Note

Estrogen derivatives of transition metal complexes for analytical detection enhancement*. Part II**

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(Received October 27, 1993; revised December 17, 1993)

Abstract

We herein describe the labelling of estradiol with ferrocenyl moiety which is useful for detection enhancement after high-performance liquid chromatography (HPLC) separation, using both UV and electrochemical detectors.

Key words: Electrochemistry; Transition metal complexes; Estrogen complexes; Analytical detection

Provided an acceptable degree of molecular recognition is retained, the labelling of a biologically active substance by an organometallic graft may offer analytical alternatives to radioisotopic assay [2]. There is a tremendous effort to develop new immunoanalytical protocol, especially in the field of organometallic markers, since these tracers are detectable by means of several analytical tools like atomic absorption, spectroscopic and electrochemical techniques. By analogy to the radioimmunoassay (RIA), this kind of approach was originally named metalloimmunoassay (MIA) by Cais [3]. These features can be exemplified in the organometallic–hormone series, where the coordination of an organometallic fragment to estradiol does not dramatically hinder the recognition properties of the resulting bio-organometallic complexes for the estradiol receptors [4]. The apparent receptor binding activity (*RBA*) values range between 1 and 33%, estradiol being taken as 100% [4]. The *RBA* values depend on the position and the steric bulk of the coordinated metal fragments [4]. The determination of physiological levels of hormonal steroids marked with such organometallic grafts has been extensively performed by means of FT-IR spectroscopy, since such complexes exhibit strong $\nu_{(CO)}$ bands in the range 2100–1850 cm^{-1}, a region where absorption due to proteins is minimal [5]. Recently, we proposed an alternative detection mode of the metal labelled estradiol by means of electrochemical techniques [6]. Progestogens have been determined polarographically (i.e. in oral contraceptives) since they have an easily electroreducible conjugated carbonyl group in the 'A' ring. On the other hand, most estrogens exhibit only ill-defined, multi-electron oxidation processes, probably involving their –OH groups. In spite of severe fouling and interference problems, this oxidation behaviour has been used for the determination of growth-promoting hormones in meat by means of high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED) [7].

The coordination of metallic moieties generally adds an independent, Nernstian reduction process to the overall electrochemical behaviour of the bioorganometallic molecules. The cathodic detection of these metal labelled molecules by using the linear sweep voltammetry (LSV) technique at solid electrodes exhibits good linear response over a range of concentrations with a sensitivity threshold of about 10^{-5} M [6], which can increase up to 10^{-7} M in the case of chemically reversible redox processes by using the square wave voltammetry (SWV) technique at the same electrodes [8]. This detection limit is far from the usual estradiol assay at physiological concentrations, but can be useful for pharmacological analyses [9].

The use of HPLC is well recognized as an ideal tool for the separation and determination of bioactive substances in biological fluids [10]. We have optimized the reversed-phase HPLC separation of free ethynylestradiol from several metallic derivatives and observed, employing a conventional fixed-wavelength UV detector ($\lambda = 254$ nm), that the metal carbonyl tracers increase the detection sensibility of the ethynylestradiol by about 30 times by virtue of their intense $d(M) \rightarrow \pi^*$ (CO), metal-to-ligand charge transfer (MLCT) transitions [1].

Finally, the electrochemical detector (ED) coupled with HPLC separation appears to be very promising

*Dedicated to the memory of Dr Mauro Arbrun, a brilliant research student and dear friend.
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SSDI 0020-1693(94)03804-W

for trace analysis of electrochemically active substance. For electroinactive substances an efficient and selective derivation is needed in order to introduce an electroactive functionality. The electrochemical cell most commonly employed for such purposes is the high-efficiency dual-electrode coulometric detector with porous graphite electrodes, which works with a satisfactory signal to noise (*S*/*N*) ratio in the anodic region only. Thus, at the first stage of this HPLC-ED investigation, we have chosen the 17α-ferrocenyl- 17β-estradiol complex, Fc–E, among the several organometallic–hormone derivatives so far synthesized, since the ferrocenyl moiety provides a fully reversible oxidation couple (0/1+) at low anodic potentials [11].

The cyclic voltammetric (CV) response of an acetonitrile solution of Fc–E at a glassy carbon electrode consists of a fully reversible 1e oxidation process at $E^{\circ\prime}=0.38$ V versus SCE, easily assigned to the Fc (0/1+) redox couple, and an irreversible multielectron oxidation at $E_p=1.51$ V versus SCE (at 200 mV s^{-1} scan rate) assigned to the oxidation of the estradiol moiety (Fig. 1). The addition of the independent, well-behaved oxidation process of Fc should render the labelled estradiol easily and selectively detectable by HPLC-ED. Indeed, the main problem encountered in determining estrogens by means of ED is the low selectivity [7], since several organic substances are electrochemically active at such very anodic potentials ($E_{appl} \geqslant 1.00$ V versus SCE). The use of ferrocene as reagent for pre-column derivation of thiols [12] and amino acids [13] has been previously reported as a tool for improving the sensitivity and selectivity of their determinations. Separation of free estradiol, E, from ferrocenyl–estradiol, Fc–E, is obtained by using a reversed-phase C-18 column and an acetonitrile mobile phase, containing $LiClO_4$ 0.02 M as supporting electrolyte, at a flow rate of 0.5 ml/min. As expected [1], the presence of the organometallic tag increases the retention volume of the adduct (4.34 ml) with respect to that of free estradiol (3.01 ml), and furthermore, increases the detection limit of the estradiol at the conventional fixed-wavelength UV spectrophotometric detector ($\lambda=254$ nm) by about 8 times, bringing the minimum amount detectable down to 5 pmol. This detection enhancement is less than those obtained with metal carbonyl tracers [1], since in the UV region the absorbance of Fc itself is weaker.

Since the coulometric cell employs a Pd pseudo-reference, in order to determine the optimum potential to be applied at the electrochemical detector, the hydrodynamic voltammogram (HDV) is obtained by repeated injections of acetonitrile solutions of Fc–E at different ED potentials. In order to increase the *S*/*N* ratio, we have chosen a fixed difference of about 300 mV between the potentials of guard and analytical cell, the relative HDV being reported in Fig. 2 along with a sketch of the dual-electrode coulometric cell employed. According to the HDV, we set the analytical cell at +400 mV versus Pd pseudo-reference, at this potential free estradiol does not undergo any oxidation process, as most of other substances of potential interference, improving the selectivity of the method (Fig. 3(a)). The linearity of ED response is excellent and the minimum

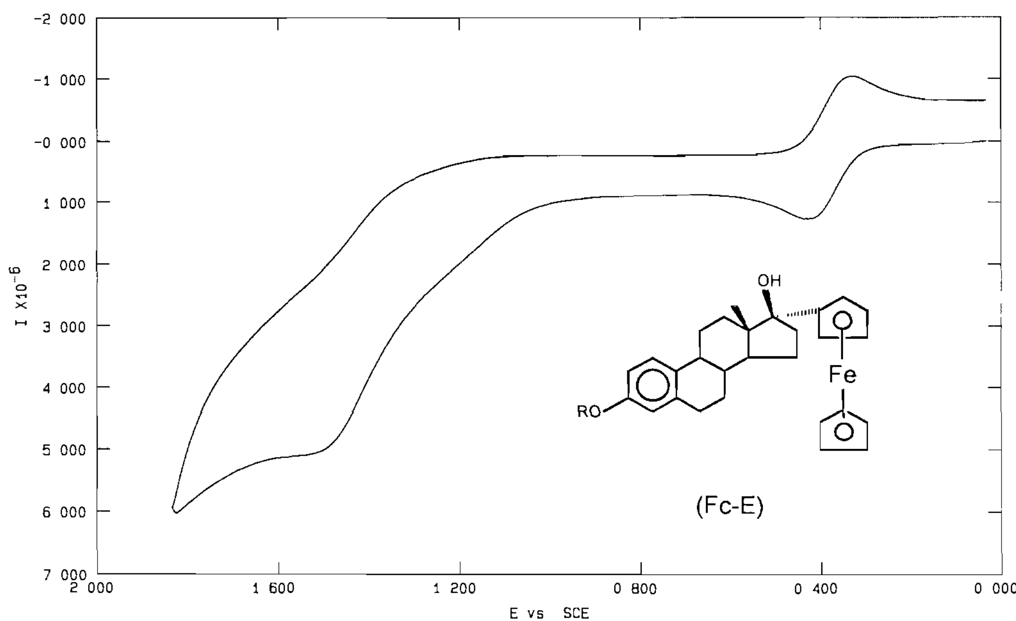

Fig. 1. Cyclic voltammetric (CV) response of an acetonitrile solution of Fc–E (1.0 mM) containing $LiClO_4$ (0.1 M) at a glassy carbon electrode. Scan rate 200 mV s^{-1} The structure of ferrocenyl–estradiol (Fc–E) is schematized in the Figure.

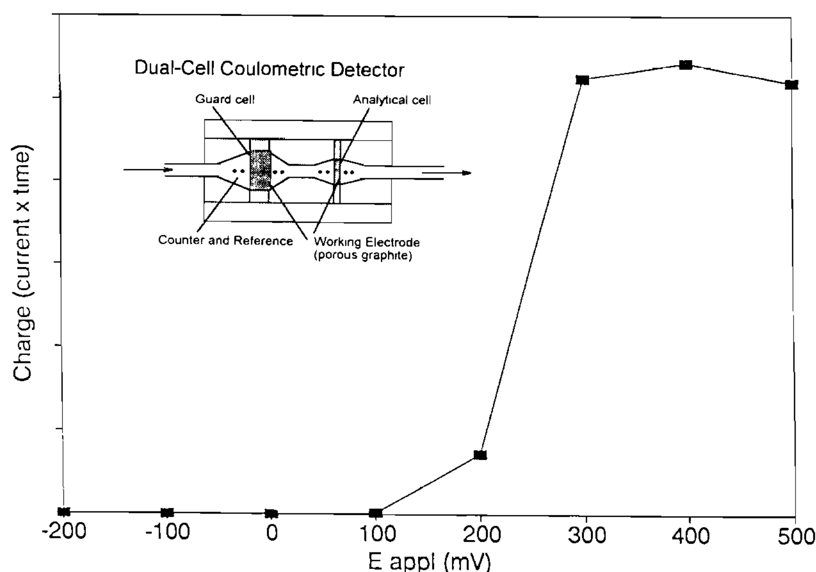

Fig. 2. Hydrodynamic voltammogram (HDV), recorded at the dual-cell coulometric detector (schematized), obtained by repeated injections containing 8 pmol of Fc–E (i.e. 20 μl of 0.4 mM solution). Mobile phase: acetonitrile containing $LiClO_4$ (0.02 M); column: LiChrosorb 100-RP18, 240×4 mm; temperature: ambient; flow rate: 0.5 ml/min.

amount detectable is 0.2 pmol of Fc–E in such experimental conditions, in good agreement with the threshold value of 0.5 pmol found for the ferrocenyl-derivation of carboxylic acids [14].

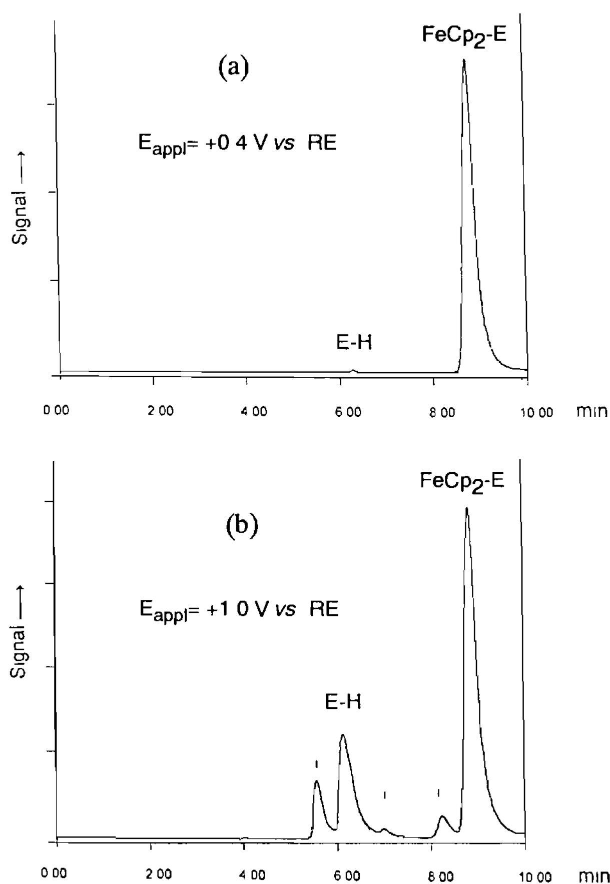

Fig. 3. Reversed phase HPLC chromatogram of E and Fc–E (both 0.4 mM in acetonitrile) recorded at the electrochemical detector with: (a) E_{appl}=0.400 V vs. Pd; (b) E_{appl}=1.00 V vs. Pd. The HPLC parameters are those reported in Fig. 2.

On the contrary, when we set the analytical potential at values sufficiently anodic that the estradiol moiety also starts to undergo its own oxidation process ($E_{appl} \geq 1.00$ V versus Pd), the signal of Fc–E smoothly increases, due to the additional contribution of such a process to the overall anodic current. However, the selectivity deeply decreases, since at this very positive potential free estradiol (E) itself and several interference substances (i) (i.e. in the actual case impurities from solvents and supporting electrolyte) begin to be revealed (Fig. 3(b)).

To conclude, the labelling of estradiol with the ferrocenyl tracer can represent a valid method within the metalloimmunoassay approach, since it allows the use of the electrochemical detector at low anodic potentials, making the detection more selective than that of unlabelled estradiol.

Work is in progress in our laboratories to test this method on crude mixtures obtained after the incubation of Fc–E with cytosol or specific antiserum, following the classical immunological protocol.

Acknowledgements

We thank CNR, MURST (Italy) and CNRS, MRT (France) for financial support. G.C.D. thanks the European Economic Community (EEC) for a studentship grant within the ERASMUS scheme.

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