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Redox Labeling of Two Antiepileptic Drugs with Metallocenes and Their Simultaneous Detection by a Nafion-modified Electrode

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Two different cationic redox labels, i.e. a ferroceneammonium ion and a cobaltocenium ion, were covalently attached to two antiepileptics, phenytoin and phenobarbital, respectively. The two labeled drugs possess distinct standard redox potentials of 0.39 V for the phenytoin derivative and −0.92 V for phenobarbital derivative (vs Ag/AgCl, Cl^- 0.05 M) at a carbon paste electrode. After preconcentration in a polyanionic Nafion-loaded carbon paste electrode the positively charged labeled phenytoin and phenobarbital derivatives could be simultaneously detected in concentration ranges which were relevant to the therapeutic ranges of the antiepileptics, with a view to a future dual-analyte immunoassay. Square-wave voltammetry permitted detection limits of 5×10^{-8} M (for the phenytoin derivative) and 2.5×10^{-8} M (for the phenobarbital derivative) for non-simultaneous detection. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

The advantages of multi-analyte assays are work simplification, reduction of analysis time and lowering of overall cost per test. Therefore there has been an increase of interest in the development of multi-analyte immunoassays in which two or more analytes are measured simultaneously in a single assay.[1, 2]

One important approach in multi-analyte immunoassays consists in the use of antibodies or antigens with different chemical labels. The main limitation to this approach is the simultaneous detection of different labels in the final-step procedure, since it is difficult to find a combination of labels which can be detected distinctly and essentially with the same sensitivity using a single technique. Detection methods typically based on dual analyte immunoassays have been reported with the simultaneous use of two radiolabels (^{125}I and ^{57}Co)[3] or two fluorescent,[4] enzyme,[5] spin[6] or metal-ion[7] labels. Examples with more than two labels are very rare and have appeared only recently,[8, 9] using either a combination of lanthanide chelate labels and their time-resolved fluorescence detection,[8] or several IR-active organometallic labels.[9]

We have recently developed an analytical technique that combines competititve homogeneous immunoassay with electrochemical detection using a Nafion-modified electrode and an antigen covalently attached to a positively charged redox label.[10–12] The anionic perfluorosulfonated polymer Nafion is able to preconcentrate selectively the cationic redox-labeled substance and therefore to enhance the sensitivity of the detection method. This technique can be used to analyze nanomolar concentrations of small haptens and was successfully applied to the homogeneous immunoassay

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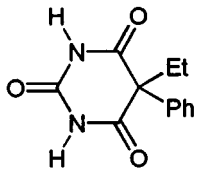
of amphetamine[10] and phenytoin.[11, 12] Since the immunoassay was conducted in homogeneous media, it was not necessary to separate the free labeled drug from the antibody-bound labeled drug, which provides an important advantage compared with other immunoassay formats. Another unexplored benefit of this technique is the possibility of developing multi-analyte immunoassays using different cationic redox labels with distinct redox potentials.

In this paper, we describe the method of attaching covalently two different cationic redox labels to the antiepileptic drugs phenobarbital (**1**) and phenytoin (**2**). It is then demonstrated that the two redox-labeled drugs are simultaneously detected at a Nafion-loaded carbon paste electrode.



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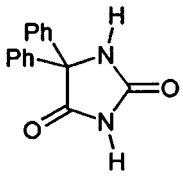

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The choice of phenobarbital and phenytoin was guided by the fact that these antiepileptic agents are frequently prescribed in combination,[13] so dual analyte assay may be required. We have chosen two redox labels which are cationic and possess distinct standard redox potentials, i.e. a cobaltocenium salt reversibly reduced at *ca* -1.1 V and a ferroceneammonium derivative reversibly oxidized at *ca* 0.35 V. Moreover, the selected labels possess several other advantages which contribute appreciably to the accomplishment of the immunoassay technique. They are non-hazardous labels, rapidly and reversibly exchanging an electron at physiological pH. Furthermore the labels are stable and soluble in aqueous solution, and they do not prevent drug recognition by antibodies when covalently attached to drugs.[10–12, 14]

EXPERIMENTAL

Starting materials

Phenobarbital (**1**) was a donation from Pr. P. Brossier, Laboratoire d'Immunoanalyse, Faculté de Pharmacie, Dijon, Fance. *N*,*N*-Dimethylaminomethylferrocene and chloroaceto-2,6-xylidide were purchased from Lancaster, Synthesis Ltd. 1,2-Dibromoethane was supplied by Merck, 1,3-dibromopropane by Fluka and phenytoin sodium salt (**7**) by Sigma. Rabbit normal serum was purchased from Sigma and titanium oxysulfate–sulfuric acid complex hydrate from Aldrich.

General

NMR spectra were recorded on a Bruker AC 400 spectrometer. Infrared spectra were obtained with a Nicolet Impact 400 FT spectrometer. Elemental analyses and mass specroscopy were performed by the Centre National de la Recherche Scientifique (CNRS) at Vernaison. Preparative column chromatography was performed on silica gel (Merck; 60-mesh). The air- and water-sensitive reactions were carried out under an argon atmosphere in purified solvents and were followed by thin-layer chromatography (TLC) (silica gel on aluminum foil; Merck 1.05554).

Electrochemical techniques

A Tacussel PRT 100-1X coupled with an IG5-N integrator was used for preparative electrolysis. An EG&G PAR 273 potentiostat interfaced to an IBM XT 286 computer system with PAR 270 software was used for square-wave voltammetry (SWV) (for parameters, see Ref. 10) and a Sirius apparatus was used for cyclic voltammetry (CV).

A three-compartment cell equipped with a magnetically stirred mercury pool as working electrode, a graphite rod as anode and a saturated calomel electrode (SCE) as reference electrode, was used for large-scale electrolyiss. The SWV experiments were carried out in a single-compartment cell (1 cm^3 of working volume, thermostated at 25 °C). A Nafion-loaded carbon paste electrode (CPE) developed by Rapicault *et al.*[12] was used as working electrode, an Ag/AgCl (50 mM NaCl) electrode as reference, and a platinum wire as counter-electrode. For the CV experiments, the volume of the cell was 10 cm^3 and the working electrode was an unloaded CPE. The SWV and CV experiments were carried out in phosphate buffer (PB: 4.35 mM NaH_2PO_4, 15.1 mM Na_2HPO_4 and 50 mM NaCl, pH 7.4) from stock solutions of **6** (5×10^{-4} M) and **9** (2×10^{-3} M) in ethanol.

m-Aminophenobarbital (4a)

$TiOSO_4 \cdot xH_2O \cdot xH_2SO_4$ ($x = 0.95$;[15] 19 mmol) dissolved in 160 cm^3 of a deaerated solution of 0.5 M H_2SO_4 in dimethylformamide (DMF) was reduced electrochemically at -0.7 V. Then a solution of 1.9 mmol of *m*-nitrophenobarbital (**3a**)[16] in 1 cm^3 of DMF was added. After being stirred for 1 h at room temperature, the solution was neutralized and filtered. Water (800 cm^3) was added to the filtrate and the mixture was extracted eight times with 200 cm^3 of diethyl ether. The course of the extraction was followed by HPLC until 96% of the product had been separated. The organic phase was dried over $MgSO_4$ and filtered. The ether was evaporated under reduced pressure and the crude product was lyophilized. The pure *m*-aminophenobarbital (**4a**) (87% yield) was precipitated from the residual viscous liquid by addition of a small volume of chloroform: m.p. 197 °C (lit.[17] 208–209 °C). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 11.65 (s, 2H, N–H), 7.03 (t, 1H, $J = 8$ Hz, phenyl C-H), 6.55 (s, 1H), 6.54 (d, 2H, $J = 8$ Hz, phenyl C–H), 6.43 (d, 1H, phenyl C–H), 5.30 (s, 2H, NH_2), 2.25 (q, 2H, $J = 7.4$ Hz, CH_2), 0.88 (t, 3H, $J = 7.4$ Hz, CH_3). MS (EI) *m/z* (relative intensity): 247 (57, [M^+]), 232 (3), 219 (43), 188 (16), 176 (7), 161 (8), 145 (11), 132 (20), 128 (16), 105 (18), 83 (31), 56 (100), 43 (30), 41 (25), 29 (100), 28 (100), 27 (13).

Ferroceneammonium–labeled xylidide (5)

To a mixture of *N*,*N*-dimethylaminomethylferrocene (2.43 g, 10 mmol) and 10 cm^3 of benzene, a solution of chloroaceto-2,6-xylidide (1.98 g, 10 mmol) in 15 cm^3 of dry tetrahydrofuran (THF) was added dropwise. The mixture was stirred for 12 h at room temperature and the reaction was followed by TLC. After evaporation of the solvent the viscous residue was purified by chromatography on silica gel (eluted with ethanol/acetic acid (80:20, v/v), and product **5** was isolated in 60% yield. Further purification by repeated recrystallization from acetone afforded very pure fine brown crystals which decomposed above 300 °C. ^{1}H NMR (400 MHz, acetone-d_6), δ (ppm): 11.70 (s, 1H, N–H), 7.05 (s, 3H, phenyl C–H), 4.90 (s, 2H, C(O)–CH_2), 4.75 (2H), 4.70 (2H), 4.40 (2H), 4.30 (s, 5H, cyclopentadienyl C–H), 3.35 (s, 6H, CH_3–N^+), 2.25 (s, 6H, phenyl CH_3). IR (KBr), ν (cm^{-1}): 3598 (w), 3536 (w), 3455 (w), 3339 (m), 3141 (m), 3107 (m), 2977 (m), 1689 (s), 1634 (w), 1546 (m), 1477 (m), 1450 (w), 1246 (w), 843 (m), 768 (m). Analysis: Calcd for $C_{23}H_{29}N_2OClFe.H_2O$: C, 60.21; H, 6.81; N, 6.11; Cl, 7.72; Fe, 12.17. Found: C, 59.83; H, 6.83; N, 5.93; Cl, 7.42; Fe, 11.12%.

Cobaltocenium-labeled phenobarbital (6)

To a solution of the mono-acid ov cobaltocenium hexafluorophosphate[18] (0.43 g, 1.14 mmol) in 30 cm^3 of dry acetonitrile were added decyclohexylcarbodi-imide (DCC) (0.27 g, 1.31 mmol) and a solution of *m*-aminophenobarbital 0.30 g, 1.21 mmol) in 5 cm^3 of dry acetonitrile. The mixture was stirred for 24 h at room temperature and the reaction was followed by TLC. A white precipitate was filtered off. The filtrate was concentrated and cooled down to 0 °C after addition of 20 cm^3 of dichloromethane. The crude product was precipitated and collected after filtration in the form of a yellow solid (0.58 g, 84% yield yield). Recrystallization from methanol afforded 0.31 g (45%) yield) of the pure product **6**: m.p. 234–235 °C. ^{1}H NMR (400 MHz, acetone-d_6), δ (ppm): 10.70–10.45 (s, 2H, cyclic N–H), 9.90 (s, 1H, acyclic N–H), 7.86 (dd, 1H, $J = 8.0$, 1.0 Hz, phenyl C–H), 7.74 (t, 1H, $J = 1.0$ Hz, phenyl C–H), 7.42 (t, 1H, $J = 8.0$ Hz, phenyl C–H), 7.23 (dd, 1H, $J = 8.0$, 1.0 Hz, phenyl C–H), 6.47 (t, 2H, $J = 1.9$ Hz, cyclopentadienyl C–H), 6.06 (t, 2H, $J = 1.9$ Hz, cyclopentadienyl C–H), 6.02 (s, 5H, cyclopentadienyl C–H), 2.40 (q, 2H, $J = 7.4$ Hz, $-CH_2-CH_3$), 0.97 (t, 3H, $J = 7.4$ Hz, $-CH_2-CH_3$). IR (KBr), ν (cm^{-1}): 3400 (m), 3216 (m), 3121 (m), 2977 (w), 2855 (w), 1760 (m), 1716 (s), 1600 (w), 1559 (m), 1436 (m), 1356 (m), 1320 (m), 850 (s), 571 (m). MS (FAB$^+$) *m/z* (relative intensity): 462 (100, [M^+]), 433 (3), 391 (3), 371 (4). Analysis: Calcd for $C_{23}H_{21}N_3O_4CoF_6P$: C, 45.49; H, 3.49; N, 6.92; Co, 9.70; F, 18.77, P, 5.10. Found: C, 44.99; H, 3.38; N, 6.70; Co, 9.40; F, 17.60; P, 4.56%.

Ferroceneammonium-labeled phenytoin (9)

The bromide **8b**[19] (0.49 g, 1.3 mmol) was dissolved in 10 cm^3 of dry THF. A solution of *N*,*N*-dimethylaminomethylferrocene (0.32 g, 1.3 mmol) in 5 cm^3 of dry THF was added slowly under stirring. The mixture was stirred at room temperature for 10 days in the dark. The

yellow precipitate was filtered, washed with THF and afforded without further purification the pure product **9** (0.63 g, 77% yield) which decomposed above 195 °C. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 9.85 (s, 1H, cyclic N–H), 7.48–7.36 (m, 10H, phenyl C–H), 4.41 (s, 2H, Fc–CH_2), 4.43 (d, 2H, J=1.5 Hz, cyclopentadienyl C–H), 4.35 (t, 2H, J=1.5 Hz, cyclopentadienyl C–H), 4.26 (s, 5H, cyclopentadienyl C–H), 3.53 (t, 2H, J=6.4 Hz, CH_2–N^+), 3.11 (mc, 2H, CH_2), 2.88 (s, 6H, CH_3-N^+), 2.04 (mc, 2H, –CH_2–CH_2–CH_2–). IR (KBr), ν (cm^{-1}): 3422 (m), 3049 (m), 1772 (m), 1730 (s), 1560 (m), 1436 (m). MS (FAB^+) *m/z* (relative intensity): 536 (22, [M^+]), 338 (8), 271 (3), 199 (100), 133 (5). Analysis: Calcd for $C_{31}H_{34}N_3O_2$ FeBr: C, 60.41; H, 5.56; N, 6.82; Br, 12.96; Fe, 9.06. Found: C, 60.40; H, 5.58; N, 6.82; Br, 12.67; Fe, 8.99%.

The ^{13}C-NMR shift of the organometallic salts **5**, **6** and **9** are given in Table 1 and tentatively assigned with the help of the ^{13}C-NMR spectra of compounds **1** and **8a**.

RESULTS AND DISCUSSION

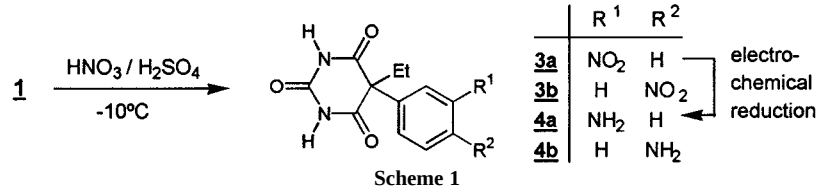
Synthesis of the cobaltocenium-labeled phenobarbital 6

The recognition of phenobarbital during the immunoreaction takes place at the hydrophilic part of the compound,[20] so we decided to attach covalently the cationic redox label to the phenyl group of phenobarbital. In order to functionalize the phenyl group we followed the nitration procedure described by Bousquet and Adams (Scheme 1).[16]

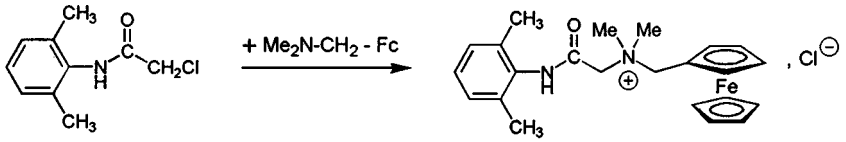
Under these conditions we observed a 3:1 ratio of *meta* (**3a**) to *para* (**3b**) substitution. The

Table 1 ^{13}C-NMR spectra (δ, ppm) of compounds **1**, **5**, **6**, **8a** and **9**

	1 in acetone-d_6	**5** in DMSO-d_6	**6** in DMSO-d_6	**8a** in DMSO-d_6	**9** in DMSO-d_6
Cyclic C=O	171.6	—	170.8	173.1	173.1
Acyclic C=O	—	162.1	160.2	—	—
Cyclic C=O	149.9	—	149.0	155.1, 154.9	155.1
Phenyl C	138.4	134.9, 133.7	139.5, 139.0	139.5	139.4
Phenyl CH	129.1, 128.2, 126.2, 125.8	127.8, 127.7, 127.0, 126.7	129.6, 122.6, 120.2, 118.1	128.6, 128.4, 128.2, 128.0, 126.8, 126.6, 126.5	128.6, 128.2, 126.6, 126.3
Cyclopentadienyl C	—	72.6	95.5	—	72.7
Cyclopentadienyl CH	—	72.2, 70.2, 69.0	86.5, 86.2, 86.1, 85.1, 84.6, 84.4	—	71.8, 70.0, 69.0
Quaternary C	60.7	—	59.9	69.3	69.2
–CH_2–N^+	—	65.1	—	—	63.7
–CH_2–Fc	—	60.9	—	—	59.5
–CH_3–N^+	—	50.2	—	—	49.2
–CH_2–	—	—	—	39.9	
–CH_2–Br	—	—	—	29.9	—
–CH_2–CH_3	28.5, 28.3	—	29.8	—	—
–CH_2–CH_3	9.7	—	9.3	—	—
–CH_2–CH_2–CH_2–	—	—	—	—	21.4
xylyl–CH_3	—	18.3, 18.2	—	—	—



Scheme 1



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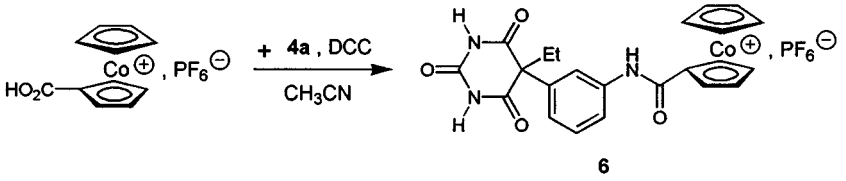
Scheme 2

catalytic hydrogenolysis of the nitrated phenobarbitals **3a**[17] and **3b**[21, 22] in the presence of palladium (on activated carbon) showed moderate to high yields (47–94%)[17, 21, 22] of aminophenobarbitals. Instead, we applied an electrochemical technique developed by Martre *et al.*[15] This electrocatalytic process involves the electrochemical reduction of Ti^{IV} to Ti^{III}. The electrolysis was carried out in DMF (0.5 M H_2SO_4). We isolated the *m*-aminophenobarbital (**4a**) in high yield (87%). In order to test our first labeling method of linking the aromatic amine **4a** to a cationic redox label we prepared the model compound **5**. The alkylation of *N*,*N*-dimethylaminomethylferrocene with commercially available chloroaceto-2,6-xylidide afforded the quaternary ammonium salt **5** in 60% yield (Scheme 2). Unfortunately the compound appeared to be unstable in solution, probably because of the electron-withdrawing effect of the carbonyl function in proximity to the ferrocene ammonium unit.



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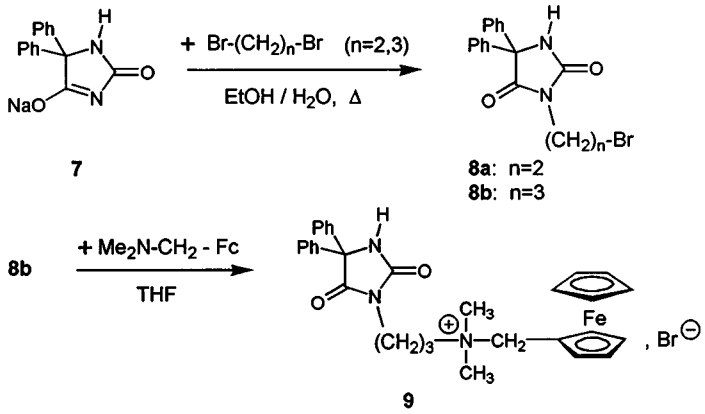
Scheme 3



7 **8b** **9**

Scheme 4

Table 2 Accumulation of **6** and **9** in a Nafion-loaded carbon paste electrode

	Linear range (M)	Detection limit (M)	Sensitivity (A M^{-1})
Ferroceneammonium-labeled phenytoin **9**	5×10^{-8}–10^{-6}	2.5×10^{-8}	*ca* 50
Cobaltocenium-labeled phenobarbital **6**	10^{-7}–3×10^{-6}	5×10^{-8}	*ca* 20

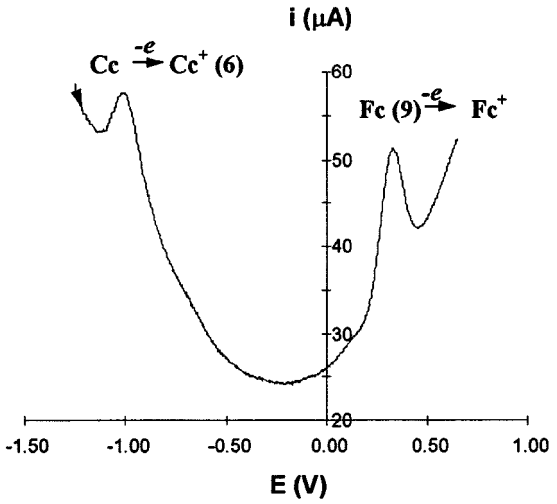


Figure 1 SWV curve of **6** (10^{-6} M) and **9** (2×10^{-6} M) at a Nafion-loaded CPE in PB (pH 7.4) containing 1% EtOH and 10% rabbit normal serum. Preconcentration time 5 min (600 rpm).

For this reason we preferred another labeling method, the formation of an amide bond. The cationic redox-labeled phenobarbital **6** was prepared from carboxycobaltocenium hyxafluorophosphate[18] and the *m*-aminophenobarbital **4a** using the procedure previously described by Beer *et al.*[23] for amide linkage of cobaltocenium salts (Scheme 3). The reaction was carried out in the presence of DCC. The final product **6** could be isolated after recrystallization in 45% yield.

Synthesis of the ferroceneammonium-labeled phenytoin 9

For the synthesis of the second labeled system we used dibromoalkanes to bind phenytoin covalently to a ferrocene derivative. The intermediate bromide **8a**[19] was prepared from the commercially available phenytoin sodium salt **7** and 1,2-dibromoethane. Our attempts to accomplish a nucleophilic substitution of the remaining bromine in **8a** by *N*,*N*-dimethylaminomethylferrocene were unsuccessful (even at temperatures as high as 70–130 °C in THF or DMF), which was probably because of steric hindrance. Instead, 3-(2-bromopropyl)-5,5-diphenylhydantoin (**8b**),[19] whose synthesis involved 1,3-dibromopropane and was carried out under the same conditions as **8a**, was reactive enough to give the quaternary ammonium salt **9** in 77% yield (Scheme 4).

Electrochemical study

The electrochemical study was carried out in PB (pH 7.4) that was not deaerated unless otherwise stated. The stability of **6** (5×10^{-4} M, 1% EtOH) and **9** (5×10^{-4} M, 10% EtOH), for 25 h and 17 h respectively, was confirmed by cyclic voltammetry (potential scan rate = 0.1 V s^{-1}) at an unloaded CPE. The anodic oxidation of the ferrocenyl group (Fc) of **9** to the corresponding ferrocenium salt (Fc^+) and the cathodic reduction of the cobaltocenium group (Cc^+) of **6** to cobaltocene (Cc) proceeded reversibly under these conditions, as shown by CV (deaerated solution in the case of **6**). Indeed, for each compound, the difference in potential between the anodic and cathodic peaks was nearly 60 mV, which is consistent with a reversible one-electron process. The standard redox potentials $E°$ are 0.39 V and −0.92 V vs Ag/AgCl(Cl^- 0.05 M) for **9** and **6**, respectively [CV determination, $E° = (E_{p,a} + E_{p,c})/2$ where $E_{p,a}$ and $E_{p,c}$ are the anodic and cathodic peak potentials].

Square-wave voltammetry is one of the most sensitive electroanalytical techniques that can be employed to detect reversible redox systems,[24] and so it was applied for the separate detection of **6**, and then **9**, at a Nafion-loaded CPE, in PB containing enough ethanol (1% and 4%, respectively) to dissolve the labeled drugs. In these measurements, the accumulation of **6** and **9** proceeded for 5 min at a rotating electrode (600 rpm) under open circuit, followed, for both these compounds, by the same voltage scan in the positive direction (−1.3 V to +0.6 V) with a view to their further simultaneous detection. Therefore the analytical response was the anodic peak corresponding to the oxidation of the cobaltocenyl group (Cc) in the case of **6** and of the ferrocenyl group (Fc) in the case of **9**. Moreover, this oxidation scan procedure was advantageous in the case of **6**, because it avoided the use of a deaerated buffer solution, since residual dioxygen was irreversibly reduced to hydrogen peroxide at the very beginning of the scan. The SWV reduction peaks of the cobaltocenium salts are often badly defined at a GCE, because the reduction of oxygen to hydrogen peroxide interferes with the reduction of the cobaltocenium salt to the corresponding cobaltocene.[12] The characteristics of the separate calibration plots of **6** and **9** are summarized in table 2. The given sensitivities indicate that accumulation occurs more readily for **9** than for

6. With a view to a further dual competitive homogeneous immunoassay, it is worth noting that the linear range of the calibration plots of **6** and **9** is relevant to the therapeutic range of **1** (6.5×10^{-5}–10.8×10^{-5} M) and **2** (2.0×10^{-5} –7.9×10^{-5} M) after dilution. Figure 1 shows that the simultaneous SWV detection of **6** and **9** is possible under experimental conditions corresponding to a dual immunoassay, i.e. in the presence of rabbit normal serum. Moreover, no significant change of the calibration curves was observed when both of the labeled drugs were simultaneously accumulated and detected at a Nafion-loaded CPE.

SUMMARY AND CONCLUSIONS

Two different cationic labels were covalently attached to two antiepileptic drugs which are frequently prescribed in combination, i.e. phenytoin and phenobarbital. These two labeled drugs would be simultaneously detected at a Nafion-loaded CPE in concentration ranges which were relevant to the therapeutic ranges of the antiepileptics. Work is in progress aimed at using these two labeled antiepileptic drugs in a dual analyte immunoassay.

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