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ELECTROCHEMICAL CHARACTERISTICS OF NITRO-HETEROCYCLIC COMPOUNDS OF BIOLOGICAL INTEREST
IV. Lifetime of the Metronidazole Radical Anion

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Electrochemical studies on metronidazole using mixed aqueous/dimethylformamide (DMF) solvents have allowed us to generate the one-electron addition product, the nitro radical anion, $RNO_2^{\cdot-}$. Cyclic voltammetric techniques have been employed to study the tendency of $RNO_2^{\cdot-}$ to undergo further chemical reaction. The return-to-forward peak current ratio, ip_r/ip_f, was found to increase towards unity with increasing DMF content of the medium, indicating the extended lifetime of $RNO_2^{\cdot-}$. Second order kinetics for the decay of $RNO_2^{\cdot-}$ were established at all DMF concentrations examined. Extrapolation has allowed the rate constant and a first half-life of $8.4 \times 10^4 dm^3/mol\text{-}sec$ and 0.059 seconds respectively, to be determined for the decay of $RNO_2^{\cdot-}$ in a purely aqueous media. This is impossible by direct electrochemical measurement in water, due to a different reduction mechanism, giving the hydroxylamine derivative in a single 4-electron step. The application of the technique to other nitro-aromatic compounds is discussed.

KEY WORDS: Cyclic voltammetry, metronidazole, nitro radical anions, lifetimes.

INTRODUCTION

The cytotoxic properties of nitro-aromatic compounds are dependent on the reduction of the nitro group, which subsequently results in DNA damage, causing strand breaks and helix destabilization.[1] We have previously employed electrolytic reduction techniques to measure the interaction of reduced nitro-aromatics with DNA. These studies have shown correlations between the extent of DNA damage and the electron affinity and rate of reduction of the drugs,[2,3] pH of the media[4] and base composition of the DNA.[5] Our present investigations involve the use of detailed electrochemical methods to probe the redox-mechanism of these bio-reductive drugs to extend our fundamental understanding of their mode of action.

We have found that the electrochemical solvent strongly influenced the reduction mechanism of the nitro group.[6] In aqueous media, a single irreversible 4-electron reduction was observed, to give the hydroxylamine. No intermediate reduction steps were identified. If a mixed aqueous/aprotic solvent system was employed, however, reduction to the hydroxylamine now occurred *via* two clearly resolved stages. The first reduction involved the reversible transfer of 1-electron to form the nitro radical anion, *i.e.* the $RNO_2/RNO_2^{\cdot-}$ couple. Subsequent reduction *via* an irreversible 3-electron

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addition occurred at more negative potentials. The ability of mixed aqueous/aprotic solvents to change the redox pathway has been used to develop a methodology for the study of lifetimes and chemical reactions of electrochemically produced $RNO_2^{\cdot-}$, a candidate for the DNA active agent. Metronidazole has been used as the model compound, with dimethylformamide (DMF) as the aprotic solvent.

MATERIALS AND METHODS

Metronidazole was supplied by May and Baker Ltd., and used without further purification. Dimethylformamide, spectroscopic grade, was purchased from the Aldrich Chemical Co..

Electrochemical measurements used the cyclic voltammetric mode exclusively, and employed a PAR 264A polarographic analyzer, interfaced with a PAR 303E cell stand and a 3-electrode cell configuration. A hanging drop mercury electrode was used as the working electrode surface, and a platinum wire as the counter electrode. All potentials were measured against an aqueous Ag/AgCl reference electrode.

Various proportions of H_2O/DMF were used as the electrochemical solvent (expressed as % v:v of the DMF content). 1.5×10^{-2} mol/dm^3 NaCl, 1.5×10^{-3} mol/dm^3 trisodium citrate buffer (0.1 ssc) was used as the supporting electrolyte. At each DMF concentration, the return-to-forward peak current ratio, ip_r/ip_f, for the reversible first electron transfer (the $RNO_2/RNO_2^{\cdot-}$ couple) was measured, varying the scan rate from 10 to 500 mVs^{-1}.

The switching potential, E_λ, was chosen so as to be well positive of the second reduction step. The routine drug concentration was maintained at 2×10^{-4} mol/dm^3 at all %DMF values. The influence of metronidazole concentration was examined at %DMF = 40, over a 6×10^{-5} to 1.7×10^{-2} mol/dm^3 range.

RESULTS

Figure 1 shows the effect of DMF addition on the CV of metronidazole. The 1-electron transfer process resulting in the generation of the nitro radical anion can be clearly distinguished in mixed aqueous/aprotic solvents (b) whereas in aqueous media, the $RNO_2^{\cdot-}$ is immediately reduced further to the hydroxylamine, the CV showing only a single, irreversible 4-electron reduction process (a). In the presence of DMF, the reverse potential sweep shows a response corresponding to oxidation of unreacted $RNO_2^{\cdot-}$ back to the original neutral material. The tendency of an electrochemically generated species to undergo chemical following reactions is reflected by the ip_r/ip_f ratio, which in the absence of all coupled reactions equals unity, but decreases if the reduction product reacts further, *i.e.* a decline in the return wave occurs. The CV mode can therefore be used to probe the lifetime of the $RNO_2^{\cdot-}$ species with changing electrochemical conditions, by measuring the ip_r/ip_f value of the RNO_2/ $RNO_2^{\cdot-}$ couple.

To allow the $RNO_2/RNO_2^{\cdot-}$ couple to be examined in isolation the switching potential (E_λ) was chosen at positive potentials relative to the second reduction step. Table I lists some typical ip_r/ip_f values so determined as a function of scan rate, %DMF and drug concentration from which a number of trends are apparent. At any particular %DMF, as the scan rate increased, the ip_r/ip_f increased towards unity,

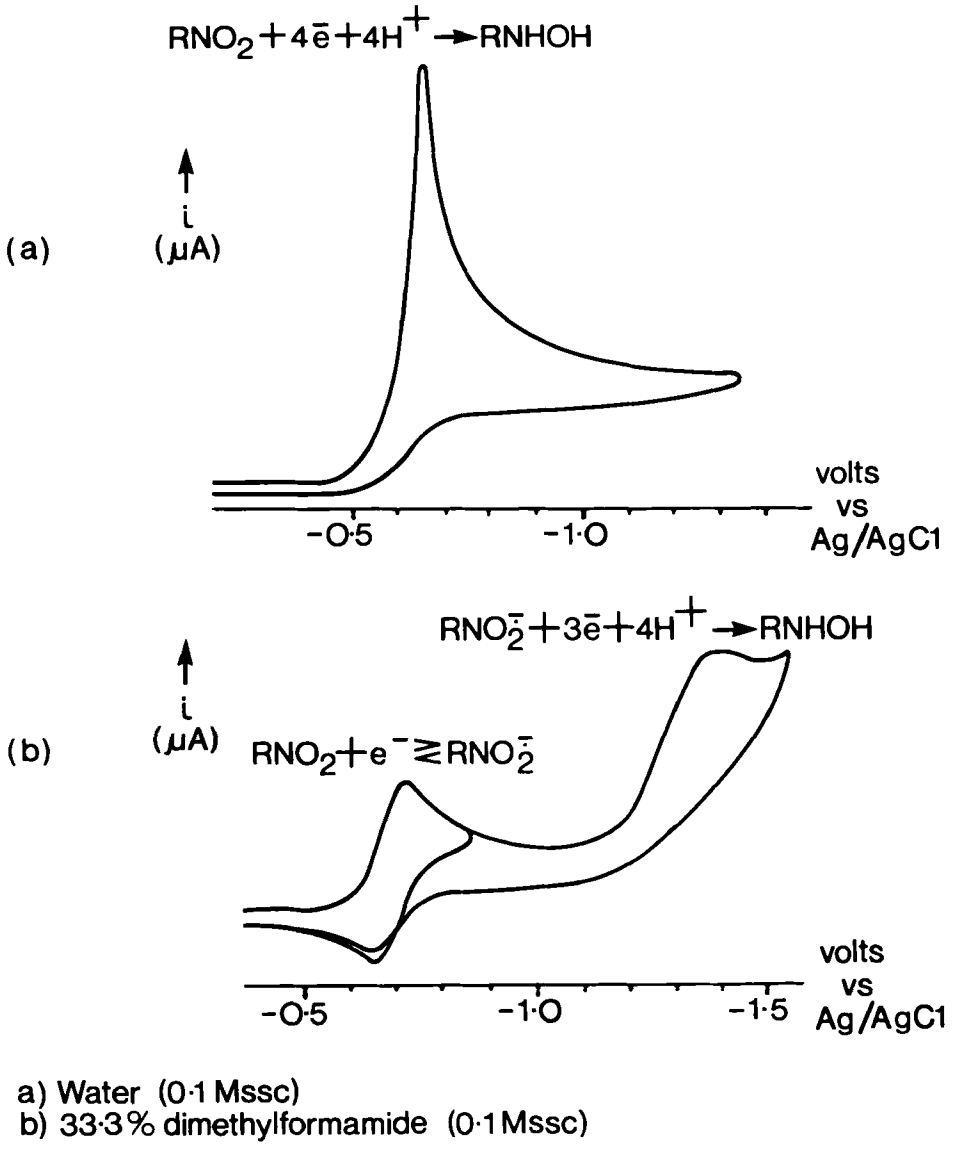

FIGURE 1 Cyclic voltammograms (scan rate, $v = 100\,mVs^{-1}$) of metronidazole as a function of solvent.

typical behaviour for an irreversible chemical reaction following a charge-transfer step. As the %DMF content was increased, the ip_r/ip_f ratio likewise increased, illustrating the extended lifetime of the RNO_2^- species. This was true up to %DMF = 50 (data not shown), above which no further changes in ip_r/ip_f were observed. An increase in metronidazole concentration, while keeping %DMF constant, resulted in a decreased ip_r/ip_f value. This would indicate that the reaction of RNO_2^- was second or higher order in nature.

TABLE I
The ip_r/ip_f ratio for the RNO_2/RNO_2^- couple for metronidazole as a function of scan rate v, the %DMF and the drug concentration

v (mVs^{-1})	metronidazole ip_r/ip_f % DMF 11.1	33.3	%DMF = 40, v = 200 mVs^{-1} [metronidazole]	ip_r/ip_f
10	–	0.592	6.0×10^{-5} mol/dm^3	1.000
20	–	0.629	2.0×10^{-4} mol/dm^3	0.981
50	0.475	0.745	9.9×10^{-4} mol/dm^3	0.743
100	0.511	0.831	2.0×10^{-3} mol/dm^3	0.654
200	0.573	0.935	9.1×10^{-3} mol/dm^3	0.550
500	0.665	1.000	1.7×10^{-2} mol/dm^3	0.518

All ip_r/ip_f ratios listed are the average ($\pm$ 5%) of four independent experimental measurements.

A more numerical approach was possible by employing various theoretical studies which have examined the effect of coupled chemical reactions on the cyclic voltammetric response.[7-9] Relationships were developed to describe the effect on the ip_r/ip_f ratio of a first and second order reaction following the charge transfer step. For first order reaction kinetics, a working curve of the ip_r/ip_f ratio was derived as a function of k_1 (where k_1 is the first order rate constant) and τ, the time constant, which equals the switching potential minus the half-wave reduction potential divided by the scan rate: *i.e.* $\tau = (E_\lambda - E_{1/2})v$. By fitting experimentally determined ip_r/ip_f ratios to the working curve, the k_1 value can be calculated. Analysis of the metronidazole data using this first order approach failed, with no continuity found for the k_1 values established.

The second order reaction of RNO_2^- was therefore investigated, where the rate equation is defined as

$$-d\,[RNO_2^-]/dt = 2k\,[RNO_2^-]^2 \quad (1)$$

The studies of Olmstead *et al.*[9] produced a working curve relating ip_r/ip_f to the parameter ω, defined by

$$\log \omega = \log (k_2 C^* \tau) + 0.034\,(a\tau - 4) \quad (2)$$

therefore incorporating the effects of k_2 ($=2k$), the second order rate constant; C*, the analytical concentration of the redox-active species; "a" given by nFv/RT; and τ – the time constant, as defined previously. If second order kinetics apply, then a plot of ω *vs* τ would give a straight line, the slope of which is given by equation 3

$$\text{slope} = k_2 C^* \exp\,[0.078\,(a\tau - 4)] \quad (3)$$

Although it is theoretically possible to calculate k_2 from a single CV measurement, our procedure has been to record the electrochemical behaviour as a function of the scan rate to give added accuracy. At each %DMF, the ω values were determined from the experimental ip_r/ip_f ratios, then plotted against the appropriate τ values. For all %DMF values, the ω *vs* τ plots were linear with correlation coefficients of 0.98 or better. It was found, however, that at low %DMF (10–20%) the ip_r/ip_f values measured were outside the range of the theoretical working curve (ω values obtainable from ip_r/ip_f from 1 to 0.6). To circumvent the substantial estimations necessary in determining ω values when ip_r/ip_f was less than 0.58, we used the following approach. A plot of ip_r/ip_f *vs* log τ was found to be highly linear. This relationship was used to establish ip_r/ip_f ratios (and their appropriate τ values) well within the range covered

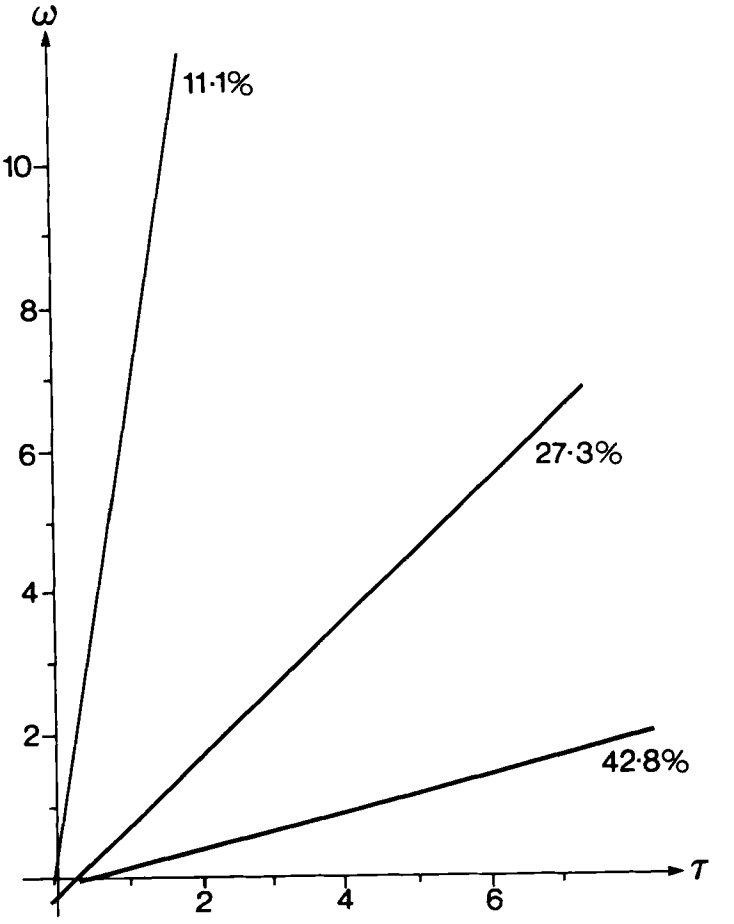

FIGURE 2 Theoretical kinetic parameter, ω, *vs* time constant, τ plots for metronidazole, showing the effect of various dimethylformamide concentrations.

by the theoretical calculations, thus allowing more realistic ω *vs* τ plots to be determined. Figure 2 illustrates the distinctive relationships found as a function of %DMF. The actual data points have been omitted for clarity, as widely differing axes would be necessary to fully represent the data. Using equation 3 the k_2 value was determined at each %DMF. As expected, the rate constant decreased as the %DMF increased, from 2.9×10^4 dm^3/mol-sec at 11.1 %DMF to 1.2×10^3 dm^3/mol-sec at 43 %DMF. By plotting $\log_{10} k_2$ *vs* %DMF, a linear relationship was found, allowing extrapolation to determine k_2 at % DMF = zero. The second order rate constant in purely aqueous media was thus found to be $k_2 = 8.4 \times 10^4$ dm^3/mol-sec ($\pm$ 10%), giving a half-life of $t_{1/2} = 0.059 \pm 0.006$ seconds (for a metronidazole radical anion concentration of 2×10^{-4} mol/dm^3).

DISCUSSION

Mixed aqueous/dimethylformamide electrochemical solvent systems have allowed a 1-electron reduction step to be identified for metronidazole, resulting in the formation of the nitro radical anion, $RNO_2^{\cdot-}$. Using cyclic voltammetric techniques, it was possible to observe the tendency of $RNO_2^{\cdot-}$ to react further; by, for example, increasing the DMF content of the medium which stabilized $RNO_2^{\cdot-}$, as observed by an increase towards unity in the ip_r/ip_f ratio.

A quantitative analysis was possible by using theoretical studies on the influence of an irreversible chemical reaction following the charge-transfer step, on the ip_r/ip_f value. The excellent correlation of our experimental data when treated using a second order kinetic approach, particularly the linearity found when plotting ω *vs* τ for all %DMF, confirmed the second order decay pathway of $RNO_2^{\cdot-}$. This is also in line with our qualitative observations on the decrease in ip_r/ip_f with increasing metronidazole concentration. Simplistically, the reaction of $RNO_2^{\cdot-}$ can be viewed as in equation 4

$$2RNO_2^{\cdot-} + 2H^+ \longrightarrow RNO_2 + RNO + H_2O \qquad (4)$$

Independent studies on the reaction pathways of $RNO_2^{\cdot-}$ generated by pulse radiolysis in aqueous media have also been found to be second order in nature.[10-12] The rate constant for metronidazole of $4.2 \times 10^4 dm^3/mol\text{-}sec$ (pH 7.4)[11] was found to be in reasonable agreement with that of $8.4 \times 10^4 dm^3/mol\text{-}sec$ determined by electrochemical methods, considering the wide differences in the two techniques.

Preliminary work on a range of nitro-aromatic compounds has demonstrated the general applicability of the electrochemical cyclic voltammetric technique, using mixed aqueous/aprotic solvents, to the study of reaction pathways and lifetimes of nitro radical anions. This presents the opportunity to compare the reaction of a range of $RNO_2^{\cdot-}$ species with biological targets, for example DNA nucleotides, to establish if $RNO_2^{\cdot-}$ is the damage-causing species. The electrochemical conditions (in terms of reduction rate and solvent requirements) employed in such studies, may be of more biological relevance than those achieved under pulse radiolysis conditions.

Acknowledgements

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