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High-performance liquid chromatography of the antihistamine pyrilamine and its N-oxide using electrochemical detection

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ABSTRACT

The electrochemical behavior of the over-the-counter antihistamine drug pyrilamine and its N-oxide analogue, have been studied by several voltammetric methods Cyclic voltammograms of pyrilamine maleate in 0 1 *M* ammonium acetate at pH 7 0 indicated a quasi-reversible electrode process by observing a wave at +0.85 V and +1 30 V in the initial anodic sweep followed by a wave at −1 30 V *versus* Ag/AgCl. Differential pulse and hydrodynamic voltammetry of pyrilamine and the N-oxide were examined to determine oxidation potentials for use in high-performance liquid chromatography with electrochemical detection (HPLC–ED). Differentiation between pyrilamine and its N-oxide was achieved in HPLC–ED analyses at a detection potential of +0.7 V and +0 9 V *versus* Ag/AgCl with tandem ultraviolet detection at 254 nm. Utility of the HPLC–ED method was demonstrated by the analysis of pyrilamine and the N-oxide in microbial biotransformation samples

INTRODUCTION

Pyrilamine, an ethylenediamine-type antihistamine, is found in many formulations of cough syrups, allergy and pain relievers, and sleep aids. The wide spread commercial use of pyrilamine, usually as the maleate or tannate salt, has increased interest in development of analytical methods for this drug in many different substrates. A majority of the methods available use high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection for analysis of pyrilamine in cough-cold liquids [1], tablets [2], suppositories [3] and expectorants [4]

Recently, pyrilamine and other related antihistamines have been the subject of laboratory animal testing for possible carcinogenicity and other adverse effects that may be extrapolated to the human consumption of this type of drug. Methods using reversed-phase HPLC with UV detection have been developed for

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analysis in animal feed [5,6] and biological matrices (plasma [7] and urine [5]) for several antihistamines and their metabolites. These methods have been shown to be quite sensitive; however, UV detection does not exhibit the specificity needed for differentiating between antihistamines and their metabolites in biological samples. For this reason, HPLC with electrochemical detection (ED) which takes advantage of the variability of oxidation and/or reduction potentials of these electroactive species, has recently become a very popular procedure for assaying antihistamines and many other drugs and their metabolites in various biological samples Promethazine and several other phenothiazine metabolites and derivatives [8–11] have been analyzed in plasma by HPLC–ED. Isoetharine, a bronchodilator used in inhalation therapy, has been detected in blood plasma of orally administered rats at an applied potential + 0.6 V by HPLC–ED [12]. A tricyclic antidepressant, trimipramine, and its demethyl, 2-hydroxy, and 2-hydroxy demethyl metabolites were analyzed by reversed phase HPLC–ED in plasma from patients administered 75- and 150-mg doses of the parent compound [13].

Similar electrochemical analytical data are lacking in the literature for analysis of pyrilamine and its metabolites in biological samples. At the National Center for Toxicological Research studies are in progress for determining the products of metabolism for pyrilamine using animal dosing and microbial biotransformation studies. In this work, we report an oxidative-mode HPLC–ED method for pyrilamine and pyrilamine N-oxide, a major biotransformation product, in extracts of cultures of *Cunninghamella elegans* incubated in the presence of the parent antihistamine.

EXPERIMENTAL

Reagents

All reagents were analytical-reagent grade and used without further purification All solvents used were HPLC grade. Stock solutions of pyrilamine maleate (100%, Hexagon Lab., Bronx, NY, U.S.A.) at a concentration of 3.5 m*M* (based on free amine) were prepared by dissolving 141 mg in 100 ml of methanol. Dilutions of the stock solution were made in HPLC mobile phase for standard injections into the HPLC system. Pyrilamine N-oxide was synthesized, purified and characterized by mass spectrometry (MS) [14]. A 3.3 m*M* stock solution of the N-oxide in methanol was used as supplied[14] and diluted with the mobile phase for HPLC analysis.

High-performance liquid chromatography

The HPLC system used in this study consisted of a Waters Model M-6000 pump equipped with an Altex 210 septumless injector. A 250 × 4.6 mm I.D. Supelco C_{18} or LC-CN reversed-phase, 5 μm particle size, column was used to provide separation of pyrilamine and its N-oxide. The mobile phases used were acetonitrile–50 m*M* ammonium acetate, pH 7 (30:70, v/v) for the LC-CN column

and acetonitrile–75 m*M* ammonium acetate with 10 m*M* trimethylamine hydrochloride, pH 6.8 (30:70, v/v) for the C_{18} column. The mobile phase was delivered at a flow-rate of 1.2 or 2.0 ml/min (depending on the analysis) through the column at ambient temperature. Injections were made using a 10- or 50-μl loop depending upon the experiment.

Ultraviolet detector

The effluent on the HPLC column was diverted into a Beckman 210 UV absorbance detector. The UV response was monitored at 254 nm using the appropriate filter. The range was set at 0.050 a.u.f.s. A Spectra Physics SP-4100 integrator was used to record the UV chromatographic trace

Electrochemical detector

The effluent of the UV detector was connected to a Bioanalytical Systems (BAS, West Lafayette, IN, U.S.A.) LC-4B amperometric electrochemical detector. The detector cell consisted of a TL-5 glassy carbon electrode (GCE) as the working electrode with an Ag/AgCl reference electrode. The auxiliary electrode was the stainless-steel block which was separated from the GCE by a TG-5M thin-layer Teflon cell gasket. The cell was operated in the oxidative mode at an applied potential ranging from + 0.3 to + 1.2 V depending on the experiment. The operating current range was varied from 5 to 100 nA according to the amounts of the compounds injected.

Cyclic and differential pulse voltammetry

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were carried out using a BAS-100A electrochemical analyzer with automatic cell stand. The reference electrode was an Ag/AgCl electrode. The working and auxiliary electrodes were a GCE and a platinum wire, respectively. Solutions were sparged with nitrogen for about 1 min prior to CV and DPV measurements to remove traces of dissolved oxygen. The GCE surface was renewed before each measurement by using a polishing pad with a few drops of an alumina slurry. The electrode was then rinsed with methanol and dried with a soft tissue. Scan-rates varied from 100 to 500 mV/s for the CV scans. The experimental conditions for the DPV scan were + 0.4 to + 1.5 V scan range, 5 mV/s scan-rate and 50 mV pulse amplitude.

IIydrodynamic voltammograms

Using the HPLC flow system (previously described), hydrodynamic voltammograms of pyrilamine and pyrilamine N-oxide were performed by varying the applied potential on the electrochemical thin-layer cell for replicate 50-μl injections of a 8.8 μM pyrilamine and 9.5 μM pyrilamine N-oxide standard admixture. The resulting current response was measured by subtracting the background current from the maximum current meter reading for each of the two compounds. Potentials were varied from + 0.3 to + 1.2 V in 0 1-V increments.

RESULTS AND DISCUSSIONS

Cyclic and differential pulse voltammetry of pyrilamine

CV of pyrilamine was performed to determine the feasibility of its ED and the oxidation and/or reduction potential at which this detection may occur. The CV of pyrilamine (as the maleate salt) at 3.5 m*M* in 0.1 *M* ammonium acetate (pH 7.0) solution is shown in Fig. 1a. The potential at a rate of 100 mV/s was scanned between + 1.5 and − 1.5 V with an initial potential of − 0.2 V. The pyrilamine exhibited a well defined oxidation wave at + 0.85 V which we suspect gives rise to an N-oxide derivative. A second ill-defined oxidation wave occured at about + 1.3 V indicating the N-oxide may be undergoing further oxidation One speculation is that this second wave indicates a demethylation product of pyrilamine. On the reverse (cathodic) sweep, a reduction wave does not occur until − 1.3 V after the initial anodic sweep. A quasi-reversible redox-electrode process is indicated by the voltammogram. The scan-rate was increased to 500 mV/s for a CV scan between − 0.2 and − 1.5 V. This resulting CV is shown in Fig. 1b. The scan-rate (mV/s) was increased to take advantage of the increase in peak current (i_p) which produces an expanded view of the voltammogram [15]. From this CV, a second oxidation wave was clearly observed with a peak potential (E_p) of + 1.35 V. In addition, a differential pulse voltammogram (Fig. 2) confirmed the dual oxidation potentials of pyrilamine at + 0.75 V (E_1) and + 1.30 V (E_2).

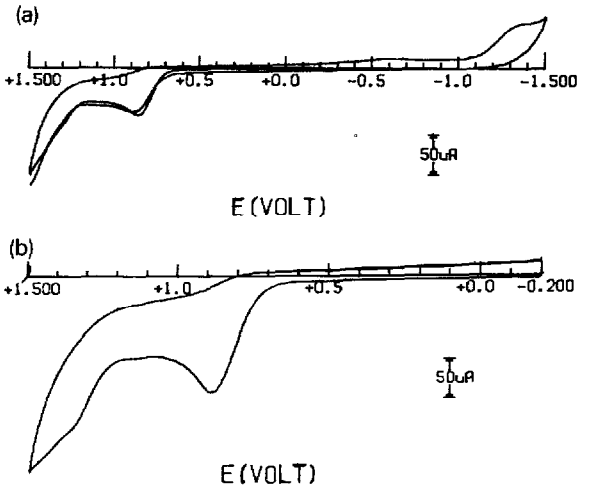

Fig 1 (a) Cyclic voltammogram of 3.5 m*M* pyrilamine maleate in 0 1 *M* ammonium acetate, pH 7.0, at 100 mV/s scan-rate from + 1.5 to − 1 5 V using a GCE *versus* Ag/AgCl reference electrode with an initial potential of − 0.2 V (b) Cyclic voltammogram of 3.5 m*M* pyrilamine maleate in 0.1 *M* ammonium acetate, pH 7 0, at 500 mV/s scan-rate from − 0 2 to + 1 5 V with an initial potential of − 0.2 V

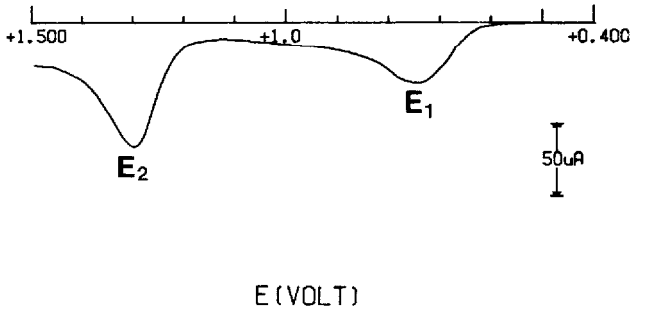

Fig 2 Differential pulse voltammogram of 3.5 m*M* pyrilamine maleate in 0 1 *M* ammonium acetate, pH 7 0, scanned from +0.4 to 1 5 V at 5 mV/s scan-rate using a GCE *versus* Ag/AgCl

HPLC with simultaneous UV and electrochemical detection

Initial HPLC experiments for separation of pyrilamine and pyrilamine N-oxide were carried out using techniques similar to those reported by others [16]. An HPLC system with UV detection and simultaneous ED was used with a reversed-phase C_{18} column and acetonitrile–75 m*M* ammonium acetate, pH 6.8 (30:70, v/v) mobile phase containing 10 m*M* trimethylamine hydrochloride, an amine modifier, to help reduce tailing. The liquid chromatographic analysis for pyrilamine using this approach is shown in Fig. 3. The UV response at 254 nm

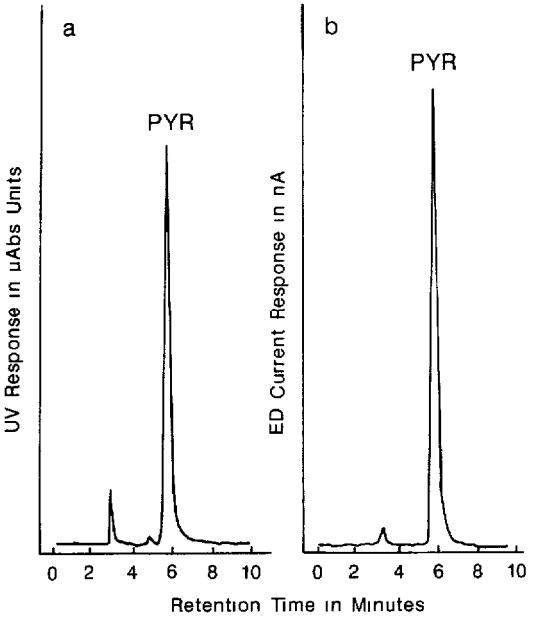

Fig 3. HPLC analysis of pyrilamine standard (10 μl injected) using an acetonitrile–75 m*M* ammonium acetate, pH 6 8 (30 70, v/v) with 10 m*M* trimethylamine hydrochloride mobile phase with (a) UV detection at 254 nm and (b) ED at an applied potential of + 0 7 V *versus* Ag/AgCl. PYR = pyrilamine

and ED response at + 0.7 V in the oxidative mode are shown in Fig. 3a and 3b, respectively. The ED background current was excessive (above 100 nA) and fouled the electrode at applied potentials above + 0.9 V. Another HPLC system consisting of a cyanopropyl (CN) column using an acetonitrile–50 m*M* ammonium acetate, pH 7.0 (30:70, v/v) mobile phase produced the best compromise between ED background current, chromatographic separation and sensitivity. Lowering the concentration of the electrolyte (ammonium acetate) from 75 to 50 m*M* and removing the amine modifier (trimethylamine) was necessary to lower the background current needed to produce hydrodynamic voltammograms and allow HPLC-ED analysis for pyrilamine and pyrilamine N-oxide.

Hydrodynamic voltammograms of pyrilamine and pyrilamine N-oxide

The electrochemical response of pyrilamine and pyrilamine N-oxide was studied further to optimize the operating voltages for HPLC–ED using the acetonitrile–50 m*M* ammonium acetate mobile phase. In this study, hydrodynamic voltammograms (HVs) were performed by varying the applied potentials from + 0.3 to + 1.2 V in 0.1-V increments for replicate 50-μl HPLC injections of an admixture of 8.8 μM pyrilamide and 9.5 μM pyrilamine N-oxide in mobile phase. However, the resulting currents were plotted *versus* the applied potentials as shown in Fig. 4. From the voltammogram it was evident that pyrilamine exhibits two voltammetric waves at + 0.7 and + 1.2 V. This was consistent with the dual anodic wave shown utilizing cyclic voltammetry on the totally aqueous solution (*i.e.* no organic modifier present) with the same pH. However, the differences in the peak potentials for the two voltammetric methods were due to the differences in the electron transfer process for a static electrode and a flow-through thin-layer

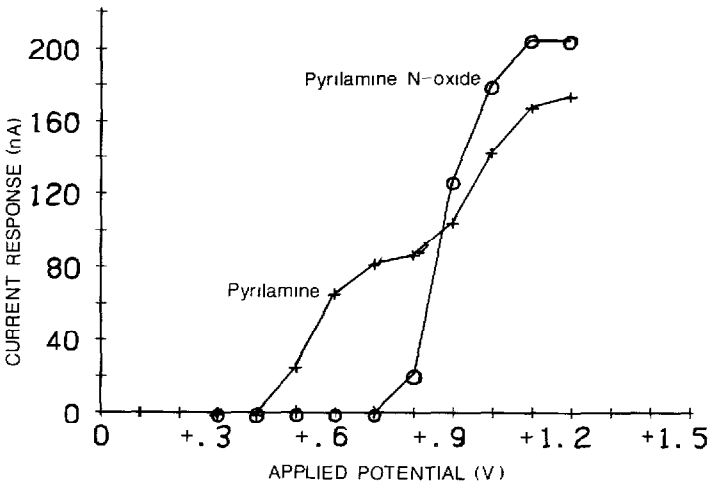

Fig. 4 Hydrodynamic voltammograms of 8.8 μM pyrilamine (+) and 9 5 μM pyrilamine N-oxide (○) using HPLC-ED with a GCE flow cell *versus* Ag/AgCl

electrode system, in the solution media, and in the type of GCE, reference, and auxiliary electrodes used. The HV for pyrilamine N-oxide exhibited a single oxidation beginning at about + 0.8 V intersecting at about + 0.85 V with the second wave shown by pyrilamine (Fig. 4) with a maximum at about + 1.1 V. The coincident oxidative behavior would indicate that the second anodic ED response of pyrilamine is due to the oxidation of its N-oxide formed in the first step. In any case, it is quite obvious that HPLC utilizing amperometric detection should be a selective technique in assaying pyrilamine in the presence of its N-oxide at +0.7 V. This will essentially eliminate current response to the N-oxide (see Fig. 4). Alternatively, both compounds can be assayed simultaneously by increasing to a more positive potential between +0.8 and +1.1 V.

HPLC–ED analysis of pyrilamine and pyrilamine N-oxide applied to microbial transformation studies

The metabolism of the ethylenediamine class of antihistamines to potential mammalian metabolites has been under investigation at the National Center for Toxicological Research for several years. Pyrilamine maleate has been shown to be metabolized essentially 100% by the microorganism *C. elegans*, in 144 h.

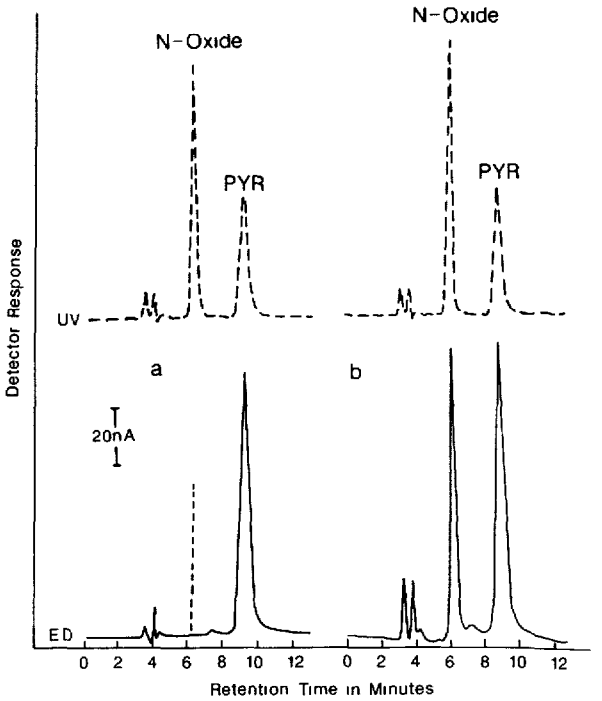

Fig 5 HPLC analysis of 10 8 pmol/μl pyrilamine N-oxide and 10.0 pmol/μl pyrilamine admixture (50 μl injected) with ED at +0 7 V (a) and +0 9 V (b) with simultaneous UV detection at 254 nm (dashed lines) Peaks N-oxide = pyrilamine N-oxide; PYR = pyrilamine

Methods for extraction, isolation and identification by HPLC–UV, MS and nuclear magnetic resonance spectroscopy (NMR) of pyrilamine and pyrilamine N-oxide, the major degradation product of *C. elegans*, have been reported previously by Hansen *et al.* [16]. Although HPLC–UV provides enough sensitivity to detect and quantify these compounds in microbial extracts, further analyses by MS and NMR are needed for confirmation and structure elucidation. ED has been coupled with UV to give added specificity to the HPLC analysis of pyrilamine and its N-oxide in microorganism biotransformation studies. As shown in Fig. 5 by varying the oxidation potential, the HPLC–ED trace of standard injections of pyrilamine and its N-oxide exhibited obvious differences at +0.7 V (Fig. 5a) and +0.9 V (Fig. 5b) with the UV traces (dashed lines) remaining the same. The pyrilamine N-oxide peak at 6.3 min retention was undetected at +0.7 V. At +0.9 V, the N-oxide peak exhibited a high ED response. Also, the pyrilamine peak at 9 1 min was found to increase approximately 13% by increasing the oxidation potential from +0.7 V (Fig. 5a) to +0.9 V (Fig. 5b). This increase in ED response for pyrilamine was expected according to its HV curve in Fig. 4 at +0.7 and +0.9 V. In Fig. 6, HPLC–ED analysis of an extract from a 144-h incubation of pyrilamine maleate (35 μmol) with a fungal culture of *C. elegans* [16] is shown at +0.7 V (Fig. 6a) and + 0.9 V (Fig. 6b) with the corresponding UV traces (dashed lines). As in Fig. 5, the pyrilamine N-oxide recorded no ED

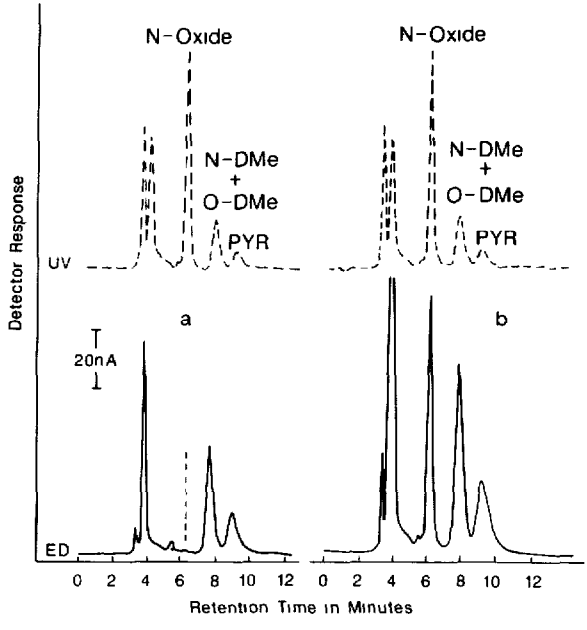

Fig 6 HPLC analysis of a 1.250 dilution of a 144-h microbial transformation of pyrilamine maleate, 35 μmol, by a fungal culture of *C elegans* using ED at applied potentials of +0 7 V (a) and +0 9 V (b) with simultaneous UV detection at 254 nm (dashed lines) Peaks: N-oxide = pyrilamine N-Oxide; N-DMe = N-demethylated pyrilamine, O-DMe = O-demethylated pyrilamine; PYR = pyrilamine

response at 6.3 min retention for +0.7 V applied potential. The peak at 7.8 min was identified as two minor fungal degradation products of pyrilamine, O-demethylated (O-DMe) and N-demethylated (N-DMe) analogues, which are not separated under these HPLC conditions. The pyrilamine was detected at both applied potentials with a slight increase in the response at +0.9 V was noted before in Fig. 5. All of the HPLC–ED analyses in this study were performed using single-electrode amperometry. With the use of dual-parallel GCEs, ED at +0.7 and +0.9 V may be accomplished simultaneously with a dual-channel recorder or integrator, thus performing the analysis of pyrilamine N-oxide by ED at +0.9 V and confirming the identity by disappearance of the N-oxide peak at +0.7 V. This concept may be applicable to N-oxide metabolites of other ethylenediamine-type antihistamines. Although HPLC–ED cannot replace MS and NMR for structure illucidation, the increased specificity over HPLC–UV alone should make it a fast, low-cost analytical tool for the determination of electrochemically active drugs in many types of microbial or biological samples.

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