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# Differential pulse voltammetric determination of 7-hydroxycoumarin in human urine

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**Abstract**: The electrochemical behaviour of 7-OH-coumarin at the bare glassy carbon electrode has been studied using differential pulse voltammetry, and based on anodic detection of this metabolite at 0.66 V (vs SCE) using DC amperometry, a method has been developed for the determination of 7-OH-coumarin levels in urine samples, and a pharmacokinetic profile established.

**Keywords**: *Coumarin; 7-OH-coumarin; differential pulse voltammetry; urine analysis.*

## Introduction

Coumarin (1,2-benzopyrone) is of significant clinical importance due to its use in the treatment of many disease states [1, 2]. It has been used in clinical practice, in post-thrombotic syndromes for treatment of varicose veins and in post-traumatic edema. Coumarin has also been shown to activate macrophages *in vitro*. This property has also been utilized clinically in the treatment of high protein lymphodemas. Activation of macrophages gives rise to an augmentation of the immune response. This has resulted in the use of coumarin in cancer therapy since it has been found to have an inhibitory effect on the induction of cancer [3, 4].

Coumarin is metabolized initially by a specific cytochrome P-450 system resulting in hydroxylation at positions 7 or 3. In man and baboons, the major metabolite formed is 7-OH-coumarin. Moran *et al.* [5] showed that on average 63% of a total dose of 200 mg coumarin was recovered as 7-OH-coumarin in the urine of human volunteers over a 24 h period. It may be inferred from this fact that a very large proportion of the dose is excreted in the urine within 24 h of administration.

Because of its therapeutic significance it has recently been the focus of intense bioanalytical study. Many methods have therefore been developed for the determination of coumarin and its derivatives. These 3- and 7-hydroxycoumarins are known to fluoresce in alkaline solution and are usually analysed by spectrofluorimetry [6]. A sensitive liquid chromatographic method was also developed for the analysis of 4-hydroxycoumarin anticoagulant rodenticides in blood with fluorescence detection [7]. A rapid and sensitive HPLC assay was also developed for the determination of coumarin, 7-OH-coumarin and its glucuronide conjugate in urine [8]. TLC has been used together with GC [9], HPLC [10] and spectrofluorimetry [11] for the separation and analysis of coumarins.

This paper describes an alternative method for the determination of 7-OH-coumarin based on its oxidative voltammetric behaviour at a glassy carbon electrode.

## Experimental

### *Apparatus*

A conventional three electrode system, with platinum gauze as counter, glassy carbon as working and saturated calomel as reference electrodes were connected to an EG&G PAR Model 174A polarographic analyser and the output currents measured using a Phillips Model PM 8251 recorder. Cyclic voltammetry was performed using an EG&G PAR Model 264A polarographic analyser connected to a JJ

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Instruments Model PL4 recorder. A batch mode of operation was employed throughout the differential pulse and amperometric experiments with a magnetic stirrer and bar providing the convective transport.

*Electrode preparation*

Before use the bare glassy carbon electrode was polished firstly with alumina slurry, after which it was sonicated in distilled water, followed by electrochemical pretreatment (cycling between −1.5 and +1.5 V for 4 min at 50 mV $s^{-1}$).

*Reagents*

Batch experiments were conducted at room temperature in 0.1 M phosphate buffer, pH 7.4, and all solutions were prepared using deionized water obtained by passing distilled water through a Milli-Q water purification system. 7-OH-coumarin (umbelliferone) was purchased from Sigma. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Riedel-de-Haen. Diethyl ether and methanol (HPLC grade) was obtained from LabScan Ltd (Dublin, Ireland).

*Procedures*

The electrochemical behaviour of the metabolite was first investigated at the bare glassy carbon electrode (previously activated as described above), by immersing the electrode in 5 ml 0.1 M phosphate buffer, pH 7.4. All measurements were performed in quiescent solution. In the case of differential pulse voltammetry, the potential range scanned was normally between 0.0 and 1.0 V using a scan rate of 5 mV $s^{-1}$ and a pulse amplitude of 50 mV.

The determination of this metabolite in urine was investigated using a modification of the extraction technique developed by Egan *et al.* [8]. A 1 ml volume of urine was spiked with the appropriate volume of 7-OH-coumarin and the sample extracted with 3.5 ml diethyl ether by inversion for 10 min. The mixture was then centrifuged at 3000 rpm for 10 min, 2.2 ml of the organic layer removed, evaporated to dryness under nitrogen, and reconstituted with 200 µl methanol. A 50 µl aliquot of this sample was then injected into a 5 ml cell for electrochemical analysis. The initial spiking range of 0–15 mM resulted in a final concentration in the cell of $3.3 \times 10^{-5}$–$4.9 \times 10^{-4}$ M after extraction.

## Results and Discussion

*Anodic voltammetric behaviour of 7-OH-coumarin*

The main reason for the relative unpopularity of solid electrodes among electroanalytical chemists is the fact that it is normally difficult to obtain reproducible results owing to the different number of active sites available at the electrode surface for successive experiments. Electrochemical conditioning of the glassy carbon electrode surface results in the formation of electroactive surface compounds which enhance the electrode response via unspecified redox mediation processes [12, 13]. Cycling the potential from −1.5 to +1.5 V appears to produce a relatively non-specific enhancement of electrode response for a wide range of irreversibly oxidized compounds [14, 15]. Electrochemical pretreatment of the electrodes was found to improve both the stability and reproducibility of the response due to the anodic oxidation of the 7-OH-coumarin which occurred at 0.66 V vs SCE in 0.1 M phosphate buffer, pH 7.4.

The cyclic voltammetric behaviour of 7-OH-coumarin at the bare activated glassy carbon electrode is shown in Fig. 1. From this it can be seen that 7-OH-coumarin exhibits an irreversible oxidation peak. The effect of scan rate on current was examined with a plot of $i$ (µA) vs scan rate$^{1/2}$ (mV $s^{-1}$), showing linearity over the range investigated (0–100 mV $s^{-1}$), proving the diffusion controlled rate of reaction.

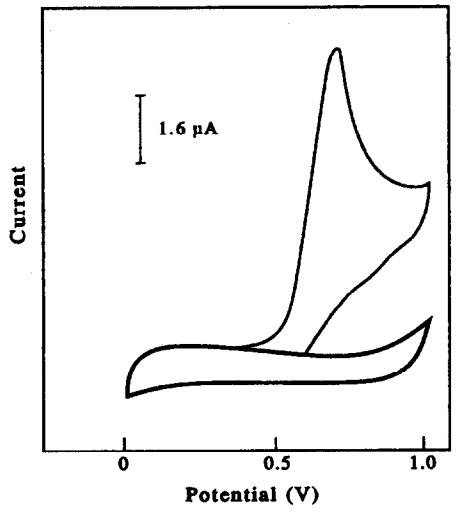


**Figure 1**
Cyclic voltammogram for 0.2 mM 7-OH-coumarin, in 0.1 M phosphate buffer, pH 7.4, at the electrochemically pre-treated glassy carbon electrode. Sensitivity, 20 µA full scale; scan rate 20 mV $s^{-1}$; scan range 0.0–1.0 V.

A typical calibration curve for the oxidation of 7-OH-coumarin at the bare glassy carbon electrode using differential pulse voltammetry, is shown in Fig. 2. It may be seen that the current measured for the oxidation process is a linear function of concentration up to 0.5 mM with sensitivity 1.1 nA $mM^{-1}$, with $r = 0.9979$ and a LOD of 10 μM. The RSD for $n = 10$ batch injections at the 0.25 mM level was 3.2%.

*Voltammetric determination of 7-OH-coumarin in human urine*

Clinical pharmaceutics is a health science discipline which deals with the application of pharmokinetics to the safe and effective therapeutic management of the individual patient. Drugs are eliminated from the body either in the unchanged form, usually via renal excretion, or as metabolites. Drug metabolites are often more polar than their parent compound and thus are readily excreted in the urine. Many drugs, including coumarin, are converted into their corresponding glucuronide conjugates by the cytochrome P450 enzyme system.

Most errors in drug analysis occur during extraction, subsequent evaporation of organic solvents and derivatization. The commonest way of isolating drugs from a biological medium is by solvent extraction. Selection of a suitable solvent depends on extraction efficiency (recovery), and selectivity with respect to co-extraction of interferents. The optimum solvent is one that extracts all or at least a large proportion of the drug but does not extract endogeneous compounds/metabolites.

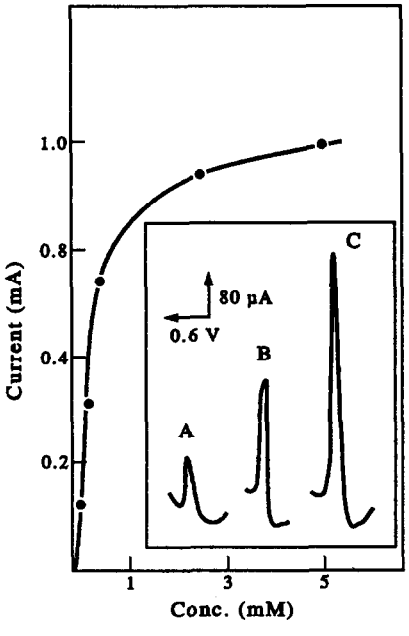


**Figure 2**
Calibration curve for the oxidation of 7-OH-coumarin at the bare glassy carbon electrode (previously activated), using differential pulse voltammetry. Scan range 0.0–1.0 V and scan rate 5 mV $s^{-1}$ with A, B and C representing 0.1, 0.25 and 0.5 mM injections, respectively. Sensitivity, 1 mA full scale; pulse amplitude, 50 mV.

Using the extraction method employed by Egan *et al.* [8], a series of control urine samples (1 ml) were spiked with 7-OH-coumarin in the range 0–15 mM, and 50 μl of the final methanol extract injected into a 5 ml cell for electrochemical analysis at a bare glass carbon electrode. The amount of metabolite present in extracted urine samples, as compared to a series of authentic standards in methanol (concentration in the cell from the final methanol extract was in the range $3.29 \times 10^{-5}$–$4.94 \times 10^{-4}$ M), describes the percentage recovery. The peak heights of authentic and extracted standards were compared at each concentration, taking into account the dilution factors (see Table 1). Linearity for both standard and sample extract calibration curves extended from 0 to 15 mM spiking concentration 7-OH-coumarin, with $r = 0.9998$ and 0.9995 with a sensitivity of $1.3 \times 10^{-1}$ mA $mM^{-1}$ and $1.2 \times 10^{-1}$ mA $mM^{-1}$, respectively. In the HPLC method developed by Egan *et al.* [8], which involved the separation of coumarin and 7-OH-coumarin following solvent extraction from a urine sample, linearity extended up to 100 μg $ml^{-1}$ (0.617 mM), with a correlation coefficient of 0.9978, a LOD of 2.0 μM 7-OH-coumarin and a precision for inter- and intra-day assay variability of <10% for $n = 5$. Percentage recoveries were in the range 84–114% for 7-OH-coumarin.

Analysis of urine samples before and after drug administration in one human volunteer

**Table 1**
Percentage recovery determined initially, by comparing the peak height of spiked control urine samples (in the range 0–15 mM) with authentic methanol standards of the same final concentration after extraction

| Spiking conc. (mM) | StD (mA) | Sample (mA) | % Recovery |
|---|---|---|---|
| 1 | 0.126 | 0.118 | 94.2 |
| 2 | 0.256 | 0.241 | 93.2 |
| 5 | 0.602 | 0.590 | 98.0 |
| 10 | 1.250 | 1.193 | 95.5 |
| 15 | 1.930 | 1.880 | 97.4 |

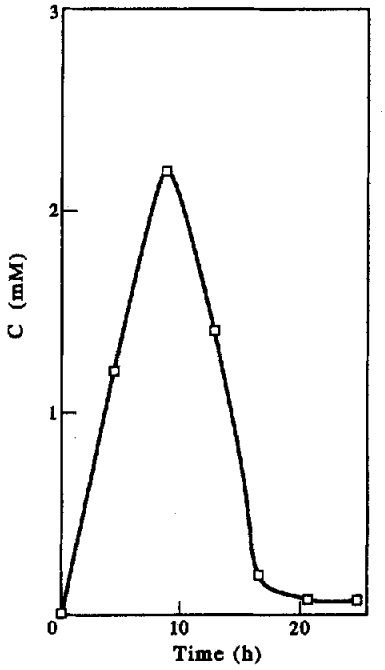


**Figure 3**
Pharmacokinetic profile for a human volunteer. Total 7-OH-coumarin determined in extracted urine samples (taken every 4 h) at the bare glassy carbon electrode.

who had received a single oral dose of coumarin (100 mg) was then carried out. The concentration of free 7-OH-coumarin excreted was determined at regular time intervals (0, 4, 8, 12, 16, 20, 24 h) (Fig. 3) from the standard curve of spiked extracted urine standards. The free 7-OH-coumarin excreted over the period studied was found to be 5.07 mM (821 μg $ml^{-1}$). The 7-OH-coumarin conjugated to glucuronide was determined following treatment of 1 ml of sample urine with 1 ml β-glucuronidase (10,000 Units $ml^{-1}$) in sodium acetate buffer, incubated at 37°C for 30 min. The conjugated 7-OH-coumarin could be calculated from the difference between the value obtained after hydrolysis and the free 7-OH-coumarin concentration. The total 7-OH-coumarin determined was found to be 918.16 μg $ml^{-1}$ (5.67 mM), of which 821.0 μg $ml^{-1}$ (5.07 mM) was free and 97.16 μg $ml^{-1}$ (0.6 mM) was conjugated. The total represents 82.8% of the coumarin excreted as the 7-OH-coumarin metabolite. It is now known that individuals have very major differences in their ability to metabolize coumarin to 7-OH-coumarin (O. Pelkonen, personal communication). Since 7-OH-coumarin may in fact be the main active pharmacological agent, it is important to monitor its production in the urine. The procedure described offers an alternative approach for such determinations.

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