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Article

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Electrochemical versus Enzymatic in Vitro Oxidations of 6-propyl-2-thiouracil: Identification, Detection and Characterization of Metabolites

by

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ABSTRACT: 6-Propylthiouracil, PTU, is a well-known antithyroid drug that has been the mainstay of treatment of Graves disease. It is, however, also associated with liver toxicity and idiosyncratic toxicity. These toxicities are generally associated with metabolites derived from its bioactivation. In this manuscript, bioactivation of PTU was studied via two separate techniques: electrochemical oxidation and through the use of human liver microsomes. The aim of this work was to compare the bioactivation products of these two techniques. The electrochemical technique was studied on-line with a mass spectrometer, EC/ESI/MS. The microsomal oxidations were studied in tandem with liquid chromatography. The EC/ESI/MS technique was devoid of the normal reducing biological matrix prevalent in microsomal incubations. The predominant product at 400 mV was the dimeric PTU species with negligible formation of other metabolites. At higher potentials, complete desulfurization of PTU was observed with formation of sulfate. No sulfonic acid was observed, suggesting that the cleavage of the C – S bond was effected at the sulfinic acid stage, releasing a highly reducing sulfur species which is known to give rise to genotoxicity. The microsomal oxidations, surprisingly, showed formation of the unstable sulfenic acid, the S-oxide. Further incubation showed both the sulfinic and sulfonic acids. None of the systems showed any adducts with nucleophiles such as glutathione, showing that none of the reactive metabolites were stable enough to be adducted to nucleophiles in both the biological matrix and the electrochemical oxidizing environment.

INTRODUCTION

6-propyl-2- thiouracil (PTU) is an antithyroid drug that was introduced close to seventy years ago.[1] This drug is effective in the treatment and management of Grave's disease, an autoimmune disease mediated by thyroid-stimulating immunoglobulins; resulting in hyperthyroidism, which is over production of thyroid hormones.[2-8] It works by inhibiting the activity of thyroid peroxidase and blocks the conversion of thyroxine (T4) to triidothyronine (T3).[9-11] The action of PTU may partly depend on its ability to function as a thiol as shown by tautomer forms below.

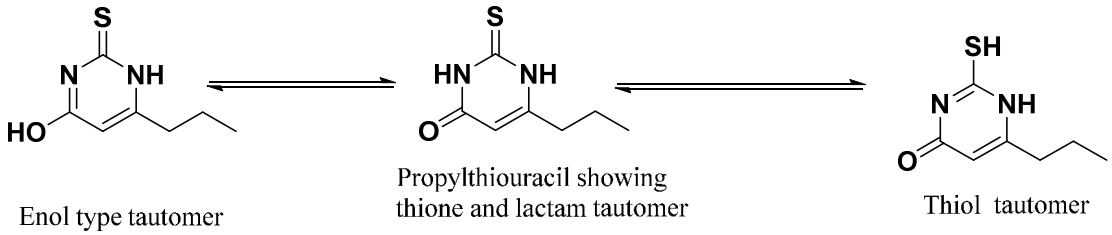


Figure 1: Tautomers of PTU

Although effective, PTU has been associated with rare but severe idiosyncratic toxicity, characterized by skin reactions, agranulocytosis, aplastic anemia, hepatitis and cholestasis. [1, 12-16] PTU is identified as being the third most frequent cause of drug-induced liver transplant in the United States.[17] In 2008, a panel of experts extensively reviewed adverse events reports and case reports of PTU-induced liver failure and deaths; they recommended that PTU should not be used as first line of therapy in

Grave's disease.[18] In 2009 the Food and Drug Administration (FDA) issued a black box warning about severe liver injury associated with treatment using PTU.[19, 20] Hepatotoxicity of therapeutic drugs is not a new phenomenon in drug therapy. A number of drugs have already been withdrawn from the market after cases of severe toxicity were noted.[21, 22] Bioactivation of drugs resulting in reactive metabolites is considered as the initial step towards drug-induced organ damage[23]. Presumably, the hepatotoxicity associated with PTU is due to its reactive metabolites in the liver, these could be radicals or electrophiles. However, as of now, there are no reports on PTU reactive metabolite(s) formation in the liver and the role of formed intermediates in the hepatotoxicity induced by this drug is ambiguous[24]. There has been a variety of metabolites observed from PTU metabolism. Thiols are metabolized to sulfenic acids, which are unstable and readily undergoes oxidation to their stable oxides; sulfinic and sulfonic acids or combine with nucleophiles. Whereas there have been some studies on electrochemical oxidation of PTU, a lot of these studies were focusing on modifying electrodes to enhance electrocatalytic oxidation of PTU using cyclic voltammetry and differential pulse voltammetry.[25, 26] Electrocheimcal oxidation of PTU is complicated by large overpotentials. Modified electrodes have been used due to their catalytic properties that lower the anodic overpotential and enhance the rate of electron transfer for the electrochemical oxidation of PTU. In the present work, the oxidation of PTU is investigated using an electrochemical flow through cell that uses porous graphite working electrode and combines electrochemistry and mass spectrometry. Metabolites generated by electrochemical oxidation were compared to those that were identified

from standard microsomal oxidation. Slight differences were noted in the identity of the metabolites.

MATERIALS AND METHODS

Chemicals. Reagent grade 6-propyl-2-thiouracil, PTU, reduced glutathione, N-acetylcysteine, acetic acid and ammonium acetate were obtained from Sigma Aldrich and were used without further purification. Ammonia, methanol, acetonitrile, phosphate monobasic and phosphate dibasic were from Fischer Chemicals. Water for all experiments was purified using a Barnstead Sybron Corp. water purification unit capable of producing both distilled and deionized water (Nanopure). Solvents used for electrochemical oxidation and mass spectrometry were HPLC grade.

On-line EC/ESI-MS Electrochemical Oxidation of Analytes. Experiments were carried out in alkaline medium. The analyte was initially dissolved in alkaline medium where it was more soluble. The media utilized a 20% methanol with 80 % 20 mM ammonium buffer solution (pH 10.2). Electrochemical oxidations were performed using Thermo Scientific Dionex 5150 Synthesis Cell^{TM} equipped with a flow-through graphite working electrode, solid state palladium reference electrode and a Palladium counter electrode (see Figure 2). 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 as described before.[27] 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 metabolites, were acquired on a high-resolution *(m/Δm* = 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.

Microsomal Enzymatic Oxidation. Human liver microsomes were obtained from Corning. A well-established protocol for microsomal incubation (provided from Corning upon purchase of the microsomes) was followed with minor changes to suit the experiment and substrates involved. The microsomal fractions were stored at -80 °C and thawed immediately before the experiment. The following quantities were used. Initially, 713 μL of purified water, 200 μL of 0.5 M potassium phosphate buffer pH 7.4, 50 μL NADPH regenerating system solution A (solution A contains NADP+ and Glucose-6-phosphate), 10 μL NADPH regenerating system solution B (Solution B contains Glucose-6-phosphate dehydrogenase) 2 μL substrate in solvent, making a final substrate concentration of 10μM, were combined before adding microsomes. When combined, solutions A and B provide the regenerating system for all NADPH-requiring oxidase assays. After warming in a 37 °C water bath for five minutes, the reaction was then

initiated by adding 25 µL (0.5 mg) liver microsomes and then was mixed and returned to a 37 °C water bath. After 60 minutes a 100 µL aliquot from the incubation was withdrawn and the reaction was terminated by adding 100 µL acetonitrile. This was then mixed and placed on wet ice followed by centrifuging at 10,000 Xg for three minutes. The supernatant from the protein pellets was withdrawn for analysis using LC/MS. Incubations without NADPH and microsomes were used as controls, to ensure that formation of metabolites was dependent on HLMs and NADPH.

LC-MS Analysis. The metabolites produced by incubation of PTU were collected and analyzed by LC-MS. The separation of the metabolites was achieved using a reverse phase Zorbax Eclipse Plus 3.5 µm, C8 column. Colum dimensions were as follows: 100 mm x 2.1 I.D. The flow rate was set at 200 µL/min and the injection volume was 20 µL/min. The gradient separation was performed by two solvents. Mobile phase A: 0.1% acetic acid in water and mobile phase B: 0.1% acetic acid in methanol. The applied gradient profile was 10% B for 5 minutes, followed by 30 % for another 5 minutes, and then 50:50 for two minutes and finally 90% B for the last six minutes. The column was operated at 35 °C. The MS based detection was carried out it positive ion mode using both FTMS and ITMS detection.

RESULTS AND DISCUSSION

Electrochemical Oxidation of PTU. Electrochemical oxidation of PTU was carried out using the EC/ESI/MS arrangement shown in Figure 2.

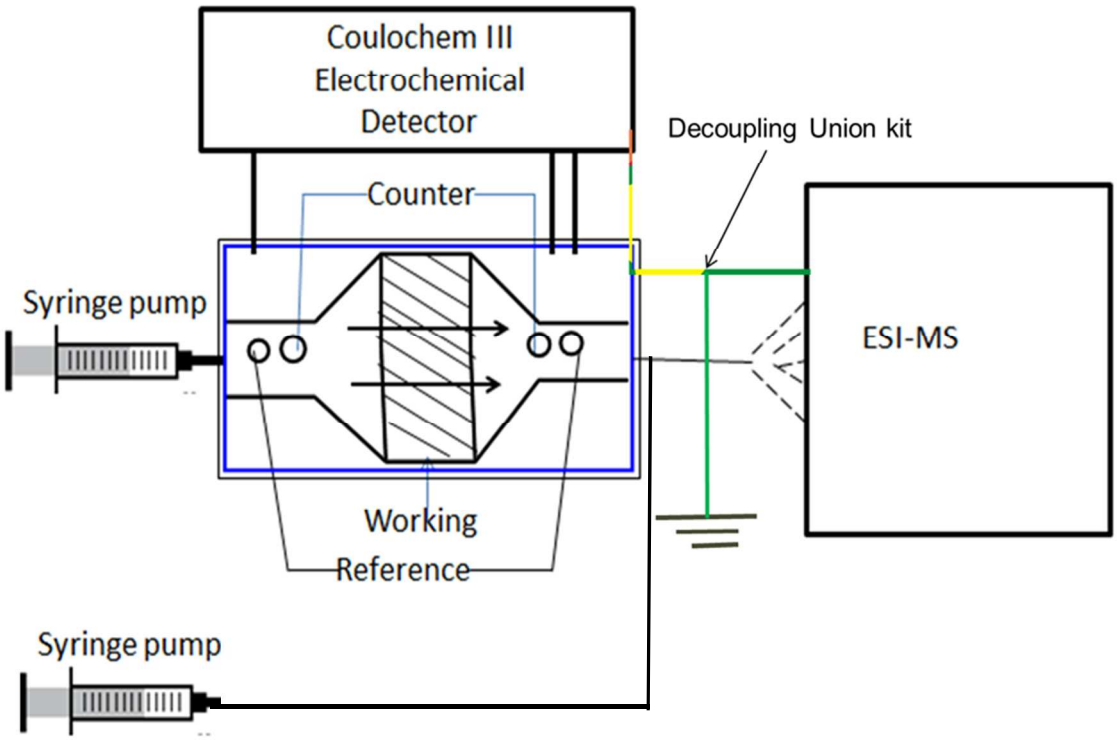


Figure 2. Schematic of EC/ESI –MS system. Coulochem III Electrochemical Detector controls potential of the flow through cell. Samples were infused at 10 µL/min.

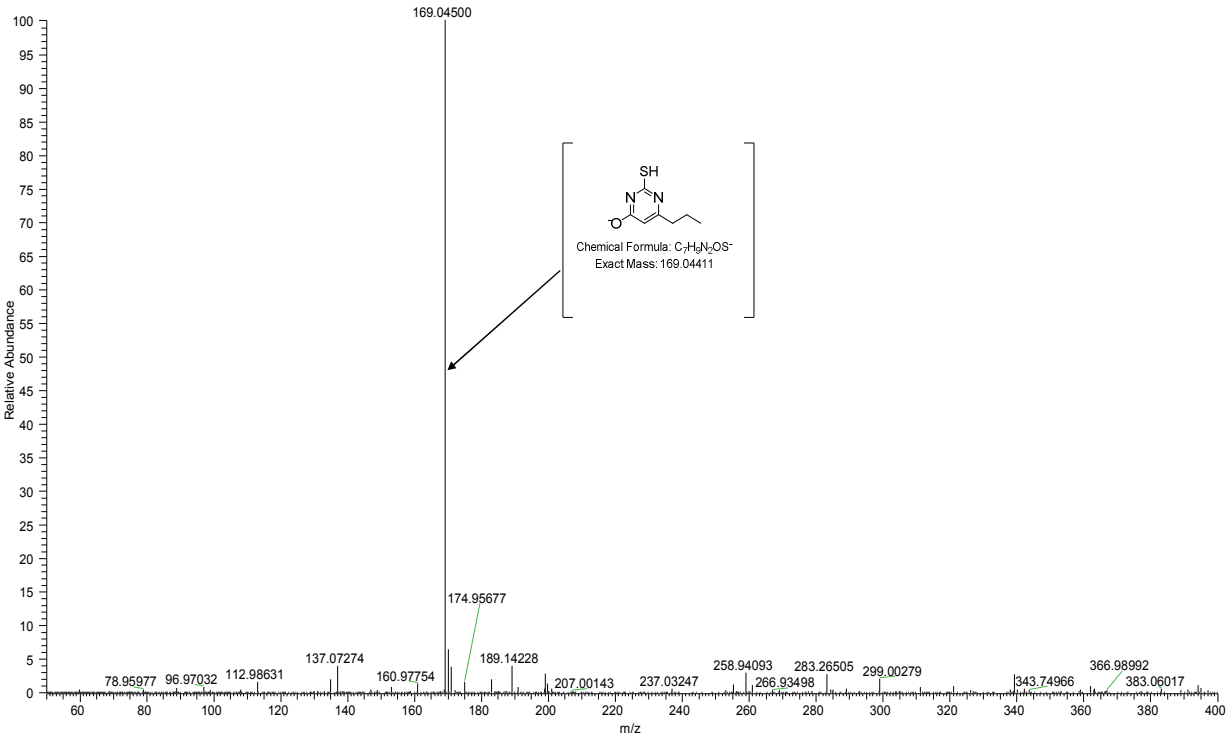


Figure 3 ESI (-) MS, with cell turned off 0 mv obtained for 0.1 mM PTU in 20 mM ammonium phosphate buffer, 20% MeOH, pH 10, Flow rate 10 µL/min.

Control experiments were initiated with the cell turned off at zero potential. Figure 3 clearly shows a clean ESI-spectrum of PTU, that was obtained in the negative mode. The only peak that is evident from the spectra above is the substrate PTU with m/z 169.04

The presence of a single peak from the solvent electrolyte mixtures shows that there were no other reactions or contaminants prior to the electrochemical oxidation. Contaminants as well as other reactions in the electrolyte mixture are usually confused with modifications that might take place on the compound of interest. As such, any

products that emerged when the reaction was initiated by applying potential could thus be attributed to transformations due to oxidation of PTU in the electrochemical cell.

When the cell was turned on, the voltage was gradually increased with a ramp of 100 mV at each step, each time. Emergence of additional peaks were evident at 400 mV. This potential was enough to initiate oxidation of PTU to a disulfide and sulfinic acid, as shown in Figure 4. PTU disulfide was the dominant peak in both acidic and alkaline medium. However, in acidic medium there were other spurious peaks that could not be easily assigned. This demonstrates the difficulty in choosing suitable solvents for EC-MS system, to avoid ion suppression.

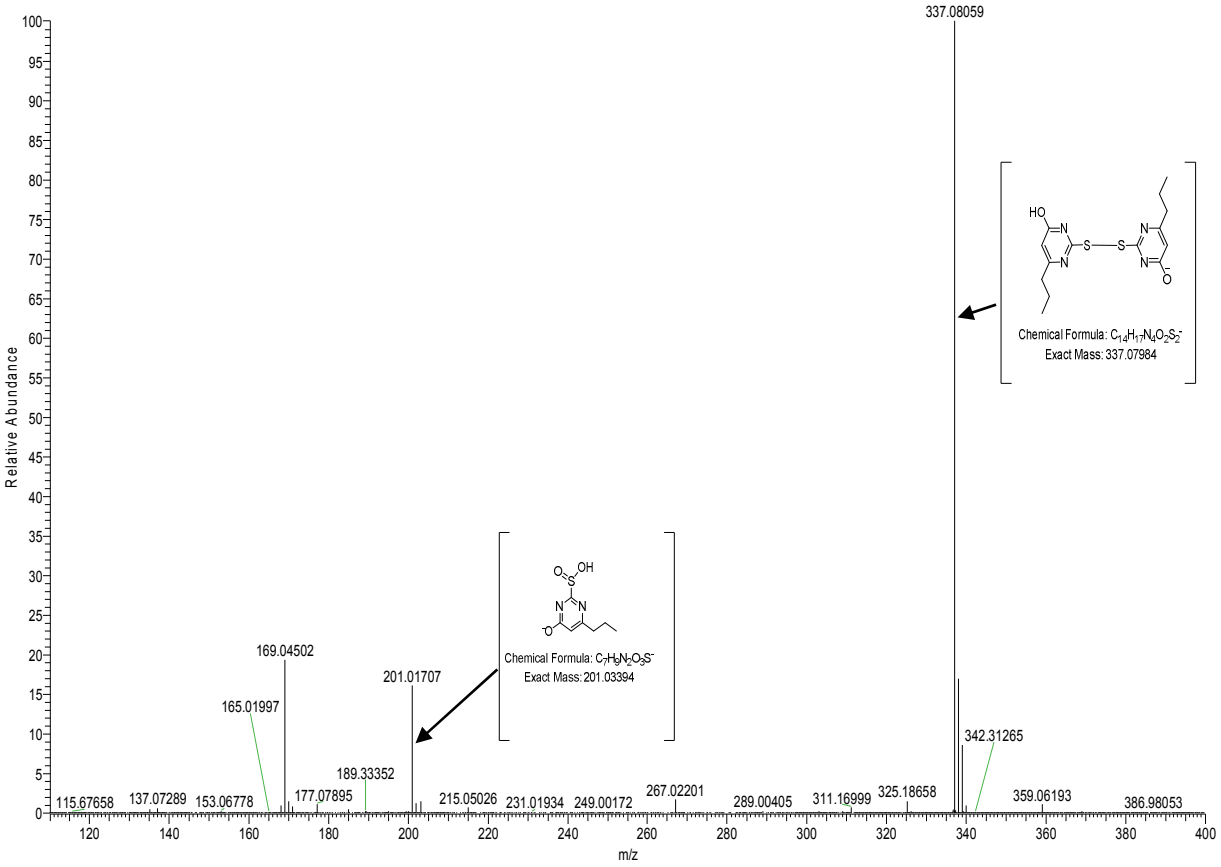
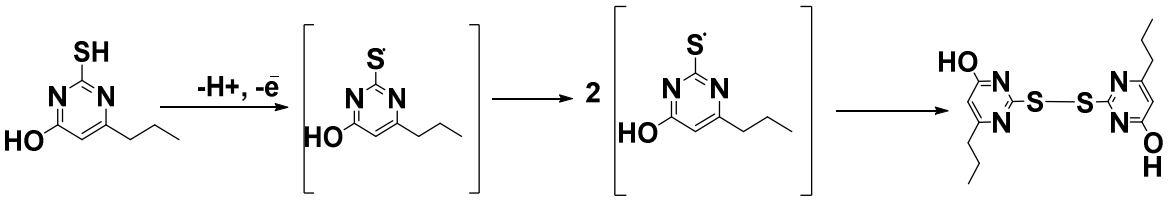


Figure 4. ESI-MS spectra generated with the cell potential at 400 mV.

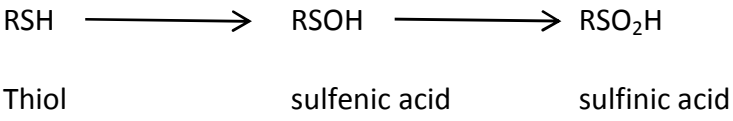
Figure 4 shows a strong peak of PTU-disulfide, which is the major product of oxidation at the given potential of 400 mV. Dimeric species from thiols are usually unreactive compared to parent thiols, hence they accumulate. Surprisingly, the electrochemical oxidation of PTU was achieved at very low potentials when compared to what has been reported previously in literature.[28] Sartori *et al*, reported higher electrode potentials of 1.42 V for oxidation of PTU to its dimer using cyclic voltammetry.[28] Electrode potential, (E_m), is pH-dependent, and has been shown to decrease at approximately 0.06 V/pH for a simple thiol such as glutathione. Therefore,

as the pH is increased, the potential required to effect oxidation decreases.[29] The data below suggests the possibility of two competing reaction pathways. Thiol oxidation can proceed through two competing pathways. Abstraction of a single electron is relatively easy leading to thiyl radicals. The two electron pathway result in formation of a sulfenic acid. These two intermediates participate in further reactions resulting in metastable products.[30] Thus the thiyl radical might initiate chain reactions resulting in disulfides. This is a coupled proton-electron transfer process resulting in a radical that dimerizes to a disulfide as shown in Scheme 1.

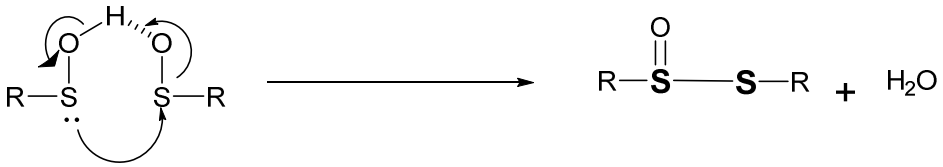


Scheme 1 Proposed oxidation scheme for PTU and its dimerization to a disulfide compound.

There is a relatively weak peak at *m/z* 201 which is attributable to a sulfinic acid. In theory one would expect to observe the oxides of PTU in the mass spectrum. Thiols are known to undergo oxidation through the sulfenic acid which further oxidizes to more stable sulfinic and sulfonic acids, respectively[31, 32]



However, there is no evidence of formation of sulfenic acid, or the S-oxide. The sulfenic acid may disproportionate to its meta stable S-oxide. Sulfenic acids exhibit potent electrophilic and relatively weak nucleophilic reactivity.[33] Thus, in small molecules such as PTU, this dual behavior can lead to self-condensation in which one sulfur atom functions as a nucleophile and the second as an electrophile to yield a thiolsulfinate ester:



The self-condensation reaction is facilitated by intermolecular hydrogen bonding. At a pH > 8, which is the case in this experiment, the thiolsulfinate esters disproportionate to form salts of sulfinic acid and disulfide.[34] This reaction can thus explain both the presence of sulfinate and the dimer observed in Figure 4. Trapping experiments were also conducted in PTU oxidations. Unlike in oxidation of methimazole,[35] there were no conjugates that formed between PTU and nucleophiles. Presumably, the reaction proceeds through formation of the dimer, the dimer then would further be oxidized to sulfinic acid. Regardless of which pathway dominates, both mechanisms have potential to result in reactive intermediates

Effect of Oxidation Potential. An analysis of ESI spectrum collected at higher oxidative potentials of 800 mV shows two additional peaks that were observed on the mass spectrum. Figure 5 shows that sulfur was fully oxidized, from -2 to +6, to yield sulfate (SO_4^{2-}) shown, with m/*z* 96. Effluent from the cell was reacted with barium chloride, resulting in formation of $BaSO_4$. There was no evidence of formation of PTU-sulfonic acid from the ESI mass spectra. Hydrolysis of sulfinic acid, should result in the cleavage of the C-S bond to give a urea-type residue, $R_1R_2C=O$ and an unstable sulfur species HSO_2^- . HSO_2^- is readily oxidized to bisulfite (HSO_3^-).[36]

$$R_1R_2CSO_2H + H_2O \rightarrow R_1R_2C=O + HSO_2^- + H^+ \quad \text{R2}$$

$$HSO_2^- + H_2O \rightarrow HSO_3^- + 2H^+ + 2e- \quad \text{R3}$$

HSO_3^- is, in turn, easily oxidized to sulfate. One would expect a significant amount of the urea-type organic residue if hydrolysis was the major pathway. However, there are only traces (of propyl uracil) at m/z 153 and an unidentified metabolite with m/z 135. If this organic residue is not stable, it would further hydrolyze through ring opening giving smaller fragments. A decrease of 32 amu is apparent from the initial mass of substrate. The exact mechanism from the sulfinic acid to sulfate and an organic residue can only be speculative, but involves no further oxidation past the formation of sulfate. Non solvent assisted cleavage of C-S bond which is either heterolytic or homolytic cleavage should result in loss of sulfur as sulfite and sulfite radical anion respectively, from sulfinic acid[37]. The organic residue observed is shown with the peak

at *m/z* of 137 thus this is 6-propyl-2, 3-dihydropyrimidin-4(1H)-one. The spectrum below still shows the dimer at m*/z* 168 which is the symmetrical PTU dimer.

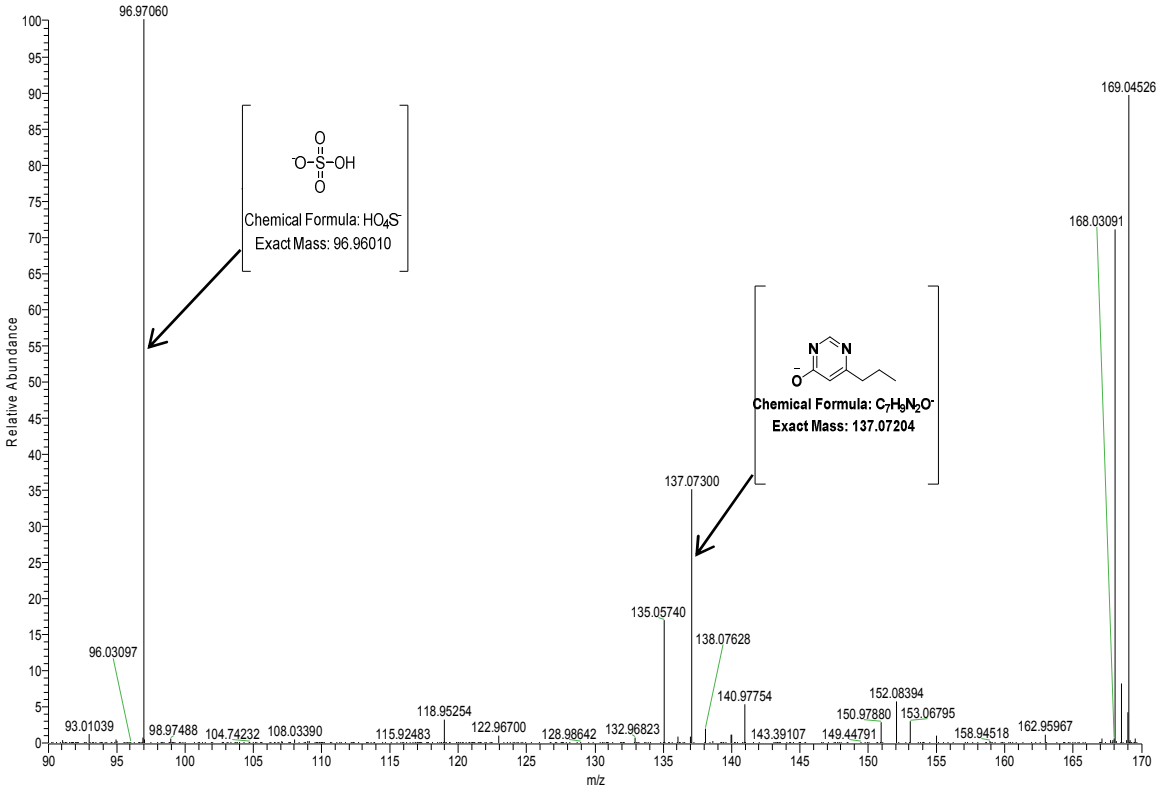


Figure 5. ESI-MS spectra generated with the cell potential at 800 mV.

The one electron pathway, resulting in thiyl radicals, appears to be the most dominant mechanism here. Radicals play a very important role in induced oxidative stress and subsequent liver injury. Waldhauser and co-workers investigated the oxidation of PTU in activated neutrophils.[38] PTU was observed to be oxidized by myeloperoxidase[39] giving sulfite as one of the products, as indicated above, which can subsequently be oxidized to sulfate. If oxidation of PTU, proceeds through

desulfurization, forming sulfite radical, one would expect the sulfite to undergo further reactions which may or may not lead to reactive metabolites. Apparently it has been shown that sulfite is oxidized by myeloperoxidase to free radicals. These prooxidant radicals, such as the reactive sulfur trioxide ($SO_3^{\bullet-}$), peroxymonosulfate ($-O_3SOO^{\bullet}$)[40] and sulfate ($SO_4^{\bullet-}$) anion radicals, can damage proteins and oxidize them to protein radicals, with consequences of allergic reactions[41]. Whereas some studies reported that there have been no reactive metabolites formation in the liver, however, in our studies in this manuscript, some intermediates e.g., 6-propyl-2,3-dihydropyrimidin-4(1H)-one , (propyluracil) and other unidentified metabolites, with prooxidant radicals were echoed in the electrochemical system. Some of the intermediates have been proposed but have not been observed in previous *in vivo* and in *vitro* studies. Radicals can also generate toxic effects through oxidative stress without forming covalent adducts with biomolecules.[42] This forms the basic framework for the danger hypothesis that leads to idiosyncratic toxicities.

***In Vitro* Enzymatic Oxidation of PTU.** The metabolism of PTU was studied on the basis of accepted standard *in vitro* techniques involving incubation of target compound with liver microsomes. The experiments reported here were performed using human liver microsomes. The results obtained from the liquid chromatography separation with mass spectrometry detection are shown below.

Oxidation of PTU by microsomes was complex. Identification of the products from metabolism was complicated by the presence of the biological matrix and a range of possible metabolites. Although not quite abundant, Figure 7 shows spurious peaks on the ESI-spectrum, some of which were unidentified. However, this observation does not really point to an inferior technique. Instead, it does show that a variety of metabolites are possible from biological oxidations versus EC-MS

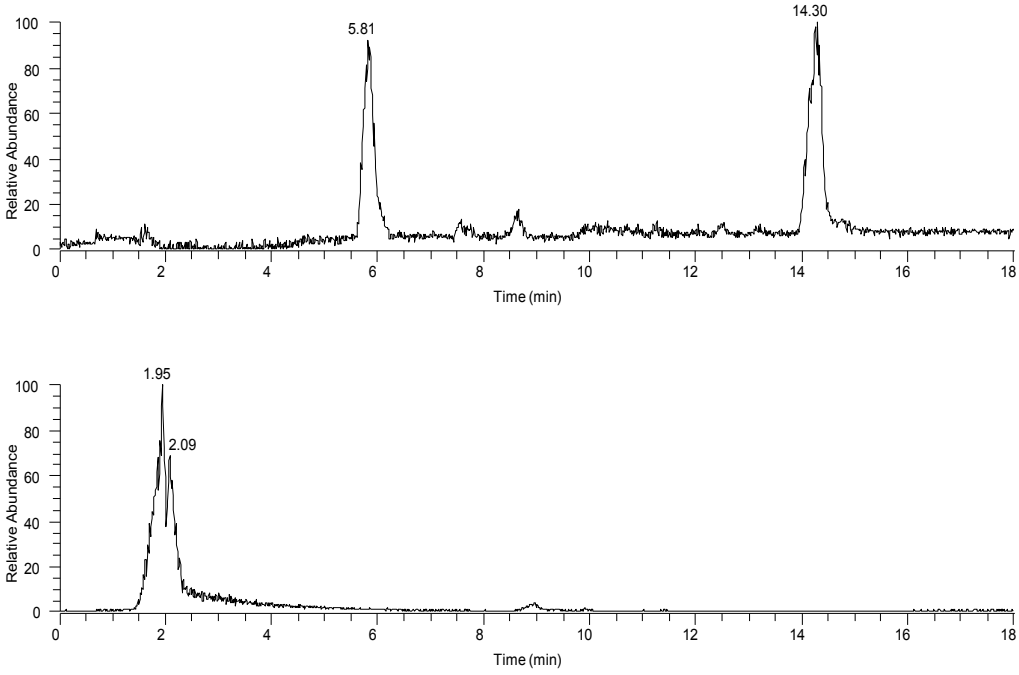


Figure 6. LC-MS chromatograms form metabolites generated by incubation with human liver microsomes (HLM).

Figure 6 shows the chromatogram, extracted from ion filtering using Xcalibur software. Retention time of 14.30 shows the unconverted substrate, PTU with m/z 171.13 shown in Figure 7. Another peak on the chromatogram, with retention time of 5.81 was associated with the unconverted substrate; this could be due to tautomer of PTU, resulting in a more polar ion form of PTU. This analysis is further complicated by appearance of another small peak that co-eluted at this same retention time. The peak had a jump of 16 amu from the substrate, thus [M + O + H] with at m/z 186.22, the putative PTU S-Oxide. The relatively low abundance of the peak, appears to show a transient molecule that is derived from unstable sulfenic, moreover it is fleeting towards sulfinic and sulfonic acids. In this biological matrix, the reactive sulfenic acid can react with protein components of microsomes. Usually, after centrifugation, the protein pellet will sediment and is not analyzed in LC-MS analysis, hence a diminished peak of the remaining metastable S-oxide can be observed. Its polarity also fits very well to the assigned retention time.

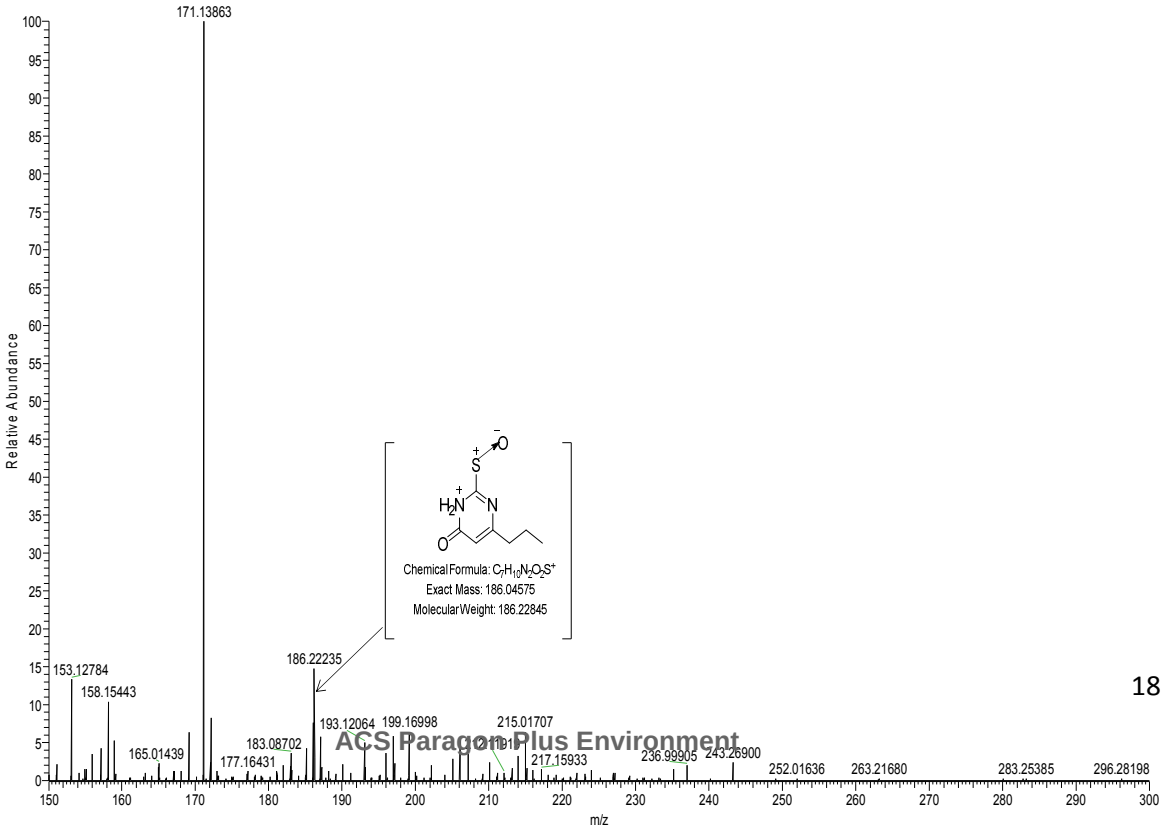
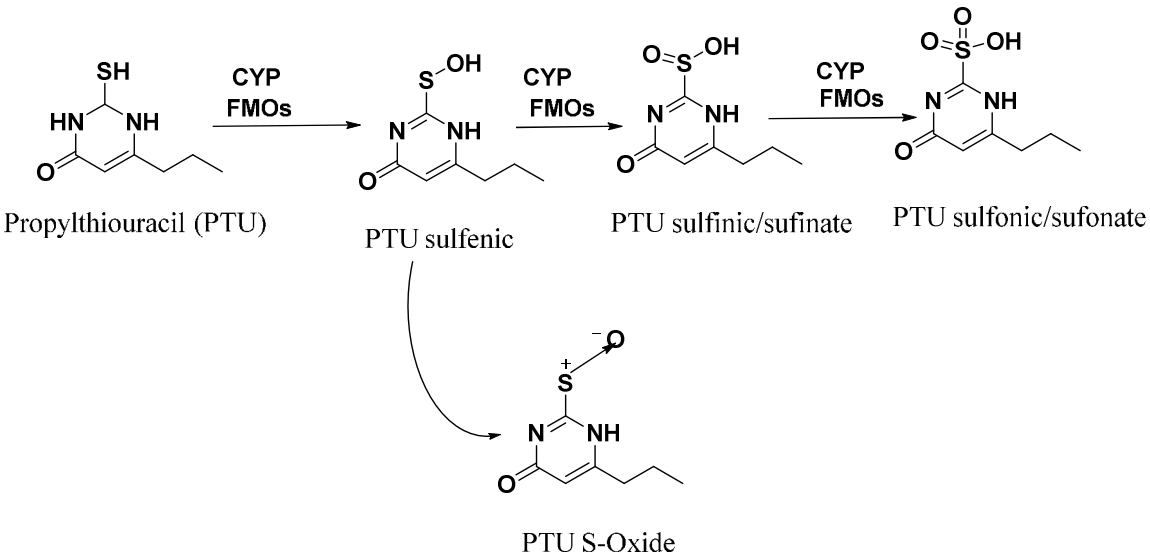


Figure 7 ESI (+) mass spectrum showing product of oxidation from microsomal incubation.

Although hepatotoxicity due to PTU has been known, it is surprising that there have not been many studies on oxidation of PTU using microsomes. Most of the studies conducted used peroxidases as oxidants, and as such there have not been known reactive metabolites produced from microsomes. Studies on oxidation of PTU with activated neutrophils showed that PTU-disulfide, PTU-sulfite/sulfinic acid and PTU-sulfate/sulfonic acid were major products of oxidation.[39]. The toxicity of thionamides, such as ethionamide has been associated with their bioactivation to reactive S-Oxides.[43, 44] Therefore, hepatotoxicity due to PTU may also arise from the observed S-oxide, this electrophilic metabolite it has the capacity to bind to liver cellular macromolecules resulting in drug in damage to cells.

Mechanism of Oxidation. Metabolism by microsomes involves primarily the

flavin-containing monooxygenases (FMO's) and the Cytochromes P450 group of enzymes. FMO's are a family of drug-metabolizing enzymes that use FAD, NADPH and molecular oxygen to catalyze the oxygenation of a large number of xenobiotics containing 'soft' nucleophiles such as sulfur, phosphorus and nitrogen.[45, 46]



Scheme 2 Proposed reaction scheme for PTU metabolism in microsomes.

The scheme above shows a simple mechanism for oxidation of PTU in microsomal incubations. FMOs convert xenobiotics into polar metabolites by adding oxygen so that they can subsequently be easily eluted through the kidneys.[47] While initially thought to be a single enzyme; FMO's are now known to contain at least 11 isoforms, with 5 of them having been characterized to date.[48, 49] It has been reported

that FMOs oxidize thionamides and thiourea functional groups to intermediate metabolites of sulfenic (RSOH) and sulfinic (RSO_2H) acids which are responsible for deactivating CYP450 enzymes.[50, 51] As mentioned earlier on (*vide supra*), sulfenic acid is a very unstable intermediate, giving rise to the metastable S-oxide. In presence of excess oxidative power, the S-Oxide and sulfinic acid will further be oxidized to sulfonic acid, as shown in figure 8.

The standard procedure for trapping reactive metabolites was also carried out using glutathione and n-acetyl cysteine as trapping agents. There were no detectable adducts/conjugates in metabolic oxidation reactions of PTU. Sulfenic acid precedes formation of S-oxide and is very reactive and is able to react with any nucleophile, especially thiols. However, even if the intermediates were formed, their conjugation with nucleophiles is not always facile, sometimes it requires enzymes, and such as glutathione transferase to assist in conjugation.[52]

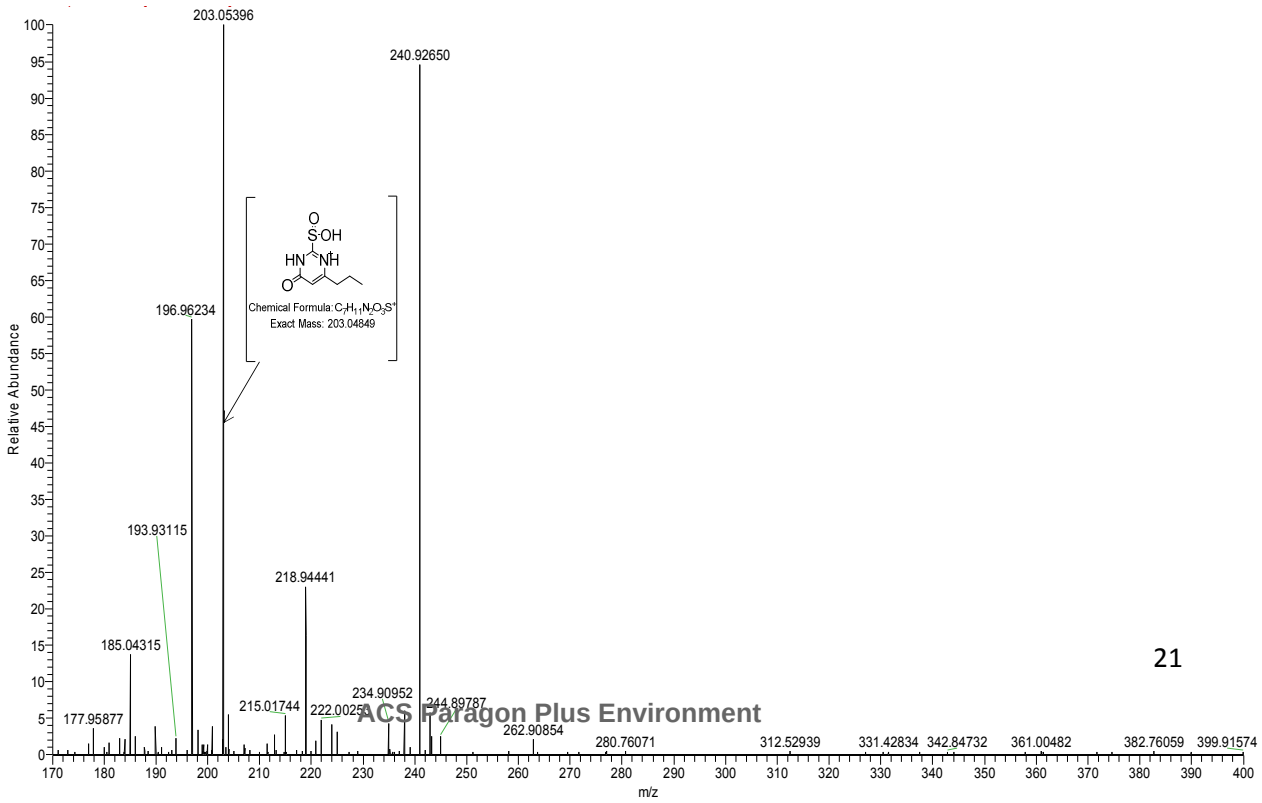


Figure 8 ESI (+) MS spectra showing sulfinic acid and sulfonic acid as products of oxidation of PTU.

PTU is less polar as compared to its products of oxidation, which co-eluted within 1.95 and 2.06 minutes. Figure 8 shows propylthiouracil sulfinic acid (PTU-SO_2H) m/z 203.04 and propylthiouracil sulfonic acid (PTU-SO_3H) m/z 218.94 and its sodium adduct m/z 240.09 as the major products of microsomal oxidation. These are stable oxides for oxidation of PTU. PTU-sulfonic acid/sulfonate, at m/z 218.94, [M +3O +H^+] and 240.09, [M +3O +Na^+] exist in zwitterionic forms before being adducted to proton or sodium respectively. Microsomal oxidations were carried at a pH of 7.4, close to neutral pH, and thus this environment can afford such neutral structures. Molecules with such structures have been observed from X-ray data. Chigwada *et al*,[53] showed the existence of this putative sulfonic acid zwitterion for tetra methyl thiourea sulfonic acid (TTTU-SO_3^-) (see Figure 9). Figure 9 shows the putative molecular ion as observed in ESI spectrum at m/z 240.02.

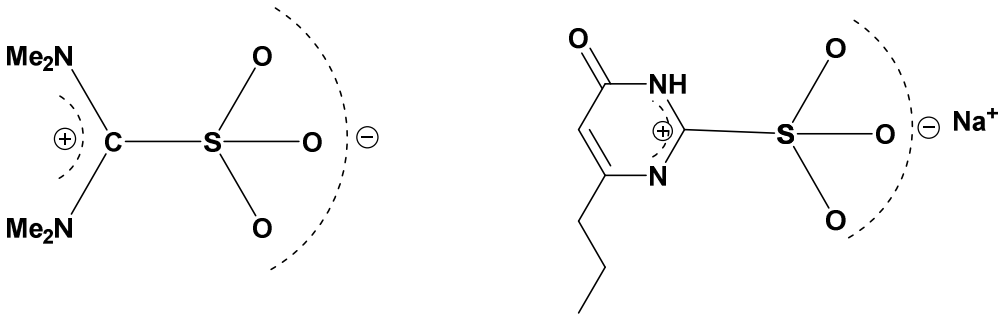


Figure 9. Putative zwitterion structures. On the left is the X-ray structure determined by Chigwada et al in their seminal manuscript, on the right is the zwitterion expected for PTU.

Figure 8 shows an undefined and unidentified metabolite at m/z 196.96. This highlights some of problems that arise from microsomal oxidations. The matrix contains different endogenous material which can co-elute with compounds of interests. Contrary to this, it also shows diversity of metabolites that can be obtained from microsomal oxidations.

Comparison of Electrochemical and Biological Metabolism. There were variations in the nature and type of metabolites that have been observed from electrochemical oxidation of the PTU compared to those observed and reported in biological studies .[54] Both Figures 7 and 8 show some unidentified peaks e.g., peak with m/z 196 could not be assigned to any metabolite in the mixture. The electrochemical process is a 'cleaner' oxidation environment than that afforded by microsomes. Thus the electrochemical oxidation platform involves the most easily oxidizable part of the molecule without any regard to other parameters present in bimolecular activations

such as stereochemistry. Oxidation of thiols through formation of their disulfides is relatively easy, compared to two electron process for the formation of an S-oxide. Contrary to EC-MS system, microsomal oxidations show non-competing reaction pathways. Successive formation of sulfur oxo-acids is through the well-established two-electron transfer mechanism of FMO S-oxygenation. Microsomes contain CYPs as well, which participate in functionalization reactions, and as such one would expect a variety of metabolites. However, there are some other factors that might prevent this; e.g., thionamides are known to be enzyme inhibitors,[55] and this has a large bearing on nature of products that can be obtained from their biological oxidation using microsomes. The kinetics for the EC-MS, two electron mechanism are quite sluggish. Electron withdrawing groups such as oxygen in the ring for PTU may reduce the nucleophilicity of S atom on the thiolate ion, making it difficult to abstract a second electron. A comparison of EC-MS oxidations to microsomal oxidations shows there is a physiological gap between the isolated microsomes and electrochemical cells. However, there were similarities in type of metabolites obtained from EC-MS with those obtained using peroxidase enzymes. Differences in metabolites may be attributed to experimental conditions.

CONCLUSION

Bioactivations, mechanism, products and intermediates of sulfur-based drugs have always been difficult to determine. PTU oxidation is complex, characterized by both free radical and two electron process mechanisms in electrochemistry and microsomal

oxidations respectively. PTU oxidation resulted in a range of metabolites. The absence of conjugates in either system, does not preclude formation of reactive metabolites. Reactive metabolites, by their nature, are short-lived, and hence elusive. Their lifetimes are very small. However, results show that an S-Oxide, which is electrophilic and hepatotoxic, is produced in liver microsomal incubations. Toxicity of PTU may be attributed to this reactive metabolite. Moreover, the mechanism entails two pathways which may be involved in toxicity attributed to PTU. EC-MS technique complements the microsomes, and is informative in evaluation of toxicities from drugs, as it gives an alternative explanation to observed physiological effects.

Acknowledgments:

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REFERENCES

1. Rivkees, S. A.; Szarfman, A. Dissimilar Hepatotoxicity Profiles of Propylthiouracil and Methimazole in Children. *Journal of Clinical Endocrinology & Metabolism* **2010,** *95*, 3260-3267.

2. Abraham, P.; Acharya, S. Current and emerging treatment options for Graves' hyperthyroidism *Ther. Clin. Risk Manag.* **2010,** *6*, 29-40.
3. Abraham, P.; Avenell, A.; Park, C. M.; Watson, W. A.; Bevan, J. S. A systematic review of drug therapy for Graves' hyperthyroidism. *Eur. J. Endocrinol.* **2005,** *153*, 489-498.
4. Abraham, P.; Avenell, A.; Watson, W. A.; Park, C. M.; Bevan, J. S. Antithyroid drug regimen for treating Graves' hyperthyroidism. *Cochrane. Database. Syst. Rev.* **2005,** CD003420.
5. Abraham, P.; Avenell, A.; Watson, W. A.; Park, C. M.; Bevan, J. S. Antithyroid drug regimen for treating Graves' hyperthyroidism. *Cochrane. Database. Syst. Rev.* **2004,** CD003420.
6. Cooper, D. S. Antithyroid drugs *N Engl. J. Med.* **2005,** *352*, 905-917.
7. Garcia-Mayor, R. V.; Larranaga, A. Treatment of Graves' Hyperthyroidism with Thionamides-Derived Drugs: Review. *Medicinal Chemistry* **2010,** *6*, 239-246.
8. Schott, M. Hyperthyroidism. *Internist* **2013,** *54*, 315-326.
9. Lee, J. H.; Park, S. H.; Koh, D. G.; Suh, B. K. Thyroid peroxidase antibody positivity and triiodothyronine levels are associated with pediatric Graves' ophthalmopathy. *World J. Pediatr.* **2014,** *10*, 155-159.
10. Calissendorff, J.; Mikulski, E.; Larsen, E. H.; Moller, M. A Prospective Investigation of Graves' Disease and Selenium: Thyroid Hormones, Auto-Antibodies and Self-Rated Symptoms. *Eur. Thyroid J.* **2015,** *4*, 93-98.

11. Katahira, M.; Ogata, H. Thyroglobulin Autoantibodies Are Associated with Refractoriness to Antithyroid Drug Treatment for Graves' Disease. *Intern. Med.* **2016,** *55*, 1519-1524.

12. Otsuka, F.; Noh, J. Y.; Chino, T.; Shimizu, T.; Mukasa, K.; Ito, K.; Ito, K.; Taniyama, M. Hepatotoxicity and cutaneous reactions after antithyroid drug administration. *Clinical Endocrinology* **2012,** *77*, 310-315.

13. Casallo, B. S.; Valero, M. A.; Marcos, S. F.; de Matias, S. L.; Blanco Gonzalez, J. J.; Martin Barranco, M. J. [Methimazole and propylthiouracil induced acute toxic hepatitis]. *Gastroenterol. Hepatol.* **2007,** *30*, 268-270.

14. Cofre, C.; Valdes, E.; Tapia, A.; Rodriguez, J. [Cholestatic hepatitis secondary to propylthiouracil]. *Rev. Med. Chil.* **1986,** *114*, 42-44.

15. Moral, M. P.; Rodriguez, S. M.; Mancheno, F. N.; Aguilera Sanchez-Tello, V.; Todoli, P. J.; Ponce, G. J.; Calabuig Alborch, J. R. [Acute cholestatic hepatitis due to propylthiouracil]. *Gastroenterol. Hepatol.* **2008,** *31*, 474-475.

16. Wang, M. T.; Lee, W. J.; Huang, T. Y.; Chu, C. L.; Hsieh, C. H. Antithyroid drug-related hepatotoxicity in hyperthyroidism patients: a population-based cohort study. *Br. J. Clin. Pharmacol.* **2014,** *78*, 619-629.

17. Russo, M. W.; Galanko, J. A.; Shrestha, R.; Fried, M. W.; Watkins, P. Liver transplantation for acute liver failure from drug induced liver injury in the United States. *Liver Transplantation* **2004,** *10*, 1018-1023.

18. Rivkees, S. A.; Mattison, D. R. Propylthiouracil (PTU) Hepatoxicity in Children and Recommendations for Discontinuation of Use. *Int J Pediatr Endocrinol.* **2009**.

19. Akmal, A.; Kung, J. Propylthiouracil, and methimazole, and carbimazole-related hepatotoxicity. *Expert. Opin. Drug Saf* **2014,** *13*, 1397-1406.

20. Rivkees, S. A. 63 years and 715 days to the "boxed warning": unmasking of the propylthiouracil problem. *Int. J. Pediatr. Endocrinol.* **2010,** *2010*.

21. Seeff, L. B. Drug-Induced Liver Injury Is a Major Risk for New Drugs. *Dig. Dis.* **2015,** *33*, 458-463.

22. Siramshetty, V. B.; Nickel, J.; Omieczynski, C.; Gohlke, B. O.; Drwal, M. N.; Preissner, R. WITHDRAWN--a resource for withdrawn and discontinued drugs. *Nucleic Acids Res.* **2016,** *44*, D1080-D1086.

23. Rousu, T.; Pelkonen, O.; Tolonen, A. Rapid detection and characterization of reactive drug metabolites in vitro using several isotope-labeled trapping agents and ultra-performance liquid chromatography/time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **2009,** *23*, 843-855.

24. Heidari, R.; Niknahad, H.; Jamshidzadeh, A.; Mohammad A, E.; Abdoli, N. An Overview on the Proposed Mechanisms of Antithyroid DrugsInduced Liver Injury. *Adv Pharm Bull,* **2014,** *74*, 1-8.

25. Moore, R. R.; Banks, C. E.; Compton, R. G. Electrocatalytic detection of thiols using an edge plane pyrolytic graphite electrode. *Analyst* **2004,** *129*, 755-758.

26. Kan, X. W.; Deng, X. H.; Zhang, W. Z.; Wang, G. F.; Li, M. G.; Tao, H. S.; Fang, B. Electrocatalytical oxidation of hydroquinone with ferrocene covalently bound to L-cysteine self-assembled monolayers on a gold electrode. *Ann. Chim.* **2005,** *95*, 593-600.

27. Chipiso, K.; Simoyi, R. H. Electrochemistry-coupled to mass spectrometry in simulation of metabolic oxidation of methimazole: Identification and characterization of metabolites. *Journal of Electroanalytical Chemistry* **2016,** *761*, 131-140.

28. Sartori, E. R.; Trench, A. B.; Rocha, R. C.; Fatibello, O. Determination of Propylthiouracil in Pharmaceuticals by Differential Pulse Voltammetry Using a Cathodically Pretreated Boron-Doped Diamond Electrode. *Journal of the Brazilian Chemical Society* **2013,** *24*, 1504-1511.

29. Madej, E.; Wardman, P. The oxidizing power of the glutathione thiyl radical as measured by its electrode potential at physiological pH. *Archives of Biochemistry and Biophysics* **2007,** *462*, 94-102.

30. Poole, L. B.; Karplus, P. A.; Claiborne, A. Protein sulfenic acids in redox signaling. *Annual Review of Pharmacology and Toxicology* **2004,** *44*, 325-347.

31. Mansuy, D.; Dansette, P. M. Sulfenic acids as reactive intermediates in xenobiotic metabolism. *Archives of Biochemistry and Biophysics* **2011,** *507*, 174-185.

32. Turell, L.; Carballal, S.; Botti, H.; Radi, R.; Alvarez, B. Oxidation of the albumin thiol to sulfenic acid and its implications in the intravascular compartment. *Brazilian Journal of Medical and Biological Research* **2009,** *42*, 305-311.

33. Gupta, V.; Carroll, K. S. Sulfenic acid chemistry, detection and cellular lifetime. *Biochimica et Biophysica Acta-General Subjects* **2014,** *1840*, 847-875.

34. Sujit, R. A. a. S. K. B. S. Formation and Disproportionation of Arene Sulfenic Acids. *Journal of Applied Chemistry* **2013,** *3*, 40-45.

35. Chipiso, K.; Simoyi, R. H. Kinetics and Mechanism of Oxidation of Methimazole by Chlorite in Slightly Acidic Media. *J. Phys. Chem. A* **2016**.

36. Svarovsky, S. A.; Simoyi, R. H.; Makarov, S. V. A possible mechanism for thiourea-based toxicities: Kinetics and mechanism of decomposition of thiourea dioxides in alkaline solutions. *Journal of Physical Chemistry B* **2001,** *105*, 12634-12643.

37. Zhang, X. Mass spectrometric and theoretical studies on dissociation of the C-S bond in the benzenesulfonic acid and benzenesulfinic acid anion series: Homolytic cleavage vs heterolytic cleavage. *Journal of Molecular Structure* **2012,** *1028*, 1-6.

38. Waldhauser, L.; Uetrecht, J. Oxidation of propylthiouracil to reactive metabolites by activated neutrophils. Implications for agranulocytosis. *Drug Metab Dispos.* **1991,** *19*, 354-359.

39. Waldhauser, L.; Uetrecht, J. Oxidation of Propylthiouracil to Reactive Metabolites by Activated Neutrophils - Implications for Agranulocytosis. *Drug Metabolism and Disposition* **1991,** *19*, 354-359.

40. Ranguelova, K.; Chatterjee, S.; Ehrenshaft, M.; Ramirez, D. C.; Summers, F. A.; Kadiiska, M. B.; Mason, R. P. Protein Radical Formation Resulting from

Eosinophil Peroxidase-catalyzed Oxidation of Sulfite. *J. Biol. Chem.* **2010,** *285*, 24195-24205.

41. Ranguelova, K.; Rice, A. B.; Lardinois, O. M.; Triquigneaux, M.; Steinckwich, N.; Deterding, L. J.; Garantziotis, S.; Mason, R. P. Sulfite-mediated oxidation of myeloperoxidase to a free radical: Immuno-spin trapping detection in human neutrophils. *Free Radical Biology and Medicine* **2013,** *60*, 98-106.
42. Nassar, A. F.; Holenberg, P. F.; Scantina, J. Drug Metabolism Handbook conepts and Applications., 39 ed. 2009; pp 302-303.
43. Henderson, M. C.; Siddens, L. K.; Morre, J. T.; Krueger, S. K.; Williams, D. E. Metabolism of the anti-tuberculosis drug ethionamide by mouse and human FMO1, FMO2 and FMO3 and mouse and human lung microsomes. *Toxicology and Applied Pharmacology* **2008,** *233*, 420-427.
44. DeBarber, A. E.; Mdluli, K.; Bosman, M.; Bekker, L. G.; Barry, C. E. Ethionamide activation and sensitivity in multidrug-resistant Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** *97*, 9677-9682.
45. Henderson, M. C.; Krueger, S. K.; Stevens, J. F.; Williams, D. E. Human flavin-containing monooxygenase form 2 S-oxygenation: Sulfenic acid formation from thioureas and oxidation of glutathione. *Chemical Research in Toxicology* **2004,** *17*, 633-640.

46. Krueger, S. K.; Williams, D. E. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol. Ther.* **2005,** *106*, 357-387.

47. Eswaramoorthy, S.; Bonanno, J. B.; Burley, S. K.; Swaminathan, S. Mechanism of action of a flavin-containing monooxygenase. *Proc. Natl. Acad. Sci. U. S. A* **2006,** *103*, 9832-9837.

48. Cashman, J. R.; Zhang, J. Human flavin-containing monooxygenases. *Annu. Rev. Pharmacol. Toxicol.* **2006,** *46*, 65-100.

49. Koukouritaki, S. B.; Hines, R. N. Flavin-containing monooxygenase genetic polymorphism: impact on chemical metabolism and drug development. *Pharmacogenomics.* **2005,** *6*, 807-822.

50. Henderson, M. C.; Krueger, S. K.; Stevens, J. F.; Williams, D. E. Human flavin-containing monooxygenase form 2 S-oxygenation: Sulfenic acid formation from thioureas and oxidation of glutathione. *Chemical Research in Toxicology* **2004,** *17*, 633-640.

51. Genter, M. B.; Deamer, N. J.; Blake, B. L.; Wesley, D. S.; Levi, P. E. Olfactory Toxicity of Methimazole - Dose-Response and Structure-Activity Studies and Characterization of Flavin-Containing Monooxygenase Activity in the Long-Evans Rat Olfactory Mucosa. *Toxicologic Pathology* **1995,** *23*, 477-486.

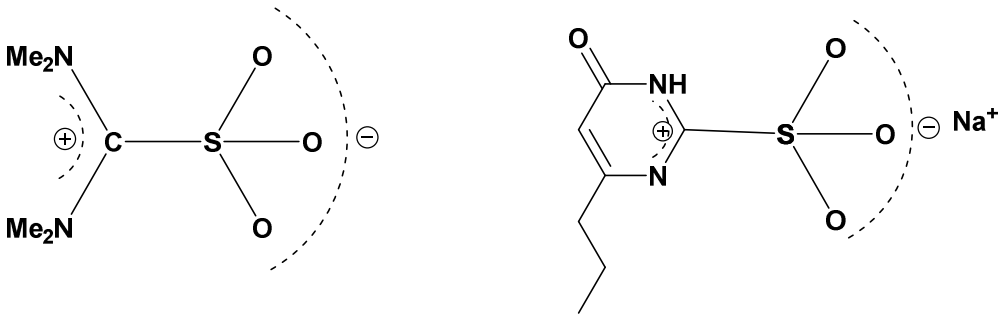
52. Shinno, E.; Shimoji, M.; Imaizumi, N.; Kinoshita, S.; Sunakawa, H.; Aniya, Y. Activation of rat liver microsomal glutathione S-transferase by gallic acid. *Life Sci.* **2005,** *78*, 99-106.

53. Chigwada, T. R.; Mbiya, W.; Chipiso, K.; Simoyi, R. H. S-Oxygenation of thiocarbamides V: Oxidation of trimethylthiourea by chlorite and chlorine dioxide. *Journal of Physical Chemistry A* **2014**.

54. Mizutani, T.; Yoshida, K.; Murakami, M.; Shirai, M.; Kawazoe, S. Evidence for the involvement of N-methylthiourea, a ring cleavage metabolite, in the hepatotoxicity of methimazole in glutathione-depleted mice: Structure-toxicity and metabolic studies. *Chemical Research in Toxicology* **2000,** *13*, 170-176.

55. Rankin, G.; Anestis, D.; Racine, C. Role of renal biotransformation in 3,4,5-trichloroaniline nephrotoxicity in vitro. *Faseb Journal* **2014,** *28*.

TOC GRAPHIC



TOC Graphic