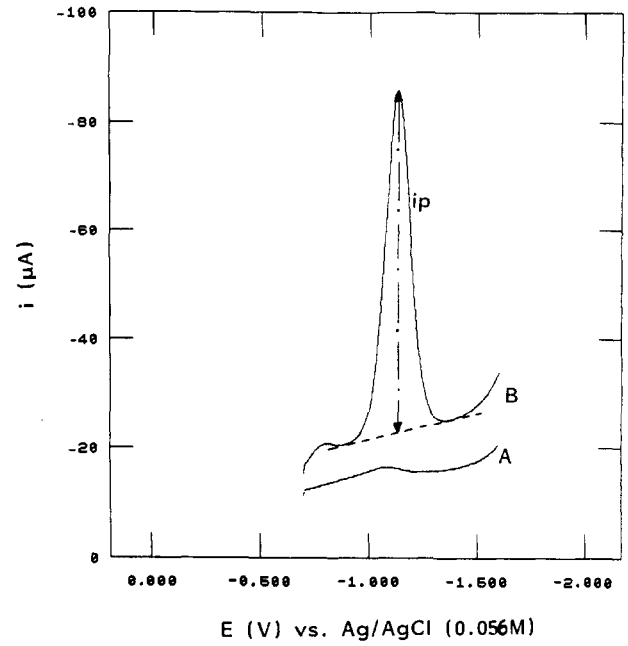

Figure 2. SWV (f = 100 Hz, E_{SW} = 50 mV, δE = 2 mV) at GC (A) and GC/Nafion (B) (film thickness 0.4 μm) immersed in a buffer solution (pH 7.4) of 0.91 μM 1 for 5 min at 600 rpm and T = 22 °C.

Optimization of Nafion Sensor for 1. Cobaltocenium 1 readily dissolves in aqueous buffer and is very stable in this medium, even at low concentrations. Using a Nafion-coated GC electrode with a film thickness of 0.4 μm, a plot of log i_p vs log [1] was made at two exposure times (Figure 3). A detection limit of 2.5×10^{-9} M (signal/noise, S/N = 3) is obtained with an exposure time of only 5 min at 600 rpm (curve A). With exposure for 15 min the sensitivity is increased 2 times (curve B). Previous work on electrodes coated with Nafion films has shown that the sensitivity can be improved by increasing the exposure time or decreasing the film thickness.[45] However, longer exposures increase the analysis time and consequently decrease sample throughput. A relatively short exposure time (5 min) was chosen and so the best results were observed for a film thickness of 0.4 μm. A thinner film (0.2 μm) gave a shorter linear response range and less reproducible measurements. Under these conditions, to compare the accumulation of the marker alone to that of the labeled amphetamine in the Nafion sensor, the detection of the organometallic cobaltocenium ion was found to be 4 times less sensitive than 1. In fact, when the cobaltocenium ion is attached to amphetamine, the hydrophobic character of the cation is increased and the aqueous/Nafion partition coefficient for the film is enhanced. This hydrophobic effect has also been observed by Martin and co-workers.[44] It is also important to note that, if the electrode is rotated, the flux of electroactive species brought to the electrode surface is rigorously constant, and hence the sensitivity and reproducibility are improved. A high dynamic range of 3 orders of

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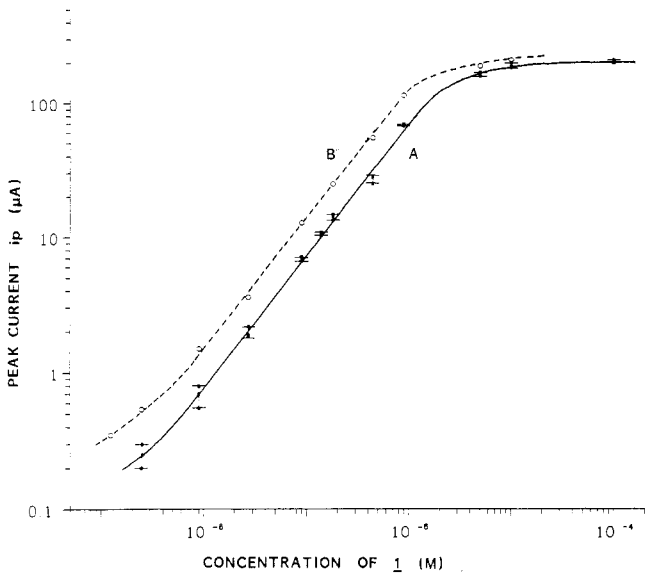

Figure 3. Calibration curves 1 at GC/Nafion for two different exposure times, 5 (A) and 15 min (B), and under the same conditions as given for Figure 2. Error bars represent 2 times the standard deviation for peak currents at three different electrodes.

Table I. Effects of the Addition of Amphetamine Excess on the Peak Current for 0.14 μM 1 in pH 7.4

amphetamine excess[a]	0	19	94
$i_p \pm$ SD[b] (μA)	10.8 ± 0.8	10.5 ± 0.6	10.7 ± 0.5

[a] Ratio of unlabeled amphetamine concentration to labeled amphetamine concentration. [b] Average peak current for two GC/Nafion (0.4 μm thick) (SD, standard deviation). Exposure time, 5 min.

magnitude is available, and at high concentration the film becomes satured; then the concentration of analyte in the film becomes fixed and independent of the solution concentration. From the high sensitivity and the large linearity range (2.5×10^{-9} to 10^{-6} M), we can conclude that a Nafion film-coated electrode is a good sensor for 1.

Influence of Various Species. Earlier studies have shown that electroinactive protonated amines can interfere with ionic exchange of electroactive species in Nafion.[38,61] Since amphetamine is protonated at pH 7.4, it was necessary to determine whether this cationic species penetrates into the Nafion film and thereby interferes with the assay of 1. At constant concentration of 1 (1.4×10^{-7} M), unlabeled protonated amphetamine does not interfere significantly, even in large excess, as shown in Table I. The partition coefficient of protonated amphetamine is probably too low to compete with the cobaltocenium-labeled amphetamine for anionic sites in the Nafion. We have also examined the influence of related drugs such as methamphetamine, epinephrine, or norepinephrine in excess (100) and observed no significant decrease of the peak current at the 95% confidence interval. Similarly, small molecules substituted by hydroxyl functions (acetaminophen) and bulky neutral molecules (carbamazepine) do not interfere whereas bulky molecules which are cationic at physiological pH can interfere. Indeed, by addition of nortriptyline (12 equiv) to 1.8×10^{-7} M 1, the peak current drops from 14.5 to 4.7 μA.

Possible interference of the serum constituents (amino acids, proteins, inorganic cations, lipids, etc.) was also examined. Figure 4 shows the electrochemical signal when normal serum (i.e., serum without antibody, Ab) is added to

(61) Degrand, C.; Blankespoor, R.; Limoges, B.; Brossier, P. Fr. Patent 9207089, 1991.

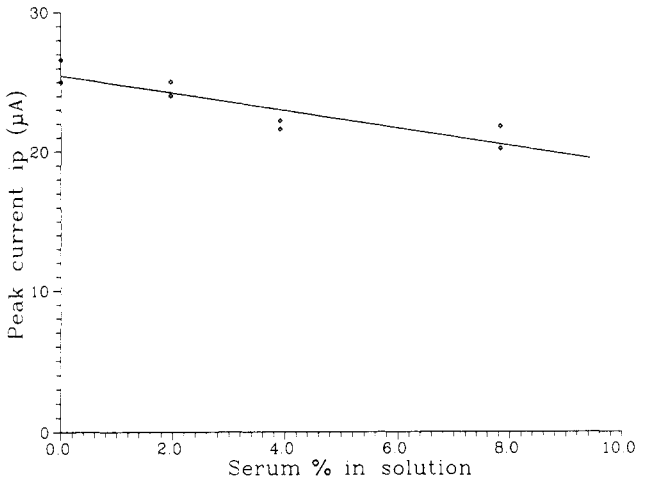

Figure 4. Effect of normal serum addition on the peak current of 0.45 μM 1 after 1 h of incubation time at GC/Nafion electrodes. Measurement conditions as given for Figure 2.

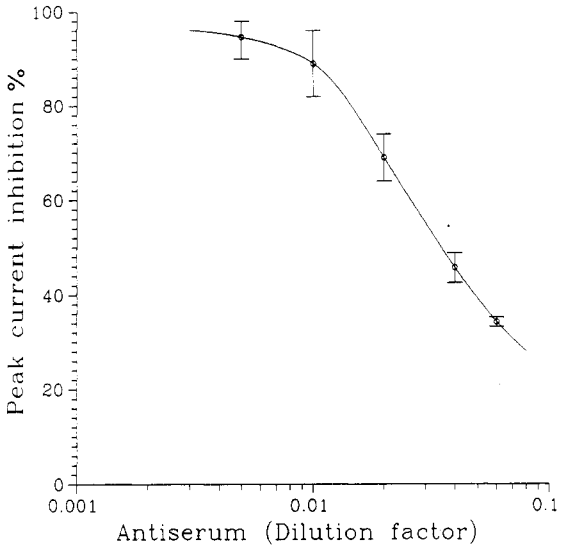

Figure 5. Antibody titration curve at GC/Nafion for 9.1×10^{-8} M 1. Incubation time 1 h at 37 °C. Final volume 1 mL. Measurement conditions and error bars as for Figure 3A.

1 and incubated 1 h at 37 °C. Even in the presence of as much as 8% serum, the signal is decreased only slightly. Perhaps the small almost proportional decrease arises from nonspecific interactions and/or modifications of the ionic strength of the solution and therefore the partition coefficient of 1 in the Nafion film. This phenomenon is taken into account in the following experiments. As already mentioned, Nafion prevents the penetration of bulky molecules such as proteins[39,48] and so presents excellent antifouling properties.

Immunoreactivity of Antibody with 1. An antibody titration curve is shown in Figure 5. The dilution factor is defined as the ratio of antiserum volume to total assay volume. Incubation of increasing amounts of amphetamine antiserum (i.e., antibody) with a fixed concentration of 1 (9.1×10^{-8} M) results in a decrease in the peak current of 1. Taking into account the result from normal serum described in the preceding section, the decrease in peak current with increasing antibody concentration suggests that immunoreaction does take place and, as expected, that 1 sequestered by the antibody (Ab/Ag-M^+) is unable to penetrate the film as a result of its large size. From the titration curve, the appropriate volume of antiserum was chosen in order to develop the best immunoassay conditions for the measurement of amphetamine. This volume, which should provide the best sensitivity and the highest signal to noise ratio, would correspond to

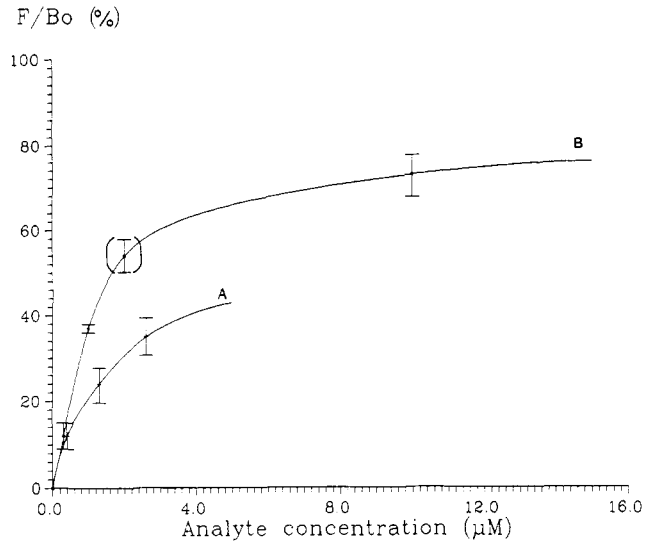

Figure 6. Standard curves for amphetamine (A) and N-succinylated amphetamine (B). In each case the analyte concentration refers to that in the final volume assay. (See assay procedure for details.)

50% inhibition. However, the antibody titration curve shows that it is necessary to have high quantities of antiserum to obtain a significant diminution of signal. It is possible that the immunogen (which produces the antibody), in which amphetamine is bound to bovine serum albumin via the amino group, produced an antiserum with a low titer and/or low specificity.[49] In our immunoassay procedure, a dilution factor of 1/25 (40 μL of antiserum in a total volume of 1000 μL) was chosen which corresponds to a peak current inhibition of 45%.

Homogeneous Competitive Immunoassay of Amphetamine. Before amphetamine can be assayed in unknown biological samples, a standard curve is needed which can be used over a range of different analyte concentrations. To calculate each point on the curve, peak current data from the SWV reduction of 1 at a fixed concentration was obtained in three different media—normal serum to give the reference peak current i_r, amphetamine antiserum to give peak current i_o, and amphetamine antiserum containing the analyte to give peak current i_a. When the concentration of amphetamine is increased, the competition between 1 and amphetamine for limited antibody increases and i_a becomes larger. So, for each analyte concentration, the percentage of inhibition reversal (F/B_o) was calculated using the equation

$$F/B_o = 100(i_a - i_o)/(i_r - i_o)$$

Physically, F corresponds to the amount of variation of unbound 1 in solution (and consequently in the Nafion film) which increases as the concentration of amphetamine is increased and B_o is the amount of 1 initially bound to the antibody (before the addition of amphetamine). A plot of F/B_o vs amphetamine concentration is given in Figure 6A, where the fixed concentration of 1 is 9.1×10^{-8} M. Once standard curves are obtained, each analysis requires only one experiment assuming an almost constant serum composition and/or a low serum content in the analyte. It is worth noting that, even in the presence of a 30-fold excess of amphetamine, a large fraction of 1 remains bound to the antibody. This suggests that the antibody has higher affinity for 1 than for amphetamine. To test this hypothesis, N-succinylated amphetamine [$PhCH_2CH(CH_3)NHCO(CH_2)_2COOH$] was prepared. Not only does this derivative of amphetamine exist as a negatively charged carboxylate at pH 7.4, which should greatly reduce its penetration into Nafion, but its larger structure should make it more similar to the immunogen than that of amphetamine. This size effect has been observed

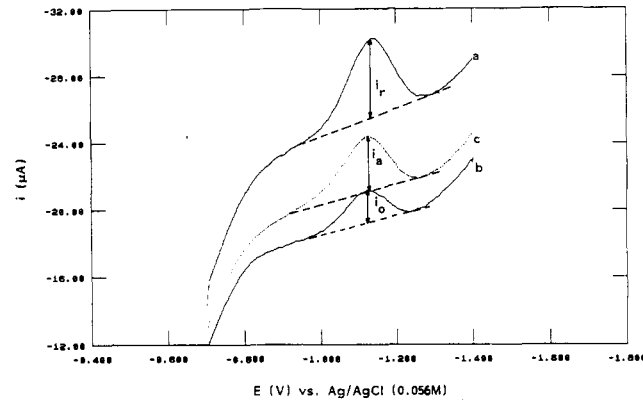

Figure 7. SWV at GC/Nafion obtained for 9.1×10^{-8} M 1 incubated 1 h at 37 °C with (a) 40 μL of normal serum, (b) 40 μL of anti-amphetamine serum or (c) 40 μL of anti-amphetamine serum and 2 μM N-succinylated amphetamine. Final volume 1 mL.

before in the immunoassay of small molecules.[62] Figure 6B shows the standard curve for N-succinylated amphetamine using peak currents such as those in Figure 7 (with 2 μM derivative) and under the same conditions as those used for amphetamine. The better sensitivity obtained for the former confirms our hypothesis that the derivatized amphetamine competes more favorably with 1 for antibody.

CONCLUSION

The sensitive electrochemical determination of cobaltocenium-labeled amphetamine 1 at a Nafion-coated electrode illustrates the potential of using an electrode coated with an anionic polymer with a cationic redox-labeled hapten. With a covalently attached, positively charged label, amphetamine is preconcentrated in the anionic film and can be detected by SWV in the nanomolar concentration range, with a relative standard deviation between 5 and 10%. Moreover, this electrochemical technique has been combined for the first time with a homogeneous competitive immunoassay using amphetamine antiserum from rabbits in a conjunction with the organometallic tracer, demonstrating that this new immunoassay can be employed in buffered solutions. This method offers a potentially valuable sensing technique for several reasons. First, it is easy to operate since it corresponds to the simplest form of immunoassay, that is, one without a separation step. Second, with a low-cost electrochemical instrumentation, a sensitive method is now available due to the preconcentrating property of the film. Finally, it should be possible to apply this technique to a wide spectrum of small analytes (e.g., drugs or pesticides) that can be covalently attached to a wide variety of redox labels that are cationic (or procationic), stable, and relatively easy to synthesize.

At this point of our research a number of significant questions remain. One concerns the competitive immunoassay and, more specifically, the antiserum cross reactivity, affinity, and titer. Concerning these parameters, the choice of amphetamine as a model drug with its available antiserum probably is far from optimum. If we want to apply this technique to the evaluation of amphetamine in real samples, the sensitivity of the calibration curve and the antiserum specificity will have to be improved. A better immune reaction would probably occur in the presence of an antiserum obtained against an immunogen connected to the para position of the drug's phenyl group, as demonstrated in ref 45. In this work, which describes the use of such an immunogen and fluorescein-labeled amphetamine, a high specificity together with a high

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titer was obtained. Furthermore, if we want our immunoassay technique to be suitable for a variety of small molecules dissolved in different media, such as serum, urine, waste water, and food, it is necessary to determine the specific influence of the matrix on the labeled hapten detection and eventual interference of compounds generally encountered in presence of analyte. Since our results have shown that, in the accumulation step, a negligible interference of several related and unrelated drugs takes place, as well as a limited effect of the matrix, we are confident that we will be able to assay real samples.

Another question is specific to the electrode technology, which has to be improved since the film-modified carbon disk is a single-use sensor. We are now testing multidisposable sensors of small size in order to carry out assays in parallel and in small volumes.

Work is in progress aimed at using this technique with other hapten and/or redox labels.

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