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Electrochemical investigations of immunologically reactive procainamide metabolites*

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Abstract: As a result of the implication of *N*-oxidized procainamide metabolites in drug-related lupus (DRL), the electrochemical behaviour of these compounds was investigated and a coulometric synthesis of the nitroso derivative developed using a previously described carbon packed bed bulk electrolysis flow cell. The electrochemical characterization of the parent *p*-substituted aromatic amine and the *N*-oxidized derivatives was achieved through systematic comparison with previously well described aromatic amine and nitro systems using cyclic voltammetry and liquid chromatography with electrochemical detection (LC–EC). Chromatographically assisted hydrodynamic voltammetry indicated current limiting plateau potentials of 0.45 and −0.2 V versus Ag/AgCl, respectively, for synthetically prepared procainamide hydroxylamine and electrolytically prepared nitrosoprocainamide. Reaction characterization and binding behaviour is described for each of the procainamide metabolites following *in vitro* incubations with cysteine, glutathione, ascorbic acid and mouse haemoglobin.

Keywords: *Procainamide electrochemistry; procainamide metabolites; drug-related lupus; bulk electrolysis; LC–EC.*

Introduction

The use of amine 4-amino-*N*,*N*-diethylaminoethylbenzamide, (procainamide PA; Fig. 1) for anti-arrhythmic therapy has been documented to elicit clinical manifestations resembling systemic lupus erythematosus in a significant percentage of those individuals taking the medication [1]. Furthermore, some 40 other medications have been implicated as the inciting agents in the autoimmune condition drug-related lupus (DRL). While extensive investi-

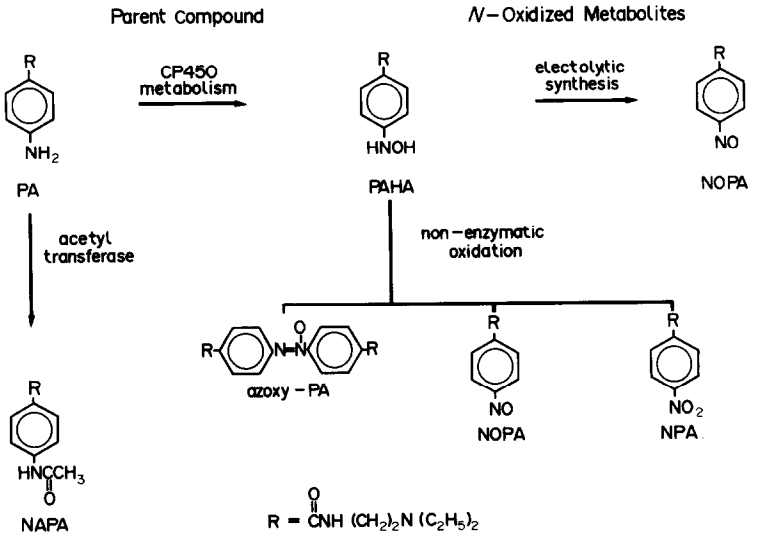

Figure 1
Schematic diagram of PA and *N*-oxidized metabolites.

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gations have been on-going for several years [2–6], no comprehensive mechanism for the cause of this drug-induced condition has yet been established.

In recent years much attention has been focused on the *N*-oxidized metabolites of PA. *In vitro* PA incubations using human and rat liver microsomes, human neutrophils and mononuclear leukocytes have demonstrated that procainamide hydroxylamine (PAHA) is produced as an *N*-oxidation product of the aromatic amine functionality (Fig. 1) [7–9]. Furthermore, PAHA and/or the nitroso metabolite (NOPA) have been postulated to bind to haemoglobin [7], and have been demonstrated to bind covalently with hepatic proteins, histones and DNA *in vitro* [5, 8]; interactions which presumably could result in the formation of complexes capable of initiating autoimmune responses [7–9].

A simple, efficient means of generating the NOPA and PAHA metabolites was needed for studying the *in vitro* binding properties of these metabolites with specific biomolecules. Chemical syntheses of PAHA have been described previously [7, 8]. Preliminary results of a rapid, controlled electrochemical synthesis of NOPA from PAHA [10] were reported. A comprehensive treatment of the electrochemical characterization of *N*-oxidized PA metabolites and the subsequent development of this synthesis are provided here, together with a description of the *in vitro* reaction behaviour of *N*-oxidized PA metabolites with several relevant biomolecules.

Experimental

Reagents

Unless otherwise noted, all chemicals obtained were of reagent grade or better, and were used as received. Aniline hydrochloride was purchased from Eastman Kodak. Mouse haemoglobin, *p*-aminobenzoic acid, nitrosobenzene and aminobenzamide were obtained from Sigma Chemical Company. HPLC grade ammonium acetate, tris(hydroxymethyl) aminomethane (THAM or Tris), acetonitrile, nitrobenzene, nitrobenzoic acid and hydroquinone were obtained from Fisher Scientific. The Division of Immunology of the University of Cincinnati College of Medicine furnished procainamide hydrochloride, PAHA and nitroprocainamide (NPA) which was recrystallized from 95% ethanol. All other materials were obtained as previously reported [10]. Deionized water (>16.5 Mohm cm) was used for all solutions as obtained by a Sybron Barnstead Nanopure purification system.

Apparatus

A bioanalytical systems (BAS) 100 electrochemical analyzer was used for cyclic- and linear-sweep voltammetry. An optically transparent quartz thin-layer electrode (OTTLE) with 100 lines/inch Au minigrid working electrode was constructed by methods previously described [11]. A BAS CV-1B and Hewlett-Packard 8452 scanning diode array spectrophotometer were used for spectroelectrochemical studies. Liquid chromatography (LC) with mass spectral detection (LC–MS) was performed through the courtesy of the Merrell Dow Research Institute (Cincinnati, OH) using a Waters HPLC system equipped with a Vestec 101 interface and a Hewlett-Packard thermospray mass selective detector. All other analytical apparatus and instrumentation were as previously reported [10]. All potentials indicated were versus a Ag/AgCl reference (3.0 M NaCl).

Voltammetry and flow-cell conditions

All solutions were purged with argon for 15 min prior to voltammetric scanning. All voltammetry studies were performed in both a 0.2 M ammonium acetate buffer, pH 5.5, and a 0.2 M THAM buffer, pH 7.4. The voltammetric scan rate was 0.1 V s^{-1}. Before each use, the bulk electrolysis flow cell [10] was flushed with 0.1 M perchloric acid followed by deionized water. Flow rates varied between 0.4–0.8 ml min^{-1}. The well-characterized two-electron oxidation of hydroquinone to benzoquinone was used as a calibration system for determining electrolysis efficiency of the bulk cell. The current generated by a 1.0 mM hydroquinone solution in a 0.2 M, pH 5.0, sodium acetate buffer was within 5% of the current calculated applying the derivative form of Faraday's Law as given in equation (1):

$$i = nF\mathrm{d}N/\mathrm{d}t. \quad (1)$$

Additionally, independent LC–EC experiments indicated a conversion efficiency of 95% for these flow-cell conditions.

Chromatography and incubation conditions

Mobile phase and sample deoxygenation,

necessary for LC–EC at negative potentials, was carried out using methods described elsewhere [12]. Two mobile phase conditions were required for well resolved chromatographic separations, as PA and its metabolites displayed vastly different retention on reversed-phase ODS columns [10]. For the more hydrophilic PAHA (3.5 min) and PA (6.0 min), the mobile phase was ammonium acetate (0.1 M, pH 5.5)–acetonitrile (9:1, v/v). NPA and NOPA exhibited much more significant band broadening, and were eluted with 30%, v/v, acetonitrile in the same buffer to give retention times of 8.0 and 10.0 min, respectively. For reaction studies with mouse haemoglobin, a Brownlee Labs 3-cm ODS guard column was used in addition to the analytical column [10]. In the LC–MS studies, a Supelco end-capped OS column (5 cm × 4.6 mm) was utilized to achieve more ideal chromatographic separations, with ammonium acetate (0.1 M, pH 5.5)–acetonitrile (92:8, v/v) to give retention times of 5.9 min for NPA and 7.0 min for NOPA. Unless indicated otherwise, metabolite–biomolecule incubations were performed for 15 min at 25°C with constant stirring. Individual PAHA or NOPA controls were used for each incubation, and blanks of each reagent solution were run within the potential region of the PAHA–NOPA couple to prevent the possibility of unidentified interferents.

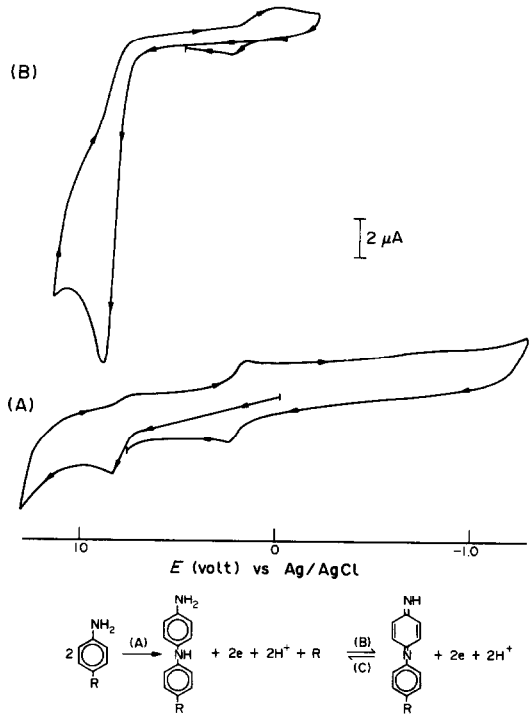


Figure 2
Cyclic voltammogram of (A) 0.05 mM *p*-aminobenzoic acid and (B) 0.5 mM PA in 0.1 M ammonium acetate buffer, pH 5.5.

Results and Discussion

Electrochemical characterization

Because *N*-acetyl procainamide has been shown to have the anti-arrhythmic properties of the parent compound without invoking the autoimmune response [13], the primary functionality of interest for potential binding reactions in the PA molecule is the *N*-oxidized aromatic amine entity. Cyclic voltammetry studies that utilized structurally similar aromatic amines as models were performed to determine the feasibility of generating the PA metabolites through a biomimetic electrochemical oxidation. Although PA and each of these aromatic compounds examined fouled the electrode surface at moderate concentrations, no detectable loss of electrode response was observed at the concentrations reported here as determined using a 0.1 mM $Fe(CN)_6^{3-}$ test solution immediately prior to, and following, each analysis.

Figure 2A shows the cyclic voltammogram of *p*-aminobenzoic acid. As described by Bacon and Adams [14], the initial irreversible oxidation (A), Ep_a = 0.83 V, was followed by a fast head-to-tail coupling reaction in which 4-amino-4′-carboxydiphenylamine was formed as the principal product. This compound then cycled as an electrochemically reversible couple (B, C) on subsequent scans, Ep_c = 0.18 V and Ep_a = 0.28 V. Voltammograms obtained of aniline oxidation demonstrated similar electrochemical behaviour.

A cyclic voltammogram of PA is shown in Fig. 2B. PA exhibited similar behaviour to the model compounds in that the initial aromatic amine oxidation, Ep_a = 0.89 V, was followed on the reverse scan by the formation of a new redox couple, Ep_c = 0.01 V, Ep_a = 0.33 V. For PA, however, this couple exhibited somewhat less reversible behaviour with much smaller peak currents for the coupled product than *p*-aminobenzoic acid (note concentrations). Since the ECE mechanism ascribed to this oxidation involved the elimination of the *para*-group in the formation of the dimerized product, PA oxidation was expected to produce a smaller amount of the diphenylamine product due to the inherently poorer

nature of the long chain amide as a leaving group, compared with the carboxylic acid. Apparently, some additional chemical coupling or hydrolysis reaction(s) followed the initial amine oxidation of PA to form a chemically irreversible product(s) which was not electroactive. *p*-Aminobenzamide solutions likewise behaved non-ideally in the formation of the electroactive coupled product. Similar poorly behaved voltammograms have been reported for other *para*-substituted aromatic amine oxidations [14].

Since the ultimate goal of this investigation was to develop an electrochemical synthetic route to the NOPA derivative, and the *in vitro* oxidation of PA did not appear to provide a suitable pathway to generate this metabolite, further studies were conducted with *para*-substituted nitroaromatic compounds and NPA. Figure 3A shows the electrochemical behaviour of nitrobenzoic acid. The irreversible four-electron reduction of nitro compounds in aqueous solution (A), $Ep_c = -0.66$ V, is well documented [15] and known to yield the hydroxylamine as the principle product. This electroactive product was then oxidized quasi-reversibly in a two-electron step to the nitroso (B) species which then cycles during subsequent scans (C), $Ep_a = 0.25$ V, $Ep_c = -0.08$ V.

A cyclic voltammogram of NPA, shown in Fig. 3B, demonstrates a similar initial four-electron reduction, $Ep_c = -0.67$ V, followed by the formation of the PAHA–NOPA quasi-reversible couple, $Ep_a = 0.44$ V, $Ep_c = -0.22$ V. Figure 4A shows the voltammetry of this couple obtained from an aqueous solution of chemically synthesized PAHA. While the electron transfer kinetics of PAHA–NOPA were noticeably slower than for the nitrobenzoic acid couple, voltammograms obtained at a 2-mV s^{-1} scan rate showed significantly less peak separation in the anodic and cathodic peak potentials of the PAHA–NOPA couple (<0.1 V). Additionally, thin-layer spectroelectrochemistry demonstrated that NPA could be reduced to PAHA, and PAHA and NOPA subsequently electrochemically interconverted by changing the applied potential and monitoring the characteristic absorbance peaks at 270 and 308 nm, respectively.

As a result of the electrochemical characterization of PA and NPA in aqueous solution, it was determined that the most feasible means of electrochemically generating the potentially reactive NOPA derivative was through the two-electron oxidation of PAHA. This oxidation was accomplished using the packed carbon bed bulk electrolysis flow cell for an electrochemical synthesis, providing much

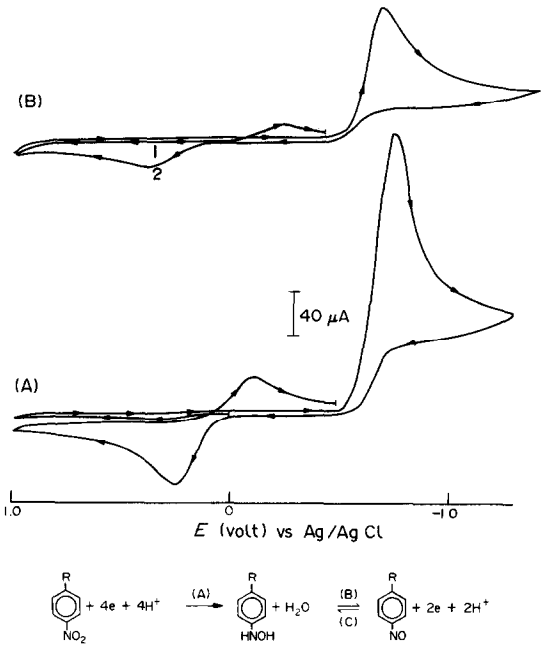


Figure 3
Cyclic voltammograms of (A) 5.0 mM nitrobenzene and (B) 5.0 mM NPA in 0.1 M ammonium acetate buffer, pH 5.5.

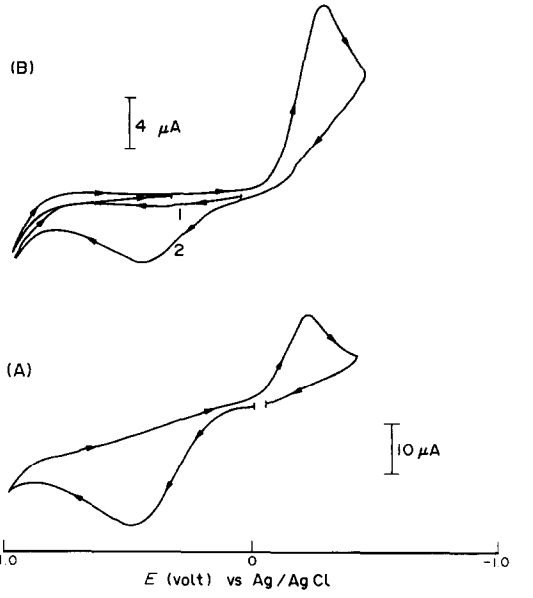


Figure 4
Cyclic voltammogram of (A) 1.0 mM PAHA; (B) 1.0 mM NOPA electrogenerated from PAHA by bulk electrolysis at 0.70 V versus Ag/AgCl, in 0.2 M ammonium acetate buffer, pH 5.5.

higher yields of known concentration of NOPA product than previously reported chemical oxidations [8]. PAHA might likewise be prepared through the four-electron reduction of NPA by methodologies previously suggested [15]; however, stringent deoxygenation procedures would be necessary to prevent large background currents due to oxygen reduction. Figures 4A and 4B show voltammograms obtained prior to and following bulk electrolysis. The disappearance of the oxidation wave on the first positive scan, followed by the large reduction wave on the reverse scan and reversibility on subsequent scans provided solid evidence for the high yield of NOPA product using the flow cell. With a 1.0-mM solution of PAHA at an applied potential of 0.7 V, an *n*-value of 1.95 (equation 1) was obtained using the packed bed flow cell. Dual-electrode LC–EC of the characteristically green NOPA solution likewise indicated essentially complete conversion.

LC–MS

To provide positive structural identification, LC–MS was employed to analyse the electrolytically prepared NOPA solution. Figure 5 shows the mass spectrum obtained for NOPA under the chromatographic conditions described above. The observed parent molecular ion peak at *m/z* 250 is in complete agreement with the theoretical value for the NOPA species; an additional small ion peak at *m/z* 266 indicates slight decomposition of NOPA to the further oxidized NPA species within the mass detector. A small amount (<5% based on total peak area) of NPA impurity resulting from the air oxidation of PAHA prior to electrolytic conversion was also evident in the total ion chromatogram of the NOPA solution, yielding a retention time of 5.9 min with a parent molecular ion peak of *m/z* 266.

LC–EC

With the electrochemical behaviour of PA and its oxidized derivatives in aqueous solution well established, dual-electrode LC–EC methods were developed for separation and quantification. Figure 6A shows the hydrodynamic voltammograms (HDV) obtained for PAHA and NOPA as detected at a single working electrode at the indicated applied potentials. The $E_{1/2}$ value obtained for this couple from the chromatographically assisted HDV is 0.08 V, which compares quite well with the average of the cyclic voltammetric peak potentials, $E^{o\prime} = 0.11$ V.

It has been shown that the series configuration of the dual-electrode thin-layer cell may be used to investigate the electrochemical behaviour of electroactive products at the downstream electrode resulting from electrochemical reactions occurring at the upstream electrode [17]. A HDV of NOPA obtained at the downstream electrode following oxidation of PAHA at the upstream electrode is shown in Fig. 6B. This is compared with the HDV of NOPA obtained directly. The shape, half-wave potential and limiting current plateau poten-

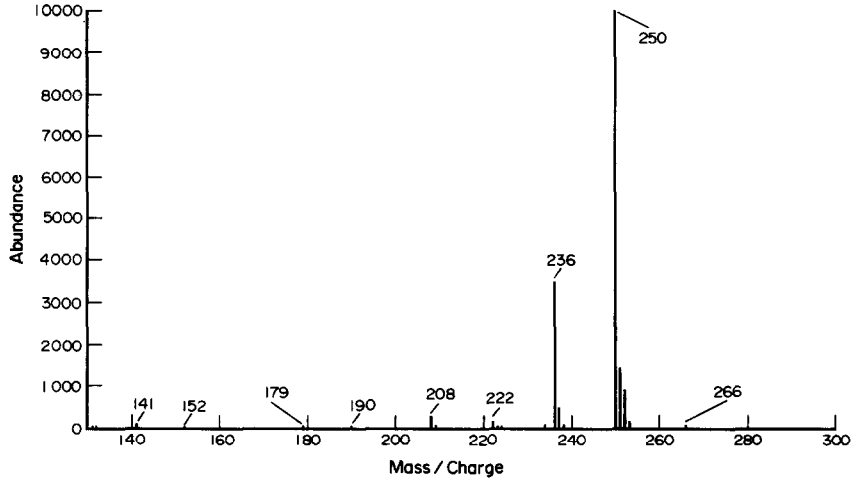


Figure 5
Mass spectrum obtained for electrolytically generated 0.1 mM NOPA in 0.1 M ammonium acetate buffer, pH 5.5, at a chromatographic elution time of 7.0 min.

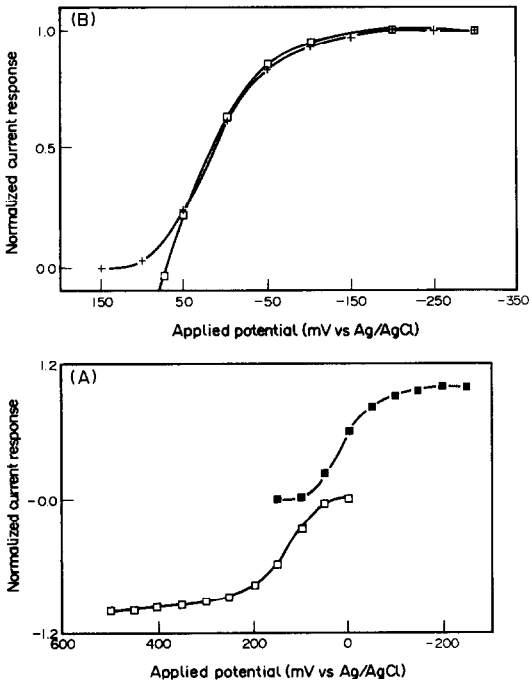


Figure 6
Hydrodynamic voltammograms of (A) □ PAHA and ■ NOPA in 0.2 M ammonium acetate buffer, pH 5.5; (B) + NOPA at upstream electrode detected directly and □ NOPA at downstream electrode detected as product of upstream PAHA oxidation in series dual-electrode configuration.

tials of these voltammograms are virtually identical, allowing chromatographic peak assignments to be established with confidence. A HDV for the four-electron reduction of NPA to PAHA was also obtained (not shown), and indicated a half-wave ($E_{1/2}$) value of −0.58 V with a current limiting plateau potential of −0.65 V. PA was found to have a plateau potential of 1.05 V at a glassy carbon electrode by linear sweep voltammetry. Electrode fouling due to the build up of oxidation products at the electrode surface prevented accurate quantitation of PA concentrations after several successive injections.

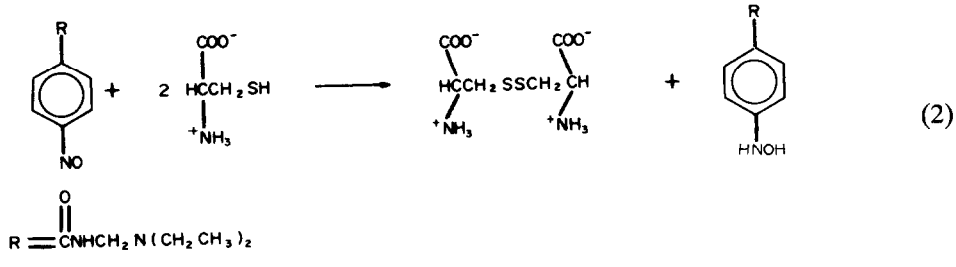
PAHA, NOPA and NPA were found to have linear working ranges between 10–10,000 pmol injected with detection limits <5 pmol, determined experimentally [signal/(background × 3)]. Although NPA and NOPA were not completely resolved chromatographically under these conditions, they were resolved electrochemically. By using an applied potential of −0.35 V, NOPA was selectively oxidized in an applied potential region where NPA is not electroactive.

Metabolite incubations

Incubations were performed to determine whether irreversible reactions could be detected between NOPA and simple biomolecules or PAHA and these molecules. As previously indicated, the only amino acid examined which resulted in a change in the measured response (i.e. loss of chromatographic peak current) was cysteine (CYS) incubated with NOPA [10]. PAHA and cystine were detected as the products of this reaction, shown stoichiometrically in equation (2), based on both LC–EC retention and hydrodynamic profiling.

Ascorbic acid has been suggested to reduce NOPA to PAHA [8], and these studies support this assumption. However, in contrast to previous reports [8], incubations of glutathione (GSH) as well as CYS with NOPA also produced PAHA at physiological pH. This observed reactivity of the nitroso functionality with sulfhydryl groups is quite interesting since many of the drugs which cause SLE have an oxidizable amine group, and most of the large macromolecules which have been reported to bind PA metabolites contain sulfhydryl groups.

It is of additional significance to report that an electroactive intermediate was observed ($t_{1/2}$ = 10.5 min) in the NOPA–GSH reaction which eluted slightly before PAHA, and that the measured current response of this intermediate decreased with an increase in the PAHA current response as a function of time (Fig. 7). A similar intermediate was seen briefly following NOPA–CYS incubations, however decomposition of the intermediate



(2)

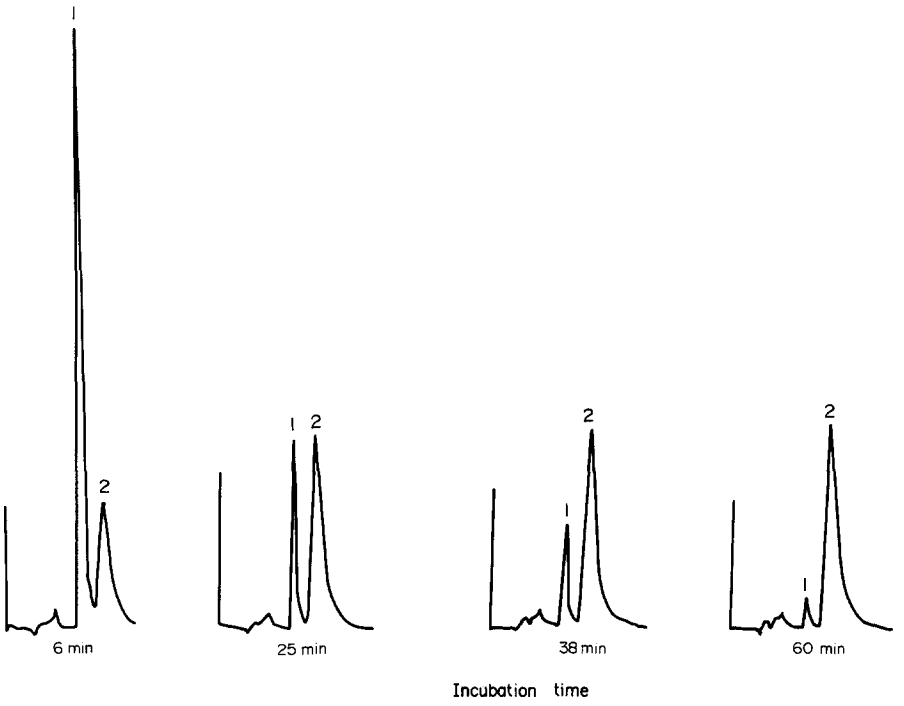


Figure 7
LC–EC chromatograms of 10 μM NOPA–20 μM GSH incubations as a function of time; peak 1 represents electroactive intermediate, t_R = 2.0 min; peak 2 represents PAHA, t_R = 3.5 min, conditions as stated in text.

product occurred much more rapidly. These intermediates may represent a bound product between the nitroso or hydroxylamine functions and sulfhydryl groups, and may be necessary for the electron transfer process to occur.

In order to establish the extent of interaction of the *N*-oxidized PA metabolites with haemoglobin and which specific PA species were directly involved in this presumed binding [7], incubations of PA, PAHA, NOPA and NPA were also conducted with mouse haemoglobin. Equimolar incubations of mouse haemoglobin with NOPA resulted in a significant irreversible reaction, with <30% NOPA remaining in a free, electrochemically detectable state within a 30-min incubation period. PAHA was also apparently "bound" to the haemoglobin conjugate, with <50% remaining in a free state within 30 min. In this case there appeared to be no interconversion of the free PAHA and NOPA species, based on the chromatograms obtained following individual incubations. The measurable concentration of PA and NPA versus control did not change during the course of these incubations, suggesting that there is no direct interaction of these compounds with haemoglobin. Further determination of the exact nature of the PA metabolite–haemoglobin conjugates is currently under investigation.

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References

[1] E.M. Tan, A.S. Cohen, J.F. Fries, A.T. Masi, D.J. McShane, N.F. Rothfield, J.G. Schaller, N. Talal and R.J. Winchester, *Arthritis Rheum.* **25**, 1271–1277 (1982).
[2] R.L. Woosley, D.E. Drayer, M.M. Reidenberg, A.S. Nies, K. Carr and J.A. Oates, *New Eng. J. Med.* **298**, 1157–1159 (1978).
[3] N.C. Henningsen, A. Cederberg, A. Hanson and B.W. Johansson, *Acta Med. Scand.* **198**, 475–482 (1975).
[4] J.P. Uetrecht, R.W. Freeman and R.L. Woosley, *Arthritis Rheum.* **24**, 994–999 (1981).
[5] R.W. Freeman, J.P. Uetrecht, R.L. Woosley, J.A. Oates and R.D. Harbison, *Drug Metab. Dispos.* **9**, 188–192 (1981).
[6] R.W. Freeman, R.L. Woosley, J.A. Oates and R.D. Harbison, *Toxicol. Appl. Pharmac.* **50**, 9–16 (1979).

[7] R.A. Budinsky, S.M. Roberts, E.A. Coats, L. Adams and E.V. Hess, *Drug Metab. Dispos.* **15**, 37–43 (1987).
[8] J.P. Uetrecht, *J. Pharmac. Exp. Ther.* **232**, 420–425 (1985).
[9] J. Uetrecht, N. Zahid and R. Rubin, *Chem. Res. Toxicol.* **1**, 74–78 (1988).
[10] J.F. Wheeler, C.E. Lunte, W.R. Heineman, L. Adams and E.V. Hess, *Proc. Exp. Biol. Med.* **188**, 381–386 (1988).
[11] T.P. DeAngelis and W.R. Heineman, *J. Chem. Ed.* **53**, 594–597 (1976).
[12] K. Bratin, P.T. Kissinger and C.S. Bruntlett, *J. Liq. Chromatogr.* **4**, 1777–1795 (1981).
[13] R. Lahita, J. Kluger, D.E. Drayer, D. Koffler and M.D. Reidenberg, *New Engl. J. Med.* **301**, 1382–1385 (1979).
[14] J. Bacon and R.N. Adams, *J. Am. Chem. Soc.* **90**, 6596–6599 (1968).
[15] A.P. Tamilov, S.G. Mairanovskii, M.Y. Fioshin and V.A. Smirnov, in *The Electrochemistry of Organic Compounds*. Halsted Press, New York (1972).
[16] H.J. Landaez-Machado, A. Darchen and C. Moinet, *Electrochim. Acta* **25**, 1519–1521 (1980).
[17] C.E. Lunte and P.T. Kissinger, *Analyt. Chem.* **56**, 658–663 (1984).

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