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Electrochemistry-coupled to mass spectrometry in simulation of metabolic oxidation of methimazole: Identification and characterization of metabolites

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ABSTRACT

Methimazole (MMI), an antithyroid drug, is associated with idiosyncratic toxicity. Reactive metabolites resulting from bioactivation of the drug have been implicated in these adverse drug reactions. Mimicry of enzymatic oxidation of MMI was carried out by electrochemically oxidizing MMI using a coulometric flow-through cell equipped with a porous graphite working electrode. The cell was coupled on-line to electrospray ionization mass spectrometry (EC/ESI-MS). ESI spectra were acquired in both negative and positive modes. In acidic medium, ESI spectral analysis showed that the dimer was the main product, while in neutral and basic media, methimazole sulfenic acid, methimazole sulfinic acid and methimazole sulfonic acid were observed as the major electrochemical oxidation products. Oxidation of MMI and subsequent trapping with nucleophiles resulted in formation of adducts with N-acetylcysteine. Some of the electrochemically generated species observed in these experiments were similar to metabolites that have been observed from *in vitro and in vivo* studies. Trapping studies also showed that bioactivation of MMI proceeds predominantly through the S-oxide and not through formation of thiyl radicals. These results show that electrochemistry coupled to mass spectrometry can be used in mimicry of oxidative metabolism and subsequent high throughput screening of metabolites.

1. Introduction

The conventional method of studying oxidative drug metabolism during preclinical experiments is through animal models (*in vivo*) or perfused organs *in vitro* [1]. However, the use of animals in experiments involving scientific research and biological testing has raised concerns over the years among animal advocates [2]. In view of this as well as the large number of drug candidates emerging and those existing, there has been a renewed interest in development of complementary tools for mimicry of oxidative metabolism. Although it remains a challenge to extrapolate data generated from such systems to actual *In vivo* systems, these biomimetic tools offer some advantages that are not inherent in conventional methods. Electrochemistry coupled on-line to liquid chromatograph/mass spectrometer (EC/LC/MS or EC/MS) has the potential to mimic redox metabolism [3–6]. Members of the cytochromes P450 class (CYP450) of enzymes are responsible for the majority of phase I biotransformations leading to reactive electrophilic intermediates [7]. The chemical reactivity of electrophilic metabolites usually prevents their detection *in vivo* since, by definition, they are short-lived and likely to undergo one or more structural modifications to form more stable final products. Jurva and co-workers investigated the extent to which this technique could be used to mimic cytochrome P450 catalyzed reactions by comparing metabolites generated from EC/LC/MS to those that were generated from the P450 system. Their results showed that reactions such as N-dealkylation, S-oxidation, P-oxidation, alcohol oxidation and dehydrogenation that proceed via a mechanism initiated by one-electron oxidation or hydrogen abstraction are amenable to electrochemical oxidation [8].

There are a number of therapeutic drugs based on simple sulfur chemistry. Our research in the past has focused on investigating the mechanism of S-oxidation of organosulfur compounds [9,10]. Extensive studies from our laboratory have shown that nearly every organic sulfur compound presents a unique reactivity and no generic oxidation pathway can be easily derived. Sulfur atom has been implicated as a site of bioactivation, resulting in formation of reactive and potentially toxic metabolites from bioactivation of sulfur containing drugs [11]. Many of the adverse reactions produced by penicillamine and other compounds with an active sulfhydryl group form a distinctive pattern when viewed as a class. Alterations in taste perception, mucocutaneous lesions, proteinuria due to immune-complex membranous glomerulopathy, and pemphigus are adverse reactions that have been encountered with all of these compounds. Classic examples include the thiol, captopril, a still-used antihypertensive drug which comes with a black box warning and is associated with hepatotoxicity [12–14]. It can only be used in low doses and after careful selection of patients to avoid idiosyncratic drug reactions and thus cannot be used for patients with severe hypertension [13]. Troglitazone, a

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thiazolidinedione, developed for diabetes mellitus type 2, had to be withdrawn from the market because it caused severe liver injury. Thiazoles such as Sudoxicam were withdrawn in Phase III trials when it was already apparent that the drug was rife with IDR's while a closely-related drug, Meloxicam, has not been associated with any IDR's.

Methimazole, an antithyroid drug used in the treatment of hyperthyroidism, has been associated with idiosyncratic toxicity, characterized by skin reactions, leucopenia, agranulocytosis, aplastic anemia, hepatitis and cholestasis [15,16]. The relationship between idiosyncratic adverse reactions and reactive metabolites is not well established. There is circumstantial evidence, however, that reactive metabolites are involved in the onset of idiosyncratic adverse reactions [17,18]. We set out to investigate formation of any unexpected or reactive intermediates from MMI. The metabolites are electrophilic in nature, are reactive, and have the capacity to bind to nucleophilic cellular macromolecules which can then elicit an immune response. EC/LC/MS method offers the advantage of generating and isolating reactive intermediates in cellular matrix free environments, since matrix components will immediately bind to the reactive metabolites resulting in evasion of detection [19–21]. A range of metabolites have been suggested during oxidation of MMI. MMI metabolism is thought to occur through a P450-mediated process, resulting in ring scission, with further S-oxidation mediated through FMO to produce the tandem of sulfenic and sulfinic acids [22]. However, some sources reported that FMO sequentially monooxygenates intact methimazole to produce unstable methimazole sulfenic and sulfinic acids without ring scission [23].

The objective of this study was to use electrochemistry and mass-spectrometry to mimic oxidative metabolism in order to generate and characterize intermediates and products using electrospray ionization. Various electrochemical methods using modified electrodes to enhance catalytic oxidation of MMI have been developed and used for the determination of MMI at sub-micromolar detection limits using cyclic voltammetry [24–26]. While most of these studies were focusing on detection and determination of MMI using electrochemical techniques, our study is designed to explore metabolic fate of MMI. The electrochemical oxidation of thiol compounds such MMI is complicated by large anodic over potential and poor voltammetric signals. In the coulometric cell used in this study, the eluent flows through the electrodes rather than by the electrodes as in convectional cells. This maximizes the contact of the electro-active compound in solution with the electrode surface, ensuring that diffusion and convection controlled process do not limit the electrochemical oxidation of the compound [27].

2. Experimental section

2.1. Reagents

Reagent grade methimazole, reduced glutathione, N-acetylcysteine and methoxylamine were purchased from Sigma Aldrich (USA) and were used without further purification. Water solutions for electrochemical oxidation were purified using a Barnstead Sybron Corp. water purification unit capable of producing both distilled and deionized water (Nanopure). ICPMS, was used to evaluate concentrations of metal ions in the reagent water. ICPMS results showed negligible amounts (< 0.1 ppb) of copper, iron and silver ions with approximately 1.5 ppb of cadmium and 0.43 ppb in lead as the highest metal ion concentrations. In previous experiments from our lab, no discernible differences in kinetics data had been obtained between experiments run with chelators (EDTA, deferroxamine) and those run without, and so all experiments were carried out without the use of chelators. Solvents used for electrochemical oxidation and mass spectrometry were HPLC grade.

2.2. Instrumentation

Electrochemical oxidations were performed using Thermo Scientific Dionex 5150 Synthesis Cell™ equipped with a flow through graphite working electrode, solid state palladium reference electrode and a Palladium counter electrode (see Fig. 1). The cell potential was controlled using Thermo Scientific Dionex Coulochem III electrochemical detector. The cell outlet was interfaced into a mass spectrometer inlet for on-line analysis using PEEK tubing. To prevent electrical damage to the detector and cell; as well as shock, the synthesis cell and the detector were decoupled from the high voltage of the mass spectrometer using a high voltage decoupling union kit. Samples were infused through a syringe pump at a flow rate of 10 μL/min for on-line experiments. Mass spectra of the electrochemical oxidation products, were acquired on a high-resolution ($m/\Delta m = 30{,}000$) Thermo Scientific LTQ-Orbitrap Discovery mass spectrometer (San Jose, CA) equipped with an electrospray ionization source. The MS ESI source parameters were set as follows: spray voltage (kV), 2.5 in negative mode and 4.5 in positive mode; spray current (μA), 1.96; sheath gas flow rate, 20; auxiliary gas flow rate, 0.01; capillary voltage (V), −16; capillary temperature (°C), 300; and tube lens (V), −115. Detection was carried out in both the negative ionization mode and positive (−ESI) for 4 min. The detection parameters were set up as follows: Analyzer; FTMS, positive and negative polarity; mass range; normal, resolution; 30,000, scan type; centroid.

3. On-line EC/ESI-MS electrochemical oxidation of methimazole

Experiments were carried out in acidic, neutral and alkaline medium. 100 μM MMI were dissolved in acidic medium which consisted of 20% methanol with 80% 20 mM formate buffer (pH 2.75). For neutral medium, a combination of 20% methanol with 80% 50 mM phosphate buffer (pH 7.4) was used. Alkaline media utilized a 20% methanol with 80% 20 mM ammonium buffer solution (pH 10.2). A 500 μL sample was infused through the electrochemical cell at a flow rate of 10 μL/min before the cell was turned on.

With the cell turned on, species generated from oxidation in each medium were monitored on the mass spectrum generated on-line. The potential was changed manually on the front panel of control module of ESA Coulochem III Electrochemical detector from 100 mv to 1200 mv. Each scan acquisition lasted for four minutes. The optimum potential was determined to be 600 mV vs Pd solid state reference. Higher potentials resulted in a large number of unidentifiable fragments.

4. Choice of supporting electrolyte and effect of pH

Larger currents were attained in phosphate and in ammonium buffers. The same species obtained at pH 7.4 in phosphate were also obtained in more alkaline media of pH 10.2. Phosphate buffer, however, was not used for subsequent experiments. This was because phosphate buffers are non-volatile and can clog the MS inlet capillary, and hence ammonium buffer was chosen as the suitable supporting electrolyte in subsequent experiments. In acidic medium the dimer was the predominant metabolite.

5. Assessment of stability, reactivity of metabolites and off-line synthesis

In order to perform off-line synthesis of intermediates, stability of the electrochemically generated species were monitored over a period of 30 h. To collect samples off-line, samples were infused at 40 μL/min, with potential maintained at 600 mV. The samples were then collected in vials with one batch kept at room temperature and the other batch kept at −20 °C. Samples were analyzed by ESI-MS. Relative abundances of the intermediates at m/z 129 and 146 decreased significantly after 26 h. for both the samples that were kept frozen at −20 °C and those maintained at room temperature. There was a 75% decrease in abundance of these intermediates over this time duration. In addition, stability of MMI was also monitored and was found to be stable for prolonged periods in solution.