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Electrochemical behaviour of clenbuterol at Nafion-modified carbon-paste electrodes ☆

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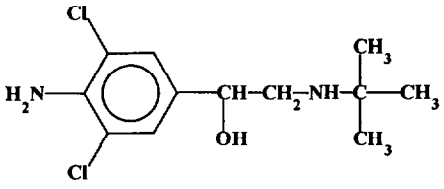
Abstract

A detailed study of the electrochemistry of clenbuterol at bare carbon-paste electrodes (CPEs) has been carried out. Results showed that clenbuterol undergoes an ECE process. This compound is irreversibly oxidised at high potentials, resulting in the formation of a product which demonstrates quasi-reversible electrochemical behaviour at less positive potentials. The amount of this chemical product formed is very pH-dependent. Investigations into the electrochemical behaviour of clenbuterol at Nafion-modified CPEs were also made. The use of a thin Nafion film cast over the CPE resulted in a large increase in peak current over bare electrodes. Linear accumulation occurred with time, the linear range increasing with decreasing concentration. This allowed the detection of low concentrations of clenbuterol. Diffusion proved to be the rate-controlling process of clenbuterol through the Nafion membrane.

Keywords: Carbon-paste electrodes; Clenbuterol; Electrochemical behaviour; Nafion-modified electrodes

1. Introduction

Clenbuterol (I; $C_{12}H_{18}Cl_2N_2O$) is a β-agonist drug used in the treatment of asthma in man, and has been under considerable investigation over the past few years. Clenbuterol and other β-agonists promote muscle growth and reduce body fat in-cattle, therefore reducing the cost of animal production. However, the clenbuterol residues which accumulate in animal tissues may have either an anti-asthmatic or tocolytic effect in humans, as



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discussed by Meyer et al. [1]. Hooijerink et al. [2] stated that "the increase in carcass weight of calves, orally treated with clenbuterol as growth promoter, is about 10% without any change in food intake". Hooijerkin also points out some of the restrictions on the therapeutic use of clenbuterol in animals. In order to prevent exploitation of the growth promotion effects, efficient screening procedures and quantification techniques, which are sensitive enough to detect the very low concentrations of the drug in biological samples, are crucial.

To date, many methods for the detection of clenbuterol in routine analysis have been reported, including HPLC coupled with UV spectrometric detection [2–4], mass spectrometric detection [5–7], and electrochemical detection [8–11], thin-layer chromatography with UV and mass spectroscopic [12] detection and gas chromatography [13–15]. None of these techniques, however, offer the very low limits of detection required for the ultratrace (subnanogram per gram) analysis of clenbuterol in biological matrices, following its use as a growth promoter.

These very low limits of detection for clenbuterol can only really be achieved at present using radioimmunoassay (RIA) or enzyme immunoassay (EIA). These immunoassays, which have been used alone [16–19] or as detection techniques coupled to HPLC [1,20], can detect clenbuterol in the subnanogram per gram range. However, such immunoassays require much reagent and sample preparation, and therefore have long analysis times.

In this paper, we have studied the electrochemical behaviour of clenbuterol in detail, with a view to developing a sensitive electrochemical approach to the analysis of clenbuterol in biological samples in the future. Bacon and Adams [21] have previously discussed the oxidation of aromatic amines at a carbon-paste electrode (CPE) and Qureshi and Erikson [11] have briefly reviewed the electrochemical behaviour of clenbuterol at bare CPEs. A comparison was also made between the electrochemical behaviour of clenbuterol at bare carbon paste and a Nafion-modified CPE. Nafion has been often used in the preparation of modified electrodes owing to its many advantageous chemical and physical properties. A very thin film of Nafion is ample to offer minimal obstruction to the diffusion of the analyte to the electrode surface, while at the same time preventing adsorption/desorption processes of organic species in biological fluids [22]. The use of Nafion-modified CPEs for the detection of other β-agonists has been examined by Boyd et al. [23]. As with the β-agonists salbutamol, fenoterol and metaproterenol, clenbuterol displayed an increased response in signal with the Nafion-modified electrode. Various investigations showed that clenbuterol accumulates linearly at the Nafion-modified electrodes, therefore promising the detection of low concentrations of clenbuterol.

2. Experimental

2.1. Reagents and materials

Perchloric acid (70–72%) and sodium hydroxide pellets were purchased from Merck. Glacial acetic acid and boric acid were obtained from Panreac, Barcelona. Purchases from Sigma included chloroacetic acid and nitric acid (60%). Nafion was purchased from Aldrich. Clenbuterol was a gift from The National Food Centre, Dublin.

Britton-Robinson buffer was prepared using 11.5 ml acetic acid (99%), 13.5 ml phosphoric acid (85%) and 12.44 g boric acid per litre. Sodium hydroxide (2 M) was used to adjust the pH of the solutions from pH 2 to 12. All solutions were prepared using deionised water and stored at 4 °C. Nafion solutions were prepared by making a 1:1 water: isopropanol dilution of the stock solution. Carbon paste was prepared by mixing 5 g of carbon with 18 ml of Nujol.

2.2. Apparatus

Cyclic voltammetry (CV) was carried out using a Metrohm VA scanner E612 and VA detec-

tor E612, coupled to a Graphic WX4421 recorder. All experiments were carried out in a glass cell designed to suit a three-electrode potentiostatic unit. The counter electrode used was a platinum electrode and the reference was an Ag/AgCl electrode.

2.3. Methods

Cyclic voltammetry at bare CPEs

To study the electrochemical processes of clenbuterol at various pH values, 1×10^{-5} M solutions of clenbuterol were prepared in Britton-Robinson buffers of pH 2–12. The CV behaviour of clenbuterol of bare CPEs was then studied using a scan rate of 50 mV s^{-1} in the potential range −0.2 to 1.35 V. After each new electrode had been prepared, it was placed in the background solution and the solution stirred for 5 min at −0.2 V. A background scan was always taken in order to ensure that there was no contamination from the previous experiment. After injection of the clenbuterol, the solution was stirred for 5 s to ensure thorough mixing of the drug within the solution.

Cyclic voltammetry at Nafion-modified CPEs

Using 1×10^{-5} M clenbuterol, a pH study (same conditions as above) was carried out using Britton-Robinson buffer solutions of pH 2–12. After having established that linear accumulation occurs, accumulation studies were carried out at various background compositions and pH values. Once a pH for analysis had been chosen, studies were carried out to establish the relevant Nafion concentration, accumulation potential, scan rate and stirring speed to give rise to the highest peak current for this pH value.

The Nafion-modified electrode was prepared by pipetting 10 μl of an appropriate concentration of the Nafion solution onto the surface of the CPE. The modifier was dried for 15 min under a domestic hair-dryer to ensure thorough drying. Nagy et al. [24] have shown that such a heat-treatment step improves both the electrochemical performance and the stability of the Nafion-coated electrode.

3. Results and discussion

3.1. Electrochemical behaviour of clenbuterol at bare CPEs

The electrochemical behaviour of clenbuterol at bare CPEs was found to be similar to that reported by Qureshi and Eriksson [11], who carried out CV in both phosphate buffer of pH 3.97 and 4.0 and acetate buffer of pH 3.98. Their studies showed that clenbuterol is irreversibly oxidised at high potentials; this process being followed by a chemical follow-up reaction, which they stated was either an EC or an ECE process. Our results prove that clenbuterol at carbon paste electrodes undergoes an ECE process. This mechanism involves the electrochemical generation of a product which then reacts with another constituent of the solution tq form a product which is more easily oxidised/reduced than the original "starting" compound and so is immediately electrolysed [25]. The oxidation product (formed at approximately 1.1 V) undergoes a chemical reaction to a second product which exhibits a quasi-reversible couple at considerably lower positive potentials. This was proven by scanning (with a new electrode) to a potential lower than the oxidation potential of clenbuterol. The absence of reduction waves on the reverse scan, and subsequent reoxidation waves on the forward scan, indicates that the quasi-reversible couple is due to a product formed at higher potentials.

Linearity of $E_p/2$ vs. pH for clenbuterol was observed in the pH range 2–12 with a negative slope of 60.9 mV per pH unit, corresponding to a Nernstian behaviour involving a process with an identical number of protons and electrons. This trend was also observed by Boyd et al. [23] in the electrochemical study of fenoterol. An experiment carried out under the same conditions as the above study, but using 0.1 M perchloric acid as the background electrolyte, displayed a shift in oxidation potential. This shift is likely to be due to the influence of the buffer solution on clenbuterol, suggesting that there is an interaction between the molecule and one of the constituents in the Britton-Robinson buffer.

A second clenbuterol oxidation peak was also observed between pH 6 and 10, which is probably related to the oxidation of the second amino group on the molecule. The single peak appearing at pH 12 indicates that this is the only process which exists at this pH.

Further studies of the quasi-reversible couple over the pH range 2–12 show that the peaks are more defined and show a current increase as the pH moves towards acidity. The amount of chemical product formed is therefore very pH-dependent. This is further evidence of the theory that the quasi-reversible couple may be due to "head-to-tail radical coupling" of para-substituted anilines, with the resulting aminodiphenylamines being oxidized at less positive potentials, as described by Bacon and Adams [21]. It was seen that the amount of "head-to-tail" coupling increases with acidity, and decreases rapidly as the pH moved towards netural values. In the case of clenbuterol, only a trace of product is seen above pH 3.

3.2. Electrochemical behaviour of clenbuterol at Nafion-modified electrodes

A similar study was carried out to study the effect of pH on the electrochemical behaviour at Nafion-modified CPEs. Conditions were identical to experiments carried out using bare carbon paste in order to permit direct comparison between the two types of electrode.

Large increases in peak current were, however, achieved for the Nafion-modified CPEs. Fig. 1 shows the electrochemical behaviour of clenbuterol at Nafion-modified CPEs. At all pH values, each initial scan revealed no quasi-reversible couple at low potentials, but a reduction peak was present on the reverse scan after the clenbuterol had been oxidised at +1.1 V, indicating that the reduction peak is due to a product of clenbuterol oxidation. On reapplication of electrolysis, a further 5 s stirring was carried out to re-establish the diffusion layer and multiple scanning in the range 0 to +1.2 V was carried out to study the accumulation behaviour on the Nafion film. From pH 2 to 6, the quasi-reversible couple displayed an increasing signal as the scan number increased. The signals decreased in intensity and clarity as the pH moved towards alkaline media, and from pH 7 to 12 no quasi-reversible couple was seen. This coincides with the pH at which the second process appears at bare carbon paste. The second process is not distinguishable at Nafion-modified CPEs, but its presence was observed by a broadening of the main clenbuterol oxidation peak. This peak decreases in intensity after the second scan (regardless of pH) due to the fact that all the clenbuterol in the Nafion layer has been coverted into products.

The half-peak potential of the clenbuterol oxidation peak remains constant between pH 1 and 5 but the oxidation potential shifted to more negative values after pH 5, with a negative slope of 45.3 mV per pH. Across the pH scale, there was a considerable increase in peak current using a Nafion-modified electrode over an unmodified CPE, which was even more significant and this current also increased as the acidity of the background electrolyte increased.

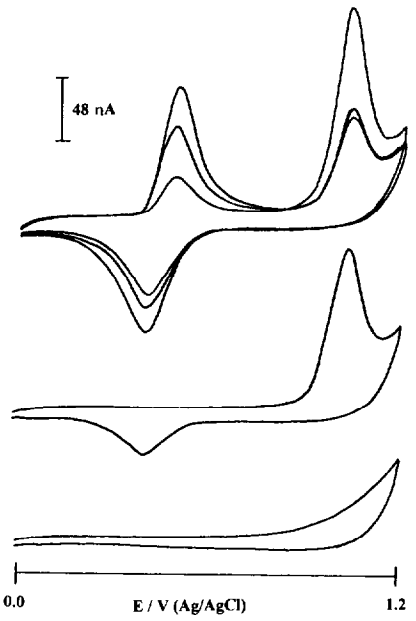

Fig. 1. Cyclic voltammetry of 1×10^{-5} M clenbuterol at Nafion-modified CPEs (0.05%) in (bottom) 0.1 M perchloric acid (background electrolye) stirred at 0 V for 5 min; (middle) initial scan following injection of clenbuterol (no accumulation); and (top) multiple scan showing accumulation of quasi-reversible couple (scan rate, 50 mV s^{-1}, scan range, 0 to +1.2 V).

3.3. Accumulation behaviour of clenbuterol at Nafion-modified CPEs

The effect of pH on the electrochemical behaviour of clenbuterol at Nafion-modified CPEs was then carried out, using 1 M, 0.1 M and 0.01 M perchloric acid, monochloroacetic acid–0.1 M sodium hydroxide buffer (pH 1.8 and 3.8), and acetic acid–0.1 M sodium hydroxide buffer (pH 3.5 and 5.6). Under these conditions, unmodified carbon paste showed no accumulation of the primary oxidation peak of clenbuterol. For the Nafion-modified CPEs, however, it was seen that the peak current (i_p) for the primary oxidation peak increased with decreasing pH based on an accumulation time of 30 s. This indicates that acidic background electrolyte provide optimum ion-exchange conditions for the analysis of samples.

Perchloric acid (0.1 M) was chosen as the best background electrolyte for further analysis based on its long linear range, high slope, peak potential and position, small peak half-width and large enhancement factor in comparison to other electrolytes. All experiments were based on a clenbuterol concentration of 1×10^{-6} M. Under identical experimental conditions, 0.1 M nitric acid was investigated as an electrolyte to establish whether the composition of the acid had an effect on the clenbuterol response. Almost identical results were obtained indicating that no constituent of the perchloric acid chemically affects the clenbuterol molecule, which may result in a different response.

A study of the effect of increasing percentage Nafion concentration showed that 0.05% was the most favourable, as it resulted in a maximum peak current at a peak potential well separated from the non-faradaic current of the background solution. It was noted that as the percentage Nafion increased, so did the peak potential. This trend was also observed by Hoyer et al. [22] in relation to the thickness of the Nafion coating and is probably due to Nafion molecules obstructing the mass transport of the analyte molecules.

Fig. 2 shows accumulation studies which were carried out for four different concentrations of clenbuterol on an electrode coated with 10 µl of

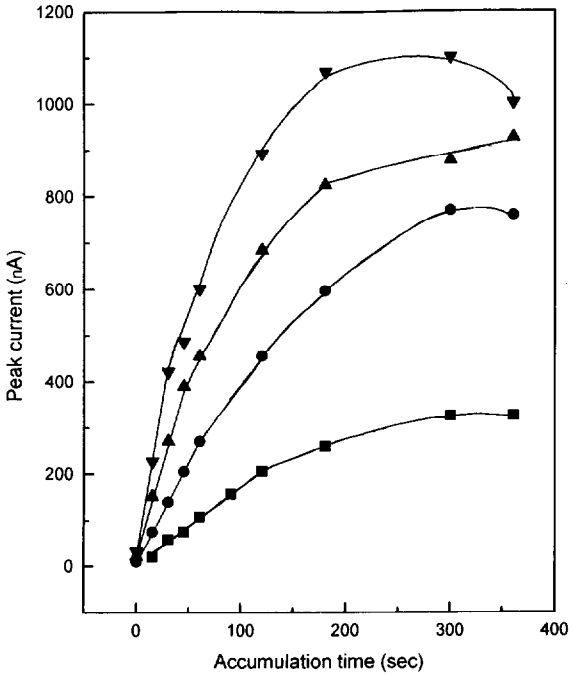

Fig. 2. Accumulation curves of various concentrations of clenbuterol at CPEs coated with a 0.05% Nafion membrane: (■) 1×10^{-7} M; (●) 2×10^{-7} M; (▲) 5×10^{-7} M; (▼) 1×10^{-6} M (scan rate, 50 mV s^{-1}).

0.05% Nafion in 0.1 M perchloric acid. The highest concentration, 1×10^{-6} M, displayed a linear accumulation response up to 30 s, whereas a concentration of 5×10^{-7} M clenbuterol was linear up to 45 s. For both concentrations, saturation occurred after 180 s. As the concentration was lowered, the linear ranges lengthened (as is usually the case) with 2×10^{-7} M and 1×10^{-7} M clenbuterol concentrations achieving linear responses up to 60 s and 120 s respectively, with saturation apparent after 300 s.

Results showed a linear increase in the slope of the current versus time curves with increasing concentration, indicating that accumulation is linear (for a chosen, fixed accumulation time) over a given concentration range. This allows the use of acumulation on the Nafion membrane in the future to develop a quantitative method to determine levels of clenbuterol in biological samples.

Reproducibility tests showed the peak to have a standard deviation of 2.4%, when $n = 10$. A calibration curve carried out in 0.1 M perchloric acid

in the range 4×10^{-8}–3×10^{-7} M proved to be linear from 4×10^{-8} to 2.4×10^{-7} M clenbuterol, following the equations

$i_p/nA = 1.25 \times 10^{+7} C/ + 0.039$ ($n = 10$; $r = 0.9991$)

where C is the concentration (mol dm^{-3}).

From this graph, a limit of detection of 5.8×10^{-9} M was calculated based on the clenbuterol concentration giving a signal equal to the blank signal plus three standard deviations of the blank. The limit of quantitation is regarded as the "lower limit for precise quantitative measurements, as opposed to qualitative detection" [26]. This was calculated based on the analyte concentration giving a signal equal to the blank signal plus ten standard deviations of the blank and was evaluated to be 2.7×10^{-8} M.

3.4. Rate-controlling process within the Nafion membrane

A study of the effect of varying both scan rate and stirring speed allowed the determination of the rate-controlling process within the Nafion layer. A plot of peak current (i_p) versus the square-root of the scan rate ($v^{1/2}$) was linear indicating the rate controlling process to be the diffusion of the clenbuterol through the Nafion layer. There was a non-linear relationship between current (i_p) and scan rate (v), concluding that adsorption is not the rate-controlling process. Confirmation of the process classification was obtained when the dependance of the peak current (i_p) on the stirring speed (rev min^{-1}) was also found to be linear, with a slope of 11.03 nA/(rev min^{-1})$^{-1}$.

Medium exchange experiments were then carried out, similar to those described by Boyd et al. [23], in 0.1 M perchloric acid using a 0.05% Nafion coating, a 1×10^{-7} M solution of clenbuterol and an accumulation time of 30 s. After having incorporated medium exchange (with 30 s accumulation) a peak was observed which was 28% of the original peak. This indicates that diffusion does indeed occur through the Nafion membrane, and from the above results it can be concluded that the rate-controlling process is the diffusion of the clenbuterol through the Nafion layer to the surface of the electrode.

4. Conclusions

The work described in this paper outlines a more detailed study of the electrochemistry of clenbuterol at bare CPEs over previously reported work [11]. Our studies confirmed that clenbuterol is irreversibly oxidised at high positive potentials, i.e. 1.1 V at pH 2. The irreversibility of the oxidation was followed by a chemical reaction, which resulted in an ECE mechanism. The oxidation gives rise to one process up to pH 5 and two processes from pH 6 to 10. Investigations into the behaviour of the quasi-reversible couple show that the amount of the chemical product formed is very pH-dependent and greatly increases in very acidic media.

The addition of a thin (0.05%) Nafion coating to the electrode surface resulted in a large increase in peak current compared to bare carbon paste, and the linear accumulation through the membrane allows the detection of clenbuterol at concentrations down to 5×10^{-9} M. This is equivalent to 1.5 μg ml^{-1} in solution. The best conditions for ion exchange at the membrane were found at acidic pHs. Studies also showed the rate-controlling process within the Nafion film to be the diffusion of clenbuterol through the membrane to the electrode surface. Accumulation studies carried out on the clenbuterol oxidation peak at various concentrations showed an increase in linear range with decreasing concentration. This knowledge aided in the construction curve of a calibration from 4×10^{-8} to 2.4×10^{-7} M with an accumulation time of 30 s. By increasing the accumulation time, and choosing a more sensitive technique such as differential pulse voltammetry for further studies, it could be possible to detect concentrations of clenbuterol in the subnanogram per gram range. The use of a Nafion-modified CPE for the detection of clenbuterol and other drugs provides a quick and easy method which is sensitive and provides an efficient barrier to negatively charged interfering compounds which may be present in biological samples.

Investigations are currently being made into the accumulation of the reduced product formed after clenbuterol oxidation at the Nafion-modified

CPE. This species proves very interesting from an analytical point of view, as the compound is reduced at a low positive potential well separated from background interferences. If linear accumulation occurs, then this process could be very important in the development of an analytical technique for the quantitation of the β-agonist in real samples, either as an analytical technique itself, or as a detection system for HPLC.

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