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IDENTIFICATION OF 9-HYDROXYLAMINE-1,2,3,4-TETRAHYDROACRIDINE AS A HEPATIC MICROSOMAL METABOLITE OF TACRINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMISTRY

HOWARD P. HENDRICKSON, DENNIS O. SCOTT and CRAIG E. LUNTE*

Department of Chemistry, The University of Kansas, Lawrence, KS 66045 (U.S.A.)

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SUMMARY

Amperometric detection using a dual-electrode thin-layer cell in the series configuration can aid in the identification of unknown components in complicated samples by voltammetric characterization. This is shown by studying the metabolism of tacrine by rat hepatic microsomes using high-performance liquid chromatography with electrochemical detection. The major metabolite detected in microsomal incubations did not co-elute with any standard acridine available and was produced in too small a quantity for mass spectral characterization. Tentative identification of this metabolite as 9-hydroxylamine-1,2,3,4-tetrahydroacridine was made by electrochemical characterization. The electrochemistry of the metabolite was compared to that of the hydroxylamine produced and studied by cyclic voltammetry.

INTRODUCTION

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine, THA) is a potent inhibitor of both acetylcholinesterase and butyrylcholinesterase [1]. It has been used for the treatment of central anticholinergic syndrome caused by drug overdose [2,3], intractable pain from terminal carcinoma [4], myasthenia gravis [5], and as a decurarizing agent [6]. Recent interest in THA has been generated by its reported efficacy as a therapy for memory loss in Alzheimer's patients [7,8]. Few toxicology studies have been reported and little information on the metabolism of THA is available. While the reported lethal dose of THA in man is relatively high, approximately 30 mg/kg extrapolated from animal data [3], hepatotoxicity and bone marrow toxicity have been noted [4,9,10]. In the light of the chronic exposure of patients to THA in the proposed treatment of Alzheimer's disease, understanding the metabolism of this drug is imperative.

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Little information has been reported in the literature concerning the metabolism of THA. Kaul [11] has reported the detection of four metabolites of THA in rat urine. These were detected by staining with methyl orange, which complexes acridines, following paper chromatography. The only structural information given was that none of the metabolites corresponded to unchanged THA and that one metabolite was probably carboxylated based on its infrared (IR) spectrum. Park et al. [12] observed both unchanged THA and a possible metabolite in the serum of a monkey dosed with THA using high-performance liquid chromatography (HPLC) and ultraviolet (UV) absorbance detection. No indication of the identity of the metabolite was given. HPLC with a variety of detection schemes has been the most popular analytical method for the analysis of THA in biological samples. Separation has been achieved using either C_{18} [13,14] or cyano [12] stationary phases with an acidic mobile phase. Detection has been by either UV absorbance [13,14] or fluorescence [12].

We describe the development of an analytical procedure to detect THA and its metabolites in preparations of rat liver microsomes. This procedure is based on HPLC using a polymer-based C_1 column coupled to both electrochemical detection (ED) and UV absorbance detection. The analytical procedure was used to detect and tentatively identify 9-hydroxylamine-1,2,3,4-tetrahydroacridine (THA-hydroxylamine) as a metabolite of THA in these microsomal preparations. As no standard was available for THA-hydroxylamine, identification could not be performed by direct comparison. However, THA-hydroxylamine could be electrochemically characterized by cyclic voltammetry of 9-nitro-1,2,3,4-tetrahydroacridine (nitro-THA). Identification of the metabolite in the incubation mixture could then be achieved by voltammetric characterization.

EXPERIMENTAL

Apparatus

HPLC was performed with a Bioanalytical Systems LC-400 liquid chromatograph (West Lafayette, IN, U.S.A.). Separation was achieved with a Hamilton PRP-1, 10 μm particle size, cartridge column (10 cm$\times$4.6 mm I.D.) with a 1.5-cm guard column using a mobile phase of 0.05 *M* ammonium phosphate buffer, pH 11, with 20% (v/v) acetonitrile. All mobile phases were prepared from distilled, deionized water and filtered through a 0.2-μm membrane prior to use. The mobile phase was deoxygenated by continuous purging with argon. A flow-rate of 2.0 ml/min was used. ED was carried out with a dual-electrode LC-4B amperometric detection system (Bioanalytical Systems) using glassy carbon working electrodes and an Ag/AgCl reference electrode. Absorbance detection was done with a Shimadzu (Columbia, MD, U.S.A.) SPD-6AV spectrophotometric detector. A 200-μl injection loop was used for all experiments.

Cyclic voltammetry experiments were performed with a Bioanalytical Systems CV-37 voltammograph. A glassy carbon working electrode, platinum auxiliary electrode and Ag/AgCl reference electrode were used. A scan-rate of 200 mV/s was used for all experiments. Solutions were carefully purged with argon prior to the experiment. All potentials are reported versus the Ag/AgCl reference electrode.

Materials

Tacrine·HCl, NADPH and *tert.*-butyl hydroperoxide were obtained from Sigma (St. Louis,MO, U.S.A.). Vanadyl acetylacetonate was purchased from Aldrich (Milwaukee, WI, U.S.A.). All chemicals were used as received.

The free base of THA was prepared by dissolving 2 g THA·HCl in 2 ml triethylamine. To this were added 5 ml each of dichloromethane and water. A white precipitate of the free base of THA formed which was collected by filtration and washed with water.

Nitro-THA was prepared from THA free base by oxidation with *tert.*-butyl hydroperoxide by a modification of the procedure of Howe and Hiatt [15]. THA free base (2 g) was dissolved in 5 ml of *tert.*-butyl hydroperoxide (70%, v/v, aqueous solution) and 5 mg vanadyl acetylacetonate were added as catalyst. This solution was heated at 60 °C. The reaction was followed by HPLC analysis. Nitro-THA was isolated from the reaction mixture by preparative chromatography on a silica column using a 50:50 (v/v) acetonitrile–water mobile phase. This isolation removed any excess THA and polymerization side-products from the nitro-THA. Confirmation of the synthesis of nitro-THA was made by mass spectrometry (MS).

Microsomal incubations

Hepatic microsomes were isolated from untreated male rats as previously described [16]. Protein content was determined by the Sigma modification of the procedure of Ohnishi and Barr [17] using micro protein determination kits (No. 690) from Sigma.

Incubations were carried out by a slight modification of the procedure previously described [18]. Incubation mixtures contained 1 ml of rat hepatic microsomal protein (4 mg/ml) in 0.1 *M* KCl–phosphate buffer pH 7.4, 100 μl of 14 m*M* NADPH in 150 m*M* $MgCl_2$, and 100 μl of 20 m*M* THA in KCl–phosphate buffer for a total volume of 1.2 ml. Reaction was initiated by addition of NADPH. All incubations were carried out at 37 °C for 1 h while exposed to the atmosphere. Incubations were quenched by the addition of 200 μl of cold 1 *M* perchloric acid, centrifuged at 10 000 *g* for 10 min and stored frozen (0 °C) until analyzed. Blanks were run identically except that buffer was added instead of THA. Metabolites were determined by direct injection of the supernatant into the chromatographic column.

RESULTS

Electrochemical characterization of nitro-THA and THA-hydroxylamine

Cyclic voltammetry of a nitro-THA solution in 0.1 *M* ammonium phosphate buffer, pH 11, gave results expected of an aromatic nitro compound (Fig. 1). Nitro-THA is reduced at −1.3 V in a $4e^-,4H^+$ step to THA-hydroxylamine. THA-hydroxylamine is reversibly oxidized at −0.05 V in a $2e^-,2H^+$ step to nitroso-THA. Therefore, THA-hydroxylamine can be electrochemically prepared and characterized in situ. This characterization can then be used for identifica-

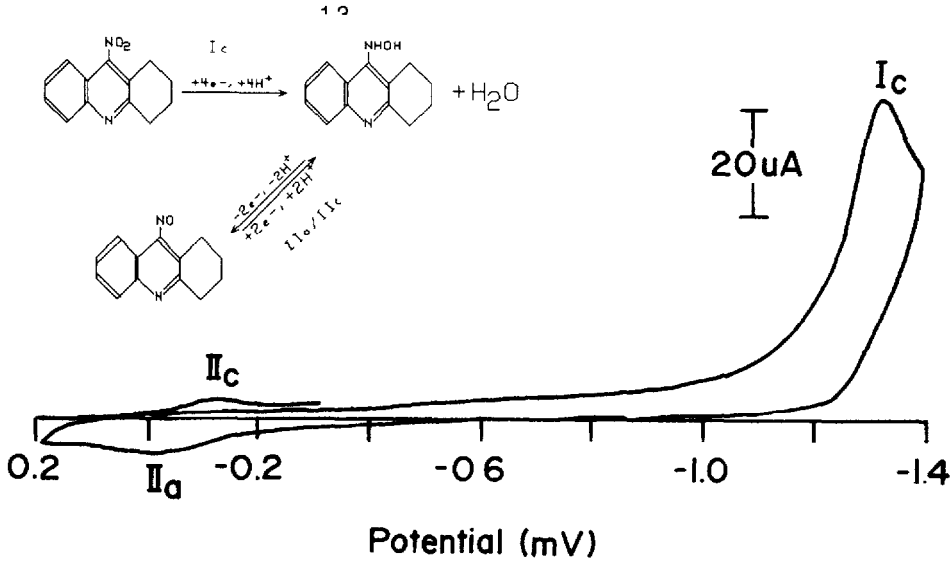

Fig. 1. Cyclic voltammogram of nitro-THA in 0.1 *M* ammonium phosphate buffer, pH 11.

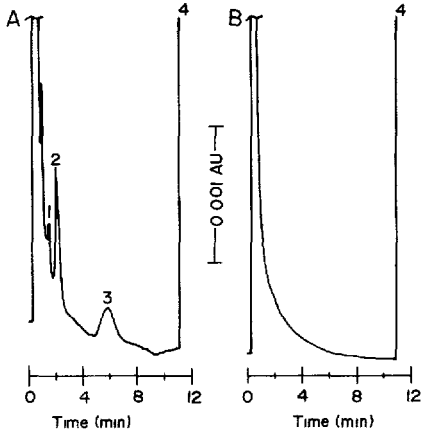

Fig. 2. Detection of THA-hydroxylamine in a microsomal incubation of THA by UV detection at 240 nm. Chromatogram A is the incubation mixture, chromatogram B is the blank (no NADPH). Detection sensitivity, 0.005 a.u.f.s. Peaks: 1 = unidentified THA metabolite; 2 = THA-hydroxylamine 3 = unidentified THA metabolite; 4 = THA.

tion of THA-hydroxylamine in metabolism studies even though no standard is available.

In vitro metabolism of THA and THA-acetanilide

A typical chromatogram with UV detection of a microsomal incubation containing THA is shown in Fig. 2. As absorbance at 240 nm is characteristic of acridines, this could be used as a screen for all metabolites produced. Three chromatographic peaks due to metabolites of THA were detected. These peaks were identified as metabolic products of THA as they were absent from incubations

which lacked either THA, NADPH or microsomes. None of the peaks eluted at the same time as any of the standard compounds.

Tentative identification of peak 2 as THA-hydroxylamine was made using the electrochemical detector in the dual-series configuration. In this configuration, products electrochemically generated at the upstream electrode can be electrochemically studied at the downstream electrode. The electrochemical behavior of a compound in a complex mixture can be obtained without the need for isolation. With adequate chromatographic separation, a single compound is introduced into the electrochemical detector at any given time. The dual-electrode detector can then provide electrochemical characterization of each component. The oxidation potential of the unknown can be obtained by stepping the detection potential of the upsteam electrode while making multiple injections. The product of this oxidation can be characterized likewise by stepping the potential of the downstream electrode. This technique has been been used previously to study the electrochemistry of the pterins [19].

Dual-electrode detection of a sample from a microsomal incubation of THA is shown in Fig. 3. The upstream electrode (W1) was operated at +0.8 V in order to detect THA and any electroactive metabolite. As can be seen, peak 1 from the UV chromatogram was not present and peak 3 was very small. Therefore, relative to THA, peaks 1 and 3 must be more difficult to oxidize. Peak 2 gave a large

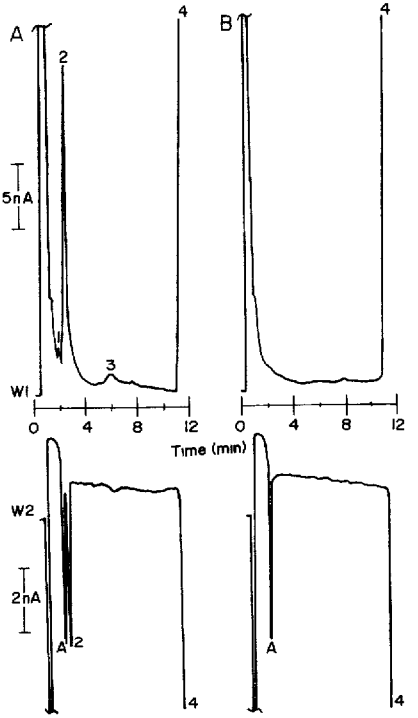

Fig. 3. Detection of THA-hydroxylamine in a microsomal incubation of THA by dual-electrode amperometric detection. Chromatogram A is the incubation mixture, chromatogram B is the blank (no NADPH). W1 is the upstream electrode operated at +0.8 V, W2 is the downstream electrode operated at −0.4 V. Peaks: 1 = unidentified THA metabolite; 2 = THA-hydroxylamine; 3 = unidentified THA metabolite; 4 = THA; A = oxygen.

response at the upstream electrode. Chromatograpically assisted hydrodynamic voltammograms (CAHDVs) of peak 2, THA and THA-acetanilide are shown in Fig. 4. From the CAHDV, peak 2 was oxidized at the same potential as THA-hydroxylamine as found by cyclic voltammetry.

Peak 2 was further characterized by considering the response at the downstream electrode (W2). The large downstream response (Fig. 3) showed that this is clearly a reversible oxidation as expected for THA-hydroxylamine. The CAHDV of the downstream process is shown in Fig. 5. The downstream response corresponds to the reduction of nitroso-THA back to THA-hydroxylamine. This is

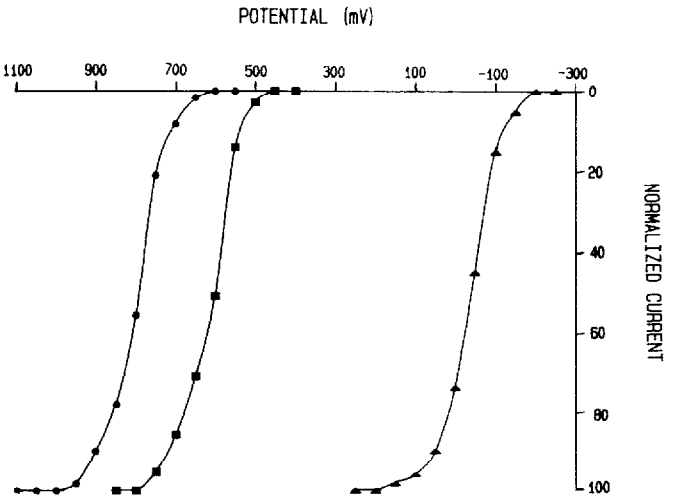

Fig 4. Chromatographically assisted hydrodynamic voltammograms of THA and its derivatives. (●) THA-acetanilide; (■) THA; (▲) peak 2.

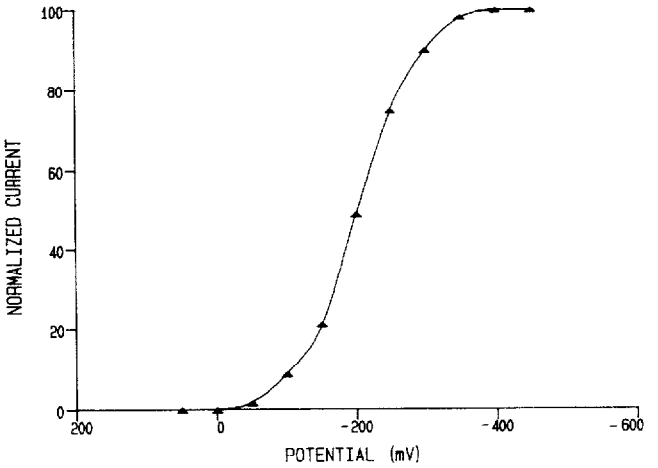

Fig. 5. Chromatographically assisted hydrodynamic voltammogram of nitroso-THA at the downstream electrode obtained when peak 2 (THA-hydroxylamine) was oxidized at the upstream electrode using a dual-electrode amperometric detector.

again the same as for THA-hydroxylamine obtained by cyclic voltammetry. That the electrochemistry of peak 2 obtained by CAHDV is the same as that of THA-hydroxylamine obtained by cyclic voltammetry provides good evidence for the identification of peak 2 as THA-hydroxylamine.

DISCUSSION

ED coupled to HPLC can provide a powerful tool to investigate trace components in complicated samples. Electrochemical techniques are capable of both extremely low detection limits and providing some qualitative information about the analyte, but typically they lack sufficient selectivity. The chromatographic step is used to provide the selectivity by presenting a single component to the electrochemical detector at any given time. ED can then be used to characterize the individual sample components. Both the direct oxidation as well as the reverse reaction can be studied. This electrochemical characterization can be coupled to cyclic voltammetric studies where labile or difficult to synthesize compounds can be prepared and studied in situ to provide a tentative identification. Two great advantages to this technique are that only small samples are required and that characterization can be performed with very complex samples. This is shown by the microsomal incubation studies where only 1 ml of sample with the analyte at micromolar concentrations could be studied. Obviously, this technique is not sufficient for conclusive identification, without an isolated standard for comparisons, but it can be used to provide a starting point for designing more complete investigations.

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