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Electrochemical, Chemical and Enzymatic Oxidations of Phenothiazines

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Abstract
The oxidation of several phenothiazine drugs (phenothiazine, promethazine hydrochloride, promazine hydrochloride, trimeprazine hydrochloride and ethopropazine hydrochloride) has been carried out in aqueous acidic media by electrochemical, chemical and enzymatic methods. The chemical oxidation was performed in acetic acid with hydrogen peroxide or in formate buffers using persulfate. The enzymatic oxidation was performed in acetate or ammonium formate buffer by the enzyme horseradish peroxidase in the presence of H_2O_2. Molecules with, in the lateral chain, two carbon atoms (2C) separating the ring nitrogen and the terminal nitrogen, showed two parallel oxidation pathways, that is (i) formation of the corresponding sulfoxide and (ii) cleavage of the lateral chain with liberation of phenothiazine (PHZ) oxidized products (PHZ sulfoxide and PHZ quinone imine). Molecules with three carbon atoms (3C) separating the two nitrogens were oxidized to the corresponding sulfoxide. The chemical oxidation of all the studied molecules by hydrogen peroxide resulted in the corresponding sulfoxide with no break of the lateral chain. Oxidation by persulfate yielded, for the 3C derivatives, only the corresponding sulfoxide, but it produced cleavage of the lateral chain for the 2C derivatives. The origin of the distinct oxidation pattern between 2C and 3C molecules might be related to steric effects due to the lateral chain. The data are of interest in drug metabolism studies, especially for the early search. In the case of 2C phenothiazines, the results predict the possibility of an in vivo cleavage of the lateral chain with liberation of phenothiazine oxidized products which are known to produce several adverse side effects.

Keywords: Promethazine, Promazine, Phenothiazine, Oxidation, Voltamperometry, HRP, TLC, MEKC, LC/EC/MS

1. Introduction

Phenothiazines represent an important group of drugs useful in the treatment of various diseases in human and veterinary medicines. Hundreds of compounds derive from the fundamental phenothiazine skeleton (PHZ). Phenothiazines are used for their antiemetic, antihistaminic, sedative, antipsychotic, neuroleptic, antiparkinson, analgesic, local anesthetic, or preoperative anesthetic effects [1]. The field of their applications is constantly growing with many research pointing out new properties such as inhibitory effect on lipid peroxidation [2], in vitro and in vivo cytostatic effects, antiproliferative effect on many tumor cells, immunosuppressive properties, interaction with the cellular differentiation [3, 4], anti-inflammatory activity [4], intrinsic antibiotic activity or in synergy with existent antibiotics [5–7] and antimalarial activity [8]. The NASA uses phenothiazines to combat space motion sickness and is studying any eventual modified pharmacokinetic behavior in space [9, 10].

Phenothiazines, however, are known to induce various adverse effects, such as endocrine alterations and cardiac and reproductive toxicity [11, 12]. The exact biochemical mechanism(s) responsible for the development of toxicity phenomena is not clearly understood [13]. It is believed to be related to the oxidation of the phenothiazines by removal of one electron at the nitrogen atom (N10) with formation of a relatively stable cation radical [13, 14]. Phenothiazines are extensively metabolized by cytochrome P-450 isoforms in human liver giving rise mainly to ring-hydroxylated, S-oxidized and *N*-demethylated metabolites [15–17].

The in vitro oxidation mechanism of these compounds, particularly of the molecules promethazine (PMTZ) and promazine (PMZ), by chemical [18, 19] electrochemical [20–25] and enzymatic [24–28] methods has been quite extensively investigated. The oxidation pattern was reported to be dependent on the number of the carbon atoms separating the two nitrogen atoms in the lateral side chain. A cleavage of the lateral chain was seldom reported during the chemical and enzymatic (HRP) oxidations of promethazine

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i.e., a molecule which possesses two carbons in the lateral chain separating two nitrogens (2C phenothiazines) [18]. Actually parallel oxidation pathways were postulated with formation of PMTZ sulfoxide along with breaking of the lateral chain [18]. This was, however, not reported to occur by electrochemical oxidation, but aromatic ring hydroxylation was suggested and PMTZ sulfoxide formation was not detected [21, 22]. Other groups identified breaking of the lateral chain but no PMTZ sulfoxide formation [24].

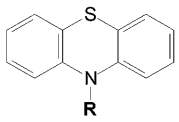
In the search for redox mediators for electrochemical biosensor development, we identified several phenothiazines as good substrates for electron transfer mediation between the enzyme horseradish peroxidase (HRP) and the electrode surface [28]. During these investigations, we noticed the distinct behavior of PMTZ compared to its structurally related isomer PMZ.

In the present work, taking into account the different literature data and with the help of powerful hyphenated instrumental techniques [23], we reinvestigate the oxidation reaction mechanisms of several structurally related phenothiazines (Fig. 1). The molecules were oxidized by enzymatic, chemical and exhaustive voltammetric means. The detection and identification of the products were performed by cyclic voltammetry (CV), micellar electrokinetic capillary electrophoresis (MEKC), thin-layer chromatography (TLC), liquid chromatography coupled to mass spectrometry (LC/MS), LC coupled to electrochemistry, and mass spectrometry (LC/EC/MS) and electrochemistry coupled to mass spectrometry (EC-MS).

2. Experimental

2.1. Reagents

Phenothiazine (PHZ), promethazine hydrochloride (PMTZ), promazine hydrochloride (PMZ), trimeprazine hydrochloride (TMP) and ethopropazine (EPP) hydrochloride (Fig. 1) were from Sigma-Aldrich (Bornem, Belgium). The standard solutions of the compounds were prepared at a concentration of 1.0×10^{-3} M in 0.1 M acetate or formate buffer. The 0.1 M acetate buffer was prepared using 0.1 M sodium acetate (Acros, Belgium) and 0.1 M acetic acid (Carlo Erba, Devos-François, Belgium). The standard solution of PHZ was prepared at a concentration of 1.0×10^{-3} M in methanol (Acros-Belgium) and diluted to the adequate concentration with a borate buffer (0.05 M, pH 9.0) containing 10 mM sodium dodecylsulfate (SDS) for MEKC. The borax salt and SDS were from Acros (Belgium) and the hydrochloric acid from Merck (Belgium). Hydrogen peroxide 30% w/v was from Vel (Leuven, Belgium). All the buffers were prepared with doubly distilled and purified water (Milli Q, Millipore system) and filtered through 0.45 μm hydrophilic polypropylene membrane filters (Gelman Sciences, Michigan, USA) before use. The solutions were kept in the dark. Ammonium formate was from Aldrich Chemie (Steinheim, Germany) and formic acid from Fluka (Buchs, Switzerland) in the highest quality available. Solvents for LC were acetonitrile "LiChroSolv gradient grade" from Merck (Darmstadt, Germany) and water from Merck eurolab (Briare le Canal, France). Peroxyacetic acid (32% solution in dilute acetic acid; *caution: strong oxidizer, dilute with water before mixing with organic substances*) was purchased from Sigma (Deisenhofen, Germany).



Molecule	Lateral side chain (R)	Oxidation pathway
PMTZ	$-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N-(CH_3)_2$	B + A
ETPZ	$-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N-(CH_2-CH_3)_2$	B + A
PMZ	$-CH_2-CH_2-CH_2-N-(CH_3)_2$	A
TMP	$-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-CH_2-N-(CH_3)_2$	A

Fig. 1. Schematic structures of the investigated phenothiazines.

2.2. Apparatus

Cyclic voltammetry (CV) was performed in a conventional three-electrode system. A glassy carbon electrode (geometric active area: 3 mm diameter) served as working electrode, a platinum wire as auxiliary and a Ag/AgCl 3 M KCl reference electrode (BAS, West Lafayette IN-USA). The glassy carbon electrode (GCE) was manually polished before use with an alumina suspension of 0.01 μm (BDH, England) and washed with doubly distilled and filtered water. A universal potentiostat programmer Model 175 (EG&G) and a polarographic analyser Model 174A (EG&G) connected to a graphical recorder Servogor XY (BBC GOERZ, Princeton, NJ, USA) were used for all the cyclic voltammetric studies. The pH of the investigated solutions was controlled by a pH-meter (Tacussel Minisis 6000-France)

Capillary electrophoresis was performed using a SpectraPHORESIS 100 system equipped with a modular injector, a high voltage power supply and a UV/vis spectrophotometric detector (Thermo Separation Products, Belgium) connected to a chart recorder Servogor 120 (BBC GOERZ). A fused silica capillary (75 μm i.d.) with a total length of 65 cm was from Alltech Associated, Inc, Belgium. The detector was placed 35.5 cm from the anode end of the capillary. Absorbance was measured through the capillary at a wavelength of 254 nm. Samples were introduced into the capillary at the anodic end by a hydrodynamic injection for

0.5 s. The temperature was kept constant at 25 °C. In MEKC, the capillary was first washed during 5 min with 1 M phosphoric acid (Acros, Belgium). At the beginning of each day, the capillary was washed, during 5 min with purified water and finally during 10 min with the borate running buffer. A separation voltage of 20 kV was applied. In order to improve repeatability, the capillary was rinsed after each experiment with the running buffer during 5 min. All the solutions were filtered through Spartan 13/0.2 μm filters (Devos-Francois, Belgium) before injection.

Thin layer chromatography (TLC) was realized with 20 × 20 cm plates of Kieselgel 60 F 254 S; layer thickness 0.5 mm, concentration zone 4 × 20 cm (Merck, Belgium). A mixture of acetone : ammonia 6 M (100 : 2) served as the mobile phase for the separation of PHZ and PMTZ, and acetonitrile : ammonia 6 M (100 : 2) served for the separation of PMZ and its oxidation compounds. The chromatographic chamber was saturated for a period of 30 min and one-dimensional ascending development was performed. The visual detection of the separated compounds was achieved using a UV lamp (254 nm).

For LC/MS measurements, the following equipment from Shimadzu was used: SCL-10Avp controller unit, DGU-14A degasser, two LC-10ADvp pumps, SIL-10A autosampler, SPD10AV UV/vis detector, LCMS QP8000 single quadrupole mass spectrometer with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) probes, and Class 8000 software Version 1.20. As stationary phase, a base deactivated Discovery RP-18 column from Supelco (Deisenhofen, Germany) was used. Column dimensions were 150 × 3.0 mm (APCI-MS) and 150 × 2.1 mm (ESI-MS). Particle size was 5 μm and pore size 100 Å. Flow rates of the mobile phase were 0.6 mL/min (APCI-MS) and 0.3 mL/min (ESI-MS). A binary gradient consisting of acetonitrile and aqueous buffer (formic acid and ammonium formate with appropriate pH) with the following profile was used:

time (min)	0.01	0.5	9	12	15	16	20
c (CH_3CN) (%)	20	25	50	95	95	20	stop

The concentration of the organic phase was not raised above 95% to ensure the presence of a sufficient concentration of the base electrolyte. The injection volume was set to 10 μL.

The MS conditions were as follows. For all measurements, the curved desolvation line (CDL) voltage −5 V, CDL temperature 280 °C, deflector voltages 40 V and detector voltage 1.65 kV were used. The APCI experiments were carried out with probe voltage 3 kV, nebulizer gas flow rate 2.5 L/min and probe temperature 500 °C. The ESI parameters were probe voltage 3 kV and nebulizer gas flow rate 4.5 L/min.

2.3. Exhaustive Electrolysis

Batch electrochemical oxidation (ECOx) was performed in a microdroplet of sample by running multiple cyclic voltammetric scannings at the GCE in upside down position. The reference electrode was a Ag/AgCl wire and a Pt wire served as auxiliary electrode and both dipped into the sample droplet. The electrolysis was carried out in 20 μL of 5.0×10^{-4} M phenothiazine derivative until the peak decreased considerably (approximately 20 cycles). After microelectrolysis, 15 μL of solution were sampled with a micropipette and studied by TLC or MEKC. The sample was diluted 10 times (PMTZ and PMZ), and 5 times (PHZ), using borate buffer and was homogenized by sonication before analysis by MEKC.

On-line electrochemical oxidation (EC) coupled to liquid chromatography (LC) with photometric and mass spectrometric detection was performed as follows: The electrochemical instrumentation from ESA, Inc. (Chelmsford, MA, USA) comprised a GuardStat potentiostat and a Model 5021 conditioning cell and a Model 5020 guard cell. The conditioning cell was used for postcolumn oxidation. The guard cell was designed to withstand high pressures and was used for the exhaustive oxidation of the injected sample plug prior to liquid chromatographic separation. The working electrode material was porous glassy carbon with a Pd counter and a Pd/H_2 reference electrode. For protection of the working electrode, a PEEK in-line filter (ESA, Inc.) was mounted ahead of the electrochemical cell. Subsequent liquid chromatographic separation and diode array detection (EC/LC/DAD) were performed on a Shimadzu (Duisburg, Germany) HPLC-system consisting of: two LC-10AS pumps, a SUS mixing chamber (0.5 mL volume), a GT-154 degasser unit, a SIL-10A autosampler and a CBM-10A controller unit with Class LC-10 software Version 1.6. and a SPD-M10Avp diode array detector. The guard cell was mounted ahead of the LC column with a 3 cm PEEK line.

Postcolumn electrochemical derivatization (LC/EC/MS) was accomplished by inserting the conditioning cell between the column and the interface of the mass spectrometer as described earlier for the oxidation of ferrocene derivatives [34]. For precolumn oxidation, the guard cell was placed ahead of the LC column.

2.4. Chemical Oxidation

An amount of 20.0 mg of phenothiazine (PHZ) was transferred into a 10.0 mL, graduated flask. The molecule was dissolved (suspended) in 1 mL of 15% v/v H_2O_2 methanolic solution and 0.2 mL of acetic acid 0.1 M. After a reaction time of 30 min in a water bath at 60 °C, the reaction mixture was diluted to 10 mL with methanol. This solution was diluted 200 times with borate running buffer for MEKC analysis, and diluted 100 times with 0.01 M acetate buffer pH 4.6 for CV analysis.

An amount of 32.3 mg of PMTZ or PMZ was transferred into a 10.0 mL graduated flask. The PMTZ or PMZ was

dissolved in 1 mL of 15% H_2O_2 solution and 0.2 mL of acetic acid. After a reaction time of 30 min in a waterbath at 60 °C, the reaction mixture was diluted to 10 mL with water. This solution was diluted 200 times with borate running buffer for MEKC analysis.

The oxidation of promazine and promethazine (2.8×10^{-4} M) with persulfate was performed in formate buffers at room temperature and was monitored by visible spectrophotometry at 520 nm (Pye Unicam, PU86650, Philips- NL). The molecules were also oxidized in acetic acid 0.1 M in the presence of hydrogen peroxide (15% v/v) at 60 °C and at room temperature.

The oxidation of the phenothiazines was also performed by the couple peroxyacetic acid/ potassium iodide (PAA/ KI) as follows: 0.4 mL phenothiazine derivative stock solution (5 mM) was diluted to 1.6 mL buffer (20 mM ammonium formate) and reacted in the presence of 18.5 mM PAA and 7.5 μM KI [35]. The oxidation products were identified by MS and LC-MS.

2.5. Enzymatic Oxidation

The enzymatic oxidation (ENZOx) of the phenothiazines was performed with horseradish peroxidase (HRP) type II (Sigma-Aldrich) and with a HRP (EC 1.11.1.7) from *Arthomyces rhamosus* (Sigma) in the presence of hydrogen peroxide. The former enzyme was used immobilized onto a cellulose acetate strip. Enzyme immobilization was made by cross-linking HRP with 2.5% v/v glutaraldehyde (GA) from Aldrich in the presence of bovine serum albumin (BSA) from Merck. Briefly, two mg HRP and one mg BSA were dissolved in 50 μL of 0.1 M acetate buffer by mixing thoroughly and spreading on a glass plate. After addition of 50 μL of GA, the cellulose acetate strip was dipped in the mix that was left at 5 °C for 1 h. Then 50 mL glycine solution (0.01 M; Merck) was added and mixed in order to react with any excess of GA. After the last rinsing with acetate buffer, this enzyme preparation was left at room temperature until dryness. The enzymatic oxidation was achieved by dipping the strip in a solution of 2.0 mL 5.0×10^{-3} M PHZ (or PMTZ or PMZ), 8.0 mL acetate buffer and 15 μL of H_2O_2 3% v/v.

The oxidation by HRP from *Arthomyces rhamosus* was performed as follows: 2 mL phenothiazine derivative stock solution (5 mM) was diluted with 10 mL buffer (20 mM ammonium formate pH 3) then addition of 0.1 mL of HRP (1 mg/L) and 12 μL of a 3% v/v H_2O_2 solution and stirring for 30 min prior injection into the EC/MS setup.

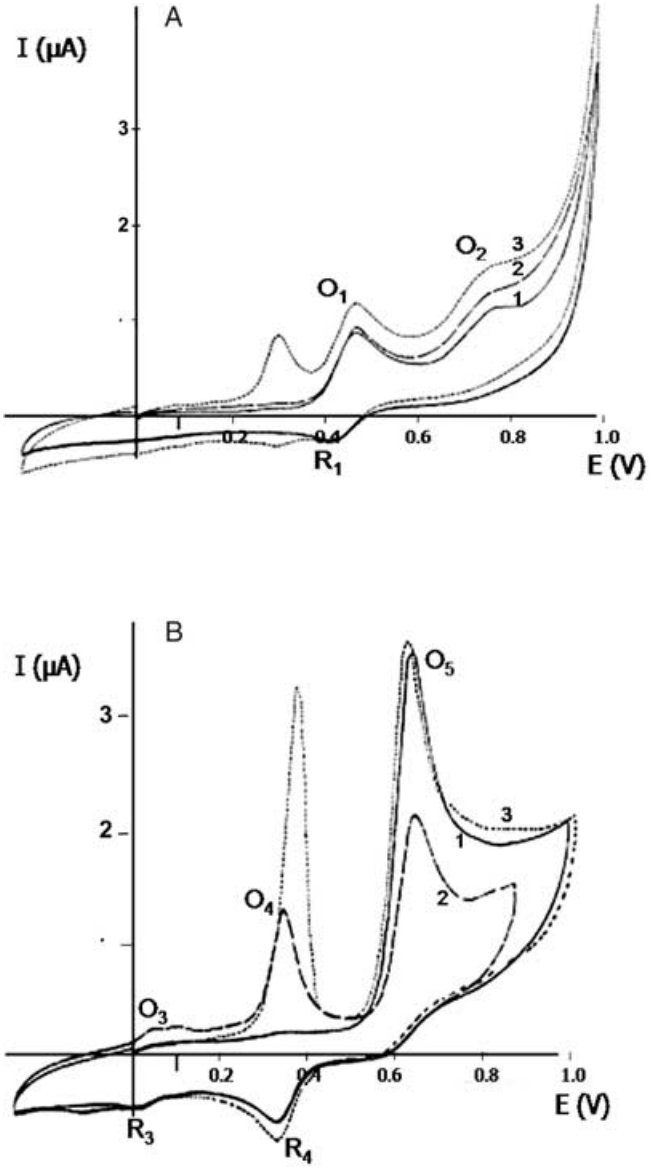


Fig. 2. A) Typical CV of 1.0×10^{-4} M PMZ, 0.01 M acetate buffer, pH 4.6; methanol 10% v/v. GCE vs. Ag/AgCl 3 M KCl. Initial potential 0.0 V, positive potential scanning direction, scan rate 50 mV/s. First and second cycles. Third cycle = in the presence of PHZ (1×10^{-5} M) in the solution (dotted line). B) Typical CV of 1.0×10^{-4} M PMTZ, 0.01 M acetate buffer, pH 4.6; methanol 10% v/v. GCE vs. Ag/AgCl 3 M KCl. Initial potential 0.0 V, positive potential scanning direction, scan rate 50 mV/s. First and second cycles. Third cycle = in the presence of PHZ (1×10^{-5} M) in the solution (dotted line).

3. Results and Discussion

3.1. Cyclic Voltammetry (CV)

All voltammograms started at 0.0 V towards positive direction and reversing scan direction at 1.0 V. A 1.0×10^{-4} M solution of the analytes showed no significant differences using formate or acetate buffer as the supporting electrolyte. In Figure 2, typical voltammograms for PMZ (Figure 2A) and PMTZ (Figure 2B) are reported.

The oxidation of promazine (PMZ) showed, in the investigated acidic media (pH 2 – 5), a well known electrochemical behavior with removal of one electron to the corresponding cation radical (peak O_1) and subsequent removal of a second electron giving the phenazothiazonium ion and formation of the sulfoxide PMZSO (peak O_2). At higher potentials (above 1.0 V), further oxidation of

PMZSO (likely at the tertiary nitrogen of the lateral chain) was inferred. By reversing the scan direction at 1.0 V, the reversible character of peak O_1 was noticed (peak R_1). No new peak formation was detected by multiple scanning. The same CV pattern was observed for trimeprazine (TMP).

The oxidation of promethazine (PMTZ) occurred at more positive potentials (ca. 150 mV more positive than promazine oxidation) showing only one irreversible peak (peak O_5) (Figure 2 B curve 1). The high magnitude of the latter (higher than for a 2 electron process) suggests a complex process with subsequent oxidation steps. By reversing the scan direction at 1.0 V, two new reduction peaks (R_4 and R_3) and their corresponding oxidation peaks (O_4 and O_3, dashed line) were detected at potentials lower than oxidation peak O_5. These new peaks correspond to molecules generated by the oxidation of PMTZ (at O_5) and which are more readily oxidized than PMTZ. The same CV pattern was observed for ethopropazine (ETPZ). This CV behavior of PMTZ was already reported in the literature, with the new redox peaks postulated to be the di- (O_3) and mono-(O_4) hydroxylated structures of PMTZ [21, 22]. The formation of PMTZ sulfoxide was not reported by these authors in the pH range studied, i.e., from 2 to 7 [22].

Taking into account literature data mentioning the break of the lateral chain during PMTZ chemical and enzymatic oxidations [18, 24], we initially performed the CV on the PMTZ solution (Figure 2B curves 1 and 2), then on the same solution but spiked with PHZ (Fig. 2B curve 3 dotted line). Experiments were performed at the GCE in acetate buffer of pH 4.6 in the presence of 10% v/v methanol due to poor solubility of PHZ. The addition of PHZ (1×10^{-5} M) gave rise to a substantial increase of the intensity of the couples O_4/R_4 and of peak O_5 (Fig. 2B curve 3 dotted line). The slight 40 mV peak shift of peak O_4 by raising the PHZ concentration is explained by the adsorptive characteristic of the PHZ oxidation peak. Spiking of PHZ was also performed on a PMZ solution (Fig. 2A curve 3). A new reversible peak was detected at lower potentials than O_1 and O_2 which corresponded to PHZ behavior. The evolution of peak potentials (O_5, O_4 and O_3) was studied as a function of pH (formate buffer, pH range between 2 and 5) for PMTZ and EPP (Figures not shown). The oxidation peak O_5 shifted towards less positive potentials by raising the pH (approximately 30 mV/pH). A good matching was obtained between the CV peaks of a pure solution of PHZ and the peaks O_3 and O_4, suggesting that the latter corresponded to the redox pattern of phenothiazine (PHZ). A good matching was also obtained between the oxidation potential (O_5) of PMTZ and ETPZ suggesting a similar oxidation pattern for the two derivatives.

From these results, and considering our previous observation at carbon paste electrodes [28], we may infer that PMTZ oxidation likely induced a breaking of the lateral chain with liberation of the phenothiazine nucleus (PHZ) giving rise to the redox couples O_3/R_3 (3-*H*-phenothiazine-3-one (PHZQI), see below) and O_4/R_4 (PHZ). This hypothesis differs, however, from the electrochemical pathway reported in the literature [21, 22] but is in agreement with literature data on the chemical and enzymatic oxidations of PMTZ [18, 24]. The electrooxidation of PMZ gave no lateral chain breaking, since no formation of the phenothiazine molecule was detected by CV.

3.2. MEKC Identification of Oxidation Products

The nature of the oxidized products was elucidated from their retention time ratio between standard compounds (PHZ, PMTZ, PMZ) and the resulting products.

3.2.1. Exhaustive Electrochemical Oxidation (ECOx)

The electropherogram of a 1.0×10^{-4} M PHZ solution issued from "micro"exhaustive ECOx at the glassy carbon electrode in acetate buffer pH 4.5 gave two peaks. The first peak corresponded to phenothiazine sulfoxide (PHZSO), and the second to residual PHZ. With the PMTZ solution, four peaks were identified, namely, PHZSO, PHZ, promethazine sulfoxide (PMTZSO), and residual PMTZ. Following PMZ oxidation, only two peaks were obtained, namely, promazine sulfoxide (PMZSO) and residual PMZ.

3.2.2. Oxidation by Hydrogen Peroxide (CHEMOx)

With PHZ, three peaks were detected in MEKC. They corresponded to residual H_2O_2, PHZSO, and residual PHZ. For a PMTZ solution, two peaks were obtained namely residual H_2O_2 and PMTZSO. Peaks corresponding to PHZ and its oxidation products were not observed. PMZSO and H_2O_2 were the two peaks obtained for the PMZ oxidized solution.

3.2.3. Enzymatic Oxidation (ENZOx)

The same electropherogram profile as for the CHEMOx was observed in the case of the PHZ solution. The PMTZ solution, gave three peaks namely, PHZSO, PHZ, and residual PMTZ (no PMTZSO was detected). PMZSO and residual PMZ were the two peaks detected after ENZOx of the PMZ solution.

3.3. TLC Identification of Oxidation Products

Two groups of compounds migrated with their spots situated at the opposite sides of the TLC plate. The molecules with only the PHZ nucleus (i.e., without lateral side chain) were observed in the upper part, while the PMTZ or PMZ derivatives (i.e., with lateral chain) were in the lower part (Fig. 3). The strong retention of the latter was most likely due to the basic tertiary nitrogen atom in the alkyl side chain, which interacted with the free silanol groups of the stationary phase.

By enzymatic oxidation of PHZ (PHZ, ENZ), two spots attributed to PHZQI (red) and PHZSO were observed. The

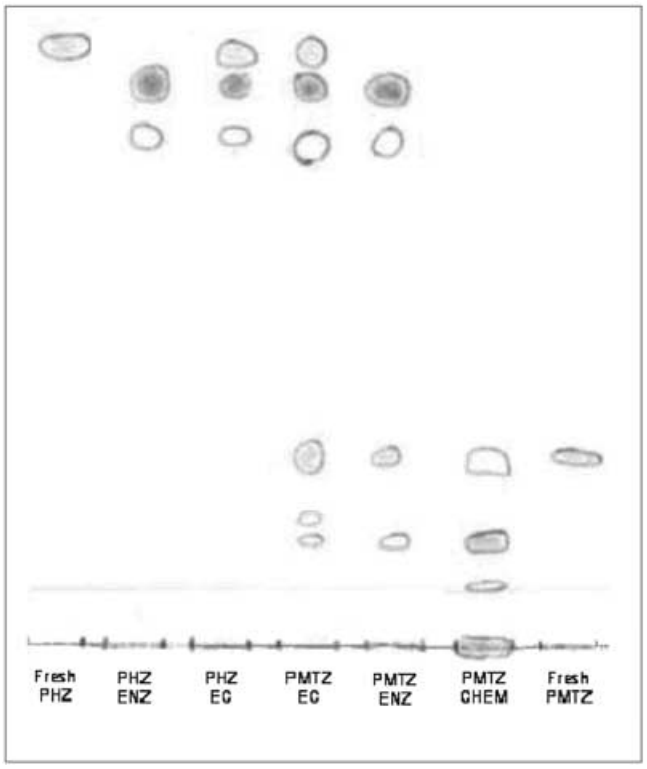


Fig. 3. Thin layer chromatography of phenothiazine (PHZ) and promethazine (PMTZ). Enzymatic (ENZ), electrochemical (EC) and hydrogen peroxide (CHEM) oxidation products.

same was obtained after electrochemical oxidation (PHZ, EC), but some residual PHZ was still detected.

Regarding PMTZ, in the lower part, a group of spots corresponding to PMTZ (residual), and likely PMTZSO, was noticed for the three oxidation modes. In the upper part, for EC and ENZ oxidations, spots due to PHZQI (red) and PHZSO were noticed. The EC row additionally showed a PHZ spot, because multiple EC cycling regenerated partly PHZ from its cation radical. Interestingly, and in agreement with previous results, the CHEM oxidation of PMTZ by hydrogen peroxide gave no visual color development and no spots in the upper part (i.e., no fission of the lateral chain). Only a pale, stable pink color was noticed by performing the peroxidation at 60 °C (Figure 4). Oxidation of PMTZ by persulfate gave a pink color which intensified drastically with time to give a stable red color. This was followed at 520 nm as a function of pH (Fig. 4) showing a high amount of the red species in acidic media (pH 3.2), in agreement with literature [18]. In order to try to identify the nature of the red spot the scratched zone of the spot was dissolved in few microliters of formate buffer of pH 4.5 and analyzed by CV at the GCE. The voltammogram gave one quasi reversible redox peak near 0.0 volt corresponding to the couple O_3/R_3 (not shown). This behavior, along with the color of the molecule, are in favor of a quinoneimine structure i.e., PHZQI [18, 29].

Regarding PMZ oxidation by hydrogen peroxide (both at 60 °C and at room temperature), the solution became pink but the color vanished rapidly with time (behavior attributed to the cation radical of PMZ) and only one spot was

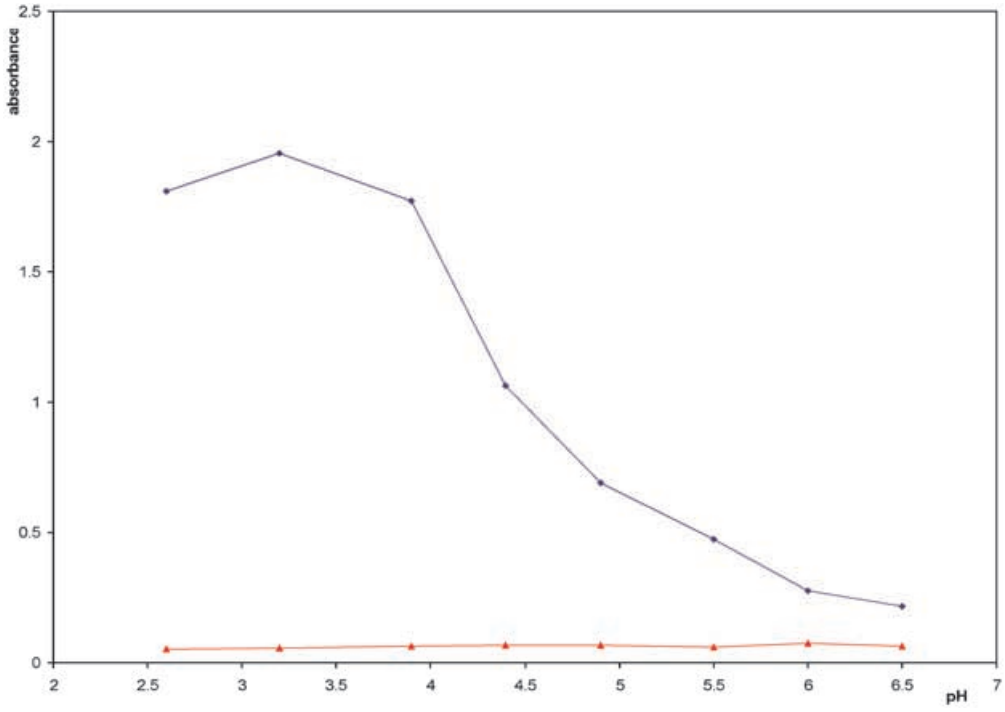


Fig. 4. Absorbance of the promethazine solution after oxidation by persulfate (room temperature oxidation, ◆) and hydrogen peroxide (oxidation at 60 °C, ▲) as a function of pH. Formate buffer.

detected in TLC likely corresponding to PMZSO. This peak was not migrating due to the strong interaction of the basic side chain with the stationary phase (TLC data not shown). Oxidation of PMZ by persulfate gave a pink color (cation radical) which gradually disappeared.

3.4. EC/MS, EC/LC/MS, and EC/LC/DAD

These hyphenated techniques allow on-line oxidation and mass spectrometric detection (EC/MS), or on-line oxidation, separation, and detection (EC/LC/MS, and EC/LC/DAD).

Experiments were realized in acidic formate buffers. The studied phenothiazines with basic side chains (pK_a around 9) were protonated under the experimental conditions, allowing their detection under electrospray conditions in the positive ion mode (ESI (+)). Mass spectra recorded on-line, following EC oxidation at 0.8 V (vs. Pd/H_2) are shown for PMZ (Fig. 5A) and for PMTZ (Fig. 5B). A more complex pattern was noticed for PMTZ than for PMZ. The mass spectrum contained many species which could readily be identified by comparison with the oxidation of the molecule phenothiazine [23]. Under identical experimental conditions the latter gave, by EC/MS at all pH values, the corresponding sulfoxide ($m/z = 216.0$), the cation radical ($m/z = 199.0$), the 3-*H*-phenothiazine-3-one ($m/z = 214.0$) plus a dimer of PHZ with $m/z = 395.0$ [23, 25]. The chemical oxidation (PAA/KI) of PHZ produced at all pH values the PHZSO plus a small amount of 3-*H*-phenothiazine-3-one. The HRP oxidation in the presence of hydrogen peroxide gave the same species as for the EC/MS assay, except that the comparably instable cation radical at $m/z = 199.0$ was not detected.

Figure 6 illustrates typical EC/LC/MS recordings for PMZ (Fig. 6A) and PMTZ (Fig. 6B). The total ion current (TIC) and the extracted mass traces of the protonated phenothiazines are shown. As noticed in Figure 6A, the PMZ electrooxidation produced the corresponding sulfoxide ($m/z = 301.1$; $R_t = 4.8$ min) with some residual PMZ

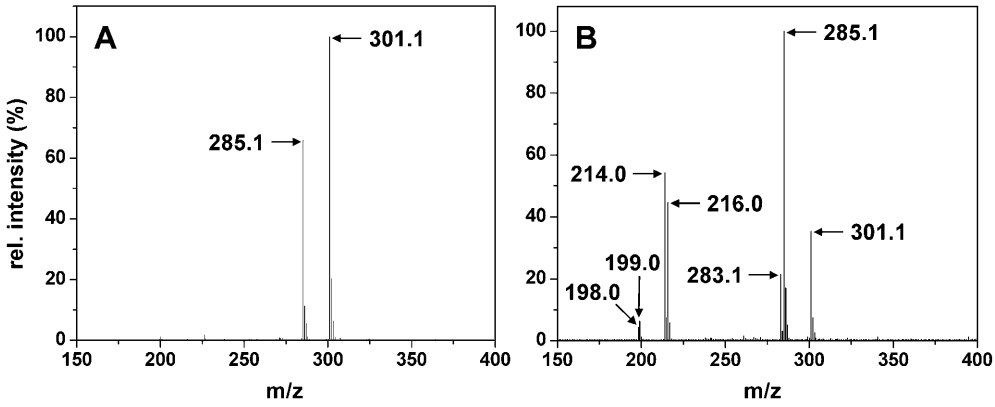


Fig. 5. ESI-MS spectra of 50 μM PMZ (A) and 50 μM PMTZ (B) following on-line EC conversion. Formate buffer 20 mM, pH 3.

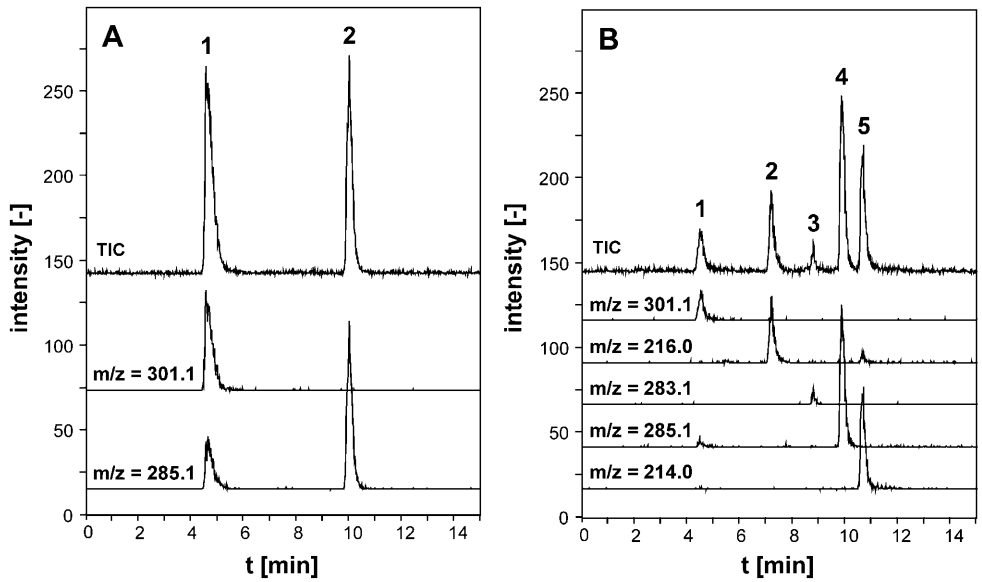


Fig. 6. LC/EC/APCI-MS chromatogram of 50 μM PMZ (A) and 50 μM PMTZ (B). Formate buffer 20 mM, pH 3.

($m/z = 285.1$; $R_t = 10.0$ min)). PMTZ electrooxidation gave rise to several species, namely PMTZSO ($m/z = 301.1$; $\lambda_{max} = 235$, 270, 300, 335 nm ; $R_t = 4.6$ min), residual PMTZ ($m/z = 285.1$; $\lambda_{max} = 240$, 300 nm; $R_t = 10.0$ min), PHZSO ($m/z = 216.0$; $\lambda_{max} = 260$, 320 nm; $R_t = 7.3$ min), 3-*H*-phenothiazine-3-one ($m/z = 214.0$; $\lambda_{max} = 225$, 290, 380, 520 nm; $R_t = 10.8$ min) as well as an unidentified peak at $m/z = 283.1$ ($R_t = 9.0$ min). The phenothiazine cation radical nucleus was detected by applying EC/MS, but could not be detected by EC/LC/MS as it was not stable enough and decomposed during the LC separation. With the EC setup used, the conversion efficiency at the porous working electrode should typically be above 90% and residual PMZ or PMTZ should be low. Here, however, the amount of detected residual molecules was quite high (see peaks at 285.1 in Figure 5A and B) and this fact is likely attributed to the well-known disproportionation of the phenothiazines cation radical into the parent compound and the corresponding sulfoxide [21, 26].

The detection of the PHZ nucleus by both EC/MS and by EC/LC/MS excludes the possibility that this molecule could originate from fragmentation of PMTZ during APCI ionization. Thermal degradation of the sulfoxide in the APCI interface (loss of oxygen, M-16) was observed since, at the retention time of the sulfoxide, the m/z of the parent compound was also obtained. This effect was not observed with the ESI(+) ionization mode as the thermal stress is lower. These results are in agreement with the MEKC and TLC data above and confirm the fact that part of PMTZ suffered from lateral chain breaking and that part of PMTZ was oxidized to its corresponding sulfoxide.

The molecules PMZ and PMTZ were studied by EC/MS as a function of pH (3, 5 and 7) and at different applied potentials (results not shown).

Both molecules were totally oxidized above 0.6 V. The amount of PMZ sulfoxide was higher at pH 3, then it decreased on raising the pH. Regarding the PMTZ oxidation, its sulfoxide formation was higher at pH 3 than at pH 7. The cleavage of the lateral chain, as inferred from the detection of phenothiazine sulfoxide (PHZSO), was higher at pH 7 than at pH 3. These results were in agreement with literature data suggesting that hydroxide ions catalyze the chain cleavage [18]. The amount of quinoneimine (PHZQI) was slightly higher at pH 3 than 5 or 7. The PHZ cation radical was only detected at pH 3 because it is too unstable above this value.

Table 1 summarizes different oxidation modes applied to the studied molecules and the resulting products identified by LC/MS. For TMP and PMZ, the three oxidation modes showed only PMZSO (plus residual PMZ) at all investigated pH values with a slightly higher conversion rate at higher pH values. The chemical oxidation (PAA/KI) of PMZ produced, at all investigated pH values, the corresponding sulfoxide and dioxygenated species, probably the sulfones, at long reaction times (several hours). The chemical oxidation of PMTZ and ETPZ by hydrogen peroxide (PAA/KI) generated quasi quantitatively their corresponding sulfoxide i.e., no cleaved species was obtained, with small amounts of dioxygenated species (most likely the sulfones) at longer reaction times. The enzymatic oxidation (by HRP in the presence of hydrogen peroxide) showed low conversion rates at pH 2 but higher rates at pH 3 and 4. The corresponding sulfoxides were obtained for TMP and PMZ. For PMTZ, the corresponding sulfoxide plus cleaved products (PHZSO, PHZQI plus an unknown species with $m/z = 283.1$) were obtained. The same pattern was obtained for ethopropazine (ETPZ) although an analogous substance to $m/z = 283.1$ was not observed. Electrochemical oxidation of the studied phenothiazines gave the same products as for the HRP oxidation. The EC/MS allowed the detection of the unstable PHZ cation radical ($m/z = 199.0$) for PMTZ and ETPZ.

Table 1. Mass-to-charge ratios of the oxidation products identified by mass spectrometry (l: large peak, m: medium peak, s: small peak)

Method	Trimeprazine (TMP) $M = 298$ Da	Promazine (PMZ) $M = 284$ Da	Promethazine (PMTZ) $M = 284$ Da	Ethopropazine (ETPZ) $M = 312$ Da
HRP	315 (l) 299 (s)	301 (l) 285 (s) 283 (s)	301 (m) 216 (m) 283 (s) 285 (m) 214 (m)	329 (m) 216 (m) 313 (m) 214 (m)
PAA/KI	315 (l) 331 (s)	301 (l) 317 (s)	301 (l) 317 (s)	329 (l) 345 (s)
EC/LC/MS ESI(+)	315 (l) 299 (s)	301 (m) 285 (m)	301 (m) 216 (m) 283 (s) 285 (m) 214 (m)	329 (m) 216 (m) 313 (m) 214 (m)
LC/EC/MS ESI(+)	315 (l) 299 (s)	301 (m) 285 (m)	301 (m) 216 (m) 283 (s) 285 (m) 214 (m) 199 (s)	329 (m) 216 (m) 313 (m) 214 (m) 199 (s)

The present data, and some of our previous results on the voltammetric oxidation of a 2C phenothiazine derivative, clearly point out a distinct pattern for the 3C and 2C phenothiazine species [30]. The oxidation process is rather complex since it is pH and potential dependent, i.e., the oxidation products may be different depending on the oxidation power of the reagents used. The unique on-line coupling between EC and MS allowed a better understanding of the oxidation pattern of phenothiazine derivatives since no extraction steps with risks of loss and degradation of products were encountered. It is clear that the 3C molecules gave no removal of the lateral chain under the different oxidation modes and pH studied, in agreement with literature data, but produced mainly the corresponding sulfoxide [22, 24]. The 2C species, however, showed upon enzymatic and electrochemical oxidation two possible parallel mechanisms, i.e., (i) fission of the lateral chain and (ii) formation of the corresponding sulfoxide. The latter, however, was not observed in the literature [24]. In the time scale of the experiments, we found no significant ring hydroxylation of the studied phenothiazines in agreement with the HRP oxidation mechanism [24] and in contrast to cyclic voltamperometric interpretations [22]. The literature data on enzymatic oxidation are quite confusing and postulate only sulfoxidation of PMTZ [2] or products issued only from cleavage of the PMTZ lateral chain [24]. The fact that the chemical oxidation of PMTZ (and ETPZ) by hydrogen peroxide gave no fission of the lateral chain but formation of the sulfoxide could be attributed to a lower oxidation power of hydrogen peroxide ($E^\circ = 1.776$ V vs. NHE) in contrast to persulfate ($E^\circ = 2.01$ V vs. NHE). Our results point out promethazine sulfoxide formation and cleavage of the lateral chain thanks to the biocatalytic action of the enzyme (HRP) in the presence of H_2O_2.

It appears thus, that both cleavage of the lateral chain and PMTZ sulfoxide formation may occur in parallel with different kinetics and product ratios, depending on the pH and oxidation strength of the method.

Figure 7 illustrates the general trends regarding the oxidation of phenothiazines taking into account literature data and our results. Deprotonation of the terminal nitrogen of PMTZ during oxidation was postulated as a phenomenon facilitating the cleavage of the lateral chain during oxidation of 2C phenothiazine derivatives [22, 24]. In addition to the deprotonation, the cleavage might be attributed to steric problems due to the lateral chain [19, 31]. Actually, the aryl rings of phenothiazine behave like butterfly wings [32] giving rise, by oxidation, to planar structures of the radical cation and the phenazothionium ion (Fig. 7 pathway A). From three dimensional images of PMZ and PMTZ (not shown), we postulate that this movement might be hindered to some extent by the bulky tertiary nitrogen of C2 derivatives. Oxidation of the latter would be possible, though, with the removal of the lateral chain via the phenazothiazonium mesomeric form (Fig. 7 pathway B). This might explain why the oxidation potential of PMTZ

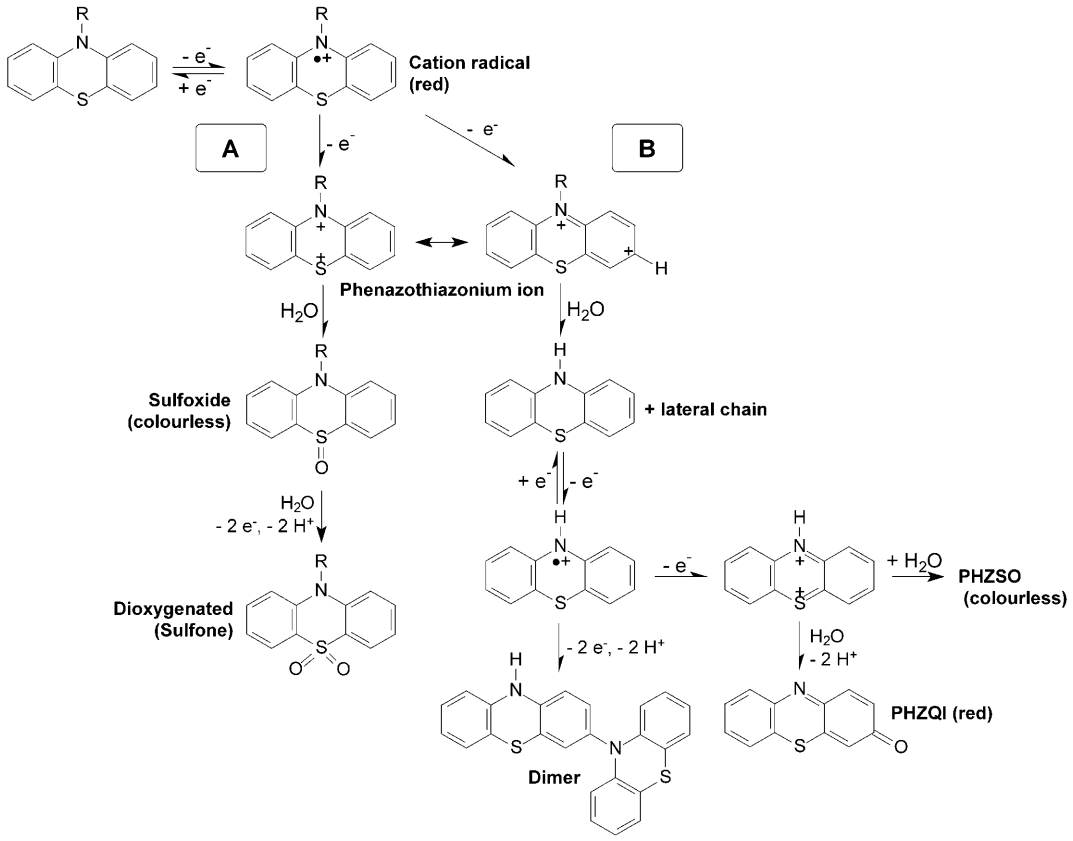


Fig. 7. Schematic drawing of the postulated oxidation pattern of promethazine and promazine.

and EPP was higher by approx. 150 mV than the oxidation of TMP and PMZ and why stronger oxidizing reagents resulted in cleavage of the alkylamine chain and generated low amounts of PMTZSO. The oxidation products identified in our study of PMTZ are in close agreement with the mechanisms reported by Underberg on the thermal degradation of PMTZ in the presence of oxygen, except that we could not detect 10-methylphenothiazine [18].

4. Conclusions

The combination of a diversity of analytical approaches has permitted new insight on the oxidation pattern of 2C and 3C phenothiazines. The former are oxidized to the corresponding sulfoxide but may also suffer from a break of the lateral chain, depending on the oxidation strength of the technique and reagents used. Most importantly, our results have permitted a reinterpretation of the cyclic voltammetry of promethazine and of the 2C phenothiazines in general. The 3C derivatives yielded, upon oxidation, the corresponding sulfoxide and no cleavage of the lateral chain was observed. Surprisingly, except by one group [29, 33], the in vivo removal of the aminoalkyl side chain of promethazine was not reported. It would be of great value to check for such metabolites since the generated species (phenothiazine, phenothiazine sulfoxide and 3-*H*-phenothiazine-3-one) have no psychotropic activity, but are physiologically and toxicologically active [14]. We should also recall that the 2C phenothiazines develop antihistaminic activity and show no psychotropic activity in contrast to the 3C phenothiazines. Although the toxicological and pharmacological significances of these in vitro findings remain to be established, it is worth evaluating if promethazine and ethopropazine chain cleavage occurs in vivo as well.

5. Acknowledgements

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